Genetic engineering and gene cloning

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Genetic engineering

Genetic engineering in plants

In general, plants are genetically engineered to increase yield or to confer resistance to drought or pests. Finding desirable genes can be difficult because multiple interacting genes usually control such traits. In addition, such genes may play other roles in plant physiology or development. So far, most successful genetic engineering of plants has relied on inserting one or a few genes that supply simple, yet useful, properties.

For example, resistance to the herbicide glyphosate is due to a single gene. Making a crop such as soybeans resistant to glyphosate allows the farmer to kill the weeds in the field without harming the soybeans. Another desirable trait often due to a single gene is the production of toxins that kill harmful insects. Also, a two-gene pathway has been engineered into rice to make it more resistant to drought. Before discussing these examples, however, we must first describe how transgenic plants are created.

Ti Plasmid

Plants suffer from tumors although they are quite different from the cancers of animals. The most common cause is the *Ti plasmid* (tumor-inducing plasmid), which is carried by soil bacteria of the *Agrobacterium* group. The most important aspect of the infection is that a specific segment of the Ti plasmid DNA is transferred from the bacteria to the plant. Because most DNA transfers occur only between closely related organisms, the ability of *Agrobacterium* to transfer DNA from one domain to another makes it an important tool for the genetic engineering of plants.

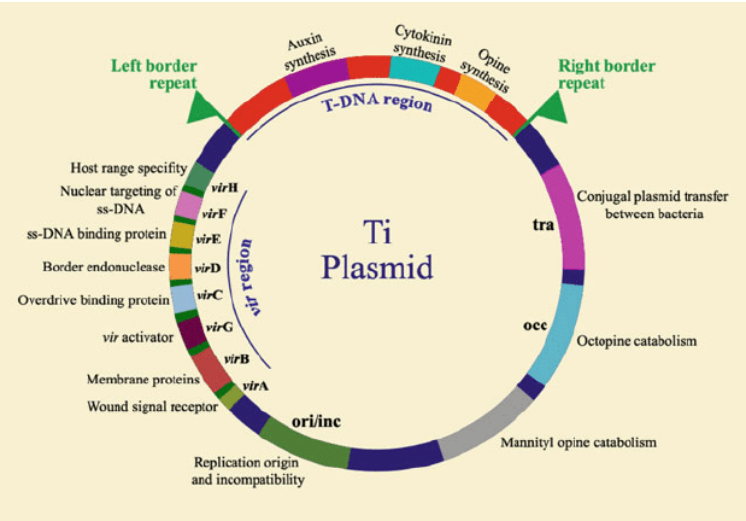

Figure 1: The structure of the Ti plasmid

In nature, *Agrobacterium* is attracted to plants that have minor wounds by

phenolic compounds such as acetosyringone, which are released at the wound. These chemicals induce the bacteria to move and attach to the plant via a variety of cell surface receptors. The same inducers activate expression of the virulence genes on the Ti plasmid that are responsible for DNA transfer to the plant. This is under control of a two-component regulatory system.

At the cell surface, the sensor, VirA, is autophosphorylated when it detects the plant phenolic compounds. Next, VirA transfers the phosphate to the DNA-binding protein, VirG, which activates transcription of the vir genes of the Ti plasmid. Two of the gene products (VirD1 and VirD2) clip the T-DNA borders to form a single-stranded immature T-complex. VirD2 then attaches to the $5'$ end of the T-DNA, and bacterial helicases unwind the T-DNA from the plasmid. The single-stranded gap on the plasmid is repaired, and the T-DNA is coated with VirE2 protein to give a hollow cylindrical filament with a coiled structure. This is the mature form of T-DNA and traverses into the plant.

The Ti plasmid is lost when Agrobacterium is grown above $28°C$. Such cured bacteria do not induce crown galls, i.e. they become avirulent. pTi and pRi share little sequence homology but are functionally rather similar. The Ti plasmids are classified into different types based on the type of opine produced by their genes. The different opines specified by pTi are octopine, nopaline, succinamopine and leucinopine.

The plasmid has 196 genes that code for 195 proteins. There is one structural RNA. The plasmid is 206,479 nucleotides long, the GC content is 56% and 81% of the material is coding genes. There are no pseudogenes.

Virulence region

Genes in the virulence region are grouped into the operons virABCDEFG, which code for the enzymes responsible for mediating conjugative transfer of T-DNA to plant cells (Stachel and Nester, 1986).

- virA codes for a receptor which reacts to the presence of phenolic compounds such as acetosyringone, syringealdehyde or acetovanillone which leak out of damaged plant tissues.
- virB encodes proteins which produce a pore/pilus-like structure.
- virC binds the overdrive sequence.
- virD1 and virD2 produce endonucleases which target the direct repeat borders of the T-DNA segment; virD4 is the coupling protein.
- virE binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes, beginning with the right border.
- virG activates vir-gene expression after binding to a consensus sequence, once it has been phosphorylated by virA.

T-DNA transfer to the plant is similar to bacterial conjugation. First, Agrobacterium forms a pilus. This rod-like structure forms a connection with the plant cell and opens a channel through which the T-DNA is actively transported into the plant cytoplasm. Both pilus and transport complex consist of proteins that

Figure 2: Crown gall tumors are caused by Agrobacterium tumefaciens

Figure 3: But plants expressing inhibitors of key proteins for Agrobacterium infection do not develop any tumors (B)

Crown gall disease in walnut (Juglans regia L.)

are vir gene products. Once inside the plant cytoplasm, T-DNA is imported into the nucleus. Both VirE2 and VirD2 have nuclear localization signals that are recognized by plant cytosolic proteins. These proteins take the T-complex to the nucleus, where it is actively transported through a nuclear pore. The single T-DNA strand is integrated directly into the plant genome and converted to a double-stranded form. The integration requires DNA ligase, polymerase, and chromatin remodeling proteins, all of which are supplied by the plant.

Once integrated, the genes in the T-DNA are expressed. These genes have eukaryote-like promoters, transcriptional enhancers, and poly(A) sites and therefore are expressed in the plant nucleus rather than in the original bacterium. The proteins they encode synthesize two plant hormones: auxin and cytokinin. Auxin makes plant cells grow bigger, and cytokinin makes them divide. The infected plant cells begin to grow rapidly and without control, resulting in a tumor (Figure 3). T-DNA also carries genes for the synthesis of a variety of different amino acid and sugar phosphate derivatives called opines. Strains of Agrobacterium are differentiated based on the kinds of opines they produce. Opines are made by plant cells that contain T-DNA, but they cannot be utilized by the plant. The Ti plasmid, which is still inside the Agrobacterium, carries genes that allow the bacteria to utilize them. Thus, the bacterium has hijacked the plant's metabolic machinery into supplying the bacteria with food. Other bacteria, which might be present by chance, cannot use opines because they do not possess the genes for their uptake and metabolism.

Molecular biologists have commandeered Ti plasmids to genetically engineer plants. The Ti plasmid has been disarmed by removing the genes for plant hormone and opine synthesis from the T-DNA and streamlined the plasmid by removing genes that are not involved in transferring the T-DNA. (These smaller plasmids are much easier to work with and can be manipulated in Escherichia coli rather than their original host, Agrobacterium.) A transgene of interest, such as an insect toxin gene, is inserted into the T-DNA region of the Ti plasmid. When the T-DNA enters the plant cell and integrates into the chromosome, it will bring in the transgene instead of causing a tumor.

The transferred region of the plasmid must also have other elements in order for this technique to be successful (Figure 1). The genetically modified T-DNA region must contain a selectable marker, such as an herbicide or antibiotic resistance gene that is used to track whether the foreign DNA has been inserted into plant cells. Expression of the transgene requires a promoter that works efficiently in plant cells. It may be one of two types. A constitutive promoter will turn the gene on in all the plant cells throughout development; thus, every tissue, even the fruit or seed, will express the gene. A more refined approach is to use an inducible promoter that acts as an on/off switch. An example of this is the cab promoter from the gene encoding chlorophyll a/b binding protein. This promoter is turned on only when the plant is exposed to light; therefore, root tissues and tubers such as potatoes will not express the gene. Many different promoters may be used, but ideally, the promoter should

turn on only in tissues that need transgene function.

In practice, Agrobacterium is used to transfer genes of interest into plants using tissue culture. Either protoplasts or a piece of callus are cultured with Agrobacterium harboring a Ti plasmid with modified T-DNA. After coculture, the plant cells are harvested and incubated with the herbicide or antibiotic used as the selectable marker. This kills all the cells that were not transformed with T-DNA or failed to express the genes on the T-DNA (Figure 4). The transformed plant cells are then induced to produce shoot and root tissue by altering the hormone conditions in the medium. The small transgenic plants can then be screened for transgene expression levels.

Recently, a method for in planta Agrobacterium transformation was developed and has revolutionized plant transgenics. In planta transformation is also known as the floral dip method (Figure 5). The method was developed using the model plant Arabidopsis but has been extended to other plants, such as wheat and maize. First, Arabidopsis plants are grown until flower buds begin to form. These buds are removed and allowed to regenerate for a few days. Once they begin to regenerate, the plants are dipped into a suspension of Agrobacterium containing a surfactant, which decreases surface tension and allows the Agrobacterium to adhere to the plant and transfer its T-DNA. Because the flower buds are just beginning to form, the T-DNA becomes part of the germline through the ovarian tissue. The plant is allowed to finish growing and produce seed. These seeds are harvested and grown in selective media to find those that have integrated and expressed T-DNA. Although the method gives a low percentage of transformants, so many seeds can be screened that the overall procedure works well.

Particle Bombardment

Another strategy for getting a transgene into plant tissue is particle bombardment (Figure 6). A gun blasts microscopic metal particles carrying DNA through the tough plant cell walls. Unlike Ti plasmid transfer by Agrobacterium, this technique works with all types of plants. Though the technique is nonspecific, it has been very successful.

First, either a leaf disk (a round piece of leaf tissue) or a callus is isolated from the plant, placed on a dish, and put in a vacuum chamber. The DNA to be inserted (carrying the transgene, proper regulatory elements, and selectable marker) is coated on microscopic gold beads. The beads are placed at the end of chamber. One variant of the method uses a blast of air or helium to drive the filter containing the gold beads toward the stop screen and sample. In the first gene guns, actual firearm blanks were used to accelerate the bullet. Between the bullet and plant tissue is a stop plate. Filter and gold beads hit this stop screen, the DNA-coated beads are thrown forward into the plant tissue. An alternative method is to accelerate the beads by a strong electrical discharge. The high voltage vaporizes a water droplet, and the resulting shock

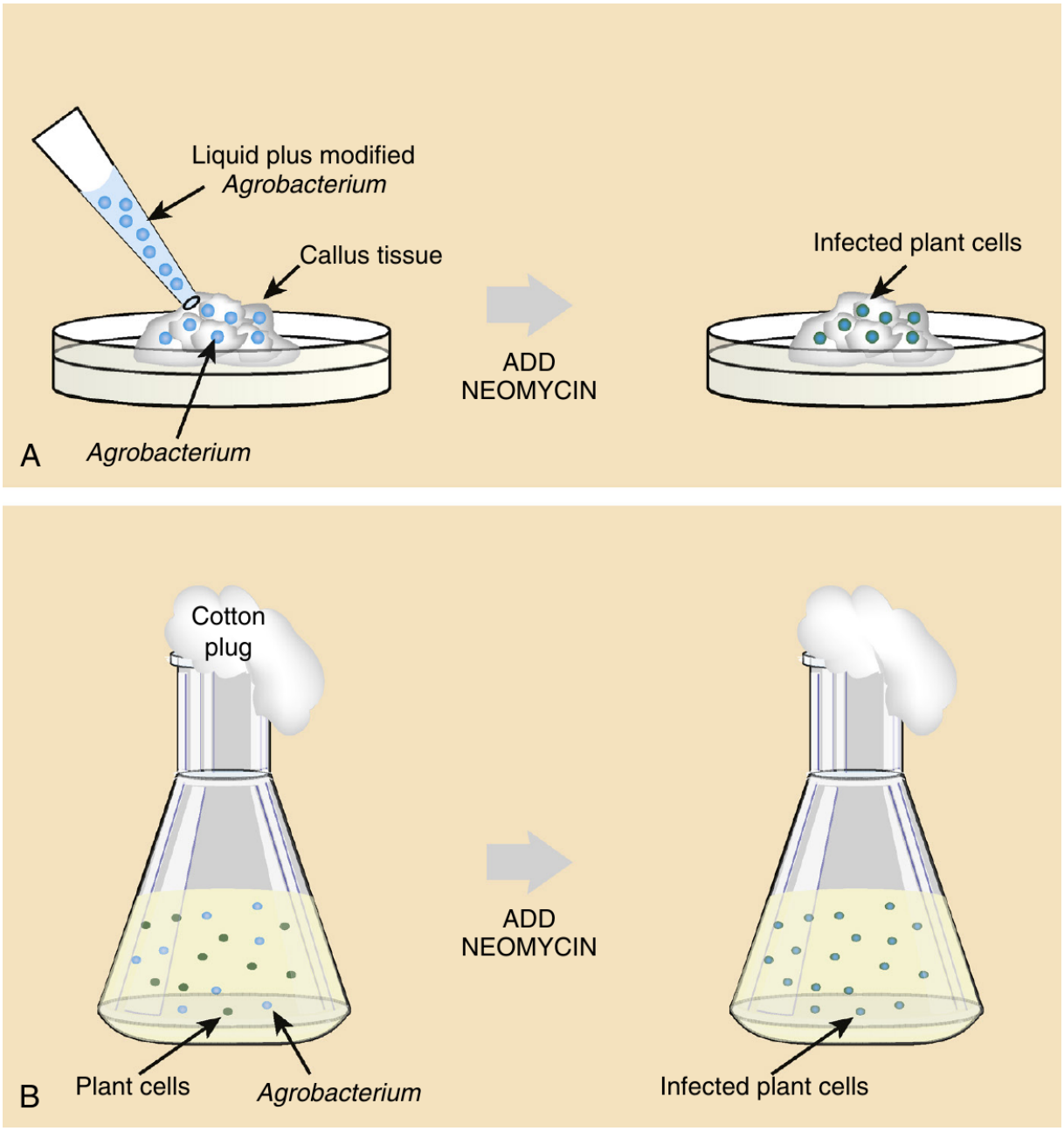

Figure 4: **Transfer of modified Ti plasmid into a plant.** Agrobacterium carrying a Ti plasmid is added to plant tissue growing in culture. The T-DNA carries an antibiotic resistance gene (neomycin in this figure) to allow selection of successfully transformed plant cells. Both callus cultures (A) and liquid cultures (B) may be used in this procedure.

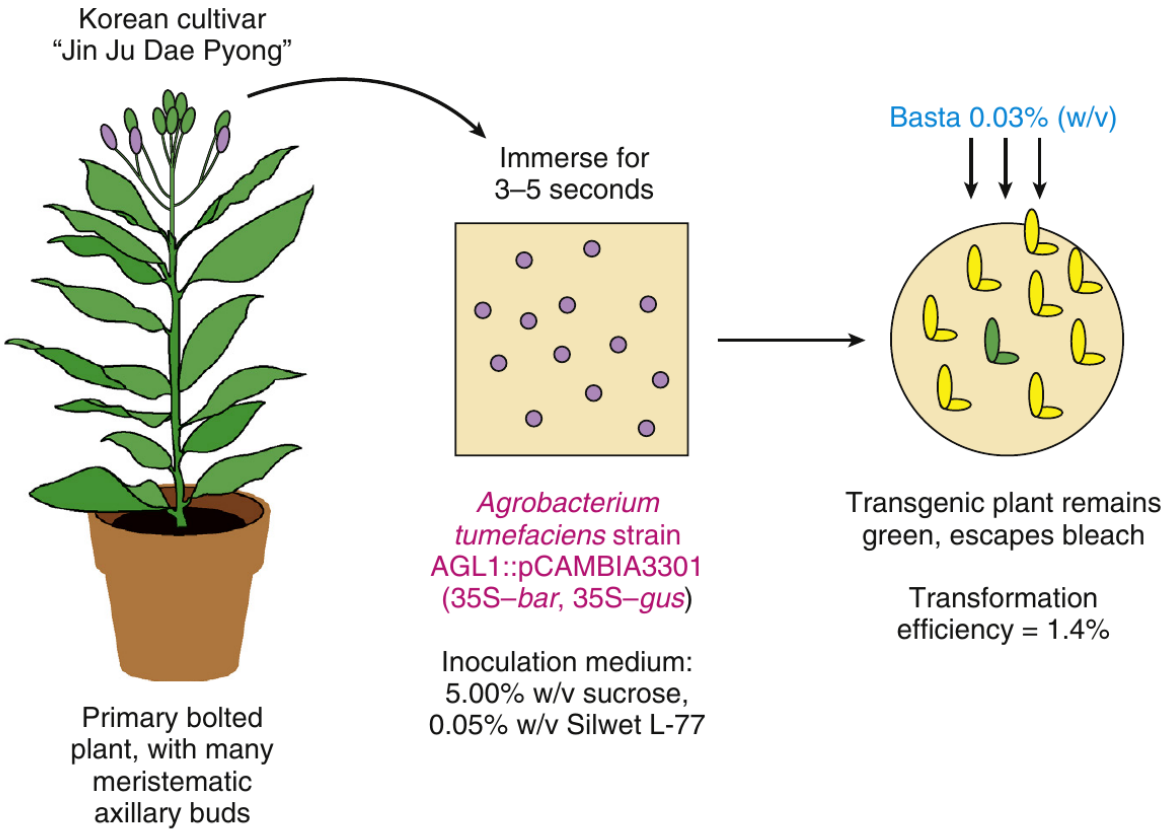

Figure 5: **Floral dip method of plant transformation** Flower buds exposed to Agrobacterium containing modified T-DNA can result in the production of transgenic seeds. Adapted from Curtis IS (2003). The noble radish: past, present and future. Trends Plant Sci 8, 305–307.

wave proels a thin metal sheet covered with the particles at a mesh screen. The screen blocks the metal sheet but allows the DNA-coated particles to accelerate through into the plant tissue. One advantage of this method is that the strength of the electrical discharge can be controlled; therefore, the amount of penetration into the tissue can be regulated.

Detecting the inserted DNA

Simplest of all ways to detect the inserted DNA is to include a selectable marker or reporter gene on the same segment of DNA as the transgene. One widely used reporter gene is *npt*, which encodes neomycin phosphotransferase. This enzyme confers neomycin resistance by attaching a phosphate group to the molecule. Transformed cells are directly selected with the antibiotic neomycin, which kills any cells that did not integrate the DNA.

An alternative reporter gene encodes for luciferase. This enzyme emits light when provided with its substrate, luciferin. Although high-level expression of luciferase can be seen with the naked eye, usually the amount of light is small and must be detected with sensitive electronic apparatus such as scintillation counter, a photocell detector, or a charge-coupled device camera. This reporter gene has a key advantage in that the luciferase protein is not stable for long time, so the amount of active protein correlates with the level of gene expression at any given time. Therefore, *luc* can be used to determine the activity of specific promoters.

Removal of selectable markers (genes), which may even have unfounded concerns in the public is catalyzed by *Cre/lox* P gene system, which produces Cre ("Causes recombination") protein, that recognized 34 base-pair DNA sequence, the loxP site. The Cre protein catalyzes recombination between two loxP sites.

The cre gene can be added to the system by cross-pollination of two different plants: One plant carrying the transgene plus a selectable marker that is flanked by two *lox* P sites is crossed with another plant carrying the *cre* gene. First, the *lox* P pollen from the plant with the *cre* gene is added to the stigma of the plant with the transgene. The resulting seeds are grown and checked for sensitivity to the selective agent (e.g., neomycin). If the Cre protein is present in the progeny, the selectable marker gene will be excised and lost during growth. This plant now has the transgene and the cre gene, but no longer has the gene for antibiotic resistance.

Genetically engineered crops

A task force of the Codex Alimentarius Commission, a U.N. agency responsible for an international code for food standardization, has advanced the following language to standardize the labeling of foods derived from GMOs for purposes of international commerce:

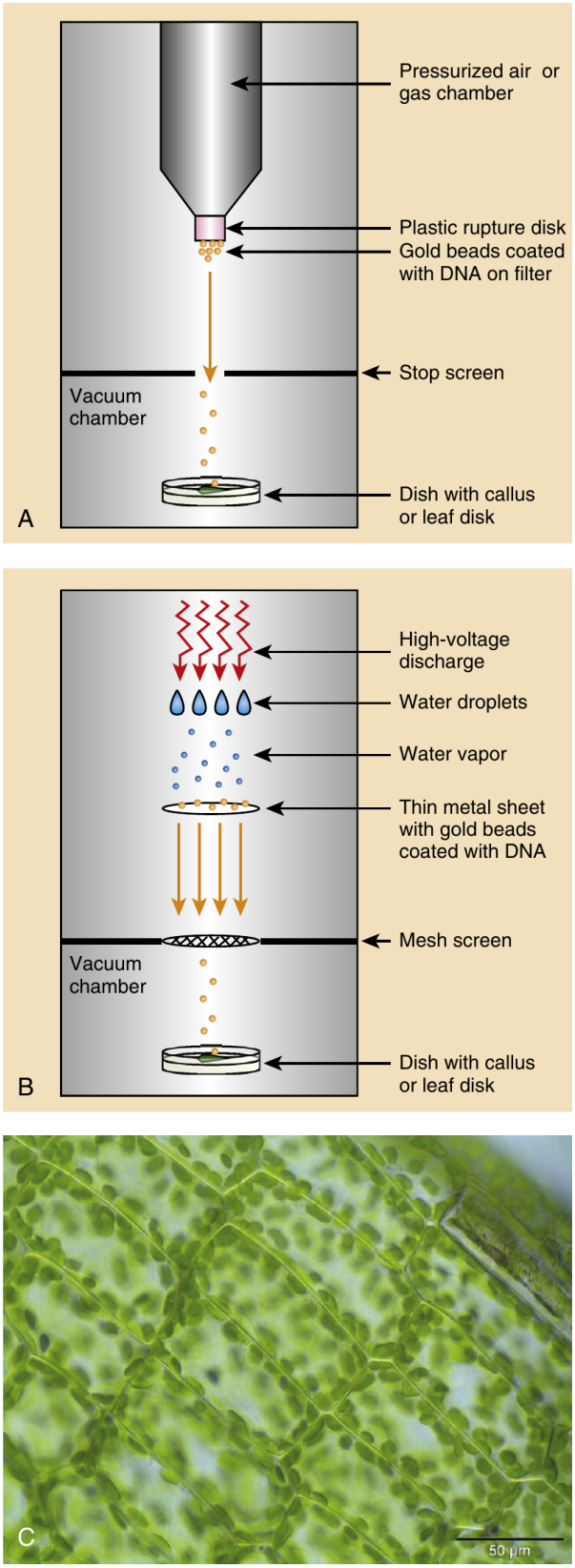

Figure 6: A gene gun that operates via (A) pressurized air or (B) high-voltage discharge is depicted. In both cases, the stop plate halts the projectile, and the microscopic metal particles carrying the DNA penetrate the plant tissue.

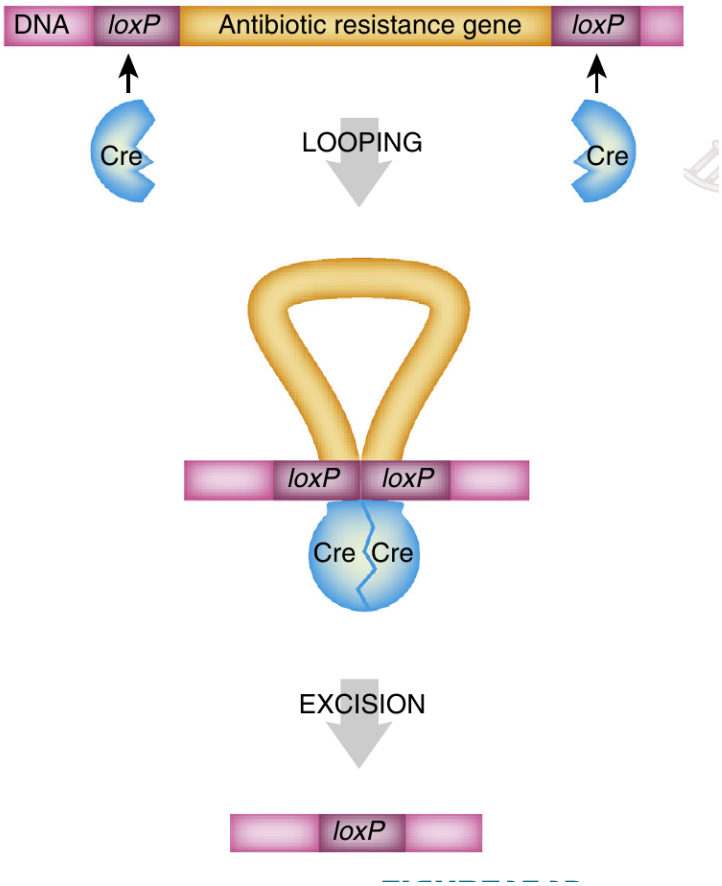

Figure 7: **The Cre/*lox*P System of Bacteriophage P1**

The Cre protein binds to *lox*P recognition sites in the DNA. Two nearly *lox*P sites are brought together, and recombination between them eliminates the intervening DNA. A single *lox*P 'scar' site remains in the target DNA molecule

"Genetically modified/engineered organism means an organism in which the genetic material has been changed through gene technology in a way that does not occur naturally by multiplication and/or any natural recombination."

Corn lines have been modified to express:

- tolerance to either the herbicide glyphosate (RoundUp(R)), or to the herbicide glufosinate-ammonium (Liberty(R));
- resistance to the pest, european corn borer (Ostrinia nubilalis) ECB;
- or a combination of herbicide tolerance to either glyphosate or glufosinate-ammonium and ECB resistance.

Soybean lines have been modified to express:

- tolerance to either glyphosate, or to (Liberty),
- modified oil (high oleic acid) content.

Cotton lines have been modified to express:

- herbicide tolerance to either glyphosate, bromoxynil or sulfonylurea,
- insect resistance to the pest, pink bollworm (Pectinophora gossypiella) PBW and tobacco budworm (Heliothis virescens) TBW.
- bromoxynil tolerance and PBW resistance.

Tomato lines have been modified to:

- delay fruit ripening
- express resistance to the pests tomato pinworm, (Kieferia lycopersicella) TPW and tomato fruitworm (Helicoverpa zea) TFW
- express a lower polygalacturonase level which makes for a more meaty tomato for processing.

Modified potato lines are:

- resistant to the Colorado Potato Beetle (Leptinotarsa decemlineata) CPB ,
- expresses resistance to the potato virus Y (PVY) in addition to being resistant to the CPB.

Chapters:

- Genetically modified crop approvals and plant acerages
  - Molecular information and approval status of selected events
- Insect resistant transgenic crops
- Transgenic technologies for insect resistance current achievements and future prospects
- Genetic engineering of crops for improved weed management traits.

References

- Crop Biotechnology, American Chemical Society, 2002
- Biotechnology, 2nd Edition, David P. Clark, Nanette J. Pazdernik

References

Scott E Stachel and Eugene W Nester. The genetic and transcriptional organization of the vir region of the a6 ti plasmid of agrobacterium tumefaciens. *The EMBO Journal*, 5(7):1445–1454, 1986.