O1 Plant breeding

Key Notes

Historical perspective

Modern crops are the result of intensive plant breeding and have many characteristics different from their wild ancestors. The process of plant breeding has resulted in increased yield and removal of undesirable characteristics, but with this is the risk of increased disease susceptibility of clonal populations and loss of biodiversity.

Plant breeding methods

Selection has resulted in recognizable varieties or landraces of crops. These varieties are heterozygous for crops that cross pollinate, but homozygous for those (like wheat) which do not. Cross hybridization gives hybrid vigor. Cross pollination between lines can be achieved by removing the anthers from one line and pollinating with a second line. Recently male sterile lines that do not make pollen have been produced. Desirable characteristics are introduced into plants by cross-fertilizing lines to produce hybrids. Back crossing allows a desirable trait to be introduced into an existing useful line.

Limits to conventional plant breeding

Plant breeding has been very successful in generating high-yielding crops. However, as well as being time consuming and labor intensive, it is also limited by natural barriers of pollination between species.

Related topics

Breeding systems (H2)
Self-incompatibility (H3)
Plant genetic engineering (O3)

Historical perspective

All the major crops world-wide are the result of a repetitive process of breeding, selection and further breeding to alter characteristics and improve yield. Alongside this process have come great increases in human population as agricultural productivity has attempted to meet the needs of that population. For some crops, the process has been carried out only recently (e.g. for oil-seed rape, Canola) while for others it has been going on for many thousands of years and in many civilizations. Wheat was domesticated first in the near-East. Breadwheat appears to have resulted from crossing primitive einkorn wheat with goatgrass to generate emmer wheat, which was crossed again with goatgrass to yield bread wheat. Corn (maize) was domesticated in Mesoamerica. One of its closest relatives is teosinte, which produces small, corn-like seed ears with hard outer husks. Rice was domesticated in Indo-China from a wild rice, *Oryza rufipogon*. Apart from yield, domestication has altered many characteristics of crop plants, including loss of dormancy from the seed and loss of dispersal mechanisms. There are also some negative effects of domestication; for instance, domesticated varieties are frequently demanding of nutrients and soil conditions. Intensive breeding programmes resulting in near clonal crops may result in loss of biodiversity and susceptibility to disease.

Plant breeding methods

Selecting plants for desirable traits resulted in the development of identifiable **varieties** or landraces of crops, each with slightly different characteristics. As some species can cross-fertilize, their landraces tend to be heterozygous (genetically mixed) while those that do not normally cross-fertilize (like wheat) will form genetically pure (homozygous) lines.

Selection breeding is the process of choosing plants showing desirable characteristics and generating seed from them. It is straightforward if the crop is self-pollinated but produces increasingly inbred lines and may result in loss of yield. Cross-pollinators can also be bred in this way, though creation of a homozygous line will not be possible. Loss of yield in inbred lines can be overcome by deliberate cross hybridization to achieve what is known as hybrid vigor. This is achieved by removing the anthers from one line and planting it adjacent to a second line with anthers. All the pollination of the first line will then be by the second line, and the seeds produced by that line will be hybrids of the two lines. Some lines are naturally male sterile (i.e. do not produce viable pollen). These plants are very valuable in plant breeding, as the anthers do not have to be removed by hand. More recently, genetic engineering (Topic O3) has been used to create male sterile lines, by linking ribonuclease gene expression with a promoter sequence (Topic E2) controlling an anther-specific gene. Ribonuclease produced in the anthers degrades the messenger RNA (mRNA) they produce. This results in plants that do not make pollen.

Desirable characteristics are introduced into plants by **cross breeding**. Lines are cross-fertilized with others (or with wild ancestors or related species) to produce hybrids containing a mixture of characteristics from both parents, some useful and some not. To eliminate the undesirable characteristics and develop useful ones, **back crossing** is carried out. In this process, the progeny of a cross (for instance between a high-yielding strain and a disease-resistant but low yielding strain; *Table 1*) are repeatedly hybridized with the high-yielding line.

Limits to conventional plant breeding

Conventional plant breeding has been very successful in generating the varieties of high-yield crops we use today. It is time consuming and labor intensive. It is also limited by the natural **pollination barriers** between species that mean desirable traits cannot be easily introduced from one species to another. **Artificial mutagenesis** (by X-rays or chemicals) has been used to generate new characteristics, together with **tissue culture** techniques (Topic O2). However, these have largely been replaced by **genetic manipulation** (Topic O3). This technique allows single characteristics to be transferred into a crop in a far more controlled and specific manner than was previously possible.

Table 1. Back crossing

Season	Action
1	Select two lines (one high yielding, one disease resistant); cross; collect seed
2	Grow seed from (1) and select plants that have good disease resistance and the best yield; cross with high yielding line and keep seed
3	Grow seed from (2) and select plants that have good disease resistance and the best yield; cross with high yielding line and keep seed
4–	Keep repeating stages 2–3 until a stable new line with acceptable yield and good disease resistance is produced (this may take eight or more repeats)
	Produce sufficient seed for large scale trials and agricultural production

O2 Plant cell and tissue culture

Key Notes

Types of cell and tissue culture

The major types of cell and tissue culture are: organ culture, embryo culture, tissue culture (production of callus and subsequent regeneration of organs or plantlets from excised pieces of tissue) and suspension culture (single cells or cell clumps in liquid media).

Methods, media and equipment

Plant cell and tissue cultures must be initiated and maintained in a sterile environment. Growth media include a carbon source (sucrose), macro and micronutrients, auxin and cytokinin, vitamins, water and, for solid media, a solidifying agent like agar.

Suspension cultures

Suspension cultures are started by breaking callus into liquid medium in conical flasks, which are agitated in an orbital incubator. Cells show logarithmic growth initially, then cell division ceases and growth rate declines. Subculturing to new medium is then required. Suspension cultures maintained for long periods may alter in properties due to mutation and altered gene expression.

Differentiation and embryogenesis

Differentiation to form roots, shoots and plantlets can be induced by selecting an appropriate auxin:cytokinin ratio for the medium. Suspension culture cells will also form embryoids (somatic embryos) in appropriate conditions.

Commercial and industrial applications

The major applications of plant cell and tissue culture are micropropagation, the production of high value products by cell cultures and as part of the genetic manipulation of plants.

Related topics

Biochemistry of growth regulation (F2)

Molecular action of hormones and intracellular messengers (F3)

Plant genetic engineering (O3)

Types of cell and tissue culture

Plant cell and tissue culture has been undertaken in various forms since the 1930s, although the techniques derive from the much older techniques of **plant propagation**, such as taking cuttings, which have been carried out by horticulturists for centuries. The techniques are based on the fact that plants show **plasticity of development**, the ability to change developmental path if suitably triggered. Thus, a stem section can regenerate roots and leaves to achieve a complete, functional plant and single cells can be caused to form an entire new embryo. Plant tissue culture requires sterile conditions as tissue growth is slow and material in culture vulnerable to fungal and bacterial infection. The major types of sterile plant culture are:

- **organ culture** in which an organ (flower bud, immature fruit) is grown isolated from the parent plant;
- **embryo culture** in which an isolated or immature embryo is grown;
- **tissue culture** in which cell material isolated from a parent plant is grown to form callus (an undifferentiated mass of cells) or to regenerate organs or into a whole plant;
- **suspension cultures** in which isolated cells or small clumps of cells are grown in a liquid medium.

Protoplasts, cells from which the cell wall has been removed by enzymic digestion, are frequently used in cell culture to separate single cells from cell clumps.

Methods, media and equipment

Cultures are prepared in a near-sterile environment, usually a **laminar flow cabinet**, in which a draught of sterile air blows constantly across the working surface, using equipment and media sterilized by heat or in an **autoclave**.

A wide range of **solid** and **liquid media** have been developed for different applications. The media have to contain certain key components (*Table 1*). Prior to use they are autoclaved and handled under sterile conditions using sterile vessels or petri dishes.

Table 1. Example of components of media for cell culture

Component	Function
Carbon source and osmotic balance (e.g. sucrose, 20 g l^{-1})	Energy supply for growth; in early stages at least, cultures will not photosynthesize; iso-osmotic medium required.
Solid medium (agar, 7 g l^{-1})	Used in solid cultures to solidify medium
Inorganic salts, macronutrients Ammonium sulphate 790 mg l^{-1} Calcium nitrate, 290 mg l^{-1} Magnesium sulphate, 730 mg l^{-1} Potassium chloride, 910 mg l^{-1} Potassium nitrate, 80 mg l^{-1} Sodium nitrate, 1800 mg l^{-1} Sodium sulphate, 450 mg l^{-1} Sodium dihydrogen phosphate, 320 mg l^{-1}	Supply essential micro and macronutrients; achieve pH balance (medium brought to pH 5.5)
Inorganic salts, micronutrients Boric acid, 1.5 mg l^{-1} Copper sulphate, 0.02 mg l^{-1} Manganous chloride, 6.0 mg l^{-1} Potassium iodide, 0.75 mg l^{-1} Zinc sulphate, 2.6 mg l^{-1} Molybdic acid, 0.017 mg l^{-1}	
Vitamins, lipids and essential amino acids *Meso*-inositol, 100 mg l^{-1} Glycine, 3.0 mg l^{-1} Thiamine hydrochloride, 0.1 mg l^{-1} Pyridoxine hydrochloride, 0.1 mg l^{-1} Nicotinic acid, 0.5 mg l^{-1}	Compounds essential for growth which cannot be synthesized by the cultures
Hormones 2,4-D (synthetic auxin), 0.15 mg l^{-1} Kinetin, 0.15 mg l^{-1}	Stimulate growth and division; altering the ratio of auxin to cytokinin influences development

In order to initiate a solid cell or tissue culture, a sterile fragment (**explant**) of plant material must be obtained and placed onto growth medium. Initially, an undifferentiated mass of cells termed a **callus** will be formed. The following list summarizes the stages involved:

(i) select a healthy plant and remove a segment of tissue in a laminar flow hood;
(ii) surface sterilize it using diluted sodium hypochlorite;
(iii) excise a segment of tissue using sterile instruments and resterilize the tissue;
(iv) place the segment onto solid medium containing hormones and nutrients to induce cell proliferation in a petri dish;
(v) seal and incubate at 22°C;
(vi) regularly observe, discard all contaminated dishes;
(vii) when callus growth sufficient, remove to fresh medium to intiate differentiation.

Frequently, a range of **auxin:cytokinin ratios** is tested to optimize growth and development. Several transfers to fresh medium may be required if the formation of plantlets is desired. The whole process may take 8–12 weeks or more to complete.

Suspension cultures

Suspension cultures are initiated by breaking cells free from a callus by gentle agitation. They consist of single cells or cell clumps suspended in aerated liquid medium. The simplest suspension culture system is a conical flask gently shaken on the platform of an orbital incubator. The swirling motion keeps the cells in suspension and oxygenates the medium. Cells in suspension culture show **logarithmic growth**, with rapid increase in fresh weight, dry weight and DNA content (indicating cell division) over the first few days. This is followed by a decline in cell division and subsequently a decrease in growth rate (*Fig. 1*). If the cells are not given fresh medium, they will then die; however, if a small aliquot is removed and **subcultured** into fresh medium the entire process can be repeated indefinitely. When the aim of the process is to harvest secondary products such as the antimicrobial dye shikonin, optimum production often occurs late in the growth cycle, when cell death is beginning to occur. The media for maintaining cells in culture is optimized for each species and cell type and will include auxin and cytokinin to stimulate cell division.

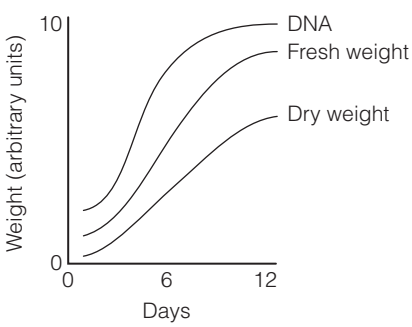

Fig. 1. Growth parameters of a suspension culture.

After prolonged culture, the cells may alter in their characteristics as a result of mutation or the activation of previously inactive genes. After repeated subculturing, for instance, genes for cytokinin biosynthesis may be activated and the cultures lose their requirement for cytokinin in the culture medium. This process is known as **habituation**.

Scaling up suspension cultures into large culture vessels is difficult as: (i) aseptic conditions must be maintained; (ii) constant aeration is required; (iii) cells are easily damaged by stirrers and changes in pressure; (iv) cells require constant agitation. These problems have been overcome in a number of designs; an example, based on using rising sterile air to agitate the cells, is shown in *Fig. 2*.

Differentiation and embryogenesis

Many experiments in cell culture are carried out with the aim of regenerating an entire plant. This is particularly important for genetic modification, where a novel gene may be inserted into a cell suspension, a protoplast or callus, and whole plants need to be produced.

Cells divide randomly to form an undifferentiated mass, known as a callus. If the auxin:cytokinin ratio of the medium is varied, the callus can be induced to differentiate to form roots and buds (Topic F1). By subculturing, intact plants can be regenerated by this method. Callus cultures may also form **embryoids** (**somatic embryos**, i.e. embryos formed in culture, as opposed to **zygotic embryos** formed sexually in a plant) on appropriate media. Cultures able to generate embryos are termed **embryogenic**.

Commercial and industrial applications

Micropropagation is the use of plant tissue culture to regenerate large numbers of plants. The technique results in genetically identical plants and is therefore **clonal propagation**. It is commonly used to produce disease-free plants, and commercially for many species including trees, potato and orchids, and as part of the procedure to genetically manipulate crops. **Somatic embryos** can be encapsulated in hydrated gel to produce a 'synthetic seed' or **propagule**.

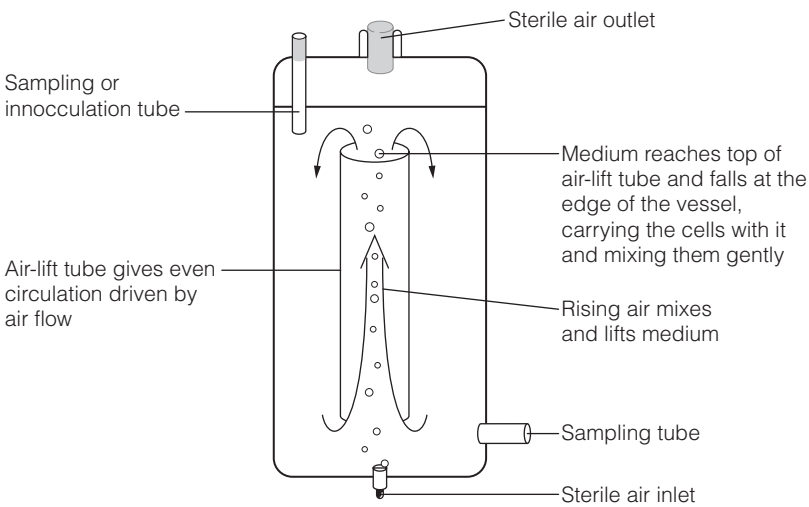

Fig. 2. An industrial-scale vessel for plant cell culture. The interior of the entire vessel is kept sterile and all the inlet and outlet ports are sealed with microparticulate filters which would prevent bacteria and fungal spores from entering.

Production of secondary products in suspension culture

Many plant secondary products (Topic J5) are difficult to produce by organic synthesis. Considerable effort has gone into using plant suspension cultures to make them. Details of important plant secondary products are presented in Topic J5; they are frequently produced by highly specialized cells. Consequently, high yields in cell culture can only be obtained if the cultures express the genes required. Frequently, this only occurs when the cells are at the end of the culture cycle. An example of a compound successfully produced in culture is the antimicrobial dye **shikonin**, produced by cultures of *Coptis japonica*.

Plant **genetic manipulation** is a widespread application of plant cell and tissue culture techniques today and without it, the commercialization of genetically modified crops would not be possible. Methods of genetic manipulation are explained in section O3.

O3 Plant genetic engineering

Key Notes

The concept

Genetic manipulation involves inserting foreign genes or modifying the activity of existing genes. Methods to insert foreign genes are coupled with the methods of plant tissue culture to regenerate identical populations of plants with novel characteristics.

Basic genetic manipulation methods

Agrobacterium tumifaciens is a soil bacterium with a plasmid that inserts foreign DNA into a plant. The plasmid contains a T-DNA transferred into the plant and a VIR region that facilitates transfer of the T-DNA. Binary vectors for genetic engineering consist of one plasmid containing the VIR region and a second containing the T-DNA including the foreign DNA. Where the *Agrobacterium* system cannot be used, direct gene transfer techniques may be employed, for instance using a DNA particle gun.

Possibilities of genetic manipulation

The aims of genetic manipulation are to enhance agriculture by modifying crop plants, to minimize inputs and losses, and maximize yields and value. To date, crops have been engineered for herbicide tolerance, insect and virus resistance and post-harvest quality. In the future, a much broader spectrum of improvements of wider benefit is proposed.

Risks of genetic manipulation

Risks identified include: environmental, such as cross pollination and gene transfer with native species; food safety, mainly the transfer of antibiotic resistance to bacteria, allergies and toxicity; and socio-economic, food supply in the hands of few multinational companies.

Related topics

Plants as food (N1)
Plant breeding (O1)
Plant cell and tissue culture (O2)

The concept

Genetic engineering (recombinant DNA technology) involves inserting foreign genes or modifying the activity of existing genes. A soil bacterium, ***Agrobacterium tumifaciens***, naturally inserts its own bacterial genes into plant genomes. The result is a crown gall, a swelling of the stem at soil level caused by over-production of auxins, and cytokinins produced by enzymes encoded by genes transferred from the bacterial genome. The regeneration of entire plants from single cells or explants (Topic O2) has been carried out for many decades to produce clonal populations of plants. Together, the two provide genetic engineers the tools for the insertion of genes from another organism into a plant and the regeneration of a clonal population of that plant. All the members of that population will express the foreign gene.

Basic genetic manipulation methods

Agrobacterium tumifaciens contains a **plasmid**, a circular piece of DNA separate from the bacterial chromosome, known as the **Ti plasmid** (*Fig. 1*). This plasmid contains genes which will be randomly inserted into the plant genome (**transferred** or **T-DNA**) and genes involved in the transfer of the DNA (the **VIR** or **virulence region for infection**). Normally, the T-DNA region contains genes for auxin and cytokinin biosynthesis and for amino acid and sugar derivative production. For genetic manipulation, the Ti plasmid is modified by the removal of the genes within the T-DNA region using restriction enzymes that cut DNA at specific nucleotide sequences. The action of the restriction enzymes leaves a linear strand of DNA with 'sticky' ends where the nucleotides are unpaired and therefore able to join to a complementary nucleotide sequence in another strand of DNA.

A gene of interest in another organism (animal, plant or bacterium) is identified and prepared for insertion into the Ti plasmid. It is cut out of its host with the same restriction enzymes used to prepare the Ti plasmid, again leaving sticky ends. This cut DNA is then mixed with the modified plasmid DNA, the two DNA strands join at their sticky ends and are sealed together by the enzyme, DNA

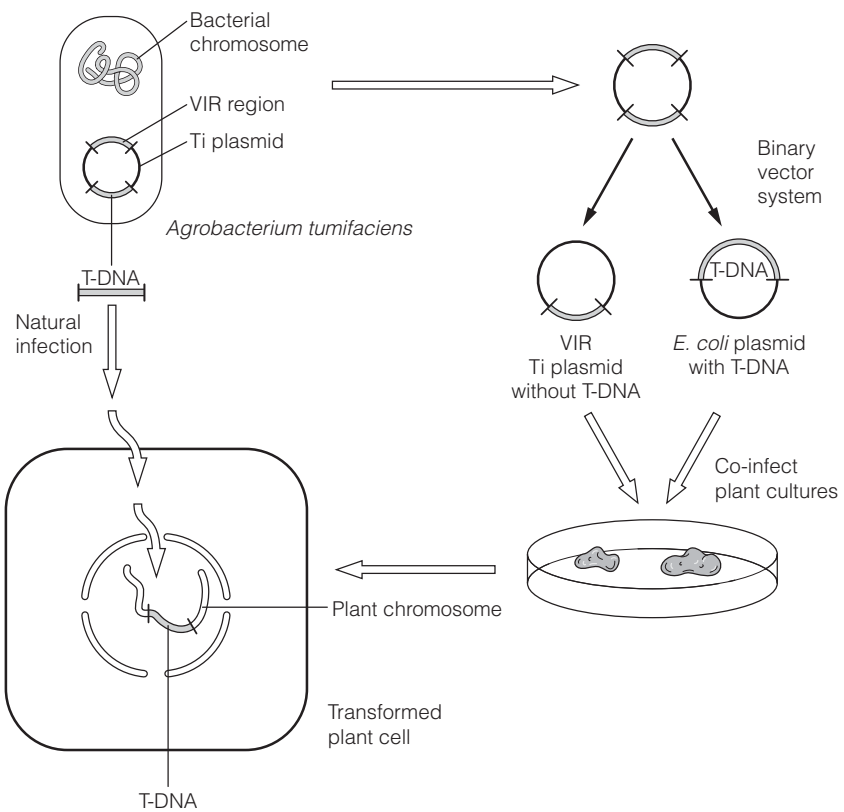

Fig. 1. Inserting a foreign gene into a plant genome using Agrobacterium tumifaciens. A. tumifaciens *contains a Ti plasmid containing a VIR (virulence region for infection) and a T-DNA region (which is transferred to the plant genome). Vectors based on the Ti plasmid contain modifications of the DNA in the plasmid. A binary vector system is shown here, in which two plasmids have been created, one a Ti plasmid without the T-DNA region, and the second an* Escherichia coli *plasmid with a T-DNA modified to contain genes to be inserted into the plant cell. In nature, transformation results in a swelling or gall at the site of infection.*

ligase, to form a recombinant plasmid (*Fig. 2*). The gene inserted must contain a promoter (Topic E2) which will allow it to be expressed in the plant.

Frequently, a second gene will also be inserted into the plasmid, in addition to the gene of interest. This is a **selectable marker gene** that will give the plant antibiotic resistance or herbicide tolerance. This gene will have also been cloned from another organism, usually a bacterium. Any plant material now expressing these genes will show the properties the marker gene confers, herbicide tolerance or antibiotic resistance, and will grow in media containing either the herbicide or antibiotic while non-transformed material cannot. In this way they can be used to select transformed plants from non-transformed ones.

Plant material (callus; Topic O2; leaf discs, suspension cultures or organs) is then infected by incubating the cells with *Agrobacterium* containing the plasmid and grown on agar plates containing antibiotic. Only material containing the gene of interest together with the antibiotic resistance marker then grows. Clonal populations of transformed plants can then be produced by micropropagation (*Fig. 3*; see also Topic O2).

Commonly, a **binary vector system** (*Fig. 1*) is used to transform plants. This system has the VIR region in a Ti plasmid modified by the removal of the T-DNA, while the T-DNA is in a second Ti plasmid. The plant is then transformed using the engineered *Agrobacterium* containing both plasmids, one including the VIR region and the other the T-DNA.

Some species cannot be transformed easily using *A. tumifaciens*. However, DNA constructs can be introduced into plant tissue directly using a DNA particle gun (*Fig. 3*). DNA is coated onto tungsten particles and fired at the specimen. The projectile is stopped by a barrier with a fine hole through which the tungsten particles carrying DNA can travel at high speed. They then penetrate the cell and some of the DNA enters the nucleus, causing transformation.

Possibilities of genetic manipulation

Production of genetically engineered crops has begun on a large scale. *Table 1* presents examples of uses of the technology, while *Table 2* presents some future prospects. The ability to transfer one or two genes into a genome has great potential in many areas of agriculture, for instance to reduce losses from pests and disease. Each situation requires a separate strategy to engineer a successful crop; *Table 1* presents some examples. Achieving the goals listed in *Table 2* may be more difficult, as some of the characteristics require the insertion or modification of more than one gene. As genes are inserted randomly into the genome by *Agrobacterium*, each transformant must be assessed separately for the consequences of transformation.

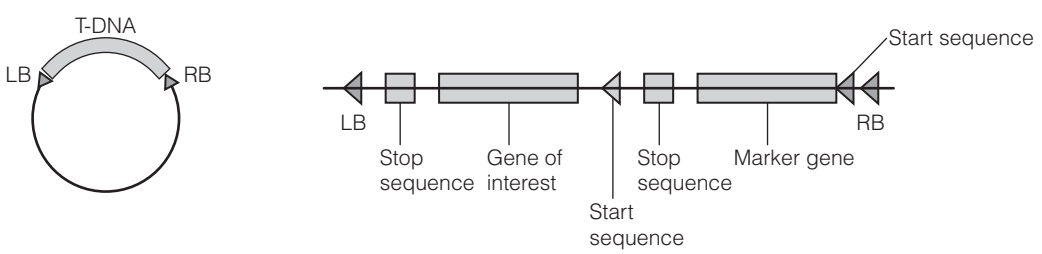

Fig. 2. A typical modified T-DNA in a Ti plasmid. The two ends of the T-DNA, known as the left border (LB) and right border (RB) remain intact as these contain repetitive DNA sequences which are important in pasting the T-DNA into the plant genome. In between, the bacterial DNA sequence has been removed and replaced with a construct of the gene of interest, transcription start and stop sequences and a marker gene (e.g. antibiotic resistance).

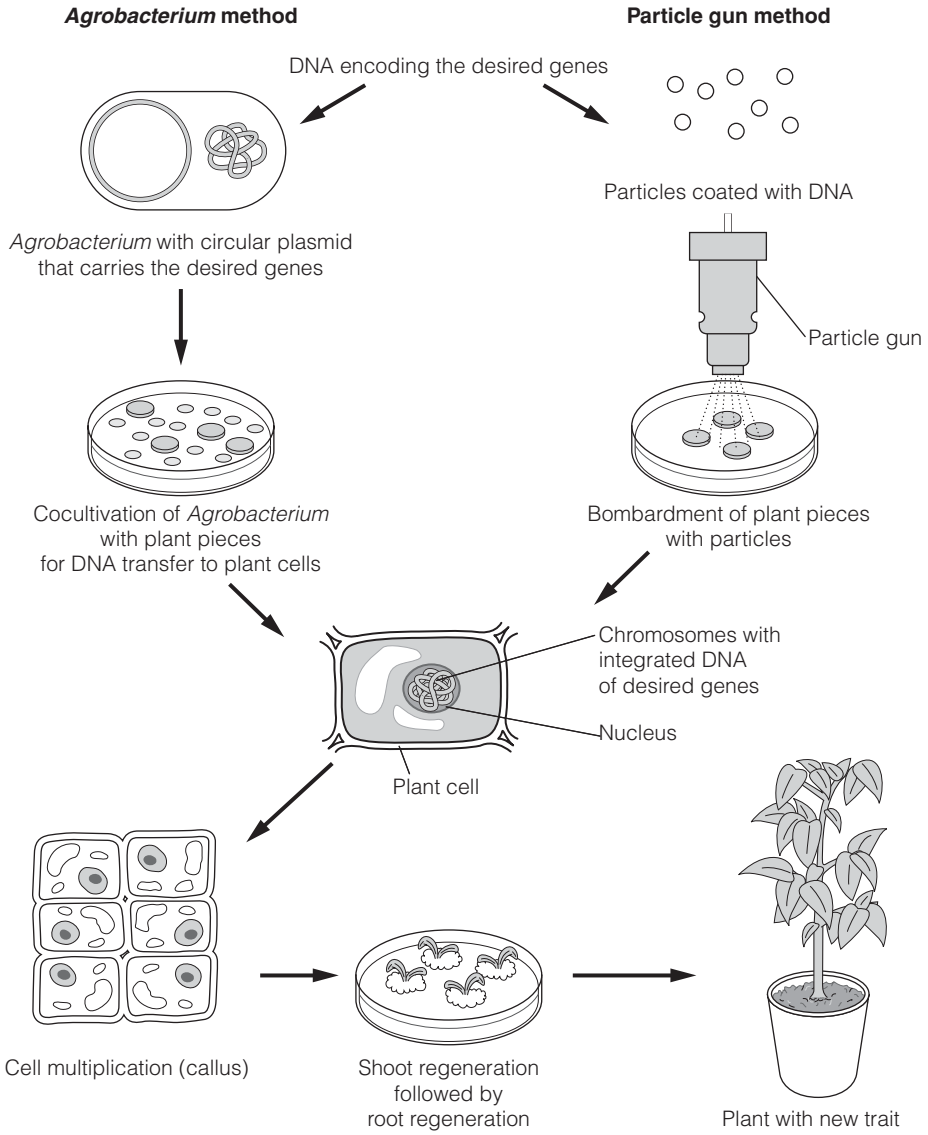

Fig. 3. Plant material transformed either by an Agrobacterium *based system or by DNA from a particle gun is allowed to form callus on agar containing antibiotic, on which only cells containing the selectable marker gene can grow. Plantlets are then regenerated on agar and mature plants expressing the inserted gene are grown.*

Risks of genetic manipulation

The risks of plant genetic manipulation may be divided into three classes: **environmental**, **food safety** and **socio-economic**. Environmental risks include (i) cross pollination of genetically modified (GM) crops with native species and (ii) damage to fauna, e.g. to insect populations or natural predators. Food safety risks include the transfer of antibiotic resistance marker genes into other organisms, e.g. bacteria that may enter the human food chain. Some fear potential but as yet unproven long-term health risks from consuming GM materials. Socio-economic risks concern the fact that GM crops are patented and largely in the

Table 1. Current applications of genetically engineered crops

Goal	Use	Typical method	Examples of crops transformed
Herbicide tolerance	Use of herbicides post emergence of seedlings at lower doses than required before seedling emergence	Introduce bacterial gene for enzyme which degrades the herbicide or which bypasses the point of plant metabolism inhibited	Soybean, canola (oil-seed rape), corn, cotton
Insect resistance	Reduce losses without pesticide spraying	Insertion of gene from the bacterium *Bacillus thuringiensis* gives resistance to a range of insect pests	Corn (against European corn borer); cotton (against boll worm, tobacco budworm etc); potato (against Colorado beetle)
Post-harvest quality	Increasing shelf-life and reduces losses in transport and harvest	Modified activity of polygalacturonase or other ripening enzymes	Tomato
Virus resistance	Reduced losses due to viral diseases	Insertion of viral coat protein gene into plant	Tobacco (tobacco mosaic virus); potato (potato viruses X and Y)

hands of a few multinational companies; that GM permits seed suppliers to have much greater control over the livelihood of agriculturalists, particularly in less developed nations; and that GM technology may benefit the rich at the expense of the poor. Against these arguments are the needs of a world population increasing by 1 billion approximately every 12 years, the rigorous testing of GM products and the potentially damaging effects of many current agricultural practices. Plant genetic modification is likely to remain controversial for some time as, in many parts of the world, considerable public opinion is against it.

Table 2. Future possibilities of genetically engineered crops

Goal	Application
Salinity tolerance	Increased crop yield in areas affected by salinity (e.g. in long-term irrigation)
Drought tolerance	Increased crop yield in marginal, semi-arid zones
Waterlogging tolerance	Improved survival in temporary flooding
Enhanced flavor, storage and properties	Improved consumer acceptance; decreased losses; decreased energy inputs to processing or storage; enhanced product value or usefulness
Enhanced amino acid content	Dietary improvement and health
Antibody and pharmaceutical production	Less energy input and cost than use of animal cell culture
Improved disease resistance	Reduced pesticide inputs; increased yields mean population can be fed using smaller land area