Annu. Rev. Nutr.

IMPACT OF PLANT GENETIC ENGINEERING ON FOODS AND NUTRITION

Luca Comai
Department of Botany KB-15, University of Washington, Seattle, Washington 98195

KEY WORDS: biotechnology, transformation, safety, storage, lipids, starch

CONTENTS

INTRODUCTION

For the purpose of this review I define genetic engineering as the manipulation of plant genomes via the introduction of a characterized DNA segment. The novel genetic information in the introduced DNA will either specify a new protein or alter the expression level of an endogenous gene. This technology differs from somaclonal variation, protoplast fusion, selection of mutants in tissue culture, and other novel technologies, in that, although grouped under plant biotechnology, these techniques are not based on the introduction of defined DNA. I am limiting my review to genetic engineering because it is

0199-9885/93/0715-0191$02.00

responsible for the most impressive advances. Readers interested in the other technologies and their products should consult existing reviews (25, 71, 77).

During the last ten years plant genetic engineering has progressed to the point where most important crop species can now be manipulated (29–31). This new and powerful approach can improve agronomic and quality traits such as nutritional value, composition, flavor, and storage ability. The imminent inclusion of an increasing number of genetically engineered plant products in our diet has raised public awareness; people wonder whether this is a safe technology (16, 103). Safety concerns also interest nutrition experts who want to learn the scientific principles of this technology as well as the potential benefits and risks (54). This review is aimed primarily at the latter group although it should help inform the concerned public.

I have divided this review into three sections. In the first, I address the technological basis of plant genetic engineering; I emphasize its potential and limitations and explain the technical details needed to evaluate safety. In the second part, I review the progress made in quality traits improvement of plant products. I have not dealt in depth with agronomic improvements, since a number of exhaustive reviews have already been published on this topic (6, 9, 29–31, 48a, 69, 110). In the last section, I discuss the safety of genetically engineered foods, including agronomic improvements.

THE TECHNOLOGIES OF PLANT GENETIC ENGINEERING

Plant Transformation

Transformation is the process of introducing DNA into the genome of an organism. Although transient transformation can occur, for the purpose of this review I am only concerned with stable, i.e. inheritable, transformation. Three approaches are routinely used to transform crop plants; the chosen method depends on the target crop. The most common plant transformation method, and probably the preferred one because of its simplicity, employs *Agrobacterium tumefaciens* (49). This bacterium is able to introduce a segment of DNA, called the T-DNA (tumor DNA), into the plant nuclear genome. The wild type *Agrobacterium* T-DNA is a plant pathogenic element, since it carries genes for plant hormone production. Expression of these genes induces proliferation of transformed cells and results in tumor growth. However, in T-DNA-based vectors all *Agrobacterium* genes have been removed, leaving only regions of DNA needed in *cis* for transfer and called borders. The borders define the start and end of the T-DNA. The T-DNA used to engineer plants is usually 5 to 10 kb, and up to 50 kb in size, with the capacity to encode 2 to 20 genes. It is borne on a plasmid capable of transfer from *Escherichia*

coli to *Agrobacterium*. It typically carries an antibiotic resistance gene for selection of transformed cells, called a selectable marker gene. The most common selectable marker confers resistance to the aminoglycoside antibiotic kanamycin and encodes the bacterial enzyme neomycin phosphotransferase (7, 50). Other genes for resistance to antibiotics or herbicides can be used for selection (22, 109). The T-DNA also carries one or rarely two additional genes. The nature of this additional gene varies according to the objective of each project.

For *Agrobacterium*-mediated plant transformation, a portion of plant material (explant) is exposed to *Agrobacterium* carrying the modified T-DNA. Proliferation and regeneration of the explant cells is induced by hormonal treatment in the presence of an antibiotic toxic to wild-type plant cells. Transformed cells expressing antibiotic resistance have a selective advantage and can regenerate into plants. Integration of the T-DNA in each transformed plant is most often at a single locus (chromosomal location), sometimes at two loci, and rarely at three or more loci (23). At each locus, one or more copies of the T-DNA can be found (68, 114).

Genetic mapping of T-DNAs inserted in plant chromosomes indicates that integration can occur at disparate positions in the genome. It is unlikely, however, that the choice of insertion sites is truly random. Rather, evidence from several laboratories indicates that insertion of the T-DNA occurs preferentially in or near genes, regardless of how much additional DNA is present in the target genome. This evidence is provided by gene fusions obtained by T-DNA insertion (64, 114). To detect gene fusions one places a promoterless reporter gene (a gene encoding an easily assayable product and lacking the region responsible for its transcription) in the proper orientation next to one terminus of the T-DNA. Upon insertion of this type of modified T-DNAs, transcriptional signals in the flanking plant DNA activated the promoterless gene in 25% of the resulting transformants. The frequency of these events is far greater than what would be expected by random insertion. Furthermore, several reports have described inactivation of genes by the T-DNA (114). Therefore, the T-DNA can function as an insertion mutagen.

Most monocotyledonous plants, like maize, wheat, and banana, cannot yet be transformed with *Agrobacterium*-based vectors (49). Instead, alternative methods using naked DNA transformation are necessary (19, 87, 88). Plants can stably incorporate exogenous DNA in their genome. However, since the cell wall prevents the entry of DNA into the cell, it must be bypassed. There are two approaches. In the first, plant cells are treated with cell wall-digesting enzymes to remove the cell wall (77). The resulting protoplasts are susceptible to transformation, although stably transformed cells are rare and must be selected. In addition, they must regenerate to form a plant, a process which is not possible with many species (77). The second approach consists of

accelerating DNA-coated microscopic metal particles and impacting target cells at high velocity (90). Since the microprojectiles penetrate cell walls and deposit DNA inside cells, disparate cell types can be targeted. For example, if the microprojectiles hit the growing apex of a plant, a few rare cells will be transformed. If a transformed cell produces pollen or eggs in its lineage, a fraction of the progeny will be transformed. DNA introduced by micro-bombardment or protoplast transformation is inserted in apparently random and usually single loci as a few copies or a single copy per insertion site (15, 33, 91).

The use of T-DNA vectors or protoplast transformation requires that a selectable marker be present in the transforming DNA (49). A few highly regenerable crops, like tobacco, constitute an exception to the rule, in that one can, by patient screening, identify rare transformed plants among those regenerated from *Agrobacterium*-treated explants. The same requirement for a selectable marker applies to techniques where DNA is introduced in cultured regenerable cells. On the other hand, one does not need to incorporate a selectable marker in micro-bombardment of meristems, since this technique generates chimeric plants (composed of transformed and nontransformed tissue) and does not allow selection of the primary transformant (15). One can identify rare transformants among the progeny of bombarded plants by several screening approaches for DNA, RNA, or protein, or, if a selectable marker was used, one has the option to select transformed plants. Genes whose products can be easily assayed are useful detectable markers. A popular one is the *uidA* gene of *E. coli* encoding the enzyme beta-glucuronidase (Gus), which allows a sensitive and convenient histochemical assay (53).

Gene Expression

Plants follow a different developmental strategy from metazoans. Briefly, three basic tissues (epidermal, cortical, and ground) originate from groups of initial cells called meristems (105). The three tissues make up all organs, such as leaves, roots, petals, and fruits. Despite the underlying identities of these tissues, each organ expresses, in addition to a common set, a specialized set of genes. This specialization continues down to the cellular level. For example, unique sets of genes are expressed in specific cell types of anthers (63), the organs producing pollen. Cell specificity is usually conferred by *cis*-acting regulatory regions in the promoter, the region responsible for transcription initiation (8). Accordingly, foreign proteins can be expressed in an organ or in a subset of cells within that organ by fusing the coding region of their genes to a tissue- or cell-specific promoter. Although some transgenes (genes introduced by transformation) can function abnormally, properly expressed ones can be readily identified (60, 67). Transgenes are stable for many generations and probably indefinitely, behaving in every respect as native

genes (74). Occasionally, difficulties are encountered in achieving sufficient expression of a foreign protein. The problem can be caused by incompatibility of the mRNA structure, of codon usage, or of the protein itself with the biochemical environment of the plant. For example, it was necessary to change the DNA sequence of a *Bacillus thuringiensis* insecticidal protein gene to achieve an expression level sufficient for insect control (84). Gene expression problems can be very serious. Often, failure to raise the expression level of a transgene prevents progress of a project from proof of concept stage to full implementation.

Gene Inactivation

The ability to remove a protein is as important as the ability to add a new protein. A gene product can be removed by conventional mutagenesis but, although there are examples of crop varieties derived by this approach, this is not always possible because of screening or mutagenesis limitations or because the same protein is encoded by more than one gene.

Genetic engineers working with fungi and mice have developed homology targeted gene inactivation, or replacement, to precisely and completely knock out target genes (26, 57). Regrettably, this approach has yet to be developed for plants although some progress has been described (43, 80, 81). Another technology, antisense RNA, has been successfully used for suppressing gene expression in plants (70). Antisense RNA refers to the transcription product of a gene whose coding region was inverted with respect to the promoter and termination regions and reintroduced into the plant. The antisense RNA molecule is complementary to the true mRNA produced by the endogenous gene. By a yet uncharacterized interaction, possibly the formation of a double-stranded RNA, the endogenous gene is suppressed in a fraction of transformants. A range of suppression phenotypes, from none to very strong, is found in different transformants and is probably caused by the influence of different neighboring chromosomal regions on the transgene. In most antisense RNA experiments reported there was no observable phenotype until expression of the target protein was decreased below 5 to 10% of wild type level. Since such a decrease can only be observed in a fraction of transformants and one is never certain of achieving complete suppression of the target locus, antisense RNA technology requires careful screening of transformed plants and is intrinsically more difficult to interpret than homology-targeted gene inactivation. On the other hand, antisense RNA suppression is effective even when multiple related genes encode identical enzymes.

Another approach to gene inactivation is called cosuppression and its basis is not understood. Simply stated, homology between an introduced gene and a resident one can lead, under certain circumstances, to mutual inactivation of the genes (56, 67). Because of developmental and epigenetic instability

and because of its mysterious basis, it has not yet been incorporated in market-targeted genetic engineering.

GENETIC ENGINEERING FOR ENHANCED QUALITY

Storage Ability of the Fresh Market Tomato

All fresh plant products are stored between harvest and consumption. The length of storage and the quality of the product at the end of storage depend on the physiology of the stored produce. Quality loss occurs due to overmaturation or senescence, pathogenesis, and water and temperature stress. Overcoming any of these problems would increase the product's market value and deliver a higher quality product to the consumer. The fresh market tomato is a classic example. Most tomatoes are harvested when hard and green, allowing about two weeks for transit and storage. After maturation, the tomato can only be kept on display shelves for about a week. Premature harvesting prevents the full accumulation of sugars and organic acids, which are important flavor components (40). Controlling some maturation processes or delaying the onset of maturation would allow harvest after the proper amounts of sugars and acids have been reached, prolonged storage, and sale of a better product.

Maturation of the tomato fruit is a dramatic process (10, 40). It initiates with ethylene gas production followed by a number of responses including the production of cell wall-degrading enzymes, the synthesis of lycopene red pigments, increased respiration, the development of a distinct aroma, and numerous other cellular and biochemical changes. Until recently, causal relationships in the process were not known, including whether ethylene was the maturation trigger and what enzymes were responsible for softening of the fruit.

The first attempt to alter the maturation process was via suppression of the enzyme polygalacturonase (95, 99). Polygalacturonase was thought to participate in the softening of the ripe fruit by degrading pectin, a glue-like polymer, in the middle lamella, a structure connecting adjacent cells. Dissolution of the middle lamella would prevent adhesion between cells and result in tissue softening. To engineer a reduced level of polygalacturonase, its coding sequence was fused in an antisense orientation to a strong viral promoter, CaMV 35S, which is expressed in most plant tissues, and introduced into the tomato. Transformants exhibited a range of polygalacturonase levels in their mature fruits from wild type to below detection. There were no phenotypic effects in fruits with polygalacturonase levels higher than 10% of wild type. However, plants with very large reductions in polygalacturonase had lower pectin depolymerization in mature fruit and had juices with greater consistency

and viscosity (39, 89). Two different groups disagree on the effect of decreased polygalacturonase on fruit softening. Smith et al (99) reported no difference in compressibility of wild type and engineered fruits exhibiting less than 1% of wild type polygalacturonase activity, while Redenbaugh et al (89) found highly significant reduction in compressibility of engineered tomatoes that had decreased polygalacturonase levels comparable to those reported by Smith et al. The two groups transformed different tomato varieties; this may account for the experimental discrepancies. Both groups, however, agree that the engineered tomato fruits were more resistant to mechanical damage and rotting by molds. There were no other phenotypic effects of polygalacturonase reduction on maturation. These studies indicate that polygalacturonase does not catalyze a crucial step of the maturation process and that softening of the tomato fruits is probably mediated by the concerted action of several cell wall-degrading enzymes, including polygalacturonase. In fact, ectopic expression of polygalacturonase in green, ripening-impaired mutant tomatoes was not sufficient to induce softening (35). While the changes in polygalacturonase-suppressed fruits were not large, they were nonetheless remarkable. Reduced softening and the increase in resistance to mechanical damage and to fungal rot will prove useful in allowing more efficient storage and distribution of vine-ripened tomatoes.

An alternative and possibly more powerful approach to engineering tomatoes for improved storage suppresses a step necessary for the initiation of ripening. The best suppression candidate was ethylene production, since its onset closely precedes ripening. Ethylene is synthesized in two steps from the precursor S-adenosylmethionine (SAM). Conversion of SAM into 1-aminocyclopropane-1-carboxylic acid (ACC) is catalyzed by ACC synthase; conversion of ACC into ethylene is catalyzed by ACC oxidase, also called ethylene-forming enzyme (117). To reduce ethylene one could either suppress a step in the biosynthesis or shunt or quench a biosynthetic intermediate. Oeller et al (79) introduced an antisense ACC synthase gene into tomato and found a transformant in which ACC synthase was severely repressed and ethylene evolution was less than 0.5% of the wild type. This plant's fruits had virtually arrested all major ripening responses including color and aroma development, softening, and increased respiration. Addition of exogenous ethylene restored the ripening process, leading to mature properties indistinguishable from normal fruits. The arrest in maturation demonstrated that ethylene is not merely associated with the onset of ripening but is a necessary signal. An attractive feature of manipulating ethylene production is that by choosing transformants with different levels of ACC synthase suppression it should be possible to obtain maturation phenotypes suitable for different marketing applications.

Hamilton et al (44) obtained suppression of ethylene formation by inhibiting

the last step of the pathway. They engineered tomatoes with antisense expression of ACC oxidase and showed that ethylene synthesis and ripening were largely inhibited although not to the level obtained by Oeller et al. Klee et al (61) took a quenching approach to ethylene suppression. They cloned a gene encoding ACC deaminase from a soil *Pseudomonas*. This enzyme degrades ACC to alpha-ketobutyric acid. Plants expressing the ACC deaminase gene were inhibited in ethylene synthesis and ripening. This approach transfers from crop to crop more easily than antisense RNA, since the latter approach needs a high degree of nucleotide sequence similarity between antisense and target genes. Therefore, an antisense DNA construct must contain target sequences from the species to be engineered, thus requiring the isolation of the target gene from each new species chosen for engineering. The ACC deaminase does not have such a requirement and, as long as expression can be achieved in the new species, it will function in any genetic background. An enzyme converting ACC to malonyl-ACC has been described and could be used in a strategy similar to the one employed with ACC deaminase (61).

There are several available approaches to manipulating tomato ripening. The antisense engineering of ACC synthase has produced the most striking laboratory results and, in general, manipulation of ethylene biosynthesis seems more promising than manipulation of downstream responses. However, it is difficult to predict which strategy will impact the consumer market most. Product development and the connected engineering optimization may reveal unexpected strengths or weaknesses. Tomatoes engineered with antisense polygalacturonase are the first genetically engineered product that will appear on the consumer market and are discussed further in the section on safety.

Cold and Freeze Tolerance

Engineered cold or freeze tolerance in plants would apply to three problems. Firstly, losses due to cold and freeze damage often occur in the field in sensitive commodities such as citrus and strawberries. In addition, losses can occur during transport of the harvested product by accidental exposure to cold temperatures. Secondly, refrigeration (above 0 C) could retard spoilage in storage but its use may be prevented by sensitivity of the stored product. Thirdly, ice crystal formation and the resulting loss of membrane and cell wall integrity compromises the quality of some plant products that are marketed frozen.

Cold tolerance is developmentally induced in cold-tolerant plants by a process called acclimation during which several biochemical changes are observed (38, 41, 66). One change, found both in animals and in plants, is the production of organic compounds and antifreeze proteins (45), which lower the freezing temperature of tissue liquids. A well-characterized antifreeze

protein about fifty amino acids in length is produced by the winter flounder (21, 72) and, interestingly, proteins with related properties have been described in plants and are good candidates for key factors of acclimation (108). Georges et al (32) expressed a fusion between chloramphenicol acetyl transferase and a synthetic gene encoding the flounder antifreeze protein in maize tissue culture cells. Hightower et al (46) described the transformation of tomato and tobacco with a chimeric gene encoding a fusion protein between staphylococcal protein A and the winter flounder antifreeze protein. They observed that tissue extracts, in which this protein could be detected immunologically, displayed a reduction in ice crystal formation. Transformation of a gene encoding the native winter flounder protein did not result in a detectable protein, which suggests that the flounder gene or protein is not compatible with some aspect of plant biochemistry. In general, expression in plants of small heterologous proteins, such as the antifreeze protein, seems to be difficult.

Information on the biochemical basis of cold tolerance is insufficient to predict whether a single change, namely the introduction of an antifreeze protein, will increase cold tolerance or keeping quality. A number of changes are associated with acclimation (18, 34) and some of the genes induced by acclimation have been cloned (42, 65). However, it is not known whether the encoded products are synergistic or even necessary for the phenotype. Mutation or antisense RNA analysis should test the contribution of each biochemical change to the cold tolerance phenotype. For example, increased desaturation of membrane lipids is also important to the establishment of cold tolerance (75).

Plant Lipids

Plant lipids, major components of the human diet, are obtained from storage organs of different crop plants and vary in composition depending on the source (100). Even broader variations are found among wild species. Contrary to what is seen in storage lipids, there is much less variation in membrane lipids, probably because of functional constraints. The variety of storage oils in different plants suggests that there should be no intrinsic barrier to changing the composition of oils by genetic engineering. To do so, one must ascertain what enzymes determine the composition of an oil. In the last ten years, biochemical and genetic studies have furthered the understanding of lipid synthesis, identifying the enzymes most likely to determine composition of a storage oil (52, 98, 100). The genes encoding these enzymes are being cloned, manipulated, introduced into oil crops, and expressed in tissues that synthesize storage lipids.

The length and degree of fatty acid unsaturation in a storage oil triglyceride determines its suitability for different applications. Fatty acid unsaturation in

storage lipids and membranes can be altered by mutations. Plant breeders are using a mutated sunflower line with storage lipids high in C18:1 fatty acids and a soybean line high in C18:0 (37). However, genetic engineering is preferable to mutation breeding because it would allow introduction of these desirable characteristics in any oil crop.

Knutzon et al (62) engineered a high stearate phenotype in rapeseed by an antisense RNA approach. Stearoyl-acyl carrier protein (stearoyl-ACP) desaturase is a soluble chloroplast enzyme that introduces a double bond in stearoyl-ACP (C18:0), converting it to oleyl-ACP (C18:1). The coding region of the gene (93, 107) for rapeseed desaturase was fused in an inverted position to the napin storage protein promoter and to the promoter of a seed-specific acyl carrier protein gene, and then introduced into rapeseed. The resulting production of antisense desaturase RNA was limited to the cotyledons (embryonic leaves), where both storage proteins and oils accumulate in the seed. The antisense RNA effectively suppressed expression of desaturase and caused the accumulation of stearate in storage triglycerides. Whereas little stearate is found in rapeseed oil, some of the antisense engineered plants had increased stearate up to 40% of total fatty acids with a corresponding decrease in oleate. A smaller but reproducible increase in longer chain fatty acids was also observed. It is not yet known whether this change affected the membrane composition of the seedling. Other changes were noted as well. In *Brassica rapa,* transformants exhibiting the highest repression of desaturase were impaired in total storage lipid accumulation and in germination. In *B. napus,* however, a comparable high stearate phenotype was not affected in germination and total lipid accumulation. These differences could be caused by the amount of desaturase suppression (complete suppression may be undesirable) or by the genetic background of the engineered plant. Whatever the cause, these observations highlight the general need to optimize the expression of engineered genes, as well as the importance of breeding a crop variety subsequent to its engineering. This experiment provided the first example of engineering plant oil composition. The high stearate oil may prove useful for production of margarine and cocoa butter substitutes.

Desaturation beyond the delta 9 position is catalyzed by membrane-bound desaturases that act on lipid esters rather than on ACP or CoA esters. Conversion of monoenoic into dienoic and further into trienoic fatty esters occurs both in the chloroplast and on the endoplasmic reticulum. However, triacylglycerols are mainly derived from the latter pathway. *Arabidopsis* mutants affected in the reticular desaturases result in major decreases not only in polyunsaturated fatty acids in storage lipids but also in the membranes of the whole plant. However, most oilseed crops appear to have seed specific desaturases that, if inactivated by mutations, reduce unsaturated fatty acid only in the storage oil and in the seed membranes without affecting the whole

plant. The recent cloning of the fad3 gene (4), encoding the Δ 15 desaturase (C18:2 to C18:3), should allow manipulations of its level in oil crops to engineer low or high C18:3 oils. In combination with the fad2 gene (100), encoding the Δ 12 desaturase (C18:1 to C18:2), the fad3 gene may allow the engineering of C18:1 oils.

Yet another approach to altering the level of unsaturated fatty acids in membrane lipids was demonstrated by Murata et al (75), who introduced and expressed in tobacco the squash and *Arabidopsis* genes for the plastid glycerol-3-phosphate acyltransferase (51). This enzyme is responsible for choosing and esterifying fatty acids to the glycerol backbone. The transferase specificity differs from squash to *Arabidopsis* to tobacco. Overexpression of the squash enzyme in tobacco resulted in a large decrease in unsaturated fatty acids, while overexpression of the *Arabidopsis* enzyme resulted in a small but significant increase in the level of unsaturated fatty acids in membrane lipids and an associated increase in cold tolerance. Engineering of different transferases by itself, or in combination with an altered desaturation pathway, is a promising approach to altering storage oils composition.

Another key characteristic of fatty acids is their length, which varies from C8 to C24 in the storage lipids of different species. The enzymatic mechanism responsible for short-chain fatty-acid production was recently elucidated. Pollard et al (85) and Davies et al (20) demonstrated the presence in developing California bay seed of a thioesterase specific for C12:0 ACP esters. The appearance of the thioesterase coincided with the onset of accumulation of C10 and C12 acyl groups in storage triglycerides. Voelker et al (112) proved that this thioesterase is responsible for the accumulation of short-chain fatty acids in triglycerides by cloning the gene and expressing it in the developing seed of *Arabidopsis* where it caused the accumulation of C12 storage lipids. While the interest in a C12-rich oil is mainly for chemical feed stock, it would also have applications in confectionery production. The isolation and engineering of thioesterases with different specificities from wild plants promises to provide broad flexibility in the design of commercial oils with various chain length fatty acids.

The field of storage lipid modification via the engineering of the fatty acid biosynthesis enzymes promises to be a very fertile one that will contribute to production of healthier foods as well as to production of chemical feed stocks (76).

From Sugars to Starch and Back

Sugars and starches are the direct products of photosynthesis and constitute major flavor and nutritional components of foods. Sugar is produced from assimilated CO_2 and stored temporarily as starch in leaf chloroplasts (the source). At night, starch is depolymerized and sugars are translocated to the

cytoplasm where sucrose is synthesized and exported through a vascular tissue called phloem to storage organs (the sink), which, depending on the plant, could be fruits, seeds, tubers, or roots. Storage organs convert sucrose to starch, or, in the case of grasses, into fructans (86).

The path of carbon interconversion from sugars to starch and vice versa and its interorgan transport is regulated at several steps (Figure 1). Plant physiologists formulated models explaining the role of different enzymes in the physiological network of sugar distribution from source to sink organs (47). Molecular biologists have recently tested these models by manipulating the properties and expression of the involved enzymes, in most cases confirming their proposed role (102). An unexpected finding is that plants are more tolerant than expected of altered sugar balances.

There are two major aims in manipulating sugars and starches: changes in

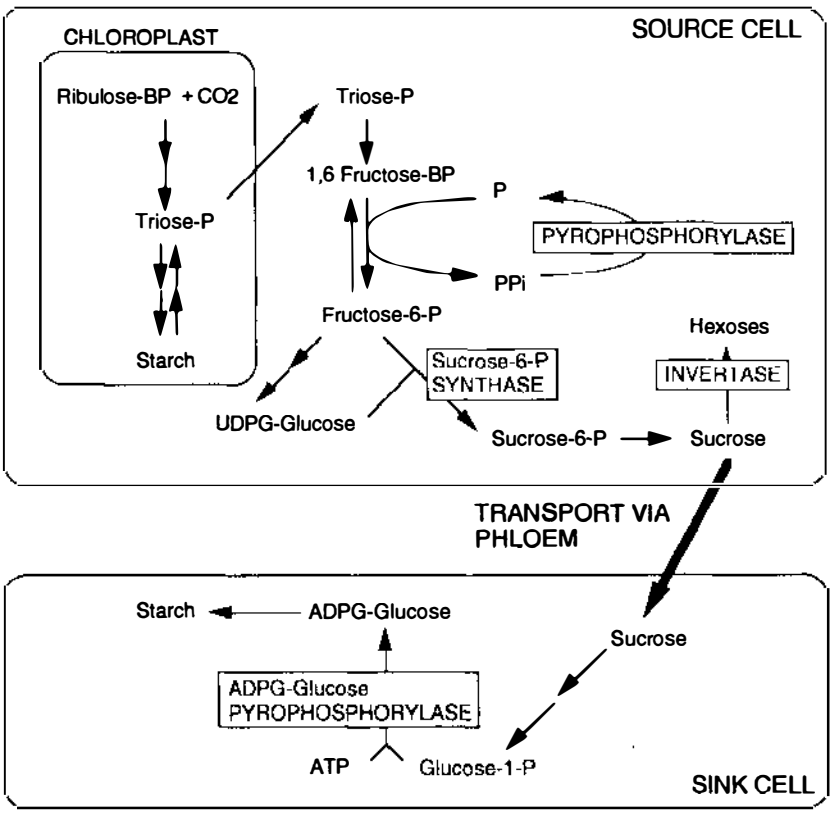

Figure 1 Interconversion and transport of assimilated carbon in plants.

concentration and changes in composition. I first review the changes in concentration.

Can sugars be increased in source tissues? Scientists reasoned that shifting the equilibrium of carbon flux toward sucrose synthesis in the source may result in an overall increase of sugars. Sucrose-P synthase converts UDPG-glucose to sucrose-6-phosphate, the direct precursor of sucrose, and it is tightly regulated in wild-type plants. Worrell et al (116) expressed the maize sucrose-P synthase gene in tomato leaves, doubling the total enzyme activity. As a result, in the engineered plants leaf starch was reduced and the sucrose level doubled. Sonnewald (101) decreased the cytosolic level of pyrophosphate in tobacco and potato leaves by expressing the *E. coli* inorganic pyrophophatase gene. Since PPi is generated during the conversion of fructose-1,6-bisP to fructose-6P, it was hoped that by decreasing PPi one could shift the reaction equilibrium toward fructose-6P and, ultimately, sucrose. Indeed, both tobacco and potato showed an increased sugar/starch ratio but they differed in how it was achieved. In tobacco, sucrose increased up to 12-fold, glucose up to 68-fold, and starch up to 8-fold. In potato, sucrose increased 2-fold and starch decreased.

Can starch be increased in source organs? Presumably, this could be achieved by interrupting the flow of sucrose out of the leaf. Von Schaewen et al (113) and Dickinson et al (24) expressed a yeast invertase in cell walls of tobacco and tomato leaves and found that it strongly reduced the export of sucrose and that it increased starch concentration in the leaves. Thus, sucrose was the main mobile form of photosynthate and its degradation to exoses prevented its transport to sink organs.

Can starch be increased in sink organs? Starch is synthesized using ADP-glucose as a substrate. The synthesis of ADP-glucose, catalyzed by ADP-glucose pyrophosphorylase, is believed to be a rate-limiting step. Decreasing the expression of ADPG pyrophosphorylase in potato tuber by antisense RNA expression diminished the sink strength of these organs and starch accumulation (73). A mutant *E. coli* ADPG pyrophosphorylase is not subject to allosteric regulation by inorganic phosphate, contrary to the plant enzyme. Expression of an amyloplast-targeted *E. coli* pyrophosphorylase in potato tubers resulted in a dry matter increase, mostly starch, of 4% (102a). These experiments, taken together, illustrate how the source-sink relationships within a plant can be manipulated by acting at different steps of a synthetic and transport pathway. A few of the phenotypes described were associated with changes in growth habit or chlorophyll accumulation and may not be compatible with agricultural productivity. However, it is conceivable that these approaches will evolve in reliable and advantageous alterations of sugars and starch content in key crops.

Changes in composition of storage carbon could affect many characteristics

of foods, including flavor and processing qualities. Changes as simple as the branching pattern of starch have important effects. There are two types of starch synthases in plants: One synthesizes a linear starch polymer (amylose) while the other synthesizes the branched polymer (amylopectin). Visser et al (111) repressed synthesis of the granule-bound starch synthase in potato by antisense RNA technology. Since this synthase is responsible for the synthesis of amylose, antisense engineered potato accumulated amylopectin. Knockout of amylose synthesis had already been achieved by mutation. However, antisense engineering established that manipulation of the two synthases should be a productive approach to obtaining variable ratios of linear to branched starch.

Changes in compositions caused by the synthesis of other polymers are also possible by introducing foreign enzymes. Bacteria produce a staggering variety of carbohydrate polymers, some of which are commonly used in food processing. Oakes et al (78) engineered production of cyclodextrins in potato tubers by expressing cyclodextrin glycosyltransferase from *Klebsiella*. Although the enzyme level was undetectable by direct assays, a measurable amount of cyclodextrins was found in the engineered tubers. Potato, being the quintessential starch crop, is a major target of genetic engineering manipulations. The prospects and potential for this important crop were recently reviewed by Vayda & Belknap (110).

A third type of composition change was described by Pen et al (82), who engineered *Bacillus licheniformis* α-amylase accumulation in the cell wall of tobacco. Since starch accumulates inside the cell, the amylase was physically separated from its substrate during the life of the plant. Contact between amylase and starch could be achieved by disrupting cell integrity, as when grinding the tissue, and starch depolymerization would occur. Compartmentation of an enzyme away from its substrate would be an effective strategy to prevent a deleterious reaction from taking place during plant growth and to initiate it during processing.

A last approach related to modifying sugar composition is to express sweet proteins in plants. Thaumatin and monellin are plant proteins that bind to taste receptors and induce a sweet response at very low concentrations. The genes for these proteins have been expressed in potato and tomato and are reported to increase the sweetness of the test tissues when they accumulated at levels of 10^{-6} to 10^{-7} M (10 to 50 μg per gram fresh weight) (83, 115). While these works demonstrate the feasibility of expressing sweet proteins in heterologous hosts, it is not yet clear which plant product would be the best target for sweetening. Another indirect approach to sweetening is the removal of bitter compounds. Citrus products such as grapefruit juice could be sweetened by the removal of flavanone glucosides, compounds responsible for bitterness.

This objective could be achieved by antisense suppression of a rhamnosyltransferase involved in flavanone glucosides synthesis (5).

Changes in Nutritionally Important Proteins

Plants storage compounds are oils, carbohydrates, and proteins. The latter class of compounds are especially important because human nutrition requires a balanced source of amino acids and the amino acid balance of many plant products is unsatisfactory. Many crops have storage proteins with unbalanced amino acids content. A second problem is the presence of antinutritional proteins in many seeds, a set of digestive enzyme inhibitors that probably evolved to deter herbivores (92). Ameliorating the nutritional quality of crops would thus involve three possible strategies (28, 97): expressing a desirable, heterologous storage protein; increasing the level of a desirable, but little expressed, endogenous protein; and suppressing the expression of antinutritional proteins.

An example of the first strategy is the cloning and expression in heterologous species of a sulfur-rich storage protein from Brazil nut (104). The Brazil nut protein contains 18% methionine and 8% cysteine and was expressed in tobacco and rapeseed seeds. In both species it accumulated to significant levels, increasing the total sulfur-containing amino acids by 300 and 30% respectively. In addition, Altenbach et al (2, 3) found that the protein was properly processed in both heterologous species from an 18,000 mol wt precursor to a 9,000 and 3,000 mol wt subunit heterodimer. The genetics and biochemistry of other storage proteins that are good candidates for engineering have been described (13, 14, 28, 59, 96).

A gene encoding a storage protein could also be modified by adding synthetic regions of DNA specifying peptides rich in desirable amino acids. Hoffman et al expressed a phaseolin gene artifically enriched in methionine residues in tobacco (48). Although this gene's mRNA was as abundant in tobacco as the mRNA of an unaltered phaseolin gene, the amount of accumulated methionine-rich phaseolin was much lower than that of native phaseolin. The engineered methionine-rich phaseolin appeared to be properly processed and was assembled in trimers. The reduced accumulation of the engineered protein must have been due to problems in a posttranscriptional process, such as transport, or protein turnover. This result emphasizes the need to understand the biochemistry of storage protein accumulation.

Could translation of a storage protein rich in a given amino acid be limited by the availability of that amino acid within the cell? While such an instance has not been reported, it may be possible to increase the synthesis of an amino acid by the engineering of a rate-limiting enzyme. For example, in a tobacco mutant lysine overproduction was caused by a lysine-insensitive dihydro-

picolinate synthase. Inspired by this observation, genetic engineers duplicated this situation by expressing in plant chloroplasts a bacterial dihydropicolinate synthase with reduced lysine feedback sensitivity and obtaining a 40-fold increase in free lysine (94).

A summary of all the discussed traits is presented in Table 1.

Table 1 Examples of genetically engineered quality traits in plants

Gene product manipulated[a]	Method[b]	Source[c]	Trait	Reference
Polygalacturonase	Antisense		Delayed softening	39, 89, 99
ACC synthase	Antisense		Delayed ripening	79
ACC oxidase	Antisense		Delayed ripening	44
ACC deaminase	Introduction	*Pseudomonas*	Delayed ripening	61
Antifreeze protein	Introduction	Flounder	Freeze tolerance	32, 46
Stearoyl-ACP desaturase	Antisense		High stearate oils	62
Δ15 desaturase[d]	Antisense or over-expression		Low or high poly-unsaturated oils	4
Glycerol-3-P acyltransferase	Introduction	Plant spp.	Low or high poly-unsaturated oils	75
Acyl-ACP thioesterase	Introduction	Bay	High laurate oils	112
Sucrose-P synthase	Introduction	Maize	Increased sugars	116
Pyrophosphatase	Introduction	*E. coli*	Increased sugars	101
Invertase	Introduction	Yeast	Increased starch	24, 113
ADPG pyrophosphorylase	Introduction	*E. coli*	Increased starch	102a
Starch synthase (granule)	Antisense		Increased amylo-pectin	111
Cyclodextrin glycosyl-transferase	Introduction	*Klebsiella*	Cyclodextrin synthesis	78
Alpha-amylase	Introduction	*Bacillus*	Starch depolymer-ization	82
Thaumatin, monellin	Introduction	Plant spp.	Sweetness	83, 115
S-rich storage protein	Introduction	Brazil nut	Increased S-amino acids	104
Dihydropicolinate synthase	Introduction	*E. coli*	Increased soluble lysine	94

[a] These examples range from projects at an early proof-of-concept phase to projects undergoing regulatory scrutiny in preparation for commercialization.

[b] Antisense mRNA production; introduction of a gene from another organism; overexpression of an existing gene.

[c] Source of the introduced gene.

[d] The fad3 gene has not been yet used to alter polyunsaturated fatty acids by genetic engineering, but it is included here because its potential appears high.

SAFETY AND PUBLIC ACCEPTANCE OF GENETICALLY ENGINEERED PLANT PRODUCTS

Most genetic engineering experiments to date have added three genetic components to the target plant: first, a novel DNA is inserted in the host genome; second, this DNA carries a selectable marker; third, the DNA carries an additional gene of various nature aimed at modifying a specific plant property. I will address the safety of each one of these components.

What risk is entailed by the insertion of a novel DNA in the host genome? This event could inactivate a resident gene. Upon self-fertilization, a mutated allele (the knocked-out gene) would become homozygous and, if the gene were to be active, not redundant and if it contributed to a relevant cellular function, it would cause a change in phenotype. That this change could result in a metabolic alteration or a pathway deregulation leading to a toxic product accumulating in harvested plant parts is possible, but extremely unlikely. Assuming that every insertion inactivates a gene and that there are 50,000 genes in a plant (58) the frequency of this event would be 1/50,000. This scenario is not a relevant risk factor for several reasons. First, a mutation rate of 1/50,000 is not different from the rate of spontaneous mutations (106), many of which are caused by plant transposable elements. Second, mutation breeding, a commonly used strategy in development of conventional varieties, is more likely to introduce deleterious mutations of this type because mutagenesis achieves mutation rates between 1/1000 to 1/10,000. Third, accumulation of a toxic product would most likely be detected during varietal testing.

A selectable marker is often a necessity for the isolation of transformants. The selectable marker gene typically encodes an antibiotic detoxifying enzyme of bacterial origin. Flavell et al (27) argued that the kanamycin detoxifying protein, encoded by the most common marker gene, is safe for human consumption, does not compromise the efficacy of oral kanamycin in humans, and that the probability that its gene may be transferred from plants to bacteria or any other organism is irrelevant. Their arguments were based on well-documented and scientifically sound evidence submitted by Calgene Inc. representatives in communications to the Foods and Drug Administration (FDA) (89). Flavell et al (27) concluded that regulations requiring the removal of the selectable marker gene from varieties destined to the market would be unjustified and would unduly complicate, or in some cases prevent, the production of such varieties. Bryant & Leather (12) agreed that the kanamycin resistance marker is safe. They concluded, however, that, for the sake of public perception, it should be removed. A technique that is capable of achieving such removal has been described by Dale & Ow (17). This technique is

laborious in that it requires specific crosses to be made and it is not yet applicable to vegetatively propagated crops nor to selectable markers that are not engineered with specific recombination-target regions. I have concluded that there is no scientific argument justifying concerns for the safety of commonly used selectable markers.

The process of modifying a trait involves the introduction of a gene that expresses a protein or an antisense RNA. The safety of either type of modification can be assessed. An example of this process is represented by the documentation submitted to FDA by Calgene Inc. in support of the safety of the Flavr Savr™ Tomato (89). This fruit is the result of antisense engineering for suppression of the softening enzyme polygalacturonase. In general, one first addresses the safety of the protein being expressed: Is it toxic or allergenic? Second, does the protein directly or indirectly cause the formation of any toxic product? The direct products of a given enzyme activity are known and can be evaluated by standard procedures. Many crops have toxic products in nonconsumed plant parts such as in tomato and potato leaves. Other crops like cassava and beans have toxic products in consumed plant parts, but the toxic components are removed or inactivated by processing. Could the expression, or suppression, of a given gene indirectly induce enhanced accumulation of these compounds? While this is unlikely, it can easily be tested. In the case of tomato, the amount of toxic tomatine in genetically engineered fruits was shown to be no different than the amount found in common varieties.

Genetic engineers have introduced into crops genes conferring herbicide and pest resistance. The products of these genes will often be present in consumed plant organs. The safety of these modifications is also an important issue.

Genes for herbicide resistance function by either of two mechanisms (9, 69). They either encode altered enzyme targets of herbicide action, such as an altered EPSP synthase conferring tolerance to glyphosate or an altered acetolactate synthase conferring tolerance to sulfonylureas, or they encode a detoxifying enzyme, such as a nitrilase conferring tolerance to bromoxynil or an acetyl transferase conferring tolerance to gluphosinate. The toxicological effects of novel enzymes introduced in the plant can be addressed by processes similar to those used with neomycin phosphotransferase. The expression level of these proteins is usually below a tenth of one percent of the total soluble proteins. They either catalyze a normal reaction of plant metabolism or a new one where they only recognize the herbicide as a substrate. The most significant factor in their safety assessment is not the presence of a new enzyme, but rather the fate and concentration of the applied herbicide residues, and, with detoxifying enzymes, the toxicological properties of the reaction

product. Thus, safety assessment follows the lines of herbicide certification. Genetically engineered herbicide tolerance will allow the use of broad range and effective herbicides when multiple applications of less effective herbicides would otherwise have been used, thereby reducing the chemical input in crop production.

Expressing viral coat protein genes in plants conferred resistance to several viral diseases (1, 6). In one case, expression of a portion of the viral replicase also effectively controlled tobacco mosaic virus (36). Many plant products sold on the consumer market are infected by plant viruses, and in some cases the titer of virus particles is very high. Thus, different coat proteins have been part of the human diet since evolutionary times and the risk to the consumer from coat protein-engineered plants seems to be very low.

A commonly used natural insecticide consists of *Bacillus thuringiensis* spores containing crystal proteins toxic to lepidopterans and certain coleopterans (48a). This insecticide is probably one of the safest available, since the bacterial toxin, which interacts with an intestinal receptor in the insect, is very specific and nontoxic to humans. In the USA, its application is allowed up to harvest time. Expressing *Bacillus thuringiensis* crystal protein genes in plants resulted in resistance to certain insect pests. Given the toxicological properties of the *B. thuringiensis* protein and the expression level in plants, it seems likely that no adverse effects will be found. Scientists are searching for fungicidal and bactericidal proteins in plants and microbes: Both bean (11) and bacterial chitinase (55) expressed in plants conferred tolerance to the fungal pathogens *Rhizoctonia* and *Alternaria,* respectively. In the future, we can expect the expression of novel proteins with pesticidal properties to substitute for most pesticides.

In conclusion, I believe that the risk posed by the majority of genetic alterations is very low. Alterations that pose a significant risk can be rapidly identified, critically examined by the process exemplified by the Flavr Savr™ document, and subjected to a rigid risk versus benefit analysis.

Public perception and acceptance of plant genetic engineering is a fundamental issue. It has its roots in the relationship between scientists and society at large and is going to influence this relationship in the future. A portion of the public mistrusts genetic engineering. Their arguments originate from a philosophy that wants to protect the genetic integrity of nature's products, or from mistrust of technology and of the safety assurances of scientists, or from the dislike of "mingling" animal and bacterial proteins in plant food products. Genetic engineers have argued that manipulations of the genes of crops and domesticated animals has been carried out since prehistoric times, that genetic engineering can actually decrease the use of hazardous chemicals on foods, and that foreign proteins in quantities exceeding those made possible by

genetic engineering are ingested daily with plant products colonized by a diverse and variable microbial flora.

A productive resolution of these concerns can only be achieved by frank and respectful communication among all involved parties.

PROSPECTIVES AND CONCLUSION

I have described advances made by plant genetic engineering in food and nutrition. The progress in this area has been substantial and suggests that any trait known to be conferred by one or few genes can be manipulated by introducing a foreign or modified gene, or by repressing an endogenous gene. I have refrained from compiling a long list of traits that are candidates for modification, because, given the theoretical premises above, the reader can easily identify such opportunities. While this is conceptually simple, it is certainly not easy to identify the combination of trait, gene, and market potential that will result in a successful new product. The reader may have been impressed by the variety of projects aimed at modifying plants for food and nutritional value. However, very few of the alterations described above will lead to commercialized products, at least in their present version.

Who will take the lead in developing new food products via quality manipulations? Genetic engineering companies are now painfully aware of the costs involved in inventing, developing, and testing new plant traits. Agronomic traits impact the seed market while quality traits address the much larger consumer market. The latter type of engineering is thus potentially more profitable and is attracting the attention of the industry (29). Public research institutions are also attracted to the modification of quality traits (100) and, while profitability will be important for them as well, they will clearly pursue the option of developing useful, but not necessarily profitable, quality traits.

An important component determining what traits will be developed is public response. Some people believe that genetic engineering is too risky to pursue, others feel that the benefits must clearly outweigh the risks. Some genetic engineering programs, such as the use of bovine growth hormone for enhanced milk production, have become very controversial because the benefits to society at large appear to be less significant than the benefits to the marketers. Developing novel products that clearly benefit the consumer is the best approach to ensure that this technology, which scientists believe is safe, will be accepted and understood by the public. Many of the projects described here would fit these parameters.

ACKNOWLEDGMENTS

I thank Lynnette Comai and Rene Fester for suggestions on manuscript style and Charlene Martinsen-Brannon for reviewing the manuscript. I also thank

the following individuals for contributing useful information: John Browse, Chris Somerville, Jean Kridl, Toni Voelker, Charles Gasser, William Hiatt, and Martin Chrispeels.

Literature Cited

1. Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., et al. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232: 738–43
2. Altenbach, S. B., Kuo, C. C., Staraci, L. C., Pearson, K. W., Wainwright, C., et al. 1992. Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol. Biol.* 18:235–45
3. Altenbach, S. B., Pearson, K. W., Meeker, G., Staraci, L. C., Sun, S. M. 1989. Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants. *Plant Mol. Biol.* 13:513–22
4. Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H., et al. 1992. Map-based cloning of a gene controlling omega-3 desaturation in *Arabidopsis*. *Science* 258:1353–55
5. Bar, P. M., Lewinsohn, E., Fluhr, R., Gressel, J. 1991. UDP-rhamnose: flavanone-7-O-glucoside-2′-O-rhamnosyltransferase. Purification and characterization of an enzyme catalyzing the production of bitter compounds in citrus. *J. Biol. Chem.* 266:20953–59
6. Beachy, R. N., Loesch-Fries, S., Turner, N. E. 1990. Coat protein-mediated resistance against virus infection. *Annu. Rev. Phytopathol.* 28:738–43
7. Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B., Schaller, H. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19: 327–36
8. Benfey, P. N., Chua, N-H. 1989. Regulated genes in transgenic plants. *Science* 244:174–88
9. Botterman, J., Leemans, J. 1988. Engineering herbicide resistance in plants. *Trends Genet.* 4:219–22
10. Brady, C. F. 1987. Fruit ripening. *Annu. Rev. Plant Physiol.* 38:155–78
11. Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., et al. 1991. Transgenic plants with enhanced resistance of the fungal pathogen. *Rhizoctonia solani*. *Science* 254:1194–97
12. Bryant, J., Leather, S. 1992. Removal of selectable marker genes from transgenic plants: needless sophistication or social necessity. *Trends Biotech.* 10: In press
13. Ceriotti, A., Pedrazzini, E., Fabbrini, M. S., Zoppe, M., Bollini, R., et al. 1991. Expression of the wild-type and mutated vacuolar storage protein phaseolin in *Xenopus* oocytes reveals relationships between assembly and intracellular transport. *Eur. J. Biochem.* 202:959–68
14. Chee, P. P., Slightom, J. L. 1991. Molecular biology of legume vicilin-type seed storage protein genes. *Subcell. Biochem.* 17:31–52
15. Christou, P. 1992. Genetic transformation of crop plants using microprojectile bombardment. *Plant J.* 2:275–81
16. Council on Scientific Affairs. 1991. Biotechnology and the American agricultural industry. *J. Am. Med. Assoc.* 265:1429–36
17. Dale, E. C., Ow, D. W. 1991. Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* 88:10558–62
18. Danyluk, J., Sarhan, F. 1990. Differential mRNA transcription during the induction of freezing tolerance in spring and winter wheat. *Plant Cell Physiol.* 31:609–19
19. Davey, M. R., Rech, E. L., Mulligan, B. J. 1989. Direct DNA transfer to plant cells. *Plant Mol. Biol.* 13:273–85
20. Davies, H. M., Anderson, L., Fan, C., Hawkins, D. J. 1991. Developmental induction, purification, and further characterization of 12:0-ACP thioesterase from immature cotyledons of *Umbellularia californica*. *Arch. Biochem. Biophys.* 290:37–45
21. Davies, P. L., Roach, A. H., Hew, C. L. 1982. DNA sequence coding for an antifreeze protein precursor from winter flounder. *Proc. Natl. Acad. Sci. USA* 79:335–39
22. De Block, M., De Brouwer, D., Ten-

ning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and neo genes in the transgenic plants. *Plant Physiol.* 91:694–701

23. Deroles, S. C., Gardner, R. C. 1988. Expression and inheritance of kanamycin resistance in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. *Plant Mol. Biol.* 11:355–64
24. Dickinson, S., Altabella, T., Chrispeels, M. 1991. Slow growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiol.* 95: 420–25
25. Evans, D. A. 1989. Somaclonal variation—genetic basis and breeding applications. *Trends Genet.* 5:46–50
26. Fincham, J. R. 1989. Transformation in fungi. *Microbiol. Rev.* 53:148–70
27. Flavell, R. B., Dart, E., Fuchs, R. L., Fraley, R. T. 1992. Selectable marker genes: safe for plants? *Bio-Technology* 10:141–44
28. Flavell, R. B., Payne, P. I. Thompson, R. D., Law, C. N. 1988. Strategies for the improvement of wheat-grain quality using molecular genetics. In *Biotechnology of Higher Plants,* ed. G. E. Russell, pp. 159–75. Dorset: Intercept
29. Fraley, R. T. 1992. Sustaining the food supply. *Bio-Technology,* 10:40–43
30. Gasser, C. S. 1992. Transgenic crops. *Sci. Am.* 266:62–69
31. Gasser, C. S., Fraley, R. T. 1989. Genetically engineered plants for crop improvement. *Science* 244:1293–99
32. Georges, F., Saleem, M., Cutler, A. J. 1990. Design and cloning of a synthetic gene for the flounder antifreeze protein and its expression in plant cells. *Gene* 91:159–65
33. Gharti-Chhetri, G. B., Cherdshewasart, W., Dewulf, J., Paszkowski, J., Jacobs, M., et al. 1990. Hybrid genes in the analysis of transformation conditions. 3. Temporal/spatial fate of NPTII gene integration, its inheritance and factors affecting these processes in *Nicotiana plumbaginifolia*. *Plant Mol. Biol.* 14:687–96
34. Gilmour, S. J., Hajela, R. K., Thomashow, M. F. 1988. Cold acclimation in *Arabidopsis* thaliana. *Plant Physiol.* 87:745–50
35. Giovannoni, J. J., Della Penna, D., Bennett, A. B., Fischer, R. L. 1989. Expression of chimeric polygalacturonase gene in transgenic rin (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. *Plant Cell* 1:53–63
36. Golemboski, D. B., Lomonosoff, G. P., Zaitlin, M. 1990. Plants transformed with a tobacco mosaic nonstructural gene sequence are resistant to the virus. *Proc. Natl. Acad. Sci. USA 87:6311–15*
37. Graef, G. L., Fehr, W. R., Hammond, E. G. 1985. *Crop Sci.* 25:1076–79
38. Graham, D., Patterson, B. D. 1982. Responses of plants to low, nonfreezing temperatures: proteins, metabolism, and acclimation. *Annu. Rev. Plant Physiol.*, 33:347–72
39. Gray, J., Picton, S., Shabbeer, J. Schuch, W., Grierson, D. 1992. Molecular biology of fruit ripening and its manipulation with antisense genes. *Plant Mol. Biol.* 19:69–87
40. Grierson, D., Kader, A. A. 1986. Fruit ripening and quality. In *The Tomato Crop,* ed. J. G. Atherton, J. Rudich, pp. 241–80. London: Chapman & Hall
41. Guy, C. L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:187–223
42. Hajela, R. K., Horvath, D. P., Gilmour, S. J., Thomashow, M. F. 1990. Molecular cloning and expression of cor (cold regulated) genes in *Arabidopsis* thaliana. *Plant Physiol.* 93:1246–52
43. Halfter, U., Morris, P. C., Willmitzer, L. 1992. Genc targeting in *Arabidopsis* thaliana. *Mol. Gen. Genet.* 231:186–93
44. Hamilton, A. J., Bouzayen, M., Grierson, D. 1991. Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. *Proc. Natl. Acad. Sci. USA* 88:7434–37
45. Hew, C. L., Yang, D. S. 1992. Protein interaction with ice. *Eur. J. Biochem.* 203:33–42
46. Hightower, R., Baden, C., Penzes, E., Lund, P., Dunsmuir, P. 1991. Expression of antifreeze proteins in transgenic plants. *Plant Mol. Biol.* 17:1013–21
47. Ho, L. C. 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:355–78
48. Hoffman, L. M., Donaldson, D. D., Herman, E. M. 1988. A modified storage protein is synthesized, processed, and degraded in the seeds of transgenic plants. *Plant Mol. Biol.* 11: 717–29

48a. Hofte, H., Whiteley, H. R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53:245–55

49. Hooykaas, P. J., Schilperoort, R. A.

1992. *Agrobacterium* and plant genetic engineering. *Plant Mol. Biol.* 19:15–38
50. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., et al. 1985. A simple and general method for transferring genes into plants. *Science* 227:1229–31
51. Ishizaki, O., Nishida, I., Agata, K., Eguchi, G., Murata, M. 1988. Cloning and nucleotide sequence of cDNA for the plastid glycerol-3-phosphate acyltransferase from squash. *FEBS Lett.* 238:424–30
52. James, D. W., Dooner, H. K. 1991. Novel seed lipid phenotypes in combinations of mutants altered in fatty acid biosynthesis in *Arabidopsis*. *Theor. Appl. Genet.* 82:409–12
53. Jefferson, R. A., Kavanagh, T. A., Bevan, M. W. 1987. Gus fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6:3901–7
54. Jones, D. D. 1988. Food safety aspects of gene transfer in plants and animal: pigs, potatoes, and pharmaceuticals. *Food Drug Cosmet. Law J.* 43:351–68
55. Jones, J., Dean, C., Gidoni, D., Gilbert, D., Bondnutter, D., et al. 1988. Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. *Mol. Gen. Genet.* 212:536–42
56. Jorgensen, R. 1990. Altered gene expression in plants due to trans interactions between homologous genes. *Trends Biotechnol.* 8:340–44
57. Joyner, A. L. 1991. Gene targeting and gene trap screens using embryonic stem cells: new approaches to mammalian development. *Bioessays* 13:649–56
58. Juergens, G., Mayer, U., Torres-Ruiz, R. A., Berleth, T., Misera, S. 1991. Genetic analysis of pattern formation in the *Arabidopsis* embryo. *Dev. Suppl.* 1:27–38
59. Kho, C. J., de Lumen, B. O. 1988. Identification and isolation of methionine-cysteine rich proteins in soybean seed. *Plant Foods Hum. Nutr.* 38:287–96
60. Kilby, N. J., Leyser, H. M. O., Furner, I. J. 1992. Promoter methylation and progressive transgene inactivation in *Arabidopsis*. *Plant Mol. Biol.* 20:103–12
61. Klee, H. J., Hayford, M. B., Kretzmer, K. A., Barry, G. F., Kishore, G. M. 1991. Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell* 3:1187–93
62. Knutzon, D. S., Thompson, G. A., Radke, S. E., Johnson, W. B., Knauf, V. C. et al. 1992. Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. *Proc. Natl. Acad. Sci. USA* 89:2624–28
63. Koltunow, A. M., Truettner, J., Cox, K. H., Wallroth, M., Goldberg, R. B. 1990. Different temporal and spatial gene expression patterns occur during anther development. *Plant Cell* 2:1201–24
64. Koncz, C., Martini, N., Mayerhofer, R., Koncz, K. Z., Korber, H., et al. 1989. High-frequency T-DNA-mediated gene tagging in plants. *Proc. Natl. Acad. Sci. USA* 86:8467–71
65. Kurkela, S., Franck, M. 1990. Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol. Biol.* 15:137–44
66. Li, P. H. 1987. *Plant Cold Hardiness*. New York: Liss. 381 pp.
67. Matzke, M. A., Matzke, A. 1990. Gene interactions and epigenetic variation in transgenic plants. *Dev. Genet.* 11:214–33
68. Mayerhofer, R., Koncz, K. Z., Nawrath, C., Bakkeren, G., Crameri, A., et al. 1991. T-DNA integration: a mode of illegitimate recombination in plants. *EMBO J.* 10:697–704
69. Mazur, B. J. 1989. The development of herbicide resistant crops. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40:441–70
70. Mol, J. N., van den Krol, A. R., van Tunen, A. J., van Blokland, R., de Lange, P., et al. 1990. Regulation of plant gene expression by antisense RNA. *FEBS Lett.* 268:427–30
71. Morrison, R. A., Evans, D. A., Fan, Z. 1991. Haploid plants from tissue culture. Application in crop improvement, *Subcell. Biochem.* 17:53–72
72. Mueller, G. M., McKown, R. L., Corotto, L. V., Hague, C., Warren, G. J. 1991. Inhibition of recrystallization in ice by chimeric proteins containing antifreeze domains. *J. Biol. Chem.* 266:7339–44
73. Mueller-Roeber, B., Sonnewald, U., Willmitzer, L. 1992. Inhibition of ADP-glucose pyrophosphorilase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage proteins. *EMBO J.* 11:1229–38
74. Muller, A. J., Mendel, R. R., Schiemann, J., Simoens, C., Inz'e, D. 1987. High meiotic stability of a foreign gene introduced into tobacco by *Agro-*

bacterium-mediated transformation. *Mol. Gen. Genet.* 207:171–75
75. Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y., et al. 1992. Genetically engineered alteration in the chilling sensitivity of plants. *Nature* 356:710–13
76. Murphy, D. J. 1992. Modifying oilseed crops for non-edible products. *Trends Biotechnol.* 10:84–87
77. Negrutiu, I., Hinnisdaels, S., Cammaerts, D., Cherdshewasart, W., Gharti-Chhetri, C. G., et al. 1992. Plant protoplasts as genetic tool: selectable markers for developmental studies. *Int. J. Dev. Biol.* 36:73–84
78. Oakes, J. V., Shewmaker, C. K., Stalker, D. M. 1991. Production of cyclodextrins, a novel carbohydrate, in the tubers of transgenic potato plants. *Bio-Technology* 9:982–86
79. Oeller, P. W., Lu, M. W., Taylor, L. P., Pike, D. A., Theologis, A. 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* 254:437–39
80. Offringa, R., de Groot, M. J. A., Haagsman, H. J., Does, M. P., van den Elzen, P. J., Hooykaas, P. J. 1990. Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium*-mediated transformation. *Embo J.* 9:3077–84
81. Paszkowski, J., Baur, M., Boguck, A., Potrykus, I. 1988. Gene targeting in plants. *EMBO J.* 7:4021–26
82. Pen, J., Molendijk, L., Quax, W. J., Sijmons, P. C., van Ooyen, A. J. J., et al. 1992. Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction. *Bio-Technology* 10:292–96
83. Penarrubia, L., Kim, R., Giovannoni, J., Kim, S.-H., Fischer R. L. 1992. Production of the sweet protein monellin in transgenic plants. *Bio-Technology* 10:561–64
84. Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L., Fischoff, D. A. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc. Natl. Acad. Sci. USA* 88:3324–28
85. Pollard, M. R., Anderson, L., Fan, C., Hawkins, D. J., Davies, H. M. 1991. A specific acyl-ACP thioesterase implicated in medium-chain fatty acid production in immature cotyledons of *Umbellularia californica*. *Arch. Biochem. Biophys.* 284:306–12
86. Pollock, C. J., Cairns, A. J. 1991. Fructan metabolism in grasses and cereals. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:77–101
87. Potrykus, I. 1990. Gene transfer to cereals. An assessment. *Bio-Technology* 8:535–42
88. Potrykus, I. 1991. Gene transfer to plants: assessment of published approaches and results. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 42:205–25
89. Redenbaugh, K., Hiatt, W. R., Martineau, B., Kramer, M., Sanders, R. A., et al. 1992. Safety assessment of genetically engineered fruits and vegetables. A case study of the Flavr Savr™ tomato. Ann Arbor: CRC Press
90. Sanford, J. C. 1990. Biolistic plant transformation. *Physiol. Plant.* 79:205–9
91. Saul, M. W., Potrykus, I. 1990. Direct gene transfer to protoplasts: fate of the transferred genes. *Dev. Genet.* 11:176–81
92. Savelkoul, F. H., van der Poel, A. F., Tamminga, S. 1992. The presence and inactivation of trypsin inhibitors, tannins, lectins and amylase inhibitors in legume seeds during germination. A review. *Plant Foods, Hum. Nutr.* 42: 71–85
93. Shanklin, J., Somerville, C. 1991. Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. *Proc. Natl. Acad. Sci. USA* 88:2510–14
94. Shaul, O., Galili, G. 1992. Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts. *Plant J.* 2:203–9
95. Sheehy, R. E., Kramer, M., Hiatt, W. R. 1988. Reduction of polygalacturonase activity in tomato fruit by antisense RNA. *Proc. Natl. Acad. Sci. USA* 85:8805–9
96. Shorrosh, B. S., Wen, L., Zen, K. C., Huang, J. K., Pan, J. S., et al. 1992. A novel cereal storage protein: molecular genetics of the 19 kDa globulin of rice. *Plant Mol. Biol.* 18:151–54
97. Shotwell, M. A., Larkins, B. A. 1991. Improvement of the protein quality of seeds by genetic engineering. In *Molecular Approaches to Crop Improvement*, ed. E. S. Dennis, D. J. Llewellyn, pp. 33–61. Wien: Springer-Verlag
98. Slabas, A. R., Fawcett, T. 1992. The biochemistry and molecular biology of plant lipid biosynthesis. *Plant Mol. Biol.* 19:169–91
99. Smith, C. J. S., Watson, C. F., Ray, J., Bird, C. R., Morris, P. C. et al.

1988. Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* 334:724–26

100. Somerville, C., Browse, J. 1991. Plant lipids: metabolism, mutants, and membranes. *Science* 252:80–87
101. Sonnewald, U. 1992. Expression of *E. coli* inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning. *Plant J.* 2:571–81
102. Sonnewald, U., Willmitzer, L. 1992. Molecular approaches to sink-source interactions. *Plant Physiol.* 99:1267–70
102a. Stark, D. M., Timmerman, K., Barry, G. F., Preiss, J., Kishore, G. M. 1991. Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase. *Science* 258:287–91
103. Straughan, R. 1990. Genetic manipulation for food production: social and ethical issues for consumers. *Br. Food. J.* 92:13–26
104. Sun, S. S., Altenbach, S. B., Leung, F. W. 1987. Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (*Bertholletia excelsa* H.B.K.). *Eur. J. Biochem.* 162:477–83
105. Sussex, I. M. 1989. Developmental programming of the shoot meristem. *Cell* 56:225–29
106. Suzuki, D. T., Giffiths, A. J. F., Miller, J. H., Lewontin, R. 1989. *An Introduction to Genetic Analysis*. New York: Freeman. 768 pp.
107. Thompson, G. A., Scherer, D. E., Foxall, V. A. S., Kenny, J. W., Young, H. L., et al. 1991. Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. *Proc. Natl. Acad. Sci. USA* 88:2578–82
108. Urrutia, M. E., Duman, J. G., Knight, C. A. 1992. Plant thermal hysteresis proteins. *Biochim. Biophys. Acta* 1121: 199–206
109. van den Elzen, P. J., Townsend, J., Lee, K. Y., Bedbrook, J. R. 1985. A chimearic hygromycin resistance gene as a selectable marker in plant cells. *Plant Mol. Biol.* 5:299–302
110. Vayda, M. E., Belknap, W. R. 1992. The emergence of transgenic potatoes as commercial products and tools for basic science. *Transgenic Res.* 1:149–63
111. Visser, R. G. F., Somhorst, I., Kuipers, G. J., Ruys, N. J., Feenstra, W. J., et al. 1991. Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. *Mol. Gen. Genet.* 225:289–96
112. Voelker, T. A., Worrell, A. C., Anderson, L., Bleibaum, J., Fan, C., et al 1992. Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science* 257:72–74
113. von Schaewen, A., Stitt, M., Schmidt, R., Sonnewald, U., Willmitzer, L. 1990. Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J.* 9: 3033–44
114. Walden, R., Hayashi, H., Schell, J. 1991. T-DNA as a gene tag. *Plant J.* 1:281–88
115. Witty, M., Harvey, W. J. 1990. Sensory evaluation of transgenic *Solanum tuberosum* producing r-thaumatin II. *NZ J. Crop Hortic. Sci.* 18:77–80
116. Worrell, A. C., Bruneau, J-M., Summerfelt, K., Boersig, M., Voelker, T. 1991. Expression of maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. *Plant Cell* 3:1121–30
117. Yang, C. F. 1985. Biosynthesis and action of ethylene. *Hortic. Sci.* 20:41–45