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# **Biotechnology in Agriculture and Forestry**

Edited by Jack M. Widholm  
Horst Lörz and Toshiyuki Nagata

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## **64 Genetic Modification of Plants**

Agriculture, Horticulture and Forestry

Frank Kempken and Christian Jung *Editors*

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Frank Kempken • Christian Jung  
Editors

# Genetic Modification of Plants

Agriculture, Horticulture and Forestry



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*This book is dedicated to  
Prof. Dr. Dr. h.c. mult. Gerhard Röbbelen  
on the occasion of his 80th birthday for  
his lifetime contribution to applied plant  
genetics.*



# Preface

Today modern agriculture is facing new challenges. Total yields have to be increased due to the continuing population growth of mankind and due to changing food consumption. However, global climate creates new problems but also new opportunities for agriculture. For more than a decade the yearly yield increases of major food staples have been on the decline, which is due to optimized production systems like the application of mineral fertilizer and crop protection measures. But also the yield increases due to genetic improvement of crops have been stagnating. Obviously we are approaching yield barriers for a number of crops, which creates a need for innovation in breeding systems.

There is no doubt that further genetic improvement of crops will be a key for increasing yields in the future. Moreover, breeding must meet the demands for increasing biomass (bioenergy) and the production of industrial raw materials. The breeding of better-adapted and higher-yielding varieties relies crucially on the available genetic variation. Broad genetic variation is a fundamental prerequisite for successful breeding. Apart from other technologies like wide crosses, mutation breeding and somatic hybridization, genetically modified plants will play an increasingly important role in future breeding systems either because natural genetic variation has been largely exploited or because natural variation is completely lacking from the primary and secondary or even from the tertiary gene pool of a crop species.

It is commonly agreed within the scientific community that genetically modified plants will be important for future breeding. Adoption rates worldwide have been increasing in the past ten years in a breathtaking manner. In the year 2008 genetically modified plants were cultivated by about 13.3 mio farmers from 25 countries worldwide on a total acreage of about 125 mio ha (<http://www.isaaa.org/>). Numerous investigations have confirmed that cultivation of genetically modified plants is safe, as far as approved plants are concerned which have passed a step by step risk assessment procedure, as is commonly applied in most countries growing genetically modified organisms (GMO) today. Instead of this, there is still a big public debate on GM plants in a number of countries. Mainly in the European Union, the production of GM plants is almost completely avoided. Low consumer acceptance

is the only reason pointed out by politicians to establish legal restrictions for GMO production, in spite of numerous studies confirming their safety towards the environment or for food and feed use. Many scientists have been frustrated due to this debate which is ignoring scientific facts and which is mainly directed by pressure groups and non-governmental organizations.

This book was written with an intention to get back to the facts. In the past years a number of books focusing on GM plants have been published. Some of these cover all aspects, including minor crop species. So, why is there a need for a new book? Our book tries to address all aspects of GM plants, including their employment in a plant-breeding procedure, and their socioeconomic implications. We try to emphasize that GM plants among others are an important tool in plant breeding to broaden the genetic variation of crop species.

The book is structured into four parts. The first part deals with technical details of plant genetic engineering. The second part introduces characters of GM plants, while the third part presents applications in agricultural production systems. The last part deals with risk assessment and economic implications, which are important aspects of GM plants. The articles are written by scientists who have a long experience in their field of expertise. We thank the authors for their excellent contributions, which make this book, we think, a valuable resource for the different aspects of GM crops. We are aware of the fact that not all topics and some minor crops could not be included in this book. We regret that this was not possible due to size limitations. Finally we are indebted to the Springer publishing company for supporting this book.

Kiel, Germany  
October 2009

Frank Kempken and Christian Jung

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**Part A**

**Generation and Analysis**

**of Transgenic Plants**



# **Chapter 1**

## **Plant Nuclear Transformation**

**John J. Finer**

### **1.1 Introduction to Plant Transformation**

“Transformation” is most simply defined as a “change”. In the plant biotechnology community, transformation can be a little more precisely defined as the process of DNA introduction into a plant cell, leading to a permanent change in the genetic makeup of the target cell and its derivatives.

The ability to produce whole plants from transformed plant cells, first reported by Horsch et al. (1985), has revolutionized the plant sciences and changed the face of the planet, through the success and rapid adoption of genetically modified crops. Although the transformation process itself was initially limiting, all crops of major interest have been successfully transformed and many if not most transformation technologies are considered routine. Some crops do remain a little recalcitrant to transformation and improvements in the methods for production of stably-transformed plants are still needed. The current limitations in the production of transgenic plants for both basic research and commercial application include more efficient production of transformed plants and obtaining more predictable insertion and expression of the introduced DNA.

#### **1.1.1 DNA Introduction Basics**

DNA introduction can impact and modify any of the organelles within the plant cell that also contain DNA. Suitable targets include the nucleus, plastid and mitochondrion. Plastid transformation is presented in the next part of this chapter while this

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portion of the chapter focuses exclusively on nuclear transformation. Transformation of the mitochondrion has been reported for some organisms (Johnston et al. 1998) but has not yet been reported for higher plants.

For (nuclear) transformation to be successful, DNA must first be introduced into the target cell. The DNA molecule is sufficiently large so that a physical entry point through the cell wall and cell membrane must be established and this can compromise the health of the targeted cell. After passage through the plant cell wall and membrane, the introduced DNA must then proceed to the nucleus, pass through the nuclear membrane and become integrated into the genome. It is believed that the introduced DNA can function for a short time in the nucleus as an extrachromosomal entity, but integration into the genetic material of the target cell is necessary for long-term functionality and expression.

To recover a transgenic plant, the single cell that is the recipient for DNA introduction must be capable of either forming a whole plant or contributing to the zygote, through either the pollen or the egg. Therefore, successful recovery of transgenic plants largely relies on the ability to either transform the pollen/egg directly (Ye et al. 1999) or the non-gametic cells (somatic cells; Horsch et al. 1985), which must be subsequently manipulated to form whole plants. In most cases, plant transformation relies heavily on the ability of the plant cells and tissues to form whole plants through the tissue culture process. Efficiencies, or at least the ease of transformation, would be tremendously increased if regeneration processes were improved. As things now stand, the methodologies for transformation that are described in this book are consistent and workable but improvements are always desirable.

## 1.2 Transient Expression

Transient expression is exactly what the phrase suggests: a short-term expression of the introduced DNA(s). Directly following the introduction of DNA into the nucleus, that DNA starts to function. Transient expression is usually studied using scorable marker genes, which report their expression via direct production of a detectable colored/fluorescent compound or an enzyme that can convert a non-pigmented substrate into a pigmented form. The most commonly used scorable markers are  $\beta$ -glucuronidase (GUS) from *Escherichia coli* which converts a colorless substrate into a blue form (Jefferson 1987) and the green fluorescent protein (GFP) (Chalfie et al. 1994) which fluoresces green upon excitation by high-intensity UV or blue light. Although expression from the *gus* gene is relatively simple and inexpensive to detect, the GUS assay itself is toxic and can therefore only be used for single time-point expression assays. In contrast, proper analysis of expression of the *gfp* gene requires costly instrumentation but gene expression can be continually observed in the target tissues over time.

Transient expression represents the first indication of successful gene introduction and function. In the development of new DNA introduction methodologies,

observation of a single blue GUS-expressing cell or a few GFP spots is usually all that is needed to suggest further investigations of an approach. Transient expression in a cell should be very clear following visualization of GUS or GFP. Expression is most often limited to the targeted cells and a demarcation of expressing and non-expressing areas should be apparent. Faint or diffuse expression of the marker genes (if regulated by the appropriate promoter) is usually an indication of improper assay conditions.

Most transformation procedures were developed based on optimization of DNA delivery using transient expression analyses. Transient expression for GUS is typically observed 24 h post-introduction (Klein et al. 1988) while GFP expression can be observed as early as 1.5 h after delivery (but peak expression usually occurs at 8–24 h; Ponappa et al. 1999). Although studies of transient expression itself are not common, these studies do provide information on the early fate of the introduced DNA.

### ***1.2.1 Optimization of Transient Expression***

Since transient expression is a direct measure of successful DNA introduction and function, development of methods to improve transient expression has often been used as a means of optimizing the transformation process itself (Klein et al. 1988). This approach has been quite useful and successful over the years. However, transient expression is only a measure of successful short-term transgene expression and it may not always perfectly reflect the ability of the cells to integrate the introduced DNA to generate stable events. As stated earlier, as the DNA molecule is so large, the process of DNA introduction itself requires that the integrity of the cell be compromised in some way. Target tissues and cells can therefore be sufficiently damaged by the DNA delivery process so that they express the transgene at high levels but not survive over the long term. This point of diminishing returns cannot be precisely defined for the different systems but it does exist. Optimization of transient expression is quite useful for the initial development of transformation methods but the efficiency of stable transformation and stability of transgene expression should be the ultimate goals of most transformation efforts.

### ***1.2.2 Transient Expression to Study Gene Expression and Stability***

In addition to using transient expression to optimize transformation and DNA introduction methods, this type of rapid transgene expression can also be used to facilitate speedy analysis of factors that influence the strength and stability of transgene expression (Sheen 2001; Dhillon et al. 2009). Once transient expression is optimized and standardized for a specific target tissue, the effects of factors that influence the level and profile of transgene expression can be reliably determined.

Quantification of transient expression, required for this type of analysis, involves either the extraction of the gene product from the targeted tissues (Klein et al. 1987) or the use of image analysis for continual monitoring of *gfp* gene expression over time (Finer et al. 2006). Tracking of GFP expression coupled with image analysis has tremendous advantages over tissue extraction as gene expression in the same piece of tissue can be followed over time.

Transient expression analysis has been utilized to study the relative strengths of different promoters and promoter fragments (Chiera et al. 2007) and to evaluate genes that modulate the introduced transgene via gene silencing (Chiera et al. 2008). Surprisingly, promoter analysis using transient expression does not appear to reflect promoter tissue-specificity (Finer, unpublished data), which suggests that large amounts of pre-integrative DNAs do not behave exactly like single- or low-copy integrated genes. However direct promoter strength comparisons do appear to be transferable from transient expression studies to expression in stably transformed tissues (Hernandez-Garcia et al. 2009). Promoter isolation and evaluation could increase tremendously with the increased availability of genome sequences from a number of different plants. Since the production of stably transformed plants can take from weeks to months, the use of transient expression may be desirable when rapid promoter analysis is needed.

Transient expression has been used to evaluate factors that influence the stability or consistency of gene expression (Dhillon et al. 2009). As gene expression variability among different events is a significant limitation in the production of transgenics, this approach may be quite useful as a preliminary evaluation tool for transgene stabilization work. The final determination of factors that modulate transgene expression must ultimately be made only following introduction to plant cells for stable transformation.

### 1.3 Agrobacterium Background

*Agrobacterium tumefaciens* is a soil-borne bacterium that causes crown gall disease in plants. Infected plants display a gall on the stem which is composed of proliferating plant cells that were transformed with bacterial DNA. The wild-type bacterial pathogen has the special ability to invade accessible areas of the target plant, adhere to certain types of plant cells and insert some of its own DNA (Bevan and Chilton 1982). This DNA is coated with different bacterial encoded proteins, which protect the DNA from degradation, direct transport to the nucleus and assist with the integration of bacterial DNA into the plant genomic DNA. The bacterial DNA that is transferred (T-DNA) is located in the bacterial cell on a native plasmid, called the tumor-inducing plasmid (Ti plasmid). In the wild-type bacterium, the T-DNA contains genes for synthesis of nitrogen-rich opines (which are metabolized by associated bacteria) and plant hormones, which cause rapid cell proliferation leading to the formation of galls.

This brief background on *Agrobacterium* is significant as the current era of plant biotechnology was born after Mary-Dell Chilton (Bevan and Chilton 1982) and Jeff Schell (Zambryski et al. 1983), along with scientists at Monsanto (Horsch et al. 1985), found that they could replace the native opine- and hormone-producing genes in the T-DNA with any gene(s) of interest and introduce those genes into plant cells. With the opine- and hormone-producing genes removed, the T-DNA becomes “disarmed”. A large number of additional discoveries enabled *Agrobacterium* to become the transformation vehicle of choice for many if not most plant transformation systems.

### 1.3.1 A String of Improvements for *Agrobacterium*

The use of *Agrobacterium*, in its original form, for the transformation of plant cells was both inefficient and unwieldy. First, the Ti plasmid was difficult to manipulate for introduction of genes of interest as it was so large. In addition, the bacterium was originally only able to infect and transform a limited number of plants and even specific cells within those plants. Due to perceived host-range limitations, grasses and monocots in general were thought to be unresponsive to *Agrobacterium*-mediated transformation. Last, wounding of the target tissue was deemed absolutely necessary as an entry point for the bacteria.

To make DNA introductions and manipulations simpler, binary vector systems were developed for use with *Agrobacterium* (DeFramond et al. 1983; Bevan 1984). The wild-type Ti plasmid contains both the T-DNA and a virulence (vir) region that encodes for genes involved in the T-DNA transfer machinery. Binary vectors allow for the separation of function on different plasmids; the Ti plasmid retains the vir region (T-DNA is removed) and the modified T-DNA is placed on the smaller binary vector, which can be more easily manipulated in the laboratory. The vir genes act *in trans*, leading to the processing of the T-DNA from the binary vector, for delivery to the targeted plant cells.

The host range limitations, originally associated with this biological pathogen and vector, have been largely overcome. As with most pathogens, different pathovars exist, which show different infectivity on different plants and cultivars of plants. Various *Agrobacterium* strains, which were selected for their high virulence, are now routinely used for plant transformation. The single advance, which had the greatest impact on increasing the host range for *Agrobacterium*, was the discovery that wounded plant tissues produced acetosyringone (Stachel et al. 1985), which subsequently induced some of the vir genes to initiate the T-DNA transfer process. Acetosyringone is now routinely included in the plant/bacterial co-culture medium at 100–200 µM. This chemical inducer of T-DNA transfer shows no deleterious effects on plant growth and development and it is always best to include this compound during co-culture, rather than risk the chance of obtaining inefficient transformation. As an alternative to including acetosyringone, *Agrobacterium* has been generated which constitutively expresses the vir genes (Hansen et al. 1994), which can give similar results.

### 1.3.2 *Agrobacterium– Plant Interactions*

The molecular mechanisms for the T-DNA transfer process have been described in detail in numerous excellent review articles (Zambryski 1992; Tzfira and Citovsky 2006) and are not presented again here. But, in order to better appreciate transformation methods that are *Agrobacterium*-based, it is best to have a basic understanding of the interaction of the bacterium with the target plant cells.

In order to transfer its T-DNA to the plant cell, the bacteria must obviously be in very close proximity to the target cell. It is well established that *Agrobacterium* binds to the plant cell and forms a pilus, which is the conduit for the transfer of DNA. It is also widely recognized that bacterial infection is mediated in most cases through wounding of the plant tissue. Wounding serves two different functions; it leads to the release of acetosyringone (in many plants) and allows the bacterium access to many different tissues. Simple preparation of the explant for culture is normally sufficient for wounding (Horsch et al. 1985) but additional wounding of some tissues with a scalpel blade is often helpful (Hinchee et al. 1988). More controlled wounding can lead to even higher transformation rates through the production of large numbers of small entry points for the bacteria (Bidney et al. 1992; Trick and Finer 1997).

Once the bacteria “enter” wounded plant tissue, it is not exactly clear where they go. In some cases, the bacteria enter the intercellular space that exists within most plant tissues and simply bind to the outside of the cell (Ye et al. 1999; Vaucheret 1994). In certain cases where the cell wall has been stripped from a plant cell, the bacteria bind to the outside of the regenerating cell wall (Deblaere et al. 1985). In the majority of cases, where the target tissue is wounded, it remains unclear whether the bacteria migrate to the intercellular spaces between cells, adhere to portions of torn/wounded cell walls, or actually colonize wounded plant cells to transform adjacent living cells (Trick and Finer 1997). Since the ultimate goal of transformation scientists and plant biotechnologists is to produce transgenic plants, the precise location of the bacteria during the transformation process is not really a nagging question. However, it is often helpful to visualize bacterial binding and the transformation process itself, when working to produce transgenic plants.

### 1.3.3 *Reducing Agents*

Although the *Agrobacterium* strains that are in common use for transformation have been engineered to achieve enhanced transformation rates, they are still perceived by many plant tissues as a pathogen. In response to pathogen invasion, plant tissues display high peroxidase activity to inhibit the growth of the pathogen and initiate localized plant cell death, so that the pathogen cannot spread through dying plant cells. During transformation with *Agrobacterium*, pathogen infection is actually desirable and inclusion of reducing agents can be used to alleviate the effects

of oxidizing agents and cell death (Olhoft et al. 2001; Finer and Larkin 2008). Reducing agents such as ascorbate, cysteine, silver nitrate and dithiothreitol have been successfully used to minimize the effects of oxidizing agents and to improve transformation efficiency.

### 1.3.4 Agroinfiltration

If there were a model plant family for *Agrobacterium*-mediated transformation and transgenic plant regeneration, it would be the Solanaceae. *Arabidopsis* is a special case and is presented in the next section. For the production of stably transformed plants, *Nicotiana tabacum* was often used in the early years of transformation as it is quite susceptible to *Agrobacterium* and it can be easily regenerated from almost all types of tissue (Bevan 1984; Deblaere et al. 1985). In some cases, the production of whole transgenic organisms may not be needed if a large number of cells within a plant can be uniformly and consistently transformed. During agroinfiltration (Vaucheret 1994), an *Agrobacterium* suspension is injected or infiltrated into leaves of *N. benthamiana*. The bacteria enter the intercellular air spaces within the leaf and transform a very large percentage of the internal mesophyll cells. The bacteria can be introduced into the internal leaf spaces by active pushing using an *Agrobacterium*-loaded syringe or by dipping the plant in an *Agrobacterium* suspension and then applying a vacuum. Agroinfiltration can give rise to very high levels of transgene expression in leaves of infiltrated plants when the T-DNA is modified to contain viral gene components to launch the viral amplification and transfer machinery (Lindbo 2007). This method can be used to rapidly generate a chimeric plant, where a large number of leaf cells contain the gene of interest. Unfortunately, this approach is not widely applied to different plants and is even limited among *Nicotiana* species. Inheritance of the transgene in agroinfiltrated plants does not occur.

### 1.3.5 *Arabidopsis* Floral Dip

Due to the small size of the genome and ease of transformation, *Arabidopsis* continues to serve as the model for plant genomics. The *Arabidopsis* floral dip method is a unique transformation method among plants. It was developed specifically for *Arabidopsis* (Clough and Bent 1998) and it has been shown to consistently work with very few other plants (Lu and Kang 2008). During floral dip, the *Arabidopsis* plant is first submerged in an *Agrobacterium* suspension, similar to one form of agroinfiltration (above). Inclusion of the wetting agent Silwet in the suspension and the application of vacuum, encourage the uptake of *Agrobacterium* by the plant. As the plant grows, the bacteria co-exist within the plant, eventually transforming the unfertilized egg within the ovule (Desfeux et al. 2000).

During the co-culture period, the *Agrobacterium* appear to proliferate at low levels within the plant. Plant infection does not lead to plant death nor does the plant invoke the hypersensitive response to limit the spread of the bacteria. The infecting *Agrobacterium* could transform leaf, petiole and other somatic cells of the plant but these transformation events are not passed onto the subsequent generation and are of limited value. The real benefit of the *Arabidopsis* floral dip is the rapid production of transgenic seed without the need to use tissue culture and in vitro regeneration from a single cell. The method is ideal, because the single cell that is targeted for transformation (the egg) is already destined to become a whole plant.

Since *Arabidopsis* can rapidly produce a large number of seeds and the plants are so small, space requirements are minimal and any inefficiencies in transformation is compensated by the ability to screen large numbers of seeds/seedlings. Seeds are simply plated on selective media, or seedlings/plants can be screened for certain characteristics or phenotypes to recover whole transgenic plants. Each transgenic seed usually represents an independent transformation event.

The inability to apply the *Arabidopsis* floral dip method to most other plants is not from lack of effort. In fact, successes using the same general approach with other plants have been reported but almost all of these have not been confirmed or repeated. It remains unclear why this method has not been widely applied to all plants. The transformation community remains cautiously optimistic that this approach will eventually be utilized for the transformation of all plants.

## 1.4 Particle Bombardment

Although *Agrobacterium* has become the method of choice for the transformation of plants, most of the first commercialized transgenic plants were generated using particle bombardment (Koziel et al. 1993; Padgett et al. 1995). Particle bombardment is a physical method for DNA delivery and the complexities of biological incompatibilities that are frequently encountered with *Agrobacterium* are completely avoided. This is also a direct DNA introduction method and it is therefore not necessary to use *Agrobacterium*-based binary vectors. DNA can be introduced as intact plasmids, isolated fragments, or PCR-generated amplicons. However, binary vectors containing genes of interest can also be used. With direct DNA introduction, DNA in any form can be utilized.

During particle bombardment, DNA is initially precipitated on small dense particles, usually 0.6–1.0  $\mu\text{m}$  tungsten or gold. The particles are accelerated at high speed towards the target plant tissue and penetrate through the cell wall to eventually lodge adjacent to, or directly in the nucleus (Yamashita et al. 1991; Hunold et al. 1994). The DNA, which was initially precipitated onto the particles, is released into the cell, finds its way to the nuclear DNA and becomes integrated into the genome.

### 1.4.1 Gene Guns

Particle bombardment does require appropriate instrumentation to propel the particles towards the target tissue. This instrumentation should provide a means to direct the DNA-coated particles, hold the target tissues in place for particle delivery and offer a mechanism for directing and controlling the force needed to accelerate the particles. With the original gene gun, that accelerative force was generated from a 0.22 caliber powder load (Klein et al. 1987) and the devices that are used today for particle bombardment are fittingly called “gene guns”. Numerous gene gun designs have been published but the two main versions in use today are the commercially available BioRad PDS1000He and the particle inflow gun (PIG; Finer et al. 1992).

The BioRad device utilizes very high-pressure helium to accelerate a lightweight mylar disc, which is layered on one side with DNA-coated gold particles. The mylar disc (macrocarrier) is accelerated into a stopping screen, which retains the mylar disc but allows the particles to pass. The PIG utilizes low-pressure helium to accelerate DNA-coated tungsten particles directly in a stream of helium. Helium is used in both cases because it is inert and its expansion coefficient is high, which means that the compressed helium gas is accelerated rapidly into a vacuum. A vacuum is not absolutely required but use of a vacuum chamber for particle bombardment is beneficial, as air drag on the accelerating particles is reduced.

### 1.4.2 Optimization of DNA Delivery

Particle bombardment, as with many of the DNA introduction methods, is rough on the target cells, as the integrity of the cell must be compromised to introduce the large DNA molecule. To get a more accurate picture of the scale of participants, the particles that enter the cell are in the range 0.6–1.0  $\mu\text{m}$  while the target plant cells are usually 20–30  $\mu\text{m}$ . If multiple particles or clumps of particles enter the same cell, damage to the target tissue increases. Ultimately, transient expression studies can be used to gauge the success of DNA introduction; living cells display transient expression while dead or severely damaged cells do not. It is unclear how many cells are moderately damaged and express transiently prior to cell death. For the optimization of DNA delivery through transient expression analysis, parameters that are evaluated usually include the following: DNA concentration, helium pressure, distance from point of particle acceleration to target tissue, DNA precipitation conditions and particle size.

The damage to the target tissue can be partly overcome through either chemical or physical drying, resulting in plasmolysis of the cells (Vain et al. 1993). Plant cells, which are normally hypertonic, push their cytoplasm through any large gaps in the cell wall. But plasmolyzed cells retain their cytoplasm following bombardment, resulting in higher transient expression and stable transformation.

### 1.4.3 *Control of DNA Integration Patterns*

One of the most interesting outcomes from particle bombardment-mediated transformation is the DNA integration pattern that can result from the introduction of plasmid DNA. Although the introduction of cassettes or amplicons is preferred over intact plasmids, the DNA integration patterns resulting from the introduction of whole plasmids has provided valuable information on the mechanism of integration following direct DNA uptake.

If intact plasmids are used for particle bombardment or any of the other methods of direct DNA delivery (see later in this chapter), the DNA integration pattern can be quite complex. Integration patterns show that plasmids can mix via both homologous and illegitimate recombination, resulting in the integration of high copy numbers of full-length plasmids, as well as pieces and parts (Finer and McMullen 1991). In addition, although the introduced DNAs segregate as a unit and are physically linked, introduced DNAs are often interspersed with plant DNA (Pawlowski and Somers 1998). One can envision the integration of introduced DNA into the plant genome using the native DNA replication and repair machinery working with the DNAs that are locally available (mixing of introduced DNAs and native genomic DNA). If desired, large amounts of DNA can be introduced (Hadi et al. 1996) and the co-introduction of two or more different pieces of DNA can be extremely efficient, leading to co-integration.

Introduction of either large amounts of different DNAs or high copy numbers of the same gene are generally undesirable as it leads to gene silencing. The use of low concentrations of isolated cassettes, generated via PCR, yields more predictable gene integration and transgene expression patterns (Agrawal et al. 2005). Apparently, the use of fragments for DNA introduction minimizes homologous recombination and concatemer formation, and the concentration of DNA used for bombardment can be reduced significantly, without reducing the recovery of transgenic events.

## 1.5 Other Direct DNA Uptake Approaches

The most commonly used method for direct DNA uptake (or naked DNA introduction) is particle bombardment. However, other methods have also been developed which are based on the same principle of passing DNA through large pores or holes in the cell wall or membrane. Some of these methods are very efficient in the introduction of DNA but inefficient for the recovery of transgenic plants. Other methods may not be very amenable for DNA introduction but generation of plants from the target tissue is more straightforward. These methods were developed either for purely scientific reasons, for unique applications, or to avoid the intellectual property restrictions of current DNA introduction methodologies.

### 1.5.1 *Protoplasts*

Protoplasts are plant cells with their cell wall removed. Since the cell wall presents the most formidable barrier to the introduction of large molecules, removal of the cell wall increases the possibilities for DNA insertion. For cell wall removal, tissues are incubated with commercial mixes of cellulases and pectinases. During protoplast liberation, protoplasts are suspended in a salt solution containing sufficient amounts of osmotic stabilizers to prevent bursting (Cocking 1972). Protoplasts can be prepared using any starting material but the selection of tissues depends on the desired outcome of the experiments. Protoplasts have been very successfully used in transient expression studies for fast analysis components that influence gene expression (Sheen 2001). For transient expression studies, leaf tissues as well as rapidly proliferating non-regenerable suspension cultures are suitable for the isolation of protoplasts. If transgenic plant recovery is desired, embryogenic suspension cultures are the preferred starting material.

Although protoplasts are devoid of their cell wall, the introduction of DNA molecules into these cells still requires that the DNA crosses the membrane. The two main methods for passing DNA through the membrane of plant protoplasts are electroporation (Fromm et al. 1985) and polyethylene glycol (PEG) treatment (Lazzeri et al. 1991). Both methods lead to temporary membrane destabilization, resulting in pore formation, which allows the DNA to pass. For electroporation, an electric charge is applied to the protoplasts, while the PEG treatment involves gradual application and subsequent dilution of a concentrated PEG solution to a protoplast/DNA suspension.

DNA introduction into plant protoplasts is relatively straightforward and efficient. Because the procedures for direct DNA uptake into protoplasts can be harsh, protoplast survival is a concern but roughly half of the surviving cells take up the foreign DNA. In spite of the difficulties associated with plant recovery from protoplasts, protoplast transformation remains a useful tool for transient expression studies. Since plant recovery from protoplasts is so technically demanding, this procedure is not often used for the recovery of transgenic plants.

### 1.5.2 *Whole Tissue Electroporation*

To avoid the technical difficulties encountered with the manipulation of protoplasts, the introduction of DNA through electroporation of whole tissues has been explored. Attempts to electroporate DNA into completely untreated target tissues have not been reliable. Although seemingly positive results have occasionally been obtained, these have not been consistent. Whole-tissue electroporation is achievable following partial digestion or removal of cell wall material (D'Halluin et al. 1992) using a nominal enzyme treatment. With a reduced or eliminated cell wall, the membrane is exposed and osmotic stabilizers are needed to prevent cell rupture.

Electroporation of treated tissues in the presence of naked DNA causes pore formation and results in the uptake of DNA by the plant cells. Although this approach would seem to offer many advantages over protoplast transformation in the ease of plant recovery from more “intact” tissue, very few valid reports of whole tissue electroporation exist in the literature (D’Halluin et al. 1992).

### ***1.5.3 Silicon Carbide Whiskers***

Silicon carbide whiskers are long thin rigid microscopic rods (1  $\mu\text{m}$  wide, 20–30  $\mu\text{m}$  long) that are used in the ceramics industry. They can be used as a vehicle for plant cell transformation when they are added to a mixture of plant cells and DNA and subsequently shaken at high speed (Kaepller et al. 1990). Although silicon carbide whiskers were originally used with a laboratory vortexer, the back-and-forth motion obtained with a paint can mixer may work as well or better. The basic concept behind this method is to penetrate the plant cell wall with the whiskers, which carry DNA along into the cell. It appears that this penetration occurs as a result of a rod being lodged between cell clusters when they collide during the mixing. An alternate suggestion, that the silicon carbide whiskers act like flying spears to penetrate the cell wall, seems less likely as the mass of the rods is so low. This method has been successfully and consistently used but the mixing treatment is fairly harsh and the target tissues are limited to cell cultures.

### ***1.5.4 Nanofiber Arrays***

The use of nanofiber arrays for DNA introduction into plants is a relatively new approach for DNA introduction into plant cells and few reports of this method exist (Finer and Dhillon 2008). Nanofiber arrays are precisely arrayed thin fibers, which are grown directly on a silica chip (Melechko et al. 2005). When viewed using electron microscopy, these chips resemble a “microscopic bed of nails”. DNA is either precipitated onto, or chemically bound to the arrays and the chip is pressed to the target tissue. Alternately, cells or clusters of cells can be forced onto the DNA-coated nanofiber array by centrifugation (the arrays are immobilized on the bottom of the centrifuge tube; McKnight et al. 2003). This approach has been more successfully employed for the introduction of DNA into animal cells as the plant cell wall barrier presents an additional hurdle for this approach. Nanofiber arrays represent a very young and inefficient technology for DNA introduction into plant cells but the approach seems logical and preliminary results look reasonable (Finer and Dhillon 2008).

### 1.5.5 Pollen Tube Pathway

In all fields of the sciences, premature claims are made which are often inadequately substantiated. The plant transformation sciences is certainly not exempt from this type of activity as new or more efficient methods for transformation are valuable and any success can accelerate career development. This “rush to publish” mentality has yielded numerous reports of new and exciting transformation methods which have not stood the test of time. The mixing of pollen with DNA and injecting DNA into the meristem and ovules have yielded some very provocative results which have not been repeated.

One method of transformation which enjoyed some major attention during the early days of plant transformation, and has seen resurgence, is transformation via the pollen tube pathway (Luo and Wu 1988). Soon after this early report with rice, the method was informally confirmed by others working with different crops. These follow-up early reports were never published. Over the years, transformation via the pollen tube pathway has been both ridiculed and praised but it has neither seen wide adoption nor been swept under the scientific carpet. This method is currently being actively used by one laboratory in China, which is quite aggressive with publication efforts (Yang 2009a, b).

For transformation via the pollen tube pathway, pollen is placed on the stigma and allowed to germinate and grow down the style to the ovary. The growing pollen tube contains the pollen nuclei and once the pollen tube grows down to the egg, one pollen nucleus fuses with the egg to form the zygote. When the pollen tube reaches the egg, the style is severed using a scalpel, supposedly leaving an open pollen tube. The success of this procedure is grounded in the concept of using a hollow pollen tube as a transport vehicle for direct DNA introduction into the freshly-fertilized egg. It is not clear whether the pollen tube is actually hollow. It is also unclear whether any DNA is able to enter the ovule. The timing of the cutting of the pollen tube and subsequent DNA introduction must be very precise, to have the DNA enter the cell with the pollen nucleus. The reported efficiency of the process is inexplicably high, considering that <1% of cells that contain DNA introduced via particle bombardment are able to integrate that DNA into their genome. Extensive analysis of soybean plants obtained through the pollen tube pathway suggests that this method is not reproducible (Shou et al. 2002).

In spite of these problems, the pollen tube pathway continues to receive positive validation in peer-reviewed literature, often in respected journals. Two recent reports (Yang 2009a, b) deserve special attention here, as the results should raise major concerns of scientific rigor. One of these papers reports a comparison of transformation via the “ovary drip” method (variant of the pollen tube pathway) and the pollen tube pathway (Yang et al. 2009a) while the other paper reports the results of a pollen tube pathway study (Yang et al. 2009b). In spite of reportedly using different methods and different maize lines in the two papers, the authors show the exact same image of a GFP-expressing root in both papers, as one piece of evidence for transformation. Further scrutiny of these papers reveals additional problems but

this one duplicated image is indicative of a basic problem with scientific accuracy. It appears that the pollen tube pathway method for DNA introduction has not yet been convincingly validated.

## 1.6 Evidence for Transformation

The premise behind DNA introduction into plant cells is the recovery of a phenotype from the activity of foreign gene(s). This phenotype is usually the ultimate goal of transformation scientists but some phenotypes can be difficult to discern; and additional means of confirming gene presence and function are necessary to determine whether a gene has been successfully introduced and is active. Proof of DNA presence and function should be relatively straightforward but the evidence can be misinterpreted. Tissues should be analyzed at a number of different levels: from the presence of the DNA, to the activity of the gene, to an altered phenotype in the transgenic plant or tissue.

### 1.6.1 *DNA Presence*

The simplest method of confirming the presence of foreign DNA is through the polymerase chain reaction (PCR). PCR is a very powerful and useful tool for amplifying fragments of DNA, using DNA primers designed to precisely bind to sites within a strand of DNA. The DNA used as a starting point for PCR can be used at low concentrations; and DNA quality issues are not paramount. PCR products are run on a gel or directly sequenced to show the appropriate size or composition of the products. PCR is an extremely reliable *preliminary* evaluation tool for determining the presence of DNA. However, because PCR allows the detection of extremely low concentrations of DNA, contamination is problematic and must be avoided or minimized. PCR alone is insufficient to prove transformation but it can be used as an efficient screening tool to select lines of interest for additional studies.

The best method for confirming the presence of introduced DNA is Southern hybridization analysis. For Southern analysis, DNAs are extracted from plant materials, digested, electrophoresed in a gel, blotted onto a membrane and finally hybridized to a labeled fragment of DNA which is complementary to the introduced DNA. The use of appropriate restriction enzymes generates precise hybridization results or patterns, which is strong evidence for successful DNA introduction. While PCR results suggest the presence of the introduced DNA, Southern analysis can also show DNA integration, which is a necessary indicator for successful transformation. In addition, Southern hybridization provides information on the number of copies of introduced DNA per nuclear genome, as well as the number of sites in the genome that the DNA integrates. To obtain meaningful results, Southern

hybridization analysis should be performed after careful assessment of appropriate restriction enzymes, which usually recognize one site within the introduced DNA. Use of the proper restriction enzyme yields sizes and patterns of hybridization signals that are unique for each transformation event. Digestion with restriction enzymes which are expected to generate uniformly sized fragments are of very limited value. The intensity of hybridization signal(s), as well as their size and shape, provides additional information on the validity of results. Although Southern analysis of PCR products is occasionally presented in the literature, results from this approach are prone to misinterpretation and should not be considered evidence for transformation.

### ***1.6.2 Gene Expression***

After the presence of the introduced DNA is verified, it is necessary to evaluate the expression of the introduced gene. This can be performed by testing for the presence of RNA, protein or an altered phenotype. Although it is not absolutely necessary to test for gene expression at all levels, an appropriate combination of analyses is usually needed. For the analysis of RNA, Northern hybridization or RT-PCR is used. For Northern analyses, the basic principles are the same as with Southern analysis but RNA is extracted instead of DNA. The hybridization signal should be a predicted size, which corresponds to the RNA transcript. For RT-PCR, DNA is generated from isolated RNA and the resulting amplicons are evaluated following electrophoresis. Again, the size and intensity of the resulting bands are important.

When the introduced DNA is a scorable marker gene, validation of gene expression is sometimes extremely simple and effective, especially if GFP is the marker. With the appropriate detection instrumentation, GFP can be directly observed in transgenic plant material. The fluorescent green color is usually unmistakable but inappropriate in-line filters and illumination can make interpretation difficult. Expression of GFP, as well as other transgenes, is dependant on the promoter used to regulate the gene. Transgene expression always shows some type of patterning, based on tissue type and inducibility. Even the use of “constitutive” promoters like the CaMV35S promoter show some patterns of gene expression. For example, expression occurs along developing cell lines, showing more intense activity in regions parallel to the longitudinal axis of the root, or close to the veinal tissue of the leaves. If the marker gene appears to weakly express throughout the tissue with no pattern, this suggests background expression or problems with the detection system.

If the gene of interest (GOI) is expected to give rise to a new phenotype, this analysis is ultimately needed. Some phenotypes can be difficult to gauge and any changes should also be clearly associated with the presence and expression of the transgene in primary transgenics and segregating progeny.

In the primary transgenics, all plants should contain the introduced DNA in a heterozygous state. Multiple copies of the transgene are commonly obtained but these copies often integrate into the same integration site, behaving like a single gene in the heterozygous condition. In the T1 progeny from single insertion events, the transgene segregates in a 1:2:1 ratio, with 25% of the progeny not containing the transgene, 50% heterozygous and 25% homozygous transgenic. When analyzing simply for the presence of the transgene, the transgene should be present in 75% of the T1 generation progeny (1:3). If ratios other than this are obtained, this suggests integration of the transgene into multiple sites (more than 75% of progeny contain the transgene) or lethality of the transgene in germ line cells or in the homozygous condition (less than 75% of progeny contain the transgene). If a very low percentage of the progeny contains the transgene, this suggests additional problems, which are a cause for concern.

## 1.7 Conclusions

The production of transgenic plants via transformation has tremendously impacted agriculture and the face of our planet. Through improvements in technology for DNA introduction, production of transgenics is no longer as limiting as it once was. Determination of potential GOIs as well as evaluation of transgenics is becoming a new bottleneck in plant biotechnology. However, improvements in DNA introduction methodology as well as developing predictable transgene expression models are still needed. Transformation efforts should not be curtailed until all plants of interest can be transformed with the same ease as *Arabidopsis*.

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# **Chapter 2**

## **Plastid Transformation**

**Heribert Warzecha and Anna Hennig**

### **2.1 Introduction**

The genome of eukaryotic cells is unevenly distributed and kept in different subcellular compartments. While the vast majority of genetic information is sheltered in the nucleus, small portions of DNA reside in organelles, namely the mitochondria and – in the case of plants – in the plastids. These unique organelles which in their most prominent manifestation are called chloroplasts, developed from cyanobacterial ancestors in a process described by the well accepted endosymbiosis theory (Gould et al. 2008). In brief, a pre-eukaryotic cell must have engulfed and taken up an ancestor of today's cyanobacteria and subsequently formed a close endosymbiotic relationship with the newly developing organelle. Residues of this evolutionary ancestry are still apparent today in certain prokaryotic characteristics retained by the plastids. There is for example the genome organization in operons, as well as the transcription and translation machinery with their 70S ribosomes, to name only a few features which resemble those of today's bacteria. However, during the adaption process which lasted several billion years the plastids lost their autonomy in that they transferred the majority of their genetic information and the capacity for its regulation. Genes were either lost or transferred to the nucleus, accompanied with the assembly of a regulatory network which operates most of the metabolic processes in plastids. What is present in contemporary plastids is a highly reduced genome retaining some integral features like DNA replication and protein biosynthesis. Furthermore, plastids and especially chloroplasts have a unique role in that they provide the primary energy source for the plants via photosynthesis and synthesize important compounds like aromatic amino acids. It has only recently become evident that plastids also have crucial roles in

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plant development and therefore additionally regulate processes in the cellular metabolism. Several reports provide evidence that plastid genes encode functions which reach beyond its borders, for example, chloroplast protein synthesis is mandatory for regular leaf development and its knock-down will result in aberrant phenotype (Ahlert et al. 2003).

Consequently, the genetic manipulation of chloroplasts became a major focus since it provides the option to study the function of this unique organelle in great detail. Also, the application of chloroplast transformation in biotechnology has advanced due to characteristics which make plastids a promising vehicle for the high-level production of recombinant proteins. The most prominent difference to nuclear genes is the mere number of transgenes which could be introduced into a single cell by transformation of the chloroplast genome. Usually, in green tissue every cell contains up to 100 chloroplasts. Every chloroplast itself contains up to 100 identical copies of the circular plastid DNA, organized in nucleoid structures of about ten aggregated copies (Thomas and Rose 1983). In total this makes up to a 10 000 copies of any gene, outnumbering every nuclear gene by far. This is one reason why chloroplast transformation often results in extraordinarily high levels of recombinant protein accumulation.

However, chloroplast transformation makes high demands on vector design, transformation method as well as plant regeneration. This is exemplified by the so far not solved problem to generate fertile transplastomic lines of the model plant *Arabidopsis thaliana* (Sikdar et al. 1998), an example depicting the obstacles of this technique which need to be overcome to gain broad applicability. On a routine basis, so far only tobacco chloroplasts are transformed, and therefore most examples given in this chapter refer to tobacco chloroplast transformation. Nevertheless, great progress has been made in expanding the range of this technique to other plant species. Today, there are reports of successful plastid transformation in about 16 species (Table 2.1) and studies with transplastomics give valuable insight into genetics and biochemistry of this unique organelle.

## 2.2 Delivery of Transforming DNA to the Chloroplast

Delivery of foreign DNA to the chloroplast requires its transport through several physical barriers: the cell wall, the cytoplasma membrane, and the chloroplast double-membrane system. Since no bacterial or viral pathogen is known which could be utilized for DNA delivery, transgene transmission needs to employ rather rigid physical methods. The most effective and widely used system utilizes micro-projectile bombardment with plasmid-coated gold or tungsten particles, the so-called biolistic method, which was first used to transiently transform onion epidermal cells (Klein et al. 1987). Subsequent refinement of the technique eventually enabled the transformation of smaller cell types as well as subcellular targets, like plastids in the unicellular algae *Chlamydomonas* (Boynton et al. 1988) or in tobacco (Svab et al. 1990). Other sophisticated methods have also been developed,

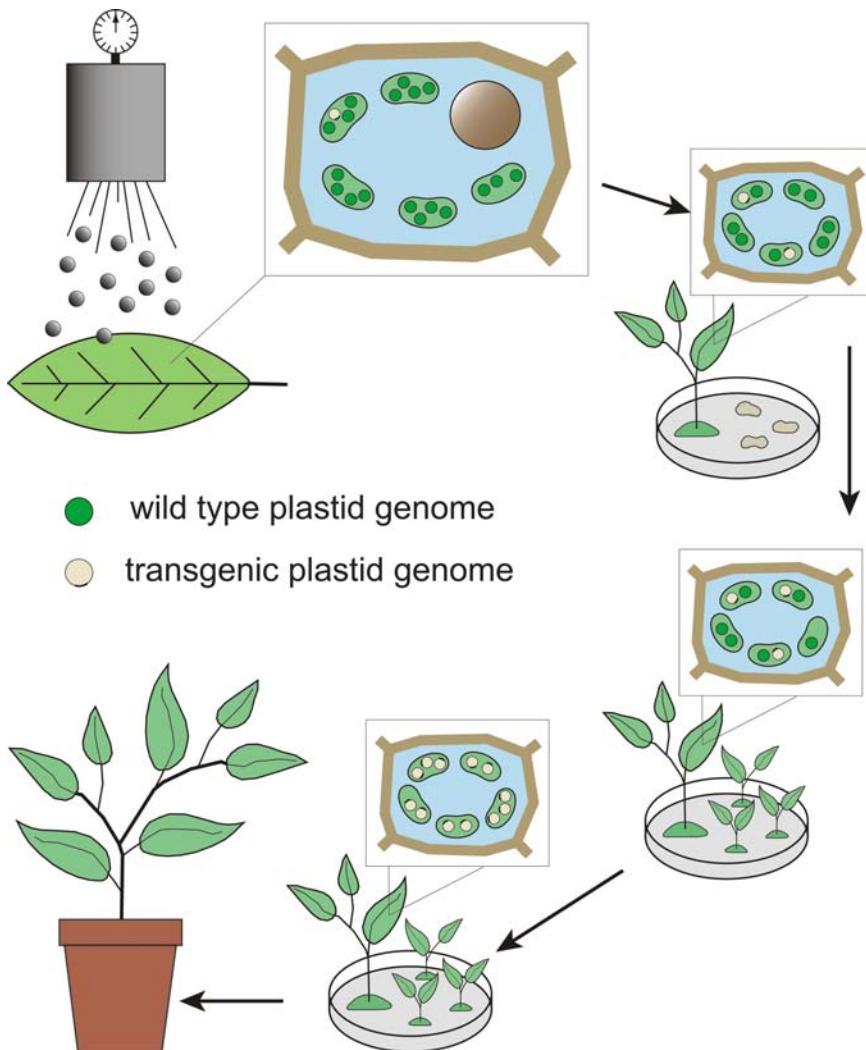
**Table 2.1** Plant species for which plastid transformation has been achieved. Common and scientific names are given, with the transgenes integrated so far, as well as the efficiency of the transformation process.

Species	Transgenes integrated	Explants per bombardment (efficiency)	Reference
Tobacco ( <i>Nicotiana tabacum</i> Petit Havana SM1)	<i>aadA</i> + <i>uidA</i> <sup>a</sup>	1/1 (100%)	Zoubenko et al. (1994) <sup>a</sup>
Potato ( <i>Solanum tuberosum</i> FL1607; <i>S. tuberosum</i> cv Desiree)	<i>aadA</i> + <i>gfp</i> <i>aadA</i> + <i>gfp</i>	3/104 (2.8%) 14/435 (3.2%)	Sidorov et al. (1999) Nguyen et al. (2005)
Tomato ( <i>S. lycopersicum</i> var. IAC-Santa Clara)	<i>aadA</i>	1–3/20 (5–15%)	Ruf et al. (2001)
Petunia ( <i>Petunia hybrida</i> var. Pink Wave)	<i>aadA</i> + <i>gusA</i>	3/31 (9.6%)	Zubko et al. (2004)
Soybean ( <i>Glycine max</i> L. cv “Jack”)	<i>aadA</i>	11/80 (13.7%)	Dufourmantel et al. (2004)
Lettuce ( <i>Lactuca sativa</i> L. cv Cisco)	<i>aadA/gfp</i>	5/85 (5.8%)	Kanamoto et al. (2006)
<i>Lesquerella fendleri</i>	<i>aadA</i> + <i>gfp</i>	2/51 (3.9%)	Skarjinskaia et al. (2003)
Carrot ( <i>Daucus carota</i> L. cv Half Long)	<i>aadA</i> + <i>badh</i>	1/7 (14%)	Kumar et al. (2004a)
Cotton ( <i>Gossypium hirsutum</i> )	<i>aphA-6</i> + <i>nptII</i>	1/2.4 (41.6%)	Kumar et al. (2004b)
Poplar ( <i>Populus alba</i> )	<i>aadA</i> + <i>gfp</i>	44/120 (36.6%)	Okumura et al. (2006)
Sugar beet ( <i>Beta vulgaris</i> ssp. <i>vulgaris</i> )	<i>aadA</i> + <i>gfp</i>	3/40 (7.5%)	De Marchis et al. (2008)
Rice ( <i>Oryza sativa japonica</i> )	<i>aadA</i> + <i>gfp</i>	2/100 (2%)	Lee et al. (2006)
Cabbage ( <i>Brassica oleracea</i> L. var. <i>capita</i> L.)	<i>aadA</i> + <i>uidA</i>	3–5/150 (2–3%)	Liu et al. (2007)
Canola ( <i>B. napus</i> )	<i>aadA</i> + <i>cry1Aa10</i>	4/1000 (0.4%)	Hou et al. (2003)
Cauliflower ( <i>B. oleracea</i> var. <i>botrytis</i> )	<i>aadA</i>	1/5 <sup>b</sup>	Nugent et al. (2006)
<i>Arabidopsis thaliana</i> ecotype RLD	<i>aadA</i>	2/201 (0.9%)	Sikdar et al. (1998)

<sup>a</sup>For tobacco, numerous more transformations have been reported

<sup>b</sup>Transformation was achieved by PEG-mediated transformation

like polyethylene glycol (PEG)-mediated transformation of protoplasts (Golds et al. 1993) or even the direct injection of DNA into the organelle via a femtoliter syringe (Knoblauch et al. 1999). Although plastid transformation with PEG requires some experience in the enzymatic digestion of the cell wall and the treatment of protoplasts as well as the regeneration of plants, it can be basically performed with standard laboratory equipment. Micromanipulation of cells on the other hand requires specialized equipment, which is limiting for its use; and so far no reports have shown the successful regeneration of transplastomic plants from this particular gene transfer method. However, the most widely and successfully used method is the biolistic transfer of DNA, depicted by the successful transformation of the plastids of numerous plant species (Table 2.1).



**Fig. 2.1** Schematic drawing of the plastid transformation and regeneration process. An explant, usually a leaf, is bombarded with DNA-coated tungsten or gold particles. When the DNA is delivered to one chloroplast, integration of the transgene takes place, generating a heteroplasmonic cell in which a small number of plastid genomes are transgenic (open circles). Subsequent differentiation and shoot regeneration from this cell results in a heteroplasmonic plantlet. To obtain a homoplasmonic transgenic plant requires several cycles of regeneration under selection

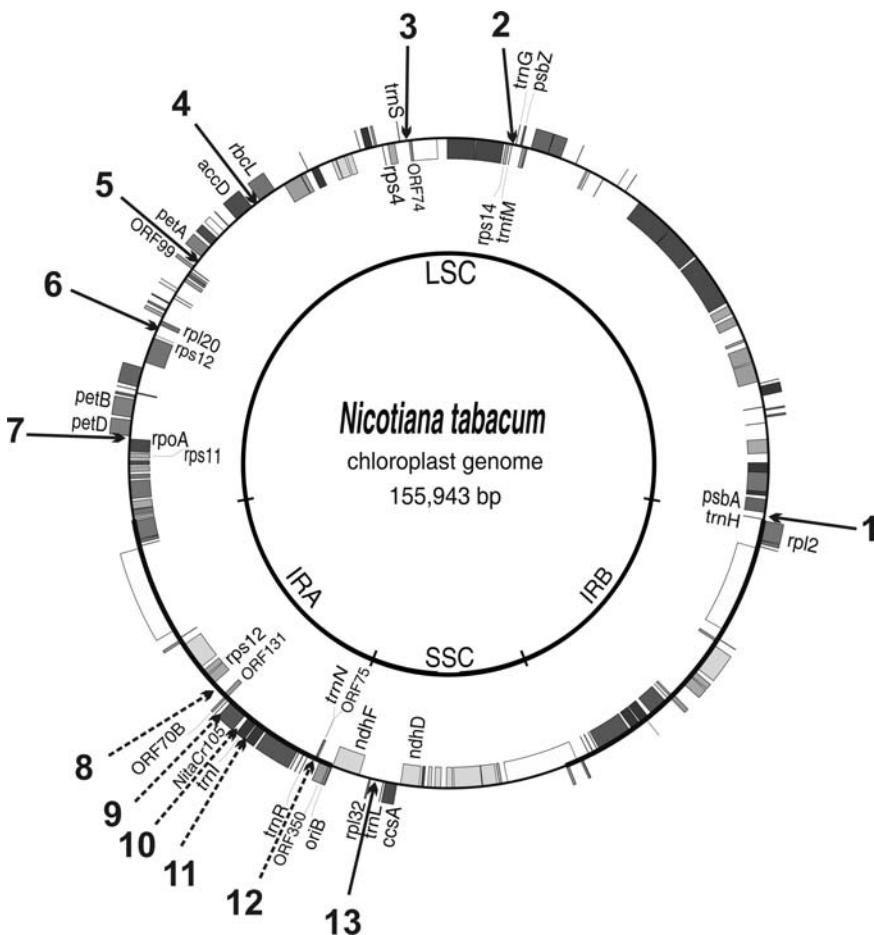
Once the transgene DNA has been delivered to the chloroplast (Fig. 2.1), stable integration via homologous recombination has to take place (see below) to generate a stable transgenic trait which will be passed on after plastid division to its descendants. Every chloroplast harbors up to a hundred copies of its genome, grouped in nucleoids representing aggregates of 7–10 copies. Since cells can contain up to

100 chloroplasts, a single integration event creates a transplastomic cell in which only a minority of genomes is altered, the so-called heteroplasmic state. For the generation of a stable transplastomic plant, wild-type plastid genomes have to be winnowed. As the sorting of plastid DNA during cell division in shoot-regeneration is a stochastic process a small percentage of altered homoplasmic plants can be generated in the absence of selection pressure (5.6%; Lutz and Maliga 2008). To increase the efficiency of transformation routinely, a homoplasmic state of the engineered plants is reached by successive regeneration under strong selective pressure with an appropriate antibiotic. It is estimated that it takes between 20 and 30 cell divisions to deplete the undesired wild-type chloroplast genomes (Maliga 2004; Verma and Daniell 2007). Since this number could not be reached in a single plant regeneration process, explants have to go through several cycles of regeneration under selection (Fig. 2.1). So far a given plants ability to regenerate from fully differentiated tissue is the biggest obstacle for applying the plastid transformation to a large number of plant species. Tobacco is by far the best analyzed system regarding plastid transformation, and therefore most experiments referred to in this section are made in tobacco.

## 2.3 Vector Design

### 2.3.1 Flanking Regions

*Agrobacterium*-mediated transformation utilizes universal vector systems (Lee and Gelvin 2008; see also Chapter 1) in which the transgene expression cassette is flanked by two rather short sequence stretches, termed left border (LB) and right border (RB). These sequences facilitate almost random insertion of the transgene cassette into the host genome, resulting in multiple individual lines differing in site and numbers of transgene integration. In absolutely contrast, insertion of foreign DNA into the chloroplast genome relies on targeted integration of transgenes by homologous recombination, facilitated by a bacterial recombination system inherited from the plastids cyanobacterial ancestors (Cerutti et al. 1992). Hence, a transgene could be targeted to virtually any site in the chloroplast genome by designing the flanking regions according to the desired location. This is not only a big advantage for the positioning of expression cassettes to defined locations but also enables the targeted inactivation of plastid genes for functional studies and gene knock-outs. For the former, preference is naturally given to intergenic regions to circumvent deleterious effects and interference with endogenous gene expression. For gene knock-out, the targeted sequence is mutated in vitro and reinserted into the plastome. In the case of tobacco, numerous studies describe the targeted knock-out of plastidal genes for functional studies (reviewed by Maliga 2004). Additionally, a total of 13 sites on the plastome has been utilized for the integration of an expression cassette (Fig. 2.2), demonstrating that modification and integration



**Fig. 2.2** Graphic map of the *Nicotiana tabacum* plastid genome (GeneBank accession number NC\_001879), made with the web-based program OGDRAW (Lohse et al. 2007). Genes on the outside of the circle are transcribed counter-clockwise, those on the inside clockwise. *IRA* Inverted repeat A, *IRB* inverted repeat B, *LSC* large single copy region, *SSC* single copy region. Numbered arrows Transgene integration sites. Dashed arrows (numbers 8–13) Site of integration into the inverted repeat region; therefore integration sites are in duplicate. First published reports are given: 1 Carrer and Maliga (1995), 2 Bock and Maliga (1995), 3 Huang et al. (2002), 4 Svab and Maliga (1993), 5 Huang et al. (2002), 6 Kuroda and Maliga (2003), 7 Suzuki and Maliga (2000) and Klaus et al. (2003), 8 Zoubenko et al. (1994), 9 Staub and Maliga (1993), 10 Svab et al. (1990), 11 Muhlbauer et al. (2002), 12 Huang et al. (2002) and Zou et al. (2003), 13 Koop et al. (1996) and Eibl et al. (1999)

of heterologous genes can be performed at many given sites in the circular plastid genome.

One of the unique features of the chloroplast genome is the presence of two large inverted repeat regions (IRA and IRB in Fig. 2.2). Integration of the transgene into this particular region leads under selection to a doubling of the gene by a process

called copy correction. Especially, sites in the *rrn* operon have been frequently chosen for transgene integration and gene expression from these sites has proved to be high in many cases (Verma et al. 2008).

To facilitate efficient recombination, flanking regions of about 1–2 kb endogenous DNA should frame the sequence to be inserted. The question whether a transformation vector for a certain plant species requires strain-specific flanking regions was clearly negated by Lutz et al. (2007). This is due to the sufficiently high sequence homology of plastid genomes between species to facilitate homologous recombination. In fact, vectors designed for transgene integration into the tobacco plastome could be also used for transformation of tomato (Ruf et al. 2001; Chapter 25), potato (Sidorov et al. 1999; Chapter 20), and petunia (Zubko et al. 2004; Chapter 19). Two recent papers describe convenient vector systems designed for chloroplast transformation (Lutz et al. 2007; Verma and Daniell 2007).

### 2.3.2 *Promoters and UTRs*

For the expression of heterologous genes, the choice of promoters and regulatory sequences is highly important. In general, chloroplasts mainly utilize a prokaryotic transcription and translation machinery, a heritage from their cyanobacterial ancestors. Gene organization in operons, an eubacterium-type RNA polymerase as well as sigma-like factors, and a specific codon-usage in the open reading frames are highly similar to those found in bacteria. However, during evolution novel mechanisms of gene organization and regulation of expression also evolved in plastids. Processes like intron splicing and RNA editing can be found in chloroplasts which are absent in bacteria. Aiming for the high-level expression of a given gene, regulatory sequences are required which provide efficient transcription, translation, and RNA stability. The strongest promoter described so far is the  $\sigma^{70}$ -type promoter of the ribosomal RNA operon (*Prrn*; Svab and Maliga 1993; Kuroda and Maliga 2001a, b). In the majority of vectors used to date, this particular promoter is used to drive the expression of the marker gene *aadA* (see below) to provide sufficient expression for selection of transformed cells. Another promoter in use is the endogenous *psbA* promoter (driving expression of the abundant D1-protein; Zoubenko et al. 1994).

In plastids there is also a high degree of translational control of gene expression which is in clear contrast to prokaryotes. Therefore, the 5'-UTRs and 5'-regulatory sequences have proven indispensable for RNA stability and efficient translation initiation. While in Eubacteria virtually all mRNAs contain a Shine–Delgarno sequence for accurate translation initiation, only 40% of chloroplast mRNAs contain such sequences, indicating that alternative pathways of regulation exist (Hager and Bock 2000). Numerous studies have investigated in detail the effect of swapping different regulatory sequences and determined the consequence on protein accumulation. For example, Eibl et al. (1999) could show that such variations in 5'-UTRs result in up to 100-fold differences in protein accumulation in the case

of the reporter gene *uidA* (encoding beta-glucuronidase). Not only the 5'-UTR but also the first codons of the open reading frame (the so-called “downstream box”) seems to contribute to translation efficiency (Kuroda and Maliga 2001a, b), demonstrating that a high variability of expression levels could be expected for any given sequence and expression cassette. The highest expression level reported so far was over 70% of the total soluble protein (TSP) of a phage derived lytic protein driven by the *rrn* promoter fused to the *Escherichia coli* phage T7 gene 10 (T7g10) 5'-untranslated region (Melanie Oey 2008). This extraordinarily high content of recombinant protein almost exhausted the protein biosynthesis capacity of the chloroplasts and resulted in plants with impaired growth. Although extraordinarily high amounts of protein are often aspired, several proteins have been reported to be toxic to the plastid at elevated levels (Hennig et al. 2007), and therefore certain threshold levels might exist which should be taken into account to preserve the plants’ viability.

## 2.4 Transgene Stacking and Control of Gene Expression

A demanding task in the generation of transgenic plants is the option of simultaneously introducing two or more genes into an organism. Conventional approaches require the combination of separate expression cassettes each containing a promoter and terminator region framing the gene of interest. In contrast, plastids are thought to offer an unique option of combining multiple ORFs under the control of one promoter, yielding a polycistronic transcript from which translation can be initiated independently (Staub and Maliga 1995). However, except for a few examples like the *cry2Aa2* operon from *Bacillus thuringiensis* (De Cosa et al. 2001; Quesada-Vargas et al. 2005), this technique has not been utilized for the simultaneous production of two or more recombinant proteins so far. This is probably due to the hitherto unpredictable secondary structure interactions in polycistronic transcripts, which determine the translatability and processing into monocistronic mRNAs and subsequently result in poor protein accumulation. Although the similarities between transcription and translation in bacteria and in plastids are striking, one cannot generally extrapolate results obtained in bacteria to plastids. For instance, an operon encoding for hemoglobin  $\alpha$ - and  $\beta$ -subunits, which worked well in *E. coli*, did not lead to detectable expression when integrated into plastids (Magee et al. 2004). A recent study identified a so-called intercistronic expression element (IEE), a short sequence that mediates the cleavage of a polycistronic precursor into stable monocistronic transcripts (Zhou et al. 2007). It will be interesting to see whether this novel element leads to concerted high-level expression of recombinant proteins.

Another challenge is the regulation of gene expression in plastids, as it is state of the art in *E. coli*. It would be highly desirable to avoid deleterious or toxic effects of recombinant proteins on plant metabolism by initiating expression at will. Most promoters used so far are more or less constitutive (like the *Prrn*) or regulated by

factors which could be hardly used for targeted expression initiation. In photosynthetically active chloroplasts especially the 5'-UTRs of several transcripts contribute to the regulation of translation. It has been shown that light regulates the translation of the *psbA* mRNA (Kim and Mullet 1994) while RNA levels are kept relatively constant (Shiina et al. 1998). However, since light cannot be withheld until the desired transgene expression needs to be initiated, it would be highly advantageous to have instead an inducible system at hand which relays on chemical or other physiological triggers. A sophisticated approach is to put the transgene under the control of the phage T7 promoter, which is per se not active in plastids. Expression can be only initiated by the appropriate T7 RNA polymerase, which needs to be introduced by genetic crossing with a plant line carrying the gene in the nucleus and fused to a plastid signal peptide (McBride et al. 1994). To add a regulatory element to this system, other studies used inducible nuclear promoters to control expression of the nuclear gene, e.g. the salicylic acid-inducible *PR-1a* promoter from tobacco (Magee et al. 2004) or an ethanol inducible promoter (Lossl et al. 2005). The disadvantage of this particular approach is that two subsequent transformations of different cellular compartments or genetic crossing of different transformants are necessary to obtain the final plant. Also the *E.coli lac* control system has been adopted for plastid expression. Therefore, the *lacI* repressor needed to be co-expressed together with the heterologous gene (*gfp*) under control of a modified *rrn/T7g10* promoter inside the plastids (Muhlbauer and Koop 2005). Spraying of plants with isopropyl thiogalactoside (IPTG) indeed induced GFP-formation, but it needs to be established whether this method is applicable on a large scale.

## 2.5 Selection

### 2.5.1 Antibiotic Resistance Markers

For the selection of plastid transformants, aminoglycoside antibiotics have proven highly useful, and especially spectinomycin has become indispensable as a selective marker. Its mode of action is plastid-specific as it binds to the prokaryotic-type plastid ribosomes and inhibits protein synthesis. Mutations in the 16S rRNA, one of the target sites of spectinomycin, confer resistance to the antibiotic and early transformation vectors contained such mutant genes (Svab et al. 1990). Since the mutant form of an endogenous gene is recessive until the homoplasmic stage is reached, transformation efficiency with such marker genes is low. The use of a gene encoding an antibiotic detoxifying protein, namely the aminoglycoside-3"-adenyltransferase (AAD, encoded by the *aadA* gene) as a marker greatly expedites the development of transplastomic plants (Svab and Maliga 1993).

Several more resistance markers have been tested for their applicability in plastid transformation, especially as it was observed that spontaneous spectinomycin resistant lines were formed under strong selection. For kanamycin, no spontaneous

resistance in higher plants was reported and thus this particular marker was also adapted for selection of plastid transformants. First successful attempts using the *neo* gene (encoding for neomycin phosphotransferase II, NPTII) generated transplastomic tobacco, but at a lower efficiency than with *aadA* (Carrer et al. 1993). Koop and co-workers used the aminoglycoside phosphotransferase gene (*aphA-6*) gene from *Acinetobacter baumannii*, which yielded a much higher transformation efficiency (Huang et al. 2002). Both selection markers are in use, even in combination (Kumar et al. 2004a, b) but have not displaced the *aadA* gene as the most frequently used antibiotic resistance marker.

### 2.5.2 Other Selection Markers

Another approach is to insert a gene which confers resistance to a toxic compound, for example herbicides. This approach is widely used for nuclear transformation (see Chapters 3, 9), and it is thought to be also functional in plastid transformation. Resistance against glyphosate could be obtained via 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; Ye et al. 2001), and integration of the *bar* gene made tobacco plants resistant to phosphinothricin (Lutz et al. 2001). However, all expression cassettes also contained the *aadA* gene for initial selection on spectinomycin and herbicide resistance was only observed when nearly homoplasmic plants were obtained.

Another novel selective agent was used for plastid transformation, namely betaine aldehyde, for which a detoxifying gene was linked to the heterologous gene. The introduction of the spinach gene encoding for betaine aldehyde dehydrogenase (BADH) into tobacco chloroplasts proved to be an useful marker (Daniell et al. 2001). Since this marker gene is of plant origin, its use is thought to obviate the concerns about the use of antibiotic resistance in plant genetic engineering. Although this approach is highly promising there is still a lack of studies confirming the broad applicability of the BADH gene as a marker in plastid transformation (Maliga 2004). For the sake of efficient and reliable generation of transplastomic plants it seems to be wise to use antibiotic resistance markers and – after establishment of the transgenic plant line – remove the gene by marker gene excision.

## 2.6 Marker Gene Excision

In principle, any marker gene becomes dispensable as soon as the homoplasmic stage is achieved. Sophisticated protocols to subsequently remove the marker flank the sequence to be removed by two directly oriented recombinase target sites. Examples include the 34-bp *loxP* sites, which are recognized by the Cre recombinase derived from the P1 bacteriophage (Corneille et al. 2001; Hajdukiewicz et al.

2001), and the *attP* (215 bp) and *attB* (54 bp) sites, recognized by the phiC31 phage integrase Int (Lutz et al. 2004). As long as no integrase is present, the genomes harboring those sites are stable. To initiate excision, a second, nuclear transformation step has to be performed. Using a construct in which the recombinase is genetically fused to a chloroplast transit peptide will target the protein to all chloroplasts and initiate site specific excision of the unwanted genes. Further backcrosses are required where also the nuclear transgene is removed to obtain a marker-free plant line. Several more methods have been described for marker gene removal, and an excellent overview is given by Lutz and Maliga (2007).

It needs to be emphasized that, in plastids, homologous recombination takes place between virtually all directly oriented sequences of sufficient length. This effect can also be used for marker gene excision (Iamtham and Day 2000), but more often it is the cause of unwanted rearrangement and transgene loss in transformation experiments (Svab and Maliga 1993). Initially this effect was observed when the integrated expression cassette contained two homologous sequences oriented in the same direction, e.g. duplicate promoter or terminator sequences, or when homologous sequences integrated were in the same direction as endogenous plastid genome sequences. When the choice of regulatory elements to be used is limited, it might be advisable to utilize interspecies regulatory elements to avoid unwanted recombination products (Nadai et al. 2008).

## 2.7 Analysis

Initial plastid transformation and subsequent regeneration of transplastomic plants need to be carefully monitored by different techniques. While presence of the transgene can be easily checked by PCR, no precise statement about the integration site can be made with gene-specific primers. To rule out an accidental insertion of the expression cassette into the nuclear genome, a PCR with a primer combination bridging the transgene/genome border is advisable. Differentiation between the homoplasmic and heteroplasmic states can be made by restriction fragment length polymorphism (RFLP) analysis. Restriction digestion of plastid genomes eventually generates fragments of variable length, regarding the integration of the transgene, and therefore resulting in a specific pattern on a subsequent DNA blot hybridized with a specific probe. The gradually disappearance of the wild-type signal and attainment of the homoplasmic condition can be monitored with this technique. However, the occurrence of promiscuous plastid DNA in the nucleus sometimes feigns a heteroplasmic state and generates a need for isolating chloroplast DNA prior to analysis (Ruf et al. 2000). An unambiguous test for plastid transformation is testing for maternal inheritance of the resistance trait. Transgenic plants pollinated with wild-type pollen generate uniformly green seedlings on a selective medium only if they are homoplasmic.

Testing the gene expression usually gives highly heterogeneous results, depending on the expression cassette and the gene itself. Accumulation rates inside the

chloroplast also greatly depend on the stability of the given protein to proteolytic degradation (Birch-Machin et al. 2004).

Once a transplastomic line has been established there is in principle no need for screening numerous other lines, as would be necessary for *Agrobacterium*-mediated nuclear transformation. This is due to the targeted integration of the transgene, resulting in uniformly modified plant lines.

## 2.8 Conclusions

During the past years tremendous progress has been made in developing and improving plastid transformation techniques and in expanding the methodology to various plant species. Although the method is time-consuming and tedious, it can be established without great effort. Especially for the production of recombinant proteins, plastid transformation has become a valuable tool, notably due to the enormous rate of protein accumulation reported. The next years will show whether this particular technique can be applied to more crop plant species as a standard method.

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# Chapter 3

## Concepts of Marker Genes for Plants

Josef Kraus

### 3.1 Introduction

Historically, plant breeding has been based on trial and error. While environmental factors originally determined selection, pre-agricultural men eventually developed a more purposeful extension of this process. This meant that selection was mainly based on appearance, yield, vigorous growth, taste and smell. Especially the past century resulted in new breeding programs that led to exceptional increases in both the quality and quantity of crops. In recent years genetic transformation techniques have been developed which complement classic breeding as it represents an additional way of generating new genetic diversity. This new technology is based on the introduction of DNA into the plant cell, followed by regeneration of the transformed cells to an entire plant (see Chap. 1). Marker genes, more exactly named selectable marker genes, are absolutely essential for the production of such transgenic plants. Despite optimization of the transformation efficiency of many crops, it is a fact that (even after three decades of agricultural biotechnology, also in model plants like *Arabidopsis*) the insertion of genes is restricted to a few cells among thousands of untransformed ones. Marker genes are required to identify, to "mark" the introduced genes and finally to enable the selective growth of transformed cells. These genes are co-transformed with the gene of interest (GOI); they are linked to the GOI and therefore remain in the transformed cell. However, once transgenic cells have been identified and regenerated to whole plants, the marker genes are no longer needed. For this reason new concepts of marker genes are discussed with regard to the safety of genetically modified plants, both for the environment and the consumer. Therefore this chapter reviews the most important marker genes

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available for gene transfer to plants, focusing particularly on recent advances, and discusses new systems for marker gene-free transformation techniques as well as marker gene deletion.

## 3.2 Criteria for Choosing the Marker Gene System

The most common type of selectable marker genes used for the efficient transformation and regeneration of plant cells are antibiotic resistance genes or herbicide resistance genes (Miki and Hugh 2004). The criteria for choosing these genes are the efficiency of the systems, their applicability to a wide range of plant species and of course their availability for the scientific community. Nonetheless criteria have changed within recent years, especially for the development of new varieties and new traits for the market. Furthermore the marker gene systems have to fulfil the requirements of regulatory and market acceptance (see Fig. 3.1).

Over the years, general opinion has accepted that using conventional transformation methods, including conventional vectors, can cause problems. Extra copies of transgenes or residual selection marker genes and their regulatory elements can increase the frequency of homology-based post-transcriptional and transcriptional gene silencing (Que and Jorgensen 1998). That implies problems due to the variability and instability of transgene expression (Matzke et al. 2000). The extra gene copies can be a result of both multi-copy insertions (during the transformation process) and trait stacking. Herbicide tolerance and insect resistance (Bt) often are introduced simultaneously to a crop in one transformation event or the combined traits are a result of re-transformation or crossing two single events. For example the third most commonly grown transgenic crop was stacked insect-resistant/herbicide-tolerant maize. Combined herbicide and insect resistance was the fastest growing GM trait from 2004 to 2005, grown on over 6.5 million hectares in the United States and Canada and comprising 7% of the global biotech area (GMO-Compass 2007). For the 2008 planting season quad stacks have been announced that protect the corn crop against both corn borer and corn rootworm while providing tolerance to various herbicides. This second generation of traits and the upcoming third generation of “output trait” products provide multi-resistance to pests and several types of pathogens, providing new products from metabolic engineering, offering new benefits to farmers and consumers (Halpin 2005).

However, that also means that the complexity of the GMO will increase in total because this includes the interaction between numerous genes. This multiple stacking of traits, also called “pyramided” traits with potential new management requirements or possible negative synergistic effects, may evoke an additional environmental safety assessment (<http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml>). It will be necessary to control these multiple genes, by developing new technologies for the coordinated manipulation of such traits. This includes cutting-back the complexity of the GMO. The more complex, the longer

- Simple efficient plant transformation system
  - Efficient method of plant transfer
    - Direct DNA transfer
    - *Agrobacterium*-mediated transfer
  - Optimal identification or selection system
    - Technically simple
    - Fast
    - Economical
    - Removable selection marker
- Optimum performance of the GMO comparable with standard varieties
  - Substantial equivalence
- Process of deregulation dependent on:
  - Acceptance by the responsible authorities
  - Biosafety
  - Complexity of the GMO
    - One or only a few insertions
    - No marker genes or completely harmless ones
    - No additional unnecessary sequences
  - Complete characterization: molecular analysis, biochemical analysis
  - Provision of quality control systems

**Fig. 3.1** Criteria for the development of new plant varieties by gene technology

the time for trait commercialization will be. In reality product development and deregulation will need a minimum of 10–15 years. Therefore, there is a demand both from the authorities and from the scientific community to improve the transformation systems, to avoid additional unnecessary sequences, to abstain from marker genes, to eliminate marker genes or to use only completely harmless ones.

### 3.3 Availability of Selectable Marker Gene Systems and Alternatives

There are different categories of selectable marker and alternative systems available, as shown in Fig. 3.2.

#### 3.3.1 Positive Selection Marker

There are sometimes different and confusing definitions in using the terms “positive selection marker” and “negative selection marker”. At present, positive selection systems are those that enable the growth of transformed cells, whereas negative selection systems kill the transformed cells (see Sect. 3.3.4).

##### 3.3.1.1 Antibiotics

The most widely used selection marker systems are based on aminoglycoside-modifying enzymes. These amino glycoside-modifying enzymes confer resistances against antibiotics as kanamycin, neomycin, gentamycin, paramomycin, streptomycin and spectinomycin.

- Positive selection marker systems by using:
  - o Toxic antibiotics
  - o Toxic herbicides
  - o Metabolic analogues
  - o Non-toxic agents
- Negative selection marker
- Alternatives
  - o Selectable marker gene elimination by:
    - Co-transformation
    - Recombinase induced elimination
    - Homologue recombination
  - o Screenable marker genes
  - o Marker-free transformation

**Fig. 3.2** Overview of selection systems

## Neomycin Phosphotransferase II

Within the aminoglycoside-modifying enzymes the *neomycin phosphotransferase II* (*nptII*), originated from transposon Tn5 of *Escherichia coli* K12 (Garfinkel et al. 1981), is the most used selectable marker gene. There are many advantages in using *nptII* in comparison with other selectable marker genes:

- The gene confers resistance against different antibiotics: kanamycin, neomycin, paramomycin, geneticin.
- The gene is efficient in model plants such as Arabidopsis, Petunia or Nicotiana tabaccum but also in most of the cultivated plants, both in monocots and dicots, in legumes and Gramineae.
- Reproducible protocols are available for the transformation of most of these crops.
- NPTII is available in combination with various regulation sequences, e.g. promoters.
- There are mutated forms of the *nptII* gene that encode enzymes with reduced activity.
- By using an intron-containing *nptII* gene only eukaryotic organisms will be able to process the gene (Paszkowsky et al. 1992; Maas 1997; Libiakova 2001). Accordingly, the potential risk of horizontal gene flow of antibiotic resistance genes from transgenic plants to bacteria is eliminated.
- *NPTII* can be used not only as a selectable marker but also as a scorable marker, as a reporter gene for studying gene expression and regulation. In vitro assays (ELISA or use of radioisotopes) for quantitative or semi-quantitative analysis of the NPTII activity are available (McKenzie 2000; Ziemiencowicz 2001).
- The patent on the *nptII* coding sequence combined with regulatory sequences will expire soon (König et al. 2003).

Most of the first-generation transgenic crops contain *nptII*, and to this date *nptII* is the best studied selectable marker with regard to safety. Already in 1994, the use of *nptII* as a marker and as a food additive for transgenic tomatoes, oilseed rape, and cotton was evaluated by the United States Food and Drug Administration (FDA). The FDA found the use of *nptII* as a selection marker safe (FDA 1994). The conclusion was based on data from Calgene (1993), Redenbaugh et al. (1994), Fuchs et al. (1993), Nap et al. (1992), Flavell et al. (1992), Kasid et al. (1990) and Bleasdale et al. (1990), among others. But unattached, without reference of scientific evaluation, the presence of antibiotic resistance genes, mainly NPTII, increases public and consumer criticism and still is dogged by controversy.

Mainly the concerns about the potential spread of antibiotic resistance genes through horizontal gene transfer led to the final recommendation that antibiotics widely used for clinical or veterinary use may not be used as selectable markers in plants (Miki 2004; FDA 1998). Also in Europe the use of antibiotics as selection marker was acknowledged as a problem and resulted in Directive 2001/18/EC, which requires the step by step phasing out of antibiotic resistance genes which may have adverse affects on human health and environment by the end of 2004 (EFSA 2004). However the European GMO Panel came to the conclusion in 2004 that the

use of the *nptII* gene as selectable marker in GM plants (and derived food or feed) does not pose a risk to human or animal health or to the environment. These safety assessments were confirmed by the EFSA in 2007 again in the light of all relevant reviews and expert consultations: Ramessar et al. (2007), Goldstein et al. (2005), Miki and McHugh (2004), Working Party of the British Society for Antimicrobial Chemotherapy (Bennett et al. 2004), FAO/WHO Consultation on Foods Derived from Biotechnology (FAO/WHO 2000), Scientific Steering Committee of the European Commission (SSC 1999), Zentrale Kommission für die Biologische Sicherheit, DE (ZKBS 1999), The Advisory Committee on Novel Foods and Processes, UK (ACNFP 1996), Nap et al. (1992).

But again, in contrast, in 2005 the WHO classified kanamycin and neomycin as critically important antibiotics (WHO 2005). To sum up there is no recommendation of a general ban of antibiotic markers, only a restricted use, but there are disagreements concerning the classification of antibiotics (mainly for kanamycin) whether they are of high, minor or no therapeutic relevance in human medicine.

### Hygromycin Phosphotransferase

Cloning of the *hygromycin phosphotransferase* (*hph*) gene and fusion with eukaryotic promoters resulted in the development of vectors that permit selection for resistance to hygromycin B in both prokaryotic and eukaryotic cells (Elzen et al. 1985). Besides kanamycin, hygromycin B is the most frequently used antibiotic for selection. In comparison with kanamycin, hygromycin is more toxic and therefore kills sensitive cells faster. However, hygromycin is the preferred antibiotic resistance marker for the selection of monocotyledonous plants, although it is not user-friendly. Extreme care has to be taken when handling hygromycin B as it is very toxic by inhalation, in contact with skin and if swallowed.

### Antibiotic Resistance Genes Beside *nptII* and *hph*

There are a lot of other marker genes, for example antibiotics like streptomycin (Maliga et al. 1988), spectinomycin (Svab and Maliga 1993), bleomycin (Hille et al. 1986) and chloramphenicol (de Block et al. 1984), which have been used in plant transformation experiences or are at least part of used transformation vectors. But most of them are under the control of a bacterial promoter and have been used for selection in bacteria not specified for selection in plants. In the end the genes are mostly integrated outside the left and right border regions of the used transformation vectors and therefore not part of the transgenic plants.

#### 3.3.1.2 Herbicides

Millions of hectares are being planted with transgenic herbicide resistant plants (see also Chap. 9), meanwhile often “stacked” with insect resistance in the same seeds to

enhance their value. The database summary *Global status of approved genetically modified plants* of AGBIOS (2009) shows 80 records for the trait herbicide tolerance. So, by far, herbicide tolerance is still the most used selection criteria. The advantages of the systems are the usage of the herbicide tolerance both as a desired trait in the field and as a selection marker (Goldstein et al. 2005) during developmental period. The most used systems comprise *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*, resistance to glyphosate), *phosphinothricin acetyl transferase* (*bar/pat*, resistance to glufosinate) *acetolactate synthase* (*ALS*, resistance to chlorosulfuron) and *bromoxinil nitrilase* (*Bxn*, resistance to bromoxinil) in descending order of AGBIOS records. In 2006 glyphosate-resistant crops have grown to over 74 million hectares in five crop species in 13 countries (Dill et al. 2008).

Meanwhile new and improved glyphosate-resistant crops are being developed. These crops will confer greater crop safety to multiple glyphosate applications and these glyphosate-resistant plants are expected to continue to grow in number and hectares planted. But there is no guarantee that new molecular stacks conferring resistance to glyphosate and ALS-inhibiting herbicides or glyphosate with glufosinate will prevent the development of resistant weeds in the future. There are already several weed biotypes with confirmed resistance to glyphosate. So the question arises whether the presence of herbicide selection markers like glyphosate resistance in some years may be undesirable when the trait is no longer necessary or inapplicable for product function. Apparently, the same conclusion is valid for herbicide resistance marker genes as for other marker genes, needed in the first place but undesirable shortly afterwards.

There are alternatives to the most used herbicide resistance genes, for example selectable marker genes which mediate resistance against the herbicides cyanamide (Weeks et al. 2000), Butafenacil (Li et al. 2003; Lee et al. 2007), Norflurazon (Inui et al. 2005; Arias et al. 2006; Kawahigashi et al. 2007) or Gabaculine (Gough et al. 2001). However, by today, most of these alternatives have not been subjected to regulatory consideration for international approvals.

### 3.3.1.3 Metabolic Analogous, Toxic, Non-Toxic Agents

Many other new approaches comprise manipulating the plant's metabolic or biosynthetic pathways. This is done by using metabolic analogous, toxic agents, non-toxic elements such as phytohormones, or carbon supplies which are natural to the plant. There is a wide range of used genes. *XylA*, *dog*, *ipt*, *tps* and *manA* are only a choice of new genes which were used to develop additional selection systems.

#### 2-Deoxyglucose-6-Phosphate Phosphatase

The deoxyglucose (DOG) system is based on the sugar 2-deoxyglucose (2-DOG) which is phosphorylated by hexokinase yielding 2-DOG-6-phosphate (2-DOG-6-P)

in plant cells. 2-DOG-6-P is toxic to plants, since it inhibits respiration and cell growth. Over-expression of the gene enzyme *2-deoxyglucose-6-phosphate phosphatase (dog<sup>R</sup>I)* in plant cells results in resistant plants (Kunze et al. 2001). Transgenic potato plants have been tested under field conditions with the result that no differences were found between the transgenic plants and the control plants. Whether the system can be applied without safety concern in the future has to be investigated further (GMO-Safety 2005).

### Xylose Isomerase

The *xylose isomerase (xylA)* system is based upon selection of transgenic plant cells expressing the *xylA* gene from *Streptomyces rubiginosus*, which encodes xylose isomerase, on medium containing xylose (Haldrup et al. 1998). In contrast to antibiotic or herbicide selection, the system is generally recognized as safe because it depends on an enzyme which is already being widely utilized in specific food processes, especially in the starch industry. But to this day selectable markers like *xylA* have not yet appeared in approved food plants.

### Isopentenyl Transferase

The enzyme *isopentenyl transferase (ipt)* is a more often used selection marker. The gene, encoded by the T-DNA of *Agrobacterium tumefaciens*, catalyzes the synthesis of isopentyl-adenosine-5' monophosphate, which is a precursor of the phytohormone cytokinin. Over-expression of *ipt* by using the gene under the control of a constitutive promoter yields enhanced cytokinin levels in transgenic plants. Cytokinins stimulate organogenesis; therefore due to the enhanced cytokinin concentrations the regeneration of transformed shoots is promoted. The combination of the *ipt* gene together with the kanamycin selection system enhances the transformation efficiency (Ebinuma et al. 1997; Endo et al. 2001). The system, also called the MAT system, is usable as a visible selection system since the transformed shoots lose their apical dominance and the ability to root. These abnormal morphologies of the shoots, so-called “extreme shooty phenotype” (ESP) prevented the development of *ipt* as a selectable transformation marker in practice, because it is only usable in combination with inducible artificial promoter systems (Kunkel et al. 1999; Zuo et al. 2002) or with marker elimination systems (Ebinuma et al. 2000, 2001). The use and removal of *ipt* were demonstrated in different plant species but the efficiency of the system was low, therefore further optimization of the selection system is required. Recently new publications (Rommens et al. 2004, 2006; Bukovinszki et al. 2006; Richael et al. 2008) give hope for an improved system. New methods (e.g. “All-native DNA transformation”) for the production of transgenic plants utilize *isopentenyl transferase* cytokinin genes in negative selection against backbone integration (e.g. see Sect. 3.4.3 and Chap. 4).

### Phosphomannose Isomerase

The *manA* gene codes for the enzyme *pmi* (*phosphomannose isomerase*). Many plants are normally not able to use the sugar mannose as a source of carbohydrate. When plants are forced to grow on mannose as the only carbon source they first convert mannose to mannose-6-phosphate, which is no longer utilizable for the plants. Transformed with the *pmi* gene, the plant converts mannose-6-phosphate to fructose-6-phosphate, which can be used in the plant metabolic pathway from there. Thus mannose can function as the only carbon source (Joersbo et al. 1998; Privalle et al. 1999). Species which have been successfully transformed using mannose as selective agent, among others, are sugar beet (Joersbo et al. 1998; Lennefors et al. 2006), sunflower, oilseed rape, pea, barley (Joersbo et al. 1999, 2000), sorghum (O'Kennedy et al. 2006), sugarcane (Jain et al. 2007), rice (Lucca et al. 2001; Ding 2006), tomato and potato (Bříza et al. 2008), apple (Degenhardt 2006), papaya (Zhu 2005), torenia (Li et al. 2007) and citrus (Ballester et al. 2008). Ballester and co-workers compared various selection systems with the same Citrus genotypes: *nptII*, *ipt* and *pmi* systems. The highest transformation rates were obtained with the *pmi*/mannose system, which indicates that this marker is also an excellent candidate for citrus transformation. So at the moment, beside the kanamycin and the glyphosate selection system, the *pmi* system is the most successful one. Regulatory approvals have been received for environment, food and feed with transgenic maize varieties in Mexico, Australia, Japan, Canada and the United States.

### 3.3.2 Alternative Systems

The rising demand both from the authorities and the public for genetically modified plants containing only foreign sequences needed for the immediate function encouraged the development of alternative systems, including:

- Subsequent elimination of marker genes by co-transformation techniques, transposon usage, specific recombination systems or homologous recombination
- Marker-free transformation without usage of any selection marker
- Combinations of different systems, e.g. usage of screenable marker, recombination systems and/or positive/negative selection in the same system

#### 3.3.2.1 Selectable Marker Gene Elimination

##### Co-Transformation

Among the techniques developed to eliminate selectable marker genes, co-transformation is the simplest one. The method is based on the strategy to introduce the

marker gene and the gene of interest into plant genome as unlinked fragments. In the progeny the selectable marker gene is segregated from the gene of interest. The introduction of the genes can occur either by using two *Agrobacterium* strains (mixed strain method), each with a binary vector (one carrying the selectable marker gene, the other carrying the gene of interest; Framond et al. 1986; McKnight et al. 1987), or by using a single *Agrobacterium* strain with two plasmids (dual binary vector system or binary vector plus cointegrate vector; Komari et al. 1996; Sripraya et al. 2008), or by using a single *Agrobacterium* strain with one binary plasmid carrying on the plasmid two T-DNAs (two-border vector system; McCormac et al. 2001; Breitler et al. 2004), one with the selectable marker gene and the second with the gene of interest. There are some prerequisites to make co-transformation functional: the efficiency of the co-transformation should be high and in the progeny the segregation efficiency should be also high. Both requirements are dependent on each other and respectively are dependent from the used co-transformation system. Comparing the systems, the mixed strain method reduces the co-transformation efficiency but enhances the integration into separate loci whereas the two border system enhances the co-transformation efficiency and reduces significantly the segregation efficiency. Overall the predisposition of plant cells for the simultaneous integration of T-DNAs naturally supports the multicopy insertions and reduces the probability to identify single copy events. Additional factors which can affect the systems are the plant varieties which have to be transformed, the *Agrobacterium* strains (nopaline or octopine strains), the size of the Ti-plasmids or the ratio of amount of used Ti-plasmid with the selection marker to the amount of the Ti-plasmid with the gene of interest (Yoder and Goldsbrough 1994; de Block 1991; Mathews et al. 2001).

To overcome some of the problems, co-transformation systems are combined with the additional usage of screenable marker genes or negative selection marker. Combination of the kanamycin resistance gene with the negative selection marker *codA* (*cytosine deaminase*) on one T-DNA enables the automatic elimination of the unwanted plants after segregation by 5-fluorcytosine treatment (Park et al. 2004). Another interesting strategy to improve or to speed up co-transformation technology is the usage of androgenetic segregation. Plant breeders require homozygous plants to ensure that the traits are passed on to all progeny. Subsequent to co-transformation with uncoupled T-DNAs, unripe pollen is isolated from the regenerated plants, androgenetic development is induced and the cells of the pollen's haploid chromosome set can spontaneously divide, diploidize and finally regenerate to completely homozygous doubled haploid plants (Goedeke et al. 2007; GMO-Safety 2007). Co-transformation technology is not restricted to *Agrobacterium*-mediated transformation but can also be used for particle bombardment. Integration of vector backbone sequences or additional unnecessary vector sequences can be avoided by applying minimal constructs containing only the promoter, coding region and terminator.

This “clean DNA transformation” using two minimal gene cassettes, one with the selectable marker gene, one with the gene of interest, was successful in various

crops (Fu et al. 2000; Breitler et al. 2002; Romano et al. 2003; Vidal et al. 2006; Zhao et al. 2007).

The advantage of the co-transformation system is based on the simplicity of the technique, the possibility to use standard vectors and the fact that no additional genes or elements are needed. But the technology is not suitable for all plant species because segregation and recombination occurs only during sexual reproduction of the plants. Therefore, this method is not applicable for cultivated plants propagated by vegetative methods or for plants with extreme long generation times, like some trees.

### Recombinase-Induced Elimination

Beside the co-transformation technology, the site-specific recombinase-mediated excision of marker genes is the most used method to get rid of marker genes. The strategy is based on the use of a two-component system comprising a specific enzyme and two short DNA sequences. The enzyme, which is a recombinase, recognizes the specific short DNA target sites and catalyzes the recombination/elimination of the sequence between the target sites. The most common systems used for the production of marker-gene-free transgenic plants are the bacteriophage P1 *Cre/loxP* system (Sternberg et al. 1981; Dale and Ow 1991), the *FLP/FRT* system from *Saccharomyces cerevisiae* (Cox 1983; Kilby et al. 1995) and the *R/RS* system from *Zygosaccharomyces rouxii* (Zhu et al. 1995; Sugita et al. 2000). All systems, reviewed by Ow (2002), Hare and Chua (2002) and Ebinuma and Komamine (2001), require the expression of the enzyme in transgenic plants. This expression can be achieved by crossing two transgenic plants: one allocates the recombinase, the other owns the gene of interest and the marker gene to excise. After segregation the next generation of recombinase- and marker-free plants are obtained (Hoa et al. 2002; Arumugam et al. 2007; Chakraborti et al. 2008). To improve and speed up the system new basic approaches were proposed, including transient expression of the recombinase and the introduction of the recombinase gene into transformed plants in combination with a negative selection marker, inducible promoters or germline-specific promoters.

Feasibility was proven for the expression of *Cre* under the control of chemical induction (Zuo et al. 2001; Sreekala et al. 2005) under the control of heat-shock promoters (Hoff et al. 2001; Zang et al. 2003; Cuellar et al. 2006; Luo et al. 2008) and for the transient expression of *Cre recombinase* by *PVX-Cre* and *TMV-Cre* recombinant viruses (Kopertekh et al. 2004). Quite recently published results from various authors give hope that, with new modified site-specific recombination vectors, it appears to be possible not only to excise the selectable marker gene but also to get single copy insertion, backbone-free integration or even marker-gene-free and homozygous plants together in one step. Verweire (2007) presented an approach where it is possible to get marker-gene-free plants via genetically programmed auto-excision without any extra handling and in the same time frame, as compared to conventional transformation without marker gene elimination. The

basic idea of the approach is to control the *Cre recombinase* by a germ-line-specific promoter. As a consequence of auto-excision of the marker gene in the male and female gametes, the plants of the next generation are marker-free. The University of Connecticut (Luo et al. 2007) has also recently developed a new technology, called “gene deleter”, or also called “GM gene deleter”. The system functions through combination and interactions of the bacterial phage *Cre* and the *Saccharomyces cerevisiae FLP* recombinases with the flanking recognition sites *loxP* and *FRT* as it was proven already by Srivastava and Ow (2004). The technology could be used to remove selectable marker genes but also all transgenes from any organs of a transgenic plant when the functions of transgenes are no longer needed or their presence may cause concerns. Results obtained by Mlynarova et al. (2006) and Bai et al. (2008), by using microspore, pollen or seed-specific promoters, also demonstrated the function of these auto-excision vectors. One advantage of all these auto-excision systems is that all extraneous DNA and multi-copy insertions (e.g. flanked by *Lox/FRT* sites in direct orientation) is eliminated. Therewith complex transgene loci can be simplified. Similar results were reported by Kondrák et al. (2006), who removed marker genes by using a binary vector carrying only the right border (RB) of T-DNA, the *Zygosaccharomyces rouxii R/Rs* recombination system and a *coda-nptII* bi-functional, positive/negative selectable marker gene. In a first step the whole plasmid was inserted as one long T-DNA into the plant genome and, after positive and negative selection, it was shown that by recombinase enzyme activity both the bi-functional marker genes as well as the backbone of the binary vector have been eliminated.

Even though a lot of work has been invested to obtain marker-free plants by site-specific recombination, the practical suitability still leaves a lot to be desired, among others due to the complex and complicated systems, due to the inefficient inducer transport, due to insufficient promoter specificity or due to insufficient Cre expression, or expression at the wrong time or at the wrong place. But, in contrast to many other marker elimination systems, regulatory approval has already been received for such a system. The maize line LY038 with enhanced lysine level, from which the selectable marker was excised by using *Cre/Lox*, received approval for food and feed in the United States, Canada and Japan (AGBios 2008).

### Transposon-Based Elimination

Some 25 years ago, Goldsborough et al. (1993) reported first about marker gene elimination by transposon usage. The most used system is the maize *Ac/Ds* transposable element system. The *Ac transposase* is able to reposition marker genes or the gene of interest located between *Ds* elements. When these plants are crossed with other plants, they produce progeny which, as a result of naturally occurring segregation processes, carries either only the target gene with minimal *Ds* sequences at both ends or the marker gene with the *Ac* gene. When the selectable marker gene is flanked with *Ds* elements, the system can also be used for

vegetatively propagated plants, because very often there is excision of the elements without subsequent reintegration (Ebinuma et al. 1993). Furthermore marker-free transgenic plants containing insect-resistant *Cry1b* gene between the Ds elements have been produced (Cotsaftis et al. 2002). Also in sugar beet it has been demonstrated in a research project that the *Ac/Ds* transposon system works in principle (GMO-Safety 2006). However, a few questions still need to be answered before a practical application of the system is possible. This includes questions concerning imprecise excisions, deletions/alterations in the DNA sequence because of many insertion and excision cycles, and low efficiency of the system.

### Homologous Recombination

Various methods have been tested to increase the efficiency of gene targeting by homologous DNA recombination in plants. One possibility is to insert DNA at specific points with the help of targeted double-strand breaks in the plant itself. The double-strand breaks are generated by a rare cutting restriction enzyme, *I-SceI*, which can enhance homologous integration frequency at the target site (Puchta et al. 2002). By using cutting sites of the *I-SceI* enzyme in the transgene construct before and after the marker gene, it is possible to induce double-strand breaks on each side of the marker gene following expression of *I-SceI* in the plant. Thereby the marker gene can then be removed. Another approach for the future can be the use of zinc-finger nucleases to target specific DNA sequences for gene modification (Lloyd et al. 2005; Wright et al. 2005). These methods can be powerful tools to modify plants genetically, but today their efficiency in plants is still not high enough for routine applications.

### 3.3.3 Screenable Marker Genes

Screenable markers encode a protein which is detectable because it produces a visible pigment or because it fluoresces or modifies the phenotype elsewhere under appropriate conditions. Screenable markers include *galactosidase* (*lacZ*; Herrera-Estrella et al. 1983),  $\beta$ -*glucuronidase* (*GusA*; Jefferson 1987), *luciferase* (*luc*; Ow et al. 1986), *green fluorescent protein* (*gfp*; Haseloff and Amos 1995), *red fluorescent protein* (*rfp*; Campbell et al. 2002) and *isopentenyl transferase* (*ipt*; Ebinuma et al. 1997). Screenable marker genes can be used as independent genes or as fusion constructs. They cannot be used for positive selection but they can help to improve transformation efficiency, they can be used as visual marker of transformation and they allow the enrichment of transformed tissue and therefore speed up the whole transformation process. Screenable markers are usable within the T-DNA, outside T-DNA on the backbone or as part of the co-transformation vector. In the meanwhile the integration of screenable marker genes outside the T-DNA borders is an often used strategy to identify vector backbone sequences in order to limit the

production of events with superfluous DNA. In a similar manner the usage of *ipt* can serve as a visual screenable marker of backbone integration within the plant genome (Bukovinszki et al. 2006).

### 3.3.4 Negative Selection Marker

Comparable with screenable marker genes, so-called negative selection markers are used to optimize transformation efficiency. Thereby negative selection systems kill the transformed cells. This allows new strategies to limit the production of vector-backbone-containing plants by flanking the T-DNA with negative selection marker genes. The most used negative selection marker gene is the *codA* gene from *E. coli* encoding *cytosine deaminase*. The usefulness of *codA* as a conditional toxic gene was explored in different *Agrobacterium*-mediated transformation protocols (Koprek et al. 1995; Schlaman 1997). Plant cells which are transgenic for *codA* show sensitivity to 5-fluorocytosine (5-FC) at different developmental stages. The negative selection marker confers a lethal phenotype on the transformant and is therefore often part of co-transformation systems. Co-transformation with *codA* is a viable method for the production of easily distinguished, selectable marker gene-free transgenic plants (Park et al. 2004). As described by Verweire et al. (2007) the *cytosine deaminase* gene can be used as a counter-selectable marker. In this system *CodA* is a component of a germline-specific auto-excision vector, in which *codA* is present in tandem with the recombinase and the positive selection marker between *lox* sites. After auto-excision of the whole *Lox*-cassette, marker-free regenerates can be identified by growing on medium containing 5-fluorocytosine.

Often mentioned in literature concerning marker genes are the *tms2* and the *daol* gene as negative selection marker genes (Upadhyaya et al. 2000; Erikson et al. 2004). The marker gene, *daol*, encoding *D-amino acid oxidase* (*DAAO*) can be used for either positive or negative selection, depending on the substrate. D-Alanine and D-serine are toxic to plants, but are metabolized by *DAAO* into non-toxic products, whereas D-isoleucine and D-valine have low toxicity but are metabolized by *DAAO* into the toxic products respectively. Hence, both positive and negative selection is possible with the same marker gene.

### 3.3.5 Marker-Free Transformation Without Usage of Any Marker Gene

De Vetten et al. (2003) reported first about transformation of potatoes without the usage of any selectable marker. Transgenic plants were analyzed and identified exclusively by PCR. In another study marker-free tobacco transformants with efficiency up to 15% of the regenerants were produced by agroinfiltration

(Jia et al. 2007). Genetic transformation of apples was also achieved without using selectable marker genes (Malnoy et al. 2007).

In summary there is proof that marker-free plants can be produced by a single-step transformation without marker genes. Whether the methods are applicable to other crops has yet to be shown.

### 3.4 Conclusions and Perspective

There have been many excellent reviews concerning selectable marker genes and marker gene elimination including biosafety aspects in recent years (Miki and Hugh 2004; Darbani et al. 2007; Ramessar et al. 2007; Sundar et al. 2008). This report is only an extract of the most important usable techniques, the marker systems which are available, but as a result it manifests how difficult it is for the user to decide which system will be the right one. The criteria for choosing the marker gene system including the marker gene elimination systems have changed within recent years. There are new requirements from the users, from the authorities and from the consumers concerning technical, regulatory and biosafety aspects. To combine all this aspects within one marker system is one of most difficult problems. A variety of selection systems seem to be essential for different plant species because no single marker gene works well in all situations. Many marker genes exist and many new marker genes have been tested in recent years, but only a few of them are widely used and still fewer have received approval from the authorities for food and feed. To this day that is the case mainly for the genes *nptII*, *pat*, *Cp4epsps* and *gus*. In an overall picture there is a wide range of marker genes in theory but in reality only some of them are used and are accepted.

Regulatory requirements for selectable marker genes in the European Union was one of the main reasons to think about the removal of marker genes from the plants once the genes have done their job. Several strategies have been developed, including co-transformation systems, site-specific recombination systems and transposon-based elimination systems, often in combination with screenable marker or negative selection marker systems. Co-transformation is technically simple but needs high transformation efficiency and the technique is usually not suitable for use in vegetatively propagated crops. Many site-specific excision systems have been proved and show promising developments for the future. But very often the problem arises that complicated systems for the marker gene elimination are needed in order to achieve the goal to simplify the GMO. Whether all these new systems can be applied without safety concern in the future is to be investigated further. The development of additional new marker technologies, including marker gene elimination technologies, will continue to be important in the production of transgenic plants.

In the end, everyone has to keep an eye on the insight that the replacement of old technologies (e.g. the use of antibiotic-resistant marker genes, herbicide-resistant marker genes) by new systems only makes sense if these new technologies can at

least ensure the same degree of scientific knowledge and safety like the old technologies.

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# Chapter 4

## Precise Breeding Through All-Native DNA Transformation

Caius M. Rommens

### 4.1 Introduction

There is a continued need for agriculture to reinvent itself, striving towards enhanced productivity, cost-efficiency, and crop quality. Farmers employ increasingly complex crop management systems and eagerly adopt new varieties that promise higher yields. Such varieties need to display a combination of exceptional traits that protect against a multitude of stresses, ensure crop uniformity and storability, and are demanded by consumers. Plant breeders attempt to combine these traits by using methods that rely on random genome modifications such as double-bridge crosses, somatic hybridization, chemical mutagenesis, and  $\gamma$ -radiation. However, such methods are often too imprecise to remove unwanted plant characteristics while they can also substantially compromise the integrity of genomes (Rommens 2008). They can also result in obscure alterations linked to reduced food quality. For instance, transfer of “high starch” and “crisp chip” traits from *Solanum chacoense* to cultivated potato (*S. tuberosum*) increased glycoalkaloid levels in the resulting variety “Lenape” to almost twice the maximum allowed concentration (354  $\mu\text{g kg}^{-1}$ ; Zitnack and Johnson 1970).

One new extension of the plant breeding process specifically alters the expression of one or several of the plant’s own genes without affecting the overall structure of the plant’s genome. This new “intragenic” approach to genetic engineering employs marker-free systems to introduce all-native transfer DNAs into plants (Nielsen 2003; Rommens et al. 2004, 2007). For instance, it increases the expression of a key gene in a biosynthetic pathway by linking this gene to the strong promoter of a different native gene. Alternatively, a silencing construct is used to down-regulate the expression of a gene that is linked to an undesirable trait.

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In this review, we provide an update on efforts to develop and implement methods for the intragenic modification of important solanaceous and cruciferous crops as well as alfalfa (*Medicago sativa*), perennial ryegrass (*Lolium perenne*), and apple (*Malus domestica*). Most of the genetic elements and marker-free transformation methods employed are available to the scientific community for research purposes.

## 4.2 Examples of the Intragenic Modification in Potato

Despite the importance of potato as the most frequently consumed vegetable, issues such as inbreeding depression, a high degree of heterozygosity, and poor fertility have hampered efforts to improve the yield and quality of this crop. Each year, millions of potato plants are evaluated in the United States for the basic input and storage traits required by the industry. The few clones selected through this rigorous process are subsequently processed and assayed for sensory traits associated with taste, texture, and color. There are currently only about ten potato varieties that display most of the traits required by the French fry, potato chip, and retail industries. Together, they occupy ~70% of the total potato acreage in the United States. Interestingly, the predominantly grown variety is also the oldest: this century-old “Russet Burbank” displays unsurpassable storage and processing characteristics, but it suffers from multiple bacterial, viral, and fungal diseases, while also displaying high levels of sensitivity against environmental stresses including salt, drought, and frost. Farmers generally prefer to grow higher-yielding and more stress-tolerant and uniform varieties. However, each variety has its own issues, most of which translate directly into specific risks for growers, processors, and retailers.

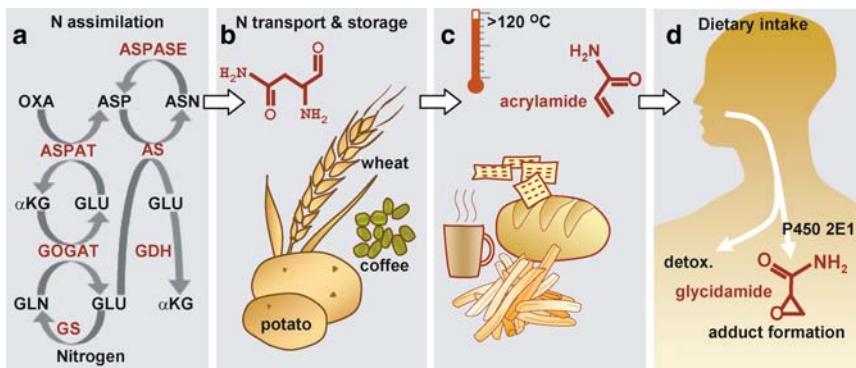
Given the urgent need for potato improvement, it may not be surprising that intragenic methods were first applied to the farmers’ favorite variety “Ranger Russet”. This variety combines superior yield with disease resistance, adaptability, tuber uniformity, and high levels of starch. However, Ranger Russet is particularly sensitive to tuber discolorations linked to impact-induced bruise, and it also accumulates high levels of glucose and fructose during cold storage. These reducing sugars react with amino acids during high-temperature processing of potato to produce Maillard reaction products that darken the color of French fries from golden-yellow to brown. The main weaknesses of Ranger Russet were turned into strengths by transforming it with a specific potato-derived transfer DNA (Rommens et al., 2006). This plant-derived transfer (P-)DNA carried a silencing construct designed to simultaneously silence the tuber-expressed polyphenol oxidase (*Ppo*), phosphorylase-L (*PhL*), and starch-associated *R1* genes (Rommens et al. 2006). Tubers of the resulting intragenic plants were down-regulated in *Ppo* gene expression and, consequently, displayed resistance to black spot bruise. Additionally, the silencing of genes involved in starch phosphorylation and degradation lowered the formation of reducing sugars during cold storage. This reduced cold-sweetening not only extended tuber storability but also limited French fry discoloration.

Interestingly, the slight modification of potato tuber starch was found to enhance crispness and French fry taste as well (Rommens et al. 2006).

The second intragenic modification of potato addressed one of the most important issues of the processing industry. This issue relates to the accumulation of large amounts of asparagine in tubers. Upon heat processing, the amide amino acid reacts with glucose and other reducing sugars to produce neurotoxic acrylamide (Fig. 4.1). Dietary intake levels of acrylamide have been rising in the Western world since the early 1900s, in part because of the increased consumption of French fries and potato chips, and are currently estimated at  $40 \mu\text{g person}^{-1} \text{ day}^{-1}$ . Indeed, acrylamide has become a signature ingredient of the modern Western diet and may represent a minor factor in the emergence of certain “modern” diseases.

A preferred route to lowering the accumulation of acrylamide would shift to crops that are naturally poor in acrylamide precursors. However, there are currently no varieties available that also display all the additional input, processing, and quality traits demanded by the processing industry. Therefore, methods were developed to reduce the acrylamide potential of existing varieties through intragenic modification (Rommens et al. 2008a). These methods were based on the finding that simultaneous tuber-specific silencing of the asparagine synthetase-1 and -2 genes lowered asparagine levels by up to tenfold. The dramatic decrease of tuber asparagine levels was associated with slightly elevated levels of glutamine but did not affect the production of other amino acids, and also did not alter total protein yield. Both French fries and potato chips from the intragenic potatoes accumulated much less acrylamide than was present in controls. This modification did not alter the color, texture, and taste of the final product. Furthermore, preliminary greenhouse data indicated that the intragenic lines displayed the same agronomic features as their untransformed counterparts. If confirmed by follow-up studies, all-native fry and chip products with very low levels of acrylamide may be offered as a new market choice within the next five years. Given the important role of processed potato products in the modern Western diet, a replacement of current varieties with intragenic potatoes would reduce the average daily intake of acrylamide by almost one-third (Rommens et al. 2008a).

A third application of intragenic potato modification is directed towards enhancing the crop’s antioxidant potential. Until recently, this quality trait was not considered in potato breeding programs. New initiatives to produce colored high-antioxidant potatoes still need to overcome many issues associated with the genetic complexity that underlies antioxidant product formation. For instance, several powerful flavonols such as kaempferol and quercetin are mainly produced in the anthers, where they support the production of viable pollen (Guyon et al. 2000). It is difficult to divert the underlying biosynthetic pathway to tubers by simply relying on random recombination processes. However, recent experiments have shown that this pathway can be activated in tubers through overexpression of the transcription factor gene *SMf1<sup>M</sup>* (Rommens et al. 2008b). The subsequent down-regulation of flavonoid-3',5'-hydroxylase gene expression limited the formation of anthocyanins and, instead, resulted in a 100-fold increased accumulation of kaempferol, to  $0.27 \text{ mg g}^{-1}$  dry weight (DW; Fig. 4.2). This genetic modification did not alter tuber yields and also had no effect on the sensory characteristics of processed food.



**Fig. 4.1** Toxicity of heat-induced acrylamide formation in starchy foods (a) Nitrogen is acquired from the environment via nitrate reduction or ammonia uptake, and assimilated through the action of enzymes such as glutamine synthetase (GS), ferredoxin-dependent glutamate synthase (GOGAT), aspartate aminotransferase (ASPAT), asparagine synthetase (AS), asparaginase (ASPASE), and glutamate dehydrogenase (GDH). GLN Glutamine,  $\alpha$ KG  $\alpha$ -ketoglutarate, OXA oxaloacetate, ASP aspartate. (b) Asparagine plays a role in the long-distance transport of nitrogen, and in the storage of this compound in sink organs such as tubers and seeds. (c) At temperatures exceeding  $120^{\circ}\text{C}$ , the  $\text{NH}_2$  group of asparagine reacts with the carbonyl group of reducing sugars to produce a Schiff base which, especially under high moisture conditions, forms the Amadori compound N-(D-glucos-1-yl)-L-asparagine. This unstable compound then forms acrylamide through decarboxylative deamination. (d) Upon intake of processed starchy foods, acrylamide is readily absorbed and distributed among tissues. Some acrylamide is detoxified (detox.) whereas another part is converted to glycidamide. Both acrylamide and glycidamide bind DNA and proteins to form adducts. This adduct formation is linked to various diseases



**Fig. 4.2** Potato phenotypes. An untransformed Bintje potato tuber (*left*) is compared with two tubers from plants expressing *StMtf1<sup>M</sup>* (*middle pair*) and a tuber that expresses *StMtf1<sup>M</sup>* but is silenced for the *F3'5'h* gene (*right*). This last tuber contains kaempferol levels that are 12-fold higher than those of the primary transformant and almost 100-fold higher than the untransformed control

Given the large amounts of potatoes that are consumed on a daily basis, estimated at 171 g person<sup>-1</sup>, replacement of currently available commodity potatoes by varieties overexpressing StMtf1<sup>M</sup> would, on average, double the average daily intake of kaempferol (Rommens et al. 2008b).

Another important target for intragenic modification relates to the lingering presence of plant-produced toxins or allergens in crops including potato. It is estimated that 0.2% of Americans are allergic to potatoes. Furthermore, consumption of potato products can occasionally result in the intake of glycoalkaloids at levels that are acutely toxic. Regulatory agencies oppose the intentional employment of genes that are known to produce allergens, toxins, or anti-nutritional compounds (Kaepller 2000) but can do little to prevent the unchecked transfer of such genes through conventional breeding (Bradford et al. 2005). Many plant-derived toxins are effective against plant pathogens and insects, and breeders may have unknowingly selected for the presence of such genes by seeking to enhance disease tolerance levels. However, given the advances in integrated strategies to control diseases and pests, it may be possible now to start lowering toxin levels in the edible parts of food crops. Although some allergen-encoding Patatin genes could be inactivated through mutagenesis, it would be difficult to eliminate all these functional genes from potato. In contrast, a carefully designed silencing approach was recently shown to substantially reduce the formation of all Patatin storage proteins in potato tubers (Kim et al. 2008).

The above examples demonstrate the significance of intragenic crop modification for quality improvements. However, it is also important to support breeders in their efforts to enhance potato's tolerance to various biotic and abiotic stresses. One of the most important diseases that threatens the potato industry is late blight. Breeding programs have not been able to markedly increase the level of resistance of current potato varieties, and chemical control is under pressure as late blight becomes increasingly aggressive and there is societal resistance against the use of environmentally unfriendly fungicides. Consequently, groups of scientists at Wageningen University, the United States Department of Agriculture, Simplot, and elsewhere have embraced intragenic approaches to transfer multiple resistance (*R*-)genes from wild potatoes to important varieties with proven adaptation, such as Desiree and Atlantic. Two *R*-genes of particular interest are the *Rpi-blb1* and *Rpi-blb2* genes from *Solanum bulbocastanum* (van der Vossen et al. 2003, 2005; see also Chap. 20). Unlike other known *R*-genes, this gene combination appears to provide durable resistance to potato.

### 4.3 Requirements for the All-Native DNA Transformation of Potato

An important aspect of the new approach to genetic modification is that it omits the use of bacterial selectable marker genes (see also Chap. 3). Initially developed procedures simply exclude a selection step, yielding potato transformation

frequencies below 0.1%. These frequencies were increased to 2% by applying supervirulent *Agrobacterium* strains such as AGL0 (de Vetten et al. 2003). An alternative method was developed by employing two different transfer DNAs, one carrying a positive and negative selectable marker gene and the other one comprising the DNA of interest. A transient positive selection step for marker gene expression followed by a negative selection step against marker gene integration yielded plants containing only the desired DNA with frequencies of about 15% (Rommens et al. 2004; Kondrak et al. 2006). The newest and most preferred method for marker-free transformation includes a quality-control mechanism that also selects for backbone-free DNA transfer (Richael et al. 2008). This method employs vectors containing the bacterial isopentenyltransferase (*ipt*) gene as backbone integration marker. *Agrobacterium* strains carrying the resulting *ipt* gene-containing vectors were used to infect explants of various solanaceous plant species. Upon transfer to hormone-free media, 1.8–6.0% of the infected explants produced shoots that contained a marker-free P-DNA while lacking the backbone integration marker. Because of the very high frequency of left border skipping in potato, these frequencies equal those for backbone-free conventional transformation.

The absence of antibiotic or herbicide tolerance genes facilitates the regulatory approval process and may also alleviate some consumer concerns about the permanent introduction of foreign DNA into the food supply. Another advantage of the use of cytokinin vectors is that the temporary formation of the natural cytokinin isopentenyl adenosine in *Agrobacterium*-infected explants is highly effective in inducing regeneration. In fact, regeneration frequencies are tenfold higher for explants on hormone-free media that were infected with an *Agrobacterium* strain carrying a cytokinin vector than for conventionally infected explants on hormone-containing media. Interestingly, the new method is immediately applicable to other dicotyledonous plant species and may represent a new step towards the development of genotype-independent transformation methods (Richael et al. 2008). The utility of *ipt*-based transformation has already been demonstrated for nightshades and canola and may likely be extended to other crops, such as rice (*Oryza sativa*), sunflower (*Helianthus annuus*), and pineapple (*Citrus sinensis*), that are known to respond to *ipt* gene overexpressing by producing adventitious shoots (Endo et al. 2002; Molinier et al. 2002; Ballester et al. 2007). In future studies we will further extend the applicability of the cytokinin vector method, not only by using hormone-free media but also by testing the utility of media containing small amounts of auxins. This modification may be required for some genotypes that need exogenously applied auxins for the regeneration of proliferated cells.

The second characteristic of the intragenic approach is, as mentioned above, that the employed transfer-DNAs are derived from within the target crop itself. The potato sequence used to support DNA transfer was isolated from pooled DNA of wild potato species that are sexually compatible with potato (Rommens et al. 2004). Its original size of 1.6 kb was reduced, through deletion of an internal fragment, to obtain a 0.4-kb P-DNA with several unique restriction sites. The 25-base pair (bp) St01 border-like elements that delineate this P-DNA were more effective than conventional T-DNA borders in mediating plant transformation

(GenBank accession AY566555; Rommens et al. 2004). Efficient initiation of DNA transfer could also be accomplished by linking a second border-like element from potato, St02, to a GC-rich region derived from DQ235183 (the inverse-complement of nucleotides 256–329; Rommens et al. 2005). Vector pSIM781, which carries this right border region, promotes similar frequencies of tobacco transformation as either a T-DNA vector or a vector containing the original P-DNA from potato. The same border-like element facilitates the termination of DNA transfer as well if linked to the 79-bp AT-rich DNA region from AF216836 (nucleotides 3231–3310) in pSIM1141.

One of the most frequently used elements for tuber-specific gene expression is the promoter of the granule-bound starch synthase gene (Visser et al. 1991). This promoter has been used extensively in transgenic research, because it delivers high levels of gene expression. Other well-known tuber-specific promoters include the patatin promoter (Jefferson et al. 1990) and the promoter of the ADP glucose pyrophosphorylase (*Agp*) gene (Du Jardin et al. 1997). For most yield-associated traits, it may be important to express the associated target genes not just in the tuber but throughout the potato plant. Examples of such traits include salt, drought, and frost tolerance. The most effective near-constitutive promoters that can be used for these purposes are the ubiquitin-3 (*Ubi3*) and ubiquitin-7 (*Ubi7*) promoters (Garbarino and Belknap 1994; Garbarino et al. 1995). The standard terminator used for the construction of gene expression cassettes was isolated from the *Ubi3* gene (Garbarino and Belknap 1994). This terminator is as effective as the frequently used bacterial terminator of the nopaline synthase (*nos*) gene.

#### 4.4 Intragenic Tomato (*S. esculentum*): Concentrating the Quality Potential of Tomato into its Fruit

Tomato is one of the most important horticultural crops and represents an important source of vitamins, minerals, and antioxidants. Intragenic methods for tomato were first applied to redesign Calgene's "FlavrSavr". The transgenic crop contained an extended *Agrobacterium* T-DNA region with an extra copy of the polygalacturonidase (*Pg*) gene inserted in the antisense orientation between the 35S promoter of cauliflower mosaic virus and the terminator of the *Agrobacterium tml* gene. It also carried a bacterial expression cassette for the neomycin phosphotransferase (*nptII*) gene. Despite its extended shelf life, opposition from non-governmental organizations resulted in an eventual withdrawal of FlavrSavr tomatoes from the market.

The intragenic version of the extended-shelf life concept was developed by first creating a silencing construct comprising *Pg* gene fragments inserted as an inverted repeat between convergently oriented promoters. The marker-free transfer DNA used to introduce this construct into tomato contained two copies of the 25-bp

border-like Le02 element (Rommens et al. 2005), positioned as a direct repeat. This element is more effective as right border than either Le01 or Le03 in supporting tobacco transformation when linked to a 164-bp fragment from tomato AY850394 (nucleotides 42723–42886). The complementing left border region was created by fusing the second copy of Le02 to a 189-bp AT-rich region similar to AP009548 (reverse sequence of 10345–10532). A binary vector carrying both the left and right tomato-derived border regions, designated pSIM894, was used for marker-free transformation to incorporate the new quality trait.

Future applications may support efforts to unleash the full quality potential of tomato. For instance, tomato plants evolved to produce high levels of antioxidant flavonols in anthers and pollen only. Replacement of the promoter of the chalcone isomerase (*Chi*) gene by a fruit-specific promoter extended flavonol production to the edible parts of tomato (Muir et al. 2001). There is a variety of promoters that can be used to direct gene expression to fruit tissues. These promoters include the ethylene-responsive fruit-ripening E8 gene (Deikman et al. 1992) and the fruit-specific 2A11 gene (Van Haaren and Houck 1993). The most frequently used promoter for near-constitutive expression was isolated from the tomato ubiquitin-3 (tUbi3) gene (Hoffman et al. 1991).

## 4.5 Exploring the Diversity of Solanaceous Crops

Potato and tomato belong to the family of nightshades, which represent economically important plants for food, drugs, alkaloids, and ornamentals, while some species are also used in academic research. The immense genetic diversity in quality traits that evolved within this group of plant species offers tremendous potential for intragenic modification. A 1.1-kb P-DNA for petunia (*Petunia hybrida*) was recently assembled by linking fragments from three different DNA fragments together (Conner et al. 2007). Effective transfer of this P-DNA from *Agrobacterium* to petunia was confirmed by PCR-based plant genotyping.

Pepper (*Capsicum annuum*) is another potential target for intragenic modification. Despite the availability of border-like elements, however, a full pepper-derived P-DNA has not yet been constructed. There are also not yet any reliable promoters for either constitutive or fruit-specific gene expression available for either pepper or any of the molecularly less well characterized solanaceous crops such as eggplant (*Solanum melongena*) and tamarillo (*Cyphomandra betacea*), and further research is required to develop the minimum toolboxes for the intragenic modification of these crops. Based on the homology among nightshades, it may not be difficult to isolate the homologs of promoters that have proven efficacious in potato and tomato. Preliminary data indicate that the cytokinin P-DNA vectors can also be used for marker-free transformation of the recalcitrant plant species pepper when small amounts of auxins are added to the tissue culture media (M, Kalyaeva, personal communication).

## 4.6 Intragenic Modification of Alfalfa: Optimization of a Forage Feed

Alfalfa is the most productive and widely adapted forage species for dairy cows; it provides fiber that effectively stimulates chewing while also functioning as important source of energy and protein for milk production. Moreover, this perennial crop plays an important role in field rotations, contributing up to  $100 \text{ kg ha}^{-1} \text{ year}^{-1}$  of soil nitrogen. Its close relative fodder crop barrel medic (*Medicago truncatula*) has emerged as a model legume due to its short generation time, diploid nature and small genome, representing a valuable system for studies on nitrogen fixation and forage quality traits. Despite intensive efforts to develop improved alfalfa varieties through traditional plant breeding, the nutritional value of this forage crop is still limited by the large quantities of lignin that complex with proteins and restricts their usability (Reddy et al. 2005).

To address alfalfa's quality issues, we developed a new marker-free transformation method for an alfalfa-derived transfer DNA (Weeks et al. 2007). This P-DNA is more efficient than the T-DNA of *Agrobacterium* in promoting transfer. *Agrobacterium* plasmids carrying this transfer-DNA can be used to transform alfalfa by first cutting 2-day-old seedlings at the apical node. These seedlings are cold-treated and then vigorously vortexed in an *Agrobacterium* suspension also containing sand as an abrasive. About 7% of the infected "decapitated" seedlings produce intragenic shoots that transfer their P-DNA to the next generation. Efficacy of the seedling method resembled that of the *Arabidopsis* in vivo flower bud transformation method (Bent 2006). This "floral dip" procedure is based on dipping immature floral buds into a suspension containing *Agrobacterium*, 5% sucrose, and 0.05% surfactant L77 (see also Chap. 1). It has only been applied successfully to *Arabidopsis* and a few related Brassicaceae (Liu et al. 1998). The *Arabidopsis* transformation method is physiologically different from the new method for alfalfa because the former method targets the interior of the developing gynoecium (Desfeux et al. 2000) whereas the latter procedure is directed towards apical meristematic cells. Given the phenotypic similarities among seedlings of different species, the vortex-mediated *Agrobacterium* transformation method should be broadly applicable to other plant systems as well. Preliminary experiments applying this approach to other species have achieved relatively low transformation frequencies to date for canola and sugarbeet (*Beta vulgaris*; T. Weeks, personal communication).

The vortex-mediated seedling transformation method was applied to transform alfalfa with an all-native P-DNA comprising a silencing construct for the caffeic acid o-methyltransferase (*Comt*) gene (Weeks et al. 2007). The intragenic plants obtained from this experiment were down-regulated in expression for the *Comt* gene and, consequently, accumulated reduced levels of the indigestible fiber component S-lignin that lowered forage quality.

Another important issue in alfalfa breeding relates to the fact that yield improvement has remained stagnant for the past 20 years. Any genes involved in yield

enhancement could be linked to the strong and near-constitutive histone H3 promoter (Kelemen et al. 2002). The alternative 1.0-kb promoter of the alfalfa plastocyanin gene mediates high levels of foliage-specific expression that equal those obtained with the 35S promoter of figwort mosaic virus (Weeks et al. 2007) and could be used to overexpress genes involved in biomass production. Furthermore, the alfalfa PRP2 promoter directs gene expression to roots and has been used effectively to increase root biomass and enhance salt tolerance through overexpression of the alfalfa *Alfin1* gene (Winicov 2000). The 0.4-kb 3' sequences of the alfalfa small subunit ribulose-1,5-biphosphate carboxylase (*rbcS*) gene represents a reliable terminator that is more effective than the standard terminator of the *Agrobacterium* nopaline synthase (*nos*) gene (Weeks et al. 2007).

## 4.7 Exploiting Native Genetic Elements for Canola Oilseed Improvements

Rapeseed (*Brassica napus*) is the second largest oilseed crop after soybean (FAO 2007). The value of the vegetable oil from this crop is based on relatively high levels of oleic acid (~61% of total fatty acid content), which is stable during heat processing but does not represent a saturated oil. Some of the most interesting rapeseed cultivars contain less than 2% erucic acid and are trademarked by the Canadian Canola Association as “canola” (Sovero 1993). There are numerous avenues in additional improvements of canola oil characteristics, depending on specific application of the final product. For example, further increasing oleic acid levels and simultaneously reducing amounts of polyunsaturated fatty acids leads to the development of cooking oil with a higher oxidative stability without the need for a partial hydrogenation, which leads to the formation of unhealthy trans fatty acids that, although enhancing the heat stability of oil, are undesirable for human consumption. Additionally, in order to remain competitive with soybean, canola also needs other agronomic and quality improvements, such as increasing the seed size, pod-shatter resistance, and oil quality, as well as seed yield enhancements. Although conventional breeding was somewhat successful in developing improved canola cultivars with better oil quality characteristics, it still remains limited to the identification and use of appropriate germplasm to enhance even further the quality of oil. This constraint on traditional plant breeding became obvious in limiting the progress in development of canola varieties with very low levels of saturated fatty acids due to the lack of genetic diversity for this trait within *Brassica* species (Scarth and Tang 2006).

Various genetic elements for high levels of either constitutive or seed-specific gene expression have been isolated predominantly from rapeseed *B. napus* and characterized in transgenic canola. This list includes constitutive promoter such as the *fad2D* gene promoter (Shorrosh 2003) as well as several seed-specific promoters isolated primarily from the genes encoding seed storage proteins, such as the

oleosin gene promoter (Keddie et al. 1994), the cruciferin (*CruI*) gene promoter (Sjödahl 1995), or the *NapA* promoter isolated from the napin gene (Kohno-Murase et al. 1994). Furthermore, a native canola terminator (cruT) was used to terminate gene transcription in transgenic canola (Shorrosh 2000). More recently, two new transcription terminators (E9T, cabT) were isolated from the canola small subunit rubisco and chlorophyll a/b binding protein genes. Both these terminators were found to be at least as effective in terminating transcription as the nos terminator in transgenic plants. A *Brassica* P-DNA was developed by isolating a fragment that contained a 25-bp border-like element, designated here as Bo01, as well as flanking sequences known to support efficient DNA transfer. This fragment represents base pairs 112744–112889 of GenBank accession AC183493. Upon substitutions of three base pairs of Bo01, the fragment was used to replace the entire right border region of a binary vector. The resulting vector was shown to initiate DNA transfer as efficiently as the original T-DNA vector in both tobacco (*Nicotiana tabacum*) and canola. Isolation of a second DNA fragment (nucleotides 112865–113068 of AC183493) yielded a left border region delineated by Bo02. The efficacy of this region in terminating the DNA transfer process was optimized through four nucleotide substitutions in the border-like sequence. In tobacco, the *Brassica* left border-derived region mediated similar frequencies of backbone-free DNA transfer as conventional T-DNA vector. Transformation vector pSIM1346 carries both the left and right border regions from *Brassica* and was found to support effective transfer of the neomycin phosphotransferase (*nptII*) selectable marker gene from *Agrobacterium* to tobacco and canola cells in proof-of-concept experiments. Current efforts employ pSIM1346 derivatives carrying constructs designed to silence fatty acid desaturase genes to increase oleic acid levels to greater than 85% (O. Bougri, unpublished data).

*Arabidopsis thaliana*, a weed-like member of the family Cruciferae, offers multiple advantages for basic and applied plant research. Its short life cycle and fully sequenced small genome, combined with an extensive knowledge on its molecular biology, make *Arabidopsis* an ideal model for related plant species belonging to the genus *Brassica*. An *Arabidopsis* P-DNA was constructed by adding a chimeric right border to a fragment from chromosome 3 that already contained an intact left border-like sequence (Conner et al. 2007). Transformation of *Arabidopsis* with a P-DNA carrying an acetohydroxyacid synthase gene expression cassette yielded chlorsulfuron-resistant transformed plants (Conner et al. 2007).

## 4.8 Drought-Tolerant Perennial Ryegrass

Perennial ryegrass is one of the most important temperate pasture grasses and forms the biological foundation of the important meat, dairy, and wool export-based sectors of New Zealand. For this reason, a large genomics program based on the use of native genes and regulatory elements for the enhancement of ryegrass and

white clover is funded by the New Zealand government and the pasture-based primary industries. The targets are: (i) drought tolerance and extended geographical range and long-term viability of pasture farming under climate change, (ii) elevated dry matter yields under temperate and typical farming through increased productivity and persistence, and (iii) improved quality of forages provided to sheep and cattle. Ryegrass is an obligate outcrosser and so the challenge is to fix the greatest improvements. While good progress has been made via conventional breeding, molecular marker technologies are beginning to be applied to enhance genetic gains (Gill et al. 2006).

Intragenic approaches will be mandatory to achieve certain targets. For instance, plants are transformed with a P-DNA carrying an expression cassette for the ryegrass *Avp1*-like gene to enhance the plant's tolerance against drought (Bajaj et al. 2006; [www.isb.vt.edu/articles/aug0601.htm](http://www.isb.vt.edu/articles/aug0601.htm)). The native promoters used to ensure high constitutive gene expression levels were isolated by employing a novel GeneThresher gene sequencing approach combined with SAGE transcriptome analysis (Sathish et al. 2007).

## 4.9 Bruise-Tolerant Apple

Various research groups developed tool boxes for the intragenic improvement of apple (see Chap. 17). This important fruit tree is currently being transformed with a P-DNA carrying a silencing construct for polyphenol oxidase (*Ppo*) genes to limit the formation of dark quinone complexes and, consequently, develop bruise tolerance ([www.okspecialtyfruits.com](http://www.okspecialtyfruits.com)). The right border region of this P-DNA was obtained by linking a synthetic 22-bp sequence, which represents the part of the border that is not transferred, to an apple DNA fragment from CV630416 (inverse complement of nucleotides 339–405). A second DNA fragment derived from apple CO754245 (nucleotides 272–334) was fused to the trinucleotide sequence CCG to create a left border region. This region was optimized by substituting the 24th base pair of the border (from C to T). Resulting plants promise to produce non-browning apples, which translates into less shrinkage and enhanced display appeal.

## 4.10 Native Markers for Intragenic Transformation

Various studies have shown that some plant genes themselves can be used as transformation markers. For instance, the acetohydroxyacid synthase gene from *Arabidopsis* has been used to develop intragenic *Arabidopsis* plants displaying chlorsulfuron tolerance (Conner et al. 2007). Another interesting native marker system is based on protoporphyrinogen oxidase genes. When the plastidic protoporphyrinogen I oxidase gene from *Arabidopsis* was overexpressed under the control of the cauliflower mosaic virus 35S promoter in tobacco, the overproduction

of protoporphyrinogen oxidase rendered plants resistant to the action of herbicide acifluorfen (Lermontova and Grimm 2000). Maize (*Zea mays*) transformants expressing a modified protoporphyrinogen oxidase were produced via butafenacil selection using a flexible light regime to increase selection pressure (Li et al. 2003). Successful tobacco chloroplast transformation with a spinach betaine aldehyde dehydrogenase gene (Daniell et al. 2001) suggests that native genes involved in the conversion of betaine aldehyde can also be used as markers for plant transformation. Several additional native markers function effectively but trigger cytokinin responses, which confer an undesirable phenotype to the transformed plant (Sun et al. 2003).

It should be mentioned, though, that the presence of native marker genes in an intragenic crop may complicate the regulatory approval process. Governmental agencies only allow deregulation when the marker gene employed does not pose any biosafety issues. One way to avoid this issue is by placing the genes-of-interest and marker gene on separate binary vectors that are compatible in the same *Agrobacterium* strain. If the associated transfer-DNAs integrate at unlinked sites in co-transformed plants, they can be physically segregated in their progenies (Miller et al. 2002). Unfortunately, marker-removal methods are labor intensive and often too inefficient to allow their widespread use in commercial product development programs, especially in asexually reproducing or vegetatively propagated crops and in cases where large numbers of primary transformation events are required.

## 4.11 Intragenic Crops Are at Least as Safe as Those Developed Through Traditional Methods

Intragenic modifications improve the agronomic performance or nutritional characteristics of crops but do not introduce traits that are new to the sexual compatibility group. The modified plants lack selectable marker genes, powerful insecticidal genes, or any other foreign genes that are new to agriculture or the food stream. Furthermore, the altered expression of one or several native genes is not expected to trigger phenotypic, biochemical, or physiological variation that is not already present in the sexual compatibility group (Rommens et al. 2007). One argument for this assumption is that any modification accomplished through all-native DNA transformation could, at least theoretically, be created by conventional breeding. Furthermore, any intragenic modification of gene expression levels is likely to fall within the extensive allele-specific differences that evolved naturally (Kliebenstein et al. 2006). At one end of the spectrum are the knock-out, or loss-of-function mutations, that can be isolated for many non-essential genes in natural populations and are obtained at higher frequency using either natural or chemical mutagens. Individuals with enhanced gene expression, at the other end of the spectrum, may be recovered during plant selection, such as those adapted to specific

environmental stresses. Both classes yield rare phenotypes pursued by breeders that can often be developed using intragenics.

Thus, new varieties developed through intragenic modification represent low-risk crops that could be cleared through the regulatory process in a timely and cost-effective manner. For example, whilst a case-by-case approach remains the pragmatic option, approval for release should not require extensive studies on potential environmental effects but should rather confirm that the nutritional profile falls within the range established for untransformed plants that belong to the same sexual compatibility group. Any increases in the amount of important toxins that exceed the biochemical variability of a species and/or recommended maximum concentrations would require further assessments. Similarly, decreases in the concentration of valuable compounds including vitamin C and essential amino acids would trigger studies on the potential impact of these changes on the nutritional value of the crop.

## 4.12 Conclusions

Traditional methods in plant breeding rely on random genome modifications and are often difficult to apply to either eliminate undesirable features or activate dormant traits. This issue is effectively addressed by precisely recombining native elements in vitro and inserting the resulting expression cassettes into plants using marker-free and all-native DNA transformation. The intragenic method is exemplified by potato varieties displaying enhanced flavor and tolerance against black spot bruise and cold-induced sweetening while accumulating greatly reduced amounts of neurotoxic acrylamide. By employing the plant's own DNA and excluding foreign DNA transfer, intragenic plants are at least as safe as those developed through traditional plant breeding. American regulatory agencies are currently considering a revamp of the regulatory approval process, by assigning new modified products and crops into risk categories. If assigned as low risk, intragenic technologies could be readily applied for numerous improvements of specialty crops.

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# Chapter 5

## Gene Silencing in Plants: Transgenes as Targets and Effectors

Andreas E. Müller

### 5.1 Introduction

Unlike animals and other non-plant systems, and with the notable exception of moss, plants lack an efficient homologous recombination system that can be used for gene targeting approaches to disrupt or replace endogenous genes by modified transgenic versions (for methodology, see Chaps. 1, 2) with altered patterns of activity or functionality (Tzfira and White 2005). While for model plants such as *Arabidopsis thaliana* and a few other species this limitation was overcome (at least in part) by the development of large T-DNA or transposon insertion mutant collections that can be conveniently screened for allelic variants and loss-of-function alleles of a gene-of-interest (Østergaard and Yanofsky 2004; Candela and Hake 2008; Jung et al. 2008), resources such as these are generally not available for crop species. A valuable alternative that is more widely pursued for applications in crop plants is the use of non-insertional mutagenesis, e.g. by the chemical agent ethyl-methane sulphonate and mutation detection by TILLING (Slade and Knauf 2005; Comai and Henikoff 2006). The random nature of mutational approaches, however, is unfavorable to the engineering of “traits by design” according to the specifications of intended applications in agriculture, horticulture, or forestry.

Genetic modification methods that do not rely on mutation in one way or another involve changes in gene expression. The level and regulation of gene expression over time and space is a major determinant of the phenotypic manifestation of a trait that a gene is causally involved with. Not surprisingly then, gene expression is controlled by elaborate regulatory pathways that ensure appropriate expression during development and under changing external conditions, and that also include mechanisms to turn off or “silence” a gene as part of developmental programs, in

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response to environmental cues, or as a defense strategy against the propagation of foreign (e.g. viral) sequences. Gene-silencing processes in plants are epigenetic in nature, i.e. they do not alter the sequence of their targets, but act through modifications of DNA and its structural context within chromatin, or by RNA-mediated control mechanisms. This chapter reviews the natural mechanisms in plants that silence gene expression and are thought to also act on transgenes, and thus attempts to highlight some of the pitfalls of genetic engineering by transgenesis. The chapter also includes a brief overview of the technical advances that have converted the natural phenomenon of gene silencing from an obstacle to genetic modification into one of the most powerful tools in biotechnology, RNA interference. Because of the vast body of research on gene silencing and the limited space available for this chapter, the reader is referred to more detailed reviews whenever possible.

## 5.2 Mechanisms of Gene Silencing

Gene silencing can be broadly attributed to processes that occur at the transcriptional (transcriptional gene silencing, TGS) or post-transcriptional level (post-transcriptional gene silencing, PTGS; now also referred to as RNA silencing).

Down-regulation of gene expression by TGS:

1. Involves inhibition of primary transcription by epigenetic mechanisms and generally correlates with DNA methylation of the promoter and chromatin condensation (heterochromatinization),
2. Is frequently induced and/or maintained by small RNA-mediated processes including RNA-directed DNA methylation and RNA-directed chromatin condensation,
3. Is not graft-transmissible, which was suggested to be due to the nuclear compartmentalization of TGS (Mourrain et al. 2007),
4. Is mitotically and meiotically heritable but may also be reversed, a process that is accompanied by loss of DNA methylation, can occur gradually over several generations, and is often observed when the initial trigger of silencing is removed.

Down-regulation of gene expression by PTGS:

1. Acts on mRNA in the cytoplasm and generally results in transcript degradation,
2. Is a sequence-specific process that is mediated by small RNA molecules,
3. Can spread systemically and is graft-transmissible,
4. Is meiotically not heritable in the absence of the initial trigger but in some cases may be maintained throughout the lifespan of a plant even when the trigger of silencing is removed,
5. Can involve RNA-directed DNA methylation of the transcribed region of a gene.

TGS and PTGS can also be distinguished by distinct enzymatic machineries and inhibitors, although some proteins appear to be involved in both processes.

While the terms TGS and PTGS have often been used to categorize silencing events, it is now well established that these processes are strongly interrelated and exert mutual effects on one another, and it has been suggested that both derive from a common ancestral mechanism (for recent reviews on RNA silencing and regulatory crosstalks between transcriptional and post-transcriptional processes in plants, see Grant-Downton and Dickinson 2005; Brodersen and Voinnet 2006; Henderson and Jacobsen 2007; Huettel et al. 2007; Eamens et al. 2008a; Hollick 2008; Pikaard et al. 2008). In particular, small RNA molecules that are an essential intermediate of PTGS are also effectors of DNA methylation and TGS, and – conversely – transcription of methylated templates is required for the maintenance of PTGS (Eamens et al. 2008b and references therein). The following section will therefore briefly review the production and significance of small RNAs as intermediates of gene-silencing pathways.

### 5.2.1 *The Role of Small RNAs*

Small RNA (sRNA) molecules entered center stage in 1999 when Hamilton and Baulcombe discovered that the presence of sRNA approximately 25 nt in length correlates with PTGS in different experimental systems in plants. The authors proposed that these molecules represent the sequence specificity determinant of PTGS. Since then, further extensive and often impressive research has confirmed this idea and revealed that there are in fact a number of different classes of sRNA molecules ranging in size from ~20 nt to ~25 nt and associated with several related but distinct RNA-mediated silencing pathways (for reviews, see Brodersen and Voinnet 2006; Chapman and Carrington 2007; Eamens et al. 2008a; Ramachandran and Chen 2008). These pathways can be distinguished according to their triggers, (sub-)specialized enzymatic activities, the sizes of the sRNA species involved, and their downstream targets, but in essence they all share a common cascade of events. In brief, sRNAs are produced from partially self-complementary or double-stranded RNA (dsRNA) that is formed as part of natural gene regulatory processes or defense mechanisms against plant pathogens. The dsRNA molecules are cleaved into sRNA duplexes by members of a family of ribonuclease III enzymes, termed Dicer-like (DCL) 1-4 in *A. thaliana*. The duplexes are unwound into single-stranded sRNA molecules and incorporated into an “RNA-induced silencing complex” (RISC). This complex contains a member of the sRNA-binding family of Argonaute proteins (of which there are ten in *A. thaliana*, AGO1-10) and is guided to single-stranded target RNA with perfect or near-perfect sequence complementarity to the sRNA component of the complex. In plants, a major mode of action following pairing of guide and target RNA is endonucleolytic cleavage by the ribonuclease activity of some Argonaute proteins and degradation of the target RNA, although translational repression of the target RNA also appears more widespread than previously recognized (Brodersen et al. 2008). Both modes of

sRNA action effectively silence target genes by preventing the synthesis of target gene-encoded proteins.

The biological functions of natural sRNA-mediated silencing pathways in plants range from endogenous gene regulation to host defenses against plant pathogens and formation of heterochromatin. Correspondingly, the dsRNA molecules that are the substrates for the sRNA production cascade outlined above originate from various sources (Sects. 5.2.1.1–5.2.1.4).

### 5.2.1.1 MicroRNA Genes

An abundant class of endogenous genes are transcribed into partially self-complementary primary transcripts with characteristic secondary structures that are further processed (in a pathway which includes DCL1) into a subclass of 21/22-nt sRNA molecules termed microRNA (miRNA; Mallory et al. 2008). Plant miRNAs have been found to silence target genes involved in developmental regulation but may also have functional roles in plant metabolism and environmental adaptation (Brodersen and Voinnet 2006; Jones-Rhoades et al. 2006).

### 5.2.1.2 Natural Sense–Antisense Gene Pairs

Genome-wide analyses have identified more than 1000 putative sense-antisense gene pairs in *Arabidopsis* (Wang et al. 2005; Jin et al. 2008). DsRNA from these gene pairs is generated by transcription from opposite strands of a single genomic locus, producing complementary pairs of sense transcripts and “*cis*-natural antisense transcripts” (*cis*-NATs). DsRNA can also be formed following transcription of sense and antisense transcripts from different loci, with the latter then being referred to as *trans*-NATs (Wang et al. 2006). Recent data suggest that one function of natural antisense transcripts is the regulation of the corresponding sense genes in response to environmental and developmental cues via a subclass of 24-nt sRNA molecules that in *Arabidopsis* are produced by DCL2 and have been dubbed nat-siRNA (natural antisense transcript – short interfering RNA; Borsani et al. 2005; Jin et al. 2008). Nat-siRNAs have also been implicated in regulation of plant immunity against bacterial pathogens (Katiyar-Agarwal et al. 2006; Jin 2008).

### 5.2.1.3 Viral Genomic RNA, Transcripts, and Replication Intermediates

During virus infection, secondary structures within viral RNA genomes, sense-antisense transcript pairs, and double-stranded RNA replication intermediates may all provide the dsRNA precursors of the sRNAs (21–24 nt) referred to as viral siRNAs or viRNAs (Ding and Voinnet 2007; Mlotshwa et al. 2008; Obbard et al. 2009). All four DCL proteins in *Arabidopsis* function in anti-viral defense pathways, although DCL4 appears to play a major role. A striking additional source of

dsRNA results from the activity of plant-encoded RNA-dependent RNA polymerases (RDRs; Wassenegger and Krczal 2006; Voinnet 2008). At least one member of this protein family in *Arabidopsis*, RDR6, is thought to recognize “aberrant” features of transcripts as they may be found in transcripts encoded by viral and other non-plant genes (including transgenes) and to use such transcripts as a template for complementary RNA synthesis. RDRs are also thought to provide one component of a self-reinforcing amplification loop of RNA silencing. According to a current model, sRNA molecules anneal to their single-stranded target RNA and serve as primers for RDR-mediated production of the complementary strand, thereby again generating dsRNA. The sRNA molecules that are processed from the newly generated dsRNA are referred to as secondary siRNAs and in some cases may also target sequences without homology to the initial trigger of silencing, a process known as transitive silencing (for details, see Voinnet 2008).

### 5.2.1.4 Heterochromatic and Repetitive Sequences

Finally, dsRNA is also produced from transposons and other repetitive sequences in heterochromatin, i.e. cytologically distinct and gene-poor chromosomal regions that remain condensed throughout the cell cycle (reviewed by Matzke et al. 2007; Girard and Hannon 2007; Huettel et al. 2007; Chan 2008). The sequence of events is believed to include transcription of methylated sequences by the plant-specific putative RNA polymerase Pol IV (Pol IVa), although the exact mode of action of this enzyme is still subject to investigation (Pikaard et al. 2008). Pol IV and the RNA-dependent RNA polymerase RDR2 by their combined action produce dsRNA and act in the same RNA silencing pathway as DCL3. The 24-nt sRNAs produced by this pathway direct DNA methylation and/or heterochromatin formation at the repeated sequences they were derived from and copies thereof elsewhere in the genome. The presumed role of Pol IV in sRNA biogenesis from methylated sequences may be part of a self-sustaining cycle that maintains the methylated and heterochromatic state of repetitive sequences and thus provides one line of defense against unchecked proliferation of transposable elements. At least at some heterochromatic loci in the *Arabidopsis* genome, this cycle also requires low-level transcription by a second plant-specific polymerase which is now referred to as Pol V (formerly Pol IVb; Wierzbicki et al. 2008).

### 5.2.2 Epigenetic Silencing of Transcription

Although many silencing phenomena are now known to involve sRNAs, it is also abundantly clear that epigenetic suppression of transcriptional activity is a potent second constituent of gene silencing. The two fundamental pillars of epigenetic regulation of transcriptional activity are chromatin modification and DNA methylation (reviewed by Chan et al. 2005; Grant-Downton and Dickinson 2005; Pfluger

and Wagner 2007; Zilberman 2008) and several hundred genes have been implicated in these processes in plants (Gendler et al. 2008). A distinctive feature of epigenetic modifications is their heritability through mitosis and meiosis, while they also retain the capacity to be reversed. The prime example of genomic regions which are subject to repressive epigenetic modifications are transposons and other repetitive sequences in heterochromatin. Both initiation and maintenance of transposon silencing are now thought to also involve sRNA-mediated processes (see Sect. 5.2.1.4). However, mutations in genes required for chromatin remodelling and DNA methylation result in reactivation of transposon activity, thus highlighting the importance of epigenetic modifications for stable repression (Lippman et al. 2003, 2004; Kato et al. 2004).

Prominent examples of endogenous genes whose transcriptional activity is silenced by epigenetic mechanisms include the *Arabidopsis* gene *FLC*, a key regulator of floral transition (reviewed by Sung and Amasino 2005; Dennis and Peacock 2007; Henderson and Jacobsen 2007), and several imprinted genes, including *FWA* and *MEDEA* (reviewed by Zilberman 2008). In brief, *FLC* acts as a repressor of flowering but can be silenced in response to prolonged exposure to cold over winter. Epigenetic silencing of *FLC* transcription involves post-translational histone modifications at the *FLC* locus including histone deacetylation and histone methylation, and further stabilization by structural protein complexes that bind to modified histones. Because these modifications are stably maintained through mitotic cell divisions upon return to warmer temperatures they provide a memory of winter, which allows the plant to flower under inductive conditions in the following spring. In contrast to mitotic divisions, however, *FLC* expression is reset by a yet unknown mechanism during meiosis.

*FWA* is a good example that demonstrates the importance of DNA methylation in epigenetic regulation of gene activity. *FWA* expression is regulated by genomic imprinting, i.e. parent-of-origin-dependent expression of only one of the parental alleles at a given locus. At the *FWA* locus, methylation of the promoter is the default state of both alleles in vegetative tissues and correlates with inactivity of the gene (Soppe et al. 2000). In the endosperm, however, only the paternal allele is silent and methylated whereas the maternal allele is expressed and non-methylated (Kinoshita et al. 2004). The significance of methylation for silencing of *FWA* was further demonstrated by the isolation of a hypomethylated epi-allele of *FWA* that is ectopically expressed (Soppe et al. 2000) and by the detection of paternal *FWA* transcripts in the endosperm of a mutant that is impaired for DNA methylation activity (Kinoshita et al. 2004). Importantly, the latter study also showed that differential regulation of methylation and expression of maternal and paternal alleles in the endosperm depends not on the de novo methylation of the paternal allele, but on the active demethylation of the maternal allele. Although these studies convincingly demonstrate the importance of methylation in *FWA* silencing, repression of *FWA* transcription also involves chromatin modifications that at least in part resemble those described for the *FLC* locus (Jiang et al. 2007). It should also be noted that, while the examples above involve epigenetic silencing at the transcriptional level, it is likely that sRNA-mediated processes contribute to gene regulation

at these loci. For *FLC*, this in fact was recently suggested (Swiezewski et al. 2007; Zhai et al. 2008).

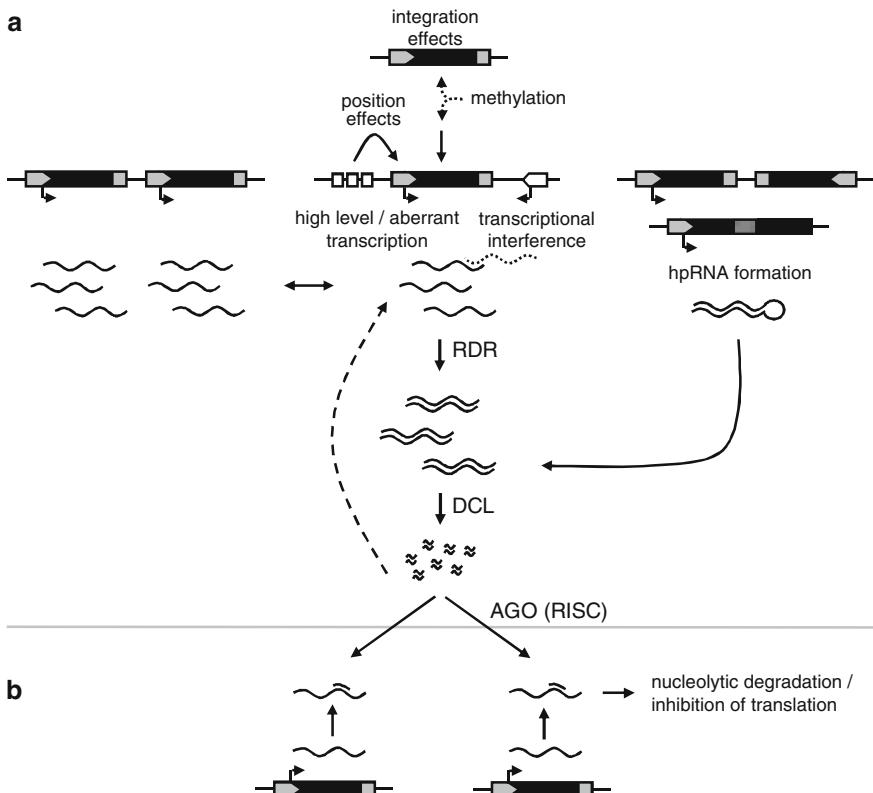
## 5.3 Silencing of Transgene Expression

The intricate natural gene-silencing mechanisms described in Sect. 5.2 provide the backdrop against which also the silencing of transgenes is to be understood. Transgene expression levels frequently follow a bi-modal distribution, with a small subset of plants showing high transgene expression but a majority of transformants in which the transgene is silenced or only weakly expressed (Butaye et al. 2005 and references therein). In addition, transgenes that are initially expressed in the primary transformants may be subject to silencing during plant development or in subsequent generations. The following sections discuss a number of risk factors that are thought to render a transgene the target of gene silencing. Induction of silencing can occur *in cis*, i.e. by processes at the transgene locus, or *in trans* by mechanistic pathways that require the presence of sequences with homology to the transgene locus (for an overview see Fig. 5.1).

### 5.3.1 Cis- and Trans-Silencing Of Multi-Copy Transgenes

It is now widely accepted that integration of multiple transgene copies into a host genome leads to an increased risk of transgene silencing (for early references, see Jones et al. 1987; Matzke et al. 1989; Hobbs et al. 1990). Multiple transgene copies often integrate as direct or inverted repeats or undergo additional rearrangements resulting in complex transgene structures at the insertion site. An early compelling example of repeat-induced *cis*-silencing in *Arabidopsis* was provided by Assaad et al. (1993), who reported that direct repeats of a transgene were silenced whereas the transgene was expressed in recombinant lines with only a single-copy of the gene at the same chromosomal position. Perhaps more frequently, however, silent transgene loci were found to be arranged as inverted repeats (for examples, see Muskens et al. 2000). Transgene repeat loci are often methylated and packaged into dense chromatin, the hallmarks of transcriptional silencing, but are also frequently found to produce dsRNA and sRNA, the occurrence of which is associated with the capacity of these loci to silence homologous genes *in trans* (e.g. Fojtová et al. 2006; Mourrain et al. 2007).

Indeed, directly or invertedly repeated transgene loci (whether the result of complex integration events or by design of the transgene construct) are often efficient effectors of sRNA-mediated *trans*-silencing if effector and target genes share homology within transcribed regions (e.g. Waterhouse et al. 1998; Muskens et al. 2000; Ma and Mitra 2002; Fojtová et al. 2006; Xu et al. 2009). Today of course they are also the basis of a standard implementation of RNA interference



**Fig. 5.1** Transgenes as targets and effectors of gene silencing. *Cis*-silencing effects are shown in **a**, *trans*-effects in **b**. Transgene integration sites in the plant genome can carry direct repeats, a single-copy insert, or inverted repeats of the transgene (depicted from left to right in **a**). All configurations may be subject to silencing. For simplicity, *cis*-silencing effects are only shown for single-copy inserts. *Cis*-silencing can be induced by integration and position effects, and in these cases involves DNA methylation (dotted arrows) and transcriptional silencing, or by high level and/or aberrant transcription, e.g. from strong or cryptic promoters. The risk of high transcript accumulation may be enhanced in multi-copy transformants such as those with directly repeated inserts (*left*). The resulting transcripts are recognized and converted into dsRNA by an RNA-dependent RNA polymerase (RDR6 in *A. thaliana*), and further processed by Dicer-like (DCL) proteins into short interfering RNA (siRNA). In a self-reinforcing amplification loop of RNA silencing, siRNA molecules may anneal to single-stranded primary transcripts (dashed arrow) and thus prime a second round of RDR-mediated dsRNA production followed by processing into secondary siRNAs. Primary and/or secondary siRNAs may also direct DNA methylation to the transgene insert or other homologous target loci (not shown). Transgene loci carrying invertedly repeated inserts (*right*) produce partially self-complementary hairpin RNA (hpRNA). Because hpRNA is partially double-stranded and a direct substrate of DCL proteins, transcription from inverted repeat transgenes bypasses the upstream events required for siRNA production from single-copy (sense) transgenes and eliminates the requirement for RDR function. Finally, siRNAs effect sequence-specific *trans*-silencing by guiding an Argonaute protein (AGO)-containing RNA-induced silencing complex (RISC) to complementary single-stranded transcripts derived from one or more transgenic or endogenous target genes (**b**). Silencing occurs by nucleolytic degradation of

technology (see Sect. 5.4). Moreover, several transgene repeat loci have been shown to *trans*-silence transgenes at non-allelic positions in a promoter homology-dependent manner, e.g. the tobacco loci *H<sub>9NP</sub>* (Mette et al. 1999, 2000) and 271 (Vaucheret 1993; Vaucheret et al. 1996; Mourrain et al. 2007). These two silencer loci consist of complex sequence arrangements including inverted and/or direct repeats of transgene promoters and have been shown to produce promoter-derived dsRNA and sRNAs (Mette et al. 2000; Mourrain et al. 2007). *Trans*-silencing may also be induced by simple transgene inserts. A classic example for which an elaborate phenomenology was developed is cosuppression of Chalcone synthase (*Chs*) trans- and endogenes in petunia (reviewed by Jorgensen 2003; Jorgensen et al. 2007). Jorgensen and colleagues established that cosuppression does not require the presence of inverted repeat or antisense transgenes but can also be mediated by transgenes that carry only a sense copy of the *Chs* coding sequence or part thereof. *Chs* cosuppression by sense transgenes results in flower pigmentation phenotypes that are distinct from those produced by either inverted repeat or antisense transgenes and requires high-level transcription of the transgene (Que et al. 1997; Jorgensen et al. 2007).

The general view that evolved from detailed molecular and genetic characterization of these and other silencing events involving two or more homologous sequences is that silenced genes that share homology within the transcribed region are silenced post-transcriptionally and non-heritably (in the absence of the inducer locus), and those that are homologous to each other within the transgene promoter are silenced at the transcriptional level and in a heritable but often reversible manner (Matzke et al. 2002). Both silencing phenomena involve sRNAs that either direct transcript degradation, or promoter methylation and chromatin condensation, respectively. While dsRNA production from inverted repeat loci by transcription across both repeat units and annealing of complementary transcript regions is thought to be a straightforward process, the production of dsRNA from direct repeats or simple sense transgenes may require complementary RNA strand synthesis by RDRs similar to the processes in anti-viral defense pathways (see Sect. 5.2.1.3; Schubert et al. 2004). Post-transcriptional transgene silencing may also be accompanied by RNA-directed DNA methylation within transcribed regions. Although methylation within transcribed regions often has no obvious effects on the rate of transcription (Elmayan et al. 1998; Mourrain et al. 2007; Lunerová-Bedřichová et al. 2008), there is recent evidence that it is required for the maintenance of post-transcriptional silencing (Eamens et al. 2008b; reviewed by Voinnet 2008).

◀ Fig. 5.1 (continued) the target RNA or inhibition of translation. *Filled boxes* Genes, *gray arrows* promoters, *gray boxes* polyadenylation signals, *white (unfilled) boxes* repetitive, heterochromatic flanking genomic sequences, *white arrow* cryptic promoter, *black arrows* transcription start sites, *dark gray box* spacer fragment in inverted repeat constructs for intentional production of hpRNA, *wavy lines* transcripts and RNA processing products, *dotted wavy line* transcripts produced from cryptic promoters

### 5.3.2 Silencing of Single-Copy Transgenes

Although silencing of transgenes is more frequently observed in plants carrying multiple transgene copies, there are reports of transgene silencing in single-copy transformants (e.g. Elmayan and Vaucheret 1996; De Wilde et al. 2001; Meza et al. 2002; De Buck et al. 2004; Eike et al. 2005). Silencing of single-copy transgenes has been variably attributed to effects of the chromosomal integration site (“position effects”), or induction of RNA-mediated processes.

Of all phenomena that have been attributed to gene silencing, position effects remain among the most enigmatic. The term is used to describe effects of the chromosomal integration site on transgene expression and are thought to be an epigenetic phenomenon which is mediated by the spreading of transcriptionally inactive chromatin and/or DNA methylation states from flanking genomic regions into the transgene (Matzke and Matzke 1998). In a comparative study of transgene expression, single-copy transgenes showed up to tenfold differences in expression levels between inserts at different chromosomal positions in the tobacco genome (Day et al. 2000). A convincing example of a position effect on a transgene in *Arabidopsis* was reported by Finnegan et al. (2004), who showed that a transgene integrated in the vicinity of the floral repressor gene *FLC*, whose expression is down-regulated by epigenetic mechanisms in response to prolonged periods of cold (see Sect. 5.2.2), also acquires a low-temperature response. However, several studies (including a comprehensive analysis of >100 independent *Arabidopsis* transgenic lines with single-copy T-DNA inserts) concluded that position effects are not a major determinant of variability in transgene expression (Hobbs et al. 1990; Jorgensen et al. 1996; De Buck et al. 2004; Schubert et al. 2004; Nagaya et al. 2005). The authors of the comprehensive study noted, however, that the selection of primary transformants was dependent on expression of a selectable marker gene and thus precluded the recovery of lines in which the transgene may have been silenced by position effects (Schubert et al. 2004). Consistent with this notion, a comparison of the genome-wide distribution and expression level of T-DNA integration sites between transgenic *Arabidopsis* plants or suspension cells identified with or without a requirement for marker gene expression suggested that selection pressure might shift the fraction of T-DNA inserts that are recovered into transcriptionally active regions of chromatin (Francis and Spiker 2005; Kim et al. 2007) and, by extrapolation, that transgenes inserted into transcriptionally inert heterochromatic regions may be prone to silencing by position effects. Moreover, recent data by Fischer et al. (2008) suggest that the chromosomal position in *Arabidopsis* also affects the susceptibility of simple transgene inserts to RNA-directed DNA methylation and transcriptional silencing induced by a *trans*-silencing locus, with low-complexity repetitive sequences in the vicinity of a transgene insertion site promoting silencing. Finally, given the large genome size of many crop plants and their comparatively high content of heterochromatin, position effects may be a more frequent occurrence in these species than in *Arabidopsis*.

In a small number of reports, single-copy transgenes appear to be silenced by processes other than those mediating position effects. In one study, Day et al. (2000) directed integration of a *gusA* transgene to a previously introduced single-copy transgenic target site in the tobacco genome by Cre-mediated site-specific recombination. Surprisingly, only some of the independent integrant lines for a given target site gave rise to the expected pattern of transgene expression (i.e. for the specific viral promoter used in this study, expression throughout the plant in vascular tissue), whereas the remaining integration events at the same locus resulted in various degrees of silencing that correlated with methylation of the integrated DNA. Models to account for the observed differences in transgene expression include somaclonal variation as a result of differential induction of methylation activity in integrants by environmental stress during regeneration in tissue culture. As alternative explanations, Day and colleagues suggested that either the integrant DNA might be particularly susceptible to methylation, possibly due to secondary structures formed prior to or during integration, or that transient production of RNA from the extrachromosomal DNA prior to integration could lead to RNA-directed DNA methylation of the newly integrated transgene. The apparent stochastic nature of silencing may derive from considerable differences in the number of DNA molecules entering individual recipient cells during the polyethylene glycol-mediated protoplast transformation procedure used in this experimental system, with cells having high initial copy numbers of extrachromosomal DNA molecules being more prone to RNA-directed methylation of the incoming DNA (Ow 2002). Because transgene silencing was not observed following biolistic transformation of rice in an otherwise similar experimental setup, it was proposed that the method of transformation and the resulting different quantities of DNA that are delivered into a recipient cell affect the probability of induction of silencing (Srivastava et al. 2004).

Single-copy transgenes that are initially expressed in seedlings can also be subject to post-transcriptional silencing during further plant development (e.g. Elmayan and Vaucheret 1996). Current models assume that silencing is triggered by transcript accumulation above a certain threshold and/or aberrant transcripts (e.g. resulting from high-level transcription or transcription from cryptic promoters at the transgene insertion site) that are recognized and converted into dsRNA by RDR6 (Brodersen and Voinnet 2006). If aberrant transcripts initiated at cryptic promoters span the transgene promoter the transgene may also become silenced by RNA-directed transcriptional silencing (Eike et al. 2005).

### 5.3.3 Reducing the Risk of Transgene Silencing

Strategies to reduce the occurrence of homology-dependent transgene silencing include selection of single-copy transformants, site-specific recombination to remove repeat structures at transgene loci or to target transgenes to pre-selected integration sites that are favorable to transgene expression, and avoidance of

sequence duplicates (e.g. promoters) within the transgene cassette (reviewed by Butaye et al. 2005). A number of precautions may also minimize the risk of single-copy transgene silencing as a consequence of aberrant or high-level transcription, including a prudent design of transgene cassettes. For example, it has been suggested to avoid the use of strong transgene promoters, to eliminate cryptic promoters from transgene cassettes, to prevent transcriptional interference (e.g. as a result of unintended transcription initiated outside the transgene and proceeding into the transgene) by inclusion of transcription terminator or “blocker” sequences, and to temporally or spatially limit transgene expression by use of appropriate promoters (Müller and Wassenegger 2004; Butaye et al. 2005; Eike et al. 2005; and references therein). Alternative approaches take advantage of our increasing understanding of the genetic basis of gene-silencing pathways. Promising strategies include the use of gene-silencing mutants as host genotypes for transgene integration; and stable, high-level transgene expression has indeed been demonstrated using *Arabidopsis* mutants impaired for RDR6 function (*sgs2*) or for a putative RNA-binding activity upstream of RDR6 (*sgs3*) in the anti-viral defense pathway (see Sect. 5.2.1.3) that is also thought to act on transgenes (Butaye et al. 2004). Although mutations in these genes do not appear to have any other phenotypic consequences in *Arabidopsis* under standard conditions in the growth chamber, their function in plant defense pathways necessitates careful evaluation of similar strategies for potential applications in crop species.

Efforts to protect transgenes from position effects have yielded mixed results. While a number of matrix attachment region (MAR) elements were shown to reduce expression variability and/or increase expression of transgenes when placed next to the transgene within an expression cassette, the extent of this effect varied widely between individual MAR elements, reporter gene systems, and plant species tested (Allen et al. 2000; Petersen et al. 2002; De Bolle et al. 2003; Levin et al. 2005; Zhang et al. 2009). A recent technical advance in *Arabidopsis* combines the use of the PTGS mutants referred to above (*sgs2* and *sgs3*) and MAR elements flanking the transgene (Butaye et al. 2004; De Bolle et al. 2007; Sels et al. 2007). This PTGS-MAR expression system showed reduced variability in transgene expression among transformants (which included plants with multi-copy transgene inserts) and an increased average transgene expression level. Both of these effects were also seen in the PTGS mutant backgrounds in the absence of the MAR elements and most likely are the result of impaired PTGS. Inclusion of MAR elements did not further reduce expression variability but led to a significant further increase in transgene expression levels.

## 5.4 Applications of RNA Interference in Transgenic Plants

Gene-silencing processes have been widely exploited as versatile experimental and biotechnological tools for functional gene studies and transgenic approaches to crop improvement, disease resistance, and metabolic engineering (see Chap. 11).

These applications first and foremost rely on the *trans*-silencing capacity and the sequence specificity of RNA-mediated silencing mechanisms and are often collectively referred to as RNA interference (RNAi) techniques, in reference to the related RNA silencing mechanism in *Caenorhabditis elegans* (Fire et al. 1998).

Homology-dependent RNA-mediated silencing techniques such as antisense suppression or sense cosuppression have been in use for a number of years and share a common mechanistic core (see Sect. 5.2.1). However, transcription of self-complementary, hairpin-like RNA duplexes from inverted repeat transgenes was shown to be a much more potent effector of target-gene silencing (Waterhouse et al. 1998; Wang and Waterhouse 2000; Smith et al. 2000). In brief, inverted repeat transgene cassettes are engineered to contain two copies of a cDNA segment, typically 300–800 bp in length, from a gene of interest so that they are arranged as inverted repeats, separated by a spacer sequence, and driven by a promoter (for details on vector construction for RNAi in various plant species, see Wesley et al. 2001; Meyer et al. 2004; Miki and Shimamoto 2004; Helliwell et al. 2005; McGinnis et al. 2005; Wielopolska et al. 2005; Dafny-Yelin et al. 2007; Luo et al. 2008). Alternative approaches include the use of a heterologous 3' untranslated region arranged as an inverted repeat and integrated into the transcription unit of a transgene (Brummell et al. 2003), transgenic expression of hairpin RNA with homology to endogenous target promoters, an approach that is based on the original discoveries by Mette et al. (1999, 2000; see Sect. 5.3.1), convergent transcription of gene fragments arranged as inverted repeats and positioned between oppositely oriented promoters (Yan et al. 2006), and the use of artificial miRNAs (Schwab et al. 2006; Warthmann et al. 2008). The latter approach is a particularly promising strategy towards maximal specificity of silencing and minimal off-target effects, and may allow the specific inactivation of alleles or splice variants of a given target gene. Considerations for the design of transgene cassettes and their potential for applications of RNAi in plants have been reviewed in detail elsewhere (Müller 2006; Ossowski et al. 2008).

The main features of RNAi that are relevant for experimental and biotechnological applications and distinguish RNAi from mutagenesis approaches are:

1. Sequence specificity, which allows targeting of individual genes without the need to generate and screen large mutant populations,
2. Genetic dominance, which allows detection of phenotypic effects in hemizygous primary transformants and provides opportunities for applications in crop species which are cultivated as hybrids,
3. The relative ease of generating multiple transgenic lines with different degrees of silencing and phenotypic effects, e.g. by adequate use of transgene promoters,
4. Inducibility and temporal or spatial confinement of target gene activity,
5. The ability to target multiple related genes simultaneously, which may help to alter phenotypes encoded by members of multigene families with redundant gene activities or in polyploid species,
6. The ability to target multiple unrelated genes simultaneously (with distinct RNAi effector sequences integrated into a single inverted repeat construct).

### 5.4.1 Applications of RNAi for Crop Protection

As described in Sect. 5.2.1, one of the biological roles of RNA silencing is its participation in host defense processes, and inverted repeat-induced RNAi is now thought to act through the anti-viral defense pathway of RNA silencing (Fusaro et al. 2006). Many successful attempts to engineer virus resistance by transgenic approaches with virus-derived sequences, including at least some of the original coat protein expression experiments, are based on RNA silencing (reviewed by Lindbo and Dougherty 2005) and merely enhance natural anti-viral defense systems in plants. Transgenic expression of inverted repeats with homology to a target virus is thought to enable a plant to respond without delay to a virus attack, thus precluding the counter-defense efforts that are mounted by the virus in the course of normal infections (Prins et al. 2008; Obbard et al. 2009). The high efficiency of RNAi approaches facilitates its application in a wide range of species including those that are notoriously recalcitrant to transformation. The use of RNA silencing is particularly valuable in the fight against pathogens for which natural resistance has not been observed or is a complex genetic trait that is not encoded by a single resistance gene (Campbell et al. 2002). Transgenic RNA-mediated resistance can be engineered against a wide variety of both RNA and DNA viruses, although the most successful examples to date target RNA viruses (Prins et al. 2008). Moreover, multi-virus resistance can be achieved relatively easily by combining sequences derived from different viruses in a single chimeric transgene (Jan et al. 2000; Bucher et al. 2006). There are, however, several limitations to the use of RNA-mediated virus resistance:

1. RNA-mediated resistance is homology-dependent, and a sequence divergence of >10% (as it may be found among related virulent strains) is sufficient to render a virus insensitive to RNA degradation (De Haan et al. 1992). To some extent, careful selection of conserved viral regions as target sequence may help to broaden the spectrum of virus resistance.
2. Resistance may be ineffective against viruses that encode suppressors of RNA silencing. This limitation may be overcome by targeting directly the viral suppressor genes (Di Nicola-Negri et al. 2005).
3. Co-infection with a virus that encodes a (different) suppressor of RNA silencing may also abolish resistance against the original target virus.
4. There are several reports that viruses may escape RNA silencing when the host plant is subjected to biotic or abiotic stresses, including low temperature (Taliansky et al. 2004; Chellappan et al. 2005; Wu et al. 2008). Despite these limitations, the capacity of RNAi to target virtually any virus certainly makes it an exciting addition to the repertoire of crop protection tools.

The use of RNAi to engineer pathogen resistance is not restricted to anti-viral applications. Expression of dsRNA from inverted repeat transgenes in plants targeting genes which are endogenous to a given pest was reported to confer resistance against the bacterial crown gall disease (Escobar et al. 2001) and was recently shown to be a promising strategy to combat plant parasitic nematodes

(Huang et al. 2006; Yadav et al. 2006; Fairbairn et al. 2007; Sindhu et al. 2009) and herbivorous insects (Baum et al. 2007; Mao et al. 2007; Price and Gatehouse 2008).

### **5.4.2 Applications of RNAi for Crop Improvement and Metabolic Engineering**

In addition to crop protection, RNAi technology has been used successfully to modify agronomically relevant traits such as, e.g. nutritional or pharmaceutical value and crop toxicity (reviewed by Tang et al. 2007; Hebert et al. 2008). Many reports on the successful application of RNAi for crop improvement take advantage of the potential of RNAi to down-regulate multiple targets. For example, a nutritionally valuable high-lysine maize variant was produced by down-regulating the entire 22-kDa  $\alpha$ -zein gene family (with the seven active members sharing >90% nucleotide sequence identity in the coding sequence; Segal et al. 2003). Similarly, a constitutively expressed RNAi effector transgene led to “decaffeination” of coffee plants by down-regulating multiple members of a caffeine biosynthesis gene family (Ogita et al. 2003, 2004). In another study, Allen et al. (2004) were able to generate opium poppy plants with high levels of the pharmaceutically valuable non-narcotic alkaloid reticuline by silencing the codeinone reductase multi-gene family that includes the key enzymes of morphine biosynthesis. Silencing of all gene family members was possible by a combination of targeting a highly conserved region among gene family members and incorporating a second gene fragment specific for a distinctive member into the same inverted repeat.

Some RNAi-based crop improvement strategies have taken advantage of the possibility to spatially restrict the induction of RNAi, e.g. by use of tissue-specific promoters. Examples include an alternative approach to engineering high-lysine maize by Houmard et al. (2007), who used endosperm-specific silencing of a lysine metabolism gene to confine increased lysine accumulation to the kernels, and the production of high-carotenoid and high-flavonoid tomato by RNAi of the photomorphogenesis regulatory gene *DET1* with fruit-specific promoters (Davuluri et al. 2005). In contrast to the spatial confinement of these compounds, overproduction of lysine also in other tissues (Houmard et al. 2007) and constitutive silencing of *DET1* (Davuluri et al. 2004) caused severe developmental defects. In an exciting report on genetic engineering of cotton that opens a path for the use of this crop species both for fiber and for food production, Sunilkumar et al. (2006) disrupted the biosynthesis of the toxic terpenoid gossypol specifically in seeds. The observed significant reduction of gossypol in cottonseed greatly improves the value of cottonseed for human consumption, without abolishing the toxin’s function in plant defense in other (non-edible) plant organs where the production of gossypol was not impaired (reviewed by Townsend and Llewellyn 2007). These reports clearly demonstrate the benefits of spatially restricted silencing. Importantly, although RNA silencing had been reported to be capable of spreading systemically, silencing targeted to either fruits or seeds did not spread to any significant extent

beyond the targeted tissues to other parts of the plant. Finally, tissue-specific RNA silencing was also employed in recent “intragenic” approaches that only use sequence elements that are native to the target species for plant transformation (Rommens 2007). In one report, Rommens et al. (2008) constructed an inverted repeat driven by tuber-specific promoters to silence two asparagine synthesis genes in potato. The resulting transformants produced low-asparagine potatoes which after heat-processing accumulated as little as 5% of the suspected carcinogen acrylamide present in wild-type controls.

## 5.5 Conclusions

The past 20 years have seen tremendous progress in our understanding of gene-silencing phenomena that initially were a great puzzlement. Ever more intensive research in both plant and animal systems led to the discovery of an intricate network of silencing pathways that revolve around small RNA molecules as the central component and may all derive from a common ancestral mechanism to protect a cell or organism against adverse effects of foreign nucleic acids. The sequence specificity provided by small RNAs, their ease of mobility, and the capacity to guide enzymatic or structural protein complexes to RNA as well as to DNA targets may all have contributed to the evolution of multiple RNA silencing pathways with subspecialized functions not only in plant defense, but also in the regulation of plant development and responses to the environment. While the processes directed towards RNA targets enable an immediate reaction to a challenge, epigenetic modifications of DNA and chromatin targets provide a second, longer-lived line of defense that may also serve as a memory of past events. Our growing knowledge of the mechanisms involved in the many facets of gene silencing is helping to define guidelines for the successful engineering of transgenic plants in which either a transgene encoding a trait-of-interest is predictably and stably expressed, or the transgene is optimized to *trans*-silence one or more endogenous or pathogenous target genes with minimal or no off-target effects. The potential applications for protection and improvement of cultivated species by gene silencing are enormous, and some of the current examples are truly inspiring. The challenge ahead in large part will be to fine-tune, and further develop, the techniques at hand for applications in particular in crop species to live up to their full promise.

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# **Chapter 6**

## **Breeding with Genetically Modified Plants**

**Christian Jung**

### **6.1 Genetic Variation in Plant Breeding**

Breeding with transgenic plants is only justified when genetic variation within the primary and secondary gene pools of a species is too small or gene transfer by conventional techniques is difficult and time-consuming. There are a number of different means for increasing or even creating new genetic variation, like species hybridization, mutation induction and protoplast fusion, which have been frequently used to breed new varieties (Fig. 6.1). It is worth mentioning that neither plant cultivation nor commercialization of commodities has any legal requirements and a public discussion about these plants is also lacking.

Therefore a breeder must carefully assess the different constraints of transgenic breeding before starting a breeding program which are the legal aspects and acceptance by farmers, consumers and stakeholders.

### **6.2 Breeding Aims**

Plant varieties must meet the requirements of plant production under special environmental and economical conditions. Moreover, the demands of food and feed industry as well as the consumers' preferences must be regarded.

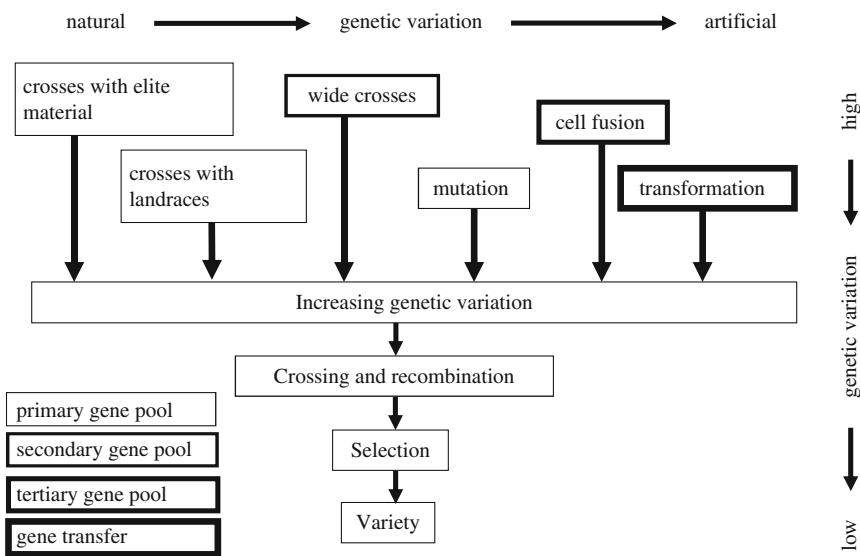
The yield potential is often the most important aim. Today's varieties are elites with high-yielding potential due to countless rounds of recombination and selection. Yield potential is a typical quantitative character controlled by many genes. Thus high yielding varieties differ substantially from their wild relatives, landrace and any other non-adapted material. Therefore, transgenic material with

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**Fig. 6.1** Methods for increasing genetic variation in plant breeding

poor-yielding capacities must be backcrossed several times with elite lines (see Sect. 6.3). The yield potential itself is not accessible to transgenic modification due to its polygenic nature.

Plants are attacked by numerous pathogens and pests and they suffer from different environmental constraints like drought, heat, frost, water, salt and low soil pH. Therefore resistances or tolerances to these stresses are needed. These measures increase the yield stability of a crop. They are often a prerequisite for crop cultivation mainly when technical measures like pesticides are unavailable. It happens quite often that modern high-yielding varieties lack resistance or tolerance genes. Transgenic technology has been tremendously successful in increasing the genetic variation in this field, mainly for virus and pest resistance (see Chap. 10). Excellent perspectives exist for drought tolerance and other environmental stresses (see Chap. 8). Transgenic technology is particularly successful in breeding varieties with tolerance or resistance to herbicides. Although non-transgenic herbicide tolerances have been used before, the availability of these genes identified from microorganisms tremendously broadens the genetic variation of crops (see Chap. 9). Moreover, this character is simply inherited and can be easily selected in segregating populations. Therefore, herbicide resistances can easily be combined with pathogen or pest resistances and a number of varieties with dual resistances are available today, e.g. corn and soybean.

Biotic stress resistance is frequently broken by the pathogen when resistance relies on single genes only (monogenic resistance). A simple mutation within the pathogen's genome can break the resistance and diminish the value of a variety. Gene stacking of resistance genes by transgenic technology offers a perspective for durable resistance breeding because double mutations on the pathogen's side are

highly unlikely (see Chap. 3). The genes can be natural ones isolated from the given species or from related or non-related species or from artificial resistance genes not present in the primary to tertiary gene pools of the species. An example is gene stacking with synthetic *cry1C* genes which gave multiple resistances to leaffolders and stemborers in rice. The transgenic line was used as a parent for hybrid rice production and the hybrids proved to be resistant as well (Tang et al. 2006).

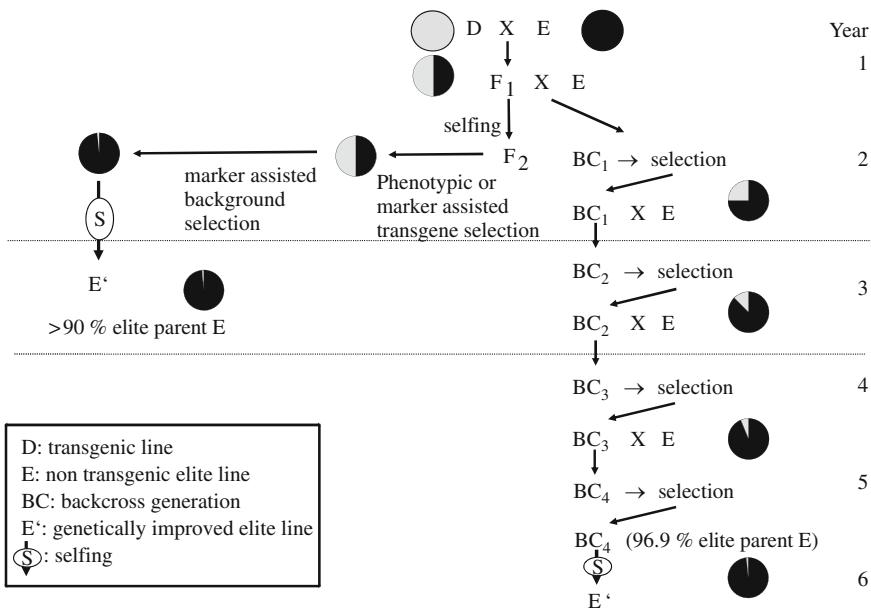
Improving the quality of harvested parts of the plant is another major aim in many breeding programs. Often single genes have a major impact on the phenotype, thus quality improvement is readily accessible to genetic modification. Consequently, the quality of major storage components like fatty acids, protein or starch has been improved by genetic modification (see Chap. 11). Moreover, transgenic plants with higher vitamin and mineral content and better feeding or processing quality have become available.

Altering the phenological development has often been a major requirement for plant production. Transgenic technology offers new perspectives by modification of flowering time genes. The onset of flowering is of uppermost importance for plant production. Often early flowering is desired to avoid stress conditions. However flowering must be avoided when the vegetative parts of a plant are harvested, like beet roots, tubers or leaves.

The key regulators for flowering time control have been cloned from *Arabidopsis thaliana*. High conservation of regulatory pathways was found among dicot species. Genes isolated from *A. thaliana* were often found to have the same function after transformation into crop species. Likewise flowering time genes were identified for monocot species, using rice as a model. These findings offer possibilities for shortening the generation cycle of crop species by using genetically modified early-flowering genotypes as crossing parents. This is of major interest for introducing genes from exotic material by repeated backcrossing (Fig. 6.2). After selfing plants from advanced backcross generations the transgene can easily be eliminated from the offspring, either by marker assisted selection, or by selecting for the phenotype itself. Many plants have long generation cycles which substantially delay the breeding progress. These are biennials with a long seed ripening phase like sugar beet or tree species which often need many years to flower. Breeding strategies employing short generation transgenics are presently discussed for forest tree and fruit tree breeding (Flachowsky et al. 2007). Manipulation of flowering time regulators also offers a possibility to produce plants with completely altered bolting or flowering behavior (see Sect. 6.6).

### 6.3 Methods for Introducing Transgenes into Elite Plant Material

Often favorable alleles are absent from the primary gene pool of a plant, therefore exotic lines are used as parents and their offspring are backcrossed several times with the elite recipient line to produce an elite line with a small



**Fig. 6.2** Introgression of a transgene from a transgenic donor (*D*) into an elite recipient plant line (*E*): backcross breeding in combination with phenotypic or marker-assisted selection. The share of the elite genome is shown in black

introgression from the donor line. In most cases these are major genes with clear phenotypes.

Genetically modified plants directly resulting from a transformation process are often not adapted to local environmental conditions because standard genotypes with inferior yielding performance but good regeneration capacity have to be used. Thus, they must be backcrossed with recipient lines to create elites carrying the genetic modification (Fig. 6.2).

Molecular markers derived from the transgene itself turned out to be helpful for backcross breeding (Bernardo 2008). They are used to select for recombinant plants in offspring generations without phenotypic analysis (marker-assisted foreground selection). In addition markers covering the rest of the genome can be used to select plants with a high proportion of the recipient (elite) genome, even in early back-cross generations (marker-assisted background selection). This saves time because several generations of backcrossing can be avoided (Fig. 6.2).

Only single-copy transgenes are desired for breeding, otherwise selection will be complicated by complex segregation patterns. There are numerous examples for successful introduction of transgenes into elite material or existing varieties, e.g. resistances to insects, virus and leaf blight in rice. Existing rice varieties were also improved for quality characters like provitamin A and ferritin content (Kang and Priyadarshan 2007). When the transformation procedure is genotype-dependent and elite genotypes are non-accessible for transformation, the backcrossing procedure is

the method of choice for introducing transgenes into a desired elite genotype. This could be an inbred line in the case of line or hybrid breeding or an inbred cultivar.

When an already existing variety has been used for transformation, the new variety is called an essentially derived variety (EDV). To protect the breeder's rights the GMO breeder needs approval from the variety's owner to commercialize the EDV. In the EC a 95% identity threshold has been established for defining an EDV.

For hybrid breeding the genetic modification can be introduced either into the male sterile parent or into the restorer parent. When however the degree of dominance is not complete, i.e. the performance of heterozygous is inferior to that of homozygous plants, the genetic modification must be introduced into both parents. In corn breeding, transformation is either done on a hybrid-by-hybrid basis or only one parent is transformed (Kang and Priyadarshan 2007).

When plants are clonally propagated elite material should be transformed. Due to the absence of recombination further improvement can only rely on extensive selection. When however the transgene cannot be incorporated into an already existing variety further crosses are needed and huge clone populations have to be created. Breeding clonally propagated transgenic varieties is further hampered by the fact that many clonally propagated plants are polyploid, which complicates selection due to their complex segregation pattern.

Today, the perspectives for transgenic breeding are rather limited because only single genes or a low number of genes can be transformed at one time. Quantitative characters are not accessible to genetic modification. Gene stacking is an interesting option to further increase genetic variability by transformation to accumulate a number of genes in an elite plant. Transgenic plants with different transgenes are crossed to each other. In the F<sub>2</sub> generation recombinant genotypes with both transgenes can be selected. In the case of single genes, double homozygous plants are expected with a frequency of one in 16, provided that the transgenes are not genetically linked. Using molecular markers homozygous plants can be easily distinguished from homozygous ones. After testing for homozygosity, these lines can be used as parents for hybrid breeding. A transgenic rice restorer line has been bred in this way, combining multiple resistances against bacterial blight and striped stem borer together with a herbicide tolerance by repeated backcrossing and hybridization of transgenic parents (Wei et al. 2008).

## 6.4 Breeding Methods

Today many crop plants can be easily transformed. However, transformation (see Chaps. 1, 2) often depends on the genotype which requires further selection to introduce the transgene into an elite background. Transgenic elites can then be grown as a new variety or they can be used as parents to produce hybrid seeds. The different methods of breeding and selection are briefly explained in this chapter, together with specific requirements for transgenic plants and new alternatives

offered by transgenic technologies. A deeper understanding of plant breeding methods is available from a number of textbooks (Sleper and Poehlmann 2006; Kang and Priyadarshan 2007; Brown and Caligari 2008).

In the following the term ‘transgene’ is used for a sequence encompassing the gene, promoter and terminator elements and in many cases a selectable marker gene. A transgene which is stably incorporated into the nuclear genome is inherited as a single Mendelian gene. When the phenotype is modified by environmental factors or by interaction with other genes the construct can be used as a molecular marker for selecting transgenic plants among a segregating offspring.

A transgene incorporated into the chloroplast genome (transplastomic plants) is inherited following a cytoplasmic inheritance (see Chap. 2). Typically the transgene is inherited only by the seed parent; thus it follows that all offspring harvested on a seed parent are transgenic. There are some important examples for cytoplasmic inheritance in plant breeding, like cytoplasmic male sterility (CMS; see Chap. 14).

Plants obtained after transformation are hemizygous for the transgene. When the phenotype can be determined unequivocally they can be used as crossing parents for a backcross program because the transgene can be easily monitored in their offspring generation. Alternatively, homozygous plants can be obtained after selfing the transgenic plants. Ideally transgenic doubled haploids (DH) are used for crossing which are 100% homozygous. Generally transgenic plants with only one insertion of the transgene are preferred due to their simpler genetic segregation patterns.

The genetic improvement of crops is a prerequisite for crop production systems. During domestication the phenotypes of crop plants have changed dramatically. Men have been selecting plants for high yield capacity, yield stability or better quality over long periods. This has resulted in landraces adapted to local environmental conditions. The classical selection technique was mass selection where favorable plants were selected by their phenotype and seeds of selected plants are grown in the next year. Later offspring of selected single plants was tested, which resulted in a much more efficient selection even for low heritable traits. For a few years genotypic selection has been possible, using molecular markers as selection tools.

The choice of a breeding method depends on the reproduction system of a plant and the heterosis. Each breeding program starts with single or multiple crosses between well adapted materials with superior yielding performance (elites) or introgression lines carrying favorable alleles from non-adapted material. The following briefly presents the main methods. Detailed descriptions of breeding methods are given by Sleper and Poehlmann (2006) and Brown and Caligari (2008).

#### **6.4.1 Line Varieties**

Self-pollinating crops are usually bred as inbred lines following three different breeding methods. The aim is to breed a pure line after several generations of

selfing or by DH production. All methods start with crosses and generation of F<sub>1</sub> populations. During bulk breeding, plants are selfed for two or three more generations to produce lines with a high level of homozygosity. Then selection starts in the F<sub>4</sub> or F<sub>5</sub> generations. Alternatively, only a single seed is harvested from each plant during selfing generations. Plants are grown under conditions where ripening is accelerated to shorten the generation time (single seed descent).

Using the pedigree method, selection starts as early as the F<sub>2</sub> generation, followed by successive selections until the F<sub>5</sub> or F<sub>6</sub> generation. Single plants are selected and their offspring tested in the field. Thus the genotype of the selected plant is determined by testing its offspring generation.

In all cases the result is an inbred line with >95% homozygosity. In the past decade the doubled haploid (DH) method became popular because plants with 100% homozygosity can be obtained from F<sub>1</sub> plants using different methods like anther or microspore culture or pollination with inductor plants. Clearly, the transformation of DHs or the combination of DH production and transformation is desirable because homozygous plants are obtained in one step.

A limited number of lines are tested in the field under different environments and the line with the best performance is selected as a new variety. When there is heterosis and a male sterility system is available, hybrid breeding is an alternative. In general, however, heterosis is low for self-pollinating crops. There are only rare examples of transgenic lineal cultivars, e.g. herbicide-tolerant soybean. One reason is that a line can be easily propagated by the farmer so that he does not have to buy seeds every year, thus reducing the profitability of a transgenic variety.

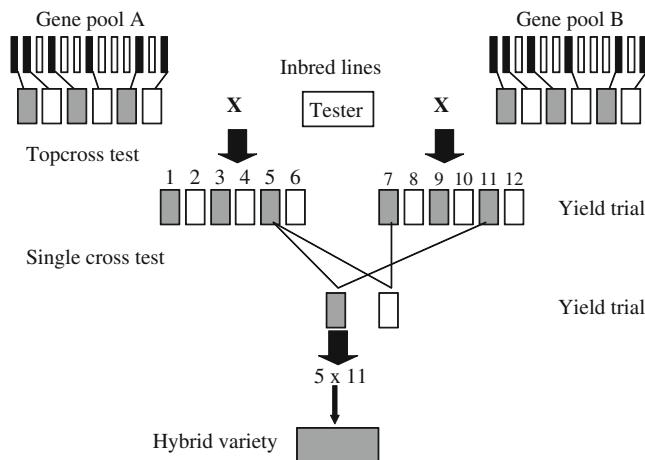
#### ***6.4.2 Open-Pollinated Varieties***

In the past, open-pollinated crops were bred as population varieties. Panmictic populations were grown and mass selection was performed. Seeds from selected plants were grown as an improved variety. Mass selection was repeated to further improve the populations.

Today open-pollinated crops are mostly bred as synthetic varieties (synthetics) or hybrids. Synthetics are open-pollinated varieties when a limited number of selected parents are used for seed production. The parents can be inbred lines or clones. This method is used when a male sterility system is lacking, however cross-pollination between parental components must be guaranteed (e.g. by self incompatibility).

#### ***6.4.3 Hybrid Varieties***

Hybrid breeding is the mostly preferred breeding method for open-pollinated crops today. Thus, many genetically modified varieties are hybrids. Hybrid breeding

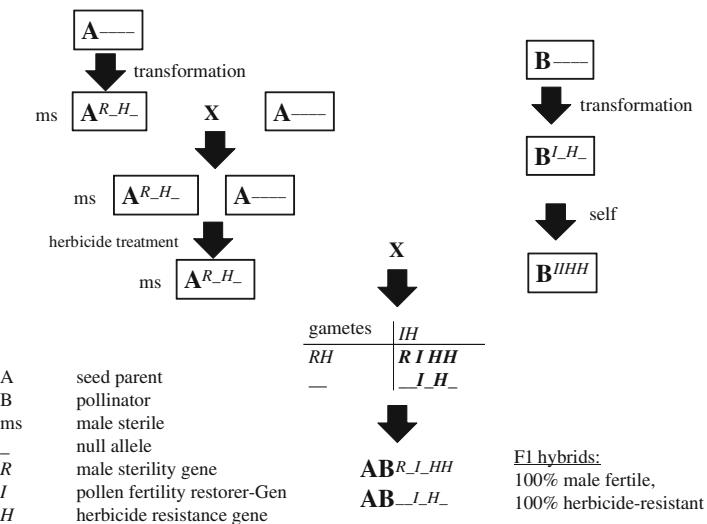


**Fig. 6.3** A simplified scheme of breeding hybrid varieties

needs a system for targeted pollen transfer. Usually CMS is used; in rare cases cross-pollination relies on self incompatibility. The main reason for breeding hybrid varieties is heterosis, which is the superior performance of hybrids as compared to their inbred parents. Substantial heterosis can be found in many open-pollinated crops. Heterosis in self-pollinated crops is much lower or non-existing.

Hybrid breeding is substantially different from line breeding because selection does not rely on the *per se* performance of a line but on the performance of its hybrid. In principle, hybrid breeding can be divided into three steps (Fig. 6.3). First inbred lines are developed by successive selfing. In a classic breeding scheme inbreds are developed from different gene pools which had been separated for generations, e.g. by geographical separation. The *per se* performance of these lines is tested in the field. A number of lines are selected for crossing with a non-related tester line or population (topcross) and the hybrids are grown in the field. Then the best inbred lines from each pool are selected and crossed with each other in a diallel crossing scheme (single cross). The best hybrid combinations are selected and their performance is tested by replicated field trials under different environments. At the end a new hybrid variety is available which can be either the product of a single cross or produced by three- or four-way crosses with three or four parents. When CMS is used for cross-pollination and reproductive organs are harvested, the pollinator must have a gene which restores pollen fertility in the hybrid (see also Chap. 14).

Transgenic lines with superior *per se* performance can be used as parents for topcross tests. Since the transgenic character is inherited in a dominant way, it is sufficient when one parental component carries the transgene. In that case the transgenic hybrid variety is heterozygous for the transgene. In the case of additive gene action both parents must carry the transgene, however in different genetic backgrounds. Thus it follows that the transgenic character must be introduced into both gene pools by successive backcross breeding (see Sect. 6.3).



**Fig. 6.4** Breeding hybrid varieties with a transgenic male sterility system and pollen restoration (Reynaerts et al. 1993)

The availability of a new male sterility system by genetic transformation offers an interesting alternative for hybrid breeding, even when natural male sterility is lacking (see Chap. 14). This kind of male sterility is called nuclear male sterility (NMS) because the male sterility gene is located in the nuclear genome. Nuclear male sterility is often found in natural populations. However, its practical use is very limited because male sterile plants cannot be maintained as pure lines. After cross-pollination their offspring segregates for male sterile and male fertile plants, which creates a need for laborious phenotypic selection of male sterile plants. By genetic modification genes have been introduced which have a deleterious effect on pollen development. When these genes are transformed together with a selectable marker which allows easy selection of male sterile plants in the offspring male fertile plants can be easily eliminated, e.g. by spraying with herbicides (Fig. 6.4). Pollen restoration can be achieved by a second gene which inhibits the male sterility system within the cell. This system has proved successful in different crops. Plants with a transgenic NMS have been cultivated on a large scale and stable pollen restoration has been demonstrated. This strategy can be applied for all plants. It offers a possibility for hybrid breeding even for crops where CMS systems are not available or their use is limited by negative pleiotropic effects or poor pollen restoration.

#### 6.4.4 Clone Varieties

Crosses are made between elite clones of clonally propagated plant species to create new genetic variation. When the transgene cannot be incorporated into an elite

clone huge populations have to be produced to get a realistic chance for selecting a genetically improved transgenic clone, because after the initial cross no further recombination takes place. Selection is further hampered by the fact that many clonally propagated crops are polyploid, which reduces the chance to find a transgenic recombinant with favorable allele composition.

As an alternative dihaploids ( $2x$ ) can be used for crossing and selection due to their simpler segregation patterns, as in the case of potato breeding. The offspring after crossing clones are highly heterogeneous and heterozygous, no matter whether diploids or polyploids were used as parents. Selection starts with the first generation with potted plants in the greenhouse. Later selected clones (often referred to as A, B or C clones) are tested in the field under different environments.

## 6.5 Safety and Legal Aspects of GMO Breeding

### 6.5.1 Separating Transgenic and Non-Transgenic Breeding Programs

Due to strict GMO legislation, breeders have to take special measures to avoid mixing seeds from GMO and non-GMO plants. Especially in Europe (where a seed directive is still missing) the absolute separation of both types of plants is an obligate requirement to avoid unforeseeable legal and financial consequences. In fact a 0% threshold is in force which creates a danger of a denial of seed lots for commercialization even when minimal amounts of GMO DNA are found. As a consequence all European breeders have strictly separated their breeding programs if they have not already abandoned their GMO activities. Separation in many cases means that GMO breeding no longer takes place in Europe. The problem of GMO introgressions not only arises from pollen transfer but also from GMO volunteers growing in non-GMO plots. This danger has to be regarded for species with a long seed survival, like rapeseed whose seeds can survive in the soil for >10 years.

For any field trial in the EC approval is needed from a national authority. According to directive 2001/18/EG this approval is limited to a certain time and location. After extensive testing under different environments an application for placing on the market can be filed. This application must include seed production and plant production on a commercial scale. When successful the transgenic plant and all its derivatives (backcross lines, hybrids) can be grown all over Europe. In practice however a number of limitations have been set by the national authorities which severely hamper the cultivation of transgenic plants in Europe. When a breeder wants to market its transgenic plant a variety approval is needed by the national authority. Alternatively seeds are distributed by biotech companies to a limited number of farmers who grow the transgenic crops after signing a license agreement. Further approvals can be needed, e.g. for herbicide

treatment in combination with a GMO variety. In fact, a decision by the EC can last >10 years and in many cases does not yield any result due to political dissent on this matter.

### **6.5.2 Breeding Marker-Free Cultivars**

Another point of concern is the presence of marker genes in transgenic plants (see Chap. 3). Although there are no safety reasons, plants with antibiotic markers are generally denied approval in the EC. It is technically impossible to delete marker sequences from transgenic plants which have been transformed *in cis* with the agronomically important gene because a recombination between both genes is extremely unlikely. There are alternatives for selecting marker-free plants. When the marker is flanked by sequences with high recombination frequency, like transposable elements, there is a dramatic increase in the probability of finding plants where the complete linkage between both genes has been broken. Likewise marker and gene can be co-transformed, which results in transgenic lines with two unlinked integration sites, so that recombinants can be easily selected from segregating generations. The applicability relies on the efficiency of co-transformation and requires time-consuming segregation studies.

### **6.5.3 ‘Cisgenic’ and Transgenic Plants**

From a scientific point of view the strict European GMO legislation is not reasonable by safety reasons. Much larger rearrangements within the genome can be achieved by non-transgenic methods (see Sect. 6.1). However, concern arises from transformations with sequences coding for harmful products or sequences from non-plant species, like mammals, or from viruses. Therefore, a concept of ‘cisgenic’ or ‘intragenic’ plants has been introduced (Schouten and Jacobsen 2008; see also Chaps. 4, 20). Cisgenes have been referred as genes from crop plants themselves or from crossable species remaining within the gene pool of the classical breeder. They should be genetically precisely defined as they are the result of a one-step gene transfer without linkage drag of other genes, whereas induced translocation and introgression breeding create many more genetic rearrangements which are largely unknown. It was suggested that cisgenic plants shall be treated like classically bred plants because of the similarity of the genes used in cisgenesis compared with classical breeding. It remains to be seen whether cisgenic plants will be treated differently from transgenic ones, which may contain genes from any other organism. Legislation today makes no difference between cisgenic and transgenic plants when gene technology has been used for incorporating the genes into the genome.

## 6.6 Non-Transgenic Versus Transgenic Breeding

What are the reasons to choose a transgenic technology for breeding a new variety? In general, breeding with transgenic plants is only justified when genetic variation for a trait is lacking within the primary and secondary gene pools of a crop species or when the period of selection can be shortened (see Sect. 6.1). There are examples where transgenic plants display a phenotype perfectly matching the requirements for crop production. However, breeders refrain from using these plants due to problems of acceptance or because a non-transgenic alternative is available. However, there are traits which never can be bred by conventional breeding. Examples are metabolic engineering, like vitamin pathway manipulation or oil crops producing fatty acids, e.g. short- or long-chain polyunsaturated fatty acids, where the corresponding genes are lacking from the primary to the tertiary gene pool of the species. This is particularly true for characters absent from the plant kingdom, like spider chain proteins, mammalian proteins or storage products only found in bacteria (e.g. polyhydroxy fatty acids, PHF; see Chap. 13). Many traits, however, which on a first glance appear to be novel can be found within the available gene pools. Neither herbicide tolerance nor amylase-free starch are new traits in plant breeding. However, they are often associated with pleiotropic effects like low yield and poor quality. In the following, a number of examples are given where transgenic plants have been successfully produced, however their commercialization has been abandoned.

A striking example is a potato (*Solanum tuberosum* L.) with resistance against virus and insect diseases. Although these clones proved to be superior in yield stability they were not commercialized due to reasons of low consumer acceptance (see Chap. 20).

Rhizomania is the most destructive disease of sugar beet (*Beta vulgaris* L.). When this virus arrived during the early 1980s no resistant varieties were available. The only source of resistance from a wild species suffered from a poor yield capacity. As soon as in the early 1990s coat protein-protected transgenic beets were available which did not show any virus replication. In parallel, new sources of resistance were found in the wild relative *B. vulgaris* ssp. *maritima* which belongs to the secondary gene pool of sugar beet. This so-called ‘Holly’ resistance proved to be almost complete with no yield penalty associated (Scholten and Lange 2000). Thus, all modern varieties rely on the ‘Holly’ resistance and transgenic resistant plants have never been employed in practical plant breeding. However, as soon as the ‘Holly’ resistance is broken, transgenic resistance will become an option.

There are examples of lacking genetic variation in the primary *and* in the secondary gene pool of a crop species. In onion (*Allium cepa* L.) production, the beet armyworm is a severe pest and natural resistances are of limited use only (Prohens and Nuez 2008). Therefore *bt* genes have been introduced into onion, resulting in highly resistant transgenic plants. However, no cultivars are grown so far due to reasons of lacking consumer acceptance. The same applies for glyphosate and glufosinate resistances. Although there is a high demand for onion production, no varieties are on the market.

Likewise, a number of tomato (*Solanum lycopersicon* L.) transgenics have been produced but since the delayed-ripening tomatoes disappeared from the market by the end of the 1990s no other varieties have been commercialized (Prohens and Nuez 2008). One reason is that substantial genetic variation exists in the primary and secondary gene pool of tomato, e.g. genotypes with ‘long shelf life’ have been found offering an alternative to transgenic breeding.

To overcome the constraints of conventional rice (*Oryza sativa* L.) breeding a number of biotechnology tools have been suggested (Kang and Priyadarshan 2007). Rice transgenics have been produced which have much higher  $\beta$ -carotene contents (*golden rice*), as found among *Oryza* species (Paine et al. 2005). Genetic variability is limited for a number of characters like resistance to stem borers and resistance to sheath blight. Low selection efficiency to pyramid genes for durable resistance to pests severely hampers the breeding progress and increases the breeding cycle of rice varieties. Moreover, the genetic variation for resistance to pests is quite narrow (Datta and Khush 2002). Therefore, many transformations have been made and numerous plant prototypes with improved disease and pest resistance have been established. The transgenes have been introduced into high-yielding lines and the agronomic performance has been demonstrated in several field trials. But for all of that, no varieties have been commercialized so far.

Squash (*Cucurbita pepo*) is highly susceptible to viruses which can be important limiting factors to summer squash production (Paris 2008). No sources of resistance are available for traditional breeding, due to crossing barriers. Moreover the virus spectrum often changes in the field, creating a danger that classical monogenic resistances are quickly broken. Therefore, transgenic squash has been produced carrying resistances for up to three viruses. Although successfully tested in the field no varieties have been commercialized so far.

Finally, eggplant (*Solanum melongena* L.) can serve as an example for the usefulness of transgenic technology. No eggplant germplasm with resistance to the Colorado beetle has been described. However, plants expressing the *bt* gene were highly resistant to two pests: the Colorado beetle and the eggplant fruit borer (Prohens and Nuez 2008). As in other plants, resistances to aphids and nematodes are difficult to handle. Eggplant with resistance to the peach tree aphid and potato aphid has been produced by transformation with a rice oryzacystatin gene. Likewise, resistance to the nematode *Meloidogyne javanica* by transformation with the *Mi-1.2* gene has been introduced to eggplant. Fruit quality has also been improved by transgenic means. Transformation with a chimeric parthenocarpic gene induced parthenocarpic fruit development. In spite of these successes no transgenic seeds have been marketed so far.

## 6.7 Conclusions

Transgenic plants can increase the genetic variation of a crop species in a way that has not been seen before in the history of plant breeding. Completely novel traits can be introduced or negative effects of natural variation can be avoided. In

most cases the primary transgenic plant is subjected to a long-lasting selection and recombination procedure to combine the new trait with superior agronomic characters. Traditional selection and breeding procedures are employed and in principle transgenic plants are bred in the same way as conventional ones. However, whenever possible, hybrids are clearly preferred. There are numerous examples of transgenic technology offering new solutions which are inaccessible by traditional plant breeding. But the constraints of public acceptance together with preponderant legal requirements and costs have largely prohibited the commercialization of transgenic varieties. However, the recent price increase on the international markets for agricultural products may suggest that the impending food crisis creates a need for the use of *all* kinds of technology for future plant breeding.

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# **Chapter 7**

## **Detection of Genetically Modified Plants in Seeds, Food and Feed**

**Lutz Grohmann**

### **7.1 Introduction**

In the context of the development and approval of a growing number of genetically modified (GM) plants which are field-tested in the environment or cultivated as crop plants, the methodology for their detection and identification has become an important issue. Detection methods and techniques used by researchers and in development laboratories for the characterisation of transformants are generally different to those applied by official testing laboratories and public analysts. Enforcement laboratories apply specific methods and analytical strategies for the detection of GM plants used in the foods, feed or seeds sectors, having in mind that the commercialisation of transgenic crop plants is regulated in different ways depending on national legal frameworks. In the European Union (EU) for example a validated transformation event-specific detection method, including sampling, extraction, identification and quantification, has to be provided by the applicant if authorisation of a certain GM event as food and feed is intended (EU 2003a). In contrast, for example in the United States, GM plants become deregulated for use as food, feed or for cultivation when they have been reviewed by the competent regulatory agencies. Moreover, according to international agreements laid down in the Cartagena Protocol on Biosafety (UN 2000), the trade and transfer of living GM organisms (e.g. seeds and propagable grains) across national borders may require information for the specific detection and identification of that GM organism.

Under certain circumstances the GM crop content needs not only to be detected and identified but also to be quantified in terms of certain thresholds for labelling the foods and feeds which contain or are produced from GM plants. Threshold levels also depend on national legislations and, for example in the EU, labelling is

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not required if the proportion of GM material is not higher than 0.9% of the food ingredient, provided that the presence of this material is adventitious or technically unavoidable, whereas for example in Japan the labelling threshold is 5%. For GM plants not authorised according to EU regulations a zero tolerance is applied, making the sensitive detection of such GM materials an emerging challenge for the official testing laboratories.

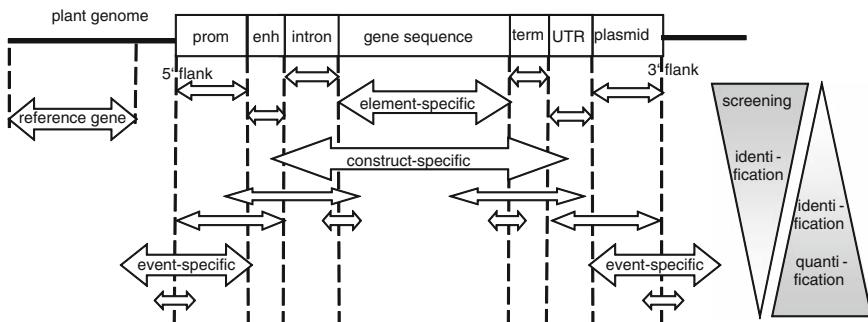
This review describes the current techniques used for detection of transgenic plant materials and the different analytical strategies applied by the official control laboratories responsible for enforcement from an European perspective. In addition, the limitations of current methodologies and finally the recent developments in GMO detection area applying advanced or alternative amplification techniques are reviewed.

## 7.2 Techniques Used to Detect a Transgenic Plant

To detect genetic modifications in plants in general (for the methods of genetic modification, see Chaps. 1, 2) two different techniques could be applied (Anklam et al. 2002; Holst-Jensen 2007). One is based on the detection of genetic material (DNA), for example by polymerase chain reaction (PCR). This technique is most versatile for the detection of GM plants and therefore preferably used and chosen for many applications (Lipp et al. 2005). The alternative approach is detecting the newly expressed protein(s) which most GM plants contain as a result of the insertion of the new gene(s). Here specific antibodies are applied and used in lateral flow strip tests or complex ELISA assays (Grothaus et al. 2007). As compared to PCR, protein techniques are more restricted in their applicability but can be very useful for certain raw commodities. DNA is relatively stable and is often still present in many products, even after processing of the plant material. Therefore genetic modifications in plants are more easily and reliably detected at the DNA level. However, this does not apply to highly processed GM materials or ingredients, such as oil, sugars or starch, which may no longer contain any DNA. Here, the EU regulations for example demand the traceability of the product through every phase of marketing, i.e. over the entire production and processing chain (EU 2003b).

### 7.2.1 DNA-Based Detection

DNA-based detection of transgenic plants targets the novel DNA sequences introduced into the crop genome. These methods show the absence or presence of GM plant material in a sample and can also measure the relative quantity (percentage) in a tested sample.



**Fig. 7.1** Analytical strategy and targeted sequences. Detection of genetically modified plants in food, feed and seed samples is generally conducted by consecutive PCR tests targeting the genetic elements (element-specific) and constructs (construct-specific). For event-specific identification and quantification of GM plants the 5' or 3' junction regions around the integration sites are targeted. A plant taxon-specific reference gene is targeted for relative quantification of the GM content. Element- and construct-specific methods are applicable mainly for screening purposes, event-specific methods are required for identification and quantification

### 7.2.1.1 Polymerase Chain Reaction

DNA-based testing for GM plants is commonly performed using PCR, amplifying specifically a short segment of the targeted DNA (Fig. 7.1). The design of specific primers depends on a knowledge of the precise and comprehensive DNA sequence information of the actually integrated DNA. If the method is to detect specifically a certain transformation event, information about the inserted DNA sequence and the 3' and 5' flanking plant genome sequences is required. For element-specific PCR-based screening and construct-specific detection the DNA sequences of the inserted elements and gene constructs are targeted, respectively.

PCR-based detection and particularly the quantitative measurement of the GM content in a sample actually involves the use of two PCR systems, one for determination of the inserted GM-derived DNA sequence and another system specific for an endogenous, plant-taxon specific reference gene sequence (Fig. 7.1). The latter is also thought to serve as a control for the quality and quantity of the extracted DNA.

### 7.2.1.2 Conventional Qualitative PCR

Conventional PCR methods are mainly used for qualitative testing to obtain yes/no answers concerning the presence of GM plant material. PCR products are analysed by agarose or polyacrylamide gel electrophoresis (Sambrook and Russel 2001) and visualised using UV fluorescence with ethidium bromide as fluorophor or by other means. It may be necessary to confirm GM-positive test results by further analyses, either by restriction analyses, Southern hybridisation or DNA sequencing (ISO 2005a).

Before the PCR method is applied the primer combination has to be optimised and validated for their performance requirements. The important performance criteria for qualitative PCR methods are the sensitivity in detecting the transgenic DNA sequences and the specificity for the targeted DNA segment. At optimal reaction conditions a limit of detection (LOD) of 1–10 copies of the target sequence can be achieved in less than 40 PCR cycles (Hübner et al. 2001). Practically the LOD of the PCR method should allow that the presence of the target sequence is detected in at least 95% of the time, with  $\leq 5\%$  false negative results (ENGL 2008). The length of the amplified product influences the PCR performance and should therefore be selected in a way that it matches to the size range of DNA fragments which can be extracted from the sample matrix. For raw materials like seeds or leaves containing less fragmented DNA a broader range of PCR product size up to maximally 250 bp is applicable, whereas for processed food or feed with higher DNA fragmentation the PCR product should be ideally 80–150 bp. The specificity of the method should be tested theoretically by sequence similarity search with the primer sequences against nucleic acid sequence databases (e.g. Blast search in EMBL, GenBank, etc.) and empirically by testing the GM target event(s), very similar non-target GM events and different non-GM plants in order to confirm that the primers can discriminate between the target and closely related non-target sequences. For the reference gene-specific PCR methods different varieties should be tested to demonstrate that the target sequence is conserved between different plant lines (Hernandez et al. 2004, 2005; Broothaerts et al. 2008).

### 7.2.1.3 Quantitative Real-Time PCR

The most preferred technique to quantify GM material in a sample is real-time PCR. It allows the detection and measurement of increasing fluorescence proportional to the amount of amplification products generated during the PCR process. Of the various chemistries TaqMan fluorogenic probes (Holland et al. 1991) are most commonly applied in real-time PCR-based detection and quantification of GM plant materials. Real-time PCR is mainly used for quantification purposes, but it is increasingly utilised also for qualitative testing to screen or to identify the GM event (Zeitler et al. 2002; Rho et al. 2004; Reiting et al. 2007; Waiblinger et al. 2007).

The limit of quantitation (LOQ) of a real-time PCR method depends on the optimisation of the PCR detection method and on the accepted standard deviation of the measurement. The LOQ is experimentally determined during method validation and should reach 30–50 target molecules, which is close to the theoretical prediction (Hübner et al. 2001). As shown in Table 7.1, the LOD/LOQ values depend primarily on the characteristic plant genome size (C value) and range from 0.004%/0.02% for papaya to 0.16%/0.7% for wheat. The obvious effect here is that PCR is inhibited when the amount of input DNA is exceeding approx. 8 ng/ $\mu$ l of reaction volume. For example for maize, according to its genome size a 200-ng DNA sample contains approximately 39 000 genome copies and thus a given sample with a GM plant content of 0.1% corresponds to 39 copies for a single-copy

**Table 7.1** Plant genome size and theoretical LOD/LOQ in real-time PCR assays

Common name	Scientific name	Nuclear DNA content <sup>a</sup>		Genome copies (in 200 ng)	LOD <sup>c</sup> (%)	LOQ <sup>d</sup> (%)
		Mbp/1C	pg/2C <sup>b</sup>			
Alfalfa/lucerne	<i>Medicago sativa</i> (2n=4X)	1.510	3.09	64 768	0.02	0.06
Barley	<i>Hordeum vulgare</i>	4.873	9.97	20 070	0.05	0.2
Cotton	<i>Gossypium hirsutum</i>	2.246	4.59	43 544	0.02	0.1
Maize	<i>Zea mays</i>	2.504	5.12	39 058	0.03	0.1
Oilseed rape	<i>Brassica napus</i>	1.182	2.42	82 741	0.01	0.05
Papaya	<i>Carica papaya</i>	0.372	0.76	262 903	0.004	0.02
Pea	<i>Opisum sativum</i>	4.172	8.53	23 442	0.04	0.2
Peanut	<i>Arachis hypogaea</i> (2n=4X)	2.813	5.75	34 767	0.03	0.1
Potato	<i>Solanum tuberosum</i> (2n=4X)	1.730	3.54	56 548	0.02	0.07
Soybean	<i>Glycin max</i>	1.115	2.28	87 713	0.01	0.05
Sugarbeet	<i>Beta vulgaris</i> ssp. <i>Saccharifera</i>	0.758	1.55	129 024	0.01	0.03
Sunflower	<i>Helianthus annuus</i>	3.030	6.20	32 277	0.03	0.1
Tobacco	<i>Nicotiana tabacum</i> (2n=4X)	4.434	9.07	22 059	0.05	0.2
Tomato	<i>Lycopersicon esculentum</i>	0.954	1.95	102 569	0.01	0.04
Rice	<i>Oryza sativa</i>	0.441	0.90	221 769	0.005	0.02
Wheat	<i>Triticum aestivum</i> (2n=6X)	15.966	32.65	6 126	0.16	0.7

<sup>a</sup>Nuclear DNA content values were taken from Arumuganathan and Earle (1991)

<sup>b</sup>1 picogram (pg) = 978×106 base pairs (Dolezel et al. 2003)

<sup>c</sup>Relative limit of detection (LOD) based on an LOD (CI=95%) of 8–12 copies of the GM target sequence (Burns and Valdivia 2008)

<sup>d</sup>relative limit of quantification (LOQ) based on an LOQ of 40 copies for the GM target sequence (Hübner et al. 2001)

transgene. A quantitative real-time PCR assay should be carefully optimised for the specific LOD/LOQ needed for GM content detection and quantification. The precision of the quantitative real-time PCR methods is commonly expressed as relative standard deviation (RSD) which can vary over 10–30% with respect to intra-laboratory repeatability and over 15–50% for inter-laboratory reproducibility, depending on the range of target copies analysed.

#### 7.2.1.4 Alternative DNA-Based Techniques

To solve the challenge that the increasing number of GM plant events is covered by appropriate analytical methodologies it is expected that multi-target analyses are necessary. The DNA microarray technology could be an option to parallelise the multi-analyte detection of several PCR products in a single run. Arrays that have been developed consist of various oligonucleotide probes that are immobilised on a glass support and used for screening of genetic elements, for constructs and events including detection of plant taxon-specific reference genes (Hamelis et al. 2007; Xu et al. 2007; Leimanis et al. 2008). However, this approach is based on the use of multiplex PCR before hybridisation of the PCR products to the microarray and, as has been shown elsewhere, PCR is limited in its multiplexing capacity within one

reaction due to the reduced sensitivities of the individual PCR systems. Therefore, alternative amplification methods are currently investigated for their potential use for GMO detection in the future, particularly to cover the increasing number of GM host plants and diversity in genetic elements and constructs. Several alternatives are being tested for improvements in GMO detection, e.g. loop-mediated isothermal amplification (LAMP; Fukuta et al. 2004), ligation-dependend probe amplification (LPA; Moreano et al. 2006), SNPlex technology (Chaouachi et al. 2008), padlock probe ligation in combination with microarray detection (Prins et al. 2008) and nucleic acid sequence based amplification using transcription techniques (NASBA) in combination with microarray detection (Morisset et al. 2008). In addition, to circumvent the limitations concerning the availability or reference materials (e.g. for unauthorised GM events), the use of multiple displacement amplification (MDA) for whole-genome amplification has been described to generate reference material for GMO detection (Roth et al. 2008).

## 7.2.2 *Protein-Based Detection*

Detection of the novel proteins expressed by GM crops is based almost exclusively on the application of immunoassay technology. Several immunoassays are available for different traits present in diverse GM plant crops and are used in a variety of applications, including testing for unauthorised events and determining the relative GM content (Grothaus et al. 2007). Immunoassays are based on the reaction of an antigen (e.g. the GM-derived protein) with a specific antibody to give a antigen-antibody complex that can be indirectly measured. The immunoassay formats commonly used for GM-protein detection are the enzyme-linked immunosorbent assay (ELISA) and the lateral flow device (LFD).

### 7.2.2.1 *Lateral Flow Strip*

Lateral flow strip devices (LFD) are used for qualitative or semi-quantitative detection of antigens and, in the case of novel GM proteins, antibodies are used in the same sandwich immunoassay format as in ELISA, except that the secondary antibody is labelled with a coloured particle such as colloidal gold rather than an enzyme as a means of generating a visible signal. A typical LFD has linked simultaneously a second antibody on the strip to provide visual control that the test has worked correctly. LFDs are available for several traits, require low instrumentation and allow rapid testing also in the field. They are show to be sufficiently specific, but concerning sensitivity only up to the 0.1% range is achievable. LFD represent a useful tool to detect GM proteins in raw materials such as seeds and leaves, however in food and feed products their applicability is restricted to samples containing sufficient GM plant material where the GM protein is expressed. The more drastic limitation for the application of LFDs for food and feed testing is

obviously the physico-chemical instability of proteins when products are processed and heat-treated. The CP4-EPSPS protein is considered as a useful GM protein marker in food/feed products and the Cry1Ab protein to a lesser extent (van den Bulcke et al. 2007).

### 7.2.2.2 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) are commonly 96-well micro-plates with removable strips of 8–12 wells coated with a primary antibody to capture a target antigen in the sample. A secondary antibody, conjugated to an enzyme such as horseradish peroxidase, is used to detect the presence of the bound antigen, which results in a sandwich of the analyte between the primary and secondary antibodies.

In general ELISAs are quantitative and provide high-throughput capability to the laboratory analysis, considering that the protein is not denatured. Detection limits for Cry1Ab protein is reported to be below 0.1% for dried maize flour (Ermolli et al. 2006). To determine the concentration of the targeted protein in a sample, standards correlating to known concentrations of the antigen are used to produce a calibration curve to determine the unknown concentration of the antigen in the sample. Either recombinant proteins, which contain a similar or identical amino acid sequence and immunoreactivity as the plant-expressed protein, or uniform preparations of actual samples with known concentrations of GM proteins (such as maize or soybean flours available as certified reference materials) may also be used as calibration standards. Since processing affects the detectability of proteins, ELISA is not applicable to most processed food or feed matrices. Furthermore, ELISA does not allow event-specific identification and may fail to detect novel GM proteins.

### 7.2.3 Method Validation and Standardisation

Validation of detection methods is an essential component to assess the reliability of test methods. By using validated and standardised methods, control laboratories assure that the analytical procedures applied are harmonised at the national or even international level. The process of validation establishes numerical values for the different performance criteria (specificity, sensitivity, applicability, robustness, etc.) and consists at the beginning of an in-house validation in the developers' laboratory followed by a collaborative trial to determine the method's repeatability and reproducibility in order to estimate the transferability of a method between laboratories (Codex 2009). If a collaborative trial-validated method is to be implemented in a laboratory, it is of course also necessary to confirm that the method performs as well under the local conditions as it did in the inter-laboratory method validation study.

To harmonise the procedures applied for the detection of GM plants in foodstuffs and derived products, the International Standardization Organisation (ISO) has

published a series of internationally agreed standards for nucleic acid extraction (ISO 2005c), for qualitative nucleic acid analysis (ISO 2005a), for quantitative nucleic acid analysis (ISO 2005b) and for protein-based methods (ISO 2004). Furthermore, general requirements and definitions involving these different working steps are described in a generic standard document (ISO 2006). These ISO standards prescribe what method performance and validation studies have to be conducted to establish data and the performance characteristics for the specific method application. At the European level a guidance document of the European Network of GMO Laboratories (ENGL) provides practical recommendations how event-specific PCR methods shall be evaluated in the context of the approval of a GM food or feed according to EU Regulation 1829/2003 and defines minimum performance requirements for acceptance of these methods (ENGL 2008).

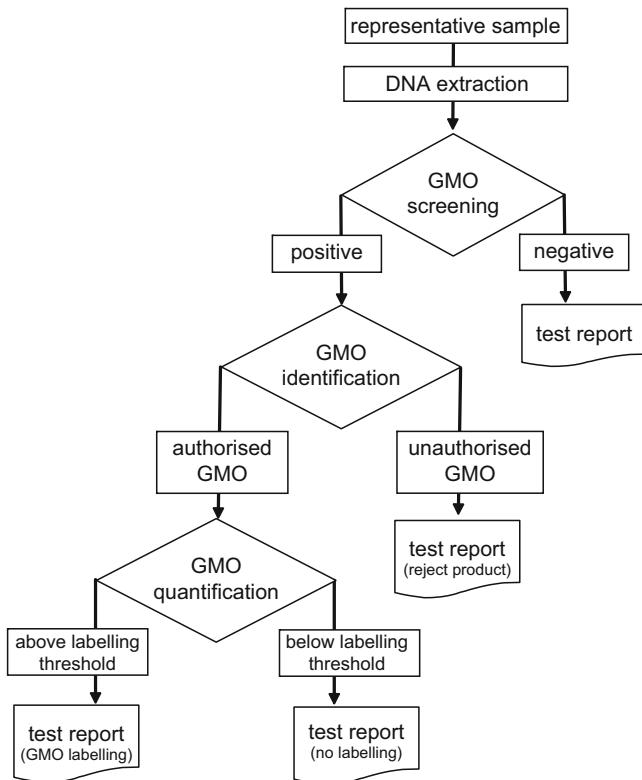
## 7.3 Detection Strategies

Detection of the presence of GM plants is an analytical process involving several working steps. It includes: (i) the sampling step, (ii) the extraction step for isolating DNA or protein fractions from the ground material and (iii) the final analysis for identification and/or quantification of GM material. The detection of GM plant DNA can be used for qualitative and for quantitative testing. In quantitative PCR assays, the amount of the specific target DNA present in the sample is estimated, whereas in qualitative PCR tests the presence or absence of a specific GM target sequence is determined.

A commonly applied strategy for testing the presence of GM plants in food, feed and seeds is to first perform screening tests with qualitative methods (Fig. 7.2). This is typically done with DNA-based PCR tests targeting the genetic elements that are most frequently present in GM plants. In the next working step the identification of the GM event is performed by construct-specific or event-specific PCR methods, followed by real-time PCR-based quantification of the relative proportion of transgene DNA copy number versus the plant taxon-specific DNA copies present in the analysed DNA sample (Holst-Jensen et al. 2003). Target sequences to be detected by analytical PCR methods include sequences integrated in the GM event (screening, construct-specific, event-specific), sequences for plant taxa-specific reference genes and occasionally sequences from the donor organisms in order to exclude false-positive results, e.g. possible plant infections with cauliflower mosaic virus (Cankar et al. 2005).

### 7.3.1 Screening

For the expression of newly integrated genes, GM plant developers use a limited number of regulatory elements (promoters and terminators). Since these elements



**Fig. 7.2** Procedure for GMO testing of food, feed and seeds from a European perspective. A stepwise approach consisting of GMO screening, identification and quantification is commonly applied for testing food, feed and seed products for compliance with European authorisation and labelling regulations

have been frequently used they are ideal candidates for the screening of a large number of samples and are useful to assess whether or not a sample under investigation is likely to contain GM-derived material (Fig. 7.2). To identify these elements Bruderer and Leitner (2003) systematically surveyed which genetic components have been introduced into GM crops at the worldwide level. Correspondingly, the widely applied screening methods target the constitutive 35S promoter (P-35S) sequence from cauliflower mosaic virus (CaMV) or derivatives of this promoter and the terminator sequence isolated from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens* which are found in 43 events (P-35S) and in 37 events (T-nos), respectively (Bruderer and Leitner 2003). The survey identified also a few genes with significant numbers of application in GM plants (see Table 7.2). Herbicide-tolerance genes like the *cp4epsps* gene derived from *A. tumefaciens* sp. strain CP4, the phosphinothricin acetyltransferase (*bar*) gene from *Streptomyces hygroscopicus* or from *S. viridochromogenes* (*pat*) have been identified to be reasonable targets for screening (Zeitler et al 2002; Waiblinger et al. 2005).

**Table 7.2** Genetic elements and constructs present in selected GM crops (Bruderer and Leitner 2003; Waiblinger et al. 2008)

Genetic element/ construct	GM crop (event name)						Sugar beet
	Canola	Cotton	Maize	Potato	Rice	Soybean	
<i>P-35S</i>	22-198 23-18-27 (Laurical) GS40/90	MON1445 MON1698 MON15985	676, 678, 680 DAS-59122-7 B16 (DL26)	ATBT04-X (NewLeaf) BT X (NewLeaf) SPBT02-5	LL62 LL06 LL601	356043 A2704-12 A2704-21	GTSB77 T120-7
Topas19/2	31807	Bt11	SPT02-7				A5547-127
OXY235	31808	Bt10					A5547-35
Liberator L62	MON531	Bt176					G94-1
T45	757	CBH-351					G94-19
	1076	DBT418					G-168
BXN		LY038					GTS40-3-2
		MON80100					GU262
		MON809					W62
		MON810					W98
		MON832					
		MON863					
		MON88017					
		MON89034					
		MS3, MS6					
		NK603					
		T14, T25					
		TC1507					
<i>T-nos</i>	MS1 RF1 RF2 MS1xRF1 MS1xRF2 MS8 RF3 MS8xRF3 OXY235	MON1445 MON1698 MON15985 MON531 757 1076 MON802 MON809	3272 Bt11 CBH-351 GA21 LY038 MIR604 MON80100 MON802 SPBT02-5, SPT02-7	ATBT04-X (NewLeaf) BT X (NewLeaf) RBMT15-X (NewLeaf) RBMT21-X (NewLeaf) RBMT22-X (NewLeaf)	Bt63	G94-1 G94-19 G-168 GTS40-3-2 W62 W98	



The  $\delta$  endotoxin (*cry*) genes from *Bacillus thuringiensis* (see Chap. 10) belong to the most frequently used genes in transgenic crops. However, screening methods targeting the different *cry* genes have not been established, because these genes and gene variants are target-organism specific, often synthetic or modified and in some cases truncated or fused, thus making this gene group less suitable for screening purposes.

If for example canola seed samples (canola see Chap. 21) have to be screened for the presence of GM events it is not advisable to use the P-35S specific method, since many GM canola events remain undetected (Table 7.2) and CaMV can infect rapeseed, thus increasing the chances of false-positive results. A screening concept for canola seeds proposed by the German official control laboratories therefore applies a combination of four different construct-specific PCR tests, allowing the detection of 13 known GM canola events (LAG 2006). As described in this concept, the combination of four PCR tests (*P35S-pat*, *pFMV-epsps*, *pSSUAra-bar*, *P35S-nptII*) covers 13 events and, if one test is positive, further analyses for identification of the GM event have to be performed.

Recently, also real-time PCR arrays based on multi-target analytical systems were developed to serve as less laborious analytical tools for the screening of unauthorised GM crops in the EU and Japan (Querci et al. 2008; Mano et al. 2009). The formats are 96-well or 384-well PCR plates prepared with primers and probes specific for the simultaneous detection of as many GM elements, constructs and events as possible.

### 7.3.2 Identification

The next step in the work flow of analysing samples which reacted positive in screening tests is the identification of the plant species and the GM events which may be present (Fig. 7.2). If the results of the screening tests indicate the presence of several different GM events, they must of course be first carefully analysed as to which specific tests have to be performed next to identify the GM plant with the most effective strategy. Depending on the sample it may thus be useful to verify first the plant taxa before numerous identification tests are performed. For example, if only DNA from one plant taxon is present, the testing scheme for GM event identification could be much less complex. Another alternative could be to first perform a sub-screening with construct-specific PCR methods targeting transgenic events containing identical gene constructs which have been used to generate several transformation events or to introduce the specific trait in different crop plants (see Fig. 7.1). If for example a construct-specific *ctp2-cp4epsps* screening is performed (Waiblinger et al. 2005, 2008), solely the different events tolerant to glyphosate (Roundup Ready, see Chap. 9) will be detected, such as canola GT73, maize MON88017 and NK603, soya MON89788 and sugar beet H7-1.

### 7.3.3 Quantification

For quantification of the GM plant material present in a sample, real-time PCR assays are commonly employed to determine the amount of sequence copies of the GM target versus the reference gene target, which obviously is not generating a direct weight-to-weight measurement (ENGL 2007). These assays use standard curves generated with a serial dilution of DNA of known GM content and target sequence concentration. In this way two calibration curves are constructed, one for the targeted GM sequence and one for the plant taxon-specific reference gene. The calibration DNA can be the DNA extracted from certified reference materials or plasmids (Block and Schwarz 2003), or hybrid amplicons carrying both target sequences can be used (Pardigol et al. 2003). The standard curves and the sample DNA are analysed in the same PCR run and, by extrapolating the Ct obtained, quantitative information for the targets is obtained. The copy numbers are calculated for the GM target sequence and the reference gene and used to estimate the relative amount and percentage of the GM plant event present in a given sample. Target DNA copy numbers of standards and quantitative positive controls must be precisely quantified before use, for example by fluorometric techniques (Ahn et al. 1996) or by spectrophotometric analysis (ISO 2005c). The DNA concentration measured is converted to copy number equivalents by using conversion factors, as reported by Arumuganathan and Earle (1991), or by referring to the plant DNA C-value database (Bennett and Leitch 2005). If certified reference materials of a certain percent GM content are used, the percentage of the material must be considered when calculating GM copy number equivalents for these materials. However, it should be also noted that quantitative PCR methods often measure the GM content in relation to specific reference materials, thus the genetic situation (zygosity, degree of ploidy, copy number per genome, etc.) is not considered, which could be an important issue particularly for maize (Papazova et al. 2005a, b; ENGL 2007).

Because of the relatively high measurement uncertainty (MU) accompanied with DNA-based quantitative analysis of the GM plant content in a given sample, it is important that testing laboratories apply procedures to calculate the combined standard deviation accumulating during the whole analytical process. Such a practical approach was recently described for the calculation of the overall MU for decision-making concerning the European 0.9% labelling threshold (Zel et al. 2007). These authors report that, for event GTS-40-3-2 (Roundup Ready soybean, see Chaps. 9, 24), the expanded uncertainty was 23.2%.

### 7.3.4 Detection of Stacked Events

A growing number of GM plant events containing stacked traits are approved and already cultivated in some countries (Tavaniers et al. 2008). Of the different approaches for the production of gene stacks, crossing GM events which express

different traits (e.g. by combining the Bt trait for insect resistance with a trait for herbicide tolerance) is preferably applied to rapidly obtain stacked events for commercialisation. This type of stacked event is indeed widely accepted by breeders and forms also the basis for the OECD definition of a unique identifier for gene stacks (OECD 2006).

In general, for the purpose of qualitative testing it is not necessary to discriminate between stacked and non-stacked events, since event-specific methods are already available for most of the commercialised parental GM lines and may be used to identify and, if necessary, to quantify the single events present in the stack. However, if a sample is positive for two or more single events which have been used for the production of a stacked event, it is hardly possible to discriminate between a mix of the single events (parents of the stack) and the hybrid (stacked) GM plant. The only currently available way to circumvent this analytical problem is to analyse single plants or seed kernels for example by using multiplex event-specific real-time PCR assays (Akiyama et al. 2005) or protein flow strips (Ma et al. 2005). However, these single kernel-based analyses are laborious and cost-intensive; sophisticated technical simplifications will be required for any routine application.

### 7.3.5 *Detection of Unauthorised/Unknown GMOs*

For GM plants not authorised for marketing as products, EU regulations stipulate a zero tolerance (Fig. 7.2). Examples of unauthorised GM products that have been identified at the European market are GM papaya ('SunUp' events 55-1, 63-1), several maize events ('StarLink' CBH-351, Bt10, 'event 32' DAS-59132-8, MIR604) and rice (LL601, LL62, 'Bt63'). One of the reasons for these incidences most likely was that protein-based ELISA and LFD tests were used by seed producers to test for the adventitious presence of GM events during scale-up and production. These tests cannot distinguish between different events, which had already potentially caused these problems with the basic seed material by contamination with unauthorised events carrying the same trait. For example, in 2005 the authorised event Bt11 maize was found to be mixed with event Bt10 which was not intended for further propagation and commercialisation and therefore not approved in the United States or in any other country at that time. A recent case (unapproved maize event DAS-59132-8 in DAS-59122-7) shows that protein-based seed quality testing is still causing problems when commercialising GM plants.

The detection for unapproved events is of course an extreme analytical challenge, since in most cases only limited information on such events is available or only partial characterisation has been reported. In these cases specific detection methods have to be developed (Mäde et al. 2006; Cankar et al. 2008), or have to be provided by the concerned seed companies and official authorities, e.g. the USDA. However any PCR-based detection strategy depends on the detailed knowledge of the genetic modification and of the DNA sequence of the insert in order to select

appropriate oligonucleotide primers. For a GM plant which is unknown to the control laboratories this approach is not applicable due to the lack of information on the genetic elements and DNA sequences. Other analytical strategies than PCR-based methods have to be applied to detect this category of GM events, e.g. finger-printing and fragment profiling techniques (AFLP, RAPD; Theuns et al. 2002), whole genome amplification (Roth et al. 2008) and extensive DNA sequencing. Recently, a pilot study with high-density microarrays showed it was applicable for the screening or profiling of discrete transgene elements present in unknown GMOs (Tengs et al. 2007). However, this method needs pure and relatively high sample DNA concentrations because no PCR amplification of target DNA is performed before the hybridisation step and these microarrays are very cost-intensive. Further optimisation of this approach will clarify whether such an array-based method could be a helpful tool not only for research on plants, but also for detection of unknown GM events in general.

### 7.3.6 *Method Databases*

Reports and public databases provide information about the genetic elements contained in GM plants (Bruderer and Leitner 2003; AGBIOS 2008). At the European level detailed information is provided on GM plants for which an application for authorisation has been submitted or which are authorised in the EU. There are also lists of methods and databases available which are valuable sources to find information on validated protein and DNA-based methods used for the identification of GM plants (Bonfini et al. 2007; CRL-GMFF 2008; Dong et al. 2008; JRC 2008).

### 7.3.7 *Sampling Issues*

The sampling procedure includes different steps and consists of: (i) taking a composite of increments from a lot to form a bulk sample, (ii) reducing the bulk sample to the laboratory sample and (iii) after grinding/homogenisation again, taking a portion for the actual analysis (test portion). An optimal sampling plan is adapted to the lot size to yield a representative laboratory sample and is of course always a compromise between costs and accepted sampling error. Guidance for the sampling of food and feed products can be found in general standards published by ISO (1999, 2002). At the European level specific documents and recommendations have been established, particularly for GMO sampling of food (CEN/TS15568; EU 2004). Sampling of seeds should follow internationally agreed practices according to the appropriate regulations of the International Seed Testing Association (ISTA). On that basis it is generally agreed that a test sample taken for the GMO analysis should contain at least 2995 seeds to detect a GM seed content of 0.1% with a

confidence level of 95%. It is noted that information on the sampling procedure is of course essential for the correct interpretation of an analytical report.

## 7.4 Conclusions

The application of appropriate methods and strategies applied for sensitive and specific GM plant detection in seeds, in food and in feed products has become a challenging issue because the global cultivation rates and species of GM crop plants, as well as the diversity of inserted genes and regulatory elements, are constantly increasing. This is currently reflected by accelerated efforts to study and develop new methods and tools with the aims of solving the technical problems, achieving scientific advancement and harmonising GMO detection approaches and testing regimes. It has to be awaited whether technical solutions can be provided for pending problems, for example like the correct distinction and correct quantification of (multiple) stacked events. Concerning the detection of unauthorised GM events it is noted that research institutions and biotechnology companies should contribute as much as possible to minimise the risk that GM plants developed and studied for research purposes are not dispersed accidentally into the environment or marketed through impurities in non-GM seed lots. As demanded for the analytical GM testing process, strict and reliable quality management systems may contribute to the positive public perception concerning the use of GM plants.

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**Part B**

**Selected Characters of Transgenic  
Plants and Their Application  
in Plant Production**



# **Chapter 8**

## **Drought Stress Tolerance**

**Dorothea Bartels and Jonathan Phillips**

### **8.1 Introduction**

Improving the drought tolerance of crops has been an important aim of plant breeders for a long time, and successful varieties have been developed. Despite this fact the issue of food security will become more serious due to the forecasted global climatic changes in combination with the increasing world population (FAO 2006). Many environmental factors are responsible for a reduced crop yield. Among them, drought is one of the major threats to agricultural production. Even in the most productive agricultural areas, periods of water deficiency are responsible for considerable reductions in biomass yield every year. This chapter focuses on drought, although exposure to drought often triggers reactions common to drought, salinity or low temperature. The consequence of all three environmental factors is cellular dehydration leading to osmotic stress, likewise the production of reactive oxygen species. Therefore plants often show tolerance to several stressors.

Drought tolerance can be achieved by different mechanisms such as by taking up as much water as possible, high water-use efficiency, and by directing photosynthesis products into harvestable material like grains (Blum 1988). Three main approaches have been used in breeding for drought tolerant varieties: (i) select for high yield potential under optimal conditions, (ii) select for maximum yield in target environments, (iii) incorporate known morphological or physiological parameters of drought stress tolerance in the selection schemes. Drought stress tolerance is a complex phenomenon and involves many genes. The existence of differences in drought tolerance between genotypes indicates that there is a genetic basis for drought tolerance mechanisms. This is the justification for the analysis of genetic

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variations through mapping quantitative trait loci (QTLs), which is often applied for crop plants. However, the time-frame from identification of QTLs to gene discovery is long, and success depends on the availability of a comprehensive genetic map.

About two decades ago molecular approaches were started to dissect the gene network determining drought stress tolerance. Many genes have been identified which are responsive to drought stress (Seki et al. 2002). Molecular studies have preferentially used the genetic model plant *Arabidopsis thaliana*, because of its small genome size and the availability of the full genome sequence. Naturally desiccation-tolerant plants like the resurrection plant *Craterostigma plantagineum* have also been exploited in order to isolate genes which confer tolerance (Sunkar et al. 2003). There have been numerous reports of genes conferring stress tolerance: The source for most of the genes has been *Arabidopsis*. Only a very few genes have successfully been tested in crop plants, because many of the potential candidate genes only led to a marginal increase of stress tolerance or the genes had side-effects on growth or morphological parameters.

Here we focus on successful strategies which have been recently applied to obtain crop plants with improved stress tolerance.

## 8.2 Transgenic Plant Strategies for Enhanced Drought Stress Tolerance in Crop Plants

Plants have evolved adaptive strategies to cope with abiotic stress conditions, such as drought. The plant stress response can be divided into perception and transduction of environmental cues through signalling components, resulting in activation of stress-related genes and synthesis of diverse proteins that function in physiological and metabolic responses. Well characterized proteins involved in the protection of plant cells from dehydration stress damage include chaperones, osmotic adjustment proteins, ion channels, transporters, and antioxidation or detoxification proteins (Bartels and Sunkar 2005).

Transgenic approaches offer a powerful means to gain information towards a better understanding of the mechanisms that govern stress tolerance. They also open up new opportunities to improve stress tolerance by incorporating genes involved in stress protection from any source into agriculturally important crop plants. To date, the transgenic approach has typically been to transfer a single gene into plants (for methods, see Chaps. 1, 2) and then observe the phenotypic and biochemical changes before and after a specific stress treatment. A limitation of this strategy is that the functions of relatively few genes involved in abiotic stress tolerance have been established, and not enough is known about the regulatory mechanisms. Complex quantitative traits such as abiotic stress tolerance have a relatively low heritability and are controlled by many genes that interact with each other, the genetic background of the organism, and the environment. Dissection of

the abiotic stress tolerance traits has resulted in the identification of multiple chromosomal segments associated with tolerance via QTL analysis (Tuberosa and Salvi 2006). Despite this complexity, notable successes have been achieved through metabolite engineering (Table 8.1; see also Chap. 11) and through the manipulation of regulatory genes (Table 8.2). Recent examples that illustrate both types of strategy to engineer drought tolerance in crop plants are described in the following sections.

### 8.2.1 *Osmoprotectants and Metabolite Engineering*

Abiotic stress such as dehydration, salt or freezing perturbs the cellular metabolism, as pointed out in numerous physiological experiments using many different plant species (Ingram and Bartels 1996; Bartels and Sunkar 2005). Therefore research strategies have been designed to counteract the metabolic imbalance provoked by abiotic stressors. It is implied in the research strategy that engineering metabolism may counteract negative consequences of stress only within a certain range. Another consideration has to be that engineering of metabolic pathways should not interfere with other pathways, e.g. those which are responsible for biomass or yield.

Most organisms, ranging from microbes to animals and plants, synthesize compatible solutes in response to dehydration. Compatible solutes are non-toxic small molecules which do not interfere with normal cellular metabolism. Depending on the organism a variety of substances have been described as compatible solutes. Examples are sugars or sugar alcohols such as raffinose, galactinol, trehalose or fructan, amino acids such as proline, amines such as glycine betaine or polyamines. Compatible solutes have their main role in turgor maintenance and in osmotic adjustment. Also additional functions have been discussed such as stabilizing cell proteins and structures, scavenging reactive oxygen species, signalling functions or induction of adaptive pathways (Hasegawa et al. 2000; Chen and Murata 2002). However, the exact function is not fully understood. Simple, preferentially one-step transformation strategies were designed to increase the accumulation of these molecules (including in plant species in which osmolytes do not accumulate naturally). This approach was partly successful and stress-tolerant plants were obtained (Table 8.1), although this strategy did not always lead to osmotic adjustment (Serraj and Sinclair 2002). Most approaches relied on transforming plants with a single gene, and this may be the reason that often only marginal stress tolerance was obtained. In nearly all examples osmolyte accumulation in the whole plant was considered but not in specific tissues or specific cells. In a detailed analysis of gene expression in *Arabidopsis* roots, Dinneny et al (2008) showed that stress genes may be restricted to particular lineages of cells in the *Arabidopsis* root. Such data may provide better targets for modifying metabolite profiles in the future.

**Table 8.1** Selected transgenic crop plants with enhanced tolerance to osmotic stress via metabolite engineering

Gene	Class	Species (origin of gene)	Transgenic crop species	Promoter	Performance of transgenic plant	Additional notes	References
<i>P5CS</i>	Δ1-pyrroline-5-carboxylate synthetase	<i>Vigna aconitifolia</i>	<i>Oryza sativa</i>	AIPC (ABA inducible promoter complex)	Dehydration and high salinity tolerance	Increased biomass (higher fresh shoot and root weight)	Zhu et al. (1998)
<i>SacB</i>	Levan sucrase	<i>Arthrobacter globiformis</i> <i>Bacillus subtilis</i>	<i>Tomato</i> <i>Beta vulgaris</i>	CaMV 35S	Various abiotic stresses	Expression targeted to chloroplasts	Park et al. (2007)
<i>otsA/otsB</i>	Trehalose-6-phosphate synthase/ Trehalose-6-phosphate phosphatase	<i>Escherichia coli</i>	<i>O. sativa</i>	AIPC (ABA inducible promoter complex); <i>rbcS</i>	Dehydration, high salinity and low temperature tolerance	Increased biomass (leaves and roots) under drought	Pilon-Smits et al. (1999)
<i>mtlD</i>	Mannitol-1-phosphate dehydrogenase	<i>E. coli</i>	<i>Triticum aestivum</i>	ZmUbi1	Dehydration and high salinity tolerance	Sustained photosynthetic rate and growth under stress	Garg et al. (2002)
<i>otsA/otsB</i>	Trehalose-6-phosphate synthase/ Trehalose-6-phosphate phosphatase	<i>E. coli</i>	<i>O. sativa</i>	ZmUbi1	Dehydration, high salinity and low temperature tolerance	Sustained photosynthetic rate and growth under stress	Jang et al. (2003)
<i>adc</i>	Arginine decarboxylase	<i>Datura stramonium</i>	<i>O. sativa</i>	ZmUbi1	Dehydration tolerance	Unaffected by PEG treatment	Capell et al. (2004)

**Table 8.2** Transgenic crop plants engineered for enhanced tolerance to osmotic stress using regulatory and signalling genes

Gene	Class	Plant species	Transgenic crop species	Promoter	Performance of the transgenic plant	Additional notes	Reference
<i>DsCDPK7</i>	Calcium-dependent protein kinase	<i>Oryza sativa</i>	<i>O. sativa</i>	CaMV 35S	Dehydration, high salinity and low temperature tolerance	Decreased wilting under stress conditions.	Saijo et al. (2000)
<i>CBF3/DRREB1A</i>	ERF/AP2 (subgroup IIIc)	<i>Arabidopsis thaliana</i>	<i>Triticum aestivum</i>	rd29A	Dehydration tolerance	Delay in germination, delayed water stress symptoms, greater number of heads, more branched root architecture.	Pellegrineschi et al. (2004)
<i>NPK1</i>	MAPKKK	<i>Nicotiana tabacum</i>	<i>Zea mays</i>	CaMV 35S	Dehydration tolerance	Sustained photosynthetic rate under drought. Delayed maturity and increased leaf number. Reduced impact of drought on kernel weight.	Shou et al. (2004)
<i>GF14<i>j</i></i>	14-3-3	<i>A. thaliana</i>	<i>Gossypium hirsutum</i>	CaMV 35S	Dehydration tolerance	Sustained photosynthetic rate and increased leaf survival under drought. "Stay-green" phenotype (delayed senescence).	Yan et al. (2004)
<i>CBF3/DRREB1A</i>	ERF/AP2 (subgroup IIIc)	<i>A. thaliana</i>	<i>O. sativa</i>	ZmUb1l	Dehydration and high salinity tolerance	Low reduction in Fv/Fm under drought and high salinity. Greater survival under drought. Low level tolerance to low temperature. No growth stunting.	Oh et al. (2005)
<i>ABF3</i>	bZIP	<i>A. thaliana</i>	<i>O. sativa</i>	ZmUb1l	Dehydration tolerance	Low reduction in Fv/Fm and greater survival under drought. No enhanced tolerance to low temperature or high salinity. No growth stunting.	Oh et al. (2005)
<i>SNAC1</i>	NAC	<i>O. sativa</i>	<i>O. sativa</i>	CaMV 35S	Dehydration and high salinity tolerance	Increased ABA sensitivity and delayed water stress symptoms. Sustained growth and greater	Hu et al. (2006)

**Table 8.2** (continued)

Gene	Class	Plant species	Transgenic crop species	Promoter	Performance of the transgenic plant	Additional notes	Reference
					survival under salinity stress.		
					Enhanced yield under drought stress in the field.		
					Deeper green, increase in leaf canopy with more tillers.		
					Enhanced water use efficiency.		
					Sustained photosynthetic rate and growth (root and shoot) under drought stress.		
					Less wilting during drought and greater recovery after stress.		
					Sustained photosynthetic rate and yield under drought stress in the field.		
					Delayed appearance of stress symptoms, rapid recovery and greater survival rate. No growth stunting.		
<i>HARDY</i>	ERF/AP2 (subgroup IIIb)	<i>A. thaliana</i>	<i>O. sativa</i>	CaMV 35S	Dehydration tolerance		Karaba et al. (2007)
<i>ZmNF-YB2</i>	CCAAT-binding	<i>Zea mays</i>	<i>Z. mays</i>	OsRAFT	Dehydration tolerance		
<i>HvCBP4</i>	ERF/AP2 (subgroup IIIc)	<i>Hordeum vulgare</i>	<i>O. sativa</i>	ZmUB1	Dehydration, high salinity and low temperature tolerance	Delayed appearance of stress symptoms, rapid recovery and greater survival rate. No growth stunting.	Oh et al. (2007)
<i>SNAC2</i>	NAC	<i>O. sativa</i>	<i>O. sativa</i>	ZmUB1	Osmotic, high salinity and low temperature tolerance	Improved growth in salinity and PEG media. No yield increase under drought stress in the field.	Hu et al. (2008)
<i>OsDREB1F</i>	ERF/AP2 (subgroup IIIc)	<i>O. sativa</i>	<i>O. sativa</i>	CaMV 35S	Dehydration, high salinity and low temperature tolerance	Sustained growth under stress conditions. Both ABA-dependent and ABA-independent pathways activated.	Wang et al. (2008)
<i>HDG11</i>	Homeodomain-START	<i>A. thaliana</i>	<i>Nicotiana tabacum</i>	CaMV 35S	Dehydration tolerance	Extensive root system, reduced leaf stomatal density and enhanced water use efficiency.	Yu et al. (2008)

### 8.2.1.1 Amino Acid-Derived Osmoprotectants

Metabolic engineering studies have mainly focused on proline and betaine. Both proline and betaine have been shown to accumulate in several plant species under stress (Sunkar and Bartels 2005). The biosynthetic pathways for both metabolites have been well studied and therefore the genes for manipulating the pathways are available (Bohnert and Shen 1999).

#### Proline

Many plants accumulate proline in response to osmotic stress (Delauney and Verma 1993). Two biosynthetic proline pathways exist in plants: the ornithine-dependent pathway and the glutamate-dependent pathway. The glutamate pathway seems to be the predominant pathway for proline synthesis, especially under stress conditions (Delauney and Verma 1993). The other important reaction that controls proline levels is the oxidation of proline by proline dehydrogenase to pyrroline-5-carboxylate (Nanjo et al. 1999). The transgenic plants overexpressing proline biosynthetic enzymes demonstrated the involvement of proline in response to water deficit (Kishor et al. 1995; Roosens et al. 2002). Increased degradation of proline via pyrroline-5-carboxylate reductase resulted in increased sensitivity to water stress (de Ronde et al. 2000). Transgenic rice (see Chap. 22) and wheat plants (Chap. 16) that accumulate proline showed better stress tolerance to dehydration and salinity, respectively (Zhu et al. 1998; Sawahel and Hassan 2002).

#### Glycine Betaine

Glycine betaine is a quaternary ammonium compound that occurs in a variety of plants, animals and microorganisms (Chen and Murata 2008). Glycine-betaine is synthesized in plants via a two-step oxidation of choline. In spinach and some other plants the oxidation is carried out by two chloroplastic enzymes: choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). The first reaction is the oxidation of choline to betaine aldehyde and the second reaction oxidizes betaine aldehyde to betaine (Fitzgerald et al. 2009). The overexpression of betaine biosynthesis genes either derived from bacteria or from plants in generating drought tolerance in plants, which do not naturally synthesize glycine betaine (Chen and Murata 2002). Examples are transgenic *Arabidopsis*, *Brassica napus*, tobacco, rice or tomato (Sakamoto et al. 1998; Mohanty et al. 2002; Shirawasa et al. 2006; Park et al. 2007). In some plants low accumulation of betaine was observed, which was explained by a limited supply of choline (Nuccio et al. 1998). The effectiveness of this approach seems also be dependent on the correct compartment in which betaine accumulates, as demonstrated for tomatoes, in which chloroplastic accumulation of betaine is more effective than cytosolic (Park et al. 2007). The different results show that understanding metabolic fluxes in plants is an important prerequisite for

successful genetic engineering. Several agronomically important crops such as wheat, potato or tomato do not accumulate glycine betaine naturally and would therefore be good targets for engineering betaine biosynthesis.

### 8.2.1.2 Sugar-Related Osmoprotectants

#### Fructans

Fructans are oligo- or polyfructose molecules that accumulate in vacuoles of many plants growing in temperate climates. Sugar beet and tobacco plants that were transformed with the bacterial fructan biosynthesis gene showed improved tolerance to drought stress (Pilon-Smits et al. 1999). However, this approach has not been transferred to other crop plants.

#### Polyols

Polyols are hydroxylated sugar alcohols with osmoprotective properties which accumulate in response to abiotic stress in various plants. The synthesis of these compounds involves simple pathways and therefore it was possible to transfer the biosynthesis genes to transgenic plants in order to test their potential for stress tolerance (Bohnert and Chen 1999; Bartels and Sunkar 2005). The overproduction of polyols such as mannitol, D-ononitol, inositol or sorbitol in transgenic plants enhanced stress tolerance (Bartels and Hussain 2008). It is assumed that the polyols confer stress tolerance through osmotic adjustment. However, the level of polyols did not always correlate with stress tolerance, therefore other mechanisms have also been suggested like reactive oxygen scavenging or signalling. Targeting polyol biosynthesis was one of the earliest concepts for engineering plants with improved stress tolerance (Bohnert and Chen 1999). Although Abebe et al. (2003) demonstrated that expressing the gene encoding mannitol dehydrogenase in wheat improved the performance under drought and salinity stress, there are no further reports of using this concept for crops. The reason for this is that some of the polyol compounds have undesired effects like growth defects and necrosis (Sheveleva et al. 1998). These authors showed that there is a competitive effect between transgene and host metabolism. This underlines the necessity to understand metabolic fluxes before successful applications to agricultural plants.

#### Trehalose

Trehalose is a non-reducing disaccharide found in many different organisms. The sugar functions as a reserve carbohydrate and as a stress protectant, particularly in yeast or microorganisms. Trehalose does not accumulate to high levels in most plants probably due to degradation by trehalase (Goodijn and van Dun 1999). The synthesis of trehalose is a two-step reaction, starting with glucose-6-phosphate and

uridine diphosphoglucose using trehalose phosphate synthase and trehalose-6-phosphate phosphatase as catalysing enzymes. By overexpressing both genes in transgenic plants, stress-tolerant plants have been obtained (Table 8.1; Bartels and Hussain 2008). Often the trehalose level did not correlate with stress tolerance and plants were observed with altered growth and morphogenic phenotypes. Investigations showed that the metabolic intermediate trehalose-6-phosphate is responsible for the aberrant phenotypes, as it functions as a signalling molecule regulating sugar and starch metabolism (Paul 2007). Recent experiments indicated that negative consequences may be overcome by choosing a suitable promoter and by transforming chloroplasts. In this way transgenic plants have been obtained with improved drought stress tolerance but no negative side-effects (Garg et al. 2002; Jang et al. 2003). Like the engineering of polyol levels, the trehalose metabolic network needs to be better understood before such an approach can be accepted as a way to improve stress tolerance in agricultural plants.

### **8.2.2 Regulatory and Signalling Genes: Tools to Engineer Drought Stress Tolerance**

Studies of abiotic stress-activated signalling cascades have resulted in the identification of potential regulatory genes, such as transcription factors and protein kinases. The transformation of plants using regulatory genes is an attractive approach for producing abiotic stress-tolerant plants. Since the products of these genes regulate gene expression and signal transduction under stress conditions, the expression of these genes can activate the expression of many stress-tolerance genes simultaneously. For example, transcription factors are able to recognize and bind to regions of DNA that have a specific sequence in the promoters of the genes they regulate. Thus, by altering the expression levels of a transcription factor, entire biological pathways can be modified. Similarly, altered expression of protein kinases may enable phosphorylation dependent changes of multiple protein substrates by changing enzyme activity, cellular location, or association with other proteins. One potential drawback of this approach is the increased likelihood of unintended or pleiotropic effects when regulatory/signalling genes are genetically engineered. Such effects tend not to be desirable in crops and strategies to ameliorate these effects may need to be considered. Here we discuss examples where transcription factors and protein kinases have been used to engineer enhanced tolerance to drought stress conditions in crop plant species.

#### **8.2.2.1 DREB/CBF: a Landmark Discovery in the Manipulation of Abiotic Stress Tolerance**

The ability to manipulate co-regulated stress tolerance-associated genes at the transcriptional level was first realised when common *cis* elements were discovered

in abiotic stress-responsive promoter regions and the associated transcription factors that specifically bound to the *cis* elements were identified. An early example was the discovery of the dehydration-responsive element/C-repeat (DRE/CRT) as a *cis*-acting element regulating gene expression in response to dehydration (salt, drought, cold stresses) in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994). Subsequently, transcription factors DREB1/CBF1-3 and DREB2, belonging to the ERF/AP2 family (subgroup IIIc; Nakano et al. 2006), were reported to bind to DRE/CRT elements (Stockinger et al. 1997; Liu et al. 1998). A major breakthrough was made when Kasuga et al. (1999) transformed *Arabidopsis* with a cDNA encoding DREB1A/CBF3 driven by either the constitutive CaMV 35S promoter or an abiotic stress-inducible promoter. The overexpression of this gene activated the expression of many stress-tolerance genes such as late embryogenesis abundant (LEA) genes and  $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS). In all cases, the transgenic plants were more tolerant to drought, salt, and freezing stresses.

*Arabidopsis* CBF/DREB proteins are also heterologously effective in crops such as *Brassica napus* (Jaglo et al. 2001), tomato (Hsieh et al. 2002), wheat (Pellegrineschi et al. 2004), and rice (Oh et al. 2005), up-regulating the corresponding target genes and enhancing stress tolerance in transgenic plants. These results established the DREB/CBF pathway as a useful target for the biotechnological improvement of abiotic stress tolerance in both monocotyledons and dicotyledons. Despite the fact that DREB/CBF related responses appear conserved, plant species vary greatly in their abilities to survive adverse effects from exposure to environmental constraints. In the initial experiments by Kasuga et al. (1999), it was observed that constitutive overexpression of DREB1A/CBF3 in *Arabidopsis* resulted in severe growth retardation under normal growth conditions. Constitutive expression of *Arabidopsis* DREB1A/CBF3 and OsDREB1F in rice, however, resulted in neither growth inhibition nor visible phenotypic alterations (Oh et al. 2005; Wang et al. 2008). A similar lack of pleiotropic effects was also observed for the basic leucine zipper (bZIP) transcription factor ABF3 when ectopically expressed in rice (Oh et al. 2005). This phenomenon may have occurred because lower levels and/or fewer numbers of target genes are activated by DREB1A/CBF3 or ABF3 in rice than in *Arabidopsis*, and hence, the effects on plant growth might be minimized in rice. Oh et al. (2005) also postulate that rice is evolutionarily more tolerant to the expression of stress-regulated genes than dicots, including *Arabidopsis*. Where pleiotropic effects are observed, however, it appears more appropriate, at least in the case of *DREB/CBF* genes, to use an inducible promoter. For example, the stress-inducible regulation of *DREB1A/CBF3* via the *rd29A* promoter repeatedly had minimal effects on plant growth under well watered conditions (Kasuga et al. 1999; Pellegrineschi et al. 2004).

*Arabidopsis* CBF3/DREB1A expression in transgenic rice increases tolerance to drought and high salinity, but with relatively low levels of tolerance to low-temperature exposure (Oh et al. 2005). These data are in direct contrast to CBF3/DREB1A expression in transgenic *Arabidopsis*, which functions primarily to enhance freezing tolerance. This is presumably because *Arabidopsis* plants that are capable of cold acclimatization have evolved differently from rice plants that are unable to undergo cold acclimatization (Jaglo et al. 2001). Furthermore, the

HvCBF4 protein from barley appears to be more efficacious than CBF3/DREB1A from *Arabidopsis* in conferring stress tolerance to transgenic rice (Oh et al. 2007). When compared with CBF3/DREB1A, HvCBF4 overexpression in rice showed similar levels of tolerance to drought and high salinity, but a higher level of tolerance to low temperature. These data suggest functional differences between members of the DREB/CBF family and highlights the variation in stress tolerance between transgenic plant species. This is probably related to the complexity and nature of the target genes or “regulon” that is present in the plant genome and the capacity of the transcription factor to activate or repress each target gene.

### 8.2.2.2 SNAC1/2: Stress-Responsive Plant-Specific Transcription Factors with Distinct Mechanisms of Action

The *NAC* gene family encodes plant-specific transcription factors that were initially linked with regulation of plant development; however a role in abiotic stress tolerance has since been established (Olsen et al. 2005). This discovery was based on the identification of a salt- and drought-induced gene, *ERD1*, that was found to be regulated in an ABA-independent manner via a novel regulatory pathway for drought and high salinity adaptation (Nakashima et al. 1997). A MYC-like *cis* element was found necessary for induction of *ERD1* that was recognized by three transcription factors of the NAC family (Tran et al. 2004). In addition to *ERD1*, many other salt and/or drought stress-induced genes were also regulated by NAC proteins which correlated with enhanced drought tolerance in *Arabidopsis* overexpression lines (Tran et al. 2004).

More recently, Hu et al. (2006) reported a NAC transcription factor significantly enhancing drought and high salinity tolerance in rice: *STRESS-RESPONSIVE NAC 1 (SNAC1)*. *SNAC1*-overexpressing rice plants exhibited significantly enhanced yield (22–34% higher seed setting than control) in field conditions under drought stress conditions at the reproductive stage, while displaying no yield penalty. The transgenic rice displayed noticeably improved drought and salt tolerance at the vegetative stage. The transgenic rice plants are more sensitive to abscisic acid (ABA) and lose water more slowly through stomatal movement, yet display no significant difference in the rate of photosynthesis. The *SNAC1*-overexpressing rice plants also showed improved salt tolerance, further emphasizing the usefulness of *SNAC1* in a broad abiotic stress tolerance improvement (Hu et al. 2006).

A closely related stress-responsive NAC transcription factor gene termed *SNAC2* was subsequently isolated from upland rice (Hu et al. 2008). Transgenic rice overexpressing *SNAC2* showed significantly improved tolerance to cold, as well as to salinity and dehydration stresses. *SNAC2* differs from *SNAC1*, however, in a several aspects, which illustrates how transcription factor gene family members can have broadly different functional characteristics. Unlike *SNAC1*, overexpression of *SNAC2* showed no significant effect on drought resistance in the field conditions even though the transgenic plant showed improved tolerance to osmotic stress by PEG treatment. In addition, *SNAC2* overexpression can enhance cold

tolerance while overexpression of SNAC1 had no significant effect on improving cold tolerance, even though *SNAC1* is induced by cold (Hu et al. 2008). Furthermore, the transcriptional target genes of SNAC1 and SNAC2 are different, such that transcript profiles of differentially regulated genes in the SNAC1- and SNAC2-overexpressing lines revealed broadly no overlap (Hu et al. 2008). This appears to be the basis for the difference of the two overexpressing transgenic plants in stress tolerance, which is further supported by the observation that flanking sequences of the core DNA binding sites in the putative SNAC1 and SNAC2 target genes are different (Hu et al. 2008). One caveat to the comparative studies involving SNAC1- and SNAC2-overexpressing lines is that the transcription factors were under different regulatory control (see Table 8.2). While both the CaMV 35S and ZmUbi1 promoter sequences are considered to be constitutive (Odell et al. 1985; Christensen et al. 1992), subtle differences in the promoter activity may influence the functional properties of each transcription factor.

### 8.2.2.3 HARDY: Engineering Water Use Efficient Rice

Water use efficiency (WUE), measured as the biomass produced per unit transpiration, describes the relationship between water use and crop production. In water-limiting conditions, it is agronomically desirable to produce increased biomass, which contributes to crop yield, using less water. Although genetic variation for WUE may vary in crop plants, so far, the engineering of major field crops for improved WUE has been challenging. One notable success involved the expression of the *Arabidopsis* subgroup IIIb AP2/ERF-like transcription factor, HARDY (HRD; Karaba et al. 2007). The *HRD* gene is normally active in inflorescence-stage tissue, however ectopic *HRD* expression leads to an enhancement in root and leaf structure, which is recognized as an adaptive mechanism for drought tolerance and WUE in crops.

The overexpression of HRD in rice generates plants with significantly higher biomass, independent of drought stress (Karaba et al. 2007). With the increase in shoot biomass in the HRD-overexpressing lines, there is a reduction in the specific leaf area and the leaf area per unit dry weight, suggesting an increase in leaf thickness or tissue density. The increased number of cells in the bundle sheath is likely to contribute to increased photosynthetic assimilation. HRD overexpression increases root biomass under drought stress, indicating an ability to adapt by inducing roots to harvest the scarce water. The increase in photosynthesizing area and carbon assimilation contributes significantly to canopy photosynthesis, resulting in high biomass. This result appears to be related to an increase in leaf mesophyll, bundle sheath, and root cortical cells, enhancing the capacity of both source and sink tissue. In rice, ectopic HRD expression causes significant increases of whole-plant WUE in well watered and drought conditions, however it remains to be determined whether such a strategy will increase WUE in different crops.

### 8.2.2.4 HD-START: a Developmental Regulator Conferring Drought Tolerance

Transpirational water loss through the stomata is a key determinant of drought tolerance. Stomatal movement is a response to environmental changes and is controlled by guard cell turgor which is influenced by many endogenous and exogenous factors (Assmann and Wang, 2001; Schroeder et al. 2001). Phenotypic screening of gain-of-function *Arabidopsis* mutants led to the discovery of *enhanced drought tolerance 1* (*edt1*) that was found to have elevated levels of *HDG11*, a gene that plays an important role in water homeostasis and encodes a homeodomain (HD)-START transcription factor (Yu et al. 2008). Overexpression of *HDG11* in tobacco resulted in improved drought tolerance, improved root architecture, and reduced stomatal density, all of which contributed to improved water homeostasis (Yu et al. 2008). In the original *edt1* mutant, *HDG11* expression resulted in higher proline levels and superoxide dismutase activity, which contributed to enhanced osmotic adjustment and reactive oxygen species detoxification. In addition, higher abscisic acid content was observed that led to a reduced rate of water loss (Yu et al. 2008). Taken together, the water homeostasis-related phenotypes conferred by ectopic expression of *HDG11*, as well as the reported absence of unwanted pleiotropic effects, make this gene an excellent candidate for genetic engineering of drought tolerance in crop plants.

### 8.2.2.5 Plant Nuclear Factor Y B Subunits: Field-Validated Drought Tolerance in Maize

Systematic analysis of *Arabidopsis* transcription factor families led to the identification of candidate genes that have the potential to improve tolerance to environmental stress in crop species (Riechmann et al. 2000; [www.mendelbio.com](http://www.mendelbio.com)). This high-throughput (HTP) screening approach resulted in the discovery of AtNF-YB1, a subunit of the nuclear factor Y (NF-Y complex), which mediates transcriptional control through CCAAT DNA elements and confers abiotic stress tolerance when constitutively expressed in *Arabidopsis* (Nelson et al. 2007). NF-Y is a conserved heterotrimeric complex consisting of NF-YA, NF-YB, and NF-YC subunits (Mantovani, 1999). Following the discovery of AtNF-YB1, an orthologous NF-YB gene from *Zea mays* (*ZmNF-YB2*) was identified that similarly coordinates plant responses to drought tolerance (Nelson et al. 2007). This discovery illustrates functional conservation of the underlying drought tolerance pathway across the dicot and monocot lineages as well as validating the HTP biotechnology discovery process. Drought tolerance was also obtained in field trials with maize lines constitutively expressing the *ZmNF-YB2* protein, demonstrating the potential of this strategy for improving drought tolerance in commercial crop plants (Nelson et al. 2007).

### 8.2.2.6 Rice Calcium-Dependent Protein Kinase 7: Multiple Abiotic Stress Tolerance with Minimal Pleiotropic Events

Cytoplasmic  $\text{Ca}^{2+}$  levels in plant cells increase rapidly in response to abiotic stress, including drought (Sanders et al. 1999). Following  $\text{Ca}^{2+}$  influx, signals are mediated by combinations of protein phosphorylation/dephosphorylation cascades, involving members of the  $\text{Ca}^{2+}$ -dependent protein kinase (CDPK) family. Overexpression of one member of the CDPK family, OsCDPK7, results in cold, salt, and drought tolerance in rice plants (Saijo et al. 2000). Analysis of the transgenic rice revealed enhanced salt/drought induction of the genes of late embryogenesis abundant (LEA) proteins, which appears to contribute, at least in part, to the improved abiotic stress tolerance in rice plants (Saijo et al. 2000). This observation is consistent with results from a previous study where the ectopic expression of the barley group 3 LEA protein, HVA1, was shown to confer both salt and drought stress tolerance to transgenic rice plants (Xu et al. 1996). OsCDPK7 is thought to be subject of post-translational control and/or requires the expression of other proteins in order to function, since the presence of OsCDPK7 is not sufficient to induce expression of stress-associated target genes. Consistent with this theory, no significant pleiotropic effects were reported with regard to development, growth and yield penalty of the OsCDPK7 overexpression lines in untreated conditions (Saijo et al. 2000).

### 8.2.2.7 Tobacco Protein Kinase: Sustained Yield in Maize under Water-Limited Conditions

Tobacco protein kinase (NPK1) is a tobacco mitogen-activated protein kinase kinase kinase (MAPKKK). The catalytic domain of NPK1 specifically activates a bypass of C kinase (BCK1)-mediated signal transduction pathway in yeast, indicating that the catalytic function of NPK1 is conserved among different organisms (Banno et al. 1993). NPK1 is located upstream of the oxidative pathway and can induce expression of heat shock proteins and glutathione-S-transferase in *Arabidopsis* and maize (Kovtun et al. 2000). Activation of oxidative stress tolerance genes is a strategy to protect the photosynthesis machinery from damage caused by drought, thus stabilizing source-sink relationships and improving the yield potential in water-limited conditions. Transgenic maize plants showed an increase in drought tolerance including higher photosynthetic rates, higher leaf numbers, and higher kernel weights compared with the control (Shou et al. 2004). Tolerance to drought stress in maize was improved through the constitutive expression of NPK1 that activates the oxidative signalling pathway, however the effect on yield components such as kernel number was less apparent in the study. This was most likely due to the application of pollen from non-stressed maize plants. Although this ensured seed set, the potential effects of NPK1 on reducing the anthesis-silking interval under drought stress was not determined. Further studies

are needed to explore the full potential of NPK1 on source–sink relationships in different maize germplasm.

### 8.3 Future Prospects: “Climate-Ready” Crops

Agriculture continues to withdraw and use the most freshwater of any economic sector. Globally, according to the United Nations Environmental Program, agricultural water use accounts for approximately 70% of all available freshwater, mainly through crop irrigation. Limited availability of water resources for agronomic uses generates important challenges in the context of how crop productivity can be elevated to meet the demands of an increasing world population. The development of crops that require less irrigation could reduce the costs of production and competition for water resources. Furthermore climate change is likely to reduce yields of primary food crops such as maize, wheat, and rice. The potential impact of climate change on agriculture will affect all global regions, however it will be most pronounced in semi-arid regions of the developing world. This prediction raises an important question: can agriculture adapt quickly enough to respond to climate change and an increasing world population?

Public and private sector research efforts are focused on developing so called “climate-ready” crops (CGIAR 2006) that sustain yield under water limited conditions. Promising examples, where efficacy has been demonstrated in crops in controlled and field conditions, have been described in this chapter. A major challenge that is faced when developing crops for sustained yield in stress conditions is the pleiotropic effect. The first generation of genetically engineered drought-tolerant plants were prone to unpredictable and unwanted effects on other key developmental traits (Kasuga et al. 1999), as also outlined above (Sect. 8.2.2.3). Spatial, temporal, and level of expression of the introduced genes are required for optimal performance. More recently, transgenic plants have been generated that display drought tolerance, however no unintended developmental abnormalities were observed. It is anticipated that these genes or combinations of these genes will lead to the environmental release of drought tolerant crops within the next five years.

The socio-economic benefits of drought tolerant crops are clear. Genetically engineered crops for abiotic stress-prone environments, however, pose questions regarding safety and environmental impact. For example, will sustained yield under water-limited conditions lead to increased competitiveness if the transgenes are introgressed into wild populations? Furthermore, will the use of regulatory genes have an unanticipated cascading effect on multiple gene pathways? It will therefore be important to understand the mechanism of action of the inserted gene(s) at the physiological level in different receiving environments as well as ascertaining whether or not the crops are substantially equivalent from a composition and toxicological point of view to conventional crop varieties.

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# **Chapter 9**

## **Herbicide Resistance**

**Micheal D. K. Owen**

### **9.1 Introduction**

Herbicide resistance is not a topic or concern specifically focused upon the relatively recent introduction and adoption of genetically modified crops. In fact, the first case of herbicide resistance in weeds was reported in the scientific literature in 1970, but the occurrence of evolved resistance to herbicides in plants was suggested in 1956 (Harper 1956; Ryan 1970). Since the original report, over 300 herbicide-resistant weed biotypes in more than 180 different plant species have been reported (Heap 2009). Generally, the recurrent use of an herbicide or herbicide mechanism of action imparts significant selection pressure on the weed population and provides an ecological advantage to those rare individuals within the population that have the heritable mutation conferring herbicide resistance (Owen and Zelaya 2005). The relatively recent introduction of crop cultivars with genetic modifications for herbicide resistance served to narrow the spectrum of herbicides used for weed control thus focusing on single herbicides (i.e. glyphosate) and increasing the potential for evolved herbicide resistance in weeds (Young 2006). It should be noted, however, that the genetically modified trait(s) typically does not impart selection pressure on the weed population, but rather the grower decision to utilize the herbicide causes the selection for resistance (Owen 2008a, b). However, despite claims that (due to a number of physicochemical characteristics related to glyphosate) glyphosate-resistant weeds would never evolve, the broad-scale adoption of genetically modified glyphosate-resistant crops globally has resulted in the evolution of glyphosate resistance in 16 weeds species, to date, and the rate of resistance evolution appears to be increasing at an increasing rate (Bradshaw et al. 1997; Heap 2009). This chapter provides a perspective of genetically modified herbicide-

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resistant crops, the implications of grower adoption of the genetically modified herbicide-resistant crops and the impact of this adoption on weed communities.

### ***9.1.1 Overview of Adoption***

Since the commercial introduction of genetically modified crops in 1996 (for methods, see Chaps. 1, 2), the area planted to these cultivars has increased globally at an increasing rate (Anonymous 2006). In 2006, approximately 100 million hectares of genetically modified herbicide-resistant crops were planted worldwide and an estimated 80% had the genetically modified trait conferring glyphosate resistance (Service 2007). The use of these cultivars increased an estimated 12% in 2007 and represented 114.3 million hectares and included 23 countries (Anonymous 2006). The primary countries that plant genetically modified herbicide-resistant crops are the United States (US), Argentina, Brazil, and Canada. North America represents 57% of the genetically modified herbicide-resistant crops planted globally while Central and South America contribute 33% of the total hectares. It is estimated that approximately 90% of the genetically modified herbicide-resistant crops grown globally are glyphosate-resistant crop cultivars and are represented primarily by soybean, cotton and maize (Duke and Powles 2008). Genetically modified herbicide-resistant canola dominates the cultivars grown in Canada and the US, representing >80% of total crop grown (Beckie and Owen 2007). Recently there has been a significant adoption of glyphosate-resistant genetically modified maize in the US, in part attributable to maize-based ethanol production. Overall, the revolution of adoption of genetically modified crops likely represents the largest man-caused biological experiment in history. This will cause enormous selection pressure that the wide-spread application of glyphosate on millions of hectares will impose on weed communities and inevitably result in significant changes by selecting for weeds that do not respond to the prevalent control tactics. Recently genetically modified herbicide-resistant (glyphosate) sugarbeets were commercially introduced in the US, which will add more selection pressure on weed communities (Duke 2005; Gianessi 2005).

### ***9.1.2 Types of Herbicide Resistance***

Herbicide resistance has evolved in weeds in several general forms. The most common type of herbicide resistance in weeds is the modification of the herbicide target site (Zelaya and Owen 2004). Target site resistance can be either monogenic or polygenic; the latter is often referred to as “creeping resistance” and may result from recurrent applications of low herbicide rates (Gressel 1995). In the case of monogenic herbicide resistance, typically resistance is accrued when there is a single-nucleotide point mutation of one amino acid, representing a substitution in

the sensitive weed population, resulting in a resistant biotype (Gressel and Levy 2006). However, recent studies suggest that weeds can also evolve monogenic herbicide resistance by “losing” an amino acid in the target protein (Patzoldt et al. 2006). A partial list of target site resistance demonstrated in weed populations includes resistant weed biotypes for acetolactate synthase (ALS) inhibiting herbicides, protoporphyrinogen oxidase (PPO) inhibiting herbicides, triazine herbicides and glyphosate (Ryan 1970; Baerson et al. 2002; Ng et al. 2003; Zelaya and Owen 2004; Patzoldt et al. 2006). Herbicide resistance in weeds also is the result of differential translocation of the herbicide to the target site (Feng et al. 2004). Weeds are also able to evolve herbicide resistance by rapidly and efficiently metabolizing the herbicide prior to the accumulation of a toxic concentration of the herbicide at the target site (Yuan et al. 2006). This is also known as non-target site resistance and is typically mediated by cytochrome P450 monooxygenases, glutathione S-transferases or glycosyltransferases, depending on the herbicide. Herbicide resistance can also be a function of ABC transporters which serve to facilitate compartmentalization of the herbicide, again protecting the target site of the herbicide (Lu et al. 1997). Finally, weeds have demonstrated other novel forms of herbicide resistance, such as morphological adaptations (i.e. leaf pubescence) and phenological changes (i.e. avoidance attributable to delayed germination) in weed populations (Owen 2001). Interestingly, weeds have demonstrated the ability to evolve multiple resistances to several herbicide modes of action (Patzoldt et al. 2005; Legleiter and Bradley 2008). Herbicide resistance in crops has been established using altered target site, the most common strategy used (i.e. glyphosate-resistant crops), enhanced metabolism (i.e. glufosinate-resistant crops) and cultivars with multiple resistances to herbicides have been developed (Green 2007; Green et al. 2008; Green et al. 2009).

### **9.1.3 Modes of Herbicide Action in Herbicide-Resistant Crops**

Most of the current herbicide-resistant crop cultivars are represented by cultivars created by transgenic modifications. (Duke 2005) These herbicide modes of action include inhibition of photosystem II (bromoxynil), inhibition of glutamine synthetase (glufosinate) and inhibition of EPSPS (glyphosate). They are facilitated by the insertion of five transgenes to confer resistance to the respective herbicides: CP4, GOX or a mutated EPSPS for glyphosate resistance, a nitrilase gene for bromoxynil resistance and the *bar* gene for glufosinate resistance. Historically, there are non-transgenic herbicide resistance traits for cyclohexanedione herbicides, imidazolinone herbicide, sulfonylurea herbicides and triazine herbicides; however the dominant herbicide-resistant trait on the market is for transgenic glyphosate resistance (Duke 2005; Duke and Powles 2008). Recently, two novel transgenes, *gat4621* and *hra*, were introduced that confer high levels of resistance to glyphosate- and ALS-inhibiting herbicides, respectively (Castle et al. 2004; Green et al. 2008; Green et al. 2009).

A gene that codes for dicamba monooxygenase (*DMO*), a Rieske non-heme monooxygenase that metabolizes dicamba, has been discovered in the soil bacteria *Pseudomonas maltophilia* and can be biotechnologically inserted into the nuclear and chloroplast genome of soybean, thus conferring these transgenic plants resistance to dicamba (Behrens et al. 2007). These cultivars are anticipated to be commercially released in several years. Furthermore, transgenes that code for resistance to 2,4-D and ACCase inhibitor herbicides are also anticipated to be inserted into the various crops in the near future. Thus, the number of herbicide modes of action with transgenic resistant crop cultivars appears to be increasing and it is anticipated that these new transgenes will improve weed management options for growers and help resolve current and future problems with the evolution of herbicide-resistant weed biotypes. However, whether or not the mitigation of current and future herbicide-resistant weed problems actually occurs depends entirely on how growers utilize the technologies and whether or not they establish appropriate integrated weed management strategies.

#### ***9.1.4 Implications of Genetically Modified Herbicide-Resistant Crops***

The wide-spread adoption of genetically modified herbicide-resistant crops has made a number of significant impacts on agricultural systems. Notably, the level of weed control and consistency of efficacy has increased compared to “traditional” soil-applied herbicides (Duke 2005). Furthermore, given that genetically modified herbicide-resistant crops are represented largely by resistance to glyphosate and to a lesser amount glufosinate, and given that these herbicides are used post-emergence to the weeds and have generally favorable edaphic and toxicological characteristics, there are likely significant positive environmental benefits. Another important environmental benefit attributable to these crops is the adoption of conservation tillage practices including no tillage production systems which result in important reductions of soil erosion, thus improving water quality and lessening the degradation of soil (Young 2006). The benefits that growers attribute to genetically modified herbicide-resistant crops reflect the perceived simplicity and convenience of weed control (Owen 2008a, b). However, an objective review of the implications of genetically modified herbicide-resistant would suggest that there are important risks that must also be considered.

##### ***9.1.4.1 Selection Pressure Indirectly Attributable to Genetically Modified Herbicide-Resistant Crops***

The consistent and widespread use of one herbicide has considerable implications on the weed community (Owen 2008a, b). Differential response of weed species to the herbicide results in some weeds that are ecologically favored in the system.

The recurrent use of a specific herbicide with a high level of efficacy on the sensitive weeds results in weeds that are favored by the system and thus become the dominant members of the weed community (Scuroni et al. 2006; Scuroni et al. 2007). For example, Asiatic dayflower (*Commelina communis*) is known to be tolerant to glyphosate and has become an increasing problem in genetically modified glyphosate-resistant crops (Ulloa and Owen 2009). The other aspect of selection pressure is the shift in a weed species that is predominantly sensitive to the herbicide to a biotype that has a mutation conferring resistance to the herbicide (Owen 2008a, b). Regardless of the ultimate type of weed shift, the greater the selection pressure that the herbicide imparts upon the agroecosystem, the more pervasive the change in the weed community; it should be recognized that it is not a matter of “if” the change in the weed community occurs but rather “when” the change is identified. Selection pressure from herbicides used in agriculture will inevitably result in changes in weed communities (Owen and Zelaya 2005).

#### 9.1.4.2 Evolved Herbicide Resistance

The evolution of herbicide resistance predates the adoption of genetically modified herbicide-resistant crops by almost four decades (Ryan 1970; Duke 2005). Resistance to 19 herbicide mechanisms of action has been documented globally, with evolved resistance to ALS inhibitors, triazines, ACCase inhibitors, synthetic auxins, bypyridiliums, ureas and amides, glycines and dinitroaniline herbicides being the most prevalent. Interestingly, some weeds demonstrate the ability to evolve resistance to multiple mechanisms of herbicide action (Preston et al. 1996; Patzoldt et al. 2005). Rigid ryegrass (*Lolium rigidum*) biotypes have been documented to resist as many as seven mechanisms of herbicide action (Heap 2009). Furthermore, a number of weed species have demonstrated the ability to evolve cross-resistance to different herbicide families with similar mechanisms of action (Hinz and Owen 1997). Despite the fact that the mutations that confer resistance to herbicides typically occur at extremely low frequencies within non-selected weed populations, resistance to any and all herbicides can evolve given the current management of weeds in most crop production systems and the strategies of resistance that weeds have demonstrated (Gressel 1996; Gressel and Levy 2006).

#### 9.1.4.3 Changes in Herbicide Use

One of the pervasive questions surrounding the adoption of genetically modified herbicide-resistant crops is the impact on herbicide use. It is well documented that, initially, the number of active herbicide ingredients used in genetically modified herbicide-resistant crops declined dramatically (Young 2006; Bonny 2007). However, whether or not the herbicide load on the environment was lessened in genetically modified herbicide-resistant crops depends on the measurement metric. It is argued that, with the genetically modified herbicide-resistant crops, fewer

applications of herbicides are required and thus less herbicide is used. However, given that the herbicides used on genetically modified herbicide-resistant crops are used at amounts that are many-folds higher than the herbicides that were replaced, it is argued that more herbicide is used compared to conventional crops (Benbrook 2001). Furthermore, the number of herbicide applications in genetically modified herbicide-resistant crops has increased steadily since the introduction of these crops (Young 2006).

#### 9.1.4.4 Lack of Integrated Weed Management

The primary benefits of the genetically modified herbicide-resistant crops, as stated by growers, is the convenience and simplicity of weed control (Bonny 2007; Owen 2008a, b). This has contributed to the dramatic decline in alternative tactics used to manage weeds and thus a loss of integrated weed management in genetically modified herbicide-resistant crops. The loss of integrated weed management then results in weed shifts in the genetically modified crops which negatively impacts crop production economics and has important long-term implications on the sustainability of cropping systems based on genetically modified herbicide-resistant crops (Owen and Boerboom 2004; Sammons et al. 2007; Owen 2008a, b).

## 9.2 Specific Crops with Herbicide Resistance

Currently there are six crops that have genetically modified herbicide-resistant cultivars. The genetically modified herbicide-resistant crops that are most widely planted include canola, cotton, maize and soybean (see also Chaps. 21, 15, 18, 24). Genetically modified herbicide-resistant sugarbeets were commercially released in 2008 and the adoption rate was reported to be exceptionally high. Genetically modified glyphosate-resistant alfalfa is also available but further commercial use is currently under review. Other important crops such as wheat, rice and turf do not have genetically modified herbicide-resistant cultivars. A short summary of the genetically modified herbicide-resistant crops follows.

### 9.2.1 Maize

Corn cultivars with resistance to herbicides include genetically modified transgenic (glyphosate and glufosinate) and non-transgenic (sethoxydim and imidazolinone) hybrids. Imidazolinone-resistant hybrids were introduced in 1993, sethoxydim-resistant hybrids in 1996, transgenic glyphosate-resistant hybrids in 1997 and transgenic glufosinate-resistant hybrids in 1998 (Dill 2005). Genetically modified glyphosate resistance in maize is the result of either the *cp4* transgene that codes for an altered EPSPS that does not allow binding of glyphosate, or *N*-acetylation of

glyphosate resulting in the non-herbicidal metabolite *N*-acetyl glyphosate (Padgett et al. 1995; Castle et al. 2004). Recently, maize cultivars with an *hra* transgene that confers 1000-fold cross-resistance to ALS-inhibiting herbicides was introduced (Green et al. 2009). The adoption of transgenic herbicide-resistant corn hybrids appears to be ever increasing (Owen and Zelaya 2005; Dill et al. 2008).

### 9.2.2 Soybean

Genetically modified herbicide-resistant soybean became commercially available in the US in 1996. The cultivars utilize the *cp4* transgene from *Agrobacterium* sp. that codes for a glyphosate-resistant form of EPSPS. Soybean cultivars with glyphosate resistance represent more than 90% of soybean planted in the US (Duke and Powles 2008). Soybean cultivars possessing the *bar* transgene from *Streptomyces hygroscopicus* thus conferring resistance to glufosinate have been developed and are now commercially available (Green 2009). A newly-reported mechanism, *N*-acetylation of glyphosate, provides considerable resistance to glyphosate and is currently under development in soybean (Siehl et al. 2005).

### 9.2.3 Cotton

Cotton resistance to glyphosate was originally due to the *cp4 epsps* transgene and grower adoption of the genetically modified glyphosate-resistant cultivars has been rapid since their introduction in 1997 (Cerdeira and Duke 2006). However, there were problems with the transgene expression in reproductive structures which resulted in the development of cultivars with two *cp4 epsps* transgenes and various promoters to provide better expression of resistance later in the development of the plants (CaJacob et al. 2007; Dill et al. 2008). Cotton with transgenic resistance to bromoxynil was introduced in the US in 1994, and glufosinate-resistant cultivars were introduced in 2003 (Duke 2005).

### 9.2.4 Canola

Genetically modified herbicide-resistant canola was introduced commercially in Canada in 1995 and approximately 80% of the herbicide-resistant canola is transgenic, primarily to glyphosate, which is much higher than the global percentage (ca. 20%; James 2008). Transgenic glyphosate-resistant canola contains the transgene that code for the mutant *cp4 epsps* but also has a transgene that codes for glyphosate oxidoreductase (GOX; Duke 2005). The other transgenic herbicide-resistant canola is resistant to glufosinate and contains the *bar* transgene that facilitates the acylation of glufosinate to herbicidally inactive metabolites (Lydon and Duke 1999).

### **9.2.5 Sugarbeets**

While there has been regulatory approval for transgenic glyphosate-resistant sugarbeets since 1998, cultivars were only recently commercially introduced. The transgenic resistance to glyphosate in sugarbeet is attributable to the mutant *cp4 epsps* transgene. It should be noted that sugarbeet weed management practices historically have been more intensive than with many other crops. Thus, given the expected intensity of herbicide selection pressure that would result from the adoption of genetically modified glyphosate-resistant sugarbeet cultivars, it is likely that weed population shifts and evolved glyphosate resistance in weed populations will rapidly ensue (Owen and Zelaya [2005](#)).

### **9.2.6 Turf**

Genetically modified glyphosate-resistant creeping bentgrass was developed (event ASR368) and field trials were established in Jefferson County, Oregon, in 2003 under a permit granted by USDA-APHIS (Anonymous [2002](#)). Independent studies demonstrated pollen-mediated transgene flow, resulting in wild plant populations expressing the transgenic glyphosate-resistant trait (Reichman et al. [2006](#); Mallory-Smith and Zapiola [2008](#)). Consequently, further production of genetically modified glyphosate-resistant creeping bentgrass was stopped (Charles [2007](#)).

### **9.2.7 Alfalfa**

Genetically modified glyphosate-resistant alfalfa cultivars were deregulated and subsequently commercialized in 2005 in the US. However, alfalfa is an open-pollinated crop and pollination is accomplished by bees which can travel considerable distances with viable pollen. Thus, contamination in non-transgenic alfalfa seed was expected. In 2007, a preliminary injunction order was issued indicating that USDA-APHIS had erred when the GLY-HR alfalfa was deregulated (US District Court for the Northern District of California, No. C 06-01075 CRB), halting seed sales and planting after 30 March 2007 (Fisher [2007](#); Harriman [2007](#)).

### **9.2.8 Rice**

Genetically modified glufosinate-resistant rice was initially developed to manage weedy red rice (*Oryza sativa* L.; Gealy and Dilday [1997](#)). However, given marketing issues, no genetically modified herbicide-resistant rice cultivars have been commercially released (rice is also covered in Chap. 22).

### 9.2.9 Wheat

There are no genetically modified herbicide-resistant wheat cultivars (see also Chap. 16). The program to develop transgenic glyphosate-resistant wheat cultivars was terminated in May 2004 (Dill 2005).

## 9.3 Implications of Genetically Modified Herbicide Resistance on Cropping Systems

Given the unprecedented global adoption of transgenic herbicide-resistant crops, it is important to consider the undeniable impact that these crops have on the respective cropping systems, pesticide use, biodiversity and ultimately the environment. Given the scope of this chapter, an in-depth review of these topics is not possible; however this should not minimize the importance of this global revolution and the impacts that have occurred. Consider that the range of topics includes the potential movement of transgenic traits to non-transgenic crops or near-relative plants, the potential for genetically modified herbicide-resistant crops to affect the soil biota and selection of best adapted species (Ammann 2005; Cattaneo, Yafuso et al. 2006; Gressel and Levy 2006; Abud et al. 2007; Pineyro-Nelson et al. 2009; Powell et al. 2009). An excellent overall review of genetically modified herbicide-resistant crop impact on the environment was published by Cerdeira and Duke (2006) and should be considered if detailed information is required. The topics addressed below include the implications of genetically modified herbicide-resistant crops on tillage, the diversity of weed management tactics and the timeliness of the implementation of these tactics.

### 9.3.1 Tillage

The cost of petrochemicals has reinforced the desirability of fewer tillage trips in the production of crops as well as the benefits attributable to improved time management. Based on these perceived and real benefits, crop production in no tillage and other conservation tillage systems increased dramatically because of genetically modified herbicide-resistant crops (Cerdeira and Duke 2006; Service 2007; Dill et al. 2008). No tillage or strip tillage cotton production increased almost threefold between 1997 and 2002 (Anonymous 2004). However, more recent data suggest that conventional tillage has returned as the dominant tillage practice in genetically modified glyphosate-resistant cotton because of important changes in weed populations (Mueller et al. 2005; Dill et al. 2008). Dramatic increases in no tillage and conservation tillage systems for maize and soybean production systems are also noted and largely attributable to genetically modified herbicide-resistant

crop cultivars (Duke 2005; Gianessi 2005; Young 2006; Dill et al. 2008). The reductions in tillage result in significant economic and time savings for growers, as well as reductions in equipment expenses (Gianessi 2005; Gianessi and Reigner 2007).

An important consideration of greater percentages of genetically modified herbicide-resistant crops produced under conservation tillage was the environmental savings from reduced soil erosion (Fawcett and Towery 2004; Gianessi 2005). Wind erosion of soil was reduced 31% and water soil erosion was reduced 30% in 1997 compared to 1982 because of the conservation tillage practices adopted in the production of transgenic herbicide-resistant crop cultivars (Fawcett and Towery 2004).

### ***9.3.2 Diversity of Weed Management Tactics***

There has been a significant decline in the use of alternative herbicides and this trend is largely attributable to the global adoption of genetically modified herbicide-resistant crops (Shaner 2000; Young 2006). Historically important herbicides have been replaced by the predominant use of glyphosate, often as the sole herbicide and weed management tactic; the lack of herbicide diversity has created an environment where changes in weed communities are inevitable. Furthermore, the size of farm has increased and the use of glyphosate for weed control provides the perception of better time utilization (Owen and Zelaya 2005). Thus the perception of simple and convenient weed management have dramatic impacts on the continued utility of glyphosate as weed populations adapt to the pervasive selection pressure imposed by the weed management system. While the lack of diversity of weed management tactics may not necessarily eliminate the use of glyphosate, it does provide a strong impetus for the development of improved weed management tactics and the adoption of a greater diversity of tactics (Green 2007).

Consider that crop rotation, while historically a strong weed management tactic, has become significantly less important given the typical crop rotations that include crops that are genetically modified and resistant to the same herbicide (Owen 2009). Furthermore, mechanical weed management has lessened in importance because of conservation tillage systems that are typically used for genetically modified herbicide-resistant crops.

Importantly, the development of new herbicide products and specifically research for new sites of herbicide action has slowed significantly (Green 2007; Green et al. 2008). It is possible that a higher glyphosate price could result in the use of alternative (older) herbicides and a greater diversity of weed management tactics. However, the desirability of weed management based on the perception of a simple and convenient weed management tactic will likely continue to slow growers from adopting a more diverse weed management system.

### 9.3.3 Timelines of Weed Management Tactics

The development of genetically modified herbicide-resistant crops, particularly those cultivars that are resistant to glyphosate has resulted in major changes in herbicide application timing (Young 2006). While historically soil-applied herbicides that provided residual control of weeds were the foundation of weed control, glyphosate applied post-emergence to weeds and crops has largely replaced the use of other herbicides. Glyphosate controls a large number of weeds, almost irrespective of weed size and environmental conditions (Sammons et al. 2007). Furthermore, growers perceive that genetically modified glyphosate-resistant crops and glyphosate provide an effective, consistent, simple and low-risk “system” for crop production with less tillage (Carpenter and Gianessi 1999; Service 2007). Thus, growers apply glyphosate almost without regard to timing (weed or crop stage of development) and presume that if the weeds have died, the tactic was successful (Owen 2007). Unfortunately, this perception of simplicity and convenience is misleading and results in significant losses of potential yield due to weed interference with the crop (Owen 2008a, b; Owen et al. 2009). Glyphosate is frequently sprayed after weeds have effectively competed with the genetically modified glyphosate-resistant crops and significant yield has been lost despite the effective “killing” of the weeds.

## 9.4 Herbicide-Resistant Weeds

Globally, new herbicide-resistant populations continue to evolve at an increasing rate. This situation has become more apparent with the evolution of weeds that are resistant to glyphosate (Powles 2008). It is difficult if not impossible to gain an accurate view of the current status of herbicide-resistant weeds. The most widely utilized and consistent source of information about the global status describing herbicide-resistant weeds is the *International Survey of Herbicide-resistant Weeds* ([www.weedscience.org](http://www.weedscience.org); Heap 2009). The current tally of herbicide-resistant weeds includes 332 resistant biotypes represented by 189 species of which 113 are dicots and 76 are monocots. These weeds are reported on over 300 000 fields. However, this website requires that reports of new herbicide-resistant weed populations are frequent and accurate. Unfortunately, weed scientists must volunteer these reports and often only individual fields are reported, which may not accurately represent the extent of the herbicide-resistant weed infestations. Regardless, it is clear that weeds are capable of evolving resistance to all herbicides, although there are several classes of herbicides for which resistant weed populations have yet to be discovered. Currently 102 weed species are resistant to ALS inhibitor herbicides, followed by 68 weed species that are resistant to PS II herbicides and 36 weeds species that have evolved resistance to ACCase inhibitor herbicides (Heap 2009). Synthetic auxin herbicides have 28 resistant weed species, 24 weed species are

resistant to the bipyridiliums herbicides, 21 species are resistant to the urea herbicides, 16 weed species have evolved resistance to glyphosate and 10 species are resistant to DNA herbicides.

As long as herbicides remain the primary if not sole tactic for weed control, weed populations will receive sufficient selection pressure to force the evolution of herbicide-resistant weed biotypes. Specifically with regard to genetically modified-herbicide resistant crops, the industry originally denied the possibility of evolved resistance to glyphosate, despite suggestions that resistance was inevitable (Gressel 1996; Bradshaw et al. 1997; Owen 2000; Zelaya and Owen 2000). Regardless of how the situation surrounding glyphosate and weed resistance was debated, there can now be no question that changes in weed populations are occurring more rapidly and are widely distributed across a number of crop production systems, despite apparent knowledge that growers have about the situation (Johnson et al. 2009; Kruger et al. 2009). Currently there are 16 weeds reported and confirmed to have evolved resistance to glyphosate (Heap 2009). In the US, nine species have been confirmed glyphosate-resistant and generally the resistance has evolved in conjunction with genetically modified glyphosate-resistant crops. Currently seven new species of glyphosate-resistant weeds have been confirmed in the US since 2004 and it is clear that the evolution of glyphosate-resistant weed species in genetically modified glyphosate-resistant crops is increasing at an increasing rate (Owen 2008a, b). Weeds in the Compositae family are represented by four species that have confirmed evolution to glyphosate and the Amaranthaceae has two species. Populations of *Xanthium strumarium*, *Chenopodium album* and *Kochia scoparia* are currently suspected to have resistance to glyphosate but have not yet been confirmed (Boerboom 2008).

#### **9.4.1 Weedy Near-Relatives to Genetically Modified Herbicide-Resistant Crops – Gene Flow**

There is considerable concern about the potential of gene flow from crops to weeds now that there are genetically modified traits included in many globally important food crops. One issue is the fear of the general public about transgenes and also the potential of increasing the prevalence of pernicious and highly invasive new weed species. Another consideration is the potential impact that transgenes may have on the genetic diversity of food crops such as maize in Mexico and soybean in China, particularly land races and wild progenitors (Gepts and Papa 2003; Lu 2004; Raven 2005). Importantly, for gene flow between crops and weeds to occur, a near-relative wild plant must co-exist spatially and temporally with the genetically modified herbicide-resistant crop. The requirement of weedy near-relatives to be available to receive the transgenic pollen makes some crops less of a risk than others. For example, soybean and maize do not have weedy near-relatives within the major production regions, while sunflower and wheat should be considered higher risk

when genetically modified cultivars are available. Obviously, crops that do not have genetically modified cultivars present no risk for transgene introgression into near-relative weeds.

Genetically modified herbicide-resistant traits generally do not affect the relative fitness of compatible weed populations and thus have little influence unless the herbicide is present. The best example of transgene movement between genetically modified herbicide-resistant crops and weedy near-relatives is genetically modified canola and the weedy Brassicaceae. Reports indicate that the transgene moves from the genetically modified crop and weedy near-relatives, but there is little effect on fitness (Hauser et al. 2003; Legere 2005). However as new genetically modified herbicide-resistant crops are released that have weedy near-relatives (e.g. *Beta vulgaris*), the lack of direct effect on weeds from transgene movement may change.

#### ***9.4.2 Implications of Herbicide Resistance – Persistence in the Agroecosystem***

The persistence of herbicide resistance in weed populations reflects the longevity of the seedbank and the relative percentage of the seedbank that contains the trait for resistance. It should be noted that seeds from genetically modified herbicide-resistant crops can also contribute to the seed bank and thus the herbicide resistance problem. However, with few exceptions (e.g. canola) the persistence of volunteer genetically modified herbicide-resistant crops is minimal, given that crop seeds generally do not last very long in the seedbank. Thus herbicide resistance attributable to volunteer genetically modified herbicide-resistant crops has a minimal effect on the seedbank. Canola, however, has demonstrated the capability to persistence in the soil seedbank and may be a factor for several years (Legere 2005).

Generally, herbicide resistance attributable to a weed population shift such that the dominant biotype is resistant will require a number of years to increase in the seedbank (Maxwell and Jasieniuk 2000). However, once established, the herbicide-resistant biotype will persist for many years, depending on the environmental conditions, the weed species and effectiveness of management tactics imposed upon the weed population. If marginal weed management (i.e. attributable to utilization of recurrent single herbicide tactics) is imparted on the weed population, the soil seedbank increases rapidly (Bauer and Mortensen 1992). If the weed species has seeds that are long-lived in the soil (e.g. *Abutilon theophrasti*) and herbicide resistance has evolved, the problem is likely to persist indefinitely regardless of the effectiveness of subsequent management tactics. If the weed species has seeds that are less persistent in the seedbank (e.g. *Amaranthus rudis*), effective management tactics can reduce the herbicide-resistant weed population relatively quickly (Steckel et al. 2007). However, it is important to consider that other production practices can also impact the seedbank and hence the persistence of herbicide resistance in the agroecosystem. For example, tillage can increase the longevity

of the weed seedbank by burying seeds and placing them in a position where they remain viable for a number of years. Thus, unless extraordinary weed management tactics are used subsequent to the establishment of a herbicide-resistant weed seedbank, it is likely that herbicide resistance will persist in the agroecosystem.

## 9.5 Conclusions

There is no question that the adoption of genetically modified herbicide-resistant crops represents the most important global revolution in agriculture. In 2007, genetically modified herbicide-resistant cultivars were planted on an estimated 114.3 million hectares and included 23 countries (Anonymous 2006). The trend of basing crop-production systems on genetically modified herbicide-resistant crops continues to escalate, in particular with maize. Fortunately, there is little evidence that many of the current genetically modified herbicide-resistant crops have impacted weed communities directly. This is attributable to the fact that there are no weedy near-relatives of the transgenic crops in the areas of major production. The exception to that is genetically modified herbicide-resistant canola (Legere 2005). However with the introduction of new genetically modified herbicide-resistant crops, there could be more exceptions in the future (e.g. sugarbeets).

While there is no direct impact of genetically modified herbicide-resistant crops on weed communities, there is a significant indirect impact; the recurrent use of the herbicide (i.e. glyphosate) for which transgenic resistance exists imposes significant selection pressure on weed populations and evolved resistance to glyphosate is increasing at an increasing rate. Please recognize that this selection process is no different than with any other herbicide and reflects the weed management strategy utilized by growers. Interestingly, as more resistance in weed populations to the herbicides used in transgenic crops develops, growers will be forced to return to older herbicides for which resistant weed populations have previously evolved (Owen 2008a, b).

While target site resistance has historically been the primary type of herbicide resistance that has evolved in weed populations, the weed populations that have evolved resistance to glyphosate often have less well understood mechanisms of resistance. In fact, it appears that there are multiple mechanisms of resistance to glyphosate and these mechanisms are subtle and difficult to identify (Gressel 1996; Feng et al. 2004). It is also concerning that many of the weeds that have evolved herbicide resistance demonstrate the ability to evolve resistance to other herbicide mechanisms of action, thus further complicating management tactics.

Perhaps the greatest concerns with regard to genetically modified herbicide-resistant crops and evolved herbicide resistance in weeds are the grower attitudes about the importance of herbicide resistance and diverse management strategies (Johnson et al. 2009; Kruger et al. 2009). It is clear that, while growers recognize the risk of evolved herbicide resistance in weed populations, they choose not to implement proactive management tactics and then strive for remediation after

the problem(s) develop. Succinctly, genetically modified herbicide-resistant crops have facilitated an attitude in adopters to neglect the appropriate use of integrated weed management tactics and thus will exacerbate future problems with herbicide resistant weeds (Boerboom et al. 2009; Owen et al. 2009).

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# Chapter 10

## Insect and Nematode Resistance

Tim Thurau, Wanzhi Ye, and Daguang Cai

### 10.1 Introduction

Crops are attacked by animal pests and nematodes, causing considerable economic losses worldwide. The global yield loss, e.g. due to herbivorous insects varies between 5% and 30% depending on the crop species, while the estimated worldwide losses due to plant parasitic nematodes are about US \$125 billion annually (Chitwood 2003). Root-knot nematodes like *Meloidogyne incognita* infect thousands of plant species, resulting in poor fruit yield, stunted growth, wilting and susceptibility to other pathogens. Factors which increase plant susceptibility to pest attacks include a lack of genetic diversity within the genomes of cultivated crop species and changes in cultivation techniques, such as large-scale cropping of genetically uniform plants and reduced crop rotation as well as the expansion of crops into less suitable regions. Use of natural resistance is a promising alternative for parasite control. Advanced understandings of natural resistance mechanisms in molecular details will broaden the horizon of crop resistance breeding programs. As resistance is often limited in many crop species and can be easily overcome by new virulent pathotypes, new genetic variability is therefore needed. Here we give an overview about recent progresses in research of plant resistance genes and the underlying molecular mechanisms as well as their potential in practice application. Today, chemical control of plant parasites depends on relatively few chemicals. These pose serious concerns of risks and hazards for humans, animals and the environment and increase the costs of growing crops. The worldwide use of pesticides increased dramatically since the early 1960s. For example, the synthetic chemical pesticides-based insecticide market is estimated at above US \$8 billion

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annually. However, restrictions and especially detriments of pesticide application for pest control (e.g. limited efficiency, inducing resistance of parasites) ask for alternative strategies to ensure a sustainable pest management in agriculture. Consequently, engineered resistance is an essential part of a sustainable parasite control and is becoming more and more important, as it offers a parasite management with benefits to the producer, the consumer and the environment. In this review we focus on the strategy for engineering parasite resistance in crops with anti-parasite genes. Genes are expressed in transgenic plants (for methods, see Chaps. 1, 2) whose products are non-phytotoxic but strongly anti-parasite, either lethally toxic or interfering with parasites after their take-up by parasites, consequently affecting their development and reproduction. Furthermore recent progress in the plant delivery of a RNAi-based gene-silencing strategy (see Chap. 5) provides new tools for engineering broad parasite resistance in crops.

## 10.2 R Gene-Mediated Resistance

### 10.2.1 *Plant Resistance and Resistance Gene*

The use of plant natural resistance mechanisms represents one of the most promising alternatives. Plants have evolved sophisticated and multi-faceted defense mechanisms. Briefly, two branches of the plant immune system exist. The older one, basal immunity (reminiscent of innate immunity in vertebrates), is triggered by pathogen-associated or microbe-associated molecular patterns (PAMP- or MAMP-triggered immunity, PTI); and the second one, effector-triggered immunity (ETI), relies on resistance (R) proteins. Once the pathogen succeeds in suppressing the insufficient basal defenses, plants evolve resistance (R) proteins which directly or indirectly interact in a specific manner with microbial effector proteins and thereby trigger plant immune responses. This is synonymous to pathogen race-plant cultivar-specific host resistance or gene-for-gene resistance (Jones and Takemoto 2004; Jones and Dangl 2006). The recognized effector is termed an avirulence (Avr) protein. Pathogens evolve further and suppress ETI, which again results in new R gene specificities so that ETI can be triggered again (Jones and Takemoto 2004; Jones and Dangl 2006).

To date, numerous R genes have been cloned which confer resistance to several classes of pathogens, including viruses, bacteria, fungi, oomycetes, insects and nematodes. R gene products can be categorized into two main classes based on conserved structural features (Dangl and Jones 2001; Chisholm et al. 2006). The largest class of R proteins (called the NBS-LRR class of R proteins) possesses in addition to a leucine-rich repeat (LRR), a central nucleotide-binding site (NBS) domain. The second major class of R genes encodes extracellular LRR (eLRR) proteins. Three subclasses of LRRs have been suggested according to their domain structures (Fritz-Laylin et al. 2005). These subclasses include receptor-like proteins

(RLP; extracellular LRR and a transmembrane (TM) domain), RLK (extracellular LRR, TM domain, cytoplasmic kinase) and polygalacturonase inhibiting protein (PGIP; cell wall LRR).

Immense progress in plant genome analysis revealed that many R genes are located in clusters that comprise several copies of homologous R gene sequences arising from a single gene family (simple clusters) or colocalized R gene sequences derived from two or more unrelated families (complex clusters). The lack of substantial evidence for direct Avr-R interaction led to the ‘guard hypothesis’ (Van der Biezen and Jones 1998), which proposes that the X induces a change in a host protein that is normally recruited by the pathogen via its Avr protein to establish a successful infection, and that this change sensed by the R-protein (guard) leads to the activation of the R protein and subsequent defense signaling (Dangl and Jones 2001; Bent and Mackey 2007; van der Hoorn 2008). This model may provide a good explanation for resistance response triggered by other resistance genes.

### 10.2.2 Plant Parasite Resistance and Resistance Genes

During evolution, different forms of natural resistance to parasites have been established. Plant innate plant defense mechanisms like morphological barriers, diverse compounds of the secondary metabolism and induced resistance mechanisms (PTI) allow only a selected number of parasitic pests to attack a specific range of plant species (Schuler 1998). Often active plant defense is induced immediately after insect attack, leading to the production of various anti-insect compounds, including anti-feedants, toxins and digestibility reducers (Korth 2003; Voelckel and Baldwin 2004a, b). Also indirect defense mechanisms are activated that recruit natural enemies from the plant’s surroundings to attack feeding insects (Turlings and Tumlinson 1992; De Moraes et al. 1998; Kessler and Baldwin 2001).

Insect resistance loci have been reported in crop plants like wheat, barley, maize, potato and rice (Yencho et al. 2000). So far, little is known about the underlying molecular mechanisms as the majority of insect resistance loci are mapped as QTLs, making the characterization and the use of these resistance traits for plant breeding difficult and time-consuming. The only cloned insect resistance gene is *Mi-1*. *Mi*, originally isolated as a root knot nematode (*Meloidogyne* spp.) resistance gene from wild tomato (*Lycopersicon peruvianum*) also confers resistance against potato aphids (*Macrosiphum euphorbiae*) and whiteflies (*Bemisia tabaci*; Vos et al. 1998; Martinez de Ilarduya et al. 2001; Nombela et al. 2003).

In contrast, a set of nematode resistance genes have been identified from various crop plants. Economically the most important plant-parasitic nematodes are cyst nematodes of the genus *Heterodera* and *Globodera* and root-knot nematodes of the genus *Meloidogyne*. Root-knot nematodes of *Meloidogyne* spp. are obligate sedentary endoparasites. Agronomically important species of cyst nematodes, mainly active in temperate regions of the world, are *G. rostochiensis* and *G. pallida*.

on potato and *H. glycines* on soybean. In addition, more than 80% of the Chenopodiaceae and Brassicaceae species are hosts of *H. schachtii* (Steele 1965), including economically important crops like sugar beet (*Beta vulgaris*), spinach (*Spinacea oleracea*), radish (*Raphanus sativus*) and rape seed (*Brassica napus*). Today *H. schachtii* is spread over 40 sugar beet-growing countries throughout the world (McCarter et al. 2008).

Nematodes completely penetrate main and lateral roots in the elongation or root hair zones of a susceptible plant as motile infective second-stage juveniles (J2) which hatch in the soil from eggs contained within a protective cyst (cyst nematodes) or egg sac (root-knot nematodes). They penetrate the plant cell walls using their robust stylet. However, before the stylet penetrates, cell walls are degraded by a number of enzymes released from the nematode's subventral glands. These include  $\beta$ -1,4-endoglucanases (cellulases; Gao et al. 2001), a pectate lyase (Doyle and Lambert 2002) and an expansin (Qin et al. 2004). J2s migrate within the root cortex towards the vascular cylinder and induce remarkable changes in a number of host cells, to establish highly metabolically active feeding cells sustaining the nematode throughout its life cycle (syncytium for cyst nematodes; giant cell for root-knot nematodes; Davis et al. 2004, 2008; Fuller et al. 2008). After three additional molts, adult males emerge from the root and are attracted to the females, where fertilization occurs. At maturity, the female of a cyst nematode dies and the body is transformed into a light brown cyst where eggs and juveniles survive and remain dormant until root exudates stimulate juveniles to hatch and emerge from the cyst. By contrast, eggs of *Meloidogyne* spp. are released on the root surface in a protective gelatinous matrix.

Chemical control of nematodes is restricted. Most of the nematicides have been withdrawn from the market due to high environmental risks. Crop rotations with non-host plants including wheat, barley, corn, beans and alfalfa as well as nematode-resistant radish and mustard are functional, but often not economically practical. In this context, the breeding of resistant cultivars is the most promising alternative.

The majority of cloned nematode resistance genes originate from crop wild relatives. The first nematode R gene to be cloned was *HsI<sup>pro-1</sup>* from sugar beet, which confers resistance against the sugar beet cyst nematode *H. schachtii* (Cai et al. 1997). Other cloned nematode R genes closely resemble known plant R genes in their domain structure. Four of these genes, *Mi-1*, *Hero*, *Gpa2* and *Gro1-4*, all cloned from tomato or potato relatives, fall into the NBS-LRR class of R genes (Williamson and Kumar 2006). The tomato genes *Mi-1* and *Hero*, respectively, confer broad-spectrum resistance to several root knot nematode species (Milligan et al. 1998; Vos et al. 1998) and to several pathotypes of the potato cyst nematodes *G. rostochiensis* and *G. pallida* (Ernst et al. 2002). *Mi* resistance was first transferred into commercial tomato cultivars in the 1950s (Gilbert et al. 1956). *Mi* also confers resistance to two totally unrelated parasites, the potato aphid *Macrosiphum euphorbiae* and the white fly *Bemisia tabaci* (Rossi et al. 1998; Nombela et al. 2003), whereas the potato genes *Gpa2* and *Gro1-4* mediate resistance to a narrow range of pathotypes of the potato cyst nematode *G. pallida* (van der Vossen et al. 2000;

Paal et al. 2004). So far, little is known about the action mode of the cloned nematode resistance genes. It is generally believed that these genes recognize nematode effectors triggering specific signaling pathways that lead to resistance responses. Agronomically more important nematode R genes are likely to be cloned in the near future, including the *H1* gene that confers resistance to *G. rostochiensis* in potato (Bakker et al. 2004) and the *Me* gene of pepper for resistance to *Meloidogyne* species (Djian-Caporalino et al. 2007).

### 10.2.3 Significance and Limitations of Plant Resistance Genes

Although the breeding of resistant cultivars is the most promising alternative for parasite control, there are several limitations for the use of natural nematode resistance genes in practice, generally.

1. Resistance is not complete. For example, *Hero A* is able to provide only partial resistance (>80%) to *G. pallida* (Ernst et al. 2002).
2. Resistance is conditionally expressed. The *Mi-1* mediated resistance is for example temperature-sensitive and breaks down above 28°C (Dropkin 1969).
3. Resistance genes are often effective against one or a limited range of species and introgression of such genes may confer yield penalties or undesirable agronomic traits (Panella and Lewellen 2007).
4. A major concern around resistance relying on a gene-for-gene relationship is when it is overcome by new virulent pathotypes even though the durability of R genes to sedentary plant nematodes has been generally high. The *H1* gene has been used in cultivated potato against *G. rostochiensis* for over 30 years in the UK but without the development of virulent population (Fuller et al. 2008).

Molecular identification and cloning of natural resistance genes make it feasible for a direct transfer of R genes into related susceptible cultivars or to other plants. Molecular markers can be developed, which can assist conventional breeding programs greatly, as demonstrated by the development of commercial soybean and potato cultivars resistant to *H. glycines* and *Globodera rostochiensis*, respectively (Starr et al. 2002). Broad resistance can be engineered by the pyramiding of different resistance genes in given species. In addition, a variety of defense-related genes from diverse sources is available for genetic engineering to enhance plant resistance to pests. These include genes specific for signaling components, defense-related genes with antimicrobial activity such as PR proteins, antifungal proteins (osmotin-, thaumatin-like), antimicrobial peptides (thionins, defensins, lectin, phytoalexins) as well as gene products that can enhance the structural defenses in the plant, such as peroxidase and lignin. The identification of global regulators of resistance response, ‘master switches’, offers the possibility to engineer broad disease resistance (Stuiver and Jerome 2001).

The techniques used to develop transgenic plants have improved dramatically in the past decade, allowing the development of new disease-resistant crops

(Dempsey et al. 1998) and transferring of the gene of interest across species that are difficult or impossible to cross. However, the transfer of resistance genes from a model to crop plants as well as between distantly related crops seems to be limited. Attempts to transfer *Mi*-mediated root-knot nematode resistance from tomato were unsuccessful (Williamson et al. 1998) and transfer of *Hero A* into a susceptible tomato cultivar conferred resistance to *Globodera* species but not in transgenic potato expressing the same construct (Sobczak et al. 2005). Exceptionally, expression of *Mi-1.2* in transgenic aubergine (*S. melongena*) resulted in significantly lower amounts of *Meloidogyne* reproduction and numbers of egg masses but had no anti-aphid effect (Goggin et al. 2006). It is generally believed that downstream components of the response cascade must be present to activate R gene-mediated resistance response in given species. Within species, significant variability in transgenic resistance may occur due to its genetic background and allelic status, the promoter used and the copy number of the transgen (Chen et al. 2006). The phenomenon termed ‘restricted taxonomic functionality’ (RTF) might reflect an inability of the R protein to interact with signal transduction components in the given host (Michelmore 2003).

### 10.3 Engineering of Insect and Nematode Resistance

Today, engineered insect and nematode resistance are becoming an essential part of a sustainable agriculture in both developing and developed countries worldwide. In 2007, insect-resistant plants based on the transgenic technology were grown on an area of 46 million hectares, more than half of it (26.9 million ha) with a stacked trait of herbicide- and insect-resistant seeds and 19.1 million hectares with insect resistance alone (James 2008). So far, several approaches are under discussion. The first one relies on expression of genes of interest in transgenic plants, whose products are non-phytotoxic but strong anti-parasitic, either lethal toxic or interfering with parasites after their take-up by parasites consequently affecting their development and reproduction. Such transgenes can encode enzymatic inhibitors that block physiological processes within the pest, toxic compounds that are then ingested, compounds that bind to signal molecules, enzymes that interfere with the nematode. Alternatively, the anti-feeding approach is aiming at breaking down the feeding structure by the introduction of genes encoding phytotoxic compounds like barnase or ribosome-inactivating proteins which disrupt feeding cells (Atkinson et al. 2003) or by the knockout of genes which are crucial for formation of the feeding structure or for nematode parasitism (Huang et al. 2006). Because this approach strictly relies on promoters as well as genes specific for nematode-feeding cells, the availability of these elements still remains the obstacle for its realization in practice (Atkinson et al. 2003). In the following, engineering insect and nematode resistance are discussed using anti-insect and anti-nematode genes.

### 10.3.1 Anti-Insect/Nematode Genes

#### 10.3.1.1 Bt Toxins

Bt toxins have been known as molecules that are active against insects and nematodes since the beginning of the previous century. They are synthesized by the soil-borne gram-positive bacterium *Bacillus thuringiensis* (Bt). About 400 Bt toxins are known so far produced by diverse *B. thuringiensis* strains (Crickmore et al. 2009). All of them have a crystal structure, therefore named Cry toxins. Because of the natural origin of the toxins, they occupy the position of the world's leading bio-pesticide.

Cry proteins bind to glycoprotein receptors that are located within the membrane of target insects' epithelium and afterwards inserted irreversibly into the membrane leading to the formation of a pore. Reasonably, alterations of these glycoprotein receptors can cause as a reason for toxin resistance of insects to a particular Bt-protein (Knight et al. 1994, 1995; Malik et al. 2001; Griffitts et al. 2005). *B. thuringiensis* strains produce different crystal proteins with specific activity against distinct species: Cry1A, Cry1B, Cry1C, Cry1H, Cry2A against lepidoptera, Cry3A, Cry6A, Cry 12A, Cry13A against nematodes, Cry3A, Cry6A against coleoptera and Cry10A, Cry11A against diptera. The toxins are effective tools for controlling lepidopteran and coleopteran insect pests, but application of Bt toxins as an insecticide by spraying is not efficient because the protein is unstable and has no systemic effect. In contrast, when synthesized by transgenic plants, Cry protoxins are taken up by sucking insects. Within the insect gut, protoxins are proteolytically cleaved to produce the active toxin, finally leading to affection on epithelial cells. So far, Bt toxins have been introduced into a wide range of crop plants like soybean, maize and cotton (see Chaps. 16, 19, 25). More than 20 transgenic crop varieties carry Cry genes (Bruderer and Leitner 2003). For instance, Cry1Ab is integrated into the genome of the transgenic maize varieties MON810 and Bt176 (Bruderer and Leitner 2003), where it is particularly active against the european corn borer (*Ostrinia nubilalis*). In cotton the variety "Bollgard" expresses the Bt toxin Cry1Ac that is efficient for controlling the cotton bollworm (*Helicoverpa armigera*). To increase the expression levels of Bt toxins in transgenic plants, considerable changes to the Bt toxin genes are required such as change in codon-usage and the use of plant-specific processing signals in different events.

Even though immense advantages have been given by the use of Bt toxin in various transgenic crop plants (Romeis et al. 2006), the utilization of Bt toxin within transgenic plants is still controversially discussed, especially in Europe. Up to now, insect resistance against Bt toxins has not been observed under field conditions, only under laboratory conditions (Christou 2006), which is thought to be caused by a decreased fitness of resistant individuals (Christou et al. 2006; Soberón et al. 2007; Tabashnik et al. 2008). For instance, monitoring the pink bollworm (*Pectinophora gossypiella*) for eight years showed no increase of resistance to Bt (Tabashnik et al. 2005). The same result come from monitoring corn

borers (*Sesamia nonagrioides*, *Ostrinia nubilalis*) in Spain over a period of five years (Farinos et al. 2004). Furthermore, an overview about environmental effects of Bt proteins was made by Clark et al. (2005). A negative effect on non-target organisms under true conditions was not observed (Romeis et al. 2006). By contrast, a meta-analysis showed an increased abundance of non-target invertebrates on Bt-transgenic cotton and maize fields, compared to non-transgenic fields managed with insecticides, as reported by Marvier et al. (2007). It is generally believed that the durability of resistance will be extended, e.g. by establishing refuges with areas of susceptible plants or by growing transgenic crops with a multi-gene, multi-mechanistic resistance (Boulter et al. 1993). The strategy of pyramiding effector genes within crops has two follow two major aims. One potential effect is to broaden insecticidal activity by combining genes with different specificity to control insect and nematode pests. The second effect is to enhance the durability of genetically engineered plant resistance because single mutation events do not break the insecticidal effect (Maqbool et al. 2001). Developing different strategies to protect the insecticidal effect of Bt toxins remains a great challenge (McGaughey et al. 1992; Frutos et al. 1999; Bates et al. 2005).

The potential for Bt toxin as a nematicide was reported by Marroquin et al. (2000). A preliminary study with transgenic tomato plants expressing the Bt endotoxin CryIAb after inoculation with *Meloidogyne* spp. resulted in a reduction in egg mass per gram of root of about 50% (Burrows and Waele 1997). The nematicidal effects were determined to result from a similar gut-damaging mechanism to that which occurs in insects: the activated toxin binds receptors in the intestine and forms a pore, causing lysis of the Gut (Wei et al. 2003; Li et al. 2007). Tomato hairy roots expressing the Bt crystal protein variant cry6A were challenged with *M. incognita* and supported significantly reduced amounts of nematode reproduction, although gall-forming ability was not affected (Li et al. 2007). The nematode feeding tube acts as a molecular sieve, permitting the uptake of certain molecules and excluding others. It is believed that root-knot nematodes are able to ingest larger molecules than cyst nematodes (Li et al. 2007). The size exclusion limit for *H. schachtii* has been determined to be approx. 23 kDa (Urwin et al. 1998). Therefore, the size exclusion limit (Böckenhoff and Grundler 1994; Urwin et al. 1997a, 1998; Li et al. 2007) severely restricts the agronomic application of transgenic Bt as a broad-spectrum nematode control strategy (Fuller et al. 2008).

### 10.3.1.2 Proteinase Inhibitors

The expression of proteinase inhibitors (PIs) of digestive proteinases in plants is a promising strategy of engineering insect and nematode resistance. Compared to Bt toxin, the beneficial properties of proteinase inhibitors are their small size and stability for their expression in transgenic plants. A direct proof of activity against insects was shown in transgenic tobacco plants which were resistant against a bud worm mediated by the expression of a trypsin inhibitor (Hilder et al. 1987).

PIs represent a well studied class of plant defense proteins which are generated within storage organs. Proteinase inhibitors are an important element of natural plant defense strategies (Ryan 1990) and are anti-feedants known to reduce the capacity of certain parasites to use dietary protein, so delaying their development and reducing their fecundity (Hilder et al. 1987). In addition, it has been shown that PIs are induced as part of defense cascades, e.g. by insect attack, mechanical wounding, pathogen attack and UV exposure (Ryan 1999). Different kinds of proteinase inhibitors are known to reduce the digestibility of the nutrients through oral uptake by insects and nematodes. The inhibitor binds to the active site of the enzyme to form a complex with a very low dissociation constant, thus effectively blocking the active site.

There are ten groups of PI characterized from plants spanning all four classes of proteinases: cysteine, serine, metallo- and aspartyl. The majority of proteinase inhibitors studied in the plant kingdom originates from three main families, namely Leguminosae, Solanaceae and Gramineae (Rao et al. 1991). The cowpea trypsin inhibitor (*CpTI*) is a serine inhibitor used in the first transgenic approach to confer insect resistance. *CpTI* in an amount of 1% of the soluble protein in the transgenic plant has an effect on the lepidopteran insect *Heliothis virens* in tobacco (Hilder et al. 1987) and inhibits insect development up to 50%. The gene was also transferred into potato, rice and other plants, where it showed similar activity. Another effective gene is the sweet potato trypsin inhibitor (*SpTI*) that is active against *Spodoptera litura* when it is expressed in tobacco and *Brassica* spp. (Yeh et al. 1997b; Ding et al. 1998). Another group of PI, cysteine proteinases, is common in animals, eukaryotic microorganisms and bacteria, as well as in plants. Recent studies have shown that other classes of proteases are also found in insect guts, such as cysteine proteinase (Wolfson and Murdock 1990). Brioschi et al. (2007) reported that adaptation of the insects to proteinase inhibitors appears through upregulation of proteinases, trypsins and chymotrypsins by insects.

The potential of plant proteinase inhibitors (PIs) for engineering nematode resistance has been demonstrated in several laboratories (Vain et al. 1998; Urwin et al. 2000; Cai et al. 2003). Both serine and cysteine proteinases are present in plant-parasitic nematodes (Koritsas and Atkinson 1994; Lilley et al. 1997). Their activities have been detected in the nematode intestine where they are involved in digestion of dietary proteins (Lilley et al. 1996). Broad nematode resistance has been achieved in potato plants by expressing a cystatin from rice, even when the proteinase inhibitor was preferentially expressed in feeding sites of *G. pallida* and *M. incognita* (Lilley et al. 2004). A cysteine proteinase inhibitor based transgenic resistance to the cyst nematode *Globodera pallida* in potato plants proved to be effective, even under field conditions (Urwin et al. 2001), demonstrating its great potential.

We demonstrated that sporamin, a tuberous storage protein of sweet potato is a functionally trypsin proteinase inhibitor. The full-length sporamin gene encodes a 23-kDa mature protein (Yao et al. 2001). It can be taken up through the feeding tube and the stylet and delivered within the nematode, where it can exhibit effective inhibition. After its transfer into the sugar beet hairy roots, a significant reduction of

developed females was observed in sporamin expressing roots but with variation in their inhibitory effects. Thereby the trypsin inhibitory activity was found to be a critical factor for nematode inhibition (Cai et al. 2003).

Nevertheless, there are no transgenic varieties carrying a proteinase inhibitor commercially available. It was discussed that parasites are able to modify their proteinase pattern and to bypass the inhibited protein digestion pathways (Broadway et al. 1997; Giri et al. 1998). Thus, the source of the PI used in transgenic plants is critical to avoid development of insect insensitivity (Ranjekar et al. 2003). Analogous to the case of Bt toxins, a combination of different PIs targeting a set of proteases would be a promising alternative to engineer a stable and broad resistance against insects and nematodes as well.

### 10.3.1.3 Lectins

Lectins are a structurally heterogeneous group of carbohydrate-binding proteins which play biological roles in many cellular processes. More than 500 different plant lectins have been isolated and (partially) characterized. Application of lectins as insecticidal protein has mainly been focused on homopteran, e.g. planthoppers, leafhoppers and aphids (Habibi J et al. 1993; Hussain et al. 2008). Because of their low level of susceptibility to proteinase inhibitors, lectins were considered to be a suitable insecticidal agent.

The toxic effect of lectins to insects and nematodes is still poorly understood. The proteins seem to bind to cells of the insect/nematode midgut disrupting the cell function like digestive processes and nutrient assimilation. Insect-feeding studies with purified lectins and experiments with transgenic plants confirmed that at least some lectins enhance the plant's resistance against insects and nematodes. Several lectins from plants have been reported to confer broad insect resistance against Lepidoptera, Coleoptera, Diptera and Homoptera (Carlini and Crossi-de-Sá 2002). A gene encoding a sugar-binding protein derived from pea (*Pisum sativum*) was the first example of a lectin which was used to generate transgenic plants with an enhanced insect resistance (Boulter et al. 1990). Another famous example of a lectin used in transgenic plants is the *Galanthus nivalis* agglutinin (GNA), which confers resistance against insects in rice, e.g. planthoppers (Rao et al. 1998; Nagadhara et al. 2004). Moreover, expression of GNA in potato has been shown to confer enhanced resistance to lepidopterans like *Lacanobia oleracea* and homopteran insects like aphids (Down et al. 1996; Gatehouse et al. 1997). Rapeseed was successfully transformed with a pea lectin, which leads to a reduced weight of pollen beetle larvae that was correlated to lectin expression (Melander et al. 2003).

Also, a significant reduction of *G. pallida* females was reported after transfer of the gene encoding the snowdrop (*Galanthus nivalis*) lectin GNA into potato plants (Burrows et al. 1997). It is believed that, analogous to insects, these proteins could be targeted to interact with the nematode at different sites: within the intestine; on the surface coat; or with amphidial secretions.

### 10.3.1.4 $\alpha$ -Amylase Inhibitors

$\alpha$ -Amylase inhibitors are inhibitory proteins that occur in the whole plant kingdom. These amylase inhibitors affect selectively  $\alpha$ -amylase from insects, animals and microorganisms, but not amylases from plants. Groups of well characterized monomeric and dimeric  $\alpha$ -amylase inhibitors were isolated from wheat, (*Triticum aestivum*) and common bean (*Phaseolus vulgaris*; Kashlan and Richardson 1981; Moreno and Chrispeels 1989; Octivio and Rigden 2002; Oneda et al. 2004). The function of these proteins within the plants/seeds has not yet been explained. They seem to be regulators of endogenous enzymes and part of the plant defense against insect attacks (Octivio and Rigden 2000). Analogous to the disruption of protein digestion by proteinase inhibitors, amylase inhibitors affect the carbohydrate metabolism of herbivorous insects. The potential of plant alpha-amylase inhibitors for engineering insect resistance was investigated in tobacco, pea and *Arabidopsis* (Carbonero et al. 1993; Schroeder et al. 1995). More promising results were obtained using the *Phaseolus*  $\alpha$ -amylase inhibitor of BAAI in pea, maize (*Zea mays*) and coffee, leading to a decreased propagation of insect pests. Expressed in transgenic maize, BAAI showed an insecticidal activity to the western corn rootworm (*Diabrotica virgifera*; Titarenko and Chrispeels 2000). In pea (*Pisum sativum*), it was possible to reach a BAAI content of up to 3% of the soluble protein which mediates strong resistance to pea weevil (*Bruchus pisorum*; Schroeder et al. 1995; Morton et al. 2000). Pereira et al. (2006) reported an effect of BAAI in coffee on the coffee berry borer (*Hypothenemus hampei*), showing the broad potential of  $\alpha$ -Amylase Inhibitors particularly against storage insect pests.

### 10.3.1.5 Chitinases and Others

A set of proteins from various organisms were tested for their activity against parasites. For instance, an insecticidal protein from scorpions enhances resistance to cotton bollworm (*Heliothis armigera*) larvae (Wu et al. 2008), toxins from endosymbionts of nematodes from the genus *Photorhabdus* and *Xenorhabdus* seems to have a broad insecticidal effect (Chattopadhyay et al. 2005).

Chitinases are known to be part of the plant defense system and are antifungal. A possible target is believed to be the nematode eggshell, which largely consists of chitin. We demonstrated recently that transgenic sugar beet roots and potato plants overexpressing a chitinase from the entomopathogenic fungus *Paecilomyces javanicus* confer broad-spectrum resistance to sedentary plant parasitic nematodes in transgenic sugar beet (*B. vulgaris*) and potato (*Solanum tuberosum*) plants (Thurau et al., unpublished data). The development of females was suppressed and the number of females was drastically reduced of both cyst nematodes *Heterodera schachtii* and *Globodera pallida*. In addition, the development of knots and egg sacks formed by root-knot nematode *Meloidogyne incognita* was also found to be severely affected. Although the mechanism underlying is not yet resolved and chitin has been reported to be present only in the egg shell of plant-parasitic

nematodes so far, our results strongly suggest an active role of chitin also in the parasitic process of various nematodes, thus providing an effective target for genetic engineering of broad nematode-resistant crops.

### 10.3.2 RNA Interference-Based Gene Silencing

The principle of RNA interference (RNAi) consists of a naturally based degradation of dsRNA as a part of protection against pathogen attack, particularly virus infection. This mechanism was discovered in the nematode *Caenorhabditis elegans*, leading to gene silencing through the occurrence of double-stranded RNA (dsRNA), mediating a downregulation of gene expression. Target RNA is degraded by enzyme complexes called DICER and RISC. The DICER endonucleases cuts double-stranded RNA into siRNAs of 21–23 nt. These small RNA molecules assemble at the RISC complex, which leads to the degradation of target RNA. Specificity of this mechanism depends on the sequence of the target-RNA molecule (for more details, see Chap. 5). For studying this mechanism in insects, *Drosophila melanogaster* functions as a model species (Wang et al. 2006).

An important aspect of RNAi in *C. elegans* is the ability to elicit phenotypic effects through the oral delivery of dsRNA molecules, either from solution or expressed within the bacteria upon which the nematode feeds, providing the new approach of engineering plant resistance to insect and nematode. Important advances have been made in the application of RNAi for nematode resistance over the past two years. Several reports demonstrated that plants expressing hairpin constructs targeting plant-parasitic nematode genes (Huang et al. 2006; Steeves et al. 2006; Yadav et al. 2006) display significant resistance to nematodes. Tobacco plant RNAi-induced silencing of *Meloidogyne* genes encoding a splicing factor and a component of a chromatin remodelling complex (Yadav et al. 2006) result in a high level of resistance to *M. incognita*. Huang et al. (2006) demonstrated the potential for engineering nematode resistance for plants by use of nematode parasitic genes. Transgenic *Arabidopsis* plants expressing the 16D10 sequence as a hairpin construct were found to be resistant to *Meloidogyne* species with a 63–90% reduction in the number of galls and as well as total egg production (Huang et al. 2006). The gene encodes a parasitism peptide which is probably involving the early signaling events in the formation of giant cells. Because of a high degree of homology between the 16D10 sequences of different *Meloidogyne* species, broad-range resistance against *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* is induced. Although there are reports of the technology being used to silence genes of cyst nematodes (Steeves et al. 2006; Valentine et al. 2007), less success has been reported by many workers attempting to engineer resistance to these species (Gheysen and Vanholme 2007). One explanation for these results could be differences in the maximum size of molecule that each species is able to ingest from the plant cell owing to the size exclusion limits imposed by the feeding tube, as

discussed above. It is reasonable to believe that the cyst nematode feeding tube may not allow an efficient uptake of the construct carrying the target molecule.

Also, RNAi proved to be a suitable method to control coleopteran insect pests, as shown by Mao et al. (2007) silencing a cytochrome 450 monooxygenase gene (CYP6AE14) in the cotton bollworm (*Helicoverpa armigera*) and impairing tolerance of bollworm larval to the cotton metabolite gossypol. Baum et al. (2007) shown similar results in the pathosystem western corn rootworm *Diabrotica virgifera*, where post-transcriptional gene silencing of several genes was induced and larval mortality was investigated in a feeding assay with transgenic corn plants and roots as well. Potential progress in the field of insect resistance, mediated by the host plant's delivery siRNA molecules, is restricted by the fact that insects lack genes encoding RNA-dependant RNA polymerase (RdRP), an enzyme necessary for the systemic activity of RNAi-mediated gene silencing (transitive RNAi; Gordon and Waterhouse 2007; Price and Gatehouse 2008). Nevertheless the possibilities of the RNAi mechanism for engineering insect resistance still have to be determined in the future.

## 10.4 Conclusions

During the past years, proteins like Bt toxins, proteinase inhibitors, lectins and amylase inhibitors were intensively investigated in respect of their anti-insect and nematode efficiency both in laboratory and in field trials. Significant control effects to parasitic pests have been achieved and demonstrated with different transgenic crop species. Crop species expressing the *Cry* proteins from *Bacillus thuringiensis* are worldwide commercialized with an enormous success. So far there is no commercialized transgenic crop for nematode resistance available. Control of insect and nematode pests, particular in developing countries, is still a great challenge for agriculture. Obvious advantages of engineered resistance like independence of genotype, reducing pesticide/nematicide application and improving human health as well as protecting the environment meet increasing demands of modern agricultural practices, especially with a global climate change for concern. Nevertheless, new genetic variability, molecular knowledge of the resistance mechanisms and new target proteins as well as novel engineering technologies is needed. In this context, fundamental research on molecular plant–parasite interaction will provide new approaches.

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# **Chapter 11**

## **Metabolic Engineering**

**Lars M. Voll and Frederik Börnke**

### **11.1 Introduction**

Quality traits, including alterations in metabolite composition of crop plants, have been major targets of traditional breeding programs. One of the most prominent examples is the introduction of rape seed varieties low in erucid acid (in seed oil) and in glucosinolates (in meal) approximately 30 years ago which was an important step towards improving the nutritional properties of rape seed products. However, conventional breeding strategies depend on the availability of significant genetic variation for a given trait within the species gene pool and are further limited by the complex genetics underlying some quality traits. Moreover, due to its untargeted nature breeding of novel traits is time-consuming and slow. The emergence of molecular biology and plant transformation technologies offers the possibility of manipulating plant metabolism by a more rapid, targeted approach. The widespread adoption of transgenic plants in the past two decades gave rise to the discipline of plant metabolic engineering and provided enormous progress concerning the manipulation of plant metabolism. Basically, metabolic engineering was defined as the alteration of metabolic output by the introduction of recombinant DNA (Bailey 1991) or, more specifically, as the genetic modification of cellular biochemistry to introduce new properties or to modify existing ones (Jacobsen and Khosla 1998). The main goals of plant metabolic engineering are to produce valuable compounds in an economically attractive format, or to increase yield of a crop plant. On the level of metabolites these goals can be achieved by: (i) an increase in the production of a specific desired compound, (ii) the deletion or reduction of a specific unwanted product and (iii) the introduction of pathways leading to new products. In contrast to conventional breeding, transgenic strategies offer a rapid way to introduce

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desirable traits directly into the genome of elite varieties. It is clear, however, that the successful manipulation of plant metabolism requires detailed understanding of the underlying factors that regulate it. Traditionally, metabolic pathways have been analyzed on a step-by-step basis and limiting enzymes have been identified according to their biochemical properties or by using metabolic control analysis (ap Rees and Hill 1994; Geigenberger et al. 2004). Subsequently, enzyme over-expression is employed to alleviate metabolic bottlenecks. However, given the enormous flexibility of plant metabolism these direct approaches are often confounded by intervention of other limiting steps within the pathway, by counter-balancing regulation and by previously unrecognized competing pathways.

Despite these hurdles considerable success has been achieved in plant metabolic engineering also using single gene strategies. In this chapter, we briefly introduce the most common molecular strategies used to modulate plant metabolism, before detailing how these approaches have been used to engineer plant metabolism. Here, we focus on primary metabolism, namely carbohydrate and lipid metabolism. Finally, we give a short impression of a few successful examples of engineering secondary metabolism.

## 11.2 Strategies for Metabolic Engineering in Plants

Metabolic engineers have access to a vast array of molecular and genetic tools to rewire plant metabolism, most of which aims at the modulation of enzyme activity either toward an increase or a decrease of metabolic flux through a given pathway. In the simplest case a single enzymatic step is the target for modulation. To increase the production of a desired compound or a novel compound, genes encoding biosynthetic enzymes of the pathway can be overexpressed. Further increases in flux can be achieved by overexpressing enzymes from heterologous sources which are not subject to regulation or which have different regulatory properties compared to the endogenous plant enzyme. A problem associated with overexpression of single enzymes is that other steps in the pathway can become limiting and thus total metabolic flux does not substantially increase. To circumvent this, several consecutive enzymes in the same pathway must be up-regulated at the same time, either by transferring several expression cassettes into the plant or by overexpression of regulatory proteins, i.e. transcription factors. The latter approach, however, requires transcriptional co-regulation of all steps in a pathway as it has been shown for a number of pathways in plant secondary metabolism (Broun 2004; Grotewold 2008).

To reduce the levels of undesirable gene products, two general approaches are commonly used: recessive gene disruption and dominant gene silencing. In gene disruption approaches, the target sequence is mutated to eliminate a particular gene function, whereas dominant gene silencing methods induce either the destruction of the gene transcript or the inhibition of transcription. So far, directed gene disruption is not efficient in higher plants. Therefore, the most widely used

technologies for the generation of loss-of-function mutants are transposon mutagenesis (Altmann et al. 1995; Kim et al. 2004), *Agrobacterium* T-DNA insertions (An et al. 2003; Jeon et al. 2000; see also Chap. 1) and, more recently, the use of chemical mutagenesis in combination with TILLING (Henikoff et al. 2004) to create disruptions in coding regions of genes. These techniques have been proven very useful for functional genomics; however, their use for metabolic engineering is limited. They are restricted to a few genetically tractable plant species and due to their untargeted nature they require the generation of large populations of mutated plants to screen for a desired mutation. In addition, genetic redundancy caused by multi-gene families and polyploidy further complicates these kinds of knock-out approaches.

RNA interference (RNAi) and related mechanisms such as ‘antisense’ or ‘co-suppression’ are homology-dependent gene-silencing technologies that possess a great potential for metabolic engineering (Mansoor et al. 2006; Tang et al. 2007; see Chap. 5 for details on the mechanism). In comparison with gene disruption, RNA silencing bears several advantages. It is a dominant trait that can be introduced into any transformable plant species, including the transfer into elite crop varieties. Owing to its targeted nature it does not require the generation of overly large populations of transgenic plants to find a suitable event. Especially in the case of antisense or co-suppression, the efficiency of silencing can vary considerably between individual transformants. This allows the manipulation of metabolic steps where a loss-of-function would be detrimental to the plant but a decrease of gene expression at 30–90% yields a desired metabolic phenotype. Furthermore, by the use of specific promoters, RNA silencing can be manipulated in a spatial and temporal manner. This is important for genes where down-regulation is good for the improvement of a specific organ, e.g. seeds or tubers, but is deleterious to the growth of other plant organs.

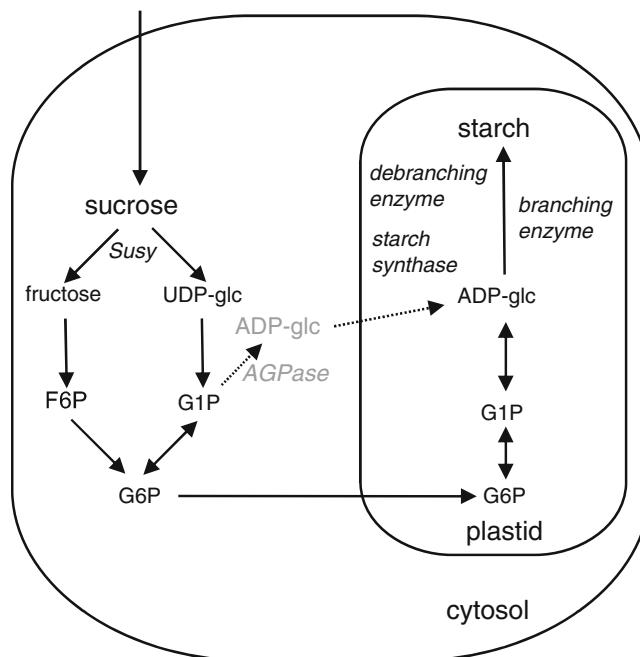
Examples are provided below to show how the above strategies are applied to manipulate the production of different classes of compounds.

## 11.3 Engineering of Primary Metabolism

### 11.3.1 Carbohydrate Metabolism

The majority of metabolic fluxes inside a plant cell center on the formation and utilization of sugars, the primary products of photosynthesis and their conversion into storage and structural carbohydrates, such as starch and cellulose. Starch is the principle constituent of many of harvestable organs, such as tubers or grain. Besides its importance as a staple in human and animal diets, it is also used as a renewable raw material for a wide range of industrial applications (Jobling 2004). Starch is a relatively simple polymer composed of glucose molecules that are linked in two different forms. Amylose is an essentially linear polymer in which the glucose

moieties are joined end-to-end by  $\alpha(1 \rightarrow 4)$  linkages. Amylopectin is a much larger branched molecule, in which about 5% of the glucose units are joined by  $\alpha(1 \rightarrow 6)$  linkages. The ratio between amylose and amylopectin is dependent on the plant species or variety, respectively, and is one determinant of the physico-chemical properties of plant derived starches which are important for technical uses. The biochemical pathways leading to starch formation are well documented and the key enzymatic steps have been identified (Fernie et al. 2002; Geigenberger 2003). Starch metabolism in potato tubers is particularly well characterized and attempts to both increase the accumulation of starch and to modify its structural properties by metabolic engineering have received considerable attention (see also Chap. 20). For starch synthesis in growing potato tubers, sucrose delivered from the phloem is cleaved by sucrose-synthase into uridine-diphosphoryl-glucose (UDP-glucose) and fructose, which are converted to hexose phosphates by UDP-glucose pyrophosphorylase and fructokinase, respectively. Glucose-6-phosphate is then imported into the amyloplast via a glucose-6-phosphate transporter (GPT; Kammerer et al. 1998) and is converted via plastidial phosphoglucomutase and ADP-glucose pyrophosphorylase (AGPase) to ADP-glucose (Fig. 11.1). This process requires



**Fig. 11.1** Principal pathway leading to the formation of starch in storage organs. The alternative route of ADP-glc via generation within the cytosol and subsequent uptake into the amyloplast, as it occurs in the endosperm cells of graminaceous species, is shown by dotted arrows. *Susy* sucrose synthase, *UDP-glc* UDP-glucose, *F6P* fructose-6-phosphate, *G1P* glucose-6-phosphate, *AGPase* ADP-glucose pyrophosphorylase, *ADP-glc* ADP-glucose

ATP, which is imported into the amyloplast via an ATP transporter (Tjaden et al. 1998). The glycosyl moiety of ADP-glucose is the substrate for the synthesis of starch via various isoforms of starch synthase.

Starch synthesis in the endosperm of cereals differs from that in other organs in that the synthesis of ADP-glucose occurs in the cytosol, via a cytoplasmic isoform of AGPase. ADP-glucose is imported into the plastid via a specific nucleotide transporter (Tomlinson and Denyer 2003).

To increase the efficiency of the pathway and thus to increase starch accumulation in crop plants, molecular strategies have initially concentrated on AGPase, the enzyme assumed to catalyze the rate-limiting step of starch synthesis. In an early attempt to increase the activity of the starch biosynthetic pathway in potato tubers, Stark et al. (1992) overexpressed a deregulated bacterial AGPase in the potato variety Russet Burbank. Overall, the transformed lines were reported to have an average of 35% more tuber starch than the controls. However, this effect was lost upon transformation of a different potato cultivar (Sweetlove et al. 1996). In the latter case, starch degradation was up-regulated in addition to starch synthesis, resulting in no net change in starch accumulation.

Attempts to increase starch contents through manipulation of AGPase in cereal seeds have made use of a variant of the maize AGPase gene (*shrunken2*) whose gene product is less sensitive to inhibition by phosphate when compared to the wild-type protein.

Smidansky and colleagues (2002, 2003, 2007) showed that maize, rice and wheat plants expressing this AGPase allele in the endosperm and grown under controlled conditions display an increase in individual seed weight as well as in seed yield per plant. However, in field trials transgenic wheat plants only showed a yield enhancement under conditions of minimal inter-plant competition and optimal water supply (Meyer et al. 2007).

Starch content in potato tubers is very sensitive to manipulation of the plastidial adenylate transporter providing the ATP necessary for the AGPase reaction. Over-expression of an adenylate transporter from *Arabidopsis* in potato tubers resulted in 16–36% more starch per gram fresh weight, indicating that ATP supply to the plastid limits starch synthesis (Tjaden et al. 1998; Geigenberger et al. 2001). Recently, a further increase in potato tuber starch content was achieved by the simultaneous overexpression of a GPT from pea and an *Arabidopsis* adenylate translocator. Double transformants exhibited an increase in tuber yield of up to 19% in addition to an increase in starch content of 28%, when compared to control plants (Zhang et al. 2008). Both effects taken together led to a calculated increase in potato tuber starch of up to 44%. The authors concluded that starch synthesis in potato tubers is co-limited by the ATP supply as well as by the import of carbon skeletons into the amyloplast (Zhang et al. 2008). Further evidence for an energy limitation of starch synthesis in potato tubers comes from transgenic plants with reduced expression of plastidial adenylate kinase (ADK; Regierer et al. 2002). In this study a strong negative influence of ADK activity on starch accumulation was found, suggesting that ADK normally competes with starch synthesis for plastidial ATP.

Taken together, successful attempts to increase starch content through metabolic engineering are scarce. The analyses so far suggest that in potato tubers considerable control of starch synthesis lies outside of the linear pathway as both the adenylate transporter as well as the plastidial ADK appear to exert higher control over the pathway than AGPase, the enzyme widely believed to be rate-limiting (Geigenberger et al. 2004).

To provide improved raw material for the starch processing industry considerable effort has been aimed at altering starch quality which is mainly defined by the amylopectin to amylose ratio (Jobling 2004). Most of the work in this direction has been done on potato tubers as these are one of the major sources for industrial starches.

The synthesis of amylose is accomplished through the activity of a particular isoform of starch synthase, GBSS, and antisense inhibition of this gene leads to amylose-free potato starch (Visser et al. 1991). Amylose-free potato starch can be expected to find application in both the food industry and in paper manufacture. Large-scale field trials with transgenic amylose-free potato varieties have been conducted in Europe and this crop is currently going through the regulatory approval process.

High-amyllose starches are also of great interest, e.g. for improved frying or for industrial use as gelling agents or thickeners. Recently, an innovative approach to increase the amylose content in potato tubers involved the inhibition of starch-branched enzyme A (SBE A) activity, the enzyme responsible for the introduction of  $\alpha 1 \rightarrow 6$  linkages into amylopectin (Jobling et al. 2003). The authors of this study expressed a single-chain antibody targeted against the active site of SBE A in transgenic potato tubers, thereby neutralizing the enzymatic activity. They found that immunomodulation of SBE A increased the amylose content of starch granules from about 20% in wild-type tubers to 74% in the best transgenic line, exceeding the concentration of amylose achieved by conventional antisense strategies (Jobling et al. 2003).

### 11.3.1.1 Production of Novel Carbohydrates in Transgenic Plants

In addition to attempts aiming at manipulating the contents and properties of endogenous carbohydrates, there have been several successful approaches for the production of novel carbohydrates in transgenic plants.

Fructans, or polyfructosylsucroses, are an alternative storage carbohydrate that are highly soluble and are stored within the vacuole. Fructans are present in approximately 15% of all flowering plants (Hellwege et al. 2000). Fructan synthesis is initiated by sucrose:sucrose 1-fructosyltransferase (SST) which catalyzes the fructosyl transfer from one sucrose molecule to another, resulting in the trisaccharide 1-kestose. In subsequent steps, fructosyltransferase (FFT) catalyzes the reversible transfer of fructosyl residues from one fructan to another, producing a mixture

of fructans with different chain length (Ritsema and Smeekens 2003). One of the simplest fructans is inulin, which consists of  $\beta(1\rightarrow2)$ -linked fructose residues while fructans of the levan type are  $\beta(2\rightarrow6)$ -linked fructose polymers.

From a biotechnological viewpoint, interest in fructans has continued to increase, as they have been recognized as beneficial food ingredients. As part of the human diet, they are considered to be prebiotics as they selectively promote the growth of beneficial intestinal bacteria. Furthermore, fructans are assumed to have anti-cancer activity, promote mineral absorption, decrease cholesterol levels and decrease insulin levels. Fructans are normally isolated from plants with low agro-economic value, such as the Jerusalem artichoke (*Helianthus tuberosus*) and chicory (vegetables are also covered in Chap. 25). Thus, attempts have been made to produce transgenic plants with higher fructan yield or making fructans with specific properties. Transformation of sugar beet with an SST gene from Jerusalem artichoke resulted in the conversion of 90% of the vacuolar sucrose into fructan (Sevenier et al. 1998), since the sugar beet accumulates to concentrations approaching 600 mM sucrose, this represents a massive fructan yield. Other researchers have introduced an SST along with an FFT from onion into sugar beet which resulted in an efficient conversion of sucrose into complex, onion-type fructans, without the loss of storage carbohydrate content (Weyens et al. 2004). Potato, as another crop naturally not accumulating fructans, was used to express plant fructosyltransferases. The SST and FFT enzymes from globe artichoke were engineered into potato and led to the accumulation of the full range of fructans found in globe artichoke itself (Hellwege et al. 2000).

Another recent example of the use of potato tubers as bioreactors is the production of isomaltulose (IM), a sucrose isomer that is an excellent sucrose substitute in foods as it shares many physico-chemical properties with sucrose but is non-metabolizable and non-cariogenic. A gene encoding a sucrose isomerase (*pall*) which catalyzes the conversion of sucrose into IM has been isolated from the bacterium *Erwinia rhamontici* (Börnke et al. 2001). Expression of the *pall* gene within the apoplast of transgenic potato tubers led to a nearly quantitative conversion of sucrose into IM. Despite the soluble carbohydrates having been altered within the tubers, growth of Pall-expressing transgenic potato plants was indistinguishable from wild-type plants. Therefore, expression of a bacterial sucrose isomerase provides a valid tool for high level IM production in storage tissues of transgenic crop plants (Börnke et al. 2002). Towards this direction, Wu and Birch (2007) introduced a sucrose isomerase gene tailored for vacuolar compartmentation into sugar cane. Transgenic lines accumulated substantial amounts of IM in their culm. Remarkably, this was not at the expense of sucrose levels, resulting in up to doubled total sugar concentration in juice harvested from sucrose isomerase expressing transgenic sugar cane lines. The reason for this boost in sugar concentration is not understood but it has been hypothesized that IM accumulation in the culm leads to enhanced sink strength that fosters import of additional carbon from source tissues (Wu and Birch 2007). It remains to be shown whether this strategy allows increasing total sugar content in other sucrose storing crops such as sugar beet.

### 11.3.2 Metabolic Engineering of Lipid Metabolism

A plant cell contains a plethora of lipid species, which are mainly represented by free fatty acids, glycolipids, phospholipids, waxes and neutral glycerolipids. Vegetable oil for human consumption almost exclusively consists of triacylglycerols (TAGs), which are composed of three fatty acids esterified to glycerol. TAGs dominate the storage lipid pool in oilseeds from which most plant oils are isolated. There are five fatty acids that are commonly esterified to triglycerides in the predominant oilseed crops (Dyer et al. 2008; see also Chap. 21), namely palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 $\Delta^9$ ), linoleic acid (18:2 $\Delta^{9,12}$ ) and  $\alpha$ -linolenic acid (18:3 $\Delta^{9,12,15}$ ). In order to obtain plant oil with improved technological or nutritional value by metabolic engineering, either unusual fatty acids that are highly abundant in exotic non-crops or novel fatty acids are produced in oilseeds, which can be cultivated on a large scale in contrast to the species from which these unusual fatty acids originate. As a prerequisite for efficient retrieval of the engineered fatty acids, these need to be targeted into storage lipids of seed oil.

Before we discuss potential applications of plants producing unusual fatty acids and the limitations and bottlenecks that were encountered upon engineering of glycerolipids in transgenic oilseed crops, we will briefly outline the route of triacylglycerol biosynthesis.

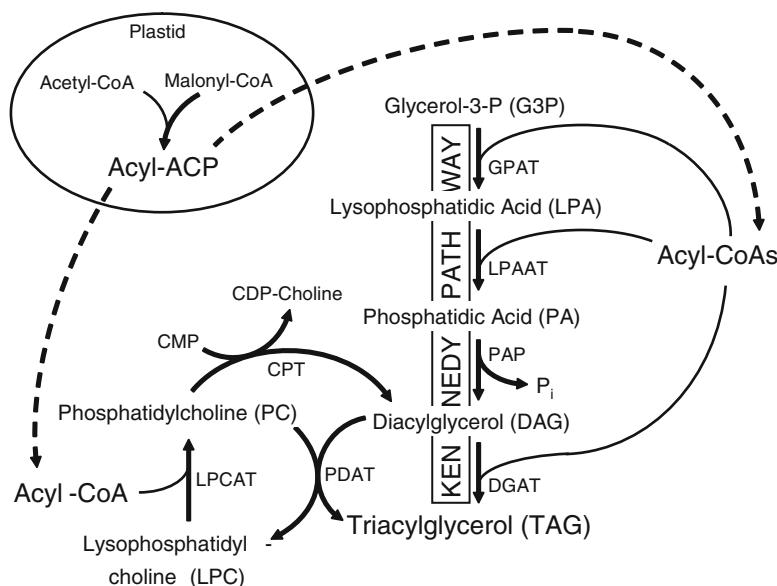
#### 11.3.2.1 Biosynthesis of Storage Lipids

The biosynthesis of glycerolipids (triacylglycerols; TAGs) is a complex, non-linear pathway that involves three subcellular compartments, the chloroplast, the cytosol and the endoplasmatic reticulum (ER). Utilizing the photochemically generated reducing power in the chloroplast stroma, the *de novo* biosynthesis of palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1 $\Delta^9$ ) from activated Acetyl-CoA building blocks takes place while the nascent acyl chain is covalently bound to acyl carrier protein (ACP) complex. Acyl-ACP can undergo two different fates:

1. For the biosynthesis of phospholipids and galactolipids at the chloroplast envelope, the acyl chains can be directly transferred from acyl-ACPs to glycerol-3-phosphate and subsequently to lysophosphatidic acid (LPA). The product, phosphatidic acid (PA), represents the substrate for the production of phospholipids via the transfer of choline, ethanolamine or serine. However, phospholipid synthesis via the prokaryotic plastidic pathway has a very minor impact on seed oil production and is not discussed further.
2. More importantly, fatty acids can be liberated from the plastidic acyl-ACP pool and transferred to the cytosol (Fig. 11.2), where they are: (i) re-esterified to coenzyme A and (ii) subsequently incorporated into the phospholipid pool at the ER. While acyl-CoAs are the substrates for elongases at the cytosolic leaflet of the ER, the cytochrome b<sub>5</sub> containing desaturases FAD2 and FAD3 utilize the phospholipid-bound fatty acid pool (Ohlrogge and Browse 1995).

Precursors and intermediates for TAG biosynthesis at the ER derive from both free acyl-CoA thioesters and phospholipids. The route of TAG synthesis via glycerol-3-P and free acyl-CoA is known as the Kennedy pathway (Fig. 11.2) and involves glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase (DGAT). In each of the three acyl transferase steps (GPAT, LPAAT, DGAT), one more acyl chain is esterified to the glycerol backbone. Alternatively (see Fig. 11.2), fatty acids can be directly transferred from the phospholipid pool into TAG by phospholipid:diacylglycerol acyltransferase (PDAT) or enter the diacylglycerol (DAG) pool by the reversible removal of the phospholipid head group via choline phosphotransferase (CPT). DAG can then be utilized by DGAT or PDAT to yield TAG.

In the production of novel plant oils, both DGAT (Jako et al. 2001; Yu et al. 2006) and PDAT (Dahlqvist et al. 2000) were found to represent rate limiting steps for the entry of heterologously produced fatty acids into the TAG pool in metabolically engineered oilseeds (Bates et al. 2007), identifying the ultimate step



**Fig. 11.2** Routes for triacylglycerol biosynthesis in oilseeds. Triacylglycerols (TAGs) can be synthesized from the glycerol-3-phosphate and the acyl-CoA pool via the Kennedy pathway by subsequent acylation of the triose backbone. Alternatively, the penultimate intermediate, diacylglycerol (DAG) and the TAG end-product can be generated via acyl transfer from the phospholipid pool. Please see the text for more detailed explanation (modified after Napier 2007 and Dyer et al. 2008). Acetyl-CoA Acetyl coenzyme A, Acyl-ACP acylated acyl carrier protein, CPT choline phosphotransferase, DGAT diacylglycerol acyltransferase, GPAT glycerol-3-phosphate acyltransferase, LPAAT lysophosphatidic acid acyltransferase, LPCAT lysophosphatidylcholine acyltransferase, Malonyl-CoA malonyl coenzyme A, PAP phosphatidic acid phosphatase, PDAT phospholipids:diacylglycerol acyltransferase

of TAG biosynthesis as a committed entry site for fatty acids. However additional bottlenecks for the flux of novel fatty acids into TAG were identified, which are discussed later.

### 11.3.2.2 Genetic Engineering of Plant Lipid Metabolism

The manipulation of lipid metabolism in genetically engineered plants provides an enormous economic potential. The world annual production of vegetable oils amounts to  $128.2 \times 10^6$  t in 2007, which is only  $30\times$  lower than the annual production of crude mineral oil of  $4100 \times 10^6$  t/year. In contrast to mineral oil, plant oils represent both a renewable resource and a versatile commodity for food, feed and industrial applications. About 14% of the annual plant oil production is being used for industrial processing, 5% are used as feed and for biodiesel production, respectively, while the rest is consumed as human food (Metzger and Bornscheuer 2006; Durrett et al. 2008).

Soybean, oil palm, rapeseed and sunflower are the predominant oil crops in the world (Dyer et al. 2008; also covered in Chaps. 21, 24). Other important oil crops are peanut, cottonseed, palm kernel, coconut and olives. However, more than 60% of the annual vegetable oil production is derived from soybean and palm oil. As summarized by Dyer et al. (2008), seed oil from these major oilseeds is mainly composed of the five major fatty acids palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 $\Delta^9$ ), linoleic acid (18:2 $\Delta^{9,12}$ ) and  $\alpha$ -linolenic acid (18:3 $\Delta^{9,12,15}$ ). Vegetable oil enriched for fatty acids uncommon to these major oilseed crops and fatty acids with additional functions provide a huge potential as chemical feedstock for the industrial production of detergents, cosmetics, drying oil, paint, ink, specialized lubricants or plastics providing a much higher versatility than mineral oil (Metzger and Bornscheuer 2006; Dyer et al. 2008). Consequently, engineering the lipid composition of seed oil has mainly followed three objectives: (i) to produce unusual fatty acids in oil crops that are of special value as chemical feedstock, (ii) to generate a fatty acid composition optimized for chemical processing and (iii) to introduce fatty acids with a special nutritional value like very long polyunsaturated fatty acids (VL-PUFAs).

In the following, we discuss the current advance in the production of: (i) unusual, short chain fatty acids like lauric acid (12:0), caprylic acid (8:0) and capric acid (10:0), (ii) long-chain fatty acids like erucic acid (22:1) and very-long-chain polyunsaturated fatty acids (VL-PUFAs) like arachidonic acid (AA; 22:4), eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) as well as (iii) various fatty acids with additional functional groups in transgenic oilseed crops.

#### Unusual Medium-Chain Fatty Acids

Glycerolipids (TAGs) containing medium-chain acyl residues are of outstanding interest for the use as biofuel. Medium-chain TAGs are devoid of two

major disadvantages intrinsic to conventional biodiesel consisting of TAG containing the five major fatty acids (Durrett et al. 2008). First, complications caused by biodiesel viscosity are alleviated when medium-chain TAGs are used, as TAG viscosity decreases with the chain length of the esterified fatty acids. The viscosity of regular biodiesel is tenfold higher compared to fossil fuel and is commonly prevented by utilizing fatty acid methyl esters (FAMEs) after the trans-esterification of the TAG fatty acids to methanol. Second, the coking index of medium-chain containing TAGs is lower, compared to other fuels.

The most distinguished example for metabolic engineering of medium-chain fatty acids in oilseeds is the generation of transgenic high-lauric acid (12:0) rapeseed, which is currently approved for commercial use. In the initial approaches, the overexpression of a laureate-specific ACP from the California bay tree (*Umbellularia californica*) in *Arabidopsis* and *Brassica napus* (rapeseed) led to an accumulation of more than 50% of lauric acid in seed TAGs (Voelker et al. 1992; Wiberg et al. 2000). However, the *sn*-2 position of glycerol barely contained lauroyl residues in these transgenics. Additional overexpression of a LPAAT from coconut with high specificity for lauroyl-CoA increased the yield of laureate in rapeseed TAG to 67%, indicating that a limitation in the Kennedy pathway (see Fig. 11.2) restricted the accumulation of lauric acid in seed oil of the transgenics (Knutzon et al. 1999). Likewise, *Cuphea lanceolata*, which accumulates more than 80% of capric acid (10:0) in seed TAG was found to contain one set of GPAT and LPAAT specific for medium-chain acyl-CoAs and an DGAT that preferentially funnels di-medium-chain DAGs into TAG (Dehesh 2001). The specificities of these three Kennedy pathway enzymes obviously leads to an effective channelling of medium-chain fatty acids into *Cuphea* TAG.

Attempts to introduce valuable medium-chain fatty acids like capric acid (10:0) or caprylic acid (8:0) into rapeseed TAG were less successful, leading to 8% and 30% medium-chain acyls residues in rapeseed oil (Wiberg et al. 2000). However, the acyl-CoA pool in the transgenic seeds was dominated by the introduced medium-chain fatty acids, again indicating that the incorporation into glycerolipids via the Kennedy pathway was the limiting step preventing a high yield of caprylic and capric acid in the TAG pool (Larson et al. 2002).

### Unusual Long-Chain Fatty Acids

Coriander and *Thunbergia alata* seed oil contain more than 80% of the unusual monoenoic fatty acids petroselinic acid (18:1 $\Delta^6$ ) and 16:1 $\Delta^6$ , respectively, both of which are valuable precursors for the production of various plastic polymers and cyclic hydrocarbon skeletons. Interestingly, both unusual fatty acids are synthesized by plastidic acyl-ACP desaturases. Palmitoyl-ACP (16:0-ACP) is utilized as a substrate for desaturation at the  $\Delta^4$  and  $\Delta^6$  position, respectively and the monoenoic fatty acid products are targeted to seed TAG via the phospholipid pool at the ER (Cahoon and Ohlrogge 1994; Schultz et al. 2000).

The  $16:1\Delta^4$  product of the coriander desaturase is then subsequently elongated to yield petroselinic acid, while the  $16:1\Delta^6$  fatty acid is a direct product of the *Thunbergia* desaturase. The accumulation of these two unusual monoenoic fatty acids in transgenic *Arabidopsis* overexpressing the coriander and *Thunbergia* ACP-desaturases amounted to less than 15% of total seed TAG (Suh et al. 2002). In coriander, specific ACP, 3-ketoacyl-ACP synthase and thioesterase are present for the synthesis of petroselinic acid in plastids (Suh et al. 2002), suggesting that an inefficient substrate channelling between the prokaryotic pathway enzymes in *Arabidopsis* and the heterologously expressed desaturase may be the cause to the relatively low abundance of petroselinic acid in seed oil of the transgenics.

In contrast to petroselinic acid, erucic acid ( $22:1\Delta^{13}$ ) is produced in high amount in oilseed rape and other Brassicaceae. However, erucic acid is largely restricted to the *sn*-1 and *sn*-3 positions of TAG. Again, the specificity of the endogenous LPAAT seems to prevent the incorporation of erucic acid at the *sn*-2 position, identifying the same bottleneck that limited lauric acid accumulation in TAG of transgenic rapeseed. When an LPAAT from *Limianthes* specific for erucic acid and the endogenous FAE1 elongase were overexpressed in parallel, the TAG pool of the resulting transgenic rapeseed contained more than 70% erucic acid (Nath et al. 2006).

The production of the very-long-chain polyunsaturated fatty acids (VL-PUFAs) AA (an  $\omega 6$ -fatty acid), EPA (an  $\omega 3$ -fatty acid) and DHA (an  $\omega 3$ -fatty acid) has drawn considerable attention due to their importance for human nutrition. Furthermore, the application of VL-PUFAs isolated from transgenic oilseed crops as a feed supplement to enable more sustainable salmon farming was supposed (Cahoon et al. 2007). Nevertheless, the production of VL-PUFAs in transgenic plants is complicated as it involves several cycles of desaturation and chain elongation of the endogenous precursors linoleic acid (18:2) and  $\alpha$ -linolenic acid (18:3). As outlined in the previous section, the substrates for fatty acid desaturases are PC bound fatty acids, while elongases use free acyl-CoAs as their substrates, necessitating a substrate shuttling between the phospholipid and the acyl-CoA pool. Metabolic engineering of transgenic plants for VL-PUFA production has been accomplished by the introduction of several desaturases and elongases in *Arabidopsis* and *Brassica juncea*, totalling to up to nine transgenes (Wu et al. 2005). However, various routes can be chosen for VL-PUFA production. Apart from the  $\Delta^6$  desaturase pathway, on which most attention has been focused to date, as it allows for the simultaneous biosynthesis of AA, EPA and DHA, the  $\Delta^8$  desaturase pathway has proven an interesting alternative for the production of AA and EPA (Qi et al. 2004). Commonly, the maximum yield of VL-PUFAs in TAG of transgenic *Arabidopsis*, *Brassica juncea* and soybean obtained to date is low and ranges between 3% for DHA (Wu et al. 2005; Kinney 2006) and 8% for EPA (Qi et al. 2004; Wu et al. 2005). Recently, desaturases that act on acyl-CoAs have been identified from microalgae and higher plants, possibly making trans-esterification between acyl lipids and the acyl CoA pool dispensable in the

future, which could also improve the yield of VL-PUFAs (Sayanova et al. 2007; Hoffmann et al. 2008).

### Fatty Acids with Additional Functional Groups

Fatty acids with additional functional groups and their chemical derivatives represent an emerging valuable resource as industrial feedstocks for the production of cosmetics, lubricants, nylon, resins, polyvinylchloride (PVC), polyurethane and drying oils in paint and ink (Metzger and Bornscheuer 2006). Here, we briefly discuss unusual fatty acids that contain hydroxyl, epoxy and stereochemically unusually conjugated hexatriene groups, which have in common that they all are synthesized by divergent forms of the ER  $\Delta^{12}$ -oleic acid desaturase FAD2 (van de Loo et al. 1995; Lee et al. 1998; Dyer et al. 2002).

Ricinoleic acid is produced by a  $\Delta^{12}$ -hydroxylase and represents almost 90% of the castor bean (*Ricinus communis*) seed oil pool. Ricinoleic acid carries a hydroxyl group at the C-12 position in addition to a cis-double bond at the C-9 position, which renders it to a versatile substrate for various organic syntheses (Metzger and Bornscheuer 2006). Vernolic acid is synthesized by a  $\Delta^{12}$ -epoxigenase and is abundant in the seed oil of, e.g. *Vernonia galamensis*, *Crepis palaestina* and *Euphorbia lagascae*. It contains an epoxy group at position C-12 in addition to the C-9 double bond and can be used as a binder in coatings and for the synthesis of enantiomerically pure products. Calendulic acid (18:3 $\Delta^{8trans,10trans,12cis}$ ) and  $\alpha$ -eleostearic acid (18:3 $\Delta^{9cis,11trans,13trans}$ ), which are abundant in the seed TAG pool of marigold (*Calendula officinalis*) and the Chinese tung tree (*Vernicia fordii*), respectively, are used as drying oils in paints, inks and coatings. The conjugated hexatrienic double bonds of calendulic and  $\alpha$ -eleostearic acid are synthesized from linoleic acid by a FAD2 conjugase (Cahoon et al. 1999).

Intriguingly, the overexpression of these three divergent FAD2 genes in *Arabidopsis* and soybean lead to less than 20% accumulation of ricinoleic, vernolic, calendulic and  $\alpha$ -eleostearic acid in seed TAG as compared to 60% to 90% in the native species (Broun and Somerville 1997; Lee et al. 1998; Cahoon et al. 1999). Instead, oleic acids contents were increased in all these transgenics and the unusual fatty acids accumulated in the PC pool (Thomaeus et al. 2001; Cahoon et al. 2006), indicating that: (i) the conversion from oleic to linoleic acid by the endogenous FAD2 desaturase is disturbed by the transgene expression and (ii) the channelling of the unusual fatty acids into the TAG pool is inefficient in the transgenics. In *Vernonia galamensis*, castor bean and tung tree, the respective DGAT2 isoforms were identified to specifically confer the transfer of vernolic, ricinoleic and  $\alpha$ -eleostearic acid into seed oil, respectively (Cahoon et al. 2006; Kroon et al. 2006; Shockley et al. 2006), identifying DGAT as the potential bottleneck for the accumulation of these fatty acids in the TAG pool.

## 11.4 Engineering of Secondary Metabolism for Human Health and Nutrition

Plants produce a large array of secondary metabolites. These are loosely defined as organic compounds with no essential role in growth and development. Although not absolutely required, these compounds confer some selective advantage for the plant and many have been implicated in the plants' interaction with its immediate environment. Plant secondary compounds are commonly consumed as part of the human diet and they play an important role as phytonutrients as they are assumed to offer protection against certain cancers, cardio-vascular diseases, act as antioxidants or bear other health promoting properties. Due to their presumed health benefits, there is growing interest in the development of food crops with tailor-made levels and composition of secondary compounds, designed to exert an optimal biological effect.

Given the wealth of plant secondary compounds relevant for human nutrition, we concentrate here on a few recent examples which highlight the potential of engineering plant secondary metabolism. For a more comprehensive overview, the reader is referred to some excellent recent reviews (e.g. Kinney 2006; Zhu et al. 2007).

### 11.4.1 Flavonoids

Flavonoids are phenolic compounds derived ultimately from phenylalanine which impart much of the color and flavor of fruits, vegetables, nuts and seeds. The first committed step in flavonoid biosynthesis is the conversion of the precursor 4-coumaroyl-CoA into chalcone by the enzyme chalcone synthase. Chalcone is then derivatized in a series of enzymatic steps to eventually form different classes of flavonoids, such as flavanones, dihydroflavonols and finally to the anthocyanins, the major water-soluble pigments in flowers and fruits (Schijlen et al. 2004). Tomato is an excellent candidate for transgenic enhancement of flavonoid content. It is an important food crop worldwide; however, its flavonoid content is generally low and largely confined to the tomato peel. Constitutive, high level overexpression of a petunia chalcone isomerase in tomato resulted in up to 78-fold increases in the levels flavonoids in the peel (Muir et al. 2001). However, since the peel accounts for only about 5% of fruit mass the overall increase was rather low. A 3.5-fold increase in fruit flavonol content of tomato was achieved by RNAi-mediated suppression of the tomato *DET1* gene, which encodes a transcription factor negatively regulating photomorphogenic responses (Davuluri et al. 2005).

Coordinate transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the final levels of secondary metabolites in plant cells. In various plant species the tissue-specific regulation of the structural genes involved in flavonoid biosynthesis is controlled by the combination of regulators

from two transcription factor families (Schijlen et al. 2004). Consequently, over-expression of *Lc* and *C1*, two transcription factors that control flavonoid biosynthesis in maize, resulted in tomato fruit containing 20-fold higher flavonol content than the respective control. In a similar approach, Butelli et al. (2008) expressed the *Del* and *Ros1* genes from snapdragon in the fruit of transgenic tomatoes. Both genes encode transcription factors that interact with each other to induce anthocyanin biosynthesis in snapdragon flowers. The fruit of the transgenic tomato plants accumulated anthocyanins at levels substantially higher than previously reported. Evidence for a health promoting effect of these engineered tomato fruits comes from a pilot study in which a cancer-susceptible mouse strain showed a significant extension of life span when fed on high-anthocyanin tomatoes (Butelli et al. 2008).

### 11.4.2 Vitamins

Vitamins are a chemically diverse group of organic compounds which are classified by their biological and chemical activity, not their structure. Humans have to acquire a number of vitamins with their diet and many of these compounds are plant derived products. Vitamin deficiency is a serious problem in the developing world and optimizing vitamin content of plants through genetic engineering has received much attention in recent years (Herbers 2003; Zhu et al. 2007). Thus far, metabolic engineering has resulted in transgenic plants that contain elevated levels of provitamin A, vitamin C, E and folate (Ye et al. 2000; Agius et al. 2003; Cahoon et al. 2003; Storozhenko et al. 2007).

The principal example of vitamin metabolic engineering in plants is the synthesis of  $\beta$ -carotene (provitamin A, a carotenoid) in rice endosperm, which led to the development of so-called ‘golden rice’ varieties (Ye et al. 2000; Al-Babili and Beyer 2005). Carotenoids do not accumulate in rice endosperm; however, the precursor geranylgeranyl pyrophosphate is abundant. By introducing heterologous activities of a phytoene synthase, a phytoene desaturase, carotene desaturase (the latter two activities were mediated by a single bacterial enzyme) and lycopene  $\beta$ -cyclase, Ye et al. (2000) were able to produce rice with up to 2  $\mu\text{g/g}$   $\beta$ -carotene dry weight. Additional golden rice varieties have been generated that contain only two recombinant enzymes (daffodil phytoene synthase and *Erwinia* phytoene desaturase; Beyer et al. 2002); and most recently, a novel variety has been developed in which the daffodil phytoene synthase has been replaced by the more efficient maize homolog, resulting in a 23-fold improvement in  $\beta$ -carotene content (Paine et al. 2005).

Whereas vitamin A is a single compound, eight tocopherols belong to the vitamin E family that differ in their methylation pattern of the polar head group and the saturation of the prenyl tail of the amphiphilic antioxidant. Due to the specificity of the retrieval system,  $\alpha$ -tocopherol has the highest vitamin E activity in humans. The pathway of tocopherol biosynthesis in plants has been characterized and the involved genes have been cloned (DellaPenna and Last 2006; DellaPenna and Pogson 2006). Work on transgenic plants has shown that the levels of vitamin E

activity can be increased either by increasing the total amount of tocochromanols or by shifting the metabolic flux towards  $\alpha$ -tocopherol. For example, expression of a *Synechocystis* PCC6803 or *Arabidopsis*  $\gamma$ -tocopherol methyltransferase in *Arabidopsis* seeds resulted in a fundamental shift from  $\gamma/\delta$ - to  $\alpha/\beta$ -tocopherol without altering total vitamin E levels (Shintani and DellaPenna 1998). Similarly, transgenic soybean with more than 95%  $\alpha$ -tocopherol in seeds that usually only contain 10%  $\alpha$ -tocopherol were generated by the simultaneous overexpression of two methyltransferases from the *Arabidopsis* tocopherol biosynthetic pathway, AtVTE3 and AtVTE4 (Van Eenennaam et al. 2003). A 10- to 15-fold increase in total vitamin E has been achieved in *Arabidopsis* leaves by overexpression of a homogentisic acid geranylgeranyl transferase (HGGT) from barley while expression of the same activity in corn seeds increased vitamin E content by a factor of six (Cahoon et al. 2003). An 8-fold increase in total leaf tocochromanol content was obtained when yeast prephenate dehydratase (PDH) and *Arabidopsis* hydroxypyphenylpyruvate dioxygenase (HPPD) were overexpressed in tobacco, thereby circumventing substrate limitation in the endogenous pathway (Rippert et al., 2004).

## 11.5 Conclusions

Recent years have seen a vast improvement of our understanding of plant metabolism at the genetic level and the interaction of metabolic pathways at the physiological level. The examples described above illustrate how this knowledge enables successful manipulation of metabolic networks via the overexpression or repression of single genes; however, these success stories are yet relatively rare. Multi-point metabolic engineering is now beginning to supersede single-gene manipulation as the most promising way to manipulate metabolic fluxes. The analytical tools available in the post-genomics era will further expand our knowledge of metabolic pathways, while advances in systems biology will help us to model the impact of different modifications more accurately. But we should bear in mind that, despite the encouraging results obtained so far, important questions remain largely unanswered to date. For instance, it remains to be shown whether the novel transgenic varieties which showed considerable improvement in certain traits under controlled conditions also outperform conventional varieties under high crop density in the field, something which has rarely been investigated so far.

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# Chapter 12

## Pharmaceuticals

Andreas Schiermeyer and Stefan Schillberg

### 12.1 Introduction

Plants and their extracts have long been used as remedies for a variety of health conditions, and many modern pharmaceuticals are still derived from plants. With recombinant DNA technology and the advent of efficient transformation technologies for plant cells it is now possible to extend the use of plants for pharmaceutical purposes by using them as production platforms for biopharmaceuticals. As of 2006, more than 150 biopharmaceuticals were approved for use in human medicine (Walsh 2006). The vast majority of biopharmaceuticals are proteins of human or animal origin and include enzymes, blood factors, thrombolytics, monoclonal antibodies, cytokines, hormones, and growth factors. These proteins are currently produced mainly in bacterial, yeast, or animal cell cultures. As the demand for and diversity of biopharmaceuticals increase, we need additional production capacities to fulfill the market requirements. Therefore, plants and plant cells have been investigated as alternative production hosts.

Since the first report of the successful production of a monoclonal antibody in transgenic tobacco plants two decades ago (Hiatt et al. 1989) a great variety of proteins with potential pharmaceutical applications have been produced in plants (Basaran and Rodriguez-Cerezo 2008; Fischer et al. 2004; Schiermeyer et al. 2004; Twyman et al. 2005). These proteins are collectively referred to as plant-made pharmaceuticals (PMPs) and a large number of plant species have been evaluated as production platforms for these molecules (Sparrow et al. 2007). These include food crops such as maize, barley, potato, and tomato, non-food crops such as tobacco and others such as duckweed and moss. As well as intact plants, certain types of plant tissue cultures (e.g. hairy roots) and cell suspension cultures from various species

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have been investigated (Hellwig et al. 2004). In the following section we discuss the basic principles required to produce PMPs, and then we consider four case studies of plant-derived biopharmaceuticals that have moved from laboratory proof-of-principle studies into clinical development.

## 12.2 Expression Systems

The properties of a pharmaceutical recombinant protein often vary according to the expression platform used to produce it, which takes into account the plant species, the tissue from which the product is extracted, and the subcellular localization of the recombinant protein. The selection of an expression system therefore depends mostly on the required properties of the recombinant protein, but also on considerations such as downstream processing and regulatory issues.

### 12.2.1 Transient Expression Systems

Transient expression systems do not involve transgenic plants or cells – the transgene encoding the pharmaceutical protein remains episomal rather than integrating into the host genome. Such systems are based either on the transient expression of episomal DNA following standard transformation (*Agrobacterium* or direct transfer by particle bombardment, see Chap. 1) or on the use of plant viruses as vectors. Although transient expression following standard transformation is mostly used as a rapid testing system to confirm gene transfer and expression, agroinfiltration (in which recombinant *Agrobacterium tumefaciens* are infiltrated into plant tissue) can be used to produce milligram amounts of recombinant pharmaceutical proteins within a short time-frame. This procedure is described in more detail by Fischer et al. (1999) and Sheludko (2008).

To date four types of expression systems have been developed using plant viruses as vectors (Lico et al. 2008). *Gene insertion vectors* are defined as those in which the pharmaceutical transgene resides within a complete viral genome and are usually based on tobacco mosaic virus (TMV) or potato virus X (PVX). In *gene substitution vectors*, an endogenous virus gene such as the coat protein gene is replaced by the pharmaceutical transgene. Both the insertion and substitution vector systems have limitations with respect to transgene size, genetic stability, and expression level. Furthermore, the elimination of the virus coat protein may impair its ability to spread systemically within a plant. *Peptide display vectors* have been employed particularly for the expression of small peptide epitopes for use as vaccines, although occasionally they have been used to display larger proteins. They are often based on cowpea mosaic virus (CPMV). When these peptides are fused with the coat protein of a virus, the resulting chimeric viral coat will display the epitope on its surface. Such viral particles have proven to be very effective for

vaccination because they are highly immunogenic, so no additional adjuvants are needed to induce an immune response in the host. The versatility of these vectors has been demonstrated with an experimental rabies vaccine that induced a humoral immune response in laboratory animals and human volunteers (Yusibov et al. 2002) and a cancer vaccine that induced a cellular immune response in mice (McCormick et al. 2006). However, while such vectors are useful for the production of chimeric viral particles, they cannot be used to synthesize intact recombinant pharmaceutical proteins.

To overcome the limitations with respect to transgene size, transgene stability and impaired virus spreading, *deconstructed vectors* have been developed that preserve beneficial virus replication functions but replace the reliance on systemic spreading with the ability to transfer DNA into many more cells (Marillonnet et al. 2004). The deconstructed vectors are based on TMV gene substitution vectors (with the transgene replacing the coat protein gene) so the virus can move to adjacent cells with the help of the movement protein but cannot spread systemically. Most virus vectors rely on systemic spreading to achieve high protein expression levels, but the deconstructed vectors take advantage of the efficiency of *Agrobacterium*-mediated gene delivery by enclosing the entire virus vector within a T-DNA, which is introduced into host cells by agroinfiltration. Under normal circumstances, episomal T-DNA is short-lived and expression is only possible for a limited time unless selection is used to propagate cells with integrated T-DNA. However, because TMV is an RNA virus that naturally replicates in the cytosol, the introduction of a virus DNA construct into the nucleus allows replication-competent virus genomes to be produced by transcription. The deconstructed vector system required several optimizations to achieve high-level expression. Efficient processing of the transcribed RNA was found when several intron sequences were added to the viral constructs to ensure correct processing and export from the nucleus. When those vectors were delivered by vacuum infiltration of recombinant *Agrobacterium tumefaciens* into whole *Nicotiana benthamiana* plants, a process termed Magnifection by its inventors, yields of up to  $4 \text{ g kg}^{-1}$  fresh weight of leaf biomass could be achieved (Marillonnet et al. 2005). Even the production of hetero-oligomeric proteins such as antibodies is possible when multiple viral vectors are co-infiltrated into the plant host. Giritch et al. (2006) achieved yields of up to  $0.5 \text{ g kg}^{-1}$  fresh weight of a human monoclonal IgG1 antibody when they expressed the heavy and light chains of the antibody on two separate non-interfering viral vectors based on TMV or PVX sequences, respectively (Giritch et al. 2006).

### 12.2.2 *Stable Expression Systems*

#### 12.2.2.1 Transplastomic Plants

The introduction of transgenes into the circular genome of plastids can be achieved by biolistic transformation methods (Verma et al. 2008), which is discussed in

detail in Chap. 2. The transgene is designed to contain flanking sequences homologous to endogenous plastid genes so that the transgene is inserted into predefined regions in the plastid genome by homologous recombination. Each plastid contains several hundred genome copies. Therefore the primary transformants must undergo multiple rounds of regeneration to achieve the homoplasmic state (in which all plastid genomes contain the transgene). This is routinely achieved by the introduction of an aminoglycoside 3'-adenylyltransferase gene that confers resistance to spectinomycin (Svab and Maliga 1993). Due to the presence of multiple genomes per plastid it is possible to achieve transgene copy numbers of up to 10 000 per cell (Bendich 1987). A further increase in the copy number can be achieved by targeting the transgene to duplicated regions of the plastid genome (Zoubenko et al. 1994). The high transgene copy number allows recombinant proteins to accumulate to high concentrations in transplastomic plants, often reaching 10% of the total soluble protein (TSP) or even higher (Daniell 2006). Since plastids are inherited maternally in most crop species, gene transfer via pollen is unlikely making this technique an important biosafety solution to outcrossing (Hagemann 2002).

Plastids are equipped with the enzymes required to assemble multisubunit proteins like the pentameric cholera toxin B subunit (Daniell et al. 2001) and to create disulfide bridges in molecules like the human growth hormone somatotropin (Staub et al. 2000). Native somatotropin has a phenylalanine residue at the N-terminus but recombinant somatotropin can only be produced in plastids with methionine at the N-terminus. To meet this challenge, the recombinant molecule was produced as a N-terminal fusion with ubiquitin, the latter being cleaved by an endogenous ubiquitin protease to yield the native N-terminal phenylalanine. Some recombinant proteins expressed in plastids are subject to N-terminal processing by the endogenous methionine aminopeptidase depending on the amino acid composition following the initiating N-formylmethionine (Fernandez-San Millan et al. 2007; McCabe et al. 2008). This has to be taken into account in transgene design when a defined N-terminus is critical for the functionality of the final protein product.

Tobacco was the first domesticated crop in which plastid transformation was achieved, and only recently has that success been replicated in other crops, such as tomato (Ruf et al. 2001) and lettuce (Lelivelt et al. 2005). This means that most reports of transplastomic plants producing pharmaceutical proteins involve the use of tobacco, yet the accumulation of recombinant subunit vaccines in the chloroplasts or chromoplasts of edible plant tissues would offer additional opportunities for vaccine production and delivery (Kamarajugadda and Daniell 2006).

Despite the high transgene copy numbers in homoplasmic plants and the absence of position effects and post-transcriptional silencing, not all recombinant proteins can be expressed in plastids at high levels. The rotavirus coat protein VP6, a potential subunit vaccine for enteric infections, accumulated to 3% TSP in the young leaves of transplastomic tobacco plants, but could not be detected in older leaves due to proteolytic degradation (Birch-Machin et al. 2004). Similarly the HIV p24 antigen could be detected in the youngest leaves of transplastomic tobacco plants but not in mature leaves (McCabe et al. 2008). With a codon-optimized

construct, homogenous expression was achieved in leaves of all ages but the transplastomic leaves exhibited a yellow phenotype and rearrangements were detected within the plastid genome. Proteolysis is a general concern irrespective of the expression system. Foreign proteins are exposed to proteolysis in planta during biomass growth phase and upon cell disruption and downstream processing, and research is ongoing to identify and hopefully inhibit the proteases involved (Doran 2006; Schiermeyer et al. 2005).

### 12.2.2.2 Nuclear Transgenic Plants

Many therapeutic proteins expressed in plants are glycoproteins, which means they must be targeted to the endomembrane system where glycan chains are added. Since plastids are unable to modify proteins by glycosylation, such proteins need to be expressed from transgenes integrated into the nuclear genome. Many plant species have been transformed successfully by either co-cultivation with *Agrobacterium tumefaciens* (Twyman et al. 2003) or by biolistic methods (Altpeter et al. 2005); see Chap. 1 for details. Gene stacking for the expression of hetero-oligomeric proteins can be achieved by crossing plants that express individual subunits, or by simultaneous transformation with multiple genes. The former approach has been used to assemble IgG and IgA class antibodies (Ma et al. 1994) as well as secreted antibodies comprising four different polypeptide chains: the heavy and light chains, the joining chain, and the secretory component (Ma et al. 1995).

Breeding programs (see Chap. 6) have been used to increase the yield of recombinant avidin in transgenic maize plants by a factor of 70 after six generations (Hood et al. 2002). Also, it is possible to introgress transgene(s) from laboratory model varieties into elite germplasm that is not readily accessible for transformation (Rademacher et al. 2008).

In contrast to plastid transgenes, nuclear transgenes can be subject to epigenetic phenomena such as position effects, transcriptional silencing (TGS) and post-transcriptional gene silencing (PTGS), the latter of which can be triggered by aberrant transcripts produced from truncated and rearranged transgenes, inverted repeats and by readthrough of tandem repeats generating mRNAs without polyadenylate tails. This is discussed in more detail in Chap. 5. Although position effects can often be ameliorated by the use of matrix attachment regions (Abranched et al. 2005), these do not protect plants from PTGS triggered by complex transgene structures, so it is usually necessary to screen populations of independent primary transformants to identify plants with high-level expression. The targeted integration of transgenes into the nuclear genome by homologous recombination could circumvent problems caused by epigenetic phenomena, but this is very inefficient in most plant species with the notable exception of the moss *Physcomitrella patens* (Decker and Reski 2004). Very recently it was shown that homologous recombination in higher plant species can be facilitated by the use of engineered zinc finger nucleases (ZFN) that introduce DNA double-strand breaks at

specific sites in the genome, thus stimulating homologous recombination events (Kumar et al. 2006). This technology could facilitate very precise engineering of transgenic plants in the future.

### 12.3 Post-Translational Modifications

The most important post-translational modification for biopharmaceuticals is N-glycosylation, since ~30% of all approved biopharmaceuticals are glycoproteins. Glycan chains affect half-life, stability, and functionality. The glycosylation machinery in plants is similar but not identical to its mammalian counterpart. In both cases, the N-glycosylation of a peptide chain starts with the co-translational transfer of an oligosaccharide precursor to asparagine residues within the consensus sequence N-X-S/T (X is any amino acid but proline) in the endoplasmic reticulum (ER). As the protein matures, the oligosaccharide precursor is trimmed to finally yield a glycoform known as the high mannose type, which is identical in plants and mammals. When the glycoprotein travels further down the secretory pathway the glycans are modified stepwise by enzyme activities located in the Golgi apparatus. The final complex-type N-glycans differ between plants and mammals. Plant glycoproteins contain  $\beta$ 1,2-xylose and  $\alpha$ 1,3-fucose, which are absent in mammals, whereas mammalian glycoproteins contain  $\beta$ 1,4 galactose and terminal neuraminic acid residues that are absent in plants. Proteins with plant-specific glycans may induce an immune response upon subcutaneous injection in mammals (Bardor et al. 2003). Although this response would be desirable in the case of plant-derived vaccines, it might limit the use of plant-derived therapeutics that have to be administered on a regular basis.

Therefore different strategies have been pursued to produce glycoproteins in plants with humanized glycans. By adding the H/KDEL sequence motif to the C-terminus of pharmaceutical proteins, they are effectively retained within the endoplasmic reticulum, which prevents the plant-specific modification in the Golgi from taking place. This strategy has been employed for the production of a mouse/human chimeric IgG1 antibody against the human chorionic gonadotropin (Sriraman et al. 2004). Other approaches aim to modify the endogenous plant glycosyltransferase system responsible for the transfer of  $\beta$ 1,2-xylose and  $\alpha$ 1,3-fucose residues. In the moss *Physcomitrella patens*, the genes for  $\beta$ 1,2-xylose-transferase and  $\alpha$ 1,3-fucosetransferase have been disrupted by gene targeting (Koprivova et al. 2004). This double knockout mutant has been used to produce secreted human erythropoietin that lacks the plant specific core  $\beta$ 1,2-xylose and core  $\alpha$ 1,3-fucose (Weise et al. 2007). In plants that are less amenable for gene targeting, an RNA interference (RNAi) approach has been used to silence the endogenous  $\beta$ 1,2-xylosetransferase and  $\alpha$ 1,3-fucosetransferase genes. By transforming the duckweed *Lemna minor* with a vector coding for the human anti-CD30 antibody MDX-060 and an inverted repeat construct homologous to  $\beta$ 1,2-xylosetransferase and  $\alpha$ 1,3-fucosetransferase, a recombinant antibody was produced lacking

plant-specific N-glycans (Cox et al. 2006). The plant-derived MDX-060 antibody was compared to its counterpart produced in Chinese hamster ovary (CHO) cells in terms of its binding characteristics with respect to the human Fc receptor and its antibody-dependent cell-mediated cytotoxicity (ADCC) activity. The plant-derived antibody had >10-fold higher affinity for the human Fc receptor and 20-fold higher ADCC activity against a tumor cell line in vitro compared to the CHO-derived antibody. Similarly the HIV-1 neutralizing antibody 2G12 that was produced in a fucosyltransferase and xylosyltransferase T-DNA *Arabidopsis* double-knockout line had a homogeneous mammalian-like N-glycosylation pattern (Schahs et al. 2007).

To further humanize the glycan structures of PMPs, the human  $\beta$ 1,4-galactosyltransferase cDNA has been introduced into tobacco (Bakker et al. 2006; Fujiyama et al. 2007) and alfalfa (Sourrouille et al. 2008). Glycan analysis of a mouse IgG antibody that was expressed in the transgenic tobacco lines revealed the presence of terminal galactose residues in a subset of the analyzed glycopeptides. Surprisingly, the level of plant-specific core fucose and core xylose residues was also significantly reduced.

Many N-glycan structures of human origin contain terminal sialic acid residues. The presence or absence of this moiety strongly affects the plasma half-life of the corresponding glycoprotein. In the case of human erythropoietin, enzymatic removal of the terminal sialic acid residues reduces the serum half-life of this protein from >5 h to <2 min when injected intravenously in rats (Erbayraktar et al. 2003). Therefore efforts are ongoing to engineer the pathway that leads to the formation of CMP-sialic acid and the transfer of sialic acid to recombinant proteins in plants. Some of the key mammalian enzymes in this pathway –  $\alpha$ 2,6-sialyltransferase, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, N-acetylneuraminc acid phosphate synthase, and CMP-N-acetylneuraminc acid synthetase – have already been expressed successfully in *Arabidopsis* (Castilho et al. 2008; Wee et al. 1998).

In contrast to the numerous reports on the significance of N-glycosylation much less is known about the O-glycosylation of PMPs. This type of glycosylation occurs on hydroxyproline residues that are formed by the action of prolyl hydroxylases. This reaction typically occurs on clustered proline residues that are found within the extensin family of hydroxyproline-rich glycoproteins (HRGPs). A similar proline-rich sequence motif is found in the hinge region of human IgA1 antibodies, and consequently it became a substrate for O-glycosylation when a recombinant antibody of this subclass was produced in maize kernels (Karnoup et al. 2005). Whether O-glycosylation limits the use of such PMPs remains to be determined. In a recent study, a synthetic O-glycosylation motif consisting of a tandem repeat of the dipeptide serine/proline (SP) was fused to the C-terminus of human interferon  $\alpha$ 2b. As expected the chimeric proteins had higher molecular masses (up to 75 kDa for IFN $\alpha$ 2-(SO)<sub>20</sub>) due to the presence of O-glycans. When injected intravenously into mice, this engineered chimeric protein had a 13-fold longer serum half-life compared to standard IFN $\alpha$ 2. The increased serum half-life was explained by slower renal clearance and greater resistance towards proteolytic degradation (Xu et al. 2007).

## 12.4 Downstream Processing

Downstream processing includes the post-harvesting steps needed to extract, purify, and formulate the active pharmaceutical ingredient. The first steps of this process are largely dictated by the expression system (Menkhaus et al. 2004). Although a secreted protein might be purified directly from the growth medium, possibly after a concentration step, an intracellular protein needs to be liberated from the surrounding cell matrix before purification. The crude extract will be subjected to clarification procedures consisting of centrifugation or filtration steps to remove cell debris and particles. During these initial steps, measures must be taken to limit the potential degradation of the target protein by proteolysis and to prevent unintended modification of the product by oxidation or the addition of phenolic groups (Pierpoint 2004). Additional adjustments of the feedstream with respect to the pH or salt concentration might be necessary to meet the needs for the subsequent chromatography steps, with two or more orthogonal separation methods typically used to maximize purity and contaminant removal (Drossard 2004). Potential contaminants include endogenous plant proteins, metabolites, and nucleic acids, and also environmental contaminants such as agrochemicals or microbes and insects associated with the plant. The actual chromatographic methods used depend on the physicochemical properties of the target protein. Common techniques include affinity chromatography with Protein A or lectins, ion exchange chromatography, hydrophobic interaction chromatography, and size exclusion chromatography. The design of the downstream process largely depends on the level of purity required for the protein's intended use, e.g. proteins for injection must be purer than proteins intended for topical administration.

When PMPs enter clinical development, the production process has to meet certain quality criteria that are defined by current good manufacturing practices (cGMP). The regulations for biopharmaceuticals (as defined by the Food and Drug Administration (FDA) in the USA and by the European Medicines Agency (EMEA) in the EU) apply for all biopharmaceuticals irrespective of the production platform. Whereas the production of biopharmaceuticals in plant suspension cells cultivated in fermenters is very similar to microbial systems and mammalian cells, the production process is quite different when whole plants are used as the expression hosts. To address these differences, guidance for the production of PMPs has been issued by the FDA and the EMEA, and consultations are underway to refine the regulations (Spok et al. 2008).

## 12.5 PMPs in Advanced Development

### 12.5.1 Glucocerebrosidase

Recombinant glucocerebrosidase is needed to replace the nonfunctional enzyme present in patients with the monogenic disorder Gaucher disease, which is characterized by the inability to degrade glucosylceramides, which therefore accumulate

in the lysosomes of phagocytes. Clinical symptoms of the disease include hepatosplenomegaly, anemia, and thrombocytopenia. Patients are currently treated with a recombinant version of the enzyme (imiglucerase, Cerezyme) that is currently produced in CHO cells. The purified recombinant enzyme needs additional in vitro enzymatic treatments to expose the mannose residues of its N-glycan chains. These terminal mannose residues facilitate uptake of the enzyme into macrophages. The complex production process of imiglucerase makes it one of the most expensive biologicals to date with an annual treatment cost of ca. US \$200 000 per patient (Kaiser 2008).

The Israeli biopharmaceutical company Protalix has developed an alternative production process using transgenic suspension cells derived from carrot roots (Shaaltiel et al. 2007). The 497-amino-acid enzyme is genetically fused to the N-terminal signal peptide from *Arabidopsis thaliana* basic endochitinase and to a C-terminal vacuolar targeting sequence from tobacco chitinase A. The purified plant-derived glucocerebrosidase (prGCD) contains two additional amino acids at the N-terminus and seven additional amino acids on the C-terminus compared to the mature human enzyme. Because prGCD is targeted to the vacuole, the complex type N-glycans are trimmed to expose mannose residues, a glycan structure known as the paucimannose type. This eliminates the need for artificial trimming of the glycans during downstream processing. At present, prGCD is undergoing a clinical phase III study to assess its safety and efficacy in Gaucher patients.

### 12.5.2 Insulin

Millions of people suffer from insulin-dependent diabetes mellitus (type I diabetes), which is now among the most common causes of death in industrialized countries. The total demand for insulin exceeds 8000 kg year<sup>-1</sup>. The mature insulin molecule is a small (5.8 kDa) non-glycosylated protein consisting of a 21-amino-acid A chain and a 30-amino-acid B chain connected by two disulfide bonds. The two chains are derived from a single precursor polypeptide (proinsulin) in which they are connected by a linking C-chain. The C-chain is cleaved off by limited proteolysis upon secretion from the Langerhans cells in the pancreas. Recombinant insulin is produced either by the separate expression of recombinant A and B chain mini-genes or by mimicking the natural route from proinsulin. Insulin was the first biopharmaceutical produced by recombinant DNA technology. Recombinant human insulin achieved market approval in 1982 and current demands are met by production processes using *Escherichia coli* (Chance and Frank 1993) and *Saccharomyces cerevisiae* (Kjeldsen 2000). Plant-derived human insulin has been produced by the genetic fusion of a mini-proinsulin polypeptide with a shortened C-chain to the C-terminus of oleosin, which allows expression in oilseed crops and accumulation in oil bodies. The chimeric protein is targeted to the ER-derived

oleosomes in the seeds, and because of the unique properties of these organelles, they can be purified easily by floating centrifugation and the recombinant insulin can be proteolytically cleaved off and further purified by chromatography. Human recombinant insulin (DesB<sub>30</sub> insulin) produced with this method in *Arabidopsis* seeds is as effective in an insulin tolerance test as human standard insulin (Nykiforuk et al. 2006). The Canadian company SemBioSys Genetics has commercialized the oleosin fusion system and uses safflower (*Carthamus tinctorius*) for the production of insulin. The company recently announced the launch of a clinical phase I/II trial in the UK with healthy volunteers to demonstrate the equivalence of the plant-derived insulin (SBS-1000) to currently marketed recombinant human insulins (Moloney et al. 2008).

### 12.5.3 *Idiotype Vaccines*

Idiotype vaccines are patient-specific vaccines developed for patients suffering from clonal diseases such as B cell lymphoma (non-Hodgkin lymphoma, NHL). The malignant cells carry individual immunoglobulins (idiotypes) on their surface which can be used to trigger a specific immune response. Currently, the vaccines are produced from patient B-cell tumor cells that are expanded as human/mouse heteromyelomas. The monoclonal idiotype antibody is subsequently fused to an immunogenic carrier protein such as keyhole limpet hemocyanin (KLH) and injected, usually together with granulocyte-macrophage colony stimulating factor (GM-CSF) as an adjuvant. These vaccines are currently in clinical development (Sinha et al. 2008).

Recently, a plant-based production platform has been established to shorten the time required to derive such a vaccine from the patient's biopsy. For this purpose, the variable domains of the idiotype are cloned as single-chain antibodies (scFv) and transiently expressed using a viral vector system in *Nicotiana benthamiana* plants (McCormick et al. 2003). Plant-derived idiotype vaccines have been tested in a phase I clinical trial on 16 NHL patients after an initial chemotherapy (McCormick et al. 2008). The safety and immunogenicity of the vaccines were analyzed at two different doses and in the presence or absence of GM-CSF. Most of the patients displayed a cellular immune response while only three patients mounted a vaccine-specific humoral immune response. There were no severe adverse reactions in these trials. The development of plant-derived idiotype vaccines was initiated by the US-based Large Scale Biology Company (which filed for Chapter 11 bankruptcy in 2006). Further research has been undertaken by the German company Icon Genetics, which is a subsidiary of Bayer. The Bayer group has announced its intention to conduct another phase I clinical trial in 2009, using the Icon Genetics Magnifection technology discussed above.

### 12.5.4 Interferon

There are three classes of interferons (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ ) with the  $\alpha$  and  $\beta$  classes grouped together as ‘type I interferons’ because they share 30% sequence identity and recognize the same receptor, and IFN- $\gamma$  representing the ‘type II interferons’ because it is more distantly related and recognizes a distinct receptor. All three interferons have been produced as recombinant proteins and are approved for the treatment of various conditions, including chronic viral infections (especially hepatitis B and C), multiple sclerosis and certain types of cancer. All the interferons have a low molecular mass ( $\sim$ 30 kDa) and are thus efficiently eliminated from the body by renal filtration. Unmodified interferons therefore have a plasma half-life of  $\sim$ 4 h. The plasma half-life can be increased significantly by attaching the polymer polyethylene glycol (PEG), up to a maximum of  $\sim$ 25 h.

IFN- $\alpha$ 2b produced in duckweed (*Lemna minor*) by the United States company Biolex Therapeutics is currently being evaluated in clinical trials (De Leede et al. 2008). IFN- $\alpha$ 2b is a 19-kDa single-chain non-glycosylated protein that is currently produced in *E. coli* for therapeutic use. In a clinical phase I dose escalation study, the plant-derived interferon (BLX-883) was administered in a controlled release formulation in poly(ether-ester) microspheres (Locteron) to healthy volunteers. The product was well tolerated at all tested doses and the most common adverse effects (influenza-like symptoms, injection site reactions, headache) were similar to those observed in test subjects receiving Peginterferon alpha-2b (PEGIntron). In a subsequent clinical phase I/II trial, the antiviral properties of Locteron are being tested in patients with chronic hepatitis C, in comparison with PEGIntron.

## 12.6 Conclusion

The large number of biopharmaceuticals now in clinical development demonstrates the need to increase current production capacities and to identify and develop alternative production systems. Plants are regarded as attractive production platforms because they can offer a virtually unlimited supply when cultivated on an agricultural basis. However the cultivation of transgenic pharmaceutical plants in the open field requires appropriate safety measures to exclude the contamination of food and feed supplies. From the above-mentioned case studies, only safflower is cultivated in the open field, whereas the others are propagated in closed systems such as greenhouses, basins, and fermenters.

As plant expression platforms continue to improve in terms of post-translational modifications and overall protein yield, more PMP processes are likely to become economically feasible in the future. The whole field will definitely profit if one of the PMPs currently undergoing clinical development receives market approval. A step in this direction has been made in the field of veterinary medicine when

Dow AgroSciences received approval in 2006 for their poultry vaccine against Newcastle disease, produced in cultivated tobacco suspension cells. It is also clear that the development and approval of PMPs will be encouraged by the establishment of solid production guidelines by the relevant regulatory bodies.

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# Chapter 13

## Biopolymers

Maja Hühns and Inge Broer

### 13.1 Introduction

The limited future of the fossil energy providers oil, natural gas and carbon represents one of the major future problems. Since additionally there are expectations that the worldwide energy requirement will increase by up to 60% by 2030 (Qaim 2006), there is an urgent need to identify alternative renewable energy sources based on wind, water or solar forces. The plants' use of solar energy via photosynthesis is extremely efficient; hence plants as renewable resources are surely an option. Nevertheless, any economic use still depends on governmental subsidies; in addition it seems to be unavoidable to intensify the agricultural land use in order to satisfy the energy demand, which might lead to a reduction of the food supply (Thrän et al. 2005). One of the main possibilities to increase harvest is plant breeding leading either to higher biomass production or to the formation of new and special ingredients of economic interest, like biopolymers. The combination of both to create a double-use plant might increase the outcome for the farmer, reduce the amount of arable land used for non-food purposes and in addition reduce CO<sub>2</sub> emission.

Naturally occurring polymers (e.g. polysaccharides, polyamides, polyesters) are produced by bacteria or plants. The most frequent polysaccharide is cellulose (glucose monomers connected by glycosidic bounds), the main component of plant cell walls, to date isolated mainly from wood, cotton, corn and wheat. Cellulose is the major constituent of paper and cardboard and also textiles made from cotton, linen and other plant fibres. Furthermore cellulose can be converted into cellophane, which is used in the packaging industry. Another important polysaccharide is starch (connected glucose–fructose dimers), the most important reserve substrate in plants, which is predominantly present as amylose and

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amylopectin. While amylase is used to create foils, amylopectin functions mainly as food additive, but also for technical applications, for example papermaking, as a thickener, glue or as raw material for biodegradable packing materials.

Polyamides are polymers containing monomers of amides joined by peptide bonds. They occur naturally as wool or silk. The best known type of silk is obtained from cocoons made by the larvae of the mulberry silkworm *Bombyx mori*, used for textile manufacture. A further type of silk naturally occurring in spiders like *Nephila clavipes* has a high tensile strength that is comparable to that of the synthetic super fibre Kevlar, but it additionally shows high elasticity (Tirrell 1996).

Polyesters occur in bacteria as a reserve substrate, having a functional ester group. They have thermoplastic, elastomeric and hydrophobic properties and are considered very suitable for consumer products such as bottles, fibres and films.

However all these groups have different drawbacks. Cellulose isolated from wood for the paper industry has some limitations, because costly and environmentally damaging processes are used to extract the lignin in order to obtain pure cellulose fibres. Therefore the paper industry is very interested in trees with lower lignin content or with modified lignin that can be more easily separated from the cellulose.

The usage of natural starch is limited due to its composition of amylose and amylopectin, components with very different characteristics and separate uses in industrial processes. Generally, only the thickening properties of amylopectin are required, while the amylose component is undesirable in many products and can additionally interfere with certain processes (Pickardt and de Katheren 2004). Unfortunately, the chemical modification or separation of amylopectin and amylose is associated with increased consumption of water and energy. However, see Chap. 11 for metabolic engineering of starch.

Naturally produced polyamids like spider silk cannot be obtained in large quantities from spiders, and the most commonly used polyamides, like nylons, aramids and sodium polyaspartate, do not even appear in nature.

The major problem for the commercial production and application of natural polyesters like polyhydroxyalkanoates (PHA) in consumer products is the high costs of bacterial fermentation, making it 5–10 times more expensive than the petroleum-derived polymers like polyethylene and polypropylene (Poirier 2002).

## 13.2 Transgene-Encoded Biopolymers

Envisioning both the drawbacks and the huge potential of plants as producers of cheap biomass, new production technologies are required to improve the competitiveness of plant-made biopolymers. Gene technology provides us with the tools to add new facilities to plant metabolic pathways, which should lead to the production of either high-quality or even new polymers in plants, possibly as a byproduct to traditional materials, such as starch, oil or sucrose. The following section discusses the production of four groups of polymers in plants in more detail: namely starch

and cellulose, PHAs, protein-based biomaterial and at least glucosyl glycerol used for cosmetics.

### 13.2.1 Starch and Cellulose

#### 13.2.1.1 Starch

Starch is the major reserve carbohydrate in plants. Potato, maize, cassava and wheat provide the main sources of energy in the human diet, but also serve for many industrial processes like adhesives, cosmetics, detergents, paper, textiles and pharmaceuticals (Davis et al. 2003). Starch is also used for the production of biodegradable plastics as an alternative to petroleum-based products. However, native starches from various plant species have limited physiochemical properties, and thus are directly suitable for only a few specific end uses. For many industrial uses, enzymatic and chemical treatments, it is necessary to improve the usability of starch. The modification of starch is possible by using biotechnology to alter starch composition or to modify starch synthesis (Chap. 11). Out of many examples for starch modification, we describe one example for altered starch composition.

Starch is composed of amylopectin and amylose, which have different characteristics for industrial purposes. Amylopectin is used as a thickener, while amylose is undesirable for many products and can interfere with certain processes. Therefore a transgenic starch potato was developed which produces exclusively the amylopectin component of starch (Kull et al. 1995). In order to do so, the gene encoding the granule-bound starch synthase (GBSS) for the biosynthesis of amylose was inactivated by post-transcriptional gene silencing (PTGS). Two subgenomic fragments of the gene were expressed in antisense orientation under control of the CaMV 35S promoter. The resulting transgenic potato plants were effective in inhibiting amylose biosynthesis in tubers, thereby leading to an increase in the branched starch component amylopectin (>98%). The phenotype was stable during vegetative propagation. For commercial use the potato variety was named “Amflora” and has been analysed in field trials for several years to test yields and resistance to pests and disease. Furthermore the allergic and toxic potential of Amflora tubers was analysed, as well as potential other impacts on human health and the environment. No increased risk to humans, animals and the environment were shown in comparison to conventional potatoes (EFSA 2005).

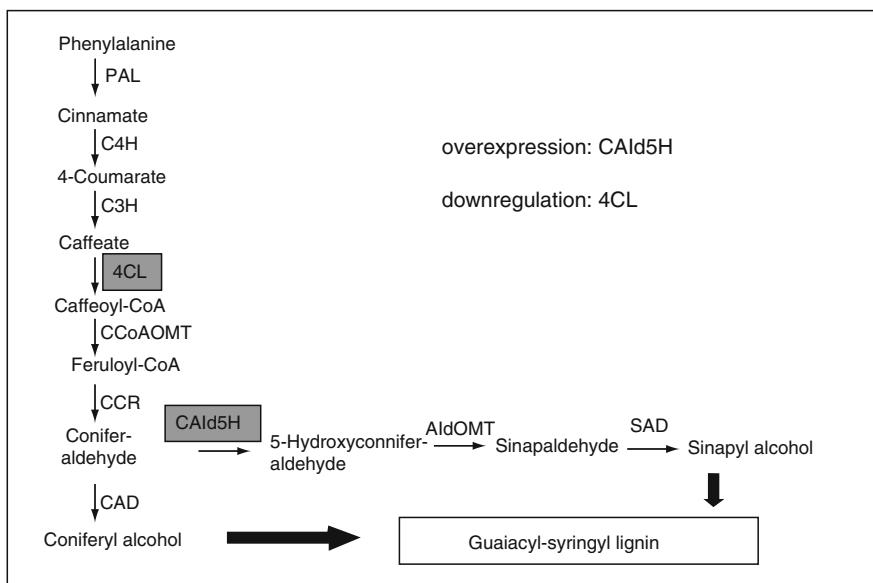
#### 13.2.1.2 Cellulose

All cell walls of higher plants contain: (i) cellulose, a homopolymer of  $\beta$ -1,4-linked glucose units, which is a flexible structural substance in the form of fibrils, (ii) hemicellulose, a heterogeneous polysaccharide, which represents a matrix in which

the cellulose fibrils are embedded, and (iii) lignin, a phenol polymer, which forms a bond between cellulose and hemicellulose. To date, cellulose is mainly isolated from trees. In order to obtain pure cellulose fibres (e.g. for the paper industry) lignins have to be eliminated. The chemical process is very expensive and pollutive under high energy consumption (Franke et al. 2000), hence reducing the lignin contents in trees might ease the isolation of cellulose.

Since the beginning of 1990 several strategies were investigated for plants to reduce lignin contents using gene technology. Mostly poplars were investigated, because there are fast-growing trees, relatively easy to modify and play an important role in paper manufacture. Up to now several effects have been achieved, due to the modification of biosynthetic pathway steps (Fig. 13.1; Pickardt and de Kathen 2004).

1. Decrease of complete lignin content by about 45%. Furthermore cellulose content was increased up to 14% (Hu et al. 1999).
2. Increase of the lignin compounds sinapyl to coniferyl units through overexpression of coniferaldehyde-5-hydroxylase. The total lignin content is equal.
3. Addition of either effects, when cumarat-CoA-ligase and coniferaldehyde-5-hydroxylase are overexpressed or downregulated in one transgenic plant. As a result the total lignin content is reduced about 52%, the part of cellulose



**Fig. 13.1** Proposed biosynthetic pathway of lignin synthesis in trees. *PAL* Phenylalanine-ammoniumlyase, *C4H* cinnamate 4-hydroxylase, *C3H* 4-coumarate 3-hydroxylase, *4CL* 4-coumarate-CoA ligase, *CCoAOMT* caffeoyl CoA O-methyltransferase, *CCR* cinnamoyl-CoA reductase, *CAld5H* coniferaldehyde 5-hydroxylase, *AldOMT* 5-hydroxyconiferaldehyde O-methyltransferase, *SAD* sinapyl alcohol dehydrogenase, *CAD* cinnamyl alcohol dehydrogenase

increased up to 30% and the proportion of sinapyl to coniferyl units increased up to 64% (Li and Quiros 2003).

4. The proportion of sinapyl to coniferyl units increased, when the coniferaldehyde-5-hydroxylase gene is regulated by the promoter of the cinnamic acid hydrolase gene. The transgenic poplar trees show no phenotypical changes compared to control plants; furthermore their wood has a higher decomposition efficiency than normal poplar trees (Huntley et al. 2003).

The interest of industries in genetically modification of forest trees is extremely higher in the United States than in Europe, caused by geographical conditions and therefore expansion of the forest industry. Furthermore, commercialization of transgenic trees in Europe seems to have fewer chances, due to their risk assessment, e.g. durability and high spreading potential (Sauter and Hüsing 2005).

### 13.2.1.3 Glucoside

The ingredients for cosmetic creams, lotions, powder, perfumes, lipstick or make-up come from a variety of sources, for example antioxidants, alcohol, oil and also polymers. Polymers serve in hair-setting products, as binders in skin creams and to keep sunscreens from washing off. One example is  $\alpha$ -D-glucosylglycerol ( $\alpha$ -GG), which is used as an anti-aging agent and moisture-regulating compound (Da Costa et al. 1998).  $\alpha$ -GG can be produced by chemical as well as by enzymatic methods and was naturally found in microorganisms as a compatible solute for providing some protection against stresses due to high salt concentrations, heat and UV radiation. It is also useful as an alternative sweetener in food stuffs, because of its low caloric value. The microbial synthesis of  $\alpha$ -GG is presently not a mature process, because it does not allow the production of  $\alpha$ -GG as a bulk chemical. The achievable concentrations are very low and also the productivity of three days is not advantageous for industrial production (Roder et al. 2005). However,  $\alpha$ -GG is enzymatically synthesized (Gödl et al. 2008) by using sucrose phosphorylase to convert sucrose with glucosyl and glycerol into  $\alpha$ -GG, which is isolated by chromatographic methods with a yield greater than 70%. Up to now, besides bacteria, GG accumulation has only been reported for the plants *Lilium japonicum* (Kaneda et al. 1984) and *Myrothamnus flabellifolia* (Bianchi et al. 1993); however nothing is known about the metabolic pathway of GG in these plants. In unpublished data GG accumulation was established in *Arabidopsis* by expression of the *ggpPS* (glucosylglycerol phosphate phosphatase/synthase) gene from the  $\gamma$ -proteobacterium *Azotobacter vinelandii*. Transgenic plants accumulated GG up to 30  $\mu\text{mol/g}$  fresh weight. However, beside increased salt tolerance, plants with higher GG content also showed growth retardation, which is not observed in plants with low GG accumulation (1–2  $\mu\text{mol/g}$  fresh weight; Klähn et al. 2008). The growth retardations might be caused by the interference of GG synthesis with trehalose biosynthesis and in turn also other carbohydrates. The improvement of the GG synthesis in plants needs more investigation by regulated gene expression.

### 13.2.2 Polyhydroxyalkanoates

A wide variety of bacteria produce polyhydroxyalkanoates (PHAs) as a carbon reserve and electron sink (for a review, see van Beilen and Poirier 2008). These PHAs consist of 3-hydroxy fatty acids with a chain length of 4–16 carbons and have wide-ranging potential for applications such as the formation of plastic bags, fibres and films. Besides their CO<sub>2</sub> neutral production, PHA products can be decomposed, which is desirable for the environmental friendly dispersal of disposable items as well as for some medical products which otherwise have to be removed from the body. Poly-3-hydroxybutyrate (PHB) is the most widespread and best characterized PHA found in bacteria like *Ralstonia eutropha* (for a review, see van Beilen and Poirier 2008). In contrast to cyanophycin synthesis, three enzymes are necessary for PHB synthesis. The first enzyme,  $\beta$ -ketothiolase, catalyses the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. The acetoacetyl-CoA reductase in turn reduces acetoacetyl-CoA to R-(–)-3-hydroxybutyryl-CoA, which is subsequently polymerized through PHA synthase to form PHB. As an alternative to petrochemicals, PHA production was established in plants, first in *Arabidopsis thaliana* by the expression of the PHB synthase in the cytoplasm leading to a maximum of 0.1% PHB present in the cytoplasm, nucleus or vacuoles (van Beilen and Poirier 2008). However, the plants showed strong growth retardation and reduced seed production. PHB synthesis in the cytoplasm of tobacco (0.01%), cotton (0.3%) and oilseed rape (0.1%; John and Keller 1996; Nakashita et al. 1999; Poirier et al. 1992) showed similar plant damage. The deleterious effects of PHB production in the cytoplasm of plants might be caused by the diversion of acetyl-CoA and acetoacetyl-CoA away from the endogenous flavonoid and isoprenoid pathways, which are responsible for the synthesis of a range of plant hormones and sterols (van Beilen and Poirier 2008). Due to their high metabolic flow of acetyl-CoA, chloroplasts might provide a more suitable production platform, although  $\beta$ -ketothiolase is not present. Therefore the required enzymes – including  $\beta$ -ketothiolase – were targeted to plastids, using signal sequences for plastid import. The highest PHB accumulation was observed in *Arabidopsis*, with a maximum of 14% of dry weight in leaves without significant effects on plant growth but visible leaf chlorosis (Nawrath et al. 1994). In seeds of oil rape up to 8% dry weight PHB accumulation was detected in leucoplasts after the transfer of all three genes (Houmiel et al. 1999), a strategy leading to even 30–40% of dry weight in leaves of *A. thaliana*. Nevertheless, in contrast to the intact canola seeds, these plants were heavily reduced in growth and did not produce any seeds. Slightly reduced amounts were detected in corn leaves (6% dry weight), sugar cane leaves (2% dry weight) and sugar beet hairy roots (5% dry weight), whereas expression of the PHB pathway in plastids of alfalfa and tobacco led to only low amounts (<0.5% dry weight; Arai et al. 2001; Saruul et al. 2002). Since nucleus-encoded proteins are expressed to a lesser extent than those encoded by plastidic genes, it was supposed that the direct expression of the PHB pathway in the plastid genome might increase the PHB yield without increasing the deleterious effects. Nevertheless, in tobacco

this strategy only leads to relatively low amounts up to 1.7% dry weight, accompanied by reduced growth and male sterility (Arai et al. 2001; Bohmert et al. 2002; Lössl et al. 2003).

In order to improve the physical properties of PHB, extensive efforts have made to synthesise co-polymers with better properties like poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(HB-co-HV)] and medium-chain-length PHA (mclPHA). P(HB-co-HV) is produced by the inclusion of 3-hydroxyvalerate in PHB, which is less stiff and tougher than PHB and also easier to process, making it a good target for commercial application (Noda et al. 2005). Coexpression of a threonine deaminase from *E. coli* along with the three PHB biosynthetic proteins in plastids led to P(HB-co-HV) accumulation up to 2.3% dry weight in seeds of oil rape and 1.6% dry weight in *A. thaliana* (Slater et al. 1999; Valentin et al. 1999). However there is a constriction providing 3-hydroxyvaleryl-CoA to the PHA synthase, which is caused by the inefficiency of the pyruvate dehydrogenase complex in converting 2-ketobutyrate to propionyl CoA (for a review, see van Beilen and Poirier 2008).

MclPHAs are described as elastomers and their physical properties depend on the monomer composition (for a review, see van Beilen and Poirier 2008). In *A. thaliana* mclPHAs were produced up to 0.4% dry weight in seedlings and consisted of 40–50 mol% of C12 and longer monomers. The production of mclPHAs with longer-chain monomers by using the conversion of the fatty acid biosynthetic intermediate 3-hydroxyacyl-ACP into 3-hydroxyacyl-CoA led to only low amounts (below 0.03% dry weight) of mclPHA in plastids of potato leaves (Romano et al. 2005).

The most useful PHA would be a polymer containing primarily 3-hydroxybutyrate with a fraction of longer-chain monomers of C6 and higher. In terms of PHA quantity, plastids are the best location for PHA synthesis. However, the synthesis, regulation of precursors (like acetyl-CoA, propionyl-CoA or 3-hydroxyacyl-ACP) and the efficacy to channel them towards PHA without deleterious effects on plant growth needs more investigation (for a review, see van Beilen and Poirier 2008).

### 13.2.3 Protein-Based Biomaterials

Protein-based biomaterials have a wide area of application, ranging from tissue engineering, drug carriers, coatings and glues to elastomers and fibres, dispersants, thickeners and additives to hydrogels. Two important target proteins are described here in detail: fibrous proteins (e.g. spider silk) and non-ribosomally produced poly-amino acids like cyanophycin.

#### 13.2.3.1 Fibrous Proteins

Fibrous proteins contain short blocks of repeated amino acids and can be regarded as elaborate block co-polymers with unique strength-to-weight, adhesive or elastic

properties (Huang et al. 2007; Sanford and Kumar 2005; Scheibel 2005). Well known fibrous proteins are elastin, resilin, collagen, keratin, mussel adhesive proteins and wheat glutenin (Kiick 2007). A huge combinatorial range is available by combining repeated sequences of the various natural fibrous proteins, or even using synthetic gene sequences or changing the linker elements between the repeated sequences (Holland et al. 2007; Nagapudi et al. 2005).

Natural silk fibres are mainly produced by a variety of silkworms (Altman et al. 2003; Shao and Vollrath 2002) and spiders (Perez-Rigueiro et al. 2003; Rising et al. 2005; Vollrath 2000). The development of novel silk-based fibres has mainly focused on the silks produced by the golden orb-weaving spider *Nephila clavipes*, which synthesizes different kinds of silks for several purposes, e.g. weaving cocoons, as a dragline or constructing a web (Hinman et al. 2000; Vollrath and Knight 2001). The main focus lays on the silks of the dragline, the main structural web silk and the spider's lifeline, because of their high tensile strength. This is comparable to that of the synthetic superfibre Kevlar, but it additionally shows high elasticity (Tirrell 1996), useful for industrial and medical purposes. Dragline spider silk consists of repeated sequence blocks of various types (Huang et al. 2007). The GGX motif probably forms a  $\beta_{10}$  helix, while the GPGXX motif is thought to form a  $\beta$ -turn spiral. Effective high-level and stable expression of silk proteins in fast-growing micro-organisms such as yeast and bacteria leads to difficulties, like the formation of inclusion bodies or distinct codon usage. In addition, bacterial production is genetically unstable due to recombination, resulting from the highly repetitive genes encoding the repetitively composed spider silk proteins. Nevertheless synthetic spider silk genes have been successfully expressed in transgenic tobacco, potato and *Arabidopsis thaliana* plants (Barr et al. 2004; Scheller et al. 2001) under control of the cauliflower mosaic virus (CaMV) promoter and targeted to the endoplasmatic reticulum. Transgenic plants were cultivated in greenhouses and in field trials (Menassa et al. 2004; Scheller and Conrad 2005). In tobacco and potato leaves up to 2% of total soluble protein (TSP) was observed (Scheller et al. 2001). The expression only in leaf apoplasts of *A. thaliana* led to spider silk production of 8.5% TSP, whereas targeting to seed endoplasmic reticulum yielded 18% TSP (Yang et al. 2005). The expression levels in plants are close to the level of 10% and 30% TSP reported in *Escherichia coli* and *Pichia pastoris*. The combination of spider silk protein and elastin polymer leads to a new biomaterial, which is used for industrial and medical purposes. For that, the expression of synthetic collagen, made from repeats of a motif found in elastin, and also of a chimeric protein composed of silk and elastin domains has been expressed in tobacco or potato (for a review, see van Beilen and Poirier 2008). The extraction of the proteins from 1 kg tobacco leaf material leads to a yield of 80 mg pure recombinant spider silk-elastin protein (Scheller et al. 2004).

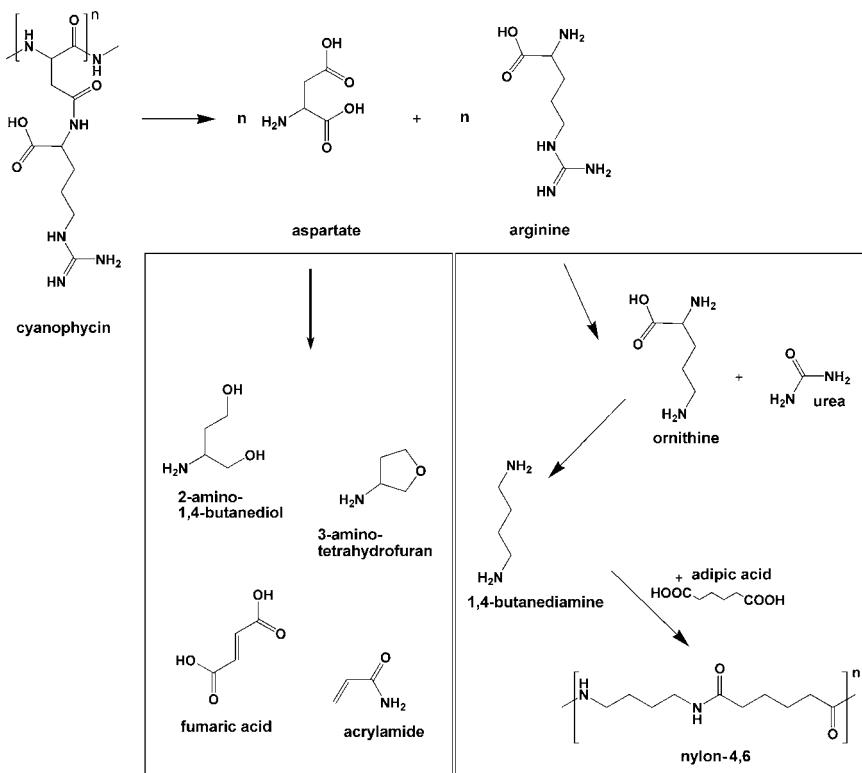
The enhancement of fibrous protein synthesis in plant requires several approaches, for example optimization of the amino acid and tRNA pools for those amino acids, which form the main part of the protein, like glycine and alanine in spider silk. Further possibilities are dislocation to compartments and tissues that are optimal for protein synthesis and storage and also the co-expression of several

fibrous proteins as found in natural silk (for a review, see van Beilen and Poirier 2008). In addition to the optimized production of fibrous proteins, the resulted fibre should have characteristics similar to the silk proteins from spider. The properties of silk fibres depend on correct assembly of the different types of proteins by spinning. Recombinant spider silk, obtained from mammalian cells, shows similar toughness to dragline silk, but with a lower tenacity (Lazaris et al. 2002).

### 13.2.3.2 Non-Ribosomally Produced Poly-Amino Acids

Polymers produced in transgenic plants include polyaminoacids such as poly- $\gamma$ -glutamate, poly- $\alpha$ -aspartate, and poly- $\epsilon$ -lysine, which have a wide range of applications, e.g. as dispersants, thickeners or additives to hydrogels (Chang and Swift 1999; Lössl et al. 2003; Oppermann-Sanio et al. 1999; Oppermann-Sanio and Steinbüchel 2002).

Polyaspartate is a soluble, non-toxic and biodegradable polycarboxylate (Tabata et al. 2000) that could replace the non-biodegradable polyacrylates in many industrial, agricultural and medical applications (Joentgen et al. 2001; Oppermann-Sanio and Steinbüchel 2002; Schwamborn 1998; Zott et al. 2001). Because no polyaspartate-producing organism has been identified up to now, the polymer is chemically synthesized (Schwamborn 1996). However, it can also be obtained from cyanophycin (multi-L-arginyl-poly-L-aspartic acid). Cyanophycin is a cyanobacterial reserve polymer composed of a poly- $\alpha$ -aspartic acid backbone with arginine residues linked via their  $\alpha$ -amino group to the  $\beta$ -carboxyl group of each aspartate residue (Simon 1976, 1987; Simon and Weathers 1976). Mild hydrolysis of cyanophycin (Joentgen et al. 2001) results in homo- and copolymers of polyaspartate and L-arginine. The basic amino acid L-arginine has been suggested to be a regulator of some immunological and physiological processes, e.g. being an immune system stimulator (Cen et al. 1999; de Jonge et al. 2002; Li et al. 2007; Nieves and Langkamp-Henken 2002; Popovic et al. 2007; Taheri et al. 2001; Tapiero et al. 2002; Yeramian et al. 2006), agrowth inductor (Lenis et al. 1999; Roth et al. 1995; Wu et al. 2007) or a tumour cell growth inhibitor (Amber et al. 1988; Caso et al. 2004; Flynn et al. 2002). Alternatively, aspartate and arginine from cyanophycin could serve as a starting point for the synthesis of a range of chemicals (Fig. 13.2). Arginine can be converted to 1,4-butanediamine, which can be used for the synthesis of nylon-4,6. Aspartate is converted in several chemicals like 2-amino-1,4-butanediol, 3-aminotetrahydrofuran (analogues of high-volume chemicals used in the polymer industry), fumaric acid (used for polyester resins) and acrylamide (used as a thickener, in manufacturing dyes or in papermaking). Cyanophycin is synthesized via non-ribosomal polypeptide synthesis in many Cyanobacteria (Simon 1987) and some other non-photosynthetic bacteria (Krehenbrink et al. 2002; Ziegler et al. 2002). For cyanophycin synthesis, only one enzyme, the cyanophycin synthetase encoded by *cphA*, is necessary to catalyse the ATP-dependent elongation of a cyanophycin primer by the consecutive addition of L-aspartic acid and L-arginine (Ziegler et al. 2002). In cyanobacteria, the polymer is variable



**Fig. 13.2** Potential products derived from the polymer cyanophycin

in length (25–125 kDa), water-insoluble and stored in membrane-less granules (Allen 1984; Simon 1987). As a first step to establish a system for mass production of the polymer in plants, the cyanophycin synthetase gene from *Thermosynechococcus elongatus* BP-1 (*cphA<sub>Tc</sub>*) was incorporated into tobacco and potato plants, with mRNA expression under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Neumann et al. 2005). The maximum amount of cyanophycin in the cytosol of tobacco leaves was 1.14% of dry weight. However, the cyanophycin-producing plants exhibited phenotypical changes like thickened cell walls, variegated leaves and slow growth. The same was true for the transgenic potato plants containing maximal amounts of 0.24% of dry weight. The clone producing the most cyanophycin did not develop eyes and could not be propagated further. Moreover, in tubers, the presence of cyanophycin could only be demonstrated by electron microscopy. A much higher capacity to produce cyanophycin was achieved by targeting the cyanophycin synthetase to plastids of *Nicotiana tabacum* (Hühns et al. 2008). Yields of up to 6.8% dry weight in leaves were obtained, without significant disturbance of plant growth and development. However, the line producing the most cyanophycin produced fewer seeds.

When the *cphA* expression is restricted to tubers, the plant fitness and cyano-phytin production in potato is further enhanced up to 7.5% dry weight, with minimal effects on growth and morphology of the plants.

The improvement of the cyanophycin accumulation in plants may require optimization of the gene sequence, adapted to the target plant and also the optimization of the pathways involved in supplying arginine and aspartic acid.

### 13.3 Conclusion

The production of biopolymers has proven to be feasible and might contribute to a sustainable agriculture. Nevertheless, application still lies in the far future. Before transgenic plants can be cultivated and used, they must undergo an authorization procedure based on a safety assessment to guarantee their safe usage. Currently, the approval of transgenic plants is an extremely cost and time-demanding process, specific for one event (one plant line derived from a single transformant with one insertion locus of the transgene). These efforts will only be undertaken if the expected gain can exceed the costs. The gain depends on: (i) the potential market of the biopolymer, (ii) the pureness and concentration of the biopolymer in the plants, (iii) the potential reduction of the primary value of the cultivar (e.g. reduction of biomass, starch content or processing quality) and (iv) the isolation costs. Except for the amylopectin potato Amflora, further investigations have to be done for all of the polymers described to optimize these parameters as far as possible, either by modification of the production compartment in the plant, selection of the production cultivar, support of plant health and biomass production, or by optimization of cultivation, harvest, storage and isolation strategies. Further, risk assessment strategies have to be optimized in order to reduce the cost and time for approval without any reduction in safety. This might be done by the development of new and specific techniques for analysis as well as by the acceptance of transgene- and cultivar-specific data to reduce effort for single events. In addition, analysis always has to be hypothesis-driven and the selection of topics addressed has to be restricted to risks specific for the event in question.

Due to the different legal frameworks in most parts of the world, it might be expected that biopolymer-producing transgenic plants will be on the market first in the United States and Canada since their regulations come under existing laws covering seed and pesticide approval as well as food and feed control and the basis for safety assessment of a new transgenic plant is by comparison to known and established plants and products. Most importantly, the final decision for approval is carried out by scientists. Nevertheless, no polymer-producing plants are close to the United States market. In contrast, in Europe, the assessment focuses on the process (i.e. genetic engineering) and the precautionary approach; and the final decision is made by the European Commission in consultation with the Member States. Therefore no transgenic polymer-producing plant has yet been authorized in the European Union, but the potato event Amflora is close to approval. Nevertheless,

although the safety assessment for cultivation and for food and feed use by the European Food Safety Authority (EFSA) was completed years ago, this event is still waiting for authorization.

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# Chapter 14

## Engineered Male Sterility

Frank Kempken

### 14.1 Introduction

The agricultural exploitation of hybrid crop varieties has enabled enormous increases in food productivity through increased uniformity and hybrid vigour. Because of hybrid vigour, or heterosis, these crops are characterized by an increased resistance to disease and enhanced performance in different environments when comparing the heterozygous hybrid progeny (called F1 hybrids) to the homozygous parents (Lefort-Buson et al. 1987). Heterotic hybrid varieties in major crops, such as cotton, maize, and rice, exhibit >20% yield advantage over conventional varieties under the same cultivation conditions. The increased vigour, uniformity, and yield of F1 hybrids has been exploited in most crops for which the pollination system allows for an economical and convenient cross hybridization (Basra 2000).

In hybrid seed production, one line is designated as the female parent and the other as the male parent. The production of hybrid seeds requires a pollination control system to prevent unwanted self-pollination of the female line, which can be a great challenge, particularly for crop species with hermaphrodite flowers. Many methods exist to prevent the self-pollination of the seed parent (female line) during hybrid seed production: the application of male-specific gametocides, such as mitomycin and streptomycin (Jan and Rutger 1988); some inter- and intra-specific crosses (Hanson and Conde 1985); the mechanical removal of male flowers or anthers, chemical treatment, i.e. the patented chemical *hybridising agent*, Croisor, and use of genetic cytoplasmic or nucleus-encoded male sterility. Generally, naturally occurring genetically male sterile plants maintain fully normal female

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functions. The phenotypic characteristics of male sterility are diverse, including the complete absence of male organs, the abortion of pollen at any step of its development, a failure to develop normal sporogenous tissues, the absence of stamen dehiscence, or an inability of mature pollen to germinate on compatible stigma.

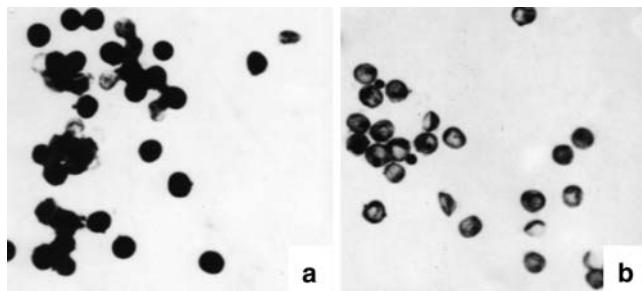
The generation of male sterility, mainly nucleus-encoded, is the basis of new, reliable, and cost-effective pollination control systems for genetic engineering that have been developed during the past decade. The propagation of male-sterile female parent lines is an important aspect for the successful application of these systems in large-scale hybrid seed production. Engineered male sterility has also been discussed in a number of recent reviews (Khan 2005; Takada et al. 2005; Chase 2006; Stockmeyer and Kempken 2006; Pelletier and Budar 2007).

## 14.2 Natural Male Sterility Systems in Plants

In order to prevent the self-pollination of female lines, pollen fertility must be controlled to permit fertilization only by pollen from the male parent. A simple way to establish a female line for hybrid seed production is to identify or create a line that is unable to produce viable pollen, similar to some lines of maize (Laughnan and Gabay-Laughnan 1983) or rice (Kadowaki et al. 1988). Therefore, this type of male-sterile line is unable to self-pollinate and seed formation is dependent upon pollen from the male line.

### 14.2.1 Cytoplasmic Male Sterility

The mitochondrion serves essential functions as the centre of energy metabolism in developing eukaryotic organisms. Pollen development in plants appears to be particularly influenced by mitochondrial function. Rearrangements of mitochondrial DNA that lead to unique chimeric genes sometimes result in an inability of the plant to produce fertile pollen (Fig. 14.1). This process, known as cytoplasmic male sterility (CMS), is particularly useful for the production of hybrid varieties for increased crop productivity and has been extensively reviewed (Schnable and Wise 1998; Kempken and Pring 1999; Linke and Börner 2005; Chase 2007). The association of CMS with abnormal mitochondrial gene expression has been established in many plant species, including maize (Levings 1990), petunia (Bino 1985), and sorghum (Pring et al. 1995; Xu et al. 1995a). It is thought that a disruption in pollen development is a consequence of mitochondrial dysfunction resulting from chimeric genes. The incorporation of the derived proteins into the mitochondrial membrane or multi-protein enzyme complexes may lead to the impairment of mitochondrial function. However, it has only been possible in a few cases to artificially introduce CMS by expressing CMS-associated chimeric genes, thus proving them to be causative agents of CMS (Hernould et al. 1993b; Gómez-Casati



**Fig. 14.1** Fertile and sterile sorghum pollen. Iodine–potassium stain of sorghum pollen from fertile and sterile lines. **A** Dark-stained fertile pollen indicating starch production. **B** Unstained pollen from the sterile line

et al. 2002), because these attempts often fail (Stockmeyer et al. 2007). However, there are ways to engineer CMS, e.g. expression of the beta-ketothiolase from *Acinetobacter* in tobacco plastids conditions maternally inherited male sterility (Chase 2006; Pelletier and Budar 2007). A unique feature of CMS is that the expression of the trait is influenced by nuclear fertility restorer genes (Schnable and Wise 1998; Kempken and Pring 1999). Nuclear restorer genes can suppress the effect of the sterile cytoplasm and restore fertility in the next generation. A number of restorer genes have been shown to encode pentatricopeptide repeat (PPR) proteins (Brown et al. 2003; Desloire et al. 2003; Kazama and Toriyama 2003; Akagi et al. 2004; Wang et al. 2006). The PPR proteins are a large family of 500–600 members in higher plants (Small and Peeters 2000).

Cytoplasmic male sterility has been utilized in some important crops, such as sunflower, rice (Chap. 22), oilseed rape (Chap. 21), and sorghum, to prevent unwanted pollinations, but CMS mutants and restorer systems are not available for all agricultural crops. In some cases, CMS has been associated with increased disease susceptibility. For example, the susceptibility of T-cytoplasm in maize to race T of the southern corn leaf blight (*Bipolaris maydis*) led to an epidemic in the United States in 1970 (Wise et al. 1987). Cytoplasmic male sterility is only transmitted maternally and all progeny are sterile. These CMS lines must be maintained by repeated crossings to a sister line, the maintainer line, which is genetically identical except for possessing normal cytoplasm and is male-fertile. The maintainer thus carries the recessive restorer alleles. Fertility restoration is essential in crops, such as corn or sunflower, where seeds are harvested.

### 14.2.2 Nuclear Male Sterility

Anther and pollen development and fertilization processes have been the subjects of much investigation (Goldberg et al. 1993). Many nuclear genes involved in pollen

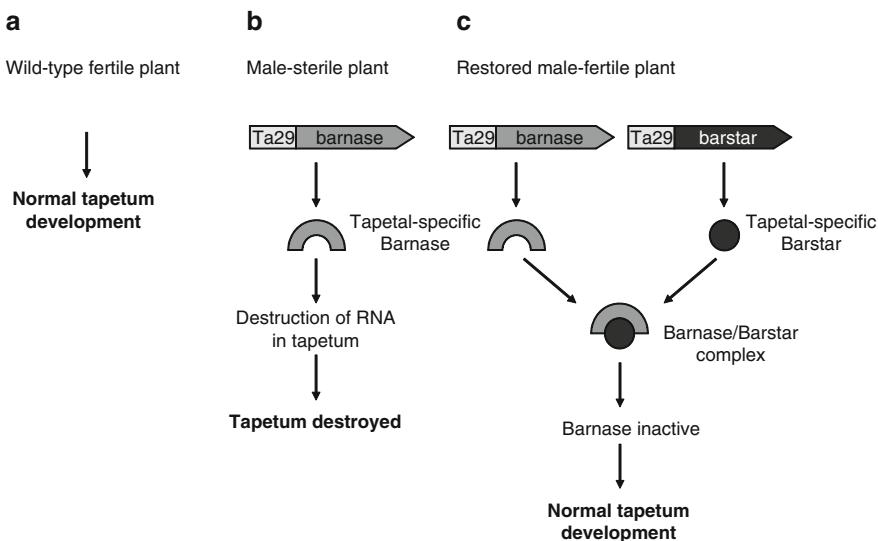
development have been identified as mutants that lead to pollen abortion and male sterility. This nuclear, or genic, male sterility is useful for hybrid seed production, but it has limitations because female parental lines are heterozygous and their offspring segregate into fertile and sterile plants in a 1:1 ratio. Nuclear male sterility in plants includes both spontaneous natural and engineered sterility. Spontaneous mutations leading to nuclear male sterility commonly occur at a high frequency. Such mutations can be easily induced by chemical mutagens or ionising radiation. Nuclear male sterility is usually controlled by a pair of recessive genes. Generally, these recessive mutations affect a large number of functions and proteins that are, for example, involved in male meiosis (Glover et al. 1998). In many crops, nuclear male sterility does not permit the effective production of a population with 100% male-sterile plants. This fact seriously limits its use in hybrid seed production (see also Chap. 6).

## 14.3 Methods of Producing Male-Sterile Plants

Many different strategies have been reported for the production of male-sterile plants by interfering with the development and metabolism of the tapetum (van de Meer et al. 1992; Hernould et al. 1998) or pollen (Worrall et al. 1992) in transgenic plants since the first transgenic male sterility system was described. Male sterility is further induced by using sense or antisense suppression to inhibit essential genes (Xu et al. 1995b; Luo et al. 2000) or by expressing aberrant mitochondrial gene products (Hernould et al. 1993a; He et al. 1996; Gómez-Casati et al. 2002). However, all of the available strategies have drawbacks, such as metabolic or general development interference or being restricted to specific species. Thus, a universal and dominant male sterility system with efficient effects on pollen growth, and offering the possibility to efficiently restore fertility, would be a great advantage for the production of hybrid seeds.

### 14.3.1 *The Selective Destruction of Tissues Important for the Production of Functional Pollen*

One way to achieve male sterility systems, is the use of a gene which encodes a protein that is able to disrupt cell function, for example a ribonuclease that destroys the RNA of the tapetal cells (Mariani et al. 1990; Mariani et al. 1992; Burgess et al. 2002). A well known example of this kind is the Barnase/Barstar system shown in Fig. 14.2. Using the PsEND1 promoter is a novel method of producing genetically engineered male-sterile plants by early anther ablation (Roque et al. 2007). The PsEND1 promoter belongs to an anther-specific gene from pea that confers very early gene expression in anther primordium cells. The authors fused this promoter to the barnase gene.



**Fig. 14.2** The Barnase/Barstar system. (a) Normal tapetum development in the wild-type plant. (b) Tapetal-specific promoter Ta29 drives expression of the barnase gene, leading to male-sterile plants. (c) Barnase inactivated by barstar inhibitor, resulting in restored male fertility. Based on data from Mariani et al. (1990, 1992)

Another way to introduce male sterility is the use of diphtheria toxin A-chain (Koltunow et al. 1990), which is expressed in a tissue-specific manner. The tapetum serves as a good target for these expression strategies because it plays a critical secretion role in the process of pollen formation. In some of these systems, sterility or fertility can be chemically regulated. For example, inducible sterility can be obtained through the expression of a gene encoding a protein that catalyses the conversion of a pro-herbicide into a toxic herbicide only in male reproductive tissues. In transgenic *Nicotiana tabacum* plants, male sterility was introduced by tapetum-specific deacetylation of the externally applied non-toxic compound N-acetyl-L-phosphinothrinicin (N-ac-Pt) (Kriete et al. 1996). Transgenic tobacco plants expressing *argE* from *Escherichia coli* under the control of the tapetum-specific tobacco TA29 promoter were produced. The gene product of *argE* represents an N-acetyl-L-ornithine deacetylase, which removes the acetyl group from N-ac-Pt, resulting in the cytotoxic compound L-phosphinothrinicin (Pt, glufosinate). The application of N-ac-Pt leads to empty anthers, resulting in male-sterile plants. Another example of tissue-specific cell ablation is the use of a bacterial phosphonate monoester hydrolase as a conditional lethal gene (Dotson et al. 1996).

In *Arabidopsis thaliana*, *pehA* from *Burkholderia caryophilli*, a glyphosate metabolizing bacterium, has been expressed using a tapetum-specific promoter. The treatment of transgenic plants with the protoxin glyceryl phosphate leads to male sterility because of the hydrolysis to glyphosate, a potent herbicide inhibiting the biosynthesis of aromatic amino acids. Another example for such

chemical control is the inducible expression of a male-sterility gene by the application of a chemical (Mariani et al. 1990; Goff et al. 1999). In order to induce fertility, the expression of a fertility restorer gene that can complement the sterility, or a male sterility gene repressor, can be chemically controlled (Cigan and Albertsen 2000).

An alternative method for fertility restoration has been suggested by Luo et al. (2000). They used a site-specific recombination system, FLP/FRT from yeast, to restore fertility in *Arabidopsis* plants that were male-sterile due to the antisense expression of the pollen- and tapetum-specific *bcpl* (Mariani et al. 1992) restored the fertility of male-sterile plants generated through the use of the bacterial extracellular ribonuclease Barnase (Paddon et al. 1989) by expressing a specific inhibitor of Barnase, called Barstar (see Fig. 14.2).

Ethylene controls many physiological and developmental processes in plants, including fruit and flower development. Ethylene exerts its effects through the ethylene receptor, which has been isolated in a variety of plant species. The over-expression of mutated melon ethylene receptor genes affects pollen development and induces a male-sterile phenotype in transgenic plants. The inducible male sterility system using mutated ethylene receptor genes could be a possible strategy for preventing pollen dispersal from these plants, thereby reducing the potential impact associated with transgenic plants. The system has been tested in tobacco and lettuce (*Lactuca sativa*; Takada et al. 2005; Ma et al. 2006; Takada et al. 2006; Takada et al. 2007).

Yet another, though quite unusual, approach is based on the nuclear expression of the mitochondrial *atp9* from wheat (see also Sect. 14.3.3). In plant mitochondria, the *atp9* transcript is subject to RNA editing. This editing process is believed to be essential for the function of the encoded peptide. To obtain male-sterile plants, the unedited sequence is fused to a mitochondrial targeting sequence and expressed under control of three different promoters in *A. thaliana*. Indeed male-sterile plants have been obtained (Hernould et al. 1993b; Gómez-Casati et al. 2002).

### 14.3.2 *Changing the Levels of Metabolites Needed for the Production of Viable Pollen*

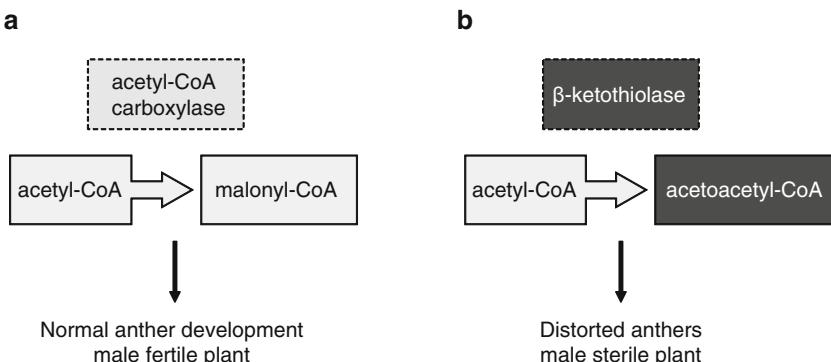
Another approach to induce male sterility in plants is the metabolic engineering of the carbohydrate supply. Carbohydrates are important for anther and pollen development. The extracellular invertase Nin88 mediates the phloem unloading of carbohydrates via an apoplastic pathway. Tissue-specific antisense repression of *nin88* in tobacco causes male sterility because early stages of pollen development are blocked (Goetz et al. 2001). McConn and Browse (1996) demonstrated that *Arabidopsis* triple mutants that contained negligible levels of trienoic fatty acids, such as jasmonate, were male-sterile and produced no seed. In that case, the fertility could be restored through the exogenous application of jasmonate.

### 14.3.3 Engineering Cytoplasmic Male-Sterile Plants

Several efforts are being made to generate engineered CMS plants (Chase 2006; Pelletier and Budar 2007). A quite promising approach was described by Ruiz and Daniell (2005) and reviewed by Khan (2005). Their approach has three advantages: (i) pollination and subsequent self-fertilisation is artificially suppressed, (ii) the trait is based on a cytoplasmic trait that cannot be transmitted via the pollen, and (iii) it allows for the selective restoration of male fertility, at least to some extent. This approach is based on inserting *phaA*, a gene that encodes  $\beta$ -ketothiolase, from the bacterium *Acinetobacter* into the chloroplast genome under control of the chloroplast *psbA* promoter. In transgenic tobacco plants, the enzyme accumulates in the leaves and anthers, altering the course of fatty acid synthesis (Fig. 14.3). By modifying lipid metabolism, pollen development is strongly impaired (Ruiz and Daniell 2005). The expression of  $\beta$ -ketothiolase also accelerates anther development and causes the pollen grains to collapse, leading to male sterility. Fertility restoration was achieved to some extent by growing the plants under continuous light. This effect is due to the light-sensitive gene expression controlled by the *psbA* promoter. Under these conditions, acetyl-CoA carboxylase gains the upper hand, thereby restoring normal fatty acid metabolism (Ruiz and Daniell 2005). However, restoration is only partial and the procedure does not appear to be applicable to field conditions.

### 14.4 Strategies for the Multiplication of Male-Sterile Lines

Although the described systems have provided important information about anther and pollen development, and ways to interfere with it, their potential use for commercial hybrid seed production is often limited because of the lack of



**Fig. 14.3** Engineering male sterility with  $\beta$ -ketothiolase. (a) In chloroplasts, acetyl-CoA is normally converted by acetyl-CoA carboxylase to yield malonyl-CoA. (b) In transgenic plants expressing high amounts of  $\beta$ -ketothiolase, this enzyme out-competes acetyl-CoA carboxylase converting acetyl-CoA into acetoacetyl-CoA. As a consequence, anther development is impaired. Based on data from Ruiz and Daniell (2005)

cost-effective and efficient methods to multiply the engineered male-sterile plants (for an overview of multiplication strategies, see Perez-Prat and van Lookeren Campagne 2002).

#### **14.4.1 Herbicide Application for Selection of Male-Sterile Plants**

One strategy for the propagation of male-sterile plants is to combine a gene conferring dominant male sterility with an herbicide resistance gene (Denis et al. 1993). After crossing the heterozygous male-sterile plants with a wild-type line of the same genetic background, the male-sterile progeny can be selected by herbicide application. It is important to eliminate all of the fertile plants to prevent any self-pollination, as this could lead to impure hybrid seeds (see Chap. 6).

#### **14.4.2 Reversible Male Sterility**

One approach for multiplying male-sterile plants is to produce plants that are conditionally fertile. During female parent multiplication, male-sterile plants are treated with a fertility-restoring chemical and can self-fertilize. For the production of hybrid seeds, chemical application is not required and the plants remain sterile. This system has some advantages over the selection of male-sterile plants by herbicide application. For example, the chemical has to be used during female parent multiplication and not during hybrid seed production and can be applied to a smaller acreage.

Based on conditional male fertility, several pollination control systems have been described. An example of the regulation of male fertility is that the manipulation of hormones in male reproductive tissues (Huang et al. 2003) induced male-sterile plants through tissue-specific expression of the *CKX1* genes and *gai*, which are involved in oxidative cytokinin degradation and gibberellin signal transduction. In this dominant male-sterility system, the male-sterile phenotype is achieved in transgenic plants that are homozygous for the transgene, and it is reversible by exogenous hormone application.

Alternatively, fertility can be induced by environmental conditions. In thermosensitive genetic male-sterile (TGMS) and photoperiod-sensitive genetic male-sterile (PGMS) mutants of rice, male sterility is influenced by temperature and photoperiod length (He et al. 1999; Dong et al. 2000). The temperature just after panicle initiation is the most critical in the expression of fertility and sterility. Most rice TGMS lines are male fertile at temperatures less than 25°C and sterile at higher temperatures (Sun et al. 1989). The seeds from TGMS lines are multiplied by selfing when exposed to the right temperature at the critical growth stage. The PGMS lines are fertile under the conditions of a natural short day and are male-sterile under long-day conditions. In this system, the male-sterile female line can be propagated by growing it under the environmental conditions that restore

fertility. This approach requires no restorer lines and no chemical treatment. However, controlled environmental conditions are needed to avoid the plants being constantly challenged by unfavourable fluctuations in their environment. Other conditional male fertility systems are based on repressing the male sterility gene or the inducible expression of a fertility restorer gene that complements the defect (Cigan and Albertsen 2000). Recently, a combination of reversible male sterility and doubled haploid production by targeted inactivation of cytoplasmic glutamine synthetase in developing anthers and pollen was established (Ribarits et al. 2007).

#### **14.4.3 Use of Maintainer Lines**

The propagation of nuclear male-sterile plants can also be achieved through cross-breeding with a maintainer plant that is male-fertile but produces 100% male-sterile progeny when used to pollinate male-sterile plants. Perez-Prat and van Lookeren Campagne (2002) developed pollen lethality and colour maintainer lines that are useful for propagating both dominant and recessive male-sterile lines. The maintainer plants are genetically identical to the nuclear male-sterile plants with the exception of a transgenic maintainer locus that renders it male-fertile. This system does not require chemical application but a fertility restorer gene and, in the case of colour maintainers, seed sorting might also be needed.

### **14.5 Commercial Use of Male Sterility**

A number of CMS systems have been and are being used in traditional plant breeding in order to generate hybrid varieties. From the many potential procedures for obtaining transgenic male sterility, only a few have been developed so far for commercially available crops. A compilation of these crops is given in Table 14.1. The events include several in canola, one in chicory, and three in maize. In almost all cases, the Barnase/Barstar system is being employed, with the notable exception of a DNA adenine methylase from *E. coli* that causes male sterility if expressed in certain plant tissues. Recently, the Barnase/Barstar system was adopted to Indian oilseed mustard (*Brassica juncea*; Ray et al. 2007), but this has not yet been developed for commercial use.

### **14.6 Conclusions and Future Perspectives**

The use of hybrid crops has been a very important agricultural advance in recent years, because hybrids have an increased yield and a wider environmental adaptability and are more insect- and disease-resistant. One strategy that has been utilized for hybrid crop production is male sterility. Biotechnology has enabled new methods for obtaining male-sterile plants and developing several new

**Table 14.1** Commercially used male-sterile plants. Data are from AGBIOS GM database (<http://www.agbios.com>). Barnase and barstar genes are from *Bacillus amyloliquefaciens*. *fr*: Fertility restoring line, *ms*: male sterile line, *PPT*: phosphinothricin-N-acetyltransferase gene from *Streptomyces hygroscopicus* or *S. viridochromogenes*

Event	Company	Description	Marker gene
<i>Brassica napus</i> (Canola)			
MS1, RF1	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNase inhibitor gene	ms + fr: PTT
MS1, RF2	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNase inhibitor gene	ms + fr: PPT
MS8xRF3	Bayer Crop Science (Aventis Crop Science; AgrEvo)	ms: barnase ribonuclease gene; fr: barstar RNase inhibitor gene	ms + fr: PPT
PHY14, PHY35	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNase inhibitor gene	ms + fr: PPT
PHY36	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNase inhibitor gene	ms + fr: PPT
<i>Cichorium intybus</i> (Chicory)			
RM3-3, RM3-4, RM3-6	Bejo Zaden BV	ms: barnase ribonuclease gene	PTT
<i>Zea mays</i> (Maize)			
676, 678, 680	Pioneer Hi-Bred International Inc.	ms: DNA adenine methylase from <i>E. coli</i>	PPT
MS3	Bayer Crop Science (Aventis Crop Science; AgrEvo)	ms: barnase ribonuclease gene	PPT
MS6	Bayer Crop Science (Aventis Crop Science; AgrEvo)	ms: barnase ribonuclease gene	PPT

pollination control systems that could be useful for hybrid seed production. However, the inability to propagate the male-sterile female parent line in a cost-effective and efficient way limits the potential application of commercial hybrid production. Future research should take into account the importance of developing solutions for propagation because, for many crops, it is the limiting factor in the large-scale production of hybrids. Male sterility systems are also being developed for tree species which in the future may be used in other tree species (Höfig et al. 2006).

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**Part C**

**Transgenic Plants in Breeding  
and Crop Production**



# **Chapter 15**

## **Cotton**

**Keerti S. Rathore**

### **15.1 Introduction**

The cotton plant is the most important source of natural fiber and has met the clothing needs of mankind for several millennia. This plant plays a major role in the economy and social structure of many countries, and in fact, has helped shape the history of some parts of the world. Although many other natural and synthetic fibers have been available, advantages related to the cost of production, many unique features offered by cotton lint, and the growing world population will ensure a continued increase in the demand for cotton. Not only will the tools offered by biotechnology help us to achieve the needed increase in the production of this crop, this technology will also be used to confer properties to the fiber that are difficult or impossible to achieve by traditional breeding. The increasing demand for food, feed, and energy will also help the cottonseed to achieve a better status than merely a byproduct of lint production. Again, there is tremendous scope for improving the quality of cottonseed through biotechnology.

Cotton is grown in over 80, mostly developing countries where it is a cash crop for many poor farmers. With its share of more than 90% of the worldwide acreage, the tetraploid *Gossypium hirsutum* or upland cotton is the predominant cultivated species. The other tetraploid species, *G. barbadense* or Egyptian cotton, is also grown in some parts of the world for its prized extra-long staple. Because of their low productivity and poor quality fiber, the two Old World, diploid species, *G. arboreum* and *G. herbaceum*, are cultivated only on limited acreage in some parts of Africa and Asia. China is the largest producer of cotton, followed by India and the United States. In 2006, the global area under cotton cultivation was estimated to be approximately 34.3 million hectares (Mha) leading to ~25 million

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metric tonnes (MMT) of fiber production (FAO 2008). Cotton is grown primarily for its fiber, however, the plant also produces large quantities of seeds. Worldwide, ~44 MMT of cottonseed was produced in the year 2006 (FAO 2008). A portion of this abundant agricultural byproduct is used to obtain edible oil; however, a large share of the cottonseed/cottonseed meal is simply used as a feed for the ruminant animals. The presence of a toxic terpenoid, gossypol, renders the protein-rich seed unfit for consumption by monogastric animals and prevents its direct use as food. Elimination of this toxin from the seed will ensure a more efficient utilization of this resource and help fulfill the growing, worldwide demand for food and feed.

## 15.2 Importance and Potential Impact of Genetic Modification in Cotton

Cotton plants are particularly susceptible to a wide variety of insect pests and nematodes, and their cultivation has traditionally relied on the use of large amounts of highly toxic pesticides. Some estimates suggest that, prior to the widespread adoption of Bt cotton, nearly 25% of all insecticides used worldwide were needed for the production of cotton (Pannetier et al. 1997). Genetically modified cotton produced by incorporating the Bt gene was therefore a huge success in the United States following its introduction in 1996 (see also Chap. 11). Amongst the cotton-producing countries, India has the largest area under cotton cultivation and yet it has ranked third in terms of production until very recently. This was because the average yield of cotton in India was one of the lowest in the world. Several factors accounted for this low productivity including insect pests. The yield of lint+cottonseed in this country averaged  $561 \text{ kg ha}^{-1}$  in 2001; however, it increased to  $1019 \text{ kg ha}^{-1}$  in 2007 (FAO 2008). In a recent publication from the International Food Policy Research Institute (IFPRI), Gruere et al. (2008) report that in the year 2007/08, India's cotton production exceeded that of the United States. Most of this rise in the production is attributed to an increasing use of Bt cotton varieties following their introduction in the year 2002 (Qaim and Zilberman 2003; James 2007; Gruere et al. 2008). It is not surprising then that, once approved by the respective regulatory agencies, cotton growers in many other countries have readily adopted GM cotton. The example of India illustrates the potential impact of biotechnology in enhancing global cotton production. Overall improvements in the production of cotton will be considerable once this technology is adopted by the rest of the cotton producing countries. Currently, Bt-mediated insect resistance and herbicide resistance are the only two transgenic traits available in cotton. When the traits that confer resistance to various other biotic and abiotic stresses become available, the combined impact of various transgenic traits on the total output will be much more substantial than what has been achieved thus far. In addition to its impact on the production, genetic engineering is likely to play a very important role in improving the quality of fiber as well as the seed.

### 15.3 Transformation of Cotton and its Improvement via Genetic Modification

There are some excellent reviews available on transgenic cotton (Murray et al. 1993; John 1997; Chlan et al. 2000; Wilkins et al. 2000; Rajasekaran et al. 2001; Kumria et al. 2003; Rathore et al. 2008). Table 15.1 provides a list of selected papers describing the key transformation methods and the introduction of certain useful traits via genetic engineering. General aspects of genetic transformation are discussed in Chap. 1.

#### 15.3.1 Methods Used to Transform Cotton

The first two reports on successful cotton transformation were published by scientists at Agracetus (Umbeck et al. 1987) and Agrigenetics (Firoozabady et al. 1987). In both cases, tissue explants obtained from a young seedling were transformed via *Agrobacterium tumefaciens*. The transformed tissues growing on selection medium were cultured for several months before recovering the transgenic plants via somatic embryogenesis. This procedure is rather long and laborious, and is limited for use in only a few genotypes that are able to regenerate via somatic embryogenesis. However, it is a robust protocol and with some modifications, is widely used to obtain transgenic cotton plants in both academic and industrial laboratories (Table 15.1). A comprehensive investigation was undertaken in author's laboratory to understand both the transformation and regeneration processes (Sunilkumar and Rathore 2001; Rathore et al. 2006). This study made use of green fluorescent protein (GFP) gene as a reporter and showed clearly that the transfer of T-DNA per se, from *Agrobacterium* to the cotton cells at the wound site in a cotyledon, hypocotyl and cotyledonary petiole, is an efficient process. In addition, its integration into the cotton genome is also quite effective. It is the culture of transformed cells to obtain a friable, embryogenic callus capable of plant regeneration, that is a highly genotype-dependent process (Trolinder and Xhixian 1989). Even with the regenerable genotypes, a high degree of tissue culture skills are required to obtain transformed cotton plants. Bearing in mind the difficulties faced by many researchers in producing transgenic cotton, a simplified protocol describing various steps in detail has been published (Rathore et al. 2006).

Thus, genotype-dependence, in terms of regeneration via somatic embryogenesis, does remain a limitation in introducing a transgenic trait directly into commercial varieties. The same constraints also apply to methods that utilize particle bombardment-mediated transformation of cultured cells. These limitations have served as an impetus to find alternative methods to produce transgenic cotton. Since regeneration of plants from shoot apical meristem is genotype-independent, relatively rapid, and a rather straightforward process, many laboratories have targeted the cells within this explant for transformation. The research involving

**Table 15.1** Summary of selected studies on transgenic cotton. C Cotyledon, ESC embryogenic cell suspension, H hypocotyls, P cotyledony petiole, SAM shoot apical meristem

Transformation method	Cultivar	Target tissue mode of transformant recovery	Transgenes	Analysis and Comments	Reference
<i>Agrobacterium</i>	Coker 310, 312, 5110	H/somatic embryogenesis	<i>cat</i> and <i>nptII</i>	Enzyme assays and Southern for confirmation	Umbeck et al. (1987)
<i>Agrobacterium</i>	Coker 201	C/somatic embryogenesis	<i>nptII</i> and OCS	Immunoblot and Southern for confirmation	Firoozabady et al. (1987)
Gene gun	Coker 310	ESC/somatic embryogenesis	<i>hpt</i>	Southern for confirmation	Finer and McMullen (1990)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	<i>CryIAc</i> , <i>CryIAb</i> and <i>nptII</i>	Western and bioassay for confirmation	Perlaik et al. (1990)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	<i>nptII</i> and <i>tfdA</i>	2,4-D monooxygenase activity, PCR, and 2,4-D resistance for confirmation	Bayley et al. (1992)
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis	<i>nptII</i> , <i>gusA</i> and <i>tfdA</i>	Southern, GUS enzyme assay and 2,4-D resistance for confirmation	Lyon et al. (1993)
Gene gun	Delta Pine 50, Delta Pine 90, Sea Island, Pima S-6	SAM from mature seed/shoot regeneration in culture	<i>gusA</i>	GUS histochemical analysis and Southern for confirmation	McCabe and Martinell (1993)
Gene gun	Coker 312, Delta Pine 50, Sea Island	SAM from mature seed/shoot regeneration in culture	Fiber-specific, FbL2A promoter driving <i>phAB</i> and <i>phaC</i> , and <i>gusA</i>	GUS assay, Southern, Western, and biochemical analyses for confirmation	Rinehart et al. (1996)
Gene gun	Delta Pine 50	SAM from mature seed/ Shoot regeneration in culture	Fiber-specific, E6 or FbL2A promoter driving <i>phAB</i> and <i>phaC</i> , and <i>gusA</i>	GUS assay, Southern, Northern, microscopic, and biochemical analyses for confirmation; fiber's thermal properties were altered	John and Keller (1996), Chowdhury and John (1998)

<i>Agrobacterium</i> and gene gun	Coker 315 and Acala varieties	C, H, ESC/somatic embryogenesis	<i>nptII</i> , mutant native AHAS genes	Southern and resistance to herbicides, imidazolinone and sulfonylurea, for confirmation	Rajasekaran et al. (1996)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	<i>nptII</i> , FMV 35S promoter driving CP4-EPSPS	Southern, ELISA, and resistance to herbicide, glyphosate for confirmation	Nida et al. (1996), Chen et al. (2006)
<i>Agrobacterium</i>	CUBQHRPIS	SAM from seedling/ shoot regeneration in culture	<i>nptII</i> , <i>gusA</i>	Resistance to kanamycin and Southern for confirmation	Zapata et al. (1999)
<i>Agrobacterium</i>	Coker 315	H/somatic embryogenesis	Tobacco basic chitinase, glucose oxidase, <i>nptII</i>	Some protection against verticillium wilt with each gene	McFadden et al. (2000)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	<i>nptII</i> , <i>gusA</i>	PCR and Southern for confirmation; a step-wise and comprehensive account of transgenic cotton production	Sunilkumar and Rathore (2001), Rathore et al. (2006)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	Mn-SOD, APX, GR, <i>nptII</i>	Enzymatic assays confirmed overexpression; chilling-induced photoinhibition of photosystem II reduced	Korriyeyev et al. (2001, 2003a, b)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	Cotton $\alpha$ -globulin promoter driving <i>gusA</i> , <i>nptII</i>	Histochemical and biochemical analyses; seed-specificity of promoter demonstrated	Sunilkumar et al. (2002a)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	CaMV 35S promoter driving GFP gene, <i>nptII</i>	Fluorescence microscopy analysis; developmental- and tissue-specific activity of promoter demonstrated	Sunilkumar et al. (2002b)
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis	Seed-specific RNAi of Cotton SAD-1	Southern, Northern and biochemical analyses; seed oil	Liu et al. (2002)

*(continued)*

**Table 15.1** (continued)

Transformation method	Cultivar	Target tissue mode of transformant recovery	Transgenes	Analysis and Comments	Reference
<i>Agrobacterium</i>	MCU5, DCH32, Coker 310FR	Shoot tip from seedling/shoot regeneration in culture	and Cotton FAD2-1, <i>nptII</i> <i>gusA, nptII</i>	with substantially higher stearic acid or oleic acid levels	Satyavathi et al. (2002)
<i>Agrobacterium</i>	Coker 312	C, H/ somatic embryogenesis	Cotton $\beta$ -tubulin promoter driving <i>gusA, nptII</i> Endochitinase gene from <i>Trichoderma virens, nptII</i>	Histochemical analyses; preferential activity in fiber and root tip observed	Li et al. (2002)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	<i>gusA, nptII</i>	Southern, Northern and biochemical analyses; protection against <i>Rhizoctonia solani</i> and <i>Alternaria alternata</i> observed	Emani et al. (2003)
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis	Sense and antisense suppression of sucrose synthase, <i>nptII</i>	Southern, immunolocalization, electron microscopy and biochemical analyses; fiber development inhibited	Ruan et al. (2003)
Gene gun	Coker 310FR	H-derived friable callus/somatic embryogenesis	Chloroplast-specific expression of <i>aphA-6</i> and <i>nptII</i>	PCR and Southern to confirm plastid genome transformation; strict maternal inheritance of Kanamycin-resistance	Kumar et al. (2004)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	Cotton <i>ghCTL2</i> promoter driving <i>gusA, nptII</i>	Preferential activity in different cell types during secondary wall deposition including lint fibers	Zhang et al. (2004)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	Seed-specific antisense of cotton FAD-2, <i>nptII</i>	Biochemical analysis; seed oil with higher oleic acid level	Sunilkumar et al. (2005)

<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis	Soybean lectin promoter or CaMV 35S promoter driving antisense <i>cda1</i> - <i>C4, npthII</i>	Southern, Northern, and Western analyses; no reduction in gossypol levels; induction of the target gene by bacterial blight was blocked	Townsend et al. (2005)
<i>Agrobacterium</i>	Coker 312	C, H/ somatic embryogenesis	Synthetic antimicrobial peptide <i>D4E1</i> , <i>npthII</i>	Southern, PCR and RT-PCR analyses; transgenic plants resistant to several fungal pathogens	Rajasekaran et al. (2005)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	<i>Arabidopsis NHX1</i> , <i>npthII</i>	PCR, Northern, and Western analyses; more biomass and more fiber produced under salt stress conditions	He et al. (2005)
<i>Agrobacterium</i>	Coker 312	C, H/somatic embryogenesis	Cotton ACTIN1 promoter driving <i>gusA</i> , RNAi of <i>ghACT1</i> , <i>npthII</i>	Histochemical analyses; preferential activity observed in the fiber; RNAi-inhibition of fiber elongation	Li et al. (2005)
<i>Agrobacterium</i>	Zhongmiansuo 35	H/somatic embryogenesis	Phloem-specific promoter driving <i>ACA</i> gene, <i>npthII</i>	Southern and Western for confirmation; resistance to cotton aphid observed	Wu et al. (2006)
<i>Agrobacterium</i>	Coker 312	H, P/somatic embryogenesis	Seed-specific RNAi of cotton $\delta$ -cadinene synthase, <i>npthII</i>	Southern, RT-PCR, Northern and biochemical analyses for confirmation; over 98% reduction in the seed gossypol level obtained	Sunilkumar et al. (2006)
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis	CaMV 35S promoter driving spinach SPS gene, <i>npthII</i>	Southern, RT-PCR, Northern, Western and biochemical analyses for confirmation; improved fiber quality	Haigler et al. (2007)

particle bombardment of isolated shoot apical meristems followed by the recovery of plants has provided unambiguous evidence for the transgenic status of the regenerants and proved the feasibility of this approach (McCabe and Martinell 1993; McCabe et al. 1998). The gene gun-based microprojectile bombardment is a direct, physical method that can deliver the genes into the epidermal cells of the L1 layer or the germline progenitor cells of L2/L3 layer within the apical meristem. As expected, the progeny plants from L1 transformants did not inherit the transgene. In contrast, the germline transformants resulting from the transformation of L2/L3 cells passed on the transgenic trait to subsequent generations. However, the primary transformants recovered from these shoot apices are chimeric and the efficiencies of recovering germline transformation events are very low. As this method is highly labor- and resource-intensive, it has not been used by others. There are reports from three laboratories on *Agrobacterium*-mediated transformation of shoot apical meristem to obtain transgenic cotton plants (Zapata et al. 1999; Satyavathi et al. 2002; Uceer and Koc 2006). The ability to tolerate kanamycin was used as a major criterion to identify the putative transformants and each report provided some molecular evidence. However, these reports did not provide any information on the type of cells that were transformed within the shoot apical meristem. The transformation efficiencies reported in these papers differ drastically, thus raising questions about the criteria used to assign transgenic status to the regenerated plants. Additional, convincing evidence that includes phenotypic analysis, molecular proof that discounts the possibility of *Agrobacterium* contamination of the plant tissue, and genetic analysis in several generations will be needed to confirm the reliability, efficiency, and robustness of this method. If an unambiguous proof of *Agrobacterium*-mediated transformation of shoot apical meristem is provided, it will ensure a widespread adoption of this technique by other researchers who are interested in a genotype-independent method to transform cotton.

### **15.3.2 Selectable Markers and Reporter Genes used for Cotton Transformation**

Chap. 3 generally discusses the use of marker genes in transgenic plants. Neomycin phosphotransferase II (*nptII*) gene in combination with kanamycin as the selection agent was used in the first two investigations reporting successful cotton transformation (Firoozabady et al. 1987; Umbeck et al. 1987). The papers listed in Table 15.1 suggest that this gene continues to be used widely to obtain transgenic cotton. Its wide popularity stems from the fact that kanamycin is relatively inexpensive and does not adversely affect regeneration from cultured cotton tissues. Hygromycin phosphotransferase (*hpt*) gene is also suitable for producing transgenic cotton and has been used as a selectable marker in some studies (Finer and McMullen 1990). Cotton has been transformed with the bialaphos resistance (*bar*) gene; however, the initial selection of transgenic tissue was based on the expression of a linked *nptII*

gene in these studies (Keller et al. 1997). Bialaphos-tolerant cotton has been also developed by Bayer CropScience and is marketed by FiberMax under the name LibertyLink (Perkins 2004). The list provided in Table 15.1 shows that  $\beta$ -glucuronidase (*gusA*) remains the gene of choice to evaluate different transformation methods as well as for the characterization of promoter activities in various tissues in cotton. This is because GUS activity assays are relatively simple and the enzyme activity can be quantitated (Jefferson et al. 1987). The utility and versatility of GFP reporter gene (allowing non-invasive monitoring of its expression) was demonstrated by observing the tissue- and development-specific activity of CaMV 35S promoter in cotton (Sunilkumar et al. 2002b).

### 15.3.3 Genetically Engineered Traits in Cotton

Bt was the first commercially useful gene introduced into cotton (Perlak et al. 1990). This cotton was later developed and marketed under the trade name Bollgard by Monsanto/Delta & Pine Land (Jones et al. 1996; Jenkins et al. 1997). These plants expressed a truncated, codon-modified *CryIAc* gene from *Bacillus thuringiensis* (Bt) encoding a  $\delta$ -endotoxin that is toxic to tobacco budworm and American bollworm (Jenkins et al. 1997). These Bt cottons were readily accepted by farmers in the United States and other countries that had allowed their cultivation. Bollgard II, introduced in 2003, contains *Cry2Ab* in addition to *CryIAc* (Micinski et al. 2006; Robinson 2006). This second *Bt* gene broadens the resistance to include fall armyworm, beet armyworm, cabbage looper, and soybean looper (Perlak et al. 2001). Syngenta has developed VIP-Cotton containing a different gene from *B. thuringiensis* that encodes a vegetative insecticidal protein (VIP; Estruch et al. 1996). The VIP is structurally, biochemically, and functionally different from the Bt  $\delta$ -endotoxins and exhibits insecticidal activity against a variety of lepidopterans (McCaffery et al. 2006). Another type of insect-resistant cotton has been developed by Dow AgroSciences by combining *CryIF* and *CryIAc* genes. This product, WideStrike cotton, also confers resistance to several Lepidopteran pests (Bacheler et al. 2006; Micinski et al. 2006). Thus, a choice of more than one insect resistance genes with different modes of action, especially if they are stacked, will help broaden the spectrum of insects that can be controlled by the genetically modified plants and also help counter the development of resistance in the target insects.

Roundup Ready cotton that is resistant to glyphosate-based herbicide (see also Chap. 10 for references) was introduced in 1997 by Monsanto (Nida et al. 1996). This trait was engineered by expressing a gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (derived from *Agrobacterium* sp. strain CP4) under the control of FMV 35S promoter. In 2006, Roundup Ready Flex cotton became available that allows safe application of the herbicide well beyond the five-leaf stage (Chen et al. 2006). Glyphosate-tolerant cottons help in the effective management of weeds and were also readily adopted by the United States cotton growers. Glufosinate- or bialaphos-tolerant cotton, developed by Bayer CropScience and

marketed by FiberMax under the name LibertyLink, is also available commercially (Perkins 2004).

As is the case with most other crop plants, no commercial, transgenic products are yet available in cotton that address the problems of biotic or abiotic stresses. However, there are some published reports describing transgene-mediated resistance to various fungal diseases in cotton (Murray et al. 1999; McFadden et al. 2000; Emani et al. 2003; Wang et al. 2004b; Rajasekaran et al. 2005). Although some of these studies appear promising, in each case, the transgene conferred protection to only a limited spectrum of pathogens. Similarly, there are a number of reports describing attempts to engineer cotton to tolerate abiotic stresses, including freezing (Kornyeyev et al. 2001, 2003a, b; Payton et al. 2001), water-logging (Ellis et al. 2000), salt stress (He et al. 2005) and drought (Yan et al. 2004).

Since cotton is grown mainly for its fiber, it is an obvious target for improvement via genetic engineering. In addition to the usual desirable properties that include strength, fineness, length, and uniformity, cotton fiber can benefit from characteristics such as better dye binding, wrinkle resistance, and shrinkage resistance. Improvements in these last three categories will help cotton fiber compete more effectively against synthetic fibers. The number of genes involved in controlling some of these traits is likely to be large and the mechanism controlling these characteristics is expected to be complex. Several laboratories are involved in identifying and isolating genes that are involved in fiber initiation, elongation, and development. As these genes become available and are characterized, their coding and regulatory sequences will be used to engineer the cotton plant to address issues related to fiber quality improvement. Nevertheless, some interesting work to modify cotton fiber has been already conducted by scientists at Agracetus and elsewhere. An early example of such research involved the synthesis of novel biological materials in the fiber. Expression of some genes derived from *Alcaligenes eutrophus* in the developing cotton fibers resulted in the deposition of poly-D-( $\text{--}$ )-3-hydroxybutyrate (PHB) in their lumens (John and Keller 1996; Rinehart et al. 1996). The modified fiber exhibited altered thermal properties resulting in improved insulating characteristics (Chowdhury and John 1998). Although this product was not developed further, the results demonstrated the feasibility of improving cotton fiber in a manner that is impossible to achieve by traditional breeding methods. Two recent studies have examined the effects of manipulating endogenous gene expression in cotton fiber cells (Ruan et al. 2003; Li et al. 2005). Although each of these studies involved suppression of a cotton gene that adversely affected fiber growth/development, the results indicate the feasibility of altering fiber properties. In a more recent study, Haigler et al. (2007) showed that constitutive overexpression of spinach sucrose phosphate synthase gene in cotton resulted in the improvements in fiber quality when the cotton plants were grown under controlled environmental conditions. Chapman et al. (2008) reported an interesting and unexpected outcome of manipulating the oil composition by overexpressing a non-functional rapeseed FAD-2 gene in cottonseed. They showed that, while the seeds from transformed lines were smaller, of poor quality and had lower oil content, the lint produced was significantly increased, suggesting a redirection of carbon reserves. These reports on

transgenic manipulation of cotton fiber are promising. However, considering the importance of this agricultural product, the progress in improving its characteristics and yield through biotechnology has been rather slow. As more fiber-specific genes and their regulatory sequences become available, transgenic technology is expected to make a significant impact on the quality and yield of this most important product of the cotton plant (Li et al. 2002; Wang et al. 2004a).

Cotton plant produces about 1.6 times more seed than fiber. Cottonseed contains ~21% oil and a substantial portion of the global production is used to obtain edible oil. Since cottonseed oil is rather low in monounsaturated fatty acid, gene-suppression technologies have been used to improve its fatty acid composition in favor of higher oleic acid. Use of antisense technology to suppress  $\Delta$ -12 desaturase gene resulted in doubling of oleic acid from a wild-type level of ~15% to ~30% and a reduction in linoleic acid level from ~55% to ~35% (Sunilkumar et al. 2005). Interestingly, RNAi-mediated suppression of the same target gene resulted in a fivefold increase in oleic acid level and a concomitant reduction in the linoleic acid (Liu et al. 2002). In a separate set of transformants, RNAi-mediated downregulation of the SAD-1 gene resulted in a >10-fold increase in stearic acid level in cottonseed oil. Importantly, it was possible to stack the two traits by intercrossing (Liu et al. 2002). These results demonstrate that transgenic technology can be used to modify fatty acid biosynthetic pathway in a tissue-specific manner to improve storage and cooking properties of the cottonseed oil. In addition to the oil, cottonseed also contains ~23% protein that is of relatively high quality. Global cottonseed output of ~44 MMT year<sup>-1</sup> can potentially meet the basic protein requirements of 500 million people. However, the ability to utilize this abundant, protein-rich resource for food is hampered by the presence of toxic gossypol. This cardio- and hepatotoxic terpenoid, present in cottonseed glands, renders the seed unsafe for human and monogastric animal consumption. Glands containing gossypol and related terpenoids are present in most parts of a cotton plant. The terpenoids are believed to play a protective role in defending the cotton plant against various insect pests and diseases (Hedin et al. 1992; Townsend et al. 2005). To avoid the weakening of defensive capability of the cotton plant, the elimination of gossypol must be strictly limited to the seed. Since traditional breeding methods have failed to achieve this goal, biotechnological approaches were tested in many laboratories around the world to solve the problem of cottonseed toxicity. Most of these attempts over the past decade have been unsuccessful (see Townsend et al. 2005 and references therein). However, in a relatively recent breakthrough, the feat of selective and significant reduction of gossypol in cottonseed was achieved by disrupting its biosynthesis through RNAi-mediated suppression of  $\delta$ -cadinene synthase activity in the developing seed (Sunilkumar et al. 2006). Some of the RNAi lines obtained showed a 98% reduction in the concentrations of gossypol in the seed. Importantly, these transformants maintained normal levels of gossypol and related terpenoids in all other parts of the plant. These studies involving alteration of oil composition and gossypol reduction suggest that a genetically modified cotton plant, in addition to meeting the clothing requirements, can also play an important role in fulfilling the nutritional needs of the growing human

population. More details on different engineered traits may be found in Sect. C of this volume.

### **15.3.4 *The Role of New Technological Advances in Cotton Improvement***

Successful transformation of the plastid genome in cotton has been achieved by Kumar et al. (2004). Although chloroplast transformation is more difficult and less efficient compared to the nuclear transformation, it does offer some advantages, including transgene containment because of maternal inheritance of the trait and a high level of consistent transgene expression. Lower efficiency and the complexity of the plastid transformation system have prevented widespread adoption of this technology. However, it may be useful for some specific applications.

The two examples provided earlier of transgene-encoded RNAi to improve cottonseed quality demonstrate the power of this gene silencing technology. Undoubtedly, it will be used to improve other properties of this important resource in the future. As the genes involved in controlling various aspects of fiber growth and development are identified, RNAi will serve as a valuable tool in the engineering of desired characteristics in the fiber. In addition to the use of RNAi to improve the quality of seed and fiber, some recent reports suggest exciting new possibilities in harnessing the power of this technology to control nematodes and insect pests of cotton. Yadav et al. (2006) transformed tobacco plants to express dsRNA against important genes of a root-knot nematode (RKN) which resulted in a virtual elimination of the target mRNA in the parasite and significant resistance in the host plant. In another report, Huang et al. (2006) describe results of a transgene-encoded expression of ingestible dsRNA in *Arabidopsis* targeting an RKN gene that encodes *16D10*, a secretory peptide essential for the nematode parasitism of the plant. The transformants showed significant resistance to four major RKN species. Corn plants expressing dsRNA against Western corn root-worm V-ATPase were effectively protected against the insect (Baum et al. 2007) and this protection was comparable to that provided by Bt. The results obtained by Mao et al. (2007) illustrate an interesting possibility to confer protection against cotton bollworm by expressing dsRNA in the plant that targets an insect cytochrome P450 monooxygenase, believed to be involved in detoxifying the natural insecticide gossypol in cotton plants. These examples illustrate that RNAi is a versatile and highly effective tool that can be used to engineer cotton plants to confer resistance to various pests.

## **15.4 Future Perspectives**

It has been two decades since the first reports on cotton transformation were published. Bt cotton was introduced in the marketplace in the United States in 1996, with herbicide-tolerant cotton a year later. In 2005, GM cotton (Bt cotton,

herbicide-resistant cotton) garnered 79% of the cotton acreage in the United States (Brookes and Barfoot 2006). Especially, Bt cotton has enjoyed the same enthusiastic acceptance by the farmers in many other countries where its use was permitted by their respective regulatory agencies. In 2006, of the 11 million small farmers who grew GM crops, most were Bt cotton farmers, including 7.1 million in China and 3.8 million in India (James 2007). However, the current GM cotton varieties offer only insect- and herbicide-resistance traits that benefit largely the growers. Availability and choice of these and other input traits is likely to increase in future. The published reports described in this chapter show the efforts underway to engineer a number of useful output traits into cotton. As new genes and their regulatory sequences become available from cotton and other species, and as the genetic modification technologies are further refined and improved, we can expect cotton plants with novel input and output traits for the benefit of growers, consumers, and the environment. The current product-line is available from just a few large companies and the farmers have to pay a premium to grow their GM cotton varieties. However, some of the basic patents on various GM-related technologies will start to expire soon. This will open up opportunities for the scientists to engineer cotton to meet the more specific, local needs of the poor farmers in the developing countries.

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# Chapter 16

## Triticeae Cereals

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### 16.1 Introduction

The Triticeae cereals include two of the most important crops worldwide – wheat (*Triticum aestivum*, *T. turgidum* conv. *durum*) and barley (*Hordeum vulgare*) – along with cereal rye (*Secale cereale*) and triticale (*xTriticosecale*), both of which are relevant in certain agricultural environments. Together with maize (*Zea mays*) and rice (*Oryza sativa*), wheat represents a major food crop, with  $>600 \times 10^6$  t (Mt) global annual production of bread wheat (*T. aestivum*) contributing about 20% of the global calorie requirement. About 100 Mt of wheat is used for animal feed, and a growing proportion of the crop provides raw material for biofuel. Wheat is cultivated in over 80 countries and is adapted to a wide range of environments. Its largest yields are obtained in temperate regions, such as in northern Europe, but large areas are grown profitably in environments which are rather dry (NRCS 2005), making China, India and the United States the world's leading producers in terms of bulk. Barley was one of the first grains to be domesticated, and is also grown over a broad environmental range. Like wheat, it is most productive in temperate zones which allow a growth period of at least 90 days, but its cultivation is also viable in the sub-arctic (e.g. in Alaska and Norway), where only a very short growing period is available. Barley is comparatively heat and drought tolerant, and is therefore preferred to wheat in arid environments. The 2008 barley harvest was about 140 Mt from a cultivated area of  $57 \times 10^6$  ha (Mha; FAO 2008; International Grains Council 2009), and the world's major producers are Russia, Canada and Germany. Most of the barley crop is used for animal feed, with about 15% supplying the malting and food sectors (US Grains Council 2006). The cereal rye crop is used mainly for animal feed, but some is used for food production since the grain has a high dietary

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value. A small amount of rye is produced for fermentation and distillation. Further, it is emerging as a feedstock for bioethanol production. In 2005, the cereal rye crop was 15.5 Mt, with the main producers being Russia, Poland and Germany (FAO 2008). As modern harvesting technology is adapted to short-stature crops, the production of cereal rye has been in decline over recent years. Unique among the Triticeae crops, cereal rye is an outbreeder, a property which complicates its handling in the light of regulations surrounding genetic engineering. Finally, triticale is a synthetic allopolyploid derived from the cross between tetraploid wheat (*T. durum*, as female) and cereal rye (as male). Its grain phenotype is intermediate between those of wheat and cereal rye. In 2005, 13.5 Mt were harvested (FAO 2008), mainly from Poland, Germany and France. Over half of the triticale crop is used for animal feed, but a small amount is used for human food.

### **16.1.1 The Generation of Transgenic Triticeae Plants**

Achieving stably transformed Triticeae plants has been a challenge, both because they are only poorly compatible with *Agrobacterium* spp., and because it has proven difficult to induce adventitious shoots from somatic tissue (for general information on transformation see Chap. 1). Nevertheless, these hurdles have largely been overcome by substantial improvements in cell culture and transformation technology over the past 15 years. The first transgenic wheat plants were obtained by Vasil et al. (1992), who used particle bombardment of callus grown from immature zygotic embryos. An intensive effort to optimize the method resulted in a marked improvement in the recovery of transgenic plants, to the extent that the method could be shown to be relatively reproducible (Becker et al. 1994; Nehra et al. 1994). Similar protocols were quickly thereafter established for barley (Wan and Lemaux 1994), cereal rye (Castillo et al. 1994), triticale (Zimny et al. 1995) and durum wheat (Bommineni et al. 1997). Particle bombardment was also successfully directed at immature embryogenic barley pollen (Jaehne et al. 1994), inflorescence primordia of tritordeum (the amphiploid formed from the cross *H. chilense* x durum wheat; Barcelo et al. 1994) and barley shoot meristematic cultures (Zhang et al. 1999).

Even though the grasses are not natural hosts for *Agrobacterium* spp., inoculation of highly totipotent immature embryos was successfully used to generate stably transgenic wheat (Cheng et al. 1997) and barley (Tingay et al. 1997) lines. Current protocols claim to deliver at least one transgenic barley line from ten inoculated embryos (i.e.  $\geq 10\%$  transformation efficiency; e.g. Hensel et al. 2008), which is much greater than what has been achieved using particle bombardment. Transformation efficiency in wheat and cereal rye lags that of barley, and currently lies at best in the region of 5% (e.g. Popelka and Altpeter 2003; Wu et al. 2003), comparing favourably with what can be achieved by particle bombardment (e.g. Rasco-Gaunt et al. 2001; Popelka et al. 2003). Other recipient cells and tissues, such as embryogenic pollen cultures in barley (Kumlehn et al. 2006), have also been

used to generate stably transgenic plants in Triticeae species. As the target cells in embryogenic pollen are typically haploid, a transformed cell (upon chromosome doubling) becomes perfectly homozygous for the transgene; this is particularly advantageous, as it removes any need for the genetic fixation required when diploid tissue is used as gene transfer recipient. A second transformation target, which has remained to date exclusive to barley, is represented by cultivated ovules at the zygote or few-celled embryo stage (Holme et al. 2006). This approach has also lent itself to dispensing with the use of a selectable marker gene, although at a cost of lower transformation efficiency. In the authors' laboratory, the optimization of this method has resulted in achieving a transformation efficiency of as high as 8% (Marthe et al., unpublished data).

A frequently noted problem with transgenesis in the Triticeae species has been the influence of recipient genotype on regeneration. Immature zygotic embryos of the barley cultivar "Golden Promise" and the wheat breeding line "Bobwhite" have proven highly amenable to gene transfer and in vitro shoot proliferation. Often however, it is desirable or even necessary to apply genetic engineering to other cultivars. To characterize genetic variation for "transformability", sets of both wheat and barley (e.g. Wu et al. 2003; Hensel et al. 2008) cultivars have been tested using the available gene transfer methods. In these studies, several lines proved in principle amenable to genetic engineering, albeit with lower efficiency as compared to the above model lines. It has only recently been shown that the isolated ovules approach is less genotype-dependent than other methods (Holme et al. 2008).

The pattern and location of integration of a transgene is relevant both for fundamental and applied research. The analysis of transgenic wheat lines produced by particle bombardment has revealed that integration sites are generally randomly distributed across the genome and that transgene expression is more dependent on the identity of the promoter than on the integration site (Jackson et al. 2001). In barley also there does not appear to be any spatial preference for transgene insertion, irrespective of whether the transformation has been effected by particle bombardment or by means of *Agrobacterium* (Salvo-Garrido et al. 2004). However, a number of studies have shown that *Agrobacterium*-mediated gene transfer is more likely to produce low-copy transgenic insertions than does particle bombardment (e.g. Travella et al. 2005). Where multiple transgenes were inserted at one locus by *Agrobacterium*-mediated gene transfer, they appeared to be generally oriented in tandem with one another (Stahl et al. 2002).

### **16.1.2 Transgene Expression Systems**

Appropriate promoters capable of effectively driving transgene expression are of central importance for the genetic engineering of plants. Many of the promoters isolated and characterized to date have originated from dicotyledonous species such as *Arabidopsis thaliana*, and since the experience has been that most of these promoters are not effective in a monocotyledonous host, so the choice of promoters

for the cereals remains relatively limited. Ubiquitous transgene expression in the Triticeae is possible using either the maize *Ubi-1* or the *CaMV35S* promoter. Thus Stoeger et al. (1999) were able to show that the *GUS* gene directed by the *ZmUbi-1* promoter generates ubiquitous expression in both barley and wheat. A more detailed functional analysis of the *CaMV35S* promoter in wheat was carried out by Furtado and Henry (2005), taking advantage of *GFP* as a reporter gene. The actin promoter *Act1*, obtained from rice, is able to drive fairly strong ubiquitous expression in both barley and wheat (Vickers et al. 2006; Primavesi et al. 2008). While all three of these promoters produce ubiquitous expression, the level of transgene expression induced varies greatly both between cell types and between developmental stages, with zero detectable reporter gene expression observed in some particular cell types.

Since the composition and yield of grain is of such interest in applied cereal research, it is scarcely surprising that most of the promoter characterization research carried out to date in the Triticeae cereals has been concerned with grain-specific expression. For example, high levels of endosperm-specific transgene expression in barley and/or wheat could be obtained by exploiting promoters derived from endosperm storage protein genes such as rice *Glu-B1* (Patel et al. 2000; Xue et al. 2003; Huang et al. 2006), barley *Hor3-1* and *Hor2-4* (Cho et al. 1999; Patel et al. 2000) and the wheat high-molecular-weight glutenin subunit genes *Glu-A1-1*, *B1-1* and *D1-1* of wheat (Lamacchia et al. 2001; Schuenmann et al. 2002; Brinch-Pederson et al. 2003). The highest level of endosperm-specific expression in barley achieved to date has involved the oat *Glo1* promoter (Vickers et al. 2006). The barley aleurone-specific *High-pI α-amylase* promoter (Jensen et al. 1996; Nuutila et al. 1999; Matthews et al. 2001; Stahl et al. 2002) and the *trypsin inhibitor T1* (Joensuu et al. 2006) promoter have also been successfully used to drive transgene expression.

The entry point for most fungal pathogens is through the outermost cell layer of the plant, namely the epidermis, so it has been of some interest to identify promoters active in this tissue. Altpeter et al. (2005) showed that the wheat-derived *TaGsta1* promoter meets this requirement. In barley, its specificity was confirmed by patterns of *GUS* expression (Himmelbach et al. 2007). A further major target for pathogen attack in the cereals is the bract including the awn. Skadsen et al. (2002) demonstrated that the *HvLem1* promoter conducts expression in the bracts, and similar patterns of expression were induced by this promoter in wheat (Somleva et al. 2006). In contrast, the promoter of *HvLem2*, a second member of the same gene family, was strongly induced by exogenously applied salicylic acid and was preferentially expressed in the lemma, palea and coleoptile (Tilahun et al. 2006).

## 16.2 Tolerance to Abiotic Stress

Plants have evolved to combat episodes of environmental stress, which inevitably affect growth and reproduction. Although conventional breeding has succeeded in improving tolerance to such stresses to some degree, one of the major promises

of genetic engineering is the opening up of additional alleviation strategies. However, as yet only a small number of examples demonstrate how a transgenic approach can improve the tolerance of the Triticeae species to abiotic stress (see also Chap. 8).

### 16.2.1 Drought and Salinity

Drought represents the single most limiting environmental factor to crop productivity worldwide. Its occurrence is frequently associated with soil salinity, which occurs naturally but has all too often been induced by poor irrigation practices. Plants respond to drought and salinity in many ways. The engineering of stress tolerance requires the identification and functional analysis of the key genes involved in the plant response. Gene expression is altered by the imposition of stress, and some of the major changes involve the increased synthesis and accumulation of signal molecules, such as abscisic acid, osmotically active metabolites and proteins acting as scavengers of free oxygen radicals (Ramachandra-Reddy et al. 2004). Comparative transcriptome analyses have identified several genes as responding to abiotic stress (reviewed by Umezawa et al. 2006), and among these, transcription factors are of particular importance. These activate genes, or groups of genes, which encode proteins directly required for the stress response. In an attempt to transgenically enhance the drought-tolerance of wheat, the *A. thaliana* *DREB1A* transcription factor was ectopically expressed under control of the stress-induced promoter of the *AtRd29A* gene (Pellegrineschi et al. 2004). In a field experiment comparing transgenic and wild-type plants, the withholding of irrigation induced drought symptoms in the wild-type plants after just 10 days, while some of the transgenic plants only expressed these symptoms after 15 days.

A further drought response pathway involves the enhanced synthesis of “compatible solutes” or “osmoprotectants”, for example mannitol, proline and glycine-betaine (Sakamoto and Murata 2000). The presence of these molecules is thought to reduce water loss via their effect on cellular osmotic potential, an action which prolongs normal cell function during episodes of stress. The *Vigna aconitifolia* gene *P5CS* ( $\Delta^1$ -pyrroline-5-carboxylate synthase) encodes an enzyme required for proline biosynthesis and has been transgenically expressed in wheat under the control of a stress-induced promoter consisting of a regulatory ABA-responsive sequence coupled to a minimal actin promoter *Act-100* (Gruszka-Vendruscolo et al. 2007). Transgenic plants accumulated more proline in their leaves than did wild-type ones, and showed an enhanced level of tolerance to water deficit. Moreover, leaves of the transgenic lines generated less malondialdehyde, a marker for the presence of oxidative stress. Overall, it appeared that the higher than normal level of proline acted to attenuate membrane damage caused by lipid peroxidation. The cellular accumulation of mannitol has also been associated with enhanced abiotic stress tolerance. The *E. coli* gene *MtlD* (encoding mannitol-1-phosphatase dehydrogenase) is part of the mannitol biosynthesis pathway. When this gene was ectopically

expressed in wheat under the control of the *ZmUbi-1* promoter, the transgenic plants showed improved growth in the face of both drought and salinity (Abebe et al. 2003).

The late embryogenesis abundant (LEA) proteins represent a group of gene products significantly up-regulated upon exposure to abiotic stress, as well as during seed desiccation. Their function is thought to be associated with the protection from modification and degradation of mRNA, enzymes and lipids brought about by water deficit. Like many other LEA protein genes, *HvAI* is induced by ABA (Hong et al. 1988), and when this gene was ectopically expressed in wheat under the control of *ZmUbi-1* promoter, both biomass productivity and water use efficiency were enhanced in the presence of a controlled water deficit (Sivamani et al. 2000). These initial glasshouse-derived results were later confirmed in a series of field experiments (Bahieldin et al. 2005). Transporter and channel proteins are known to accumulate under water deficit conditions, and their role is believed to involve the stabilization of cell membrane integrity. The *A. thaliana* gene *NHX1* encodes a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter, and when ectopically expressed in wheat under the control of the *CaMV35S* promoter T1 seedlings had enhanced tolerance to salt stress, while T2 plants produced more biomass than their non-transgenic counterparts in the presence of salt stress. Under saline field conditions, the mean grain yield of T3 lines surpassed that of the non-transgenic controls (Xue et al. 2004).

### 16.2.2 Aluminium Toxicity

About 30% of arable land in the tropics and subtropics has acidic soil. The low pH encourages the solubility of the  $\text{Al}^{3+}$  ion, which is toxic to root growth and function. Plants counteract this challenge by accumulating organic acids (such as malic acid) in their roots, and these are able to detoxify  $\text{Al}^{3+}$  by chelation and subsequent secretion of the chelated complexes (Delhaize et al. 1993). The *TaALMT1* gene, which co-segregates with aluminium tolerance in wheat, was cloned by Saskia et al. (2004). This gene then was shown to encode a membrane-bound aluminium malate transporter. Barley is particularly sensitive to aluminium damage, so an attempt was made to constitutively express *TaALMT1*. The outcome of this experiment was that the rate of malate efflux from the roots was dependent on the concentration of aluminium ion in the hydroponic solution. Homozygous T2 plants were better able to grow in a high aluminium acidic soil than were their wild-type counterparts. In turn, the neutralization of the soil by application of  $\text{CaCO}_3$  resulted in comparable root growth in transgenic and non-transgenic lines (Delhaize et al. 2004).

## 16.3 Resistance to Fungal Infection

Besides the pre-formed constitutive barriers against pathogen infection, such as the cuticle and the cell wall, the resistance of plants against fungal attack requires the sensing of the presence of the pathogen by the plant, followed by a signalling

process which induces specific defence mechanisms. The expression of pathogenesis-related (PR) genes induced by the recognition of pathogen-associated molecular patterns (PAMPs) constitutes the basal layer of plant defence, which is generally effective against a broad spectrum of pathogens. However, the recognition of a fungal pathogen can be suppressed or evaded by the presence of fungal avirulence factors. Plants, in turn, have evolved race-specific resistance (R) proteins. Activation of the R genes typically results in a locally restricted hypersensitive reaction, in which cell death occurs rapidly around the infection site, thereby isolating the pathogen from the rest of the plant. The complex plant–pathogen interactions offer a multitude of opportunities to genetically engineer plants with improved resistance to fungal pathogens.

### 16.3.1 Regulators of Plant Defence

Key regulators which control an entire pathway of defence represent prime candidates for genetic engineering approaches, especially with a view to establishing broad spectrum and durable resistance. The term “systemic acquired resistance” (SAR) describes the resistance which develops throughout the whole plant following an earlier localized exposure to a pathogen. As the *A. thaliana* gene *NPR1* is a key regulator of SAR, an attempt was made to elevate the resistance of wheat to fusarium head blight (FHB) by ectopic expression of *AtNPR1* in a susceptible wheat host. The transgenic wheat showed an enhanced level of resistance to infection with *Fusarium graminearum*, which it was possible to associate with a more rapid and stronger expression of the defence protein PR1 (Makandar et al. 2006). Glutathione reductases (GRs) belong to the flavoprotein oxidoreductase family, and play a pivotal role in the plant’s response to oxidative stress which is often associated with pathogen attack. The Triticeae species *Haynaldia villosa* shows a particularly strong resistance to the powdery mildew pathogen, so was targeted as a source of GRs which could improve the level of resistance of wheat to this pathogen. When a particular *H. villosa* GR gene was constitutively expressed in a susceptible wheat, the resulting transgenic lines accumulated both *PR1* and *PR5* transcript associated with reduced susceptibility to the pathogen (Chen et al. 2007).

### 16.3.2 Pathogenesis-Related Proteins

The expression of the PR proteins is up-regulated upon the recognition of a pathogen (Bol et al. 1996). The PRs do not prevent the establishment of an interaction between the host and the pathogen, but rather attenuate fungal reproduction. Some PR proteins are thaumatin- or thionin-like, and others have glucanase or chitinase or peroxidase activity. The over-expression of some of these genes in

the Triticeae has achieved enhanced basal resistance to various pathogenic fungi. Chitinases and  $\beta$ -1,3-glucanases catalyse the hydrolysis of, respectively, chitin and glucan, which together represent the major structural components of the fungal cell wall. Many plant chitinases and glucanases possess anti-fungal activity in vitro (Cornelissen et al. 1996). The barley *chitinase II* gene constitutively expressed in wheat under the control of *ZmUbi-1* promoter improves the level of resistance to a number of fungal diseases, including powdery mildew, FHB and leaf rust under glasshouse conditions (Bliffeld et al. 1999; Oldach et al. 2001), and the FHB effect could be confirmed under field conditions (Shin et al. 2008). Similarly the expression of a barley  $\beta$ -1,3-glucanase gene in wheat also reduced the severity of FHB under both glasshouse and field conditions (Mackintosh et al. 2007).

Plant ribosome-inactivating proteins (RIPs) are translation inhibitors which impede protein elongation by depurinating conserved adenine residues of the 28S rRNA of the eukaryotic ribosome (Stirpe et al. 1992). RIPs which specifically inhibit other species' ribosomes have been recruited as a defence mechanism against certain pathogens (Jensen et al. 1999). Activity against various phytopathogenic fungi could be demonstrated in vitro by RIP30, which is present in the barley caryopsis (Leah et al. 1991). A transgenic wheat constitutively expressing *HvRIP30* displayed an enhanced level of resistance against powdery mildew (Bieri et al. 2000). In a similar approach, the use of maize *RIP b-32* resulted in attenuated disease symptoms in wheat infected by *F. culmorum* (Balconi et al. 2007).

Thaumatin, a molecule extracted from the African shrub *Thaumatooccus daniellii*, is used as a sucrose substitute in human food. Thaumatin-like proteins (TLPs, such as PR5) are produced in some plants as a response to either pathogen attack or osmotic stress. Their anti-fungal activity is based on an ability to destabilise fungal cell membranes. The constitutive expression of a rice *TLP* gene in barley and wheat gave enhanced resistance to *F. graminearum* under glasshouse conditions (Chen et al. 1999; Anand et al. 2003). Moreover, the constitutive expression of a barley *TLP* gene in wheat was able to reduce the severity of FHB in both the glasshouse and the field (Mackintosh et al. 2007). The TLPs and thionins (such as PR13) also interact with the fungal cell membrane. Mackintosh et al. (2007) have shown that over-expression of an endogenous  $\alpha$ -1-purothionin gene leads to a reduction in *F. graminearum* induced FHB severity in wheat.

In order to engineer durable resistance in Triticeae cereals, several attempts have been made to stack several transgenes in a single line. In one example, barley and rice *chitinase*,  $\beta$ -1,3-glucanase and *RIP* genes were co-expressed in wheat in various combinations, but the synergistic effects which had been expected from cognate experiments in dicotyledonous species were not statistically significant (Chen et al. 1999; Bieri et al. 2000, 2003). When Anand et al. (2003) co-expressed a *chitinase* and a  $\beta$ -1,3-glucanase gene cloned from the partially FHB resistant wheat cultivar "Sumai 3" in an FHB susceptible wheat, plants in which both transcripts were expressed showed delayed spread of FHB symptoms under glasshouse conditions. A recent report described the stacking of rice *TLP* and *chitinase*

genes in barley, but no pathology phenotypes are as yet available (Tobias et al. 2007). The over-expression of a wheat *peroxidase* (*TaPERO*) gene driven by the epidermis-specific *TaGstA1* promoter was attempted by Altpeter et al. (2005). The TaPERO protein is assumed to contribute to the modification of cell wall components. Experimentally, the transgenic lines showed an enhanced level of resistance to powdery mildew infection.

The phytoalexins are low-molecular, anti-microbial plant metabolites. In grape, groundnut and pine, reduced susceptibility to pathogen attack has been associated with high levels of resveratrol, the synthesis of which relies on the expression of stilbene synthase. The ectopic expression of the grape *Stilbene synthase* gene *VST1* under the control of its own promoter resulted in a heightened resistance of wheat to the necrotrophic fungus *Botrytis cinerea* (Leckband and Loerz 1998).

The wheat puroindolines (PINs) are endosperm-specific lipid-binding proteins with a unique tryptophan-rich domain, and have a pronounced effect on grain hardness, a trait which is of major importance for end-use quality. However, they also have been shown to exhibit anti-bacterial and anti-fungal activity (Jing et al. 2003). When the *PIN* genes of hexaploid wheat were constitutively expressed in a tetraploid (durum) wheat lacking any endogenous PIN, the appearance of leaf rust infection symptoms was delayed in the transgenic lines. In addition, when disease control was effected by fungicide application, the disease symptoms disappeared more rapidly in the transgenic lines than in the non-transgenic controls (Luo et al. 2008).

### 16.3.3 R Proteins

Most R proteins have a characteristic nucleotide-binding leucine-rich repeat (NB-LRR) domain. A typical example is barley *RPG1*, which mediates resistance against several isolates of stem rust. When the resistant barley cultivar "Morex" *RPG1* sequence was stably expressed under the control of its own promoter in the susceptible barley cultivar "Golden Promise", over-expressing lines showed a significantly improved level of stem rust resistance (Horvath et al. 2003). The constitutive over-expression in a susceptible wheat of *LR10*, a wheat R gene which interacts with leaf rust, resulted in an improved level of leaf rust resistance under both glass house and field conditions (Feuillet et al. 2003; Romeis et al. 2007). The endopolygalacturonases secreted by fungi act to degrade plant cell walls by cleavage of the  $\alpha$ -(1-4)-bond of D-galacturonic acid residues, and plants have evolved the so-called "polygalacturonase-inhibiting proteins" (PGIPs), which are cell wall glycoproteins able to inhibit this fungal enzyme activity. The PGIPs belong to a class of R proteins which have an extracellular LRR domain (Chisholm et al. 2006). When a common bean *PGIP* gene was constitutively expressed in wheat, Janni et al. (2008) observed an enhancement in the level of resistance to the fungus *Bipolaris sorokiniana*.

### 16.3.4 Fungal Proteins

FHB is a serious disease in barley and wheat, because many of the causative pathogens produce the trichothecene toxin deoxynivalenol (DON). The synthesis and catabolic pathways of trichothecene are well explored. The *TRI101* gene encodes a 3-OH-trichothecene acetyltransferase which catalyses the conversion of trichothecene into less toxic products. It is assumed that this reaction constitutes a protective mechanism for *F. graminearum* against damage from the activity of its own toxin (Kimura et al. 2003). When *F. sporotrichoides* *TRI101* was constitutively expressed in wheat and barley under control of the *ZmUbi-1* promoter, glasshouse grown plants were partially protected from FHB infection and the amount of DON accumulating in their grain was less than in comparable non-transgenic control plants. Under field conditions, however, no significant reduction in DON accumulation was recorded (Okubara et al. 2002, Manoharan et al. 2006).

### 16.3.5 Viral Proteins

Many fungi contain virus-like particles, some of which exhibit anti-fungal activity. The presence of persistent, non-infectious viral RNA sequences which encode the so-called “killing proteins” (KPs) is of considerable interest. In maize, some corn smut strains secrete KP4, a toxin which inhibits the development of competing fungi. The constitutive expression of recombinant *KP4* in transgenic wheat resulted in a significant decrease in the level of colonization in the grain by both loose smut and common bunt, which are both readily disseminated during harvest and grain storage (Clausen et al. 2000). The enhancement of resistance to common bunt was verified in a field trial by Schlaich et al. (2006), who were also able to show that the toxicity of KP4 is highly specific for *Ustilaginales* fungi, and was non-toxic for in vitro cultures of hamster or human cells. The presence of recombinant KP4 did not appear to interfere with any endogenous defence mechanisms in wheat.

## 16.4 Resistance to Viral Infection

Certain viruses can cause spectacular yield losses in the Triticeae cereals. To date, very little progress has been made in improving virus resistance in the Triticeae by transgenic approaches unlike the situation in dicots, such as potato. Little unambiguous evidence has been generated to demonstrate that virus load can be reduced by transgene expression. However, the ectopic expression in barley of the coat protein-encoding gene of Barley yellow dwarf virus (BYDV) and some other BYDV or

Cereal yellow dwarf virus genes did result in a reduction in virus titre and reduced disease symptoms. This resistance could not, unfortunately, be unequivocally associated with the presence of the transgene product, and no clear evidence of trait heritability has been provided (McGrath et al. 1997; Wang et al. 2001). Transgenic wheat lines expressing a viral coat protein or an RNA-dependent RNA polymerase sequence of Wheat streak mosaic virus were resistant to virus infection under glasshouse conditions, but the resistance phenotype was not reproducible under field conditions (Sharp et al. 2002).

## 16.5 Resistance to Insects

Widespread outbreaks of insect pests do not occur very frequently in Triticeae cereals, yet they can cause substantial crop losses. In order to enhance the insect resistance (see also Chap. 10) of wheat, Stoeger et al. (1999) generated transgenic lines which expressed the insecticidal lectin-encoding *GNA* gene from snowdrop (*Galanthus nivalis*). The transgene was directed either by the ubiquitous maize *Ubi-1* promoter or by the phloem-specific *sucrose synthase 1* promoter of rice. By means of an infestation bioassay using the grain aphid *Sitobion avenae* under glasshouse conditions, they showed that the generative reproduction of aphids on *GNA*-expressing plants was significantly reduced.

The barley trypsin inhibitor BTI-CMe, encoded by the *HvITR1* gene, is a protease inhibitor putatively involved in defence against herbivores. Purified gene product was shown to reduce the in vitro activity of trypsin and trypsin-like proteases of fall armyworm (*Spodoptera frugiperda*) gut extracts (Alfonso et al. 1997). Ubiquitous expression of the *ITR1* gene in wheat significantly inhibited the development and reduced the survival rate of Angoumois grain moth (*Sitotroga cerealella*) neonate larvae reared on mature grains. In contrast, survival or weight gain of the grasshopper *Melanoplus sanguinipes*, which is another important pest in cereals, was not significantly reduced upon feeding on CMe-expressing wheat leaves compared to administration of non-transgenic control leaves (Altpeter et al. 1999).

## 16.6 Grain Quality

Grain yield and quality depend on complex physiological processes occurring during plant development. However, protein content is a major determinant of the feed value of cereal grains. The protein content of wheat can be increased by conventional breeding, but not without compromising yield, so genetic engineering approaches have been suggested as having some potential in this context (see also Chap. 11). In an attempt to channel greater amounts of major substrates of protein

biosynthesis into the developing grain, the broad bean (*Vicia faba*) amino acid permease *AAP1* gene and the barley sucrose transporter *Sut1* gene were ectopically expressed in wheat caryopses. The grain of the resulting transgenic plants had a higher protein content under glasshouse conditions, and a field validation of this result is currently underway (Biosicherheit 2008).

Proteins which can enhance the availability of nutrients or facilitate the downstream processing of cereal grains are of particular interest. Xylans and glucans are major components of the cereal grain cell wall. Neither is readily digested by monogastric animals (such as poultry and pigs), because the monogastric intestine lacks the appropriate hydrolases (Bedford 1995). In addition, the high viscosity of solutions containing xylans and glucans inhibits the digestion and absorption of some of the other nutrients present, and so can result in a poor feed conversion ratio. A common solution to this problem is the supplementation of animal diets by the appropriate enzymes (Bedford 1995; Malathi and Devegowda 2001; Juanpere et al. 2004), but a transgenic approach, where the necessary recombinant hydrolases (glucanases, xylanases, phytases) are accumulated in the endosperm, is also possible (Jensen et al. 1996; Nuutila et al. 1999; Horvath et al. 2000; Patel et al. 2000; Xue et al. 2003). While those glucanases and xylanases can contribute to the digestibility of the grain, the availability of phosphorus, iron and zinc can be improved by the incorporation of a recombinant phytase from *Aspergillus niger* (Brinch-Pedersen et al. 2000).

Glucanases are also important for the malting and brewing processes. The ectopic expression of glucanases in the endosperm enhances not only the utilization of the glucans themselves, but also the accessibility of other grain constituents. Plant glucanases are irreversibly inactivated at temperatures above 55°C, which limits their relevance in conventional grain processing. Thus, thermo-tolerant enzymes obtained from fungi and bacteria are preferred for this application (Jensen et al. 1996; Nuutila et al. 1999). Kihara et al. (2000) ectopically expressed a thermostable  $\beta$ -amylase gene in barley, and this led to an improvement in kilning and mashing, as well as to increased fermentability later in the brewing process.

Baking quality in wheat depends strongly on the amount and composition of the endosperm storage proteins. Each of the high molecular weight glutenin subunit genes (*Glu-1*) encodes a pair of subunits, termed *x* and *y*. The gene encoding the A genome *Glu-1 x* subunit (1Ax1) was over-expressed in a wheat cultivar lacking this allele and, while mixing time, loaf volume and water absorbance were all improved in some of the transgenic lines, there were also substantial disturbances observed in the abundance of other storage protein fractions (Altpeter et al. 1996). Barro et al. (1997) undertook a similar analysis involving the D genome *Glu-1 x* and *y* subunits (respectively, 1Dx5 and 1Dy10) under the control of their own regulatory sequences. Some of the resulting transgenic lines had improved dough elasticity. Under field conditions, Shewry et al. (2006) were able to show that these transgenes were consistently expressed, without any negative effects on overall plant performance. Similar results have been obtained in both cereal rye (Wieser et al. 2005) and durum wheat (Gadaleta et al. 2008).

### 16.6.1 Production of Recombinant Proteins

Crop plants offer an inexpensive and convenient production system for high value recombinant molecules, an approach currently termed “molecular farming” (see Chaps. 12, 13). The Triticeae cereals have some specific advantages in this context (Ma et al. 2003). Firstly, the grain itself constitutes a natural storage organ, which can be composed of up to 20% protein. Second, breeding and cultivation of these cereals is well advanced, and commercial yields of up to 10 t/ha are not uncommon under intensive cultivation conditions. Third, long-term storage post-harvest is enabled by the low moisture content of the mature grain, which limits the exposure of recombinant proteins to degradation from hydrolases and proteases. The binding activity of a recombinant antibody expressed in the wheat endosperm remained stable over several months of grain storage (Stoeger et al. 2000; Brereton et al. 2007). Unlike microbial production systems, therefore, there is no requirement for low temperature and sterile storage conditions for the harvested product; and furthermore the processing of the recombinant protein can be de-coupled from its harvesting. Another advantageous feature relevant to downstream processing is the lack of phenolics in the grain, which can complicate extraction from other expression systems (Ma et al. 2003). Finally, from a biosafety viewpoint, since wheat and barley are both self-pollinating, the uncontrolled spread of transgenes by out-crossing can be relatively easily controlled by physical isolation (Commandeur et al. 2003).

Barley has emerged as a preferred expression system for recombinant proteins for industrial purposes. The *T. daniellii Thaumatin I* gene has been expressed in barley endosperm, driven by the *D-hordein* promoter, and a number of transgenic production lines have been grown in the field over several years (gmoinfo 2008). Stahl et al. (2002) generated transgenic barley plants expressing human antithrombin III,  $\alpha$ 1-antitrypsin and serum albumin. Some therapeutic proteins, such as the human proteins lactoferrin and lysozyme, which exhibit antibacterial, antifungal and antiviral effects, have already been produced from transgenic field-grown barley (Huang et al. 2006; Ventria Bioscience, Fort Collins, USA). In a rather different approach, a barley suspension cell-based expression system has been developed to determine the patterns of intracellular accumulation, and the structure and composition of barley-derived recombinant full-length human collagen I alpha I chain, in order to optimize constructs for monocot *in planta* expression (Ritala et al. 2008).

Recombinant antibodies can be used for diagnostic as well as for therapeutic purposes. The first stable expression in wheat of a medically relevant antibody concerned the single-chain antibody scFvT84.66, which recognizes carcinoembryonic antigen, a well characterized tumour-associated marker. Brereton et al. (2007) constitutively expressed in wheat two recombinant single chain antibody genes, whose gene products reduce the incidence of corneal graft rejection. The first report of a recombinant antibody-fusion protein which could be used directly as a medical-diagnostic assay was described by Schuenmann et al. (2002). This

antibody was expressed in the endosperm of barley, and is designed to replace a commercial diagnostic reagent for the detection of HIV-1 antibodies in human blood.

The administration of immunogenic proteins via the ingestion of transgenic barley grain is much simpler than having to use oral vaccination, although a vaccine produced in barley can be administered intravenously as well as orally. Joensuu et al. (2006) produced the F4 fimbrial subunit protein FaeG in transgenic barley endosperm. F4-positive enterotoxigenic *E. coli* strains are a frequent cause of porcine post-weaning diarrhoea. Orally applied FaeG induces a strong protective mucosal immune response. A second example of this technology has involved the production in barley endosperm of E2, the major envelope glycoprotein present on the surface of classic swine fever virus and the testing of the transgene product by oral vaccination (Maltagen, Andernach, Germany). A number of other animal and human growth factors are currently being commercially produced in barley (ORF Genetics, Reykjavik, Iceland).

The efficiency of molecular farming is highly dependent on the yield of recombinant protein in the plant. In addition to relying on effective endosperm-specific promoters, the expression of transgenes in barley has been further enhanced by modifying codon usage to better suit expression in barley (Jensen et al. 1996; Horvath et al. 2000; Xue et al. 2003). The efficient accumulation of transgene products also requires their targeting to sub-cellular storage compartments, such as protein bodies or protein storage vacuoles, by linking appropriate signal peptides to the coding sequence (Horvath et al. 2000). Brereton et al. (2007) have described how protein accumulation can be further increased by the ligation of an H/KDEL peptide tag to the C-terminal end of the transgene, as this ensures the retention of the gene product within the endoplasmic reticulum lumen, thereby minimizing protein loss via exocytosis. In barley and wheat, respectively, as much as 0.15 g/kg and 0.18 g/kg of recombinant antibody has been produced (Schuenmann et al. 2002; Brereton et al. 2007).

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# Chapter 17

## Fruit Crops

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### 17.1 Introduction

“An apple a day keeps the doctor away” is a popular adage which shows the importance of fruit crops for the human diet. It is a matter of common knowledge that a diet rich in fruits and vegetables and low in saturated fats is healthy and protective against cardiovascular diseases and certain cancers (Block et al. 1992; Ferro-Luzzi et al. 1994; Morris et al. 1994). The World Health Organization (1990; WHO) therefore recommends a daily intake of more than 400 g of vegetables and fruits per person. The worldwide mean daily fruit consumption of one person is approximately 170 g at the moment, which results in a total world consumption of about 390 million t year<sup>-1</sup> (exclusive of grapevine; <http://faostat.fao.org>; data from 2003; newer data not available). Fruit crop production is thus of particular economical importance. In 2007, a total of about 500 million t of fruit crops (exclusive of melons) were produced in the world on approximately 47 million ha (<http://faostat.fao.org>). Highly productive cultivars are one of the most important basic requirements which must be available to cover such a demand. Fruit breeders are therefore always in search of new genotypes, which are more productive, highly resistant and better adapted to existing environmental conditions. So far so good, but the breeding of fruit crops is never easy. Many of the agronomically important fruit crops are woody plants, characterized by a long juvenile period. This fact makes breeding cycles very time-consuming and expensive (Flachowsky et al. 2009). Directed gene transfer via *Agrobacterium tumefaciens* or particle bombardment could offer an exciting tool to overcome most of the existing problems of classic breeding. In recent years many protocols have been established for the transformation of nearly all economical important fruit crops. A number of field trials with

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GM fruit crops have been performed in Europe and in the United States, but only in two cases have GM fruit crops found their way into the market.

## 17.2 Temperate Fruit Crops

### 17.2.1 Top Fruit

#### 17.2.1.1 *Malus* Species (Apple)

The domesticated apple *Malus domestica* Borkh. is the most important pome-fruit worldwide. It ranked fourth within the fruit crops in 2006 behind bananas, grapes and citrus, with approximately 64 million t produced on 4.7 million ha (<http://faostat.fao.org>).

The genus *Malus* (apples) comprises approximately 55 species, including *M. domestica* Borkh. and numerous wild species (Phipps et al. 1990). Only the domesticated apple is economically important. All *Malus* species are grouped into infrageneric groups (section, series) and each species can be divided into intraspecific groups (cultivars; Harris et al. 2002; Qian et al. 2006). The taxonomy within the genus *Malus* is problematic, because of the intimate association that humans have with apples. Sometimes it is not as easy to distinct between wild and cultivated apples and hence between distinct categories (Harris et al. 2002).

Genetic transformation in apple and apple rootstocks was recently summarized by Bulley et al. (2007), Gessler and Patocchi (2007) and Dolgov and Hanke (2006). For general aspects of transformation see Chap. 1. The first report on apple transformation was published in 1989 by James et al. Since that time many protocols have been described for the transformation and regeneration of at least 24 apple scion cultivars, 12 rootstock cultivars and three apple wild species (Bulley et al. 2007; Dolgov and Hanke 2006; Szankowski et al. 2009). Initial studies were often focused on the improvement of the transformation efficiency by testing different transformation technologies using particle bombardment (Gercheva et al. 1994), *Agrobacterium tumefaciens* (James et al. 1989) and *A. rhizogenes* (Lambert and Tepfer 1991, 1992), respectively. Other studies were focused on the effect of different antibiotics on the regeneration, proliferation and morphogenesis of transgenic tissue (Norelli and Aldwinckle 1993; Yepes and Alwinckle 1994a, b). Different tissue, like leaf blades, stem internode explants or shoot apices were also tested to find out the best starting material for plant regeneration (Liu et al. 1998; Caboni et al. 2000). The most effective and reproducible method for apple regeneration has remained through adventitious shoot formation. In most cases in vitro plant leaves were inoculated with *A. tumefaciens* strains containing binary plasmid vectors with chimeric gene constructs. The transformation efficiency ranged between 0.02% and 20% (Bulley et al. 2007), depending on the apple genotype used for transformation, the type of tissue, the transformation method and the *A. tumefaciens* strain.

The selection of transgenic apple plants is still performed by using the *nptII* selectable marker gene and kanamycin as selective agent (see also Chap. 3). Only a few studies have been published that focus on alternative selection strategies. The *bar* gene of *Streptomyces hygroscopicus*, which confers resistance to phosphinothricin, was used several times (de Bondt et al. 1996; Dolgov and Skryabin 2004; Lebedev et al. 2002; Szankowski et al. 2003). The *manA* gene of *Escherichia coli* was also tested on apple (Degenhardt et al. 2006, 2007; Flachowsky et al. 2004; Zhu et al. 2004). The *manA* gene encodes for a phosphomannose-isomerase that catalyses the conversion of mannose-6-phosphate to fructose-6-phosphate. Transgenic cells expressing the *manA* gene can utilize mannose as a carbon source. Although it is good to have alternative selectable marker genes, especially in view of future field trials, the ultimate aim is a marker-free transgenic plant (Gessler and Patocchi 2007). Marker-free transgenic plants can be produced by using clean vector technologies or by transformation without marker genes. A first study using a clean vector system was recently published on apple (Krens et al. 2004). The transformation without the use of marker genes has been reported twice (Flachowsky et al. 2004; Malnoy et al. 2007a), but with different results. However, both technologies offer the possibility to produce marker-free GM plants.

Beside the establishment of the transformation technology, work has also focused on the improvement of agronomical important traits like resistance to biotic and abiotic stress and herbicides. Other studies have been engaged with self fertility, dwarfing, rooting ability or precocity. Furthermore much work was done to improve traits, which are related to the fruit (Table 17.1).

Much effort has been made to improve the resistance to fire blight caused by *Erwinia amylovora*. Initial studies were focused on transgenic expression of antimicrobial peptides (AMPs). In apple the AMPs attacin, lysozymes, and cecropin analogs were used. The results of these studies were summarized by Norelli et al. (1999). The best fire blight resistance was thereby observed with attacin E transgenic plants. However, the acceptance by growers and consumers of such GM plants is low, because of the bacterial and animal origin of the transferred genes (Norelli et al. 2003). Recent studies on GM apple plants were mainly focused on promoting plant defense reactions. The theory of this approach is based on the fact that *E. amylovora* secretes effector proteins into plant host cells, which are involved into suppressing host defense responses, redirecting normal host metabolism to facilitate pathogen multiplication and initiating cell necrosis. Different effector proteins (e.g. *hrpN*, *eop1*, *hopC\_Ea*) were overexpressed in apple to simulate an infection and to induce defense mechanisms. Other strategies were based on silencing of apple genes (e.g. *HIPM*, *DIPM*), which interact with the effector proteins or on overexpression of apple genes (e.g. *MdNPR1*, *mbr4*) involved in the pathogen defense (for method, see Chap. 5). All strategies were successful, but most acceptable to consumers and growers are likely to be alterations in the expression of apple genes, resulting in enhanced resistance. No fire blight resistance gene has yet been isolated from resistant apple wild species until now, but several scientific groups are working on it.

Similar strategies as for fire blight were used to improve the resistance to apple scab. Beside genes of the biocontrol organism *Trichoderma harzianum*, AMPs from

**Table 17.1** Summary on studies conducted on the transformation of apple and apple rootstocks for agronomically important traits. Overexpr. Overexpression

Selected trait	Genes used	Type of expression	Reference <sup>1</sup>
Insect resistance	<i>E. postvittana</i>		
	pPLA2 ( <i>chicken avidin</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	pSAV2α ( <i>S. avidinii</i> streptavidin)	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	<i>Na-PI</i> ( <i>N. alata</i> )	Overexpr.	Maheswaran et al. (2007)
	<i>C. pomonella</i>	Overexpr.	
	<i>CpTI</i> ( <i>V. unguiculata</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
Fungal resistance	<i>cryIA(c)</i> ( <i>B. thuringiensis</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	<i>V. inaequalis</i>		
	<i>MpNPR1</i> ( <i>M. domestica</i> )	Overexpr.	Bulley et al. (2007), Malnoy et al. (2007b)
	<i>ech42</i> ( <i>T. harzianum</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	<i>Nag 70</i> ( <i>T. harzianum</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	<i>SB-37</i> ( <i>H. cecropia</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
Bacterial resistance	<i>AttE</i> ( <i>H. cecropia</i> )	Overexpr.	Gessler and Patocchi (2007)
	HEWL (hen egg white lysozyme)	Overexpr.	Gessler and Patocchi (2007)
	T4 lysozyme (bacteriophage T4)	Overexpr.	Gessler and Patocchi (2007)
	<i>Rs AFP2</i> ( <i>R. sativus</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007), Dolgov and Hanke (2006)
	<i>Ace AMP1</i> ( <i>A. cepa</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	<i>PinB</i> ( <i>T. aestivum</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007)
	<i>Hcvfl</i> , 2 and 4 ( <i>M. floribunda</i> )	Overexpr.	Gessler and Patocchi 2007, Bulley et al. 2007
	<i>G. juniperi-virginianae</i>		
	<i>MpNPR1</i> ( <i>M. domestica</i> )	Overexpr.	Bulley et al. (2007), Malnoy et al. (2007b)
	<i>E. amylovora</i>		
	<i>SB-37</i> ( <i>H. cecropia</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007), Dolgov and Hanke 2006
	<i>Shiva 1</i> ( <i>H. cecropia</i> )	Overexpr.	Bulley et al. (2007), Dolgov and Hanke (2006)
	<i>AttA, AttE</i> ( <i>H. cecropia</i> )	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007), Dolgov and Hanke (2006)
	T4 lysozyme (bacteriophage T4)	Overexpr.	Gessler and Patocchi (2007), Bulley et al. (2007), Dolgov and Hanke (2006)

(continued)

**Table 17.1** (continued)

Selected trait	Genes used	Type of expression	Reference <sup>1</sup>
	HEWL (hen egg white lysozyme)	Overexpr.	Gessler and Patocchi <a href="#">(2007)</a> , Dolgov and Hanke <a href="#">(2006)</a>
	<i>Dpo</i> (bacteriophage ΦEa1h)	Overexpr.	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a> , Dolgov and Hanke <a href="#">(2006)</a> , Flachowsky et al. <a href="#">(2008b)</a>
	<i>hrpN</i> ( <i>E. amylovora</i> )	Overexpr.	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a>
	<i>eop1</i> ( <i>E. amylovora</i> )	Overexpr.	Lalli et al. <a href="#">(2008)</a>
	<i>hopC_Ea</i> ( <i>E. amylovora</i> )	Overexpr.	Lalli et al. <a href="#">(2008)</a>
	<i>DspF</i> ( <i>E. amylovora</i> )	Overexpr.	Malnoy et al. <a href="#">(2008a)</a>
	<i>MpNPR1</i> ( <i>M. domestica</i> )	Overexpr.	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a> , Malnoy et al. <a href="#">(2007b)</a>
	<i>mbr4</i> ( <i>M. baccata</i> )	Overexpr.	Flachowsky et al. <a href="#">(2008c)</a>
	<i>DIPM</i> ( <i>M. domestica</i> )	Silencing	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a>
	<i>HIPM</i> ( <i>M. domestica</i> )	Silencing	Malnoy et al. <a href="#">(2008b)</a>
	<i>A. tumefaciens</i>		
	<i>iaaM, ipt</i> ( <i>A. tumefaciens</i> )	Silencing	Bulley et al. <a href="#">(2007)</a>
Stress resistance	Heat, drought, cold and UV-B		
	Cytosolic ascorbate peroxidase (pea)	Overexpr.	Bulley et al. <a href="#">(2007)</a>
	<i>Osmyb4</i> ( <i>O. sativa</i> )	Overexpr.	Pasquali et al. <a href="#">(2008)</a>
	Zinc deficiency		
	<i>ZNT1</i>	Overexpr.	Swietlik et al. <a href="#">(2007)</a>
	<i>ZIP4</i>	Overexpr.	Swietlik et al. <a href="#">(2007)</a>
	Iron deficiency		
	<i>LeIRT2</i> ( <i>L. esculentum</i> )	Overexpr.	Bulley et al. <a href="#">(2007)</a>
Herbicide resistance	Basta		
	<i>bar</i> ( <i>S. hygroscopicus</i> )	Overexpr.	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a> , Dolgov and Hanke <a href="#">(2006)</a>
Self fertility	S3, S5 (S-alleles of <i>M. domestica</i> )	Silencing	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a>
Dwarfing and rooting ability	<i>PhyB</i> ( <i>A. thaliana</i> )	Overexpr.	Bulley et al. <a href="#">(2007)</a> , Dolgov and Hanke <a href="#">(2006)</a>
	<i>rolA, rolB, rolC</i> ( <i>A. rhizogenes</i> )	Overexpr.	Gessler and Patocchi <a href="#">(2007)</a> , Bulley et al. <a href="#">(2007)</a> , Dolgov and Hanke <a href="#">(2006)</a>
	GA 20-oxidase ( <i>M. domestica</i> )	Silencing	Bulley et al. <a href="#">(2007)</a>
	<i>gai</i> ( <i>A. thaliana</i> )	Overexpr.	Dolgov and Hanke <a href="#">(2006)</a> , Zhu et al. <a href="#">(2008)</a>
Precocity	<i>BpMADS4</i> ( <i>B. pendula</i> )	Overexpr.	Bulley et al. <a href="#">(2007)</a> , Hanke et al. <a href="#">(2007)</a> , Flachowsky et al. <a href="#">(2009)</a>
	<i>API</i> ( <i>A. thaliana</i> )	Overexpr.	Hanke et al. <a href="#">(2007)</a> , Flachowsky et al. <a href="#">(2009)</a>

*(continued)*

**Table 17.1** (continued)

Selected trait	Genes used	Type of expression	Reference <sup>1</sup>
	<i>LFY</i> ( <i>A. thaliana</i> )	Overexpr.	Hanke et al. (2007), Flachowsky et al. (2009)
	<i>MdMADS5</i> ( <i>M. domestica</i> )	Overexpr.	Hanke et al. (2007), Flachowsky et al. (2009)
	<i>MdAPI</i> ( <i>M. domestica</i> )	Overexpr.	Hanke et al. (2007), Flachowsky et al. (2009)
	<i>AFL1</i> and <i>AFL2</i> ( <i>M. domestica</i> )	Overexpr.	Hanke et al. (2007), Flachowsky et al. (2009)
	<i>MdTFL1</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007), Flachowsky et al. (2009), Hanke et al. (2007)
Traits related to the fruit	Flavor		
	Thaumatin II protein ( <i>T. daniellii</i> )	Overexpr.	Bulley et al. (2007), Gessler and Patocchi (2007)
	<i>S6PDH</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007), Gessler and Patocchi (2007)
	Fruit ripening		
	<i>ACS</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007), Gessler and Patocchi (2007)
	<i>ACO</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007), Gessler and Patocchi (2007)
	<i>MdPG</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007)
	Reduced browning potential		
	<i>PPO</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007), Gessler and Patocchi (2007)
	Color and health properties		
	<i>Vst1</i> ( <i>V. vinifera</i> )	Overexpr.	Bulley et al. (2007)
	<i>Lc</i> ( <i>Z. mays</i> )	Overexpr.	Li et al. (2007)
	<i>MdMYB10</i> ( <i>Malus</i> ssp.)	Overexpr.	Bulley et al. (2007)
	<i>MdANS</i> ( <i>Malus</i> ssp.)	Silencing	Szankowski et al. (2009)
	Allergens		
	<i>Mal d1</i> ( <i>M. domestica</i> )	Silencing	Bulley et al. (2007), Gessler and Patocchi (2007)

<sup>1</sup>References include review articles (if available) and original research articles

insects and other plant species as well as genes involved in the pathogen defense were expressed in apple. In contrast to fire blight, the first scab resistance gene of apple (*HcrVf2* of *M. floribunda* 821) has been isolated and transformed (Gessler et al. 2009). This gene should now be used in combination with a clean vector system to produce cisgenic scab-resistant apple varieties.

Another promising strategy was recently published by Flachowsky et al. (2009). A breeding program has been established at the Institute of Breeding Research on Horticultural and Fruit Crops (Dresden-Pillnitz, Germany), which uses transgenic early flowering apple plants (Fig. 17.1) to speed-up breeding cycles. Using this system one crossbred generation per year is feasible. This system can be used to introgress resistance genes from apple wild species into the cultivated apple by natural crossing, to realize several backcross generations in a few years and to



**Fig. 17.1** *BpMADS4* transgenic apple seedling. First flowers were obtained approximately four months after seeding. The seedling was obtained after crossing a F1 plant of the cross T1190 (*BpMADS4* transgenic line of the apple cv. ‘Pinova’; Flachowsky et al. 2007) by *M. fusca* (fire blight resistant apple wild species) and the scab resistant apple cv. ‘Topaz’

produce a new and highly resistant apple cultivar, free from any transgenic sequences, within a manageable amount of time.

With the focus on biosafety research several studies have been performed on transgene stability (Briviba et al. 2004; Flachowsky et al. 2008a; Reim and Hanke 2004; Zhu et al. 2007), inheritance of the transgenic trait (Flachowsky et al. 2009; James et al. 1995), pollen fertility (Du et al. 2007) and gene flow (Reim et al. 2006; Soejima et al. 2007).

Since the early 1990s, many field trials with GM apples have been performed worldwide, but no GM apple cultivar is yet on the market, neither in the United States nor in Europe.

A total of 47 field test records were found for the United States within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>).

Most of these were focused on GM apple plants with improved fruit quality (fruit ripening, low-browning, storability) or improved resistance to insects or bacterial and fungal diseases. Twelve out of the 47 field test records were focused on plants with altered polyphenol oxidase (PPO) levels. Especially Okanagan Specialty Fruits (Summerland, B.C., Canada) is working on the development of new apple varieties by genetic modification. This company has developed a low-browning GM apple for processing and apple chip production through silencing of PPO genes. The commercialization of the first PPO silenced low-browning apple cultivar is planned for 2009/2010 (<http://www.okspecialtyfruits.com>).

In Europe, releases of GM plants have to be notified according to Directive 2001/18/EC. A total of nine summary notifications can be found for apple (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>): four from the Netherlands, two from Belgium, two from Sweden and one from Germany. GM apples are still quite a long way from commercial use in Europe.

However, scientific groups in Italy and in the Switzerland are working on the development of cisgenic (see Chaps. 4, 6) apple plants with improved resistance to apple scab using the *HcrVf2* resistance gene from the crab-apple *Malus floribunda* 821. A nearly identical project was started in the Netherlands. The Plant Research International (PRI), in cooperation with the private fruit breeding company Inova Fruit BV and two further partners, is also working on the development of *HcrVf2* cisgenic apple varieties. Both projects are promising, but the commercialization of the first cisgenic apple cultivar in Europe is expected in 2013 at the earliest. Further objectives of ongoing projects are the development of low allergenic GM apple cultivars by silencing the major apple allergen *Mal d1* and cisgenic apple cultivars with an increased amount of healthy compounds through up-regulation of the flavonoid biosynthesis using the recently identified *MYB10* transcription factor of apple.

### 17.2.1.2 *Pyrus* Species (Pear)

The genus *Pyrus* belongs to the subfamily *Maloideae* of the *Rosaceae* and comprises at least 22 species, which are distributed in East and West Asia, Europe and Africa. Commercially used are mainly the two pear species *P. communis* L., the European pear, and *P. pyrifolia* (Burm.) Nakai (= *P. serotina* Rehder), the Japanese pear or 'nashi' (Katayama and Uematsu 2003; Monte-Corvo et al. 2000; Oliveira et al. 1999). Pears ranked at eighth within the fruit crops in 2006 with about 20 million t produced on 1.7 million ha (<http://faostat.fao.org>). More than 80 countries produce pears, with China, Italy, USA, Spain and Argentina as the main producers. The lion's share (60%) of the world's production is produced in China.

Since the beginning of pear transformation many protocols have been published for *Agrobacterium*-mediated transformation and transgenic plant regeneration from in vitro leaves (Merkulov et al. 1998; Mourguet et al. 1996; Yancheva et al. 2006), axillary shoot-meristem explants (Matsuda et al. 2005) and cotyledons (Kaneyoshi et al. 2001).

The selection of transgenic pear plants is still performed by using the *nptII* selectable marker gene. A few other marker genes like the *bar* gene of *Streptomyces hygroscopicus* or the *Vr-ERE* of *Vigna radiata* have been tested on pear, but their efficiency was too low (Chevreau et al. 2007; Lebedev et al. 2002a). An increased transformation frequency compared to *nptII* was found in transformation experiments on a pear rootstock using the *hpt* gene for selection, which confers resistance to hygromycin (Lebedev and Dolgov 2000). However, the dream for the future is a GM pear plant which is free from any marker genes.

Equally important as the establishment of highly efficient transformation protocols on different pear species and cultivars are studies which were focused on the improvement of agronomical important traits (Table 17.2). As in apple, pears are affected by fire blight. Improvement of fire blight resistance is therefore one of the most important aims of research. Beside this, the resistance to abiotic stress and fungal diseases, the improvement of flavor and the delay of maturity are also traits of interest. Dwarfing and improvement of rooting ability are traits, which are of importance for pear rootstocks.

Since the early 1990s, several field trials with GM pears have been performed worldwide. A total of five field test records were found for the United States within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field test records were focused on GM pears with altered fruit ripening and improved resistance to fire blight.

**Table 17.2** Summary on studies conducted on the transformation of *Pyrus* for agronomically important traits

Selected trait	Genes used	Type of expression	Reference <sup>1</sup>
Fungal resistance	<i>Rs-AFP2</i> ( <i>R. sativus</i> )	Overexpr.	Lebedev et al. (2002b)
Fire blight resistance	<i>attE</i> ( <i>H. cecropia</i> )	Overexpr.	Petri and Burgos (2005)
	<i>SB-37</i> ( <i>H. cecropia</i> )	Overexpr.	Reynoird et al. 1999
	<i>Shiva-1</i> ( <i>H. cecropia</i> )	Overexpr.	Mourguès et al. 1999
	bovine lactoferrin	Overexpr.	Malnoy et al. 2003
	<i>dpo</i> (bacteriophages	Overexpr.	Malnoy et al. 2005a
	$\Phi$ Ea1h) <i>hrpN</i>	Overexpr.	Malnoy et al. 2005b
	( <i>E. amylovora</i> ) <i>hrpN</i>	Overexpr.	Malnoy et al. 2008
	( <i>E. amylovora</i> )		
Traits related to the fruit	Flavor: thaumatin II protein ( <i>T. daniellii</i> )	Overexpr.	Lebedev et al. (2002c)
	Fruit ripening: <i>ACO</i> (pear)	Overexpr. Silencing	Gao et al. (2007) Murayama et al. (2003)
	Health properties: <i>Vst1</i> ( <i>V. vinifera</i> )	Overexpr.	Flaishman et al. (2005)
Abiotic stress resistance	<i>MdPDS1</i> ( <i>M. domestica</i> )	Overexpr.	Wen et al. (2008), He et al. (2008)
Dwarfing and rooting ability	<i>rolB</i> , <i>rolC</i> ( <i>A. rhizogenes</i> )	Overexpr.	Petri and Burgos (2005)

<sup>1</sup>References include review articles (if available) and original research articles

For Europe only one summary notification was found for pear, which was carried out in Sweden with plants with improved rooting ability (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>). GM pears are still quite a long way from commercial use. A commercial use of GM pears is not to be expected for the next years.

### 17.2.1.3 *Prunus* Species (Almond, Apricot, Sweet and Sour Cherry, Cherry Rootstocks, Peach, Plum)

The genus *Prunus* comprises several important stone fruit and nut species, including almond (*P. dulcis* Mill.), apricot (*P. armeniaca* L.), sweet and sour cherry (*P. avium* L. and *P. cerasus* L.), peach [*P. persica* (L.) Batsch] and plum (*P. domestica* L., *P. salicina* Lindl.). The economically most important stone fruit species are peach and plum, which ranked 10th and 12th within the fruit crops in 2006, with approximately 18 million t and 9.7 million t produced on 1.5 million ha and 2.3 million ha, respectively (<http://faostat.fao.org>).

Since the early 1990s many protocols have been published for the regeneration of adventitious shoots from various *Prunus* explants. Good overviews about this work are given by Burgos et al. (2007) and Canli and Tian (2008). Protocols for regeneration from leaf tissue are available for plum, almond, sour and sweet cherry as well as for wild cherry and black cherry. Regeneration of adventitious shoots from immature cotyledons was described for apricot, peach, plum, sour cherry and almond. Further studies have described the regeneration of peach, sweet and ornamental cherries using mature cotyledons or the regeneration of plum using hypocotyl slices (Burgos et al. 2007; Canli and Tian 2008).

Genetic transformation of *Prunus* species was recently summarized by Burgos et al. (2007), Petri and Burgos (2005) and Scorza and Ravelonandro (2006). Protocols for genetic transformation have been reported for plum (Mante et al. 1991; Petri et al. 2008a), almond (Miguel and Oliveira 1999), sour cherry (Dolgov and Firsov 1999; Song and Sink 2005), apricot (Laimer da Câmara Machado et al. 1992; Petri et al. 2008b) and peach (Padilla et al. 2006; Pérez-Clemente et al. 2004; Wu et al. 2006). The selection of GM *Prunus* plants was mostly performed using the *nptII* or the *hpt* gene as selectable marker genes, which confer resistance to kanamycin and hygromycin, respectively (Burgos et al. 2007). Only a few studies have been published that focused on alternative selection strategies such as the *bar* gene of *Streptomyces hygroscopicus* (Druart et al. 1998) or the *manA* gene of *Escherichia coli* (Ramesh et al. 2006). The mannose/*pmi* selection system was tested on almond and compared to the traditionally used *nptII*/kanamycin selection system (Ramesh et al. 2006). The transformation efficiency was higher with the mannose/*pmi* system (5.6% for kanamycin, 6.8% for mannose/*pmi*), which led to the conclusion that the mannose/*pmi* system could be a usable tool to avoid the *nptII*/kanamycin selection system.

Most studies on GM *Prunus* plants have been concerned with the establishment and improvement of regeneration and transformation protocols using *Agrobacterium tumefaciens*. A few studies have been performed on *Prunus* rootstocks using *A. rhizogenes* for transformation (Gutiérrez-Pesce et al. 1998;

**Table 17.3** Summary on selected studies conducted on the transformation of *Prunus* for agronomically important traits

Selected trait	Genes used	Species	Type of expression	Reference <sup>1</sup>
Virus resistance	Sharka resistance (plum pox virus)	Apricots	Overexpr.	Petri and Burgos (2005), Scorza and Ravelonandro (2006)
	cpPPV (plum pox virus)	Plum	Overexpr.	Petri and Burgos (2005)
	PRSV (papaya ring-spot virus)	Plum	Overexpr.	Petri and Burgos (2005)
Cold resistance	<i>afp</i> ( <i>P. americanus</i> )	Sweet cherry	Overexpr.	Petri and Burgos (2005)
Herbicide resistance	<i>bar</i> ( <i>S. hygroscopicus</i> )	Cherry rootstocks	Overexpr.	Petri and Burgos (2005)
Altered habit	<i>ipt</i> ( <i>A. tumefaciens</i> )	Peach	Overexpr.	Petri and Burgos (2005)
Nematode resistance	<i>Meloidogyne incognita</i> <i>gafp-1</i> (antifungal protein from <i>G. elata</i> )	Plum	Overexpr	Nagel et al. (2008)
Delayed maturity	<i>ACO</i> ( <i>P. persica</i> )	Peach	Silencing	Wu et al. (2006)
Functional genomics	<i>PDS</i> ( <i>P. armeniaca</i> )	Plum	Silencing	Petri et al. (2008a)

<sup>1</sup>References include review articles (if available) and original research articles

Gutiérrez-Pesce and Rugini 2004). Nevertheless, several studies were published, which were focused on the production of GM plants with improved agronomically important traits using *A. tumefaciens*-mediated transformation. Traits of interest were virus, nematode or cold resistance, herbicide resistance and altered tree habit. GM plants were also produced with focus on studies of gene functions in the field of functional genomics (Table 17.3).

The agronomically most important trait in *Prunus* is virus resistance. Especially the *Plum pox virus* (PPV), the etiological agent of sharka, is one of the most devastating diseases of stone fruits. The sharka disease is responsible for extensive economic losses (Németh 1994; Roy and Smith 1994) and the PPV virus has quarantine status in many countries (Scorza and Ravelonandro 2006). Alone in Europe there are about 100 million stone fruit trees currently infected with the virus (Kegler and Hartmann 1998). The breeding of sharka resistant *Prunus* trees is not as easy because of the polygenic and strain-specific nature of the PPV resistance and the long juvenile period of seedlings. Genetic engineering offers an exciting tool to overcome these problems and during the past decade several studies have been published in which researchers have tried to introduce resistance to PPV into apricots and plums via direct gene transfer.

Previous studies were performed on GM apricot and plum plants overexpressing coat proteins of the Plum pox virus (Laimer da Câmara Machado et al. 1992; Scorza et al. 1994) or the papaya ring-spot virus (Scorza et al. 1995a, b). From GM plum plants overexpressing the PPV coat protein, the line C5 (today named ‘Honey Sweet’) was selected, because of the high level of resistance. This line contains a multicopy insert of the *cpPPV* gene. The expression level of this gene is reduced in

C5 as a follow of post-transcriptional gene silencing (PTGS, reviewed by Scorza and Ravelonandro 2006; see also Chap. 5). Based on inoculation studies, it was found that C5 is highly resistant to the major serotypes of PPV. The stability and durability of the PTGS-based PPV resistance of C5 was tested in field trials in different countries for several years (Fuchs et al. 2007; Hily et al. 2004; Malinowski et al. 2006). Based on the results obtained from the numerous studies on C5, Scorza and Ravelonandro (2006) concluded that PTGS-based strategies could be used in future approaches to produce PPV-resistant stone fruits.

First PTGS-based strategies for resistance to PPV were recently tested in heterologous systems (Nicola-Negri et al. 2005; Zhang et al. 2006). Different PPV-specific hairpin constructs were developed and evaluated for their effect on the PPV resistance in transgenic *N. benthamiana* plants. The majority of the transgenic lines were significantly less susceptible than control plants. The silencing constructs will now be tested on different *Prunus* species. A similar strategy to induce multivirus resistance in *Prunus* was recently published by Liu et al. (2007). The authors created a chimeric gene (*PTRAP6*) by fusion of gene fragments (400–500 bp) from six major *Prunus* fruit viruses (American plum line pattern virus, peach mosaic virus, plum pox virus, prune dwarf virus, prunus necrotic ringspot virus, tomato ringspot virus). Using this chimeric gene, a hairpin construct (*PTRAP6i*) was developed and constitutively overexpressed in *N. benthamiana* plants. Tests on transgenic plants of homozygous R<sub>3</sub> generation lines with three out of the six viruses presented evidence that transgenic expression of *PTRAP6i* could be a powerful tool to produce virus-resistant *Prunus* fruit trees.

Since the early 1990s several field trials with GM plums and cherries have been performed. For the United States a total of seven field test records were found for GM plums within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field tests were focused on plants with a reduced juvenile stage, delayed maturity, improved resistance to nematodes or fungal and virus diseases. A first petition for deregulation of a GM fruit tree was approved in the United States in June 2007. The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) excluded the GM plum line C5 ‘Honey Sweet’ from the regulations at 7 CFR part 340 (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>).

In Canada two field trials have been performed with GM cherries, which were focused on trees with improved fruit quality (<http://www.gmo-compass.org/eng/home/>).

In Europe, a total of five and three summary notifications can be found for GM plums and cherries, respectively (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>). Field trials with GM plums were performed in Spain (two), in Poland (one), in the Czech Republic (one) and in Romania (one). All plum field trials were focused on PPV resistant plants. Three field trials with GM cherries have been performed in Italy. These field trials were carried out with plants with a better rooting ability. However, GM plums and GM cherries are still quite a long way from commercial use in Europe. A commercial use of GM plums and GM cherries in Europe is not to be expected in the long run.

## 17.2.2 Small Fruit

### 17.2.2.1 *Fragaria* Species (Strawberry)

The genus *Fragaria* consists of approximately 20 species. The majority of these species are diploid. Commercially important is the octoploid species *Fragaria x ananassa* Duch. (Hadonou et al. 2004; Sargeant et al. 2003). In 2006 approximately 3.9 million of strawberry fruits were produced on 263 000 ha worldwide (<http://faostat.fao.org>).

The cultivated strawberry (*F. x ananassa*) is a rapidly growing herbaceous perennial with a small genome, short reproductive cycle and facile vegetative and generative propagation for genetic transformation. The development of in vitro regeneration systems using a range of explants, including leaves, petioles, peduncle tissue, sepals, stipules, roots, runners, ovaries, protoplasts, stems and callus, and culture conditions has opened up the opportunity for strawberry improvement through genetic engineering (summarized by Mercado et al. 2007b).

Recently, the state of the art in strawberry transformation was reviewed by several authors (Debnath and Teixeira da Silva 2007; Folta and Davis 2006; Folta and Dhingra 2006; Graham 2005; Qin et al. 2008; Quesada et al. 2007). Enormous advances have been made in strawberry genetic transformation since the first transgenic plants were obtained in 1990 by two independent groups (James et al. 1990; Nehra et al. 1990). Besides *F.x ananassa*, transformation systems were also developed for related species, like *F. vesca* (Alsheikh et al. 2002; El-Mansouri et al. 1996; Haymes and Davis 1998; Oosumi et al. 2006; Zhao et al. 2004) and *F. moschata* (Mezzetti et al. 2002a).

The most important approach in strawberry relies on *Agrobacterium tumefaciens*-mediated leaf disk transformation (Barcelo et al. 1998; du Plessis and Brand 1997; Gruchala et al. 2004; Martinelli et al. 1996; Mathews et al. 1995a, b; Mezzetti 2003; Ricardo et al. 2000). Direct gene delivery into protoplast by electroporation was also reported (Nyman and Wallin 1988). A combined *Agrobacterium*-biolistic method was described later (Cordero de Mesa and Jimenez-Bermudez 2000). A new methodology to produce transgenic strawberries was developed using a temporary immersion bioreactor system (Hanhineva and Karenlampi 2007). Regardless of the sufficient regeneration levels achieved from leaf explants, the regeneration of transformed strawberry plants remains difficult and seems to be strongly genotype dependent. Since the 1990s, reliable protocols using *Agrobacterium tumefaciens*-mediated transformation were established for several commercial cultivars. Detailed surveys of literature are given by Mezzetti (2003) and Mezzetti and Constantini (2006). The effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional marker gene was recently described by Schaart (2004). There are several promoter studies in strawberry. A tissue specific expression using the floral binding protein 7 promoter from *Petunia* was used by Schaart et al. (2002). Transgene expression driven by a heterologous phloem-specific promoter was

published by Zhao et al. (2004). Agius et al. (2005) used a transient expression system to conduct a functional analysis of homologous and heterologous promoters in fruit. A near root-specific promoter was described recently (Vaughan et al. 2006). Transformation studies in strawberry are focused on modification of selected traits (Table 17.4).

There are a few studies related to environmental risk assessment of transgenic plants (see Chap. 27) in strawberry. The formation of chimeras during transformation has been reported in strawberry by several authors (Mathews et al. 1998; Monticelli et al. 2002) and is considered to be one of the major problems for strawberry transformation. Abdal-Aziz et al. (2006) described high frequencies of non-T-DNA sequence integrations in transgenic strawberry plants obtained through *Agrobacterium* transformation. An environmental risk evaluation of transgenic strawberry expressing a rice chitinase gene was performed in greenhouse, semi-greenhouse and field and revealed no effect on other plants, microflora, morphological characteristics and yield (Asao et al. 2003). The sexual transmission of transgenes to R1 generation progeny was reported for *F. x ananassa* (James et al. 1995) and for *F. vesca* (Haymes and Davis 1998).

The fact that the garden strawberry *Fragaria x ananassa* contains an octoploid genome made it difficult to use this species as a model for molecular studies and the interpretation of the transformation events. The wild strawberry *F. vesca* that contains a diploid genome represents an ideal model for functional genomics research in Rosaceae (Oosumi et al. 2006). However, recently transformation protocols were developed for a rapid-cycling genotype LF9 of *F. x ananassa* which allows high-throughput studies of gene function in the octoploid genetic background (Folta et al. 2006).

Transformation in strawberry is also used to study the function of genes, especially those related to fruit ripening (Hoffmann et al. 2006).

Several field trials with GM strawberries have been performed in the United States and in Europe. For the United States a total of 42 field test records were found within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field test records were focused on plants with improved resistance to herbicides and fungal diseases, respectively or on plants with altered agronomic properties.

In Europe, a total of eight summary notifications can be found for GM strawberries (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>). Summary notifications for the release of GM strawberries were submitted in Estonia (two), in Great Britain (one) and in Italy (five). GM strawberries are still quite a long way from commercial use in Europe. A commercial use of GM strawberries is not to be expected in the next years.

### 17.2.2.2 Grapevine

The genus *Vitis* comprises about 70 species, which are distributed over Southern Europe, Asia Minor, East Asia and North and Central America (Alleweldt and

**Table 17.4** Summary of studies conducted on the transformation of strawberry for agronomically important traits

Selected trait	Genes used	Type of expression	Reference
Insect resistance <i>Otiorhynchus</i> spp.	Cowpea trypsin inhibitor	Overexpr.	Graham et al. (1995, 1997, 2002), James et al. (1992), Watt et al. (1999)
Virus resistance Mild yellow edge virus	Coat protein	Overexpr.	Finstad and Martin (1995)
Fungal resistance <i>V. dahliae</i>	Chitinase ( <i>L. chilense</i> )	Overexpr.	Chalavi et al. (2003)
<i>B. cinerea</i>	Chitinase ( <i>P. vulgaris</i> )	Overexpr.	Vellicce et al. (2006)
	Thaumatin II ( <i>T. daniellii</i> )	Overexpr.	Shestibratov and Dolgov (2005)
<i>S. humuli</i>	Chitinase (rice)	Overexpr.	Asao et al. (1997)
<i>C. acutatum</i>	Chitinase and glucanase ( <i>T. harzianum</i> )	Overexpr.	Mercado et al. (2007a)
Herbicide resistance Glyphosate	EPSP ( <i>A. tumefaciens</i> )	Overexpr.	Morgan et al. (2002)
Glufosinate	Phosphinothricin acetyl transferase	Overexpr.	du Plessis and Brand (1997)
Abiotic stress Salt tolerance	Late embryogenesis abundant protein (barley)	Overexpr.	Wang et al. (2004)
	Osmotin	Overexpr.	Husaini and Abdin (2008)
Freezing tolerance	<i>CBF1</i> ( <i>Arabidopsis</i> )	Overexpr.	Owens et al. (2002), Owens (2005)
	Acidic dehydrin (wheat)	Overexpr.	Houde et al. (2004)
	Type III antifreeze protein (fish)	Overexpr.	Khammuang et al. (2005)
Fruit quality Reduced softening	Strawberry pectate lyase	Silencing	Jimenez-Bermudez et al. (2002)
	Strawberry glucanase <i>cel1</i> S-adenosylmethionine hydrolase (T3 bacteriophage)	Silencing Overexpr.	Palomer et al. (2006) Mathews et al. (1995)
Sugar content	ADP-glucose pyrophosphorylase	Silencing	Park et al. (2006)
Fruit color	Strawberry chalcone synthase	Silencing	Lunkenbein et al. (2006a)
Fruit flavor	Strawberry methyltransferase	Silencing	Lunkenbein et al. (2006b)
Fruit size/ ripening	Strawberry <i>GAST</i> gene	Overexpr.	de la Fuente et al. (2006)
Fruit size/yield	<i>defH9-iaaM</i> (snapdragon/ <i>P. syringae</i> )	Overexpr.	Mezzetti et al. (2004)
Plant morphology	IAA-glucose synthase (maize)	Overexpr.	Wawrzynczak et al. (2005)

Possingham 1988; Grando et al. 1996). The most renowned species is *Vitis vinifera* L., the European or bunch grape, which was domesticated 5000 years ago in Asia Minor or Armenia (Grando et al. 1996). In 2006 about 67 million t of grapes were produced on 7.5 million ha worldwide (<http://faostat.fao.org>).

Since the first transgenic grape plant was reported in 1990 (Mullins et al. 1990), a lot of successful transformations have been reported (for a review, see Yamamoto et al. 2003). Early attempts to transform grape using *Agrobacterium tumefaciens* met with difficulties and a biolistic transformation using coated microprojectiles was established and improved (Hebert et al. 1993, 2005a; Kikkert et al. 1996; Scorzetta et al. 1995a, b, 1996). Presently, *Agrobacterium*-mediated methods are the predominantly employed protocols for grape transformation worldwide (Perl et al. 1996). The use of high-quality embryogenic cultures has allowed the transformation of grape to become routine. A range of methods was published to improve transformation efficiency, to optimize protocols for rootstock and scion cultivars and to avoid selectable marker systems (Dhekney et al. 2005, 2008; Dutt et al. 2007; Li et al. 2006; Lopez-Perez et al. 2008; Nakajima et al. 2006; Olah et al. 2003; Reustle et al. 2003; Xue et al. 1999). Agronomic genes introduced into grapevine (for a review, see Deng and Duan 2006) were focused on virus resistance (Barbier et al. 2000; Golles et al. 2000; Gribaudo et al. 2003; Jardak-Jamoussi et al. 2003; Krastanova et al. 2000; reviewed by Laimer 2006; Martinelli et al. 2000; Radian-Sade et al. 2000; Reustle et al. 2005; Spielmann et al. 2000), fungal resistance (Aguero et al. 2005; Hinrichsen et al. 2005; Kikkert et al. 2000, 2005b; Reisch et al. 2003; Vidal et al. 2003, 2006; Yamamoto et al. 2000), bacterial resistance (Aguero et al. 2005; Holden et al. 2003; Vidal et al. 2003, 2006), herbicide resistance (Mulwa et al. 2007), stress tolerance (Gutaronov et al. 2001; Olah et al. 2004; Tsvetkov et al. 2000), seedlessness (Colova-Tsolova et al. 2003; Perl et al. 2000) and morphology (Geier et al. 2008). Most significant progress in grape genetic engineering was the obtaining of transgenic grape cultivars resistant to grapevine fanleaf virus (GFLV). Transgenic plants were tested under field conditions and assessment of the field safety has been performed (Vigne et al. 2004). Field evaluation was also reported for *DefH9-iaaM* plants, expressing an auxin synthesizing gene which influences fruitfulness and berry quality (Mezzetti et al. 2005). The state of the art in genetic transformation in viticulture was recently summarized by Perl and Eshdat (2007).

A total of 54 field test records were found for the United States within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field test records were focused on GM grape vine plants with improved resistance to bacterial, fungal and virus diseases or with improved tolerance to herbicides. Other field trials were focused on GM plants with improved traits related to product quality.

In Europe, a total of six summary notifications (four for France, one for Italy, one for Germany) can be found for GM grapes (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>). No commercial use of GM grape vine plants is expected for the next years.

### 17.2.2.3 *Ribes* Species (Blackcurrant, Redcurrant, Gooseberry)

The cultivated forms of currants and gooseberries which belong to a number of *Ribes* species have in recent years been the subject of increased interest due to the perceived health benefits. There are a few publications in *Ribes* on regeneration, but no information on transformation. First report was given by Graham and McNicol (1991) in black currant. Transformation methods were optimized and aimed on the development of virus resistant plants (Karjalainen et al. 2001).

### 17.2.2.4 *Rubus* Species (Raspberry, Blackberry)

Raspberry and blackberry are genetically diverse with several *Rubus* species of the *Rosaceae* in their background. Regeneration of adventitious shoots from different type of explants has been reported for several *Rubus* spp. (reviewed by Mezzetti 2003; Swartz and Stover 1996). Transformation methods were developed using the *Agrobacterium*-mediated system (de Faria et al. 1997; Hassan et al. 1993; Kokko and Karenlampi 1998; Mathews et al. 1995a, b). Transformation was aimed on the delay of fruit decay (Mathews et al. 1995a, b), resistance to raspberry bushy dwarf virus (Martin and Mathews 2001) and parthenocarpic fruit development (Mezzetti et al. 2002b, 2004). Transformed plants have been successfully field-trialed, although they were not commercialized (Finn and Hancock 2008). While regeneration systems have been developed for blackberries (Meng et al. 2004; Swartz and Stover 1996), no transgenic blackberries have been produced to date (Finn and Hancock 2008).

A total of 16 field test records were found for GM raspberries for the United States. within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field test records were focused on GM raspberry plants with improved resistance to fungal and virus diseases and with a better product quality. In Europe, only one summary notification (Italy) can be found for GM raspberry plants (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>).

### 17.2.2.5 *Vaccinium* Species (Blueberry, Cranberry)

Several species of *Vaccinium* are important commercially, like highbush, lowbush and rabbiteye blueberries as well as large cranberry. A number of studies were published to improve a regeneration system for blueberry using in vitro leaves as well as seedling explants as source material (summarized by Hancock et al. 2008). Transformation in blueberry was reported for the first time by Graham et al. (1996). Later Song and Sink (2004) published transformation in different highbush blueberry cultivars. Transformation in highbush blueberry was aimed on herbicide resistance, field trials were also performed (Song et al. 2006, 2008). An efficient regeneration system has been developed for cranberry (Qu et al. 2000) The first

transgenic cranberry was obtained by particle bombardment by Serres et al. (1992). In these experiments the *Bt* gene from *Bacillus thuringiensis* (see Chap. 10) was used to obtain resistance to *Rhopobota naevana*. Zeldin et al. (2002) transformed cranberry for herbicide resistance. Polashock and Vorsa (2002) summarized knowledge on transformation and regeneration in cranberry.

Three and one field test records were found for GM blueberry and cranberry plants, respectively, for the United States within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field test records were focused on GM plants with improved resistance to insects and herbicides, respectively.

## 17.3 Tropical and Subtropical Fruit Crops

### 17.3.1 Avocado

The commercially used avocado (*Persea americana* Mill.) is a major fruit crop of the tropics and subtropics. The avocado belongs to the subgenus *Persea* of the genus *Persea*, which consists of approximately 80 species (Mhameed et al. 1997). In 2006 about 3.3 million t of avocado were produced on approximately 400 000 ha worldwide (<http://faostat.fao.org>).

The procedure that has been developed for genetic engineering has been based upon somatic embryogenesis from embryogenic suspension cultures (Cruz-Hernandez et al. 1998). Recently, transformation in avocado was reported using embryogenic cultures and a plant defensin gene aimed on herbicide resistance (Raharjo et al. 2008). Only one field test record was found for GM avovado plants for the United States within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). This field test record was focused on GM plants with improved resistance to fungal diseases.

### 17.3.2 Banana

The genus *Musa* L. is divided into five sections with 30–40 species (Ude et al. 2002). The section *Eumusa* is the largest section and includes the wild ancestors of the modern bananas, *M. acuminata* L. and *M. balbisiana* Colla. Banana grown in tropical and subtropical regions is the fourth most important global food crop after rice, wheat and maize. In 2006 about 80 million t of banana were produced on approximately 4.4 million ha worldwide (<http://faostat.fao.org>). Transformation studies in banana were recently reviewed by several authors (Arvanitoyannis et al. 2008; Pua 2007; Rout et al. 2000). First transformation in banana were performed using electroporation of protoplasts and particle bombardment of

embryogenic cell suspensions (Becker et al. 2000; Sagi et al. 1994, 1995). *Agrobacterium*-mediated transformation was first reported by May et al. (1995) using apical meristems and corm slices. *Agrobacterium*-mediated transformation of embryonic cell suspensions and apical shoot tips were also published (Ganapathi et al. 2001; Tripathi et al. 2005). The techniques are described by Ganapathi et al. (2003). There are several reports on improving transformation efficiency and techniques and verifying gene integration (Acereto-Escoffie et al. 2005; Huang et al. 2007; Khanna et al. 2004, 2007; Perez-Hernandez et al. 2006; Tripathi et al. 2008; Valerio and de Garcia 2008). Genetic engineering tools have also found their place for the improvement of this crop (Sagi et al. 1998). Transformation studies are carried out to confer resistance to nematodes (Atkinson et al. 2004) and fungal pathogens (Chakrabarti et al. 2003; Li et al. 2007; Pei et al. 2005; Remy et al. 1998; Sreeramanan et al. 2006), to improve fruit quality and shelf life and to produce pharmaceutically important peptides in fruit, i.e. edible vaccines, antibodies and therapeutic proteins (Kendurkar et al. 2006; Kumar et al. 2005).

Three field trials with GM bananas with improved resistance to fungal diseases have been performed in the United States (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). A commercial use of GM bananas is not to be expected in the next years.

### 17.3.3 Citrus Species

The genus *Citrus* includes several species of economic importance as sweet oranges (*C. sinensis* L.), mandarins (*C. reticulata* Blanco, *C. deliciosa* Ten.), satsumas (*C. unshiu* Marc.), clementines (*C. clementina* hort. ex Tanaka), grapefruits (*C. paradisi* Macfad.), pummelos (*C. grandis* L. Osbeck), lemons (*C. limon* L. Burm. f.) and limes (*C. aurantifolia* Christm. Swingle). Citrus grown in tropical and subtropical regions is the largest fruit crop in the world. Trifoliate orange (*Poncirus trifoliata* L. Raf.) is a member of the family *Rutaceae* closely related to Citrus and used as a rootstock for Citrus. The citrange (*Citrus sinensis* x *Poncirus trifoliata*) is a hybrid also used for citrus varieties as a rootstock (Kaneyoshi and Kobayashi 2000).

Genetic transformation in citrus was recently summarized by Pena and Navarro (2000) and Pena et al. (2007). There are many in vitro protocols such as on callus and cell suspension cultures, organogenesis induction and protoplast isolation that are viable for genetic transformation in citrus. Transgenic citrus plants at low frequencies have been obtained by direct DNA transfer into protoplasts, co-cultivation of internodes or epicotyl segments with *Agrobacterium* and particle bombardment of nucellar embryonic cell suspensions (Gutierrez et al. 1997; Hidaka et al. 1990; Hidaka and Omura 1993; Kobayashi and Uchimiya 1989; Li et al. 2002, 2003a; Moore et al. 1992; Moore and Cline 1987; Niedz et al. 2003; Vardi et al. 1990; Yao et al. 1996). The first reliable protocol was reported by Kaneyoshi et al. (1994). The most widely used method of gene transfer in citrus is the *Agrobacterium*-mediated transformation of epicotyl and intermodal stem segments. Using this system,

transgenic plants were produced for citrus species and relatives, including Carizzo citrange, Washington navel orange, Tarocco sweet orange, Duncan grapefruit, Rio Red grapefruit, Mexican lime, Xuegan sweet orange, Rangpur lime, Valencia and Natal sweet oranges, *P. trifoliata* (for a review, see Pena et al. 2007; Duan et al. 2007; Orbovic et al. 2008). The procedure of transformation using stem segments of greenhouse grown and epicotyl segments of in vitro grown young seedlings and shoot tip grafted plant material is described in Pena and Navarro (2000) and Pena et al. (2007). Among the transformation methods it is worthy to note the reliable method for the production of mature transgenic citrus plants transforming adult tissue of selected genotypes from *C. sinensis* and *C. clementina*, extended later to other citrus species and genotypes (Almeida et al. 2003; Cervera et al. 1998a, 1998b, 2008; Pena et al. 1997). There are several other factors beside the genotype used that effect transformation (summarized by Molphe Balch 2003). Recently, it was reported that the same treatment for transgenic shoot regeneration can elicit the opposite effect in closely related citrus genotypes (Rodriguez et al. 2008).

The availability of efficient transformation and regeneration systems allows transferring agronomical important genes into citrus plants. Research was focused on:

1. Biotic stress resistance: resistance to the citrus tristeza virus (Ananthakrishnan et al. 2007; Dominguez et al. 2000, 2002; Fagoaga et al. 2005, 2006; Febres et al. 2008; Ghorbel et al. 2000, 2001; Iwanami et al. 2004; Rai 2006) and other viruses (Zanek et al. 2008), bacterial resistance (Boscariol et al. 2006; Gonzalez-Ramos et al. 2005; Guo and Grosser 2004; Omar et al. 2007), fungal resistance (Azevedo et al. 2006; Fagoaga et al. 2001; Gentile et al. 2007), citrus blight (Kayim et al. 2004), broad spectrum disease resistance (Kunta et al. 2008)
2. Abiotic stress tolerance, like salinity (Cervera et al. 2000a)
3. Tree and fruit quality traits: plant architecture (Fagoaga et al. 2007), seedlessness (Koltunow et al. 2000), male sterility (Li et al. 2002), metabolics of the fruit (Costa et al. 2002; Guo et al. 2005; Wong et al. 2001)

One of the main problems in citrus is the long juvenility. With the aim of accelerating flowering time, a range of flowering related genes from *Arabidopsis thaliana* were introduced into citrus. The permanent expression of *LFY* and *API* significantly shortened the juvenile phase in citrus trees (Pena et al. 2001; Pena and Seguin 2001). Endo et al. (2005) reported that transgenic poncirus trees showed very early flowering and fruiting when overexpressing *CitrusFT*, a homolog of *FT* from *A. thaliana*.

There are several publications on biosafety issues in citrus. Selection strategies alternative to *nptII* were evaluated (Ballester et al. 2007, 2008). Production of silenced and chimeric plants after *Agrobacterium*-mediated transformation were described (Dominguez et al. 2004). Stability of integration and expression of the transgenes was confirmed for all the transformants grown under natural environmental conditions (Cervera et al. 2000b).

**Table 17.5** Field tests in fruit crops in the United States (<http://www.isb.vt.edu>). *BR* Bacterial resistance, *FR* fungal resistance, *IR* insect resistance, *PQ* product quality, *VR* virus resistance

Crop	Number of field tests	Phenotype
Citrange	2	BR
Grapefruit	17	IR, VR, BR, PQ
Lime	2	VR
Sweet orange	1	BR

In recent years several field trials with GM citrus plants have been performed in the United States (Table 17.5). These field trials were performed with GM plants of different species mainly improved for resistance to biotic pathogens.

Eight summary notifications can be found for the release of GM citrus plants in Europe: four for sweet orange, three for citrange and one for lemon. All summary notifications for GM citrus plants have been submitted in Spain, except for lemon. This notification has been submitted in Italy.

### 17.3.4 Kiwifruit

The genus *Actinidia* contains about 60 species. Large-fruited kiwifruit is a relatively minor crop selected from two closely related species: *Actinidia deliciosa* A. Chev. and *A. chinensis* Planch. In 2006 about 1.2 million t of kiwifruit were produced on approximately 75 000 ha worldwide (<http://faostat.fao.org>).

*Actinidia* species are amendable to tissue culture techniques (reviewed by Atkinson and MacRae 2007; Oliveira and Fraser 2005). Transformation in kiwifruit was first reported in 1991 (Uematsu et al. 1991). Compared with other fruit crops, relatively high rates of transformation can be achieved in kiwifruit (Li et al. 2003b). Agronomic genes were introduced into kiwifruit such as to promote rooting (Rugini et al. 1997), to enhance resistance to *Botrytis cinerea* (Nakamura et al. 1999), to study morphogenesis (Kusaba et al. 1995, 1999), to provide beneficial effects on health (Kobayashi et al. 2000). Transmission of transgenes to progeny plants was studied in (Fung et al. 1998). Transformation in *Actinidia eriantha* Benth. revealed this species as a potential tool in functional genomics (Wang et al. 2006).

A total of three summary notifications can be found for GM kiwifruit plants (<http://bgmo.jrc.ec.europa.eu/deliberate/dbplants.asp>). These field test records submitted in Italy were focused on plants with improved resistance to fungal diseases or with plants with increased root formation. A commercial use of GM kiwifruits is not to be expected in the next years (<http://www.gmo-compass.org/eng/home/>).

### 17.3.5 Mango

Mango (*Mangifera indica* L.) grown in tropical and subtropical regions is an important fruit crop in Asia. In 2006 about 32 million t of mango, mangosteens

and guavas were produced on approximately 4.5 million ha worldwide (<http://faostat.fao.org>).

Biotechnological advances in mango and their applications described by Krishna and Singh (2007) and Rivera-Dominguez (2006). The first reports of the recovery of mango genetic transformants were described by Mathews et al. (1992, 1993). The system is based on embryogenic suspension cultures transformed by *Agrobacterium tumefaciens*. The major aims of genetic engineering in mango are metabolic manipulations in fruit ripening, lipid metabolism, plant architecture, flower formation, reduction of juvenility, fruit quality and disease resistance (Gomez Lim and Litz 2007).

No field trials have been performed on GM mango to date and a commercial utilization of GM mangos is not expected at present (<http://www.gmo-compass.org/eng/home/>).

### 17.3.6 Papaya

Papaya (*Carica papaya* L.) native to southern Mexico and Central America is an economically important fruit crop of tropical and subtropical regions. In 2006 about 7 million t of papaya were produced on approximately 370 000 ha worldwide (<http://faostat.fao.org>).

However, most of the papaya plantations of the world suffer from the destructive disease caused by Papaya ringspot virus (PRSV). Transformation in papaya is mainly based on an *Agrobacterium tumefaciens*-mediated method into callus, somatic and zygotic embryos because of their high potential of regeneration. However, direct gene delivery through biolistics is an alternative procedure. The most successful case of modifying perennial fruit trees by genetic engineering came from papaya. In order to solve problems caused by PRSV, Gonsalves' group at Cornell University and Hawaii started to develop transgenic papaya since the late 1980s (Fitch et al. 1992, 1993; Gonsalves 1998; 2002; Lius et al. 1997). The transgenic papaya lines Rainbow and SunUp were deregulated by 1998 and granted approval for commercial application. This is the first successful case of a transgenic fruit tree being commercialized in the world (Robischon 2006). The successful application of transgenic papaya in Hawaii, the adaption of this technology to Taiwan, attempts to establish multiple and durable resistance to different viruses and the generation of transgenic papaya in other geographic areas is summarized by Yeh et al. (2007). In 2007 GM papayas were produced in Hawaii on approximately 2000 ha. The Hawaiian papayas are an important export article. They are exported mainly to Japan (<http://www.gmo-compass.org/eng/home/>). In 2006 a virus-resistant GM papaya was deregulated in China. The cultivation of GM papaya is expected for several Asian countries in the near future (<http://www.gmo-compass.org/eng/home/>). Beside virus resistance, papaya was also transformed to improve resistance to aphids (McCafferty et al. 2006, 2008), to improve fungal resistance

(Zhu et al. 2007), to increase cold tolerance (Dhekney et al. 2007) and to produce pharmaceutical important substances, like vaccines (Hernandez et al. 2007).

Since the early 1990s several field trials with GM papaya have been performed. For the United States a total of 34 field test records were found within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field trials were focused on plants with improved resistance to insects, bacterial, fungal and virus diseases, and on plants with improved product quality. In Europe, neither the cultivation nor the import of GM papaya is allowed.

### 17.3.7 Persimmon

Japanese persimmon (*Diospyros kaki* Thunb.) native to East Asia is gaining popularity worldwide, especially as an out-of-season fruit for the northern hemisphere. In 2006 about 3.2 million t of persimmon were produced on approximately 730 000 ha worldwide (<http://faostat.fao.org>).

An efficient plant regeneration system is available for persimmon shoot, callus and protoplast cultures (see Tao and Dandekar 2000). The first transgenic persimmon was reported by Tao et al. (1994). Transformation in persimmon is based on *A. tumefaciens* and *A. rhizogenes* into leaf explants, callus and hypocotyl segments. The protocol for *A. tumefaciens* leaf disk transformation is described in Tao and Dandekar (2000). Focus of genetic engineering in persimmon is on increased insect resistance (Tao et al. 1997) and fruit ripening (Tamura et al. 2008).

GM persimmon plants have been also tested in the field. A total of nine field test records, which were focused on plants with improved resistance to insects, to fungal diseases or with improved agronomic properties, were found for the United States within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>).

### 17.3.8 Pineapple

Pineapple (*Ananas comosus* L. Merr.) follows banana, mango and citrus in terms of world tropical fruit production. In 2006 about 19 million t of pineapples were produced on approximately 950 000 ha worldwide (<http://faostat.fao.org>).

Firoozabady et al. (2006) developed efficient methods for plant regeneration, via both organogenesis and embryogenesis, of Smooth Cayenne pineapple. Success has been reported in transforming pineapple by *Agrobacterium*-mediated gene delivery into friable embryogenic tissue and chunky non-dispersable embryogenic tissue. Thousands of plants were transferred to the greenhouse and to the field to evaluate clonal fidelity and somaclonal variation. Particle bombardment was also a method of choice in pineapple transformation. Sripaoraya et al. (2001) introduced herbicide tolerance into the Thai pineapple by microprojectile-mediated delivery. These

plants were evaluated under field conditions (Sripaoraya et al. 2006). Pineapple transformation is also aimed on increased disease resistance, like nematode resistance (Botella and Fairbairn 2005; Rohrbach et al. 2008), blackheart disease (Graham et al. 2000; Ko et al. 2006) and controlled flowering (Botella et al. 2000; Botella and Fairbairn 2005). Recently, Davey et al. (2007) published a detailed summary on regeneration and transformation in pineapple.

In recent years several field trials with GM pineapples have been performed in the United States. A total of 12 field test records were found within the Environmental Releases Database (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). These field test records were focused on plants with improved resistance to nematodes and virus diseases. Other field trials were focused on plants with altered agronomic properties and improved product quality.

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# Chapter 18

## Maize

David D. Songstad

### 18.1 Introduction

Conventional plant breeding, molecular breeding and transgenic plant technology are coming together in a productive and meaningful way, allowing for rapid delivery of value added traits over the past 13 years. It is the synergy among these technologies that has allowed for the rapid advances in yield gain and introgression of novel traits into diverse germplasm much more rapidly than was possible in the recent past. Over these past 13 years, society has witnessed the introduction of herbicide resistant soybeans, starting in 1996 (Padgett et al. 1996) followed by the generation of other crops, notably herbicide-resistant cotton (*Gossypium hirsutum*; Chen et al. 2006), herbicide-resistant canola (*Brassica napus*; Stringham et al. 2003) and maize (*Zea mays*) resistant to European maize borer (Armstrong et al. 1995) and the herbicide glyphosate (Heck et al. 2005). The perennial growth in biotech acreage is testimony of the acceptance and benefits delivered to farmers. In 2007, 282 million acres (approx. 114 million ha) of biotech crops were planted world-wide resulting in US \$7 billion of added economic benefit to farmers resulting from the biotech traits (James 2007).

Maize is the premier monocotyledonous species for biotech research based on its positive tissue culture and transformation characteristics, conventional and molecular breeding advances and cash value in the agronomic marketplace. The advances in maize tissue culture and biotechnology have emerged from its infancy in the 1970s and 1980s to its current status where nearly 75% of the maize grown in the United States contains biotech traits (James 2007). The track record for maize biotechnology dates back to mid-1970s with the first documented regeneration of maize plants from callus cultures by Green and Phillips (1975). Approximately 15 years elapsed until the first reported regeneration of transgenic maize plants by

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Fromm et al. (1990) and Gordon-Kamm et al. (1990). More recently, Ishida et al. (1996) reported the first stable transformation of maize by *Agrobacterium tumefaciens* (see Chap. 1). This chapter focuses upon the advances which contributed to the current state of transgenic maize, the acceptance of biotech enhanced traits in the marketplace and the benefits delivered to the customer that include enhanced yield to help ensure our food security.

## 18.2 Culture Media and Supplements

A variety of basal media have been used to initiate maize callus cultures and regenerate plants. These include the MS (Murashige and Skoog 1962) formulation for induction of the first regenerable maize callus by Green and Phillips (1975). Since this time, plant regeneration has been reported from callus initiated from explants on N6 (Chu et al. 1975), D [N6/B5 combination] (Duncan et al. 1985), LS (Linsmaier and Skoog 1965) and SH (Schenk and Hildebrandt 1972) media.

A common aspect to all of these culture media is the incorporation of an auxin such as 2,4-D (2,4-dichlorophenoxyacetic acid) or dicamba (3,6-dichloro-o-anisic acid). The level of these auxins in the tissue culture media typically varies from approximately 1  $\mu\text{M}$  to 10  $\mu\text{M}$ . In addition, other chlorinated benzoic acid growth regulators have been used to initiate maize callus cultures from various genotypes (Close and Ludeman 1987).

Amino acids are another important component of maize tissue culture media. The beneficial effects of L-proline were first reported by Armstrong and Green (1985) for the induction of type II callus from cultured immature embryo explants of inbred line A188. The exact role for proline is not known although the addition of casamino acids (mixture of amino acids from casein hydrolysate) was beneficial for a type I embryogenic response from cultured immature embryos of several maize inbreds (Duncan et al. 1985).

Another important addition to maize tissue culture medium that promotes somatic embryogenesis from explant tissues is silver nitrate ( $\text{AgNO}_3$ ). The mechanism is likely silver ion blocking the action of ethylene without affecting its biosynthesis. The first report of evaluating  $\text{AgNO}_3$  was by Songstad et al. (1988) in promoting plant regeneration from type I callus cultures of Pa91 and H99. Inclusive with this publication was the first demonstration of effective plant regeneration through the use of norbornadiene, another ethylene action blocker. Subsequently, Vain et al. (1989a, b) reported an increase in the type II callus induction rate from maize immature embryo explants of inbred A188 cultured on medium containing  $\text{AgNO}_3$ . Enhanced type II callus induction attributed to the presence of  $\text{AgNO}_3$  was also reported by Songstad et al. (1991) using immature embryo explants of the agronomic elite inbred B73. Songstad et al. (1992) also reported beneficial effects of  $\text{AgNO}_3$  on the type II callus response from cultured immature tassel explants, indicating that explants other than immature embryos responded favorably to blocking ethylene action.

### 18.3 Genotype

Genotype is also an important factor in obtaining high-quality embryogenic callus cultures. Originally the focus on maize embryogenic callus was to identify the conditions by which genotypes would produce “type II” embryogenic cultures. Type II callus cultures consisted of a friable embryogenic nature capable of plant regeneration and also suitable for formation of suspension cultures. Fransz and Schel (1991) showed that the type II response from inbred line A188 originated from the abaxial scutellar cells, including the epidermis. Similar findings were reported by Songstad et al. (1996), showing histological evidence of cell division at the scutellar surface of pre-cultured immature embryos leading to an embryogenic response. These friable cultures were desirable as starting material for a variety of transformation experiments including protoplasts, biolistics and selection for somaclonal variants. The most widely used type II embryogenic culture is the “Hi-II” system (Armstrong et al. 1991) derived from A188 X B73. Aside from Hi-II, the two inbreds that produced type II cultures were A188 (Armstrong and Green 1985) and B73 (Lowe et al. 1985; Songstad et al. 1991).

Over time, it became apparent that it was possible to transform maize inbreds that produced type I callus cultures (Wan et al. 1995; Ishida et al. 1996; Armstrong and Rout 2003; Ishida et al. 2003). Type I cultures are similar to type II in that both are embryogenic and capable of plant regeneration. The difference is in the culture morphology in that type I cultures are not friable but composed of a compact embryogenic phenotype. The ability to transform maize inbreds capable of producing a type I callus response is profound because the type I callus morphology is more common from elite inbred lines than is type II.

### 18.4 Explant

The most commonly used explant in maize tissue culture is the immature embryo. The first reported use of this explant was by Green and Phillips (1975) and the specifications for optimized response in culture have been refined. Typically, immature embryos are harvested from maize ears approximately 9–11 days after pollination when they are between 1.5 mm and 2.5 mm long. Staging the immature embryo within this developmental window and orientating the explants with the embryonic axis facing the medium allows for callus proliferation, typically from the scutellar surface of the embryo.

Germination of excised embryos also has been used to initiate adventitious bud culture (Lowe et al. 1985). Approximately 3–4 days after germination, the nodal meristem was excised and served as the explants to form organogenic callus cultures. Similar in vitro morphogenic cultures were produced by Zhong et al. (1992a, b). Application to transformation research is discussed later in this chapter.

Immature tassel explants are also a source of embryogenic callus. These explants were first reported by Rhodes et al. (1986) with the observation of type II callus cultures, but at a low frequency. Subsequent reports by Pareddy and Petolino (1990) and Songstad et al. (1992) reported type I and type II callus from cultured immature tassel explants, respectively. In both cases, the incorporation of  $\text{AgNO}_3$  was critical for approximately 60% of the cultured immature tassel segments to produce type II calli. Within the immature tassel tissue, Suprassanna et al. (1986) also reported totipotent callus cultures from anther-free glume explants cultured in vitro. Zhong et al. (1992b) reported use of immature tassel explants to initiate embryogenic callus and multiple shoot clumps based on the nature of the auxin–cytokinin ratio.

Leaf tissue explants have also been reported to produce somatic embryos when cultured in vitro (Chang 1982; Conger et al. 1987; Ray and Ghosh 1990; Ahmadabadi et al. 2007). In general, the frequency of callus initiation and plant regeneration potential from leaf explants appears to be less when compared to the immature embryo counterpart – although no published reports comparing side-by-side evaluation of these explants sources is in the literature. However, Ahmadabadi et al. (2007) reported the use of hybrid ( $\text{Pa91} \times \text{H99}$ ) seedlings approximately 5–10 cm high, from which 1–2 cm leaf tissues were isolated from the basal region of the shoot and used in vitro to produce regenerable callus cultures. After 4–6 weeks incubation in the dark at 25°C, two types of calli were observed. The first was friable, non-embryogenic and not capable of regeneration of plants. The second was dense dark yellow appearing embryogenic type-I callus capable of plant regeneration.

## 18.5 Transformation

### 18.5.1 Free DNA Delivery in Protoplasts

*Agrobacterium* was first utilized to transform dicotyledonous species in the early 1980s before it was possible to transform monocots (Songstad et al., 1995). Therefore, it became imperative to find alternative means to transform monocot species. The first breakthrough occurred when Chourey and Zurawski (1981) first described callus formation from isolated protoplasts of the maize line Black Mexican Sweet (BMS). Although these BMS cells were not capable of regenerating plants, it did allow for the demonstration that the survival of isolated protoplasts was at such a frequency that cell wall formation could occur and viable calli were initiated. Other scientists realized the importance of this advancement in that transient removal of the plant cell wall allowed for a means to deliver free DNA across the cell membrane. Fromm et al. (1985) utilized this BMS protoplast system to demonstrate that stable transgenic callus cultures could be produced by using electroporation to deliver the reporter DNA across the cell membrane. Electroporation is a physical means for DNA transfer and is different from the chemical means of DNA transfer described by Armstrong et al. (1990) where BMS cells were

treated with polyethylene glycol (PEG), which allowed for efficient transfer of the reporter gene.

Overcoming the inability to regenerate plants from protoplast culture was alleviated by use of maize lines capable of plant regeneration. This was first demonstrated by Rhodes et al. (1988a) when embryogenic maize cell cultures were used as a source for protoplast isolation. However, no fertile plants capable of seed production were reported. The following year, Shillito et al. (1989) published results describing production of fertile plants capable of seed production from protoplast-derived callus cultures. Although seed were produced, the resultant progeny showed morphological and reproductive abnormalities (Shillito et al. 1989). Rhodes et al. (1988b) reported the regeneration of transgenic plants from protoplast culture but did not report evidence for fertile plants or seed production. This problem appeared to be due to a combination of many factors including culture induced variability, protoplast culture and the transformation process.

### ***18.5.2 Intact Tissue Electroporation***

Attention then turned to developing techniques for free DNA transfer that does not require removal of the plant cell wall. The logical extension from electroporation of protoplasts is the electroporation of intact tissues. The first reports describing free DNA delivery into intact tissues were by Abdul-Baki et al. (1990) and Matthews et al. (1990) using tobacco pollen electroporated in buffer containing the  $\beta$ -glucuronidase (GUS) reporter gene. DeKeyser et al. (1990) reported successful electroporation of monocot leaf base tissues resulting in GUS and NPTII gene expression. Rice, maize, wheat and barley leaf bases were electroporated and transient GUS activity was demonstrated. Songstad et al. (1993) applied electroporation conditions to intact immature embryos resulting in transient GUS gene delivery. Electroporation conditions were optimized and an average of 40 GUS spots per embryo was reported. However, stable transformation from this technique was not observed. D'Halluin et al. (1992) described the successful electroporation of maize immature embryos partially digested with 0.3% maceroenzyme for 1–3 min, leading to production of stable callus lines and regeneration of transgenic plants. This indicates that the incorporation of cell wall-digesting enzyme along with electroporation provides the synergy for successful transformation.

### ***18.5.3 Silicon Carbide***

Another physical means of plant transformation involves free DNA delivery to cells via silicon carbide fibers. These fibers are single crystals with an average diameter of 0.6  $\mu\text{m}$  and a length ranging from 10  $\mu\text{m}$  to 80  $\mu\text{m}$  (Songstad et al. 1995). These fibers have high tensile strength and it is likely a combination of their shape, size and strength that allows for the physical delivery of DNA to plant cells. The first

demonstration of silicon carbide fiber-mediated DNA delivery to plant cells was by Kaepller et al. (1990), using BMS cell suspension cultures. The first stable transformation of plant cells was by Kaepller et al. (1992), also with BMS. This process for DNA delivery involves placing BMS cells in a liquid mixture containing silicon carbide fibers and DNA and using a laboratory vortex to agitate the cells and fibers to facilitate penetration and DNA delivery. Kaepller and Somers (1994) described further optimization of silicon carbide-mediated DNA delivery parameters and BMS cell culture treatment to result in 3600 transient events per 300  $\mu\text{l}$  packed volume of cells which led to 316 stable events following selection (Songstad et al. 1995).

Frame et al. (1994) was the first to report stable transformation of maize via silicon carbide fiber-mediated DNA delivery leading to regeneration of transgenic plants. Instead of using BMS, a regenerable maize cell line derived from A188  $\times$  B73 was utilized as recipient for DNA delivery. Pre- and post-transformation treatment of cells on high osmotic medium was a key to the successful transformation. Transgenic plants that reached maturity were fertile and produced progeny. Wang et al. (1995) described testing substances similar to silicon carbide fibers and silicon nitride fibers mixed with DNA also resulted in DNA transfer but at a fraction of that observed with the silicon carbide control. However, comparison of free DNA delivery methods to maize inbred A188 and the “Hi-II” revealed that particle bombardment and intact tissue electroporation resulted in high levels of transient GUS activity that far exceeded that of either silicon carbide fiber or cell electrophoresis (Southgate et al. 1998).

#### **18.5.4 Microprojectile Bombardment**

The search for an effective DNA delivery method resulting in stable transformation of monocot species began in earnest with the first production of transgenic dicot plants with *Agrobacterium* (Fraley et al. 1983). Many attempts to transform various cereal species with *Agrobacterium* failed and the infertile transgenic plants from protoplast-mediated transformation created doubt that monocot species such as maize and wheat could ever be transformed (Potrykus 1989). However, others viewed this as an opportunity to discover new DNA delivery methods with application to monocots. A promising approach first described by Klein et al. (1987) involved bombardment of living onion (*Allium cepa*) cells with 4- $\mu\text{m}$  tungsten microprojectiles carrying free DNA, resulting in transient expression of the chloramphenicol acetyltransferase (CAT) reporter gene. The particle bombardment device used in this study involved acceleration of microprojectiles with a 0.22 caliber gunpowder charge. Further refinement of the bombardment process for free DNA delivery to maize involved use of 1.2- $\mu\text{m}$  tungsten microprojectiles, use of the GUS reporter gene, filter paper to anchor target cells and the use of spermidine and calcium chloride to precipitate an optimized amount of DNA in association with microprojectiles (Klein et al. 1988).

Klein et al. (1989) reported the stable transformation of maize BMS cell cultures via gunpowder-propelled microprojectile bombardment. The selection for stably transformed BMS was achieved by successfully delivering the GUS and neomycin phosphotransferase-II (NPT-II) genes to these cells and allowing for growth and survival on medium containing the antibiotic kanamycin. Up to 117 kanamycin-resistant calli were recovered from a single bombardment of approximately 200 000 cells. Histochemical GUS expression from transgenic calli was observed, with a high degree of event to event variability. Similar results were obtained by Spencer et al. (1990) by following bombardment of BMS cells for delivery of the phosphinothricin acetyl transferase (PAT) gene and selection on medium containing bialaphos. The GUS gene was also delivered along with PAT but on a separate plasmid and a co-transformation rate of 50% was reported.

This research provided the foundation for further advances in maize transformation. Two nearly simultaneous publications by Fromm et al. (1990) and Gordon-Kamm et al. (1990) described the use of microprojectile bombardment to produce fertile transgenic maize plants and progeny. Both efforts utilized A188 × B73 cell lines established from immature embryo-derived friable type II callus which was used as inoculums to establish suspension cultures. These cultures were maintained on solid or liquid medium for several months prior to bombardment. The two publications differ with regard to the selectable marker used to produce the transgenic cultures and regenerated plants. Fromm et al. (1990) utilized the pEC9 vector that conferred ectopic resistance to the herbicide chlorsulfuron. Gordon-Kamm (1990) utilized a vector encoding ectopic expression of the PAT gene conferring resistance to the herbicide bialaphos. The transformation protocol from both publications were similar in that tungsten microprojectiles approximately 1 µm in diameter were used in conjunction with DNA precipitated by the spermidine/calcium chloride method described by Klein et al. (1988). Transgenic calli were observed about 6–8 weeks after culture on selection medium containing either chlorsulfuron (50 nM) or bialaphos (1 mg/l or 3 mg/l). Plants were regenerated and confirmed to be transgenic by Southern hybridization and by evaluation for the visible marker luciferase (Fromm et al. 1990) and GUS (Gordon-Kamm et al. 1990) expression in leaf tissue. Fertile transgenic plants were produced and progeny were also analyzed and confirmed to contain the transgenes. These two papers clearly demonstrated that microprojectile bombardment was a tool that opened the door for genetic modification of maize and other monocots. Dennehey et al. (1994) described transformation experiments comparing four different phosphinothricin-based selective agents where Hi-II callus targets were bombarded with DNA carrying the bar and GUS genes. Bialaphos at 1 mg/l was optimal for selection because it hindered wild-type cell growth even when 25 mM L-proline was added to the medium.

One of the issues associated with bombardment of long-term suspension cultures is the effect of somaclonal variation in the regenerated plants. Phenotypic abnormalities were reported by both Fromm et al. (1990) and Gordon-Kamm et al. (1990) and abnormalities further highlighted by Spencer et al. (1992) by analysis of segregation patterns in transgenic maize produced from suspension culture bombardments. Research then focused on finding target tissues other than suspension

cultures. Often overlooked is the fact that the first bombardment of maize callus cultures leading to transgenic plants was also described by Fromm et al. (1990) using a plasmid vector containing the Bar gene encoding phosphinothricin resistance and the GUS reporter gene. Walters et al. (1992) also published production of transgenic maize plants following bombardment of callus cultures to deliver the hygromycin phosphotransferase gene and selection on a medium containing hygromycin. However, the plants produced also showed phenotypic abnormalities and the authors cite that the age of the callus cultures used in these bombardments (approximately 12 months old prior to bombardment) was responsible for this effect.

These initial publications highlighted the use of friable type II callus cultures in the successful transformation of maize. The first demonstration of successful transformation of type I maize callus cultures by microprojectile bombardment was by Wan et al. (1995). Double-haploid-derived maize inbred lines were used as donor plants for immature embryos and subsequent type I callus. Gold microprojectiles (1.0  $\mu\text{m}$  diameter) were used to deliver plasmid DNA containing the Bar (phosphinothricin resistance) and GUS genes to the type I callus via the PDS1000 helium gun. Medium containing bialaphos was used for the selection of transgenic calli that were confirmed positive by histochemical GUS staining. Plants regenerated were confirmed to be transgenic by application of basta herbicide to leaf tissue and by Southern hybridization. The importance of the Wan et al. (1995) publication is that most tissue culture-responsive maize genotypes produce type I callus and this bombardment breakthrough allowed for a less genotype-dependent maize transformation system.

This led to the need to reduce the time cultures were maintained prior to bombardment as a means to reduce somaclonal variation in transgenic plants. Since callus cultures were initiated from immature embryo explants, this tissue became the target of interest. The first report of bombarding maize immature embryos was by Klein et al. (1988) and demonstrated the transient expression of GUS delivered by microprojectile bombardment to these tissues. It was not until 1993 that the first reports appeared that described production of transgenic maize plants from immature embryo bombardment. Armstrong and Songstad (1993) used Hi-II pre-cultured immature embryos and bombardment with the pEC9 construct described by Fromm et al. (1990) and produced transgenic plants tolerant to the herbicide chlorsulfuron. Pre-culturing the immature embryos for up to 4 days on callus initiation medium prior to bombardment was important for the successful transformation of maize using these tissues. Histological examination revealed that rapidly dividing cells were observed on the scutellar surface of 2-day and 4-day pre-cultured immature embryos (Songstad et al. 1996). These rapidly dividing cells had a dense cytoplasm and appeared to be microcalli. Koziel et al. (1993) also reported production of transgenic maize plants through bombardment of immature embryos with a vector containing an insecticidal gene from *Bacillus thuringeinsis* that conferred tolerance to the European maize borer.

Microprojectile bombardment was also utilized to transform maize by bombardment of adventitious meristematic tissues. Lowe et al. (1995) bombarded

embryonic axes of coleoptilar-stage embryos with a plasmid vector containing the GUS and NPTII genes and germinated plants from these embryos and observed chimeric sectors of GUS gene expression and the majority did not result in transmission of this trait to progeny. To increase the likelihood of obtaining a transgenic event with germline transmission of the GUS trait, embryo axes were bombarded and germinated and apical meristem excised approximately 2–3 weeks after bombardment (when plantlets were 5–8 cm tall) and cultured on a shoot multiplication medium containing the cytokinin 2-benzyladenine (2-BA) and kanamycin and subcultured every two weeks. After two months, plants were regenerated and confirmed to be transgenic by GUS expression and Southern hybridization and this trait was also transmitted to progeny. The frequency of event production capable of germline transmission using this technique was two events out of 160 bombarded embryo axes. Furthermore, the authors state that applying this technique to an elite inbred resulted in one germline event out of 240 bombarded axes and attempts to transform three other inbreds was not successful. Similar results involving bombardment of the maize shoot apical meristem were reported by Zhong et al. (1996). Zhang et al. (2002) also successfully transformed maize by bombardment of shoot meristematic cultures and reported modification of shoot proliferation medium which improved the ability to transform recalcitrant elite inbreds.

The last explant to be used in successful maize transformation is leaf base tissues. Ahmadabadi et al. (2007) utilized compact type-I callus derived from Pa91 × H99 hybrid seedlings as biolistic target material. Leaf-derived callus was bombarded with DNA carrying the phosphomannose isomerase (*pmi*) gene. Selection of stable callus lines occurred by growing bombarded calli on medium containing mannose after which transgenic plants were regenerated. Expression of *pmi* was indicated using the chlorophenol red assay (Wright et al. 1996, 2001) and also by Southern and Northern blots for the *pmi* gene and its transcription product, respectively. The clear benefit of leaf explants is that it offers an alternative to the standard immature embryo explants, which requires either field or greenhouse resources.

Improvements in immature embryo bombardment efficiency also occurred by modification of the culture medium prior to and following bombardment. Vain et al. (1993) first reported the positive effect of osmoticum treatment consisting of culturing suspension cells on medium containing mannitol or sorbitol for 4 h prior to bombardment. It is believed that the effect of osmoticum is by causing cell plasmolysis and reducing the amount of damage due to microprojectile penetration during bombardment. Brettschneider et al. (1997) followed a similar approach but used elevated sucrose concentration to provide the osmotic effect and also utilized gold microprojectiles and the PDS-1000 helium gun to bombard immature embryos of various inbred lines and hybrids.

Control of ethylene has also been shown to be beneficial in the genetic transformation of maize. The first report of this was by Morrish et al. (1993) where B73 × A188 callus cultures showed a three- to 20-fold increase in ethylene emanation when comparing bombarded to non-bombarded controls. Furthermore, there was an inverse relationship between the ethylene emanation rate and the reporter gene

expression from bombarded maize callus cultures. Since ethylene may be partially responsible for cell death and diminished viability after bombardment, Morrish et al. (1993) recommended inclusion of an ethylene antagonist (e.g. AgNO<sub>3</sub>) in the culture medium to limit the detrimental influences of the transformation process. This was successfully demonstrated by Songstad et al. (1996) where microprojectile bombardment of maize immature embryos, pre-cultured for 2–4 days on a medium containing AgNO<sub>3</sub>, resulted in the production of transgenic embryogenic callus cultures and fertile transgenic plants. Following this application, Zhao et al. (2001) described the use of silver nitrate to enhance the *Agrobacterium*-mediated transformation of maize. Additional details regarding the genetic transformation of maize are found later in this chapter.

In addition to the Biolistics device developed by John Sanford, other devices also capable of free DNA delivery via microprojectile penetration have been reported. McCabe and Christou (1993) reported the use of ACCELL technology for electric discharge particle acceleration resulting in the delivery of free DNA to maize and other plant species. This technology is different from that described above in that it uses a shock wave to propel the microprojectiles. This shock wave is generated through the delivery of an electric arc across two electrodes which accelerated a carrier sheet containing the DNA-coated microprojectiles into a retaining screen, allowing the microprojectiles to proceed and penetrate the target cells. The advantage is that no combusting gases are used in this process.

Another gene delivery device that uses gas inflow for particle acceleration is the particle inflow gun (PIG) described by Finer et al. (1992). This device consisted of a steel vacuum chamber fitted with copper tubing coming from a tank of Helium gas linked with an in-line solenoid valve controlled by a timer. The copper tubing extended through the top of the vacuum chamber and was connected to a filter unit housing a screen on which the microrprojectile/DNA mixture was placed. The amount of pressure used to propel the microprojectiles was determined by the pressure regulator on the helium tank (typically set at 40–80 psi; approx. 276–552 kPa). The solenoid allowed for a burst of helium gas to be released (for 50 ms) which delivered the particles and DNA into the target cells. Transient GUS expression was obtained using this tool by bombardment of maize embryogenic suspension cultures (Finer et al. 1992). Stable transformation of maize using the PIG was achieved by microprojectile/DNA delivery to embryogenic suspension cells pretreated with osmoticum (Vain et al. 1993).

### 18.5.5 *Agrobacterium*

Microprojectile bombardment is an effective method in the production of transgenic maize plants. However, there has been great interest in developing an *Agrobacterium*-mediated transformation system for maize similar to that available for dicots. Several early studies were published regarding preliminary aspects of

*Agrobacterium* transformation of maize. Convincing results involving transient GUS expression in 3- to 5-day-old sterile A188 seedling tissues inoculated with *Agrobacterium* was reported by Ritchie et al. (1993). GUS expression was observed in leaf and coleoptiles tissues and also in the vascular cylinder. Attempts to observe transient GUS expression in other genotypes were not successful indicating a genotype effect.

Use of the A188 genotype by Ishida et al. (1996) resulted in the first journal publication report of stable transformation of maize using *A. tumefaciens* strain LBA4404 carrying pTOK233. Immature embryos approximately 1–2 mm in length were excised and co-cultivated with *A. tumefaciens* at 25°C in the dark at a concentration of  $1 \times 10^9$  colony-forming units (cfu)/ml. The right and left borders contained the Bar and GUS genes, each driven by the cauliflower 35S promoter. Following inoculation, immature embryos were co-cultured for three days and then transferred to medium containing 10 mg/l phosphinothricin for two sequential three-week subculture cycles. Following plant regeneration, plantlets were evaluated for histochemical GUS expression and an average of 11.8% of the inoculated embryos produced transgenic events. Analysis of regenerated plants indicated that nearly all were of normal phenotype with 70% fertility and approximately 40% were single copy for the Bar gene. Ishida et al. (1996) state that the reason why maize transformation via *Agrobacterium* had not yet been reported is because of the multiplicity of factors that are required including, genotype, type and stage of tissue, selectable marker, LS medium (Linsmaier and Skoog 1965) for tissue culture and nature of the vector. Subsequently, Ishida et al. (2003) demonstrated production of transgenic H99 plants using their transformation system now modified by the addition of silver nitrate to the selection medium and replacing cefotaxime with carbenicillin for the elimination of *Agrobacterium* following infection. More recently, Ishida et al. (2007) reported successful transformation of A634 and W117 at a frequency of 15% where about half the plants produced contained one or two copies of the transgenes.

The same LHA4404 *Agrobacterium* strain with pTOK233 described above was employed by Zhao et al. (2001) to produce transgenic maize plants from the Hi-II germplasm. Immature embryo explants approximately 1.0–1.5 mm long were excised and immersed into *A. tumefaciens* inoculums at a density of  $1.0 \times 10^9$  cfu/ml. *Agrobacterium* contained a binary plasmid with 35:Bar and Ubiquitin:GUS within a single T-DNA. Following inoculation, immature embryos were transferred to co-culture and “resting medium” for up to seven days. The key component of these media was use of N6 salts with silver nitrate (in addition to 2,4-D, acetosyringone and carbenicillin). Immature embryos were then transferred to N6-based selection medium containing 2,4-D and 3.0 mg/l bialaphos. Explants were transferred to fresh selection medium every two weeks and after a total of two months stable transgenic putative callus was observed on the selection medium. Transgenic plants were regenerated from approximately 40% of the inoculated immature embryos that produced callus. A majority of the plants regenerated were low copy number (one or two copies) for the bar gene.

Other strains and/or binary vectors of *Agrobacterium* have also been successfully used in the genetic transformation of maize. Frame et al. (2002) described a standard binary vector system in *Agrobacterium* strain pEHA101 carrying pTF102 with bar and GUS genes capable of transforming the Hi-II germplasm. Following inoculation with *Agrobacterium*, immature embryos (1.5–2.0 mm long) were co-cultured at 20°C or 23°C in the dark on a medium containing the amino acid cysteine. Although there was no difference between 20°C or 23°C co-culture temperature, incorporation of cysteine to this medium resulted in an increase in transient GUS expression and also an increase in the stable transformation efficiency. Frame et al. (2006) reported the use of this same strain and binary vector system with N6 and MS media in the genetic transformation of three maize inbreds: B104, B114 and Ky21. Armstrong and Rout (2003) also described the use of several strains and vectors in successful transformation of maize.

*A. tumefaciens* carries a plasmid in which the DNA between the right and left borders is transferred to the plant chromosome. Ordinarily a single insert is desired when T-DNA is inserted. However, sometimes it is desirable to deliver two genes of interest at two different locations such that they can be segregated away from each other. This was first described in maize using *Agrobacterium* by Miller et al. (2002) by the delivery of two inserts at two different locations by use of two independent sets of right and left borders. This was accomplished by designing two plasmids within *Agrobacterium*, the first containing the Bar selectable marker gene and the other plasmid containing the GUS reporter gene. Each gene had its own right and left border sequences. It was observed that the two independent T-DNA regions integrated at different loci and segregated from each other in 64% of the independent transgenic plants produced. This approach was shown to be more practical than mixing two independent strains of *Agrobacterium* with each containing a unique T-DNA in order to achieve two sites of insertion.

In addition to immature embryos, other tissues have been used in inoculation with *Agrobacterium* to transform maize and in the production of transgenic plants. Rout et al. (1996) first described the use of maize tissue culture in *Agrobacterium*-mediated transformation of maize. Maize suspension cultures were inoculated with *Agrobacterium* and selection of stable transformed tissues was achieved. This created an opportunity for transformation of maize from other tissues and Sidorov et al. (2006) described transformation of maize from seedling-derived callus tissue. In this technique, seedlings were germinated and nodal region of 7- to 10-day-old seedlings were split and cultured on medium containing picloram and 2,4-D. Approximately 40% of the split nodal explants produced embryogenic calli that originated from primarily from the axillary buds and not the apical meristem. These calli were inoculated with *Agrobacterium* containing the green fluorescent protein reporter gene and NPTII selectable marker. Following a two-day co-culture, calli were divided into smaller pieces (2–3 mm) and placed onto selection medium containing 100 mg/l paromomycin. Stable GFP sectors were observed two weeks after selection and plant regeneration occurred after two months on selection medium. Transformation efficiency as high as 11% was reported and approximately 60% of the plants regenerated had one or two copies of either GFP or NPTII.

## 18.6 Benefits

Since its first introduction in the 1990s, maize hybrids carrying either herbicide-resistant or insect tolerant-trait have seen constant acceptance by farmers and consumers throughout the world. The total global acreage of maize with biotech traits has increased to approximately 87 million acres in 2007 (James 2007; approx. 35 million ha). This represents 24% of the total acreage of maize planted globally in 2007 and this represents nearly 31% of the 282 million acres (approx. 114 million ha) planted globally for all crops with biotech traits in 2007. However, on a value basis, the maize biotech plantings in 2007 represent 47% of the total value of biotech crops planted that year (James 2007), which highlights the important monetary aspect of this agronomic crop.

In 2007, over 17 million acres (approx. 7 million ha) of maize were planted with genes conferring herbicide tolerance, and insect-resistant traits accounted for nearly 23 million acres (approx. 9 million ha). It is important to note that these values are for each stand-alone trait and does not represent the acreage when these traits are stacked. The acreage for herbicide tolerance and insect resistance in the same hybrid accounted for over 46 million acres (approx. 18.5 million ha) in 2007 and indicates the clear trend that farmers prefer the stacked traits over the stand-alone in their agronomic production systems (James 2007).

The introduction of herbicide resistant maize (see also Chap. 10) to the commercial market has resulted in clear benefits to farmers. First, use of herbicide resistant maize allows the farmer to implement a simpler farming practice that utilizes a more effective means of weed control and also allows for large-scale application of no-till farming. Combining herbicide resistant maize with no-till has resulted in a farming practice that requires less time in the field and also one that requires less diesel fuel leading to a reduction in overall overhead costs and a reduction in carbon emission. Furthermore, the USDA has developed an energy calculator based on *Revised universal soil loss equation, ver. 2* (RUSLE2) runs within the United States that compares fuel requirements based on farming systems and reports an approximate 45% fuel savings associated with a change in maize agricultural practice from conventional tillage to no-till (USDA, NRCS, Energy Tools; <http://ecat.sc.egov.usda.gov>). Tables 18.1, 18.2 give an example (crop management zone 17, spanning the southern regions of Missouri, Illinois and Indiana) which illustrates the savings of diesel fuel (in gallons and US dollars) for 1000 acres (approx. 405 ha) of maize. The diesel fuel savings for 1000 acres of no-till versus conventional till was an impressive US \$9000 and extending this to the entire 2008 maize planting acreage in the United States ( $86.9 \times 10^6$  acres, approx.  $35.2 \times 10^6$  ha; USDA National Agriculture Statistics Service) represents a potential savings in diesel fuel that approaches US \$800 million. The key to this widespread application of no-till is planting herbicide-resistant maize hybrids.

This significant shift to no-till farming has also resulted in positive steps towards top soil conservation (Duke and Cerdeira 2005). No-till is made practical by the use of a pre-plant application of herbicide (e.g. glyphosate) for weed burndown

**Table 18.1** Total diesel fuel cost estimate (US \$/year for 1000 acres of maize) based on US \$4.00/gallon (<http://ecat.sc.egov.usda.gov>; 1 acre = approx. 405 ha; 1 US gallon = 3.78 l)

	Conventional tillage	Mulch tillage	Ridge tillage	No till
Total fuel cost	\$20,240	\$16,760	\$13,320	\$11,080
Potential cost savings over conventional tillage	–	\$3,480	\$6,920	\$9,160
Savings	–	17%	34%	45%

Source: <http://ecat.sc.egov.usda.gov>

**Table 18.2** Total farm diesel fuel consumption estimate (gallons/year) for 1000 acres of maize (<http://ecat.sc.egov.usda.gov>)

Crop	Conventional tillage	Mulch tillage	Ridge tillage	No till
Total fuel use	5,060	4,190	3,330	2,770
Potential cost savings over conventional tillage		870	1,730	2,290
Savings		17%	34%	45%

Source: <http://ecat.sc.egov.usda.gov>

followed by drill planting of herbicide-tolerant (Roundup Ready) seed. Aside from the overhead savings, no-till farming is an environmental savings due to reduced soil erosion and reduced risk of soil compaction (Duke and Cerdeira 2005). Equally important, Gianessi (2005) reported that herbicide-resistant crops (e.g. Roundup Ready) generally require less herbicide than non-biotech crops and estimated that Roundup Ready crops have reduced overall herbicide use by approximately 17 million kg/year herbicide.

Obvious benefits are also attributed to the increased planting of insect resistant maize hybrids (see also Chap. 11). A documented average increase in yield of approximately 5% in the United States has been attributed to insect resistant biotech traits and the total economic income benefit to farmers stood at US \$306 million for the 2005 planting year (Brookes and Barfoot 2006). Many farmers experience higher yield gains that vary according to pest density.

Finally, it is the continued development of new technologies in the area of conventional breeding, molecular breeding and molecular biology/maize transformation that allows for a continual increase in yield that is required by our growing global population. Continuing to discover and develop new technologies in the agricultural sciences will provide the food security that is desired and expected by consumers (Brookes and Barfoot 2008). The future does look very promising. Development of second- and third-generation herbicide- and insect-resistant traits that stack multiple modes of action will insure the beneficial aspects of this technology for years to come. This includes development of insect control technology based on RNA interference (Baum et al. 2007). Also, development of drought-tolerant maize is becoming reality (Nelson et al. 2007; Castiglioni et al. 2008). A variety of different approaches have been discovered that confer tolerance to water-limiting conditions that involve either transcription factor that mediates

stress (Nelson et al. 2007) or performs as a RNA chaperone to limit impact of water stress on transcription and translation (Castiglioni et al. 2008).

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# **Chapter 19**

## **Ornamentals**

**Thomas Debener and Traud Winkelmann**

### **19.1 Introduction**

The global consumption of flowers and floricultural products at the consumer level (even without considering woody species for gardening and landscaping) is estimated to be 100–150 billion Euros/year (Chandler and Tanaka 2007). With novelty being a major driving force for the ornamental industry, attempts to use biotechnology for the manipulation of ornamental plant characters started soon after the first plants could be transformed (Brand 2006; Meyer et al. 1987). For example, one of the first European field trials was done with transgenic petunias with modified flower colour, although this had no commercial background (Meyer et al. 1992), and ornamental species from more than 40 genera have been successfully transformed (Brand 2006). For general information on plant transformation see Chap. 1. Interestingly, the public acceptance for genetically modified ornamentals does not seem to differ from that of genetically modified organism (GMO) food in the United States (Klingeman et al. 2006) and can be considered to be on a low level similar to that for GMO food in Europe. In contrast to transgenic food crops transgenic ornamentals do not have to be tested for their safety for human consumption, a major obstacle for commercialisation of transgenic crops. Although this might facilitate the commercial application of genetic engineering to ornamental crops, there are also some particular problems. One is the highly diverse group of ornamental crops on the market leaving the individual crop with fewer resources than the major “cash crops”.

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Other obstacles are sometimes recalcitrance to transformation and regeneration. Finally, for some of the most important ornamental crops as for example roses, the occurrence of natural populations of cross-compatible wild species in the major consumer regions complicate potential field trials and releases to the market significantly.

In contrast to the high potential of biotechnological strategies in ornamental plant breeding commercialisation is vastly lagging behind the major agricultural crops (Clark et al. 2004a). This is reflected by the small number of applications for field releases of ornamental plant species in Europe and the United States (Kuehnle and Mudalige-Jayawickrama 2007). The stagnation in using biotechnology in floriculture is also expressed by the fact that, by the end of 2008, no new releases of transgenic ornamentals were listed by the European Commission as compared to the status of 2004, which is given by Kuehnle and Mudalige-Jayawickrama (2007). However, new developments in using plant transcription factors to redirect parts of the stress transcriptome recently led to increased efforts to engineer stress response in ornamentals (Boehm 2009).

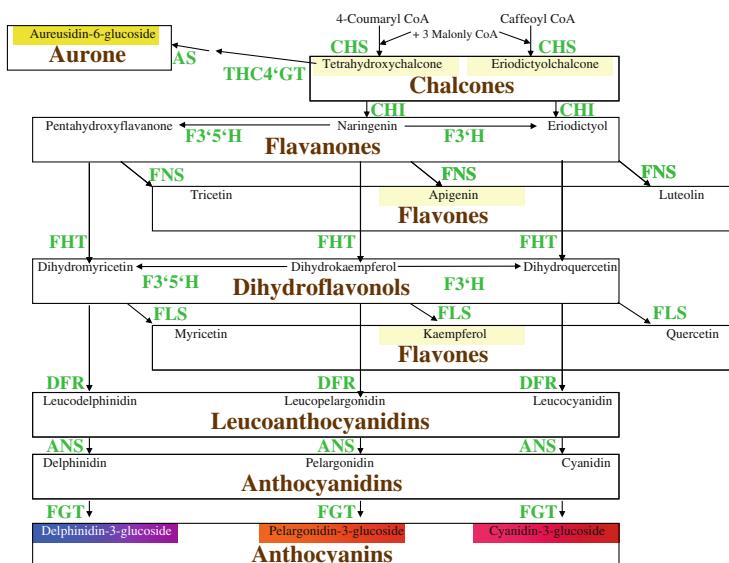
In the following, the main achievements in genetic modification of floricultural species are reviewed according to the different characters which are important breeding aims in ornamental plants.

## 19.2 Flower Colour Modifications

Genetic engineering of flower colour was among the first applications of gene transfer in ornamentals (Meyer et al. 1987) and up to now is the only example for commercialised ornamental GMOs, namely the Moon series of carnation with mauve to bluish flower colours introduced by Florigene company (Chandler and Tanaka 2007). Flower colour is the result of the type and amount of pigment found in a respective organ, the amount and type of co-pigments, the vacuolar pH and the presence of metal ions. Among the three main groups of plant pigments, flavonoids, carotenoids and betalains, flavonoid biosynthesis and metabolism is best understood and many structural as well as regulatory genes have been isolated from different species. Some excellent reviews recently summarized the current knowledge available for floral pigment biosynthesis and genetics (Grotewold 2006; Rosati and Simoneau 2006; Tanaka 2006; Tanaka and Ohmiya 2008; Tanaka et al. 2008).

Many important ornamental species lack either yellow or orange flowers, like *Pelargonium*, azaleas, *Cyclamen*, African violets and others, while in others blue hues are missing, as for instance in rose or carnation. Therefore, considerable efforts have been and are currently made to create these novel colours by plant breeders. Often conventional cross-breeding is not successful due to missing biochemical pathways or substrate specificities of certain enzymes within a

particular species and its relatives. Thus, genetic engineering provides a possibility to overcome these limitations. Moreover stability of gene expression over time and after several cycles of sexual or vegetative propagation can be directly observed if the transferred gene results in altered flower colour, which can reveal valuable fundamental information. Since the approaches undertaken so far have been intensively reviewed (Chandler and Tanaka 2007; Kuehnle and Mudalige-Jayawickrama 2007; Potera 2007; Rosati and Simoneau 2006; Tanaka et al. 2005) in the following we highlight only very briefly the main achievements in modification of floral pigmentation to red, yellow, blue and white colours. For a better understanding the main steps and enzymes involved in flavonoid biosynthesis are illustrated in Fig. 19.1. Changes in flower colour can be obtained by introduction of a foreign structural or regulatory gene, by over-expression of an endogenous gene or by down-regulation of biosynthetic genes by antisense or co-suppression or most efficiently by RNAi (Nakamura et al. 2006; see Chap. 5 for details). However, unexpected results were sometimes reported, due to feedback control of some reactions, substrate competition and switches to other biochemical pathways. Moreover the promoter influences the magnitude and spatial distribution of pigments (Nakatsuka et al. 2007).



**Fig. 19.1** Biosynthesis of important anthocyanins and copigments in schematic presentation. AS Aureusidin synthase, *THC4'GT* tetrahydroxychalcone 4'-O-glucosyltransferase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *FNS* flavone synthase, *FLS* flavonol synthase, *FHT* flavanone 3-hydroxylase, *F3'H* flavonoid 3'-hydroxylase, *F3'5'H* flavonoid 3',5'-hydroxylase, *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanidin synthase, *FGT* flavonoid 3-O-glucosyltransferase

### 19.2.1 Red and Pink Flowers

Pigments responsible for red colour can be found in all three major pigment classes, betalains, carotenoids and flavonoids, however only for flavonoids have transgenic approaches been published to date. Among the flavonoids, anthocyanins as water-soluble compounds in the vacuole lead to red flower hues, from brick red and orange red (pelargonidins) to magenta and pink (cyanidins) and purple, violet and blue (delphinidins; Fig. 19.1; Tanaka et al. 2008). In some genera like *Petunia* or *Cyclamen* no pelargonidins are formed naturally, but for *Petunia* this pathway has been established by introducing a dihydroflavonol reductase (*DFR*) gene from maize (Meyer et al. 1987). While the endogenous *Petunia DFR* did not accept dihydrokaempferol as substrate, the maize gene was able to convert it to pelargonidin and its derivatives. By crossing transgenic lines to other genotypes, Oud et al. (1995) achieved more intense orange-red flowering offspring which expressed the new trait in a more stable way and combined it with positive horticultural traits.

Another example of new reddish colours can be listed for *Forsythia*, normally accumulating yellow carotenoids (Rosati et al. 2003). The combined action of two transgenes, a *DFR* gene from *Antirrhinum majus*, and an anthocyanidin synthase (*ANS*) gene from *Matthiola incana* resulted in cyanidin-derived anthocyanin accumulation and bronze flower colour. Both examples proved that the introduction and overexpression of genes coding for key enzymes in anthocyanin synthesis led to new types of floral pigments. In some cases additional down-regulation of endogenous gene expression is needed, as reported recently for *Osteospermum*, in which predominantly delphinidin-derived anthocyanins are formed (Seitz et al. 2007). A shift to more orange red hues by insertion of *Gerbera* or *Fragaria DFR* genes was not observed until the endogenous flavonoid 3',5'-hydroxylase (*F3'5'H*) was silenced by RNAi.

### 19.2.2 Yellow and Orange Flowers

In nature, yellow flowers contain either carotenoid pigments or flavonoids. As stated above, the stability and appearance also depend on other factors like glycosylation, co-pigments or metal ions. In *Camellia chrysanthia* petals, aluminium ions in combination with the quercetin-derived pigments were shown to contribute to the deep yellow colour (Tanikawa et al. 2008).

Modifications of carotenoid flower pigments by genetic engineering were rarely reported except for transgenic *Lotus japonicus* expressing a bacterial  $\beta$ -carotene ketolase which resulted in orange instead of yellow flowers, but also more lime-green leaves (Suzuki et al. 2007). The biosynthetic pathway of carotenoids becomes better and better understood and by adding signal peptide sequences the gene products can be directed to chromoplasts.

Another strategy to engineer yellow flower colour has been tested for *Torenia* by aurone flavonoids (Ono et al. 2006). Chalcones are substrates for a

tetrahydroxychalcone 4'-O-glucosyltransferase (*THC4'GT*) in the cytoplasm and can be finally converted to aurones by aureusidin synthase (*AS*) in the vacuole (Fig. 19.1), leading to bright yellow colours in *Torenia* flowers. Although the authors of this study propose this approach to be applicable to many ornamental species, because of chalcones being broadly present (Ono et al. 2006), Chandler and Tanaka (2007) have not so far succeeded in producing yellow *Petunia* applying this strategy.

### 19.2.3 Blue Flowers

The old dream of a blue rose has not yet fully become reality, but recently rose flowers with novel blue hues were achieved by genetic modification (Katsumoto et al. 2007). They are currently under investigation for their horticultural performance before they are commercialised (expected to be in 2010). Since roses naturally lack delphinidin-derived anthocyanins, a *Viola F3'5'H* gene was introduced into rose cultivars that had been selected for a high vacuolar pH value. However, novel types of coloration were not observed, until the rose *DFR* gene was down-regulated by RNAi and a *DFR* from *Iris hollandica* was transformed, resulting in nearly exclusive delphinidin production in the petals.

The second prominent example for genetically engineered bluish flowers is that of carnation, which exhibited new delphinidin-type pigmentation after transformation of a *Viola F3'5'H* and a *Petunia DFR* gene (Fukui et al. 2003). These so-called blue carnations of the Moon series ([www.florigene.com](http://www.florigene.com)) have been marketed in the United States, Australia, Japan and Europe and turned out to be stable in appearance over ten years of propagation and production (Chandler and Tanaka 2007).

### 19.2.4 White Flowers

In conventional ornamental plant breeding white flowers have often been reached by mutation breeding by which, most probably, key genes in flavonoid biosynthesis were knocked out. Nowadays down-regulation of genes can be efficiently achieved by biotechnological strategies. For example, in *Torenia* white flowers were created in a male and female sterile cultivar ‘Summerwaves Blue’ by silencing the *ANS* gene, and RNAi was proven to be more effective than sense or antisense suppression (Nakamura et al. 2006).

### 19.2.5 Pigmentation Patterns

In addition to modifications in flower colour, new types of patterns would be extremely interesting for ornamental plant breeders. Unexpected patterning was

observed in *Petunia* (Napoli et al. 1990) and lisianthus (*Eustoma grandiflorum*) plants transgenic for an antisense chalcone synthase gene (Deroles et al. 1998). Flower patterns are caused by the action of transcription factors (Schwinn et al. 2006), by transposons (Iida et al. 1999) or by chimeral plant composition. The first two causes could be used also in transgenic approaches, however successful applications are hardly found in literature (for transposons, see Liu et al. 2001) and stability as a key requirement for approval of plant breeder's rights will be essential.

In addition to manipulation of structural genes, the transformation of regulatory genes, i.e. transcription factors offer further possibilities. The best studied transcription factor is *Leaf colour* (*Lc*) from maize belonging to the basic helix-loop-helix (*Myb*) family. It has been constitutively expressed in different plant species, causing enhanced pigmentation due to up-regulation of several structural genes in some (*Petunia*, tobacco, tomato), but not in other species (lisianthus, *Pelargonium x domesticum*; Bradley et al. 1999). Recently a new technology was proposed for dominant repression of transcription factors called chimeric transcriptional repressor (*CRES-T*; Shikata and Ohme-Tagati 2008). By applying this method interesting new floral colours and shapes have been obtained with the *Arabidopsis thaliana SEPALLATA* (*SEP3*) transcription factor in *Torenia* (Shikata and Ohme-Tagati 2008).

### 19.3 Postharvest Quality

After the production phase, the postharvest phase starts which includes storage, trading and the shelf life at the consumer and which can be defined for cut flowers as well as for potted plants. Aims during this postharvest phase are to keep the plants or flowers, i.e. its flowers and vegetative parts, in a decorative appearance for as long as possible and to slow down natural senescence processes. Senescence is genetically controlled and mainly mediated by three classes of plant hormones, ethylene, cytokinins and abscisic acid. Especially ethylene plays an important role in postharvest physiology. As its biosynthesis and signal transduction pathways as well as its receptors are well understood, biotechnological modifications of ethylene synthesis and perception have been described several times (reviewed by Bleeker and Kende 2000). Other authors have summarized genetic modulations of ethylene biosynthesis and signalling for plants in general (Czarny et al. 2006; Lers and Burd 2007; Stearns and Glick 2003) and ornamental species in particular (Serek et al. 2006). In general, two approaches have been undertaken to reduce the deleterious effects of ethylene, firstly modifications in endogenous ethylene synthesis and secondly expression of mutated ethylene receptor genes. The two steps in ethylene biosynthesis are catalysed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase. Down-regulation of endogenous ACC synthase by sense or antisense suppression in carnation resulted in suppressed ethylene

production (Iwazaki et al. 2004). Other publications deal with repression of ACC oxidase, for example in carnation (Savin et al. 1995) or in *Torenia* (Aida et al. 1998). In these *Torenia* plants, ethylene production was reduced resulting in prolonged flower longevity. Florigene commercialised a long-life carnation in which ethylene synthesis was repressed, but according to Chandler and Tanaka (2007) this product failed, because it could not be differentiated from other products during marketing chains and the benefits for consumers were not clear at the point of sale.

Ornamentals are often exposed to exogenous ethylene during storage and trading, and the blocking of plants' own ethylene synthesis does not protect them from the exogenous hormone. Therefore, the second strategy of transferring mutated ethylene receptor genes seems to be more promising at the moment. Since ethylene receptors are negative regulators of the signal pathway, a mutant receptor will result in a dominant phenotype of ethylene insensitivity. First experiments with ornamentals used the mutated ethylene receptor gene *etr1-1* from *Arabidopsis thaliana* under control of the duplicated CaMV35S promoter in *Petunia* (Wilkinson et al. 1997). However, ethylene plays important roles in several processes in the plant like growth, development of roots or defence against pathogens. Shaw et al. (2002) observed diminished disease resistance against fungal and bacterial pathogens in *Petunia* carrying the mutated ethylene receptor gene *ers* from *Brassica oleracea* under the CaMV35S promoter. Therefore, in later studies the *etr1-1* under the control of the flower specific promoter *FBP1* from *Petunia hybrida* was transferred. Bovy et al. (1999) were the first to use this construct to produce carnation with a longer display life, without the undesired side effects. Recently potted plants, i.e. *Campanula carpatica* (Sriskandarajah et al. 2007) and *Kalanchoë blossfeldiana* (Sanikhani et al. 2008), were transformed with the *etr1-1* gene driven by the *FBP* promoter. In both cases the activity of the promoter was shown to be restricted mainly to floral organs as expected, and the morphology of the plants was unchanged in comparison to non-transgenic controls. Transgenic lines were identified in both species which expressed markedly improved flower longevity if the plants were exposed to exogenous ethylene (Sanikhani et al. 2008; Sriskandarajah et al. 2007). For both species the registration process for commercialisation is in progress (Serek et al. 2007). Transgenic orchids (*Oncidium* and *Odontoglossum* hybrids) have been obtained carrying the *FBP::etr1-1* construct and await their testing for flower longevity (Raffeiner et al. 2009). In order to reduce leaf yellowing which is provoked by ethylene in *Chrysanthemum*, Narumi et al. (2005) compared different single nucleotide mutations in the *Chrysanthemum* ethylene receptor gene *ers1* corresponding to other known mutations of the *etr-1* gene and found the *etr1-4* mutation to reduce ethylene sensitivity most efficiently in transgenic plants. By the time that more information and gene sequences become available also other parts of the ethylene signal transduction pathway may be used, one example being *Petunia* with repressed expression of ethylene insensitive 2 (*EIN2*) gene, that mediates ethylene signals, showed longer postharvest life of the flowers, but also negative effects on rooting and root hair formation (Shibuya et al. 2004).

Besides biotechnological modifications of ethylene perception and synthesis, cytokinin synthesis has also been the target of transgenic approaches aiming at improved postharvest quality. The isopentenyl transferase *IPT* gene from *Agrobacterium tumefaciens* has been used to overexpress cytokinins in particular plant organs or developmental stages in order to benefit from the senescence retarding effects of cytokinins. Chang et al. (2003) developed transgenic *Petunia* plants containing the *IPT* under the senescence associated gene 12 (*SAG12*) promoter. They were able to show that the flowers of transgenic *Petunia* expressed delayed senescence and were less sensitive to exogenous ethylene. The horticultural performance of these transgenic lines revealed pronounced differences between lines, and Clark et al. (2004b) identified one line with delayed leaf senescence and no alterations in other horticultural traits. The  $P_{SAG12}\text{-}IPT$  construct had originally been developed by Gan and Amasino (1995) and was shown to lead to autoregulatory control of cytokinin biosynthesis and to delay chlorophyll degradation and leaf senescence.

When the *IPT* gene under control of a cold inducible promoter (*cor15*) was transferred into *Petunia* and *Chrysanthemum*, it was shown that a cold treatment of 72 h at 4°C induced the gene and resulted in less senescence symptoms during a dark storage simulation experiment at 25°C in cuttings and young transplants (Khodakovskaya et al. 2005). This might not be the ideal inducible promoter system but demonstrated the feasibility of the idea. From the increasing insights in cytokinin reception and signaling (e.g. Kim et al. 2006) and in the elements which are required for the delay of senescence by cytokinins like extracellular invertases (Lara et al. 2004), new ways to improve postharvest quality in ornamentals seem practicable in the future.

## 19.4 Plant Architecture

Plant growth habit is of special interest for ornamental potted plants. Here the consumers as well as the horticultural industry prefer pot plants with compact growth. This is traditionally achieved by choosing adequate cultivation conditions mainly regarding light and temperature regimes, by pruning, by nutrition and irrigation and by chemical growth retardants. The latter are substances which inhibit biosynthesis of gibberellic acids, many of which are under debate due to their toxicity to humans and negative impacts on the environment and are already prohibited in many countries. Therefore, genetically compact varieties gain increasing importance, achieved in some species by conventional breeding, but also obtained in transgenic approaches.

Different ways to modify plant architecture in ornamental plants have been suggested and they can be grouped into: (i) use of hairy-root phenotypes induced by *Agrobacterium rhizogenes*, (ii) transformation of *rol* genes and (iii) changes in gibberellic acid biosynthesis, degradation or sensitivity. However, since increasing knowledge is arising in genes controlling plant architecture, including shoot apical meristem activity, axillary meristem formation and outgrowth and

inflorescence architecture (reviewed for example by Ongaro and Leyser 2008; Wang and Li 2006, 2008), in future much more specific and precise alterations of plant growth habit seem to become possible. With regard to compact ornamental plants, there is particular interest in recent results on the process of stem elongation, which is thought to be regulated by four pathways, mediated by: (i) gibberellin acid, (ii) auxin, (iii) skeleton and (iv) brassinosteroid (summarized by Wang and Li 2008).

*Agrobacterium rhizogenes* transfers part of its Ri plasmid into plant cells resulting in the hairy-root phenotype (Tepfer 1984), which in general comprises a range of morphological changes including dwarfness due to reductions in internode length and leaf size, increased branching, increased flower number, better rooting ability and others. Transformation with *A. rhizogenes* induces hairy roots, from which transgenic plants can be regenerated via adventitious shoot formation (Christey 2001). An overview of examples for *A. rhizogenes* transgenic ornamental species is presented by Casanova et al. (2005). One interesting aspect in *A. rhizogenes* transgenic plants is that they are derived from a process which also occurs in nature and might not be considered as a GMO by the authorities, as indicated for Japan by Mishiba et al. (2006). These authors report on gentians transformed by *A. rhizogenes* with reduced plant height and thereby possibly usable as potted plants, which did not need the approval by the Japanese authorities. The same approach was recently reported for *Kalanchoe blossfeldiana* (Christensen et al. 2008). Among *A. rhizogenes* transgenic lines, some were identified with reduced internode length while flowering behaviour did not differ from non-transformed control plants. Interestingly, the flowers of transgenic plants had an improved postharvest quality and increased ethylene tolerance (Christensen and Mueller 2009).

While many of the changes observed in *A. rhizogenes* transgenic plants might be beneficial for ornamental crops, other effects (e.g. smaller flowers) are not desired. Therefore, several research groups concentrated on the transfer of genes of the Ri plasmid, the so-called *rol* (root loci) genes, singly or in combination (reviewed for ornamental species by Casanova et al. 2005; Smith et al. 2006). Up to now the biochemical functions of all four *rol* genes (*rolA*, *rolB*, *rolC*, *rolD*) remain unclear. Most promising results have been obtained with *rolC* transgenic plants which share several phenotypic alterations like reduced apical dominance and thus increased branching, reductions in internode length, smaller, mostly more narrow leaves, wrinkled leaf area, earlier flowering, reduced flower size and reduced male fertility (Casanova et al. 2005; Smith et al. 2006). Especially for woody ornamental plants the increased rooting ability as demonstrated in *rol*-transgenic rose (van der Salm et al. 1997) could be an interesting effect. For many investigated species a marked variation in levels of expression of the new phenotype was commonly observed, so that many independent transgenic lines have to be developed and possibly crossed back to other breeding lines. Among the ornamental species for which field trials have been performed in Europe are *rol*-transgenic *Limonium* (Mercuri et al. 2001) and *Osteospermum* (Giovannini et al. 1999). Moyal-Ben Zvi et al. (2008) reported on *Gypsophila paniculata* carrying the *rolC* gene under control of the CaMV35S

promoter, which were characterized by a bushy phenotype and enhanced lateral shoot development accompanied by extensive rooting.

Gibberellic acids (GAs) are one important class of phytohormones responsible for stem elongation. Transgenic approaches aiming at reduced internode elongation or compact habitus involve either a mutated gene *GAI* (*gai* = gibberellic acid insensitive) which affects GA signal transduction or enzymes inactivating GAs. Transgenic *Petunia* (Tanaka et al. 2005) and *Chrysanthemum* (Petty et al. 2003) overexpressing the *GAI* gene had dwarf phenotypes, but in the case of *Chrysanthemum* pleiotropic effects were described concerning prolonged time to flowering and reduced number and size of flowers. Unexpected results were reported for carnations expressing the *Arabidopsis gai* gene under control of the CaMV35S promoter: the plants showed increased stem length instead of dwarfness and flowered earlier than control plants (Li-Hua et al. 2007). Genes for GA2-oxidases inactivating bioactive GAs from *Phaseolus coccineus* were recently transferred to two *Solanum* species and effectively reduced the GA contents when driven by the CaMV35S promoter. Transgenic *S. melanocerasum* and *S. nigrum* plants were clearly reduced in height, but not delayed in flowering. However, side effects on chlorophyll and carotenoid levels were found (Dijkstra et al. 2008). Another type of modification of changing GA metabolism was tested by Topp et al. (2008) who tried to silence an endogenous GA20-oxidase in *Kalanchoë blossfeldiana*. This gene is responsible for the formation of active GAs. However, the ethanol treatment applied to *Kalanchoë* cuttings to induce the promoter had negative effects on elongation of control plants as well, so that the system needs further testing.

For the future, improvements can be expected from using promoters which specifically allow to control gene expression spatially as well as temporarily, an aspect which is significant for all kinds of transgenic approaches, but particularly important for manipulation of the phytohormone status. Furthermore, from the increased understanding of cross talk between phytohormones and the discovery of new plant hormones, e.g. the terpenoid strigolactones, which were shown to be involved in shoot branching (Umeshara et al. 2008), novel possibilities to modify plant architecture will arise.

## 19.5 Disease Resistance

Infections with pests and pathogens cause significant losses in ornamental crop production (Daughtrey and Benson 2005). Although agrochemicals are effective against a range of pathogens and vectors, their use is increasingly restricted due to rising costs, legal restrictions and environmental concerns of the public. As an alternative either resistance breeding or biotechnological strategies gain in importance (see Chap. 10). However, in some crops resistance breeding is complicated by the limited natural sources of resistance genes, narrow gene pools of cultivars or complex genetics (as e.g. in roses, carnation, *Cyclamen*).

In ornamentals the application of biotechnological methods to enhance disease resistance has several specific aspects. In most cases the ornamental value of the crop is severely affected even by mild disease symptoms so that under commercial conditions even these mild disease symptoms cannot be tolerated (Daughtrey and Benson 2005). Furthermore, many ornamental crops are vegetatively propagated based on stock populations so that some pathogens (e.g. viruses) impose an enhanced risk of accumulation after many generations of vegetative propagation, making disease resistance increasingly important.

Due to intensive research and development conducted over the past two decades, several approaches for the manipulation of disease resistance/tolerance via biotechnology are available (Punja 2001; Strange and Scott 2005). Available strategies as well as applications to ornamental biotechnology were recently reviewed by Hammond et al. (2006). Strategies for virus resistance include the expression of viral coat proteins, the silencing of viral genes via antisense or RNAi constructs, the expression of virus specific antibodies as well as the expression of defective viral replicase genes. Resistance to bacteria and fungi has been achieved via the over-expression of antifungal proteins and by upregulation of transcription factors. Resistance to insects and nematodes (Chap. 10) via biotechnology has been achieved by overexpression of genes encoding either metabolic inhibitors or toxins (like the *Bacillus thuringiensis* endotoxins). Effects reported from expression of cry endotoxin genes from *B. thuringiensis* are the most successful examples of increased insect resistance through biotechnology of agricultural crops so far.

Here, selected examples are presented from only those approaches where transgenic plants were tested for resistance in bioassays.

### 19.5.1 Virus Resistance

Virus resistance is an important producer trait as many vegetatively propagated ornamental crops are propagated from stocks of mother plants which have to be kept disease-free, as the curation of virus infection is extremely difficult. For many clonally propagated ornamental crops rigorous virus testing of different steps in the production chain via ELISA or PCR are applied, as virus infections cause significant losses for the producer.

The generation of virus resistant transgenic crop plants is one of the most successful applications of biotechnology to resistance management strategies (Fuchs and Gonsalves 2007; Goldbach et al. 2003). The transformation of plants with genes coding for viral coat proteins or silencing virus specific genes was demonstrated to result in highly resistant plants. After the first approaches utilised sequences from viral coat proteins, more recent strategies increasingly rely on the silencing of viral genes via the RNAi mechanism (Hammond et al. 2006).

A first report was published by Sherman et al. (1998) who transferred three constructs with full-length, truncated sequences or antisense sequences of the nucleocapsid (*N* gene) gene of the tomato spotted wilt virus (TSWV) into

*Chrysanthemum*. The sequence of the *N* gene had been derived from a *Dahlia* isolate of TSWV. Inoculation with a highly virulent strain of TSWV isolated from *Chrysanthemum* revealed one transgenic line expressing a truncated and two transgenic lines expressing the antisense version of *N* with no symptoms and no virus accumulation. These lines also had no detectable levels of the *N* protein in ELISA assays (Sherman et al. 1998).

Later, Kamo et al. (2005) used particle bombardment to transform *Gladiolus* plants with the bean yellow mosaic virus coat protein (*CP*) gene in either sense or antisense orientation. Transgenic plants (four with the *CP* gene in sense orientation, seven in antisense) were obtained with both constructs. After artificial inoculation of the transgenics a delay in virus infection was reported in comparison to non-transgenic controls for some of the transgenic lines carrying either type of construct.

In a different approach the expression of double-stranded RNA-specific ribonuclease gene *pac1* derived from *Schizosaccharomyces pombe* was transferred to chrysanthemum (*Dendranthema grandiflora*). Three transgenic lines stably expressing *pac1* were inoculated with chrysanthemum stunt viroid (CSVd) and showed decreased disease symptoms and virus accumulation when compared to control plants (Ogawa et al. 2005). In addition, they showed lower infection rates when inoculated with tomato spotted wilt virus (TSWV).

Recently Clarke et al. (2008) developed an *Agrobacterium*-mediated transformation system for poinsettia (*Euphorbia pulcherrima* Willd. Ex Klotzsch) and transferred a virus-derived hairpin (hp) RNA to silence the poinsettia mosaic virus. Based on DAS-ELISA assays, transgenic plants with increased resistance to the virus were detected and these plants also expressed small interfering RNAs (si RNAs), indicating that resistance was based on the silencing process as intended (see Chap. 5).

### 19.5.2 Resistance Against Fungi and Bacteria

As for other stress response traits, strategies applied for enhancing fungal and bacterial disease resistance in plants involved the expression of antifungal genes (e.g. lytic enzymes) and more recently so-called “master switch genes”. These are mostly transcription factors, such as *WRKY* genes (containing the amino acid sequence WRKY), MAPK kinases (mitogen-activated protein kinases) or *NPRI* (non-expressor of pr genes), which modulate whole defence pathways (Gurr and Rushton 2005). In ornamentals only approaches utilising genes coding for antimicrobial peptides have been reported so far (Hammond et al. 2006).

Bi et al. (1999) transformed scented geraniums with a gene encoding the antimicrobial protein *Ace-AMP1* from onion via *Agrobacterium*. The plants with the highest expression levels for *Ace-AMP1* showed increased resistance to *Botrytis*

*cinerea*. The same gene was used to transform roses via *Agrobacterium*-mediated transformation and several transgenic genotypes showed enhanced resistance to rose powdery mildew (*Podosphaera pannosa*), both in excised leaf assays and in greenhouse experiments (Li et al. 2003).

Two other approaches led to transgenic roses with enhanced resistance to *Diplocarpon rosae*, the causal agent of rose black spot. Marchant et al. (1998) transformed embryogenic callus of roses via particle bombardment to express a basic class I chitinase from rice under control of the CaMV35S promoter. The expression of the transgene reduced symptom development after black spot inoculation by 13–43%. Dohm et al. (2001a, b, 2002) transferred combinations of barley chitinase with barley glucanase, barley chitinase with barley ribosome inhibiting protein (RIP) and all genes as single constructs into roses via *Agrobacterium* mediated transformation of somatic embryos. All genes were expressed under CaMV35S control with or without a transit peptide which directed the secretion into the intercellular space. Although most of the plants with integration of the transgene expressed the antimicrobial peptides only some showed enhanced resistance to black spot. The best line expressing barley RIP with the transit peptide showed a reduction of infection by 60%.

Takatsu et al. (1999) expressed a rice chitinase gene (*RCC2*) in chrysanthemum. Eleven lines expressing *RCC2* showed different levels of resistance to *Botrytis cinerea*. Three lines displayed very high resistance with only very little symptoms.

Another type of gene was used by Esposito et al. (2000) who transferred a combination of *ech.42* a gene from the antagonist fungus *Trichoderma harzianum* encoding an endochitinase and an osmotin gene from *Nicotiana tabacum* to *Petunia*. Among the 24 plants for which the integration and expression of both transgenes could be confirmed by Northern, Southern and Western blot analyses increased resistance to *Botrytis cinerea* ranged from 20% to 50%.

Recently a synthetic cecropin-based lytic peptide (*Shiva-1*) with the secretory signal of the pathogenesis related protein 1b (*PR1b*) and under the control of the CaMV35S promoter was transformed into two *Anthurium* cultivars (Kuehnle et al. 2004). Testing for blight tolerance by inoculation with a strain of *Xanthomonas axonopodis* revealed significantly enhanced tolerance in two lines, whereas two other lines showed no improvement and one even higher susceptibility. Another report on engineered bacterial resistance was published by Liau et al. (2003) who transformed *Oncidium* orchids with a sweet pepper ferredoxin-like gene (*PFLP*). Transgenic *Oncidium* plants expressing *pflp* showed a significantly decreased degree of soft rot symptoms when inoculated with *Erwinia carotovora*.

### 19.5.3 Insect Resistance

Successful engineering of resistance to insects in ornamental crops has only reported a single time so far. Shinoyama and Mochizuki (2006) introduced the

*CRYIAb* endotoxin gene from *Bacillus thuringiensis* into five popular chrysanthemum cultivars. An insect bioassay using larvae of the oriental tobacco budworm (*Helicoverpa armigera*) and the common cutworm (*Spodoptera litura*) was conducted. Larvae died during the first or second instar when supplied with leaves of transgenic lines expressing *CRYIAb*.

## 19.6 Flowering Time

The control of flowering time has been intensively studied in model plant species and a number of genetic switches influencing flowering time have been characterised (Turck et al. 2008). Although flowering time is one of the major traits contributing to the ornamental value of a crop, few results have been published to date. This might be due to the fact that many factors identified in models (e.g. *Arabidopsis*) contribute quantitatively to changes in flowering time and they might function in a slightly different molecular context in other species. Giovannini et al. (2002) reported the transfer of the *Arabidopsis CONSTANS* gene (*CO*) to *Osteospermum ecklonis* and the analysis of a single transgenic clone which produced 30% more flowers over an extended flowering time. However, due to the small sample number no general conclusion can be made from this report.

In the previously mentioned study Li-Hua et al. (2007) expressed the *Arabidopsis gai* gene under control of the CaMV35S promoter in carnations. Analysis of five transgenic clones showed an increase in shoot length and also early flowering in the greenhouse as compared to non-transgenic controls. However, in this approach the change in flowering was more like a side effect to the general change in hormonal balance mainly aiming at general changes in the plant habit.

## 19.7 Modification of Flower Structure

Flower morphology is one of the key traits for selection of new ornamental cultivars. For example only double-flowered roses are marketable as cut roses and flower size is an important criterion for variety development in orchids. Although several orthologues to floral homeotic genes have been isolated from a variety of ornamental species (Kitahara and Matsumoto 2000; Kitahara et al. 2001; Mondragon-Palomino and Theissen 2008; Tzeng et al. 2002) no attempt has been made to manipulate flower morphology with these genes in ornamental crops. Instead these genes have been transferred to model species for functional analyses (Hibino et al. 2006; Kitahara et al. 2004).

In a recent publication Verdonk et al. (2008) used a somewhat non-specific approach in expressing the *Agrobacterium tumefaciens* isopentenyltransferase

(*IPT*) gene under control of the flower specific *Arabidopsis APETALA 3* promoter in *Petunia hybrida*. This increased flower size (corolla diameter, flower fresh weight) but had no effect on vegetative development.

## 19.8 Improvement of Abiotic Stress Tolerance

The response of ornamental crops to abiotic stresses (see Chap. 8) is of particular importance because a large number of species are derived from climatic areas different from their place of cultivation and/or consumption. As an example tropical orchids like *Phalaenopsis* have a major market in temperate areas like the northern United States and central Europe. Therefore, significant amounts of energy are needed to provide optimal temperatures for growth of these crops in the production process. Likewise, many bedding and pot plants for outdoor cultivation suffer not only from a lack of freezing tolerance but also chilling tolerance (with temperatures above 0°C) which limits the growth period for consumers significantly (Park and Chen 2006). In addition, drought tolerance was recently identified as being an important trade and consumer trait that would provide an added value to bedding and potted plants which would need less care by the consumer (Chylinski et al. 2007).

In model plants a significant body of literature has been published about genetic mechanisms involved in the response of higher plants to abiotic stresses (Chinnusamy et al. 2005; von Koskull-Döring et al. 2007). In these studies a large number of genes were identified that are involved either in the biosynthesis of protecting metabolites or in regulating stress response pathways. Consequently, several approaches have been tested to improve abiotic stress tolerance by genetically engineer both model plants and agricultural crops (Chinnusamy et al. 2005). As an example, some stresses like freezing, salinity and drought all reduce the osmotic potential of stressed cells. Therefore, osmotic adjustment by engineering the over-production of osmolytes and/or osmoprotectants (e.g. glycine betaine, trehalose, fructans) have been successfully used in *Arabidopsis*, tobacco and a number of agricultural crops (Park and Chen 2006). Alternatively transcription factors (e.g. *CBF1*, *CBF4*) that up-regulate whole stress response pathways have been successfully used to engineer freezing and drought tolerance simultaneously in a number of species (Park and Chen 2006).

However, in contrast to the vast potential that the enhancement of abiotic stress tolerance might have on ornamental markets, no published report on successful engineering of any abiotic stress tolerance has appeared to date. Recently some activities of private companies ([www.ornamental-bioscience.com](http://www.ornamental-bioscience.com)) have aimed at the engineering of four major vegetatively propagated ornamental crops for drought, heat, freezing and salinity stress by overexpression of transcription factors from *Arabidopsis*. First experiments with different *Petunia* genotypes have already shown increased tolerance to drought stress (Boehm 2009).

## 19.9 Modification of Floral Scent

More than 700 secondary metabolites contributing to the scent of angiosperm flowers have been characterised most of which fall into four major substance classes: terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives (Dudareva and Pichersky 2008). Pioneering work in *Clarkia breweri* a Californian desert plant led to the identification of several genes involved in terpenoid biosynthesis. Additional structural genes have been isolated from other genera including *Petunia*, *Rosa*, *Fragaria* and *Citrus* (reviewed by Dudareva and Pichersky 2008).

One of the first scent-related genes to be transferred to an ornamental plant was S-linalool synthase (*LIS*) from *Clarkia breweri* which was constitutively expressed in *Petunia* plants (Lucker et al. 2001). However, instead of free linalool only non-scented derivatives could be detected in various tissues.

Carnation (*Dianthus cariophyllus*) flowers emit a number of volatiles but lack monoterpenes. In order to change the volatile pattern of carnation flowers by the additional production of monoterpenes, the *C. breweri LIS* gene under control of the CaMV35S promoter was transformed into carnation via *Agrobacterium* (Lavy et al. 2002). Although headspace GC-MS analyses revealed that linalool and some of its derivatives were emitted from the flowers of transgenic lines, no changes in the flower scent were detectable to the human nose.

In a third attempt another *Clarkia* gene, benzyl alcohol acetyltransferase (*BEAT*), under control of the CAMV35S promoter was transformed to *Eustoma grandiflorum* (Aranovich et al. 2007). Feeding assays with externally applied benzyl alcohol to flowers lead to five to seven times higher concentrations of benzyl acetate compared to non-transformed plants but other alcohol substrates supplied were also converted to acetates.

In order to characterise the function of a cloned rose acyltransferase (*RhAAT1*) Guterman et al. (2006) transformed *Petunia* with this gene under the control of the CaMV35S promoter. The resulting transgenic flowers produced phenylethylacetate and benzylacetate not produced by control plants. As the preferred substrate of *RhAAT1* in *in vitro* assays is geraniol, this demonstrates that the production of volatiles by some enzymes is dependent on both substrate availability and specificity. However these experiments were not conducted to reengineer the *Petunia* volatile profile but rather for analytical reasons, as roses are much more difficult to transform.

A completely different strategy was followed by transforming *P. hybrida* with the *Pap1* Myb transcription factor from *Arabidopsis thaliana* (Ben Zvi et al. 2008). In contrast to genes coding for enzymes converting a particular substrate into a volatile this transcription factor was shown to regulate non-volatile phenylpropanoids, including anthocyanins. As a result an increase in pigmentation intensity and a tenfold increase in the emission of volatile phenylpropanoid/benzenoid compounds was measured in the transgenic versus non-transgenic *Petunia* flowers. Subsequent analyses of transgenic lines via expression profiling showed that the

increase in pigmentation and volatile emission is most likely due to increased metabolic fluxes redirected by the transcription factor.

## 19.10 Conclusion

In the near future the commercialisation of transgenic ornamentals will be impeded by two factors: (i) the high costs for the development of lines with stable expression and which fulfil the criteria of the regulatory authorities (Chandler and Tanaka 2007) and (ii) the lack of public acceptance. However, if genetically modified ornamentals become available that carry traits with added values beyond simple colour modifications (e.g. combinations of colour modification and extended vase life or combinations involving disease resistances) biotech approaches have the potential to become an accepted method for the development of new ornamental products.

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# Chapter 20

## Potato

Jens Lübeck

### 20.1 Introduction

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after maize, rice, and wheat in terms of total production quantity and is the world's number one food crop in terms of productivity relating to yield (322 million tonnes from 19.3 million hectares; FAO 2007). It is one of the most expanding crops, especially in developing countries. In 2008 the United Nations celebrated the 'International Year of the Potato' to raise awareness of the potato's fundamental importance as a staple food of humanity and the potato's place in agriculture, the economy and world food security (<http://www.potato2008.org>).

Potato shows a tremendous variability in geographical adaptation. It is grown in more than 150 countries from latitudes 65°N to 50°S and at altitudes from sea level to 4000 m. The current edition of the *World catalogue of potato varieties* (Hils and Pieterse 2007) lists more than 4200 different potato varieties from more than 100 countries, a remarkable achievement of 150 years of traditional potato breeding, given the fact that this extraordinary variation relies on a relatively narrow genetic base (Bradshaw et al. 2006).

However, the increasing worldwide demand in sustainable potato production is still a challenge to modern potato breeders. The breeding of potato is hampered in its efficiency by its tetraploidy associated with a high degree of heterozygosity and tetrasomic inheritance. Furthermore, during variety development more than 50 traits have to be considered and sufficient tubers for the evaluation of tuber quality traits or agricultural performance are only available years after crossing, due to the low multiplication factor of this vegetatively propagated crop.

Genetic engineering can be regarded as an important tool in expanding the genetic base available to breeders, in the improvement of agricultural and quality

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traits, and in opening new possibilities for innovative uses. Potato became transformable in 1986 (Ooms et al. 1986), shortly after the generation of the first transgenic plant was reported in 1983 (Herrera-Estrella et al. 1983). Transformation in general is discussed in Chap 1. Since then methods have been refined (Rocha-Sosa et al. 1989) and transgenic potatoes have served as a favourite model system for a wide range of fundamental studies in plant molecular physiology and molecular genetics. A search on Google Scholar for ‘transgenic AND potato’ revealed 43,300 results by the end of November 2008. The fundamental studies, e.g. on sink–source interactions, especially with regard to carbon partitioning, or on the expression of foreign proteins for pharmaceutical use, are not discussed here and have been reviewed elsewhere (Lytovchenko et al. 2007; Pribylova et al. 2006). This chapter highlights some examples of how genetic engineering can address the increasing worldwide demands for potato varieties with higher resistance to pests, improved tuber quality, more nutritional value, and an enhanced capacity for the production of (new) biopolymers. Additional aspects may be found in Chap 4.

## 20.2 Pathogen Resistance

### 20.2.1 Insects

The Colorado potato beetle (*Leptinotarsa decemlineata*) is the major insect pest of potato plants in large regions of the temperate climates. It often becomes resistant to repeatedly used insecticides, which makes intricate and costly spraying regimes necessary, including the rotation of active agents of pesticides. See also Chap. 10 for references.

Transgenic potato plants constitutively expressing a synthetic *cry3A* gene were able to control all stages of the Colorado potato beetle (Perlak et al. 1993). Cry endotoxin proteins from the bacterium *Bacillus thuringiensis* (Bt), when incorporated by insects directly or by feeding on leafs of transgenic plants, passes into the insect’s gut and thus causes death by its pore-forming activity at intestine epidermal cells (Whalon and Wingerd 2003). The action of Cry proteins is highly specific to coleopteran or lepidopteran insects and does not affect other insects or natural enemies to the pest (Naimov et al. 2003). For this reason the Bt toxin is one of the few pesticides used in organic farming.

Potatoes expressing *Cry3A* were the first transgenic potatoes commercialised. Monsanto started the marketing of their Bt potato under the trade mark NewLeaf in 1995 through the newly founded subsidiary Nature Mark.

The Bt approach was also applied to provide transgenic resistance to the potato tuber moth (*Phthorimaea operculella*), a notorious threat to potato fields and stored tubers in tropical and subtropical climates. The capability of the *cry5* gene to control larval feeding on stored tubers was investigated by Mohammed et al. (2000) using three different promoters. Constitutive expression or expression under the control of the light-inducible *Lhca3* promoter of either the *cry1Ac9*

gene (Davidson 2004) or the *cry9Aa2* gene (Meiyalaghan 2005) led to transgenic potato lines resistant to the larvae of the potato tuber moth under laboratory and field conditions. In South Africa there are ongoing activities to deregulate and release Bt transgenic potato cv. Spunta resistant to the potato tuber moth (Douches 2007; Estrada et al. 2007).

An additional more recent approach to engineering insect resistance in potato is the use of RNAi constructs. dsRNAs targeting the V-ATPase A and V-ATPase E orthologues each caused significant larval mortality in the Colorado potato beetle bioassay (Baum et al. 2007).

## 20.2.2 Viruses

Potato leafroll virus (PLRV) and potato virus Y (PVY) are the major devastating viral diseases in potato. They are a severe problem, not only due to the damage caused by primary infection, but also because of the vegetative propagation of potato. During the years of multiplication for seed potato production they are transmitted through the tubers to subsequent generations, leading to downgrading of seed crops and to high costs for insecticides to control virus-transmitting aphids.

Many attempts have been made to confer virus resistance to potato by means of genetic engineering. The strategies used are mainly based on pathogen-derived resistance (PDR), e.g. the expression of viral sequences encoding structural and non-structural proteins or RNA transcripts conferring post-transcriptional gene silencing (see Prince et al. 2008 for a general review; see Solomon-Blackburn and Barker 2001 for a review on potato).

Coat protein-mediated resistance was achieved for PVY (Lawson et al. 1990) as well as PLRV (Kawchuk et al. 1991), although the latter approach proved to be strain-specific and failed when tested in the field (Thomas et al. 1997). Broad and stable resistance to PLRV was generated by expressing the *orf1* (replicase) and *orf2* (helicase) of PLRV under the constitutive figwort mosaic virus 35S promoter (Thomas et al. 2000).

Polymerase-mediated PLRV resistance and coat protein-mediated PVY resistance were each combined with Bt *Cry3A* resistance to Colorado potato beetle by Monsanto scientists and the resulting potatoes were commercialised as NewLeaf Plus and NewLeaf Y in 1998. Because of increasing consumer concern since 1999, McDonald decided to ban genetically modified crops from its food chain, forcing Monsanto to withdraw their NewLeaf potato lines and to back out of the potato business (Kaniewski and Thomas 2004). The Nature Mark company was dissolved in 2001.

Apart from the multitude of PDR strategies towards virus resistant potatoes, the use of antiviral antibodies has been reported recently (Gargouri-Bouzid et al. 2006; Nickel et al. 2008). ScFv antibodies raised against the C-terminal part of the PLRV P1 protein were expressed constitutively in transgenic potatoes. When greenhouse-grown transgenic plants were challenged by viruliferous aphids, a reduction of virus accumulation was observed 5 weeks post-infection from levels of around 50%

compared to wild-type plants to no measurable accumulation. However, growth of the transgenic plants was slightly slower than the wild-type controls and also displayed a chlorotic phenotype (Nickel et al. 2008).

### 20.2.3 *Phytophthora infestans*

Potato late blight, caused by the oomycete *Phytophthora infestans*, is one of the world's most destructive agricultural diseases (Fry 2008; Garelik 2002). However, 150 years of potato breeding have contributed next to nothing to potato resistance to this disastrous pest and only the application of chemicals provides reasonable levels of disease control. Hence, countless efforts have been made to strengthen the resistance of potato to *P. infestans* by means of genetic engineering.

The strategies used are versatile, including the expression of antifungal compounds (Liu et al. 1994; Wu et al. 1995), the mimicry of a hypersensitive response (HR) (Strittmatter et al. 1995), the induction of plant innate HR by (co-)expressing resistance (R) genes and the corresponding Avr genes (Pain et al. 2003) following the concept of de Witt (1992) or the modification of signalling pathways (Yamamizo et al. 2006). Whereas antifungal compounds were expressed constitutively, the latter three strategies strictly rely on the use of pathogen-inducible and pathogen-specific promoters with appropriate spatial and temporal expression pattern, but promoters fitting all these requirements have up to now not been found. The combination of defined regulatory elements in synthetic promoters may be an interesting option to this end (Rushton et al. 2002). Nevertheless, in contrast to approaches to generate insect or virus resistance, attempts to genetically engineer broad spectrum durable *P. infestans* resistance on a level sufficient for agricultural practice have failed.

The use of naturally occurring resistance genes (R genes) has been neglected during the past decades, because R genes introgressed from *S. demissum* in the early 20th century have quickly been overcome by resistance-breaking strains, an observation which eventually could well be explained by the race-specificity of R genes after formulation of the gene-for-gene hypothesis (Flor 1971). However, recent advances in the cloning of R genes from wild *Solanum* species, like *RB* and its allelic variant *Rpi-blb1* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003), has raised expectations to find race-non-specific and durable R genes. Additional R genes, *Rpi-blb2* (van der Vossen et al. 2005) and *Rpi-blb3* (van der Vossen et al. 2008) from *S. bulbocastanum*, have been cloned and many others from different wild species have been located on genetic maps. Recently, effector genomics resulted in the rapid cloning of *Rpi-stol1* and *Rpi-ptal1*, functional homologues of *Rpi-blb1* from *S. stoloniferum* and *S. papita* (Vleeshouwers et al. 2008).

Whether these genes are durable or not remains to be elucidated. Transgenic potato lines carrying the *Rpi-blb1* and *Rpi-blb2* genes have been tested in the field throughout Europe by BASF Plant Science (e.g. notification numbers: B/DE/07/191, B/NL/07/07, B/GB/07/R42/01, B/CZ/07/01, B/FR/06/12/15) since 2006. Field trials performed in the United States showed that *Rpi-blb1* (*RB*) does not confer

resistance to tubers in field trials with *RB* transgenic potato lines (Halterman et al. 2008) and a first virulent *P. infestans* strain to *Rpi-blb1* has been detected in functional *Agrobacterium* infiltration assays (Vleeshouwers et al. 2008).

Many more R genes from wild relatives of the potato are expected to be cloned in the upcoming years and the only realistic option for making use of them is the cisgenic approach, i.e. plants transformed with cisgenes are exempted from the GMO regulation (Jacobsen and Schouten 2008; see also Chap. 4). In order to cope with the extraordinary dynamics of *Phytophthora infestans*, going through the regulatory approval process for every gene would be by far too time-consuming, as is the pyramiding of these genes by marker-assisted introgression.

## 20.3 Tuber Quality Traits

### 20.3.1 Blackspot Bruise

Impact-induced bruise of potato tubers causes an enzymatic discolouration which is highly undesirable for nearly all uses of potatoes. The leakage of polyphenol oxidase (PPO) from damaged amyloplasts and the subsequent oxidation of phenolic compounds in the cytosol lead to the precipitation of black melanin-like pigments (Friedman 1997). Downregulation of PPO has successfully been applied to diminish discolouration, by transforming potato with either antisense (Bachem et al. 1994) or sense (Coetzer et al. 2001) PPO RNA constructs under the control of constitutive or tuber-specific promoters.

Whilst on the one hand these approaches relied on silencing the family of homologous PPO genes and on the other hand PPOs operate in activation of pathogen defences, Rommens et al. (2004) used constructs specifically targeting POT32, a PPO gene predominantly expressed in mature tubers, not expressed in leaves and not induced upon infection. Additionally, the used vectors were assembled only from potato derived sequences, resulting in ‘intragenic’ plants. In a more recent study, this approach was extended using a construct with inverted repeats of the POT32 gene, the starch-associated *R1* gene and the phosphorylase-L gene, leading to multigene silencing (Rommens et al. 2006). Suppression of the latter two genes aimed at reducing cold-induced sweetening.

### 20.3.2 Cold-Induced Sweetening

The potato processing industry would like to store tubers at low temperatures (4–6°C) in order to limit sprouting and concurrently avoid the need for dormancy-prolonging chemicals. However, at low temperatures potato tubers undergo the process of cold-induced sweetening (Müller-Thurgau 1882). They accumulate reducing sugars, i.e. glucose and fructose, and these in turn give rise to a non-enzymatic browning

through a Maillard reaction upon frying at high temperatures, thereby making them unsuitable for processing (Burton 1969). Additionally, the Maillard reaction was found to form the carcinogen acrylamide in heated foodstuff (Mottram et al. 2002; Tareke et al. 2002).

To avoid hexose accumulation in cold-stored tubers, two strategies have been successfully used: (i) inhibition of starch degradation and (ii) reduction of the activity of invertase(s). Inhibition of starch degradation was found in transgenic potatoes with antisense suppression of the gene encoding the starch granule-bound protein R1, due to reduced starch phosphate content which resulted in a less degradable starch. The reduced starch degradation led to an up to ninefold lowered amount of reducing sugars after 2 months of storage at 4°C, when compared to wild-type tubers (Lorberth et al. 1998). Reduction in the activity of invertase was achieved by the constitutive expression of a tobacco vacuolar invertase inhibitor homologue (*Nt-inhh*). Cold-induced hexose accumulation could be reduced by up to 75%, without altered starch quality (Greiner et al. 1999). Recently, Chen et al. (2008) reported an 84% decrease in cold-induced hexose accumulation in tubers of transgenic potatoes with RNAi-mediated repression of sucrose phosphatase (SPP). The plants showed a strongly lowered hexose-to-sucrose ratio, a high accumulation of sucrose-6-phosphate, and a blocked cold-induced expression of vacuolar invertase.

## 20.4 Nutritional Value

### 20.4.1 Amino Acids/Protein

Essential amino acids are a major factor of malnutrition of children in developing countries. Thus, modifying potato as a staple crop with regard to total tuber protein level or increasing the tuber's content of essential amino acids may be beneficial (check also Chap. 11).

Chakraborty et al. (2000) transformed potato with a gene coding for the seed albumin AmA1 from *Amaranthus hypochondriacus*, which is characterised by a well balanced amino acid composition and has no known allergenic properties. By using the sink-specific GBSS promoter the total amino acid content of the transgenic tubers was increased by up to 45%. The content of the particularly important amino acids cysteine, lysine, methionine, and tyrosine was raised two- to fourfold.

In addition, several attempts have been made to raise the level of sulfur-containing amino acids, in particular methionine. Methionine is the most limiting essential amino acid and, beside its role as a component of proteins, it serves as a precursor for the hormone ethylene or polyamines; it is also the primary methyl group donor for multiple biological processes (Amir et al. 2002).

The major regulatory enzyme of methionine biosynthesis is cystathione  $\gamma$ -synthase which competes with threonine synthase for -phosphohomoserine, the common substrate for both enzymes (Hesse et al. 2004). Antisense inhibition of threonine synthase in potato was reported to increase methionine levels in tubers up

to 30-fold compared with non-transformed plants, whereas in contrast to leaves threonine levels were not reduced in tubers (Zeh et al. 2001).

An alternative strategy to increased methionine levels in potato tubers is the expression of ectopic genes for cystathionine  $\gamma$ -synthase. Transgenic potato plants over-expressing the cystathionine  $\gamma$ -synthase gene from *Arabidopsis thaliana* showed a sixfold increase in the soluble methionine level in tubers compared to the wild-type plants (Di et al. 2003). This is in contrast to over-expression of the endogenous potato gene which left methionine levels unchanged although enzyme activity was elevated 2.7-fold (Kreft et al. 2003).

Dancs et al. (2008) combined the expression of an N-terminal deleted version of cystathionine  $\gamma$ -synthase from *Arabidopsis* (Hacham et al. 2006) and the methionine-rich protein 15-kDa  $\beta$ -zein (Golan et al. 2005). Co-expressing lines showed a twofold increase in free methionine and ethanol-soluble protein, respectively.

Expression of an ectopic feed-back insensitive key enzyme was also a strategy used to raise the levels of other limiting amino acids in potato tubers. The rice *OASAID* transgene, which encodes a point mutation of an alpha-subunit of rice (*Oryza sativa*) anthranilate synthase induced a two- to 20-fold increase in the amount of free tryptophan in transgenic potato plants, compared to wild-type plants (Matsuda et al. 2005; Yamada et al. 2004). Constitutive expression of the *cysE* gene from *Escherichia coli* encoding a serine acetyltransferase led to levels of cysteine and glutathione both in leaves and tubers which were 1.5-fold higher on average than in control plants (Stiller et al. 2007).

#### 20.4.2 Carotenoids

Plant carotenoids are lipid soluble pigments that play essential roles in plants and also play significant roles in the human diet. Humans do not produce carotenoids and therefore rely on a dietary supply of e.g.  $\beta$ -carotene which serves as a vitamin A precursor. Vitamin A deficiency is a serious problem in large parts of the developing world and led to the pioneering work of Ye et al. (2000), who engineered the  $\beta$ -carotene biosynthetic pathway into carotenoid-free rice endosperm.

As a major food crop also potato raised increasing interest for enhancing or modulating its carotenoid levels (for a review, see Giuliano et al. 2008). Strategies for increasing pro-vitamin A levels included silencing of the endogenous lycopene  $\varepsilon$ -cyclase (Diretto et al. 2006) or  $\beta$ -carotene hydroxylase genes (Diretto et al. 2007a; van Eck et al. 2007) as well as overexpression of ectopic bacterial genes for early steps in carotenoid biosynthesis such as phytoene synthase (*crtB*, *Erwinia uredovora*; Ducreux et al. 2005) or 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*, *Escherichia coli*; Morris et al. 2006a). However, until now, the most successful approach with regard to  $\beta$ -carotene content in potato tubers was the joint over-expression of the *Erwinia herbicola* phytoene synthase (*crtB*), phytoene desaturase (*crtI*), and lycopene  $\beta$ -cyclase (*crtY*; Diretto et al. 2007b), which resulted in

$\beta$ -carotene accumulation (47 µg/g dry weight) sufficient to deliver 50% of the recommended daily allowance in one 250 g serving.

Zeaxanthin has been another target for modification of carotenoid levels in potato tubers. This carotenoid accumulates in the macula lutea, the yellow spot near the centre of the retina of the eye and protects retinal cells from blue light damage. However, zeaxanthin intake from food sources is low, and increasing its content in staple foods such as potatoes could contribute to a decreased risk of age-related macular degeneration. Römer et al. (2002) succeeded in the genetic engineering of zeaxanthin-rich potatoes by silencing the zeaxanthin epoxidase gene (*zep*) by either antisense inhibition or co-suppression. The zeaxanthin content was raised to up to 130-fold in tubers compared to the wild type. A recently published study indicated that consumption of this zeaxanthin-rich potatoes significantly increased chylomicron zeaxanthin concentrations, suggesting that they could serve as an important dietary source of zeaxanthin (Bub et al. 2008).

Retransforming the zeaxanthin-rich potato line 47-18 with the ketolase gene *crtO* from *Synechocystis* 6803 led to the formation of astaxanthin among other ketocarotenoids (Gerjets and Sandmann 2006). Astaxanthin – commercially one of the most important ketocarotenoids – was also produced by expressing an algal  $\beta$ -ketolase gene in naturally zeaxanthin-enriched *Solanum phureja* (Morris et al. 2006b).

It should be noted here that all of the approaches described above rely on tuber-specific expression of the transgenes, due to the essential role of their targets in leaves. Thus, classical breeding is not an alternative and the proposed goals can exclusively be reached by genetic engineering.

#### 20.4.3 Fructan/Inulin

Fructans (fructooligosaccharides) are an important constituent in functional foods because they act as a prebiotic agent and positively influences the composition of the gut microflora (Roberfroid and Delzenne 1998). Additionally, there are observations of beneficial effects on mineral absorption, blood lipid composition, and prevention of colon cancer (van Loo et al. 1999).

Expression of bacterial fructosyltransferase genes in potato led to accumulation of considerable amounts of fructans with a very high degree of polymerisation (van der Meer et al. 1994), but almost all bacterial fructosyltransferases produce levan-type fructan, which is characterized by  $\beta$ -2-6 linkages of fructose molecules.

Inulin-type fructans have  $\beta$ -2-1 bonds in fructose chains and are of particular interest because they reduce the energy density of food and are used to enrich food with dietary fibre or to replace sugar and fat. Hellwege et al. (2000) succeeded in establishing a transgenic potato synthesising the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots. The ability to synthesise high molecular weight inulin was transferred to potato plants via constitutive expression of the *I-SST* (sucrose:sucrose 1-fructosyltransferase) and the *I-FFT* (fructan:fructan 1-fructosyltransferase) genes of globe artichoke. Inulin

made up 5% of the dry weight of transgenic tubers, without adverse effects on tuber number or on the fresh or dry weight of the tubers.

## 20.5 Production of Biopolymers

### 20.5.1 Starch

Starch, the natural carbohydrate-based biopolymer of potato tubers, is composed of ~80% amylose, a linear chain of  $\alpha$ 1-4-linked glucose units, and ~20% amylopectin, a highly branched chain of glucose units with additional  $\alpha$ 1-6 linkages. Potato starches modified in branching or phosphorylation have been produced by genetic engineering, resulting in novel characteristics to broaden their already tremendous range of industrial applications (Jobling 2004).

By constitutive antisense suppression of the granule-bound starch synthase (GBSS) Visser et al. (1991) were able to obtain transgenic potatoes that are devoid of amylose and contain only amylopectin. This approach was extended to tuber-specific downregulation of GBSS using antisense constructs under the control of either the gbss promoter or the patatin promoter (Hofvander et al. 1992). Various companies and institutes conducted large-scale field trials of amylopectin potatoes throughout Europe. The event EH92-527-1 (Amflora, BPS-25271-9), which derives from one of the first experiments in the early 1990s, is currently going through the regulatory approval process according to EU Directive 2001/18 and Regulation (EC) 1829/2003 on genetically modified food and feed (<http://www.gmo-compass.org>) and may become the first transgenic potato commercialised in Europe.

Further approaches to modify potato starch for commercial uses were the production of short-chain amylopectins with improved freeze–thaw stability by downregulation of the starch synthase genes GBSS, SSII, and SSIII (Jobling et al. 2002) or the production of amylose starch with high gelling strength via inhibition of starch-branched enzymes SBEI and SBEII (Schwall et al. 2000).

The sole covalent modification of starch is phosphorylation and the high degree of phosphate makes potato starch unique among commercial starches. Lorberth et al. (1998) found modified phosphate levels by repression of the starch granule-bound protein R1 which later was identified as an alpha-glucan, water dikinase (Ritte et al. 2002).

### 20.5.2 Polyhydroxyalkanoates

Polyhydroxyalkanoates, polyesters of hydroxyacids are naturally synthesised by various strains of bacteria as intracellular storage molecules for carbon and energy. Their characteristics are identical with those of elastomers and adhesive materials, and therefore these biodegradable plastics have a broad range of technical

applications (Moire et al., 2003). However, bacterial fermentation of the most abundant representative of this class of polymers poly(3-hydroxybutyrate) (PHB) is by far more expensive than chemical synthesis of polyethylene. Plants have been proposed as an alternative for the low-cost production of PHB; and the general feasibility was shown in *A. thaliana* by constitutive expression of the three genes of the *Ralstonia eutropha* PHB operon [ $\beta$ -ketoacyl-CoA thiolase (*PhbA*), acetoacetyl-CoA reductase (*PhbB*), PHB synthase (*PhbC*)]; Poirier et al. 1992].

Several attempts have been made to produce PHB in transgenic crop plants, including oilseed rape, corn, cotton, sugar beet, sugar cane, flax, tobacco, and potato (van Beilen and Poirier 2008). Directing the PHB biosynthesis pathway to plastids by nuclear transformation of a triple construct aiming at parallel expression of transit peptide fusions of the three PHB operon genes led to a considerable accumulation of PHB (up to 40% of leaf dry weight) in *A. thaliana* (Bohmert et al. 2000). However, using the same construct in potato transformation experiments resulted in not a single transgenic line and Bohmert et al. (2002) attributed this effect to the *PhbA* gene.

Inducible expression of the PHB enzymes was shown as a possible way to overcome some constraints associated with constitutive expression (Bohmert et al. 2002; Lössl et al. 2005). This approach is particularly interesting for potato, because post-harvest induction in stored and physiologically active tubers is an interesting option.

Besides PHB production the production of medium-chain-length polyhydroxylalkanoates (mclPHAs) was tested in potato. Constitutive expression of a *P. putida* 3-hydroxyacyl-ACP-CoA transacylase in combination with the *P. oleovorans* Pha-C1 polymerase, both genes fused to the potato rbcS transit peptide, resulted in measurable but very low amounts of mclPHAs (below 0.03% of leaf dry weight; Romano et al. 2005).

### 20.5.3 Cyanophycin/Poly-Aspartate

Cyanophycin (multiarginyl-poly[L-aspartic acid]) is a non-ribosomal protein-like polymer which is arranged as a poly-aspartic acid backbone to which arginine residues are linked. Poly-aspartate can serve as a biodegradable substitute for synthetic polycarboxylates in various technical processes (Mooibroek et al. 2007) and can be obtained by mild hydrolysis from cyanophycin, which in nature accumulates in cyanobacteria.

Neumann et al. (2005) constitutively expressed the cyanophycin synthetase gene (*cphATE*) from *Thermosynechococcus elongatus* in tobacco and potato. Aggregates of cyanophycin were detected in the cytosol of transgenic potato leaves and tuber cells by electron microscopy, which made up to 0.24% of the dry weight in leaves. In potato plants grown from tubers the cyanophycin content increased eightfold. However, cyanophycin-accumulating transgenic lines showed an undesirable phenotype and reduced plant fitness.

A possibility to avoid the phenotypic abnormalities and to increase polymer accumulation may be the targeting of cyanophycin synthesis to other cellular compartments. An attempt in this regard was made recently by fusing the functional cyanophycin synthetase gene to different transit peptide sequences for import into plastids. Transgenic tobacco lines showed polymer accumulation up to 1.7% of dry weight in primary transformants and up to 6.8% in T2 plants. However, the problem of reduced fitness remained (Hühns et al. 2008).

#### 20.5.4 Spider Silk

Spider silks display extraordinary mechanical features that makes them attractive for numerous industrial or medical applications (Gosline et al. 1999; Kluge et al. 2008). Spider silk proteins (termed spidroins) consist largely of glycine and alanine and are of high molecular weight (200–350 kDa in size; Hayashi et al. 1999). The highly repetitive sequences in genes encoding the repetitively composed spidroins are a major constraint to the production of spider silk proteins in bacteria, due to genetic instability resulting from recombination events.

To overcome these and other limitations, Scheller et al. (2001) used potato (and tobacco) as a plant biofactory to express a synthetic *MaSp1* gene homologue of the spider *Nephila clavipes* fused to a gene for fibroin from larvae of silkworm moth (*Bombyx mori*). An N-terminal signal peptide together with the KDEL signal at its C terminus, provide ER retention of the transgenic spidroins in plant cells. Synthetic hybrid protein could be detected in potato leaves and tubers at amounts up to 2% of the total soluble protein.

### 20.6 Conclusions

Over the past 20 years, the application of transgenic approaches has disclosed the enormous potential of genetic engineering techniques with regard to the potato's improvement for many commercially relevant traits. However, the only transgenic potatoes which have been commercialised so far, the NewLeaf lines of Monsanto, were withdrawn after only a few years of marketing, due to a lack of consumer acceptance (especially in Europe), which in turn forced the major global food processors to ban genetically modified potatoes from their food chains. The general public remains afraid about unexpected risks supposedly associated with the use of genetically modified plants in agriculture. This issue needs to be addressed, not only by technical improvements like marker-free transformation, the use of all-native P-DNA vectors, or cisgenesis, but also by communicating the valid economical and environmental advantages of transgenic traits on a case-to-case basis and last but not least by countervailing against the 'cult of the amateur' (Trewavas 2008).

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# Chapter 21

## Rapeseed/Canola

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### 21.1 Introduction

Oilseed rape (*Brassica napus* L.) is the major oilseed crop in temperate regions and ranks second among oilseed crops produced worldwide (FAO 2008). Depending on the climate it is grown as a winter annual or as a spring annual crop. Important growing areas are Northern and Southern America, Northern Europe, China and Australia. Interest in oilseed rape has recently increased due to its diversified utilization in food and feed production and its growing economic importance as a novel source of renewable energy, mainly as biodiesel (Durrett et al. 2008). Most parts of the world grow ‘canola’- or ‘double-low’-quality type oilseed rape with low seed oil contents of erucic acid and low seed glucosinolate contents. Canola or double-low quality types are available not only in *Brassica napus*, but also in the closely related species *B. rapa* and *B. juncea*. In all those species, canola quality has been achieved by conventional breeding using spontaneous mutants. Currently, specialty oil quality oilseed rape types like high oleic acid/low linolenic acid (HOLL) or high erucic acid oilseed rape (HEAR) are also cultivated on a smaller scale as identity-preserved crops. Conventional breeding programs mainly focus on improving yield, yield stability and oil content. Recently, increasing attention has also been paid to minor seed constituents like carotenoids (vitamin A; Shewmaker et al. 1999), tocopherols (vitamin E; Marwede et al. 2004), sinapate esters (Hüsken et al. 2005a, b; Zum Felde et al. 2006), phytosterols (Amar et al. 2008) and to other input and output traits (Dunwell 2005; Cardoza and Stewart 2007). Traditional oilseed rape (*Brassica napus* L.) has a black seed coat. Yellow seeded types are available which have a thinner seed coat and therefore show enhanced contents of

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seed oil and seed protein (Rahman 2001). Currently, the agronomic performance of those yellow seeded material is enhanced by conventional breeding.

With respect to the worldwide cultivation of transgenic crops, canola ranks fourth, following soybean, maize and cotton (ISAAA 2008). In 2007 transgenic canola was grown on 5.5 Mio hectares worldwide, which accounts for 5% of the total biotech crop area; 20% of the worldwide grown canola is transgenic. Currently, transgenic canola is cultivated in Canada, the United States and in Chile, but other countries like China, Argentina or Australia may follow soon. The only commercialized transgenic traits in canola so far are herbicide resistance (glyphosate and glufosinate; see Chap. 9) and the Barnase/Barstar hybrid seed production system (see Chap. 14).

## 21.2 Transformation Using Direct Gene Transfer Methods

There are various direct gene transfer methods that can be used to transform *Brassica* genotypes (see Bhalla and Singh 2008 and references therein; see Chap 1). However, since *Brassica* species are comparatively easy to transform with *Agrobacterium*, none of the direct transformation techniques has so far received greater importance. The often cited disadvantage of direct gene transfer methods (that they frequently lead to integration of multiple linked transgene copies) is invalidated since it has been shown that direct gene transfer with only the gene of interest – and not with the whole plasmid – leads to single and low copy number integrations (Fu et al. 2000). Direct gene transfer methods like PEG- or electroporation-mediated transformation of protoplasts may be advantageous, if it is necessary to circumvent certain transformation patents. Despite their low efficiency, direct transformation techniques may become important for the engineering of specific traits in oilseed rape which require a high expression. Here, the expression of foreign genes in plastid genomes has led to a high expression of proteins from plastid transgenes (Bock 2007; see Chap. 2). In *Brassica* species, stable delivery of DNA to plastids either via particle gun or PEG-mediated transformation has been reported for oilseed rape (Hou et al. 2003), cauliflower (Nugent et al. 2006) and cabbage (Liu et al. 2007, 2008a, b).

## 21.3 Transformation Using *Agrobacterium tumefaciens*

### 21.3.1 *Explant Type, Additives and Genotype Dependence*

*Brassica napus* and related species belong to the model crops which have been transformed with *Agrobacterium tumefaciens* for more than twenty years. First reports about successful stable transformation date back to 1987 (Fry et al. 1987;

Pua et al. 1987; Radke et al. 1988; Moloney et al. 1989). Already at that time different types of explants were used for co-cultivation with *Agrobacterium tumefaciens*. Pua et al. (1987) used longitudinal stem sections excised from internodes of 4- to 5-week-old in vitro shoot cultures. Fry et al. (1987) took stem segments of 6- to 7-week-old greenhouse-grown plants before flowering. Radke et al. (1988) and De Block et al. (1989) used hypocotyl segments of in vitro germinated seedlings. Moloney et al. (1989) employed the cut end of cotyledonary petioles for infection with *Agrobacterium*. The addition of acetosyringone to the co-cultivation medium may induce bacterial virulence genes and may enhance transformation efficiency (Charest et al. 1988). De Block et al. (1989) emphasized the importance of adding silver nitrate to the regeneration medium and the use of micropore tape instead of parafilm for sealing Petri dishes to reduce humidity and to avoid accumulation of the stress hormone ethylene. Hypocotyl segments are very sensitive towards *Agrobacterium* co-cultivation and react with necrosis. It was shown that preconditioning the segments before inoculation can increase the transformation rate (Ovesná et al. 1993; Cardoza and Stewart 2003). However, preconditioning represents an additional labor-intensive work step and hence is not always routinely performed.

Following the pioneering fundamental work at the end of the 1980s and beginning of the 1990s, transformation became routine in *B. napus* and in many of the related species, like *B. rapa*, *B. oleracea* and *B. juncea* (see Bhalla and Singh 2008 and references therein). In *B. napus* the spring canola cultivar Westar yielded high transformation efficiencies in independent laboratories and is currently used in many experiments. Attempts were made to extend transformation protocols to other modern spring (Seifert et al. 2004; Zhang et al. 2005) and winter oilseed rape cultivars (Damgaard et al. 1997), but transformation frequencies usually remained below the results obtained with Westar. Recently, updated transformation protocols for hypocotyl segments (Cardoza and Stewart 2006) and for cotyledonary petioles were published (Bhalla and Singh 2008).

### 21.3.2 *Agrobacterium* Strains

There is still some debate about the effectiveness of different *Agrobacterium* strains for *Brassica* transformation. The work of Charest et al. (1989) suggested that transformation was most efficient with nopaline strains, less efficient with the succinamopine strain A281 and least efficient with octopine strains. However, the earlier work of Hood et al. (1986) indicated that strain A281 is hypervirulent and derivatives like AGL0, AGL1 and EHA101 were later used successfully in *Brassica* transformation (e.g. Hüsken et al. 2005a). The utilization of *Agrobacterium* strains with different combinations of chromosomal background, virulence plasmid (vir regions) and binary plasmid (size, *Agrobacterium tumefaciens* replication of origin, gene(s) of interest, etc.) complicate meaningful comparisons. The type of replication of origin used for binary plasmid replication in *Agrobacterium* has an

influence on binary plasmid copy number and may also have an influence on transformation efficiency (Hausmann and Töpfer 1999). In addition, the type of selectable marker gene and the types of promoter and terminator sequences used to regulate expression of the selectable marker can affect transformation efficiency. Clearly, promoters enabling a higher expression of the selectable marker gene (e.g. CaMV35S vs nopaline synthase promoter) should allow a better selection of transformed cells. The efficiency of nopaline strains has been confirmed in later studies (Damgaard et al. 1997). However, octopine strains (LBA4404) may also give good results (Bhalla and Singh 2008), though success seems to depend on the genotype (Damgaard et al. 1997).

### 21.3.3 Transformation Using Protoplasts

The transformation of *Brassica* species through co-cultivation of protoplasts with *A. tumefaciens* has received little attention (Thomzik and Hain 1990; Wang et al. 2005). However, protoplasts from *Brassica* species can be regenerated to plantlets fairly easy. Once the transformation and regeneration system is established, the protoplast system has advantages when large numbers of transgenic plants need to be regenerated in order to identify the elite event which is further used in breeding the transgenic crop.

### 21.3.4 Transformation Using Haploids

The above-mentioned transformation approaches using explants from seedlings or young plantlets have the disadvantage that first generation transgenic plants (T1 generation) are hemizygous for the integrated transgenes. If more than one transgene copy is inserted, segregation in T2 and later generations becomes complex and it may be difficult to distinguish hemizygous from homozygous plants. Hence, attempts were made to use haploid microspores (Pechan 1989) and microspore derived embryos (Swanson and Erickson 1989) for *Agrobacterium* transformation. Although some initial success was reported, the results are difficult to reproduce. Direct gene transfer into microspores is technically demanding and proved to be of low efficiency (Fukuoka et al. 1998; Bhalla and Singh 2008 and references therein).

Isolated microspore culture is routinely applied in oilseed rape breeding to produce doubled haploid homozygous lines (Möllers and Iqbal 2008). Diploidization is achieved by colchicine treatment of either the microspores or the regenerated haploid plantlets. However, haploid plantlets can be easily obtained and clonally propagated. Surprisingly, only a few attempts have been made to use explants from haploid plantlets propagated in vitro or in the greenhouse in transformation experiments, although equivalent explant types from diploid plantlets were used successfully in earlier studies (Fry et al. 1987; Pua et al. 1987; Kuvshinov et al. 1999).

In a more recent study, petioles and internode segments obtained from in vitro propagated haploid oilseed rape plantlets showed a better callus and shoot regeneration capacity than leaf explants in *A. tumefaciens* transformation experiments (Wijesekara 2007). Using explants from haploid plantlets for transformation have the advantage that upon colchicine treatment regenerated transgenic haploid plantlets become homozygous in one step, regardless of their transgene copy number. Furthermore, the genetic background is also completely homozygous, which is not necessarily the case, when seeds from inbred cultivars are used for transformation.

### **21.3.5 Transformation Avoiding Tissue Culture**

More recently, interest in improving the *Agrobacterium* transformation of *Brassica* species focussed on the development of infiltration techniques which allow the generation of transgenic plants without the need of going through the laborious and time-consuming tissue culture procedure. Following pioneering work in *Arabidopsis*, some progress has been achieved for species in the genera *Raphanus* (Curtis and Nam 2001) and *Brassica* (Liu et al. 1998; Cao et al. 2000). For two spring-type canola varieties Wang et al. (2003) reported transformation efficiencies ranging from 0.05% to 0.2%, following optimization of the conditions for adult flowering plant infiltration. However, in a more recent work with pakchoi (*B. rapa* ssp. *chinensis*) the transformation frequency in the harvested seeds consistently varied only between 0.01% and 0.03% (Xu et al. 2008), which is clearly too little to represent a tempting alternative to the conventional tissue culture approach.

### **21.3.6 Plant-Selectable Marker Genes and Marker Gene-Free Transgenic Plants**

Plant selectable marker genes located on the T-DNA of *Agrobacterium tumefaciens* are required to enable cell division of transformed cells and to inhibit the cell division of untransformed cells in the presence of the selective agent, thus increasing the percentage of transformed plantlets among the total number of regenerated plants. Although a number of different plant selectable marker genes have been used (see Chap. 3), it appears that the kanamycin resistance gene (*neo* or *nptII*) and the BASTA resistance gene (*bar* or *pat*) have been used more frequently in *Brassica* transformation experiments than others. However, commercialization of genetically modified plants is hampered by public and scientific concerns about possible risks related to the expression of antibiotic and/or herbicide resistant marker gene products in those plants. There are several strategies that can be used to develop marker gene-free transgenic plants (Chakraborti et al. 2008, and references therein). A co-transformation approach, in which a single *Agrobacterium* strain with a binary

plasmid with two to three T-DNA was used, led to a high frequency of individual and independent integrations of those T-DNAs in tobacco (McCormac et al. 2001). The following approaches have been applied successfully to *Brassica* species: (i) co-transformation by double infection with two *A. tumefaciens*, each carrying a different T-DNA on its binary plasmid (De Block and De Brouwer 1991), and (ii) co-transformation with one *A. tumefaciens* strain containing two binary plasmids (Daley et al. 1998). In both cases, independent integration of the two transferred transgenes could be observed, which allows the recovery of marker gene free transgenic plants. A selectable marker gene system for transformation of oilseed rape that does not require the use of antibiotics or herbicides has also been developed (Sonntag et al. 2004).

Another approach to generate marker gene free transgenic plants is to perform transformation without selectable marker genes. Although transformation of cells is thought to occur only at very low frequencies, this approach led to the regeneration of 1–5% transgenic potato plants as identified by PCR among the total number of regenerated plants (De Vetten et al. 2003). Such an approach requires hypervirulent *Agrobacterium* strains like A281 or derivatives and a high transformation rate of cells capable of regeneration into plantlets. It would be interesting to test this marker gene-free approach to the oilseed rape system. The marker gene-free transformation system seems to have a number of advantages compared to the other methods available for generating marker gene-free transgenic plants (see De Vetten et al. 2003).

In general, the presence of a selectable marker gene may be regarded as a disadvantage. However, when directly screening for the new transgenic trait is difficult in a breeding program, the marker gene phenotype may be more easily screened. A good example for this is the Barnase/Barstar hybrid system (Chap. 14), in which the male sterility and the restorer gene are linked to glufosinate tolerance. In segregating populations non-transgenic self fertile and non-restorer genotypes can be eliminated by herbicide spraying. Engineering the resveratrol glucoside biosynthetic pathway in oilseed rape by co-transformation of two genes of interest located on two different binary plasmids (one with the *pat*-selectable, the other with the *nptII*-selectable marker gene) is a good example where the achieved metabolic modifications are analytically demanding to follow and where the two marker genes served as more easily detectable tags in an ELISA test (Hüsken et al. 2005b). However, the number of suitable selectable marker genes is very limited and their permissive use meets public and scientific concern.

## 21.4 Employment of Transgenic Oilseed Rape in Breeding

If the proof of concept has shown that a specific trait can be modified in the desired way, it has to be decided whether the new trait is going to be commercialized. For a proof of concept study, usually only about ten independent transgenic events are generated and characterized. It is unlikely that among those there is already the one

single transgene copy elite event that shows a maximum and stable trait expression. If a new trait is chosen to be commercialized, new transformation experiments are usually performed to produce up to 100 independent transgenic events (Crosbie et al. 2006). It is advisable to perform transformations in winter oilseed rape, when the new trait is to be commercialized in a winter oilseed rape growing area. This is not only because backcrossing from spring into winter oilseed rape is time-consuming, but also because effective transgenic trait selection in the nursery may be strongly influenced by differences in the winter hardiness, flowering and maturation time of the segregating plant material. Once a population of primary transgenic plants has been generated, this is tested for transgene copy number and in field experiments for transgene trait expression. For the sake of public opinion and regulatory issues, it is advantageous for the transformation to be performed without an antibiotic resistance gene. When co-transformation has been performed, segregating T2 and later generations have to be screened for marker gene-free genotypes. It has been proven a fairy tale that *A. tumefaciens* transformation leads to clean integration of transgenes. Hence, promising marker gene-free single-copy transgenic candidates need to be further analyzed for the presence of unwanted binary vector backbone sequences and *Agrobacterium* chromosomal DNA (Ülker et al. 2008). It seems recommendable to cross and back-cross the putative elite event to the actual breeding material to get rid of any unwanted gene sequences. Furthermore, it is advisable to enter the regulatory approval process and the breeding program with only a single selected elite event. If two or more elite events are used, this leads to complex segregation patterns due to different transgene integration sites. Once, a suitable elite event is identified and regulatory approval has been achieved, the new transgenic trait is introduced into current breeding material by conventional crossing.

When the new transgenic trait is to be stacked with other already existing transgenic traits is to be considered, this is done by conventional crossing, or a new transformation construct is created carrying the new transgene together with previous transgenes on the same T-DNA. This has the advantage that multiple genes stacked on a single T-DNA segregate as a single Mendelian trait. This simplifies breeding very much and should speed up cultivar development. There are already many examples in *Brassica* in which two or more transgenes have been put onto a single T-DNA (e.g. Houmiel et al. 1999, Nath et al. 2009).

In oilseed rape breeding and cultivation, the importance of hybrid cultivars is steadily increasing. For breeding hybrid cultivars it is relevant to know whether the transgenic trait is inherited in an intermediate, partially dominant or dominant fashion. If the trait is expressed in the vegetative parts of the plant and is inherited in a dominant fashion, the transgenic trait needs to be incorporated only in either one of the two hybrid breeding pools (see also Chap. 6). However, when the transgenic trait concerns a seed specific modification, e.g. oil quality, the hemizygous transgene segregates and leads to homo- and hemizygous transformed and untransformed F2 seeds (growing on the F1 hybrid plant). In this case, it is necessary to incorporate the new transgene in both hybrid breeding pools to achieve a high and uniform expression of the trait. Transgenic glyphosate and glufosinate

**Table 21.1** Examples of transgenic oilseed rape events for which regulatory approval has been achieved in at least one country (AGBIO 2008)

Event	Applicant	Introduced trait/genetic element	Copies
T45 (HCN28)	Bayer Crop Science	Tolerance to glufosinate/ <i>pat</i> gene – phosphinothricin N-acetyltransferase from <i>S. viridochromogenes</i>	1
GT73 or RT73	Monsanto	Tolerance to glyphosate/CP4 <i>epsps</i> 5-enolpyruvylshikimate-3-phosphate synthase ( <i>Agrobacterium tumefaciens</i> CP4) <i>goxv247</i> glyphosate oxidoreductase ( <i>Ochrobactrum anthropi</i> )	1
MS8	Bayer Crop Science	Barnase/Barstar pollination control system in combination with tolerance to glufosinate MS8 lines contain the <i>barnase</i> gene from <i>Bacillus amyloliquefaciens</i>	1
MF3		MF3 lines contain the <i>barstar</i> gene from the same bacteria Both lines contain the phosphinothricin N-acetyltransferase (PAT) encoding <i>bar</i> -gene from <i>Streptomyces hygroscopicus</i>	1
Oxy-235	Aventis Crop Science	Tolerance to the herbicides Bromoxinil and Ioxynil/Bxn – nitrilase ( <i>Klebsiella pneumoniae</i> ssp. <i>ozanae</i> )	1
23-18-17, 23-198	Monsanto (formerly Calgene)	High laurate (12:0) and myristate (14:0) canola produced by inserting a thioesterase encoding gene from the California bay laurel ( <i>Umbellularia californica</i> )	15

resistance is a good example where a single transgene copy in the hemizygous form is sufficient to confer adequate tolerance to recommended herbicide dosages. If the Barnase/Barstar pollination control system is used for breeding hybrids (see below and Table 21.1), the male sterile and the male fertile lines used for hybrid seed production both contribute one *bar* gene allele conferring glufosinate tolerance, although at two different loci.

However, in many cases, particularly when quantitative changes in the concentration of already existing or newly introduced constituents is intended, multiple transgene copies are required to achieve maximum trait expression. The most popular example from oilseed rape is probably laurate canola. According to the petition for deregulation of laurate canola, the transgenic event pCGN3828-212/86-23 contained 15 transgene copies which were integrated at five different sites ([http://www.aphis.usda.gov/brs/aphisdocs/94\\_09001p.pdf](http://www.aphis.usda.gov/brs/aphisdocs/94_09001p.pdf)). The presence of multiple copies in the homozygous form is clearly required to achieve full trait expression, although in this specific case not all transgene copies may have been functional. In similar approaches, transformation of the plastid genome probably would have some advantages (see above and Chap. 1). When expressing phytase (phyA) in transgenic oilseed rape, Ponstein et al. (2002) found that phytase expression depended on the homozygosity and dosage of the transgene. In an approach to

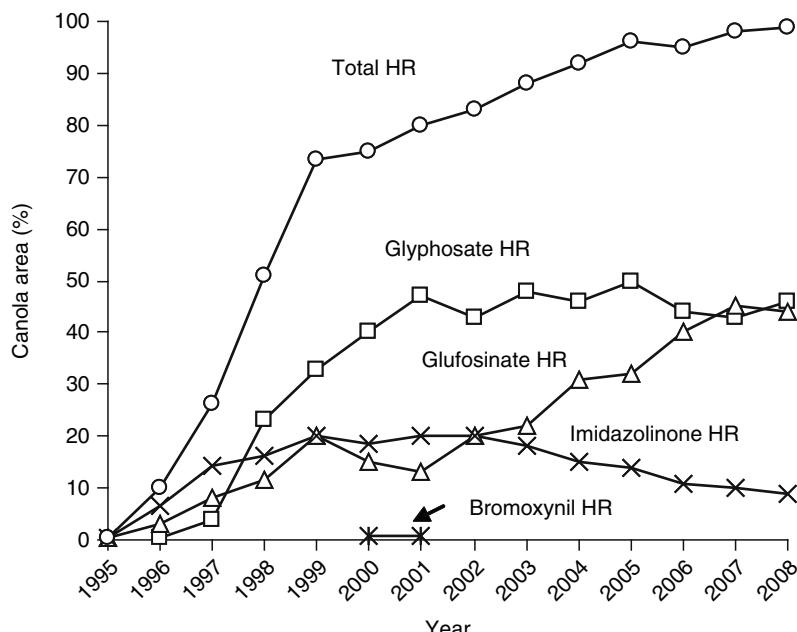
reduce anti-nutritive sinapate ester content in transgenic oilseed rape, Hüsken et al. (2005a) reported a reduced sinapate ester content for homozygous single-copy transgenic lines, compared to hemizygous ones. Similar results were obtained in a follow-up study, in which the highest resveratrol glucoside contents were found in homozygous transgenic oilseed rape plants (Hüsken et al. 2005b).

## 21.5 Employment of Transgenic Oilseed Rape in Crop Production

The AGBIOS database (AGBIOS 2008) shows 12 transgenic events for oilseed rape/canola – plus three events obtained following chemical mutagenesis – for which some type of regulatory approval has been obtained in at least one country. The achieved modifications mainly concern resistance to the herbicides glyphosate and glufosinate and the Barnase/Barstar pollination control system. There is one modification concerning resistance to the herbicides bromoxynil and ioxynil by incorporation of the nitrilase gene from *Klebsiella pneumoniae* (Tan et al. 2006) and one modification of the oil quality (laurate canola). Table 21.1 gives some examples of transgenic oilseed rape for which some type of regulatory approval has been obtained. Resistance to glufosinate is either achieved by transformation with the *pat* gene from *Streptomyces viridochromogenes* (T45, Table 21.1) or by transformation with the *bar* gene from *Streptomyces hygroscopicus* (MS8 and MF3, Table 21.1). In event GT73, resistance to glyphosate is achieved by two different genes which are located on the same T-DNA. The Barnase/Barstar pollination control system consists of the two independent transgenic events MS8 and MF3, each containing in addition the *pat* gene on the same T-DNA.

Herbicide-resistant oilseed rape was rapidly adopted by Canadian farmers (Fig. 21.1; Beckie et al. 2006). In 2008 oilseed rape was grown in Canada on 6.4 million hectares, of which 99% were herbicide-resistant. To this, transgenic glyphosate (Roundup Ready)- and glufosinate (Liberty Link)-resistant oilseed rape contributed 46% and 44%, respectively. Non-transgenic Clearfield (imidazolinone)-tolerant oilseed rape was grown on 9% of the total oilseed rape area under cultivation (Hugh Beckie, personal communication).

A look at field trials currently performed in the United States (ISB 2008) gives some impression which types of transgenic traits may come next to commercialization. The field -tested modifications concern plant height (dwarf types), increased yield, improved nitrogen utilization efficiency, insect resistance, herbicide tolerance and seed quality improvement (Table 21.2). Only in some cases have the identity and origin of the transferred genes been revealed. Improved nitrogen utilization efficiency under limited nitrogen fertilizer levels was achieved by transformation of the oilseed rape cultivar Westar with the barley alanine amino-transferase gene (Strange et al. 2008). Similar results can be found for field trials performed with transgenic canola in Canada (Canadian Food Inspection Agency 2008).



**Fig. 21.1** Adoption of herbicide-resistant (HR) canola in Canada (from Beckie et al. 2006). Updated according to Hugh Beckie (personal communication)

**Table 21.2** Examples of recent United States field trial applications for oilseed rape 2006–2008 (ISB 2008)

Aphis number	Applicant	Gene	Phenotype
07-234-102N	University of Tennessee	GA-insensitive dwarfing gene Bt Cry1Ac acetohydroxyacid synthase (ahas)	Dwarf corn, earworm-resistant, sulfonylurea-tolerant
08-065-106N	BASF	CBI <sup>a</sup>	Imidazolinone-tolerant, seed quality improvement
08-059-115N	Monsanto	CBI <sup>a</sup>	Increased yield, glyphosate-tolerant
07-255-103N	Pioneer Hi-Bred International	GAT4621 – <i>Bacillus licheniformes</i>	Glyphosate-tolerant
07-250-105N	Targeted Growth, Inc.	CBI <sup>a</sup>	Increased yield
07-253-101N	Arcadia Biosciences	Alanine amino transferase from <i>Hordeum vulgare</i> , CBI <sup>a</sup>	Nitrogen utilization efficiency increase

<sup>a</sup>Confidential business information

There are currently comparatively few applications for field trials with transgenic oilseed rape in Europe (Table 21.3). The applicant in all cases is Plant Science Sweden AB. Modifications concern oil composition and oil content. GMO

**Table 21.3** Examples of field trials with transgenic oilseed rape currently performed in the European Union (GMO Compass 2008)

SNIF number	Origin of trait genes	Phenotype
B/SE/08/1613	Fungal (3), moss (1), algae (1)	Improved oil composition
B/SE/07/10450	Arabidopsis thaliana, 3 genes; Brassica napus, 2 genes; Saccharomyces cerevisiae, 1 gene	Increased oil content
B/SE/07/10746	Arabidopsis thaliana, 3 genes; Brassica napus, 4 genes; Saccharomyces cerevisiae, 1 gene	Increased oil content
B/SE/07/107	Arabidopsis thaliana, 7 genes; Brassica napus, 1 gene; Escherichia coli, 1 gene	Increased oil content
B/SE/07/108	Physcomitrella patens, 2 genes	Increased oil content

Compass gives a good overview of which transgenic modifications in oilseed rape have been approved for food and feed, import and processing, or cultivation in the European Union and the status of ongoing applications (GMO Compass 2008).

## 21.6 Conclusions

In summary, in oilseed rape so far only a few transgenic herbicide resistance traits and one transgenic pollination control system have been commercialized in Canada, the United States and in Chile. Transgenic canola has been adopted rapidly by Canadian farmers and to date 90% of the canola area under cultivation is transgenic. New promising transgenic traits, like improved nitrogen efficiency, increased yield and oil content, are currently being tested in field experiments. If results are positive, there are good prospects for their successful commercialization.

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# Chapter 22

## Rice

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### 22.1 Introduction

Rice is one of the most important staple crops for more than half of the global population. Yield improvement was historically the most important target for many breeding programs. There were two big leaps of rice yield in the past half-century, primarily as the result of genetic improvement: increasing harvest index by reducing plant height making use of the semidwarf gene, and utilization of heterosis by producing hybrids. Consequently, rice yield was more than doubled in most parts of the world and even tripled in certain countries within a period of four decades from the 1960s to 1990s (<http://faostat.fao.org/>). However, a number of challenges of rice production are emerging in the new century. These include: (i) increasingly severe occurrence of insects and diseases, (ii) environmental pollution and ecological disruption caused by the overuse of chemical pesticides and fertilizers, (iii) high yield pressure due to global population increase and the reduction of arable land, (iv) water shortage and increasingly frequent occurrence of drought, (v) extensive cultivation in marginal lands. Zhang (2007) outlined the strategies for addressing these challenges by developing new rice cultivars referred to as Green Super Rice (GSR), taking the following traits as the targets: adequate resistances to multiple insects and diseases, abiotic stresses such as drought and salinity, high use-efficiency of nitrogen (N) and phosphorus (P), on the basis of continuous improvement of grain yield and quality. Although the goal of GSR looks very arduous, modern biotechnology (especially transformation technology) offers new opportunities for rice genetic improvement, while the progress of rice functional genomics is deepening our understanding of rice genetics and providing more available rice gene resources for rice genetic improvement. Advances in both fronts will facilitate the

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utility of genetic engineering approaches to achieve the final goal of GSR. Some genetically modified (GM) rice with partial GSR characteristics such as insect- and disease-resistance were developed more than ten years ago. In this chapter, we first review briefly the progress of rice transformation technology and rice functional genomics research, and then present and discuss the status of GM rice with improvement of the traits relevant to GSR.

## 22.2 Rice Transformation Technology and Functional Genomics

A highly efficient *Agrobacterium*-mediated rice transformation system was established by Hiei et al. (1994). For general information of genetic engineering, refer to Chap. 1. Nevertheless, the transformation of *indica* rice was still difficult. Several important modifications based on this protocol shortened greatly the transformation procedure and made transformation of *indica* rice much more amenable (Lin and Zhang 2005; Hervé and Kayano 2006; Hiei and Komari 2006; Toki et al. 2006). Recently, protocols of *Agrobacterium*-mediated transformation for a wide range of rice genotypes (including both *japonica* and *indica* varieties) were provided by Hiei and Komari (2008), indicating the establishment of high throughput and widely adaptable transformation system of rice. According to Hiei and Komari (2008), transformation of *japonica* rice can be completed within 2 months using callus with 50–90% transformation efficiency, while transformation of *indica* rice can be done within 2.5 months using immature embryo with extremely high transformation efficiency (a single immature embryo may produce 5–13 independent transformants). However the disadvantage of the protocol is that transformation of *indica* rice must use immature embryo, which is not convenient because collection of immature embryo is laborious and limited by the season. Therefore, other protocols for *indica* transformation using callus from mature seed is still useful in some cases (Lin and Zhang 2005; Datta and Datta 2006).

The ultimate goal of rice functional genomics research is to determine the functions of all genes especially agronomically important genes in the rice genome. Currently, the rice genome has been completely sequenced (International Rice Genome Sequencing Project 2005). The finished quality sequence based on Nipponbare revealed that the rice genome is 389 Mb and encodes ~32 000 rice genes according to the results of The Rice Annotation Project (2007, 2008), both of which are smaller than previous estimates. To identify biological function of each coding gene, large-scale insertional mutagenesis libraries of T-DNA or transposon have been established and totally about 200 000 flanking sequence tags (FSTs) have been isolated by more than ten independent groups (Krishnan et al. 2009). A reverse genetic strategy is commonly applied for gene identification using these insertional mutagenesis libraries. Moreover, a collection of >50 000 full-length cDNA sequences of both *japonica* and *indica* rice are available, which facilitates greatly the discovery of novel genes (The Rice Full-Length cDNA Consortium 2003;

Xie et al. 2005; Liu et al. 2007). A goal to determine the function of every rice gene by the year 2020 was proposed recently for the international rice functional genomics research community (Zhang et al. 2008). Completion of this goal will tremendously enrich available gene resources for rice genetic improvement.

## 22.3 Insecticidal Rice

### 22.3.1 *Bt Rice*

As an innoxious alternative to chemical insecticides, biological insecticide formulations of *Bacillus thuringiensis* (Bt) have been used in plant protection for over 60 years (see Chap. 10). With the development of plant genetic engineering, Bt insecticidal genes have been introduced into GM crops (including rice) to develop insect resistant crops. Nowadays, Bt crops have become a major part of commercialized GM crops worldwide. Many studies and evaluations of Bt rice have been conducted in both laboratory and field (Fujimoto et al. 1993; Wünn et al. 1996; Ghareyazie et al. 1997; Nayak et al. 1997; Wu et al. 1997; Cheng et al. 1998; Maqbool et al. 1998; Tu et al. 2000b; Maqbool et al. 2001; Ye et al. 2001; Khanna and Raina 2002; Bashir et al. 2004; Ramesh et al. 2004; Chen et al. 2005, 2008; Tang et al. 2006). All the results consistently confirmed that Bt rice is highly effective against rice borers and leaf folders, which are the two major classes of rice lepidopteran pests that cause severe yield loss in all rice-growing countries. Bt rice was temporarily commercialized in Iran in 2005.

Although a number of *Bt* genes have been identified ([http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/)), only a small fraction of them have been applied in Bt rice. The mostly used Bt genes in rice are *Cry1A* genes, such as *Cry1Ab*, *Cry1Ac*, or a fusion gene *Cry1Ab/c* (Fujimoto et al. 1993; Wünn et al. 1996; Ghareyazie et al. 1997; Nayak et al. 1997; Wu et al. 1997; Cheng et al. 1998; Breitler et al. 2000; Tu et al. 2000b; Khanna and Raina 2002; Bashir et al. 2004; Ramesh et al. 2004). Some studies of Bt rice using other *Bt* genes such as *Cry1C*, *Cry2A*, and *Cry9C* have also been characterized (Maqbool et al. 1998, 2001; Bashir et al. 2004, Chen et al. 2005, 2008; Tang et al. 2006).

A concern for the widespread application of Bt crops is insect resistance management, because insects have the potential to evolve resistance to Bt toxins and Bt crops (Frutos et al. 1999; Ferré and Van Rie 2002; Bates et al. 2005). Several strategies have been proposed to prevent or delay the occurrence of pest resistance (Frutos et al. 1999; Ferré and Van Rie 2002; Bates et al. 2005). Among them, high dose/refuge and gene pyramiding/stacking are two promising strategies that have been adopted in practice (Bates et al. 2005). Gene pyramiding is supposed to be more practical than high-dose/refuge for Bt rice due to the small-scale planting model of most rice-growing countries (High et al. 2004). This strategy is based on the assumption that a single mutation in a pest is unlikely to confer simultaneous resistance to two different Bt toxins, and thus two-toxin rice cultivars would require

smaller refuges and delay the development of resistance more effectively than single-toxin cultivars (Zhao et al. 2003). However, the commonly used *Cry1Ab* and *Cry1Ac* are not suitable for gene pyramiding due to their highly amino acid homology. Binding tests of insect midgut brush border membrane vesicles showed that *Cry1Aa*, *Cry1Ab*, and *Cry1Ac* toxins share a common binding site (Escriche et al. 1997; Ballester et al. 1999; Karim and Dean 2000), which means that a mutant of insects that is able to overcome one of the *Cry1A* genes is also likely to be resistant to other *Cry1A* genes as well. Based on the binding assay of brush border membrane vesicles from rice stem borers, it would be effective to combine *Cry1A* genes with *Cry1C*, *Cry2A*, or *Cry9C* for gene pyramiding (Alcantara et al. 2004). Recently, transgenic rice lines harboring *Cry1C*, *Cry2A*, and *Cry9C* have been evaluated in the field (Chen et al. 2005, 2008; Tang et al. 2006), all of which exhibited high resistance to rice stem borers and leaf folders. These genes have now been stacked in various combinations, including *Cry1Ab+Cry1C*, *Cry1Ac+Cry1C*, *Cry1Ac+Cry1C*, *Cry1Ac+Cry2A*, and *Cry1C+Cry2A*. The bioassay in the laboratory showed that rice lines with two Bt genes had higher toxicity to rice stem borer than ones with single Bt toxins (Yang et al., unpublished data).

Results from large-scale germplasm screening of rice indicated that there are no germplasm resources available for stem borer and leaf folder resistance. Bt rice is clearly the most cost-effective, if not the only, way to control these insects. Field tests of Bt rice have been extensively conducted in China and India (High et al. 2004), and either or both countries may commercialize Bt rice in the near future.

### 22.3.2 GNA Rice

Snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) is another important insecticidal gene resource for transgenic insect-resistant breeding due to its particular capacity to control rice sap-sucking (hemipteran) pests, which cannot be controlled by Bt toxins. Powell et al. (1993) initially reported that GNA was toxic to two major rice sap-sucking pests, brown planthopper (*Nilaparvata lugens*, BPH), and green leafhopper (*Nephrotettix cincticeps*, GLH), by a feeding assay with artificial diet. Subsequent studies indicated that transgenic rice plants expressing GNA had various levels of resistance to all the rice major sap-sucking insects tested, including BPH (Rao et al. 1998; Foissac et al. 2000; Nagadhara et al. 2003), GLH (Foissac et al. 2000; Nagadhara et al. 2003), white-backed plant hopper (*Sogatella furcifera*, WBPH; Nagadhara et al. 2004), and small brown plant hopper (*Laodelphax striatellus*, SBPH; Sun et al. 2002). However, toxicity of GNA rice to rice sap-sucking insects is not comparable to that of Bt rice to rice lepidopteran insects. Bioassay showed that transgenic GNA rice plants could significantly reduce the growth, development, and fecundity of the infested pests, but did not lead to high mortality of infested sap-sucking pests (Rao et al. 1998; Sun et al. 2002; Nagadhara et al. 2003, 2004).

Recently, transgenic rice expressing another mannose-binding lectin from garlic leaf (*Allium sativum* agglutinin from leaf, ASAL) also exhibited enhanced

resistance to BPH and GLH (Saha et al. 2006). Moreover, expressing ASAL in transgenic rice plants significantly reduced the infection incidence of rice tungro diseases, which is a prevalent viral disease in many rice producing areas of Southeast Asia, caused by co-infection of GLH-vectorized rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (Saha et al. 2006).

It should be noted that transgenic approach should not be considered as the only way to control BPH. Naturally occurring resistance to BPH should also be exploited in the development of insect resistance cultivars. At least 19 BPH-resistant genes have been identified from cultivated and wild rice species (Zhang 2007). Molecular mapping of these genes has facilitated introgression of these genes into the desired rice cultivars by marker-assisted selection (MAS).

## 22.4 Disease-Resistant Rice

### 22.4.1 Resistance to Bacterial Blight

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *Oryzae* (Xoo) is one of the most devastating rice diseases worldwide. Fortunately, this disease can be effectively controlled by resistant varieties. Approximately 30 BB resistance (*R*) genes or loci against Xoo have been identified in cultivated and wild rice so far (Gu et al. 2008). Amongst them, six *R* genes (*Xa1*, *Xa3/Xa26*, *xa5*, *xa13*, *Xa21*, *Xa27*) have been cloned and many have been fine-mapped (Gu et al. 2008), which facilitates introgression or pyramiding of these *R* genes in desired cultivars by conventional breeding and MAS. MAS has been successfully used for *R* gene introgression and pyramiding against BB (Huang et al. 1997; Chen et al. 2000; Zhang et al. 2006). There are also efforts to develop transgenic rice using *Xa21* (Tu et al. 2000a; Datta et al. 2002, Maruthasalam et al. 2007), a gene with a broad resistance spectrum. Although the transgenic approach usually needs less workload, GM varieties are practically much more difficult to be commercialized at present.

### 22.4.2 Resistance to Fungal Diseases

Blast and sheath blight caused by *Magnaporthe grisea* and *Rhizoctonia solani*, respectively, are two of the most important fungal diseases in rice. Overexpressing pathogenesis-related proteins (PRs), including chitinase (PR-3),  $\beta$ -1,3-glucanases (PR-2), and thaumatin-like proteins (PR-5), and other plant- or microorganism-derived antifungal proteins, is a common strategy to develop transgenic fungus-resistant rice. PRs are a battery of proteins encoded by the host plants but induced exclusively in pathological or related situations, and many PRs showed antifungal activity in vitro (Van Loon and Van Strien 1999). Chitinases are among the most well known PRs used to develop fungus resistant plants, which can catalyze the

hydrolysis of chitin, which is one of the major cell wall components of many fungi. Transgenic studies have confirmed that overexpressing chitinases in transgenic rice enhanced the resistance against both sheath blight (Lin et al. 1995; Datta et al. 2000, 2002; Nandakumar et al. 2007) and blast (Nishizawa et al. 1999). Transgenic rice have been reported which express  $\beta$ -glucanase with enhanced resistance against *M. grisea* (Nishizawa et al. 2003) and thaumatin-like protein with enhanced resistance against *R. solani* (Datta et al. 1999). Combinations of chitinase with a modified maize ribosome-inactivating protein (Kim et al. 2003) or a thaumatin-like protein (Maruthasalam et al. 2007) were also attempted to enhance resistance to sheath blight. Constitutively expressing a rice-derived defense-related gene *Rir1b* showed enhanced resistance against rice blast fungus (Schaffrath et al. 2000). Kanzaki et al. (2002) reported that transgenic rice overexpressing a wasabi (*Wasabia japonica*) defensin gene conferred effective resistance against rice blast fungus. Defensins are low-molecular-weight (5 kDa) proteins occurring in the seeds, stems, roots, and leaves of a number of plant species that can cause permeabilization of fungal membranes and therefore lead to antifungal activity. Krishnamurthy et al. (2001) reported that constitutive expression of wheat-derived antimicrobial peptides purindolines PINA and/or PINB in rice conferred significantly increased fungal resistance against both *M. grisea* and *R. solani*. Coca et al. (2004) demonstrated that a microbial antifungal protein AFT from *Aspergillus giganteus* had the potential for developing fungal resistant rice against *M. grisea*.

Expressing pathogen-derived protein elicitors in transgenic rice to induce the plant general defense response and system-acquired resistance (SAR) is another strategy for developing transgenic rice with enhanced disease resistance. This strategy generally leads to non-specific resistance against fungi. The study of Shao et al. (2008) showed that expression of a harpin-encoding gene (*hrf1*) from Xoo enhanced expression level of a range of defense-related genes in transgenic rice and conferred high non-specific resistance to rice blast fungus *M. grisea*. The transgenic rice plants exhibited high resistance to all the major *M. grisea* races in rice-growing areas along reaches of the Yangtze River, China.

### 22.4.3 Resistance to Viral Diseases

Several strategies have been proposed to develop transgenic virus resistant rice. Some of these strategies are based on the concept of pathogen-derived resistance (Sanford and Johnston 1985), for which complete or partial viral genes are introduced into the rice plants to interfere with one or more essential steps of viral life cycle through protein-mediated or RNA-mediated mechanism. Expressing viral coat protein (CP) genes in transgenic rice plants that often referred to as coat protein-mediated resistance is the earliest approach to develop viral resistant transgenic plant (Powell-Abel et al. 1986). Hayakawa et al. (1992) demonstrated that transgenic rice overexpressing CP of rice stripe virus exhibited significant resistance to viral infection. To overcome tungro disease, CP proteins CP1, CP2,

and CP3 from RTSV were transformed individually or together into rice plants by Sivamani et al. (1999). While transgenic rice plants showed moderate resistance to RTSV, transgenic plants expressing all three CP genes together did not enhance the resistance compared to those expressing individual CP genes (Sivamani et al. 1999). Recently, the study of Kouassi et al. (2006) demonstrated that overexpressing antisense CP or untranslatable CP mRNA in transgenic rice induced moderate resistance to rice yellow mottle virus (RYMV), while overexpressing wild-type CP or deleted CP gene enhanced the virus infection. These results indicated that the resistance mechanism of transgenic plant accumulating antisense CP or untranslatable CP mRNAs was possibly related to RNA-mediated post-transcriptional gene silence (PTGS; Kouassi et al. 2006). Moreover, expressing the viral nucleocapsid protein gene from rice hoja blanca virus (RHBV; Lentini et al. 2003) and the spike protein gene from rice ragged stunt oryzavirus (Chaogang et al. 2003) were also confirmed to enhance resistance to the corresponding viruses compared to the non-transgenic controls.

It is known that PTGS and RNA interference (RNAi, see Chap. 5) play an important role in natural resistance of host plants against viral infection (Baulcombe 2004). Some RNA-mediated antiviral tactics associated with PTGS have been reported. Enhanced viral resistance was acquired in transgenic rice by expressing full-length or truncated RTSV replicase gene in both sense and antisense orientations (Huet et al. 1999). Nevertheless, the resistance of transgenic rice expressing replicase gene in the sense orientation is higher than that expressing replicase gene in antisense orientation (Huet et al. 1999). Similarly, RYMV resistant transgenic rice was obtained by expressing deleted or full-length RYMV RNA-dependent RNA polymerase (RdRp) genes (Pinto et al. 1999). More recently, RNAi strategy was used to obtain RTBV resistance in transgenic rice plants (Tyagi et al. 2008). RTBV ORF VI was simultaneously expressed in both sense and antisense orientation to form double-stranded RNA and trigger RNAi in transgenic rice. Transgenic rice showed mild to similar Tungro symptoms compared to the non-transgenic control when challenged by viruliferous GLH, although virus titer in transgenic plants was reduced (Tyagi et al. 2008). Moreover, Han et al. (2000) demonstrated a strategy to develop viral resistant rice by expressing a hammerhead ribozyme, which is a small catalytic RNA molecule with sequence-specific RNA cleavage activity.

Recently, Niu et al. (2006) demonstrated a new strategy to use artificial miRNA to suppress expression of viral genes which gained viral resistance in transgenic *Arabidopsis*. Although similar studies have not been reported in rice, this strategy enlightens a new way for viral resistance in transgenic crop plants, including rice.

## 22.5 Abiotic Stress Tolerance

Abiotic stresses such as drought, salinity, and high temperature are among the major factors causing crop yield loss (see Chap. 8). It is expected that abiotic stresses like drought and salinity will become more severe in the future (Vinocur

and Altman 2005; Bhatnagar-Mathur et al. 2008). Improving the tolerance of the crops against different abiotic stresses to expand the adaptation to various environments is crucial to produce more food, using limited land and natural resources in changing environments. Improvement of the crops for abiotic stress tolerance has become one of the main objectives of modern plant biotechnology.

One distinct feature of higher plants from other multicellular organisms is that plants are sessile and have to endure environmental challenges such as drought, salinity, and excessive temperature. Correspondingly, plants have evolved a complex reactive network to respond and form acclimation to environmental stresses. The plants' response to abiotic stresses comprises cascades of physiological and metabolic reactions involving many genes. The primary stresses such as drought, salinity, and extreme temperature cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial signals are perceived by stress sensors (e.g. histidine kinases) and then transduced to second messenger molecules, such as  $\text{Ca}^{2+}$ , inositol phosphates, and reactive oxygen species (ROS). The second messenger molecules subsequently activate a downstream signal cascade by phosphorylating transcription factors to regulate the expression of many down-stream functional or structural genes that help the plant re-establish osmotic homeostasis, scavenge harmful compounds, protect and repair damaged proteins and membranes (Vinocur and Altman 2005; Gao et al. 2008).

Due to the complex mechanism of abiotic stress tolerance, it is very difficult to rely on conventional approaches for breeding abiotic stress-tolerant crops. Current genetic engineering technologies have been broadly applied to crop improvement of abiotic stress tolerance including rice. As shown in Table 22.1, numerous transgenic trials have attempted to improve rice abiotic stress tolerance. A common strategy is to express abiotic stress-responsive or related genes in transgenic rice driven by constitutive or inducible promoters (Table 22.1). The applied transgenes can be roughly classified into two groups according to their action patterns. One group can be referred to as functional or structural genes, functioning in the downstream parts of plant abiotic stress-responsive network. These include protective chaperon proteins, such as heat-shock proteins (HSP) and late embryogenesis abundant (LEA) proteins, detoxifying genes including superoxide dismutase (SOD), glutathione S-transferase (GST), water channel proteins, ion transporters, and various catalytic enzymes that synthesize osmoprotectants (compatible solutes), including proline, trehalose, glycinebetaine, and polyamines, etc. This group was generally used in the initial transgenic studies because the mechanism is comparatively simple and the manipulations are also more amenable. However, these genes are thought to be less effective because of their simple action model compared to the complexity of abiotic stress tolerance.

Recent studies focus more on using the group of regulatory genes, which function in the upstream of the response network, such as signal perception, transduction, and transfer pathways. This group includes genes for calcium-dependent protein kinase (CDPKs), calcineurin B-like protein-interacting protein kinases (CIPKs), mitogen-activated protein kinases (MAPKs), and transcription factors. Modifying the expression level of these genes can generally activate a battery of

**Table 22.1** Summary of recent transgenic trials to enhance abiotic stress tolerance. Increases and enhancements are indicated by ↑; no significant difference compared to non-transgenic controls is indicated by →. O Constitutive overexpression, i stress-inducible expression

Gene type	Transgene	Source	Effects	Reference
Proline synthesis	<i>P5CS</i> (o, i)	Mothbean	Proline ↑; drought- and salt-tolerance ↑	Zhu et al. (1998), Su and Wu (2004)
Trehalose synthesis	<i>TPSP</i> (o, i)	<i>E.scherichia coli</i>	Trehalose ↑; drought-, salt-, and cold tolerance ↑	Garg et al. (2002), Jang et al. (2003)
	<i>OsTPP1</i> (o)	<i>Indica</i> rice Nona Bokra	Trehalose →; salt- and cold-tolerance ↑	Ge et al. (2008)
Glycine betaine synthesis	<i>CodA</i> (o)	<i>Arthrobacter globiformis</i>	Glycine betaine ↓; drought-, salt- and cold-tolerance ↑	Sakamoto et al. (1998), Mohanty et al. (2002), Sawahel (2003)
	<i>COX</i> (o, i)	<i>Arthrobacter pascens</i>	Glycine betaine ↑; salt-tolerance ↑	Su et al. (2006)
	<i>CMO</i> (o)	Spinach	Glycine betaine ↓; salt- and temperature stress-tolerance ↑	Shirasawa et al. (2006)
	<i>adc</i> (o)	Oat, <i>Datura stramonium</i>	Putrescine ↓; drought-tolerance ↑	Nouri et al. (2000); Capell et al. (2004)
Polyamine synthesis	<i>HAV 1</i> (o, i)	Barley	Drought- and salt-tolerance ↑	Xu et al. (1996), Rohila et al (2002), Babu et al. (2004)
LEA protein	<i>PMAs0, PMAl959</i> (o)	Wheat	Drought- and salt-tolerance ↑	Cheng et al. (2002)
	<i>OsLEA3_1</i> (o)	<i>Indica</i> rice Minghui 63	Drought-tolerance ↑	Xiao et al. (2007)
	<i>Hsp101</i> (o)	<i>Arabidopsis</i> Rice	Heat-tolerance ↑	Katiyar-Agarwal et al. (2003)
HSP	<i>sHSP17.7</i> (o)	Minghui 63	Heat-tolerance and UV-B resistance ↑; drought-tolerance ↑	Murakami et al. (2004); Sato and Yokoya (2008)
	<i>GPAT, SGPAT</i> (o)	Arabidopsis, spinach	Unsaturation of fatty acids ↑; cold-tolerance ↑	Yokoi et al. (1998), Ariizumi et al. (2002)
GPAT		Rice	Salt-tolerance ↑	Hoshida et al. (2000)
		Rice	Cold-tolerance ↑	Takesawa et al. (2002)
Detoxification genes	<i>GS2</i> (o)	Pea	Drought-tolerance ↑	Wang et al. (2005)
	<i>GST</i> (o)	<i>Avicennia mrayana</i>	Drought- and salt-tolerance ↑	Prashanth et al. (2008)

(continued)

**Table 22.1** (continued)

Gene type	Transgene	Source	Effects	Reference
	<i>katE</i> (o)	<i>Escherichia coli</i>	Salt-tolerance †	Nagamiya et al. (2007), Moriwaki et al. (2008)
Water channel protein and ion transporter	<i>RWC3</i> (i)	Upland rice Zhonghan 3	Drought-tolerance †	Lian et al. (2004)
	<i>AgNHHX1</i> (o)	<i>Atriplex gmelini</i>	Salt-tolerance †	Ohta et al. (2002)
	<i>SsNHHX1</i> (o)	<i>Suaeda salsa</i>	Salt-tolerance †	Zhao et al. (2006a)
	<i>SsNHHX1+AVP1</i> (o)	<i>Suaeda salsa</i> , <i>Arabidopsis</i>	Greater salt-tolerance than single <i>SsNHHX1</i> †	Zhao et al. (2006b)
	<i>OsNHHX1</i> (o)	Rice	Salt-tolerance †	Chen et al. (2007)
	<i>PgNHHX1</i> (i)	<i>Penicillium</i> <i>glaucum</i>	Salt-tolerance †	Verma et al. (2007)
Gene of signal transduction	<i>OsCDPK7</i> (o)	Rice	Drought-, salt-, and cold-tolerance †	Saijo et al. (2000)
	<i>OsMAPK5</i> (o)	Rice	Drought-, salt-, and cold-tolerance †	Xiong and Yang (2003)
	<i>Calcineurin A</i> (o)	Mouse	Salt-tolerance †	Ma et al. (2005)
	<i>OsCIPK 3</i> (o)	Rice	Cold-tolerance †	Xiang et al. (2007)
	<i>OsCIPK 12</i> (o)	Rice	Drought-tolerance †	Xiang et al. (2007)
	<i>OsCIPK 15</i> (o)	Rice	Salt-tolerance †	Xiang et al. (2007)
	<i>CBF1/DREB1b</i> (o)	<i>Arabidopsis</i>	Cold-tolerance →	Lee et al. (2004)
	<i>CBF3</i> (o)	<i>Arabidopsis</i>	Drought-, salt- and cold-tolerance †	Oh et al. (2005)
	<i>ABF3</i> (o)	<i>Arabidopsis</i>	Drought-tolerance †	Oh et al. (2005)
Transcription factor	<i>OsDREB1A/1B; DREB1A, 1B, and 1C</i> (o)	Rice, <i>Arabidopsis</i>	Drought-, salt- and cold-tolerance †; growth retardation	Ito et al. (2006)
	<i>OsDREB1F</i> (o)	Rice	Drought-, salt- and cold-tolerance †	Wang et al. (2008)
	<i>ZFP252</i> (o)	Rice	Drought- and salt-tolerance †	Xu et al. (2008)
	<i>SNAC1</i> (o)	Upland rice	Drought- and salt-tolerance †	Hu et al. (2006)
	<i>SNAC2</i> (o)	IRAT109	Salt- and cold-tolerance †	Hu et al. (2008)
Stress-associated proteins	<i>OsiSAP8</i> (o)	Rice	Drought-, salt- and cold-tolerance †	Kanneganti and Gupta (2008)

downstream abiotic stress-related genes to defend plants against environmental stresses. Application of the regulatory genes is thought to be more effective than those genes with simple functions. Recently, Hu et al. (2006) reported that overexpression of the stress-responsive gene *SNAC1* (STRESS-RESPONSIVE NAC 1) from IRAT109 (a drought-resistant upland rice variety) significantly enhanced drought-resistance in transgenic rice (22–34% higher seed setting than the control) in the field under severe drought stress conditions at the reproductive stage, without phenotypic changes or yield penalty. This is one of the rare cases that the stress tolerance of transgenic plants was tested in the field.

A number of attempts have been conducted (as shown in Table 22.1) and certain progresses have been achieved. Two points should be noted: (i) although strong constitutive promoters, such as CaMV35S, rice *actin 1*, and maize *ubiquitin* promoters, were used to drive the expression of transgenes in most studies (Table 22.1), some studies showed that stress-inducible promoters seemed to be better than constitutive promoters (Su and Wu 2004; Su et al. 2006); (ii) a combination of multiple stress-tolerant transgenes in a transgenic plant may perform better than single ones (Zhao et al. 2006b).

As a complex trait, abiotic stress tolerance is genetically controlled by quantitative trait loci (QTLs). Recently, two major rice QTLs of abiotic stress-tolerance, *SKC1* (salt-tolerance), and *Sub1* (submergence-tolerance), were cloned (Ren et al. 2005; Xu et al. 2006) facilitating a fast QTL-pyramiding through MAS or a transgenic approach. We believe that more transgenic rice with better abiotic stress tolerance will be generated in the future with the advance of molecular mechanism research of plant abiotic stress tolerance and the discovery of new related gene sources.

## 22.6 Quality Improvement

The grain quality of rice consists of several components: cooking quality, eating quality, appearance quality, milling quality, and nutritional quality. The cooking, eating, and appearance qualities of the rice grain represent a major problem for rice production in many rice-producing areas of the world. Nutrition improvement is crucial for many developing countries where people's dietary food is mostly rice, and therefore micronutrient (iron, zinc and vitamin A) malnutrition is prevalent (see Chap 11).

Transgenic approaches have been successfully applied to improve nutritional quality of rice. GM rice with enhanced β-carotene was an outstanding paradigm. This GM rice has an impressive name "Golden Rice" (GR) for the distinguished yellow or orange hue of its grain. A carotenoid biosynthesis pathway was established in rice endosperm by introducing two foreign genes into transgenic rice: phytoene synthase gene (*psy*) from daffodil (*Narcissus pseudonarcissus*), and bacterial phytoene desaturase (*crtI*) from *Erwinia uredovora* (Ye et al. 2000). Two versions of GR were developed successively, and referred to as GR1 and GR2.

GR2 contains much more  $\beta$ -carotene ( $20\times$  higher) than GR1 through replacing the daffodil *psy* gene with a maize-derived ortholog (Paine et al. 2005), which should be effective to alleviate vitamin A deficiency that prevails in the target areas.

Goto et al (1999) reported iron-biofortified rice, in which the iron storage protein ferritin derived from soybean was overexpressed under an endosperm-specific promoter in transgenic rice to increase iron content in rice grains. Several groups attempted similar strategies and obtained similar results: two- to threefold increase of iron in rice grains was observed compared to the non-transgenic controls (Lucca et al. 2001; Vasconcelos et al. 2003; Qu et al. 2005).

There is a strong emphasis in China currently on improving the eating, cooking, and appearance qualities of hybrid rice. The cooking and eating qualities are mostly determined by the amylase content (AC), gelatinization temperature (GT), and gel consistency (GC) of the grain starch. Appearance quality is mainly specified by grain shape as defined by grain length, grain width, the length:width ratio, and the translucency or chalkiness of the endosperm. Molecular marker-based genetic analysis in the past decade established that each of the quality traits is mainly conditioned by a major locus. For example, the *Wx* locus on chromosome 6 plays major roles in specifying AC and GC plus a minor role in GT (Tan et al. 1999; Wang et al. 2007), and the *Alk* locus, tightly linked to *Wx*, has a major effect on GT (He et al. 1999; Wang et al. 2007). For the appearance quality traits, grain length is mostly controlled by the *GS3* locus on chromosome 3, and grain width is largely conditioned by *GS5* on chromosome 5 (Tan et al. 2000). A major locus for chalkiness (*Chk5*) was also identified on chromosome 5 (Tan et al. 2000). Several genes for these traits have been cloned (Wang et al. 1990; Gao et al. 2003; Fan et al. 2006; Song et al. 2007). The single-locus inheritance indicated that MAS can play a major role in quality improvement. Zhou et al. (2003) simultaneously improved the cooking, eating, and appearance quality of Zhenshan 97, the female parent of a number of widely used hybrids in China with poor quality, through introgressing the *Wx* gene region from Minghui 63 by MAS.

## 22.7 Nutrient-Use Efficiency

### 22.7.1 Nitrogen-Use Efficiency

Nitrogen (N) is an essential nutrient that plants require in the most quantity and is thus a major limiting factor in crop production. N uptake and assimilation pathways in higher plants are well documented. Nitrate and ammonium are two major inorganic N compounds present in agricultural soils. Nitrate is converted to ammonium by two reductases (nitrate reductase, nitrite reductase) after it is absorbed from the soil. The following assimilation of ammonium is regulated by two key enzymes [glutamine synthetase (GS) and glutamate synthetase (GOGAT)] which convert ammonium to glutamine (Gln) and glutamate (Glu), respectively. Glu is a

central amino acid and is transferred to many amino acids by different aminotransferases.

In rice, a major source of inorganic N is ammonium. The ammonium is actively taken up by the roots via various ammonium transporters and subsequently assimilated by GS and NADH-GOGAT in the roots. In the rice plant, approximately 80% of the total N in the panicle is remobilized through the phloem from senescing organs. Thus, GS in senescing organs and GOGAT in developing organs are important for N remobilization and reutilization, respectively, because Gln is the major form of N in the phloem sap (Tabuchi et al. 2007).

There are several reported attempts to improve N-use efficiency (NUE) by genetic manipulation. Overexpression of a *NADH-GOGAT* gene from *japonica* rice under the control of a *japonica* rice *NADH-GOGAT* promoter in an *indica* cultivar Kasalath increased grain weight up to 80%, indicating that NADH-GOGAT is indeed a key step for N utilization and grain-filling in rice (Yamaya et al. 2002). Shrawat et al. (2008) reported that introducing a barley *alanine aminotransferase* (*AlaAT*) cDNA driven by a rice tissue-specific promoter (*OsAnt1*) into rice significantly increased the biomass and grain yield under a N well-supplied condition. Moreover, transgenic rice plants showed the changes of key metabolites and total N content, indicating enhanced N uptake efficiency. Zhou et al. (2009) over-expressed separately all of three rice aspartate aminotransferase (AAT) genes from (*OsAAT1-3*) and one *Escherichia coli*-derived AAT gene (*EcAAT*) in transgenic rice. The transformants overexpressing *OsAAT1*, *OsAAT2* and *EcATT* showed significantly increased leaf AAT activity and higher grain amino acid and protein contents, compared to the non-transgenic controls. No significant changes were found in leaf AAT activity, seed amino acid content, or protein content in *OsAAT3* over-expressed rice plants. These results indicated that overexpression of AAT altered N metabolism in transgenic rice plants.

As a trait related to a complex metabolic pathway, approaches using the analysis of QTLs and microarray analysis have been applied for NUE research. Dozens of QTLs for low-N tolerance were detected using a population of 239 recombinant inbred lines from a cross between Zhenshan 97 and Minghui 63 (Lian et al. 2005). A total of 471 low-N responsive expressed sequence tags (ESTs) were determined in the root tissue using a microarray of 11 494 rice expressed sequence tags, representing 10 422 unique genes (Lian et al. 2006). All of these detected QTLs and low-N responsive genes provided potential gene resources for developing high NUE rice via genetic manipulation or MAS.

### 22.7.2 *Phosphorus-Use Efficiency*

The overwhelming majority of soils in the rice-producing areas are phosphorus (P)-deficient with a high P-fixing capacity (Li 1985). Most of the arable soils are either acidic (tropics and subtropics) or calcareous (temperate regions). In acidic soils free iron and aluminum oxides bind native and applied P into forms unavailable to

plants, whereas in calcareous soils the abundant calcium and magnesium compounds bind inorganic phosphates into forms highly unavailable to plants. The high P-fixing capacity in both types of soils results in very low P-availability and thus low rates of uptake by the plants. Moreover, it is highly alarming that the global P resources will be exhausted before the end of this century (Vance et al. 2003). Thus, improving the uptake efficiency of the rice plant in P-fixing soils is a major research target.

From a cDNA library constructed by the suppression subtractive hybridization method, Yi et al. (2005) identified *OsPTF1*, a P-deficiency responsive transcription factor from Kasalath, a P-efficient *indica* landrace. Transgenic plants of the low-P sensitive rice variety Nipponbare overexpressing *OsPTF1* showed enhanced P efficiency in both solution and soil cultures. Tillering ability, root and shoot biomass, and P content of the transgenic plants were >30% higher than the wild-type plants in P-deficient culture solution. In soil pot and field experiments at low-P levels, tiller number, panicle weight, and P content increased >20% in transgenic plants, compared with wild-type plants.

Most of high-affinity P transporter genes are expressed predominantly in roots and are induced by P depletion, indicating that they are involved in the acquisition of P through the roots under low external P concentrations. Seo et al. (2008) identified a Pi transporter gene *OsPT1* that is expressed constitutively in the shoot regardless of external P concentration and is slightly inducible in the root by P depletion. Transgenic rice plants overexpressing *OsPT1* under the control of the CaMV 35S promoter accumulated almost twice as much phosphate in the shoots compared with the wild-type controls under both normal and P-null fertilizations. The transgenic plants had more tillers and better roots, indicating high P content in the plants. The results demonstrated that overexpression of *OsPT1* in rice enhanced P acquisition. However, transgenic rice overexpressing *OsPT1* showed 30% shorter than the wild-type controls, which was supposed to be caused by the comparative deficiency of other nutrients such as N and potassium (K) because they were not concomitantly increased with an enhanced P acquisition.

Wissuwa and Ae (2001a) analyzed P uptake of 30 rice varieties representing a wide diversity of the cultivated rice germplasm on normal and P-deficient soils. The analysis revealed very wide variation among the genotypes in low-P tolerance, as measured by P uptake on P-deficient soil relative to that on normal soil. Clearly there is a tremendous potential of using natural variation for improving P efficiency of rice cultivars. Wissuwa and Ae (2001b) further developed near isogenic lines (NILs) for two QTLs, a major one on chromosome 12 and a minor one on chromosome 6, by introgressing the alleles from Kasalath, a P-efficient variety, to Nipponbare, a P-inefficient variety. P uptake of the NIL carrying the Kasalath allele of the QTL on chromosome 12 on a P-deficient upland soil was three to four times that of Nipponbare, whereas the advantage of NIL carrying the Kasalath allele of the QTL on chromosome 6 was in the range of 60–90%. These genes hold promise for improving P uptake efficiency of the rice crop, although further study is needed to evaluate their effectiveness in the genetic backgrounds of elite cultivars under diverse field conditions.

## 22.8 Yield

In rice, yield is multiplicatively determined by three component traits: number of panicles per unit surface, number of grains per panicle, and grain weight. Hundreds of QTLs for yield and yield component traits have been identified during the past decade ([www.gramene.org](http://www.gramene.org)). Several QTLs for yield components have been cloned, including those for number of tillers per plant (Li et al. 2003), number of grains per panicle (Ashikari et al. 2005), and grain size (Fan et al. 2006; Song et al. 2007; Shomura et al. 2008). Recently, an important QTL *Ghd7*, which has major effects on three distinct traits: number of grains per panicle, plant height, and heading date, has been cloned (Xue et al. 2008). The major effects observed between the NILs and the cloning of QTL have fundamental implications for yield improvement, suggesting that yield, like other traits, can also be improved by individually manipulating the component traits using both MAS and transformation.

Much of the effort to develop high-yield rice has concentrated on seeking so-called C4 rice in the past decade, primarily using a transgenic approach. The majority of terrestrial plants, including many agronomically important crops such as rice, wheat, barley, and soybean, assimilate CO<sub>2</sub> through the C<sub>3</sub> photosynthetic pathway (Calvin cycles). In this pathway, ribulose 1, 5-biphosphate carboxylase/oxygenase (Rubisco) is the key enzyme for CO<sub>2</sub> fixation. However, Rubisco can also react with O<sub>2</sub>, releasing CO<sub>2</sub> and leading to photorespiration with a dual activity of both carboxylase and oxygenase. Photorespiration is estimated to consume approximately 40% of photosynthetic products, and the extent can further increase under stress conditions such as drought, high light, and high temperature (Ku et al. 1999).

Another plant type, referred to as C<sub>4</sub> plants including maize, sorghum, and sugarcane, have evolved a CO<sub>2</sub>-concentrating mechanism to overcome photorespiration. C<sub>4</sub> plants divide the C<sub>4</sub> cycle between two different cell types: mesophyll cells (MCs), and bundle sheath cells (BSCs). The initial CO<sub>2</sub> fixation occurs in the MC cytosol by phosphoenolpyruvate carbocylase (PEPC) to form C<sub>4</sub> acid compounds. C<sub>4</sub> acid compounds are transported to BSCs and release CO<sub>2</sub> to the vicinity of Rubisco in BSCs through decarboxylation reaction, by which mechanism the CO<sub>2</sub> concentration is significantly elevated around Rubisco and therefore can suppress photorespiration. Associated with two-cell C<sub>4</sub> process, C<sub>4</sub> plants generally possess a characteristic leaf structure known as “Kranz” anatomy, which comprises thick-walled BSCs immediately adjacent to veins surrounded by thin-walled MCs. C<sub>4</sub> plants have competitive advantages over C<sub>3</sub> plants because of these features (higher photosynthetic capacity, N and water use efficiencies; Matsuoka et al. 2001; Leegood 2002; Hibberd et al. 2008).

Undoubtedly, transferring C<sub>4</sub> traits into rice to develop C4 rice will be one of the most ambitious goals of GM rice. Genes encoding all of the key photosynthetic enzymes for C4 cycle are present in C<sub>3</sub> plants, but their expression levels are much lower than that in C<sub>4</sub> plants (Hibberd et al. 2008). Thus, overexpressing genes for single enzymes involved in C<sub>4</sub> cycle in rice is a strategy at an early stage to produce C<sub>4</sub> rice. Indeed, most of these enzymes have been overexpressed at present

individually or in combination in rice by genetic manipulation. Maize PEPC is the most common enzyme that was overexpressed in transgenic rice as a key enzyme for initial CO<sub>2</sub> fixation in C<sub>4</sub> plants. The effects of overexpressing PEPC were not consistent in different studies. Some studies reported overproduction of PEPC improved rice photosynthesis and further increased yield (Jiao et al. 2002; Bandyopadhyay et al. 2007), whereas others reported few (Ku et al. 1999; Suzuki et al. 2006) or even slightly negative effects (Fukayama et al. 2003; Taniguchi et al. 2008) on photosynthesis. Overexpression of maize C<sub>4</sub>-specific pyruvate, orthophosphate dikinase (PPDK), in transgenic rice was reported to have no impact on photosynthesis and rice growth, or slight negative effects because an extremely high accumulation of PPDK probably caused N deficiency (Fukayama et al. 2001; Taniguchi et al. 2008). Overexpression of maize C<sub>4</sub>-specific NADP-malic enzyme (ME) resulted in serious stunting, leaf chlorophyll bleaching, and enhanced photo-inhibition of photosynthesis (Takeuchi et al. 2000; Tsuchida et al. 2001; Taniguchi et al. 2008), while overexpressing rice C<sub>3</sub>-specific NADP-ME isoform did not show any changes of plant photosynthesis and growth (Tsuchida et al. 2001; Taniguchi et al. 2008). Overexpression of sorghum NADP-malate dehydrogenase (MDH) had little effect on plant growth (Taniguchi et al. 2008). Overproduction of a PEP-CK from *Urochloa panicoides* targeting to chloroplast of transgenic rice leaves showed some characteristics of the carbon flow of C<sub>4</sub> plants. <sup>14</sup>CO<sub>2</sub> labeling experiments showed that about 20% radioactivity was incorporated into C<sub>4</sub> compounds, which was much higher than that of a non-transgenic control (Suzuki et al. 2000). Moreover, co-expressing PEPC and PEP-CK did not further enhance C<sub>4</sub>-like carbon flow, compared to PEP-CK transgenic rice. In contrast, PEPC/PEP-CK transgenic rice showed aberrant phenotypes such as lower chlorophyll concentration and swollen thylakoid membranes (Suzuki et al. 2006).

It seems unlikely that introducing a single enzyme or even a portion of the C<sub>4</sub> cycle could have a large impact on photosynthesis, considering the complex C<sub>4</sub> photosynthesis mechanism and Kranz anatomy. Different from classical terrestrial C<sub>4</sub> plants, some aquatic C<sub>4</sub> plants lacking Kranz anatomy can accomplish the C<sub>4</sub> cycle in single cells (Leegood 2002). Among them, the best studied is the *Hydrilla verticillata* mechanism for C<sub>4</sub> metabolism, which is relatively simple (Taniguchi et al. 2008). Currently, some researchers focus on installing single-cell C<sub>4</sub> mechanism like *H. verticillata* in rice because it avoids the complex Kranz anatomy. To establish a *H. verticillata* C<sub>4</sub>-like pathway in transgenic rice, Taniguchi et al. (2008) overexpressed the maize C<sub>4</sub>-specific PEPC, the maize C<sub>4</sub>-specific PPDK, the sorghum NADP-MDH, and the rice C<sub>3</sub>-specific NADP-ME in combination. However, photosynthesis and growth analysis demonstrated that these transgenic rice plants only exhibited slightly improved photosynthesis accompanied with slight but reproducible stunting phenotype (Taniguchi et al. 2008).

Taken together, no really exciting breakthrough of C<sub>4</sub> rice has been achieved so far. Although Jiao et al. (2002) reported an increased grain yield of transgenic rice by 22–24% through co-expressing C<sub>4</sub>-specific PEPC and PPDK, their result has not been confirmed by other groups. An international consortium of C<sub>4</sub> rice comprising ten research groups was formed in 2006, which aims to develop C<sub>4</sub> rice and increase rice yield to 50% (Normile 2006). The members of the consortium are optimistic to

create C<sub>4</sub> rice and some potential strategies have been proposed (Hibberd et al. 2008). Probably, the question whether C<sub>4</sub> rice is really feasible will be answered in the near future.

## 22.9 Herbicide-Tolerant Rice

Herbicide tolerance has been continuously the number one trait of GM crops, with the largest growing area since GM crops were first commercially grown in 1996 (see Chap. 9). Many studies for developing herbicide-tolerant rice cultivars have been conducted in the past. The *bar* gene from *Streptomyces hygroscopicus* is the first herbicide-resistant gene used in transgenic rice (Datta et al. 1992; Oard et al. 1996). The *bar* gene encodes a phosphinothricin (PPT) acetyltransferase (PAT) that catalyzes the transfer of an acetyl moiety from acetyl-coenzyme A to the amino group of PPT. Herbicide glufosinate ammonium (trade names: Liberty, Finale, Basta) is an ammonium salt of PPT that can non-selectively kill various plants by inhibiting glutamine synthetase (Oard et al. 1996). Recently, a comprehensive evaluation of herbicide-tolerant GM rice with the *bar* gene including a field trait, environmental risk assessment, and socio-economic analysis was conducted in Costa Rica, which will probably promote the commercialization of the herbicide-tolerant GM rice in that country (Espinoza-Esquível and Arrieta-Espinoza 2007).

Studies have also been conducted to produce herbicide-tolerant GM rice by overexpressing cytochrome P450 (P450 or CYP) monooxygenases. The P450 monooxygenases exist in all organisms from bacteria to humans, and they play an important role in detoxifying hydrophobic xenobiotic chemicals through an oxidative reaction to make xenobiotics more reactive and hydrophilic (Ohkawa et al. 1999). Overproduction of some P450 species in plants can accelerate the metabolism of xenobiotic compounds, including herbicides, and therefore improve herbicide tolerance. Plants have hundreds of P450 species, but the functions of most plant P450 genes have not been identified and the herbicide-metabolizing activity of plant P450 species is relatively low, compared with some mammalian isoforms (Inui and Ohkawa 2005). Most P450 genes used to produce herbicide-tolerant rice are from mammals so far. Inui et al. (2001) introduced human P450 genes *CYP2C9* and *CYP2C19* driven by the CaMV35S promoter and Nos terminator into rice, and the result showed that transgenic rice with human *CYP2C9* was tolerant to the sulfonylurea herbicide chlorsulfuron, while that with human *CYP2C19* exhibited cross-tolerance to herbicides with different modes of action, including mefenacet, metolachlor, norflurazon, and pyributicarb. Further studies with the similar strategy showed that individually expressing human *CYP1A1* and *CYP2B6* or co-expressing human *CYP1A1*, *CYP2B6*, and *CYP2C19* in transgenic rice can also enhance the tolerance of transgenic rice to various herbicides with different chemical structures and modes of action (Kawahigashi et al. 2005a, 2006, 2007; Hirose et al. 2005). Other than human P450 species, transgenic rice plants overexpressing pig P450 *CYP2B22* and *CYP2C49* also exhibited enhanced tolerance to various herbicides with different modes of actions. Transgenic rice with pig *CYP2B22* was tolerant to 12 herbicides, including chlortoluron, amiprofos-methyl, pendimethalin,

metolachlor, and esprocarb. *CYP2C49* rice showed tolerance to 13 herbicides, including chlortoluron, norflurazon, amiprofos-methyl, alachlor, and isoxaben (Kawahigashi et al. 2005b). Transgenic rice overexpressing P450 genes may be useful for phytoremediation of environmental pollutants because some P450 species metabolize not only herbicides but also insecticides and other organic chemicals (Inui and Ohkawa. 2005; Kawahigashi et al. 2005c, 2006, 2007). However, the composition of the secondary metabolites in these transgenic rice plants possibly varies due to the alteration of P450 species and activities, and the transgenic rice plants should be analyzed completely before release into the environment.

There are other strategies to produce herbicide-tolerant rice. Protoporphyrinogen oxidases (protoxes) are required for biosynthesis of heme and chlorophyll in plants, which catalyzes oxidation of rotoporphyrinogen IX (protoxin IX) to protoporphyrin IX (proto IX). Protox is the primary target site of action for diphenyl ether herbicides such as oxyfluorfen and acifluorfen (Jung et al. 2004; Jung and Back 2005). Overexpressing heterologous protoxes is a strategy to produce diphenyl ether herbicide-resistant transgenic rice. Lee et al. (2000) acquired oxyfluorfen-resistant transgenic rice by overexpressing *Bacillus subtilis* protox targeting to the cytoplasm or plastids. Moreover, transgenic rice plants overexpressing bacterial protox targeted to the plastid exhibited higher herbicide resistance than those targeted to the cytoplasm (Lee et al. 2000). Transgenic rice with high resistance to oxyfluorfen by expressing *Myxococcus xanthus* protox dual targeting to chloroplasts and mitochondria has also been developed (Jung et al. 2004; Jung and Back 2005). Acetolactate synthase (ALS) catalyzes the first step in the biosynthesis of the branched-chain amino acids leucine, isoleucine, and valine, which are the primary target site of action for at least four classes of herbicides (sulfonylureas, imidazolinones, triazolopyrimidine sulfonamides, pyrimidinyl carboxy herbicides; Chipman et al. 1998). ALS-inhibiting herbicides account for an essential part of global weed control market because they are highly effective, selective, and non-toxic to animals. Transgenic rice tolerant to ALS-inhibiting herbicide was generated through introducing two amino acid mutations in the rice ALS gene (Endo et al. 2007). The mutant ALS gene was introduced into rice by *Agrobacterium*-mediated transformation, however the target gene was substituted “in situ” and no additional DNA fragments were inserted into the genome of transgenic rice plants because the authors adopted a gene targeting strategy. The transgenic rice was supposed to be equivalent to non-GM herbicide tolerant rice produced by conventional breeding approaches (Endo et al. 2007).

## 22.10 Prospects

The past two decades have shown tremendous progresses in the development of transgenic research in rice, both in transformation technology and in rice genetic improvement. From a technology perspective, there are still urgent needs for further improvement, at least in the following fronts:

1. Homologous recombination, or gene targeting, is generally regarded as a means for precise replacement and delivery of DNA. In rice, however, it may still take considerable work before this becomes a method for general purpose, although large effort was made for establishing this technique (Terada et al. 2002, 2007).
2. Transformation of multiple genes with a single construct is highly desirable for many purposes, which should be explored especially for simultaneous improvement of multiple traits in breeding programs.
3. Tissue-specific expression is generally preferred not only for the normal growth and development of rice plants, but also because it is extremely important for the rice grain to be used as a staple food. There are reasons to be optimistic that technology will advance sufficiently to satisfy the need for breeding purposes.

Zhang (2007) outlined the strategies for developing GSR by integration of genomic, transgenic, MAS, and conventional breeding technologies in rice breeding, using genes from various sources. From a global perspective, there has been tremendous progress in the identification of genes for most of the traits for the development of GSR, thanks to the international effort in functional genomics research. However, there is a huge need for the enrichment of these genes, especially for some traits, such as resistance to sheath blight, the most serious disease especially for areas with high productivity, and nutrient use efficiency. Special attention should be paid to those traits, both for gene discovery and the underlying biology. Moreover, although the rice cultivars have attained a very high yield level due to a combination of breeding technologies and field management, there is always demand for further increase of yield potential. Given the current achievements in breeding for heterosis and population structure which has generated a huge “pool”, the next feasible leap might stem from the improvement of the “source”, or increasing photosynthetic rate, which may also require a combination of approaches.

In conclusion, it can be expected that the rapid advances in the transformation technology together with the accelerated pace of gene discovery will greatly facilitate the development of GSR. And the realization of GSR will generate a huge impact on sustainable rice production on a global scale.

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# Chapter 23

## Sugarcane

Fredy Altpeter and Hesham Oraby

### 23.1 Introduction

Sugarcane (*Saccharum* sp. hybrids) is a highly polyploid and frequently aneuploid, interspecific hybrid (Sreenivasan et al. 1987; Grivet et al. 1996). This highly productive C<sub>4</sub> grass is used as the main source of sugar and more recently to produce ethanol, a renewable transportation fuel. There is increased interest in this crop due to the impending need to decrease the dependency on fossil fuels. Despite its economic importance, efforts in sugarcane breeding and genomics are lagging behind other important crops. This is caused by the complexity of the sugarcane genome, narrow gene pool, poor fertility and the long breeding cycle. Transgenic sugarcane plants with improved agronomic and value-added traits have been reported. Future developments are expected to lead to commercial release of transgenic sugarcane and may include its development into a biofactory for high-value products.

### 23.2 Origin

One of the earliest records describing sugarcane goes back to 326 BC (Purseglove 1972). Modern sugarcane varieties that are cultivated for sugar production are complex interspecific hybrids (*Saccharum* sp.) between the species *S. officinarum* and *S. spontaneum* with contributions from *S. robustum*, *S. barberi*, *S. sinense* and related grass genera such as *Miscanthus*, *Erianthus* and *Narenga* (Brandes 1958;

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Purseglove 1972; Daniels and Roach 1987). *S. officinarum* (or noble cane) most likely originated in New Guinea from a domesticated, thick-stalked, high-sugar, low-fiber form of *S. robustum* (Daniels and Roach 1987). *S. officinarum* is low in fiber and accumulates very high levels of sucrose in the stem, but has poor disease resistance (Sreenivasan et al. 1987). *S. spontaneum* occurs in the wild from eastern and northern Africa, through the Middle East, to India, China, Taiwan and Malaysia, and through the Pacific to New Guinea. The center of origin is probably in northern India where forms with the smallest chromosome numbers occur. *S. spontaneum* has a greater stress tolerance than *S. officinarum* (Sreenivasan et al. 1987). *S. robustum* is found along river banks in New Guinea and some of its adjacent islands and is indigenous to the area. *S. barberi* probably originated in India. *S. sinense* occurs in portions of India, Indo-China, southern China and Taiwan (Purseglove 1972).

### 23.3 Sugarcane Breeding, Biotechnology and Biosafety

Although conventional breeding programs in sugarcane are relatively recent compared to other major crops, interspecific hybridization within the genus *Saccharum* supported significant improvements in yield, ratooning ability, sugar content and disease resistance, while maintaining acceptable fiber levels for milling (Jackson 2005; Lakshmanan et al. 2005; Ming et al. 2006). Most modern sugarcane cultivars originate from crosses between a relatively small number of original progenitor clones compared with the large number of basic clones that exist in the *Saccharum* genus, resulting in a narrow gene pool (Jackson 2005). It remains challenging to exploit the large genetic variation existing among clones of different *Saccharum* species (Ming et al. 2006). Molecular markers may assist breeders in incorporating useful genes from sexually compatible sources into the gene pool of the advanced cultivars. Modern cultivars contain between  $2n = 100$  and  $2n = 130$  chromosomes, with 5–10% consisting of the wild *S. spontaneum* contribution and less than 5% of these being recombinant or translocated chromosomes (Ming et al. 2006). The high ploidy (5x to 14x; Burner and Legendre 1994) and the complex genome structure of sugarcane create challenges for marker development and genome characterization (D'Hont et al. 1996; Cuadrado et al. 2004). Sugarcane is a prime candidate for genetic transformation to enhance sugar production (general methods of transformation are reviewed in Chap. 1), conversion of biomass to biofuels or production of value-added products (see also Chap. 11). Vegetative propagation of sugarcane prevents segregation of multiple transgenes needed for trait stacking or pathway engineering. The transfer of stress tolerance genes into a crop generates concern that the transgenic plant will become a weed, that the gene will be transferred to wild relatives increasing their weediness, or that intraspecific gene transfer by pollen may prevent effective segregation of transgenic and non-transgenic products (see Chap. 27). Probability of transgene escape from sugarcane to related species is discussed by Bonnett et al. (2008). In general, sugarcane offers a high level of transgene containment. Many cultivars do not produce viable pollen

or seeds under typical commercial growing conditions. In addition sugarcane is vegetatively propagated. This prevents transgenes from unintended entering into new plantings following intraspecific gene transfer by pollen. This helps to ensure segregation of transgenic and non-transgenic products if value-added products are produced. Also, sugarcane usually does not persist under non-cultivated conditions. Further, sugar the primary product of non-transgenic sugarcane is essentially free of DNA and protein. These attributes contribute to the biosafety of sugarcane for the production of value-added products. A sugar mill is in a sense already a biorefinery producing sugar and molasses as products and generating bagasse, used as a fuel for use in sugar mill boilers. Some natural pharmaceutical compounds are derived from sugarcane (Menendez et al. 1994). A slate of products including transgenic, value-added products may be produced from sugarcane biomass in the future, particularly fuels and chemicals, which together provide additional revenue. The concept converts a sugar mill into a sugarcane processing plant for sugar and/or value-added products (Rein 2007).

## 23.4 In Vitro Culture

### 23.4.1 *In Vitro Culture for Sugarcane Improvement*

In vitro culture plays a crucial role in the conservation, creation and utilization of genetic variability of sugarcane, including cryopreservation, in vitro selection, genetic engineering and commercial mass production of disease-free sugarcane. Young meristematic tissues such as immature leaf, immature inflorescence or basal shoot meristems are required in sugarcane and many other monocotyledonous species to induce regenerable tissue cultures. After establishing meristematic tissues on a culture medium, these can be stimulated to regenerate into whole plants in vitro via organogenesis or embryogenesis, with or without a callus phase. Avoiding or shortening the callus phase may reduce the occurrence of genetic abnormalities in regenerated plants, a phenomenon referred to as somaclonal variation (Burner and Grisham 1995). However, in rare cases, somaclonal variation can lead to desirable traits such as improved stress tolerance (Liu 1990; Leal et al. 1996; Zambrano et al. 2003; Singh et al. 2008). The developmental pathway that the regenerating tissue will follow is determined by explant type and exogenously applied growth regulators, with particular importance of the auxin and cytokinin types and their balance (Lakshmanan et al. 2006). The generation of embryogenic sugarcane cultures (Ho and Vasil 1983a) was critical for the development of transgenic sugarcane plants (Bower and Birch 1992). The regeneration of sugarcane plants via direct organogenesis from transverse thin cell layer sections of immature leaves is currently the most efficient method of achieving fast, large-scale production of disease-free varieties (Lakshmanan et al. 2006). Micropropagated sugarcane plants are disease-free, vigorously growing and superior to seed cane in yield and sugar recovery under field agronomic practices (Sood et al. 2006).

### 23.4.2 Sugarcane Somatic Embryogenesis

The first successful plant regeneration from sugarcane callus cultures was reported by Barba and Nickel (1969) and Heinz and Mee (1969). Many authors described the regenerative structures with terms such as ‘meristemoids,’ ‘shoot meristem,’ ‘embryoids’ and ‘somatic embryos’. However, convincing evidence of somatic embryo development and sugarcane plant regeneration was first presented by Ho and Vasil (1983a, b). The embryogenic sugarcane callus was formed by divisions in mesophyll cells situated primarily in the abaxial half of the leaf and also from cells of the vascular parenchyma. Somatic embryos arise either from single cells (Ho and Vasil 1983a) or indirectly from pro-embryogenic masses which themselves are derived from single cells (Vasil and Vasil 1994). In indirect somatic embryogenesis, embryos are produced from callus initiated from meristematic tissues, such as immature leaves (Ho and Vasil 1983a; Snyman et al. 2001), immature inflorescences (Heinz and Mee 1969; Liu 1993; Gallo-Meagher and Irvine 1996), or basal shoot meristems (Arencibia et al. 1998). More rapid tissue culture and regeneration protocols via direct embryogenesis minimize somaclonal variation and reduce the time required to produce a transgenic plants. Such protocols were reported for sugarcane (Snyman et al. 2001; Desai et al. 2004), following earlier reports in other graminaceous monocots (Altpeter et al. 1996; Denchev et al. 1997). 2,4-Dichlorophenoxyacetic acid (2,4-D) is considered the most effective auxin for embryogenic callus induction in sugarcane (Ho and Vasil 1983a; Lakshmanan 2006). To promote regeneration, the callus is transferred to medium with either a reduced auxin concentration or containing no auxin in combination with or without a cytokinin. Different cytokinines (including 6-benzylaminopurine, kinetin, zeatin, and thidiazuron, TDZ) were evaluated for induction of regeneration from embryogenic callus. Thidiazuron in the absence of an auxin was superior in inducing shoot regeneration from callus. However, shoots regenerated on kinetin-containing medium elongated faster (Gallo-Meagher et al. 2000; Chengalrayan and Gallo-Meagher 2001).

### 23.4.3 Sugarcane Organogenesis

Organogenesis in vitro consists of dedifferentiation of differentiated cells to acquire organogenic competence following hormone perception, re-entry of quiescent cells into the cell cycle and organization of cell division to form specific organ primordia and meristems (for a review, see Sugiyama 1999). Organogenesis occurs either directly from the explant or indirectly from a callus culture and bypasses the formation of a somatic embryo. Large number of plants can be produced directly from the apical shoot meristem (Hendre et al. 1983; Lee 1987; Burner and Grisham 1995) or axillary buds (Sauvaire and Galzy 1978). Light can switch the regeneration pathway from embryogenesis to organogenesis and activate different cell types

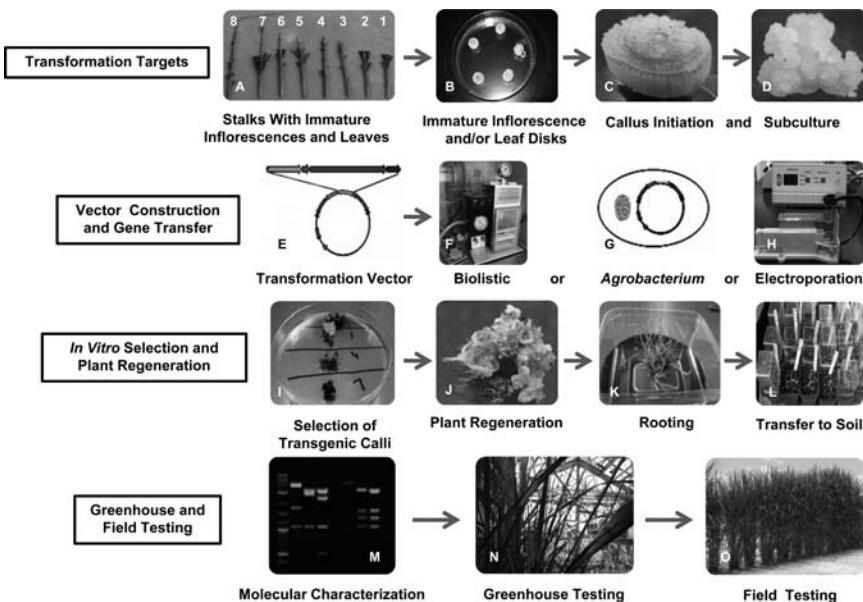
in response to the same auxin. This probably reflects the influence of endogenous levels of phytohormones, which can result either from different levels of uptake or from alterations on their metabolism (Garcia et al. 2007). Plant regeneration through organogenesis was more efficient when using naphthaleneacetic acid (NAA) than when using picloram or 2,4D alone (Garcia et al. 2007). NAA combined with cytokinins such as 6-benzylaminopurine (BA; Lakshmanan et al. 2005) or kinetin (Gill et al. 2006) resulted also in a large number of regenerated plants. Direct organogenesis is the preferred regeneration procedure and is extensively used for commercial mass propagation of sugarcane in the United States and Brazil (Snyman 2004; Lakshmanan et al. 2005).

## 23.5 Genetic Engineering of Sugarcane

Genetic engineering of monocotyledonous crops, including sugarcane requires an in vitro culture system for regeneration of plants from totipotent tissues or cells. Such cells can be targeted with an efficient gene delivery system. Successful gene transfer events can be identified during callus growth and/or plant regeneration with the help of a selectable marker and the corresponding selective agent (Fig. 23.1).

### 23.5.1 Methods of Transformation

A number of reports (summarized in Table 23.1) described alternative genetic transformation protocols and/or introduction of transgenes for sugarcane improvement. Most common steps in these protocols are illustrated in Fig. 23.1. The first transgenic sugarcane calli were obtained via treatment of protoplasts by polyethylene glycol (PEG; Chen et al. 1987) or electroporation (Chowdhury and Vasil 1992; Rathus and Birch 1992). In these studies lack of regeneration from protoplast prevented production of transgenic plants. Transgenic sugarcane plants were reported following electroporation of meristematic tissues of in vitro grown plants (Arencibia et al. 1992) or intact embryogenic cells (Arencibia et al. 1995, 1997). Biolistic gene transfer is one of the most reproducible and versatile gene transfer systems (reviewed by Altpeter et al. 2005) and was successfully used for the generation of the first transgenic plants from a commercial sugarcane cultivar (Bower and Birch 1992). Subsequently biolistic gene transfer became the most widely used method for sugarcane transformation (Table 23.1) due to its high reproducibility and applicability to easily established target tissues including embryogenic callus (Gallo-Meagher and Irvine 1996; Ingelbrecht et al. 1999; Zhang et al. 1999; Falco et al. 2000; Gilbert et al. 2005; Weng et al. 2006) and immature leaf transverse section explants (Snyman et al. 2006). *Agrobacterium*-mediated gene transfer results frequently in simpler transgene integration patterns



**Fig. 23.1** Generation of transgenic sugarcane plants. (A) Stalks in the transition from vegetative to flowering. Most responsive immature inflorescence material is found in stages 3 and 4. (B) Transverse sections of surface-sterilized stalks (shown in A, stages 3, 4) include both immature leaf and inflorescence segments. (C) Callus initiation from immature inflorescence segments (center) and surrounding leaf segments. (D) Subculture of embryogenic sugarcane callus used as target for transfer of (E) a recombinant plasmid by (F) biostatic gene transfer, (G) *Agrobacterium*-mediated gene transfer or (H) electroporation. Transgenic events are identified by active callus growth (I) and by plant regeneration on culture medium containing the selective agent corresponding to the co-introduced selectable marker gene (J). Following rooting and elongation (K), regenerated sugarcane plantlets are (L) transferred to soil. Following molecular characterization (M), transgenic plants are evaluated physiologically and agronomically under (N) greenhouse and (O) field conditions

than biostatic gene transfer. However, biostatic gene transfer is superior in gene stacking or pathway engineering, when multiple expression cassettes need to be co-expressed (reviewed by Altpeter et al. 2005). The biology and biotechnology of *Agrobacterium*-mediated genetic transformation of plants as well as factors influencing transformation efficiency are reviewed by Tzfira and Citovsky (2006) and Opabode (2006). *Agrobacterium*-mediated transformation of sugarcane was successfully achieved by co-cultivation with embryogenic callus (Arencibia et al. 1998; Elliot et al. 1998), immature leaf sections (Enriquez-Obregon et al. 1998) or axillary buds (Manickavasagam et al. 2004). Many of the primary transformants obtained from axillary buds by bypassing a callus phase were chimeric, but continuous shoot multiplication on the same selection medium progressively eliminated chimeras and escapes, and this resulted in the so far highest reported transformation efficiency for sugarcane (11.6–49.6%; Manickavasagam et al. 2004).

**Table 23.1** Examples of reported transgenic sugarcane plants

Trait	Gene	Explant	Gene transfer	Selection and screening	Field trials	Reference
Reporter and selection systems						
NPTII/GUS	<i>npt-IIuidA</i>	Immature leaves	Bombardment	Geneticin/GUS	No	Bower and Birch (1992)
NPTII/GUS	<i>npt-IIuidA</i>	Intact meristems	Electroporation	Kanamycin/GUS	No	Arenchiba et al. (1992)
GUS	<i>uidA</i>	Immature leaves	Electroporation	GUS	No	Arenchiba et al. (1995)
HPT/GUS	<i>hpIuidA</i>	Immature leaves	<i>Agrobacterium</i>	Hygromycin/GUS	No	Arenchiba et al. (1998)
PPT/GFP	<i>bar/gfp</i>	Immature leaves	<i>Agrobacterium</i>	Bialaphos/GFP	No	Elliott et al. (1998)
Posphomannose isomerase	<i>pmi</i>	Immature leaves	Bombardment	Mannose	No	Jain et al. (2007)
Herbicide resistance						
Glufosinate ammonium	<i>bar</i>	Immature inflorescences	Bombardment	Bialaphos	No	Gallo-Meagher and Irvine (1996)
Glufosinate ammonium	<i>bar</i>	Immature leaves	<i>Agrobacterium</i>	Phosphinothricin	Yes	Enriquez-Oregon et al. (1998)
Glufosinate ammonium	<i>bar</i>	Immature leaves	Bombardment	Geneticin	No	Falco et al. (2000)
Glufosinate ammonium	<i>pat</i>	Not specified	Bombardment	Glufosinate ammonium	Yes	Leibbrandt and Snyman (2003)
Glufosinate ammonium	<i>bar</i>	Axillary buds	<i>Agrobacterium</i>	Phosphinothricin	No	Manickavasagam et al. (2004)
Insect resistance						
Sugarcane stem borer	<i>cry1Ab</i>	Meristematic tissue	Electroporation	GUS	Yes	Arenchiba et al. (1997, 1999)
Cane grub	<i>gnl/pinn</i>	Immature leaves	Bombardment	Geneticin	No	Nutt et al. (1999)
Mexican rice borer	<i>gna</i>	Immature leaves	Bombardment	Geneticin	No	Selamou et al. (2002)
Sugarcane stem borer	<i>SKT/SBRI</i>	Immature leaves	Bombardment	Geneticin	No	Falco and Silva-Filho (2003)
Sugarcane stem borer	<i>cry1Ac</i>	Immature leaves	Bombardment	Geneticin	No	Weng et al. (2006)
Aphid resistance	<i>gna</i>	Immature leaves	<i>Agrobacterium</i>	Hygromycin, G418, PPT	No	Zhangsun et al. (2008)
Disease resistance						
Sorghum mosaic virus	<i>SCMV-CP</i>	Immature leaves	Bombardment	Geneticin	No	Joyce et al. (1998)
Sorghum mosaic virus	<i>ScMV-CP</i>	Immature leaves and inflorescences	Bombardment	Geneticin and bialaphos	Yes	Ingelbrecht et al. (1999)
Sugarcane leaf scald	<i>athD</i>	Immature leaves	Bombardment	Geneticin	No	Zhang et al. (1999)
Fiji disease virus	<i>FDV/S9 ORF 1</i>	Not specified	Bombardment	Geneticin	No	McQualter et al. (2004)
Sugarcane mosaic virus	<i>SCMV-CP</i>	Immature inflorescences	Bombardment	Geneticin	Yes	Gilbert et al. (2005)
Abiotic stress tolerance						
Drought	<i>TPS1TPP</i>	Immature leaves	<i>Agrobacterium</i>	Phosphinothricin	Yes	Zhang et al. (2006)
Drought	<i>P5CS</i>	Immature leaves	Bombardment	Glufosinate ammonium	No	Molinari et al. (2007)
Drought	<i>dreb2b</i>	Immature leaves	Bombardment	Hygromycin	No	Wu et al. (2008)

(continued)

**Table 23.1** (continued)

Trait	Gene	Explant	Gene transfer	Selection and screening	Field trials	Reference
Carbohydrate metabolism and value-added traits						
Sucrose accumulation	Invertase activity PPO suppression	Immature leaves Not specified	Bombardment Bombardment	Geneticin Geneticin	No	Ma et al. (2000)
Juice and raw sugar color	<i>UQ68J SI</i>	Not specified	Bombardment	Geneticin	Yes	Vickers et al. (2005)
Isomaltulose	<i>mdsSynth</i>	Immature leaves	Bombardment	Paromomycin	No	Wu and Birch (2007)
Sorbitol	PFP suppression	Immature leaves	Bombardment	Geneticin	Yes	Chong et al. (2007)
Sucrose accumulation	$\beta$ -Amylase expression, AGPase suppression	Not specified	Bombardment	Not specified	No	Groenewald and Botha (2008)
Reduction of starch accumulation	<i>hch</i> and <i>cpl</i>	Immature leaves	Bombardment	Geneticin	No	Ferreira et al. (2008)
<i>p</i> -Hydroxybenzoic acid production	<i>gm-cfs</i>	Immature leaves	Bombardment	Geneticin	Yes	McQualter et al. (2005)
Human Cytokine GM-CSF	<i>phuA, phuB</i> and <i>phuC</i>	Immature leaves	Bombardment	Geneticin	No	Wang et al. (2005)
Polyhydroxybutyrate production						Petrarouts et al. (2007), Purnell et al. (2007)

### 23.5.2 Selection of the Transformed Tissues

Most of the published sugarcane transformation reports employ antibiotics or herbicides to select for the transgenic events (Table 23.1). One of the most frequently used selection system is the neomycin phosphotransferase (npt-II) selectable marker gene in combination with geneticin selection (Table 23.1). Transgenic lines grew rapidly and produced roots on medium containing 0.04 mM (25 mg/l) geneticin which eliminated all escapes (Bower and Birch 1992). Falco et al. (2000) reported only 3% of the total transgenic sugarcane plant population escaping geneticin selection, whereas the escape rate was 42% in the case of plants regenerated in the presence of bialaphos for resistance to the *bar* gene in immature leaf and immature inflorescence derived calli (Gallo-Meagher and Irvine 1996). However for axillary bud transformation a comparison of kanamycin, geneticin and phosphinothricin (PPT) selection showed that PPT was the most effective selection agent (Manickavasagam et al. 2004). Consumer concern over the presence of antibiotic and herbicide resistance genes in genetically modified crops prompted the development of alternative selection procedures. A ‘positive’ selection regimen essentially incorporates a physiologically inert metabolite as the selection agent and a corresponding selectable marker gene that confers a metabolic advantage, thus alleviating the growth inhibitory effects of selection for the transformed cells. The utility of the phosphomannose isomerase (PMI)/mannose-based selection system has been established for sugarcane by Jain et al. (2007) with an escape rate of 44%. As an alternative to ‘positive’ selection, selectable marker genes including antibiotic or herbicide resistance genes can be excised following selection by site-specific recombination. This technology is reviewed by Hare and Chua (2002; see also Chap. 3).

### 23.5.3 Traits of Interest

Reports on transgene introduction into sugarcane for crop improvement target herbicide resistance, abiotic and biotic stress tolerance, and metabolic engineering of the carbohydrate metabolism (an introduction into genetic engineering of these traits is provided in Chaps. 9, 8, 10, 11 respectively). Sugarcane is also considered an attractive target for production of value added traits due to the high level of transgene containment found in this vegetatively propagated crop.

#### 23.5.3.1 Herbicide Resistance

The first herbicide resistant transgenic sugarcane plants were described by Gallo-Meagher and Irvine (1996) following biolistic gene transfer. Stable integration and expression of the *bar* gene referring glufosinate resistance was confirmed through

three vegetative cycles in the greenhouse (Gallo-Meagher and Irvine 1996) and under USDA-Aphis permit 95-143-01R in the first field test of any transgenic sugarcane (Irvine et al. 1996). Glufosinate resistant transgenic sugarcane lines were also described by Enriquez-Obregon et al. (1998), Falco et al. (2000), Leibbrandt and Snyman (2003) and Manickavasagam et al. (2004). Integration of the *bar* gene following *Agrobacterium*-mediated gene transfer resulted in high-level herbicide resistance in most of the lines (Enriquez-Obregon et al. 1998; Manickavasagam et al. 2004). However, some lines did not resist the herbicide despite simple transgene integration of one or two copies following *Agrobacterium*-mediated gene transfer (Enriquez-Obregon et al. 1998; Manickavasagam et al. 2004). Interestingly, a transgenic sugarcane line with nine copies of the *pat* gene showed stable and high-level glufosinate resistance under field conditions without yield penalty despite complex transgene integration following biolistic gene transfer (Leibbrandt and Snyman 2003). To assess the financial advantage of glufosinate-resistant sugarcane a comparison was made between the transgenic and a conventional weed control strategies under field conditions (Leibbrandt and Snyman 2003). No significant differences were observed in sugarcane yield following glufosinate application to transgenic sugarcane and non-transgenic cane using conventional herbicide treatment including pre- and post-emergence applications. Therefore, the costs of the individual herbicides, the application cost associated with the necessary multiple applications for conventional herbicides and the premium price for transgenic seeds determine the profit margin for the individual weed control strategy (Leibbrandt and Snyman 2003).

### 23.5.3.2 Biotic Stress Tolerance

Sugarcane is susceptible to many diseases and insects including fungal, bacterial, phytoplasmas, viral diseases, stem borers, canegrubs, earth perls and others accounting for dramatic yield losses of sugarcane worldwide (Allsopp and Suasa-ard 2000; Rott et al. 2000). A truncated *Bacillus thuringiensis* (Bt) insecticidal crystal protein (*cry*) *cryIAb* was constitutively expressed in sugarcane under control of the 35S promoter to provide resistance against lepidopterous sugarcane stem borer (*Diatraea saccharalis* F.). Transgenic lines with significant larvicidal activity were identified despite the very low expression of CryIAb (0.5–1.4 ng/mg soluble protein; Arencibia et al. 1997). Results of field trials confirmed the expression of the resistance trait. Limited but consistent morphological, physiological and phytopathological somaclonal variation was also detected in several transgenic lines, along with DNA polymorphisms (Arencibia et al. 1999). A truncated and codon-optimized *cryIAc* gene was expressed in sugarcane under control of the constitutive maize ubiquitin promoter. Expression levels of CryIA(c) between 1.8 ng/mg and 10.0 ng/mg total soluble protein were detected in transgenic lines. Highest expressing lines were resistant to *Proceras venosatus* (W), the main sugarcane borer in China, resulting in protection of the stems from damage (Weng et al. 2006).

However, higher levels of *cry1Ac* expression may be desirable to prevent the development of resistant insect populations. Constitutive expression of the soybean Kunitz trypsin inhibitor (SKTI) and soybean Bowman–Birk inhibitor (SBBI) in sugarcane resulted in retardation of larval growth of the sugarcane stem borer *Diatraea saccharalis* (F.) in laboratory bioassays. However, lines with the highest expression of these proteinase inhibitors did not display elevated insect mortality or did not prevent insect damage in greenhouse trials (Falco and Silva-Filho 2003). Bioassays with sugarcane leaf tissues expressing the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) resulted in resistance to the Mexican rice borer (*Eoreuma loftini* (D.), while sugarcane stem borer (*Diatraea saccharalis* F.) was not negatively effected in its development or reproduction (Setamou et al. 2002). However, expression of GNA or potato proteinase inhibitor II significantly reduced the weight gain of canegrubs feeding on transgenic sugarcane in a greenhouse trial (Nutt et al. 1999). Preliminary bioassays on GNA-expressing sugarcane also suggest increased resistance to woolly aphid *Ceratovacuna lanigera* (Z.), a vector of viral sugarcane diseases (Zhangsun et al. 2008).

The major viral pathogens of sugarcane include sugarcane mosaic virus (SCMV), Fiji disease virus (FDV), sorghum mosaic virus (SrMV), sugarcane streak virus (SSV) and sugarcane yellow leaf virus (SCYLV). Genetic engineering of virus resistance relies on transgenes derived from the pathogen's genome, termed pathogen-derived resistance (PDR; Sanford and Johnstone 1985). Several strategies have been used to engineer virus resistance in plants (for a review, see Baulcombe 1996). Transgenic sugarcane with resistance under greenhouse conditions to SCMV (Joyce et al. 1998), FDV (McQualter et al. 2004) or field conditions to SrMV (Ingelbrecht et al. 1999) were generated by transforming plants with either the viral coat protein (Joyce et al. 1998; Ingelbrecht et al. 1999), or the translatable S9 ORF 1-derived transgene (McQualter et al. 2004). A population of 100 transgenic plants with integration of an untranslatable SCVM coat protein gene was evaluated for virus resistance and agronomic performance under field conditions. Phenotypic variation was very high. However, several transgenic accessions combining SCMV resistance under field conditions with good agronomic performance were identified (Gilbert et al. 2005).

Leaf scald disease of sugarcane is caused by the systemic, xylem-invading pathogen (*Xanthomonas albilineans*) producing a family of low molecular weight toxins (albicidins) that cause the characteristic chlorotic symptoms by blocking chloroplast development. Leaf scald disease symptoms and yield losses in the field are observed only in sugarcane stalks with high pathogen populations (Rott et al. 1994). Expression of a gene for albicidin detoxification *albD* in transgenic sugarcane conferred a high level of resistance to chlorotic symptom induction and to multiplication and systemic invasion by *X. albilineans* (Zhang et al. 1999).

Many elite sugarcane clones are susceptible to multiple fungal and/or bacterial diseases limiting their commercial exploitation (Lakshmanan et al. 2005). A number of transgenic strategies including the introduction or modification of expression of genes that activate, regulate or directly contribute to antimicrobial defenses in plants have resulted in enhanced disease resistance in several crops reviewed by

Campbell et al. (2002). The molecular basis of pathogenesis in the numerous fungal and bacterial diseases of sugarcane is just being explored with the help of an extensive sugarcane EST database (reviewed by Ming et al. 2006) and will support efforts to specifically engineer resistance against these pathogens.

### 23.5.3.3 Abiotic Stress Tolerance

Abiotic stress, particularly water deficit or cold, severely affects sugarcane productivity. Targets for genetic engineering of abiotic stress include entire cascades of molecular networks involved in stress perception, signal transduction and the expression of specific stress-related genes and metabolites which activate stress-responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes (reviewed by Vinocur and Altman 2005). Transcription factors (TF) of stress response genes are particularly interesting targets for improving stress tolerance due to their ability to simultaneously control expression of a multitude of stress response genes. Negative effects on plant growth under non-stress conditions are overcome by using stress-inducible promoters (e.g. rd29A, HVA1) to drive transcription factor activity (Kasuga et al. 1999; Dubouzet et al. 2003; James et al. 2008). This strategy is currently being investigated in sugarcane, where the expression of the *dreb2b* transcription activator under control of the drought inducible rd29A promoter was recently described. Data on abiotic stress tolerance of these transgenic lines have not been reported yet (Wu et al. 2008).

Compatible solutes (e.g. proline, trehalose, glycinebetaine) contribute to maintaining cell turgor during water deficits, detoxification of reactive oxygen species and/or protection of membrane integrity.  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS) catalyzes the rate-limiting step in proline biosynthesis. The *p5cs* gene was overexpressed in sugarcane under the control of a stress-inducible promoter. Interestingly, physiological data suggested that proline accumulation acts as a component of antioxidative defense system during dehydration stress rather than as an osmotic adjustment mediator. Transgenic plants expressing P5CS accumulated more biomass under drought stress in the greenhouse than non-transgenic plants (Molinari et al. 2007).

Trehalose has been shown to stabilize dehydrated enzymes, proteins and lipid membranes efficiently, as well as to protect biological structures from damage during desiccation (Drennan et al. 1993). Constitutive expression of trehalose synthase (TSase) from *Grifola frondosa* in sugarcane resulted in up to 13 mg/g fresh weight accumulation of trehalose. Transgenic plants showed increased drought tolerance and agronomic performance under drought conditions in greenhouse and field (Zhang et al. 2006).

Physiological knowledge of the processes of environmental stress tolerance in sugarcane and other grasses is still developing (reviewed by Tester and Bacic 2005). Interfacing physiological and molecular-genetic research in future efforts will enhance both breeding and genetic transformation projects.

### 23.5.3.4 Metabolic Engineering of the Carbohydrate Metabolism and Value-Added Products

Sugarcane is an attractive target for metabolic engineering to enhance the yield of the current commercial product sucrose or to achieve the economical production of novel value-added products. Increasing sucrose yield in sugarcane is one of the priorities for conventional and molecular breeding programs. Several strategies focus on changing the expression levels of different enzymes in carbohydrate metabolisms, including invertase activity in different cellular compartments (Ma et al. 2000), pyrophosphate-dependent phosphofructokinase (PFP) activity (Botha and Groenwald 2001a, b, c), pyrophosphate:fructose 6-phosphate 1-phosphotransferase activity (Groenewald and Botha 2008) and polyphenol oxidase activity, which correlates with sugar color (Vickers et al. 2005). However, so far only regulating pyrophosphate-dependent phosphofructokinase activity has been claimed as a successful strategy to increase sucrose yield in mature sugarcane plants (Botha and Groenwald 2001c). Reduction of the starch content of sugarcane suspension cells by suppression of ADP-glucose pyrophosphorylase (AGPase) or over-expression of  $\beta$ -amylase does not influence sucrose concentrations (Ferreira et al. 2008). Sucrose storage involves a complex coordination of sucrose biosynthesis, transport, compartmentation and partitioning between storage and consumption to drive all other metabolism within sink tissues. Potential target genes in sugarcane have been suggested (Grof and Campbell 2001; Watt et al. 2005) but our understanding of the regulation of these processes is very limited (Koch 2004). Plant-scale kinetic and structural modeling of sucrose accumulation in sugarcane (Rohwer et al. 2007) is a powerful approach and should lead to the rational design of genetic engineering strategies.

Isomaltulose is used in food as a sucrose substitute and has the benefit that it does not support the growth of bacteria associated with oral tooth decay. Vacuolar targeting of a highly efficient bacterial isomerase enzyme that converts sucrose to its isomer isomaltulose supported isomaltulose accumulation without reduction in sucrose, resulting in twice the total sugar concentration in selected transgenic lines relative to their parent cultivar under greenhouse conditions (Wu and Birch 2007). This remarkable step above the former ceiling in stored sugar concentration provides a new perspective into plant source–sink relationships and may lead to enhanced sugar and biofuel production (Smith 2008).

Transgenic sugarcane expressing the *Malus domestica* sorbitol-6-phosphate dehydrogenase gene (*mds6pdh*) produced on average 120 mg/g dry weight sorbitol (equivalent to 61% of the soluble sugars) in the leaf lamina and 10 mg/g dry weight in the stalk pith without affecting sucrose accumulation in the culm. However sorbitol-producing sugarcane generated 30–40% less aerial biomass and was 10–30% shorter than control lines. This work also demonstrated that impressive yields of alternative products can be generated from the intermediates of sucrose metabolism in *Saccharum* spp. (Chong et al. 2007).

Expression of an ER-targeted human cytokine protein GM-CSF under control of the constitutive ubiquitin promoter yielded a maximum of 0.02% TSP in leaves,

without negative phenotypic effect on the field grown transformed sugarcane lines (Wang et al. 2005).

The aromatic hydroxybenzoic acid (pHBA) used in the manufacture of polymeric resins is currently produced industrially by chemical synthesis and is also a natural intermediate in several plant and bacterial biosynthetic pathways. pHBA-glucose conjugates were detected at 7.3% and 1.5% dry weight in sugarcane leaf and stem tissue, respectively, following constitutive expression of 4-hydroxycinnamoyl-CoA hydratase/lyase. No phenotypic abnormalities were detected under greenhouse conditions (McQualter et al. 2005).

The production of polyhydroxyalkanoates (PHAs) such as poly-3-hydroxybutyrate (PHB) in plants has been the focus of major research efforts, as these biodegradable polyesters are considered as possible alternatives to polymers currently synthesized from non-renewable resources (Snell and Peoples 2002). Plastid-targeted expression of the PhaA-PhaB-PhaC pathway resulted in PHB accumulation up to 2% of leaf dry weight in sugarcane without adverse effects on plant growth and sugar accumulation under greenhouse conditions (Petrasovits et al 2007; Purnell et al. 2007). The minimum yield for commercial production has been estimated at more than 15% of plant dry weight for the higher-value PHBV copolymer (Slater et al. 1999).

## 23.6 Future Trends

Improvement of agronomic traits will be accelerated by rational design of genetic engineering strategies through progress made in functional genomics and systems biology approaches. Potential improvements include higher sucrose content (Watt et al. 2005; Rohwer et al. 2007) and longer harvest seasons along with enhanced sustainability. The latter would include varieties that use water and nitrogen more efficiently and by decreasing the dependence on applied chemicals to control pests and diseases. The advantages of high biomass production and carbon flux through useful precursor pools, high level of transgene containment (discussed in Sect. 23.3) and the option to develop sugarcane processing plants into biorefineries (Rein 2007) indicate a bright future for renewable biomaterials production aided by metabolic engineering. This will support the transition from a non-sustainable petrochemical-based economy to a renewable carbohydrate-based economy. To capitalize on the advantage of sugarcane as an efficient biomass producer, reasonable alternative target compounds would typically be required in large quantity. Most promising targets include certain industrial enzymes (Howard and Hood 2005), proteins with potential large-scale medical uses (Ma et al. 2005), proteins with valuable fibrous or adhesive properties or a bioplastic precursor (Scheller and Conrad 2005) combined with biofuel production (Smith 2008). Target compounds that can be produced as co-products with sucrose for the food sector or that use sugarcane residues like surplus bagasse or leaves are also attractive. Possibilities include also alternative sugars with health benefits or compounds derived from the

aromatic and wax biosynthetic pathways in sugarcane (reviewed by Birch 2007). The fuel ethanol industry has been growing extensively worldwide and considerable efforts have been exerted towards improving ethanol yield and reducing its production costs during the past three decades. Enhanced conversion of plant biomass into fermentable glucose for ethanol production was recently reported following in planta expression of a thermostable endoglucanase (Sicklen 2006; Oraby et al. 2007) or after reduction of lignin content by RNAi suppression of genes involved in lignin biosynthesis (Chen and Dixon 2007). Strategies to modify cell wall composition or to enhance de-polymerization of complex carbohydrates will likely enhance the production of cellulosic ethanol from abundant sugarcane residues or high biomass-producing energycane cultivars. Integration of physiology, genetics and biotechnology will allow us to maximize returns out of this remarkable photosynthetic biofactory as we understand and tailor the required developmental expression patterns, cellular compartmentation, signalling and control of source–sink relationships.

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# **Chapter 24**

## **Soybean**

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### **24.1 Introduction**

The soybean, *Glycine max* (L.) Merr., continues to be the crop that provides the single largest source of vegetable protein to the human diet and the second-largest source of vegetable oil, having been recently surpassed by oil palm. Soybean products are consumed directly, used as feed for animal production, or as substrates for a wide variety of industrial substrates, ranging from ink to rubber. Therefore, soybean modification is a high priority in order to maintain and improve upon its agronomic characteristics, as well as modifying the seed composition to increase the usefulness of the various seed components. The soybean once suffered the reputation of being a recalcitrant crop when it came to regeneration from tissue culture and transformation (for general aspects of plant transformation, see Chap. 1). Today, soybean boasts an array of transformation methods perhaps unequaled for any other crop. These range from organogenic and *Agrobacterium*-based methods to embryogenic and microprojectile bombardment-mediated transformation. There have been earlier reviews of soybean transformation, including Trick et al. (1997),

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Dinkins et al. (2002), Clemente and Klein (2004), Widholm (2004) and Olhoft and Somers (2007).

## 24.2 Methodology

### 24.2.1 *Cot Node and other Organogenic Transformation Systems*

#### 24.2.1.1 Cot Node

The first report of transformation and regeneration of soybean utilized *A. tumefaciens* inoculation of the cotyledonary node (cot node; Hinchee et al. 1988). The cot node system represents one of the two main methods used for soybean transformation (see embryogenic culture system below). The cot node is a small piece of seedling tissue that contains a few millimeters of hypocotyl tissue above and below the node, along with a few millimeters of the petiole from the cotyledon. For this method, seeds are germinated on a medium containing cytokinin for 4–7 days and the cotyledonary node is excised. Use of cytokinin in the germination medium preconditions the tissue to form shoots during the next step of culture. This cot node explant is precisely prepared and wounded to allow access of the *A. tumefaciens* to the tissue, which then gives rise to multiple shoots (Wright et al. 1986). These multiple shoots are close to the bud in the axis of the cotyledonary node and accurate wounding may actually discourage the primary bud from developing. Production of a proliferative mass of newly formed shoots is desirable for this approach and the initials that give rise to these shoots are the actual target for transformation. The cot node is usually wounded by a series of parallel, shallow slices with a scalpel blade, longitudinal to the hypocotyl. After inoculation with *A. tumefaciens*, the shoot-producing tissues are placed under moderate selection where transgenic shoots are produced. Subsequent rooting of the shoots leads to transgenic plants.

For success with the cot node system, soybean lines should be used that are both susceptible to *A. tumefaciens* and responsive to shoot induction. The most commonly-used line is cv. “Thorne”, although other lines are also responsive. Improvements in the cot node system have been made by including acetosyringone to induce various *A. tumefaciens vir* genes and reducing agents, which lessen the effects of pathogen-induced stress responses (Olhoft et al. 2001).

#### 24.2.1.2 Stem Node

Using a similar approach, Olhoft et al. (2007) recently developed a stem node (primary node) system for producing multiple soybean shoots which can also be targeted for transformation via *A. rhizogenes*. In this approach, proliferating shoots

from this explant can be targeted as well (Hong et al. 2007). Selection and rooting of shoots is the same as the cot node system.

#### 24.2.1.3 Bombardment of the Shoot Tip

Although particle bombardment of the shoot tip is not being used by many laboratories for soybean transformation at present, it is worth mentioning here. The description of this method (McCabe et al. 1988) was published at the same time (same issue of *Bio/Technology*) as the cot node method, resulting in “shared” first reports of soybean transformation. This approach, although inefficient and quite costly, also produced the event that led to the first generation of RoundupReady soybean (see also Chap. 9), which had a profound effect on agricultural practices. For this method, the shoot tip was targeted using a particle gun that had the unique ability to allow penetration of the microcarriers through numerous cell layers which is necessary in order to obtain germline transformants. Shoot tips of germinating seedlings were laboriously prepared and multiple shoots were generated and screened. Selection ability is limited in this system so a large effort was placed into screening large numbers of chimeric shoots for the presence of the transgene.

#### 24.2.2 Embryogenic Culture Transformation System

Embryogenic cultures of soybean are obtained by first culturing cotyledons from immature zygotic embryos of soybean on a medium containing very high levels of a synthetic auxin (2,4-dichlorophenoxyacetic acid). The appropriate levels of auxin induce embryo formation but inhibit embryo development. If auxin levels drop below a threshold, the embryo continues its development, assuming the proper nutrient levels and growth conditions are present. If the auxin level remains high enough, the development of the somatic embryos is arrested after the globular phase, and the embryos give rise to a second set of somatic embryos instead of continuing their development. Proliferative embryogenic cultures of soybean are ideally suited for transformation because new embryos are formed from the cells on the apical surface of older embryos (Finer 1988), which are easily targeted for DNA introduction. Transformation of proliferative embryogenic cultures is most often achieved using particle bombardment along with *hygromycin phosphotransferase*, *hpt*, as the selectable marker gene.

Over the years, improvements in the media and the protocols have made this an efficient and flexible system, with alternative methods and media available at many of the steps. An up-to-date annotated protocol is always maintained at <http://mulch.cropsoil.uga.edu/soy-engineering/>. This system is easily scalable, and it is now possible to recover up to 20–50 independent transgenic events per 100 mg of tissue shot. Though most work has been done on the cultivars Fayette and Jack, several

other genotypes have also been used including Williams 82, which is the standard genotype used for soybean genomics work.

### **24.2.3 Whole-Plant Transformation Systems**

#### **24.2.3.1 Floral Dip**

If the ideal transformation system could be developed for soybean, it would be very similar to the *Arabidopsis* floral dip method (Clough and Bent 1998). For floral dip, bolting *Arabidopsis* plants are submerged in an *Agrobacterium tumefaciens* suspension. The bacteria survive within the plant, eventually transforming the ovules. After fertilization, transgenic seed are recovered, without the need for tissue culture. For soybean and other legumes, in spite of large efforts to develop the floral dip method, no confirmed transgenics have ever been recovered.

#### **24.2.3.2 Pollen Tube Pathway**

For the pollen tube pathway approach, pollen is first placed on the stigma, where it germinates and grows down the style to fertilize the egg. Directly after fertilization, the stigma is severed, exposing the pollen tube, which is supposed to act as a conduit for delivery of a DNA solution to the freshly fertilized egg. Although there are numerous reports of using the pollen tube pathway for transformation of soybean, it is disappointing that convincing molecular evidence for transformation has not been shown. The only believable reports on pollen tube pathway for soybean transformation show no positive results and suggest that the procedure is not very promising (Shou et al. 2002; Li et al. 2002).

#### **24.2.3.3 Composite Plants**

Composite plants of soybean (Collier et al. 2005) are not transgenic plants, which can transmit the transgene to the progeny. They are instead non-transgenic shoots that are supported by transgenic roots. To generate these plants, *A. rhizogenes* is inoculated onto radicles of susceptible soybean seedlings. Mixtures of transgenic and non-transgenic roots are produced at the site of inoculation and these roots are used to support growth of the plant. Composite plants can be used to study promoter activity in the roots or expression of a transgene of interest in roots. Although production of composite plants does provide a non-tissue culture method for the study of transgene expression in roots, use of *A. rhizogenes* gives “hairy roots” that may or may not behave like normal roots on a soybean plant. Nevertheless production of composite plants may have good applications for the study of transgenes in roots.

#### 24.2.3.4 Virus-Induced Gene Silencing

Although this is not a true transformation system since no genes are stably integrated into the soybean genomes, the technique called virus-induced gene silencing (VIGS) can be very useful for testing gene and promoter function. Such a system was used by Nagamatsu et al. (2007) using a wide-host-range viral vector, cucumber mosaic virus, as carrier of an antisense sequence of chalcone synthase. Infection of soybean plants caused no viral symptoms, but the seed that were normally brown were yellow, indicating silencing of the chalcone synthase genes. When antisense flavonoid 3'-hydroxylase was used the leaf quercetin levels decreased, indicating the correct targeting. In both cases a decrease of the target mRNAs and accumulation of short interfering RNAs was found (see also Chap. 5).

#### 24.2.4 Other Considerations for Transformation

While various methods are available to transform soybean, they do vary in their labor requirements, genotype specificity and efficiency. The cotyledonary node system and the somatic embryogenic system are widely used in the public sector. The private sector tends to select its transformation methodology primarily on intellectual property and freedom-to-operate issues.

Thus far, the cot node has been most effective with *A. tumefaciens*, and the embryogenic system with microprojectile bombardment. Attempts have been made to couple the somatic embryogenic system with *Agrobacterium*-mediated transformation that have resulted in some success (Trick and Finer 1998), but the methodology has not been widely reproducible or widely adopted (Ko et al. 2004a, b).

Concerns are frequently expressed that microprojectile bombardment results in transgene copy numbers that are too high to be useful. The claim was true with the original protocols for microprojectile bombardment, which delivered about 625 ng of DNA per shot (calculated from the DNA concentration described by Kikkert (1993)). By contrast, current protocols have eliminated the problem by delivering about 50 ng of DNA per shot. Further, even the use of *A. tumefaciens* can result in multiple insertions. For example, see the Southern blots published by Olhoft and Flager (2003).

Finally, the somatic embryo system is especially useful to assess transgenic seed traits, as the transgene can be assayed without need to obtain a whole plant (Mazur et al. 1999). For example the modifications of oil composition section below describes how oil composition changes were measured in mature somatic embryos.

#### 24.2.5 Multi-Gene Insertions and Marker-Free Plants

There are several possible strategies to insert multiple genes and to also produce selectable marker-free progeny (see Chaps. 3, 4). Xing et al. (2000) and Sato et al. (2004)

used a two T-DNA binary vector where the gene of interest is placed in one T-DNA element and the selectable marker gene in another on the same binary vector in *A. tumefaciens*. Analysis of transformed progeny lines showed that 20–40% were marker-free while at the same time showing expression of the transgene of interest.

When a plasmid containing six genes, or the same genes on different plasmids were used to bombard embryogenic soybean cultures, very few of the selected  $T_0$  plants contained all the genes and none expressed all the genes (Schmidt et al. 2008). On the other hand, with slightly different bombardment conditions using higher levels of DNA, 12 different plasmids were successfully co-introduced at high frequency (~75%) into embryogenic soybean tissues (Hadi et al. 1996). In other work, three isolated gene cassettes plus a selectable marker gene cassette were bombarded into embryogenic cultures and eight of ten selected lines that produced plants with fertile seed contained all four genes (Zernova et al., personal communication). Two of the lines showed segregation of the transgenes in the  $T_1$  progeny including loss of the selectable marker gene, *hpt*, but the line showed no expression of the two antisense isoflavone biosynthetic enzymes still present. When Furutani and Hidaka (2004) bombarded embryogenic cultures with the *hpt* and *GFP* genes and selected with hygromycin, 18% of the selected clones also expressed GFP.

#### 24.2.6 Selectable Markers

The first successful production of transgenic soybean plants using the *A. tumefaciens* cot node system used the *nptII* gene from *Escherichia coli* that encodes neomycin phosphotransferase that detoxifies kanamycin as the selectable marker (Hinchee et al. 1988; see Chap. 3). Most of the cot node transformation is now done using the *bar* gene from *Streptomyces hygroscopicus* that encodes the enzyme phosphinothricin acetyltransferase that detoxifies the herbicides phosphinothricin and bialaphos (Zhang et al. 1999). Phosphinothricin is a glutamine analog that inhibits glutamine synthetase. Olhoff and Flagel (2003) reported efficient selection of transformants using the cot node system with hygromycin as the selection agent. The *CP4* gene from *A. tumefaciens* that encodes the glyphosate herbicide target gene (see below) was also used for selection of transformants using the cot node system (Clemente et al. 2000). The CP4 enzyme is not inhibited by glyphosate and the plants produced showed tolerance to the herbicide.

As mentioned above, the selectable marker gene, *hpt* from *E. coli* that encodes hygromycin phosphotransferase for hygromycin resistance, has been used in most cases for the selection of transformed embryogenic suspension cultures. However, the *aada* gene that provides resistance to the antibiotic spectinomycin has been used for selection of plastid transformants (Dufourmantel et al. 2004, 2005, 2006, 2007).

The feedback-resistant tryptophan biosynthetic control enzyme anthranilate synthase (ASA2) from tobacco (Song et al. 1998), that has been used as a selectable marker since it can impart resistance to toxic tryptophan analogs, was inserted into soybean using embryogenic suspension cultures. Free tryptophan was increased

significantly in leaves and embryogenic suspension cultures, while total tryptophan was only slightly increased in seeds (Inaba et al. 2007a). So far attempts have not been successful using ASA2 as a selectable marker with soybean.

The fungal gene that encodes the enzyme cyanamide hydratase (*Cah*) is another possible selectable marker gene for soybean. The enzyme detoxifies cyanamide and has been used as a selectable marker with *Arabidopsis*, potato, rice and tomato (Damm 1998) and wheat (Weeks et al. 2000). Expression of *Cah* in soybean callus, embryogenic cultures and whole plants leads to cyanamide resistance (Zhang et al. 2005) and metabolic profiling studies of leaves show that the expression does not affect normal metabolism but does lead to rapid conversion of cyanamide to urea (Ulanov and Widholm 2007).

All of the transformation examples given in this chapter involve insertion of DNA into the nuclear genome, but there have been reports of gene insertion into the soybean plastid genome. The advantages of such insertions are high level expression, no silencing and in most species no transmission through pollen. Zhang et al. (2001) selected transformed cultures. Fertile plants containing a *Bacillus thuringiensis* *Cry1Ab* protoxin that imparted insect resistance were produced by Dufourmantel et al. (2004, 2005). In all cases embryogenic suspension cultures were used with the *aadA* gene encoding spectinomycin resistance as the selectable marker. The stability of the plastome containing the *aadA* gene was evaluated after six generations without spectinomycin selection in plants of three of the original lines produced by Dufourmantel et al. (2004). The plants remained homoplasmic with no evidence for the presence of untransformed plastomes and the plants were also resistant to spectinomycin.

Soybean was also plastid genome transformed with a bacterial 4-hydroxyphenylpyruvate dioxygenase gene, an enzyme in the plastoquinone and vitamin E biosynthetic pathway (Dufourmantel et al. 2007). This enzyme is the target for certain herbicides such as sulcotrione and isoxaflutole. Expression of the gene caused accumulation of the enzyme up to 5% of the total soluble protein and imparted high levels of tolerance to isoxaflutole. The tolerance was greater with the plastid transformants in comparison with nuclear transformants in relation to the greater enzyme levels present.

### 24.2.7 Homozygosity Determination

Once transgenic events have been obtained it is advantageous to obtain true breeding or homozygous events within a short time frame so as to better ascertain the effect of the transgene. Traditionally, a selfed T<sub>1</sub> seed is planted and its progeny scored for a phenotype either visually or by molecular analyses (e.g. PCR). Mendelian segregation predicts that one-fourth of the descendants will be homozygous for the presence of the transgene. Maintaining the remaining progeny increases

labor and greenhouse costs. Usually progeny are grown until ten out of ten of their progeny are positive for the trait, indicating homozygosity.

Real-time (RT)-PCR methods have been successfully used to determine zygosity in soybean (Schmidt and Parrott 2004). RT-PCR relies on the signal strength of the transgene relative to that of a calibrator gene in a bplex reaction. An alternative technology, Invader, has been developed for gene copy number and zygosity determination (Lyamichev et al. 1999; Gupta et al. 2008). Invader also employs a fluorescent bplex reaction, but since it is isothermal and non-PCR, the potential of false determinations arising from trace contamination of PCR targets is eliminated. The assay is accurate and can be performed in 96-well plates or automated with 384-well plates. Using a probe to the hygromycin resistance gene, this system has been able to reliably identify homozygous soybean plants (P. LaFayette, unpublished data).

## 24.3 Applications of Transformation Technology

### 24.3.1 *Herbicide Resistance*

Unquestionably the most commercially important trait that has been introduced into soybean is resistance to the non-selective herbicide glyphosate (Roundup, see Chap. 9). Over 90% of the soybeans grown in the United States in 2008 carried this trait that was introduced to farmers in 1996. Glyphosate inhibits an enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), in the shikimic acid pathway that produces the aromatic amino acids phenylalanine, tryptophan and tyrosine and many other important compounds. An EPSPS from *A. tumefaciens* that was glyphosate-resistant was isolated and inserted into soybean using the apical meristem particle acceleration method (McCabe et al. 1988) where *gusA* reporter gene expression was used for visual selection to produce the glyphosate-resistant soybean line (Padgett et al. 1995). While there were early predictions that glyphosate-resistant weeds would be difficult to select for, due apparently to the great amount of use, there are now at least 12 resistant weed species that have been selected around the world (Service 2007).

### 24.3.2 *Modification of Oil Composition*

Due to the importance of oil in soybean a number of transformation events have been made to alter the composition. Soybean seed contains about 20% oil, mostly triglycerides, with a high content of the polyunsaturated fatty acids linoleic and linolenic (near 70%). Recently the consumption of the trans configurations of the

fatty acids, which are produced by the hydrogenation that is needed to cause solidification for use as margarine and increase stability for cooking oils, has been linked to cardiovascular disease. Thus it is desirable to decrease the polyunsaturated fatty acid level and increase oleic acid that has only one double bond. It would also be useful to have omega-3 fatty acids, such as docosenoic acid and stearidonic acid, that can be utilized readily by humans (Damude and Kinney 2008). There are also a number of specialty fatty acids for commercial use that could be produced in soybean, thus increasing use and value.

Some of the first soybean transformations to change oil used the embryogenic system so that the modifications could be measured in mature embryos before plant regeneration. These include the expression of  $\Delta^{12}$ desaturases from *Momordica* and *Impatiens* to produce 18:3 and 18:4 fatty acids with conjugated double bonds (Cahoon et al. 1999), expression of a fatty acid elongase and desaturase from *Limnanthes douglasii* to produce the 20:1  $\Delta^5$  fatty acid  $\Delta^5$ -docosenoic acid (Cahoon et al. 2000) and expression of an oxygenase from *Euphorbia lagascae* to produce 12-epoxy fatty acids (Cahoon et al. 2002).

Buhr et al. (2002) were able to use a self-cleaving ribozyme system to down-regulate the  $\Delta^{12}$  fatty acid desaturase *FAD2-1* and the palmitoyl-thioesterase *FatB* and produced some lines with over 85% oleic and less than 6% saturated fatty acids compared to the normal levels of 18% for both.

Soybean plants that expressed the *Borago officinalis*  $\Delta^6$  desaturase that can convert linoleic and  $\alpha$ -linolenic acids into  $\gamma$ -linolenic and stearidonic acid, respectively, contained up to 29%  $\gamma$ -linolenic and 4.1% stearidonic acids in the seeds while the untransformed control had none (Sato et al. 2004). A two T-DNA binary vector with the selectable marker in the second element was used for the *A. tumefaciens*-cot node transformation and four of the 17 lines produced that contained  $\gamma$ -linolenic and stearidonic acid did not contain the selectable marker gene due to segregation. When both the *B. officinalis*  $\Delta^6$  desaturase and *Arabidopsis*  $\Delta^{15}$  desaturase were expressed in soybean seeds the content of the omega-3 fatty acids  $\alpha$ -linolenic and stearidonic could be increased to about 30% each from about 10% and 0%, respectively, in the control (Eckert et al. 2006).

Chen et al. (2006) inserted the fatty acid elongase and the  $\Delta^6$  and  $\Delta^5$  desaturases from a fungus *Mortierella alpina* and a RNAi silencing structure for the soybean  $\Delta^{15}$  desaturase gene, all under the control of the  $\beta$ -conglycinin promoter, into soybean. Analyses of transformed somatic embryos and mature seed from transformed plants showed that several long chain fatty acids including arachidonic (20:4) could accumulate up to about 10% of the total fatty acids while the control had none.

When soybean was engineered with genes from *Arabidopsis* that convert  $\gamma$ -tocopherol to  $\delta$ -tocopherol and  $\gamma$ -tocopherol to  $\alpha$ -tocopherol using the seed-specific  $\beta$ -conglycinin promoter, the vitamin E human most biologically active component  $\alpha$ -tocopherol was increased eightfold, resulting in a fivefold increase in seed vitamin E activity (Van Eenennaam et al. 2003). Tavva et al. (2007) inserted the  $\gamma$ -tocopherol methyltransferase gene from *Perilla frutescens* under the control of the seed-specific vicilin promoter into soybean and found a tenfold increase in

$\alpha$ -tocopherol in the seed. The enzyme can convert the less active form  $\gamma$ -tocopherol into  $\alpha$ -tocopherol.

These and other results not presented indicate that soybean oil can be manipulated in many commercially desirable ways and transgenic lines with decreased polyunsaturated fatty acids and increased oleic will be available for commercial use in 2009 (Damude and Kinney 2008) to compete with the lines carrying an induced mutation that are presently being grown. The advantage is that the transgenic lines are expected to have more stable phenotypes.

### 24.3.3 Nematode Resistance

Plant-parasitic nematodes such as the soybean cyst nematode (SCN, *Heterodera glycines*) and root knot nematodes (RKN, *Meloidogyne* spp.) are the primary biotic factors negatively affecting soybean yield (see Chap. 10). Soybean loss for SCN infestation alone has been estimated to be over 93 million bushels in 2007 (Wrather and Koenning 2008; one bushel is approx. 27 kg). Population densities of nematodes can be managed through the use of resistant cultivars and crop rotation. The primary issue confronting resistance is durability (Diers et al. 1997; Dong et al. 1997). For example, one of the most widely used sources for resistance for SCN (PI88788) is beginning to break down as nematode populations change (Hershman et al. 2008). Clearly, novel approaches to nematode resistance are needed to ensure the future profitability and yield stability of soybean production.

RNA interference (RNAi, see Chap. 5) shows promise for control of soybean parasitic nematodes. Using *Arabidopsis* as a model system for RKN, Huang et al. (2006) expressed a root-knot nematode parasitism gene *16D10* dsRNA in transgenic *Arabidopsis* and obtained resistance against four major root-knot nematode species. Specific and quantitative silencing of the target transcript and potential off-target effects on the plant host were also demonstrated (Sukno et al. 2007). For SCN the number of cyst nematode females on plant roots were significantly reduced by host-derived RNAi targeted to cyst nematode parasitism genes in *Arabidopsis* (Hussey et al. 2007). Urwin et al. (2002) fed dsRNA for a putative C-type lectin to J2 stage SCN and recovered 41% fewer nematodes from plants. Steeves et al. (2006) successfully targeted dsRNA to a major sperm protein *msp1* of *H. glycines*. Bioassay data indicated transgenic plants had up to 68% reduction in eggs per gram of root tissue. The effects of plant-derived dsRNA molecules appear to continue to the next generation. A non-transgenic susceptible cultivar was inoculated with eggs derived from both control plants and from eggs propagated from transgenic *msp-1* plants. The *msp-1* derived eggs displayed approximately 75% reduction in egg production compared to the control. This result implies the RNAi phenotype can be transmitted to progeny similar to that documented in *Caenorhabditis elegans* (Vastenhouw et al. 2006) and has implications on the successful deployment of RNAi technology for soybean parasitic nematode control.

### 24.3.4 Isoflavones

Soybean seed have high levels of the isoflavones daidzein, genistein and glycine and their conjugates and these are associated with a number of human health benefits. Several attempts have been made to manipulate the isoflavone content including those of Yu et al. (2003) who transformed soybean with a fusion of the maize *C1* and *R* transcription factor genes driven by the seed-specific phaseolin promoter. Plants expressing these genes had a large increase in daidzein, a decrease in genistein and a twofold increase in total isoflavones in the seed. These seed also showed increased expression of a number of phenylpropanoid pathway genes, but not isoflavone synthase. When the anthocyanin branch of the pathway was blocked by cosuppression of flavanone-3-hydroxylase in the *C1*- and *R*-expressing lines the total isoflavone content could be increased by up to 300%. Jung et al. (2003) transformed soybean with the isoflavone synthase gene driven by the seed-specific  $\beta$ -conglycinin promoter and found only small changes in seed isoflavone levels.

Soybeans have also been transformed by bombardment with a mixture of three gene cassettes containing phenylalanine ammonia lyase, chalcone synthase and isoflavone synthase, all in either sense or antisense orientation, all driven by the soybean seed-specific lectin promoter (Zernova O, Lygin A, Widholm J, Lozovaya V, personal communication). Many of the lines contained these three genes as well as the *hpt* selectable marker gene. No line produced seeds with increased isoflavone levels while seven of the ten lines analyzed had lower levels. When soybean were transformed with the isoflavone synthase gene (sense) controlled by the constitutive cassava vein mosaic virus promoter, the isoflavone levels in leaves were increased by 30% in some cases or decreased by 75% in other cases (Zernova et al. 2009). These results show that isoflavone concentrations can be changed by expression of key phenylpropanoid biosynthetic genes.

Subramanian et al. (2005) induced hairy roots on soybean cotyledons with *A. rhizogenes* carrying an RNAi silencing construct for both soybean isoflavone synthase genes and found that silencing could occur both in the transformed roots and in the cotyledon itself. This prevented accumulation of isoflavones induced by wounding or elicitation and caused enhanced susceptibility of the cotyledons to *Phytophthora sojae* infection.

### 24.3.5 Insect Resistance

Expression of Bt in soybean has provided useful levels of resistance against defoliating caterpillars (Walker et al. 2000; Macrae et al. 2005; Miklos et al. 2007), particularly when breeding is used to combine it with resistance genes already found in soybean (Zhu et al. 2008). Both of these events are highly resistant to defoliation by the velvetbean caterpillar (*Anticarsia gemmatalis*), which is the

major defoliating insect pest of soybean in the Southeastern United States and in South America (see also Chap. 10).

### 24.3.6 Disease Resistance

It has been possible to obtain soybean resistant to bean pod mottle virus (Di et al. 1996) and to soybean mosaic virus (Wang et al. 2001; Tougou et al. 2006). Although resistance appears to be effective, it is not known to what extent, if any, these transgenic soybeans are being used in breeding programs. Virus resistance may well represent a trait for which the cost of deregulation exceeds the added value of the trait. For example, in 2006, all viral diseases combined led to a 0.11% yield loss across 16 States according to Koenning (2006).

Sclerotinia stem rot (white mould) is a serious soybean disease caused by the fungus *Sclerotinia sclerotiorum*. Oxalic acid is secreted by the fungus as a pathogenicity factor damaging the plant tissue. Thus there was interest in inserting a gene for oxalate oxidase that degrades oxalic acid to carbon dioxide and hydrogen peroxide. Soybean was transformed with the wheat *germin* gene that encodes oxalate oxidase activity driven by the 35S promoter, and plants expressing the gene showed resistance to *S. sclerotiorum* equal to that of known resistant cvs. Yield was not decreased by the transgene (Donaldson et al. 2001; Cober et al. 2003). Interestingly, the oxalate oxidase gene can be used as an easily measured reporter gene by measurement of the hydrogen peroxide produced both histochemically and spectrophotometrically (Simmonds et al. 2004).

### 24.3.7 Phytase

Phytic acid is a phosphorous-containing storage compound, found in the soybean seed, which is normally released to the germinating seed through the activity of the enzyme phytase. When consumed by non-ruminant animals, the large amount of phytic acid in untreated soybean meal is not efficiently digested and is excreted by animals, leading to serious environmental consequences. *Phytase* genes from fungi (Denbow et al. 1998), bacteria (Bilyeu et al. 2008) and soybean (Chiera et al. 2004) have all been successfully introduced into soybean, in attempts to express phytase ectopically in the seed and reduce phytic acid levels there. Although phytic acid levels were decreased and phosphorous availability was improved in all cases, potential problems exist with reduced seed germination frequencies in the highest expressing transgenics (Bilyeu et al. 2008). Use of low phytic acid mutants in soybean (Wilcox et al. 2000), with or without a transgenic approach, may

ultimately be the best solution for reducing phytic acid levels and increasing phosphorous availability.

### 24.3.8 Seed Protein Composition

Soybean seed provides a reliable and inexpensive protein source for many domesticated animals as well as humans. Although the amino acid content in soy protein is fairly well balanced, it is somewhat deficient in the sulfur amino acids cysteine and methionine (Young 1991). To counter this deficiency, transgenics have been utilized to introduce genes, which encode for high-sulfur amino acid-containing proteins, which are targeted for seed-specific expression (see also Chap. 11). Maughan et al (1999) were the first to successfully generate transgenic soybean with the intent of producing altered sulfur amino acid content in the seed. Although expression of a *bovine β-casein* gene was shown in a seed-specific manner, alterations in amino acid profiles were not reported. Dinkins et al (2001) introduced a *15-kDa zein protein* gene into soybean and generated seed that showed a moderate increase in both methionine and cysteine levels. Li et al. (2005) also reported a slight increase in sulfur amino acid levels in transgenic soybean seed following introduction of a maize  $\gamma$ -zein protein. These modest increases in sulfur amino acids may be limited by the availability of free sulfur-containing amino acids. If the pool of sulfur amino acids can be increased, the likelihood of generating soybean seed with higher amounts of sulfur-containing amino acids would increase (Krishnan 2005).

Herman et al. (2003) reported the silencing of the major immunodominant allergen in soybean seed, a papain protease denoted P34, by expressing a full-length copy of the gene driven by the seed-specific  $\beta$ -conglycinin promoter. The seed composition, development, structure and ultrastructure was unchanged except for removal of the P34 protein.

The expression of the  $\alpha$  and  $\alpha'$  subunits of one of the major seed storage proteins,  $\beta$ -conglycinin, was suppressed by 5' untranslated region cosuppression (Kinney et al. 2001). The protein content of the mature seed was unaffected since more of the glycinin storage protein was made and new endoplasmic reticulum-derived protein bodies appeared. When a line containing a glycinin promoter driving a GFP gene was crossed into this line with suppressed  $\beta$ -conglycinin, GFP accumulated to >7% of the total seed protein showing that the proteome rebalancing can be exploited to produce very high levels of foreign proteins in soybean seeds (Schmidt and Herman 2008).

Staswick et al. (2001) suppressed the expression of two vegetative storage protein genes using an antisense construct driven by the 35S promoter by 50-fold in soybean plants and found that these plants grew normally under field conditions and showed no yield loss or seed composition changes. These proteins had previously been thought to be important in N storage in vegetative tissues during soybean plant growth but these results indicate that these proteins are dispensable.

## 24.4 Gene Discovery and Promoters

### 24.4.1 *Genomic Resources for Selection of Promoters and Genes for Modification*

In order to discover the function of soybean genes and the promoters that regulate them, the generation and application of genomics resources for soybean was crucial. Less than ten years ago, there were only 100 expressed sequence tags (ESTs) in the public databases and now there are over 300 000 as a result of a soybean grower association sponsored project, the “Public EST project for soybean”. Over 80 cDNA libraries were made from mRNAs extracted from numerous tissue and organ systems of the soybean plant (Shoemaker et al. 2002; Vodkin et al. 2004).

Rapid development of microarray resources followed from the EST information as part of the NSF-sponsored "A functional genomics program for soybean". Arrays were developed with a total of 36 864 single-spotted PCR products derived from the low-redundancy cDNA representing many genes expressed in the developing flowers and buds, young pods, developing seed coats and immature cotyledons, as well as roots of seedlings and adult plants, tissue-culture embryos, germinating cotyledons and seedlings subjected to various stresses, including some challenged by pathogens or infected with the nodulating bacterium *Bradyrhizobium japonicum*. The cDNA array platforms are entered in the Gene expression omnibus database at <http://www.ncbi.nlm.nih.gov/geo>. The second generation of microarrays have also been constructed containing 38 000 unique “long oligos” of 70 bases and information can be found at <http://soybeangenomics.cropsci.uiuc.edu>.

The arrays have been used to investigate many biological questions, including: (i) the processes of somatic embryogenesis (Thibaud-Nissen et al. 2003), (ii) soybean seed development and germination (Dhaubhadel et al. 2007, Gonzalez and Vodkin 2007; Vodkin et al. 2008), (iii) the responses to pathogen or symbiont challenge (Zou et al. 2005; Zabala et al. 2006; Brechenmacher et al. 2008; Li et al. 2008), (iv) herbicide response (Zhu et al. 2008a) and (v) response to elevated carbon atmospheric conditions (Ainsworth et al. 2006). In addition, they proved useful for identifying single gene differences between isogenic lines (Zabala and Vodkin 2005; O'Rourke et al. 2007). Data from the soybean arrays allows selection of genes that occur in various tissues and organ systems or respond to various challenges. Promoters can then be selected that operate in the desired tissue and developmental program and gene pathways and networks constructed.

An ambitious series of experiments from the laboratory of Robert Goldberg using laser capture microscopy (LCM) is currently underway to determine the genes required to “make a seed” (Le et al. 2007; <http://estdb.biology.ucla.edu/seed>). These and other ongoing studies of early soybean seed development (Vodkin et al. 2008) are likely to yield transcription factors critical in seed formation that will then be tested for function by transgenics.

In the future, many transgenic plants will likely be analyzed by microarray data to assess whether the introduced gene causes any major changes in the global

expression profiles in the plant. For example, a recent study showed no major transcriptome changes associated with currently used glyphosate-resistant soybean during the 24 h after application of the herbicide or in the developing seed of resistant plants (Zhu et al. 2008).

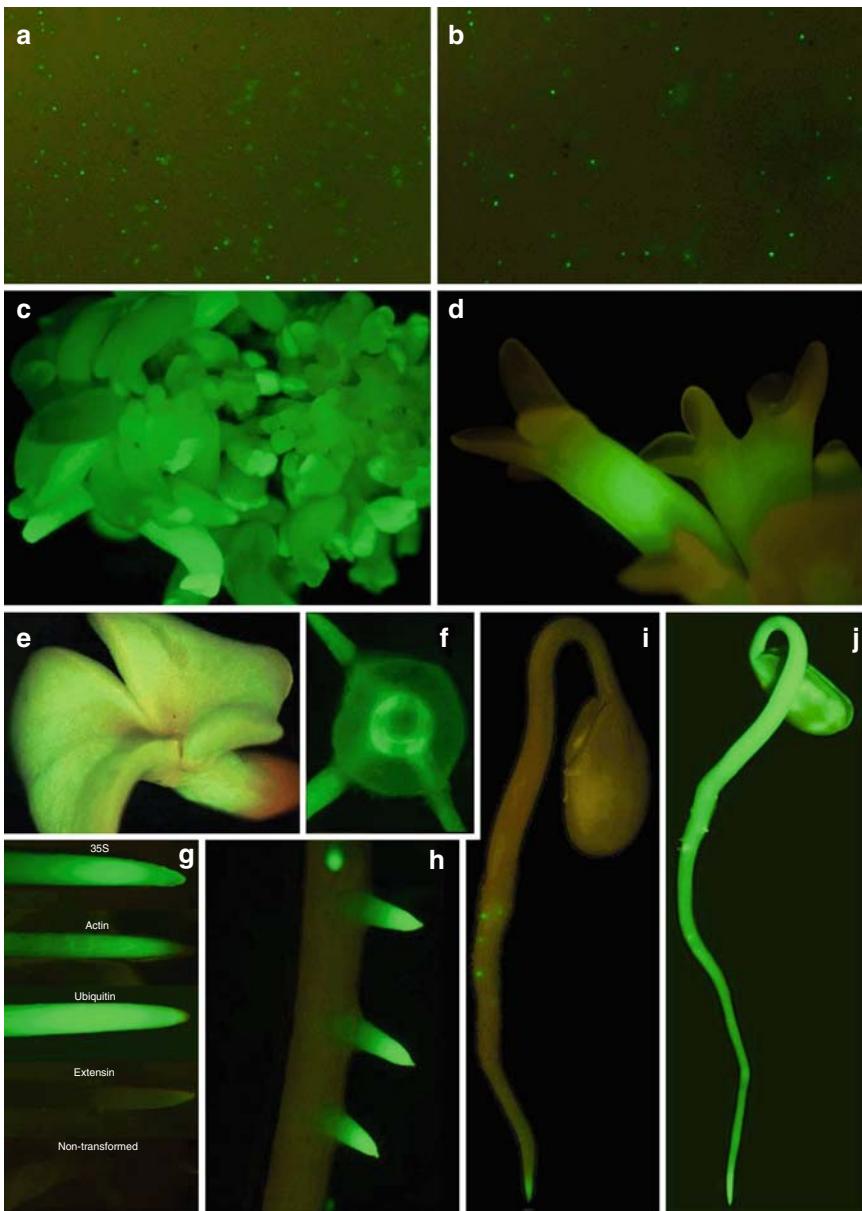
The discovery of endogenous small RNAs classes including the microRNAs (miRNAs) and short interfering RNAs (siRNAs) in 1999 (Hamilton and Baulcombe 1999; reviewed by Matzke and Matzke 2004; see Chap. 5) led to an entirely new class of molecules that need to be delineated by high-throughput sequence methods. The target genes regulated by the small RNAs must be delineated; and application of transgenics to determine the function of the small RNAs is likely to be an expanding area in the future.

There is already a non-transgenic example of regulation of a soybean trait by siRNA downregulation. Naturally occurring duplications of the chalcone synthase (CHS) genes in soybean silence other members of the CHS gene family, including CHS7 and CHS8, leading to shut-down of the pigmentation pathway, resulting in yellow seed coats. The mechanism is mediated by small RNAs (Todd and Vodkin 1996; Clough et al. 2004; Senda et al. 2004; Tuteja et al. 2004, 2008). Thus, gene regulation by endogenous siRNAs can be an important recent addition to plant allelic diversity and control that is also agriculturally useful. Understanding the mechanism of this endogenous system should aid attempts to create stable and tissue-specific downregulation of plant genes by genetic engineering.

#### 24.4.2 Promoter Evaluation

Although selection of the proper promoter is crucial for regulating transgenes of interest, few soybean promoters have been extensively evaluated in soybean. This shortcoming results from the historical inefficiency of soybean transformation, along with the emphasis placed on introduction of genes of interest. Up to this point, most soybean promoters have preferentially been evaluated in tobacco (Lindstrom et al. 1990; Philip et al. 1998) and *Arabidopsis* (Stromvik et al. 1999; Darnowski and Vodkin 2002; Thirkettle-Watts et al. 2003; Saeed et al. 2008) rather than soybean, due to the ease of producing tobacco and *Arabidopsis* transgenics. However, some soybean promoters have been evaluated in soybean (Buenrostro-Nava et al. 2006; Fig. 24.1) and this approach is preferred over validation of promoter activity in heterologous systems.

To perform proper promoter characterization, promoter constructs should direct expression of a marker gene; usually  $\beta$ -glucuronidase (GUS), luciferase or the green fluorescent protein (GFP). Introduction of marker genes under regulatory control of various promoters permits both qualitative and quantitative determinations of promoter strength, tissue specificity and inducibility. Quantification of promoter activity can be obtained using classical enzymatic assays or colorimetric/fluorometric assays of extracted tissues. Alternatively, quantification of the fluorescence from GFP in intact tissues can be performed using image analysis



**Fig. 24.1** GFP expression in transiently-expressing and stably-transformed soybean tissues. (a) Transient expression in bombarded soybean cotyledonary tissue, 10 h after introduction of the *gfp* gene, under regulatory control of the CaMV35S promoter. (b) Same coordinates/area as A, after 24 h. (c) Developing somatic embryos containing GFP with a soybean HSP90-like promoter (Chiera et al. 2007). (d) GFP expression in developing soybean somatic embryos containing a soybean phytase promoter. (e) Mature soybean somatic embryo containing GFP expression in cotyledons driven by the soybean lectin promoter (Buenrostro-Nava et al. 2006). (f) Immature

(Buentrostro-Nava et al. 2006). The images shown in Fig. 24.1A, B show the utility of image analysis over a time course. Although the potential for detailed promoter characterization using GFP is great, interference from chlorophyll remains problematic in most cases (Wu et al. 2008).

#### 24.4.2.1 Characterization of Soybean Promoters

Although heterologous promoters are often used for production of soybean transgenics, native promoters will probably generate more normal and predictable expression profiles in transgenic plants. Soybean promoters, which show activity in roots, nodules, seeds and pods or induction with alcohol or auxin, have been isolated and characterized over the past decade. Figure 24.1 shows examples of soybean promoter-driven GFP expression patterns in various soybean tissues, including HSP90-like and phytase promoters in developing somatic embryos (C, D), lectin promoter in a mature somatic embryo (E), ubiquitin promoter, Gmubi, in a root cross-section (F), 35S, actin, Gmubi and extensin in seedling roots (G), HSP90-like promoter in root initials (H), S11 promoter in root initials (I) and Gmubi promoter in germinated seedling (J). However, the seed-specific promoters regulating lectin (Cho et al. 1995; Maughan et al. 1999) and  $\beta$ -conglycinin (Goldberg et al. 1981) are the most extensively studied native soybean promoters.

Soybean lectin is a protein that accumulates in protein bodies of cotyledons from 2–5% of the total seed protein. The promoter was shown to drive GUS expression measured histochemically mostly in cotyledons of embryos developing from transformed embryogenic suspension cultures (Cho et al. 1995) and the expression of the casein gene in transgenic soybean (Maughan et al. 1999). Buenrostro-Nava et al. (2006) used an automated tracking system to show that GFP expression driven by the lectin promoter was not detected in early stages of somatic soybean embryo development but increased gradually to equal that seen with GFP driven by the constitutive CaMV 35S promoter at late stages of development (Fig. 24.1E). Studies of lectin promoter driven sense-suppression of isoflavone biosynthesis genes show that isoflavone levels in cotyledons can be decreased dramatically while the embryo axis, which contains about 30% of the total seed isoflavones, is not affected (Zernova et al. 2009) thus showing that the lectin promoter is active in cotyledons and not the embryo axis. Lectin promoter driven expression of the phytoene synthase (*crtB*) gene from *Erwinia uredovora* increased the  $\beta$ -carotene content of soybean seed and transgenic plants producing orange seed were recovered (B. Joyce, P. LaFayette, D. Tucker, W. Parrott, unpublished data).

←  
root cross-section showing GFP expression from a soybean ubiquitin promoter (Gmubi; Chiera et al. 2007). (g) Expression in roots of transformed seedlings, using various promoters, CaMV 35S and the rest from soybean. (h) GFP expression in root initials, from the HSP90-like promoter. (i) Seedling root initials displaying GFP expression with a soybean S11 promoter. (j) High levels of constitutive expression in all tissues of a soybean seedling using the soybean Gmubi promoter

Several of the studies that manipulated soybean oil synthesis utilized the seed-specific  $\beta$ -conglycinin  $\alpha'$  subunit promoter from soybean for high-level seed expression. There are 3  $\beta$ -conglycinin subunits,  $\alpha'$ ,  $\alpha$  and  $\beta$ , that make up about 30% of the total seed protein and the highest mRNA expression found in developing seeds are the  $\alpha'$  and  $\alpha$  subunits (Goldberg et al. 1981). The  $\beta$ -subunit synthesis is stimulated by sulfur deficiency and depressed by added methionine (Ohkama et al. 2002).

The promoters of the two soybean *isoflavone synthase* genes were cloned and tested as GUS fusions in soybean normal and hairy root tissues (Subramanian et al. 2004). The IFS1 promoter responded to *Bradyrhizobium japonicum* inoculation in certain root cells and to salicylic acid in all root tissues.

Another promoter that might be useful with soybean is the promoter of the tobacco anthranilate synthase (ASA2) gene described in the selectable markers section above and in Chap. 3. The ASA2 gene expression was shown to be tissue culture-specific in tobacco (Song et al. 1998) and when the 2.3-kb promoter was used to drive the *gusA* gene in soybean, GUS expression was seen, using the histochemical assay only in pollen, seed and tissue cultures (Inaba et al. 2007b). Very low expression was also detected using the very sensitive 4-methylumbelliferyl-glucuronide (MUG) assay in leaves and stems. When a construct containing the ASA2 promoter driving the selectable marker gene *hpt* was used to bombard soybean embryogenic cultures, it was possible to select transformed cultures relatively efficiently (Zernova et al. 2008). The *hpt* gene was only expressed at a level high enough to be measured by Northern hybridization in developing seeds but low-level expression could be detected by RT-PCR in all tissues except roots and mature seeds.

A recently identified soybean ubiquitin promoter (Gmubi; Chiera et al. 2007) provides high levels of constitutive expression and may have more immediate applications as a replacement for the constitutive CaMV35S promoter in transgenic soybean (Fig. 24.1F, G, J). The availability of soybean promoters should expand tremendously with the recent release of the soybean genome (Soybean Genome Project, DoE Joint Genome Institute).

## 24.5 Future of Soybean Transformation

The future of soybean transformation is bright, given the advances in reliable production and throughput of transgenic plants that have occurred in the past 12 years. The ability to rapidly transform soybean and test function of all soybean genes will be needed and increasing throughput is required for such an effort. Bioinformatics tools can determine a comparative gene relationship to model plants, such as *Arabidopsis* and rice, but there likely will be a substantial number of soybean genes whose function is best determined by direct transformation of soybean. Using siRNA approaches to knockout gene function is an attractive approach and will be used much more often in the future. Transformation with transposable elements to develop gene-tagging approaches is also under way as a

functional genomics approach (Mathieu et al. 2009). Soybean research has been primed to enter a golden age with the completion during 2008 of the soybean genome sequence. Using the data from transcriptomic projects, numerous transcription factors and the coding regions for genes involved in pathways can be extracted readily from the soybean genome sequence and cloned easily for testing in transgenic plants. Likewise, the sequence of upstream promoter regions is available and can be tested directly in soybean using various reporter systems. Now is the time to merge the soybean genome information with increased efforts on transformation to understand the function of soybean genes especially for transcription factors and pathways functioning in important economic traits as seed composition and disease resistance.

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# Chapter 25

## Vegetables

Evelyn Klocke, Thomas Nothnagel, and Günter Schumann

### 25.1 Introduction

Vegetables are a target for many transformation purposes. From the first trials for herbicide resistance until now, transformation protocols have been developed for almost all important vegetable crops. *Agrobacterium*-mediated transfer is the base for most transformation protocols for vegetables, as in other crops. Some special method investigations like plastid transformation (see also Chap. 2) and others are outlined below.

With rapidly rising capacities for DNA sequencing, databases for plant genomes are expanding very fast. The abundance of genomic data has an influence on projects for the genetic transformation of various vegetables. The availability of genes is no longer a bottleneck for this work. Increasing knowledge about genomes and a broad public access to DNA data banks boost new possibilities of creating gene constructs for transformation of vegetables. Moreover, the latest RNAi technology (see Chap. 5) will affect the transformation techniques for vegetable crops.

This chapter gives a short overview of GM technology in vegetables. Particularly vegetable crops for the temperate climate in Europe and America are considered (Table 25.1). Special emphasis is placed on the current trends of vegetable transformation, focusing especially on potential practical applications. Some of the investigations belonging to fundamental research are important for an understanding of processes like gene expression, plant development and production of metabolites in vegetables.

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**Table 25.1** Review of genetically engineered vegetables, the aim (or target character) and the description of the transgene. This table contains experiments with established transgenic plants only. Experiments with marker or reporter genes exclusively are only listed as examples.

Character	Transgene	Transgene description	Aim	References
<i>Solanum lycopersicum</i> L.				
Virus resistance	TMV CP	Tobacco mosaic virus (TMV) coat protein	Tolerance to TMV and tomato mosaic virus (ToMV)	Nelson et al. (1988)
V1		Tomato yellow leaf curl virus (TYLCV) capsid protein	Delayed disease symptoms	Kunik et al. (1994)
TYLCV C1, -T-Rep		Truncated C1 and T-Rep genes of tomato yellow leaf curl virus (TYLCV)	Resistance to TYLCV	Brunetti et al. (1997), Antignus et al. (2004), Yang et al. (2004), Fuentes et al. (2006)
TL CV Rep		Replicase – tomato leaf curl virus (TL CV)	Resistance to TL CV	Praveen et al. (2005)
TL CV CP		TL CV coat protein	Variable resistance to TL CV	Raj et al. (2005)
TSWV N		Tomato spotted wilt virus (TSWV) nucleoprotein	Resistance to TSWV	Kim et al. (1994), Utzen et al. (1995)
N		Gene from <i>Nicotiana tabacum</i>	Resistance to TMV and ToMV	Whitham et al. (1996)
N/TSW-5		Lettuce isolate of TSWV (TSWV-BL)	Resistance to TSWV	Gubba et al. (2002)
CMV-CP		Cucumber mosaic virus (CMV) coat protein	Resistance to TMV, <i>Verticillium</i> and <i>Phytophthora</i>	Providenti and Gonsalves (1995), Tomassoli et al. (1999)
CMV		Truncated replicase from CMV	Moderate resistance in T1 progeny to CMV	Nunome et al. (2002)
Fungal resistance	Chi-I,II/Glu-I, II	Class I chitinase and class I $\beta$ -1,3 glucanase	Resistance to <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	Jongedijk et al. (1999)
	pch28	Chitinase	Resistance to <i>V. dahliae</i> race 2	Tabaeizadeh et al. (1999)
	tplD34,	Pathogenesis-related protein (PRP)	<i>Alternaria solani</i> resistance	Radhajeeyalakshmi et al. (2005), Schaefer et al. (2005)
	M-GLU, Mj-AMP1	PRP	<i>Phytophthora capsici</i> enhanced tolerance	Sarowar et al. (2006)
	CABPP1, CAPOA1		SAR to <i>F. oxysporum</i> f. sp. <i>lycopersici</i>	Lin et al. (2004)
	NPR1	<i>Arabidopsis</i> gene; systemic acquired resistance (SAR)	SAR to <i>F. oxysporum</i> f. sp. <i>lycopersici</i>	Chan et al. (2005)
pRB7/Thi2.1		<i>Arabidopsis</i> thionin; SAR		

Bacterial resistance	bO NPR1 pRB7/Thi2.1	Bacterio-opsin <i>Arabidopsis</i> gene; SAR <i>Arabidopsis</i> thionin; SAR Gene from <i>Lycopersicon peruvianum</i>	SAR SAR to <i>Ralstonia solanacearum</i> SAR to <i>R. solanacearum</i>	Rizhsky and Mittler (2001) Lin et al. (2004)
Nematode resistance	Mi-1, a, Mi-1,2		Resistance to <i>Meloidogyne incognita</i>	Chan et al. (2005) Vos et al. (1998), Goggin et al. (2004)
Insect resistance	HD-1	<i>Bt</i> resistance	<i>Bt</i> resistance to <i>Manduca sexta</i> , <i>Heliothis virescens</i> , <i>H. zea</i> and <i>Keiferia lycopersicella</i>	Fischhoff et al. (1987), Delannay et al. (1989)
		Gene from <i>L. peruvianum</i>	Resistance to potato aphid ( <i>Macrosiphum euphorbiae</i> )	Vos et al. (1998)
	Mi-1, a	Protease inhibitors	Increased resistance to <i>H. obsoleta</i> and <i>Liriomyza trifolii</i>	Abdeen et al. (2005)
Abiotic stress	rbcs1A::PCI coda	Choline oxidase from <i>Arthrobacter globiformis</i>	Temperature tolerance (chilling tolerance)	Park et al. (2004a)
	LegPAT	Glycerol-3-phosphate acyltransferase	Temperature tolerance (chilling tolerance)	Sui et al. (2007)
	cAPX	Cytosolic ascorbate peroxidase	Temperature tolerance (heat stress) and UV-B tolerance	Wang et al. (2006)
	CBF1	<i>Arabidopsis C</i> repeat/dehydration-responsive element binding factor 1	Water stress (drought)	Hsieh et al. (2002a)
	ABRC1/CBF1	ABRC1-stress-inducible promotor from barley HAV22 and CBF1	Chilling, drought and salt tolerance	Lee et al. (2003b)
	bsPA	Boiling stable protein from <i>Populus tremula</i>	Water stress (drought)	Roy et al. (2006)
	HLA1	Gene from <i>Saccharomyces cerevisiae</i>	Salt tolerance	Gisbert et al. (2000), Rus et al. (2001), Muñoz-Mayor et al. (2008)
	AtNHX1 BADH DefH9-iaaM	Gene from <i>Arabidopsis</i> Gene from <i>Atriplex hortensis</i> Genes from <i>Pseudomonas syringae</i> pv. <i>savastanoi</i> and <i>Antirrhinum majus</i>	Salt tolerance Salt tolerance	Zhang and Blumwald (2001) Jia et al. (2002) Ficcadenti et al. 1999
Parthenocarpny	roIB	A <i>rhizogenes</i> -derived gene		Carmi et al. (2003)

(continued)

**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
Fruit ripening	CaCell ACC GAD	Endo-1,4- $\beta$ -D-glucanase from pepper RNAi gene silencing Glutamate decarboxylase	Prolonged shelf life Antisense	Harpster et al. (2002b) Xiong et al. (2005) Kisaka et al. (2006)
Taste/flavour	E8-monellin Thaumatin $\Delta$ -9 Desaturase gene	Gene from <i>Discorephyllosum cuminissii</i> Gene from <i>Thaumatooccus daniellii</i> Desaturase gene from <i>S. cerevisiae</i>	Sweetness Sweet taste and liquorice aftertaste Changes in the profile of flavour compounds	Penarrubia et al. (1992) Bartoszewski et al. (2003) Wang et al. (1996)
Nutritional value	Adh 2 ctrl chi	Alcohol dehydrogenase cDNA Phytoene desaturase from <i>Erwinia uredovora</i> Chalcone isomerase from <i>Petunia</i>	Improved flavour characteristics Threefold increased $\beta$ -carotene content	Speirs et al. (1998) Römer et al. (2000)
	LC/C1	Maize transcription factors	Elevated flavanol end-products in the fruit peel	Muir et al. (2001)
	HQT	Hydroxycinnamoyl transferase	Tenfold higher flavonoid glycoside content	Le Gall et al. (2003)
	DETI	Endogenous photomorphogenesis gene, RNAi gene silencing	Increased levels of antioxidant chlorogenic acid (CGA)	Niggeweg et al. (2004)
	tLcy-b	Lycopene $\beta$ -cyclase	Carotenoid and flavonoid content	Davuluri et al. (2005)
	Del/Ros1	Transcription factor from <i>Antirrhinum majus</i> that regulates anthocyanin production	Conversion of lycopene to $\beta$ -carotene under field conditions	Giorio et al. (2007) Butelli et al. (2008)
Processing quality	ipt LepG, LeExpl ySAMdc	Isopentenyl transferase Ripening regulated fruit PG gene and expansin Adenosylmethionine decarboxylase from yeast	Higher fruit solids Increased fruit firmness and juice viscosity Increased lycopene content and enhanced fruit quality	Martineau et al. (1995) Kalamaki et al. (2003a, b), Powell et al. (2003) Mehta et al. (2002)
Pharmaceuticals	Miraculin	Gene from <i>Richadella dulcifica</i>	Twentyfold higher miraculin content, low-calorie sweetener for diabetic	Sun et al. (2007)

ACEI	Angiotensin-I-converting enzyme inhibitor, TMV-mediated transformation	Antihypertensive tomato fruits	Hanamoto et al. (1993)
Gp	Rabies glycoprotein, ( <i>Agrobacterium tumefaciens</i> ) ( <i>A.t.</i> )-mediated	Vaccine, oral animal immunization, e.g. raccoons	McGarvey et al. (1995)
P1-2A3C	Polyprotein + protease gene from foot-and-mouth disease virus	Oral immunization, e.g. guinea pigs	Pan et al. (2008)
RSV-F	Respiratory syncytial virus fusion gene	Vaccine	Sandhu et al. (2000)
ctxB	Cholera toxin B subunit, <i>A.t.</i> -mediated	Vaccine against cholera	Jani et al. (2002), Jiang et al. (2007), Sharma et al. (2008)
VPI	Coat protein of enterovirus 71 (EV71)	Vaccine against hand-foot-and-mouth disease	Chen et al. (2006a)
PRSS1S2S	Synthetic hepatitis B virus (HBV)	Large surface antigen gene	Lou et al. (2007)
ORF2 (HEV-E2)	Partial gene of hepatitis E virus	Vaccine	Ma et al. (2003)
Aβ	Human β-amyloid	Vaccine against Alzheimer's disease	Youm et al. (2008)
sDPT	Synthetic immunoprotective exotoxin epitopes	Vaccine against diphtheria–pertussis–tetanus (DPT)	Soria-Guerra et al. (2007)
AChE	Human acetylcholinesterase	Preventing organophosphate intoxication	Mor et al. (2001)
IL-12	Mouse interleukin-12	Recombinant protein for mucosal administration	Gutiérrez-Ortega et al. (2005)
TaxK	Taxadiene from <i>Taxus baccata</i>		Kovacs et al. (2007)
AAT	Modified human α-1-antitrypsin	Therapeutic protein	Agarwal et al. (2008)
GmIFS2	Isoflavone synthase from <i>Glycine max</i>	Isoflavone production for health benefits	Shih et al. (2008)
Antiallergenicity	Lyc e1, Lyc e3 RNAi gene silencing	Low allergenic tomato fruits	Le et al. (2006a, b), Lorenz et al. (2006)
<i>Capsicum annuum</i> L.			Tsafaris (1996)
Herbicide	pat	Basta resistance	
resistance			(continued)

**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
Virus resistance	CMV-CP	Cucumber mosaic virus coat protein (CMV-CP) gene		Zhu et al. (1996)
	CMV	cDNA of CMV satellite RNA	Resistance against cucumber mosaic virus	Kim et al. (1997)
	CMV-CP	CMV-CP gene, tomato mosaic virus CP gene		Shin et al. 2002a)
	ToMV-CP	Tobacco mosaic virus CP gene, pepper PMMV interaction 1 transcription factor gene		Lee et al. (2004)
	TMV-CP, PPI1	CMV-CP gene, TMV-CP gene	Field performance	Cai et al. (2003)
Fruit ripening	TMV-CP	Suppression of endo-1,4- $\beta$ -D-glucanase from pepper	Influence on cell wall	Harpster et al. (2002a)
	CaCell			
Flower development	Osmads1	Rice OsMADS1 gene	Phenotypic effect	Kim et al. (2001)
<i>Solanum melongena</i> L.				
Insect resistance	cry	<i>Bt</i> genes	Resistance against <i>Lepinotarsa decemlineata</i>	Arpaia et al. (1997, 2007), Iannaccone et al. (1997), Jelenkovic et al. (1998)
			Against <i>Lepinotarsa decemlineata</i> , field test	Acciari et al. (2000), Mennella et al. (2005)
	<i>Bt</i> gene		Against <i>Leucinodes orbonalis</i>	Kumar et al. (1998)
	cry		Impact on <i>Tetraonychus urticae</i> and <i>Phytoseiulus persimilis</i> , laboratory test	Rovenská et al. (2005)
	<i>Bt</i> gene		Effect <i>Myzus persicae</i> and <i>Macrosiphum euphorbiae</i>	Ribeiro et al. (2006)
	cry		Resistance against <i>Verticillium dahliae</i> , changes in fatty acids	Xing and Chin (2000)
Fungal resistance	$\Delta 9$ -Desaturase	$\Delta 9$ -Desaturase gene from yeast	Against <i>Botrytis cinerea</i>	Turini et al. (2004)
	Dm-AMP1	Antimicrobial defensin from <i>Dahlia merckii</i>		

mtlD	<i>E. coli</i> mtlD gene	Against <i>Fusarium oxysporum</i> , <i>Verticillium dahliae</i> and <i>Rhizoctonia solani</i>	Prabhavathi and Rajam (2007)
Nematode resistance	Mi-1.2	Mi-1.2 gene	Resistance against <i>Meloidogyne javanica</i>
Abiotic stress	mtlD	Mannitol-1-phosphodehydrogenase gene	Tolerant against osmotic stress by salt, drought and chilling
Embryo development	AtgpP-5	<i>A. thaliana</i> glycin-rich gene 5	Controlling embryo development
Parthenocarpy	DefH9-iaaM	<i>Pseudomonas syringae</i> gene +regulatory sequences of ovule-specific gene from <i>Antirrhinum majus</i>	Rotino et al. (1997), Donzella et al. (2000)
<i>Raphanus sativus</i> L.			
Flower development	GI	Antisense GIGANTEA (GI) gene fragment	Delayed bolting
Abiotic stress	LEA	Late embryogenesis abundant gene from <i>Brassica napus</i>	Salt tolerance, water deficit
<i>Brassica oleracea</i> L.			
Virus resistance	B22IV, B22VI	Capsid gene and antisense gene VI of <i>Cauliflower mosaic virus</i> (CaMV)	Passelgue and Kerlan (1996)
Insect resistance	PVY-cr	<i>Potato virus Y</i> capsid gene	
	cry	<i>Bt</i>	Insect resistance of broccoli against e.g. <i>Pieris rapae</i> , <i>Plutella xylostella</i>
	cry	<i>Bt</i>	Insect resistance of broccoli against <i>P. xylostella</i> using chemically inducible promoter
	cry	<i>Bt</i>	Insect resistance of cabbage against <i>P. xylostella</i>
	cry	<i>Bt</i>	Insect resistance of cauliflower against <i>P. xylostella</i>

(continued)

**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
	cry	<i>Bt</i>	Insect resistance of cabbage against <i>P. xylostella</i> , chloroplast transformation	Liu et al. (2008)
TI	Trypsin inhibitor gene from <i>Ipomoea batatas</i>	Tests against <i>P. xylostella</i> and <i>Spodoptera littoralis</i>	Ding et al. (1998)	
CpTI	Cowpea trypsin inhibitor	Tests against <i>Heliothis armigera</i> and <i>Pieris rapae</i>	Hao and Ao (1997), Lv et al. (2005)	
sporamin, spoaMAR	Use of promoter pPspoA/cassette with matrix-attached region (MAR)	Tests against <i>Helicoverpa armigera</i>	Chen et al. (2006b)	
Fungal resistance	ThEn42	<i>Trichoderma harzianum</i> endochitinase gene (cDNA)	<i>Alternaria</i> resistance	Mora and Earle (2001)
Bacterial resistance	GO	Glucose oxidase gene from <i>Aspergillus niger</i>	<i>Xanthomonas campestris</i> resistance	Lee et al. (2002)
Abiotic stress	CUP1	Structural gene of yeast metallothionein gene	Heavy metal tolerance	Hasegawa et al. (1997)
	betaA	Bacterial gene for biosynthesis of glycinebetaine	Salt tolerance	Bhattacharya et al. (2004)
	vhb	<i>Vitreoscilla haemoglobin</i> overexpression	Tolerance to a prolonged submergence	Li et al. (2005)
Ripening	ACC	Tomato antisense 1-aminocyclopropane-1-carboxylic acid oxidase gene	Ethylene biosynthesis	Henzi et al. (1999, 2000)
	ACO II/IPT	Broccoli antisense ACC oxidase II and isopentenyl transferase gene	Ethylene/cytokinin biosynthesis	Gapper et al. (2002, 2005)
	ACC	ACC oxidase (sense/antisense), ACC synthase (cDNAs)	Ethylene production, delay of chlorophyll loss	Higgins et al. (2006)
	ipt boers	Retarding effect on post-harvest yellowing	Cytokinin biosynthesis	Chen et al. (2001)
	BoCP5	Mutant broccoli ethylene response sensor gene	Ethylene biosynthesis	Chen et al. (2004)
	BoINV2	Broccoli antisense gene of cystein protease	Influence of post-harvest protease activity	Eason et al. (2005)
		Antisense construct of BoINV2 (soluble acid invertase)	Retarding effect on post-harvest yellowing	Eason et al. (2007)

BoCLH1	Antisense chlorophyllase gene		Retarding effect on post-harvest yellowing and chlorophyll degradation	Chen et al. (2008a)
Self incompatibility	SLG	S locus glycoprotein gene	Self incompatibility	Sato et al. (1991), Toriyama et al. (1991a, b)
	SLR1	Antisense SLR1 glycoprotein gene	Self incompatibility	Franklin et al. (1996)
	SRK, SLG	S locus receptor kinase gene, S locus glycoprotein gene	Self incompatibility	Conner et al. (1997)
Male sterility	DTx-A	Cytotoxic diphtheria toxin A-chain (DTx-A) gene + tapetum-specific promoter	Male sterility	Lee et al. (2003c)
Pharmaceuticals	B5/SARS-CoV	Vaccinia virus glycoprotein B5, human SARS coronavirus glycoprotein	Production of antigens	Pogrebnyak et al. (2006)
Gene function	Ac Tpase	Ds-based two-element transposon system	Transposon activity, insertion mutagenesis	McKenzie et al. (2002), McKenzie and Dale (2004)
<i>Brassica rapa</i> L.				
Herbicide resistance	bar/TuMV-Nla	Basta resistance, <i>Turnip mosaic virus</i> Nla protease	Method in planta	Qing et al. (2000), Xu et al. (2008)
Virus resistance	TMV-L	<i>Tobacco mosaic virus</i>	L coat protein gene	Jun et al. (1995)
	TuMV-Nlb	Antisense <i>Turnip mosaic virus</i> Nlb	TuMV-resistance, method in planta	Yu et al. (2007)
Insect resistance	Bt cry		Insect resistance against <i>Pieris rapae</i> , <i>Plutella xylostella</i> , <i>Trichoplusia ni</i>	Cho et al. (2001)
	Cry	<i>Bt</i>	Insect resistance, influence on nontarget insects	Kim et al. (2008)
	CpT1	Cowpea trypsin inhibitor + antibacterial peptide gene	Resistance against <i>P. rapae</i> and <i>Erwinia arradiae</i>	Zhao et al. (2006a, b)
Bacterial resistance	Antibacterial gene	Antibacterial peptide gene	Resistance against <i>E. arradiae</i>	Wang et al. 2002
Self incompatibility	SLG, SRK	S locus glycoprotein gene, S receptor kinase gene	Self incompatibility	Shiba et al. (1995, 2000), Takasaki et al. (1999, 2000, 2001)
	SP11	S locus protein 11	Self incompatibility	Shiba et al. (2001), Sato et al. (2003, 2004)

(continued)

**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
Male sterility	BcMF6	Antisense pollen-expressed polygalacturonase gene BcMF6	Pollen development, A9 promoter	Zhang et al. (2008)
	CYP86MF	Antisense fragment of the CYP86MF gene and the rapetum-specific A9 promoter		Yu et al. (2004), Cao et al. (2006b)
Flower development	OsMADS1	Rice floral development gene (MADS box gene)		Shin et al. (2003)
Plant physiology	BrFLC1, 2, 3 pRiA4, pRi1855	Floral repressor gene Genes in pRiA4 and pRi1855	Flowering time Auxin synthesis, root and plant growth	Kim et al. (2007a) He et al. (1994, 2000)
Abiotic stress	otsA/LEA	Trehalose-6-phosphate synthase/late embryogenesis abundant protein	Environment stress tolerance	Park et al. (2003), (2005b)
	Cu/ZnSOD, CAT SOD, CAT	Maize superoxide dismutase and/or catalase gene <i>E. coli</i> superoxide dismutase and/or catalase gene	Resistance to SO <sub>2</sub> (chloroplast transformation) Resistance to SO <sub>2</sub>	Tseng et al. (2007) Tseng et al. (2008)
Metabolic engineering	MAMI, CYP79F, CYP83A1 CYP79B2, CYP79B3, CYP83B1 GLO,JMT	Arabidopsis cDNAs Arabidopsis cDNAs	Aliphatic glucosinolate biosynthesis Indol glucosinolate metabolism, plant defence	Zang et al. (2008a) Zang et al. (2008b)
<i>Lactuca sativa</i> L.	Herbicide resistance	L-Gulono-γ-lactone oxidase (vitamin C metabolism)/jasmonic methyl transferase bar, glu, EPSPS	Fungal resistance Basta and Roundup resistance	Min et al. (2007) McCabe et al. (1999), Mohapatra et al. (1999), Torres et al. (1999), Nagata et al. (2000)
Fungal resistance	glu oxdc	β-1,3-Glucanase from <i>Arthrobacter</i> spp. Decarboxylase gene from mushroom	Resistance against <i>Bremia lactucae</i> Resistance against <i>Sclerotinia sclerotiorum</i>	Dede (1998) Dias et al. (2006)

Virus resistance	LMV-0	<i>Lettuce mosaic virus</i> (LMV) coat protein gene	Sense and antisense orientation	Gillbertson (1996), Dinant et al. (1997)
	LBVaV	Coat protein gene of <i>Lettuce big-vein associated virus</i> (LBVaV)		Kawazu et al. (2006)
	TSWV-BL	Nucleocapsid protein gene of <i>Tomato spotted wilt virus</i> (TSWV) and <i>Lettuce infectious yellow virus</i> (LIYV)		Falk (1996), Pang et al. (1996)
Insect resistance	SaPIN2a	Proteinase inhibitor II (PIN2) from <i>Solanum americanum</i>	Against cabbage looper ( <i>Trichoplusia ni</i> )	Xu et al. (2004), Chye et al. (2006), Xie et al. (2007)
Abiotic stress and plant physiology	ABF3, ABA LfA	Abscisic acid Late embryogenesis abundant protein gene from <i>Brassica napus</i>	Tolerance to drought and cold stress Tolerance to salt stress and water stress	Vanjiidorj et al. (2005) Park et al. (2005c)
	p5CS	δ-(1)-Pyrroline-5-carboxylate synthetase	Water stress resistance (drought, salt, cold)	Pileggi et al. (2001)
	rolAB GA20	<i>A. rhizogenes</i> rolAB genes Overexpression of a pumpkin gibberellin (GA) 20-oxidase gene	Response to auxin Controlling plant stature	Curtis et al. (1996a) Niki et al. (2001)
	etr1-1	Ethylene mutant receptor etr1-1 confers ethylene insensitivity	Effect on the regeneration properties	Kim et al. (2004)
	NR	Post-transcriptional gene silencing	Nitrate content	Curtis et al. (1999), Dubois et al. (2005)
	Ferritin	Iron storage protein	High yield, high iron content and rapid growth rate	Goto et al. (2000)
Metabolic engineering and functional food	Monellin Miraculin	Single-chain monellin gene Synthetic miraculin gene	Flavour and quality Taste-modifying proteins, sweetness-inducing activity	Penarrubia et al. (1992) Sun et al. (2006)
	STS	Stilbene synthase gene from <i>Parthenocissus henryana</i>	Key enzyme in resveratrol biosynthesis	Liu et al. (2006)
	asnA	<i>E. coli</i> asparagine synthetase A gene	inulin content increased	Sobolev et al. (2007), Giannino et al. (2008)
	CAX1	<i>A. thaliana</i> cation exchanger1 H <sup>+</sup> /Ca <sup>2+</sup>	Increased Ca content	Park et al. (2009)

(continued)

**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
	R2R3-MYB	Flavonoid biosynthesis factor from <i>A. thaliana</i>	Anthocyanin biosynthesis	Park et al. (2008)
Male sterility	TC/VTE1, $\gamma$ -TMT	Tocopherol cyclase, $\gamma$ -tocopherol methyltransferase	Maturity regulation	Cho et al. (2005), Lee et al. (2007a)
Pharmaceuticals	ipt PR-Glu	Pathogenesis-related glucanase gene linked to a tapetum-specific promoter	Human therapeutic protein	McCabe et al. 2001 Curtis et al. (1996b)
	CTB-Pins, sCTB	Cholera toxin B subunit (human proinsulin)		Kim et al. (2006), Ruhlman et al. (2007)
	sLTB, SARS-CoV	<i>E. coli</i> heat-labile enterotoxin B subunit, severe acute respiratory syndrome coronavirus		Li et al. (2006), Kim et al. (2007b)
	MV-H E2-CSFV, CP	Measles virus hemagglutinin Glycoprotein of swine fever virus, cystein protease from <i>Fasciola hepatica</i>	Oral animal vaccination	Webster et al. (2006) Legocki et al. (2005)
	HBsAg	Antigen of hepatitis B virus		Kapusta et al. (1999, 2001), Kawashima et al. (2001)
	hITF ChfFN- $\alpha$	Human intestinal trefoil factor Chicken $\alpha$ -interferon against vesicular stomatitis virus	Preventing infectious diseases of poultry	Zuo et al. (2001) Song et al. (2008)
	IFS	Soybean isoflavone genistein	Phytotoestrogen	Liu et al. (2007b)
Carrot ( <i>Daucus carota</i> L.)	pat		Glufosinate resistance, Liberty resistance	Dröge et al. (1992), Drogelaser et al. (1994), Chen and Punja (2002)
Herbicide resistance	ALS	Mutant acetolactate synthase gene	Imazapyr resistance	Aviv et al. (2002)
Fungal resistance	Chiit	Chiitase genes from tobacco, petuna, bean	Against <i>Rhizoctonia</i> , <i>Alternaria</i> , <i>Botrytis</i> , <i>Sclerotinia</i>	Linhorst et al. (1990), Broglie et al. (1991)
	chi-2	Chiitase genes from tobacco, bean, barley	Against <i>Rhizoctonia</i> , <i>Alternaria</i> , <i>Botrytis</i> , <i>Sclerotinia</i>	Gilbert et al. (1996), Punja and Raharjo (1996), Jayaraj and Punja (2007)
	CHT36	Microbial endochitinase <i>Trichoderma harzianum</i>	Against <i>Alternaria</i> , <i>Botrytis</i>	Baranski et al. (2008)

MF3	Microbial factor from <i>Pseudomonas fluorescens</i>	Against <i>Alternaria, Botrytis</i>	Baranski et al. (2007)
tlp	Rice thaumatin-like protein		Chen and Punja (2002), Punja (2005)
lip	Wheat lipid transfer-protein (PR)	Resistance against <i>Erysiphe heraclei</i>	Jayaraj and Punja (2007)
HLP	Human lysozyme protein	Against <i>Alternaria, Cercospora, Erysiphe</i>	Takaichi and Oeda (2000)
AP24	Tobacco PR-5 osmotin + chitinase + glucanase	Increase Ca content, functional food	Tigelaar et al. (1996), Melchers and Suiver (2000)
Functional food	<i>A. thaliana</i> cation exchanger 1 H <sup>+</sup> /Ca <sup>2+</sup> β-carotene ketolase gene from alga <i>Haematococcus pluvialis</i>	Functional food, neutraceutical	Park et al. (2004b)
bkt	E. coli heat-labile enterotoxin (LTB)	Against cholera and diarrhoea	Jayaraj et al. (2008), Jayaraj and Punja (2008)
LTB			Rosales-Mendoza et al. (2007, 2008)
GAD65	Autoantigen in human insulin-dependent diabetes mellitus (IDDM)		Poreddu et al. (1999), Avesani et al. (2003)
MPT64	<i>Mycobacterium tuberculosis</i> gene		Wang et al. (2001)
HepB	Hepatitis B virus surface protein		Imani et al. (2002)
tt830-844	Measles-unrelated T cell epitope (tt830-844)		Bouché et al. (2003, 2005)
MV	Immunodominant antigen of the measles virus		Marquet-Blouin et al. (2003)
<i>Cucumis melo</i> L.			
Virus resistance	CMV-WL	Coat protein-mediated resistance (CP-MR)	Resistance to <i>Cucumber mosaic virus</i> (CMV)
ZYMV, WMV	CP-MR		Resistance to <i>Watermelon virus 2</i> (WMV 2) and <i>Zucchini yellow mosaic virus</i> (ZYMV)
ZYMV, WMV2, CMV	CP-MR		Resistance to WMV 2, ZYMV and CMV
Abiotic stress	HLA1	Salt tolerance	Bordas et al. (1997)
Fruit ripening	CmACO1-AS	ACC oxidase antisense	Núñez-Palenzuela et al. (2006)

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**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
<i>Cucumis pepo</i> L. Virus resistance	MEL1 ACC CMV-CP	Melon ACC oxidase antisense Apple ACC oxidase antisense Coat protein-mediated resistance (CP-MR)	Extended shelf life Ten days longer shelf life	Ayub et al. (1996), Guis et al. (2000) Silva et al. (2004)
<i>Cucumis sativus</i> L. Virus resistance	ZYMV, WMV CMV-C-CP, CMV-O- CP pCAMS V RCC2	CP-MR 54-kDa replicase gene of CFMMV Rice chitinase cDNA Cucumber class III chitinase gene	Resistance to <i>Cucumber mosaic virus</i> (CMV) Resistance to <i>Watermelon virus</i> 2 (WMV 2) and <i>Zucchini yellow mosaic virus</i> (ZYMV)	Tricoli et al. (1995), Fuchs et al. (1998) Clough and Hamm (1995), Fuchs and Gonsalves (1995), Tricoli et al. (1995)
Fungal resistance	CH12	Gene from <i>Saccharomyces cerevisiae</i>	Resistance to <i>Botrytis cinerea</i>	Gonsalves et al. (1992), Nishibayashi et al. (1996a)
Abiotic stress	HLA1 DHN10, DHN24 DefH9-iaAM	Dehydriin from <i>Solanum surrandium</i> Genes from <i>Pseudomonas syringae</i> pv. <i>savastanoi</i> and <i>Antirrhinum majus</i> Gene from <i>Thaumatococcus daniellii</i>	Resistance strategy to gray mould ( <i>Botrytis cinerea</i> ) Salt tolerance under in vitro conditions	Gal-On et al. (2005) Tabei et al. (1998), Kishimoto et al. (2002, 2003) Kishimoto et al. (2004)
Parthenocarpy	Taumatin II	Temperature tolerance (increased chilling tolerance)	Bordas et al. (1997)	Yin et al. (2006a)
Taste	mSOD1 <i>Citrullus lanatus</i> (THUNB.) MATSUN. & NAKAI. CGMMV-CP	Superoxide dismutase (SOD) from cassava Genes from <i>Saccharomyces cerevisiae</i>	Yin et al. (2004), Yin et al. (2006b)	Szwacka et al. (2002), Gajc-Wolska et al. (2003, 2005) Lee et al. (2003a)
Pharmaceuticals				
Virus resistance	HLA1			Park et al. (2005d)
Abiotic stress				Ellul et al. (2003)

<i>Pisum sativum</i> L.					
Herbicide resistance	bar		Basta resistance	Schroeder et al. (1993), Shade et al. (1994)	
Fungal resistance	Vst1, PGIP	Stilbene synthase gene (Vst1) from grape, polygalacturonase inhibiting protein (PGIP) from raspberry		Richter et al. (2004)	
Virus resistance	AMV-CP	Chimeric coat protein		Grant et al. (1998), Timmerman-Vaughan et al. (2001)	
Insect resistance	Al-1, Al-2	$\alpha$ -Amylase inhibitors 1, 2 from <i>Phaseolus vulgaris</i>	Resistance to <i>Bruchus pisorum</i> (pea weevil)	Schroeder et al. (1995), Morton et al. (2000), Collins et al. (2006)	
<i>Phaseolus vulgaris</i> (L.)					
Herbicide resistance	bar		Glufosinate ammonium resistance	Aragão et al. (2002)	
Virus resistance	BGMV-BR, bar	Coat protein from <i>Bean golden mosaic virus</i> and Basta resistance		Russell et al. (1993)	
	rep-TrAP-Ren, BC1	Antisense of genes from Brazilian isolate <i>Bean golden mosaic virus</i> (BGMV-BR)		Aragão et al. (1998)	
	AC1, AC2, AC3, BC1	Antisense of AC1, AC2, AC3 and BC1 genes from BGMV		Aragão et al. (1996)	
	Rep, bar	Rep gene mutant of BGMV and Basta resistance		Faria et al. (2006)	
	AC1-RNAi	Post-transcriptional gene silencing (RNAi) of the AC1 gene from BGMV	High-resistance progenies	Bonfim et al. (2007)	
Abiotic stress	LEA	Late embryogenesis abundant protein gene from <i>Brassica napus</i>	Drought stress resistance	Liu et al. (2005)	
Protein content	be2s2	Methionine-rich 2S albumin from the Brazil nut	Increased methionine content	Aragão et al. (1996, 1999)	
<i>Cichorium intybus</i> L. & <i>C. endivia</i> L.					
Herbicide resistance	csr1-1	Mutant acetolactate synthase gene from <i>A. thaliana</i>	Resistance to sulfonylurea herbicides	Vermeulen et al. (1992), Lavigne et al. (1995)	
Metabolic engineering	6G-FFT	6G-Fructosyltransferase from onion	Synthesized fructan of the inulin neoseries and linear inulin	Vijn et al. (1997)	

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**Table 25.1** (continued)

Character	Transgene	Transgene description	Aim	References
	6-SPT	6-Fructosyltransferase from barley	Synthesized branched fructans and tetrasaccharide bifurcose	Sprenger et al. (1997)
Male sterility	barnase and bar	Tapetum-specific promoter and barnase gene from <i>Bacillus amyloliquefaciens</i>	Hybrid breeding	Mariani et al. (1992), Williams (1995), <a href="http://www.agbios.com">www.agbios.com</a>
<i>Spinacea olereacea</i> L.	Herbicide resistance	Pat gene from <i>Steptomyces hygroscopicus</i>	Glufosinate ammonium resistance	Wells (1999), Burgos et al. (2001)
Virus resistance	CMV-CP	Coat protein genes	Against <i>Cucumber mosaic virus</i> (CMV)	Yang et al. (1997)
<i>Asparagus officinalis</i> L.	Herbicide resistance	Phosphinothricin acetyl transferase	Basta resistance	Cabrera-Ponce et al. (1997)
Onion ( <i>Allium cepa</i> L.)	Herbicide resistance	bar, CP4	Basta and Roundup resistance	Eady et al. (2003a)
Insect resistance		Cry1Ab, Cry1Ca	<i>Bt</i> hybrid genes	Zheng et al. (2005)
<i>Allium tuberosum</i> L. & <i>A. porrum</i> L.	Herbicide resistance	ALS	Acetolactate synthase (ALS) gene from chlorsulfuron resistant <i>Arabidopsis mutant</i>	Park et al. (2002)
Insect resistance		cry	<i>Bt</i> hybrid gene which encodes domains I and II of Cry1Ab and domain III of Cry1Ca	Zheng et al. (2004)

## 25.2 Economically Important Vegetable Families

### 25.2.1 Solanaceae

#### 25.2.1.1 *Solanum lycopersicon* L.

In the family Solanaceae, besides tobacco, tomato has played a key role in genetic engineering techniques in the past years. Among the other vegetable crops, tomato fulfills the basic requirements for gene transfer, which includes its character as a model object for in vitro culture techniques (Bhatia et al. 2004), its moderately sized genome with 950 Mb (Shibata 2005) applicable to recent sequencing technology and its importance as vegetable crop for the fresh market and for processing. Hence, it is not surprising that the first commercialized transgenic food crop ever brought to market was Calgene's 'Flavr Savr' tomato in 1994. It was followed in 1995 by DNA Plant Technology's 'Endless Summer'. 'Flavr Savr' was a success with consumers but failed economically for a variety of reasons (Martineau 2001). In 1996 Zeneca launched a transgenic processing tomato product that was the best selling tomato paste in the United Kingdom during 1999—2000. The paste reduced processing costs and resulted in a 20% lower price (Redenbaugh and McHughen 2004).

Considerable success has been achieved in introducing virus resistance (Kunik et al. 1994; Whitham et al. 1996; Gubba et al. 2002), fungi resistance (Jongedijk et al. 1995; Tabaeizadeh et al. 1999; Radhajeyalakshmi et al. 2005; Sarowar et al. 2006) and bacteria resistance based on systemic acquired resistance (SAR; Rizhsky and Mittler 2001; Lin et al. 2004; Chan et al. 2005). Insect resistance (see also Chap. 10) has been engineered by using bacterial genes derived from *Bacillus thuringiensis* ssp. *kurstaki* (*Bt* genes; Fischhoff et al. 1987; Delannay et al. 1989) or a proteinase inhibitor from potato (Abdeen et al. 2005) which is a part of the plant natural defence mechanism against herbivores. Furthermore *Mi-1*, a *Lycopersicon peruvianum* gene which confers resistance against the three economically important root-knot nematode species (*Meloidogyne incognita*, *M. javanica*, *M. arenaria*; Roberts and Thomason 1986; Goggin et al. 2004), is also active against the potato aphid, *Macrosiphum euphorbiae* (Vos et al. 1998).

Other limiting factors in the horticultural production are abiotic stresses (see Chap. 8), such as extreme temperature, drought and salinity. A transformation system with chloroplast-targeted *codA* gene of *Arthrobacter globiformis* (for method, see Chap. 2), which encodes choline oxidase to catalyse the conversion of choline to glycinebetaine, was successfully established with tomato cv. 'Money-maker' (Park et al. 2004a). The study demonstrates a better fitness of transgenic plants after chilling at 3 °C for 7 days with regard to their survivability and the fruit set. Other efforts were made to engineer chilling tolerance by ectopic expression of *Arabidopsis CBF1* (Hsieh et al. 2002a, b).

Most commercial tomato cultivars are sensitive to salinity. Considerable genetic knowledge of salt tolerance (Foolad 2004) is the basis for transgenic strategies

to overcome this problem (Gisbert et al. 2000; Rus et al. 2001; Jia et al. 2002; Muñoz-Mayor et al. 2008). Due to the complexity of the trait in many cases the increased transgenic salt tolerance was only marginal. However, advancement was the creation of transgenic tomato plants by overexpressing a vacuolar  $\text{Na}^+/\text{H}^+$  antiport with the *AtNHX1* gene from *Arabidopsis* (Zhang and Blumwald 2001). Transgenic plants grown in the presence of 200  $\mu\text{M}$  sodium chloride flowered and produced fruits.

While most of the above-mentioned traits were agronomical and benefitted primarily the grower and the producer, currently significant efforts are also being made to improve nutrients and consumer qualities. Although technically more difficult and therefore not ideal for the grower, there are many potential opportunities for enhancing nutritional value (Bird et al. 1991; Römer et al. 2000; Muir et al. 2001; Le Gall et al. 2003; Giorio et al. 2007) and organoleptic qualities such as taste (Penarrubia et al. 1992; Bartoszewski et al. 2003) and aroma in the tomato fruits. Important quality parameters of fresh fruits are volatile compounds, which often do not meet the high standards of flavour required by the consumer. For instance the  $\Delta$ -9 desaturase gene from *Saccharomyces cerevisiae* expressed in tomato showed changes in certain flavour compounds (Wang et al. 1996). The overexpression of a non-specific alcohol dehydrogenase gene in tomato fruits (Speirs et al. 1998) altered the levels of aroma determining aldehydes and alcohols. In a preliminary taste trial, the authors identified fruits with elevated alcohol dehydrogenase activity and higher level of alcohols as having a more intense ‘ripe fruit’ flavour.

Tomato plants have been designed to produce a range of proteins and biomolecules. The cholera toxin B protein has been expressed in tomato plants, and the feasibility to elicit an immune response in mice has been demonstrated (Jiang et al. 2007). Recently Butelli et al. (2008) expressed two transcription factors from *Antirrhinum majus* L. in tomato; the fruit of the plants accumulated anthocyanins at levels substantially higher than previously reported for efforts to engineer anthocyanin accumulation in tomato and at concentrations comparable to the anthocyanin levels found in blackberries and blueberries.

Tomato fruits contain proteins with high allergenic potential (Jäger and Wüthrich 2002). Genetic engineering could be an approach to remove allergens. This was demonstrated in a remarkable way by Le et al. (2006a, b), who designed tomatoes with reduced allergenicity by dsRNAi-mediated inhibition of *ns-LPT* (*Lyc e 1* and *Lyc e 3*, respectively) expression (for details on gene silencing, see Chap. 5). Furthermore it was demonstrated that silencing of the *Lyc* genes by means of RNAi contributes to reducing skin reactivity and is passed on to the next generation of fruits (Lorenz et al. 2006).

### 25.2.1.2 *Solanum melongena* L.

Eggplant (aubergine) is native to India. Today it is an important crop in tropical and warm parts of the temperate zone. Like other plants of the family Solanaceae it

suffers from severe diseases, insect attacks and abiotic stress, leading to high crop loss every year.

In vitro culture methods were used comprehensively to improve the eggplant cultivars (for reviews, see Collonnier et al. 2001; Kashyap et al. 2003). Due to the good response in tissue culture the first attempts at genetic engineering for eggplant were accomplished soon after the first reports on plant transformation of *Arabidopsis* and tomato (Guri and Sink 1988; Rotino and Gleddie 1990). So far, a number of useful genes have been introduced to eggplant. General aspects of genetic modification of plants are discussed in Chap. 1.

Parthenocarpic transgenic eggplants have been successfully achieved by transferring a gene construct consisting of bacterial *iaaM* gene and *DefH9* promotor, specifically to the placenta and ovules (Rotino et al. 1997). Donzella et al. (2000) reported on the field performance of the transgenic parthenocarpic hybrids. They concluded that the transgenic parthenocarpic hybrids allowed an increase in productivity up to 25%.

It was shown that an introduced bacterial mannitol-1-phosphodehydrogenase (*mtlD*) gene evokes a multifactor abiotic stress tolerance (Prabhavathi et al. 2002). Transgenic eggplants featured an improved tolerance to salt, drought and chilling stress. Recently, Prabhavathi and Rajam (2007) described that mannitol-accumulating transgenic eggplants exhibit resistance to fungal wilts. The data suggest that the *mtlD* gene could be useful for both plant biotic and abiotic stress tolerance.

Further efforts are being made to develop eggplant cultivars with resistance against fungal diseases. The fatty acid composition has an impact on resistance to *Verticillium dahliae*. Transfer of yeast  $\Delta$ -9 desaturase gene in eggplant displayed the linkage between plant fatty acid content and the resistance traits (Xing and Chin 2000). After successful transformation with an antimicrobial defensin gene from *Dahlia merckii*, Turrini et al. (2004) found transgenic eggplants had an improved resistance against *Botrytis cinerea*.

In tomato the *Mi-1.2* gene confers resistance against nematodes, whiteflies and potato aphids (Nombela et al. 2003). Expression of the tomato *Mi-1.2* gene in eggplants causes resistance against nematodes only, not aphids (Goggin et al. 2006). There is the assumption that the genetic background plays an important role for gene function.

Under the tropical climate eggplant is infested by a number of insect pests. Plant protease inhibitors have a defensive function, targeting leaf-feeding insects like aphids. Transgenic eggplants with an *oryzacystatin* gene coding for an inhibitor of cystein proteinases have been obtained by *Agrobacterium*-mediated transfer (Ribeiro et al. 2006). In feeding tests the population growth and the survival of *Mycus persicae* Sulzer and *Macrosiphum euphorbiae* Thomas were reduced.

The most destructive insects on eggplants are the Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) and the eggplant shoot and fruit borer (ESFB; *Leuconodes orbonalis* Guen.). There are a number of reports about *Bt* transgenic eggplants, describing the transformation procedure. Furthermore, the impact of transgenic *Bt* eggplants on the target insects (CPB or EFSB) as well as on non-target arthropods has been examined thoroughly (Chen et al. 1995; Rovenská et al. 2005;

Arpaia et al. 2007). Connected with current announcements to introduce *Bt* eggplant in commercial use, there is a comprehensive analysis about the potential impacts of *Bt* eggplants on economic surplus in India (Krishna and Qaim 2007, 2008). Safety tests for the *Bt* eggplant have been conducted in India, starting in greenhouses and now moving on to large-scale field trials.

### 25.2.1.3 *Capsicum annuum* L.

Peppers are cultivated and used around the world as sweet peppers, such as the bell pepper, or as pungent chilli peppers. Pepper originated in the tropics. Today pepper is cultivated also in the subtropics and in temperate climates as a staple vegetable crop. Belonging to the family Solanaceae well known for plants with an excellent tissue culture and transformation capability, pepper is a recalcitrant exception. First, Liu et al. (1990) reported about *Agrobacterium*-mediated transformation of bell pepper. They showed the principal possibility of pepper transformation with foreign genes like *nptII* and *gus*. In 1993, US patent 5262316 (Engler et al. 1993) described the co-cultivation of explant material from the pepper plant with *A. tumefaciens* or *A. rhizogenes* carrying an exogenous DNA sequence. Therefore the invention related to a method for genetically transforming and regenerating pepper plants. Despite a detailed description of the transformation procedure, the patent gives no clearness about the regeneration efficiency. Over the past 15 years a few other groups (e.g. Zhu et al. 1996; Manoharan et al. 1998; Pozueta-Romero et al. 2001; Li et al. 2003; Lee et al. 2004) have been working on the improvement of the transformation system for pepper. In summary it should be stated that the pepper transformation is not a routine method and is highly dependent on genotype and explant source.

Due to the importance of pepper, genetic engineering is (despite the low efficiency of the transformation protocols) a promising tool to improve some cultivars. Pepper yields are endangered every year by severe virus diseases. Kim et al. (1997) induced cDNA of the satellite RNA of the *Cucumber mosaic virus* (CMV) into the pepper genome. The authors described an attenuation of the symptoms in T<sub>1</sub> hot pepper plants. In spite of the positive results there are no more publications with such strategy. Some concerns about the biosafety could be the cause for that.

Another strategy, the virus coat protein mediated protection, was more widely applied (Zhu et al. 1996). Shin et al. (2002a) reported about the testing of transgenic pepper plants expressing the coat proteins of CMV and *Tomato mosaic virus* (ToMV). Cai et al. (2003) gave a detailed report about the development of CMV- and TMV-resistant transgenic chilli pepper, the field performance of some progenies and a biosafety assessment.

It was demonstrated that the expression of tobacco stress-induced gene 1 (*Tsi 1*) in pepper enhanced the resistance of the transgenic pepper plants to various pathogens, including viruses, bacteria and oomycetes (Shin et al. 2002b). Transcriptional regulatory genes may have an impact on the overall disease resistance in pepper.

The risk to overcome such broad resistance should be low, therefore it is a strategy worth further investigation.

The Chinese government approved commercialization of pimientos (Spanish pepper) in the late 1990s, although more detailed information is missing ([http://www.chinadaily.com.cn/english/doc/2006-02/14/content\\_519769.htm](http://www.chinadaily.com.cn/english/doc/2006-02/14/content_519769.htm)).

In India the performance of transgenic bell pepper and chilli with snowdrop lectine gene has been examined in field trials in 2002 (<http://www.indiaresource.org/issues/agbiotech/2003/fieldsoftrial.html>). The additional lectine gene should evoke resistances against lepidopteran, coleopteran and homopteran pests. Experiments have been performed under the umbrella of Rallis India Ltd and the Bangalore Tata Group. Common knowledge about some results is strictly limited.

Due to its simplicity, herbicide resistance was often the first published genetically engineered trait. Surprisingly that is not correct for pepper. There exists a brief mention by Tsafaris (1996). A Korean team (Lee et al. 2007b) reported on a conference about the environmental evaluation of herbicide-resistant peppers.

Korean scientists (Kim et al. 2001) introduced rice MADS box genes into pepper, studying the impact of such genes on the plant development.

Harpster et al. (2002a) investigated the function of the *CaCell* gene by silencing in transgenic pepper. The consequences for fruit ripening process in T<sub>3</sub> plants in a greenhouse were examined. This is the only example that genes isolated from pepper are used for the investigation of their function in pepper. But there are plenty of isolated and notified pepper genes and cDNAs used for further gene expression studies in plants easily accessible for transformation, like *Arabidopsis*, tobacco or tomato; some of the latest of such works were published by e.g. An et al. (2008), Hong et al. (2008), Hwang et al. (2008), Oh et al. (2008).

## 25.2.2 *Brassicaceae* (*Brassica oleracea* L., *B. rapa* L., *Raphanus sativus* L.)

Substantial work on the elaboration and application of genetic transformation for *Brassica* vegetable crops is in progress throughout the world. *Brassica* vegetables encompass important vegetables, such as cauliflower, broccoli, cabbage and Brussels sprouts. In the Asian cuisine in countries like China, India and Korea *Brassica rapa* L. vegetables play an important role. The high variability of crucifers, their economic impact and their good responsiveness to biotechnological approach are considerable factors so that, from the first possibilities for genetic engineering to date, *Brassica* species are a promising object for such techniques. The development of plants with useful traits is relatively advanced. Despite this only a few field testings with transgenic brassicas have been performed. Commercial cultivars seem to be not in sight.

Early after the first reports of successful transformation of *B. oleracea* using *A. tumefaciens* with marker genes (David and Tempé 1988; Srivastava et al. 1988; De Block et al. 1989) this technique was applied for the investigation of

self-incompatibility (Sato et al. 1991; Thorsness et al. 1991; Toriyama et al. 1991a, b). Due to difficulties in transforming *B. rapa*, similar works for *B. rapa* were published later (Takasaki et al. 1999, 2000, 2001). A valuable trait for breeding purposes, self-incompatibility in Brassicaceae is genetically controlled by some *S* locus genes. Transformation technology has opened up new possibilities to investigate the expression and interaction of the *S* locus genes.

Male sterility is another breeding feature of great worth, enabling F<sub>1</sub> hybrid production on a large scale. In the past decade researchers reported about new approaches concerning the male sterility of *Brassica* species. It should be mentioned that this is a cutting-edge topic with regard to environmental concerns about possible transgene escape. No pollen development could be a solution for safe plant containment. Lee et al. (2003c) obtained several transgenic plants from cabbage, *B. oleracea* ssp. *capitata*, by way of *Agrobacterium*-mediated transformation to test the activity of anther-specific promoter isolated from Chinese cabbage. With that promoter, the expression of the cytotoxic diphtheria toxin A-chain (*DTx-A*) gene resulted in male-sterile cabbages. Using RNA antisense technology (see Chap. 5) and a tapetum-specific promoter (Yu et al. 2004; Zhang et al. 2008) could develop male-sterile Chinese cabbage.

Another possibility to get transgenic plants without dissemination of transgenes via pollen could be chloroplast transformation (Nugent et al. 2006; Liu et al. 2007a, 2008). Liu et al. (2008) reported the acquired insect resistance of cabbage after chloroplast genetic engineering with a *Bt* gene, demonstrating the efficiency of the genetic modification of plastids. They cited Bock (2007) that the plastid transformation is a prerequisite method to produce vaccines or therapeutic proteins in plants. So far, this general statement has not been realized for *Brassica* vegetables. Although the *Brassica* vegetable crops are important, to date only Pogrebnyak et al. (2006) has reported the *Agrobacterium*-mediated transformation of collard and cauliflower with, respectively, a smallpox vaccine candidate gene and a gene coding for SARS coronavirus spike protein.

Every year the yield losses caused by diseases and by insect attacks are high. For the whole complex of engineering disease and pest resistance, many reports are available for both *B. oleracea* and *B. rapa*. Table 25.1 gives a brief overview about the latest publications in that field. Generally, the methods of transformation are well established and a number of scientific teams are performing the transformation of *Brassica* with a high efficacy.

There is a great interest in having a controlled influence on postharvest physiological processes. To gain a deep understanding of the role of ethylene, cytokinin and other factors, broccoli was used as a model species (Henzi et al. 1999, 2000; Chen et al. 2001; Gapper et al. 2005; Higgins et al. 2006). In connection with the improved availability of isolated genes and cDNAs, new studies for postharvest yellowing show the effect of additionally introduced *Brassica* genes in broccoli (Chen et al. 2004, 2008a; Eason et al. 2007). Kim et al. (2007a) transferred floral repressor genes isolated before from *B. rapa* to Chinese cabbage. The results demonstrate that it is feasible to control the flowering time and the undesirable bolting of Chinese cabbage.

Improved access to genes originating from sequencing projects is also reflected in other current works for *Brassica* transformation. For instance, *Arabidopsis* cDNAs were used for metabolic engineering of aliphatic or indole glucosinolates of *B. rapa* (Zang et al. 2008a, b).

Since various factors of abiotic stress seriously impair the growth and development of *Brassica* crops, approaches for improved abiotic stress tolerance are an objective for a number of transformation projects. So far, the investigations have encompassed bacterial, yeast and plant genes. The genetic improvement of heavy metal tolerance in cauliflower by transfer of the yeast metallothionein gene (*CUP1*) was demonstrated by Hasegawa et al. (1997). Li et al. (2005) delivered the gene coding for *Vitreoscilla haemoglobin* (*vhb*) into cabbage. They observed that the overexpression of VHb protein affects the plant's tolerance of submergence stress. The introduction of the bacterial *betaA* gene for the synthesis of glycinebetaine causes a higher salinity tolerance in transgenic cabbage (Bhattacharya et al. 2004). For Chinese cabbage Tseng et al. (2007, 2008) explored the possibility of overcoming the phytotoxic effect of sulfur dioxide and salt stress. They transferred genes coding for superoxide dismutase and catalase from maize and *Escherichia coli*, respectively.

Belonging to the family Brassicaceae, radish (*Raphanus sativus* L.) is a further most common crucifer vegetable consumed worldwide. Radish is greatly recalcitrant in tissue culture. For that reason there are only a few reports about radish transformation. Moreover these reports describe transformation protocols trying to overcome difficulties with tissue culture and regeneration efficiency. Curtis et al. (2002) used the floral-dip method for producing transgenic radish plants with the *GIGANTEA* (*GI*) gene from *Arabidopsis*. Park et al. (2005a) elaborated a transformation protocol via sonification and vacuum infiltration of germinated seeds with *Agrobacterium*, successfully transferring a *LEA* gene (late embryogenesis abundant) from *B. napus*. The accumulation of the foreign protein in radish conferred an increased drought and salt tolerance.

### 25.2.3 Fabaceae (*Pisum sativum* L., *Phaseolus vulgaris* L.)

Whereas most crop species of the Fabaceae are used as protein or oil plants in food industry or animal nutrition, e.g. soybean, chickpea, pea, bean, lentil and others, a few species are also used as vegetables. Two examples are reviewed in this chapter: the garden pea (*Pisum sativum* L.) and the snap bean (*Phaseolus vulgaris* L.). For fresh, frozen or canning purposes, green premature seeds or juvenile pods of the garden pea are harvested and green pods in an early seed development stage of the snap bean are harvested.

After overcoming a number of difficulties during in vitro culture and regeneration, the first transgenic pea plants were reported by de Kathen and Jacobsen (1990) and Puonti-Kaerlas et al. (1990). The transfer of herbicide resistance (*bar*) as a potentially useable trait was reported but not carried through to commercial release

(Schroeder et al. 1993; Shade et al. 1994). Partial resistance to *Alfalfa mosaic virus* (AMV) was observed in transgenic pea engineered with a chimeric virus coat protein (Grant et al. 1998; Timmerman-Vaughan et al. 2001).

Another strategy focused on conferring resistance to pea weevil (*Bruchus pisorum* L.) by expression of an  $\alpha$ -amylase inhibitor ( $\alpha$ -AI) and the phytohemagglutinin promoter from *Phaseolus vulgaris* (Shade et al. 1994; Schroeder et al. 1995; Morton et al. 2000; De Sousa-Majer et al. 2004; Collins et al. 2006).

A fungal resistance approach was reported by Richter et al. (2006) who transformed via *Agrobacterium tumefaciens* two antifungal genes coding for a polygalacturonase-inhibiting protein (PGIP) from raspberry (*Rubus idaeus* L.) or the stilbene synthase (*Vst1*) from grape.

Analogous to pea, genetic engineering in bean was for a long time limited by the absence of efficient methodologies, from in vitro regeneration systems up to transformation systems. Now, transformation approaches via *Agrobacterium*, electroporation and particle-gun have been achieved (Genga et al. 1991; McClean et al. 1991; Dillen et al. 1995; Kim and Minamikawa 1997).

The first transgenic plant progeny was published by Russell et al. (1993). In a biolistic approach they transferred marker and reporter genes (*pat*, *gus*) and also a coat protein gene isolated from the *Bean golden mosaic virus* (BGMV).

The team of Aragão et al. (1996, 1998) obtained transgenic plants using different genes of BGMV in antisense orientation and showed resistance. Faria et al. (2006) achieved transgenic beans with a vector that contained a mutated virus replication gene (*rep*). Stability of the transgene loci and BGMV resistance were observed in some plant progenies. Bonfim et al. (2007) explored the concept of using an RNA interference construct to silence the ACI viral gene region of BGMV.

The methionine content was significantly increased in transgenic lines engineered via biolistic methods with a gene coding for the methionine-rich storage albumin from the Brazil nut (Aragão et al. 1996, 1999). The same group (Aragão et al. 2002) reported the transfer of herbicide resistance mediated by the *bar* gene to bean.

Transgenic kidney bean with the late embryogenesis abundant (*LEA*) protein gene from *Brassica napus* was produced by using a sonication and vacuum infiltration *Agrobacterium*-mediated transformation approach. Plants expressed a high level of the *LEA* gene showed a high tolerance to salt and water deficit stress (Liu et al. 2005). Whereas a commercial exploitation of GM peas in the medium term is expected especially for dry (seed) pea production (herbicide tolerance, resistance to insects, fungi and virus diseases), a commercial usage of the GM beans is in the long term not expected.

Meanwhile, genetic transformation has been reported in all the major legume crops, like *Cicer arietinum* L., *Cajanus cajan* L., *Vigna*, *Phaseolus*, *Lupinus*, *Vicia* and *Lens* species, but with the exception of soybean, transgenic plants have not yet been commercially released. A translation of knowledge of genomics or functional genomics in the model legumes *Medicago truncatula* and *Lotus japonicus* will open new transgenic approaches in future.

### 25.2.4 *Cucurbitaceae* [*Cucumis sativus L.*, *C. melo L.*, *Cucurbita pepo L.*, *Citrullus lanatus* (THUNB.) Matsun. & Nakai., and other *cucurbit* species]

The cucurbit family (*Cucurbitaceae*) includes three genera of valuable crop species: *Cucumis*, *Cucurbita* and *Citrullus*. In the genus *Cucumis*, cucumber (*C. sativus*) and melon (*C. melo*) are the two main crops. Squash, pumpkin and zucchini belong to the genus *Cucurbita*, which includes the cultivated species *C. pepo*, *C. moschata*, *C. maxima*, *C. argyrosperma* and *C. ficifolia*. In the genus *Citrullus*, watermelon is the only species of economic importance (Bates et al. 1990).

Since the first report about successful transformation of cucumber using *A. rhizogenes* (Trulson et al. 1986), a lot of work has been done to establish and improve transformation efficiency not only in *C. sativus* (Schulze et al. 1995; Nishibayashi et al. 1996b; He et al. 2008), but also in *Cucumis melo* (Fang and Gurmet 1990; Valles and Lasa 1994; Galperin et al. 2003; Cürik et al. 2005; Rhimi et al. 2007; Nuñez-Palenius et al. 2007), *Cucurbita pepo* (Katavic et al. 1991; di Toppi et al. 1997), *Citrullus lanatus* (Choi et al. 1994; Cho et al. 2008) and *C. colocynthis* (Dabauza et al. 1997).

The progress made with the application of this technique is reviewed by Yin et al. (2005). The use of viral coat protein genes to confer resistance has been approved for several virus diseases (Gaba et al. 2004). The commercially most successful has been zucchini engineered for resistance to the *Zucchini yellow mosaic virus* and *Watermelon mosaic virus 2* with coat protein genes. The transgenic zucchini traded firstly by Seminis is a cross with Asgrow's transgenic crookneck squash. The Asgrow Company received permission for commercial use in the United States in 1995.

During the past several years, genetic engineering approaches have been employed to develop transgenic cucurbit plants with enhanced tolerance to abiotic stress. In order to induce chilling tolerance in cucumber, the expression pattern of a *Solanum sogarandinum* pGt::*Dhn10* gene encoding a dehydrin DHN10 protein was analysed (Yin et al. 2004). The transgenic lines exhibited a slight enhanced chilling and a freezing tolerance either comparable to or less than the non-transgenic control. Another significant advancement was the transformation of different watermelon [*Citrullus lanatus* (THUNB.)] cultivars expressing the *Saccharomyces cerevisiae HAL1* gene related to salt tolerance (Ellul et al. 2003). The halotolerance observed in T<sub>3</sub> lines confirmed the inheritance of the trait and supports the potential usefulness as a tool for genetic engineering of salt-stress protection.

From a commercial aspect, parthenocarpy is a cost-effective solution to improve fruit set. Moreover, the seedlessness of fruits can increase consumer acceptance. In cucumber the pDefH9::*iaaM* construct was successfully introduced into the genome and 70—90% of the fruits produced by the transgenic lines were parthenocarpic (Yin et al. 2006a).

## 25.2.5 Asteraceae

### 25.2.5.1 *Lactuca sativa* L.

Lettuce (*Lactuca sativa* L.) is a major fresh vegetable and is becoming increasingly more important in Europe in the convenience area, e.g. salad mixtures. In Egypt and Asian countries lettuce stems and leaves are consumed in dishes of various kinds, in cooked, raw, pickled or dried form (Ryder 1986). Lettuce belongs to the family Asteraceae, with approximately 100 species of *Lactuca*. Only the four species *L. sativa* L., *L. serriola* L., *L. saligna* L. and *L. virosa* represent the important breeding pool. They are self-fertilized diploids and can be crossed with each other. Modern lettuce breeding is geared towards the areas of disease/insect resistance, improved quality and increased yield.

First, Michelmore et al. (1987) transferred a *nptII* gene for kanamycin resistance using *A. tumefaciens*. Chupeau et al. (1989) transformed lettuce protoplasts with the *nptII* gene using electroporation. Later an iceberg lettuce was successfully transformed with the reporter gene *gus* (Torres et al. 1993). Today transformation using *A. tumefaciens* has become routine in lettuce.

Herbicide-resistant transgenic lettuce was reported by several authors using the *bar* gene (McCabe et al. 1999; Mohapatra et al. 1999) and a glyphosate oxidase gene (*GOX*; Torres et al. 1999; Nagata et al. 2000).

Plants transformed with genes encoding enzymes that hydrolyse fungal cell walls, such as the  $\beta$ -1,3-glucanase from *Arthrobacter* spp. (Dede 1998) or an oxalate decarboxylase gene from edible mushroom (Dias et al. 2006), showed increased resistance against downy mildew (Dede 1998) and *Sclerotinia sclerotiorum* (Dias et al. 2006).

The virus coat protein strategy was successfully applied to enhance resistance to the *Lettuce mosaic virus* (LMV; Dinant et al. 1993, 1997, 1998; Gilbertson 1996) and the *Lettuce big vein associated virus* (LBVaV) and the *Mirafiori lettuce virus* (MLV; Kawazu et al. 2006). A transferred nucleocapsid protein gene of the lettuce isolate of *Tomato spotted wilt virus* (TSWV) increased the resistance to TSWV (Pang et al. 1996) and *Lettuce infectious yellow virus* (LIYV; Falk 1996).

A proteinase inhibitor (*PIN2*) gene from *Solanum americanum* Mill. was used to generate resistance to cabbage looper caterpillars (*Trichoplusia ni* Hübner; Xu et al. 2004; Chye et al. 2006; Xie et al. 2007).

Male sterility (see also Chap. 14) as prerequisite of hybrid breeding could be induced by expressing a  $\beta$ -1,3-glucanase gene linked with a tapetum-specific promoter, resulting in the dissolution of the callose wall during the microsporogenesis (Curtis et al. 1996b).

Another research area is designed to influence plant physiology and tolerances to environmental stress. Lettuce engineered with genes coding enzymes of the proline biosynthesis resulted in salt- and temperature-tolerant plants (Curtis et al. 1996a; Pileggi et al. 2001). Overexpression of an *Arabidopsis ABF3* gene (Vanjildorj et al. 2005),

or the late embryogenesis abundant protein (*LEA*) gene from *Brassica napus* (Park et al. 2005c) enhanced cold, salt and drought tolerance, too.

A number of examples for the transgenic improvement of horticultural and nutritional quality were reported, especially in the past decade, such as monellin or miraculin synthesis for changes in flavour components (Penarrubia et al. 1992; Sun et al. 2006), increased tocopherol (Cho et al. 2005; Lee et al. 2007a), iron and Ca content (Goto et al. 2000; Park et al. 2009), or the anthocyanin biosynthesis (Park et al. 2008).

Analogous to other crops, pharmaceuticals could be an interesting area for application of genetic engineering in lettuce. Reports so far include the transfer of genes coding the cholera toxin B protein (Kim et al. 2006; Ruhlman et al. 2007), a measles virus hemagglutinin (Webster et al. 2006), an antigen of the hepatitis B virus (Kapusta et al. 1999, 2001; Kawashima et al. 2001) or a human intestinal trefoil factor (Zuo et al. 2001). Further potential applications for oral animal vaccinations were tested, such as against the *Swine fever virus* (Legocki et al. 2005) or the *Vesicular stomatitis virus* of poultry (Song et al. 2008).

Contrary to the high input in transgenic research, transgenic lettuce has not been commercialized so far.

#### 25.2.5.2 *Cichorium intybus* L., *C. spinosum* L., *C. endivia* L.

*Cichorium intybus* L. (chicory, radicchio) is cultivated as biennial crop widespread in Europe and the world, whereas *C. endivia* L. and *C. spinosum* L. are annuals predominately grown in Europe and North Africa.

First, Sun et al. (1991) reported *A. rhizogenes*-mediated transgenic *C. intybus* which was converted from biennial to annual flowering. Later Genga et al. (1994) and Abid et al. (1995) described the transfer of *gus* gene to radicchio, using *A. tumefaciens*. Herbicide resistance was engineered by an acetolactate synthase gene from *A. thaliana* (Vermeulen et al. 1992; Lavigne et al. 1995). Herbicide resistance is of economic interest because the growth rate of the chicory seedlings in the field is low and fast-developing weeds can suppress them.

A transgenic approach to engineer male sterility as a prerequisite for hybrid breeding was developed and first demonstrated by Mariani et al. (1990, 1992). Next, Bejo Zaden B.V. (The Netherlands) engineered male sterile chicory and radicchio, using a chimeric gene construct of *barnase* gene from *Bacillus amyloliquefaciens*, a tapetum-specific promoter and the selective marker gene *bar*. Bejo received the license to produce F<sub>1</sub> hybrids of chicory and radicchio in 1995; however the licence is not longer valid. Another request for the authorization of salad and GM chicory or radicchio was withdrawn. Today the marketing of these GM vegetables is not allowed in the European Union (EU).

Other approaches focused on metabolic engineering. Transgenic chicory with a 6G-fructosyltransferase from onion (Vijn et al. 1997) or barley (Sprenger et al. 1997) synthesized fructan of the inulin neoseries or branched fructans of the graminan type, respectively. Both may be interesting as potential functional food for diet or in diabetic therapy.

### 25.2.6 Apiaceae (*Daucus carota L.*)

The family Apiaceae contains approximately 113 cultivated species distributed worldwide. About 21% are used as vegetables, but only carrot, celery and fennel with greater commercial importance (Rubatzky et al. 1999; Pistrick 2002).

Carrot has been extensively studied as a model species for tissue culture, plant somatic embryogenesis and protoplast fusion (Ammirato 1986) and was therefore predestined for transformation approaches. The first transgenic carrots were reported after *A. rhizogenes* infection by Tepfer (1984). Shortly after, Langridge et al. (1985) obtained transgenic plants by electroporation of suspension protoplasts with naked DNA. Later, transgenic plants were obtained by *A. tumefaciens* infection of various carrot plant explants and cells (Scott and Draper 1987; Thomas et al. 1989; Wurtele and Bulka 1989).

Herbicide resistance was first introduced into carrot via direct gene transfer of the *pat* gene (Dröge et al. 1992; Drogelaser et al. 1994). Chen and Punja (2002) introduced the *bar* gene and Aviv et al. (2002) a mutant acetolactate gene (ALS) from *Arabidopsis thaliana* causing resistance to herbicide Imazapyr.

A number of genes have been introduced to enhance resistance to fungal pathogens, such as chitinases, glucanases, thaumatin-like protein, osmotin and lysozyme. Resistance has been engineered by using chitinases cloned from petunia and tobacco (Linthorst et al. 1990), from beans (Broglie et al. 1991) or from *Trichoderma harzianum* (Baranski et al. 2008). A thaumatin-like protein from rice was expressed in carrot and showed enhanced tolerance to six fungal pathogens (Chen and Punja 2002; Punja 2005). Transgenic carrots with the tobacco osmotin (AP24) in combination with a chitinase and a glucanase gene also expressed broad-spectrum tolerance (Tigelaar et al. 1996; Melchers and Stuiver 2000). Carrot lines which constitutively expressed a human lysozyme showed enhanced resistance to *E. heraclei* and *A. dauci* (Takaichi and Oeda 2000). The microbial factor (MF3) from *Pseudomonas fluorescens* enhanced the resistance to *Alternaria* sp. and *Botrytis cinerea* (Baranski et al. 2007).

An interesting field is the production of biopharmaceuticals. A number of transgenic carrots have been engineered to produce proteins or potential human vaccines, such as enterotoxin (LTB) against cholera and diarrhea (Rosales-Mendoza et al. 2008), the *MPT64* gene of *Mycobacterium tuberculosis* (Wang et al. 2001), the major hepatitis B virus surface protein (Imani et al. 2002), an immunodominant antigen of the measles virus (Bouche et al. 2003, 2005; Marquet-Blouin et al. 2003) and glutamic acid decarboxylase (GAD65) as an autoantigen in autoimmune type 1 diabetes mellitus (Porceddu et al. 1999; Avesani et al. 2003).

Currently two approaches focus on functional foods or nutraceuticals. It was demonstrated that transgenic carrots expressing the *Arabidopsis* H<sup>+</sup>/Ca<sup>2+</sup> transporter *CAX1* increase their calcium content up to 50% compared with the control. Enhancing the concentration of bioavailable calcium in vegetables could prevent calcium malnutrition and reduce the incidence of osteoporosis (Park et al. 2004b). Furthermore, carrots have been engineered into the ketocarotenoid biosynthetic

pathway by introducing a  $\beta$ -carotene ketolase gene from the alga *Haematococcus pluvialis*. Transgenic carrots converted up to 70% of total carotenoids to novel ketocarotenoids, showing that carrots are suitable for applications to the functional food, nutraceutical and aquaculture industries (Jayaraj et al. 2008; Jayaraj and Punja 2008).

Transgenic plants have also been obtained in celery (*Apium graveolens* L.; Catlin et al. 1988) and caraway (*Carum carvi* L.; Krens et al. 1997). Both papers describe the establishment of an *Agrobacterium*-mediated transformation protocol, at the moment only of academic value.

At the present time, there are no transgenic carrot cultivars or other Apiaceae commercially available on the market.

### 25.2.7 *Chenopodiaceae* (*Spinacia oleracea* L.)

Spinach (*Spinacia oleracea* L.) is one of the most nutritious vegetables, due to a high content of  $\beta$ -carotene and folate; furthermore it is a rich source of vitamin C, calcium, iron, phosphorous sodium and potassium. Current breeding is mainly focused on a number of pests, bacterial and fungal diseases and viruses, as well as on improved nutrition. To increase the resistance level, particular emphasis is given to biotechnological approaches.

The first transformed spinach was reported by Al-Khayri (1995) after introduction of the *gus* gene. Other researchers used these protocols to engineer spinach that carried the coat protein gene for the *Cucumber mosaic virus* (Yang et al. 1997), the *nptII* and *gfp* gene (Zhang and Zeevaart 1999), or the gene for glyphosate tolerance (Wells 1999; Bevitori 2000; Burgos et al. 2001).

No transgenic plants have been commercialized so far.

### 25.2.8 *Liliaceae*

#### 25.2.8.1 *Allium cepa* L., *A. porrum* L., *A. sativum* L.

The onion (*Allium cepa*) and its close relatives leek (*A. porrum*) and garlic (*A. sativum*) are very important vegetable crops on a worldwide scale. As monocotyledons, *Allium* species have proven to be recalcitrant to in vitro regeneration and genetic engineering (Eady 1995; Eady et al. 1996; Barandiaran et al. 1998). So it took until 2000, when Eady et al. (2000) published the first repeatable protocol for the production of transgenic *A. cepa* plants, followed by a successful garlic transformation (Kondo et al. 2000). The latter is of particular interest, because garlic breeding has been limited to the clonal selection of wild varieties or mutants, due to the loss of fertile flowers.

Transgenic onion plants tolerant to herbicides (see Chap. 9) containing glyphosate or posinotrothricin were recovered by Eady et al. (2003a). The same group

(Eady et al. 2003b) demonstrated that the integration and expression of foreign genes are essentially not different to the Mendelian fashion. The results suggest that the herbicide resistance transformed in elite onion germplasm is expressed and inherited in such a way that it will have a normal agronomic function.

With respect to the beet armyworm (*Spodoptera exigua* Hübner), the most important pest in *Allium* cultivation for (sub)tropical zones, a transgenic pest management strategy seems to be the only way to overcome this problem. Garlic and shallot plants (Zheng et al. 2004, 2005) have been engineered with synthetic *Bt* gene. The produced transgenic *A. cepa* plants grew well in the greenhouse, had a normal phenotype, produced bulbs and were completely resistant to the beet armyworm (Zheng et al. 2005).

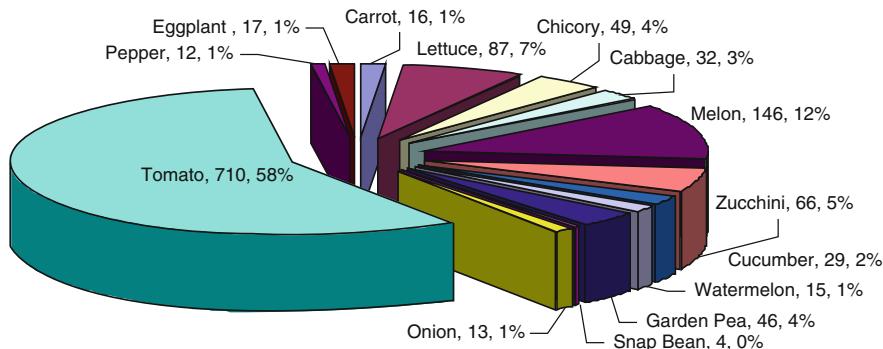
#### 25.2.8.2 *Asparagus officinalis* L.

Transgenic asparagus (*Asparagus officinalis* L.) was successfully achieved by *A. tumefaciens*-mediated transformation (Delbreil et al. 1993; Limanton-Grevet and Jullien 2001), microprojectile bombardment (Cabrera-Ponce et al. 1997; Li and Wolyn 1997) and electroporation of protoplasts (Mukhopadhyay and Desjardins 1994). In most experiments the *nptII* marker gene and the *gus* reporter gene were transformed and expressed. Additionally, transgenic asparagus with the *bar* gene was reported by Cabrera-Ponce et al. (1997). A commercial application is not known.

### 25.3 Conclusions

The commercial applications of genetic engineering technology to vegetables lag far behind those of agricultural crops. As the global acreage of transgenic agricultural crops has expanded dramatically since their introduction in 1996, it is paradoxical that the trend in vegetables is the opposite.

Within the past 15 years alone in the United States and the EU, over 1240 transgenic field trials for vegetables have been documented (Fig. 25.1). Although the number of trials is indicative of who is working on what vegetable, it does not accurately reflect the absolute activity. On the trial number basis, tomato accounts for over half. Transformation technology is potentially an effective tool for vegetable breeding in fields that are not easily accessible by conventional breeding techniques. Nevertheless no more commercial utilization is expected in the near future in Europe or the United States. Only a few GM cultivars are licensed for different countries, such as tomato, zucchini, chicory and eggplant. Despite the transgenic zucchini cultivation in the United States on probably 10 000 ha, no market launch is expected in the EU. In China, GM peppers are supposed to be cultivated. However, reliable information is not yet available, because a lot of the research is being done in the private sector. Commercial utilization of *Bt*-eggplants



**Fig. 25.1** Deliberate releases of GM vegetables into the environment for field trials (1992–2007). Data are presented as: vegetable name; number of field trials worldwide; percentage (sources: <http://www.transgen.de>, <http://www.gmo-compass.org>, [http://usbiotechreg.nbii.gov/database\\_pub.asp](http://usbiotechreg.nbii.gov/database_pub.asp), <http://www.agbios.com>)

in India and the Philippines will start in 2009; and the use of GM garden peas is expected in the medium term.

For the whole complex of engineering disease and pest resistance, as well as abiotic stress tolerance, a lot of reports are available. It could be assumed that in the future transgenic methods will be increasingly used for that purpose, due of the growing awareness of the problems connected with the global climate changes.

While the first transgenic vegetables were strongly tailored to the needs of the producers, incentives are needed to share the benefits. Vegetables with clear benefits for the consumers are needed to develop demand. Although technically more difficult, there are many potential opportunities for enhancing the nutritional value or consumer appeal of vegetables through genetic engineering. In addition to modification of flavour, research projects to increase the content of vitamins, minerals or nutraceuticals in vegetables are in progress. Despite the fact that transformation is a powerful approach to plant improvement, the major impediment to genetically engineered vegetables is the reluctance of the consumer and subsequently the market.

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**Part D**

**Risk Assessment and Economic  
Applications**



# **Chapter 26**

## **Regulatory Oversight and Safety Assessment of Plants with Novel Traits**

**Yann Devos, Karine Lheureux, and Joachim Schiemann**

### **26.1 Introduction – From Foragers to Genetic Modification in a Genomic Era**

Because plants are a fundamental constituent of the human diet, either as a direct source of nutrients, or indirectly as feed for animals, scientific and technical advances in agriculture have played a crucial role in ensuring food security, in meeting feedstock demands, and in providing various benefits to society at large. Over the past few centuries, techno-scientific progress in agriculture has improved the reliability and quality of the world food and feed supply, allowing fewer farmers to feed more and more people, with less labour. The whole package of genetically improved plants, irrigation, fertilisers, pesticides and tillage operations has revolutionised agriculture.

Throughout the history of plant breeding, various plant breeding techniques have been used to develop and select new gene combinations for improving the performance of plants (Moose and Mumm 2008). Initially, wild plants with useful characteristics were gathered and cultivated. In this domestication process, preferential characteristics observed in phenotypes of wild plant individuals or spontaneous variants were selected and reproduced. This gradual evolution allowed former foragers to increasingly control when, where, and in what quantities food plants were grown, rather than to depend upon vagaries of nature. This evolution went together with the adoption of more sedentary lifestyles.

Through the selection of observable phenotypes, farmers and plant breeders have been modifying the underlying genotype of plants to adapt them to human needs (e.g., more favourable characteristics in terms of yield and agronomic,

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nutritional, and/or processing quality). By performing wide crosses and extensive backcrosses, genetic variation has been increased, whilst novel traits have been introgressed into food crops. More recently, plant biotechnology-based breeding techniques such as plant cell and tissue culture have helped plant breeders in obtaining fertile generations from normally sterile crossings, which has enabled the transfer of desired traits between crops and more distantly related plant species. The problem of desired traits lacking within a plant species or within distantly related plant species has been overcome through chemical and irradiation mutagenesis, as both techniques induce mutants that might have the desired characteristics. Chemicals have been used not only to generate new mutations, but also to double the number of chromosomes of plants, in turn facilitating the hybridisation of plants that would not naturally cross-breed. With the fusion of protoplasts, somatic hybridisation between sexually incompatible individuals also has become possible.

Since the 1980s, genetic engineering has provided a new means of generating genetic variation, which simultaneously overcomes the barrier of species. Genetic engineering allows inserting a well identified and subsequently isolated gene into the plant genome, in turn generating genetically modified (GM) plants with the desired trait (Akhond and Machray 2009). Due to the large pool of possible genes available to plant breeders, genetic engineering offers promising opportunities for the incorporation of genes into crop plants. Genes with desired characteristics no longer have to belong to the same species as the recipient, but can come from biologically unrelated species. For general aspects related to the development and analysis of GM plants see Chaps. 1–7. Characteristics of GM plants are covered in Chaps. 8–14, and specific GM plants in Chaps. 15–25.

With molecular markers, molecular biology has provided another biotechnology-based technique to support plant breeding programmes. These markers have increased knowledge of and ability to characterise genetic diversity in the germplasm pool for many crop plants. Marker-assisted selection and backcrossing have facilitated the direct selection of target genes in individuals or populations as molecular markers are located in or are very closely linked to genes expressing desired traits. Because genes can be selected, either directly or indirectly, the selection of desired traits is no longer solely carried out through observable phenotypes, but is also becoming genotype-based. In the current era of gene sequencing, mapping, molecular genetics and genomics, the knowledge about plant genes, genomes, and their biological functions is continuously being increased and refined.

Further progress in plant breeding to continue contributing to the needs of an ever-expanding human population, while keeping pace with the decreasing area of available arable land and water, might be achieved by the association of plant breeding based on experimental field work with biotechnology-based techniques (Moose and Mumm 2008). Because both approaches complement one another, their association might offer promising possibilities for agriculture, nutrition, industry, and even for medicine. In this respect, the next generation of GM plants, which is currently under study or in the developmental pipeline, provides a first glance at the anticipated possibilities (Chapotin and Wolt 2007; Akhond and Machray 2009). These developments in plant breeding have had and will continue to have

implications for the regulatory oversight and risk assessment of plants with novel traits. Though the introduction of novel traits, including the stacking of several genes (Halpin 2005; De Schrijver et al. 2007; EFSA 2007), may result in extensive changes of metabolic pathways, composition, toxicity, nutritional value, environmental impact, etc., a rigorous risk assessment is only required for GM plants in the European Union (EU). Whether this approach is science-based or not is discussed by the scientific community (EFSA 2008). In this chapter, implications for the regulatory oversight and risk assessment are addressed in more detail for GM plants.

## 26.2 Regulatory Oversight of GM Plants and Their Derived Food and Feed Products

### 26.2.1 *Process-Based Versus Product-Based Approach*

In Europe, a *process-based* system was put in place for the regulation of genetically modified organisms (GMOs) as the breeding techniques used for their production were considered new and raised specific safety concerns. A GMO is thus mainly characterised by the breeding techniques used to produce it and is defined as an organism in which the genetic material has been altered in a way that does not occur naturally by crossing and/or natural recombination (EC 2001). Breeding techniques falling under the EU GMO definition are:

1. Recombinant nucleic acid breeding techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation
2. Breeding techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation
3. Cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally

In vitro fertilisation, natural transformation processes (e.g., conjugation, transduction, transformation), and polyploidy induction are currently excluded from the GMO definition.

In the United States (US) and Canada, a *product-based* approach is followed for the regulation of GMOs (Macdonald and Yarrow 2003; McHughen and Smyth 2008; Smyth and McHughen 2008). Legislation focuses on the risks of products, and not the breeding techniques of production, as genetic engineering per se is not considered inherently risky. Because the focus is on novel traits or attributes

introduced into a plant, rather than the method of production, plants and their derived food and feed products are regulated under the existing regulatory system.

### **26.2.2 Regulatory Framework for GMOs in the EU**

In the early 1990s, two European Directives for the use of GMOs were adopted to ensure the protection of human and animal health and the environment, and to guarantee consumers' freedom of choice without misleading consumers/users. Directive 90/219/EEC, which has been amended by Directive 98/81/EEC, regulated the contained use of GM micro-organisms, whilst Directive 90/220/EEC regulated the deliberate release of GMOs into the environment, covering both the release for research purposes (part B) and for commercial use as or in products (part C). This triad reflects the stepwise process GM plants go through, beginning with experiments under contained use (e.g., laboratory, greenhouse), through experimental release, up to the placing on the market. According to the step-by-step principle, the containment of GMOs can be reduced and the scale of release increased gradually, if assessment of earlier steps indicates that the next step can be taken.

On 15 May 1997, Regulation (EC) No 258/97 – the so-called *Novel food regulation* – removed food products derived from GM plants from the *Deliberate release directive's* scope. Regulation (EC) No 258/97 covered risk assessment procedures, marketing, and labelling of all types of novel food products, including those produced by new plant breeding techniques such as genetic engineering, as well as food without a history of safe use in the EU.

On 17 October 2002, Directive 2001/18/EC replaced (the older) Directive 90/220/EEC. With it, the precautionary principle was explicitly adopted as a guide, risk assessment criteria were broadened to include direct, indirect, immediate, delayed, and cumulative long-term adverse effects, post-market environmental monitoring (PMEM) became obligatory, the need for a common methodology for the environmental risk assessment was established, an additional rigorous risk assessment of antibiotic resistance marker genes was introduced, the existing labelling provisions applying to GM food were extended to all marketed products containing GMOs, the general concept of traceability at all stages of commercialisation was introduced, the transparency in the decision-making process was increased, the consultation of the public became mandatory in the authorisation procedure, the possible consultation of an ethics committee was confirmed, and the implementation of national cultivation registers was required, recording the locations where GM plants have been grown.

Adding to Directive 2001/18/EC, Regulation (EC) No 178/2002 laid down general principles of food law and procedures in food and feed safety. With this regulation, the application of the precautionary principle was further extended to risk analysis of all food and feed products in the EU, whether or not of GM-origin. In response to a multiple wave of food crises that caused considerable concerns in European publics about food safety and the ability of regulatory authorities to fully

protect consumers, the European Food Safety Authority (EFSA) was created as a European-wide risk assessment body. By providing ‘independent, objective, and transparent’ science-based advice, EFSA aims to ensure a high level of consumer protection and to restore and maintain confidence in the EU food supply.

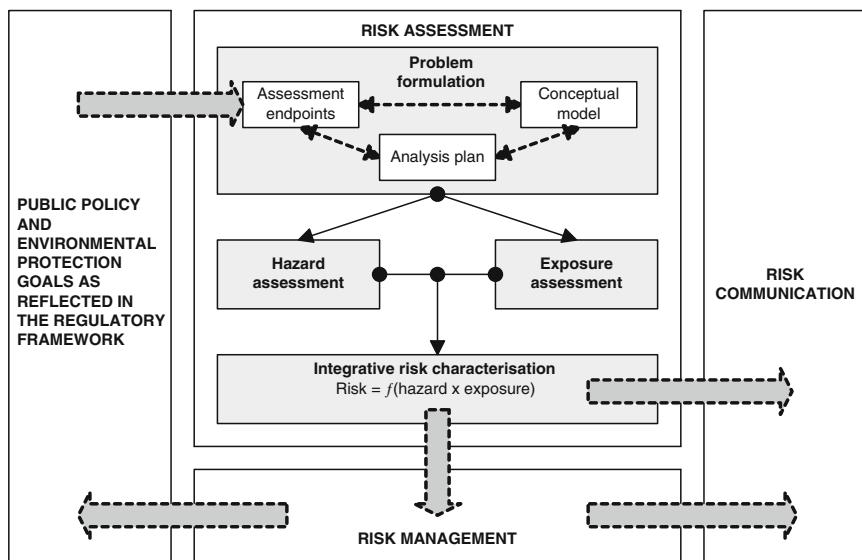
Issued on 18 April 2004, Regulation (EC) No 1829/2003 on GM food and feed covers the commercialisation and risk assessment of GM food and feed, such as food/feed containing or consisting of, food/feed produced from, and food/feed containing ingredients produced from GMOs, as well as seed-propagating material. Prior to this date, approvals for human food use were required under the *Novel food regulation*, whereas feed use was assessed under Directive 2001/18/EC and its predecessor. The amended approval procedure is centralised around EFSA and based on a ‘one door—one key’ approach whereby all commercial uses can be covered in a same GM crop market registration dossier. Moreover, it also introduces the need for a GM crop market registration dossier to cover both food and feed uses, as it avoids market approval for a single use in case a product is likely to be used both for food and feed uses (e.g., Demeke et al. 2006). Regulation (EC) No 1830/2003 complements, clarifies, and makes operational some of the labelling and traceability objectives of previous legislation.

In addition, based on Regulation (EC) No 1946/2003, transboundary movements of GMOs across the EU and elsewhere are being established in accordance with the international obligations of the *Cartagena protocol on biosafety* (see Chap. 29).

## 26.3 Risk Assessment Principles

### 26.3.1 *Interplay of Risk Assessment, Risk Management and Risk Communication*

GMOs and their derived food and feed products are generally subjected to a risk analysis before they can be commercialised (Craig et al. 2008; Paoletti et al. 2008). In the EU, the risk analysis consists of three components: risk assessment, risk management and risk communication (Fig. 26.1). In risk assessment, potential adverse impacts associated with a specific activity are scientifically characterised on a case-by-case basis, whilst in risk management, policy alternatives to accept, minimise or reduce the characterised risks are weighed and, if needed, appropriate prevention and control options are selected. Because risk managers and regulators rely on risk assessments to make an informed decision on whether or not to approve a certain use of a GM plant, it should explain clearly what assumptions have been made during the risk assessment, and what is the nature and magnitude of uncertainties associated with the characterised risks. The decision whether a certain risk is acceptable and/or tolerable under a particular set of conditions is not part of the risk assessment itself, as this choice is not only based on scientific criteria, but also involves political, social, cultural and economic considerations. Theoretically, there is a functional and temporal separation between risk assessment and risk



**Fig. 26.1** Risk analysis. The diagram depicts the main components of risk analysis and the successive steps comprising the environmental risk assessment of GM plants

management in order to reduce any conflict of interest and to protect the scientific integrity of risk assessment (Johnson et al. 2007). Risk communication is defined as an interactive exchange of information and opinions on risk throughout risk analysis, running between risk assessors, risk managers and other interested parties. It includes the explanation of risk assessment findings and of the basis on which risk management decisions are made (EFSA 2006a).

Even though there are considerable differences between countries in regulatory requirements for GM plants, environmental priorities (including the preservation of biodiversity) as well as risk terminology, most risk assessments of GM plants follow a science-based assessment process that estimates the level of risk through comparison with a non-GM counterpart (Hill 2005; Paoletti et al. 2008). In addition, regulatory requirements involve consideration of a range of issues relevant to the overall risk assessment in order to determine the impact of the GM plant on human/animal health and the environment relative to the non-GM plant, and thus its relative safety (Conner et al. 2003; Craig et al. 2008). Some of these elements are discussed in the next section.

### 26.3.2 Risk Assessment Methodology and Terminology

Despite the considerable variation among risk assessment frameworks for GM plants regarding risk assessment steps, risk assessment generally comprises several sequential steps (Fig. 26.1): (i) problem formulation as critical first step, (ii) hazard

assessment that examines potential hazards and their magnitude, (iii) exposure assessment that covers levels and likelihood of exposure, and (iv) integrative risk characterisation in which the magnitude of consequences and the likelihood of occurrence are integrated (EFSA 2006a). In the EU, the consideration of mitigation options such as PMEM is not included as a fifth step in the risk assessment framework, as risk assessment is kept separate from risk management (Hill 2005). The terms hazard and risk are often interchangeably used in the EU (see e.g., Johnson et al. 2007), but have different meanings. The term hazard is associated with the potential of an agent or situation to cause adverse effects. It refers to an inherent property of that agent or situation. Risk is recognised as a function of the probability and severity of an adverse effect occurring to human and animal health or the environment following exposure to a hazard, under defined conditions.

### **26.3.3 Problem Formulation**

In order to identify the areas of greatest concern or uncertainty related to risks, each risk assessment begins with the identification and formulation of the problem, usually in the context of regulatory decision-making (Hill and Sendashonga 2003). In this respect, the most important questions to be solved and merit detailed risk characterisation are identified (Wolt et al. 2009).

On the one hand, the problem formulation phase involves defining assessment endpoints, which are explicit and unambiguous targets for protection extracted from public policy goals. On the other hand, it involves the development of a methodology that will help to direct the risk characterisation and to produce information that will be relevant for regulatory decision-making. This is generally done on the basis of a conceptual model and an analysis plan (EPA 1998, Hill and Sendashonga 2003; Raybould 2006, 2007; Nickson 2008; Romeis et al. 2008; Storkey et al. 2008; Wolt et al. 2009).

The information that is considered during problem formulation takes many forms, including published scientific literature, expert opinions, stakeholder deliberations, and data generated during product development by applicants and submitted to the regulatory authority as part of market registration dossiers (Romeis et al. 2008). As such, existing knowledge of the system (plant/stressor/environment/hazard/exposure) is summarised during the problem formulation. This means that, when the level and quality of the available information is high, the risk assessment can build on existing knowledge, in turn reducing the number of risk hypotheses that will need to be tested for risk characterisation. In the following, we focus on environmental risk assessment of GM plants.

#### **26.3.3.1 Assessment Endpoints**

To allow regulatory decision-making, assessment endpoints should be defined as far as possible using measurable criteria relevant to the casus under study, so that

change in these endpoints can be identified. If protection of biodiversity is a public policy goal, a typical assessment endpoint is the abundance and species richness of certain groups of organisms at a relevant life stage within a landscape or region (Romeis et al. 2008).

Assessment endpoints are operationally defined by an ecological entity (i.e., arthropod natural enemies) and attributes of that entity (i.e., regulation of arthropod pest populations) that could potentially be impacted by the GM plant or its associated farm management practice (stressor) and that require protection from harm (Suter 2000). It is not an abstract goal such as ecosystem health or sustainability, but a real, operationally definable property of a component of the environment that reflects management or protection goals set by public policy. Because arthropod natural enemies fulfil relevant ecological functions by contributing to the natural regulation of arthropod pest populations within crop fields in agricultural landscapes, they can be identified as the entity to be preserved with the biological control functions they perform as attribute (Sanvido et al. 2008).

Once assessment endpoints have been set, the environmental quality to be preserved needs to be defined (limits/thresholds for concern, trigger values, decision criteria), as it enables defining and identifying the level of difference between the GM plant and its comparators that may lead to harm and trigger regulatory concern. This process includes defining the magnitude and both the spatial and the temporal scales relevant for the entity and the attribute to be preserved. The magnitude describes to what extent the environmental quality should be preserved (or above what threshold a change would be considered a disturbance in environmental quality). The spatial and temporal scales are the habitats in which the environmental quality and the period during which the environmental quality should be preserved, respectively (Sanvido et al. 2008; Storkey et al. 2008).

### 26.3.3.2 Conceptual Model

The conceptual model describes the consequential exposure scenario of how harm to the assessment endpoint (valued entity) may arise from GM plant deployment and this in a way that allows for a characterisation of risks. Thereby, key relationships are described between the GM plant, the valued entity, pathways of exposure through which the GM plant may affect the valued entity either directly or indirectly (= exposure profile), and potential impact of the GM plant to the environment (Wolt et al. 2009). The conceptual model includes the available information on the nature of the stressor, its proposed use (including the intended scale of cultivation), reasonable exposure profiles, and potential responses of the assessment endpoint as a result of exposure.

A well structured conceptual model in which the components of the system are detailed will allow the identification and formulation of relevant risk hypotheses that arise from the consideration of potentially significant risks. These risk hypotheses are necessary to make assumptions and predictions about how a stressor could affect an assessment endpoint (Raybould 2006; Nickson 2008). It is important to

bear in mind that risk hypotheses are not null hypotheses, but rather proposed answers to reasonable questions about how the assessment endpoint(s) will respond to the stressor(s) (Raybould 2007; Nickson 2008; Storkey et al. 2008). Within the analysis phase of the environmental risk assessment, risk hypotheses are translated into one or more rigorous statistical hypotheses which are amendable to testing and corroboration (Wolt et al. 2009). Conceptual models can take an array of forms going from simple statements towards complex flowcharts and diagrams.

### 26.3.3.3 Analysis Plan

The last step of the problem formulation comprises an analysis plan in which decisions are made about the most appropriate way to measure the response of each assessment endpoint to GM plant deployment. In this planning phase, data needed and the approach to be taken for data acquisition and synthesis are delineated in order to test risk hypotheses formulated in the conceptual model. Reasonable scenarios are placed in an analysis plan by describing and selecting (i) the various measures to be used (measurement endpoints) in the assessment and subsequent risk characterisation, and through the description of (ii) methods and criteria for measurement.

Measurement endpoints define the indicator of change that will actually be recorded as part of a comparative risk assessment study (Storkey et al. 2008). These endpoints usually constitute estimates of *exposure* or *hazard*. Measures of exposure cover properties of the GM plant and are described in terms of the route, frequency, duration, and intensity of exposure relative to the valued entity (Wolt et al. 2009), whilst measures of hazard represent the measurable change to the valued entity in response to a changed attribute (e.g., transgenic protein) of the GM plant to which it is exposed (Storkey et al. 2008). Measures of hazard may be an acute lethal concentration resulting in the death of 50% of the organisms tested ( $LC_{50}$ ), or a chronic *no observable adverse effect level* (NOAEL) measured for the valued entity (Wolt et al. 2009). The way the exposure measurement relates to the hazard measurement is described in the risk formulation.

Once specific measurement endpoints are chosen and given a priority, appropriate methods and criteria of measurement are selected and described in the analysis plan (EPA 1998). This includes information on studies to be conducted, the appropriate tier for analysis, the design of protocols, and statistical power (Marvier 2002; Lövei and Arpaia 2005; Romeis et al. 2008; Storkey et al. 2008). The selection and prioritising of both measures to be used and testing needed enable to allocate human and financial resources in a proper way (Qi et al. 2008), so that only essential data for risk characterisation are collected (Raybould 2006). It is important to realise that for practical reasons not all potentially exposed terrestrial arthropods can be considered for regulatory testing (Romeis et al. 2008). Therefore, it is necessary to select appropriate species that can be tested effectively under laboratory conditions or that are available in sufficient numbers in the field to give statistically meaningful results (Gathmann et al. 2006; Todd et al. 2008).

This selection of species is based on several criteria: ecological relevance, susceptibility to known or potential stressors (sensitivity and exposure), anthropocentric value that is usually defined in public policy through management goals, and testability (Todd et al. 2008). The environmental risk assessment may also consider species with special aesthetic or cultural values or species of conservational importance and that are classified as threatened or endangered. The number and type of species that are to be tested will depend upon the risk hypotheses generated during the conceptual model.

The information from the problem formulation and the processes described above is the crucial starting point for risk assessments, as it enables detecting effects that indicate a potential risk in a structured and logic way. Having a properly constructed analysis plan based on a conceptual model that is clearly linked to assessment endpoints helps to guide the collection of data that are relevant to demonstrate the safety of a GM plant. Moreover, it helps to make the risk assessment process comprehensive by summarising existing knowledge of the system under study and transparent by explicitly stating significant assumptions underlying the risk assessment, and ultimately regulatory decision-making. In contrast, poor problem formulation in risk assessments may fail to identify the most important questions to be solved and can lead to the collection of data that might be irrelevant for demonstrating the safety of a GM plant (Raybould 2006).

### **26.3.4 Risk Assessment Principles and Concepts**

Several principles and concepts are to be considered during the risk assessment of GM plants. Risk assessment of GM plants should: (i) be science-based where quantitative information is available and use qualitative information in the form of expert judgment, (ii) use a comparative approach, (iii) be case-specific, (iv) be iterative and examine conclusions already made based on new information, and (v) follow a tiered approach.

#### **26.3.4.1 Comparative Risk Assessment and Familiarity Concept**

According to the comparative risk assessment concept, the importance of risks posed by a GM plant is placed in the context of risks posed by current non-GM comparators (e.g., non-GM recipient or parental organism). As such, differences between the GM plant and comparator are established. The underlying assumption of this comparative assessment approach for GM plants is that traditionally-bred plants have a history of safe use for the consumer or animals and the environment, and familiarity for the consumer. The concept of familiarity is based on the fact that most GM plants are developed from crop plants, the biology of which is well known. The knowledge about the non-GM plant, gained through experience over time, can therefore be used in a risk assessment to establish differences associated

with the genetic modification and the subsequent management of the GM plant. According to the Organisation for Economic Co-operation and Development (OECD), familiarity is derived from the knowledge and experience available from conducting a risk analysis prior to scale-up of any new plant line or crop plant variety in a particular environment, and from previous market registration dossiers for similar constructs and traits in similar or different crop plants (OECD 1993). However, it is important to bear in mind that familiarity is not an endpoint in risk assessment and does not necessarily mean safety. If differences between the GM plant and comparator have been identified, it needs to be defined whether these differences have any significance for the assessment endpoints (Raybould 2007).

#### **26.3.4.2 Case-by-Case Principle**

According to the case-by-case principle, the source and target environments, biological and ecological characteristics of a GM plant, the scale and frequency of deliberate release, and the interactions among these elements should be considered when performing an environmental risk assessment (Andow and Zwahlen 2006; Garcia-Alonso et al. 2006).

#### **26.3.4.3 Iterative and Adaptive**

It is recognised that an environmental risk assessment (see also Chap. 27) is framed within the scientific knowledge available at the time it is conducted, and that regulatory decisions must be made acknowledging that these shortcomings may not be resolved. Therefore, under current EU legislation, it is recommended to describe these scientific uncertainties, which generally relate to possible cumulative and long-term risks due to the large-scale exposure of different environments to GM plants when grown at a larger scale over long periods (EFSA 2008). In this respect, PMEM of GM plants, which became mandatory under current EU legislation, allows for the collection of additional data during the commercialisation phase of a GM plant. The scientific knowledge derived from the monitoring of GM plants, experiences gained from their cultivation, and any other new knowledge (generated through, for instance, biosafety research) provide valuable information for risk assessors who will use this information for continually updating environmental risk assessments and reducing the remaining uncertainties.

PMEM of GM plants is mandatory in all market registration dossiers for deliberate release submitted under Directive 2001/18/EC and Regulation EC1829/2003, and aims at: (i) studying any possible adverse effects of the GM plant identified in the formal pre-market risk assessment procedure, and (ii) identifying the occurrence of adverse effects of the GM plant or its use which were not anticipated in the environmental risk assessment (Sanvido et al. 2005; EFSA 2006a; see also Chap. 27).

Risk assessments are always iterative in the sense that regulatory decisions are temporary, reversible, and adaptable in the light of new information that becomes available. Under Directive 2001/18/EC, the duty of re-examination has been strengthened by limiting the duration of market consent to a maximum period of ten years.

#### 26.3.4.4 Tiered Approach

An environmental risk assessment is generally conducted in a tiered manner, where information collected in lower tiers directs the extent and nature of the experimentation conducted in higher tiers. Thereby, both hazards and exposure are evaluated within different tiers that progress from worst-case scenario conditions framed in highly controlled laboratory environments to more realistic conditions in the field (Dutton et al. 2003; Wilkinson et al. 2003; Andow and Zwahlen 2006; EFSA 2006a; Garcia-Alonso et al. 2006; Bartsch et al. 2008; Nickson 2008; Romeis et al. 2008). The conclusion regarding potential risks drawn at each tier will lead to a regulatory decision after the residual uncertainty of the assessment has been defined or to additional investigations (Romeis et al. 2008). If a risk is identified, decision-making can consider whether risk management should be implemented to reduce risk. It is important that throughout the assessment, the problem being addressed remains appropriate and is revised if necessary.

Lower-tier tests serve to identify and test potential hazards under worst-case scenario conditions and thus involve conservative assumptions. By exposing target and non-target biota likely to be directly exposed to the GM plant or its products to high levels of the GM plant or its products, the likelihood increases for detecting potential adverse effects on these organisms. These studies are conducted under controlled laboratory or growth room conditions in order to quantify effects in relation to known exposure levels, to provide high levels of replication and control, and to increase the statistical power for testing the established hypotheses. Indirect effects of the GM plant on organisms not directly exposed to the GM plant, but are one or two steps behind in the food chain (e.g., predators and parasites of primary phytophagous or plant pathogenic organisms) are generally assessed in the second tier. Second-tier studies are also generally conducted under controlled laboratory, growth room or glasshouse conditions in order to measure effects in relation to known exposure levels (EFSA 2006a). If no hazards are identified and the GM plant is not different from the comparator, the tested product is regarded as safe.

However, in case potential hazards are detected in early-tier tests or if unacceptable uncertainties about possible hazards remain, additional information is required to confirm whether the observed effect might still be detected at more realistic rates and routes of exposure (EFSA 2006a; Garcia-Alonso et al. 2006; Bartsch et al. 2008; Nickson 2008; Romeis et al. 2008). Progression to larger-scale experiments in higher tiers aims to provide increasingly refined estimates of exposure. Field trials are then established in which the cultivation of the GM plant is conducted with greater environmental realism. As such, actual levels of exposure of different

biota can be quantified. In comparison with the comparator plant and its management, likely ecological adverse effects due to the GM plant and its management can be determined. While higher-tier studies offer greater environmental realism, they may have lower statistical power due to the higher variability of environmental conditions (e.g., climate) that can mask effects generated by the GM plant or its product (Bartsch et al. 2008). In exceptional cases, higher-tier studies may be conducted at the initial stage when early-tier tests are not possible or meaningful. As such, many risk assessments are conducted in a tiered manner, meaning that risk assessment studies increase in complexity depending upon the findings at each level of assessment (Hill and Sendashonga 2003). In cases where uncertainty about the risk remains after higher-tier studies, one can always return to lower tiers to conduct additional studies (Romeis et al. 2008).

The tiered approach is consistent with the iterative or adaptive nature of risk assessment where conclusions are reviewed when new information is obtained. As such, the uncertainty in risk assessment is reduced because each tier is guided by results obtained in the previous tier, and specific, testable, and relevant hypotheses are formulated based on these data (Andow and Zwahlen 2006; EFSA 2006a; Garcia-Alonso et al. 2006; Bartsch et al. 2008; Nickson 2008; Romeis et al. 2008).

## 26.4 EFSA GMO Panel Guidance and Further Prospectives

The EFSA Scientific Panel on GMOs has developed guidance documents for the risk assessment of GM plants (EFSA 2006a) and micro-organisms (EFSA 2006b). These guidance documents assist applicants in the preparation and presentation of their market registration dossiers. The EFSA guidance document for the risk assessment of GM plants follows specific EU regulatory requirements, is based on the comparative assessment approach (developed by the OECD 1993; further elaborated by the Food and Agriculture Organization of the United Nations and the World Health Organization; FAO/WHO 2000), and is in line with the recommendations of Codex Alimentarius (2003).

The EFSA guidance document is based on two-step logic: (i) the identification of possible differences between the GM and non-GM plant, and (ii) the assessment of the environmental and food/feed consequences as well as the nutritional impact of identified differences, if any. The guidance document defines data requirements and provides a detailed description of both issues and principles to be considered when performing the risk assessment of GM plants. These include the molecular characterisation of the genetic modification, the assessment of the modification with respect to agronomic characteristics of the GM plant, and the evaluation of food/feed safety aspects of the GM plant and/or derived food and feed. Data on composition, toxicity, allergenicity, nutritional value, and environmental impact provide, on a case-by-case basis, the cornerstones of the risk assessment process. Key elements for the environmental risk assessment are potential changes in

interactions of the GM plant with the biotic and abiotic environment resulting from the genetic modification.

The environmental risk assessment of a GM plant is based on the characteristics of the plant, the nature of the introduced trait(s), the receiving environment in which the plant will be introduced, and the interaction between the plant and the receiving environment, depending on the intended uses. An assessment of direct and indirect, as well as immediate and delayed effects is required. In line with Directive 2001/18/EC, the EFSA guidance document considers the following issues in the risk assessment of each GM plant:

1. Changes in the persistence and invasiveness of the GM plant
2. Would the genetic modification provide a selective advantage or disadvantage as compared to the conventional counterpart
3. The potential for gene transfer
4. Interactions between the GM plant and target organisms
5. Interactions between the GM plant and non-target organisms (NTO)
6. Potential effects on human and animal health due to accidental exposure
7. Potential effects on biogeochemical processes
8. Impacts of specific cultivation, management, and harvesting techniques associated to the cultivation of the GM plant
9. The potential interaction with abiotic environment
10. The scientific quality of the proposed PMEM plan (EFSA 2006a) including farm questionnaires (Schmidt et al. 2008).

Since the EFSA GMO Panel is continuously considering any new scientific information, it has taken several initiatives to further advance the science of GMO risk assessment and to address specific scientific issues (see also Paoletti et al. 2008). In the context of the environmental risk assessment of GM plants, some initiatives have been taken to consider the latest experience gained as well as technological progress and scientific developments made.

#### ***26.4.1 EFSA Scientific Colloquium on Environmental Risk Assessment of GM Plants***

In June 2007, EFSA organised a scientific colloquium to discuss approaches and challenges related to the environmental risk assessment of GM plants (EFSA 2008). This colloquium aimed to stimulate discussions on: (i) approaches for NTO testing, (ii) the use of models for predicting potential risk outcomes of moving to a higher scale of GM plant deployment based on outcomes of risk assessment studies performed at a lower scale of GM plant deployment, (iii) the use of models for predicting long-term effects, and (iv) broadening the scope of the environmental risk assessment to enable the integration of a much wider range of influences and drivers of agricultural systems. While it was agreed that the environmental risk

assessment as outlined in the current EFSA guidance document on the risk assessment of GM plants and derived food and feed products is at the forefront of recent developments in this area, further guidance was found useful in some fields. In this respect, participants from academia, the public research sector, national advisory bodies, non-governmental organisations, the private sector, and competent authorities from Member States made a list of recommendations to EFSA (2008).

#### ***26.4.2 Self-Tasking Working Group on NTO Testing***

Following the discussions held and recommendations made on NTO testing at the EFSA scientific colloquium (EFSA 2008) and acknowledging the different NTO testing approaches debated in the scientific literature (for the ecological approach including obligatory field tests, see Andow and Hilbeck 2004; Andow and Zwahlen 2006; Andow et al. 2006; for the tiered ecotoxicological approach using surrogate species, see Romeis et al. 2008), EFSA has established a working group on NTO testing. This working group is responsible for harmonising different NTO testing approaches and for developing more detailed guidance in this area. The focus of the working group is on the development of criteria for species and ecological functional group selection, experimental design of field studies, statistical power of NTO tests, receiving environment, arthropod diversity, and PMEM.

#### ***26.4.3 Update of Environment Sections of the EFSA Guidance on the Risk Assessment of GM Plants and Derived Food and Feed Products***

Since March 2008, the EFSA GMO Panel has been working to further update the sections on the environmental risk assessment of its guidance document on the risk assessment of GM plants and derived food and feed products. Following some of the recommendations made at the EFSA scientific colloquium and following a request of the European Commission, EFSA will establish not only more detailed guidance on NTO testing, but also on: (i) the design of field studies to assess potential ecological effects of the GM plant in its receiving environment, (ii) the identification of EU geographical regions where GM plants may be released, (iii) the selection of appropriate techniques for assessing potential long-term effects of GM plants, (iv) the assessment of environmental fitness of GM plants and their progeny and the impact of specific farm management practices, and (v) specific requirements for the assessment of GM stacked events. The EFSA GMO Panel will continue to further update the environmental risk assessment sections of its guidance document on GM plants in light of recent scientific developments and relevant scientific publications.

At international level, several activities are carried out in the area of methodology for the environmental risk assessment of GMOs and development of scientific methodologies and teaching tools that can be used for environmental risk assessment and management of transgenic plants, in accordance with the *Cartagena protocol on biosafety* and other international agreements (GMO ERA Project and related publications). In an attempt to harmonise risk assessment methodologies such as protocols and processes for measuring impact, the OECD '*Environmental considerations for risk/safety assessment for the release of transgenic plants*' working group is currently discussing what type and kind of information is needed for risk assessments (McCammon 2006).

## 26.5 Discussion and Conclusions

The debate on whether regulatory regimes using breeding techniques instead of the product as a trigger for regulatory oversight provide the best framework for an adequate safety assessment of plants is ongoing. On the one hand, it is questioned whether newly developed plant breeding techniques will outgrow GMO legislation in the EU (COGEM 2006; Morris and Spillane 2008). On the other hand, it is questioned why traditionally-bred plants and their derived products are not subjected to a similar safety assessment as those obtained through genetic engineering (Batista et al. 2008; Kok et al. 2008) or, on the opposite, why GM plants are regulated more strictly than traditionally bred ones in the EU (Bradford et al. 2005; Morris 2007).

At the European level, Member States and the European Commission are considering recent developments in plant breeding and currently are discussing whether the European regulatory framework is appropriately covering these new techniques and their application. With the increasing knowledge about plant genes and both their regulation and functions, and with the development of new plant breeding techniques for rapidly inducing or selecting desired plant characteristics, the distinction between genetic engineering and other plant biotechnology-based breeding techniques has been anticipated to fade away. According to the Dutch Commission on Genetic Modification, which issued a report on newly developed plant breeding techniques and their application, this evolution might have implications on the scope of GMO legislation, as it might not always be clear whether plants with novel traits obtained through some of these new plant breeding techniques would be captured by the EU GMO definition and be subjected to regulatory requirements (COGEM 2006). Recently, in an answer to a parliamentary question, the European Commission informed that a specific working group of external experts has been created to determine which of the newly developed plant breeding techniques would result in genetic engineering and would thus be captured by or excluded from the EU GMO definition (cf., Parliamentary question P-6606/07 2008).

Amongst the broad array of newly developed plant breeding techniques (e.g., reverse breeding, agro-inoculation, grafting on GM rootstock, gene silencing by DNA methylation, the use of oligonucleotides, specific mutagenesis with homologous recombination; discussed by COGEM 2006), cisgenesis and the development of cisgenic plants are an interesting case (Jacobson and Schouten 2007; Rommens et al. 2007). While cisgenic plants have been modified using the same genetic engineering-based plant breeding technique as transgenic plants, the introduced gene(s) comes from the plant itself or from a close, sexually compatible relative. Since only genes from the natural gene pool of the recipient species are introduced, COGEM argued that cisgenic plants should be regulated less strictly than transgenic plants in some cases (COGEM 2008), whilst other authors requested the complete exemption of cisgenic plants from current GMO regulatory oversight (Myskja 2006; Schouten et al. 2006a, b; Rommens et al. 2007). In Australia, cisgenic plants are explicitly excluded from regulatory oversight, whilst under Canadian and US law they are expected to be considered in a similar way to any other new plant variety. However, since environmental risks might be associated with the use of cisgenic plants, some authors have argued that these plants should have the same standard of regulatory oversight as transgenic plants. For example, Russell and Sparrow (2008) strongly recommended cisgenesis to be explicitly included in the GMO legislation's scope by broadening the GMO definition to include cisgenesis, as is currently the case in New Zealand.

Apart from newly developed plant breeding techniques, currently used techniques are also generating questions on the limits of the process-based approach of current GMO legislation in the EU. Following the regulatory distinction between food products derived from GM plants and those derived from traditionally bred ones, two separate approaches are followed for the safety assessment of novel food products in the EU (Kok et al. 2008). For GM food products, stringent regulatory requirements apply on a case-by-case basis, irrespective of the nature of the actual changes in comparison with direct parental lines, whilst those derived from plants obtained by plant breeding strategies other than genetic engineering (e.g., classic hybridisation, mutational breeding) are only subjected to requirements described under the *Novel food regulation*. Even though plant breeding techniques other than genetic engineering are known to cause genetic alterations in the plant genome, an assessment of potential unintended side effects of the plant breeding process on a routine basis is legally not required so far (Kok et al. 2008). However, microarray analysis on four rice lines (two mutagenised, two transgenic) revealed that plant mutagenesis may induce more transcriptomic changes than transgene insertion (Batista et al. 2008). The improvement of a crop plant variety through the acquisition of a new desired trait can cause stress and lead to an altered expression of untargeted genes, with observed alterations being more extensive in mutagenised than in GM plants. Therefore, Batista et al. (2008) recommended the safety assessment of improved plants to be performed on a case-by-case basis without restricting it to GM food products. Because traditionally bred food products might elicit similar safety concerns than those obtained from genetic engineering, Kok et al. (2008) suggested screening all new plant varieties for their new characteristics

by applying a comparative safety assessment. As such, regulatory requirements for traditionally bred plants would be comparable to and compatible with those applied on GM plants when similar characteristics and uncertainties are involved. With the increasing use of targeting induced local lesions *in genomes* (TILLING) that provides a low-cost, high-throughput reverse genetic technique that combines random chemical mutagenesis with PCR-based screening of gene regions of interest (McCallum et al. 2000a, b; Colbert et al. 2001), it is anticipated that the number of plants with novel traits obtained through induced mutagenesis will continue to increase.

Other authors have pointed out the current disparity in the regulatory assessment of the environmental impact of GM plants in comparison with conventional counterparts with similar phenotypic characteristics (Bradford et al. 2005; McHughen 2007; Morris 2007). So far, plants obtained through plant breeding techniques other than genetic engineering fall outside the scope of EU legislation from the perspective of environmental risk, though their cultivation might pose environmental safety concerns similar to those of GM plants with similar phenotypic characteristics (Chassy et al. 2003). In this respect, plants that are resistant to non-selective herbicides are an interesting case, as they can be produced not only by genetic engineering, but also by traditional plant breeding techniques (Tan et al. 2005). Moreover, the adoption of the so-called Clearfield varieties could result in similar environmental impacts than those induced by GM herbicide-resistant (HR) plants: both plants allow the application of herbicides that control a broad range of grasses and broadleaf weeds (Duke 2005; Beckie et al. 2007; Sanvido et al. 2007). Given that HR plants produced by traditional plant breeding techniques are not produced through genetic engineering, they are not considered genetically modified under current EU GMO legislation and are thus not subjected to particular safety assessments prior to their commercial release. Some authors therefore have argued that the EU regulatory approach lacks consistency. According to these authors, there are no convincing arguments in favour of applying more stringent regulatory requirements for one particular plant breeding technique if another technology might result in similar environmental impacts (Raybould 2006; ACRE 2007; Morris 2007; Sanvido et al. 2007). Canada is generally cited as an example, as all novel plants or products developed through genetic engineering and other plant breeding techniques are covered by the same legislation. Bradford et al. (2005) went a step further by proposing the deregulation of certain GM plants, and by stratifying various kinds of risks of genetic constructions and experiments into risk classes that could be subjected to different regulatory requirements. In doing so, the regulatory requirements applying to GM plants would depend upon the risk associated with the traits and gene functions, rather than the production method itself.

New plant breeding techniques for inducing or selecting desired plant characteristics are being developed rapidly and might offer new opportunities for plant breeding. Besides the benefits that the use of new plant breeding techniques might offer, plants with novel traits might also pose risks to human and animal health and the environment. When analysing these potential risks, it is important to bear in mind that the real choice is not between novel plant varieties that are

inherently risky and traditionally bred ones that are completely safe. Both existing and new plant varieties will have positive and negative outcomes (Sanvido et al. 2007). To fully acknowledge these outcomes and to assess and manage more effectively the environmental footprint of agriculture as a whole, it has been suggested that broader and more balanced regulatory oversight might be needed in the EU (ACRE 2007; EFSA 2008). At the EFSA scientific colloquium on challenges and approaches for the environmental risk assessment of GM plants (EFSA 2008), the discussion group on broadening the scope of the environmental risk assessment (discussion group 4) provided the following recommendations:

*“A paradigm shift would be required to change from risk assessment as it is currently practiced, to a more sophisticated assessment which balances risks and benefits: (i) The focus on only GM crops defies scientific evidence. In the longer term, risk assessors could develop an alternative approach on a scientific basis. ‘Novelty’ is one option. (ii) The status quo, in which risk assessment is interpreted very narrowly in terms of adverse impacts, is not sustainable, and perceptions of the quality of environmental risk assessments suffer as a result. A framework for the future is required. (iii) There is a need to build decision support tools for the risk assessors to better consider impacts of whole farming systems”.*

Since plants with novel traits might pose risks to human and animal health and the environment, according to the precautionary approach followed at the EU level, actions will be taken to avoid that adverse effects would remain undetected. In this respect, the European Commission has taken a first initiative through the creation of a specific expert working group that will determine which of the newly developed plant breeding techniques would be captured by or excluded from the EU GMO definition and thus be subjected to a safety assessment in accordance to EU legislation on GMOs (cf., Parliamentary question P-6606/07 2008).

**Disclaimer** Opinions and views expressed in this chapter are strictly those of the authors, and may not necessarily represent those of the organizations where the authors are currently employed.

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# **Chapter 27**

## **Environmental Impact of Genetically Modified Maize Expressing Cry1 Proteins**

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### **27.1 Introduction**

Before being placed on the market, genetically modified (GM) plants need to undergo an assessment of their impact on the environment as well as human and animal health. The (environmental) risk assessment (ERA) strategy for GM plants seeks to deploy appropriate methods and approaches to compare GM plants and their derived food and feed products with their non-GM comparators (Chap. 26). For more than a decade, genes of *Bacillus thuringiensis* ('Bt') that encode lepidopteran-specific protein toxins (Cry1Ab, Cry1F)<sup>1</sup> have been engineered into maize for

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<sup>1</sup>For the purpose of this chapter, all these Cry proteins are summarized as 'Cry1' class, although most of the results published and cited are based purely on the Cry1Ab protein.

protection against lepidopteran pests like the European corn borer (*Ostrinia nubilalis*) and the Mediterranean corn borer (*Sesamia nonagrioides*; Fishhoff 1996; see Chap. 10). However, questions have been raised on the potential environmental impact of these transgenic plants (Jepson et al. 1994; Poppy 2000; Dale et al. 2002). Generally, the ERA should consider unintended effects on plant fitness, gene transfer consequences (including those for crop relatives), resistance development in target organisms, adverse effects on non-target organisms, effects on the health of humans and animals exposed to the GM plant, effects due to altered cultivation and management, and potential impacts on biogeochemical cycles and the abiotic environment. The following literature survey addresses results published so far.

## 27.2 Potential Unintended Effects on Plant Fitness Due to the Genetic Modification

Maize is highly domesticated and generally unable to survive in the environment without cultivation. Maize plants are not winter hardy in many regions of the world: they have lost their ability to release seeds from the cob and they usually do not occur outside cultivated land or disturbed habitats in agricultural landscapes, despite cultivation for many years. While Cry1-expressing maize (for more details on transgenic maize, see Chap. 18) provides a potential advantage in cultivation under infestation conditions of certain lepidopteran pests (Gómez-Barbero et al. 2008), survival of maize outside of cultivation is mainly limited by a combination of low competitiveness, absence of a dormancy phase, and susceptibility both to diseases and to cold climate conditions. Since these general characteristics of Cry1-expressing maize are unchanged, the inserted insect resistance trait is not likely to provide a selective advantage outside of cultivation. Therefore, it is considered very unlikely that volunteers of Cry1-expressing maize, or the progeny, will differ from conventional maize varieties in their ability to survive until subsequent seasons or to establish feral populations under environmental conditions (e.g., EFSA 2008b, c; Palaudelmas et al. 2009). Maize seeds and seedlings do not generally survive away from cultivated land and are only winter hardy in southern European countries or other parts of the world with similar climate. In Mediterranean regions, for instance, maize kernels remaining on the soil after harvest can germinate, grow and flower, and can locally cross-pollinate neighbouring maize plants (Melé et al. 2007; Gruber et al. 2008).

For the ERA it is necessary to consider data on compositional analysis and field performance generated from field trials at several locations. During these trials, phenotypic characteristics and plant environment interactions are studied and compared with those of control maize. If no biologically meaningful differences between studied maize varieties are observed, field data do not show increased invasiveness or enhanced weediness or fitness of Cry1-expressing maize. So far, studies conducted by applicants (Monsanto, Syngenta, Pioneer), published

literature on the cultivation of numerous varieties of Cry1-expressing maize, and national monitoring observations in France (Delos et al. 2006, 2007) and Spain (Eizaguirre et al. 2006) confirm that Cry1-expressing maize (event MON810) behaves like non-GM maize.

## 27.3 Potential for Gene Transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer of DNA, or vertical gene flow via seed and pollen dispersal.

### 27.3.1 *Plant to Micro-Organism Gene Transfer*

Current scientific data (EFSA 2007b) suggest that gene transfer from GM plants to micro-organisms under natural conditions is extremely unlikely, and its establishment would occur primarily through homologous recombination in micro-organisms.

Exposure of micro-organisms to transgenic DNA derived from GM maize would take place during natural decay of GM plant material and/or pollen in the soil of areas where GM plants are cultivated. In addition, food and feed products derived from the GM maize could contain transgenic DNA. Therefore, micro-organisms in the digestive tract of humans and animals may be exposed to transgenic DNA.

The modified *cry1* genes in Bt maize are under the control of the prokaryotic regulatory elements. Taking into account the origin and nature of the *cry1* genes and the lack of selective pressure in the intestinal tract and the environment, the likelihood that horizontal gene transfer of *cry1* genes would confer selective advantage or increased fitness to micro-organisms is very limited. For this reason it is very unlikely that genes from Cry1-expressing maize would become transferred and established in the genome of micro-organisms in the environment or human and animal digestive tract. In the very unlikely event that such horizontal gene transfer would take place, no adverse effects on human and animal health or the environment are expected, as principally no new traits would be introduced or expressed in microbial communities.

### 27.3.2 *Plant to Plant Gene Transfer*

Since substantial literature shows that vertical gene transfer characteristics of Cry1-expressing maize are similar to those of non-GM maize, pollen dispersal and consequent cross-pollination are not considered as environmental hazards in

themselves (EFSA 2008b, c). The ERA is primarily concerned with assessing the environmental consequences of transgene flow on ecosystems by assessing the spread and fitness of hybrids and backcross progeny as well as exposure to non-target organisms. Theoretically, seeds originating from the cross-pollination of certain cross-compatible wild/weedy relatives can mediate the potential spread and establishment of hybrids and backcross progeny (Wilkinson et al. 2003; Morales and Traveset 2008; Devos et al. 2009).

The extent of cross-pollination of other maize varieties will mainly depend on the scale of cultivation. For maize any vertical gene transfer is limited to other *Zea mays* plants except in Central America and Mexico where populations of sexually compatible wild relatives of maize are known (OECD 2003; Baltazar et al. 2005). In addition, a possible consequence of planting Bt maize is unintended gene transfer into traditional maize land-races (Pineyro-Nelson et al. 2008; Bitocchi et al. 2009; Snow 2009). Since general characteristics of Cry1-expressing maize (low competitiveness, absence of a dormancy phase, susceptibility to diseases and to cold climate conditions) are unchanged, the inserted trait is not likely to provide a selective advantage outside of cultivation.

However, in the European Union (EU), the only recipients of cross-pollinated transgenes from maize are other cultivated maize varieties and types. Therefore, cross-pollination in maize is not considered an environmental risk, but an agricultural management and coexistence issue. Moreover, even though seed dispersal of Cry1-expressing maize (event MON810) in the EU occurs at high frequencies due to its cultivation in several countries (Spain >> Czech Republic >> Portugal >> Germany >> Romania >> Slovakia >> Poland; see e.g., Devos et al. 2008), the seed-mediated establishment of Cry1-expressing maize and its survival outside of cultivation have not been reported yet. Therefore, the likelihood of unintended environmental effects as a consequence of spread of genes from Cry1-expressing maize is therefore considered to be low.

## 27.4 Potential Interactions of the GM Plant with Target Organisms

Transgenic Cry1 proteins are pore-forming toxins producing ion channels in lipid membranes of gut of targeted lepidopteran pests (Rausell et al. 2004; Bravo et al. 2007; Gomez et al. 2007; Pigott and Ellar 2007). Because insect pests have been able to develop resistance to chemical insecticides applied to control them (Whalon et al. 2008), the potential development of insect resistance to Cry proteins constitutively expressed in GM crops is considered as a relevant concern in the ERA (e.g., EFSA 2008b, c; Gassmann et al. 2009). Resistance development generally refers to a genetically based decrease in a population's susceptibility to a toxin and can be evaluated with laboratory bioassays estimating the resistance ratio, which is the LC<sub>50</sub> (concentration of toxin killing 50% of the larvae) of a field-derived strain

divided by the LC<sub>50</sub> of the susceptible strain (Saegritz et al. 2006; Andow 2008; Bravo and Soberón 2008). Susceptibility is usually measured by sampling insects from a field population and determining how their progeny respond to the toxin in laboratory experiments (Tabashnik et al. 2008a).

Major lepidopteran target pests of Cry1Ac-expressing cotton and Cry1Ab-expressing maize (such as MON810) have been monitored worldwide for potential resistance development against specific Cry1 proteins. A recent meta-analysis of available monitoring data indicated that neither in the EU, nor in the United States (US), have populations of resistant European and Mediterranean corn borer been found in regions where Cry1-expressing maize is grown (Tabashnik et al. 2008a), confirming previous observations (Andow et al. 2000; Bourguet et al. 2003; Farinós et al. 2004; Eizaguirre et al. 2006; Schuphan 2006; Stodola et al. 2006; Andreadis et al. 2007). In Spain, for instance, after many years of field exposure of corn borer populations to Cry1-expressing maize, no indications of resistance development were found (Farinós et al. 2004; Eizaguirre et al. 2006; Andreadis et al. 2007). So far, F<sub>2</sub> screenings (Andow and Alstad 1998) performed on mated females collected from the field across Mediterranean EU countries and their progeny reared under confined conditions failed to detect major resistance alleles in corn borer populations (Bourguet et al. 2003; Schuphan 2006; Andreadis et al. 2007). These data indicate that dominant resistance alleles are extremely rare in populations of corn borers and also that the initial frequency of recessive resistance alleles is low (Andow et al. 1998, 2000; Bourguet et al. 2003; Schuphan 2006; Stodola et al. 2006; Andreadis et al. 2007). In contrast, laboratory selections for resistance with Cry1-toxins yielded partial resistance levels in some corn borer strains after many generations (Chaufaux et al. 2001; Huang et al. 2002; Farinós et al. 2004; Alves et al. 2006; Schuphan 2006). While resistance levels fluctuated between generations for each strain, toxin susceptibility decreased significantly over generations for all selected strains. However, none of the laboratory-selected resistant corn borer larvae studied by Farinós et al. (2004) survived on Bt maize seedlings. It is thus questionable whether these levels of resistance will reflect potential resistance development upon exposure of field populations to Bt crops (e.g., Bourguet 2004). Moreover, even though partial resistance has been shown to be reasonably common in some European corn borer populations (Bourguet et al. 2003), the polygenic nature of resistance in tested laboratory strains suggests that major genes for resistance to the Cry1-protein are rare in founding populations of the European corn borer (Alves et al. 2006).

Similar observations have been made in other maize target pests that are not representative of the European fauna. Huang et al. (2007), for instance, did not detect major resistance alleles in F<sub>2</sub> populations of the Southwestern corn borer (*Diatraea grandiosella*), which is a major maize stalk borer pest in central and southern parts of the US and in Mexico. However, a level of ‘resistance’ to maize MON810 has been reported in a Bt maize-derived population of the African stem borer (*Busseola fusca*) in South Africa where some larvae were able to survive in the presence of the Bt toxin, but had reduced larval growth rate (Van Rensburg 2007). Another example of field-evolved resistance in Bt maize concerns resistance

of fall armyworm, *Spodoptera frugiperda*, to the Cry1F protein. Larvae surviving on Cry1F-expressing maize in two fields in Puerto Rico (US) were collected and exposed to high concentrations of the Cry1F protein in laboratory bioassays, showing no mortality at these concentration levels (Moar et al. 2008; Tabashnik 2008; Tabashnik et al. 2008b).

Available data indicate that recessive resistance alleles are rare in populations of European and Mediterranean corn borers. Moreover, according to the EU research project ProBenBt, which studied various aspects of European and Mediterranean corn borer genetics and Cry1 resistance in targeted lepidopteran pest species, gene flow among European populations of both pest species is likely to be high enough to delay resistance development to Cry1 proteins in maize (Schuphan 2006). The fact that some adults of the European corn borer mate at a more restricted spatial scale (Hunt et al. 2001; Qureshi et al. 2005; Dalecky et al. 2006; Bailey et al. 2007) than previously assumed in the high dose/refuge strategy might under certain circumstances (e.g., crop-rotated landscape) decrease its efficiency (Dalecky et al. 2006; Schuphan 2006). However, predictions generated by a recently developed demographic dynamic model confirm that applying the high dose/refuge resistance management strategy is likely to maintain sensitivity to Cry1 proteins in the European corn borer (Tyutyunov et al. 2008).

To delay or prevent the potential development of insect resistance to Bt crops, a resistance management tactic, relying on a high dose/refuge strategy, has been endorsed in the US and EU (Bates et al. 2005; Andow 2008; Bravo and Soberón 2008; Gassmann et al. 2009). The high dose/refuge strategy intends to reduce the selection pressure for resistance alleles by combining Bt maize that produces a high dose of toxin with non-Bt maize plants that are grown nearby as a refuge (Ives and Andow 2002). To ensure that individuals heterozygous for a resistance allele are killed by the Cry1-protein produced in plant tissues, the increase in fitness conferred by resistance alleles must be recessive. The second assumption of the high dose/refuge strategy is that resistance alleles must be rare, so that only few homozygotes survive on Bt crops. Finally, it is assumed that the few resistant insects emerging in Bt crops must mate randomly or preferentially with the larger pool of susceptible insects preserved on non-Bt crops (Alstad and Andow 1995; Andow 2008).

The large-scale cultivation of Cry1-expressing maize over several years will increase the selection pressure on corn borers, which could result in the potential development of resistance. An analysis of global monitoring data, collected in Australia, China, Spain, and the US, revealed an increased frequency of resistance alleles in some field populations of *Helicoverpa zea* (a pest of cotton) to the Cry1Ac protein (Tabashnik et al. 2008a). Field-evolved resistance has also been documented for two maize pests that are not representative of the European fauna: *S. frugiperda* (Moar et al. 2008; Tabashnik 2008; Tabashnik et al. 2008b) and *B. fusca* (Van Rensburg 2007; Tabashnik 2008). However, no field-evolved resistance has been reported to Bt proteins for other lepidopteran pests (*Helicoverpa armigera*, *Heliothis virescens*, *O. nubilalis*, *Pectinophora gossypiella*, *S. nonagrioides*; Ferré et al. 2008; Tabashnik et al. 2008a; Gassmann et al. 2009).

The likelihood of occurrence is low in corn borer populations since, under field conditions and several years of cultivation, no resistance has been reported. However, the cultivation of Cry1-expressing maize in the EU is currently on a small scale and limited to a few geographic regions. In addition, as potential resistance development is dependent upon multiple factors, predicting future responses of corn borer populations in Europe is case-specific (Tyutyunov et al. 2008). Dispersal distances, for instance, have been shown to be influenced by plant size, weather conditions during the flight, pheromonal patterns in the field, and the timing of the flight (Hunt et al. 2001; Engels et al. 2008). Therefore, the potential development of resistance in target pests should be monitored in order to detect potential changes in resistance levels in pest populations. Applicants are generally requested to monitor resistance development in target pests in the US and Canada. In the EU, the monitoring of Cry1-expressing maize demands case-specific insect resistance management and considers further general surveillance through farmer questionnaires (Schmidt et al. 2008).

## 27.5 Potential Interactions of the GM Plant with Non-Target Organisms

Considering the high diversity of non-target organisms, many species of which inhabit agricultural landscapes, and the high complexity of interactions even in agricultural biocoenoses, an in-depth analysis of available literature on Cry1-expressing maize interaction with non-target organisms is indicated.

### 27.5.1 Persistence of Cry1 Proteins in Soil: Exposure Assessment

In order to assess the potential adverse impact of Cry1-expressing crops on soil organisms, both the exposure and sensitivity of non-target soil organisms to the Cry1 protein need to be established. It is well documented that during plant growth Cry1-expressing maize can contribute to the presence and persistence of plant-produced Cry proteins in soil via root exudation (e.g., Saxena et al. 2002, 2004). A second route for potential accumulation and persistence of Cry1 proteins in soil relates to dead plant material remaining on fields after harvest and which is incorporated into the soil during tillage operations (Stotzky 2004).

The persistence of the Cry1 protein in soil is dependent upon multiple factors, varying among different experimental conditions (e.g., type of crop, soil, pH, microbial activity, temperature, method used for quantification of the protein). In a recent review paper, Icoz and Stotzky (2008) discuss the variability in persistence of the Cry1 protein in soils. Half-lives (the time until the amount of a substance

remaining is 50% of the original amount) of the Cry1 protein ranged from 1.6 days in a soil amended with biomass of Cry1-expressing maize (Sims and Holden 1996) up to 34.0 days in soil amended with biomass of and planted to Bt rice (Wang et al. 2006). Schrader et al. (2008) observed a strong decline of immunoreactive Cry1 in plant residues of maize event MON810 in microcosm experiments. After 5 weeks, in leaf material, it was reduced to 14.1% and in root material to 12.8% of the initial concentration, which was approximately 5 µg/g.

Although Cry1 proteins are degraded or inactivated in soil within weeks, a small fraction can persist far longer under certain conditions. Laboratory studies have shown that the Cry1 protein can bind on clay minerals and humic substances in soil, thereby reducing its availability to micro-organisms. This reduced availability decreases degradation of the Cry1 protein, so the insecticidal activity is retained during the growing season (e.g., Tapp et al. 1994; Tapp and Stotzky 1995; Crecchio and Stotzky 2001). In this respect, Zwahlen et al. (2003a) showed that the Cry1 protein is still detectable in decaying maize material after a soil exposure in litter bags for 200–240 days. The Cry1 protein in low concentrations was detected for up to 56 days in soil amended with purified or biomass of Bt cotton (Donegan et al. 1995; for more details on transgenic cotton see Chap. 15), up to 234 days in soil amended with purified protein (Tapp and Stotzky 1998), and for up to 180–350 days in soil amended with Cry1 maize biomass or residues of Cry1 maize (Saxena and Stotzky 2002). Stotzky (2004) reported that the Cry1 protein released in root exudates and from biomass of Cry1 maize persisted in low concentrations in soil microcosms for at least 180 days and 3 years, respectively.

The potential accumulation of plant-produced Cry1 proteins in soil following repeated and large-scale cultivation of Cry1-expressing maize has been studied. The Cry1 protein was recorded in soil during four consecutive years of Cry1-expressing maize cultivation, and no accumulation was observed (Icoz et al. 2008). In addition, Baumgarte and Tebbe (2005) and Andersen et al. (2007) reported that concentrations of the Cry1 protein found in soil were higher in a given season for plots with varieties derived from the maize event MON810 in comparison with non-Bt maize varieties, but concentrations did not seem to increase from year to year. Hopkins and Gregorich (2003, 2005) and Dubelman et al. (2005) also reported that Cry1 proteins from GM plants do not persist in soil three months after harvest, and they found no evidence of accumulation of the Cry1 protein in soil from fields planted for at least three consecutive years with Cry1-expressing maize, regardless of soil type, geographic region, and climatic conditions (Dubelman et al. 2005). Despite the fact that Cry proteins can bind rapidly on clay minerals and humic substances, there is no evidence for accumulation of the Cry1 protein in soils in the field, even after three years of continuous cultivation of Bt crops (e.g., Marchetti et al. 2007; Höinemann et al. 2008).

In the ERA, the exposure of non-target soil organisms to the Cry1 protein needs to be combined with an impact assessment. In this respect, the primary concerns are the susceptibility of non-target soil fauna to the Cry1 protein, effects on micro-organisms, and impacts on soil organism diversity and soil functioning. These aspects are discussed in the following sections.

### 27.5.2 Biological Effects in Soil: General Impact Assessment

Multi-year experiments conducted with GM maize at four sites across three European climatic zones in the context of the EU-funded ECOGEN project (Andersen et al. 2007; Krogh and Griffiths 2007) showed that no or only few effects on snails, microarthropods or mycorrhizal fungi could be attributed to Cry1-expressing maize (event MON810; Cortet et al. 2007; de Vaulfleur et al. 2007; Griffiths et al. 2007a; Krogh et al. 2007). Field experiments revealed that Cry1-expressing maize could have a significant, but small and transient, effect on soil protozoa, nematodes, and micro-organisms (Griffiths et al. 2005, 2007a). Even though the presence of the Cry1 protein in snail faeces was identified as a novel route of exposure into the soil food web (de Vaulfleur et al. 2007), no direct effects could be detected related to maize event MON810 in mesocosm experiments. The ECOGEN experiments allowed for a comparison of results ensuing from different scales and for an assessment of their utility since the same organisms and soils were studied in laboratory, glasshouse, and field. Although useful information and insights from each of the experimental approaches and scales were gathered, predicting outcomes to one scale from results obtained from another still remains difficult (Birch et al. 2007). One reason for the difficulty to demonstrate predictions from tier 1 to tier 2 is that the testing substance basically has no effect, and it is obvious that no-effect at a lower level can only predict no-effect on a higher level. If a testing substance has a pronounced effect on tier 1, then it will be much easier to demonstrate effects on higher tier levels. Based on the ECOGEN analyses, the authors concluded that Cry1-expressing maize does not have adverse effects on soil biota, since effects observed were most likely to be caused by season, soil type, tillage, crop type or variety (Cortet et al. 2007; de Vaulfleur et al. 2007; Griffiths et al. 2007a; Krogh et al. 2007). Similarly, effects on soil microbial community structure, micro-arthropods, and larvae of a non-target root-feeding Dipteran (*Delia radicum*) observed in a glasshouse experiment were most likely due to soil type and plant growth stage, rather than Cry1-expressing maize (event MON810). Although statistically significant effects of Cry1-expressing maize on soil microfauna populations (e.g., overall increase in protozoa (amoebae) and nematode numbers) were observed, these effects were relatively small, especially when compared with effects of soil type, plant growth stage, insecticide application, and variety (Griffiths et al. 2006, 2007b).

Several other studies did not show any consistent effect of Cry1-expressing maize on soil species. For example, in an eight-month field study consisting of litter-bag experiments with Cry1-expressing maize (event Bt11), Zwahlen et al. (2007) did not detect major changes in the composition of the soil fauna community, collembolans, mites, and annelids during the experiment. Similar conclusions were drawn by Hönenmann et al. (2008), who observed similar meso- and macrofauna soil communities between the tested maize varieties (including two varieties containing event MON810). In summary, as concluded by Icoz and Stotzky (2008),

few or no toxic effects of the Cry1 protein on woodlice, collembolans, mites, earthworms, nematodes, and protozoa have been reported.

### 27.5.3 Assessment of Impact on Earthworms

Earthworms can be exposed to the Cry1 protein through root exudates and decomposing plant material. However, laboratory and field studies performed on a few earthworm species, such as *Aporrectodea caliginosa* (Vercesi et al. 2006; Schrader et al. 2008), *Eisenia fetida* (Clark and Coats 2006), and *Lumbricus terrestris* (Saxena and Stotzky 2001a; Zwahlen et al. 2003b; Schrader et al. 2008) did not reveal significant adverse effects on earthworm survival, growth, and reproduction following protein ingestion. The detection of the Cry1 protein in the gut and faeces of earthworms confirmed protein ingestion (reviewed by Icoz and Stotzky 2008).

Based on laboratory experiments, Saxena and Stotzky (2001a) concluded that the uptake of the Cry1 protein (event MON810) by earthworms is of no safety concern, since no adverse effects on mortality or weight were observed on *L. terrestris* exposed to soil planted to or amended with plant material from Cry1-expressing maize after 40 or 45 days, respectively, compared with non-Bt maize. However, as pointed by Clark et al. (2005), growth is probably not an appropriate assessment endpoint for adults: individuals used by Saxena and Stotzky (2001a) were already mature, with fully developed clitella (i.e., sexually mature) and thus less likely to exhibit changes in growth. Zwahlen et al. (2003b) investigated mortality and growth of *L. terrestris* in laboratory and field experiments by exposing juveniles and adults to maize event Bt11 (expressing the Cry1 protein) during a period of 200 days. Field experiments did not reveal any differences in growth rate between Cry1-based and near-isogenic maize material exposure. In laboratory experiments, the growth of adults, expressed as mean fresh weight, was similar for 160 days, but declined thereafter in Cry1-exposed earthworms up to 200 days. It is difficult to attribute this biological effect to the Cry protein or to unanticipated changes in plant characteristics that could have altered microbial composition in such confined soil samples. Experimental conditions in the laboratory were quite different from those encountered under field conditions, moreover earthworm reproductive activity was not recorded and therefore it is not possible to make any inference on long-term effects on natural populations.

Laboratory toxicity studies, in which *E. fetida* were fed leaf material from Cry1-expressing maize (events Bt11, MON810) or the isogenic counterpart in a soil system and monitored for 28 days, did not reveal adverse subacute effects on survival or reproduction due to the ingestion of Cry1-expressing maize leaf material. However, differences in nutritional parameters of Cry1-expressing maize lines and isolines were anticipated to lead to differences in effects on non-target organisms (Clark and Coats 2006).

Vercesi et al. (2006) studied effects of maize event MON810 on important life-history traits (survival, reproduction, growth) of *A. caliginosa* under various

experimental conditions. In a series of experiments, the authors investigated the growth of juveniles until maturity as well as cocoon production and hatchability. Finely ground leaves of maize event MON810 added to soil had no adverse effects on these life-history traits in *A. caliginosa*, even when they were exposed to high worst-case scenario concentrations. In addition, growth of juvenile *A. caliginosa* was unaffected when they were kept in pots with a growing Cry1-expressing maize plant for four weeks. Only when considering cocoon hatchability was a slight, but statistically significant, negative effect observed for a high concentration of Cry1-expressing maize residues. However, due to the addition of high concentrations of finely ground Cry1-expressing maize residues, Vercesi et al. (2006) questioned whether the negative effect would have any ecological significance under field conditions. It cannot be excluded that the added cow dung food could have led to lower exposure to Cry1, as this gave the worms an alternative food-source, but the authors stress that *A. caliginosa* by nature feeds on the soil with the ground maize leaves. In experiments performed by Schrader et al. (2008), the two tested earthworm species, *A. caliginosa* and *L. terrestris*, survived incubation for five weeks, irrespective of whether they received event MON810 or non-transgenic maize material.

Other papers (e.g., Krogh et al. 2007) confirmed that no effects on earthworms were detected in field surveys during the cultivation of Cry1-expressing maize. No significant differences were reported in the population density or biomass of *Lumbricidae* between soils with Cry1 (events MON810, Bt176) and non-Cry1-expressing maize and between soils with maize treated with or without insecticide at five sites during four years of maize cultivation in field, though both the site and sampling years had a significant influence on both assessment endpoints (Anonymous 2006).

#### 27.5.4 Assessment of Impact on Isopods

Woodlice (*Porcellio scaber*), considered a model decomposer organism, have been used in laboratory feeding studies for detecting potential adverse impacts related to exposure to plant material from Cry1-expressing maize. Exposure for and assimilation of the Cry1 protein by *P. scaber* were demonstrated by lower concentrations of the protein in faeces than in the consumed plant material (Wandeler et al. 2002; Pont and Nentwig 2005). No adverse effects of the Cry1 protein on consumption, survival and growth of *P. scaber* were observed when fed plant material of Cry1-expressing maize and non-Bt maize (Escher et al. 2000). The survival and growth of *Trachelipus rathkii* and *Armadillidium nasatum*, two abundant isopods in maize-growing regions, were not adversely affected after exposure to the purified Cry1 protein or leaves of Cry1-expressing maize (events Bt11, MON810) under laboratory conditions for eight weeks (Clark et al. 2006). Detected differences in mortality, weight gain and consumption by isopods, and in digestibility of plant material were generally attributed to differences in the nutritional quality of maize

varieties used (Escher et al. 2000; Wandeler et al. 2002; Pont and Nentwig 2005; Clark et al. 2006).

### 27.5.5 Assessment of Impact on Nematodes

Nematodes are considered useful indicators of soil quality due to their great diversity and participation in many functions at different levels of food webs in soil and due to their presence in almost all soils with a high population density and a large number of species (Anonymous 2006; Icoz and Stotzky 2008).

A recent review on the effects of Bt crops on soil ecosystems illustrated that, depending upon experimental conditions, the Cry1 protein might have different effects on nematodes (Icoz and Stotzky 2008). Saxena and Stotzky (2001a) found no significant differences in the number of nematodes in the rhizosphere soil of Cry1 and non-Bt maize grown in a plant-growth chamber or between soil amended with biomass of Cry1 and non-Bt maize. An overall comparison of event MON810 versus non-Bt maize across three different field sites in different European regions revealed a significant, but transient, reduction in numbers of nematodes under Cry1-expressing maize as compared with non-Bt maize (Griffiths et al. 2005). Nematode community structure was different at each site and the effect of Cry1-expressing maize was not confined to specific nematode taxa. The authors concluded that the effect of Cry1-expressing maize was small and within the normal variation range expected in the considered agricultural systems. In contrast, Griffiths et al. (2006) reported significantly higher nematode populations of *Acrobeloides* spp. and *Pratylenchus* spp. under Cry1-expressing maize than non-Bt maize in a greenhouse study. There was an overall increase in nematode numbers under Cry1-expressing maize when all data were pooled, but no significant effect at any individual plant growth stage or in any particular soil type. Moreover, the effect of Cry1-expressing maize was no greater than that of an insecticide treatment. Reasons for the differences between the two studies are not clear, but the authors attribute it to the fact that plants grown in pots probably had a higher density of roots than would be expected in the field. This difference in environmental conditions in the greenhouse and the field might have affected interactions between plants and soil organisms (Griffiths et al. 2006; Birch et al. 2007). In addition, based on a glasshouse study involving eight different paired varieties of maize (Cry1 – including event MON810 – and near-isogenic), Griffiths et al. (2007b) reported that: (i) nematode abundance varied mainly between maize varieties, rather than between Cry1- and non-Cry1-expressing maize, and (ii) differences in previously published soil nematode studies under Cry1-expressing maize were smaller than varietal effects.

Effects of Cry1-expressing maize (events MON810, Bt176) on two nematode species, plant-parasitic *Pratylenchus* spp. and the bacterivorous *Caenorhabditis elegans*, have also been studied in field trials in Germany (Anonymous 2006). No adverse Cry1 effects were observed with respect to population density of

*Pratylenchus* spp., whilst growth, number of eggs, and reproduction rate of *C. elegans* were negatively affected. In a laboratory bioassay, Höss et al. (2008) studied potential toxic effects of the Cry1 protein on *C. elegans* by exposing *C. elegans* either to rhizosphere and bulk soil from experimental fields cultivated with Cry1 maize (event MON810) or to different solutions of the Cry1 protein expressed in *Escherichia coli*. Nematode reproduction and growth were significantly reduced in rhizosphere and bulk soil of Cry1-expressing maize as compared with soil from isogenic maize, and were significantly correlated with concentrations of the Cry1 protein in soil samples. However, because concentrations of the Cry1 protein measured in soil samples from Cry1-expressing maize were low and not sufficiently high to produce direct toxic effects on *C. elegans* (see also Baumgarte and Tebbe 2005), adverse effects on the reproduction and growth of *C. elegans* were assigned to indirect effects. The authors concluded that further investigations are needed to assess whether there are potential indirect effects of the protein on reproduction and growth of *C. elegans* and to clarify the reasons thereof (Höss et al. 2008). Any observed effects would then have to be compared with other factors limiting populations such as cultivation and other fluctuations in the physical soil environment.

Experiments conducted in the context of the ECOGEN project showed that changes to nematode communities due to Cry1-expressing maize were small and transient, and smaller than those induced by seasonal, soil type, tillage, crop type or varietal effects (Griffiths et al. 2007a). Reduced abundance of nematodes was only observed at the field site in Denmark in October 2005 and not at the other sampling occasions. No significant differences in nematode abundance in field sites in France were shown.

The ERA on Cry1-expressing maize can conclude that any changes in the nematode community structure associated with Cry1-expressing maize and their products are likely to be minor compared with effects of agricultural practices, environmental stresses or differences between localities and maize varieties. Rearrangements of nematode populations, which are associated to several sources of variation in the agricultural environment, occur frequently and are not necessarily an indication of environmental harm.

### **27.5.6 Assessment of Impact on Collembolans**

Because collembolans are important in the breakdown and recycling of crop residues, they are key indicator species of soil fertility and health. In general, no negative effects of the Cry1-protein on collembolans have been observed (reviewed by Icoz and Stotzky 2008). The addition of four purified Bt insecticidal proteins (Cry1Ab, Cry1Ac, Cry2A, Cry3A) at concentrations of 200 mg/g to the diet of the collembolans, *Folsomia candida* and *Xenylla grisea*, for 21 days did not affect their survival or reproduction compared with the unamended diet (Sims and Martin 1997). No deleterious effects on survival and reproduction of *F. candida* were

observed when fed leaves of Bt maize expressing the Cry1-protein, compared with leaves of non-Bt isolines (Clark and Coats 2006). While Bakonyi et al. (2006) showed that Bt maize was less preferred as food by *F. candida* than near-isogenic non-Bt maize, this effect was not observed for *Heteromurus nitidus* and *Sinella coeca*. *F. candida* defecated 30% less around Bt maize, but did not show a preference to stay on any plant material. Preference was not linked to consumption, so the tendency to stay on the plant material was not linked to palatability. For well fed *F. candida*, the consumption was 30% less on Bt diet, but when they were starved, they indiscriminately consumed both diets. An interpretation of the study in toxicological terms relies on the value of an avoidance of toxic substances for predicting the toxic potential in a realistic field situation. Hitherto the Cry1-protein has not been shown to be toxic to collembolans. In addition to the presence of the assumed toxicant, the Cry1-protein, there were differences in C/N ratio in the plant material. Such differences are common because Bt maize is an F<sub>1</sub> hybrid and comparators are of similar hybrid origin or single lines and therefore not fully isogenic. Different varieties have been shown previously to elicit various responses related to their background genetic composition and not to the GM event or its products (Griffiths et al. 2007b). The different consumption of Bt maize may be due to nutritional differences, as suggested by the C/N ratio. The study shows that *F. candida*, which responded with a lower consumption of the Bt toxin, did not discriminate between the two diets under starved condition. Heckmann et al. (2006) reported that the growth and reproduction of the collembolan, *Protaphorura armata*, reared on ground roots of Cry1-expressing maize were not significantly different from those reared on ground roots of non-Bt maize for four weeks. *P. armata* performed significantly better on a diet of yeast amended with purified Cry1Ab protein than on ground root tissue of Bt and non-Bt maize. The choice of *P. armata* was based on its root herbivory while *F. candida* would be a surrogate eating mainly micro-organisms. No significant differences in the population density of collembolans were found in soils cultivated with Bt and non-Bt maize and between the application of an insecticide (Baythroids) and no insecticide (Anonymous 2006).

### 27.5.7 *Cry1 Genes in Water: Exposure Assessment in Aquatic Environments*

The occurrence and persistence of the *cry1* gene from *Bacillus thuringiensis* (Bt var. kurstaki) and Cry1-expressing maize (event MON810) have been examined in aquatic environments near fields where Cry1-expressing maize was cultivated by Douville et al. (2007). The authors reported that the Cry1 gene persisted for more than 21 and 40 days in surface water and sediment, respectively, and detected the Cry1 gene in surface water samples taken at long distances downstream from the maize plot. In addition, Douville et al. (2009) report in a preliminary study

that Cry1 genes from Bt bacteria and Cry1-expressing maize occur in mussels, but there is no indication of environmental impact presented in this publication. However, DNA presence alone is not considered a reliable indicator of toxicity to non-target organisms. A more reliable indicator of toxicity to non-target organisms would be the presence and concentrations of the Cry1 protein in surface water and sediment. In a previous study by the same group of researchers, it was reported that the presence of the Cry1 protein in water bodies was either absent or just above the detection limit (Douville et al. 2005), suggesting that Cry1 protein concentrations would remain far below any toxic level.

### **27.5.8 *Presence of Cry1 Proteins in Water: Impact Assessment in Aquatic Environments***

Due to their specific mode of action, Cry1 proteins were not regarded to be toxic to aquatic organisms (Glare and O'Gallaghan 2000). Recently two publications appear to report some side effects: Bøhn et al. (2008) and Rosi-Marshall et al. (2007).

The laboratory experiment performed by Bøhn et al. (2008) revealed that *Daphnia magna* fed with a Cry1-expressing maize flour-containing suspension (event MON810) had a higher mortality and a lower proportion of females reached sexual maturity as compared with the non-Bt maize treatment, suggesting toxic effects of Cry1-expressing maize. However, since maize flour is not part of the natural diet of *Daphnia*, the unusual delays in development of *Daphnia* fed non-Bt maize might have been caused by nutritional deficiencies related to a maize-based diet. Moreover, internationally accepted guidelines for toxicity and reproduction testing of *Daphnia* were not followed. Due to these methodological weaknesses, it is doubtful that any substantive conclusion on potential risks of Cry1-expressing maize can be drawn from the study.

Rosi-Marshall et al. (2007) reported that byproducts of Cry1-expressing maize entered headwater streams and claimed that this would reduce growth and increase mortality of some non-target stream insects such as trichopterans. Even though the study has its strengths in quantifying the exposure of headwater streams by maize biomass (Cry1 or non-Bt) in general, the EFSA GMO Panel (EFSA 2007a) and other scientists (Beachy 2008; Parrot 2008) have indicated that the study shows weaknesses that prevent clear conclusions. The authors measured degradation rates in aquatic systems and found no difference between Cry1 and non-Bt maize plant material. Concentrations of Cry1 protein in leaves and pollen were not measured, so no dose-response relationship with Cry1 protein can be made. It is thus unclear how the degradation rate of Cry1 protein is related to that of plant material. In addition, the identity of the Cry1-expressing maize event used in the feeding test is not clear and no isogenic controls to compare with the GM material were used. Also, there is no detailed information given on the amount of maize material fed to test

organisms, and the effects reported are relatively minor in comparison with known toxic chemicals. Finally, there is no information on the reproducibility of the feeding test. Therefore, important background information on the levels of exposure and plant material used is missing and critics consider that the conclusions made by Rosi-Marshall et al. (2007) are not supported by the data presented in the paper. It can only be concluded that a potential hazard for trichopterans has been identified under laboratory conditions when exposed to high doses of Cry proteins. However, due to the low level of exposure to trichopterans in aquatic ecosystems, it is unlikely that Cry1 proteins in Cry1 maize products would cause toxic effects.

### 27.5.9 Exposure and Impacts on Non-Target Lepidoptera

Although maize is not considered an important resource of food for indigenous lepidopteran species in the EU, larvae of lepidoptera consuming the Cry1-expressing plant or its products can be exposed to the Cry1 protein. In the vicinity of Cry1-expressing maize fields, larvae can be exposed to the Cry1 protein when feeding on host plant leaves naturally dusted with pollen and anthers of Cry1-expressing maize during anthesis. In a theoretical exposure assessment, Schmitz et al. (2003) estimated that approximately 7% of German macrolepidopteran species occur in farmland areas where maize is grown and thus could be potentially affected by exposure to Cry1-containing maize pollen.

Larvae of a range of lepidopteran species are susceptible to the Cry1 protein and can be adversely affected by the protein after ingestion of significant amounts (Losey et al. 1999; Jesse and Obrycki 2000; Hellmich et al. 2001; Felke et al. 2002; Anderson et al. 2004, 2005; Dutton et al. 2005; Lang and Vojtech 2006; Prasifka et al. 2007). Dutton et al. (2005) showed that the pest species, *Spodoptera littoralis*, fed either on Cry1-expressing (event Bt11) plant material or Bt-sprayed plants (Dipel) is adversely affected with young *S. littoralis* larvae being the most sensitive to the Bt protein. Compared with larvae maintained on control plants, larvae maintained on transgenic or sprayed plants had a higher mortality and a slower development time, confirming that certain herbivore lepidopterans, including *S. littoralis*, are sensitive to the Cry1 protein (Dutton et al. 2005). Sensitivity to the Cry1 protein was also shown for the stored-product moth pest species *Ephesia kuehniella*, *Ephesia elutella*, *Cadra cautella*, and *Plodia interpunctella* (Hubert et al. 2008). The anticipated effects of GM maize on secondary lepidopteran pests largely depend upon the maize event, its expression pattern, the type of ingested plant material, and the phenology of the species in field conditions.

In laboratory studies, lethal and sublethal effects of Cry1-containing maize pollen consumption by larvae have been demonstrated for some non-target butterfly species, depending upon the GM maize event and the lepidopteran species used, as well as the amount of pollen consumed and the toxin amounts contained in it. Concentrations of the biologically active Cry1 protein in pollen of maize events Bt11 and MON810 were shown to be relatively low, resulting in similar toxicological

effects on non-target lepidopteran populations exposed to pollen from these maize events (Mendelsohn et al. 2003), in contrast to maize Bt176 pollen which contains much higher concentrations of the Cry1 protein (Hellmich et al. 2001). A laboratory assay revealed toxicity to monarch butterfly larvae (*Danaus plexippus*) that consumed Cry1-containing maize pollen deposited on milkweed plants (*Asclepias* spp.) compared with those reared on leaves dusted with non-transformed maize pollen or on leaves without pollen (Losey et al. 1999). Larvae of the pest species *Pieris brassicae*, *Pieris rapae*, and *Plutella xylostella* also fed less, grew more slowly, and showed a higher mortality when larvae ingested their food plant material dusted with pollen of maize Bt176, compared with larvae of an untreated control group (Felke et al. 2002).

The toxicity of pollen from maize Bt176 has also been tested on butterfly species of conservation concern in some EU Member States, such as the common swallowtail (*Papilio machaon*) and the peacock butterfly (*Inachis io*). Lang and Vojtech (2006) reported a lower survival rate of larvae of *P. machaon*, exposed to the highest levels of Bt maize pollen densities (event Bt176) that might be experienced under field conditions. The ingestion of Bt maize pollen led to reduced plant consumption, lower body weight, longer development time of larvae, and smaller wing size of adults. Felke and Langenbruch (2005) revealed that the ingestion of a small number (ten) of pollen grains of maize Bt176 reduced the speed of larval development of *I. io* and resulted in a significant reduction in average weight, as compared with individuals that received pollen from non-Bt maize. However, the pollen of MON810 showed no effect on mortality of larvae of *P. xylostella*, which is known to be a more sensitive species to Cry1 (Felke and Langenbruch 2005).

Besides the assessment of the impact of Cry1-containing maize pollen on Lepidoptera, an exposure assessment is needed for assessing potential risks for a given lepidopteran species. An extensive study of field experiments conducted in the US reported that the risk of Cry1-containing maize pollen on monarch butterfly populations is likely to be negligible for maize event MON810 (Hellmich et al. 2001; Oberhauser et al. 2001; Pleasants et al. 2001; Sears et al. 2001; Stanley-Horn et al. 2001; Oberhauser and Rivers 2003; Wolt et al. 2003). Lethal and sublethal effects were only observed when monarch butterfly larvae consumed significant concentrations of maize event MON810 pollen (Sears et al. 2001; Stanley-Horn et al. 2001; Dively et al. 2004). Because the proportion of butterfly population exposed to toxic levels of Cry1-containing maize pollen is small (e.g., due to the lack of temporal overlap between larval development and pollen shed; Oberhauser et al. 2001) and the amount of Cry1 protein contained in maize event MON810 pollen is low as compared with maize Bt176 (Hellmich et al. 2001), it was concluded that impacts on *D. plexippus* populations are negligible (Sears et al. 2001; Dively et al. 2004), especially when considered against the wide range of existing environmental and agronomic stressors currently influencing butterfly populations (Aviron et al. 2006; Gathmann et al. 2006b). Pollen concentrations exceeding the toxicity level mainly occur on leaf surfaces in Cry1 expressing maize fields and within 1–3 m of the edge of the Cry1-expressing maize field (Jesse and Obrycki 2000; Pleasants et al. 2001; Zangerl et al. 2001; Wolt et al. 2003; Dively

et al. 2004; Lang et al. 2004), whilst susceptibility to the Cry1 protein declines with older instars (Hellmich et al. 2001; Felke et al. 2002). Even though Dively et al. (2004) detected a higher mortality and a decreased fitness to monarch larvae consuming event MON810 pollen in laboratory and semi-field tests, these sublethal effects on the monarch population due to long-term exposure to Cry1-containing maize pollen were considered small (~0.6% to 2.5%) by the authors and much lower than those attributed to natural variability.

Decreased larval feeding and weight of monarch butterfly larvae have been reported after exposure in the laboratory to a high density of Cry1-expressing anthers (MON810) as compared with larvae exposed to milkweed leaf disks with no anthers or non-Bt anthers (Hellmich et al. 2001; Anderson et al. 2004, 2005). However, an examination of anthers in and near maize fields showed that toxic levels of anthers rarely occur under normal field conditions, so that exposure of monarch butterflies to toxins from intact anthers from Bt maize alone or in combination with pollen from Bt maize is likely to be very low (Anderson et al. 2004). Although Anderson et al. (2004) and Prasifka et al. (2007) reported a reduction in feeding and weight gain due to behavioural changes under laboratory conditions, a point that still remains to be explained is how this change might translate to the field. Under field conditions early instar larvae, which are most susceptible to the Cry1 protein, are less exposed, as they mainly feed on the upper third of milkweed plants where the lowest densities of anthers occur (Pleasants et al. 2001; Anderson et al. 2004). In addition, larvae can move to the underside of leaves where they would avoid any contact with anthers (Pleasants et al. 2001; Jesse and Obrycki 2003).

Extrapolating observations made on certain non-target lepidopteran species to other butterflies remains difficult due the variability in acute sensitivity among butterfly species to the Cry1 protein (as determined in artificial diet studies reported by Wolt et al. 2003). Moreover, data on the distribution and hence the exposure of European lepidopteran species in agricultural landscapes on a population level are limited (Schmitz et al. 2003; Anonymous 2006; Gathmann et al. 2006a, b). In this respect, a three-year field study performed in Germany revealed no difference in abundance of larvae of the butterfly species *P. rapae* and *P. xylostella* between the Cry1-based treatment (event MON810) and control treatment on weed strips artificially sown in maize field plots (Gathmann et al. 2006b). Although seven other butterfly species were observed in the study, their low abundance did not enable suitable statistical analysis, confirming that studying all lepidopteran species that could be potentially exposed to Cry1-containing maize pollen may be difficult in practice, especially if small effects are to be detected (Lang 2004; Gathmann et al. 2006b) against a wide range of existing environmental and agronomic stressors currently influencing lepidopteran populations (Aviron et al. 2006; Gathmann et al. 2006b). It is thus important to clarify the representativeness of indicator non-target butterfly species used in the frame of an environmental risk assessment in order to draw conclusions on risk to other species (see e.g., Schmitz et al. 2003). A recent ERA on MON810 maize concluded that the likelihood of adverse effects on non-target organisms (including non-target lepidoptera) is very low (EFSA 2009).

### 27.5.10 Global Analysis of Impacts on Non-Target Entomofauna

Nine years of experience of Cry1-expressing maize cultivation in Spain revealed no adverse effects on non-target arthropods (de la Poza et al. 2005; Pons et al. 2005; Eizaguirre et al. 2006; Farinós et al. 2008). Two different field studies in which the potential impact of Cry1-expressing maize (event Bt176) on predatory arthropods was studied over at least three consecutive years in Spain did not show clear differences in predatory arthropod abundance among Cry1-expressing maize and the isogenic counterpart, though their abundance varied between years and sites (de la Poza et al. 2005; Eizaguirre et al. 2006). Focussing on effects of Cry1-expressing maize in species richness, diversity, and seasonal phenology of ground-dwelling arthropods, Farinós et al. (2008) reported that no significant differences among the most abundant arthropod groups (e.g., spiders, ground beetles, rove beetles) could be attributed to the Cry1-expressing maize treatment. Both Pons et al. (2005) and Eizaguirre et al. (2006) showed that Cry1-expressing maize did not have an adverse impact on non-target pest species in the field: overall, more aphids and leafhoppers were found in Cry1-expressing maize fields as compared with non-Bt maize fields, whilst numbers of cutworms (*Agrotis segetum*) and wireworms (larvae of click beetle *Agriotes lineatus*) remained similar.

In a field monitoring study performed in Germany from 2000 to 2005, field pairs (half-fields) planted with Bt-maize (event MON810) and a conventional maize variety were studied to determine densities of arthropod taxa on plants, activity densities and diversity of ground-dwelling arthropods (Schorling and Freier 2006). Density comparisons of different taxa (such as aphids, thrips, heteropterans, aphid-specific predators, spiders, carabids) revealed a few significant differences for specific taxa between Bt and conventional maize fields, but no general tendencies over the six years. No effects due to the growing of maize event MON810 on non-target communities (including butterfly larvae) were observed during a field study performed in Germany over three consecutive years (Gathmann et al. 2006b; Eckert et al. 2006; Toschki et al. 2007). In another study, monitoring of foliage-dwelling spiders was carried out in Cry1 maize fields and adjacent margins over three successive years in Germany (event Bt176) and compared with non-Bt maize fields. Results did not reveal consistent adverse effects on individual numbers, species richness, and guild structure of spiders due to the cultivation of Cry1-expressing maize (Ludy and Lang 2006a). Ludy and Lang (2006b) also reported that web-building spiders such as the garden spider (*Araneus diadematus*) can be exposed to and thus ingest high amounts of Cry1-containing maize pollen via recycling of pollen-dusted webs. However, a laboratory study showed that the garden spider is not affected in its weight, survival, moult frequency, reaction time, and various web variables following consumption of high amounts of Cry1-containing maize pollen.

Results of a meta-analysis of 42 independent field experiments carried out across different continents by Marvier et al. (2007) indicated that non-target invertebrates are generally more abundant in near-isogenic control fields where no insecticide

treatments are applied than in fields cropped with Bt cotton or Cry1-expressing maize (events MON810, Bt176, MON863). However, when non-Bt cotton or maize fields are managed conventionally with the application of insecticides, non-target taxa are less abundant than in fields cropped with Bt cotton or maize.

A more recent meta-analysis of published field studies on non-target effects of Bt-crops made the differentiation among functional guilds of non-target arthropods. Thereby, the abundance of predators, parasitoids, omnivores, detritivores, and herbivores was compared under scenarios where (i) neither, (ii) only the non-Bt-crops, or (iii) both Cry1 and non-Bt crops received insecticide treatments showed different effects of Cry1 maize among functional guilds of non-target arthropods (Wolfenbarger et al. 2008). As expected, fewer specialist parasitoids of the target pest occurred in Cry1-expressing maize fields, as compared with unsprayed non-Bt controls, but no significant reductions were detected for other parasitoids. In comparison to sprayed non-Bt controls, numbers of predators and herbivores were higher in Cry1-expressing crops, with the magnitude of the difference being influenced by the type of insecticide. Due to reductions in their predator numbers in sprayed non-Bt maize, omnivores and detritivores were more abundant in insecticide-treated controls. However, no differences in abundance were found when both Cry1 and non-Bt crops were sprayed. Predator-to-prey ratios were unchanged by either Bt crops or the use of insecticides; ratios were higher in Cry1-expressing maize relative to the sprayed non-Bt control. These data indicate that a decreased abundance of some target and non-target invertebrate taxa in a maize agro-ecosystem might be observed in areas of cultivation where no alternative pest control measures are adopted. However, the use of and type of insecticides influence the magnitude and direction of observed effects, and insecticide effects were reported to be larger than those of Cry1 crops.

### **27.5.11 Trophic Chain Effects on Predators**

Invertebrate predators can be exposed to the Cry1 protein not only by feeding on plant material or on honeydew excreted from sap-sucking species, but also by feeding on prey organisms that have previously fed on Bt maize (Romeis et al. 2008a, b). Harwood et al. (2005), for instance, studied exposure to the Cry1 protein (event Bt11) for certain groups of non-target organisms, namely Diptera, Hymenoptera, Coleoptera (including predatory Coccinellidae), Hemiptera, Homoptera, Neuroptera, Heteroptera (including herbivore species), Orthoptera, Collembola, Lepidoptera, Dictyoptera, and Araneae. The authors reported levels of Cry1 protein observed within non-target herbivores and their natural enemies such as spiders and predatory insects under field conditions, showing that significant quantities of the Cry1 protein can move into higher trophic levels. Similarly, Obrist et al. (2006a) investigated the transmission of the Cry1 protein through the food chain and thus the exposure of predatory species to the Cry1 protein (event Bt176). These studies showed that the Cry1 protein from GM maize passed along trophic chains up to the

third trophic level, and that in some cases it accumulated in concentrations that were higher than on leaves. The Cry1 protein was detected in certain predators (such as *Orius* spp., *Chrysoperla* spp., *Stethorus* sp.), whilst its presence was negligible in others (e.g., hemerobiids, *Nabis* sp., *Hippodamia* sp., *Demetrias* sp.). Another tritrophic study performed by Obrist et al. (2006b) not only confirmed protein uptake by larvae of the green lacewing, *Chrysoperla carnea*, via its herbivore preys, *Tetranychus urticae* and *Spodoptera littoralis*, after Cry1 maize consumption (see also Dutton et al. 2002), but also confirmed maintenance of the biological activity of the Cry1 protein after ingestion by both herbivore species. Harwood et al. (2007) showed the presence of the Cry1 protein in gut samples of certain predatory coccinellids (e.g., *Coleomegilla maculata*, *Harmonia axyridis*, *Cycloneda munda*, *Coccinella septempunctata*). The fact that the presence of the Cry1 protein was not always confined to periods of anthesis suggested that tritrophic linkages in the food chain facilitated the transfer of the Cry1 protein into higher-order predators.

Hence, the uptake of the Cry1 protein by predators not only occurs by direct feeding on Cry1-expressing plant material (such as pollen), but also indirectly through the consumption of arthropod prey that contains the Cry1 protein, especially for species preying on spider mites (e.g., Andow et al. 2006a, b; Romeis et al. 2008a, b).

Potentially toxic effects on predators fed with preys containing levels of the Cry1 protein might occur when predators are sensitive to the protein. However, direct toxic effects on predators are unlikely due to the specific toxicity of the Cry1 protein to lepidopterans. Based on the current literature, Romeis et al. (2006) suggested that there are few or no indications of direct adverse effects of Cry1-expressing maize on natural enemies. Hence, several studies confirm that the Cry1 protein is not toxic to non-target organisms less closely related to targeted pests. Studying the impact of exposure to the Cry1 protein through prey organisms, Dutton et al. (2002) did not show direct adverse effects of Cry1 maize on predatory lacewings, *C. carnea*, fed on spider mites (*T. urticae*, *S. littoralis*, *Rhopalosiphum padi*) containing different levels of the Cry1 protein. The significant increase in mortality and delay in development observed on *C. carnea* when fed *S. littoralis* were assigned to poor prey quality (Dutton et al. 2002). Likewise, Meissle et al. (2005) related the adverse effects on the generalist predator, *Poecilus cupreus*, fed *S. littoralis* larvae (which had been raised on Cry1 maize; event MON810) to the nutritional quality of the prey and not to the direct effect of the Cry1 protein. In another study, the presence of Cry1 in both prey *T. urticae* and a ladybird (*Stethorus punctillum*) predator collected from commercial fields of maize event MON810 had no adverse effect on the survival of the predator, nor on the developmental time through to adulthood. Furthermore, no subsequent effects on ladybird fecundity were observed (Alvarez-Alfageme et al. 2008). Likewise, Obrist et al. (2006c) concluded that the predatory mite, *Neoseiulus cucumeris*, is not sensitive to the Cry1 protein as no effects were detected when offered Cry1-containing spider mites (such as *T. urticae*). Observed effects on *N. cucumeris* when fed pollen of Cry1

maize (event Bt11) were assigned to differences in the nutritional quality of Cry1 and non-Bt maize pollen, rather than sensitivity to the Cry1 protein.

Hilbeck et al. (1998a,b 1999) indicated significantly prolonged larval development and increased mortality when *C. carnea* larvae were fed lepidopteran larvae reared on Cry1-expressing maize under laboratory conditions. However, key experiments on what caused the significantly higher mortality in Bt-exposed lacewings larvae in these studies are still missing. Because Rodrigo-Simón et al. (2006) reported that the Cry1 protein does not show specific binding in vitro to brush border membrane vesicles from the midgut of *C. carnea* larvae, which is a prerequisite for toxicity, the higher mortality reported by Hilbeck et al. (1998a, b, 1999) is likely to be due to the lepidopteran prey apparently being of lower nutritional quality (Romeis et al. 2004, 2006). This conclusion is supported by data showing that *C. carnea* larvae are unaffected when feeding on non-susceptible *T. urticae* containing large amounts of biologically active Cry1 protein (Dutton et al. 2002). In addition, *C. carnea* larvae in the field are known to feed mainly on aphids, whereas lepidopteran larvae are not considered an important prey, especially after their first moult (Romeis et al. 2004). Because aphids do not accumulate the Cry1 protein (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002), the risk they pose to *C. carnea* larvae can be regarded as negligible. Even though chronic effects cannot be completely excluded, the continuous exposure of *C. carnea* to diets exclusively based on lepidopteran larvae is unlikely under field conditions where a variety of prey is available (Dutton et al. 2003). In addition, Li et al. (2008) demonstrated that adults of *C. carnea* are not affected by Bt maize pollen and are not sensitive to the Cry1 protein at concentrations exceeding those observed in the pollen of Bt maize.

### 27.5.12 Trophic Chain Effects on Parasitoids

In general, invertebrate parasitoids appear to be more sensitive than predators to diets that contain Cry proteins (Lövei and Arpaia 2005), though effects are possibly associated with the poor quality of their hosts. Parasitoids can be exposed to the Cry1 protein through one or more trophic levels (e.g., their host organisms feeding on Bt plant tissue). Indirect host-mediated effects were observed when effects of Cry1 maize on the non-target lepidopteran herbivore, *S. littoralis*, and on the hymenopteran parasitic wasp, *Cotesia marginiventris*, were investigated. *C. marginiventris* survival, developmental times, and cocoon weights were significantly adversely affected when their *S. littoralis* host larva had been fed Cry1 maize. Because *S. littoralis* larvae are significantly affected by the Cry1-expressing maize in terms of development time and survival (e.g., Dutton et al. 2002, 2005; Vojtech et al. 2005), it is likely that these slower-developing hosts might not provide sufficient nutrients for the normal development of parasitoid larvae. Even though direct effects to parasitoid larvae cannot be excluded, as host larvae contain the Cry1 protein, these direct toxic effects seem very unlikely due to the specificity of

the Cry1 protein (Vojtech et al. 2005). However, another study suggested that the Cry1 protein present in the host, *Spodoptera frugiperda*, fed Cry1 maize may have a direct effect on *C. marginiventris*, though confirmatory research on direct exposure (i.e., not mediated by the host) was not carried out in this study (Ramirez-Romero et al. 2007). Ramirez-Romero et al. (2007) observed that the exposure to Cry1 protein via hosts fed Cry1 maize tissue affected parasitoid developmental times, adult size, and fecundity, but not cocoon-to-adult mortality and sex ratio. These effects occurred even when concentrations of the Cry1 protein were low in hosts. The fact that *C. marginiventris* females were smaller and less fecund when fed Cry1-containing hosts as compared with conventional maize, led the authors to suggest a direct effect of the Cry1 protein, though effects on parasitoids of direct exposure to the Cry1 protein were not studied (Ramirez-Romero et al. 2007). However, the specific toxicity of the purified Cry1 protein batch used by Ramirez-Romero et al. (2007) was not measured and compared with the effectiveness of Bt plant material to susceptible target organisms. The effectiveness of different purified Cry1 batches can vary considerably from source to source by a factor of ten (Saeglitz et al. 2006), so that the influence of host quality cannot be excluded in the Ramirez-Romero et al. (2007) study.

By contrast, the performance of *C. marginiventris* feeding on aphid honeydew was observed to increase due to positive effects of Cry1-expressing maize (events Bt11, MON810, Bt176) on the performance of the maize leaf aphid, *Rhopalosiphum maidis* (Faria et al. 2007). Even though aphid performance was within the normal variation observed among conventional maize varieties, different studies reported that aphids perform better on Cry1-expressing maize than on near-isogenic counterparts (e.g., Bourguet et al. 2002; Dutton et al. 2002; Lumbierres et al. 2004; Pons et al. 2005; Eizaguirre et al. 2006). With the larger colony densities of aphids on Cry1 maize, more honeydew was produced, in turn increasing parasitoid longevity and rate of parasitism. Based on the observations made, Faria et al. (2007) concluded that as long as aphid numbers do not reach pest status, the increase in aphid susceptibility in Cry1 maize may pose an advantage in maintaining beneficial insect fauna in Cry1 maize. Because phloem sap of Cry1-expressing maize does not contain the Cry1 protein, the protein is not ingested or excreted by sap-sucking species (e.g., *R. maidis*, *Rhopalosiphum padi*; Head et al. 2001; Raps et al. 2001; Dutton et al. 2002). Parasitoid species feeding on honeydew excreted by sap-sucking species are thus not likely to be exposed to the Cry1 protein (Romeis et al. 2008b).

### 27.5.13 Assessment of Impacts on Pollinating Insects

Maize pollen can be collected, stored, and consumed by honeybees, especially in regions where there are limited sources of pollen when maize is flowering. Pollen feeding is a route of exposure of honeybees to Cry1 protein expressed in maize

event MON810, and potential adverse effects need to be considered in the ERA (EFSA 2008b).

Reviewing available scientific data on potential adverse effects on honeybees of the Cry1 protein or Cry1 pollen of maize gathered under either laboratory or semi-field conditions, Malone (2004) concluded that none of the Cry1-expressing plants commercially available at the time of the publication have significant impacts on the health of honeybees. Other feeding studies performed in controlled conditions with honeybees being fed either with Cry1-containing pollen or mixtures of honey or sugar syrup containing purified endotoxin have indicated no direct adverse effects on larvae and adult survival (Malone and Pham-Delègue 2001; Ramirez-Romero et al. 2005, 2008; Rose et al. 2007). Based on a meta-analysis of 25 independent laboratory studies assessing direct effects on honeybee survival of Cry proteins from currently commercialised Cry1-expressing crops, Duan et al. (2008) concluded that the assessed Cry proteins do not negatively affect the survival of either honeybee larvae or adults in laboratory settings. However, Duan et al. (2008) considered that, in field settings, honeybees might face additional stresses, which could theoretically affect their susceptibility to Cry proteins or generate indirect effects.

Since exposure to Cry1-containing pollen could have potential indirect adverse effects on the development of the whole honeybee colony, some studies focussed on the hypopharyngeal gland development in honeybees. Hypopharyngeal glands are considered an important indicator of bee life history and thus for colony development, as worker (nurse) bees use their hypopharyngeal gland to prepare brood food (jelly) for the larvae. In this respect, Babendreier et al. (2005) fed young adult bees for 10 days with maize pollen expressing Cry1 protein (event MON810) or with purified Cry1 protein solubilized in sugar solutions. No significant differences either in diameter or weight development of hypopharyngeal glands of control bees and bees fed Cry1-containing pollen or Cry1-containing sugar solutions were found. By contrast, protease inhibitors caused significant differences which indicated the sensitivity of the method.

In a field study where colonies foraged on Cry1-expressing maize (event Bt11) and were fed Cry1 pollen cakes for 28 days, Rose et al. (2007) did not observe adverse effects on bee weight, foraging activity, and colony performance. Similarly, in a flight cage study maintained in controlled conditions, no significant differences were reported in honeybee mortality, syrup consumption, and olfactory learning performance when honeybee colonies were exposed to different syrups containing Cry1 protoxin (Ramirez-Romero et al. 2005). In this respect, Ramirez-Romero et al. (2008) recently concluded that negative effects of the Cry1 protein on foraging behaviour and olfactory learning performance of honeybees are unlikely in natural conditions. Feeding behaviour and olfactory learning performance were disturbed only when honeybees were exposed to extremely high concentrations of Cry1 protein (5000 ppb), which do not occur under normal apicultural or field conditions (Ramirez-Romero et al. 2008).

As pollen shedding in a given maize field usually takes place for approximately 10 days each season, potential bee exposure to pollen from maize event MON810

will be limited under normal apicultural conditions. In most cases, the proportion of maize pollen as a total of all pollen collected and fed to larvae during a summer will be low. Babendreier et al. (2004), for instance, reported that fully grown worker bee larvae contain between 1720 and 2310 maize pollen grains in their gut before defecation, corresponding to 1.52–2.04 mg of pollen consumed per larva. On average, 74.5% of pollen grains were completely digested, while 23.3% were partially digested, and 2.2% remained undigested. Since pollen consumption of honeybee larvae is minimal when compared with adults, larval stages are far less exposed to Cry1 proteins. Babendreier et al. (2004) indicated that the contribution of the protein by directly feeding larvae with pollen is less than 5% in relation to the total amount of protein necessary for complete larval development. Moreover, due to the low concentration of Cry1 in event MON810 pollen, honeybees are only exposed to very low concentrations of the protein. In conclusion, the low exposure level of Cry1-containing pollen combined with its low toxicity is unlikely to result in any adverse effects on honeybees under normal apicultural conditions. In addition, sufficient scientific evidence gathered from laboratory and semi-field studies demonstrated the absence of impacts of maize event MON810 pollen on honeybees.

## 27.6 Potential Impacts on Human and Animal Health

The ERA requires addressing potential immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GM plant and persons working with, coming into contact with, or in the vicinity of the GM plant release(s). In addition, an assessment is required of the possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from exposure to or consumption of the GM plant and any products derived from it, when it is intended to be used as animal feed.

Potential long-term effects of Cry1-expressing maize on animal or human health have not been reported in the scientific literature so far (e.g., see reviews by Flachowsky et al. 2007; Hammond 2008). One reason might be that environmental exposure of Cry1 proteins derived from food and feed sources would be negligible, as studies by Lutz et al. (2006; see references therein) indicate that the majority of Cry proteins are rapidly degraded in the gastrointestinal tract of mammals.

## 27.7 Potential Interaction with the Abiotic Environment and Biogeochemical Cycles

Potential effects on the abiotic environment and biogeochemical cycles of Cry1 are unlikely, as the level of Cry1 protein environmental exposure would be low, and no adverse effects of Cry1-expressing maize on the abiotic environment and

biogeochemical cycles are known. For example, the environmental exposure of Cry proteins in soils may be considered as an initial interaction pathway with the abiotic environment and biogeochemical cycles. Cry proteins can bind to humic acids, clays, and the organomineral complex found in soil which may give some protection from degradation (OECD 2007). However, a number of studies provide data that there is no persistence and accumulation of Cry proteins from GM crops in soil (Herman et al. 2001, 2002; Head et al. 2002; Ahmad et al. 2005; Baumgarte and Tebbe 2005; Dubelman et al. 2005; Hopkins and Gregorich 2005; Krogh and Griffiths 2007).

Due to the close interaction between crops and microbe-mediated soil processes (including biogeochemical processes), soil organisms in the rhizosphere are exposed to the Cry1 protein released from Cry1-expressing maize in root exudates. Some studies demonstrated consistent significant differences in relation to micro-organisms between soils with Cry1 and non-Bt maize. Root exudates of Cry1 maize (event Bt176) were shown to reduce presymbiotic hyphal growth of the arbuscular mycorrhizal fungus, *Glomus mosseae*, as compared with those of another Cry1 maize (event Bt11) and control maize (Turrini et al. 2004). Castaldini et al. (2005) also reported consistent differences in rhizosphere heterotrophic bacteria and mycorrhizal colonization (including *G. mosseae*) between Cry1 maize (event Bt176) and its conventional counterpart. According to the authors, the genetic modification in maize Bt176 might have led to changes in plant physiology and composition of root exudates, which in turn may have affected symbiotic and rhizosphere micro-organisms. In this respect, Widmer (2007) suggested that effects observed on symbiotic micro-organisms will only be disadvantageous for the crop itself, without representing a concern for the ecosystem. In addition, a number of other studies (reviewed by Widmer 2007; Filion 2008; Icoz and Stotzky 2008), performed under laboratory, glasshouse or field conditions covering a large array of classic and more recent analytical tools, revealed only some minor changes in soil microbial community structure with Cry1-expressing maize compared with non-Bt maize (Blackwood and Buyer 2004; Brusetti et al. 2004; Griffiths et al. 2006; Mulder et al. 2006) or generally show no adverse effects of the Cry1 protein released by Cry1 maize in root exudates or from biomass incorporated into soil micro-organisms or micro-organism-mediated processes (Saxena and Stotzky 2001a; Flores et al. 2005; Anonymous 2006; Hönenmann et al. 2008; Icoz et al. 2008). Where effects on microbial communities have been reported, these effects were in general considered spatially and temporally limited and small compared with those induced by differences in geography, temperature, seasonality, plant variety, and soil type (Fang et al. 2005, 2007; Griffiths et al. 2005 2006; Lilley et al. 2006; Filion 2008; Icoz and Stotzky 2008). Factors such as plant growth stage and field heterogeneities produced larger effects on soil microbial community structure than Cry1-expressing maize (event MON810; Baumgarte and Tebbe 2005; Griffiths et al. 2007b).

Mulder et al. (2006) reported short-term effects of Cry1-expressing maize (event MON810) which induced ecological shifts in microbial communities of cropland

soils in laboratory tests. However, significant differences in macronutrients between the tested Cry1 maize and the near isogenic comparator are likely to have caused the shift in microbial communities, so that no conclusions on the impact of the genetic modification can be made. Microbial activity was mainly affected by sugar content rather than the Cry1 protein. Percent differences in sugar content were relatively higher than those observed in levels of the Cry1 protein. The highly enhanced soil respiration during the first 72 h after the addition of residues of Cry1-expressing maize, reported by Mulder et al. (2006), can be interpreted as being related to the presence of other macronutrient crop residues. However, three weeks after the addition of the maize residues to the soil, no differences were detected between the activity of specific bacterial guilds in soils amended with transgenic maize and bacteria in soils amended with conventional maize.

Studies in which the decomposition of Cry1-expressing maize was compared with that of non-Bt isogenic lines mostly show that Cry1-expressing maize does not affect decomposition rate or mass of C remaining over time (e.g., Cortet et al. 2006; Tarkalson et al. 2008). Litter-bag experiments with Cry1 maize (event Bt11) reported by Zwahlen et al. (2007) did not reveal major changes in the decomposition rate of Cry1-expressing maize residues. Similarly, various studies on maize event MON810 found no evidence of effects related to the genetic modification when examining the decomposition rate of Cry1-expressing maize (Griffiths et al. 2007b; Hönenmann et al. 2008; Lehman et al. 2008; Tarkalson et al. 2008). These recent findings confirm that previously reported decreases in decomposition rate (e.g., Saxena and Stotzky 2001b; Flores et al. 2005; Fang et al. 2007; Raubuch et al. 2007) do not result from an inhibition of soil micro-organisms by the Cry1 protein, but rather from increased lignin contents in certain maize varieties. Altered lignin content in maize varieties has been shown not to be a generic effect of the Cry1 gene insertion (Griffiths et al. 2007b).

The ERA can conclude that potential effects on biogeochemical processes (e.g., via soil micro-organisms) due to maize event MON810 if they occur, will be transient, minor, and localised in different field settings and are likely to be within the range currently caused by a range of other agronomic and environmental factors.

## 27.8 Impacts of the Specific Cultivation, Management and Harvesting Techniques

An assessment is required of the possible immediate and/or delayed, direct and indirect environmental impacts of the specific cultivation, management, and harvesting techniques used for the GM plant where these are different from those used for non-GM plants. There are reports from Spain indicating that insecticide use is less on Bt maize than on non-GM maize in some areas (Gomez et al 2008) and that

Bt maize comprises a higher proportion of later sown maize than earlier sown maize due to pest infestation times and higher damage reported in later sown maize. There are no other indications that cultivation of Cry1-expressing maize has an impact on the specific management and harvesting techniques. However, there are reports that there are lower levels of *Fusarium* infestation and mycotoxins present in some comparable Bt and non-Bt maize crops and harvest samples and so this may be causing some differential management to control fungal infestations. At present it is not considered likely that any of these changes have detrimental environmental effects and could be indicative of environmental benefits.

The specific herbicide programmes associated with GM herbicide-tolerant crops have been identified as having environmental impacts (EFSA 2008a) and will need to be evaluated if these traits are combined with Bt traits in future GM crops.

## 27.9 Monitoring

In the European Union, the objectives of a monitoring plan according to Annex VII of Directive 2001/18/EC are: (i) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the environmental risk assessment are correct, and (ii) to identify the occurrence of adverse effects of the GMO, or its use, on human or animal health or the environment which were not anticipated in the environmental risk assessment.

A plan for post-market environmental monitoring (PMEM) of GM plants is mandatory in all applications for deliberate release submitted under EU Directive 2001/18/EC and EU Regulation 1829/2003. PMEM aims at identifying possible unanticipated adverse effects on human health or the environment which could arise directly or indirectly from GM plants. PMEM is composed of case-specific monitoring and general surveillance. Case-specific monitoring is not obligatory but may be required to verify risk assessment assumptions and conclusions, whereas a general surveillance plan must be part of the application. Due to different objectives between case-specific monitoring and general surveillance, their underlying concepts differ (Sanvido et al. 2005). In the former, foreseen potentially adverse changes are to be related to specific causes, whereas in the latter the detection of unforeseen changes without known specific cause is the aim. Case-specific monitoring is mainly triggered by scientific uncertainties that were identified in risk assessment. Hence, a hypothesis is established that can be tested on the basis of newly collected monitoring data. In contrast, in general surveillance, the general status of the environment that is associated with uses of GM plants is monitored without any preconception in order to detect any effects that were not anticipated in the environmental risk assessment. When effects are observed they are studied to determine whether the effect is adverse and whether it is associated with the use of a GM plant (EFSA 2006). General surveillance data may originate from the applicants' own surveys as well as from contracted third parties and from existing

compatible agro-environmental monitoring programmes that generated baseline data. Questionnaires for farmers to report on observations of effects linked with the cultivation of GM crops can form a useful part of a general surveillance regime (Schmidt et al. 2008). The EFSA GMO Panel provides guidance for and assesses the scientific quality of case-specific monitoring and general surveillance of for adverse effects of GM plants (EFSA 2006), whilst the final endorsement of PMEM is done by risk managers. The only identified environmental risk of Cry1 GM maize so far is the development of resistance in target insects, and thus case-specific monitoring is required to detect resistance evolution (EFSA 2008b, c).

## 27.10 Conclusions

An extensive body of research data has been assembled on the environmental impacts of Cry1-expressing maize. The available literature so far suggests only minor environmental effects. Toxic effects of Cry1 maize within tier 1 and tier 2 laboratory studies rarely result in significant effects in tier 3 field studies. However, an inherent uncertainty remains to extrapolate from ecotoxicological laboratory experiments in order to make conclusions on long-term environmental effects. The majority of laboratory studies and all the field studies reviewed did not reveal any unexpected adverse or long-lasting environment effects. Negative effects observed in the laboratory do not necessarily translate to field conditions. As negative effects have rarely been observed in the laboratory, tier 1 studies have had a good predictive effect, even for long-term field observations. There is at least ten years experience of cultivating GM crops worldwide and only few established long-term effects have yet been reported (e.g., insect resistance development in Cry1 crops; reviewed by Sanvido et al. 2007).

The BEETLE report (2009) concludes that research studies, modelling, and monitoring are appropriate tools to investigate long-term environmental effects of commercial GMO cultivation. It proposes the development of indicators and databases for appropriate surveillance of long-term effects on soil and other biodiversity resulting from GM crop cultivation and management. Potential indicators should be further developed over time by risk assessors and risk managers. The indicators for environmental monitoring should be selected in accordance with the crop/trait combination and the receiving environment.

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# **Chapter 28**

## **Benefits of Transgenic Plants: A Socioeconomic Perspective**

**Matin Qaim and Arjunan Subramanian**

### **28.1 Introduction**

The global area under transgenic crops grew from 1.7 million hectares in 1996 to 125 million hectares in 2008. Today, over 13 million farmers worldwide grow transgenic crops in 25 countries, including 15 developing countries (James 2008). So far, most of the commercial applications involve herbicide tolerance (see Chap. 9) and insect resistance (see Chap. 10), but other transgenic traits are in the research pipeline and might be commercialized in the short- to medium-term future (Halford 2006).

The rapid global spread of transgenic crops has been accompanied by an intense public debate. Supporters see great potential in the technology to raise agricultural productivity and reduce seasonal variations in food supply due to biotic and abiotic stresses. Against the background of increasing demand for agricultural products and natural resource scarcities, productivity increases are a necessary precondition for achieving long-term food security. Second-generation transgenic crops, such as crops with higher micronutrient contents, could also help reduce specific nutritional deficiencies among the poor. Furthermore, the technology could contribute to rural income increases, which is particularly relevant for poverty reduction in developing countries. And finally, supporters argue that reductions in the use of chemical pesticides through transgenic crops could alleviate environmental and health problems associated with intensive agricultural production systems.

In contrast, biotechnology opponents emphasize the environmental and health risks associated with transgenic crops. Moreover, doubts have been raised with respect to the socioeconomic implications in developing countries. Some consider

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high-tech applications per se as inappropriate for smallholder farmers and disruptive for traditional cultivation systems. Also, it is feared that the dominance of multinational companies in biotechnology and the international proliferation of intellectual property rights (IPRs) would lead to the exploitation of poor agricultural producers. In this view, transgenic crops are rather counterproductive for food security and development.

While emotional public controversies continue, there is a growing body of literature providing empirical evidence on the impact of transgenic crops in different countries. This chapter reviews recent socioeconomic impact studies, focusing on peer-reviewed academic papers. Claims and studies by narrow interest groups are not included, as they are not objective and usually build on unrepresentative information. In the following, we separately review studies on the impacts of insect-resistant and herbicide-tolerant crops, building on available ex-post research. Subsequently, we also briefly discuss the potential effects of future transgenic crop applications from an ex-ante perspective. This includes crops with other improved agronomic traits as well as nutritionally enhanced staple foods.

## 28.2 Impacts of Insect-Resistant Crops

Transgenic insect-resistant crops commercially grown so far involve different genes from the soil bacterium *Bacillus thuringiensis* (Bt) that make the plants resistant to certain lepidopteran and coleopteran pest species. The most widely used examples are Bt maize and Bt cotton. In 2008, Bt maize was grown on 30 million hectares in 17 different countries. The biggest Bt maize areas are found in the United States, Argentina, South Africa, Canada, and the Philippines. Bt cotton was grown on almost 15 million hectares in 2008, mostly in India, China, and the United States, but also in a number of other countries (James 2008).

### 28.2.1 Agronomic Effects

If insect pests are effectively controlled through chemical pesticides, the main effect of switching to transgenic Bt crops is a reduction in insecticide applications, as the genetic resistance mechanism substitutes for chemical control agents. However, there are also situations where insect pests are not effectively controlled by chemical means, due to the unavailability of suitable insecticides or other technical, financial, or institutional constraints. In those situations, Bt technology adoption can help reduce crop damage and thus increase effective yields. Table 28.1 confirms that both insecticide-reducing and yield-increasing effects of Bt crops can be observed internationally.

In conventional cotton (see Chap. 15), high amounts of chemical insecticides are normally used to control the bollworm complex, which is the main Bt target pest.

**Table 28.1** Average farm level effects of Bt crops

Country	Insecticide reduction (%)	Increase in effective yield (%)	Increase in profit (US \$/ha)
<b>Bt cotton</b>			
Argentina <sup>a</sup>	47	33	23
Australia <sup>b</sup>	48	0	66
China <sup>c</sup>	65	24	470
India <sup>d</sup>	41	37	135
Mexico <sup>e</sup>	77	9	295
South Africa <sup>f</sup>	33	22	91
United States <sup>g</sup>	36	10	58
<b>Bt maize</b>			
Argentina <sup>h</sup>	0	9	20
Philippines <sup>h,j</sup>	5	34	53
South Africa <sup>h,k</sup>	10	11	42
Spain <sup>l</sup>	63	6	70
United States <sup>m</sup>	8	5	12

Sources: <sup>a</sup> Qaim and de Janvry (2003, 2005); <sup>b</sup> Fitt (2003); <sup>c</sup> Pray et al. (2002); <sup>d</sup> Qaim et al. (2006), Sadashivappa and Qaim (2009); <sup>e</sup> Traxler et al. (2003); <sup>f</sup> Thirltle et al. (2003), Gouse et al. (2004); <sup>g</sup> Carpenter et al. (2002), Falck-Zepeda et al. (2000); <sup>h</sup> Brookes and Barfoot (2008); <sup>j</sup> Yorobe and Quicoy (2006); <sup>k</sup> Gouse et al. (2006); <sup>l</sup> Gómez-Barbero et al. (2008); <sup>m</sup> Naseem and Pray (2004).

Accordingly, Bt cotton adoption allows significant insecticide reductions, ranging between 30% and 80% on average. However, yield effects are also quite pronounced, especially in developing countries. In Argentina, for instance, conventional cotton farmers under-use chemical insecticides, so that insect pests are not effectively controlled (Qaim and Janvry 2005). In India and China, chemical input use is much higher, but the insecticides are not always very effective, due to low quality, resistance in pest populations, and sometimes also incorrect timing of sprays (Huang et al. 2003; Qaim et al. 2006).

For Bt maize (see Chap. 10), similar effects are observable, albeit generally at a lower magnitude. Except for Spain, where the percentage reduction in insecticide use is large, the more important result of Bt maize is an increase in effective yields. In the United States, Bt maize is mainly used against the European corn borer, which is often not controlled by chemical means.<sup>1</sup> In Argentina and South Africa, mean yield effects are higher, because there is more severe pest pressure. The average yield gain of 11% in South Africa shown in Table 28.1 refers to large commercial farms. These farms have been growing yellow Bt maize hybrids for several years. Gouse et al. (2006) also analyzed on-farm trials that were carried out with smallholder farmers and white Bt maize hybrids in South Africa; they found average yield gains of 32% on Bt plots. In the Philippines, average yield advantages of Bt maize

<sup>1</sup>More recently, a different Bt maize technology was commercialized in the United States to control the corn rootworm complex, against which significant amounts of chemical insecticides are used in conventional agriculture. However, representative studies on the impacts of this new Bt maize technology under farmer conditions are not available.

reach 34%. These patterns suggest that resource-poor smallholder farmers face bigger constraints in controlling insect damage in their conventional crops.

### 28.2.2 *Economic Effects*

The profit effects of Bt technologies are also shown in Table 28.1. Bt seeds are more expensive than conventional seeds, because they are mostly sold by private companies that charge a special technology fee. The fee is positively correlated with the strengths of IPR protection in a country. In all countries, Bt-adopting farmers benefit financially, that is, the economic advantages associated with insecticide savings and higher effective yields more than outweigh the technology fee charged on transgenic seeds. The absolute gains differ remarkably between countries and crops. On average, the extra profits are higher for Bt cotton than for Bt maize, and they are also higher in developing than in developed countries. Apart from agro-ecological and socioeconomic differences, the transgenic seed costs are often lower in developing countries, due to weaker IPRs, seed reproduction by farmers, subsidies, or other types of government price interventions (Basu and Qaim 2007; Sadasivappa and Qaim 2009).

Agricultural policies are also partly responsible for the different profit effects. In the United States, China, and Mexico, the cotton sector is subsidized, which encourages intensive production schemes and high overall yields. The situation is similar for maize in Spain. In Argentina, by contrast, farmers are not subsidized, but face world-market prices. Especially for cotton, world-market prices have been declining over the past ten years, thus eroding the economic benefits resulting from technological yield gains. But also within countries, farmer conditions are heterogeneous so that the effects are variable (Qaim et al. 2006; Pemsl and Waibel 2007).

There are also studies that have analyzed the benefits of Bt crops from a macroeconomic perspective for individual countries and for the world as a whole. For example, using a computable general equilibrium (CGE) model and Bt cotton adoption data from 2001, Anderson et al. (2008) showed that the global welfare gain was in a magnitude of US \$0.7 billion. Since Bt cotton adoption has increased since then, the welfare gains have increased, too. The same study showed that global benefits of Bt cotton could be further boosted to US \$2.3 billion through widespread technology adoption in developing countries, including in sub-Saharan Africa. Apart from direct positive effects on farm profits, the agricultural labor saved through lower pesticide applications in Bt cotton is partly channeled to other activities, including the production of food crops, thus resulting in higher labor productivity and household incomes. Such second-round effects are captured in CGE analyses.

Partial equilibrium models have been used to analyze surplus distribution effects resulting from Bt crops (e.g., Falck-Zepeda et al. 2000; Pray et al. 2001). Farmers benefit through higher farm profits, while consumers benefit from technology-induced price decreases. In addition, innovating companies generate rents through

the technology fee charged on transgenic seeds. With strong patent protection and high seed prices, companies sometimes capture a benefit share of 50% or more. However, in developing countries, where IPR protection is often weak, farmers, and in some cases also consumers, are the main beneficiaries of Bt crops (Qaim et al. 2008).

### ***28.2.3 Poverty and Distribution Effects***

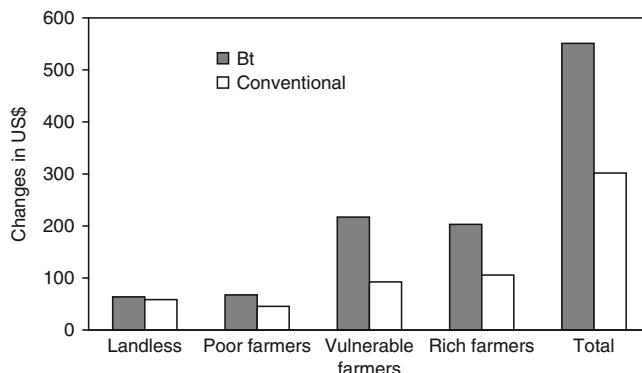
Since 70% of all poor people in the world are smallholder farmers and agricultural laborers, transgenic crops might also have important implications for poverty and income distribution in developing countries. Bt crops are very suitable for the small farm sector. Especially in China, India, and South Africa, Bt cotton is often grown by farms <3 ha. In South Africa, many smallholders grow Bt white maize as their staple food. Several studies show that the Bt technology advantages for small-scale farmers are in a similar magnitude as for larger-scale producers, in some cases even higher (Pray et al. 2001; Morse et al. 2004; Qaim et al. 2008).<sup>2</sup>

Subramanian and Qaim (2009) analyzed wider socioeconomic outcomes of Bt cotton at the micro-level in India, including effects on rural employment and household incomes. Building on a village-modeling approach, they showed that Bt technology is employment-generating, especially for hired female agricultural laborers, which is due to significantly higher yields to be harvested. But employment is also generated in other local rural sectors, like trade and services, which are linked to cotton production. Impacts on household incomes are shown in Fig. 28.1. Each additional hectare of Bt cotton produces 82% higher aggregate incomes than conventional cotton, implying a remarkable gain in overall economic welfare through technology adoption. For landless households, the positive income effects are relatively small: more female employment for cotton harvesting is counteracted by less male employment for pest control. However, all types of farm households – including those below the poverty line – benefit considerably more from Bt than from conventional cotton. These findings demonstrate that transgenic crops can contribute significantly to poverty reduction and rural development.

### ***28.2.4 Environmental and Health Effects***

Bt crops also have environmental and health implications. In the public debate, potential environmental risks, such as undesirable gene flow or impacts on

<sup>2</sup>Especially for India, there are still reports by biotech critics that Bt cotton ruins smallholder farmers. However, such reports do not build on representative data. Gruère et al. (2008) showed that the occasional claim of a link between Bt cotton adoption and farmer suicide cannot be substantiated.

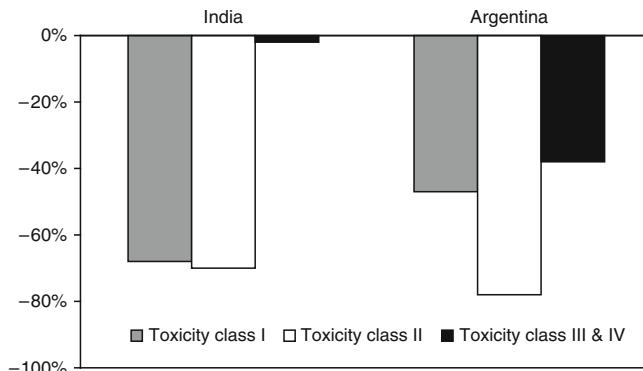


**Fig. 28.1** Household income effects of Bt cotton compared to conventional cotton in India. Results are based on model simulations for a typical cotton-growing village in the state of Maharashtra. Two simulations were run, both considering a 1-ha expansion in the village cotton area. The first scenario assumes that the additional 1 ha is cultivated with Bt cotton, while the second assumes that it is cultivated with conventional cotton. Differences between the two scenarios can thus be interpreted as net impacts of Bt technology adoption (adapted from Subramanian and Qaim 2009)

non-target organisms, are often to the fore. Also, food safety concerns are being raised. Shelton et al. (2002) and Bradford et al. (2005) reviewed such risks, concluding that most of them are not connected to the technique of genetic modification but would be present for any conventionally produced crops with the same heritable traits. While potential risks need to be further analyzed and managed, Bt crops can also bring about substantial environmental and health benefits.

The main environmental benefits are related to reductions in chemical insecticides, so far especially in cotton. Worldwide, cotton is the biggest pesticide-consuming crop, so that the percentage reductions discussed above also translate into huge reductions in absolute quantities. Brookes and Barfoot (2008) estimated that between 1996 and 2006 Bt cotton was responsible for a global saving of 128 million kg of pesticide active ingredients, reducing the environmental impact of total cotton pesticides by 25%. Figure 28.2 shows that Bt adoption leads to over-proportional reductions in the most toxic insecticides.

In the first years of Bt crop deployment it was predicted that insect populations would soon develop Bt resistance, which would undermine the technology's effectiveness and lead to declining insecticide reductions over time. However, until now Bt resistance development has not been observed under field conditions, which might partly be due to successful resistance management strategies, such as the planting of non-Bt refuges. But even in countries where no such strategies are implemented, Bt resistance has not been reported. There are also other factors that can lead to changes in Bt effects over time. In China, for instance, insecticide applications somewhat increased again after several years of Bt cotton use, in spite of the absence of Bt resistance. Wang et al. (2006) attributed this to secondary pests, which might have become more important through the Bt-induced reduction in



**Fig. 28.2** Insecticide reductions through Bt cotton by toxicity class. Results are based on within-farm comparisons obtained from surveys in different cotton-growing regions of India and Argentina. Following the international classification of pesticides, toxicity class I comprises the most toxic, while toxicity class IV comprises the least toxic products (based on data from Qaim and Zilberman 2003; Qaim et al. 2006; Qaim and de Janvry 2005)

broad-spectrum insecticides. Their analysis, however, was based only on one year of observations with increased insecticide applications, so that conclusive statements are premature (Wang et al. 2009). Sadashivappa and Qaim (2009) did not find any evidence of secondary pest outbreaks in India, using data collected over a period of five years.

Bt crops are also associated with health benefits. Direct health advantages for farmers occur due to less insecticide exposure during spraying operations. Often, the health hazards for farmers applying pesticides are greater in developing than in developed countries, because environmental and health regulations are more lax, pesticides are mostly applied manually, and farmers are less educated and less informed about negative side effects. Pray et al. (2001) and Huang et al. (2003) showed for China that the frequency of pesticide poisonings was significantly lower among Bt cotton adopters than among non-adopters. Hossain et al. (2004) used econometric models to establish that this observation is causally related to Bt technology. Bennett et al. (2003) obtained similar results for Bt cotton in South Africa.

For consumers, Bt crops can bring about health benefits through lower pesticide residues in food and water. Furthermore, in a variety of field studies, Bt maize was shown to contain significantly lower levels of certain mycotoxins, which can cause cancer and other diseases in humans (Wu 2006). Especially in maize, insect damage is one factor that contributes significantly to mycotoxin contamination. In the United States and other developed countries, maize is carefully inspected so that lower mycotoxin levels might primarily reduce the costs for testing and grading. But in many developing countries, strict mycotoxin inspections are uncommon. In such situations, Bt technology could contribute to lowering the actual health burden (Wu 2006; Qaim et al. 2008).

## 28.3 Impacts of Herbicide-Tolerant Crops

Herbicide-tolerant (HT) crops are tolerant to certain broad-spectrum herbicides like glyphosate or glufosinate (see Chap. 9), which are more effective, less toxic, and usually cheaper than selective herbicides. HT technology is so far mostly used in soybean (Chap. 24), maize (Chap. 18), cotton (Chap. 15), and canola (Chap. 21). The dominant crop is HT soybean, which was grown on 66 million hectares in 2008, mostly in the United States, Argentina, and Brazil, but also in a number of other countries. Likewise, HT maize is cultivated primarily in North and South America, with smaller areas in South Africa and the Philippines. In maize, HT is often stacked with Bt genes. The same is true for HT cotton in the United States. HT canola is predominantly grown in Canada and the United States (James 2008).

### 28.3.1 Agronomic and Economic Effects

HT-adopting farmers benefit in terms of lower herbicide expenditures. Total herbicide quantities applied were reduced in some situations, but not in others. In Argentina, herbicide quantities were even increased significantly (Table 28.2). This is largely due to the fact that herbicide sprays were substituted for tillage. In Argentina, the share of soybean farmers using no-till almost doubled to 80% since the introduction of HT technology. Also in the United States and Canada, no-till practices expanded through HT adoption (Fernandez-Cornejo and Caswell 2006). In terms of yields, there is no significant difference between HT and conventional crops in most cases. Only in a few examples, where certain weeds were difficult to control with selective herbicides, the adoption of HT and the switch to broad spectrum herbicides resulted in better weed control and higher crop yields.

**Table 28.2** Average farm level effects of HT soybeans in Argentina (Qaim and Traxler 2005)

	Conventional soybeans	HT soybeans	Change (%)
Herbicide expenditure (US \$/ha)	33.64	19.10	-43.2
Herbicide quantity	2.68	5.57	107.8
Toxicity classes I—III (l/ha)	1.10	0.07	-93.6
Toxicity class IV (l/ha)	1.58	5.50	248.1
Share of farmers using no-till practices	0.42	0.80	90.5
Number of tillage passes per plot	1.66	0.69	-58.4
Labor time (h/ha)	3.92	3.30	-15.8
Machinery time (h/ha)	2.52	2.02	-19.8
Fuel (l/ha)	53.03	43.70	-17.6
Cost of production (US \$/ha)	212.99	192.29	-9.7
Soybean yield (t/ha)	3.02	3.01	-0.3
Profit (US \$/ha)	271.66	294.65	8.5

Examples are HT soybeans in Romania and HT maize in Argentina (Brookes and Barfoot 2008).

Overall, HT technology reduces the cost of production through lower expenditures for herbicides, labor, machinery, and fuel. Yet, the innovating companies charge a technology fee on seeds, which varies between crops and countries. Several early studies for HT soybeans in the United States showed that the fee was in a similar magnitude or sometimes higher than the average cost reduction, so that profit effects were small or partly even negative (Naseem and Pray 2004). Comparable results were also obtained for HT cotton and HT canola in the United States and Canada. The main reason for farmers in such situations to still use HT technologies was easier weed control and the saving of management time. Fernandez-Cornejo et al. (2005) even showed that the saved management time for United States soybean farmers translated into higher off-farm incomes. Moreover, farmers are heterogeneous, that is, many adopters have benefited in spite of zero or negative mean profit effects. The average farm level profits seem to have increased over time, partly due to seed price adjustments and farmer learning effects.

In South American countries, the average profit effects of HT crops, especially HT soybeans, are larger than in North America. While the agronomic advantages are similar, the fee charged on seeds is lower, as HT technology is not patented there. Many soybean farmers in South America even use farm-saved transgenic seeds. Qaim and Traxler (2005) showed for Argentina that the average profit gain through HT soybean adoption is in a magnitude of US \$23/ha (Table 28.2). The technology is so attractive for farmers that HT is now used on almost 100% of the Argentine soybean area. In Brazil and other South American countries, where the technology was commercialized more recently, adoption rates are also increasing rapidly.

While farmers in developing countries benefit significantly from HT soybeans, most soybean growers are relatively large-scale and fully mechanized farms. So far, HT crops have not been widely adopted in the small farm sector. Smallholders often weed manually, so that HT crops are inappropriate, unless labor shortages or weeds that are difficult to control justify conversion to chemical practices. In this respect HT crops differ remarkably from Bt crops, which are very suitable for small farmer conditions, as shown above.

Also for HT crops, macroeconomic models have been used to analyze broader welfare effects. Partial equilibrium analyses have shown that the global welfare gains of HT soybeans were in a magnitude of US \$1.2 billion in 2001 (Qaim and Traxler 2005). Due to increasing adoption rates, gains have been further rising since then. At the global level, downstream sectors and consumers are the main beneficiaries capturing over 50% of the benefits, although the effects vary strongly by country. Within the United States, farmers capture about 20% of the national welfare gains, as compared to almost 60% accruing to the innovating company. By contrast, in Argentina the farmer benefit share is 90%. These differences are largely due to different levels of IPR protection (Qaim and Traxler 2005). Using a CGE model, Anderson and Yao (2003) estimated global welfare effects of US \$7 billion per year for HT soybean and HT/Bt maize.

### 28.3.2 *Environmental Effects*

Adoption of HT crops does not lead to reductions in herbicide quantities in most cases; but selective herbicides, which are often relatively toxic to the environment, are substituted by much less toxic broad-spectrum herbicides (Table 28.2). Glyphosate, for instance, has very little residual activity and is rapidly decomposed to organic components by microorganisms in the soil. According to the international classification of pesticides, it belongs to toxicity class IV, the lowest class for “practically non-toxic” pesticides. Also the reduction in tillage operations and the expansion of no-till practices through HT technology adoption brings about environmental benefits in terms of a reduction in soil erosion, fuel use, and carbon dioxide emissions (Qaim and Traxler 2005; Brookes and Barfoot 2008).

Nonetheless, a conclusive environmental evaluation is not possible at this stage. Over the long run, weed species might develop resistance to glyphosate and other broad-spectrum herbicides, which would require increasing amounts of pesticides to be applied. Glyphosate resistance in certain weed species has already been reported in some locations. Furthermore, the high profitability of HT soybeans led many farmers in Argentina and Brazil to convert bush and grass land into soybean land and cultivate the crop as a monoculture. Although the soybean area in these countries had been growing anyway over the last decades, growth has accelerated since the introduction of HT technology. Area conversions and monocultures might contribute to biodiversity loss and other environmental problems. These are not technology-inherent risks, as they might occur in any situation where the relative profitability of one particular crop increases considerably. Still, appropriate policies are required to avoid undesirable externalities.

## 28.4 Potential Impacts of Future Transgenic Crops

### 28.4.1 *Crops with Improved Agronomic Traits*

While Bt is so far mainly used in maize and cotton, there are also other Bt crops that are likely to be commercialized soon (Romeis et al. 2008). Especially Bt rice (Chap. 22) and Bt eggplant (Chap. 25) have already been field-tested extensively in China and India. Data from these field trials are in line with results for already commercialized Bt crops: insecticide-reducing and yield-increasing effects can lead to significant economic, social, environmental, and health benefits (Huang et al. 2005; Krishna and Qaim 2008). Also for other pest-resistant traits that are being developed in different crops – such as fungal, virus, nematode, or bacterial resistance – similar effects can be expected. Qaim and Zilberman (2003) argued that yield effects of pest-resistant transgenic crops will generally be more pronounced in the tropics and subtropics, where pest pressure is often higher and farmers face more severe constraints in controlling pest damage (Table 28.3).

**Table 28.3** Expected yield effects of pest-resistant transgenic crops in different regions (Qaim and Zilberman 2003)

Region	Pest pressure	Availability of chemical alternatives	Adoption of chemical alternatives	Yield effect of transgenic crops
Developed countries	Low to medium	High	High	Low
Latin America (commercial)	Medium	Medium	High	Low to medium
China	Medium	Medium	High	Low to medium
Latin America (non-commercial)	Medium	Low to medium	Low	Medium to high
South and Southeast Asia	High	Low to medium	Low to medium	High
Sub-Saharan Africa	High	Low	Low	High

The effects of transgenic crops with tolerance to abiotic stresses will also be situation-specific. A drought-tolerant transgenic variety, for instance, will lead to higher yields than conventional varieties under water stress, whereas the outcome might be vice versa when sufficient water is available. Especially in the semi-arid tropics, many small-scale farmers are operating under drought-prone conditions, so that the benefits of drought tolerance (Chap. 8) could be sizeable. Using a CGE model, Hareau et al. (2005) estimated that the global annual welfare gains of drought-tolerant rice could be in the magnitude of US \$2.5 billion, with a significant share of these gains occurring in India and other parts of Asia. But also crop tolerance to salinity, flood, and other abiotic stresses could bring about substantial benefits, especially in developing countries. Climate change seems to be associated with more frequent weather extremes, so that more tolerant transgenic crops could help reduce the risks of crop failures and food crises.

#### 28.4.2 *Crops with Improved Nutritional Traits*

Nutritionally enhanced transgenic crops (Chap. 11) that researchers are working on include oilseeds with improved fatty acid profiles, and staple foods with enhanced contents of essential amino acids, minerals, and vitamins. Enhancing food crops with higher nutrient contents through conventional breeding or transgenic approaches is also called biofortification (Qaim et al. 2007). A well known example of a transgenic biofortified crop is Golden Rice, which contains significant amounts of provitamin A. Golden Rice could become commercially available in some Asian countries by 2012 (Potrykus 2008).

Biofortified crops do not involve direct productivity and income effects for farmers or consumers, so that the benefits need to be evaluated differently. Especially in developing countries, micronutrient deficiencies are widespread. Children and women in poverty households are particularly affected; adverse health

outcomes include impaired physical and mental development, higher incidence of infectious diseases, and premature deaths. If biofortified staple crops were widely grown and consumed in developing countries, micronutrient deficiencies could be reduced, entailing important health advantages and economic benefits. Stein et al. (2006) suggested a framework for evaluating the potential benefits: since micronutrient malnutrition causes significant health costs, which could be reduced through biofortification, they quantified the health costs with and without biofortified crops and interpreted the difference – that is, the health cost saved – as the technological benefit.

In their ex-ante analysis of the impact of Golden Rice, Stein et al. (2008) used representative household data from India to show that this technology could reduce the health costs of vitamin A deficiency by up to 60%. They also calculated a high cost-effectiveness of Golden Rice, which compares favorably with other nutrition and health interventions. Anderson et al. (2005) used a macroeconomic CGE model to simulate the benefits of Golden Rice at the global level. Modeling consumer health effects among the poor as an increase in the productivity of unskilled laborers, they estimated worldwide welfare gains of over US \$15 billion per year, with most of the benefits accruing in Asia.

Significant economic and health benefits can also be expected for other biofortified crops, like iron- and zinc-dense staple foods or crops containing higher amounts of essential amino acids (Qaim et al. 2007). The high potential cost-effectiveness of biofortification in developing countries is due to the fact that the approach is self-targeting to the poor, with biofortified seeds spreading through existing formal and informal distribution channels. However, possible issues of consumer acceptance have to be considered. And, especially when no price premium is paid in the output market, suitable strategies to convince farmers to adopt such crops are needed. A combination of nutritional traits with interesting agronomic traits might be a practicable avenue.

## 28.5 Conclusions

Transgenic crops have been used commercially for over ten years. So far, mostly HT and Bt crops have been employed. Available impact studies show that these crops are beneficial to farmers and consumers and produce large aggregate welfare gains. While HT crops lead to cost savings in weed control and tillage operations, Bt crops entail significant pesticide reductions and higher effective yields. Average economic benefits for adopting farmers are sizeable. Moreover, Bt crops bring about environmental and health advantages. They are well suited also for small-scale farmers, contributing to more employment and higher household incomes. In many cases, farmers in developing countries even benefit more than farmers in developed countries, because of weaker IPR protection and differences in agroecological and socioeconomic conditions.

Transgenic technologies that are still in the research pipeline include crops that are tolerant to abiotic stresses and crops that contain higher amounts of nutrients. The benefits of such applications could be even bigger than the ones already observed. Against the background of a dwindling natural resource base and growing demand for agricultural products, transgenic crops could contribute significantly to food security, poverty reduction, and sustainable development at the global level. New technologies will have to play the main role for the necessary production increases in the future. So far, multinational companies dominate transgenic developments, mostly focusing on crops with large international markets. More public research and public-private partnerships will be necessary to ensure that technologies that are particularly relevant for the poor in developing countries are also made available.

In spite of the large potentials of transgenic crops, the technology lacks public acceptance, especially in Europe. Concerns about new risks and lobbying efforts of anti-biotech groups have led to complex and costly biosafety, food safety, and labeling regulations, which slow down innovation rates and lead to a bias against small countries, minor crops, small firms, and public research organizations. Over-regulation has become a real threat for the further development and use of transgenic crops. The costs in terms of foregone benefits might be large, especially for developing countries. This is not to say that zero regulation would be desirable, but the trade-offs associated with regulation have to be considered. In the wider public, the risks of transgenic crops seem to be overrated, while the benefits are underrated.

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# **Chapter 29**

## **Risk Assessment and Economic Applications – the Cartagena Protocol on Biosafety: GMO Approval and Import on a World-Wide Scale**

**Joachim Bendiek and Hans-Jörg Buhk**

### **29.1 Introduction**

The development of techniques to generate genetically modified organisms (GMOs) via transformation with genetic material from a different organism (see Chaps. 1, 2) regardless of their taxonomic relation opens the opportunity to broaden the gene pool of any receiving species. Depending on the trait transferred to the GMO, and regarding the environment the GMO is exposed to, this possibility might offer environmental and/or economical benefits, but might also be related to risks to humans and the environment. Countries that participated in the development of genetic engineering from the beginning were among the first to settle binding rules and legal frameworks for handling of GMOs. To date, a number of different genetically modified crop plants are increasingly cultivated in more and more parts of the world. International trade in commodities and seeds leads to transboundary movement of GMOs and their introduction into different environments. In this context, the Cartagena Protocol on Biosafety (CPB) is welcomed in many parts of the world as it defines standards of requirements that should apply in the case of the transboundary movement of GMOs. These requirements are implemented into national legislation in many parts of the world. This chapter briefly characterises the legal requirements and approval concepts of the European Union (EU) and the United States (US) as two different approaches of GMO regulation in relation to the CPB. The consequences of unsynchronised authorisations of a given GMO related to international trading of commodities are briefly presented.

The CPB uses the term “living modified organism” (LMO). It is defined as “any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology” (UN 2000). “Modern biotechnology”

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means “the application of in vitro nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection” (UN 2000). This definition of the term LMO is compatible with the term “genetically modified organism” (GMO) in legal frameworks of the EU and its Member States (Directive 90/219/EEC, Directive 2001/18/EC).

The narrow definition of the term LMO differs from that of the Convention on Biological Diversity (CBD). Although not explicitly defined in the Convention itself, it is originally understood as an organism resulting from biotechnology. “Biotechnology” in the sense of the CBD means “any technological application that uses biological systems, living organisms, or derivates thereof, to make or modify products or processes for specific use” (UN 1992). Thus, in the Convention the term is defined in a broad sense which includes organisms derived by traditional techniques such as plant breeding and natural processes such as conjugation, transduction and transformation as well as the use of in vitro recombined nucleic acids – a method which is characteristic of genetic engineering. However, when defining the terms of reference for the negotiation of what later became the Cartagena Protocol, the Conference of the Parties to the CBD decided during their second Conference in Jakarta, Indonesia, in 1995, to narrow down the term LMO to modified organisms derived from “modern biotechnology” (Mackenzie et al. 2003). For the purpose of this chapter, the term LMO is used in the context of CBD and CPB whereas the term GMO is used in the context of EU and US legislation.

## 29.2 The Cartagena Protocol on Biological Safety

### 29.2.1 *The Convention on Biological Diversity as the Basis for the Cartagena Protocol on Biological Safety*

The Cartagena Protocol on Biosafety (CPB) is a protocol to the international Convention on Biological Diversity (CBD). A protocol is a binding international instrument for the parties to the CPB, separate from, but related to, another treaty, in this case the CBD (Mackenzie et al 2003).

The CBD is an international treaty aimed at the conservation of biological diversity, the sustainable use of the compounds of biological diversity and the fair and equitable sharing of benefits arising out of the utilisation of genetic resources (UN 1992; Mackenzie et al. 2003). The CBD was adopted in Nairobi, Kenya in May 1991. It was opened for signature at the UN Conference on Environment and Development (*Rio Earth Summit*) in Rio de Janeiro, Brazil, in June 1992, and it entered into force on 29 December 1993.

Countries and regional economic integration organisations that access the Convention become Parties to the CBD. They convene in Conferences of the Parties (COP).

As of January 2009, the CBD comprises 191 parties including regional economic integration organisations such as, for example, the European Union. Only a couple of states (Andorra, Holy See, Iraq, Somalia, US) have not yet joined the Convention (CBD 2009). Only Parties to the Convention may become Parties to the Protocol.

The CBD contains three provisions directly related to biotechnology and LMO. Article 8(g) invites all contracting parties to “regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health”. This article can be understood as a request directed at all Parties to the Convention to define a legal framework for the use and application of LMOs in their country.

According to Article 19 (3), the Parties of the Convention are asked to consider the need for and modalities of a protocol setting out appropriate procedures in the field of the safe transfer, handling and use of any LMO resulting from biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity. Hence, it is this article that forms the basis for the development and, ultimately, adoption of the Cartagena Protocol.

Finally, the third provision, Article 19 (4), refers to the establishment of a clearing house mechanism for the supply and exchange of any available information on the use and safety regulations required by the contracting parties in handling LMO, as well as the supply of any available information on potential adverse effects of the organisms concerned to the contracting party to whom those organisms are to be consigned. The implementation of this article by the contracting parties contributes to transparency in international trade. The sharing of information and options about the potential (adverse) effects of specific LMOs in a specific environment facilitates capacity building and hence leads to advanced expertise in assessing the potential effects of a given LMO.

In interpreting the Cartagena Protocol it is important to understand that the term “biological diversity” implicitly applies to living organisms only, including viruses and viroids. As a protocol to the CBD, the Cartagena Protocol is restricted to living organisms, which explains why LMO-derived products (e.g. food and feed produced from LMO) do not fall under its scope.

In summary, the CBD considers the development of the Cartagena Protocol, fixes the scope of the Cartagena Protocol and determines the access of parties to the Cartagena Protocol.

## **29.2.2 *The Cartagena Protocol on Biosafety and the Biosafety Clearing House***

The Cartagena Protocol on Biosafety to the Convention on Biological Diversity sets an international standard for a minimum set of requirements in the field of safe transfer, transport, handling and use of living modified organisms resulting from

modern biotechnology that might have adverse effects on the conservation and sustainable use of biological diversity. The precautionary approach contained in Principle 15 of the Rio Declaration on Environment and Development shall be applied, human health shall be taken into account and it specifically focuses on the transboundary movement of LMOs from one country to another (UN 2000).

From the outset there has been intensive debate on the possibilities and benefits that genetic engineering might offer to different sectors of society on the one hand and the potential impacts on and threats to human health and the environment from an unconsidered and interest-driven application of genetic engineering on the other (Mackenzie et al. 2003). It is the inherent potential of so-called modern biotechnology that fosters both positions:

1. Advocates of this technology herald a new green revolution with less resource input, and higher food and feed output – even from currently unproductive land.
2. Concerned observers of the technology warn of the potential invasiveness of GMOs into new environments, their potential impacts on non-target species, on soil organisms and, consequently, on cycles of materials, and of the potential direct or indirect effects on human health.

In fact, gene technology opens the possibility to transfer any given genetic information into any organism, regardless of the taxonomic distance between donor and recipient organism. Theoretically, the possibilities that arise from this potential are infinite. However, laboratory practice and the complexity of living organisms still seem to set considerable limitations to the application of the technology. To date, the majority of GMOs used on a commercial scale under unconfined conditions are still developments of rather simple genetic structure: The analysis of data on farm land covered with GMO crops shows that GMOs prevail which express herbicide tolerance genes or insect resistance conferred by the expression of one or several pest-specific proteins of *Bacillus thuringiensis* or a combination of these traits (stacked events; Kinderlerer 2008; USDA 2008).

In the mid-1990s, when negotiations on the Cartagena Protocol officially began, the majority of countries had not yet put any LMO regulations into force. However, important commodity exporting countries had started to produce GMO crops and it was only a matter of time before these products entered the international trade chain. Many countries, particularly food- and feed-importing countries in the developing regions of the world, feared that they would be confronted with the import of essential products without these having been evaluated for their potential effects on the environment of the country in question or on human health. The Cartagena Protocol strengthens the position of these importing countries, as it defines basic requirements for the transboundary movement of LMOs. The basic idea is that any Party to the Protocol has the opportunity to decide on a scientific basis whether a given LMO may be imported before the transboundary movement occurs. To this end, the exporting entity must provide the importing country with a set of information required for a scientifically sound risk assessment before the import takes place. Thus, referring to the CPB, the exporter can expect a science-based decision of the importing country within a given time frame.

**Table 29.1** Major LMO-producing countries and their status in relation to the Cartagena Protocol (James 2007; BCH 2009a)

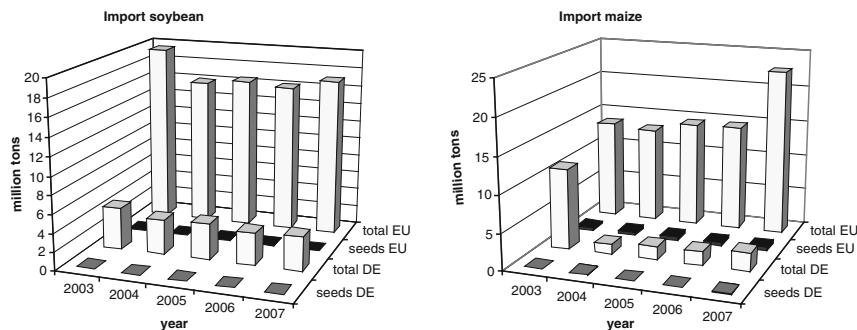
Country	GM acreage (10 <sup>6</sup> ha)	LMO crops	CPB party
US	57.7	Soybean, maize, cotton, canola, squash, papaya, alfalfa	No
Argentina	19.1	Soybean, maize, cotton	No
Brazil	15.0	Soybean, cotton	Yes
Canada	7.0	Oilseed rape, maize, soybean	No
India	6.2	Cotton	Yes
China	3.8	Cotton, tomato, poplar, papaya, sweet pepper	Yes
Paraguay	2.6	Soybean	Yes
South Africa	1.8	Maize, soybean, cotton	Yes
Uruguay	0.5	Soybean, maize	No
Philippines	0.3	Maize	Yes
Australia	0.1	Cotton	No
Spain	0.1	Maize	Yes

The Cartagena Protocol was adopted on 29 January 2000 in Montreal, Canada, and entered into force on 11 September 2003. The CPB is only open to Parties to the CBD. As of January 2009, it has 153 Parties (BCH 2009a). However, several major LMO-producing countries are not party to the CPB (Table 29.1).

The CPB requests a positive decision of the importing country before the import of a LMO occurs. These decisions result from a scientifically sound risk assessment. An information-sharing system via the Biosafety Clearing House (BCH) is implemented and, in cases of unintentional or illegal transboundary movement of LMO, the CPB defines the necessary information procedures to the country of import. Still contentious are the implementation of measures mentioned in the Cartagena Protocol regarding the handling, transport, packaging and identification of LMO, labelling requirements, liability and redress.

In general, LMOs are exported to a country with two different objectives. On the one hand, LMOs are exported and imported for the purpose of sowing/planting as propagation material. This propagation material is destined to be introduced into the environment. Hence, this material and the resulting plants interact with the environment throughout their lifecycle. Propagation material represents a considerably smaller fraction of exported and imported LMO for example from the US to the EU (Fig. 29.1).

On the other hand and representing the vast majority of volume, there is the import of LMOs for direct use as food, or feed, or for processing (Fig. 29.1). These commodities are shipped in huge quantities (e.g. maize, soybeans, cereals, oilseed rape) and after unloading they are soon processed into intermediate or final food, feed, or technical products, according to their intended use. Very often they represent inhomogeneous mixtures of various origins (Kalaitzandonakes 2004, 2006). One of the common features of this material is that, during processing, it loses its biological potential to germinate and hence to propagate. Thus, the possibility of interaction between the environment and these LMOs, which are imported for the purpose of direct food, feed, or processing, is generally limited to a



**Fig. 29.1** Proportion of seed import on total import of maize and soybean into Germany and the EU in 2003–2007 (BMELV 2009)

short interval between unloading at the point of import and processing at the respective facility.

Consequently, according to the expected use of the LMO in the country of import, two different procedures of entrance and hence of authorisation procedure are defined in the Cartagena Protocol. The advanced informed agreement procedure (AIA) applies to LMOs intended for the introduction into the environment (cultivation, field trials). LMOs intended for direct use as food, feed, or for processing (ffp) do not need an agreement according to the AIA. However, they do require a positive decision of the importing country before the first import can take place.

The Cartagena Protocol defines the minimum set of information that the entity of export shall deliver to the competent authority of the party of import. Both the necessary information required for the notification documents to the different procedures and a brief guideline on how to perform a LMO risk assessment are given in the annexes of the Cartagena Protocol.

The core of the Cartagena Protocol is the Biosafety Clearing House (BCH). This is a huge web-accessible database (BCH 2009b), developed and maintained by the Secretariat of the Convention on Biological Safety (SCBD) in Montreal which contains, among other data, the decisions of the Parties regarding the domestic use of LMOs, summaries of risk assessments carried out to provide the scientific basis for the decisions of the Parties regarding the domestic use of LMOs, brief descriptions of the evaluated LMOs, brief descriptions of the genes and genetic elements transferred into the LMO, information about the legal framework that applies to the domestic use of LMOs, individual Party contact information (institutions, websites) and a biosafety information resource centre (BIRC).

The entry of data mentioned above is an obligation for all Parties to the Protocol. However, the BCH is also open for data input to non-Parties to the Protocol. As it is the national legitimised entities that deliver relevant information, this is one of the very few databases accessible to the public with official and reliable information on the legal status of a LMO and the legal framework in a given country. Regarding information exchange, the BCH is a virtual meeting point and brings together parties that want to share their experience in risk assessment and decision-making.

However, non-Parties that are LMO producers (e.g. US, Canada, Argentina) submit extensive information about their LMO decisions and legal frameworks, providing evidence of the importance of this information tool (BCH 2009b).

How to put the requirements of the Protocol into practice is within the responsibility of each Party to the Protocol. According to the information offered in the “Laws and Regulation” section of the BCH (2009b) as of January 2009 there are LMO legal frameworks in force in approximately 70 countries; and another 35 countries are on the way to passing legal frameworks. In contrast to many developing countries, practically all industrialised countries have legal frameworks on LMO in force. This particularly is the case for the large LMO-exporting countries that are non-Parties to the CBP.

## 29.3 GMO Approval

As pointed out in the previous section, the GMOs and products thereof in many countries around the world are subject to legal restrictions different from those that apply to traditionally bred varieties (Kinderlerer 2008). Generally, a GMO or product thereof needs an active authorisation for an intended use. This is the case, for example, for GMOs and certain products thereof in the EU (e.g. food, feed, medicinal products) and many other countries. In the US, however, there is no GMO-specific legal framework (USA 2009). There, it is evaluated whether a GMO needs to be regulated according to existing legal frameworks. The differences between both legal approaches are outlined in detail below.

### 29.3.1 European Union

In the EU, legislation for the development, handling, storage, use and destruction of GMOs have been in place since 1990. It were *Council Directive 219/90/EEC on the contained use of genetically modified micro-organisms* and *Council Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms* that paved the way to an ever-increasing and exhaustive list of legal requirements that now apply to the use of GMOs and products thereof. To date, there are several basic legal rules for handling GMOs in the EU.

Council Directive 219/90/EEC and its amendment, Council Directive 98/81/EC, on “the contained use of genetically modified micro-organisms” sets the frame for any activity under contained conditions in which micro-organisms are genetically modified or in which such genetically modified micro-organisms are cultured, stored, transported, destroyed, disposed of or used in any other way, and for which specific containment measures are used to limit their contact with the general population and the environment (EU 1990, 1998).

Directive 2001/18/EC on “the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC” applies to any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with and to provide a high level of safety for the general population and the environment (EU 2001). Hence any organism introduced into the environment of a Member State of the EU requires prior authorisation. Details of the authorisation processes are given below (Fig. 29.2).

Both directives need to be implemented into national legislation. In the case of Germany, the *Gentechnikgesetz* [Gene Technology Act] and the corresponding *Gentechnik-Verordnungen* [National Regulations on Gene Technology] represent the main instruments to implement the above mentioned EU directives into national legislation to regulate GMOs (DE 2009; BCH 2009b).

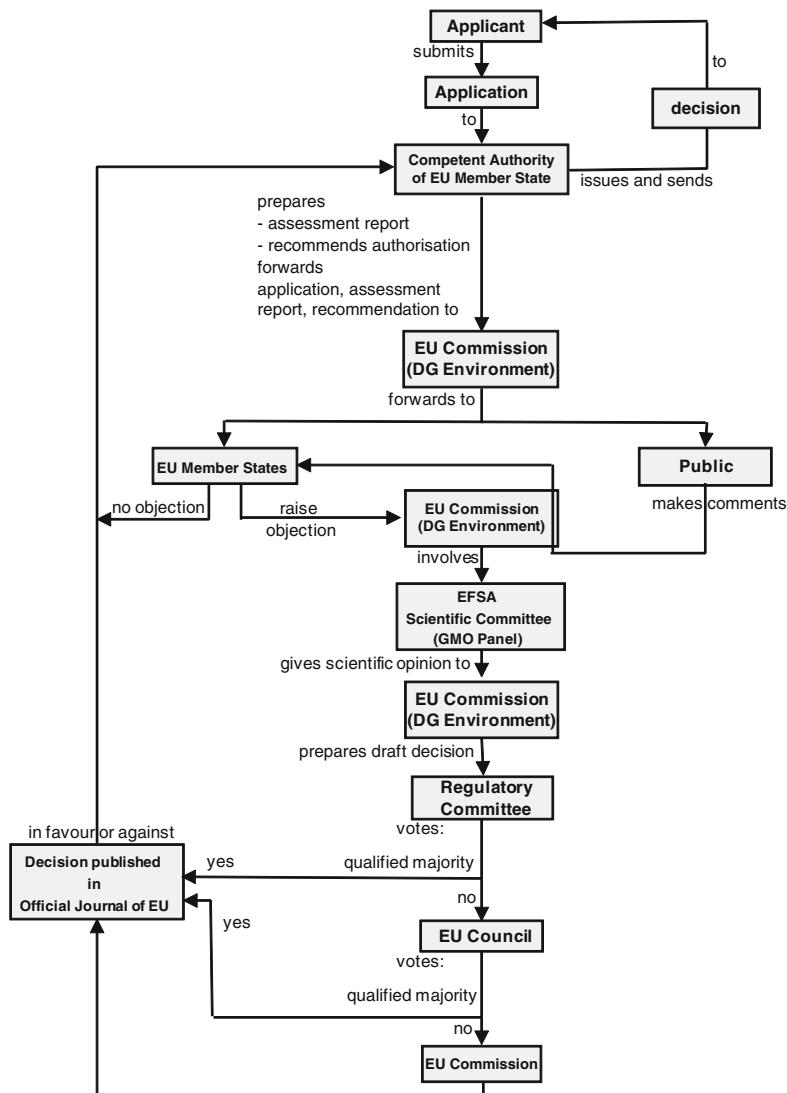
Regulation (EC) No 1829/2003 on genetically modified food and feed (EU 2003a) applies to genetically modified food and feed (GMFF) to be introduced onto the market in the EU. It also applies to genetically modified crop plants to be used as food, or feed, or as food/feed derived from GMO. Regarding aspects of a necessary environmental risk assessment as to be applied for living GMO including their cultivation, this regulation refers to the respective Directive 2001/18/EC and thus highlights the strong interrelation between both legal rules.

Regulation (EC) No 1830/2003 concerns the traceability and labelling of GMO and the traceability of food and feed products produced from GMO (EU 2003b). It basically contains the rules for the labelling of GMO and products thereof, and guarantees tracing a product from “farm to fork” and from “fork to farm” (EU 2009b, c). The labelling of GMO and products thereof is compulsory, except labelling thresholds are defined.

Labelling of GMO and GMFF authorised for commercial use is compulsory according to any of the existing legal frames (Directive 2001/18/EC, Regulation (EC) No. 1829/2003, Regulation (EC) No. 1830/2003). However, labelling of GMFF is not necessary if the genetically modified proportion is not higher than 0.9% of the food or feed ingredient, provided that this presence is adventitious or technically unavoidable (see also Chap. 7). Hence, the often-quoted 0.9% threshold is in fact a labelling threshold. This exception for labelling does not apply to seeds. Consequently, even seed lots with a low level (adventitious) presence of GMO that are authorised for cultivation, need to be labelled. This issue is referred to as “zero tolerance” of labelling. GMO and GMFF not authorised in the EU are illegal, regardless the proportion might be. Here, the term “zero tolerance” is applied as well, although in a different context and with different consequences.

Regulation (EC) No. 1829/2003 and Regulation (EC) No. 1830/2003 are embedded in a number of further EU regulations, decisions and recommendations which apply to general inspection and control requirements of food and feed and to the necessary sampling and detection procedures of food and feed, putting special emphasis on requirements of GMO detection (EU 2002, 2004a, b, c, d, 2006).

With respect to the CPB and the BCH, EU Regulation (EC) No. 1946/2003 (EU 2003c) is of fundamental importance. The European Community relies on its



**Fig. 29.2** Brief display of the decision-making process for genetically modified organisms (GMO) in the European Union according to Directive 2001/18/EC. An applicant submits an application for authorisation to the national competent authority of a Member State of the EU (“rapporteur”). The national competent authority acknowledges the receipt of the application and informs the European Commission about the application. The competent authority performs the risk assessment and prepares an assessment report according to the provisions of the Directive and the corresponding national legal framework. This assessment report contains a recommendation for authorisation of this application. According to national legal frameworks, in many Member States the competent authority respects opinions of further institutions (e.g. advisory bodies) for their assessment report. The application and the assessment report are sent to the other EU Member States via the EU Commission. Then the other EU Member States evaluate the submitted documents and decide whether they support the recommendation of the rapporteur

existing legislative framework for intentional movements of GMOs within the European Community and for imports of GMOs into the European Community (BCH 2009c). In the EU, Regulation (EC) No. 1946/2003 specifically connects the extensive EU legal framework with the requirements of the Cartagena Protocol. Additionally, EU exporters of GMOs which are intended for deliberate release into the environment in third countries (outside the EU) must inform the EU Commission and the Competent Authority of the Member State of export before the export takes place.

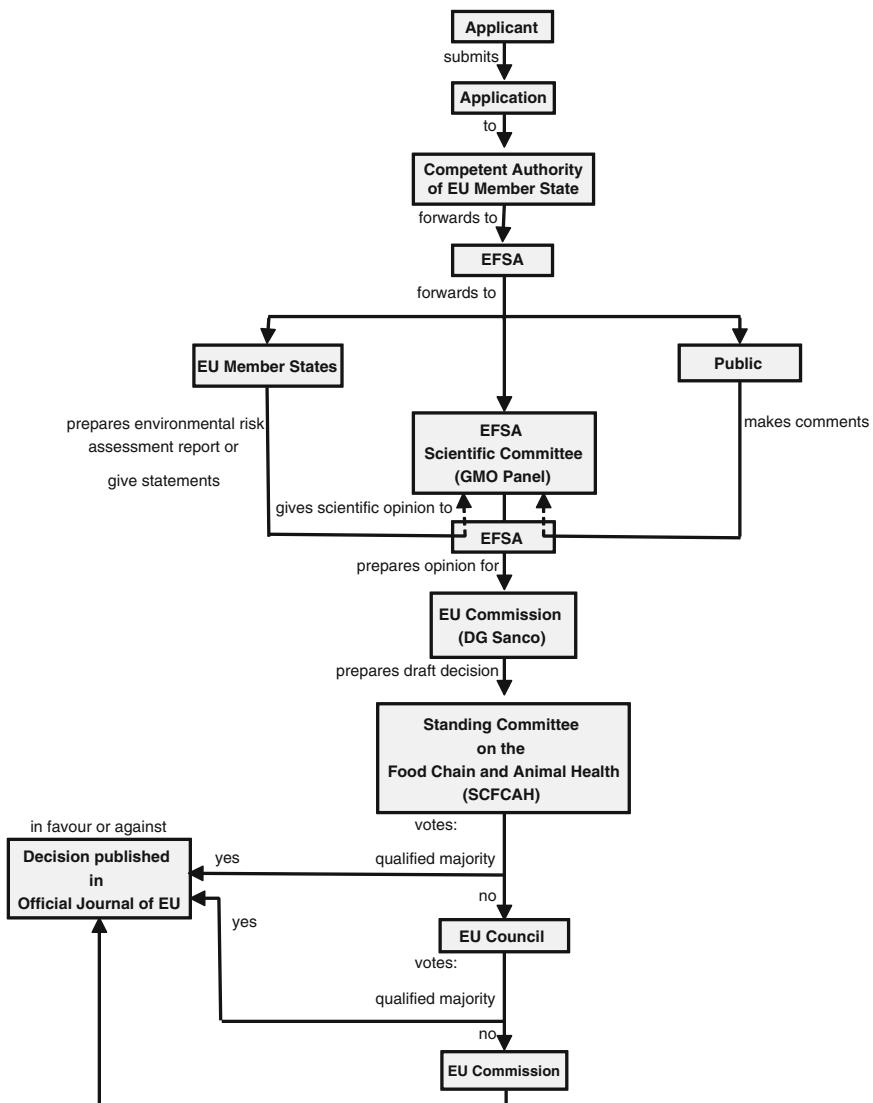
### 29.3.1.1 Authorisation of Genetically Modified Food and Feed in the EU

In the EU, food and feed consisting of, containing or produced from GMO (genetically modified food and feed, GMFF) need to undergo a safety assessment through a European Community procedure before being placed on the market within the European Community (EU 2003a). No GMFF shall be placed on the market within the European Community unless it is covered by an authorisation granted in accordance with Regulation (EC) No. 1829/2003 and the relevant conditions of the authorisation are satisfied. To obtain an authorisation, a complex procedure applies that is basically processed at EU level (EFSA 2006). The European Food Safety Authority (EFSA) is the central institution during the risk assessment process in these procedures. The decision-making procedure is briefly described in Fig. 29.3.

The evaluation process of applications of GMFF comprises two different layers: the evaluation of the use of the GMFF for food and feed purposes and the assessment of the potential environmental risk (see Chap. 27) of the corresponding GMO. The first is done by institutions experienced in food and feed evaluation, the latter by institutions experienced in GMO environmental risk assessment according to Directive 2001/18/EC, as this Directive regards the deliberate release of GMO into the environment. Member States are directly involved in the evaluation process. In the Member States different institutions might be responsible for the respective evaluations. This is the background why Regulation (EC) No. 1829/2003 defines different application scopes and makes a difference to the extent of involvement of different institutions.

If the application concerns GMOs to be used as seeds or other plant-propagating material (cultivation), the EFSA *must* ask a national competent authority of one Member State designated in accordance with Directive 2001/18/EC to carry out the environmental risk assessment according to this Directive. The national competent authorities under Directive 2001/18/EC of the other Member States are consulted by the EFSA during this process.

If the application concerns GMOs to be used for import and processing (no cultivation), the national competent authorities regarding Directive 2001/18/EC are consulted by the EFSA. Additionally, the EFSA *may* ask a national competent authority described above to carry out the environmental risk assessment according to Directive 2001/18/EC. To date, the EFSA has not used this option in such cases.



**Fig. 29.3** Brief display of the decision-making process for genetically modified food and feed (GMFF) in the European Union according to Regulation (EC) No. 1829/2003. An applicant submits an application for authorisation to the national competent authority of a Member State of the EU. The national competent authority acknowledges receipt of the application and forwards the application to the European Food Safety Authority (EFSA). The EFSA makes the application available to the other Member States and the Commission, and makes the summary of the application available to the public. The scientific assessment of the application will be undertaken under the responsibility of the EFSA. The application is subject to a science-based risk evaluation. The results of the risk assessment are expressed in an EFSA opinion that is sent to the EU Commission. In order to prepare the opinion, the EFSA has to consider statements and comments from different institutions and entities depending on the scope of the application and the type of product to be evaluated (EU 2003a)

If the application concerns food derived from, but not consisting of or containing GMOs, the EFSA *may* ask the appropriate food assessment body of a Member State to carry out a safety assessment of the food in accordance with Regulation (EC) No. 178/2002. This option applies, for example, to sugar (saccharose) derived from genetically modified plants like sugar beet or sugar cane. The sugar, although chemically identical to sugar of non-genetically modified plants, is subject to an authorisation process under Regulation (EC) No 1829/2003. For applications that correspond to this example, the EFSA offers the possibility to the competent authorities under Regulation (EC) No. 1829/2003 to give a statement to the application.

In order to develop their opinion, the EFSA is supported by an independent Scientific Committee that gives scientific advice in the area of new and harmonised approaches for the risk assessment of food and feed (EU 2002). It also provides strategic advice to the EFSA's Executive Director. The Scientific Committee is organised into several Panels that attend to specific issues of food and feed risk assessment. The "GMO Panel" is dedicated to GMO. It carries out risk assessments in order to produce scientific opinions and advice for risk managers. Its risk assessment work is based on reviewing scientific information and data in order to evaluate the safety of a given GMO.

The public has access to the application for authorisation, excluding the confidential information, to the opinions from the competent authorities of the Member States and to other information, according to Regulation (EC) No. 1829/2003. The public may give comments and statements to the application within a given time limit, which ends before the EFSA adopts its opinion. The EFSA considers these comments in its final opinion, which is also published.

The EU Commission prepares a draft of the decision to be taken in respect of the application, taking into account the EFSA opinion. This draft is presented to an EU executive body, the Standing Committee on the Food Chain and Animal Health (SCFCAH), composed of representatives of the Member States (Fig. 29.3). The SCFCAH decides upon the draft with weighted votes: depending on their population, Member States have a different number of votes. Decisions are taken with "qualified majority" in favour or against the draft.

Adoption with qualified majority leads to the publication of the decision in the Official Journal of the European Commission. This publication is the official authorisation for the market placement of GMFF (Fig. 29.3).

It is worth mentioning that frequently this Standing Committee does not find a qualified majority in favour or against an application. In these cases, a regulatory procedure set up by the European Council applies (EU 1999). The draft decision is forwarded to the Council of the European Union. The Council may or may not decide with qualified majority upon the draft. In the case when the Council does not decide, the Commission adopts a decision (Fig. 29.3).

Experience shows that these decision-making processes may take a very long time, because the Member States do not reach a qualified majority at the different steps of decision-making.

### 29.3.1.2 Placing on the Market of Genetically Modified Organisms According to Directive 2001/18/EC

GMOs need to undergo a safety assessment through a European Community procedure before being placed on the market within the European Community (EU 2001). In this respect, there is no difference to GMFF. As for the GMFF, a complex procedure applies in order to obtain an authorisation. However, the procedure for placing on the market of GMOs according to Directive 2001/18/EC differs in several respects from the procedure described in the previous section.

Applications are limited to organisms, i.e. living entities, including viruses and viroids. If during processing a GMO turns out to lose its ability to replicate, it is no longer considered an organism. Resulting products, even if derived from GMOs, do not fall under the scope of this Directive.

The competent authority of the Member State receiving the applicant's application form drives the procedure (rapporteur). This competent authority hands the final decision document to the applicant.

Since Regulation (EC) No. 1829/2003 on GMFF is in place, procedures according to Directive 2001/18/EC apply mainly to those organisms that are not used for food and feed. Examples are ornamental plants, shrubs, trees, micro-organisms (for technical purposes), pet animals, etc. However, applications regarding GMO intended to be released into the environment as well (e.g. for cultivation) and to be used for food and feed purposes might still be authorised according to this Directive. But independent of such an application, an additional application under Regulation (EC) No. 1829/2003 is still necessary for food and feed approval and hence this leads to a double procedure. However, Regulation (EC) No. 1829/2003 offers the possibility to cover food and feed purposes as well as cultivation within one application. This possibility is referred to as the "one door, one key" principle (EU 2009a). However, such a procedure under Regulation (EC) No. 1829/2003 refers to Directive 2001/18/EC regarding environmental risk assessment. This decision-making procedure is briefly described in Fig. 29.2.

If all Member States agree to the decision recommendation of the rapporteur, the authorisation is issued by that national competent authority of the Member State to whom the application was first submitted (Fig. 29.2). In the past, very few applications were authorised this way. These procedures required 15–16 months from the submission of the application to receipt of the authorisation and were the fastest ever decided regarding placing on the market in the EU. All of them regarded genetically modified carnations.

In most cases, however, one or several competent authorities of the other Member States raise objections to the approval of the application. In such cases a decision-making procedure at the Member State level applies, similar to the one described under Sect. 29.3.1.1 (Figs. 29.2, 29.3). The regulatory committee referred to in Fig. 29.2 is composed of the representative of each Member State and is chaired by the representative of the Commission. But still, it is the competent

authority of the Member State receiving the application that certifies the decision document (Fig. 29.2).

### 29.3.2 United States of America

The United States (US) apply a different approach to assess genetically engineered products. In contrast to other countries, the US has not issued a specific legislation that particularly addresses GMOs or products thereof, but amended existing health and safety laws. This system was delineated in 1986 under the Coordinated Framework for Regulation of Biotechnology (USA 1986). To this end, regulations and guidelines were developed as needed to address GMO-specific aspects under already existing laws. The approach of the USA is often characterised as being product-specific, whereas other countries apply a technique-specific (method-specific) approach. Currently, the laws applied to regulate the products of modern biotechnology are the Plant Protection Act (PPA), the Federal Food, Drug, and Cosmetic Act (FFDCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA).

If necessary, new regulations and guidelines are developed as the need arises. Actually, a guideline is underway towards adoption that will govern approval of the use of genetically engineered animals (Wadman 2008; Ledford 2009).

Under the Coordinated Framework, agencies that were responsible for regulatory oversight of certain product categories or for certain product uses are also responsible for evaluating those same kinds of products developed using genetic engineering. The US government agencies responsible for oversight of the products of agricultural modern biotechnology are the Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS), the Environmental Protection Agency (EPA) and the Department of Health and Human Services' Food and Drug Administration (FDA). Depending on its characteristics, a GMO or GM product may be subject to review by one or more of these agencies. Depending on its intended use, a product may or may not be reviewed by all three regulatory agencies.

A food crop plant developed using genetic engineering to produce a pesticide in its own tissue provides an example that is reviewed by all three regulatory agencies. A common example of this type of product is maize into which a gene isolated from the soil bacterium *Bacillus thuringiensis* (Bt) has been inserted (see Chap. 10). The Bt gene encodes a protein with insecticidal effects which is expressed in the tissue of the genetically modified plant in order to protect the plant against a particular feeding insect such as, for example, the European corn borer (*Ostrinia nubilalis*). This particular maize plant is reviewed under different aspects from the three US agencies mentioned above.

Under the Plant Protection Act (PPA) USDA-APHIS in principle regulates a genetically modified maize plant (import, interstate movement, release into the environment, as in field trials), regarding it as a potential plant pest risk similar to

foreign species. For deregulation (placing on the market), the developer of the plant has to provide data to show that the plant is not a plant pest. If proven, the developer is granted de-regulated status for the GM plant. Consequently, movement (import, interstate movement, release into the environment) is no longer subject to regulation by authorisation.

The EPA regulates the distribution, sale, use and testing of the pesticidal substance. For example, in the case of Bt in maize, the EPA regulates the Bt because the Bt protein expressed in the genetically modified plant is a pesticide. The EPA generally regulates the field testing of pesticides through an Experimental Use Permit. In order to legally distribute the pesticide in commerce, the company must register the pesticide with the EPA. Through this registration, the EPA can establish the conditions of commercial use. The EPA is also responsible for setting the amounts or levels of pesticide residue that may safely be in food or feed (i.e. establishing a tolerance). The EPA may allow an exemption from the requirement to set such a tolerance level if it can be shown that there are no food or feed safety issues associated with the pesticide (USA 2009).

Developers of Bt crops also consult with the FDA about possible other unintended changes to the food or feed, for example possible changes in nutritional composition or levels of native toxicants. Although this consultation is voluntary, all of the food/feed products commercialized to date have gone through the consultation process. The consultation with the FDA serves to ensure that safety or other regulatory issues that fall within the agency's jurisdiction. In general, there are no labelling requirements in the USA for food and feed derived from genetically modified plants (USA 2009).

## 29.4 GMO Approval, GMO Labelling and GMO Trade

The introduction of approval systems and labelling requirements in different countries of the world for GMOs and GMO products has influenced international trade. Unsynchronised authorisation for the commercial use of GMOs and GMO products in different trading countries rigorously impedes the interchange of certain products. GMOs that are authorised for commercialisation in commodity-producing and exporting countries, such as the US, may not be exported to countries where these GMOs are not authorised as, for example, into the EU. Consequently, either the export of certain products from the US to the EU decreases, or the producing country implements a system that enables the separate production and processing of GMO-containing and GMO-free products. In fact, the import of rice from the US into the EU dropped drastically after a genetically modified rice event was detected in 2006 in rice originating from the US but not authorised in the EU (Table 29.2). However, the European demand for rice could be covered with imports from other regions of the world. The import of rice from, for example, Guyana, India, Thailand and Uruguay increased by approximately 20% (India) to

**Table 29.2** EU imports (t) of rice and rice products (2005–2007) from selected exporting countries (BMELV 2009)

Source	Year		
	2005	2006	2007
Total	2 660 126	2 824 249	3 026 320
United States	291 633	205 205	44 177
Guyana	106 249	90 888	133 404
India	234 763	304 741	365 437
Thailand	260 274	309 955	438 833
Uruguay	10 073	50 314	144 838

approximately 290% (Uruguay) between 2006 and 2007 and thus easily compensated the existing demands in the EU (Table 29.2).

## 29.5 Conclusions

The Cartagena Protocol on Biosafety is a binding international instrument under the Convention on Biological Diversity. It defines general requirements in the field of safe transfer, transport, handling and use of those LMOs that might have adverse effects on the environment. It specifically focuses on the transboundary movement of LMOs. Different approaches are followed to implement legal frameworks regarding the regulation of GMOs. Many countries have implemented specific legislation to regulate GMOs, e.g. the EU, often referred to as a method-triggered or technique-specific approach. Another example is provided by the US. Instead of establishing GMO-specific legislation, existing legislation is applied as a co-ordinated framework for the regulation of Biotechnology. Products are regulated according to their intended use, often referred to as a product-triggered approach. Whether these approaches are fundamentally different can be questioned. Regulating a genetically modified maize plant as a potential plant pest (invasive species) because of the applied method of genetic engineering can also be seen as a method-triggered approach. The legal requirements to be respected in different regions of the world influence the international trade due to asynchrony in GMO authorisation.

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# **Chapter 30**

# **Public Perceptions of Modern Biotechnology and the Necessity to Improve Communication**

**Roger J. Busch**

## **30.1 Introduction**

This chapter deals with public perceptions of modern biotechnology in Europe. The European situation, in this regard, seems to be quite different from situations in the United States, in the Far East and, perhaps, in the Arab countries. Public dialogue about technological development can be regarded as a European peculiarity. Nevertheless, readers from non-European countries could get some preventive information in the case of an export of organized critique into their countries.

It may be trivial to remind people that the use of genetic engineering – whether in the field of medical science, the development of pharmaceuticals or in the field of agricultural development – was and is part of heavy social disputes. But if all people involved have already realized this and initiated appropriate consequences, it is not clear why these disputes revive regularly.

The reaction of natural scientists to social challenges is often incomprehension. In fact, reservations against genetic engineering expressed by citizens, which are sometimes brought forward by consumer protection agencies and NGOs in representation, are making research, development and, above all, the use of this technology difficult, not only in Germany.

Are consumers not enough informed? Do they need more information? There have already been many attempts to provide better information, implemented by research associations along the lines of the “public understanding of science”. However, the desired results have not yet been obtained – probably not least because the public cannot escape the feeling of being trained to accept. But how can people accept something they do not understand or cannot understand? Enlightenment proves indispensable. The question, however, is whether enlightenment

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ends with the mere transfer of information. This is definitely not the case. It is a matter of trust! Or rather, it is the attempt to overcome the lack of trust in promoters of science and technology.

The “public understanding of science” is a mutual process. It is a question of comprehension, understanding and/or sympathy – on both sides. The will to co-operate should not be lacking, besides some people who are presumably interested in keeping up the conflict and all its concomitant phenomena due to economic reasons. It is inevitable to structure the conflict and thus make clear at which level of the debate the individual parties actually are. The conflict is not only a conflict of interests but it includes elements of a conflict of conviction. It is a matter of *interpreting* truth and the way things ought to be. Specific reduction mechanisms are effective, which make communication on the basis of facts more difficult. Complexity is reduced to simple patterns (with recourse to specific values) – in order to be able to recapture orientation in the face of the new. This does not reflect reality but it helps to be able to make first cautious steps and at the same time minimize risks. This reduction becomes problematic if it is increasingly understood as a reproduction of reality. Related to the technique of reduction, it can be observed that each party adopts the attitude to suspect the other of offending the common good and interest (*interpretation open!*) in case of doubt. In addition, there are permanently hints to our – by definition – unlimited lack of knowledge. As a result of our lack of knowledge we are then advised to prefer not to act. This is intuitively comprehensible but hardly practical.

The question about the parties’ attitudes to morality is absolutely relevant. All decisions whether specific information (i.e. interpreted data) can be understood and digested are taken on this level. The way to co-operation can only be paved by mutual concession of moral legitimacy. To escape this question may be possible on the level of an experts’ discourse. This, however, has only little chance of success in bioethical debates. Or it leads to ignoring technical expertise in specific communication – a phenomenon which has been only too well known in the debate about genetic engineering of the past decades.

All in all, research and application of modern biotechnology are facing the challenge of fundamentally modifying their dialogue with the public. This applies to methods as well as to contents – and not least to the attitude of representatives of biotechnology. This is discussed in detail in the following.

## 30.2 Societal Debate and Its Problems

It is essential for scientists and promoters of modern biotechnology to know the context they address with their messages. How do customers and citizens think, act and judge? Which values do they support? How would they cope with their lack of understanding facing information which might be important for their future life?

Different methods in public perception research are at hand. Quantitative data achieved by questionnaires provide sociological overviews. It is important,

however, to extend knowledge about *how* people build their personal attitudes leading to specific answers about biotechnology in general and especially about the genetic modification of plants. This can be achieved by qualitative analysis in the realm of social psychology. Furthermore, it is necessary to know under which conditions people would be ready to modify their former attitudes. Here, methods of communication analysis can be used.

The following refers to all three disciplines above-mentioned, starting with sociology, then referring to social psychology and last focusing on communication analysis.

Regularly, statistical data are collected using sociological methods to provide Eurobarometer surveys in order to give an overview on shifts and changes in citizens' judgments about the genetic modification of plants. Eurobarometer surveys can be seen as the best source in this respect.

Some social psychologists in Switzerland and the United Kingdom are specialized on the analysis of the influence of trust and confidence in the building of personal attitudes. Thus, Michael Siegrist and colleagues in this chapter serve as the "first address" for an evaluation of this issue.

Communication analysis has to work with sociological and social psychological evaluations as well. Practical experience and experiments have to be used to better understand the building of attitudes under specific conditions. In the given context, experiments conducted by TTN Institute for applied ethics are used for reference.

George Gaskell and colleagues analysed data collected by a Eurobarometer survey in 2005<sup>1</sup> (Gaskell et al. 2006) which clearly shows a wide scope of different judgements on modern biotechnology by citizens all over Europe. One subliminal message from their evaluation is that a "broadcasting" mode of delivering information about biotechnology is insufficient and, even worse, might provoke misunderstanding and thereby foster rejection. In general, this concerns promoters of biotechnology as well as their critics whereas critics seem to have an advantage to reach irritated citizens by providing simple patterns of ethical judgements.

### ***30.2.1 Statistic Data on Public Attitudes Towards Biotechnology***

Eurobarometer 64.3 focused on biotechnology issues which actually are under discussion: stem cell research, the use of genetic information in human medicine, nanotechnology, pharmacogenetics and agricultural biotechnologies. The latter issue was not addressed as such but narrowed down to applications in GM foods. This reduces the scope (biotechnology is more than just GM foods) but nevertheless improved the collectability of statistic data on how citizens perceive the importance and admissibility of personal benefits to judge biotechnology.

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<sup>1</sup>Eurobarometer 64.3;

“Europeans are generally optimistic about the contribution of technology to our way of life. An index of optimism shows a high and stable level for computers and information technology and solar energy from 1991 to 2005. Over the same period the index for biotechnology declined sharply from 1991 to 1999. From 1999 to 2005 the trend reversed, and now biotechnology is back to the level of 1991” (Gaskell et al. 2006). Even though a crisis of trust in actors involved in biotechnology could not be claimed by the survey’s data (Gaskell et al. 2006), these actors should not relax. Only nuclear energy is judged worse than biotechnology. Even nanotechnologies find better support – in spite of lots of unsolved risks and open questions on security and unintended and unpredictable effects of use.

A short reading of the Eurobarometer’s data could invite promoters of biotechnology to focus their communication on younger generations. Citizens aged below 25 tend to be associated with optimism about technological developments. This also includes biotechnology. However, GM food as an application of modern biotechnology is not generally supported by young citizens either: more than 50% stated not to support this application as such, whereas asked on their willingness to purchase GM food, more than 50% answered that they would buy GM foods “if approved by relevant authorities”, “if more environmental friendly”, “if containing less pesticide residues” and “if healthier” (Gaskell et al. 2006). These data show that *future* applications might have a positive influence on biotechnology’s perception and judgement. However, regarding the citizens’ readiness to transfer this attitude on agricultural biotechnology as such would run too short. The main promoters of agricultural biotechnology – i.e. industry – are observed sceptically, even though scepticism was reduced from 1999 to 2005 – except in Germany and – looking at the absolute figures – in Sweden (Gaskell et al. 2006).

Eurobarometer 64.3 also investigated the role and reputation of solicitors of public interests. Consumer organizations and university scientists are regarded as doing “a good job for society”. Scientists in industry doing research in biotechnology are judged slightly worse, but environmental groups campaigning against biotechnology definitely lost reputation between 2002 and 2005 (Gaskell et al. 2006).

Looking at the engagement with biotechnology, four modes have been figured out: the “active European” (“has heard about biotechnology before on TV or radio, has talked about it, has searched the internet for information, has probably attended a meeting about it”) accounts for 12% of all Europeans. The “attentive European” (“has heard about biotechnology before on TV or radio, has talked about it, is likely to have a lot of ‘textbook’ knowledge about biotechnology and genetics”) accounts for 14%. The “European Spectator” (“has read about biotechnology in newspapers, heard about it on TV or radio, might have talked about it before”) accounts for 33%. The “unengaged European” “has not heard, read or talked about biotechnology, nor searched the internet, nor attended a meeting (and is) unlikely to have much ‘textbook’ knowledge of biotechnology and genetics”. This type accounts for 41% of the European population (Gaskell et al. 2006).

This means that only about 26% of all Europeans can be regarded as significantly interested in biotechnology, whereas more than 70% keep their distance from that

issue. Taking into account that most of the “active Europeans” and the “attentive Europeans” live in countries which can be seen as more critical against biotechnology, promtors of biotechnology face a difficult task trying to improve their communication.

### **30.2.2 *Frames of Reference by Promotors and Critics***

Citizens’ activity levels, as outlined above, do not suffice to understand the development of their attitudes towards agricultural biotechnology. In many cases, the evidence of personal (or societal) benefits might trigger personal support or at least general acceptance. This might have been the hope of agricultural biotechnology’s promotors. They outlined the potential of their work to enhance agricultural productivity. Input-trait as the initial target of genetic modification of plants were explained to farmers as well as to consumers. Even though benefits would be provided for farmers at first hand, consumers were expected to accept this – just like they did in the past regarding technological developments in agricultural production. However, promotors overrated the customers’ readiness to accept. Some NGOs were very successful in combining agricultural biotechnology with the impression of an upcoming crisis: agriculture will exhaust the soil, big biotech companies will control agricultural production totally and natural environments will be poisoned for a very long time. As a consequence, societal discussion could no longer be led within a cost–benefit containment that had worked pretty well up to then. Instead, issues like global responsibility and specific interpretations of sustainability prevailed, which could not easily be answered by promotors aiming at providing production enhancement. Citizens were confronted with a technology they could not assess. They felt threatened by the matter’s complexity. Indeed, “in a crisis, most people do not have the knowledge that they need for making an informed decision. Here, people may need trust in order to reduce the complexity that they face” (Siegrist et al. 2007).

#### **30.2.2.1 Risk Perception and the Role of Confidence and Trust**

The phenomenon of crisis and crisis communication can help to understand, to some extent, how citizens develop their attitudes towards new technologies. Indeed, they cannot cope with the novelty of scientific data because of the lack of scientific knowledge. They must not be blamed for that. It is not their duty to learn all about biotechnology. It is the duty of scientists and promotors of biotechnology to provide information that could foster mutual understanding and – in the long run – facilitate the public’s concession to use this new technology.

As individual benefits for consumers were, and are, not at hand, the focus of societal debates has been set on the potential *risks* of agricultural biotechnology.

According to the work of Michael Siegrist and Heinz Gutscher – along with studies by Carmen Keller, Timothy Earle and others – two different types of trust, which are highly influential in risk communication, should be distinguished: confidence and social trust. “*Confidence* is based on familiarity, experience and past performance. *Social trust*, in contrast, refers to the willingness to rely upon others. We trust a person whose values are compatible with our own main goals” (Siegrist et al. 2007). This distinction goes with Michael A. Hoog, who analysed the relations between social identity and the group context of trust (Hoog 2007). If we are forced to make judgements under uncertainty, “we tend to take cognitive short cuts (heuristics), which deliver the best solution in the shortest time and with minimum cognitive effort. . . . Research has shown that, in the absence of reliable diagnostic information about a specific person, people base trust and subsequent co-operation on the perception of value similarity. . . . The perception of value or attitude similarity between self and others is a potent prime for social identification; and the process of social identification produces trust”<sup>2</sup> (Hoog 2007). In this context, the distinction between in-group and out-group is essential. Value similarity is attributed to be important. The difficulty arises to assess value similarity of members of different social groups – in other words: to identify values they share across the borders and solidarities of their groups. Hoog indicates a possible solution: “one should create conditions in which the two groups categorize themselves as one superordinate group – inter-group relations are transformed into intra-group relations and the trust problem is solved” (Hoog 2007). But he continues: “sadly, although this can be done in controlled laboratory experiments, it is very difficult to do in the real world.”

Nevertheless, Hoog’s concept of a superordinate social group might fit to what will be outlined in Sect. 30.4 (*Improvements*).

Siegrist and colleagues, in their above-quoted article, discuss three case studies that have one thing in common: well established companies (Shell PLC, Coca-Cola Co., Sara Lee Corp.) faced severe crises that threatened their public and economic performance. Their good reputation was endangered by contamination problems. “For the functioning of most institutions, the absence of social trust is not critical; the absence of mistrust suffices. Social trust is not a necessary condition for people’s co-operation. As long as uncertainty is low (indicating constancy), confidence is sufficient for co-operation. What is frequently overlooked, however, is that the situation becomes very different when disruptions turn into crisis situations and lead to the shattering of confidence” (Siegrist et al. 2007). People not familiar with scientific facts and not able to fully understand the crisis issue – in psychological terms – experience stress. “In a desire to get relief from that stress, we turn to members of our group, to people for whom similar values are important” (Siegrist et al. 2007). Value similarity gains utmost importance. Even stronger: “when

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<sup>2</sup>Whether trust is the cause or – contrary – a manifestation of attitudes (cf. Scholderer and Hagemann 2008) can be left open in this context, because here we focus on trust as an influential factor as such.

people possess absolute values (i.e. values which can't be refuted by third parties; RJB), cost-benefit analyses are impossible because a given value will always be more important than any other value. How society should deal with such absolute values is still an open question" (Siegrist et al. 2007).

Adopting this to societal discussions about agricultural biotechnology, confidence is not an easy reference point for biotech companies – because there has not been any positive track record for consumers. Instead, it would have been essential to build social trust – which was blocked by NGOs with their reproach that these companies would induce an environmental crisis. So risk – and specific perceptions of risk triggered by NGOs – became the central issue of societal discussion, which was, as an intended effect, biased.

Promoters of agricultural biotechnology could no longer be perceived in terms of value similarity. NGOs were estimated as solicitors of public morality. Most of science communication had been ineffective because it could not overcome the hurdle: scientists were talking about statistic risk exposure while non-scientists stuck to interpretations of stochastic risk exposures, frightened by unintended potential consequences of biotechnological applications.

The conclusion? "Communicators should not only be knowledgeable about 'the facts of the case', they should also, and primarily, be knowledgeable about the concerns and values of their target audience. ... International companies, in particular, cannot do without knowledge of local value preferences" (Siegrist et al. 2007).

### 30.2.2.2 Scepticism Against Technology and Progress?

It is not only risks that have to be dealt with in societal discussion about biotechnology. It is also the citizens' attitudes towards technology and progress.

Many people do not have any problem with technologies. However, many people who are generally seen as being technophiles have a spot of bother with accepting the use of modern biotechnology in nature. "Nature" stands for self-regulation, autonomy and – not least – for the guarantee of the resources we depend on. In addition, there are reductive interpretations, which euphemize what should be described as struggle of survival in reality.

Modern biotechnology interferes in these correlations. This was also the case with every change by the breeding of plants, which then became "crops" and every utilization of land for the cultivation of arable crops. Biotechnology – as the "genetic modification of plants" – however, in many people's perception, embodies a problematic quantum jump of human interference with nature. In addition to the above-delineated two statements about the perception and interpretation of certain risk expositions, it is necessary to bear in mind that the specific genetic modification of plants may clash with the conception of a morally acceptable conformation of nature.

In this context, "nature" and "natural" are terms which are, on the one hand, used as synonyms for "good", "innocent" and "pure-minded". On the other hand,

however, they seem to be also descriptive terms, e.g. in science. This makes the problem all the more controversial. Bernard Williams coined the term “thick concepts”. According to Williams, this term includes concepts that seem to describe only a feature or an action but, in fact, impart moral valuation, which claims universality.

It seems to be the interference in natural processes itself which in this case shows many people’s anxiety towards continuing technization and economization of their lived-in world, which are interpreted as “advancements” by third parties. In this case, genetic modification of plants serves as the crystallization point of a vague anxiety in view of these processes.

As a result, scientific or economic arguments about security or risks, economic advantages or disadvantages are not able to reach into the deepness of individual interpretations.

These individual conceptualizations – even if absolutely incomprehensible for third parties – must be taken into consideration if social communication about modern biotechnology is to be improved.

### 30.3 Insufficient Approaches

In general, we have to acknowledge that societal communication about modern biotechnology in plants divides people into two “parties”: those who are actors and drivers and those who just feel driven by them and exposed to novelty without consultation(Busch 1998). This results in a very uncomfortable setting for promoters of biotechnology: they feel obliged to inform or even teach the reluctant or hesitating people to bring them – and biotechnology – forward. Is that not what had been described as “Aufklärung” (enlightenment) by Immanuel Kant?: “enlightenment is man’s emergence from his self-incurred immaturity. Immaturity is the inability to use one’s own understanding without the guidance of another. This immaturity is self-incurred if its cause is not lack of understanding, but lack of resolution and courage to use it without the guidance of another. The motto of enlightenment is therefore: *sapere aude!* Have courage to use your own understanding! (Kant 1784). Is it a phenomenon of “self-incurred immaturity” not to vote in favour of biotechnology? Is it the job of biotechnologists to give guidance to release people from ignorance? Supposingly, a lot of communication activities in the past seem to have been shaped by this kind of job description for communicators. It is evidently predictable that these efforts would be in vain. The “enlightenment discourse” never worked. People – as uninformed or perhaps mis-informed they might be – do not want to be taught if this could be combined with or at least perceived as casting doubt on their personal attitudes and/or values.

In this respect, broadcasting “communication” – one message for all, use it or forget about it – might seem more appropriate. People could decide whether to use or to reject information. But in fact, the latter would not meet the expectations of

biotechnologists to induce a rapid shift towards acceptance or at least towards social concession to use an appropriate space to do a good job.

Better than broadcasting would be to address target groups. One could invite e.g. teachers, politicians and/or legislators as not deeply involved in the issue to make them acquainted with biotechnology. This is, however, a very expensive and time-consuming approach and nobody could predict the results. This might be one reason why target-group communication with the mentioned stakeholders did not get the premium format.

All in all, a patent remedy does not seem to be at hand. There is no communication quick-fix to overcome societal blockades against the implementation of genetically modified plants into normal agriculture. But, perhaps, there are chances to improve communication.

## 30.4 Improvements of Communication with the Public

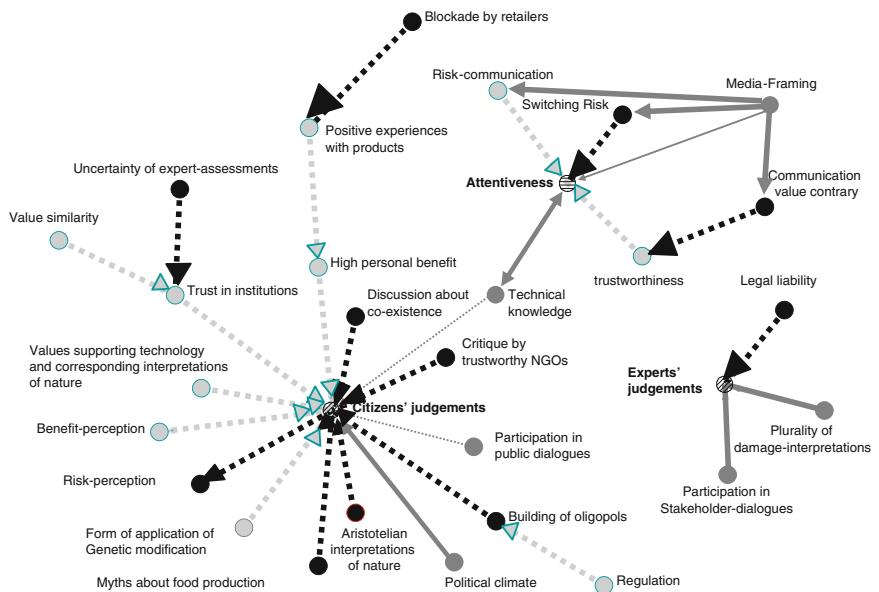
Taking into account what has been analysed so far, “improvements” cannot be achieved by merely adding some new gimmicks, pictures or testimonials to classical communication approaches. Marketing is communication, but communication goes far beyond marketing. Thus, if a fair and transparent communication with consumers/citizens is targeted, biotechnology communication has to be reframed.

“Classical” communication is shaped by transferring information about new scientific achievements to the public. For example, scientists discovered a new pathway to genetically modify potatoes. Then, other scientists tried to enhance this modification or to add another. It is like “more of the same” – plausible to those scientists but irritating to people who are not familiar with this sort of work and its perspectives.

As a result, when those scientists tried to communicate their success publicly – not only within scientific conferences – they would possibly face critical questions not on scientific data but on the right to genetically modify potatoes, such as: why should we genetically modify potatoes? To which challenge do genetically modified potatoes give an answer? The societal relevance of the project has to be made evident. Comparing the mentioned issue with projects in the realm of environment protection using biotechnological methods (so-called “white biotechnology”), we can see that these projects are significantly more accepted than projects of the realm of enhancement of agricultural production. This is due to a socially shared perception of a challenge to be dealt with. Here, people would not feel a problematic interference with their personal values. It is important to protect the environment – so why not use modern, even biotechnological, methods to achieve this?

It has been observed that the interplay of personal values and social contexts which have an impact on value and attitude-building is of utmost importance.

At the TTN Institute for Applied Ethics (University of Munich) a map of normative patterns was developed in 2007, providing a framework to build attitudes based on empirical data from surveys, as shown in Fig. 30.1 (adopted from Busch



**Fig. 30.1** Influential factors in attitude building (based on empirical data from surveys). *Thick black broken lines* Hindering effect on acceptance, *thick light-grey broken lines* positive effect, *grey lines* indifferent effect, *grey dotted lines* only a very weak effect

and Prütz 2008a). In general, thick black broken lines indicate a hindering effect on acceptance, thick light-grey broken lines indicate a positive effect, grey lines indicate an indifferent effect and grey dotted lines indicate that there is only a very weak effect. It is important to take the directions of arrows into account: Citizens' risk perceptions, in particular, are highly influenced by, for example: (i) trust in institutions (positive effect on acceptance of biotechnology) or (ii) NGOs who are perceived as trustworthy (negative effect on acceptance of biotechnology). Thus, risk perceptions appear as a result of interactions and not as a result of individual reflection alone.

Fig. 30.1 also shows that expert judgements on biotechnology in plants do not directly influence the building of citizens' attitudes on that subject. It is the media which get access to citizens' attentiveness – and the (mass-)media preferably refer to so-called “switching risks”, which possess a low cognitive presence in everyday life. But they can be stimulated and thus remain on the individual or social agenda for longer terms – until they get erased by opposite information. Most pervasive risks (e.g. over-indebtedness, employment problems), however, are constantly present and influence personal reflections and well being over longer periods. They cannot be “switched away” easily. Mass media operate well directed with switching risks. They address consumers' worries to be hit by negative effects stochastically. While (scientific, economic) normality regularly does not get access to media presentation, the conspicuous does.

### ***30.4.1 Respect to Sustainability and Ethics***

How to escape from this deficiency? Some marketing advisors would propose trying to hand-over the “bad guy’s textbook” to another actor: show that you are doing right whereas the other does not! But perhaps it would not be easy to succeed. A crucial precondition should be respected: actors who want to import something new and unfamiliar into a social context must refer to moral values important and valid in this social context. They cannot (or should not) start from *their* own perception of the advantages of their product (product-induced perspective) – trying to convince people to adopt it or at least concede to its use. They have to take care of contextual perceptions (context-related perspective) – including the valid value definitions of those they address. Evidently, it is not feasible to prove accordance of genetical modification projects with every single value of every single citizen. However, indisputably, importance can be ascribed to sustainable development as social obligation that individuals would support. Thus, the scientist’s task would be to prove the accordance of the innovation with the obligation of sustainable development. Over the past years, criteria have been developed to “measure” sustainability (cf. Coenen and Grundwald 2003). Without having to go deeper into this, it can be emphasized that sustainability as a societal obligation is evidently value-based. And it refers to relative enhancements of the present status. Not the good and true as such is accessible to human action but only the relative best. Consequently, if a genetically modified plant provides a *relative* positive change in environmental effects – without massively and irreversibly interfering with the right of citizens to be protected against harm and/or with economic welfare of third parties – then this innovation would fit into the society’s obligation to sustainably develop the ecological, social and economic use of nature.

Actually, it can be observed that the dramatic development of worldwide food shortage and the growing use of biomass to produce energy instead of food and feed are not taken into account properly by opponents of biotechnology. If climate change confronted agricultural production with severe problems to grow traditional plants, agricultural production would have to use biotechnological methods to cope with new challenges. This would be compatible with the idea of sustainable development. Nevertheless, opponents of biotechnology avoid giving answers to this challenge.

### ***30.4.2 Involvement of Consumers***

As a result, a message could be easier to formulate. Still unsolved, however, is the task of efficiently reaching people with this message. As outlined above, broadcasting information about innovations proved to be insufficient to influence individual attitudes on unfamiliar applications. People experience themselves as pushed by unwanted and irritating information which would not be useful in their everyday

life. Therefore, the task emerges to engage people, to involve them and to make them keen on assimilating this information. Inducing this, at least two approaches can be used, which can accumulatively increase their effects (cf. Busch et al. 2008b).

The first approach starts with confronting people with an evident and inevitable challenge and invites them to participate in finding an appropriate solution. To become able to contribute, people feel motivated to gather information because they want to prove that they act as mature citizens. They consider costs and benefits – which they would not do when addressed by classic and general information broadcasting. In a first step of getting acquainted with the issue, people select and assimilate information according to their personal attitudes on the subject. This makes it necessary to motivate them to assess playfully their own rationality by inviting them to take over a contrary rationality for a certain amount of time (role-play). It is likely that they would experience a shift of their attitudes towards curioseness or perhaps even acceptance of the unfamiliar – just because of the fact that they experienced their attitudes as partially inappropriate.

This approach – which requires a protected social setting – potentially results in individual reassessment of attitudes.

The second approach can be easily combined with the first – and thereby provide a cumulative effect – or can also be used as “stand alone”: it is personal encounter. Societal discussions about controversial technologies are widely shaped by talking *about* promoters – not by talking *with* them. Thus, personally encountering the people who are promoting applications (which – as a precondition – are legally admissible) and overcoming personal insecurity and/or uncertainty regarding the subject being promoted can influence insecure people to achieve a wider perspective – and thereby to make concessions to those applications.

These two approaches can certainly be combined with and prepared by more general concepts of involving citizens. In the UK, science festivals are organized and find societal attention. They potentially provide a first opportunity for many people to get in contact with unfamiliar but nevertheless interesting scientific issues – without being obliged to give statements or assess an application. Science festivals are more than, for example “days of open labs”, as promoted in Germany. And in fact, it is more interesting and at the same time less selective to visit a science festival than a specific laboratory. Low access conditions foster the visitors’ receptiveness.

All this could be mis-interpreted as instruction to immorally manipulate people. In fact, it would be an immoral manipulation if invited people were not informed about the host’s target for the encounter. As mature citizens, it is up to them to handle their experiences. Thereby, the setting would be fair and transparent. It would not be paternalistic because the host would not claim to possess the one and only truth but only to provide the tools to try out the better.

This sort of communication efforts is certainly as time-consuming as some of the above-mentioned. But scientists and/or promoters of biotechnology have to remember that, up to now, a lot of time (and money) has been spent without result, addressing people by using ineffective teaching methods while assuming to use

the appropriate ones. Involving people successfully has to take account of their interest to participate as mature citizens. Then they are likely to follow the invitation: "come in and find out".

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