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The Introduction and Expression of Foreign Genes in Plants

D. M. SHAH, N. E. TUMER, D. A. FISCHHOFF, R. B. HORSCH, S. G. ROGERS, R. T. FRALEY AND E. G. JAWORSKI

Division of Biological Sciences, Monsanto Company, 700 Chesterfield Village Parkway, St Louis, MO 63198, USA

Introduction

During the past few years, there has been a vast accumulation of knowledge regarding the structural and functional organization of genes in higher plants, and factors that control their expression. This explosion of interest in plant molecular biology has been greatly facilitated by the development of gene transfer systems which allow genes to be introduced into cells of a variety of plant species. As it is possible, in several instances, to regenerate fertile plants from single transformed cells, engineered genes integrated stably into plant chromosomes can serve as powerful tools to study important physiological and developmental processes. A variety of methods for introducing foreign DNA into plant cells have been attempted over the past several years; however, the stable integration and functional expression of the introduced genes has only recently been successful. Of fundamental importance for stable transformation of plants has been the development of the *Agrobacterium* tumour-inducing plasmid (Ti) system. The other proven approach relies on delivery of free DNA across the plasma membrane of protoplasts (cell wall-less plant cells). In this review, we briefly describe the *Agrobacterium*–Ti plasmid-mediated plant transformation systems; we summarize the available information on the expression of developmentally regulated tissue-specific and inducible genes introduced into plants and, finally, we present the current status of engineering agronomically important

Abbrevations: ADH, alcohol dehydrogenase; AlMV, alfalfa mosaic virus; *B.t.*, *Bacillus thuringiensis*; Cab, chlorophyll *a/b* binding protein; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyl transferase; +CP, with coat protein; −CP, without coat protein; EPSP, 5-*enol*pyruvyl shikimate-3-phosphate; IAA, indolecacetic acid; lbc, leghaemoglobin; LIH, left internal homology; NOS, nopaline synthase; rbc, ribulose-1,5-bisphosphate carboxylase; Ri plasmid, root-inducing plasmid; Ti plasmid, tumour-inducing plasmid; TMV, tobacco mosaic virus.

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traits into higher plants. Although several fascinating problems of agronomic significance are currently being tackled with the aid of gene transfer technology, we describe a few examples, some of them from our own work, which illustrate remarkable prospects for using genetic transformation in crop improvement.

Transformation of plants

The successful development of methods to transfer genes into plants was made possible by engineering genes to confer a selectable phenotype to transformed cells. The first such selectable marker gene for plant cells was a chimaeric construction using the bacterial coding sequence for neomycin phosphotransferase (kanamycin kinase, EC 2.7.1.95) with 5′ and 3′ sequences from the nopaline synthase gene from *Agrobacterium tumefaciens* (a gene already shown to express well in plant cells). This gene construction permitted plant cells to grow on medium containing otherwise inhibitory concentrations of kanamycin. The other key requirements for producing transgenic plants are a DNA delivery method and procedures for regenerating whole plants from the transformed cells. A detailed history of the construction of selectable marker genes and other aspects of the *A. tumefaciens* transformation system has been presented in a recently published review article (Fraley, Rogers and Horsch, 1986).

A. tumefaciens, causative agent of crown gall disease, has evolved a highly sophisticated mechanism for gene transfer, as the *modus operandi* of its pathogenic behaviour. Salient features of the *Agrobacterium*–Ti system are presented below. For a detailed discussion of this system, the reader is referred to excellent earlier reviews (Bevan and Chilton, 1982; Kahl and Schell, 1982; Ream and Gordon, 1982; Schell and Van Montagu, 1983; and references therein). *A. tumefaciens* is commonly found in soil and causes crown galls, or tumours, on a wide range of dicotyledonous plants. Oncogenic strains of this bacterium harbour larger (150–250 kb) tumour-inducing or Ti plasmids. A small portion of Ti plasmid DNA, called T-DNA, is stably maintained in axenic cultures of crown galls. This T-DNA is covalently attached to, and thus stably maintained in, the nuclear DNA of cells transformed during infection. Expression of specific T-DNA genes has been shown to be responsible for the phytohormone-independent growth and the novel metabolic capacities (opine synthesis) of the crown gall tumour cells. The most common opines, octopine and nopaline, are conjugates of arginine and α-ketoglutarate, or arginine and pyruvate, respectively. *Agrobacterium* strains determine the specific opine synthesized by the tumour cells and the ability of these strains to catabolize opines is used to classify different types of Ti plasmids. Thus, the T-DNA is a natural vector for gene transfer.

Some 13 genes have been identified in the 23 kb nopaline T-DNA segment by transcript mapping, transposon insertion and deletion analysis. The octopine T-DNA is not continuous and instead is comprised of two separate T-DNAs, T_L and T_R. Eight genes have been identified on a 13·2 kb T_L-DNA, whereas five genes have been mapped on the 7·9 kb T_R-DNA. T-DNA

genes have been shown to be transcribed into RNA that is polyadenylated. Translation products of some of these genes have been shown to be the enzymes involved in the synthesis of opines and the enzymes involved in the biosynthesis of phytohormones, indoleacetic acid (IAA) and cytokinin. Expression of the phytohormone biosynthetic enzymes in the transformed plant cells allows the transformed plant cells to proliferate in the absence of exogenously added hormones. Because high-level expression of phytohormones prevents regeneration of intact plants from the transformed tissue, deletion of the phytohormone biosynthetic genes is essential to enable the Ti plasmid to be used as a vector to transfer genes into plant cells that can be regenerated into plants.

Sequence analysis of the T-DNA flanking regions in the Ti plasmid and that of the integrated T-DNA segments isolated from the DNA of crown gall tumours has shown that direct repeats of 25 base pairs flank the T-DNA region. The right T-DNA border is essential for tumour formation in most plant species, whereas the left border is not. The T-DNA border sequences may serve as recognition sites for a recombination event which generates a T-DNA intermediate for transfer and integration into plant DNA. The T-DNA border sequences are the only sequences required in *cis* for T-DNA transfer to plants. None of the T-DNA resident genes are essential for T-DNA transfer to the plant genome. Thus, the resident genes which are solely responsible for the crown gall disease can be removed and any DNA sequences desired can be inserted between the T-DNA border sequences for transfer into transformed plant cells. A region of the Ti plasmid, called the *vir*-region, outside the T-DNA is essential for the excision and transfer of T-DNA to plant cells and subsequent integration into plant DNA. The functions of the *vir*-region genes remain largely unknown; however, in a recent study it has been shown that the T-DNA border sequences are specifically nicked after *vir* gene activation by plant signal molecules such as acetosyringone, and this nicking is due to acetosyringone-induced specific single-stranded cleavage within the 25 bp repeat sequence (Wang *et al.*, 1987). The generation of nicks at the T-DNA borders is probably the first T-DNA-associated molecular reaction in the transfer process. The border endonuclease activity is specified by the proximal two cistrons of *vir*D (Yanofsky *et al.*, 1986).

To harness this natural gene transfer mechanism, we designed a shuttle vector that can replicate in *Escherichia coli* where recombinant DNA manipulations are easily handled, and is then transferred into *A. tumefaciens* in preparation for transfer into plants. There are two basic modes of maintenance of the shuttle vectors in *Agrobacterium*: either by integration into the Ti plasmid by recombination at a region of DNA homology, or by autonomous replication in *trans* to the Ti plasmid. The former type of vector is referred to as a *cis* or integrating vector while the latter is called a *trans* or binary vector. In most cases, they accomplish the same goal of shuttling genes from *E. coli* to *A. tumefaciens* in a T-DNA package that can then be transferred to plants.

The essential components of our vectors include plasmid functions for replication (pBR322, RK2 Replicon) and/or integration (LIH; left internal

homology) in bacteria as well as spectinomycin and streptomycin resistance (Spc/Str^R) genes for genetic manipulations in bacteria. They also include a chimaeric selectable marker for plant antibiotic resistance such as neomycin phosphotransferase II (NOS-NPTII-NOS) which makes transformed plant cells resistant to kanamycin, and a border sequence (RB) to identify the end of the new T-DNA. There is also a scorable marker, the gene for nopaline synthase (NOS) (D-nopaline dehydrogenase, EC 1.5.1.19), that provides a powerful genetic tool for monitoring the presence of T-DNA in plants and their progeny. The final feature is the most important of all: a site to add your favourite gene (YFG).

Development of *A. tumefaciens* Ti plasmid as a vector for transfer and expression of foreign genes was made possible by the construction of chimaeric genes that function as dominant selectable markers and intermediate vector systems that shuttle genes between *E. coli* and *A. tumefaciens*. The selectable markers allowed the construction of vectors with disarmed T-DNA in which the phytohormone biosynthetic genes interfering with plant regeneration have been removed. Techniques to grow plant cells and tissues in culture and to regenerate intact plants from them provides the basis for the stable genetic modification of plants. Somatic cells or tissues of several plant species can be maintained in culture and subsequently induced to regenerate mature, fertile plants from single transformed cells. The simplest and most widely practised approach is based on variations of the leaf disc technique (Horsch *et al.*, 1985) where explants are infected with *Agrobacterium* strains and then selected and regenerated in a single step. For further information on the technique and application of *A. tumefaciens*-mediated transformation, the reader is referred to the review articles by Rogers, Horsch and Fraley (1986) and by Lichtenstein and Draper (1986).

Plant transformation in some instances involves enzymatic removal of the cell wall to produce protoplasts which can take up exogenously added DNA (Davey *et al.*, 1980; Krens *et al.*, 1982; Paszkowski *et al.*, 1984). These protoplasts are then cultured so that they form new cell walls and divide to form colonies. The individual colonies are then induced to regenerate shoots or embryos that will give rise to mature plants. The ability to detect successful uptake of exogenous DNA into protoplasts depends on the presence of a selectable genetic marker. Several solanaceous plant species such as tobacco, petunia, *Datura*, potato as well as plants belonging to other species (for example *Brassica*, carrots, alfalfa, clover and several others) are amenable to regeneration from protoplasts. Significant progress has also been made in the area of plant regeneration from protoplasts in the graminaceous species. Reports of regeneration of plantlets or plants from rice protoplasts have been published recently (Fujimura *et al.*, 1985; Abdullah, Cocking and Thompson, 1986; Coulibaly and Demarly, 1986; Yamada, Yang and Tang, 1986). With the demonstration of stable transformation of monocotyledonous cells using chemically or electrically stimulated uptake of genes (Lörz, Baker and Schell, 1985; Potrykus *et al.*, 1985; Fromm, Taylor and Walbot, 1986), genetic manipulation of cereals such as rice using protoplast technology may soon be possible. In future, it may be possible to bypass the use of tissue

culture by novel methods of transferring genes into meristematic or reproductive tissues of intact plants. In a recently published report, successful transformation of rye plants has been demonstrated by direct injection of DNA into the developing floral tillers of rye plants (de la Pēna, Lörz and Schell, 1987). The prospects for gene transfer in a wide range of monocotyledonous or dicotyledonous crop species are indeed very promising.

Gene expression

Any rational programme of plant improvement by gene transfer methods must be based on a knowledge of how genes are organized in a plant genome, and how their expression is regulated during development. Although the current level of understanding of plant gene organization and expression is substantially less than that in fungal, mammalian or *Drosophila* systems, plant molecular biologists are rapidly closing the gap. Transformation systems have served as powerful tools for examining the tissue-specific and developmentally regulated expression of genes in all organisms where they have been developed. Plant transformation has sparked tremendous interest in the structural as well as functional dissection of plant genes. Exciting results have already emerged from these studies during the past three years and are briefly reviewed here.

DEVELOPMENTALLY REGULATED ORGAN-SPECIFIC GENES

The development of plants follows a broad and plastic pathway and is dependent on differential expression of many genes in the diverse cell types of a developing plant. DNA/RNA hybridization experiments which compared the mRNA sequence sets of all organ systems of a tobacco plant have demonstrated that qualitative changes in gene expression programmes are required both to maintain and to establish distinct differentiated states in higher plants (Kamalay and Goldberg, 1980). These experiments have further shown that each organ contains a set of approximately 25 000 mRNAs; however, the sequence composition of each set differs significantly. Some of the mRNAs are organ-specific; some are expressed in more than one organ; and some are expressed in all organs. A number of genes that are expressed in an organ-specific manner have been transferred to transgenic plants with the aim of examining the *cis*-acting sequences governing organ-specific programming of the transferred genes.

The first of these is the phaseolin storage protein gene from the French bean, *Phaseolus vulgaris* (Slightom, Sun and Hall, 1983). This gene is normally expressed in the embryo of the developing bean seed. The gene is expressed at very low levels in other organs. The intact phaseolin gene with 860 bp of the 5′ flanking sequence was introduced into tobacco plants and its expression was analysed in various tissues (Sengupta-Gopalan *et al.*, 1985). This analysis showed that the gene was expressed at very low levels in tobacco leaves, callus and seedlings; seeds, however, contained 0·1% of the total mRNA as phaseolin mRNA. Thus, this gene was expressed in a tissue-

specific and developmental-stage-specific way in tobacco, as in the French bean.

In another study, expression of the α-subunit of β-conglycinin, a seed storage protein of soybean (*Glycine max*), has been examined in the seeds of transformed petunia plants (Beachy *et al.*, 1985a). The gene (Gmg 17.1), containing 8·5 kb of the 5′ flanking sequence, was expressed in immature embryos but not in leaves of the transformed petunia plants. The protein was detected immunologically in proteins extracted from embryos at 10 days post pollination, concurrent with the accumulation of the subunits of the major petunia seed proteins. In order to define the regulatory elements that control the expression of this embryo-specific gene, a series of deletion mutants was constructed in the 5′ flanking region of the gene and each deletion mutant was introduced into the genome of petunia cells (Chen, Schuler and Beachy, 1986). The mutant genes containing only 42 and 69 nucleotides of the 5′ flanking sequence were not expressed in immature embryos. The gene containing 159 nucleotides of the 5′ flanking sequence was expressed at a low level in immature embryos. When the gene was flanked by 257 nucleotides of the flanking sequence, the level of expression increased at least 20-fold. However, additional nucleotides to ~8·5 kb 5′ of the gene did not increase the level of expression. Analysis of the sequence spanning the nucleotides 159 to 257 revealed four repeats of a 6-base-pair GC-rich sequence: $(AGCCCA)$ (A/C); this may play an important part in determining the level of expression of this gene in transgenic plants. These results indicate that a soybean gene encoding β-conglycinin is appropriately regulated in transgenic petunia plants and that a small sequence in the promoter region has an important role in controlling the expression of this embryo-specific gene.

In a recently published report, a 17·1 kb fragment of soybean DNA containing the seed lectin gene and at least four non-seed protein genes was introduced into tobacco plants (Okamuro, Jofuku and Goldberg, 1986). As in soybean, lectin mRNA is detected in tobacco seeds, accumulates and decays during tobacco seed development, and is translated into protein that accumulates before dormancy. The non-seed protein genes are also expressed in a manner similar to that in soybean plants. These results indicate that a differentially expressed gene cluster of soybean is correctly regulated in a heterologous plant and that sequences controlling its expression are recognized by heterologous regulatory factor(s).

The nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) (*rbcS*) from a number of higher plants have been studied extensively for tissue-specific and light-induced regulation in heterologous and homologous transgenic plants. The *rbcS* genes from pea are best characterized in this regard. The *rbc*-E9 gene is light regulated and expressed predominantly in leaves, but not in stems or roots of the pea plant. The expression of this gene was first examined in transgenic petunia

calli (Broglie *et al.*, 1984). The gene was expressed to an extent 20–50 times higher in light-grown petunia calli than in dark-adapted calli. The transcripts had retained their authentic 5′ ends, were spliced correctly and were polyadenylated at the same site as in peas. This gene was expressed from its own promoter in a light-dependent manner similar to that observed in pea leaves. In a follow-up study, the 1050 bp 5′ flanking fragment of this gene was found to be sufficient to confer light-inducible expression on a heterologous coding sequence encoding neomycin phosphotransferase in petunia calli (Morelli *et al.*, 1985). In addition, a series of 5′ deletion mutants of the promoter was constructed to localize sequence elements required for maximal expression and light inducibility. A deletion mutant containing only 35 bp of the 5′ flanking sequence was still responsive to light. A similar study performed with another pea *rbcS* gene (SS3.6) showed that a deletion mutant containing 90 bp of the 5′ flanking sequence retained a low level of light inducibility (Timko *et al.*, 1985).

The expression of the transferred *rbcS*-E9 gene with 1052 bp of the 5′ flanking sequence was also light inducible and organ specific (that is, high in leaves, low in stems and undetectable in roots) in transgenic tobacco and petunia plants (Nagy *et al.*, 1985; Fluhr *et al.*, 1986). The expression of two other pea *rbcS* genes (3A and 3C) was also regulated correctly in transgenic petunia. Furthermore, an enhancer-like element contained within a 240 bp fragment of the *rbcS* genes conferred phytochrome-induced transcription and organ specificity on non-regulated promoters such as cauliflower mosaic virus 35S promoter. An enhancer-like element in the *rbcS* gene was first reported by Timko *et al.* (1985). In another study, the expression of the *Nicotiana plumbaginifolia rbcS* gene (*rbcS*-8B) in homologous vs heterologous transgenic plants has been compared (Poulsen *et al.* 1986). The 1·07 kb 5′ flanking sequence of the *rbcS*-8B gene was sufficient to confer light-regulated expression on the coding sequence of the bacterial chloramphenicol acetyltransferase (EC 2.3.1.28) (CAT) in transgenic plants. Expression levels of the chimaeric gene were uniform among transgenic plants of *N. plumbaginifolia* and *N. tabacum*, but some variation was observed in the expression levels of this gene among *Petunia hybrida* plants. These observations suggest that chromosomal insertion sites have little effect on the quantitative and qualitative aspects of the gene expression in a homologous system. In addition to *rbcS* genes, expression of the chlorophyll *a/b* binding protein (*Cab*) genes from pea and petunia has also been examined in transgenic plants (Jones, Dunsmuir and Bedbrook, 1985; Simpson *et al.*, 1985). A 400 bp upstream region of the pea *Cab* gene is sufficient for light-regulated and organ-specific expression of the chimaeric bacterial *CAT* gene in transgenic plants. In other studies, the promoter residing on a 1·8 kb 5′ flanking fragment of the *Cab* gene from wheat was found to be regulated correctly in transgenic tobacco and petunia plants (Lamppa, Morelli and Chua, 1985; Lamppa, Nagy and Chua, 1985). Plastid development has also been shown to have a crucial role in the activation of expression of *rbcS* and *Cab* genes in transgenic plants (Simpson, Van Montagu and Herrera-Estrella, 1986). These studies illustrate

remarkable conservation of the mechanisms for regulated transcription of the photosynthesis-associated gene families in higher plants.

In general, it appears that regulated genes are expressed appropriately in transgenic plants despite their integration at a different chromosomal location, and that *cis*-control elements programming their expression are in close proximity to the coding sequence. This implies that *trans*-acting proteins involved in regulating the expression of these genes are capable of finding their cognate sequences and activating transcription at different chromosomal locations. In transgenic mice, very poor correlation exists between gene copy number and expression (*see* Palmiter and Brinster, 1986 for a review). This also appears to be the case in transgenic plants for a limited number of genes that have been examined, suggesting that only a few of the transferred genes are expressed. As the introduced genes are regulated appropriately in heterologous plants, species specificity does not appear to be a critical factor, suggesting remarkable evolutionary conservation of regulatory factors governing tissue-specific expression of genes.

INDUCIBLE GENES

The expression of a number of genes is modulated by environmental or biological stimuli in higher plants. This type of regulation is also determined by tightly-linked *cis*-control elements present in the promoter region. Examples of well-characterized elements are those that are involved in regulation by heat shock. These short highly conserved elements are repeated several times in the well-characterized heat-shock genes of *Drosophila*, mammals as well as higher plants (*see* Craig, 1985; Lindquist, 1986 for reviews). Expression of soybean and maize heat-shock genes has been found to be heat inducible in heterologous plant tissue (Schöffl and Baumann, 1985; Gurley *et al.*, 1986; Rochester, Winter and Shah, 1986). The conservation of the heat-shock regulatory elements is suggested by a recent observation that the promoter of the *Drosophila* heat-shock gene encoding 70 kd protein is thermo-inducible in plant cells (Spena *et al.*, 1985).

Another striking example is the wound-induced proteinase inhibitor II gene of potato. This gene is normally expressed in tubers of a non-wounded potato plant, but is not expressed in leaves of a non-wounded plant. It is rapidly induced in leaves upon wounding by either mechanical means or by a pathogen (Rosahl *et al.*, 1986; Sanchez-Serrano *et al.*, 1986). High levels of correctly initiated potato proteinase inhibitor II mRNA were detected in leaves of transgenic tobacco plants after mechanical wounding as well as after treatment of detached leaves with oligosaccharides (Sanchez-Serrano *et al.*, 1987). These observations suggest that tobacco plants, which do not contain genes homologous to the potato proteinase inhibitor II gene, have the capacity to regulate the expression of this gene appropriately in the complex manner similar to that in potato. The tobacco plants contain *trans*-acting regulatory proteins which recognize the wound-responsive elements of the potato gene.

Light is one of the most important effectors of differentiation in plants.

Many of the photomorphogenic changes that take place during plant development are triggered by alteration in gene expression. Three different photoreceptors are known to control differentiation processes induced by light: the best-characterized photoreceptor, phytochrome, regulates the expression of genes associated with photosynthesis discussed earlier; the other two photoreceptors, blue light and UV-B photoreceptors, have not been identified. One of the most intensively studied families of genes, regulated by phytochrome as well as UV-B photoreceptors, encodes enzymes involved in flavonoid biosynthesis (Schröder *et al.*, 1979; Kreuzaler *et al.*, 1983). The genes encoding the key enzyme of the flavonoid pathway, chalcone synthase (naringenin-chalcone synthase; EC 2.3.1.74) have been isolated and characterized extensively (Wienand *et al.*, 1982; Kreuzaler *et al.*, 1983; Sommer and Saedler, 1986). Recently, the promoter of the chalcone synthase gene of *Antirrhinum majus* was fused to the neomycin phosphotransferase coding sequence and the resulting chimaeric gene was introduced into tobacco plants with the help of Ti plasmid-derived vectors (Kaulen, Schell and Kreuzaler, 1986). The 1·2 kb 5′ upstream region of this gene was found to be sufficient for the UV-B light-regulated expression of the chimaeric transcripts. The analysis of the various deletions of this 5′ upstream sequence has revealed a sequence motif that has a quantitative effect on gene expression and contains a direct repeat of 47 bp and the enhancer core sequence of animal genes (Gluzman and Schenk, 1983).

A set of genes is turned on in root nodules following the symbiotic association between soil bacteria of the genus *Rhizobium* and plants of the family Leguminosae. One gene family encodes leghaemoglobin in soybean nodules (Brisson and Verma, 1982; Hyldig-Nielsen *et al.*, 1982). A chimaeric leghaemoglobin gene (*lbc*3 promoter/*CAT*/*lbc*3 3′) was introduced into the genome of another legume species, *Lotus corniculatus*, and its expression was monitored in root nodules formed on transgenic plants inoculated with the *Lotus* microsymbiont, *Rhizobium loti* (Jensen *et al.*, 1986). The chimaeric gene was expressed in a nodule-specific manner and its expression was regulated at the level of RNA. The chimaeric gene was also activated at the correct stage of nodule development. This study illustrates remarkable conservation of the induction mechanism for the leghaemoglobin genes in legumes. Thus, in conclusion, genes introduced into transgenic plants respond to environmental or biological signals in a manner analogous to that of the endogenous genes as long as appropriate *cis*-acting sequences are included in the introduced gene. One exception to this has been recently reported for the expression of the maize alcohol dehydrogenase I (ADH I; EC 1.1.1.1) promoter in transgenic tobacco plants (Ellis *et al.*, 1987). Maize ADH I promoter is turned on in response to anaerobiosis in maize. The promoter region was linked to the coding sequence of the CAT enzyme and transferred into tobacco cells. Little or no CAT enzyme activity could be detected in transgenic tobacco plants after anaerobic induction. The enhancer elements from the constitutively expressed D-octopine synthase (D-octopine dehydrogenase, EC 1.5.1.11) gene or the cauliflower mosaic virus 35S promoter had to be supplied in addition to the ADH I promoter to detect the enzyme

activity. The first 247 bp upstream of the translation initiation codon of the maize ADH I gene were sufficient to confer the anaerobic regulation of the hybrid gene. At present, it is not clear whether the lack of quantitative expression of the maize ADH I promoter in tobacco is an exception or whether it represents a more general behaviour of the monocotyledonous promoters in the nuclear background of dicotyledonous plants. If species-specific gene expression in plants is common, it may pose problems for the use of interspecific gene transfer in genetic modification of crop species.

AGRONOMICALLY USEFUL GENES

Genetic engineering promises to have an enormous impact on the improvement of crop species. Plant breeding has played a major part in providing plants with better resistance to insect pests and with enhanced yields. Genetic transformation can rapidly accelerate plant breeding efforts for crop protection and enhanced yield. Efforts to identify and transfer genes of agronomic significance are already under way. Some degree of success has already been realized in engineering selective resistance to herbicides and in engineering resistance to viral diseases and insect pests. We present here a summary of the results of these studies with emphasis on our own work.

Herbicide resistance

Several herbicides used in world agriculture today inhibit plant growth by blocking the biosynthesis of essential amino acids. These include: glyphosate, *N*-(phosphonomethyl)-glycine, which inhibits the synthesis of aromatic amino acids; the sulphonylureas and imidazolinones, which block branched-chain amino acid biosynthesis; and phosphinothricin which inhibits glutamine biosynthesis. Detailed knowledge of the sites of inhibition for these herbicides has resulted in preliminary efforts to engineer resistance using gene transfer strategies.

Glyphosate, the active ingredient of the herbicide, Roundup®, is a broad-spectrum non-selective herbicide used to control annual and biennial sedges, grasses and broad-leaved weed species. It is rapidly absorbed by foliar tissues and roots and is quickly translocated to various plant organs. Once inside the plant, glyphosate is not metabolized to a significant degree; in soil, however, the herbicide is rapidly degraded by micro-organisms. The mode of action of glyphosate was first reported in 1972 (Jaworski, 1972). It was found that glyphosate inhibited aromatic amino acid biosynthesis in a bacterium (*Rhizobium japonicum*) and in duckweed (*Lemna gibba*). This conclusion was based on the reversal of glyphosate-induced growth inhibition by aromatic amino acids. Similar reversal of growth inhibition by aromatic amino acids was subsequently reported in *Escherichia coli, Chlamydomonas reinhardtii*, plant-cell cultures and intact plants. Several years later, the shikimate pathway enzyme, 5-*enol*pyruvylshikimate-3-phosphate (EPSP) synthase (3-phosphoshikimate 1-carboxyvinyltransferase; EC 2.5.1.19), involved in aromatic amino acid biosynthesis was identified as the specific target of

this herbicide in bacteria (Steinrücken and Amrhein, 1980). Subsequent studies have shown that glyphosate also inhibits this enzyme in higher plants (Mousdale and Coggins, 1984; Nafziger *et al.*, 1984; Rubin, Gaines and Jansen, 1984). Thus, glyphosate has the same site of action in both bacteria and higher plants. Direct evidence that EPSP synthase is the primary target of the herbicide stems from the observation that, in *E. coli*, amplification of the cloned EPSP synthase gene results in increased tolerance to glyphosate (Rogers *et al.*, 1983). Glyphosate-resistant mutants of *S. typhimurium* (Comai, Sen and Stalker, 1983; Stalker, Hiatt and Comai, 1985), *A. aerogenes* (Schultz, Sost and Amrhein, 1984; Sost, Schultz and Amrhein, 1984), and *E. coli* (Kishore *et al.*, 1986) have been isolated and shown to contain glyphosate-resistant forms of EPSP synthase. Glyphosate-resistant plant-cell cultures have been described that have developed resistance to high concentrations of the herbicide as a result of elevated levels of EPSP synthase (Nafziger *et al.*, 1984; Amrhein, Jöhanning and Smart, 1985; Smart *et al.*, 1985; Smith, Pratt and Thompson, 1986; Steinrücken *et al.*, 1986). Despite some early controversy, it has now become clear, from the studies described below, that EPSP synthase is the primary target of the herbicide in plants.

Initial attempts to engineer glyphosate resistance in transgenic plants have employed two different strategies. In the first study, a mutant gene encoding glyphosate-resistant EPSP synthase was isolated from *S. typhimurium* and was shown to contain a single base pair change resulting in a proline-to-serine amino acid substitution at position 101 of the protein (Comai, Sen and Stalker, 1983; Stalker, Hiatt and Comai, 1985). The isolation of this gene allowed the construction of two chimaeric genes, in one of which the EPSP synthase coding sequence was flanked by the regulatory regions from the octopine synthase gene and in the other of which the coding sequence was flanked by the mannopine synthase (D-mannopine dehydrogenase, EC 1.5.1.–) promoter and *tml* (T-DNA transcript 6B) 3′ region (Comai *et al.*, 1985). The chimaeric genes were then introduced into tobacco cells using Ri plasmid vectors (Ri plasmids are root-inciting plasmids harboured by virulent *A. rhizogenes* strains carrying genes essential for root production) and plants were regenerated from the transformed cells. The expected size chimaeric mRNA and bacterial EPSP synthase protein and level of enzyme activity were detected in the leaves of the transformed plants. The transformed plants carrying the chimaeric genes were two- to threefold more resistant to glyphosate than were control plants.

In the second study, overproduction of the glyphosate-sensitive plant EPSP synthase in transgenic petunia plants was shown to confer substantial resistance to glyphosate (Shah *et al.*, 1986). In this study, the full-length cDNA clone encoding petunia EPSP synthase was isolated from a glyphosate-resistant petunia cell line that overproduces (15- to 20-fold) the herbicide-sensitive form of EPSP synthase. A chimaeric gene was constructed in which the coding sequence of the petunia EPSP synthase precursor enzyme was placed under the control of the cauliflower mosaic virus 35S promoter (*Figure 1*). This promoter directs high-level expression of foreign genes in transformed plants (Odell, Nagy and Chua, 1985; Sanders *et al.*, 1987).

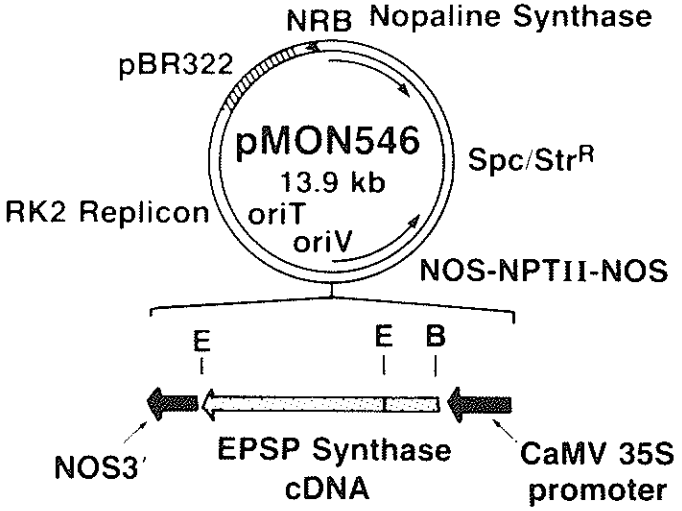

Figure 1. The plant expression vector (pMON546) containing the chimaeric petunia EPSP synthase gene for transformation into petunia cells. This vector contains a full-length EPSP synthase coding sequence flanked by the cauliflower mosaic virus 35S promoter and the polyadenylation signal from the nopaline synthase gene. The EPSP synthase coding sequence encodes the precursor enzyme consisting of 72 amino acids of the transit peptide and 444 amino acids of the mature enzyme. The vector contains the chimaeric neomycin phosphotransferase gene (NOS-NPTII-NOS) as a selectable marker gene and nopaline synthase gene (NOS) as a scorable marker gene. For details on the construction of this vector, refer to the article by Shah *et al.* (1986). E = *Eco*RI; B = *Bgl*II; NRB = nopaline right border sequence.

The chimaeric gene was introduced into petunia cells using the leaf disc transformation method (Horsch *et al.*, 1985). Leaf discs transformed with the chimaeric gene produced calli that grew on medium containing 0·5 mM glyphosate, whereas the control calli lacking the gene did not. Petunia plants containing the chimaeric gene were regenerated from the transformed calli and tested for glyphosate resistance by spraying them with Roundup® herbicide (formulated glyphosate with surfactant) at a dose equal to 0·8 lb/acre (≈0·9 kg/ha). This is two to four times the dose required to kill wild-type petunia plants. All plants containing the chimaeric gene survived glyphosate spraying and grew to maturity (*Figure 2*).

The major difference between the two strategies is that, in the first study, the bacterial enzyme was not targeted to chloroplasts, whereas the second study utilized the transit peptide sequence of 72 amino acids, which is encoded by the full-length cDNA, to target the plant enzyme to chloroplasts. Recent studies have shown that the plant EPSP synthase activity is localized in chloroplasts which represent a major site of aromatic amino-acid biosynthesis (Mousdale and Coggins, 1985). The *in vitro* chloroplast uptake studies further show that the transit peptide is required for the import of the enzyme into chloroplasts (Della-Cioppa *et al.*, 1986). Given that EPSP synthase is normally localized in the chloroplasts, future attempts to enhance the level of herbicide resistance in transgenic plants using glyphosate-resistant EPSP synthase genes must be based on targeting the enzyme to chloroplasts.

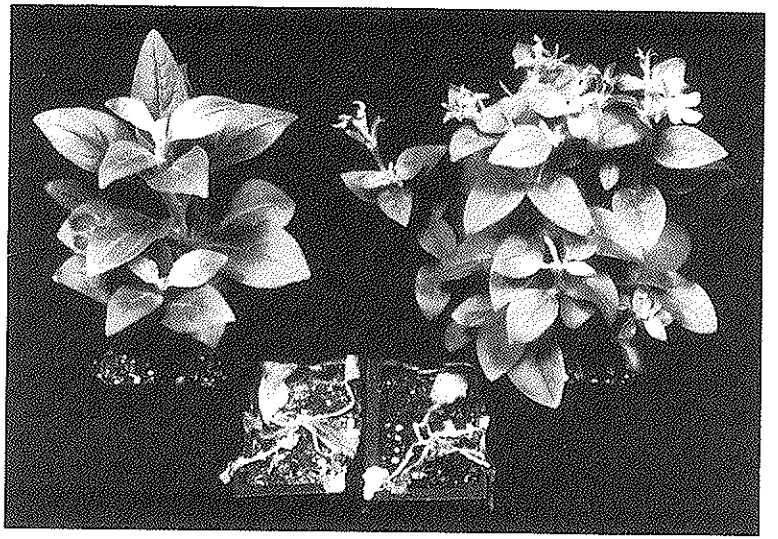

Figure 2. Glyphosate-tolerant transgenic plants. Transgenic plants containing the chimaeric petunia EPSP synthase gene (pMON546) were regenerated from the transformed calli selected for kanamycin resistance. These plants and the wild-type control plants were sprayed with Roundup® (formulated glyphosphate with surfactant) at a dose equal to 0·8 lb/acre (≈0·9 kg/ha). The plants containing the chimaeric gene (upper) survived glyphosate spraying and grew to maturity, while the control plants (lower) stopped growing and died. The photograph shows plants 3 weeks after spraying.

The studies described above represent a major step towards establishing selective resistance to the herbicide glyphosate using gene transfer strategies. There are also reports of using gene transfer methods to engineer selective resistance to sulphonylurea, phosphinothricin and atrazine herbicides in transformed plants. Genetically engineered herbicide resistance may have commercial utility in the near future.

Virus cross-protection

An important application of genetic engineering technology is in producing resistance to pathogens. At present, very little is known about the genetic mechanisms of disease resistance in plants. A possible way to engineer disease resistance would be to isolate the resistance genes from plants that are resistant to a pathogen and to transfer them to susceptible plants. This approach is complicated by the fact that resistance could be a polygenic trait. Even if resistance is encoded by a single gene, identifying and isolating such a gene from the host could be very difficult, largely due to the complexity of the host–pathogen interactions.

Observations made 40–50 years ago pointed to another mechanism for increasing host resistance to pathogens. In 1929 McKinney observed that tobacco plants infected with one strain of a virus resist infection by a second related strain (McKinney, 1929). This phenomenon, termed 'cross-protection', could be demonstrated with a number of viruses for which

distinct strains could be found (reviewed by Hamilton, 1980; Palukaitis and Zaitlin, 1984; Sequeira, 1984; Fulton, 1986).

Cross-protection has been successfully applied in agriculture and has been effective with a number of viral diseases. Some of the examples include protection of citrus plants from citrus tristeza virus, protection of glasshouse-grown tomatoes from tomato mosaic virus and protection of papaya plants from papaya ringspot virus (Fulton, 1986). The use of cross-protection in agriculture, however, is not widespread because of potential drawbacks. Some of these are that it requires isolation and characterization of an appropriate mild strain, which involves considerable effort; a virus present in the plant could react synergistically with another virus or it might mutate to a more severe form; mild protecting virus may spread to other hosts in which its effects may be more severe; inoculating an entire crop is difficult and costly and the mild strain itself might cause a reduction in yield.

Although the mechanisms of cross-protection are not fully understood, several investigators have suggested that protection from viral disease could be achieved with the help of gene transfer methods (Hamilton, 1980; Palukaitis and Zaitlin, 1984; Sequeira, 1984; Beachy *et al.*, 1985b; Sanford and Johnston, 1985). Several research groups have successfully introduced viral genes into plants and have verified their expression (Bevan, Mason and Goelet, 1985; Baulcombe *et al.*, 1986; Powell Abel *et al.*, 1986; Tumer *et al.*, 1987).

Powell Abel *et al.* (1986) constructed an expression vector containing the 35S promoter from cauliflower mosaic virus (CaMV), a cDNA encoding the coat protein gene of the U1 strain of tobacco mosaic virus (TMV) and a polyadenylation signal from the nopaline synthase gene. This vector was introduced into tobacco and tomato cells and plants were regenerated. Progeny of self-fertilized transgenic plants expressing high levels of the coat protein gene (0·05–0·1% of total soluble cell protein) were inoculated with TMV. These plants either did not develop an infection, or disease symptoms developed more slowly than in the control plants, after inoculation with the U1 strain of TMV. The plants expressing the coat protein (+CP) were also delayed in symptom development after inoculation with a severe TMV strain, PV230, which is immunologically related to the U1 strain (Nelson, Powell Abel and Beachy, 1987). The numbers of chlorotic and necrotic local lesions on the inoculated leaves of transgenic *Xanthi* and *Xanthi 'nc'* plants inoculated with PV230 or U1 were 70% and 90% lower, respectively, than the numbers observed on the control plants. As observed in classic cross-protection, the decrease in the numbers of chlorotic and necrotic lesions could be overcome, at least in part, by inoculation with viral RNA. Thus the presence of CP on the challenge virus was necessary for maximum protection to occur (Nelson, Powell Abel and Beachy, 1987).

The phenomenon of genetically engineered cross-protection was extended to another virus, alfalfa mosaic virus (AlMV) (Tumer *et al.*, 1987). AlMV differs from TMV in many respects. TMV is rigid rod-shaped virus which encapsulates a single RNA molecule; it encodes at least four proteins in three open reading frames (Hirth and Richards, 1981). In contrast, AlMV

is a bacilliform virus which has a tripartite genome consisting of RNA molecules 1, 2 and 3. RNA 4, a subgenomic RNA of RNA 3, encodes the coat protein (Jaspars, 1985). Unlike infection with TMV RNA, which does not require coat protein, infection by AlMV RNA requires addition of RNA 4 or coat protein (Bol, Van Vloten-Doting and Jaspars, 1971; Houwing and Jaspars, 1978). Thus, TMV and AlMV are clearly distinguished by their morphology, genome structure, strategies of viral gene expression, early steps in replication and modes of transmission (AlMV is transmitted mechanically and by aphids; TMV is primarily mechanically transmitted).

Tumer *et al.* (1987), have cloned RNA 4 encoding the CP of AlMV and engineered it into an expression vector downstream from the CaMV 35S promoter. The cDNA was flanked at the 3′ end by the nopaline synthase polyadenylation site. Leaf discs of tobacco and tomato were transformed with *A. tumefaciens* containing this construct, selected for kanamycin resistance and regenerated into plants (Horsch *et al.*, 1985; McCormick *et al.*, 1986). The amount of AlMV CP expressed in different transgenic tobacco plants varied between 0·1% and 0·4% of total extractable leaf protein. The amount of AlMV CP expressed in transgenic tomato plants was between 0·1% and 0·8% of the total extractable leaf protein. The segregation ratio of the CP gene was determined in the seedling progeny of the self-fertilized transgenic plants. Progeny from a single transgenic tobacco plant were then inoculated with different concentrations of AlMV (20 μg/ml, 10 μg/ml and 5 μg/ml) and symptom production was monitored in a growth chamber. Typical symptoms of AlMV infection appeared in 3–4 days on the inoculated leaves of the control seedlings (*Figure 3*). Of the seedlings that expressed the AlMV CP, 80% of the plants inoculated with 20 μg/ml of AlMV and 90% of the plants inoculated with 5 and 10 μg/ml of AlMV did not develop symptoms by the end of 2 weeks (*Figure 4*). As expected, decreasing the inoculum concentration decreased the number of plants with (+CP) or without (−CP) the coat protein gene that showed symptoms on the inoculated leaves and increased the delay of symptom production by 1–12 days.

Seedlings which were inoculated with 20 μg/ml and 10 μg/ml crude AlMV suspension were observed over a 45-day period. At the end of 45 days, 81% of the plants which were inoculated with 10 μg/ml of AlMV and 60% of the plants which were inoculated with 20 μg/ml of AlMV did not develop symptoms. The severity of symptoms, in the small percentage of plants that showed any, was much less than those shown by the control plants.

In a similar experiment, the progeny of self-fertilized transgenic tomato plants were inoculated with AlMV. One week after inoculation, plants that expressed the CP gene did not produce any lesions, while the plants that did not have the CP gene and the wild-type plants showed substantially higher numbers of lesions on the inoculated leaves. Most of the plants that did not develop symptoms on the inoculated leaves, did not accumulate the virus in the systemic leaves.

Replication of AlMV and TMV was examined in the inoculated leaves of the AlMV and TMV CP-expressing plants, respectively, by monitoring the virus accumulation (Nelson, Powell Abel and Beachy, 1987; Tumer *et al.*,

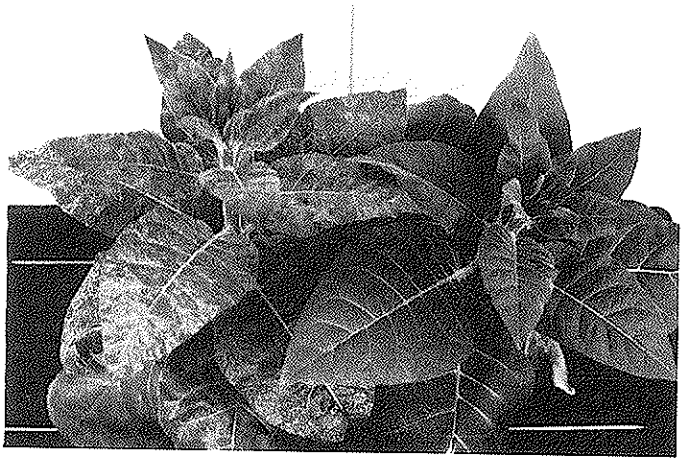

Figure 3. Suppression of AlMV symptoms on transgenic tobacco plants containing the chimaeric AlMV coat protein gene. The photograph shows the upper leaves of a +CP (right) and a −CP (left) tobacco plant four weeks after inoculation with 10 μg/ml AlMV. The upper systemically infected leaves of the −CP plant show typical vein clearing and mosaic symptoms of AlMV infection, whereas the +CP plant does not show any symptoms. +CP = plant containing the coat protein gene; −CP = plant lacking the coat protein gene.

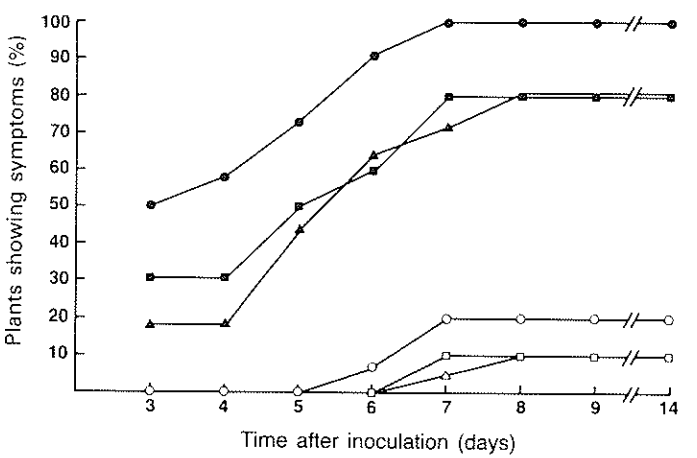

Figure 4. The percentage of plants showing symptoms on inoculated leaves on successive days after inoculation with different concentrations of AlMV. The progeny of one +CP plant were inoculated 6 weeks after planting, with the concentrations of AlMV indicated. Eleven −CP (●) and 15 +CP (○) seedlings were inoculated with 20 μg/ml AlMV; 10 −CP (■) and 23 +CP (□) seedlings were inoculated with 10 μg/ml AlMV; 11 −CP (▲) and 17 +CP (△) seedlings were inoculated with 5 μg/ml AlMV. The seedlings were kept in a growth chamber and symptom development was monitored daily. +CP = progeny, from transgenic plant, that contain the coat protein gene. −CP = progeny, from transgenic plant, that do not contain the coat protein gene.

1987). During the phase of rapid virus replication (3–7 days after inoculation), inoculated leaves of transgenic tobacco plants which expressed the TMV CP gene contained less than 30% of the virus found in the inoculated leaves of the control plants. Transgenic tobacco plants expressing the AlMV CP gene contained levels of AlMV that were at least 100 times lower than those in the control plants. Similar results were obtained with transgenic tomato plants expressing the AlMV CP (Tumer *et al.*, 1987) or TMV CP (R. S. Nelson, P. Powell Abel and R. N. Beachy, unpublished work). These results show good correlation between the decreased amount of replication in the inoculated leaves of the +CP plants and the decrease in the numbers of symptoms observed.

An important question which remains to be answered is whether the protection observed in transgenic plants is the same as that found in plants that are cross-protected against virus infection. Although the answer is not known, the two types of protection show similarities: both types of protection have been demonstrated against RNA viruses; they both result from a delay in disease development. In the case of the transgenic plants, the delay is the result of a 90–100% reduction in the numbers of lesions on the plant. Some plants escape infection completely and, if infection is established, spread of virus is slowed in the transgenic plants. In the case of TMV, protection could be partly overcome both in the transgenic and cross-protected plants by increasing the concentration of the virus in the challenge inoculum. In the case of AlMV, the protection was not overcome with the highest concentration of inoculum used (50 μg/ml). The protection observed against TMV could be overcome, at least in part, in both tomato and tobacco plants if the challenge inoculum contained the virus RNA rather than virions (Nelson, Powell Abel and Beachy, 1987).

The decrease in the number of transgenic plants that became infected following inoculation, and the decrease in the number of local lesions that develop on leaves of +CP plants, support the hypothesis that viral infection is prevented on the inoculated leaves of the transgenic plants. However, the delay observed in the development of lesions on inoculated leaves of the transgenic plants expressing the AlMV CP, and the remaining protection observed in transgenic plants expressing the TMV CP upon challenge by TMV RNA, suggest that additional steps of the viral infection cycle could be blocked.

The protection observed in the transgenic plants expressing the TMV CP is effective against the common strain U1, a severe strain PV 230, as well as several strains of TMV. Cross-protection is also most effective against viral strains that are closely related and, in fact, has been used by plant virologists for virus identification. In transgenic plants, however, there is some degree of protection against a more distantly related strain of TMV, referred to as C_cTMV or SHMV-n (R. S. Nelson and R. N. Beachy, personal communication). It is still too soon to tell whether the protection observed in transgenic plants and that in classic cross-protection result from similar mechanisms. Regardless of the precise mechanism of the resistance observed in transgenic plants, genetically engineered cross-protection provides a generally applicable way of producing virus-resistant plants.

Because plant viral genomes are small and most of the genome is involved in pathogenicity, it should be possible to confer virus resistance by blocking the expression of viral gene products. This could be achieved by antisense inhibition of viral RNA (inhibition of gene expression via formation of stable hybrids between sense and antisense RNAs). Antisense RNA can be produced in transgenic plants and the formation of a stable duplex between complementary RNAs may interfere with the normal function of viral RNA in transgenic plants challenged with the virus. Alternatively, modification of viral genes such as the replicase gene and the expression of the modified gene in plants may confer protection. All of these strategies are approachable at the molecular level with different plant viruses.

Insect resistance

Another application of genetic engineering with important implications for crop improvement has been the production of insect-resistant plants. Progress in engineering insect resistance in transgenic plants has been achieved through the expression in plants of the insect toxin gene of *Bacillus thuringiensis (B.t.)*. An excellent review by Dean (1984) of the biochemical genetics of *B.t.* can be found in volume 2 of this series. *B.t.* is an entomocidal bacterium which produces a parasporal protein crystal. Most strains of *B.t.* are toxic to lepidopteran larvae (Dulmage, 1981), although some strains with toxicity to dipteran (Goldberg and Margalit, 1977) or coleopteran (Krieg *et al.*, 1983; Herrnstadt *et al.*, 1986) larvae have also been described. The insect toxicity of *B.t.* resides in the parasporal protein crystal which, in the case of lepidopteran-active strains, is composed of toxin protein subunits of approximately 130 000 kD (Bulla *et al.*, 1981). Genes encoding the lepidopteran-specific toxins from several strains of *B.t.* have been cloned and sequenced (Adang *et al.*, 1985; Schnepf, Wong and Whiteley, 1985; Shibano *et al.*, 1985; Hofte *et al.*, 1986; Thorne *et al.*, 1986; Wabiko, Raymond and Bulla, 1986; D. A. Fischhoff *et al.*, unpublished work). These genes are largely similar; however, there are significant regions of variability at both the nucleotide sequence and amino-acid sequence level. Deletion variants of several of these genes have been constructed *in vitro* and tested for toxicity after expression in *E. coli* (Adang *et al.*, 1985; Schnepf and Whiteley, 1985; Shibano *et al.*, 1985; Hofte *et al.*, 1986; Wabiko, Raymond and Bulla, 1986; D. A. Fischhoff *et al.*, unpublished work). Taken together, these experiments indicate that the region essential for toxicity resides in the *N*-terminal portion of the protein extending approximately from amino acid 29 to amino acid 610.

D. A. Fischhoff *et al.* (unpublished work) have engineered and expressed in tomato plants a lepidopteran-specific toxin gene from *B.t.* subsp. *kurstaki* HD-1 (Watrud *et al.*, 1985). DNA sequence analysis has shown that this gene is very similar to genes reported from *B.t.* subsp. *berliner* 1715 (Hofte *et al.*, 1986; Wabiko, Raymond and Bulla, 1986). A fragment of this gene encoding amino acids 1–725 was isolated and shown to encode a functional

toxin by expression in *E. coli*. This truncated toxin gene was engineered by the addition of synthetic oligonucleotide linkers and inserted into the expression cassette vector pMON316 (Rogers *et al.*, 1987) at the *Bgl*II site to create pMON9711. In pMON9711 the *B.t.* toxin gene is flanked by the CaMV 35S promoter and the 3'-end of the nopaline synthase gene. pMON9711 also contains a chimaeric neomycin phosphotransferase gene which confers kanamycin resistance on transformed plant cells.

Tomato plants transformed by pMON9711 were recovered by the method of McCormick *et al.* (1986). Tomato leaf discs were transformed by *Agrobacterium tumefaciens* which contained pMON9711 co-integrated into a disarmed Ti plasmid (Fraley *et al.*, 1985). Transformed tomato cells were selected by their ability to grow on kanamycin-containing medium. Callus derived from these transformed cells developed shoots. The shoots were induced to form roots in the presence of kanamycin, and the plants were then transferred to soil. Plants were assayed for expression of the *B.t.* toxin gene by Northern analysis, and a polyadenylated RNA of the size expected of a full-length transcript was detected. The plants were also analysed for toxicity to *Manduca sexta* (tobacco hornworm), an insect which is sensitive to the *B.t.* toxin and is known to feed on tomato leaves. All of the larvae applied to the transgenic plants died within a few days, and the plants showed very little evidence of feeding damage. Larvae applied to control plants survived and eventually consumed the plants completely. These experiments demonstrate the feasibility of producing transgenic plants resistant to some lepidopteran insects through the expression of the *B.t.* toxin gene.

Future prospects for genetically engineering insect-tolerant plants will probably continue to focus on the protein insect toxins from *B.t.*, for several reasons. The *B.t.* toxins show a high degree of specificity: individual toxins are active only against a single order of insects (e.g. Lepidoptera or Coleoptera), and within a given order not all insects are equally sensitive. In spite of this high degree of specificity, the *B.t.* toxins are active against some insect pests of major agronomic importance, such as *Heliothis zea* (corn earworm/cotton bollworm) in the case of lepidopteran-specific toxins and *Leptinotarsa decemlineata* (Colorado potato beetle) and *Anthonomus grandis* (boll weevil) in the case of the coleopteran-specific toxins. The lepidopteran-specific toxins, which have been intensively studied, are considered to be very safe: they have no known activity against mammals, fish or non-target invertebrates.

The *B.t.* toxins represent one of the few known examples of insecticidal proteins. The toxins are active after oral ingestion by sensitive insects, but the mode of action of *B.t.* toxins at the molecular level is not known. Because the toxins are encoded by single genes and retain activity after expression in *E. coli*, they are amenable to study using recombinant DNA techniques. Through analysis of cloned toxin genes and production and expression of variants of these genes, we are likely to gain insight into the mode of action and insect specificity of *B.t.* toxins. Ultimately, it may be possible to increase the activity of the toxins against sensitive insects, as well as to create modified toxins active against pests which are currently insensitive.

Future prospects

Gene transfer technology has provided a powerful new approach to the problem of differential expression of plant genes. Precise localization of the *cis*-acting elements for tissue-specific and environmentally modulated expression of genes has just begun for a small number of plant genes. The next few years will witness a large increase in the number of genes introduced into transgenic plants to study the normal developmental regulatory processes. Progress in the area of the identification of new genes and their controlling elements has been overwhelming. Although the identification of new genes continues, the development of techniques for chromosomal gene replacement, phenotypic inactivation of gene expression, and optimal expression of the introduced genes is needed. Development of novel inducible promoters will permit tightly controlled expression of genes. The prospects for the genetic improvement of crop species through gene transfer are quite promising. The genes for herbicide resistance and disease resistance have been identified and expressed in different plants to obtain the desired phenotypic effects. The practical application of genetic engineering to a broader range of agronomically important problems will clearly depend on the identification of agronomically useful genes, the improvement and broadening of gene transfer techniques, extension of these techniques to other crop species and the ability to control the expression of the introduced genes.

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