GENES FOR CROP IMPROVEMENT

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INTRODUCTION

Between 1987 and 1991, the United States Department of Agriculture received well over 100 proposals for field trials of transgenic plants. The majority of trials were designed to test for enhanced resistance to biotic and abiotic stresses (Table 1). Other trials were concerned with alterations in product quality: delayed fruit ripening, modified seed proteins, increased carbohydrate content of tubers, increased sterol content, modification of oil content to reduce rancidity, and alteration of flower pigmentation. The common theme was crop improvement and the desired phenotype was conferred by transformation, usually with a single foreign gene.

Plant breeders depend on variation as the raw material for crop improvement. The three main sources of variation for conventional plant breeding are: (a) segregation and recombination following hybridization, (b) chemical or physical mutagenesis, and (c) germplasm collections of related wild varieties and species. The advent of transformation gives plant breeders access to a new and broader gene pool. Plants have been transformed with foreign genes from bacteria, viruses, animals, and of course other plants (1), and chemically synthesized genes have also been transferred to plants (2,3). This illustrates the power of genetic engineering to expand the range of gene transfer beyond that obtainable by conventional breeding.

This review of genes for crop improvement begins with a summary of the current status of plant transformation, especially the recent progress in transformation of cereals. There follows a survey of the potentially useful foreign genes that have been introduced into plants by transformation to enhance stress resistance or alter product quality. Finally, I consider the structural modifications that may be needed in foreign genes to ensure their efficient expression in plants. I shall not discuss in any depth the important and

J. K. Setlow (ed.), *Genetic Engineering*
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rapidly expanding applications of transformation as a tool to understand the regulation of plant growth and metabolism (4–7).

RECENT DEVELOPMENTS IN PLANT TRANSFORMATION

The transfer of foreign DNA to plants was first reported in 1983, when three groups described the recovery of kanamycin-resistant calli from tobacco and petunia plants (8–10). The transferred gene was a chimera composed of the coding sequence of the neomycin phosphotransferase (*neo*) gene from the bacterial transposon Tn*5* and the promoter and transcriptional terminator of the nopaline synthase (*nos*) gene from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens.* The *neo* gene provided for inactivation of the antibiotic kanamycin, while the *nos* regulatory sequences permitted expression of the gene in plant cells. During induction of the crown gall disease, *A. tumefaciens* naturally transforms its host, and the *nos* gene is expressed as part of the redirection of host metabolism toward the supply of C and N to the bacterium in the form of nopaline. Thus, the first successes in plant transformation were achieved by commandeering some of the machinery involved in the only known example of routine gene transfer between kingdoms of cellular organisms.

Agrobacterium-mediated transformation is now used to introduce foreign genes into the nucleus of many dicots (11,12). For some species such as tobacco, tomato and potato, specific varieties have been found to be extremely readily transformed by *Agrobacterium,* that is, transgenic calli are produced efficiently and are regenerated with high frequency into viable, fertile plants. However, for other dicotyledonous plants *Agrobacterium*-mediated transformation may proceed but regeneration may be infrequent, or transformation itself may prove a stumbling block. For this reason, tobacco, tomato and potato feature prominently in the list of plants engineered for crop improvement.

Many attempts have been made to determine whether *Agrobacterium*-mediated transformation of cereals is feasible. Two groups have reported *Agrobacterium*-mediated transformation of rice. Raineri et al. (13) presented Southern and western blots as evidence for stable transformation with the β-glucuronidase (*gus*) reporter gene but did not recover fertile plants. The frequency of transformation was highest with 2- to 4-day-old embryos. Chan et al. (14) reported expression of the *gus* and *neo* genes. Southern blots and enzyme assays were included in their evidence for success. Transformation was best with 3- to 4-day-old seedlings but was dependent on the prior exposure of rice tissue to medium conditioned by a potato suspension culture. The key substance secreted by potato is most likely to be a flavonoid capable of triggering the *Agrobacterium*-plant interaction (11,12).

Over the last 6 to 7 years, the most successful methods for transformation of cereals involved the use of protoplasts (15). DNA was introduced into protoplasts via electroporation (16,17) or PEG-mediated uptake (18). After transformation, the protoplasts were allowed to form callus and then to regenerate into plantlets (19–21).

Table 1
Applications to USDA to Field Test Genetically Engineered Plants
1987–1991

Crop	Type of Study						
	Virus Resistance	Bacterial Resistance	Fungal Resistance	Insect Resistance	Herbicide Resistance	Freezing Resistance	Product Quality
Potato	+	+		+	+		+
Tomato	+		+	+		+	+
Corn				+	+		+
Tobacco	+			+			+
Cotton				+	+		
Rapeseed				+			+
Rice				+			+
Alfalfa	+						
Cantaloupe	+						
Chrysanthemum							+
Cucumber	+						
Soybean					+		
Sunflower							+

Table 2
History of Rice Transformation

Year	Advance	Ref.
1986	Transient expression of foreign gene in japonica protoplasts:	
	- after electroporation	17
	- after PEG-mediated uptake	18
1988	Regeneration of transgenic japonica plants after transformation of protoplasts	19,20,21
	Transient expression after biolistic transformation	25
1989	Recovery of fertile transgenic plants from japonica protoplasts	22
1990	Transformation of indica protoplasts	23
	Recovery of fertile transgenic plants from indica protoplasts	24
1991	Recovery of fertile transgenic plants from biolistic transformation of indica and japonica callus and embryos	27

Table 2 summarizes the transformation history of rice, the first cereal to be regenerated from protoplasts to give fertile, transgenic progeny (22). The main problem with protoplast-based methods is the low frequency with which most varieties regenerate viable, fertile plants. While certain japonica varieties show a high capacity for regeneration, indica varieties are more difficult. Even so, several elite indica cultivars have now been transformed as protoplasts and regenerated to give fertile plants (23,24).

In the last five years, direct delivery of DNA into plant cells by the biolistic method has come to prominence (25). Gold or tungsten particles coated with DNA are fired into intact plants, tissue explants, callus or cell suspensions. Acceleration of the particles has been achieved by such methods as gunpowder (25), helium gas (26) and electric discharge (27,28). The biolistic approach is successful for transient expression studies (25,29) and for nuclear transformation of rice (27,28) and maize (29–31), together with sugarcane, wheat and sorghum (28). The biolistic method appears to be independent of variety and has been successful with both japonica and indica varieties of rice (27,28).

Plants contain three distinct genomes (Table 3). In addition to the nuclear genome, genetically functional DNA exists in the chloroplast and the mitochondrion. Most transformation studies attempt to integrate foreign genes into the nuclear genome, but there is increasing interest in organellar transformation. Chloroplasts have been transformed in the green alga *Chlamydomonas* (32,33) and in tobacco (34). So far, no report of successful transformation of the mitochondrial genome has been reported for plants, although it has been successful for yeast (35,36). The biolistic method is

Table 3
Transformation of Nuclear, Chloroplast and Mitochondrial Genomes of Plants (mid-1992)

Genome	*Agrobacterium*	Protoplasts		Biolistic	
	(stable)	(stable)	(transient)	(stable)	(transient)
Nucleus	+	+	+	+	+
Chloroplast	-	-	-	+	+
Mitochondrion	-	-	-	-	-

currently the only successful method for chloroplast transformation. It is also the method by which the mitochondria of yeast cells were transformed.

In principle, foreign genes could exist in host cells in three states: transiently as the introduced plasmid, on a longer term basis as an autonomously replicating plasmid, or permanently as a segment of DNA integrated into one of the three genomes by way of homologous or nonhomologous recombination. The third state is clearly preferable but depends on a recombination event. Most nuclear transformation studies to date have relied on nonhomologous recombination to integrate the foreign gene into nuclear DNA. Since the site of integration of a foreign gene could affect its own expression and also inactivate a host gene at or near that site, there is growing interest in homologous recombination or targeted integration (37). Should this procedure become routine, gene replacement would become possible and would be of considerable academic and applied interest.

MARKER GENES AND REPORTER GENES FOR TRANSFORMATION

Foreign genes are widely exploited in the plant transformation process itself (Table 4). Since very few cells in a target population become transformed, selection of transformants demands the use of selectable markers. Selectable marker genes usually encode enzymes which inactivate either an antibiotic or a herbicide. Only transformed cells survive and grow on media containing an appropriate concentration of the antibiotic or herbicide. The *neo* gene is used during transformation (10) to detoxify antibiotics such as kanamycin and G418, whereas the bacterial hygromycin phosphotransferase gene (38) inactivates hygromycin. The use of herbicides as selectable markers is discussed below.

Colorimetric, fluorometric, luminometric, or radiometric monitoring of transformation also exploits certain foreign genes known collectively as reporter genes. The most commonly used reporter genes are those encoding β-glucuronidase (39), luciferase (40) and chloramphenicol acetyl transferase (CAT) (10). Their principal uses are in facilitating the development of transformation protocols and in the characterization of plant promoters.

Table 4
Genes of Use for Crop Improvement

Transformation protocols	Abiotic stress
Antibiotic resistance:	Heavy metal:
neomycin phosphotransferase (B)	Metallothionen II (A)
hygromycin phosphotransferase (B)	Salt:
	Mannitol-1-phosphate dehydrogenase (B)
Herbicide resistance:	
phosphinothricin acetyl transferase (B)	Freezing:
	Fish antifreeze protein (A)
EPSP synthase (B,P).	Oxidation:
	Mn-Superoxide dismutase (P)
Reporter genes:	
chloramphenicol acetyl transferase (B)	Modified Product Quality
β-glucuronidase (B)	Fatty acid composition:
luciferase (A)	ACP thioesterase (P)
	Amino acid composition:
Pest & Disease Resistance	Methionine-rich protein (P)
Virus:	Dihydropicolinate synthase (B)
coat protein (V)	Delayed fruit ripening:
RNA-binding protein (V, antisense)	Polygalacturonase (P, antisense)
Satellite RNA (V)	ACC oxidase (P, antisense)
AL1 virus replication gene (V, antisense)	ACC synthase (P, antisense)
	ACC deaminase (B)
	Flower pigments
Fungus:	Chalcone synthase (P, antisense)
Chitinase (P)	
Ribosome inactivating protein (P)	Other Properties
	Polyhydroxybutyrate formation:
Insect:	Acetoacetyl CoA reductase (B)
Bt Cry 1A(b) (B,S)	PHB synthase (B)
Bt Cry 1A(c) (B,S)	Male sterility:
Cowpea trypsin inhibitor (P)	Barnase (B) and Barstar (B)
Potato inhibitor II (P)	Antibodies:
	Heavy chain (A)
	Light chain (A)

(Source of genes: A = animal, B = bacterium, P = plant, S = synthetic, V = virus)

Herbicide Tolerance

Transgenic mechanisms of herbicide resistance have obvious commercial significance in addition to their use as selectable markers for transformation. Considerable progress has been made in devising strategies for increasing herbicide tolerance in plants (41). One of these strategies envisions the

transformation of plants with foreign genes which detoxify herbicides. An example is provided by the herbicide Basta/phosphinothricin, which inhibits glutamine synthetase, leading to accumulation of ammonia. DeGreef et al. (42) obtained field resistance to Basta through use of the *bar* gene from *Streptomyces hygroscopicus* (38). The *bar* gene encodes an acetyltransferase which acetylates the active component of Basta, and renders it inactive as a herbicide. This strategy is reminiscent of the mechanism to which certain corn lines owe their natural tolerance of atrazine: detoxification of the herbicide through conjugations with glutathione-S-transferase (43). In the context of selectable marker genes, the *bar* gene is probably the most popular of the herbicide resistance genes.

The second strategy for obtaining transgenic resistance to herbicides is to overexpress the target protein of the herbicide, while the third strategy is to express a desensitized target protein. Both of these strategies may be illustrated by reference to the herbicide glyphosate [N-(phosphonomethyl)glycine]. This herbicide inhibits aromatic amino acid biosynthesis in plants and bacteria by binding to the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Resistance to glyphosate has been achieved in plants both by overexpression of EPSPS (so that more herbicide is required to achieve a given level of killing) (44) and by expression of a modified EPSPS that is relatively insensitive to the herbicide (45,46).

Basta and glyphosate belong to the new generation of relatively benign herbicides with very low mammalian toxicity. However, their post-emergence use has been limited because of their nonselective (broad spectrum) mode of action. The development of Basta- and glyphosate-resistant crop plants will permit the use of these herbicides in new situations and encourage the phasing-out of the highly toxic herbicides currently in use. Of course, this strategy would eventually be rendered ineffective if there were gene transfer between the transgenic crop and wild relatives. To reduce the chance that weeds would acquire herbicide tolerance through cross-pollination, it would be desirable to integrate genes for herbicide resistance into the chloroplast DNA of the transgenic plant. In most crop plants, chloroplast DNA is inherited maternally, i.e., not transmitted through pollen (47). Since some progress toward chloroplast transformation has already been made (29,32–34), it is likely that the transfer of herbicide resistance genes to the chloroplast genome will be a focus of future work.

GENES FOR RESISTANCE TO PESTS AND DISEASES

Virus Resistance

It has been known for many years that plants can be protected against a virulent strain of certain viruses by prior infection with an attenuated strain of the same virus or a related virus. This phenomenon is known as cross-protection (48). In the case of positive-strand RNA viruses, a similar protective effect is observed in transgenic plants expressing the coat protein gene of the

virus (49–52). The genes used in this type of transformation are double-stranded cDNAs derived from the viral RNA. Although the mechanism of cross-protection is not fully understood, it does not always appear to require high levels of expression of the coat protein itself (53,54).

Genetically-engineered resistance has also been obtained for a negative-strand RNA virus (55). Tomato spotted wilt virus is an enveloped virus containing an internal RNA-binding protein known as the nucleocapsid protein. Double-stranded cDNA derived from the nucleocapsid gene was introduced into tobacco plants by the *Agrobacterium* route and was found to be expressed and to confer considerable protection from a later challenge by the virus itself. This approach may be useful for producing plants resistant to infection by other negative-strand viruses. This mechanism of cross-protection is also not understood but could involve anti-sense inhibition of viral transcription or replication.

Virus resistance has been enhanced through transformation of plants with double-stranded DNA derived from satellite RNAs (56,57). Again, the mechanism of cross-protection is unclear but may involve competition between infective and satellite RNA sequences for cellular components.

A transgenic cross-protection method has been developed for a DNA-containing virus, tomato golden mosaic virus, one of the gemini viruses (58). Expression of an antisense construct derived from the viral AL1 gene conferred resistance in transgenic tobacco. The AL1 gene is involved in DNA replication. It is to be expected that many other viral genes can be used to give cross-protection.

Fungal Resistance

Many plants, animals and microorganisms produce proteins which are toxic to fungi. The genes for some of these proteins have been cloned and will presumably be employed in transgenic approaches to enhance fungal resistance in plants. Two examples relate to plants showing enhanced resistance to the important soil-borne fungal pathogen *Rhizoctonia solani.* In one case, transgenic plants expressed bean chitinase under the control of the CaMV 35S promoter (59), while in the other case plants expressed a ribosome inactivating protein (RIP) from barley under the control of the *wun* 2 promoter from potato (60).

R. solani causes disease symptoms on roots, stems and leaf sheaths of a wide range of plant species. For example, it causes leaf scurf in potato and sheath blight in rice. Fungal cell walls depend on chitin (a β-1,4-polymer of N-acetylglucosamine) for mechanical strength; treatment of fungal mycelia with chitinase leads to the bursting of the cells at the growing hyphal tip (61). Broglie et al. (59) found that, following transformation with the bean chitinase gene, tobacco seedlings expressed elevated levels of chitinase and showed better survival rates than control plants in soil heavily infested with *R. solani.* In contrast, transgenic plants showed no protection against *Pythium aphanidermatum,* a pathogen lacking a chitin-containing cell wall.

Canola plants (*Brassica napus*) transformed with the chitinase gene also showed enhanced survival in the presence of *R. solani* (59). The extent of

disease resistance observed in the transgenic tobacco or canola plants varied with the amount of fungal inoculum, a property characteristic of quantitative resistance. However, the delay in the appearance of symptoms as well as the lower severity of disease may enable young seedlings to survive the critical period during stand establishment in the field when they are most susceptible to attack by soil-borne pathogens.

Logemann et al. (60) transformed tobacco with the barley RIP protein, which inactivates some eukaryotic ribosomes by hydrolyzing a N-glycosidic bond in 28S rRNA. There is considerable specificity in the action of RIP because its expression in transgenic tobacco does not impair tobacco growth but does retard growth of *R. solani.* Since *in vitro* studies (62) with *Trichoderma reesei* and *Fusarium sporotrichioides* demonstrated that combinations of barley RIP and barley chitinase inhibit growth more efficiently that either enzyme does alone, it is possible that the access of RIP to fungal cells is impeded by chitin. It would be interesting to know whether the simultaneous expression of both chitinase and RIP in transgenic plants leads to a synergistic enhancement of resistance to *R. solani.*

Insect Resistance

Insects are a major source of yield loss, especially in the countries of the humid and sub-humid tropics. The potential attractiveness of the transgenic approach to insect control arises from five considerations: (a) the economic and human health costs of insecticide use, (b) the development of insecticide resistance in pests, (c) the counter-productive effects of insecticides on many of the natural enemies of crop pests, (d) the absence of effective host plant resistance to many insect pests, and (e) the tendency of effective host plant resistance, when it does exist, to break down in the face of adaptive changes in the pest population.

Resistance to several insects has been enhanced through expression in plants of *Bacillus thuringiensis* (BT) toxin genes (2,3,63,64) and also genes encoding proteinase inhibitors (65,66). To be effective, these two types of inhibitor must be expressed in tissue consumed by the insect. The inhibitors interfere with aspects of insect digestion. BT toxins bind to epithelial glycoproteins of the intestine, especially the midgut, and cause fatal leakage of fluids between the intestine and the hemocoel (67). BT toxin genes of the *Cry*IA class have been effective against certain lepidopteran insects in transgenic tobacco, tomato and cotton but ineffective against other lepidopterans. However, their effectiveness has been enhanced 10- to 100-fold by chemical synthesis of the gene sequence to eliminate many of the adenine-thymine (AT)-rich sequence motifs which cause instability in the mRNA of transgenic plants (2,3) (see below for a fuller discussion of this problem).

In transgenic tobacco, the cowpea trypsin inhibitor enhances resistance to *Heliothis virescens* (65), and potato inhibitor II enhances resistance to *Manduca sexta* (66). It seems likely that proteinase inhibitors act by sequestering digestive proteinase but this remains to be established; they might act in a more subtle manner.

GENES FOR RESISTANCE TO ABIOTIC STRESS

Heavy Metal Tolerance

The first example of stable transgenic resistance to an abiotic stress was provided by the *Agrobacterium*-mediated introduction of the human metallothionen-II gene into *Brassica napus* and tobacco (68). The CaMV 35S promoter was used in conjunction with the *nos* terminator. The growth of root and shoot of transformed seedlings was unaffected by up to 0.1 mM $CdCl_2$, whereas control seedlings showed severe inhibition of root and shoot growth and chlorosis of leaves. The tolerance phenotype segregated as a dominant, single-locus Mendelian character.

Salt Tolerance

Many low molecular weight substances have been found to accumulate in living cells to provide protection from salt stress. In the case of plants, various species accumulate glycine betaine, proline and sugar alcohols such as mannitol and sorbitol. Tarczynski et al. (69) introduced into tobacco the *E. coli* gene encoding mannitol-1-phosphate dehydrogenase under the control of the CaMV 35S promoter and the *nos* terminator. In *E. coli* this reversible enzyme acts primarily to oxidize mannitol-1-phosphate to fructose-6-phosphate as part of growth on mannitol. In transgenic tobacco, however, this sequence of reactions is driven in reverse by excess fructose-6-phosphate, the mannitol-1-phosphate is hydrolyzed by a nonspecific phosphatase, and mannitol accumulates to more than 6 µmol/g fresh weight. When transgenic and control tobacco plants were compared for tolerance to 25 mM NaCl, mannitol accumulation provided significant protection to mature transgenic plants, enabling them to flower and set seed, whereas control plants died before flowering (H. Bohnert, personal communication). Protection was not observed in younger seedlings.

Freezing Tolerance

Many fruits and vegetables suffer considerable damage when exposed even for one night to freezing conditions. One transgenic approach to achieving freezing tolerance in plants was initiated by Hightower et al. (70), who introduced into tobacco the gene encoding an alanine-rich "anti-freeze" protein from fish. Although this class of protein has not yet been detected in plants, its action in fish is well established. Such proteins depress the freezing-point of water and thereby reduce the probability of cellular damage. The protein is expressed in tobacco but whether it depresses the freezing point of water in plant tissues and prevents ice formation has not been reported.

Oxidative Stress

Atmospheric oxygen and oxygen produced by photosynthesis can be reduced in plant cells to highly toxic chemicals known collectively as active

oxygen species. Hydrogen peroxide, the hydroxyl radical and superoxide are examples of this group. Plants have evolved enzymatic mechanisms (catalases, peroxidases and superoxide dismutases) to deal with these molecules, which can be produced under a variety of stress situations, particularly those that block photosynthetic electron transport under moderate to high light intensities (e.g., cold, herbicides, presence of gaseous pollutants such as SO_2 and O_3, and certain fungal toxins). Bowler et al. (71) found that overexpression of manganese superoxide dismutase and targeting of the enzyme to the chloroplast led to significant protection from paraquat-induced damage in the light. Targeting of the same enzyme to the mitochondrion did not lead to protection, a result consistent with the fact that in the light paraquat-induced superoxides are formed in the chloroplast as a result of electron transfer from photosystem I.

GENES FOR MODIFIED PRODUCT QUALITY

Alteration of Fatty Acid Composition

Voelker et al. (72) have redirected the synthesis of fatty acid chains in *Arabidopsis* away from C_{16} or C_{18} molecules to the C_{12} laurate, which is of industrial importance. They transferred to *Arabidopsis* the gene for 12:0-acyl-carrier protein thioesterase from the oilseed plant, California bay (*Umbellularia californica*). The thioesterase, by prematurely hydrolyzing the growing acylthioesters, is thought to play a crucial role in the production of medium-chain fatty acids. To ensure that the thioesterase was active in seeds of *Arabidopsis* at the time of triacylglycerol production, it was fused with the promoter from the napin gene. Napin is a seed storage protein of *Brassica napus*. The transgenic plants were found to produce seeds which contained C_{12} laurate as their major fatty acid in triacylglycerols.

Alteration of Amino Acid Quantity

Humans and other animals utilize a full complement of 20 amino acids for protein synthesis. Although animals can synthesize some of these compounds from the citric acid cycle, there are 10 so-called essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) which must be obtained in the diet, that is, directly or indirectly from plant sources. Since methionine and lysine are often deficient in grains or seeds, it is interesting to note two recent studies on these amino acids: a chimeric gene encoding a methionine-rich seed protein from Brazil nut has been used to enhance by 30% the methionine content of tobacco seed proteins (73), and the lysine content of tobacco plants has been increased by expressing bacterial dihydropicolinate synthase in their chloroplasts (74). Dihydropicolinate synthase is the first enzyme of the lysine biosynthetic pathway; since the bacterial enzyme is not subject to the same regulatory mechanisms as the endogenous plant enzyme, its presence leads to a deregulation of lysine biosynthesis.

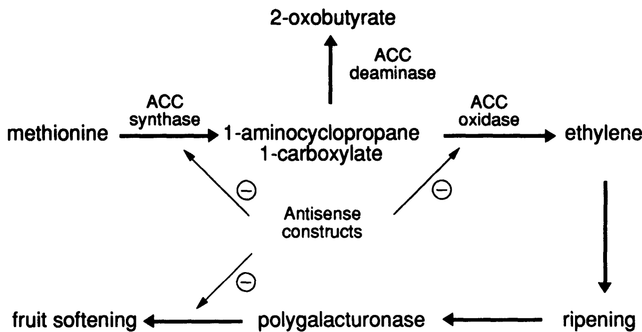

Figure 1. Transgenic modifications of the ethylene biosynthetic pathway to prolong the shelf life of tomato fruit.

Delayed Fruit Ripening in Tomato

Polygalacturonase is one of the key enzymes in the softening of fruit during ripening (Figure 1). Sheehy et al. (75) and Smith et al. (76,77) demonstrated that the antisense gene approach could lower the polygalacturonase levels in transgenic tomato fruit and prolong shelf life.

Hamilton et al. (78) also used an antisense gene approach to delay tomato fruit softening. However, in their case, the antisense construct produced RNA that was complementary to mRNA encoding ACC oxidase, the enzyme responsible for oxidizing 1-aminocyclopropane-1-carboxylate (ACC) to ethylene (Figure 1). It has been known for decades that ethylene is the plant hormone which regulates ripening. Oeller et al. (79), in a third antisense approach, succeeded in lowering the ethylene level in ripening tomato fruit by 99.5% by using the antisense construct from the gene encoding the first enzyme in the ethylene biosynthetic pathway, ACC synthase (Figure 1).

In a further attack on the ethylene biosynthetic pathway in tomato fruit, Klee et al. (80) employed a bacterial gene encoding ACC deaminase. Expression of this gene in transgenic tomato reduced the ethylene content of fruit by as much as 90%. The fruit showed delays in softening of up to 6 weeks.

Flower Pigmentation

The antisense approach was used by van der Krol et al. (81) to alter flower pigmentation patterns in petunia. The construct contained in the reverse orientation the gene for chalcone synthase, one of the early enzymes in the production of flavonoids from phenylalanine.

OTHER GENES FOR ADDED VALUE

Polyhydroxybutyrate Production

Polyhydroxybutyrate (PHB), a high molecular weight polyester of industrial importance, is accumulated as a storage form of carbon in many species of bacteria and is a biodegradable thermoplastic. Poirier et al. (82) have used genes from the bacterium *Alcaligenes eutrophus* that encode two enzymes required to convert acetoacetyl-coenzyme A to PHB (acetoacetyl-CoA reductase and PHB synthase). These genes have been placed under the control of the CaMV 35S promoter and introduced into *Arabidopsis.* Transgenic plant lines that contained both genes accumulated PHB as electron-translucent granules. This illustrates the potential of using plants for the production of bioplastics and other biopolymers.

Male Sterility

Hybrid vigor has proved of immense value in increasing the yield of several outbreeding crops. The commercial importance of maize hybrid seed production is based entirely on this phenomenon. Its exploitation in inbreeding crops such as rice is relatively recent. Cytoplasmic male sterility (cms) is a key element in the intensive commercial production of hybrids. A maize line showing cms is employed as the female parent. If the cms is stable, no selfing of the female parent will occur and all seed produced by it after cross-pollination by a male-fertile line will be hybrid seed. Since hybrid seed can provide large increases in yield, farmers are often prepared to buy such seed each year in preference to multiplying lower-yielding, male-fertile inbred lines.

Commercial producers of hybrid maize propagate cms lines by crossing them with maintainer lines. They can also restore male fertility by crossing the cms line with a restorer line. Much of the effort being expended in establishing hybrid seed production in crops other than maize is devoted to the search for stable cms lines and for maintainer and restorer lines.

An alternative approach is the development of artificial systems of reversible nuclear male sterility through genetic engineering. In one such approach, a bacterial ribonuclease (Barnase) is expressed under the control of a promoter that is specific for the tapetal cells that feed the developing pollen sacs. The resultant death of the tapetal cells induces male sterility (83). To complete this system, it would be necessary to find a mechanism of reversing sterility. Reversal has been achieved through expression of Barstar, an inhibitor of Barnase (84). Expression of an anti-sense Barnase construct might also prevent accumulation of the mRNA for Barnase. An intriguing alternative way of artificially down-regulating expression of the Barnase gene could be to use genes encoding ribozymes. The latter degrade specific mRNA molecules by RNA-catalyzed splicing reactions (85). It is not yet clear whether the antisense approach or the ribozyme approach would give sufficiently marked and stable down-regulation to act as restorers of male fertility.

The advantage of artificial nuclear male sterility is that it gives the breeder much more flexibility in his breeding program. He is not restricted to a few lines showing stable cms and responsiveness to restorers and maintainers.

Antibodies

One of the most intriguing possibilities in plant genetic engineering is to exploit the selectivity of antibodies to inhibit or interrupt specific processes. Assembly of functional antibodies within transgenic plants has already been demonstrated (86,87). Since antibodies are composed of two types of subunit, light chains and heavy chains, it is necessary to express both proteins in plants to obtain a functional antibody. Hiatt et al. (86), prepared two transgenic lines of tobacco, one transformed with a light chain gene and the other transformed with a heavy chain gene. Both lines produced their respective immunoglobulin chain but only hybrid plants assembled functional antibody containing both chains. Düring et al. (87), transformed tobacco with a single construct containing both genes under the control of separate promoters; functional antibody accumulated within cells. By analogy with experiments conducted in other systems (88), it should be possible to fuse the heavy and light chain genes into a single open reading frame and still recover active antibody.

MODIFICATION OF GENES FOR ENHANCED EXPRESSION

Promoters and Terminators

Among the principal requirements for foreign gene expression in plant cells are a suitable promoter and terminator (Figure 2). The promoter most commonly used in plant transformation is the cauliflower mosaic virus 35S promoter. This viral promoter controls the synthesis of a 35S RNA during infection of plants by CaMV and is highly expressed in many tissues of monocotyledonous and dicotyledonous plants (39,89,90). Another generally expressed promoter is the actin promoter (91). Such promoters are useful for initial transgenic studies when the basic efficacy of the foreign gene is to be assessed. However, subsequent studies would in all likelihood require more specific expression of the foreign gene: expression in specific tissues, at specific times or in response to specific plant hormones (92–94) or specific environmental cues, such as heat shock (95), light (96), wounding (97) and/or fungal elicitors (98). Many such promoters are known and there are methods available for identifying additional promoters with interesting properties (99). Promoters frequently have complex structures; it may be possible to obtain various patterns of expression with the use of different permutations and combinations of regions of a single promoter. This is seen clearly with the CaMV 35S promoter (88). Promoter strength may also be enhanced or modulated by placing two or more copies of a promoter in tandem or by combining promoters (100,101).

Transcriptional terminators in plants contain at least one polyadenylation signal (eg., AATAAA, AATTAA or AACCAA) (102,103) but may also need

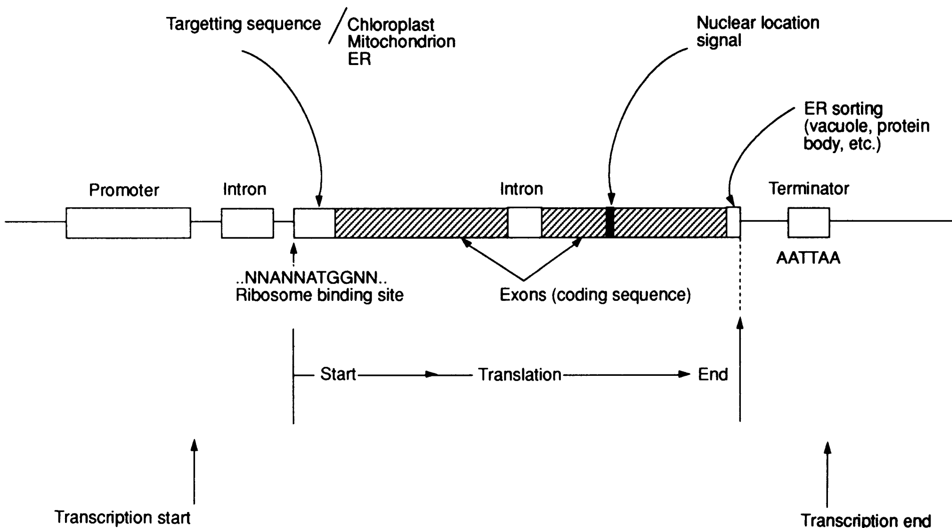

Figure 2. Modifications of a foreign gene for optimal expression in plants.

other 3′ sequences as well (104,105). The two most commonly-used terminators for plant transformation are the CaMV 35S terminator and the *nos* terminator (from the nopaline synthase gene of the T-DNA of *Agrobacterium*). The polyA tail may stabilize mRNAs against degradation (106).

Ribosome-binding Site

Genes may have to be modified to provide for appropriate ribosome binding sites in mRNA if high levels of expression are desired. The bacterial ribosome binding site (the Shine-Dalgarno sequence) is quite well defined but plant ribosome binding sites are rather different from their bacterial counterparts and less conserved. From a study of 75 published genomic DNA sequences from several higher plants, Joshi (107) concluded that the consensus context of the initiating ATG of plant mRNAs is TAAACA<u>ATG</u>GCT. It appears that the most important residue is the third residue before the initiating ATG. This residue should be a purine (usually A) for an abundantly expressed protein (107–109). Translational efficiency can be enhanced by inclusion of a viral untranslated leader sequence between the promoter and the coding region of the gene (110,111).

Introns

An intriguing phenomenon is the effect of introns on foreign gene expression in monocots. Callis et al. (112) were the first to report that introns

increase gene expression in cultured maize cells. Tanaka et al. (113) reported as much as 90-fold higher levels of GUS expression in transgenic rice when an intron from the castor bean catalase gene is inserted into the GUS coding sequence. Enhanced expression was correlated with an increased level of mRNA and efficient splicing of the intron. This enhancement was not observed in transgenic tobacco. A stimulation of gene expression was observed in transgenic rice when the first intron of the *ActI* actin gene was inserted between the CaMV 35S promoter and the *gus* gene (114). Maas et al. (115), studied the effects of both exon 1 and intron 1 of the maize *Shrunken-1* gene on expression of CAT gene in transient expression experiments in rice and maize. The exon alone stimulated expression 10-fold, the intron stimulated 100-fold, and the two elements in combination stimulated 1000-fold when placed between the 35S promoter and the CAT gene. The exon is also stimulatory in tobacco protoplasts but the intron is inhibitory. It is not clear why monocots and dicots differ in their response to the presence of introns in transformation constructs.

In Figure 2, two introns are included in the gene to indicate that the intron may be located in the coding sequence itself, which is then split into two exons, or in the upstream region between the transcriptional start site and the translational start site. The former location is the more usual in plant genes but the latter location appears to be equally effective and has the distinct advantage of providing for easier construction of transformation vectors. It can be quite tricky to place an intron within a coding sequence without altering the coding properties of the gene.

Potential Glycosylation Sites

It may also be important to make other specific modifications of genes to assist in their successful expression in plant cells. Many proteins that enter the endoplasmic reticulum undergo glycosylation at specific short amino acid sequences (Asn-X-Ser/Thr) which are recognized as glycosylation sites by glycosyl transferases. Similar sequences may also be present in proteins which never enter the endoplasmic reticulum. Should these proteins be forced to enter the endoplasmic reticulum (e.g., by transformation of plants with appropriate constructs), those previously unglycosylated sites might become glycoslyated and the protein might be inactivated. This problem was actually foreseen for the β-glucuronidase of *E. coli* (116). Based on the DNA sequence of the *gus* gene, two putative N-linked glycosylation sites were predicted: N^{358}LS and N^{423}IS. The *gus* gene is satisfactorily expressed in plants if the enzyme is directed to the cytosol (39), the chloroplast (117) or the mitochondrion (118), but if the protein is directed to the endoplasmic reticulum it becomes glycosylated, resulting in loss of activity and poor staining of tissue with X-gluc (119). The problem was solved through alteration of both sites, N^{358} being mutated to serine, threonine or proline and N^{423} being eliminated through a spontaneous alteration in sequence (12). The modified GUS protein can be directed through the endoplasmic reticulum to the vacuole and recovered with high activity.

AT/GC Bias

Modification on a larger scale has been necessary for BT toxin genes (2,3). *Cry* IA(b) and *Cry* IA(c) BT toxin genes were found to be expressed very poorly in transgenic tobacco and tomato. This was attributed to three main problems: codon bias, out-of-position polyadenylation/termination signal sequences and mRNA instability. In fact, all three problems were consequences of the high AT content of BT toxin genes. Some AT-rich codons are commonly used in the toxin genes but are rarely used in plant genes (e.g., TTA coding for leucine). Thus, in plants the translation of a foreign gene rich in TTA codons would lead the ribosome to pause, thereby increasing the probability of premature termination of the polypeptide. Premature transcriptional termination would be expected also in such a situation because of the high frequency within the BT toxin gene of sequences such as AATTAA resembling polyadenylation signal sequences (103). Finally, ATTTA sequences are suspected to destabilize mRNA. Direct evidence for the instability of *cry* IA(b) mRNAs in transgenic carrot plants and protoplasts has been obtained by Murray et al. (121). By changing 21% of the residues in the 1845 bp gene through chemical synthesis (without changing the predicted amino acid sequence), Perlak et al. (3) were able to increase expression of the BT toxin by 10-fold for *cry* IA(b) and 100-fold for *cry* IA(c), and thereby increase the toxicity of the transgenic plants to insects.

Subcellular Targeting

The site of function of every plant protein is quite specific. Most proteins are found in the cytosol. Such proteins are usually synthesized on free cytoplasmic ribosomes and need no special signals to remain in the cytosol. Many other proteins that are synthesized on free cytoplasmic ribosomes enter the chloroplast, the mitochondrion or the nucleus. Specific signals are required for this targeting (122–124). The signals of chloroplast and mitochondrial targeting are 35 to 60 amino acids in length, are located at the N-terminus of proteins and are usually removed after entry into the organelle. Suborganellar targeting such as between the different compartments of the chloroplast require additional targeting signals (123,125).

For nuclear proteins too large to diffuse through nuclear pores (> 60 kD), a short internal targeting sequence may be required. The sequence Pro-Lys-Lys-Lys-Arg-Lys-Val of the large T-antigen of Simian Virus 40 has been shown to be sufficient to target several proteins to the mammalian nucleus (126). The same sequence is able to transport the bacteriophage T7 RNA polymerase (100 kD) (124) and the GUS enzyme (homotetramer of 68 kD subunits) (127) to the nucleus of transgenic tobacco. The highly basic DNA-binding domains of three nuclear proteins of plants were found to facilitate the import of GUS into nuclei of transgenic tobacco (127), suggesting a close association or overlap of the DNA binding and nuclear targeting domains of B-ZIP proteins.

Many proteins are synthesized on cytoplasmic ribosomes that are initially free but rapidly become bound to the endoplasmic reticulum as the N-terminus

of the growing polypeptide emerges from the ribosome. The N-terminus of these proteins forms the signal peptide for entry of the protein into the ER (128–131). The signal peptide (20 to 25 residues) consists of three regions designated n, h and c. The n-region of 3 to 5 residues contains a single positively charged amino acid residue, the h-region contains 7 to 15 hydrophobic residues, and the c-region of 3 to 5 small neutral residues is important for recognition by a processing protease which removes the signal peptide very soon after it enters the lumen of the ER. Proteins which contain no other targeting sequence follow a so-called default pathway through the ER system and are secreted from the cell (132). Proteins with additional specific targeting sequences are deposited in protein bodies, lysosomes, vacuoles, peroxisomes and other membrane-bound derivates of the ER (133–136). A minority of chloroplast and mitochondrial proteins is encoded and synthesized in the organelles themselves (137,138).

Scaffold-associated DNA Sequences

Plant nuclear genes operate within a complex and highly organized chromosomal structure (139). Specific DNA sequences contribute to this structure by providing binding sites for scaffolding proteins involved in the bending and packing of chromosomes (140). Studies on animal cells indicate that inclusion of DNA sequences (about 3 kb) of scaffold-associated regions (SARs) in transfection vectors increases the transcription of foreign genes and dampens the position effects that arise from the uncontrolled insertion of foreign genes into different sites within the genome (141,142). It is likely that future plant transformation vectors will include SARs to achieve these same benefits.

CONCLUDING COMMENTS

Genetic engineering will lead to the improvement of many key characteristics of crop plants. It is already evident that the resistance of plants to several biotic stresses such as weeds, viruses, insects and microorganisms can be significantly enhanced by this approach. Less progress has been made towards resistance to abiotic stress but this situation will improve as we learn more about the biochemistry of stress responses. Improvements are also being made in the quantity and quality of plant products, and it has become possible to introduce a form of nuclear male sterility and thereby assist the production of higher-yielding hybrid plants in species where this has not yet been achieved.

Much concern has been expressed about the potential dangers of plant genetic engineering. Relatively few countries have adopted regulatory policies to deal with this problem. It is important that both industrialized and developing countries have in place clear and specific guidelines that cover containment for laboratory work, confinement for field-testing and certification for release to national agricultural research systems or the private sector. The guidelines should allow for rapid approval in non-controversial cases and

thorough analysis in more difficult cases. The definition of what is non-controversial will itself evolve over time (143).

Of special concern is the spread of genes from transgenic crops to weedy wild relatives following cross-pollination. As we have seen, in those crop species which show maternal inheritance of plastids, the problem might be solved through integration of foreign genes into chloroplast DNA. However, before such a possibility can be made a reality, it will be necessary to develop efficient mechanisms of chloroplast transformation. Furthermore, a chloroplast location for foreign genes will make biochemical sense in only a minority of cases, e.g., some herbicide resistance genes. The dumping of foreign genes in the chloroplast should not be seen as a substitute for a realistic assessment of likely dangers.

Another vexing question is the impact of large-scale cultivation of transgenic crops on the genetic constitution of populations of field pests and diseases (144). Intensive cultivation of insect-resistant plants can be expected to lead to the selection of resistant strains. This problem is of course not unique to transgenic plants. It is seen also for insect-resistant plants developed by classical breeding techniques and finds a parallel in the development of insecticide-resistance in many pest populations following the excessive use of pesticides. Considerable insights will be needed into the population dynamics of insects and their mechanisms of genetic change, if appropriate strategies for deployment of engineered plants are to be formulated. Are weak resistance mechanisms likely to provide less selection pressure than strong mechanisms? Does security and sustainability lie in the deployment of several diverse resistance mechanisms, and should these be deployed simultaneously or sequentially? It should be noted that the best answers to these questions can be provided by small-scale field experimentation with transgenic plants.

Will transgenic plants display yield depression or reduction in fertility as a result of passage through tissue culture? Careful agronomic studies will have to be undertaken to determine whether transgenic plants suffer genetic changes as a result of the transformation protocol and whether the losses resulting from these changes outweigh the benefits accruing from the presence of the foreign gene.

Acknowledgments. I thank Dr. Pia Bennett, Mrs. Shirley Raymundo, and Ms. Minnie Bernardino for assistance in the preparation of the article.

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