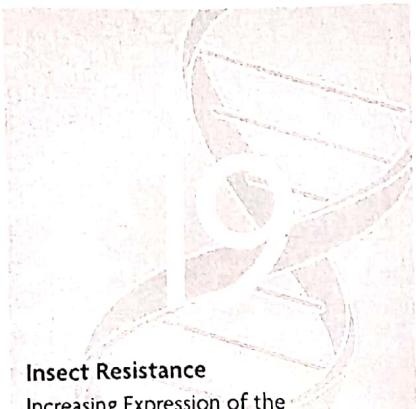

Engineering Plants To Overcome Biotic and Abiotic Stress

THE PRINCIPAL OBJECTIVE OF PLANT BIOTECHNOLOGY is to create new varieties of cultivated plants (cultivars). The majority of the initial studies of transgenic plants have focused on developing strains that give better yields. Genes that confer resistance to insects, viruses, herbicides, environmental stress, and senescence have been incorporated into various plants. A considerable amount of this work has been commercialized and has been the subject of much public scrutiny and discussion. Some of this work is discussed below.

Insect Resistance

The genetic engineering of crop plants to produce functional insecticides makes it possible to develop crops that are intrinsically resistant to insect predators and do not need to be sprayed (often six to eight times during a growing season) with costly and potentially hazardous chemical pesticides. It has been estimated that in 2007 the amount spent on chemical insecticides worldwide was approximately $15 billion to $20 billion. The cost of maintaining such genetically engineered insect-resistant crops is lower than that for nonresistant crops. Moreover, biological insecticides are usually highly specific for a limited number of insect species, and they are generally considered to be nonhazardous to humans and other higher animals. In addition, by reducing the damage to plants from insect predation, a corresponding decrease in the damage to plants from a number of fungal diseases should result, since many pathogenic fungi often invade a plant either together with or as a consequence of insect infection.

Several different strategies have been used to confer resistance to insect predators. One approach involves a gene for an insecticidal protoxin produced by one of several subspecies of the bacterium *Bacillus thuringiensis* (see chapter 16). Other common strategies use genes for plant proteins, such as α-amylase inhibitors, protease inhibitors, and lectins, that have been shown to be effective against a wide variety of insects. After an insect ingests one of these inhibitors, it is not able to digest food (i.e., plants)

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because the inhibitor interferes with the hydrolysis of starch or plant proteins. Thus, the insect will feed less and eventually die.

Increasing Expression of the *B. thuringiensis* Protoxin

B. thuringiensis protoxin does not persist in the environment, nor is it hazardous to mammals. Thus, it is a safe means of protecting plants. It is both simpler and less costly to express the genes for *B. thuringiensis* toxins in plants than to spray *B. thuringiensis* preparations onto the surface of the plant. This mode of insecticidal-toxin delivery limits the environmental distribution of the toxin and avoids problems associated with spraying *B. thuringiensis* preparations, such as limited environmental stability and the timing of the toxin application.

The scientific challenge in utilizing the *B. thuringiensis* protoxin is to create a transgenic plant that expresses and synthesizes a functional form of this prokaryotic insecticide at sufficient levels to prevent damage by insect predation. In initial experiments, the *B. thuringiensis* subsp. *kurstaki* insecticidal-protein genes, *cry1Aa*, *cry1Ab*, and *cry1Ac*, were not particularly well expressed in plants (Table 19.1). This is problematic, because high levels of expression of these insect control proteins are needed in order to produce commercially viable insect-resistant plants. To raise the level of the expressed protein, scientists truncated the gene so that only the N-terminal portion of the insecticidal protoxin—the part of the protoxin that contains the toxin (see chapter 16)—was produced and inserted a strong plant promoter to direct gene expression. Under these conditions, there was a significant increase in the level of insecticidal toxin produced, affording transgenic plants some protection against damage from insect predation.

The minimum sequence that encoded toxin activity had to be determined. To this end, the amino acid sequences of protoxins from various strains of *B. thuringiensis* were compared to determine whether there is a common insecticidal (toxin) domain. This analysis showed that the N-terminal portion of the protoxin molecule is highly conserved (~98%) and the C-terminal region is more variable (~45% conserved). Further work showed that all of the insecticidal-toxin activity resides within the first 646

TABLE 19.1 Expression of some *B. thuringiensis* insecticidal toxin genes in transgenic plants

Plant(s)	Gene	% Expression	Insecticidal
Tobacco	*cry1Ab*, full	0.0001–0.0005	No
Tobacco	*cry1Ab*, truncated	0.003–0.012	Yes
Tobacco	*cry1Aa*, full	Not detected	No
Tobacco	*cry1Aa*, truncated	0.00125	Yes
Tobacco	*cry1Ac*, truncated	<0.014	Yes
Tomato	*cry1Ab*, truncated	0.0001	Yes
Cotton	*cry1Ab*, truncated, WT	<0.002	No
Cotton	*cry1Ab*, truncated, PM	0.05–0.1	Yes
Tomato, tobacco	*cry1Ab*, truncated, WT	0.002	Yes
Tomato, tobacco	*cry1Ab*, truncated, PM	0.002–0.2	Yes
Tomato, tobacco	*cry1Ab*, truncated, FM	0.3	Yes

Adapted from Fly, p. 105–124, *in* Entwistle et al. (ed), *Bacillus thuringiensis, an Environmental Biopesticide: Theory and Practice* (John Wiley & Sons, Chichester, United Kingdom, 1993)

Terms and abbreviations: full, the complete protoxin gene; truncated, a shortened version of the protoxin gene; WT, wild-type codons; PM, partially modified codons; FM, fully modified codons.

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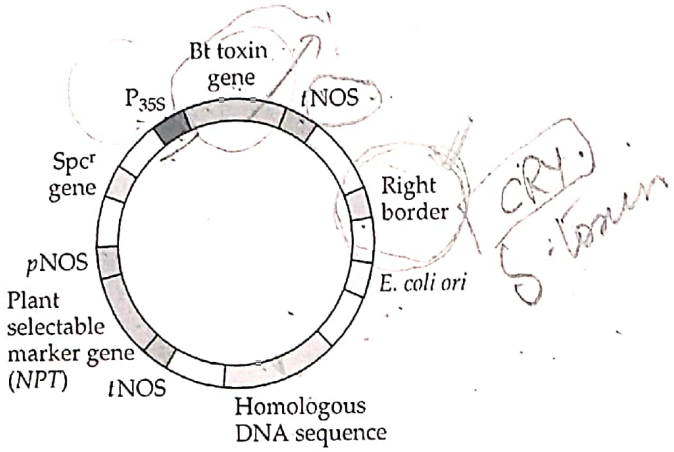

FIGURE 19.1 Cointegrate cloning vector carrying a *B. thuringiensis* (Bt) insecticidal-toxin gene. The toxin gene is under the control of the strong, constitutive 35S promoter (P_{35S}) from cauliflower mosaic virus and the nopaline synthase transcription terminator–polyadenylation site (*t*NOS). The vector has an *E. coli* origin of DNA replication *(ori)* and an Spc^r gene, which allow the vector to be maintained and selected in *E. coli* cells; a T-DNA right border; a plant selectable marker gene; and a region of DNA that is homologous to DNA in the disarmed Ti plasmid, for integrating the two plasmids. The neomycin phosphotransferase gene *(NPT)*, which acts as a plant reporter gene, is under the transcriptional control of nopaline synthase gene sequences (*p*NOS and *t*NOS) and is used to select for kanamycin-resistant transformed plant cells.

amino acids from the N terminus of the 1,156-amino-acid protoxin. When the segment of the protoxin gene that encodes the highly conserved amino acid sequence was cloned and expressed in bacteria, the shortened protein was as active as the native (protoxin) form in protecting plants against lepidopteran insects in laboratory trials.

Transgenic tomato plants with a truncated *B. thuringiensis* protoxin gene were produced to test whether the shortened protoxin would be able to protect the plants from damage by various insect pests. The shortened version of the protoxin gene was put under the transcriptional control of the strong constitutive 35S promoter from cauliflower mosaic virus and the nopaline synthase transcription termination–polyadenylation site and was then cloned into the T-DNA (transferred DNA) region of a cointegrate-type Ti plasmid vector (Fig. 19.1). The vector contained a spectinomycin resistance (Spc^r) gene that allowed it to be selected in either *Escherichia coli* or *Agrobacterium tumefaciens*, an *E. coli* origin of DNA replication, and a neomycin phosphotransferase gene that was under the control of the nopaline synthase promoter and transcription termination–polyadenylation sites and enabled the selection of transformed plant cells in the presence of kanamycin. In addition, the cointegrate cloning vector had the right border of the T-DNA from a nopaline Ti plasmid and a segment of the octopine Ti plasmid that provides a region of homology for cointegrate formation by homologous recombination with a disarmed Ti plasmid. The plasmid was constructed and manipulated in *E. coli* before it was transferred by conjugation to a strain of *A. tumefaciens* that contained a disarmed Ti plasmid. After recombination in *A. tumefaciens*, the short form of the protoxin gene was transferred to the chromosomal DNA of tomato plants.

In both greenhouse and field trials, transgenic tomato plants that expressed the short form of the protoxin were protected to some degree against damage caused by tobacco hornworms *(Manduca sexta)*, tomato

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fruitworms (*Heliothis zea*), and tomato pinworms (*Keiferia lycopersicella*). The extent of the protection was not the same for each of the insects, nor was it complete. The transgenic plants were protected to some extent from damage caused by tobacco hornworms and tomato fruitworms and to a lesser degree from damage by tomato pinworms. A combination of a low dose of chemical insecticide and production of the protoxin by the plants increased the level of protection afforded by the protoxin.

In an effort to dramatically increase the level of expression, an isolated insecticidal-toxin gene was modified by site-directed mutagenesis to change any DNA sequences that could inhibit efficient transcription or translation in a plant host (Table 19.1). This "partially" modified gene had a nucleotide sequence that was 96.5% unchanged from that of the wild-type gene and encoded the identical insecticidal-toxin protein. Transgenic plants that expressed this partially modified sequence produced a 10-fold-higher level of insecticidal-toxin protein than did plants that were transformed with the wild-type gene. Subsequently, a "fully" modified version of the insecticidal-toxin gene was designed and chemically synthesized. This fully modified gene contained codons more commonly used by plants, as opposed to those favored by gram-positive bacteria, such as *B. thuringiensis*. This gene was also modified to eliminate any potential messenger RNA (mRNA) secondary structure or chance plant polyadenylation sequences that might decrease gene expression. After modification, it had a G+C content of 49% (the wild-type gene is 37% G+C) and a nucleotide sequence that was only 78.9% identical to that of the wild-type gene.

Transgenic plants that were transformed with this highly modified synthetic protoxin gene had an approximately 100-fold-higher level of toxin protein than did plants transformed with the wild-type gene.

MILESTONE

Light-Inducible and Chloroplast-Associated Expression of a Chimaeric Gene Introduced into *Nicotiana tabacum* Using a Ti Plasmid Vector

L. Herrera-Estrella, G. Van den Broeck, R. Maenhaut, M. Van Montagu, J. Schell, M. Timko, and A. Cashmore
Nature 310:115–120, 1984

After researchers had established the Ti plasmid system as an effective means of transforming many different plants, their attention turned to the development of procedures for the expression of foreign genes in plants. Initially, most of the genes that were introduced into plant cells were under the transcriptional control of either the relatively strong constitutive 35S promoter from cauliflower mosaic virus or the nearly as strong constitutive promoter for the nopaline synthase gene that is encoded within some T-DNAs. However, the development of plants with useful new and modified traits often requires that a specific protein be expressed only in certain tissues, e.g., leaves or roots, or only at certain times in the life of the plant, e.g., during early seedling development, fruit formation, or high-temperature stress. As a first step toward the development of plants that expressed foreign genes in a tissue-specific or time-specific manner, Herrera-Estrella et al. constructed a chimeric gene that included the 5′-flanking region from the pea gene for the small subunit of ribulose bisphosphate carboxylase containing transcriptional regulatory sequences, the coding region of a bacterial chloramphenicol acetyltransferase gene as an easily selectable gene, and the 3′-flanking region from the nopaline synthase gene containing signals both for termination of transcription and for polyadenylation of the mRNA. Normally the gene for the small subunit of ribulose bisphosphate carboxylase is expressed only in green or photosynthetic tissue; as expected, the chloramphenicol acetyltransferase gene under the regulatory control of this DNA sequence was also expressed only in photosynthetic tissues. This work provided one of the first demonstrations that, despite their complexity, plant promoters could direct the transcription of heterologous proteins accurately and with tissue specificity. Since this study was done, researchers have used a wide range of plant promoters to direct tissue- and development-specific heterologous gene expression in transgenic plants.

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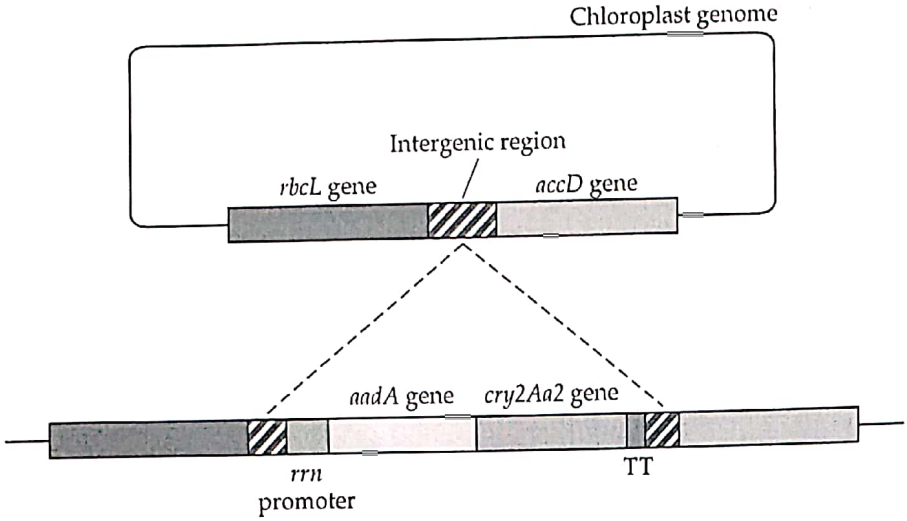

FIGURE 19.2 Site on the chloroplast genome where a foreign gene encoding the *B. thuringiensis* Cry2Aa2 protoxin is integrated by homologous recombination. The genes *rbcL* and *accD* are both present in a single copy per chloroplast genome. The intergenic region between these two genes, which is the site of insertion of the foreign genes, is smaller than it appears in this representation. The *aadA* gene (encoding spectinomycin and streptomycin resistances) and the *cry2Aa2* gene are both under the transcriptional control of the constitutive chloroplast *rrn* promoter and transcription terminator (TT), and each contains its own ribosomal binding site. Integration of foreign DNA into the intergenic spacer region prevents insertion of a foreign gene from interfering with the expression of any endogenous chloroplast genes. Adapted from Kota et al., *Proc. Natl. Acad. Sci. USA* 96:1840–1845, 1999.

Moreover, this higher level of insecticidal-toxin synthesis was directly correlated with increased insecticidal activity.

In another approach to increasing the expression of the protoxin, one group of researchers expressed the fully modified protoxin gene under the control of the promoter for the gene that codes for the small subunit of the plant enzyme ribulose bisphosphate carboxylase and downstream from the chloroplast transit peptide sequence of this enzyme, so that the overproduced protoxin became localized within the chloroplast. This strategy led to a very high level of expression (nearly 1% of the total leaf protein) of the insecticidal protoxin. Other researchers have introduced an insecticidal-protoxin gene directly into the chloroplast DNA of the host plant. The *B. thuringiensis* protoxin gene was integrated into a specific site on the chloroplast DNA by constructing a vector that contained the protoxin gene flanked by two single-copy chloroplast genes (Fig. 19.2). Integration of the introduced genes occurs by homologous recombination. Once integrated into the chloroplast DNA, a protoxin gene under the transcriptional control of a strong chloroplast promoter may be expressed at high levels, so the protoxin may compose as much as 2 to 3% of the total soluble protein in the leaf, yielding a very high level of insecticidal activity. In addition, even this level of foreign-protein expression could be dramatically increased (by 10- to 20-fold) by coexpressing (as part of the same operon that was introduced into the chloroplast DNA) a *B. thuringiensis* gene that encodes a chaperonin protein that facilitates the correct folding of the insecticidal-protein protoxin.

Integration of the *B. thuringiensis* protoxin gene into chloroplast DNA has a number of potential advantages over inserting it into the chromosomal

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DNA. First, the protoxin gene does not have to be modified, because the chloroplast transcriptional and translational apparatuses are typically prokaryotic. Second, because there are many chloroplasts per cell and many copies of chloroplast DNA per chloroplast, the protoxin gene is present in multiple copies and therefore is more likely to be expressed at a high level. Third, in most plants, chloroplasts are transmitted only through the egg and not through pollen, which means that plants receive all of their chloroplast DNA from their female parent. Consequently, there is no risk of unwanted transfer of the protoxin gene to other plants in the environment by pollen. The disadvantage of expressing the *B. thuringiensis* protoxin in chloroplasts is that insects that attack stems or fruit will not encounter the protoxin, since these tissues do not have any chloroplasts.

To date, some form of the gene for the protoxin has been introduced and expressed in a wide variety of plant species, including alfalfa, apple, broccoli, cabbage, canola, corn (maize), cotton, cranberry, eggplant, grape, hawthorn, juneberry, peanut, pear, poplar, potato, rice, rutabaga, soybean, spruce, sugar cane, tobacco, tomato, walnut, white clover, and white spruce. Following several seasons of successful field trials, these transgenic plants were approved for commercial release in the United States, Canada, and Argentina, and large-scale growth of the plants in the field began in 1996. Although insect populations still have to be monitored to keep track of the frequency of resistant organisms, the use of crops expressing *B. thuringiensis* insecticidal proteins has already exceeded the length of time that it typically takes for resistance to arise in insects to conventional pesticides. A number of transgenic plants that express an insecticidal toxin or protoxin are currently being used commercially—it is estimated that worldwide, in 2007, farmers planted approximately 40 million hectares of transgenic *B. thuringiensis* insecticidal-protein-containing crops. This technology has more than lived up to the hopes and expectations of scientists. Notwithstanding the initial concerns about the technology, especially in Europe, this approach to crop protection has gained widespread acceptance throughout much of the world.

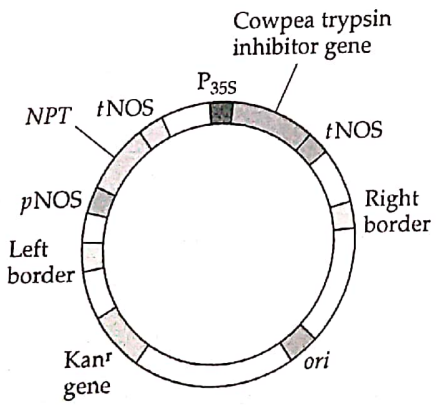

FIGURE 19.3 Binary cloning vector carrying a cowpea trypsin inhibitor gene. The vector contains a broad-host-range origin of DNA replication *(ori)* and a kanamycin resistance (Kan^r) gene, which function in both *E. coli* and *A. tumefaciens*. Between the T-DNA left and right borders, there are (1) a neomycin phosphotransferase gene *(NPT)* under the transcriptional control of nopaline synthase signals (*p*NOS and *t*NOS), which enables kanamycin-resistant transformed plant cells to be selected, and (2) the cowpea trypsin inhibitor gene, which is under the control of the 35S promoter (P_{35S}) from cauliflower mosaic virus and the transcription terminator–polyadenylation region from the nopaline synthase gene (*t*NOS).

Other Strategies for Protecting Plants against Insects

No single *B. thuringiensis* protoxin is effective against a broad range of insect species. This may limit the overall usefulness of these protoxins. However, plants have evolved general insect defense mechanisms that are sufficient for plant survival but not always effective enough to keep the damage to a level that would be acceptable for crop plants. For example, some plants produce protease inhibitors that, when ingested, prevent the feeding insect from hydrolyzing plant proteins, thereby effectively starving the predator insect. Consequently, it seemed reasonable to isolate a plant gene for a protease inhibitor, add a strong promoter, and create transgenic crop plants that produce sufficiently high levels of the protease inhibitor to reduce damage from insect predation.

Protease inhibitors. In one study, researchers isolated a clone that encodes cowpea trypsin inhibitor from a complementary DNA (cDNA) clone bank by using a chemically synthesized DNA probe based on the amino acid sequence of the cowpea trypsin inhibitor protein. The full-length cDNA was subcloned onto a Ti plasmid binary cloning vector (Fig. 19.3) and introduced into a strain of *A. tumefaciens* carrying a disarmed Ti plasmid that

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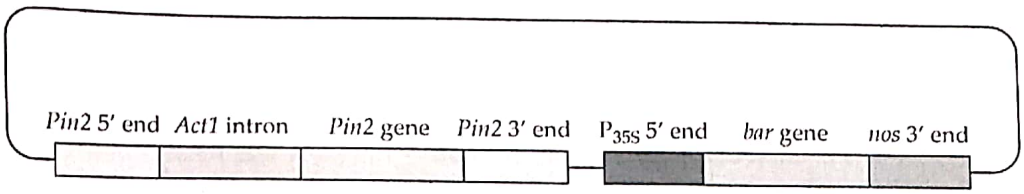

FIGURE 19.4 Plasmid vector carrying the potato proteinase inhibitor II gene (*Pin2*). 5′ end, the region of DNA preceding the gene; 3′ end, the region of DNA following the gene; *Act1* intron, the first intron from the rice actin 1 gene; P_{35S} 5′ end, the 35S promoter from cauliflower mosaic virus; *bar* gene, the bacterial phosphinothricin acetyltransferase gene; *nos* 3′ end, the region of DNA following the nopaline synthase gene. The *bar* gene serves as a selectable marker for transgenic plants, conferring resistance to the herbicide Basta (ammonium glufosinate).

contained active *vir* genes. Following *A. tumefaciens* infection of tobacco leaf disks with this vector, cells that incorporated the cloned DNA were selected for growth on kanamycin, and transgenic plants were regenerated. The damage caused by *Heliothis virescens* (tobacco budworm) larvae to transgenic plants that expressed more than 2 mg of cowpea trypsin inhibitor per mg of protein was significantly less than the damage inflicted on nontransformed plants.

Cowpea seeds that contain approximately 2 mg of inhibitor per mg of plant protein are not toxic to either animals or humans. However, if the amount of protease inhibitor produced by a transgenic plant is determined to be a potential hazard, then it is possible to limit the expression of the protease inhibitor to the plant tissues that the major insect pests prefer but that are not used as food by humans or animals. In other words, a cloned protease inhibitor gene could be active in the leaves and roots of a plant but not in the commercially valuable fruit.

Introduction of the potato proteinase inhibitor II gene provides rice plants with protection against the pink stem borer (*Sesamia inferens*), a major insect pest of rice. Infestation of rice plants by pink stem borers causes severe damage to the plants, often resulting in a hollow stem and dead panicles with no seeds. A plasmid carrying the potato proteinase inhibitor II gene under the control of its own promoter and transcription termination region was constructed. The plasmid also contained the first intron from the rice actin gene inserted between the promoter and the potato proteinase inhibitor II coding region (Fig. 19.4). This construct was introduced into rice suspension cells by microprojectile bombardment, and transgenic plants were generated. When pink stem borer larvae were artificially applied, 70 to 100% of the wild-type plants were severely damaged by insect predation, while only 15 to 20% of the transgenic plants were damaged. Since plant proteinase inhibitors are common components of both human and animal food and are readily inactivated by cooking, their introduction into new crops can be regarded as safe.

Another strategy that is designed to increase the effectiveness of relatively low levels of *B. thuringiensis* insecticidal-toxin activity entails combining the toxin with a serine protease inhibitor. In laboratory trials, investigators found that when the amount of purified *B. thuringiensis* insecticidal toxin that causes minimal insect mortality was mixed with a low concentration of protease inhibitor, the insecticidal activity of the mixture was 20-fold greater than that of the *B. thuringiensis* protoxin alone. To test whether this scheme would function in transgenic plants, a DNA fragment

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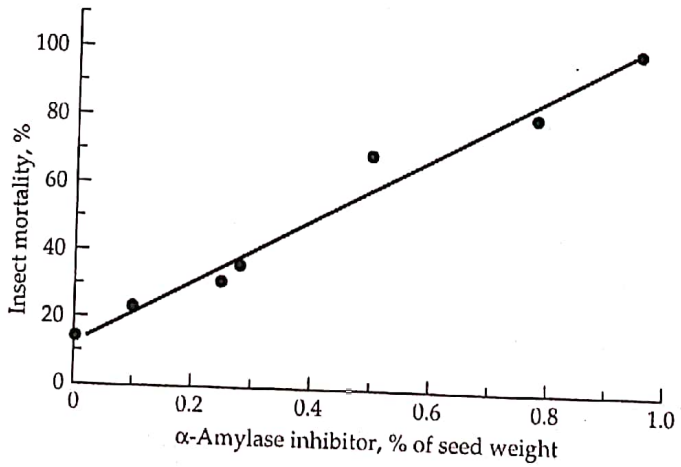
that encoded a fusion protein consisting of both a protease inhibitor and a truncated toxin was constructed. Transgenic tobacco plants that produced small amounts of this fusion protein were protected from insect attack.

α-Amylase inhibitor. Another way of imparting insect resistance to susceptible plants entails using a gene that encodes an α-amylase inhibitor. The cowpea weevil *(Callosobruchus maculatus)* and the azuki bean weevil *(Callosobruchus chinensis)* are seed-feeding beetles that cause considerable economic loss of these legume crops, especially in developing countries. When larvae of these insects are fed a diet that includes the common bean *(Phaseolus vulgaris)*, insect growth is inhibited. This growth inhibition is attributable to the presence of an α-amylase inhibitor in the seed proteins of the common bean. Accordingly, the gene for the α-amylase inhibitor from the common bean was isolated, placed under the transcriptional control of the strong seed-specific promoter for the bean phytohemagglutinin gene, and used to transform pea plants *(Pisum sativum)*. Peas are usually quite susceptible to damage by both cowpea weevils and azuki bean weevils. However, transgenic pea plants that expressed the α-amylase inhibitor were resistant to both of these insects. The level of resistance to cowpea weevils was found to be proportional to the amount of α-amylase inhibitor that the transgenic plant produced (Fig. 19.5).

Cholesterol oxidase. Another approach to developing insect-resistant transgenic plants makes use of a bacterial cholesterol oxidase gene. Cholesterol oxidase, which is present in a range of different bacterial genera, catalyzes the oxidation of 3-hydroxysteroids to ketosteroids and hydrogen peroxide. This enzyme is commonly used in assays to determine the levels of cholesterol in human serum. Low levels of the enzyme have a high level of insecticidal activity against larvae of the boll weevil (*Anthonomus grandis grandis*) (Fig. 19.6), a common and economically important insect (Coleoptera) pest of cotton, and have lower levels of activity against some lepidopteran pests. Cholesterol oxidase probably acts by disrupting the insect's midgut epithelial membrane, thus killing the insect. A cholesterol oxidase gene encoding a protein with a molecular mass of approximately 55,000 daltons

FIGURE 19.5 Mortality of cowpea weevil larvae reared on transgenic pea plants that produce different amounts of α-amylase inhibitor.



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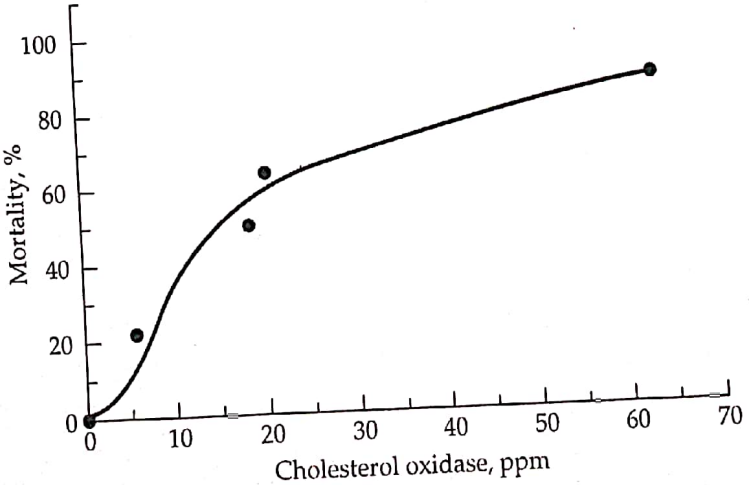
with a length of 504 amino acids plus a leader peptide of approximately 5,000 daltons (43 amino acids) was isolated from a strain of *Streptomyces* and cloned into a vector under the control of a plant virus (figwort mosaic virus) promoter and a termination sequence from the 3′ region of the *A. tumefaciens* nopaline synthase gene. When this construct was introduced into tobacco cell protoplasts, the transformed cells actively expressed the cholesterol oxidase. When the gene is introduced into cotton plants on a commercial scale, either by itself or in combination with genes for other biological insecticides, it should be an effective means of protecting plants against damage from insect predation.

Vegetative insecticidal toxins. In addition to the well-characterized Cry insecticidal toxins—over 350 of which have been identified—*B. thuringiensis* produces a secreted insecticidal protein during its vegetative growth stage. To date, two major groups of vegetative insecticidal proteins (Vip) have been identified. One group consists of the proteins Vip1 and Vip2, which are not toxic to lepidoptera, and Vip3, which targets several major lepidopteran pests. The less-well-characterized Vip proteins may act synergistically with Cry proteins to kill their target insects, providing a double-barreled approach to insect toxicity, so that it is extremely difficult for susceptible insects to develop resistance. It would therefore be advantageous if transgenic plants expressing both Cry and Vip proteins could be created. As a first step, researchers shuffled the two major domains of two Vip3 proteins, Vip3Ac1 and VipAa1 (Fig. 19.7). One of the hybrid Vip3 proteins (i.e., Vip3AcAa) displayed the highest activity of the four proteins against fall armyworms, cotton bollworms, and silkworms. Moreover, only the Vip3AcAa construct was toxic to a strain of cabbage looper that was resistant to the well-characterized *B. thuringiensis* insecticidal protein Cry1Ac. The chimeric toxin Vip3AcAa enriches the diversity of Vip toxins that can be used together with conventional Cry proteins to generate transgenic plants that are highly unlikely to select for resistant insects.

Other proteins. The activities of several other proteins have been utilized in an effort to protect plants from insect predation. For example, some

FIGURE 19.6 Effect of increasing amounts of cholesterol oxidase on the mortality of boll weevil larvae. ppm, parts per million. Adapted from Corbin et al., *Appl. Environ. Microbiol.* 60:4239–4244, 1994.



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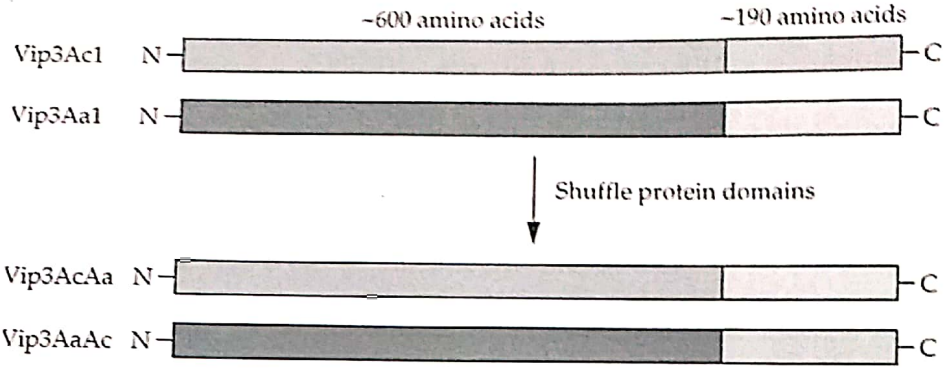

FIGURE 19.7 Generation of two hybrid insecticidal proteins constructed by domain shuffling. The approximate sizes of the amino- and carboxy-terminal fragments are indicated. The hybrid protein Vip3AcAa was found to have the greatest level of insecticidal activity with the insect larvae tested.

lectins, i.e., carbohydrate-binding proteins found in the seeds and storage tissues of a variety of plant species, are toxic to certain species of insects. While many plant lectins are toxic to mammals as well as insects, the lectin from the snowdrop plant *(Galanthus nivalis)* is toxic only to insects. With this in mind, the snowdrop lectin gene has been introduced into approximately a dozen different plants, with the result that plants that expressed this protein were damaged by aphids to a lesser extent than nontransformed plants. However, although the snowdrop lectin significantly lowered the amount of leaf material eaten, insect mortality was only slightly increased.

When the gene encoding the enzyme tryptophan decarboxylase from periwinkle *(Catharanthus roseus)* is expressed in tobacco, the plants are protected from damage by the whitefly *(Bemisia tabaci)*. While the precise mechanism of this protection is unknown, it has been suggested that the tryptamine that is produced by this enzyme, following the decarboxylation of tryptophan, is used in the production of insect-inhibiting plant alkaloids.

The gram-negative bacterium *Photorhabdus luminescens* produces a 283-kilodalton protein, toxin A, that is highly toxic to a variety of insects. When this protein was expressed in transgenic *Arabidopsis thaliana* (Box 19.1) plants in amounts of ≥700 ng/mg of extractable plant protein, it was found to be highly toxic to the tobacco hornworm, as well as the southern corn rootworm, with insect mortality typically 100%.

Finally, transgenic corn plants that express avidin—a glycoprotein isolated from chicken eggs that binds the coenzyme biotin with extremely high affinity—caused biotin deficiency that led to stunted growth and death in a number of different insect species. Importantly, the levels of avidin that are toxic to insects are not toxic to mice, suggesting that protecting plants with an avidin transgene is not necessarily a problem for humans.

RNA interference. The ingestion or microinjection of double-stranded RNA into some worms and insects has been used to silence genes in these organisms. This gene silencing works through the generation of RNA interference (RNAi) (see chapter 11). In one study, when 290 different double-stranded RNAs thought to encode essential or important functions were

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fed (one at a time) at a low level to larvae of the western corn rootworm (*Diabrotica virgifera* LeConte), growth was significantly inhibited with 67 of the RNAs. Fourteen of the double-stranded RNAs had 50% lethal concentrations of ≤5.2 ng/cm^2. Based on these results, transgenic corn expressing one of the double-stranded RNAs, targeting the transcript for vacuolar ATPase, was constructed (Fig. 19.8). The transgenic plants were protected against the western corn rootworm to an extent comparable to the protection afforded by a *B. thuringiensis* transgene. The demonstration that it is possible to produce RNAi in coleoptera following oral delivery of double-stranded RNA is an important first step in the development of a completely novel approach to developing a wide range of insect-resistant plants, including resistance to some insects that are refractory to the *B. thuringiensis* insecticidal toxin.

In a variation on the above-mentioned strategy, another group of researchers first identified the mechanism that the cotton bollworm uses to protect itself against the compound gossypol, which is produced by cotton plants to prevent insect predation. Gossypol is a yellow polyphenolic aldehyde that permeates cells and acts as an inhibitor of several of the insect's dehydrogenase enzymes. It has been used as a male oral contraceptive in China, possesses antimalarial properties, and may have anticancer properties. The cotton bollworm protects itself from the toxic effects of gossypol by inactivating the gossypol with the enzyme cytochrome P450 monooxygenase. Thus, transgenic plants were constructed to synthesize an RNAi molecule that would silence the insect's gene for the cytochrome P450 monooxygenase. By preventing the expression of cytochrome P450 monooxygenase, the insect was exposed to the full toxic effects of the plant-produced gossypol, so that it was either killed or at least debilitated, and the damage to the plant was limited (Fig. 19.9). Plants produce a myriad of allelochemicals to protect themselves against insects, and many insects have developed strategies to overcome the toxic effects of these compounds. Therefore, the mechanisms utilized by insects to overcome the toxicity of the plant-produced compounds are attractive targets for developing insect-resistant plants in the future.

BOX 19.1

Arabidopsis thaliana

Arabidopsis thaliana (thale cress) is a small weed in the same family (Brassicaceae) as canola, mustard, and broccoli. It is native to Europe, Asia, and northwestern Africa. *A. thaliana* is popular with scientists as a model organism in plant biology and genetic studies. It has one of the smallest genomes—at 7×10^7 bp, it is similar to the size of the yeast genome, which is approximately 1.5×10^7 bp—of any flowering plant, which makes it readily amenable to molecular genetic studies. The small size of its genome has made *A. thaliana* useful for the generation and selection of mutants, and it was the first plant genome to be sequenced, in 2000.

The plant's small size and short life cycle are also advantageous for research. Laboratory strains of *A. thaliana* take about 6 weeks from germination to mature seed. The small size of the plant is convenient for cultivation in limited space, and it produces many seeds—an individual plant can produce several thousand seeds.

Plant transformation in *Arabidopsis* is straightforward and has become a routine procedure in many laboratories, using *A. tumefaciens* to transfer DNA to the plant genome. The current *Arabidopsis* transformation protocol, termed "floral dip," involves dipping a flower into a solution containing *Agrobacterium*, the DNA of interest, and a detergent. This method avoids the need for tissue culture or plant regeneration. The idea is that some of the *Agrobacterium* cells will transfer their T-DNA containing the target DNA into the reproductive tissue of the plant. As a consequence of the above-mentioned traits and the relative complexity of most other plants, *Arabidopsis* has become the *E. coli* of the plant world.

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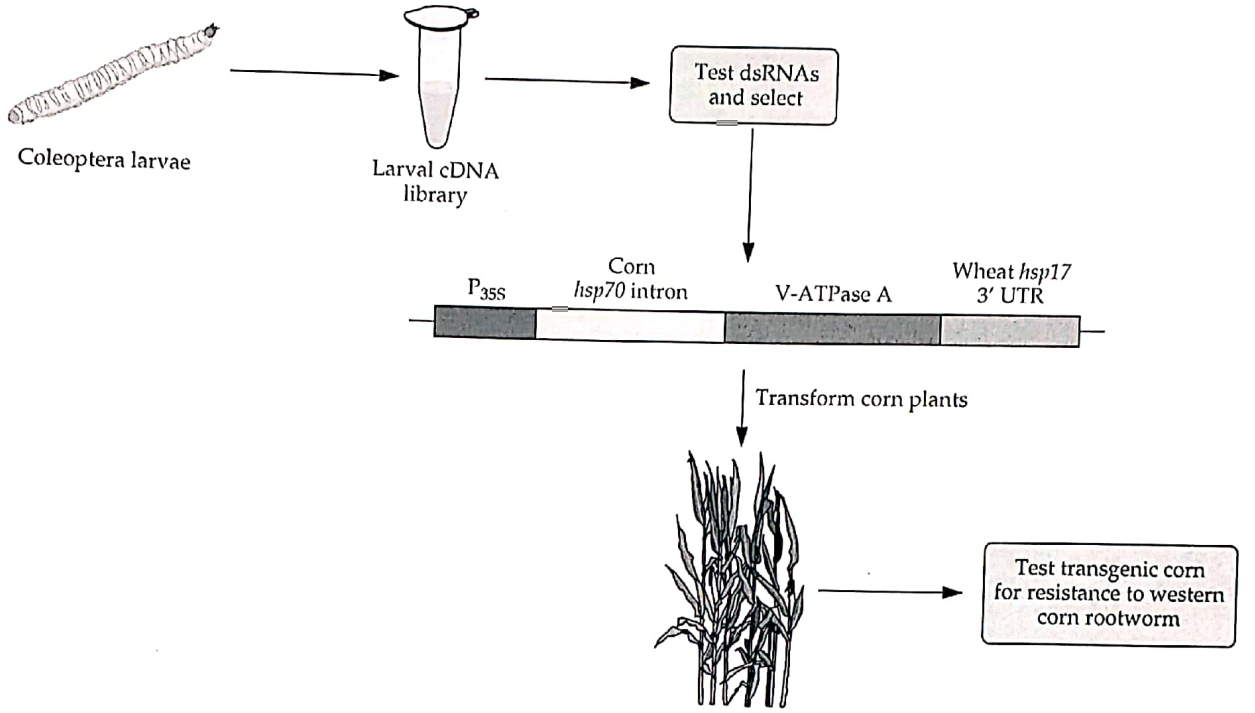

FIGURE 19.8 Use of RNAi to protect plants against insect predation. The double-stranded RNAs (dsRNAs) were produced using a commercial in vitro transcription system developed for this purpose. A number of double-stranded RNAs from essential insect genes were tested for the ability to elicit RNAi and inhibit insect larval proliferation. One of the most effective double-stranded RNAs, which encodes a portion of an ATPase gene, was spliced into a Ti plasmid vector and used to transform corn plants. The transformants with the highest levels of resistance to the western corn rootworm were selected. UTR, untranslated region.

Preventing the Development of *B. thuringiensis*-Resistant Insects

There is little doubt that insects have the genetic potential to develop resistance to *B. thuringiensis* insecticidal toxins, and the more that *B. thuringiensis* insecticidal toxins are used, the greater the likelihood that populations of target insects will accumulate resistant individuals. Experimental strategies have been devised to prevent transgenic plants that express the *B. thuringiensis* protoxin gene from acting as selection agents for resistant insects. In one approach, the expression of the insecticidal toxin in transgenic plants was limited to a short period. The gene for the *B. thuringiensis* protoxin was cloned downstream of the promoter of a gene from tobacco called the pathogenesis-related protein 1a (PR-1a) gene. The expression of the PR-1a gene is part of a natural defense mechanism that combats pathogens. The PR-1a gene is normally induced by any one of a variety of pathogenic organisms or by chemicals, such as salicylic acid and polyacrylic acid. When transgenic plants with the *B. thuringiensis* protoxin gene under the control of the PR-1a promoter were treated with a chemical inducer, they synthesized detectable levels of insecticidal toxin within 1 day of application, which protected the plants against insect attack. Therefore, it is conceivable that the protoxin could be induced by the administration of an inexpensive and safe chemical inducer only when it is required during the

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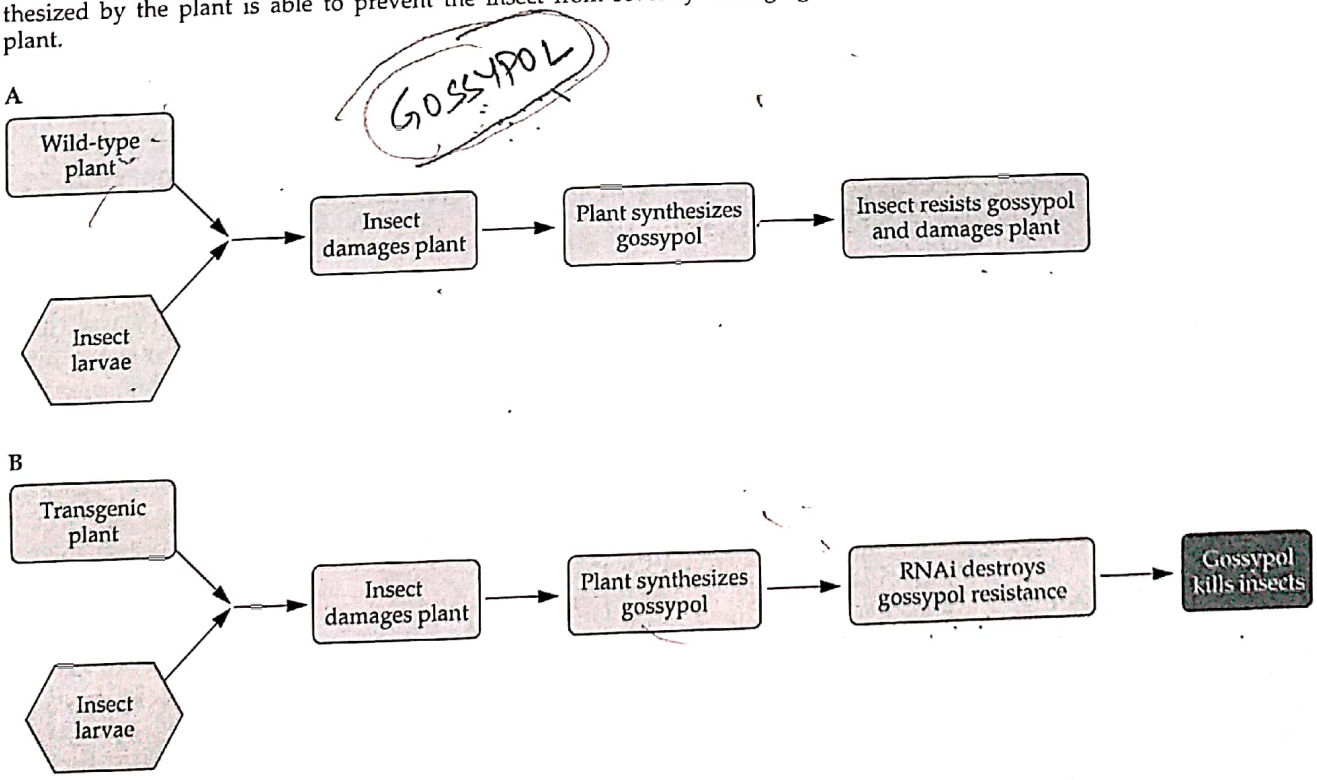
growing season, e.g., when insect larvae are feeding. Such periodic production should lower the selection pressure for resistant insects.

One approach that might increase the insecticidal effectiveness of *B. thuringiensis* expressed in transgenic plants and also decrease the development of insect resistance is to fuse the bacterial insecticidal gene with another protein that increases the binding of the insecticide to the target intestinal cellular receptor. With this in mind, a fusion protein consisting of an N-terminal *B. thuringiensis* insecticidal toxin and a C-terminal peptide consisting of the nontoxic B-chain of the protein ricin was constructed. Ricin is a protein toxin that is extracted from castor beans. It consists of an A-chain of 267 amino acids that contains the toxin activity and a B-chain of 262 amino acids that is catalytically inactive but serves to mediate entry of the complex into the cytosol. The *B. thuringiensis* insecticidal toxin binds to a receptor located within the membrane of the insect midgut (Fig. 19.10). Normally, since each insecticidal toxin interacts with a single receptor, the loss or modification of the receptor leads to resistance to the insecticidal toxin. However, since the ricin B-chain binds with very high affinity to *N*-acetylgalactosamine residues (which are adjacent to the *B. thuringiensis* insecticidal-toxin receptor), the fusion protein has two separate and independent means by which it is targeted to the receptor. With this fusion protein, it becomes extremely unlikely that both targeting mechanisms will cease to be effective at the same time. It has been suggested that this approach may be most effective in field situations where it is difficult or

FIGURE 19.9 Use of RNAi to inhibit the synthesis of a P450 monooxygenase enzyme that inactivates the plant secondary metabolite gossypol. **(A)** In wild-type plants, the P450 monooxygenase inactivates the gossypol, the plant is defenseless, and the insect can severely damage the plant. **(B)** In transgenic plants that produce an RNAi that directs the degradation of the P450 monooxygenase mRNA, the gossypol synthesized by the plant is able to prevent the insect from severely damaging the plant.



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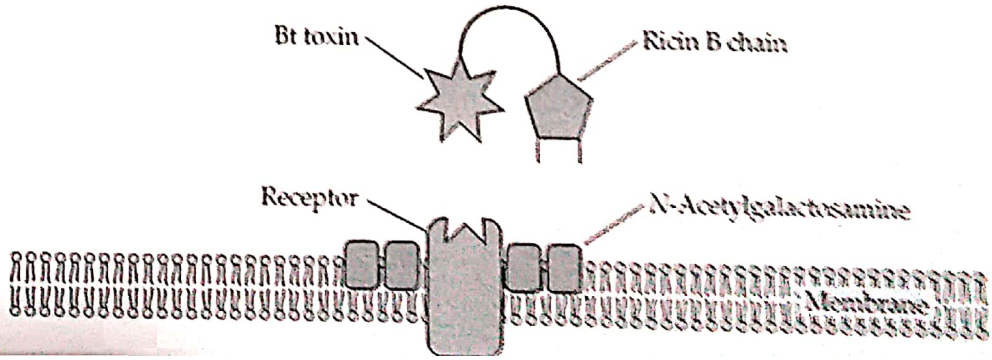
impossible to implement a spatial-refuge (refugium) strategy (such as for transgenic rice).

A number of additional strategies designed to prevent the development of insects that are resistant to the *B. thuringiensis* insecticidal toxin have been devised. They include the following.

- Using spatial-refuge strategies. In this approach, a certain fraction of each farmer's land, generally around 20%, is planted with a nontransgenic crop, while the remainder of the land is planted with a transgenic version of the same crop expressing a high level of the *B. thuringiensis* insecticidal toxin. The idea behind this strategy is that the very small number of insects that are able to survive on the transgenic insecticidal crop—a high dose of toxin kills 99.9% of susceptible insects—will mate with the much larger number of toxin-sensitive insects from the nontransgenic crop. Thus, the gene for resistance is effectively diluted—a high dose of the toxin kills 99% of the heterozygotes—and the pest population remains sensitive to the insecticide.
- Using two or more different *B. thuringiensis* insecticidal toxins (sometimes called gene stacking) or fusing portions of the active regions of two different toxin genes to generate novel hybrid protein insecticidal toxins (Box 19.2; also see chapter 16). This approach assumes that resistance to two control methods is much less likely to develop simultaneously. This approach has been found to be effective in the field when it is combined with spatial refugia.
- Transforming plants with both a *B. thuringiensis* insecticidal-toxin gene and another form of biological insecticide (e.g., an α-amylase inhibitor gene). This also assumes that resistance to two control methods is much less likely to develop simultaneously.
- Spraying low levels of chemical insecticides at the same time that transgenic plants expressing a *B. thuringiensis* insecticidal-toxin gene are used. This also assumes that resistance to two control methods is much less likely to develop simultaneously.

The insect resistance management strategies that have been used up to now appear to have been successful. For example, in one large study, researchers monitored the level of resistance of the pink bollworm (*Pectinophora gossypiella*) to *B. thuringiensis* in cotton fields over the course of 8 years, from

FIGURE 19.10 Schematic representation of a hybrid protein consisting of the Cry1Ac insecticidal-toxin protein (at the N terminus), the B-chain of ricin (at the C terminus), and an insect midgut insecticidal-protein receptor. The Cry protein recognizes and binds to the insect midgut receptor, while the B-chain of ricin acts as an *N*-acetygalactosamine-specific lectin that binds very tightly to these residues, which are located adjacent to the receptor. Bt, *B. thuringiensis*.



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BOX 19.2

Managing Insect Resistance to *B. thuringiensis* by Gene Stacking

Although various strains of *B. thuringiensis* produce hundreds of different bacterial insecticidal proteins, transgenic field crops engineered to produce these proteins have utilized only a few types of insecticidal toxin. This has led to concerns that the expression of a single toxin throughout the growing season could result in insects evolving resistance to the toxin's effects. One of the strategies that has been employed to prevent or delay the development of insect resistance to *B. thuringiensis* insecticidal toxins includes transforming plants with two unrelated *B. thuringiensis* insecticidal-toxin genes. Recent data indicate that gene stacking (also called gene pyramiding) of two genes encoding proteins with different modes of action significantly delays the development of insect resistance to these insecticidal toxins.

Bollgard II is a strain of genetically engineered cotton that was developed by the Monsanto Corporation to produce both the Cry2Ab2 and Cry1Ac insecticidal proteins. This strain was produced by retransformation of the previously commercialized Bollgard cotton, which produces only the Cry1Ac insecticidal protein. The commercial use of Bollgard cotton began in 1996, and by 2003, it had been grown globally on more than 32 million acres with the benefits of reduced insecticide use, improved control of target insect pests, increased yield, and reduced production costs accruing to farmers. The two insecticidal proteins produced by Bollgard II provide protection against several major lepidopteran pests of cotton, including the cotton bollworm, tobacco budworm, pink bollworm, and armyworm. In addition to an expanded insecticidal range, Bollgard II is expected to significantly delay (or prevent) the development of insect resistance in the field. Nevertheless, the use of Bollgard II requires the concomitant employment of refugia.

1997 to 2004. They found that the frequency of resistance did not change over this period and attributed this result to the use of refugia, the recessive inheritance of the resistance, the fact that the resistance that developed was incomplete, and the fitness costs associated with the development of insect resistance. It is nevertheless essential that new strategies to prevent the development of insect resistance continue to be developed. The amount of land that is devoted to the growth of transgenic crops that express a *B. thuringiensis* insecticidal toxin continues to increase rapidly worldwide. The types of crops that have been engineered to express a *B. thuringiensis* insecticidal toxin are also continuing to expand. Thus, a responsible approach dictates that we continue to be careful and vigilant in avoiding the development of insect resistance so as not to waste this resource.

Virus Resistance

Plant viruses often cause considerable crop damage and significantly reduce yields. Therefore, in the absence of effective chemical treatments, plant breeders have attempted to transfer naturally occurring virus resistance genes from one plant strain (cultivar) to another. However, resistant cultivars often revert to virus sensitivity, and resistance to one virus does not necessarily confer resistance to other, similar viruses. Natural virus resistance can be achieved in different ways: viral transmission can be blocked, establishment of the virus can be prevented, or viral symptoms can be bypassed or resisted. Genetic engineering has been used to develop nonconventional types of virus-resistant transgenic plants.

Viral Coat Protein-Mediated Protection

When transgenic plants express the gene for a coat protein (which usually is the most abundant protein of a virus particle) of a virus that normally infects those plants, the ability of the virus to subsequently infect the plants and spread systemically is often greatly diminished. For a long time, the

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precise mechanism by which the presence of coat protein genes inhibits viral proliferation was not understood; however, it is now thought that it likely works through the generation of RNAi. Moreover, the antiviral effect occurs early in the viral replication cycle and, as a result, prevents any significant amount of viral synthesis. This feature is an advantage because it decreases the probability of selecting for spontaneous viral mutants that can overcome this resistance and replicate in the presence of viral coat protein. The viral coat protein gene approach has been used to confer tolerance for a number of different plant viruses (Table 19.2). With this approach, researchers have developed virus-resistant transgenic plants for a number of different crops. Although complete protection is not usually achieved, high levels of virus resistance have been reported. In addition, a coat protein gene from one virus sometimes provides tolerance for a broad spectrum of unrelated viruses. The utility of this strategy is supported by the observation that transgenic plants that encode viral coat proteins do as well in field trials as in the laboratory studies.

In both eukaryotes and prokaryotes, an RNA molecule that is complementary to a normal gene transcript (mRNA) is called antisense RNA. The mRNA, being translatable, is considered to be a sense RNA. The presence of antisense RNA can decrease the synthesis of the gene product by forming a duplex molecule with the normal sense mRNA, thereby preventing it from being translated. The antisense RNA–mRNA duplex is also rapidly degraded, a response that diminishes the amount of that particular mRNA in the cell. Theoretically, it should be possible to prevent plant viruses from

TABLE 19.2 Some transgenic plants engineered to have viral coat protein-mediated protection against viral infection

Viral source of coat protein	Transgenic plant(s)
Alfalfa mosaic virus	Alfalfa, tobacco, tomato
Arabis mosaic virus	Tobacco
Beet necrotic yellow vein virus	Sugar beet
Cucumber mosaic virus	Cucumber, tobacco
Cymbidium ringspot virus	Tobacco
Grapevine chrome mosaic virus	Tobacco
Maize dwarf mosaic virus	Sweet corn
Papaya ringspot virus	Papaya, tobacco
Plum pox virus	Tobacco
Potato aucuba mosaic virus	Tobacco
Potato leafroll virus	Potato
Potato virus S	Potato
Potato virus X	Potato, tobacco
Potato virus Y	Potato, tobacco
Rice stripe virus	Rice
Soybean mosaic virus	Tobacco
Tobacco etch virus	Tobacco
Tobacco mosaic virus	Tobacco, tomato
Tomato mosaic virus	Tomato
Tomato rattle virus	Tobacco
Tomato streak virus	Tobacco
Tomato spotted wilt virus	Tobacco
Watermelon mosaic virus 2	Tobacco
Zucchini yellow mosaic virus	Muskmelon, tobacco

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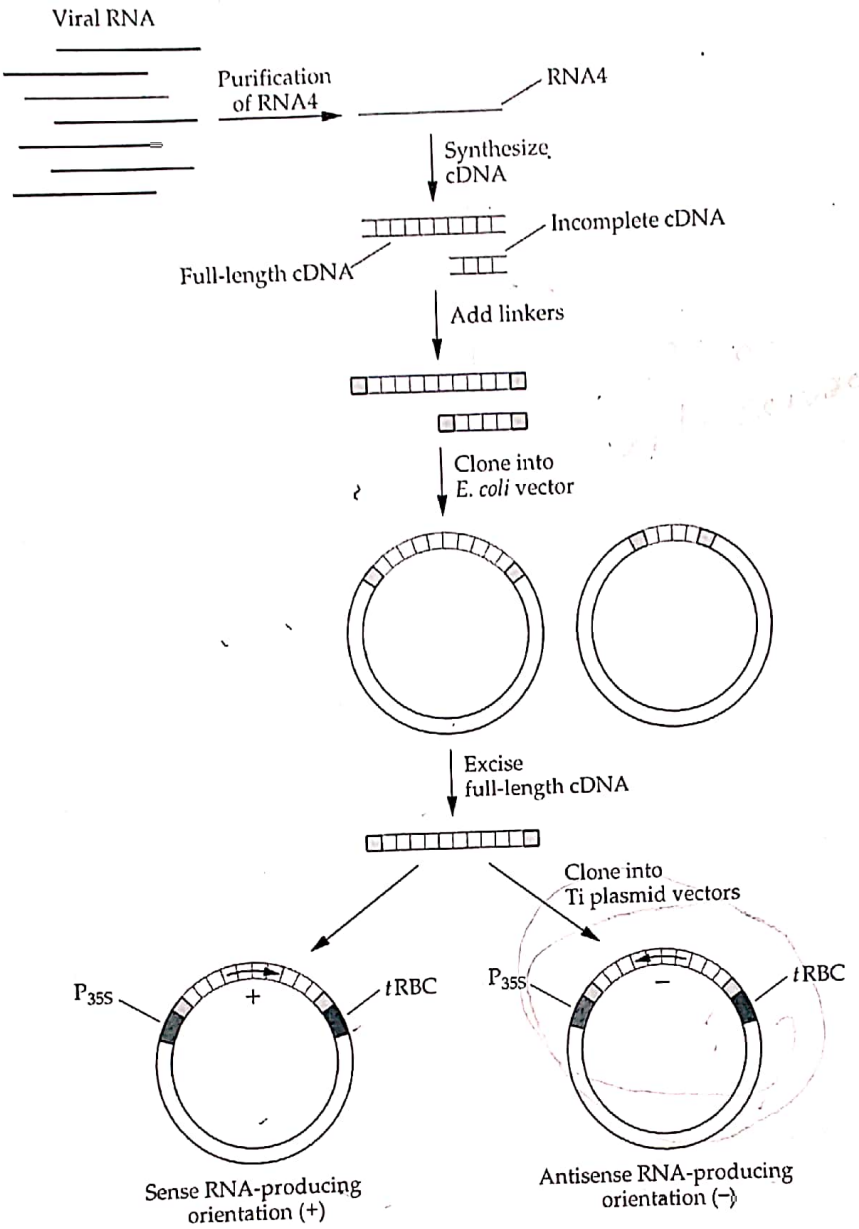

FIGURE 19.11 Procedure for introducing CuMV coat protein cDNA into plant cells. RNA4, which encodes the coat protein, is isolated from a viral RNA preparation and used as the template for the synthesis of double-stranded cDNA. Linkers are added to the cDNA preparation, and the cDNAs are cloned into an *E. coli* plasmid vector. A full-length cDNA clone is identified, excised from the *E. coli* vector, and subcloned into a Ti plasmid cloning vector between the 35S promoter from cauliflower mosaic virus (P_{35S}) and the transcription terminator from the gene for the small subunit of ribulose bisphosphate carboxylase (*t*RBC). This cloning step creates two orientations for the RNA4 cDNA. In one case, the RNA that is transcribed is translated into coat protein (sense RNA), and in the other case, the transcribed RNA is complementary to the mRNA for the coat protein (antisense RNA).

replicating and subsequently damaging plant tissues by creating transgenic plants that synthesize antisense RNA that is complementary to viral coat protein mRNA.

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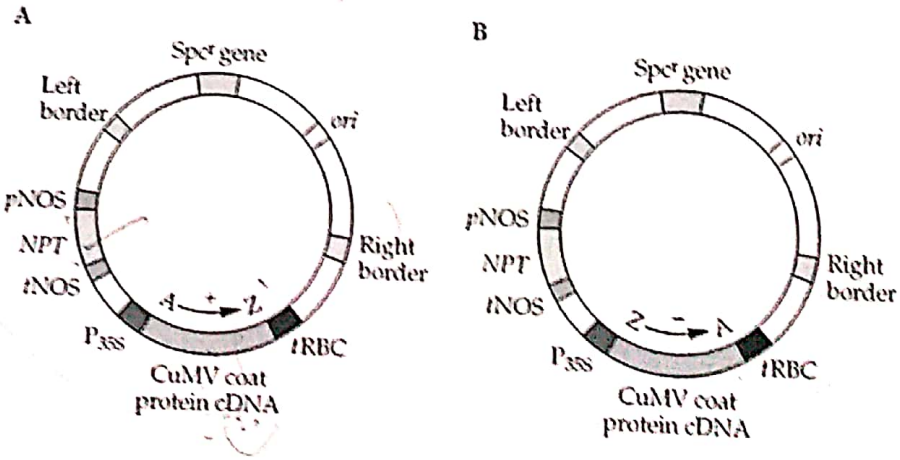
In one of many studies, the efficacies of the viral coat protein gene and antisense RNA approaches were compared by cloning the cDNA for the coat protein of cucumber mosaic virus (CuMV) into tobacco plants in two orientations (sense and antisense; one orientation per plant) and then testing transgenic plants for sensitivity to viral infection (Fig. 19.11). The genome of CuMV consists of three separate single-stranded pieces of RNA, each coding for a specific viral protein. In vivo, one of these pieces, RNA3, is processed to remove a portion of its sequence, thereby generating RNA4, which encodes the viral coat protein. To create transgenic plants that either produced normal mRNA and expressed the viral coat protein or produced its antisense RNA, the following steps were carried out:

1. Isolation of RNA4
2. In vitro enzymatic conversion of RNA4 into a double-stranded cDNA
3. Addition of linkers onto the cDNA
4. Insertion of the full-length cDNA sequences into cloning vectors in both orientations, with each oriented sequence under the control of the 35S promoter sequence from cauliflower mosaic virus and the termination-regulatory sequences from the plant gene for the small subunit of ribulose bisphosphate carboxylase
5. Formation of separate transgenic plants carrying the cDNA sequence in one of the two possible orientations

The Ti plasmid binary vector system was used to transfer both protein-producing sense and antisense RNA-producing cDNA sequences to separate tobacco cells, from which transgenic plants were regenerated (Fig. 19.12). The transgenic tobacco plants that expressed the CuMV coat protein were protected from viral-particle accumulation and did not show symptoms of viral infection, regardless of whether the inoculum of the challenge

FIGURE 19.12 Ti plasmid binary cloning vectors containing either the protein-producing sense (A) or the RNA-producing antisense (B) orientation of the CuMV coat protein cDNA. Each cDNA sequence is under the control of the 35S promoter (P_{35S}) from cauliflower mosaic virus and the transcription terminator–polyadenylation site (*t*RBC) from the gene for the small subunit of ribulose bisphosphate carboxylase. The vector also contains a neomycin phosphotransferase (*NPT*) gene under the control of nopaline synthase transcription signals (*p*NOS and *t*NOS), an Spc^r gene, a T-DNA right-border sequence, a T-DNA left-border sequence, and a broad-host-range origin of DNA replication (*ori*). The protein-producing sense (+) orientation is shown by the A→Z arrow, and the RNA-producing antisense (−) orientation is shown by the Z→A arrow.



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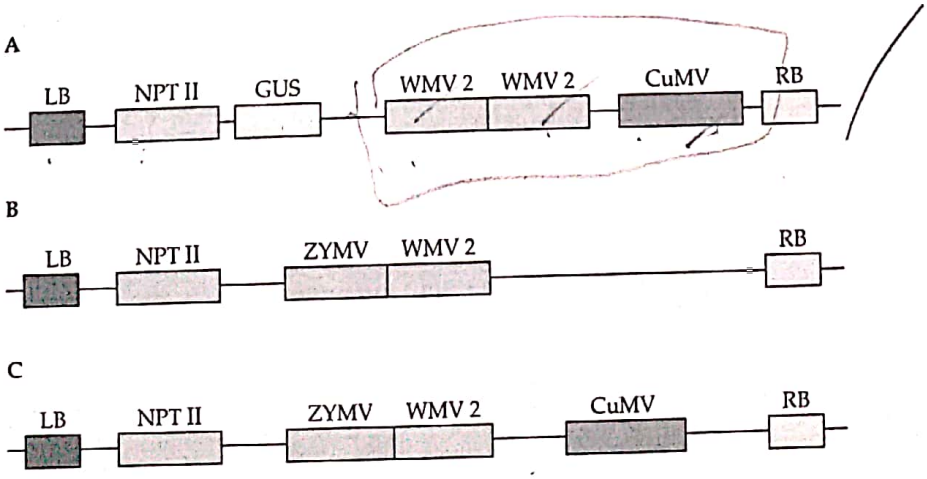
virus was high or low, whereas the antisense orientation construct protected transgenic plants only against low viral doses.

Several groups of scientists have constructed transgenic plants that synthesize antisense RNA copies of viral coat protein genes and tested whether these plants can withstand a viral challenge. In all instances, the plants were protected against the invading virus only when low concentrations of the virus were used. At high concentrations, the plants were damaged by the virus. In addition, antisense RNA copies of viral coat protein genes generally afforded a much lower level of protection to transgenic plants than did sense versions of the viral coat protein genes. Although the antisense RNA approach may not be an effective means of creating virus-resistant plants, it may be possible to use small interfering RNA (double-stranded RNAs about 21 nucleotides long) to protect plants against invading viruses. In this case, the interfering RNA would act to target specific mRNAs (e.g., mRNAs encoding viral coat proteins) for nuclease digestion.

Often field crops are exposed to several different viruses, any one of which may damage the plant and lower the final yield. Ideally, transgenic plants should be resistant to more than one virus. With this in mind, Ti plasmid binary vectors expressing one or more coat protein genes for CuMV, zucchini yellow mosaic virus, and watermelon mosaic virus 2 were used to transform yellow crookneck squash (*Cucurbita pepo*) plants (Fig. 19.13). Transgenic plants that contained the coat protein genes from all three viruses were resistant to damage by all three viruses under laboratory conditions. Initially, transgenic plants expressing coat protein genes for zucchini yellow mosaic virus and watermelon mosaic virus 2 were tested under field conditions by using aphids, which are small insects that naturally transmit these viruses to developing plants. The transgenic plants that expressed both coat protein genes were completely resistant to infection when the two viruses were transmitted at the same time (Fig. 19.14). On the other hand, while transgenic plants expressing only one of the two viral coat proteins were

FIGURE 19.13 (A) A T-DNA construct with a neomycin phosphotransferase (NPT II) gene as a selectable marker, a β-glucuronidase (GUS) gene as a reporter gene, two copies of the coat protein gene from watermelon mosaic virus 2 (WMV 2), and the coat protein gene from CuMV. The left and right borders of the T-DNA are indicated by LB and RB, respectively. (B) Similar to panel A without CuMV and GUS, with one copy of WMV 2, and with the coat protein gene from zucchini yellow mosaic virus (ZYMV). (C) Same as panel B with the addition of CuMV. All of the genes in these constructs include both promoters and transcription terminator regions.



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Micro-RNAs. One approach to developing plants that are resistant to a range of different viruses might include engineering the plants to produce micro-RNAs (miRNAs) that interfere with viral replication by targeting the viral RNA (or the viral mRNA) for degradation. In a recent series of experiments, starting with a 273-nucleotide precursor of a naturally occurring plant miRNA, scientists used PCR to replace a small portion of the existing sequence so that the precursor could be processed to yield a 20- to 24-nucleotide-long miRNA that was complementary to viral RNA (Fig. 19.18). The newly synthesized artificial miRNA (amiRNA) became part of an RNA-induced silencing complex (see chapter 11) in which the viral RNA (mRNA) was specifically bound and cleaved. It is also possible to clone two or more different pre-amiRNAs in tandem—this was done using turnip yellow mosaic virus and turnip mosaic virus—so that plants transformed with this construct become resistant to two separate viruses. This approach can be made even more effective by targeting more than one portion of each viral RNA using several pre-amiRNAs. Despite its intriguing possibilities, this system still requires a considerable amount of development before it is shown to be effective under field conditions.

Herbicide Resistance

A significant fraction of global crop production is lost through weed infestation every year, despite the expenditure of $10 billion on more than 100 different chemical herbicides. In addition, many herbicides do not discriminate weeds from crop plants; others must be applied early, before the weeds

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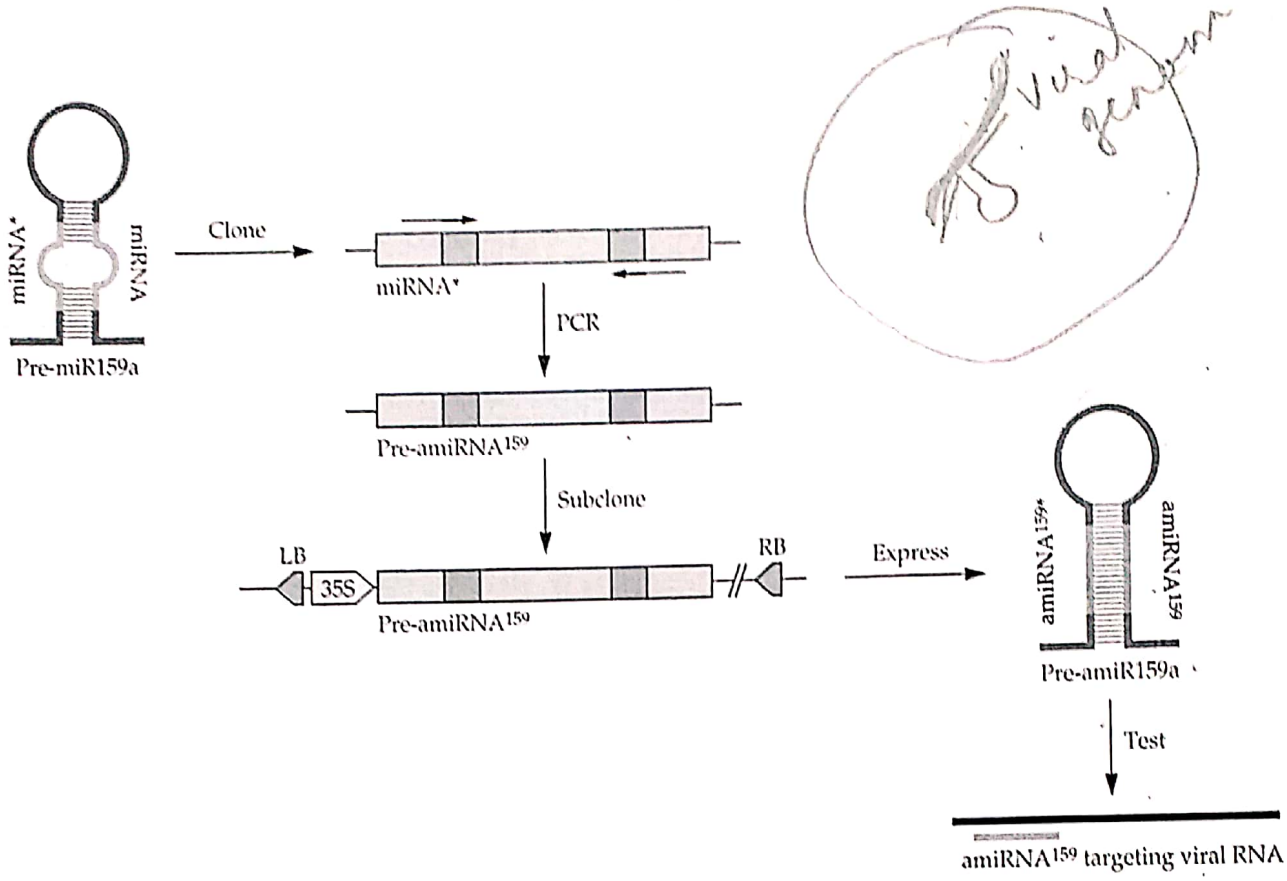

FIGURE 19.18 Use of amiRNAs to provide protection against viruses. The 273-nucleotide-long precursor (labeled pre-mR159a) of a naturally occurring miRNA, which is 20 to 24 nucleotides long, is cloned, and then PCR is used to alter a portion of the pre-miRNA sequence. The modified amiRNA sequence is cloned into a binary vector under the control of the 35S promoter, which is then used to transform *Arabidopsis* plants that now produce a pre-amiRNA that, when it is processed, targets a specific viral RNA for cleavage. Adapted from Niv et al., *Nat. Biotechnol.* 24:1420–1428, 2006.

take hold; and some persist in the environment. The creation of herbicide-resistant crop plants is one way to overcome some of these drawbacks.

A number of different biological manipulations that would cause a crop plant to be herbicide resistant can be envisioned.

1. Inhibit uptake of the herbicide.
2. Overproduce the herbicide-sensitive target protein so that enough of it remains available for cellular functions despite the presence of the herbicide.
3. Introduce a bacterial or fungal gene that produces a protein that is not sensitive to the herbicide but performs the same function as the plant (herbicide-sensitive) protein.
4. Reduce the ability of a herbicide-sensitive target protein to bind to a herbicide.
5. Endow plants with the capability to metabolically inactivate the herbicide.

A number of these strategies have been implemented to produce herbicide-resistant transgenic plants (Table 19.3). This approach has been so successful that more than 75% of the transgenic crops that are currently planted worldwide have been engineered to be herbicide resistant. By far, the most widely

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TABLE 19.3 Some examples of gene-based herbicide resistance

Herbicide(s)	Mode of development of herbicide resistance
Triazines	Resistance is due to an alteration in the *psbA* gene, which codes for the target of this herbicide, chloroplast protein D-1.
Sulfonylureas	Genes encoding resistant versions of the enzyme acetolactate synthetase have been introduced into poplar, canola, flax, and rice.
Imidazolinones	Strains with resistant versions of the enzyme acetolactate synthetase have been selected in tissue culture.
Aryloxphenoxypropionates, cyclohexanediones	These herbicides inhibit the enzyme acetyl coenzyme A carboxylase. Resistance, selected in tissue culture, is due either to an altered enzyme that is not herbicide sensitive or to the degradation of the herbicide.
Glyphosate	Resistance is from overproduction of EPSPS, the target of this herbicide. Resistance has been engineered by transforming soybean with the gene for a glyphosate-resistant EPSPS and tobacco with a glyphosate oxidoreductase gene, which encodes an enzyme that degrades glyphosate.
Bromoxynil	Resistance to this photosystem II inhibitor has been created by transforming tobacco and cotton plants with a bacterial nitrilase gene, which encodes an enzyme that degrades this herbicide.
Phenoxycarboxylic acids (e.g., 2,4-D and 2,4,5-T)	Resistant cotton and tobacco plants have been created by transformation with the *tfdA* gene from *Alcaligenes*, which encodes a dioxygenase that degrades this herbicide.
Glufosinate (phosphinothricin)	Over 20 different plants have been transformed with either the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *S. viridochromogenes*. The phosphinothricin acetyltransferase that these genes encode detoxifies this herbicide.
Cyanamide	Resistant tobacco plants were produced when a cyananide hydratase gene from the fungus *Myrothecium verrucaria* was introduced. The enzyme encoded by this gene converts cyanamide to urea.
Dalapon	Tobacco plants transformed with a dehalogenase gene from *Pseudomonas putida* can detoxify this herbicide.

used herbicide is glyphosate, which is considered to be safe, cheap, effective, and "environmentally friendly" because it is readily degraded to nontoxic compounds in the soil. Glyphosate, trademarked as Roundup by the Monsanto Corporation, inhibits a key enzyme in the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), that plays an important role in the synthesis of aromatic amino acids in both bacteria and plants. Plants resistant to this herbicide have been developed by putting an EPSPS-encoding gene from a glyphosate-resistant strain of *E. coli* under the control of plant promoter and transcription termination–polyadenylation sequences and cloning the construct into plant cells. Transgenic soybean,

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corn, canola, tobacco, petunia, tomato, potato, and cotton plants that produce an amount of the resistant *E. coli* EPSPS sufficient to replace the inhibited plant enzyme are resistant to the effects of glyphosate. Thus, in these cases, the crop plant would not be affected by glyphosate treatment, whereas the weeds would be. Crops that have been engineered to be resistant to glyphosate by this approach are said to be "Roundup ready."

Notwithstanding the many years of successful use of glyphosate and Roundup-ready plants, two important factors are now changing people's thinking about this approach. In the first instance, the herbicide patent has now expired and other companies are very actively pursuing the development of plants that are resistant to glyphosate using other approaches. Secondly, there is a realization that worldwide agriculture has become too dependent upon a single herbicide and that alternative strategies need to be developed.

To find an enzyme that can inactivate glyphosate, one group of researchers assayed a collection of several hundred *Bacillus* sp. strains for the ability to acetylate glyphosate (Fig. 19.19). The assay was based on the ability to measure *N*-acetylglyphosate in the supernatant of permeabilized cells (Fig. 19.20). The three strains (all *Bacillus licheniformis*) that had the highest level of glyphosate *N*-acetyltransferase activity were isolated, and

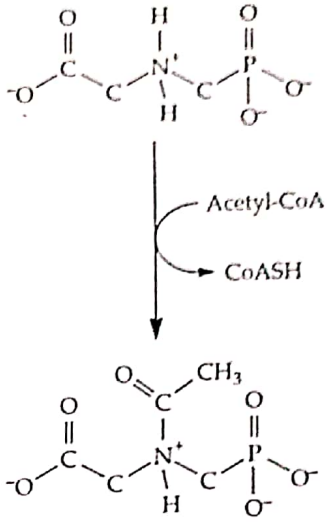
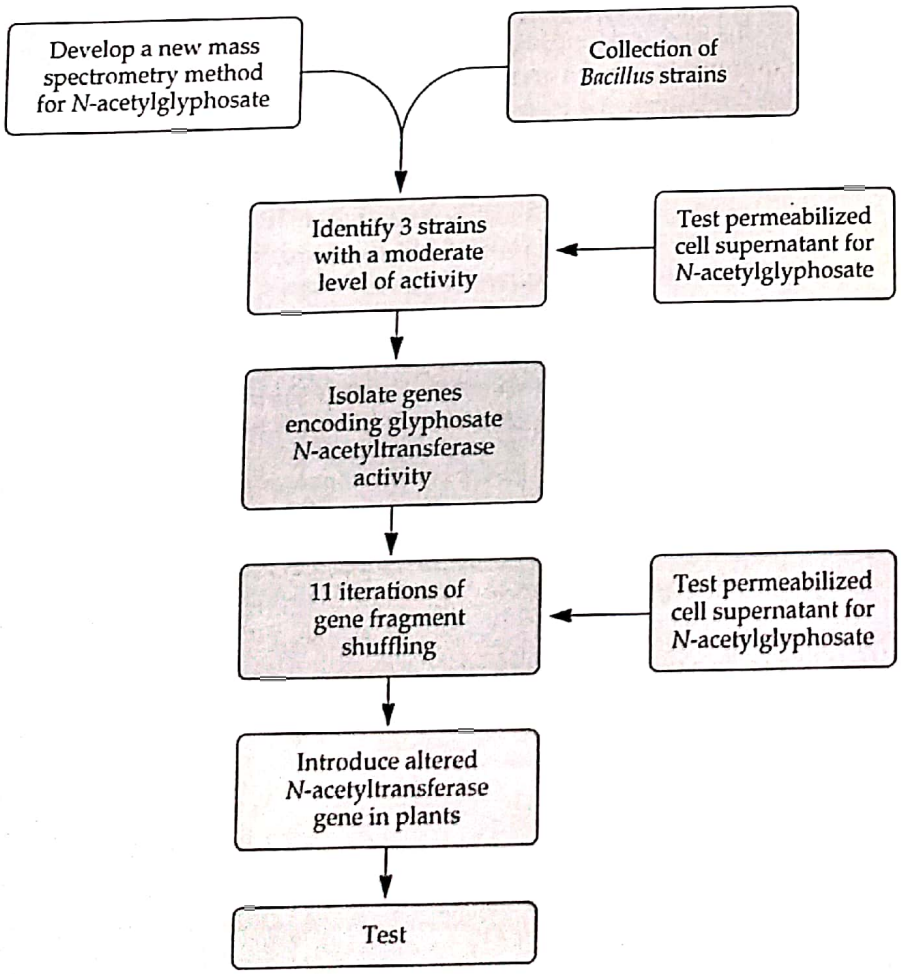


FIGURE 19.19 N-acetylation of the herbicide glyphosate by the bacterial enzyme glyphosate *N*-acetyltransferase. CoASH, coenzyme A.

FIGURE 19.20 Overview of a scheme to isolate a bacterial enzyme with a sufficient level of glyphosate *N*-acetyltransferase activity to allow it to be used to engineer plants so that they are resistant to high levels of glyphosate.



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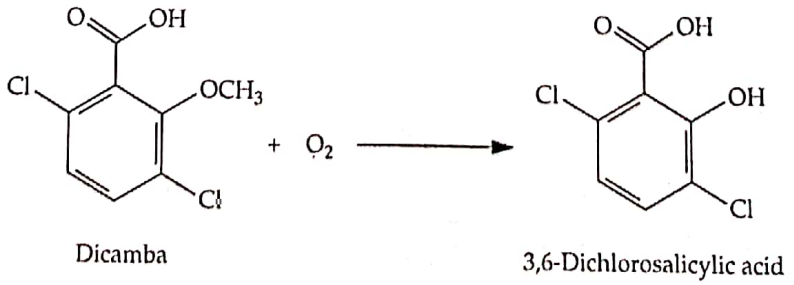
the enzymes were characterized. In all cases, these strains exhibited only a very low level of enzyme activity. Subsequently, the genes from each of the three selected strains encoding glyphosate *N*-acetyltransferase activity were isolated. These genes were then shuffled (see chapter 8) numerous times, each time selecting the strain with glyphosate *N*-acetyltransferase with the highest level of activity. In fact, following 11 iterations of DNA shuffling, the enzyme catalytic efficiency (see chapter 8) improved by nearly 10,000-fold. Interestingly, while the modified enzyme still functions as a glyphosate *N*-acetyltransferase, after so many rounds of modification, the amino acid sequence of the modified enzyme is only 76 to 79% identical to the amino acid sequences of the parental enzymes. Finally, the modified glyphosate *N*-acetyltransferase gene was introduced into *Arabidopsis*, tobacco, and corn plants. The transgenic plants, which expressed the enzyme in the plant cytosol and were both morphologically normal and fertile, were tolerant of approximately six times the dose of glyphosate that killed the parental nontransformed plants. This work is an important first step in developing plants that can act as an alternative to Roundup-ready plants. However, the efficacy of this approach remains to be proven in the field.

The herbicide dicamba has been used since the 1960s to control a wide range of broadleaf weeds. When it is applied to dicotyledonous plants, dicamba acts by mimicking the effects of high levels of the plant hormone indole-3-acetic acid and binding to indole-3-acetic acid receptors, which are essential for normal growth and development of the plant. The herbicide is widely used, relatively inexpensive, and environmentally friendly in that it does not persist in soils and has no toxicity to humans or other animals. Moreover, the widespread use of the herbicide has not led to the development of any dicamba-resistant weeds. Researchers have therefore sought to develop crop plants that are resistant to dicamba. To do this, a dicamba monooxygenase gene was expressed in *Arabidopsis*, tomato, and tobacco

FIGURE 19.21 **(A)** Conversion of dicamba to 3,6-dichlorosalicylic acid by dicamba monooxygenase. **(B)** The genetic construct used to express the dicamba monooxygenase gene within the chloroplasts of transgenic plants. The promoter was from peanut chlorotic streak virus, the enhancer was from tomato etch virus, the transit peptide was from the small subunit of pea ribulose 1,6-bisphosphate carboxylase, the diooxygenase gene was from the soil bacterium *P. maltophilia*, and the terminator sequence was from the small subunit of pea ribulose 1,6-bisphosphate carboxylase. Adapted from Behrens et al., *Science* 316:1185–1188, 2007.

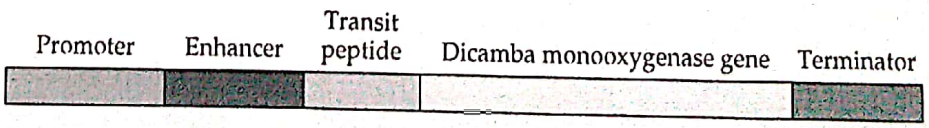
A



Dicamba

3,6-Dichlorosalicylic acid

B



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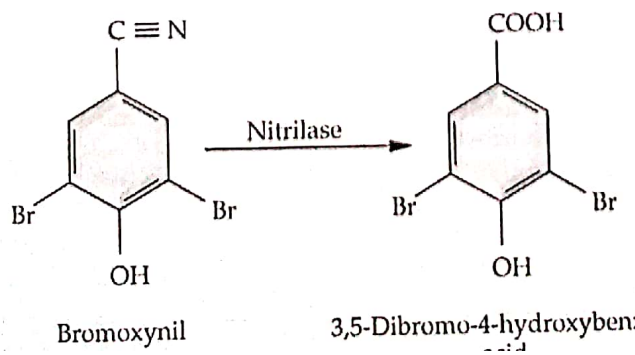
plants (all as test systems). The dicamba monooxygenase is part of the three-component enzyme dicamba *O*-demethylase, from the bacterium *Pseudomonas maltophilia*, that converts dicamba to 3,6-dichlorosalicylic acid, a compound without any appreciable herbicidal activity (Fig. 19.21). In transgenic plants, only dicamba monooxygenase is needed for the inactivation of the herbicide, since the enzyme can be targeted to the chloroplast, where there is a ready source of reduced ferredoxin (the product of the other two genes in the dicamba *O*-demethylase complex). The reduced ferredoxin supplies electrons for the monooxygenase reaction. As expected, transgenic plants expressing dicamba monooxygenase are resistant to high levels of the herbicide when grown in both the greenhouse and the field. It is speculated that it may be possible to "stack" plants with genes encoding both glyphosate and dicamba resistance so that farmers can either alternate the use of the two herbicides or else apply them at the same time. In this way, it is anticipated that unwanted weeds are unlikely to develop resistance to both herbicides, while the transgenic crop plant is uninhibited by the herbicide.

Resistance due to inactivation of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), a herbicide that acts by inhibiting photosynthesis, has been achieved for some plants. In this case, resistant plants were created by the introduction of a bacterial gene that encodes the enzyme nitrilase, which can inactivate bromoxynil before the herbicide can act (Fig. 19.22). The gene for nitrilase was isolated from the soil bacterium *Klebsiella ozaenae* and placed under the control of the light-regulated promoter from the small subunit of the enzyme ribulose bisphosphate carboxylase before it was transferred to tobacco plants. As expected, the transgenic plants expressed nitrilase activity in their shoots and leaves, but not in their roots, and were resistant to the toxic effects of the herbicide.

Fungus and Bacterium Resistance

Extensive damage and loss of crop productivity are caused by phytopathogenic fungi. It has been estimated that one fungal disease of one major crop, i.e., fungal rice blast, a disease that affects rice plants, costs farmers in Southeast Asia, Japan, and the Philippines more than $5 billion per year. At present, the major way of controlling the damage and losses to crop plants that result from fungal infection is through the use of chemical agents that may persist and accumulate in the environment and that are subsequently hazardous to animals or humans. It would therefore be beneficial if a

FIGURE 19.22 Detoxification of the herbicide bromoxynil by the enzyme nitrilase from *K. ozaenae*.



Bromoxynil 3,5-Dibromo-4-hydroxybenzoic

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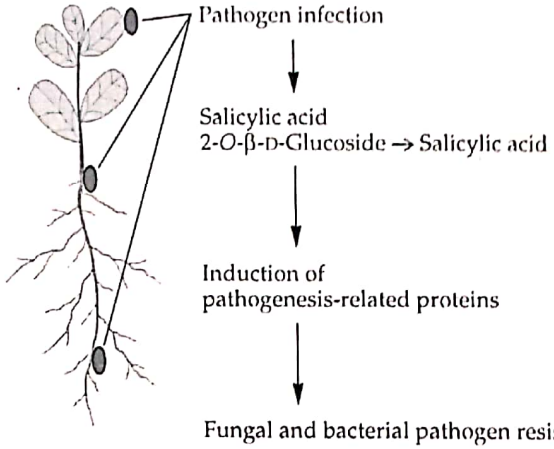
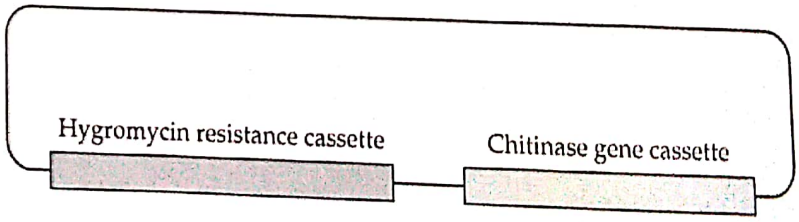


FIGURE 19.23 Overview of systemic acquired resistance in plants. After infection of a plant by a pathogenic fungus or bacterium (shown in blue), the inactive storage compound salicylic acid 2-*O*-β-D-glucoside is converted to salicylic acid and/or salicylic acid is synthesized. The salicylic acid activates or induces the *NPR1* gene, whose protein product acts as a "master" regulatory protein to turn on the expression of the PR proteins, which have enzyme activities directed against various pathogenic organisms.

simple, inexpensive, effective, and environmentally friendly nonchemical means of preventing fungal damage to crop plants could be found.

Plants often respond to fungal or bacterial pathogen invasion or other environmental stresses by converting a conjugated storage form of salicylic acid (salicylic acid 2-*O*-β-D-glycoside) to salicylic acid, which induces a broad systemic defense response in the plant. This "systemic acquired resistance" to pathogens extends to plant tissues that are far from the site of the initial infection and may last for weeks to months. It results from the synthesis of a group of proteins called pathogenesis-related (PR) proteins (Fig. 19.23). The PR proteins include β-1,3-glucanases, chitinases, thaumatin-like proteins (thaumatin is a small, very sweet protein), and protease inhibitors that protect the plant-invading pathogens. To develop plants resistant to fungal pathogens, researchers have attempted to utilize parts of the systemic acquired resistance system. For example, transgenic plants that constitutively express high levels of one or more PR proteins, such as chitinase, which can hydrolyze the β-1,4 linkages of the *N*-acetyl-D-

FIGURE 19.24 Plasmid vector containing a rice chitinase gene cassette and a hygromycin resistance gene cassette (in both cases including transcriptional regulatory sequences) used to transform rice protoplasts. Rice cell protoplasts were transformed by polyethylene glycol treatment in the presence of this plasmid. Transformed cells were selected for their resistance to hygromycin. Later, they were tested for the presence of chitinase genes by Southern hybridization and for chitinase by Western blot analysis; then, they were used to regenerate transgenic plants.



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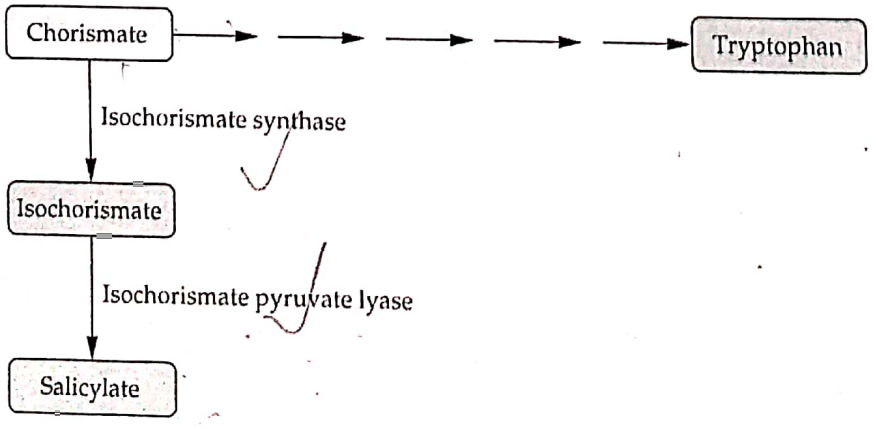
glucosamine polymer chitin, a major component of many fungal cell walls, have been engineered (Fig. 19.24).

The *NPR1* gene from the plant *A. thaliana* encodes a "master" regulatory protein that controls the expression of the PR proteins, and it can be activated or induced by the addition of salicylic acid. In *A. thaliana*, overexpression of the *NPR1* gene can lead to the generation of broad-spectrum disease resistance against both fungal and bacterial pathogens. Moreover, scientists have observed that overproduction of this "master switch" is an effective strategy in several plants other than *A. thaliana*, including rice, sugar beet, apple, and corn.

Another approach to engineering plants with broad-spectrum disease resistance involves overproducing salicylic acid. Theoretically, this can be done by transforming plants with bacterial genes that encode the enzymes isochorismate synthase and isochorismate pyruvate lyase, which catalyze salicylate synthesis (Fig. 19.25). Salicylate is synthesized from chorismate, which is produced in large amounts in the chloroplast and is also an intermediate in the biosynthesis of the amino acid tryptophan. The two bacterial genes for salicylate synthesis were fused to chloroplast-targeting sequences from the gene for the small subunit of ribulose bisphosphate carboxylase—the small subunit of ribulose bisphosphate carboxylase is encoded within the nuclear DNA, but following its synthesis, this protein is transmitted to the chloroplast (Fig. 19.26). The result of this genetic manipulation was that when both of these enzymes were localized in the (tobacco) plant chloroplast, salicylic acid was produced constitutively. Consequently, the plants constitutively expressed a number of PR proteins. The plants appeared normal but exhibited enhanced resistance to both viral and fungal pathogens. Since it is not necessarily advantageous to the plant to constitutively express PR proteins, there is some question as to how effective this strategy of conferring protection against a broad range of pathogens will be in the field.

Transgenic plants that have been engineered to constitutively express chitinase under the control of the cauliflower mosaic virus 35S promoter include rice, tobacco, and canola. Transgenic plants that expressed chitinase were more resistant to damage by fungal pathogens than control plants, even though the control plants synthesized their own PR proteins in response to the fungal infection. Presumably, this resistance reflects the higher level of chitinase expressed by the transgenic plants than by non-

FIGURE 19.25 Use of bacterial enzymes to convert plant chloroplast chorismate to salicylate.



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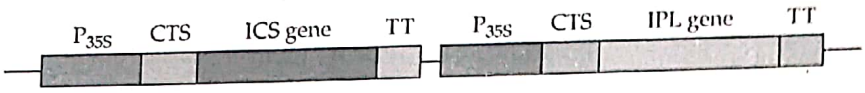
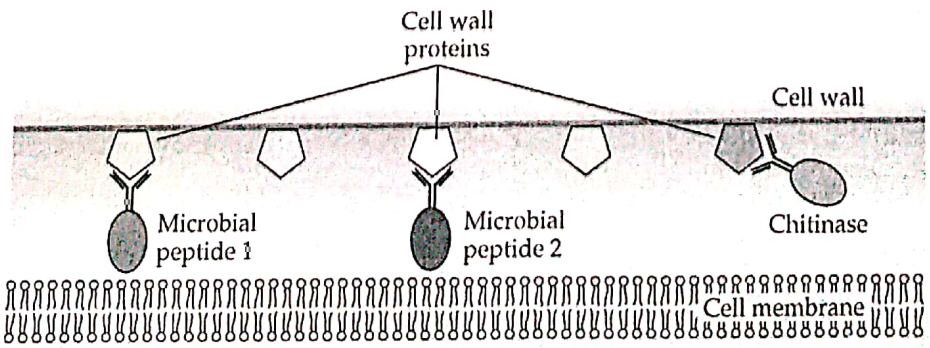


FIGURE 19.26 Construct used to transform plants so that they constitutively overproduce salicylate. P_{35S}, the 35S promoter from cauliflower mosaic virus; CTS, chloroplast targeting sequence; ICS, isochorismate synthase; TT, transcription termination region; IPL, isochorismate pyruvate lyase.

transformed plants. In addition, while transgenic plants constitutively expressing chitinase were resistant to fungal pathogens, binding of the beneficial root fungus *Glomus mosseae* to the plant roots was not affected. This was probably a consequence of a difference in the cell wall compositions of different fungi. Importantly, a transgenic plant constitutively expressing chitinase has been found to be effective at resisting fungal damage under field conditions. In a variation on the strategy described above, a cDNA encoding chitinase from the biocontrol fungus *Trichoderma harzianum* was isolated and introduced, under the control of the 35S promoter, into tobacco and potato plants. As expected, these transgenic plants were resistant to both soil-borne fungal pathogens (primarily affecting roots) and foliar fungal pathogens (primarily affecting shoots and leaves). In sum, the overexpression of some PR-like proteins, such as chitinase, appears to be an effective strategy for protecting plants against damage from pathogenic fungi.

Pathogenic fungi belonging to the genus *Fusarium* are the causative agents of some of the most costly and devastating plant diseases in the world. Therefore, a strategy that targeted various *Fusarium* spp. would be quite important for agriculture worldwide. A number of different antimicrobial peptides (which can disrupt the cell membrane) and the enzyme chitinase (mentioned above) are inhibitory to the growth of *Fusarium* spp. However, these biological approaches (regardless of how they are administered) are not as effective as spraying plants with chemical fungicides. To overcome this limitation, workers have fused the genes (cDNAs) for two different antimicrobial peptides (one from the radish *Raphanus sativus* and one from the mold *Aspergillus giganteus*) and a chitinase (from wheat) to a single-chain Fv antibody that binds to a *Fusarium* cell wall protein (Fig. 19.27). Although the single-chain Fv antibody was originally selected from a library constructed from chickens that had been immunized with

FIGURE 19.27 Schematic representation of three different anti-fungal fusion proteins. Each protein includes a single-chain Fv antibody that binds to a *Fusarium* sp. cell wall protein and an antifungal peptide/protein directed against either the cell membrane or the chitin component of the cell wall. Adapted from Bohlmann, *Nat. Biotechnol.* 22:682–683, 2004.



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Fusarium graminearum, the selected antibody cross-reacted with cell wall antigens from nine different species and subspecies of *Fusarium*. Constructs encoding the three fusion proteins were used to transform *A. thaliana*, and the transgenic plants were tested for resistance to infection and growth inhibition by *Fusarium oxysporum*. When either the selected single-chain Fv antibody or any one of the three antipathogenic peptides/proteins was expressed in transgenic plants, it endowed the plants with a low to moderate level of resistance to damage by the pathogen (Table 19.4). However, all three of the antibody-peptide/protein fusions conferred a high level of resistance to the pathogen, suggesting, in each case, that the two components of the fusion protein were acting synergistically. This is an interesting and potentially quite useful approach that merits further development.

The annual worldwide losses to farmers from potato diseases caused by the pathogenic soil bacterium *Erwinia carotovora* are approximately $100 million. Moreover, potato breeders have not identified any resistance traits that can be bred into commercial cultivars. To address this problem, transgenic potato plants that actively express bacteriophage T4 lysozyme were developed. The lysozyme was targeted for secretion into the apoplast (the intercellular spaces inside the plant but outside the plant cells) in potato plants, since this is the part of the plant where *E. carotovora* enters and spreads. More specifically, the T4 lysozyme gene was fused to the barley α-amylase signal peptide coding sequence and placed under the transcriptional control of the cauliflower mosaic virus 35S promoter, transcription terminator, and polyadenylation site. Although the T4 lysozyme gene was under the control of this strong promoter, only a very low level of lysozyme was synthesized, perhaps reflecting differences in codon usage between a bacteriophage gene and the potato genome. This result notwithstanding, under laboratory and greenhouse conditions, transgenic plants with this construct were significantly protected from damage by high levels of *E. carotovora*. Since much lower levels of the pathogen than were used in these laboratory experiments are present in the field, this type of genetic construct should provide a high level of protection under natural conditions. To avoid killing plant-beneficial bacteria in the vicinity of the roots, researchers have employed hen egg lysozyme instead of T4 lysozyme because it is more specific for various phytopathogenic *Erwinia* spp. Moreover, researchers have found this strategy to be useful for protecting

TABLE 19.4 Resistance of transgenic *A. thaliana* to the phytopathogen *F. oxysporum*

Transgene	Disease index (%)
None (wild type)	100
Fv antibody	55
Peptide 1	50
Peptide 2	60
Chitinase	52
Antibody–peptide 1 fusion	10
Antibody–peptide 2 fusion	0
Antibody–chitinase fusion	5

Adapted from Peschen et al., *Nat. Biotechnol.* 22:732–738, 2004.

The extent of disease was assessed 2 weeks after infection with *F. oxysporum*. A disease index of 100% indicates that all of the plants are dead, 50% indicates that the average plant has disease symptoms but is alive, and 0% indicates that all plants are disease free.

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many different plants, including potato, rice, tomato, and tobacco, from various bacterial pathogens. Finally, in addressing the concern that proteins that are present in the apoplast of root cells may be exuded from the roots (see chapter 18) and kill plant growth-promoting bacteria, as well as pathogenic bacteria, researchers have noted that the endogenous rhizosphere microbial community (containing many beneficial bacteria) was essentially unchanged when a lysozyme transgene was expressed. Despite this apparent success, it remains to be determined whether this sort of genetic manipulation, which functions well in the laboratory, will be useful in the field.

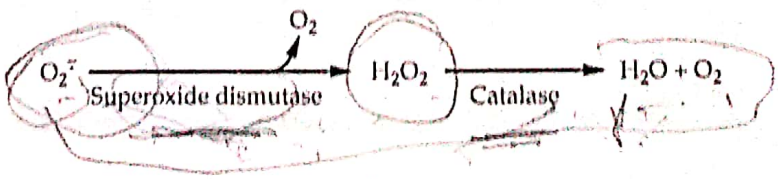
Oxidative Stress

Unlike many animals, plants cannot physically avoid adverse environmental conditions, such as high levels of light, ultraviolet (UV) irradiation, heat, high salt concentrations, or drought, so physiological strategies have evolved to cope with these stresses. At the molecular level, one of the undesirable consequences of physiological stress is the production of oxygen radicals. Thus, investigators reasoned that if they could create plants that were able to tolerate increased levels of oxygen radicals, these plants should also be able to withstand various forms of environmental stress.

A variety of biotic stresses, including salt, freezing, and drought, as well as exposure to pollutants, stimulate the formation of reactive oxygen species in plant cells. These toxic molecules damage membranes, membrane-bound structures, and macromolecules, including proteins and nucleic acids, especially in the mitochondria and chloroplast, resulting in oxidative stress. A common type of potentially damaging oxygen radical is the superoxide anion. Within a cell under oxidative stress, the enzyme superoxide dismutase detoxifies superoxide anion by converting it to hydrogen peroxide, which in turn is broken down to water by various cellular peroxidases or catalases (Fig. 19.28). In one study, tobacco plants that were transformed with a superoxide dismutase gene that was under the control of the 35S promoter from cauliflower mosaic virus had reduced oxygen radical damage under stress conditions compared with control plants.

Plants have several different isoforms of the enzyme superoxide dismutase. The Cu/Zn superoxide dismutases are found primarily in chloroplasts and to a lesser extent in the cytosol. The Mn superoxide dismutase is located in the mitochondria, and some plants also have an Fe form of superoxide dismutase. Transgenic tobacco plants that carried the cDNA for a chloroplast-localized Cu/Zn superoxide dismutase under the control of the 35S promoter from cauliflower mosaic virus were much more resistant to high-light damage than nontransformed plants. When they were tested, the transgenic plants retained 94% of their photosynthetic activity under

FIGURE 19.28 Conversion of superoxide anion to hydrogen peroxide and then to water and oxygen.



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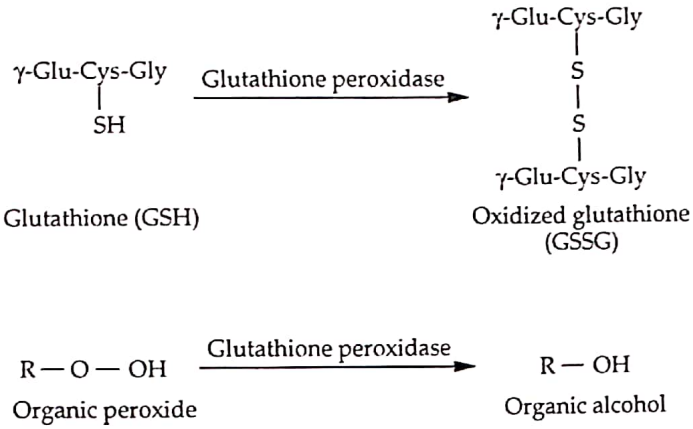

FIGURE 19.29 Oxidation of glutathione to oxidized glutathione and simultaneous reduction of an organic peroxide to an organic alcohol, catalyzed by glutathione peroxidase. γ-Glu, glutamic acid residue linked to the next amino acid through the gamma carboxyl group rather than through the alpha amino group, as in a usual peptide linkage; Cys, cysteine; Gly, glycine.

conditions in which nontransformed plants lost all of their activity. In another experiment, transgenic plants with cloned Mn superoxide dismutase targeted to their chloroplasts were three- to fourfold less sensitive to oxidative damage caused by ozone than nontransformed plants.

Oxidative stress may also be reduced if the level of oxidized glutathione within a plant is increased. Glutathione peroxidase catalyzes the conversion of glutathione to oxidized glutathione by reacting with organic peroxides and reducing them to organic alcohols (Fig. 19.29). To test this idea, a tobacco cDNA encoding an enzyme with both glutathione *S*-transferase and glutathione peroxidase activities was isolated. Transgenic tobacco plants that expressed glutathione peroxidase were created using the isolated cDNA under the control of the 35S promoter, and the construct was introduced into plants with a binary Ti plasmid system. The transformed plants had approximately twice the level of enzyme activity found in nontransformed plants. Seedlings of these transgenic plants grew significantly faster than control seedlings when exposed to either chilling or salt stress. The efficacy of this system remains to be demonstrated in the field.

Salt and Drought Stress

Many plants live in environments where growth is severely impaired by either drought or high salinity. With increasing dependence on irrigation in agriculture and more frequent salting of icy and snowy roads in the winter, increased soil salinity has become a common problem worldwide. Approximately one-third of the world's irrigated land has become unsuitable for growing crops because of contamination with high levels of salt. Irrigation typically increases the amount of salt present in soil. To survive under these conditions, many plants synthesize low-molecular-weight nontoxic compounds collectively called osmoprotectants. These compounds facilitate both water uptake and retention and also protect and stabilize cellular macromolecules from damage by high salt levels. Some well-known osmoprotectants are sugars, alcohols, the amino acid proline,

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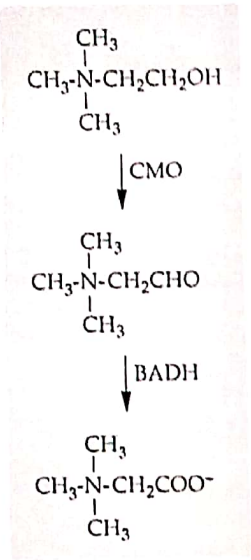


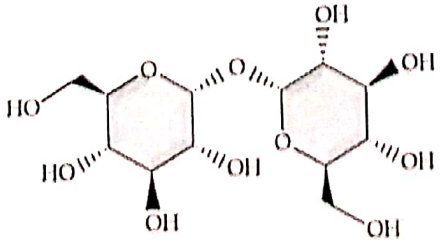
FIGURE 19.30 Conversion of choline to glycine betaine. CMO, choline monooxygenase; BADH, betaine aldehyde dehydrogenase.

and quaternary ammonium compounds. To create more salt-tolerant plants, scientists have tried to engineer an increase in the cellular accumulation of the following osmoprotectants: trehalose, proline, D-ononitol, mannitol, sorbitol, glycine betaine, and 3-dimethylsulfoniopropionate.

The quaternary ammonium compound betaine is a highly effective osmolyte that accumulates in some plants during periods of water stress or high salinity. However, several important crops, including potatoes, rice, and tomatoes, do not accumulate betaine. Thus, the introduction of betaine-biosynthetic enzymes into these plants might enable them to withstand water stress and/or high salinity. Betaine is synthesized from choline in two steps in both plants and bacteria (Fig. 19.30). In plants, such as spinach, choline is converted to betaine aldehyde by the enzyme choline monooxygenase and then to betaine by betaine aldehyde dehydrogenase. In bacteria, such as *E. coli*, both steps of betaine biosynthesis are catalyzed by the same enzyme, choline dehydrogenase. To create a more salt-tolerant tobacco, plant cells were transformed with a Ti plasmid vector carrying the *E. coli betA* gene, which encodes choline dehydrogenase, under the control of the cauliflower mosaic virus 35S promoter. In laboratory tests, tobacco plants expressing this gene were up to 80% more tolerant of a high (300 mM) salt concentration than were nontransformed tobacco plants. While it may be possible to improve the osmoprotection afforded by the *E. coli betA* gene by using a plant tissue-specific promoter to direct the expression of the gene, this experiment is an important step in the development of plants that are more tolerant of high levels of salt.

It is also possible to increase the trehalose (Fig. 19.31) concentration in plants (where trehalose is a natural alpha-linked disaccharide formed by an α or α-1 bond between two α-glucose units) and thereby protect the plants against inhibition by high levels of salt in the soil. To do this, rice plants were transformed, using a binary vector, with one of two different DNA constructs (Fig. 19.32). In *E. coli*, trehalose-6-phosphate is first formed from uridine diphosphate (UDP)-glucose and glucose-6-phosphate, and then the trehalose-6-phosphate is converted to trehalose. A fusion of the genes encoding the two enzymes that normally catalyze the two steps in the biosynthesis of trehalose in *E. coli* was constructed so that a single protein contained both activities. This simplifies the transformation of plants in that only one target gene needs to be introduced and ensures that the two enzyme activities necessary for the synthesis of trehalose are present at identical levels. In one genetic construct, the fusion protein gene is under the transcriptional control of an abscisic acid-inducible promoter and is expressed in the cytosol. In the other construct, it is under the control of the promoter for the small subunit of ribulose bisphosphate carboxylase, and the fusion protein is expressed in plant chloroplasts. In transgenic rice

FIGURE 19.31 Structure of trehalose.



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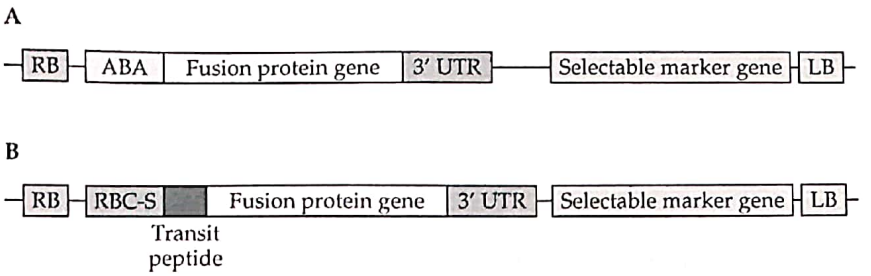

FIGURE 19.32 Two genetic constructs used to engineer rice plants to synthesize trehalose to protect them against growth inhibition by high salt levels. The fusion protein is under the control of an abscisic acid (ABA)-inducible promoter where ABA synthesis increases in the presence of salt **(A)** or the promoter from the small subunit of ribulose bisphosphate carboxylase **(B)**. In panel B, the transit peptide facilitates the localization of the fusion protein in the chloroplasts. RB, right border; LB, left border; UTR, untranslated region.

plants that contain either DNA construct, the level of trehalose is 3 to 10 times higher than in nontransformed rice plants in the presence of salt. Moreover, the biomass of the transgenic plants is four to six times that of the nontransformed plants in the presence of salt. Thus, by increasing the amount of trehalose that a plant synthesizes, the plant acquires increased tolerance for moderate levels of salt in the environment.

Researchers have engineered the plant *A. thaliana* to be salt tolerant by sequestering sodium ions in the large intracellular vacuole (Fig. 19.33). The strategy consisted of overproducing the endogenous *A. thaliana* gene encoding an Na^+/H^+ antiport protein. The Na^+/H^+ antiport protein transports Na^+ into the vacuole using the electrochemical gradient of protons generated by vacuolar H^+-translocating enzymes. When tested, the transgenic plants that overproduced the Na^+/H^+ antiport protein thrived in soil that was watered with a solution of 200 mM salt. This approach to the manipulation of salt stress in plants is effective with corn, canola, cotton, rice, tobacco, and tomato plants, as well as with *A. thaliana*. In transgenic tomato plants, the salt is localized in the leaves, and therefore, the transgenic tomato fruits do not accumulate salt and are quite normal in all respects, including taste. In addition to Na^+ toxicity, plants that live in saline environments have to contend with water loss caused by osmotic stress. By concentrating the salt in the plant's large vacuole, water that is free of salt should be driven into the plant cells, resulting in plants that use water more efficiently. This system has been quite successful in greenhouse trials and in the limited number of field trials where it has been tested. It provides researchers with the potential, especially in combination with other approaches, such as the overproduction of certain osmolytes, to engineer a wide range of salt-tolerant crop plants that can be grown on marginal land or possibly watered with seawater or other salt-containing water.

Many of the strategies that have been used to engineer plants to become more salt tolerant are also effective at making the plant drought tolerant; however, some strategies are specific for one stress or the other. In fact, a very large number of different genes have been employed in attempts to create drought-tolerant transgenic plants. These approaches have included introducing genes encoding overproduction of various osmolytes (e.g., trehalose, proline, glycine betaine, and polyamines), plant stress proteins (e.g., chaperones and heat shock proteins), reactive-oxygen-scavenging proteins

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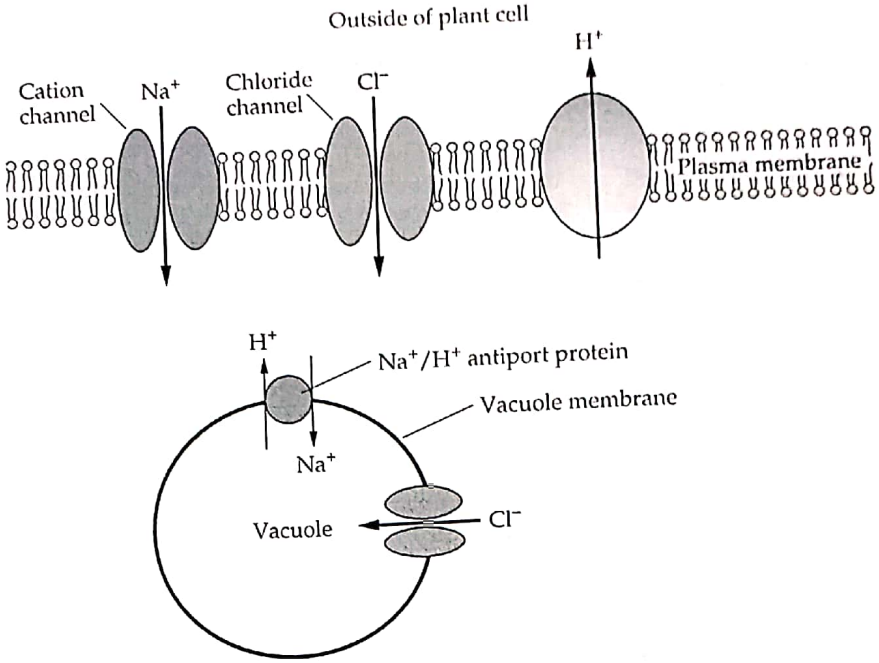

FIGURE 19.33 Schematic representation of ion transport in the plant *A. thaliana* showing the Na^+ ions being sequestered in the large vacuole.

(e.g., superoxide dismutases), hormone biosynthesis and catabolism proteins (e.g., affecting cellular levels of abscisic acid, cytokinin, and ethylene), transcription factors that turn on the synthesis of a host of other proteins, and signaling proteins that activate the synthesis of other proteins.

One group of researchers reasoned that in order to increase the tolerance of plants for drought, it is necessary to delay the onset of drought-induced senescence during the drought episode. Moreover, by suppressing this response, plants would be more likely to resume normal growth when water became available. Of course, it is necessary to keep in mind that regardless of their genetic makeup, plants cannot exist for indefinite periods in the absence of water. Prior to this work, it had been observed that leaf senescence could be delayed in transgenic plants expressing a foreign gene encoding isopentenyltransferase, an enzyme that catalyzes the rate-limiting step in cytokinin biosynthesis. Therefore, tobacco plants were transformed to express isopentenyltransferase under the control of a *SARK* (senescence-associated protein kinase gene) promoter (Fig. 19.34). This regulatable promoter is induced during late maturation and decreased during the development of senescence. When these transgenic tobacco plants were watered with only 30% of the amount of water used under normal conditions, the suppression of leaf senescence resulted in a four- to fivefold-higher level of biomass in the transgenic versus the nontransformed plants. This result suggests that it may be possible to get irrigated crops to grow normally with only one-third the amount of water that is usually used.

Fruit Ripening and Flower Wilting

A major problem in fruit marketing is premature ripening and softening during transport. These changes are part of the natural aging (senescence)

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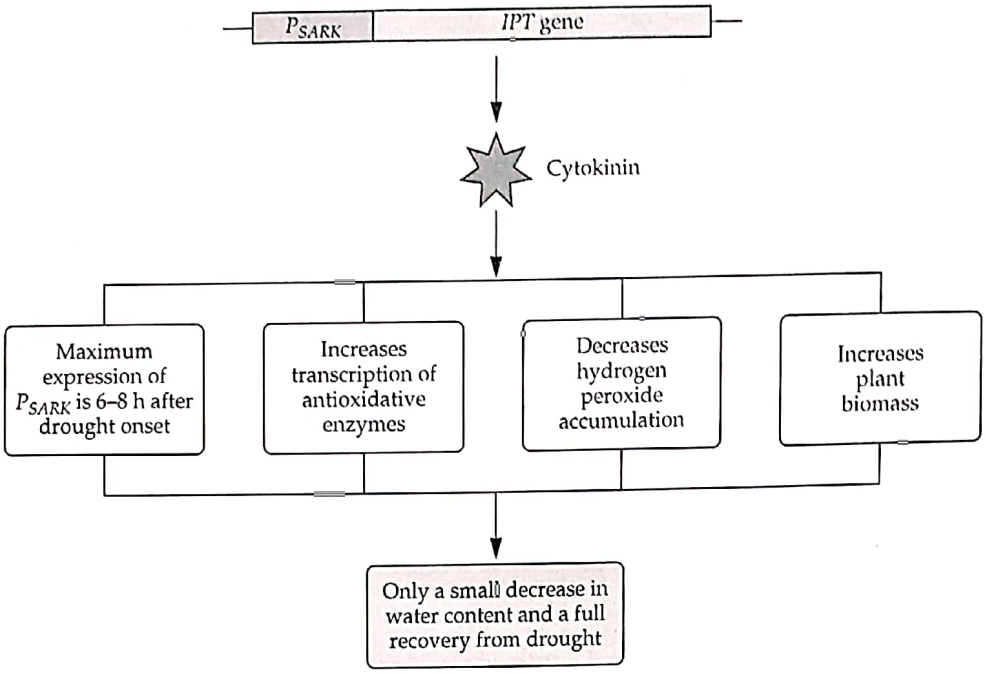

FIGURE 19.34 Overview of how overexpressing cytokinin protects plants against drought. P_{SARK} is a senescence-induced promoter. The *IPT* gene encodes isopentenyltransferase, which catalyzes the rate-limiting step in cytokinin biosynthesis.

process of the fruit. Some of the genes that are induced during ripening encode the enzymes cellulase and polygalacturonase. It has been postulated that by interfering with the expression of one or more of these genes, the ripening process might be delayed. This interference could be achieved by creating transgenic plants with antisense or sense (cosuppression) RNA-producing versions of these genes. In fact, when an antisense RNA-producing gene for polygalacturonase was introduced into tomato plants, a $1.3 billion-a-year crop in the United States, both polygalacturonase mRNA and enzymatic activity were reduced by 90%. The lowering of polygalacturonase production inhibited fruit ripening in tomatoes, permitting the tomatoes to ripen on the vine instead of being harvested while they were still green. These tomatoes were claimed to have a long shelf life while retaining the flavor of the tomato. This genetically engineered tomato is known as the Flavr Savr (pronounced "flavor saver") tomato. On 18 May 1994, the U.S. Food and Drug Administration ruled that the Flavr Savr tomato was as safe for human consumption as tomatoes that were bred by conventional means, and because Flavr Savr tomatoes were essentially the same as other tomatoes, special labeling was not required.

The plant growth regulator ethylene induces the expression of a number of genes that are involved in fruit ripening and senescence and in flower wilting. It is synthesized from methionine by way of the intermediate compounds *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC) (Fig. 19.35). Treatment of plants with chemical compounds that block ethylene production delays fruit ripening, senescence, and flower wilting. Thus, premature fruit ripening and flower wilting might be prevented by inhibiting the synthesis of ethylene. This can be achieved by blocking several different steps in the ethylene biosynthesis pathway (Fig. 19.35). For

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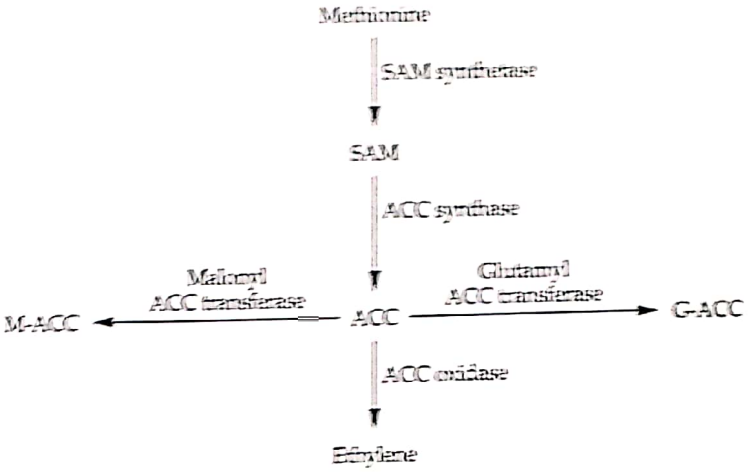
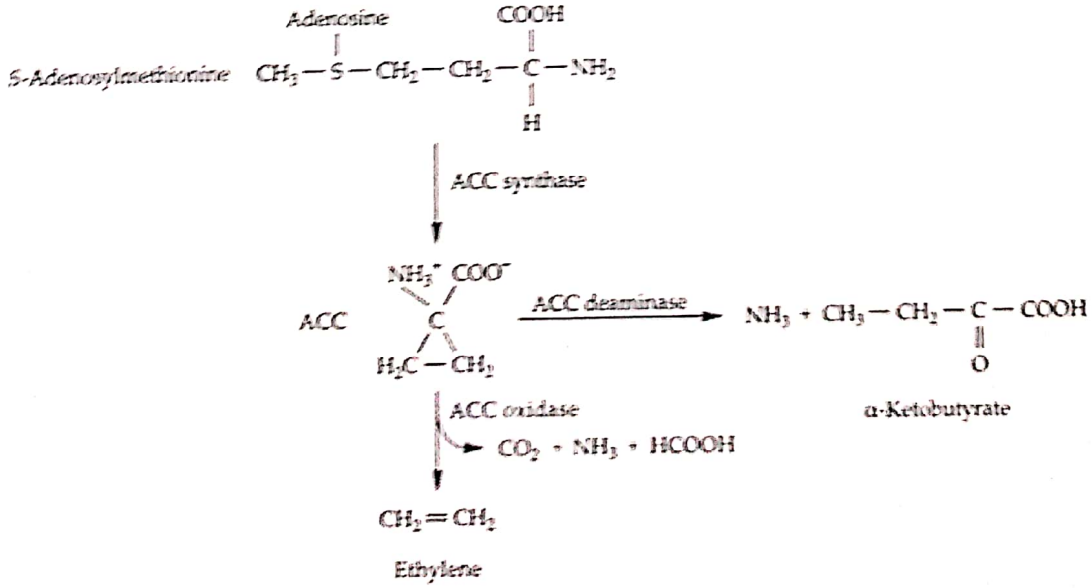


FIGURE 19.35 Centrality of ACC in the synthesis of ethylene. M-ACC, 1-(malonylamino)-cyclopropane-1-carboxylic acid; G-ACC, 1-(γ-1-glutamylamino) cyclopropane-1-carboxylic acid.

example, transgenic plants that have been engineered to contain antisense RNA versions of S-adenosylmethionine synthetase, ACC synthase, or ACC oxidase have much lower than normal levels of ethylene. Similarly, the amount of ACC, and hence the amount of ethylene, that can be synthesized may be decreased by increasing the activity of either malonyl ACC transferase or glutamyl ACC transferase, thereby converting ACC into one of these dead-end storage compounds (Fig. 19.35), or by transforming plants

FIGURE 19.36 Inhibition of ethylene biosynthesis by genetic manipulation. Normally, ACC is synthesized from S-adenosylmethionine by the enzyme ACC synthase, and then ACC oxidase converts ACC to ethylene. Ethylene synthesis may be inhibited in transgenic plants either by an antisense mRNA version of ACC synthase; by ACC oxidase, which inhibits the synthesis of these enzymes; or by the enzyme ACC deaminase, which competes with ACC oxidase for the available ACC, producing ammonia and α-ketobutyrate rather than ethylene.



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with the bacterial enzyme ACC deaminase, which converts ACC to α-ketobutyrate (Fig. 19.36). Each of these genetic manipulations results in a decreased level of ethylene and thereby extends the storage life of fruit and flowers.

By screening a large number of soil bacteria for the ability to utilize ACC as a sole source of nitrogen, strains that degrade ACC were identified. From one of these strains, the gene for the enzyme ACC deaminase was isolated based on the ability of transformed *E. coli* strains that expressed this gene to grow on minimal medium containing ACC. This gene was subcloned, put under the control of the 35S promoter from cauliflower mosaic virus, and expressed in tomato plants. The transgenic plants synthesized a much lower level of ethylene than did normal plants, and the fruit of the transgenic plants had a significantly longer shelf life. These bioengineered changes result in fewer losses due to spoilage because of the much lower levels of ethylene. Similar results have been observed with transgenic cantaloupes that have lowered ethylene levels. This strategy to delay fruit ripening is effective with a range of different fruits.

In another experiment, researchers isolated an 850-base-pair (bp) DNA fragment that corresponded to a portion of the cDNA for ACC oxidase from the tropical plant torenia (*Torenia fournieri* Lind.)—the complete cDNA is about 1 kilobase pair. The cDNA fragment was cloned in both the sense and antisense orientations into a binary vector and then used to transform torenia. In wild-type plants, the flowers lasted an average of 2.0 days before they wilted; transgenic plants with the ACC oxidase cDNA fragment in the antisense orientation lasted 2.7 days—a small but significant difference—and transgenic plants with the ACC oxidase cDNA fragment in the sense orientation lasted around 4.4 days. With both types of transgenic plants, not only did the flowers last longer, but also more flowers bloomed per stem than with the wild-type plant, yielding a more aesthetically pleasing plant.

In the future, a large number of plants will be engineered to have lower ethylene levels, primarily so that fruit ripening and flower wilting, or abscission, are inhibited. Fruits that are likely to be the targets of such genetic manipulation include melons, pineapples, and bananas; targeted flowers might include roses, carnations, tulips, chrysanthemums, and orchids.

SUMMARY

By using a variety of techniques (see chapter 18), it has become relatively straightforward to transform plants with foreign genes. Plants have been engineered to be resistant to a range of environmental stresses, including insects, viruses, herbicides, pathogens, and oxidative and salt stress.

Several different strategies have been used to confer resistance against insect predators, including introducing a gene encoding an insecticidal protoxin produced by one of several subspecies of *B. thuringiensis;* plant proteins, such as α-amylase inhibitors, lectins, or protease inhibitors; or other bacterial insecticidal proteins.

Transgenic plants expressing the gene for a viral coat protein are protected against infection by that virus. They may also be protected against damage from infective viruses by expression of other genes, such as an *E. coli* gene for RNase III, pokeweed antiviral proteins, and single-chain antibodies directed against various viruses.

To permit crop plants to proliferate in the presence of weeds, many plants have been engineered to be resistant to one or more "environmentally friendly" herbicides. This approach has become enormously successful and is the basis for the largest number of transgenic plants that are used in the field.

To develop plants resistant to fungal and bacterial pathogens, several approaches have been tested. For example, transgenic plants have been engineered to express high levels of chitinase or lysozyme or to overproduce PR proteins.

Different foreign proteins protect plants against different stresses. Superoxide dismutase and oxidized glutathione protect plants against oxidative stress, betaine overproduction

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