

Genetic modification of plants: significant issues and hurdles to success¹⁻³

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ABSTRACT Transformation and regeneration is routine for many crop plants. A genetically engineered tomato with a longer shelf life at full ripeness was introduced in the United States in 1994, and other soon-to-be-released products, both foods and fibers, incorporate genes for resistance to pests, diseases, and environmentally benign herbicides. Other possibilities are altered plant fats and oils, methionine- and lysine-enhanced grain and legume proteins, plant foods that can deliver immunizing antigens, and other ways of controlling fruit ripening. Food safety concerns include the inadvertent production of toxicants and allergens. Foreign DNA can be introduced into plants by bacterial vectors, direct uptake by protoplasts, and mechanical introduction on metal particles or other materials. Limitations include little or no control of copy number or site of integration of the introduced DNA, dependence on selectable markers for recovery of traits, and inadequate knowledge of how to control key metabolic steps to maximize desirable traits. Directed genetic change still requires conventional crop breeding to deliver benefits to farmers and consumers. *Am J Clin Nutr* 1996;63:651S-6S.

KEY WORDS Plant transformation, genetically engineered foods, allergens, toxicants, plant breeding, fatty acids, phytase

INTRODUCTION

Ever since the discoveries of Mendel, geneticists have been interested in the prospect of directed genetic change. Conventional plant breeding always requires progeny large enough to recover forms that recombine the desired features from the parents of a cross. Recombination results from random segregation due to chromosome reassortment and from crossing-over during gamete formation at meiosis. The gametes, pollen and egg cells, fuse randomly to form fertilized eggs, or zygotes, that develop into seeds. Hundreds of different crosses are made each year in large-scale crop-breeding programs, and individual segregating (F_2) populations of ≥ 1000 of each cross are grown in successive years for selecting recombinants in later generations. In a major program of breeding winter wheat in the United Kingdom, several miles of single-row segregating families are grown each year to recover desirable forms for selection and evaluation. The number of families is progressively reduced by selection in each succeeding generation and the number of the plants of each family increases so that after 3 or 4 y yield trials are performed, first in small plots and then in larger plots in different locations.

After World War II there was some interest in producing mutations by exposing seeds to ionizing radiation. This was followed by treatment with chemical mutagens. Much time and effort was spent in searching for useful variants but few were found. Most of the mutants were inferior in vigor and in other respects compared with the original parental material.

PLANT TRANSFORMATION

The discovery of DNA transformation in bacteria by Avery et al (1) in 1944 and the realization that genetic information is encoded in the nucleotide base sequence of DNA suggested that it might be possible to transform higher organisms with DNA-coding sequences to effect directed genetic changes. One of the first successful methods harnessed a circular DNA molecule, or plasmid, carried by the pathogenic plant bacterium *Agrobacterium tumefaciens*. The bacterium invades wounds in plant tissue, where it stimulates rapid host cell growth, resulting in the production of a plant tumor or gall. This occurs because a tumor-inducing (Ti) segment of the plasmid DNA becomes integrated in a chromosome of the host cell nucleus. Deleting the genes that control the production of tumor cells from the plasmid leaves right and left border fragments, each 24 nucleotides long, that mark the ends of the integrating Ti segment. This segment of bacterial plasmid DNA can be used as a vector to introduce foreign DNA. If a bacteriophage lambda *cos* site is included in the vector, up to ≈ 40 kb of DNA can be packaged in vitro between the borders. Transformation vectors include marker genes that can be detected in bacterial and plant host cells and controlling elements, or promoters, to drive the expression of the desired gene in plant tissue at the appropriate phase of development.

A drawback of the Ti vector system is that it is restricted to plants that are hosts for *Ag. tumefaciens*. Another method uses protoplasts that are prepared by treating plant cells with enzymes that dissolve cell walls. Held in a suitable osmoticum, such as a 4-mol/L solution of mannitol, they are stable and do not burst. The protoplasts are treated with a solution of transforming DNA, some of which is taken up and may become

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integrated in the host cell genome. The uptake of DNA can be increased by treatment with polyethylene glycol or by electrical disruption of the protoplast membrane. The protoplasts must form new cell walls as the osmoticum is gradually replaced with culture medium. Embryogenic cell lines are selected from the resulting unorganized callus that is formed.

The biolistic, or gene gun, method uses very small DNA-coated metal particles (gold or tungsten) that are rapidly accelerated toward a target of plant cells or tissues. The particles penetrate the plant cell walls and some enter the cell nuclei. In a small proportion of these cells the introduced DNA becomes integrated in a host chromosome. A selectable marker is needed to recover putative transformants.

USES OF PLANT TRANSFORMATION

In the context of food-crop improvement several benefits may be achieved by strain modification. High yields reduce the costs of raw materials, quality improvements can reduce processing costs, and agronomic characteristics such as herbicide tolerance and resistance to pests and diseases may reduce both farm costs and agrochemical residues in raw materials. Novel characteristics that add value to raw materials can be introduced by transformation.

Herbicide tolerance

Research on the mechanisms of herbicide toxicity led to the isolation of genes that encode target enzymes that are not inhibited by the agent. Incorporating these specific genes into crops makes it possible to use nonselective herbicides with desirable qualities such as a short half-life in the environment and very low or no toxicity to nontarget organisms, especially mammals, on a wide range of crops. The herbicide Roundup (glyphosate; Monsanto, St Louis) inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase, which is involved in the synthesis of aromatic amino acids in the chloroplast. Until a gene for glyphosate tolerance was available, Roundup was used to keep land free from plant growth or to prepare soil for crop cultivation. It is rapidly decomposed in the soil and thus has little residual toxicity. Genes for herbicide tolerance provide an alternative to chemical screening tests for identifying selective herbicides (2).

The sulfonyl urea and imidazolinone herbicides interfere with amino acid biosynthesis in plants by inhibiting the enzyme acetohydroxyacid synthase (AHAS), which is responsible for the synthesis of leucine and valine. Mutations at two different sites in the AHAS gene create enzyme forms that are resistant to both types of herbicides (3). Phosphinothricin (PPT) inhibits glutamine synthetase in plants. The *bar* gene, from *Streptomyces hygroscopicus*, encodes an enzyme, phosphinothricin acetyltransferase, that converts PPT to a nontoxic derivative, thus conferring resistance to PPT herbicides in transgenic plants (4).

An alternative method of creating herbicide-tolerant crop forms is to select herbicide-tolerant mutants from plant cell tissue cultures. Herbicide-tolerant corn lines recovered from tissue culture mutants were used to make F₁ hybrids tolerant to the herbicide Pursuit (American Cyanamid Co, Princeton, NJ) (5).

Pest resistance

The development of transformation as a breeding tool has increased interest in identifying genes for resistance to pests and diseases. Attempts to clone the resistance genes already in use by breeders proved to be unexpectedly difficult and breakthroughs have occurred only within the past year (6).

The soil bacterium *Bacillus thuringiensis* forms a delta endotoxin protein. Ingested by the larva of a sensitive insect, the toxin induces a loss of the semipermeability of the intestinal wall so that hemolymph leaks into the intestinal cavity (7). The larva loses fluids and dies. Several endotoxin proteins have been identified that are active specifically against different groups of insects. The cryI protein, active against many lepidopteran larvae, was first used as the biological insecticide BT. Suspensions of *B. thuringiensis* resting cells containing the cryI protein were shown to be safe and effective in large-scale applications over many years. Expressed in transgenic crops, the *Bt* gene that encodes the cryI protein is effective in controlling the larvae of hornworm, cabbage looper, diamondback moth, and leaf miner. Because the toxin gene came from a prokaryote, the codon usage had to be adjusted to maximize expression in a eukaryote.

Transgenic plants that express *Bt* genes may have a short life in agriculture because of the strong selection pressure they exert for insects that are insensitive to the endotoxin protein. Resistance to BT was reported in diamondback moths after extensive use of this insecticide (8). Special management practices such as using several different control methods together and reducing the level of expression of the *Bt* gene or the numbers of plants carrying it may be necessary if BT is to remain a useful biological control agent.

The seeds of an exotic collection of cowpea germplasm that is unusually resistant to the larvae of moths that attack stored grain were found to contain a trypsin inhibitor. The gene that encodes this inhibitor was isolated and cloned from the cowpea accession and was introduced into transgenic plants (9). By interfering with the digestion of the plant proteins ingested by insect larvae feeding on the plants, transgenic plants that express this gene show moderately high resistance. Trypsin inhibitors and other protease inhibitors are commonly found in food and feed crops.

Virus resistance

Many plant viruses are transmitted by insects that feed by sucking plant sap. In 1986 it was reported that when the coat protein gene for the RNA virus that causes tobacco mosaic was introduced into the host plant its expression interfered with the replication and systemic spread of the virus, making the plant resistant (10). This phenomenon has also been observed in other plant viruses and their hosts (11). Naturally occurring virus resistance is uncommon, and the discovery showed much promise. The first commercial application of this discovery is the use of viral coat protein genes of cucumber mosaic, zucchini yellow, and watermelon mosaic viruses in squash (12). The engineered squash contains viral coat proteins that account for <0.1% of the total protein of the squash fruit. This compares favorably with the somewhat greater virus content of commonly consumed fruit from plants that are naturally infected with virus.



Other forms of virus resistance include viral genes that when expressed in plants interfere with replication of the viral genome or impede virus movement from cell to cell (13, 14). The pokeweed plant produces several ribosomal inhibitor proteins. One of these expressed in transgenic plants protects them against a broad range of viruses (15).

Resistance to pathogenic fungi

Many fungal pathogens have cell walls made of chitin. Novel transgenic plants that express bean or *Serratia marcescens* chitinase genes have been reported to show resistance (16). Four genes for resistance to different plant pathogens were recently cloned from higher plants (6). Their structural similarity—all had regions of DNA with repeat lysine encoding motifs—suggests that they have a role in signaling biochemical defense responses. An earlier example was of a corn gene that appears to encode the structure of a membrane receptor protein to which a fungal pathotoxin binds (17). General methods for recovering and redeploying such genes will help to reduce our dependence on pesticides.

FOOD APPLICATIONS

Controlled ripening

The first food product from this recombinant DNA technology, genetically engineered chymosin (rennin), is widely used in Europe and the United States for cheese making. The first genetically engineered food plant to be introduced in the United States, the Flavr Savr tomato (Calgene, Davis, CA), was released early in 1994. These tomatoes incorporate a Ti vector that carries an antisense form of the tomato gene that encodes polygalacturonase (PG). Produced during fruit ripening, this enzyme breaks down pectin, the cement that holds the cells of the fruit tissue together, and is responsible for fruit softening. The PG antisense gene reduces the mRNA transcript formed and hence the amount of PG. The result is a fruit with a longer shelf life at full ripeness. Because transformation is inefficient and very few treated cells are transformed, it is necessary to have a marker to select cells that carry integrated and expressed foreign DNA. The Ti vector thus carries a gene that encodes neomycin phosphotransferase II, which confers resistance to the antibiotic kanamycin. Kanamycin kills untransformed tomato cells. The kanamycin resistance gene, driven by a constitutive promoter, is always expressed. The PG antisense gene is expressed only after flowering and fertilization, late in plant development. The Food and Drug Administration concluded that the expression of the neomycin phosphotransferase II gene is of no significance for consumers of the fruit.

An alternative means of delaying fruit ripening is by cosuppression. The addition of sense copies of a gene by transformation often results in the failure of the resident gene to be expressed. The mechanism of cosuppression is not fully understood. Cosuppression is also being used to delay ripening in tomatoes, a process that is regulated by the production of ethylene. In this case, additional copies of the gene that encodes the enzyme 1-aminocyclopropane-1-carboxylate synthase, which is important in ethylene synthesis, blocks ethylene production by cosuppression. The produce industry routinely triggers the ripening of fruit that are harvested before they are ripe by treating them with ethylene gas in storage.

Antisense, cosuppression, or both can also be used to control the amounts of other materials that plants produce. The candidates include raffinose and stachyose, oligosaccharides in legume seeds (beans and soybean) that are responsible for flatulence. Naturally occurring variants of the bean (*Phaseolus vulgaris*) with low concentrations of stachyose have been used to produce bean cultivars that cause less flatulence.

Phytase production

Genetic engineering promises improvements in the nutritional value of livestock feed. Phytate (myoinositol hexaphosphate) is the principal storage form of phosphorus in plant seeds. Inorganic phosphate is released from phytate by an endogenous enzyme, phytase, at seed germination. However, when plant seeds are fed to monogastric animals, such as pigs and poultry, these animals cannot use phytate and thus cannot access the phosphorus. After the oil has been extracted from oilseed rape (canola) in Europe the meal is used as animal feed with inorganic phosphate added as a supplement. Phytase, prepared from *Aspergillus niger*, can replace the phosphate supplement (18). A gene from *As. niger* that encodes the enzyme was used to transform tobacco with an agrobacterium vector (19). Phytase-expressing plants were normal in growth and development and their seeds were enriched in phytase. Seeds from transgenic tobacco were tested in feeding trials with male broiler chicks. This supplemented diet was compared with diets supplemented with and without inorganic phosphate and *As. niger* phytase. Growth in animals fed diets containing milled transgenic phytase-expressing seeds was significantly greater than that in animals fed control seeds or diets without supplementation. The production of transgenic oilseed rape and soybean that constitutively overexpress phytase in their seeds is now under way. Other work has shown that phytase added to soil increases the availability of phosphorus to plant roots (20). These findings suggest that the expression of the enzyme in the roots of transgenic plants may have a similar effect.

Reducing phytate in soybean and other vegetable products may play a role in human nutrition. Phytate is an effective chelator of divalent cations, such as iron and zinc. Consuming protein sources that contain relatively high concentrations of phytate results in decreased mineral bioavailability, and consuming vegetable proteins with low concentrations of phytate promotes mineral absorption.

Phytochelatin

Another class of plant compounds with potential importance in human nutrition is the phytochelatin, a class of inducible peptides that bind metals and may determine the availability from plant foods of trace metals such as zinc and copper that are important in human nutrition. However, phytochelatin is also active in the uptake of toxic heavy metals. Phytochelatin contains four to six repeated units of γ -glutamyl cystine. Recent research has shown that cadmium sensitivity in *Arabidopsis thaliana* is caused by mutations that reduce the synthesis of glutathione, the substrate for phytochelatin biosynthesis (21). This suggests that selection for greater cadmium sensitivity might provide a method for reducing unwanted phytochelatin in food plants, thereby reducing their heavy metal content.

Expression of antigens

The ability of gut mucosal cells to respond to antigens (22) has prompted attempts to create inexpensive oral vaccines by expressing antigens in transgenic food plants. Mason et al (23) showed that tobacco that was transformed with a gene encoding hepatitis B surface antigen expressed this protein in its leaves in a form that was antigenically similar to that in human serum. Because so much of our food is cooked and because heating may degrade the antigenic proteins, this approach will have limitations for human vaccination. There is also the prospect of engineering forage for livestock so that cow milk is induced to include antibodies that are effective against diseases of human infants, but, again, heating to sterilize or process such milk may render the antibodies ineffective.

Edible plant oils

Vegetable oils consist largely of triacylglycerols and their compositions vary considerably among genera and species. Plant breeding has exploited this variation to produce a variety of improved forms. The composition of canola oil was changed by breeding and selection to reduce the high amounts of erucic acid (22:1) that characterized early varieties. At the same time, glucosinolates (goitrogenic glycosides) were removed from canola seed so that the meal left after oil extraction in a crushing mill has greater value as livestock feed. In recent reviews Somerville (24) and Ohlrogge (25) described increasing the value of plant edible oils in human nutrition by using cloned genes to alter the activities of specific enzymes responsible for lipid and fatty acid biosynthesis. The most common plant fatty acids have chain lengths of 16 or 18 carbon atoms and from 1 to 3 double bonds. They are liquid at room temperature, and for making margarine, in which a higher melting temperature is required, the double bonds must be reduced by hydrogenation. However, this not only increases the saturated fat content of the oil but also converts many of the double bonds from the *cis* to the less desirable *trans* configuration. Because the enzyme steryl-acyl carrier protein desaturase introduces the first double bond, it has been a target for antisense RNA. This approach was successful in two species of *Brassica* (*B. napus* and *B. rapa*) in which the content of stearic acid, which has no double bonds (18:0), in the seed oil was increased from 2% to 40%.

Although vegetable oils have less saturated fatty acid than do animal fats, the 10–20% (depending on species) that they do contain is a target for further reduction by molecular biology. The major saturated fatty acid in plants is palmitic acid (16:0). One approach depends on manipulating a branch point in fatty acid synthesis in which either the precursor palmitoyl-acyl carrier protein is elongated to form stearic acid or the acyl carrier protein is released to form palmitic acid. A reduction in the palmitic acid content was achieved by overexpressing the enzyme that is responsible for elongation. Another successful approach was to use cosuppression to reduce the enzyme that releases palmitic acid.

Hypoallergenic rice

Rice grains contain a 16-kDa globulin protein that is heat-stable and that resists proteolytic enzymes in the human gut. This protein is the cause of an ectopic dermatitis in sensitive Japanese children. The allergen can be destroyed by enzyme treatment, but the cost

is excessive. Scientists recovered mutants in which the amount of allergenic protein was reduced after the rice seeds had been treated with a chemical mutagen (26). In one mutant with good agronomic qualities the allergenic protein content was reduced by $\approx 50\%$. Two other mutants with trace amounts of the allergen were largely sterile and were of little use in breeding and seed production. The allergen has been characterized and the gene that encodes it sequenced (27) so it is theoretically possible to produce transgenic rice with an antisense gene or a cosuppressing construct that would be hypoallergenic.

Seed proteins

Soybean and canola seed storage proteins, although important in human and livestock nutrition, could be of more value if their balance of amino acids were improved by increasing the methionine content. Altenbach et al (28) used a gene from Brazil nut that encodes a 2S storage protein with a methionine content of 18% and introduced it by transformation into canola. It increased the methionine content of the seed meal by $>30\%$. The same gene expressed in the seeds of transgenic soybean also increased their nutritional quality but, unfortunately, made them allergenic to people who are sensitive to Brazil nuts (29). Evidently, the 2S protein is a major allergen in Brazil nuts. In view of this, it seems unlikely that the transgenic soybean will be commercialized in its present form.

Much attention is now paid in wheat breeding to the genes that encode high-molecular weight glutens and gliadins in the grain endosperm. Some of these proteins are responsible for the viscoelastic properties of doughs for making bread. Some progress has been made in cloning and expressing genes for low-molecular weight wheat storage proteins in tobacco seed (30). Engineering high-molecular weight proteins in wheat has yet to be achieved but once done offers prospects of improving the flour of other cereals, such as sorghum and millet, that are important in some developing countries.

A CRUCIFEROUS WEED AS A CROP PLANT ANALOGUE

Many of the spectacular advances made in molecular biology in the past 25 y have depended on the availability of a wide variety of model organisms, such as *Escherichia coli*, yeast, mice, *Caenorhabditis elegans* (a nonparasitic nematode), and corn. During this time there has been growing interest in the small cruciferous weed *Ar. thaliana*. This plant has a haploid number of four chromosomes and a DNA content of $\approx 0.1 \times 10^9$ nucleotide bases. Because this is much lower than the DNA content of crop plants, which is up to 100 times greater, there is a concerted effort to sequence the entire genome of this plant. *Ar. thaliana* has other advantages: it completes its life cycle from seed to seed in ≈ 6 wk, each flower produces several hundred seeds, and large plant populations can be grown in growth chambers or greenhouses in little space (31, 32). It is now an important tool for research in plant molecular biology. Genes of interest may be more readily identified and cloned from *Ar. thaliana* than from a crop plant. These genes may also be altered or their expression adjusted before they are used in an economically important plant. *Ar. thaliana* is thus a crop plant analogue that makes it possible for molecular biologists to try out systems that can later be applied to economic

plants (33). One of the first examples of such a use was the isolation of the acetolactate synthase gene from *Ar. thaliana* followed by the recovery of mutant forms that are insensitive to the herbicide imidazolinone. The resultant gene for tolerance could then be used to confer tolerance to unrelated crops after it had been introduced by transformation.

CONCLUSIONS

Biotechnology is opening up many new opportunities for crop-plant development, but many problems still have to be solved. The acceptance of new forms by the general public has been reviewed by several sociologists (34). There are encouraging indications from surveys that public anxiety and concerns about the safety of food products are declining; however, it is still unclear how widely the products of genetic engineering will be accepted. Innate conservatism on the part of farmers and the food-processing industry will also slow widespread adoption.

New products include crop plants with tolerance to environmentally benign herbicides and resistance to insect pests and viruses, fruit with delayed ripening, and oil seeds with improvements in the balance of fatty acids. Other possibilities include food plants resistant to fungal pathogens, foods that express antigens that can immunize consumers through the gastrointestinal tract, hypoallergenic rice, and seeds that express phytase before germination to make stored phosphate available to monogastric animals that are fed them. Care must be taken to ensure that no allergenic proteins are introduced inadvertently by engineering. Some of these developments owe much to advances made in studies of the small weed *Ar. thaliana*, which has promise as a crop plant analogue.

New crops that make claims to enhance health and nutrition will encounter regulatory barriers to commercialization. Industry is unwilling to pay for raw materials that have been improved if there are cheaper, alternative means of adjusting the quality of feed stocks. The costs of high-value specialty chemicals and pharmaceuticals will likely be reduced by using engineered crops to produce them.

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