Genetic Engineering of Plants: Methodology

CONSIDERABLE EFFORT HAS GONE INTO DEVELOPING VARIETIES of plants that produce increased yields and have enhanced nutritional value. Although much of this endeavor has been directed toward the three major grains—corn (maize), wheat, and rice—successful breeding programs for other food plants and horticultural species have also been established. Recombinant DNA technology, which has been used extensively with microbial systems, is also an important tool for the direct genetic manipulation of plants. There are a number of effective DNA delivery systems and expression vectors that work with a range of plant cells. Furthermore, most plant cells are totipotent—meaning that an entire plant can be regenerated from a single plant cell—so fertile plants that carry an introduced gene(s) in all cells (i.e., transgenic plants) can be produced from genetically engineered cells. If the transgenic plant flowers and produces viable seed, the desired trait is passed on to successive generations.

There are three major reasons for developing transgenic plants. First, the addition of a gene(s) often improves the agricultural, horticultural, or ornamental value of a crop plant. Second, transgenic plants can act as living bioreactors for the inexpensive production of economically important proteins or metabolites. Third, plant genetic transformation (transgenesis) provides a powerful means for studying the actions of genes during development and other biological processes.

Some of the genetically determined traits that can be introduced into plants by a single gene or, possibly, a small cluster of genes are insecticidal activity, protection against viral infection, resistance to herbicides, protection against pathogenic fungi and bacteria, delay of senescence, tolerance of environmental stresses, altered flower pigmentation, improved nutritional quality of seed proteins, increased postharvest shelf life, and self-incompatibility. In addition, transgenic plants can be made to produce a variety of useful compounds, including therapeutic agents, polymers, and diagnostic tools, such as antibody fragments. Alternatively, they can be engineered to synthesize viral antigenic determinants and, after ingestion, can be used as edible vaccines. To date, over 150 different plant species have been genetically transformed, including many crop and forest species,

BOX 18.1

Is the Debate over Genetically Modified Foods Effectively Over?

Rice is the most important crop in the world. In 2004, China, the world's largest producer of rice, announced a ramping up of efforts to commercialize genetically modified rice. India, the second-largest producer of rice, was quick to follow suit. With the two largest rice producers in the world (with ~40% of the world's population) switching to genetically modified rice, the rest of the world will eventually have little choice but to accept genetically modified foods.

From 1996 to 2007, the global area devoted to transgenic crops has increased from 1.7 million to 114 million hectares (ha). In 2007, the major producers of transgenic plants were the United States (57.7 million ha planted), Argentina (19.1 million ha), Brazil (15.0 million ha), Canada (7.0 million ha), India (6.2 million ha), China (3.8 million ha), Paraguay (2.6 million ha), and South Africa (1.8 million ha). Major transgenic crops worldwide include (in order of number of hectares planted) soybean, corn, cotton, canola, rice, squash, papaya, alfalfa, wheat, and eggplant. The major traits that have been introduced into plants include herbicide tolerance and insect resistance.

In 2002, the Food and Agriculture Organization of the United Nations endorsed the development and use of genetically modified crops. According to researcher Florence Wambugu of Nairobi, Kenya, "The African continent urgently needs agricultural biotechnology, including transgenic crops, in order to improve food production. Famine provides critics with an opportunity to promote an antibiotech message that only results in millions of people, who urgently need food, starving to death." Wambugu urged the public to recognize the difference in needs between Europe and Africa. Europe, with a population that is under control, has surplus food and does not experience hunger, whereas Africa, in contrast, experiences mass starvation and death. By mid-2008, South Africa was the only country in Africa to have approved the commercial use of genetically modified crops.

in over 50 countries worldwide. Plant biotechnology is having an enormous impact on plant-breeding programs because it significantly decreases the 10 to 15 years that it takes to develop a new variety using traditional plant-breeding techniques.

By mid-2008, researchers had reported the complete DNA sequences of hundreds of microorganisms and dozens of animals, but only three plants: *Arabidopsis thaliana*, rice, and poplar. At that time, the genome sequencing of several other plants, including corn, soybean, canola, tomato, cotton, potato, cassava, sorghum, grape, and peach, had been initiated. While the study of plant genes and genomes clearly lags behind studies of animals, it is gaining momentum, so that within the next 5 to 10 years, a wealth of information, with an enormous impact on plant biotechnology, is expected to become available.

Despite all of the progress that has been made in the development of transgenic plants for a wide variety of purposes, a vocal minority of individuals in North America and a larger number in Europe still oppose the use of this technology. Nevertheless, with each succeeding year since the mid-1990s, the use of transgenic crops has continued to increase both in absolute terms and in the number of countries using this technology (Box 18.1). It is expected that in the not too distant future, the majority of agricultural crops worldwide will be transgenic.

Plant Transformation with the Ti Plasmid of *A. tumefaciens*

The gram-negative soil bacterium *Agrobacterium tumefaciens* is a phytopathogen that, as a normal part of its life cycle, genetically transforms plant cells. This genetic transformation leads to the formation of crown gall tumors, which interfere with the normal growth of an infected plant (Fig.

18.1). This agronomically important disease affects only dicotyledonous plants (dicots), including grapes, stone fruit trees (e.g., peaches), and roses.

Crown gall formation is the consequence of the transfer, integration, and expression of genes of a specific segment of bacterial plasmid DNA—called the T-DNA (transferred DNA)—into the plant cell genome. The T-DNA is actually part of the "tumor-inducing" (Ti) plasmid that is carried by most strains of *A. tumefaciens*. Depending on the Ti plasmid, the length of the T-DNA region can vary from approximately 10 to 30 kilobase pairs (kb). Strains of *A. tumefaciens* that do not possess a Ti plasmid cannot induce crown gall tumors.

The initial step in the infection process is the attachment of *A. tumefaciens* to a plant cell at the site of an open wound, often at the base of the stem, i.e., the crown, of the plant. After the initial attachment step, *A. tumefaciens* produces a network of cellulose fibrils that bind the bacterium tightly to the plant cell surface. Originally, it was thought that *A. tumefaciens* infected wounded plants because the physical barrier of the cell wall had been breached by injury, thereby facilitating entry of the bacterium. However, it is now recognized that these bacteria respond to certain plant phenolic compounds, such as acetosyringone and hydroxyacetosyringone (Fig. 18.2), which are excreted by susceptible wounded plants. These wound response compounds resemble some of the products of phenylpropanoid metabolism, which is the major plant pathway for the synthesis of plant secondary metabolites, such as lignins and flavonoids. These small molecules (i.e., acetosyringone and hydroxysyringone) act to induce the virulence *(vir)* genes that are carried on the Ti plasmid (Fig. 18.3).

The *vir* genes are located on a 35-kb region of the Ti plasmid that lies outside of the T-DNA region. There are 25 *vir* genes arranged in seven operons on the plasmid. The products of the *vir* genes are essential for the transfer and integration of the T-DNA region into the genome of a plant cell.

After a Ti plasmid-carrying cell of *A. tumefaciens* attaches to a host plant cell and the *vir* genes are induced, the T-DNA is transferred by a process that is similar to plasmid transfer from donor to recipient cells during bacterial conjugation. In this model, the T-DNA is transferred as a linear, single-stranded molecule from the Ti plasmid, enters the plant cell, and eventually becomes integrated into the plant chromosomal DNA.

The formation of the single-stranded form of T-DNA is initiated by strand-specific cutting, by an enzyme encoded by one of the *vir* genes, at both borders of the intact T-DNA region. The 5′ end of the single-stranded T-DNA carries the right-border sequence, and the left-border sequence is at the 3′ end. The integration of the T-DNA into the plant genome depends on specific sequences that are located at the right border of the T-DNA. This border contains a repeating unit that consists of 25 base pairs (bp) (Fig. 18.4). Although the left border contains a similar 25-bp repeat (Fig. 18.4), deletion studies have shown that this region is not involved in the integration process.

During the insertion of the T-DNA into the plant chromosomal DNA, short deletions of the plant DNA are often produced at the junction between the T-DNA and the plant chromosomal DNA. In addition, while the insertion of the T-DNA into the plant DNA occurs at random sites, the T-DNA borders exhibit some homology with the plant DNA at the site of insertion.

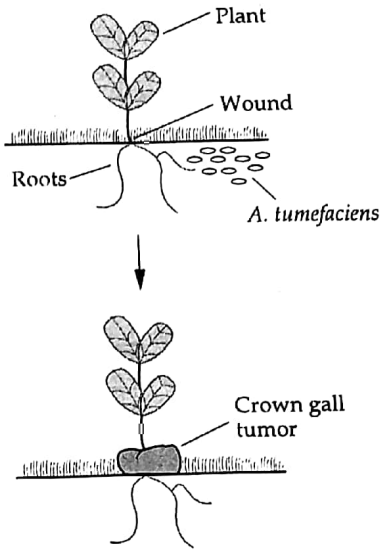
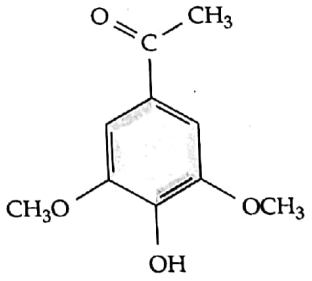
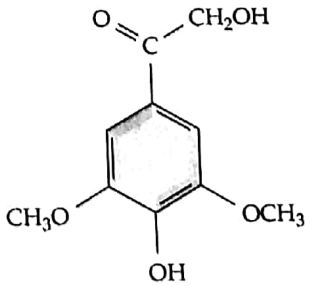


FIGURE 18.1 Infection of a plant with *A. tumefaciens* and formation of a crown gall.

FIGURE 18.2 Structures of the plant molecules acetosyringone and hydroxyacetosyringone. These compounds are released in response to wounding and can induce the *vir* genes of the Ti plasmid.



Acetosyringone



Hydroxyacetosyringone

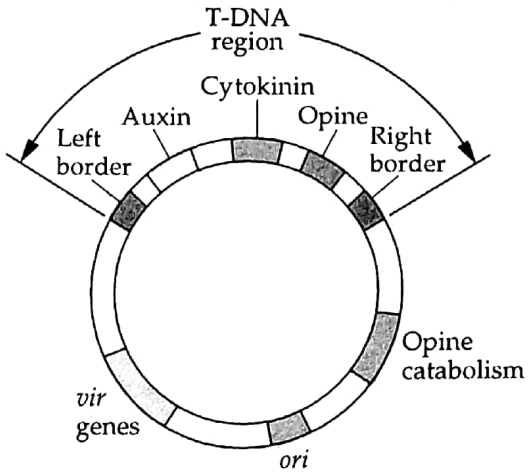


FIGURE 18.3 Schematic representation of a Ti plasmid. The T-DNA is defined by its left and right borders and includes genes for the biosynthesis of auxin, cytokinin, and an opine; these genes are transcribed and translated only in plant cells. Outside of the T-DNA region, there is a cluster of *vir* genes, a gene(s) that encodes an enzyme(s) for opine catabolism, and an origin of DNA replication *(ori)* that permits the plasmid to be stably maintained in *A. tumefaciens*. None of these features is drawn to scale.

Most of the genes that are located within the T-DNA region are activated only after the T-DNA is inserted into the plant genome. This reflects the fact that these are essentially plant genes, which cannot be expressed in bacteria because of the differences in transcriptional and translational regulatory sequences between the two types of organisms. The products of these genes are responsible for crown gall formation. The T-DNA region includes the genes *iaaM* and *iaaH*. This pair of genes encodes enzymes that synthesize the plant hormone auxin (indoleacetic acid). Specifically, *iaaM* codes for the enzyme tryptophan 2-monooxygenase, which converts tryptophan to indole 3-acetamide, and *iaaH* encodes indole 3-acetamide hydrolase, which converts indole 3-acetamide to indoleacetic acid (Fig. 18.5A). In addition, the T-DNA region carries the *tmr* gene (also known as *ipt*), which encodes isopentenyltransferase. This enzyme adds an isopentenyl side chain to 5′-AMP to form isopentenyladenosine 5′-phosphate, the first committed step in the synthesis of the cytokinin isopentenyladenine (Fig. 18.5B). Hydroxylation of these two molecules by plant enzymes generates the cytokinins called transzeatin and transribosylzeatin, respectively. Both auxin and the cytokinins regulate plant cell growth and development. In excess, they can cause the plant to develop tumorous growths, such as crown galls.

In addition to auxin and cytokinin biosynthesis genes, the T-DNA region from each specific Ti plasmid carries a gene for the synthesis of a molecule called an opine. Opines are unique and unusual condensation products of either an amino acid and a keto acid or an amino acid and a

FIGURE 18.4 Conserved bases on the right and left borders of the T-DNA of Ti plasmids. N indicates any one of the four nucleotides, i.e., there is no sequence conservation at these positions.

Right	5′-TGNCAGGATATATNNNNNNGTNANN-3′
Left	5′-TGGCAGGATATATNNNNNTGTAAAN-3′

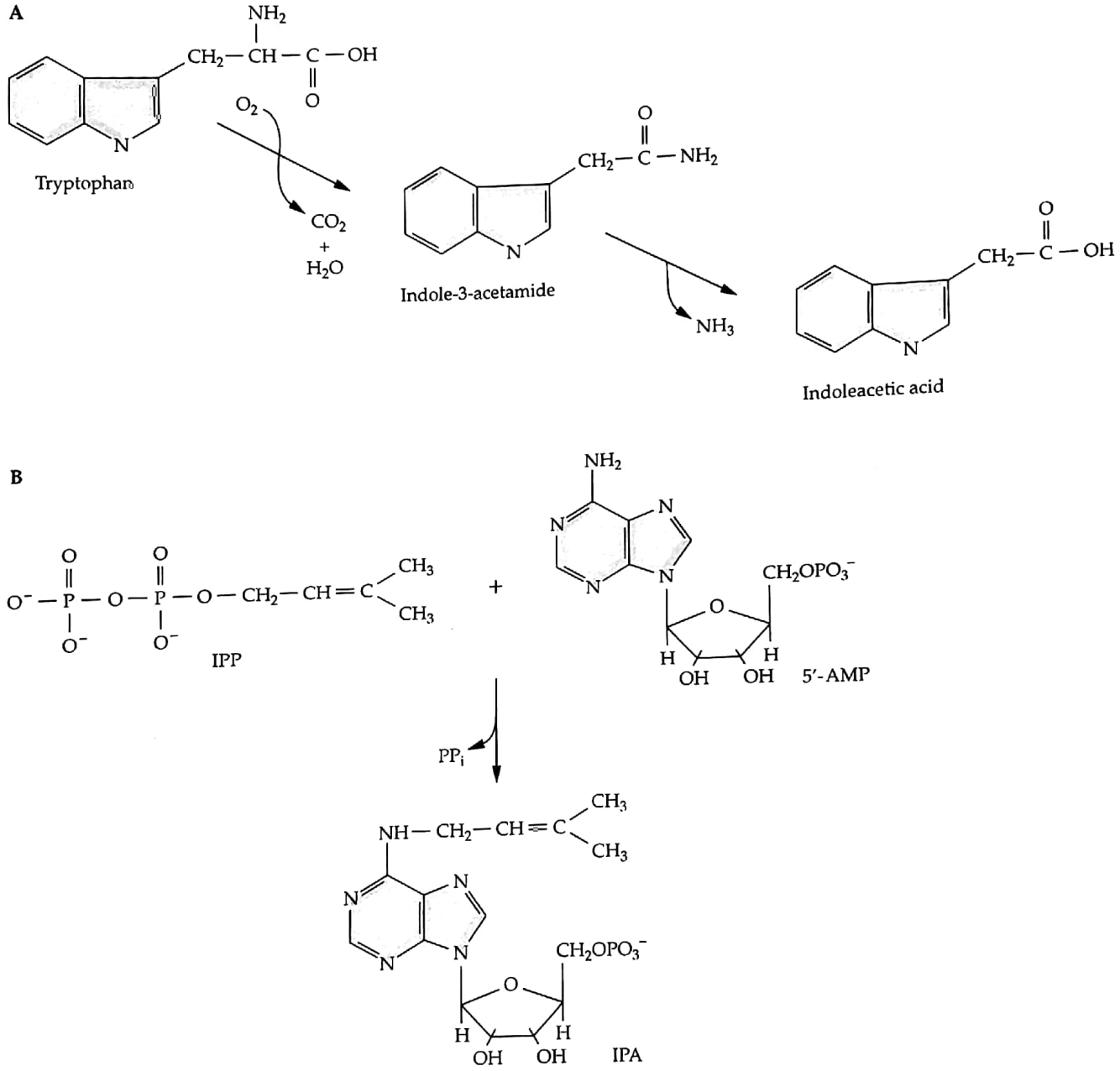


FIGURE 18.5 Biosynthesis of auxin and cytokinin by the enzymes encoded by the T-DNA genes of the Ti plasmid of *A. tumefaciens*. **(A)** The auxin pathway involves the conversion of tryptophan to indole 3-acetamide by tryptophan monooxygenase and then indole 3-acetamide to indoleacetic acid by indole-3-acetamide hydrolase. **(B)** The cytokinin synthesis reaction entails the attachment of an isopentenyl moiety from isopentenyl diphosphate (IPP) to 5′-AMP by the enzyme isopentenyltransferase to form isopentenyl adenosine monophosphate (IPA).

sugar. For example, the condensation product of arginine and pyruvic acid is called octopine, arginine with α-ketoglutaraldehyde is nopaline, and agropine is a bicyclic sugar derivative of glutamic acid (Fig. 18.6). The opines are synthesized within the crown gall and then secreted. They can

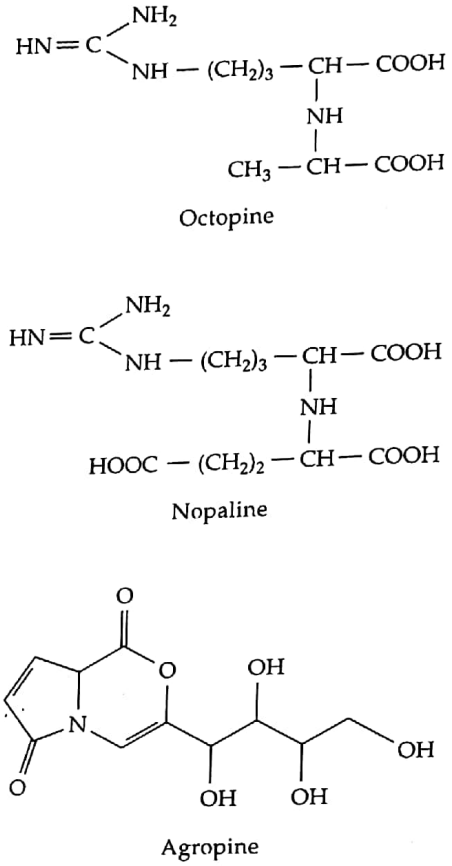


FIGURE 18.6 Chemical structures of three opines: octopine, nopaline, and agropine.

be used as a carbon source, and sometimes also as a nitrogen source, by any *A. tumefaciens* cell that carries a Ti plasmid-borne gene for the catabolism of that particular opine (Fig. 18.3). The opine catabolism gene(s) is on the Ti plasmid and is not part of the T-DNA region. All other soil microorganisms that have been tested are incapable of utilizing opines as a carbon source. Thus, a unique set of mechanisms has evolved whereby each strain of *A. tumefaciens* genetically engineers plant cells to be biological factories for the production of a carbon compound that it alone is able to use.

Ti Plasmid-Derived Vector Systems

The simplest way to exploit the ability of the Ti plasmid to genetically transform plants would be to insert a desired DNA sequence into the T-DNA region and then use the Ti plasmid and *A. tumefaciens* to deliver and insert this gene(s) into the genome of a susceptible plant cell. However, although the Ti plasmids are effective as natural vectors, they have several serious limitations as routine cloning vectors.

- The production of phytohormones by transformed cells growing in culture prevents them from being regenerated into mature plants. Therefore, the auxin and cytokinin genes must be removed from any Ti plasmid-derived cloning vector.

- A gene encoding opine synthesis is not useful to a transgenic plant and may lower the final plant yield by diverting plant resources into opine production. Therefore, the opine synthesis gene should be removed.
- Ti plasmids are large (approximately 200 to 800 kb). For recombinant DNA experiments, however, a much smaller version is preferred, so large segments of DNA that are not essential for a cloning vector must be removed.
- Because the Ti plasmid does not replicate in *Escherichia coli*, the convenience of perpetuating and manipulating Ti plasmids carrying inserted DNA sequences in that bacterium is not available.
- Transfer of the T-DNA, which begins from the right border, does not always end at the left border. Rather, vector DNA sequences past the left border are often transferred, although the transfer of these sequences is not often tested for.

To overcome these constraints, recombinant DNA technology was used to create a number of Ti plasmid-based vectors. These vectors are similarly organized and contain the following components.

- A selectable marker gene, such as neomycin phosphotransferase, that confers kanamycin resistance on transformed plant cells. Because the neomycin phosphotransferase gene, as well as many of the other marker genes used in plant transformation, is prokaryotic in origin, it is necessary to put it under the control of plant (eukaryotic) transcriptional regulation signals, including both a promoter and a termination–polyadenylation sequence, to ensure that it is efficiently expressed in transformed plant cells.
- An origin of DNA replication that allows the plasmid to replicate in *E. coli*. In some vectors, an origin of replication that functions in *A. tumefaciens* has also been added.
- The right border sequence of the T-DNA region. This region is absolutely required for T-DNA integration into plant cell DNA, although most cloning vectors include both a right and a left border sequence.
- A polylinker (multiple cloning site) to facilitate insertion of the cloned gene into the region between T-DNA border sequences.
- A "killer" gene encoding a toxin downstream from the left border to prevent unwanted vector DNA past the left border from being incorporated into transgenic plants. If this incorporation occurs, and the killer gene is present, the transformed cells will not survive.

Because these cloning vectors lack *vir* genes, they cannot by themselves effect the transfer and integration of the T-DNA region into recipient plant cells. Two different approaches have been used to achieve these ends. In one approach, a binary vector system is used (Fig. 18.7A). The binary cloning vector contains either *E. coli* and *A. tumefaciens* origins of DNA replication, i.e., an *E. coli–A. tumefaciens* shuttle vector, or a single broad-host-range origin of DNA replication. In either case, no *vir* genes are present on a binary cloning vector. All the cloning steps are carried out in *E. coli* before the vector is introduced into *A. tumefaciens*. The recipient *A. tumefaciens* strain carries a modified (defective, or disarmed) Ti plasmid that contains a complete set of *vir* genes but lacks portions, or all, of the

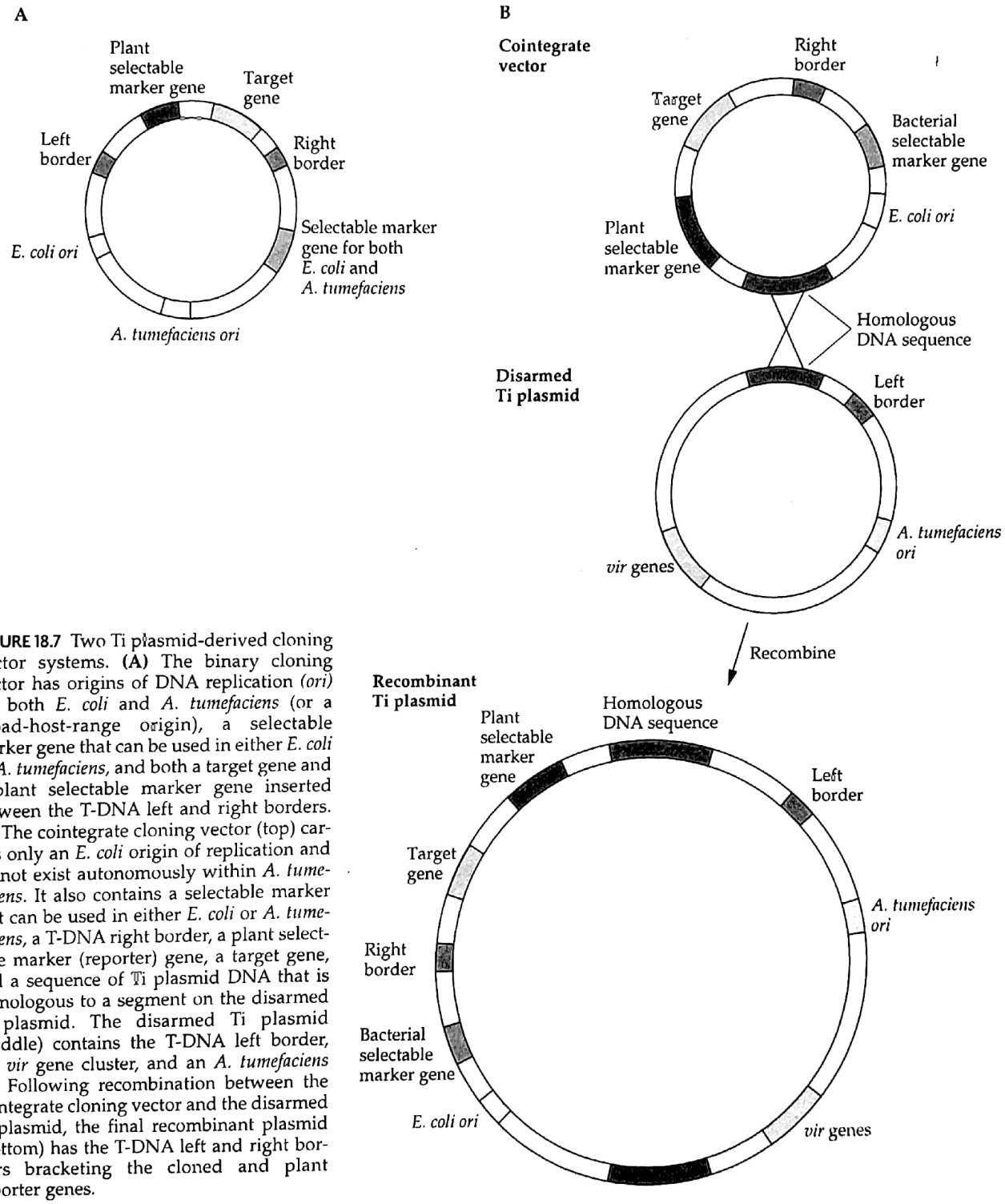


FIGURE 18.7 Two Ti plasmid-derived cloning vector systems. **(A)** The binary cloning vector has origins of DNA replication *(ori)* for both *E. coli* and *A. tumefaciens* (or a broad-host-range origin), a selectable marker gene that can be used in either *E. coli* or *A. tumefaciens*, and both a target gene and a plant selectable marker gene inserted between the T-DNA left and right borders. **(B)** The cointegrate cloning vector (top) carries only an *E. coli* origin of replication and cannot exist autonomously within *A. tumefaciens*. It also contains a selectable marker that can be used in either *E. coli* or *A. tumefaciens*, a T-DNA right border, a plant selectable marker (reporter) gene, a target gene, and a sequence of Ti plasmid DNA that is homologous to a segment on the disarmed Ti plasmid. The disarmed Ti plasmid (middle) contains the T-DNA left border, the *vir* gene cluster, and an *A. tumefaciens ori*. Following recombination between the cointegrate cloning vector and the disarmed Ti plasmid, the final recombinant plasmid (bottom) has the T-DNA left and right borders bracketing the cloned and plant reporter genes.

T-DNA region, so that this T-DNA cannot be transferred. With this system, the defective Ti plasmid synthesizes the *vir* gene products that mobilize the T-DNA region of the binary cloning vector. By providing the proteins encoded by the *vir* genes, the defective Ti plasmid acts as a helper plasmid, enabling the T-DNA from the binary cloning vector to be inserted into the plant chromosomal DNA. Since transfer of the T-DNA is initiated from the right border, the selectable marker, which will eventually be used to detect the presence of the T-DNA inserted into the plant chromosomal DNA, is usually placed next to the left border. If the selectable marker were adjacent to the right border, transfer of only a small portion of the T-DNA would yield plants that contained the selectable marker but not the gene of interest. A few binary vectors have been designed to include two plant selectable markers, one adjacent to the right border and the other adjacent to the left border.

In the second approach, called the cointegrate vector system, the cloning (cointegrate) vector has a plant selectable marker gene, the target gene, the right border, an *E. coli* origin of DNA replication, and a bacterial selectable marker gene. The cointegrate vector recombines with a modified (disarmed) Ti plasmid that lacks both the tumor-producing genes and the right border of the T-DNA within *A. tumefaciens*, and the entire cloning vector becomes integrated into the disarmed Ti plasmid to form a recombinant Ti plasmid (Fig. 18.7B). The cointegrate cloning vector and the disarmed helper Ti plasmid both carry homologous DNA sequences that provide a shared site for in vivo homologous recombination; normally these sequences lie inside the T-DNA region. Following recombination, the cloning vector becomes part of the disarmed Ti plasmid, which provides the *vir* genes necessary for the transfer of the T-DNA to the host plant cells. The only way that this cloning vector can be maintained in *A. tumefaciens* is as part of a cointegrate structure. In this cointegrated configuration the genetically engineered T-DNA region can be transferred to plant cells.

A practical problem that arises when using binary vectors is that their relatively large size (usually >10 kb) often makes it difficult and inconvenient to manipulate them in vitro. In addition, larger plasmids tend to have fewer unique restriction sites for cloning purposes. For these reasons, it is advantageous to develop and use smaller binary vectors. Based on the DNA sequence of a commonly used binary vector, pBIN19, it was predicted that more than half of the DNA could be deleted and the vector would still be completely functional. Thus, instead of the 11.8-kb size of the original vector, a 3.5-kb mini-binary vector (pCB301) was constructed (Fig. 18.8). This minivector, which can be used to clone DNA fragments to be transferred into the plant genome, cannot be introduced into *A. tumefaciens* by conjugation because certain regions of DNA required for conjugal transfer have been deleted. However, electroporation can be used as an alternative means. To facilitate the use of the minivector, a number of derivatives of pCB301 were constructed. For example, a *bar* gene, together with a plant promoter and transcription termination region, encoding the enzyme phosphinothricin acetyltransferase was inserted into the multiple cloning site so that transformants expressing this gene would be easily selected. Adjacent to the *bar* gene but in the opposite orientation is an expression cassette which includes a 35S promoter, a DNA sequence to target the protein for expression in either chloroplasts or mitochondria, a translational enhancer element (not shown in Fig. 18.8) that increases the level of expression of the protein encoded by the cloned gene, a portion of the multiple cloning site,

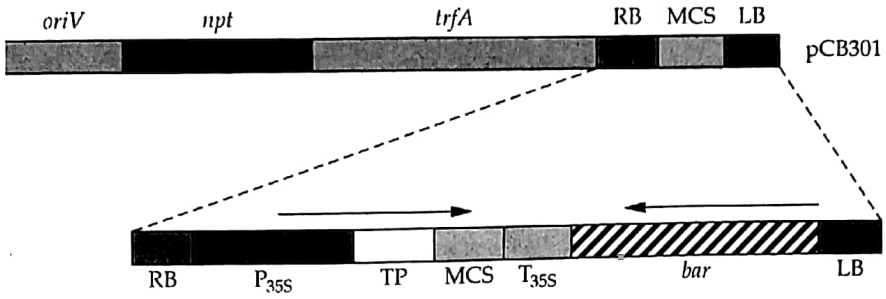


FIGURE 18.8 The mini-binary vector pCB301 system. *oriV*, part of the origin of replication; *npt*, neomycin phosphotransferase gene; *trfA*, part of the origin of replication; RB, right border of T-DNA; MCS, multiple cloning site; LB, left border of T-DNA; P_{35S}, 35S constitutive promoter from cauliflower mosaic virus; TP, targeting protein sequence; T_{35S}, transcription termination sequence from the cauliflower mosaic virus 35S gene; *bar*, gene for phosphinothricin acetyltransferase. By varying the DNA sequence of the TP, the protein encoded by the introduced gene may be targeted to either the mitochondria or chloroplast. Adapted from Xiang et al., *Plant Mol. Biol.* **40**:711–717, 1999.

and a transcription termination sequence (Fig. 18.8). These derivatives of the minivector pCB301 are flexible and easy to use and contain a variety of unique restriction enzyme sites in the multiple cloning site. After the target gene has been cloned into the multiple cloning site, the final construct is introduced into *A. tumefaciens* by electroporation.

In many instances it may be advantageous to transform plants with several foreign genes, for example, genes that encode an entire biochemical pathway. While this is not yet commonly done, it is nevertheless possible to introduce a large amount of foreign DNA into plants. Although the transformation efficiency is low, plants have been successfully transformed with large DNA fragments ranging from 30 to 150 kb.

Although *A. tumefaciens*-mediated gene transfer systems are effective in several species, monocotyledonous plants (monocots), including the world's major cereal crops (rice, wheat, and corn), are not readily transformed by *A. tumefaciens*. However, by refining and carefully controlling conditions, protocols have been devised for the transformation of corn and rice by *A. tumefaciens* carrying Ti plasmid vectors. For example, immature corn embryos were immersed in an *A. tumefaciens* cell suspension for a few minutes and then incubated for several days at room temperature in the absence of selective pressure. The embryos were then transferred to a medium with a selective antibiotic that allowed only transformed plant cells to grow. These cells were maintained in the dark for a few weeks. Finally, the mass of transformed plant cells was transferred to a different growth medium that contained plant hormones to stimulate differentiation and incubated in the light, which permitted regeneration of whole transgenic plants. Many of the early plant transformation experiments were conducted with limited-host-range strains of *Agrobacterium*. However, more recently, broad-host-range strains that infect most plants have been tested and found to be effective, so many of the plant species that previously appeared to be refractory to transformation by *A. tumefaciens* can now be transformed. Thus, when setting out to transform a new plant species, it is necessary to determine which *Agrobacterium* strain and Ti plasmid are best suited to that particular plant. In addition, modification of the tissue culture conditions by the inclusion of antioxidants during transfor-

mation of grape, rice, corn, or soybean has been found to increase the transformation frequencies of those plant cells.

A systematic examination of the conditions that are used in *Agrobacterium*-mediated plant transformation revealed that ethylene significantly decreased the transfer of genes to plant genomes. Ethylene is produced as a consequence of *Agrobacterium* infection of plants. To remedy this, a bacterial gene encoding aminocyclopropane-1-carboxylate (ACC) deaminase, which when expressed can lower plant ethylene levels (see chapter 15), was introduced into an *A. tumefaciens* strain that is utilized to introduce foreign DNA into plants. When melon cotyledon segments were genetically transformed using the *A. tumefaciens* strain expressing ACC deaminase, the transformation frequency of the plants (as judged by the level of introduced marker enzyme activity) increased significantly (Fig. 18.9). Although this innovation has yet to be tested with other plants, it is hoped that the introduction of this ethylene-lowering gene will increase the transformation frequencies for a wide range of different plants.

Physical Methods of Transferring Genes to Plants

When the difficulties in transforming some plant species first became apparent, a number of procedures that could act as alternatives to transformation by *A. tumefaciens* were developed (Table 18.1). A number of these methods require the removal of the plant cell wall to form protoplasts. Plant protoplasts can be maintained in culture as independently growing cells, or with a specific culture medium, new cell walls can be formed and whole plants can be regenerated. In addition, transformation methods that introduce cloned genes into a small number of cells of a plant tissue from which whole plants can be formed, thereby bypassing the need for regeneration from a protoplast, have been developed. At present, most researchers

FIGURE 18.9 Effect of lowering ethylene levels on the transformation of melon cotyledons. Following transformation, the activity of the marker enzyme β-glucuronidase was measured. Treatments: 1, no *A. tumefaciens*; 2, *A. tumefaciens* carrying the marker gene on a Ti plasmid; 3, *A. tumefaciens* carrying the marker gene on a Ti plasmid with aminoethoxyvinylglycine (AVG), a chemical inhibitor of ethylene synthesis, added to the system; 4, *A. tumefaciens* carrying the marker gene on a Ti plasmid and an ACC deaminase gene on a separate plasmid.

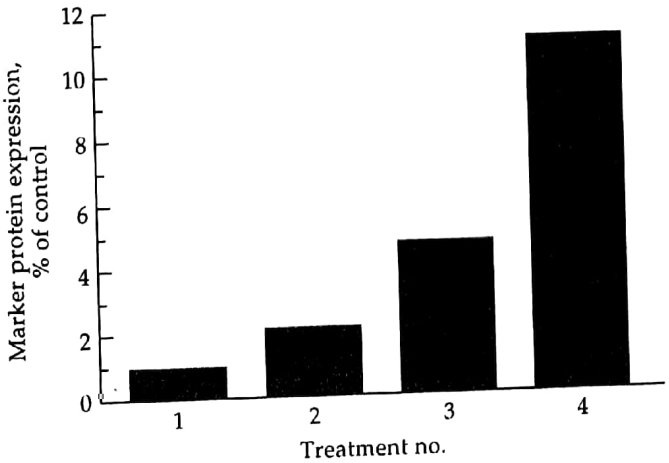


TABLE 18.1 Plant cell DNA-delivery methods

Method	Comment
Ti plasmid-mediated gene transfer	An excellent and highly effective system that is limited to a few kinds of plants
Microprojectile bombardment	Used with a wide range of plants and tissues; easy and inexpensive
Viral vectors	Not an effective way to deliver DNA to plant cells
Direct gene transfer into plant protoplasts	Can be used only with plant cell protoplasts that can be regenerated into viable plants
Microinjection	Has limited usefulness because only one cell can be injected at a time; requires the services of a highly skilled individual
Electroporation	Generally limited to plant cell protoplasts that can be regenerated into viable plants
Liposome fusion	Can be used only with plant cell protoplasts that can be regenerated into viable plants

favor the use of either Ti plasmid-based vectors or microprojectile bombardment to deliver DNA into plant cells. A very large number of different plants have been genetically transformed with these various techniques (Table 18.2).

Microprojectile Bombardment

Microprojectile bombardment, also called biolistics, is the most important alternative to Ti plasmid DNA delivery systems for plants. Spherical gold or tungsten particles (approximately 0.4 to 1.2 μm in diameter, or about the size of some bacterial cells) are coated with DNA that has been precipitated with $CaCl_2$, spermidine, or polyethylene glycol. The coated particles are accelerated to high speed (300 to 600 meters/second) with a special apparatus called a particle gun (or gene gun). The original version of the gene gun used a small amount of gunpowder to provide the propelling force. The device that is currently used employs high-pressure helium as the source of particle propulsion (Fig. 18.10). The projectiles can penetrate plant cell walls and membranes; however, the particle density used does not significantly damage the cells. The extent of particle penetration into the target plant cells may be controlled by varying the intensity of the explosive burst, altering the distance that the particles must travel before reaching the target cells, or using different-size particles.

Once inside a cell, the DNA is removed from the particles and, in some cells, integrates into the plant DNA. Microprojectile bombardment can be

TABLE 18.2 Plants that have been genetically transformed

Alfalfa	Carnation	Kiwi fruit	Papaya	Potato	Sunflower
Apple	Carrot	Lettuce	Pea	Red fescue	Sweet potato
Arabidopsis	Corn (maize)	Licorice	Peanut	Rice	Tall fescue
Asparagus	Cotton	Lily	Pear	Rye	Tobacco
Banana	Cranberry	Lotus	Pearl millet	Sorghum	Tomato
Barley	Cucumber	Norway spruce	Peony	Soybean	Wheat
Bean	Eggplant	Oat	Petunia	Strawberry	White spruce
Cabbage	Flax	Orchard grass	Plantain	Sugar beet	
Canola	Grape	Orchid	Poplar	Sugarcane	

used to introduce foreign DNA into plant cell suspensions, callus cultures, meristematic tissues, immature embryos, protocorms, coleoptiles, and pollen in a wide range of different plants, including monocots and conifers, plants that are less susceptible to *Agrobacterium*-mediated DNA transfer (Table 18.3). Furthermore, this method has also been used to deliver genes into chloroplasts and mitochondria, thereby opening up the possibility of introducing exogenous (foreign) genes into these organelles.

Typically, plasmid DNA dissolved in buffer is precipitated onto the surfaces of the microprojectiles. Using this procedure, it is possible to increase the transformation frequency by increasing the amount of plasmid DNA; however, too much plasmid DNA can be inhibitory. It is estimated that there are approximately 10,000 transformed cells formed per bombardment. With this technique, cells that appear to be transformed, based on the expression of a marker gene, often only transiently express the introduced DNA. Unless the DNA becomes incorporated into the genome of the plant, the foreign DNA will be degraded eventually.

The configuration of the vector that is used for biolistic delivery of foreign genes to plants influences both the integration and expression of those genes. For example, transformation is more efficient when linear rather than circular DNA is used. Moreover, large plasmids (>10 kb), in contrast to small ones, may become fragmented during microprojectile bombardment and therefore produce lower levels of foreign-gene expression. However, large segments of DNA may be introduced into plants

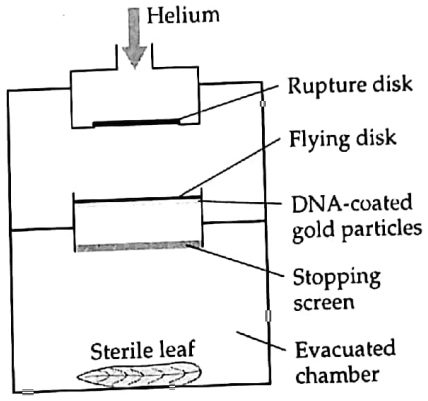


FIGURE 18.10 Schematic representation of a microprojectile bombardment apparatus. When the helium pressure builds up to a certain point, the plastic rupture disk bursts, and the released gas accelerates the flying disk with the DNA-coated gold particles on its lower side. The gold particles pass the stopping screen, which holds back the flying disk, and penetrate the cells of the sterile leaf.

TABLE 18.3 Transgenic plants formed by microprojectile bombardment of various plant cells

Plant(s)	Cell source(s)
Corn	Embryonic cell suspension, immature zygotic embryos
Rice	Immature zygotic embryos, embryogenic callus
Barley	Cell suspension, immature zygotic embryos
Wheat	Immature zygotic embryos
Turfgrass	Embryogenic callus
Rye	Meristems
Sorghum	Immature zygotic embryos
Pearl millet	Immature zygotic embryos
Orchid	Protocorms
Banana and plantain	Embryonic cell suspension
Poplar	Callus
Norway and white spruce	Somatic embryos
Pea	Zygotic embryos
Cucumber	Embryogenic callus
Sweet potato	Callus
Cranberry	In vitro-derived stem sections
Peony and lily	Pollen
Alfalfa	Embryogenic callus
Bean	Zygotic embryos
Cotton	Zygotic embryos
Grape	Embryonic cell suspension
Peanut	Embryogenic callus
Tobacco	Pollen

Adapted from Southgate et al., *Biotechnol. Adv.* 13:631–651, 1995.

using yeast artificial chromosomes (YACs) (see chapter 7). The YACs were engineered to contain plant selectable markers, as well as yeast selectable markers, which were already present on the YAC vector (Fig. 18.11). As a test system, various amounts of DNA from the fungus *Cochliobolus heterostrophus* were cloned so that the total size of the engineered YAC ranged from 80 to 550 kb. Following biolistic transfer of the engineered YACs with fungal DNA to plant (tobacco) cells, a number of transformants resistant to the antibiotic kanamycin were isolated. These transformed plant cells were then tested for the presence of the second plant selectable marker gene (encoding resistance to the antibiotic hygromycin), which was located on the other arm of the YAC vector. The presence of both plant selectable marker genes in transformed plant cells indicated that the entire YAC, along with all of the inserted foreign DNA, was probably transferred. DNA hybridization experiments revealed that YACs up to 150 kb in total size have a good chance of being transferred to plant cells and that the transferred DNA can be stably integrated into the plant cell. Thus, the production of transgenic plants that contain several foreign genes is feasible; eventually, entire biosynthetic pathways may be introduced into plant cells.

Chloroplast Engineering

While the vast majority of plant genes are found as part of the nuclear DNA, both the chloroplast and mitochondrion contain genes that encode a number of important and unique functions. However, not all of the proteins that are present in these organelles are encoded by organellar DNA. Some chloroplast and mitochondrion proteins are encoded in the nuclear DNA, synthesized in the cell's cytoplasm, and then, by a special mechanism, imported into the appropriate organelle. Accordingly, there are two ways that a specific foreign protein can be introduced into the chloroplast or mitochondrion. In one way, a fusion gene encoding the foreign protein and additional amino acids that direct the transport of the protein to the organelle can be inserted into the nuclear chromosomal DNA, and after synthesis, the recombinant protein can be transported into the targeted organelle. In the other way, the gene for the foreign protein can be inserted directly into either the chloroplast or mitochondrial DNA.

Most higher plants have approximately 50 to 100 chloroplasts per leaf cell, and each chloroplast has about 10 to 100 copies of the chloroplast DNA genome. Stable genetic transformation of chloroplasts in order to modify chloroplast functioning or to produce foreign proteins requires insertion of the foreign DNA into the chloroplast genome rather than into the much

FIGURE 18.11 Schematic representation of a YAC vector used to transfer large pieces of DNA to plant genomes. TEL, telomere; SM, selectable marker; CEN, centromere. The various elements are not drawn to scale; the foreign DNA, especially, is much larger than shown. Each of the plant selectable marker genes contains its own promoter and transcription terminator (not shown). The plant selectable markers are a hygromycin resistance gene and a kanamycin resistance gene. Adapted from Mullen et al., *Mol. Breed.* 4:449–457, 1998.