80th Annual Meeting
of The American Phytopathological Society
November 14, 1988, San Diego, California

# Applications of Plant Genetic Engineering to Crop Protection

David A. Fischhoff

Monsanto Company, Chesterfield, MO 63198

The work described here summarizes research in plant genetic engineering at Monsanto Company over the past few years, focusing on our work to introduce agronomically important traits into crop plants. Basic plant transformation technology is described and its application to genetic engineering of crop plants is illustrated. In particular, transgenic crop plants that are herbicide tolerant, virus resistant, and insect tolerant are described. The three examples demonstrate the features of and the power of the transformation and genetic engineering approach in creating new plant varieties with improved characteristics. The three traits have utility in crop protection from three types of pests: weeds, viruses, and insects. One of these, genetically engineered resistance to plant viruses, is of great interest to APS members and is the subject of many presentations at the APS annual meeting. Based on these examples, it is clearly possible to extrapolate to many other traits of agronomic utility, including resistance to a wide variety of plant pathogens.

## Plant Transformation

Ability to transform a wide range of plants depends on a plant pathogen, *Agrobacterium tumefaciens*, the casual agent of crown gall disease of plants. The bacterium causes disease by a novel mechanism that is an example of natural genetic engineering. *Agrobacterium* carries a large plasmid, Ti, that contains a segment of DNA known as T-DNA. When *Agrobacterium* infects a plant cell, the T-DNA is transferred from the Ti plasmid into the plant cell where it integrates at random into the plant chromosomes. The T-DNA carries genes for the biosynthesis of phytohormones, and these genes have evolved to function efficiently in plants. It is the production of plant hormones by the action of the T-DNA genes that leads to uncontrolled plant cell growth or the gall. The transfer of the T-DNA is under control of Ti plasmid genes (*vir* genes) that are separate from the T-DNA. Transfer requires only short DNA sequences (borders) at the ends of the T-DNA and the *vir* genes. Because of this, it was possible to "disarm" the T-DNA by removing the phytohormone biosynthetic genes while still leaving intact the ability of *Agrobacterium* to transfer DNA into plant chromosomes. Using techniques of microbial genetic engineering, the phytohormone biosynthetic genes were deleted from the interior of the T-DNA, removing the disease-causing ability while leaving just one of the border sequences intact on the Ti plasmid.

A system was also developed that permitted the manipulation of genes in *Escherichia coli*, where recombinant DNA techniques are most easily performed, followed by the transfer of the genes into disarmed *Agrobacterium*. The system involves the use of intermediate plasmid vectors that have several noteworthy features. These vectors contain DNA sequences that allow them to replicate in *E. coli* and other DNA sequences that allow the vector to be mobilized and transferred by bacterial conjugation into *Agrobacterium*. The vectors also contain a T-DNA border and a region that allows homologous recombination with the disarmed T-DNA in *Agrobacterium*. This recombination creates a novel, functional T-DNA containing the intermediate vector flanked by two borders. This novel T-DNA is capable of transfer to plants under the control of the *Agrobacterium vir* genes.

Because the novel T-DNA contains the intermediate vector, any gene can be manipulated in *E. coli*, prepared for expression in plants and transferred to plants via *Agrobacterium*. In order to facilitate expression of genes in plants, we have developed a series of cassette vectors that contain plant expressible promoters to initiate mRNA transcription coupled with plant gene 3′ ends containing signals for messenger RNA termination and polyadenylation. Between the promoter and the 3′ end is a series of restriction enzyme cleavage sites into which genes can be inserted placing them under the control of the plant promoter. One promoter that has been used for expression of all three traits described below is the 35S promoter derived from cauliflower mosaic virus (CaMV). This promoter is expressed at a high level in plant cells, and it is expressed well in most plant tissues. Genes from any source can be inserted in these cassettes for expression in plants. In the examples described below the genes for the traits of interest have been derived from plants, viruses, and bacteria.

Once T-DNA has been transferred into plant cells, it is necessary to have a method for selecting those plants cells that have taken up and integrated the DNA into their genome. For this, a selectable marker gene, resistance to the antibiotic kanamycin, was engineered for plant expression and placed on the intermediate vector. The product of this gene, neomycin phosphotransferase, detoxifies kanamycin and allows transformed plant cells to grow on medium containing concentrations of kanamycin that are lethal to nontransformed cells. The kanamycin-selected cells all contain the intermediate vector including the gene of interest, but each transformation event involves the random insertion of the T-DNA into a different location in the plant genome. The difference in location of the T-DNA lead to different levels of expression of the same gene in independent transformation events. This is known as the position effect. Because of the position effect, it is sometimes necessary to screen multiple independent transgenic plants to recover one in which the trait is expressed at useful levels.

To achieve a useful system for the production of transgenic plants, it is necessary to couple the *Agrobacterium* T-DNA transfer process to explant systems capable of regeneration of whole plants. An example of this is the leaf disk transformation process developed for petunia, tobacco, tomato, and other plant species. In this system, small disks are punched from a leaf and mixed with liquid *Agrobacterium* cultures to allow infection and DNA transfer to take place. When the leaf disks are incubated on growth medium containing kanamycin, only the transformed cells in the disk grow and form kanamycin-resistant callus tissue. The selected transformed callus tissue can then be induced to regenerate yielding trangenic plants.

This general system is efficient and can be tailored to match the explant and regeneration requirements of a variety of plant species. With this approach, more than a dozen plant species have been transformed to yield transgenic plants including tobacco, tomato, potato, lettuce, alfalfa, flax, oil seed rape, cotton and soybean. Some of these species such as tobacco and tomato are relatively easy to transform and yield large numbers of independent transformants, whereas other species such as soybean and cotton are more difficult. There are likely to be substantial improvements in transformation efficiency in all of the major crop species in the

coming years.

Several other crops are noteable by their absence from this list, especially the monocots such as corn, wheat, and rice. This absence is due to the biological specificity of *Agrobacterium*. *Agrobacterium* can infect a variety of dicot plant species, but it is essentially not infectious on monocots. For monocot transformation, recent work has focused on direct DNA delivery into potentially regenerable protoplasts or explants using methods such as electroporation or particle guns. There have been preliminary reports of successful production of transgenic rice and corn plants derived from direct DNA delivery procedures. We can look forward to even more rapid progress in monocot tranformation in the future.

### Herbicide Tolerance

Glyphosate is the active ingredient in Roundup herbicide. Glyphosate is a broad spectrum, potent, postemergent herbicide with unusually attractive environmental qualities and effectiveness. It does not enter groundwater, is rapidly degraded in soil, and yet effectively controls a large variety of weeds. It is a very attractive herbicide for expanded use as a weed control agent in crops, but its broad spectrum means that most crop plants are too sensitive to glyphosate. Genetic engineering of glyphosate tolerance into plants would allow additional crop uses of this herbicide.

Glyphosate acts by inhibiting a key enzyme, known as EPSP sythase or EPSPS, in the aromatic amino acid biosynthetic pathway in plants. Synthesis of aromatic amino acids is essential for plant growth. A key discovery in our efforts to engineer glyphosate tolerance came from the observation that a petunia cell culture could be selected for growth on normally inhibitory concentrations of glyphosate. Analysis of the resistant cell line showed that the resistance was due to the overproduction of the EPSPS mRNA and enzyme. A similar discovery had been made with the analogous bacterial gene, *aroA*, in *E. coli*. The approach to engineering glyphosate tolerance into plants was based on mimicking this discovery by deliberately overproducing the EPSPS enzyme in plants.

The gene for EPSPS was isolated from petunia and analyzed. It was discovered that the EPSPS enzyme contained at its N-terminus a transit peptide that directs the EPSPS protein synthesized in the cytoplasm into the chloroplasts where the enzyme functions in amino acid biosynthesis. The petunia EPSPS gene including the transit peptide was engineered to be expressed from the CaMV 35S promoter. Transgenic petunia, tobacco, and tomato plants containing the engineered EPSPS gene were recovered and tested for tolerance to glyphosate. These plants overproduced EPSPS based on enzyme assays, and this overproduction was responsible for a significant degree of tolerance to glyphosate compared to nontransformed control plants. The engineered plants survived and showed only minor damage under conditions where the control plants were killed by the herbicide.

However, in some species the plants that overexpressed the petunia EPSPS were not totally protected from herbicide damage at typical field use rates, so an alternative approach to engineering glyphosate tolerance was also utilized. It has proven possible to isolate variants of the EPSPS enzyme that have intrinsic tolerance to inhibition by glyphosate. The petunia EPSPS gene was engineered to encode one variant enzyme. This variant enzyme is many times more tolerant to glyphosate inhibition than the wild type EPSPS. The variant gene was also engineered in the CaMV 35S promoter cassette and used to transform plants. Plants producing the variant enzyme are highly tolerant to spray by Roundup.

We have field tested herbicide-tolerant plants during the past 2 years. Tomato plants were tested in Illinois and oil seed rape plants were tested in Canada. In all field tests we have found that the laboratory results are duplicated in the field. That is, the engineered crops in the field showed a significant level of tolerance to Roundup under conditions where weeds were effectively controlled by the herbicide.

### Virus Resistance

It has long been known in plant pathology that infection of a plant by a mild strain of a virus can often protect the plant from superinfection by a more virulent strain. This phenomenon of cross-protection has had applications in plant protection; however, the mechanism by which it works is uncertain. With Professor Roger Beachy of Washington University, we observed that expression in tobacco plants of the coat protein of tobacco mosaic virus (TMV) protected transgenic plants from infection by TMV. The protection resulted in dramatically reduced levels of virus and in a substantial reduction and delay in symptom development in transgenic plants compared to wild type controls. TMV coat protein and several other viral coat proteins have been cloned from plant RNA viruses as cDNA copies and then inserted into the standard plant expression cassettes. We and others have demonstrated resistance to infection conferred by expression of the homologous viral coat protein for at least six viruses from several families.

Tomato plants were engineered to express TMV coat protein, and these plants were field tested over the past 2 years. Inoculation with TMV led to infection of essentially all control plants in the field, while transgenic plants remained nearly symptom free and virus free. Depending on the inoculating strain, infection of control plants by TMV caused up to 60% yield loss. The protected transgenic plants showed no yield decrease after infection.

We have extended our work to include some major viral diseases in other crops. Potato has been genetically engineered for resistance to potato virus X. Viral diseases are currently controlled in potato through the control of insect vectors of some viruses and through the seed certification process. In the certification process, seed potatoes infected by potato virus X and some other viruses are identified and removed from commerce. Viral resistance could be an important adjunct to current agricultural practices in potato. The coat protein gene of potato virus X was cloned and engineered behind the CaMV 35S promoter for plant expression. This gene was used to transform Russet Burbank potato, the single largest variety grown in the United States. Plants expressing potato virus X coat protein were recovered and challenged by inoculation with potato virus X. Even at the highest dose tested, where 100% of the control plants showed virus production and symptoms, the transgenic plants were essentially free of virus.

### Insect Tolerance

Tolerance to damaging insect pests is another agronomic trait where there is a good fit with plant genetic engineering technology. Our approach to engineering of insect tolerance is based on plant expression of genes from bacteria—the insect control protein genes from *Bacillus thuringiensis*. *B. thuringiensis* is a naturally occurring insect pathogenic soil microbe. Upon sporulation, *B. thuringiensis* produces a protein crystal composed of subunits of a single protein, the insect control protein. This crystal protein is a potent oral insecticide and is responsible for the insecticidal activity of *B. thuringiensis*. Preparations of *B. thuringiensis* spores and crystals have been sold as commercial insecticides for many years.

Most strains of *B. thuringiensis* are active against larvae of lepidopteran insects, the caterpillar larvae of moths and butterflies. Lepidoptera that are sensitive to the action of the *B. thuringiensis* proteins include some important agronomic pests such as cotton bollworm and pink bollworm, European corn borer, and beet armyworm. *B. thuringiensis* proteins are potent but highly specific insecticides; no nontarget organisms (invertebrate or vertebrate) are sensitive. The proteins are also highly selective among insects. For example, the lepidopteran active proteins do not affect any other order of insect, and even among lepidoptera, some species are much more sensitive than others. Because they are single polypeptides, the *B. thuringiensis* proteins are encoded by single genes. These attributes have made expression of *B. thuringiensis*

genes a technically feasible approach for engineering selective insect tolerance into crop plants.

We cloned the gene for a lepidopteran-specific protein from *B. thuringiensis* var. *kurstaki* HD1 (*B.t.k.*), the strain that is used in one of the commercial microbial insecticides. This gene encodes a 130 kDa protein that is insecticidal when expressed in *E. coli*. Using the *E. coli* expression system deletion variants of the gene were analyzed. It was found that only the N-terminal half of the native *B.t.k.* protein was needed for insecticidal activity. Truncated active *B.t.k.* genes encoding about 50–60% of the full length protein were generated in *E. coli*, engineered for plant expression in the CaMV 35S promoter cassette, and used to transform tomato plants.

The transgenic tomato plants were challenged with larvae of tobacco hornworm, which is sensitive to *B.t.k.* After larval feeding for several days, the control plants were completely defoliated. In contrast, on the transgenic plants no hornworm larvae survived and there was very little evidence of feeding damage. The absence of feeding damage is due to the mode of action of *B.t.k.* protein. *B.t.k.* protein acts on the midgut of sensitive insects causing a disruption of the midgut cells. One of the earliest symptoms of action of *B.t.k.* is cessation of feeding by treated insects leading to the low levels of damage observed on the transgenic plants.

These insect-tolerant plants have also been field tested, and the field results confirm or surpass greenhouse experience. Insect-tolerant plants in the field were infested with tobacco hornworm larvae and again showed complete protection under conditions where the controls were defoliated. In addition, the plants were infested with tomato fruitworm. (*Heliothis zea* is also known as the cotton bollworm and the corn earworm). Fruitworm is a major agronomic pest in tomato, but it is substantially less sensitive than hornworm to *B.t.k.* After fruitworm infestation, 18–23% of the tomato fruits on control plants had holes caused by fruitworm; such holes make the tomatoes unmarketable. The transgenic plants showed only 4–8% fruit damage, a substantial level of control. Work is underway to optimize insect control via *B.t.k.* expression and to apply this approach to additional crop species.

In order to extend the potential of genetically engineered insect tolerance to additional pest targets, it is necessary to have insect control proteins active against a broad range of insects. As mentioned above, the majority of *B. thuringiensis* strains are active against lepidopterans; however, in the past few years *B. thuringiensis* strains active against coleopterans (beetles) have been isolated. We have cloned the gene from one of the coleopteran active strains, *B. thuringiensis* var. *tenebrionis* (*B.t.t.*). This gene produces a protein of 60 kDa that is structurally quite distinct from the lepidopterans-specific *B. thuringiensis* proteins. The *B.t.t.* protein has no activity against lepidopterans, but it is quite active against Colorado potato beetle, a major pest of potato. The *B.t.t.* gene has also been engineered for plant expression and used to transform both tomato and potato. Biochemical assays and insect feeding assays of engineered plants of both species showed that the *B.t.t.* gene is expressed, and the plants show substantial tolerance to Colorado potato beetle.

## Summary

The successful genetic engineering into crop plants of tolerance to the herbicide glyphosate, resistance to plant viruses and tolerance to certain insect pests has been described. There are many features common to these three examples. All three traits are encoded by single genes. Al three utilized the CaMV 35S promoter cassette for plant gene expression. And, transgenic plants expressing the three traits were produced via *Agrobacterium*-mediated transformation. However, the three traits were engineered with genes from diverse sources (plants, plant viruses, and bacteria), and each of the three has presented unique challenges in terms of gene isolation and optimization of gene expression. These examples can be considered a paradigm for the engineering of other traits of agronomic interest. A premium is clearly placed on having a sufficient understanding of the biology and biochemistry of the plant-pest system to allow for the identification of proteins important to the trait of interest. This knowledge forms the basis for gene isolation and analysis, engineering for plant expression, and eventual testing of transgenic plants. It is obvious from the above that genes derived from a variety of organisms can be successfully engineered to function in plants. In the future we can expect to see similar approaches applied to other agronomic traits including those of interest to plant pathologists, such as resistance to fungal and bacterial plant pathogens. We will also begin to approach more complex multigenic traits such as yield and food quality.