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Plant genetic engineering for crop improvement

G. Kahl* and P. Winter

Plant genetic engineering has long since left its experimental stage: transgenic plants with resistance to viruses, bacteria, fungi, various pests and abiotic stresses have already been released in their hundreds. Transgenic plants can produce better fruits and food of higher quality than wild-types, and can be used as bioreactors for the synthesis of pharmaceutically important compounds. This review portrays some of the achievements in this field of plant molecular biology.

Key words: Bioreactor plants, crop improvement, genetic engineering, molecular flower breeding.

The past 10 years have seen unprecedented progress in the field of genetic engineering of plants, particularly crop species. This has been based on the development of new techniques, the detection of genes that can be used for plant improvement, the perfection of gene-transfer techniques, successes in regeneration of plants from single cells or embryogenic precursors, the evaluation of first-generation transgenic plants in the field, and a subtle change in public perception of the new technologies. This review will focus on selected topics that demonstrate the great potential of gene technology (Table 1).

Pathogen Resistance

The various plant pathogens that cause severe disease symptoms in commercially important crops fall into three basic categories: viruses; bacteria; and fungi. Whereas several effective and successful approaches to engineering virus tolerance in plants have been developed, the generation of transgenic plants tolerant to bacterial or fungal pathogens is still in its infancy. Nevertheless, some promising strategies are emerging and will be discussed here.

Virus Resistance

Various protocols for engineering virus resistance in host plants have been designed, including coat-protein-mediated resistance, expression of satellite RNA or replicase sequences, interference by defective RNA or DNA sequences, and the use of antisense RNA. Of these, coat-protein-mediated protection was the first and is the most successful way of generating virus-resistant plants (see Pappu *et al.* 1995).

Coat-protein-mediated Virus Resistance. The concept of coat-protein-mediated protection is based on cross-protection: if a plant is infected by a mild virus prior to infection with a serologically related aggressive virus, then it is less affected by the secondary infection than naive controls. Although cross-protection was observed many years ago (Sequeira 1984), the exact mechanism involved remains unclear. One possible explanation is that excessive amounts of coat proteins (cps) that are not bound to viral RNA accumulate in the infected cell. These cps inhibit the uncoating of the RNA of the aggressive challenger virus. As a result, viral RNA expression and replication are inhibited and symptom development is prevented or delayed. Such resistance has been found in more than 20 species of transgenic plants that have been transformed with the sense coat-protein gene, including crop plants such as tomato (Nelson *et al.* 1987; Tumer *et al.* 1987), potato (Hoekema *et al.* 1989; Van Den Elzen *et al.* 1989; Kaniewski *et al.* 1990; Kawchuk *et al.* 1990; MacKenzie & Tremaine 1990; Van Der Wilk *et al.* 1991), alfalfa (Hill *et al.* 1991) and rice (Hayakawa *et al.* 1992). Most related experiments have, however, been performed with tobacco (Powell-Abel *et al.* 1986; Loesch-Fries *et al.* 1987; Nelson *et al.* 1987; Tumer *et al.* 1987; Van

The authors are with Plant Molecular Biology, Biozentrum, Frankfurt University, Marie-Curie-Strasse 9, D-60439 Frankfurt, Germany; fax: 69 7982 9268.* Corresponding author.

Table 1. Plant genetic engineering: an overview.

Engineered trait	Strategy used
Resistance	
Viral	Coat protein
	Satellite RNA
	Replicase
	Anti-sense
	Immunoprotection
Bacterial	Lysozyme
	Thionin
	Detoxification
Fungal	Phytoalexin gene insertion
	Immunoprotection
	Programmed cell death
Insect	*Bacillus thuringiensis* endotoxins
	Protease-inhibitor gene insertion
Abiotic stress tolerance	
Salt resistance	Mannitol-1-phosphate-dehydrogenase gene insertion
Cold tolerance	Arctic flounder gene insertion
Drought tolerance	Abscisic acid-regulated gene engineering
Heat tolerance	Heat-shock gene engineering
Fruit quality	
Ripening	Interference with ethylene biosynthesis
	Anti-sense
Softening	Anti-sense
Nutritional quality	
Balanced amino-acid content	Synthetic gene
	Amino-acid complementation
Gene farming	
Peptide and protein production	Gene insertion
Oil and carbohydrate production	Anti-sense
	Gene insertion
	Gene amplification
Production of degradable polymers	Gene insertion
	Gene amplification
Production of biochemicals	Gene insertion (mannitol, cyclodextrin)
Heavy-metal trapping	Gene insertion
Flower colour change	Gene insertion
	Anti-sense

Dun *et al.* 1987; Cuozzo *et al.* 1988; Hemenway *et al.* 1988; Van Dun & Bol 1988; Anderson *et al.* 1989; Gielen *et al.* 1991; De Haan *et al.* 1992; Lindbo & Dougherty 1992; Brault *et al.* 1993; Strittmatter & Wegener 1993; Willmitzer 1993). In each case, resistance was strongly correlated with the amount of intact coat protein in the transgenic plants (Loesch-Fries *et al.* 1987; Hemenway *et al* 1988).

The sense coat-protein gene is not alone in being able to confer virus resistance; its antisense counterpart can do so as well. For example, plants containing antisense coat-protein RNA from cucumber mosaic virus (Cuozzo *et al.* 1988) or potato virus X (Hemenway *et al.* 1988) were tolerant to low density viral inocula. Defective coat-protein genes have also been used successfully, including a coat-protein cistron of potato virus Y from which the translational start signal had been deleted (Van Der Vlugt *et al.* 1992). Transferring coat-protein genes of tobacco etch virus, whose products were untranslatable, into tobacco plants conferred a certain tolerance to viral infection (Lindbo & Dougherty 1992).

Coat-protein-mediated protection is not always specific. In some cases, the transgenic plant is not only protected against the virus from which the coat-protein gene originated, but also against other, serologically unrelated viruses (Stark & Beachy 1989; Nejidat & Beachy 1990; Ling *et al.* 1991). The coat-protein strategy is effective in the field (Nelson *et al.* 1988; Beachy *et al.* 1990; Kaniewski *et al.* 1990) and against mixed infections with two different viruses (Lawson *et al.* 1990). Although the exact molecular

mechanism of coat-protein-dependent protection is unknown, and more than one mechanism may be involved (Golemboski *et al.* 1990; MacKenzie & Tremaine 1990), this strategy has great potential in the genetic engineering of plants (see Baulcombe 1994).

Satellite RNA. Satellite RNAs are small, extra-genomic components of some RNA viruses that require the intact genome of a helper virus for their replication and propagation within a plant. Such satellite RNA can modulate the disease symptoms caused by the helper virus. The genomes of the satellite RNA from cucumber mosaic virus and tobacco ringspot virus have been transferred to target plants, and expressed under the control of a strong constitutive promoter (Baulcombe *et al.* 1986; Gerlach *et al.* 1987; Harrison *et al.* 1987). In both cases, disease symptom development after a challenge with the corresponding helper virus was delayed. However, there is a risk that a mutation could convert a benign satellite RNA molecule into a virulent one when this technique is used.

Replicase. Virus-resistant plants can also be engineered by the introduction of full-length coding sequences for non-structural proteins such as proteases or replicases (RNA-dependent RNA polymerase). For example, transgenic tobacco expressing a truncated replicase gene from a specific tobacco mosaic virus (TMV) strain was highly tolerant to TMV and to closely related viral strains (Golemboski *et al.* 1990). The corresponding sequence of pea early browning virus replicase also conferred resistance to TMV and to two related strains (MacFarlane & Davies 1992). However, the expression of functional alfalfa mosaic virus replicase did not lead to resistance in transgenic tobacco (Taschner *et al.* 1991) and barley protoplasts were not immune against brome mosaic virus (BMV) after transfer of the intact BMV replicase gene (Mori *et al.* 1992). Nevertheless, there is much potential in this approach, especially since the design of effective replicase mutants is feasible (Longstaff *et al.* 1993).

Anti-sense Technology. Introduction of various antisense sequences of viral genes into target plants can result in partial or complete resistance towards the original virus or related viruses. Expression of antisense RNA from the *al* gene, which encodes a replication protein, protected plants against tomato golden mosaic virus (Day *et al.* 1991). The presence of antisense coat-protein genes in potato conferred considerable resistance to potato leafroll virus (Kawchuk *et al.* 1990), and an antisense transcript of the RNA 3 intercistronic region of brome mosaic virus blocked viral RNA replication (Huntley & Hall 1993). Generally, however, only weak protection has been achieved using antisense sequences (Cuozzo *et al.* 1988; Hemenway *et al.* 1988; Rezian *et al.* 1988; Powell *et al.* 1989).

Alternative Strategies. A very effective but risky method of increasing viral resistance involves the transfer of genes encoding ribosome-inactivating proteins (RIP) into target plants to interfere with virus replication. For example, a single-chain RI protein from pokeweed (PAP), which excises a single adenine residue from a conserved region of the 26S ribosomal RNA, has broad-spectrum antiviral activity. Transgenic plants with high-level PAP gene expression were tolerant to viral infection (Lodge *et al.* 1993).

Immunoprotection of plants may also be possible in the future, if antibodies directed towards essential viral components (e.g. coat proteins and replicases) are synthesized by transgenic plants. Transgenic plants can produce complete antibodies (Hiatt *et al.* 1989; Düring *et al.* 1990).

The engineering of virus tolerance in plants has left its experimental stage, and coat-protein-mediated protection in particular is now a fairly reliable strategy. We expect that an increasing number of crops will be protected in this way.

Bacterial Resistance

In spite of the progress in engineering virus resistance in plants, there are only a few reports of the successful generation of bacteria-resistant transgenic plants (Herrera-Estrella & Simpson 1995). Basically, three approaches have been used. Bacterial genes encoding enzymes degrading bacterial cell walls have been introduced into plants. For example, lysozyme genes from hen egg white or bacteriophage T4 have been transferred into tobacco and potato plants. High-level expression of the lysozyme and its secretion into the intercellular spaces seem to be protective, e.g. slices of tubers from transgenic potato plants were protected against heavy infection by the pathogenic *Erwinia carotovora* sp. *atroseptica* (Trudel *et al.* 1992; Düring *et al.* 1993). Genes encoding anti-microbial, cysteine-rich thionins have been transferred into tobacco (Bohlmann & Apel 1991). High-level expression of α-thionin genes driven by cauliflower mosaic virus (CaMV) 35S promoters reduced the disease symptoms caused by *Pseudomonas syringae* pv *tabaci* or *Ps. syringae* pv *syringae* and their severity (Carmona *et al.* 1993). The third approach, conferring the ability to detoxify bacterial toxins, seems to be the most promising system. For example, *Ps. syringae* pv *tabaci* produces the phytotoxic dipeptide tabtoxin, which induces chlorotic wildfire disease in tobacco, probably by inhibiting the host's glutamine synthetase, leading to the accumulation of toxic ammonia. When the bacterial gene *ttr*, which encodes a tabtoxin-inhibiting acetylase, was transferred into tobacco and constitutively expressed, the symptoms of wildfire disease were less pronounced than in naive controls (Anzai *et al.* 1989). A similar strategy was used by Herrera-Estrella and co-workers, who transferred a bacterial gene encoding a toxin-resistant target enzyme, in this case ornithine carbamoyl

transferase, into the plastids of plants; the presence of the toxin-insensitive enzyme made the host resistant to the phaseolotoxin of *Ps. syringae* pv *phaseolicola* (De La Fuente-Martinez *et al.* 1992).

Engineering Resistance against Fungal Pathogens

A demanding challenge for plant gene technology is the engineering of traits that are encoded by two or more genes in so-called quantitative trait loci (QTL). The resistance of plants towards pathogenic fungi was originally thought to be encoded by oligogenes. However, in some cases it needs only one gene to confer appreciable tolerance to susceptible host plants. Genetic engineering of resistance to pathogenic fungi is now following several promising routes, some of which are mentioned below (see Herrera-Estrella & Simpson 1995).

The Phytoalexin Route. Phytoalexins are low-molecular-weight organic compounds which are rapidly synthesized in plant cells infected by a fungus. Depending on their concentrations, the different phytoalexins exhibit fungistatic or fungicidal activities, and are part of the plant's defence machinery (Bailey 1987). Any change in the composition of the phytoalexins could add to the defence potential of the host plant. The transfer of a single gene encoding stilbene synthase from grape to tobacco allowed the transgenic plants to synthesize the antifungal 3, 4, 5-trihydroxystilbene (resveratrol), a potent inhibitor of fungal growth (Hain *et al.* 1990). The presence of this phytoalexin confers partial resistance towards *Botrytis cinerea*. The phytoalexin strategy promises the potential for engineering fungus tolerance in at least some pathosystems (e.g. in the potato–*Phytophthora infestans* system; Hain *et al.* 1993).

Antifungal Proteins. Small proteins with distinct antifungal activity *in vitro*, such as thionins (Bohlmann & Apel 1991), osmotins (Vigers *et al.* 1991; Woloshuk *et al.* 1991) and zeamatins (Roberts & Selitrennikoff 1990) are potential intracellular fungicides, but have not yet been tested in transgenic plants.

Chitinases and β-1.3-glucanases are constituents of most plant cells and belong to the so-called pathogenesis-related proteins (PR proteins), because their synthesis increases markedly after attack by phytopathogens (Bol *et al.* 1990). The two types of enzyme together destroy fungal cell walls containing β-1.3-glucans and chitin and thereby inhibit fungal growth (Schlumbaum *et al.* 1986). Of the various classes of chitinases and β-1.3-glucanases, only vacuolar class I hydrolases are potent fungal inhibitors (Mauch *et al.* 1988; Cornelissen & Melchers 1993: Sela-Buurlage *et al.* 1993). The improvement of resident chitinase and/or β-1.3-glucanase gene expression by strong constitutive promoters has been attempted, with limited success (Lund *et al.* 1989; Broglie *et al.* 1991; Neuhaus *et al.* 1991). Though the over-expression of a chitinase gene from bean in transgenic tobacco lead to distinct resistance against *Rhizoctonia solani*, probably due to the hydrolysis of newly formed chitin in growing infection hyphae, substantial resistance can probably only be engineered using a combination of several genes (pyramiding). The simultaneous expression of class I or class V chitinase, class I β-1.3-glucanase and additional genes (e.g. the chitin-binding protein [CBP] gene and the ribosome-inhibiting protein [RIP] gene) therefore promises a far better level of resistance than has been achieved so far. As the extracellular (apoplastic) space is probably the first site of encounter between pathogen and host, fungitoxic hydrolases should be targeted to this compartment. Preliminary targeting experiments have been successful; class I hydrolases from tobacco were modified and correctly excreted into the extracellular space, where they retained their antifungal activity (Melchers *et al.* 1993).

Plantibodies. The strategy of directing plant antibodies against fungal proteins (e.g. secretory enzymes) is new but will no doubt be developed in the future. For example, plantibodies raised against fungal cutinases and secreted by a secretory signal peptide into the apoplastic space will interfere with the activity of these key fungal enzymes and probably protect host plants.

Artificial Cell Death. Race-specific resistance of potato cultivars against *Ph. infestans* is mediated by a programmed cell death at the infection site, which prevents the fungal hyphae from penetrating neighbouring cells (hypersensitive reaction). In this incompatible interaction, the fungus is restricted to the necrotic areas, whereas in compatible interactions no such effective defence reaction occurs and the fungus can overgrow the host tissue. Though apparently a complex process, programmed cell death can be engineered. The barnase gene from *Bacillus amyloliquefaciens*, encoding a cytotoxic RNase and driven by a fragment of the *prp 1-1* gene promoter has been transferred into potato (the promoter mediates rapid and localized transcription of the linked gene and is highly specific for fungal elicitors). The expression of this gene induced necrosis of host cells at infection sites, mimicking the hypersensitive response, and restricted the growth of pathogenic fungi. The potentially suicidal effects of ba-RNase in non-affected plant cells, as the result of leaky promoters, were minimized by the simultaneous transfer and constitutive expression of barstar genes encoding a highly specific barnase protein inhibitor (Hartley 1989). Localized cell death has been observed in transgenic potato plants, with concomitant increase in resistance to *Ph. infestans* (Taylor *et al.* 1990; Martini *et al.* 1993; Strittmatter & Wegener 1993).

Resistance towards Insect Pests

As an alternative to the present methods of insect control, involving externally applied, unspecific, hazardous or poten-

tially hazardous organochemicals (which have a negative ecological impact), the expression of insecticidal compounds in transgenic plants is clearly superior. Therefore much effort has been invested in conferring insect resistance to commercially important crop plants. Resistance may be successfully engineered via two strategies: the exploitation of insecticidal δ-endotoxin proteins from *Bacillus thuringiensis* and the use of proteins interfering either with the insect's metabolism or its development.

Bacillus thuringiensis *Endotoxins.* Probably more than 600 different strains of the Gram-positive soil bacterium *Ba. thuringiensis* exist World-wide. Each strain harbours a plasmid, and each plasmid probably encodes a specific protein, the δ-endotoxin protein, which is absolutely necessary for the construction of the spore walls during endospore formation. The surplus δ-endotoxin is deposited as a paracrystalline protein body. Once ingested by feeding insects, these protein bodies are solubilized in the insect midgut's alkaline milieu, releasing one or more proteins. Certain midgut proteases cleave these protoxin proteins, generating highly specific and toxic compounds. The high specificity of the endotoxins (endotoxins from specific bacterial strains only being toxic to a few specific insect species and close relatives) is mediated by specific high-affinity receptor proteins on the brush border membrane in the insect's midgut. Their extreme toxicity, in turn, is a consequence of the blockage of the receptors, with subsequent pore formation, paralysis and total disruption of the mid-gut (Hofmann *et al.* 1988; Höfte & Whitely 1989; Van Rie *et al.* 1990). The specificity of the endotoxins has attracted the interest of many researchers. For example, about 12 endotoxin proteins, with slight differences in their amino-acid sequences, are known to be toxic only to Lepidoptera. Of these, the so-called cry IA (b) and cry IB (endotoxins) are both toxic to *Pieris brassicae* larvae, whereas cry YIA (b) kills *Manduca sexta*.

The first successful isolation, modification and transfer of *Ba. thuringiensis* endotoxin genes into target plants was in 1987 (Barton *et al.* 1987; Fischhoff *et al.* 1987; Vaeck *et al.* 1987). The insecticidal protein gene from *Ba. thuringiensis* var. *kurstaki* conferred far-reaching resistance to larvae of certain Lepidoptera species (*Manduca sexta, Heliothis virescens* and *H. zea*). A series of other δ-endotoxin genes from other *Ba. thuringiensis* strains have been used with the same positive result. For example, the endotoxin of *B. thuringiensis* strain *tenebrionis* is mainly active against the Colorado beetle (*Leptinotarsa decemlineata*), transgenic plants expressing the corresponding endotoxin gene being highly resistant to this insect (Brunke & Meeusen 1991). The endotoxin strategy has proven successful in many host plant-insect interactions, including those involving tobacco (Barton *et al.* 1987; Vaeck *et al.* 1987), tomato (Fischhoff *et al.* 1987), cotton (Perlak *et al.* 1990) and potato (Chen *et al.* 1992). δ-endotoxin-mediated insect resistance is effective under field conditions (Delannay *et al.* 1989) and, most attractively, holds promise for the control of nematodes, trematodes, mites and protozoa, as well as insects (Feitelson *et al.* 1992).

The expression of endotoxin genes in plants is generally low but it can be improved by tailoring the genes [trimming the coding regions to remove plant polyadenylation signals (ATTTA sequences), intron/exon splice sites, polymerase II termination signals and altered codon usage]. These mutations generally booster the expression of endotoxin genes in plants, and consequently the plants' level of resistance (Perlak *et al.* 1990, 1991; Koziel *et al.* 1993). Two technical improvements have added to the effectiveness of the endotoxin strategy. Firstly, as translational fusions, for example between *cry IA* and *cry IC* genes, are superior to wild-type endotoxin genes, such translational fusions should ideally be engineered in the target plants. Secondly fully synthetic endotoxin genes, appropriately designed, also confer better resistance in crops than their wild-type counterparts, and are also effective in the field (Koziel *et al.* 1993).

Two major concerns remain. Firstly, the insect population may develop resistance against the toxin by mutations in the receptor protein genes. In fact, several important pests have been made resistant in the laboratory (e.g. *Plodia interpunctella, Plutella xylostella, H. virescens* and *Leptinotarsa decemlineata*) and in the field (e.g. *Plutella xylostella*; McGaughey & Whalon 1992). An obvious alternative to employing only one endotoxin gene is to transform the target plant with two (or more) different genes, so that the insect would have more than one endotoxin protein with which to cope. Secondly, the presence of relatively high concentrations of δ-endotoxin proteins in crop plants may stress the plants' energy balance. One way to overcome this problem is to use promoters linked to the endotoxin gene that are normally silent but become active after the pest insect attacks (e.g. wound-inducible promoters).

Protease Inhibitors and other Proteins. About eight non-related protease-inhibitor families are present in plants. These serve to inhibit serine, cysteine, aspartic acid and metalloproteases. They reach especially high concentrations in seeds and tubers. Since they have little if any activity against endogenous plant proteases, they are probably involved in defence mechanisms, inhibiting the proteases of insect pests and so exerting an anti-nutritional effect. There is some evidence in favour of this hypothesis (Sanchez-Serrano *et al.* 1986; Peña-Cortes *et al.* 1989; Pearce *et al.* 1991). Genetic engineering of insect resistance could similarly involve protease inhibitor genes. In fact, the transfer and expression of a cowpea trypsin inhibitor cDNA, controlled by a 35S CaMV promoter, into tobacco plants conferred a certain resistance against the tobacco budworm, *H. virescens* (Hilder *et al.* 1987, 1990). The same basic strategy has been applied to engineer resistance to *M. sexta* in tobacco, using

the potato serine protease inhibitor PI-II (Johnson *et al.* 1989). However, the usefulness of this technique suffers from the fact that only high concentrations of inhibitor show an effect. On the other hand, protease inhibitors act unspecifically and could protect against a broad spectrum of insect species.

The same strategy has been used to engineer insect resistance in target plants using α-amylase inhibitor or bifunctional α-amylase/serine protease inhibitor genes. Other genes encoding other anti-nutritional proteins have also been transferred into plants and expressed there, at least conferring weak insect resistance. Such genes include those encoding neuropeptides such as proctolin, which interferes with insect development and that encoding tryptophan decarboxylase, which converts tryptophan to tryptamine, a potent serotonin precursor whose presence hampers the mating, feeding or development of insects.

Improvement of Crop Qualities

Gene technology is also being used to improve a series of agronomically important traits in crop plants. We do not consider the engineering of herbicide resistance here (see Mazur & Falco 1989; Oxtoby & Hughes 1990), but rather focus on present achievements in controlling abiotic stresses and improving the quality of the crop itself.

Abiotic Stress Resistance

Although abiotic stresses such as excessive salinity, heat or cold and drought limit the full development of a crop's potential World-wide, the genetic engineering of anti-stress capacities in plants has been cumbersome and is certainly still in its early days. The reasons are manifold but the main one is our ignorance of how a plant manages stress. In consequence, only a limited number of genes that could confer abiotic-stress resistance is available for use. Nevertheless, there are some very encouraging developments. For example, a gene from *Escherichia coli* encoding mannitol 1-phosphate dehydrogenase has been transferred into tobacco and constitutively expressed. This enzyme catalyses the reversible interconversion of fructose-6-phosphate and mannitol-1-phosphate. The transgenic plants accumulated mannitol in leaves and roots (non-transformed tobacco does not contain this sugar alcohol), and showed appreciable tolerance to high-salinity stress in comparison with control plants. It is encouraging that a single gene can confer tolerance to 250 mM NaCl, since this trait was previously thought to be multigenic (Tarczynski *et al.* 1992 a, b). These results support a classical concept, that in response to drought, high salinity, or low temperature, many plants accumulate osmolytes (osmoprotectants), including low-molecular weight compounds such as proline, glycine-betaine and sugar alcohols such as mannitol. These osmolytes are thought to increase salt or drought tolerance. At least for mannitol, this property has been demonstrated. Genetic engineering of other stress tolerances is already under way: a gene from an Arctic flounder confers cold tolerance in tomato plants; drought tolerance can be increased with abscisic-acid-regulated gene engineering: and heat tolerance can be increased using heat-shock genes. We expect major break-throughs in this area in the near future.

Improvement of Crop Quality

Basically, two parameters of crop quality have been targeted by genetic engineers: fruit quality and the nutritional quality of major crop plants.

Fruit Quality. One of the major constraints for enhancing the yield of many crops is the premature, ethylene-induced ripening of their fruits, which occurs before they are shipped or consumed. Around 50% of all fresh fruits and vegetables are thought to be lost due to such spoilage. The producers use various means to prevent, or at least reduce, this spoilage, for example by harvesting unripened green fruits or sequestering ethylene using chemicals. Consumers have become concerned about the chemicals used. A goal in the genetic engineering of fruit quality was therefore to depress ethylene content. This has been achieved by two ways. Firstly, a gene for bacterial enzyme degrading 1-amino-cyclopropane-1-carboxylic acid (ACC), a precursor in ethylene biosynthesis, has been transferred into tomato plants. Its expression significantly reduced ethylene concentrations and delayed fruit ripening by 2 weeks (Dilworth 1991). Secondly, the anti-sense version of the gene for the ethylene-forming enzyme, ACC oxidase, under the control of the constitutive 35S CaMV promoter, has been expressed in transgenic tomato. This also reduced ethylene concentrations appreciably (almost totally in ripening fruits; Hamilton *et al.* 1990). These strategies for interfering with fruit ripening by blocking ethylene synthesis still have to be developed.

Yet another approach targets the enzymes that cause fruit softening. Polygalacturonase (PG), whose activity leads to the degradation of pectins and the softening of the cell walls of fruits, is the most prominent of these (Giovannoni *et al.* 1989). Fruit softening would be delayed or prevented if PG activity could be blocked. In fact, an almost complete inhibition of PG activity has already been engineered using anti-sense technology. A chimeric gene encoding the anti-sense RNA of PG has been transferred into tomato plants and, when expressed under the control of the 35S CaMV promoter, it strongly interfered with PG synthesis (Sheehy *et al.* 1988; Smith *et al.* 1988). As a result, the transgenic fruits did not soften, and were more resistant to mechanical stresses that occur during harvesting, packaging and transport, but other processes, such as lycopene or ethylene production and pulp formation, were not affected (Smith *et al.* 1990). The tomatoes could be left on the plant to develop their full aroma.

Improving Nutritional Quality. Genetic engineering can help to improve otherwise deficient contents of essential amino acids in plants. A gene has been synthesized that encodes a protein with a high methionine content (Jaynes *et al.* 1986). After transfer of this gene into potato and its expression the overall methionine content of the plant's protein increased (Yang *et al.* 1989). This synthetic gene approach should however, be improved by over-expression of the synthetic transgene. An increase in the methionine content of seed proteins has also been achieved by inserting a heterologous gene encoding a methionine-rich protein into tobacco. The gene used, from Brazil nut (*Bertholletia excelsa*), was expressed at high levels in the target plant's seeds and increased the methionine content of the seed protein by some 30% (Altenbach *et al.* 1989; Altenbach & Simpson 1990). Though initially of practical value for fodder improvement only, this strategy could also be used to improve the methionine content in crops, and to balance the contents of other essential amino acids, such as lysine and tryptophan.

Transgenic Plants as Bioreactors

During the past century, major domains of conventional agriculture, such as the production of food, feed, fibre and fuel, have been partially lost to the petrochemical industry. This is especially true for fuel production. However, because petrochemical resources are limited and non-renewable and the use of petrochemicals and their derivatives is frequently hazardous to man and the environment, interest in the use of plants as factories to produce fuel or other renewable products is increasing. The genetic engineering of plants to convert them into highly productive, relatively cheap and easy-to-handle bioreactors and is one of the prime goals of many companies and institutions World-wide. A few examples may illustrate the versatility of this approach.

Production of Peptides and Proteins

Peptides of pharmaceutical interest have already been produced in transgenic plants. One of the first plant bioreactors, oilseed rape, synthesized the pentapeptide opiate leuenkephalin, after a fused gene created from the 2S albumin-seed-protein gene of *Arabidopsis thaliana* and leuenkephalin gene sequences was transferred into it. The fusion product, 2S albumin-leuenkephalin, accumulated in the producer plants to relatively high levels (10 to 200 g/hectare), and leuenkephalin could be recovered from rape seed extracts after protease treatment and HPLC purification (Vanderkerckhoeve *et al.* 1989; Krebbers & Vanderkerckhoeve 1990).

The production of high-molecular-weight proteins in plants is also feasible. Human serum albumin has been synthesized in transgenic potato and tobacco (Sijmons *et al.* 1990), monoclonal antibodies (plantibodies) have been produced in tobacco (Hiatt *et al.* 1989; Düring *et al.* 1990), and many other proteins, including antigenic proteins for vaccine production (Mason *et al.* 1992), are now being produced in plants. The gene farming of pharmaceutical peptides and proteins is about to begin (Swain 1991).

Production of Oils and Carbohydrates

Higher plants synthesize over 200 different fatty acids, most of which are non-edible and only of interest for industrial purposes (Murphy 1992). In major crops, fatty acids with acyl-chain lengths of C_{16} to C_{22} are bound to glycerol in the form of triacylglycerols. Generally, the value of such fatty acids to man would be higher if certain functional groups could be introduced or if their degree of unsaturation could be changed. Since some suitable desaturases have been cloned (Shanklin & Somerville 1991; Arondel *et al.* 1992; Cahoon *et al.* 1992), gene-transfer techniques could be used to change the level of saturation. Seed-specific expression of a stearoylacyl-carrier-protein (ACP) desaturase anti-sense gene lead to a decrease in desaturase concentrations and a concomitant accumulation of stearate in rapeseed embryos (Knutzon *et al.* 1992). Although only a first step, this achievement indicates that the production of plant oils with practically any degree of unsaturation will be possible in the future.

The chain length of fatty acids may also be engineered (Voelker *et al.* 1992) and production of complex wax esters is a possibility (Kishore & Somerville 1993).

Plants normally produce a whole series of carbohydrates with various degrees of complexity (e.g. sucrose, β-1.3 → 1.4-glucans, hemicelluloses, pectins, cellulose and starch). Engineering a plant's carbohydrate content and composition is a long-standing goal of food and chemical companies and has recently involved gene technology. For example, sucrose is synthesized in photosynthetically active tissues of plants, transported to sink tissues and converted to various polymeric carbohydrates. The enzyme catalyzing the first unique step in sucrose biosynthesis, sucrose phosphate synthase (SPS), is a potential target for engineers. A maize SPS cDNA has been expressed in transgenic tomato plants, driven by the small subunit of the Rubisco promoter, and increased the sucrose level by 50% in leaves, at the expense of starch. This is evidence that SPS is involved in carbon partitioning and it will therefore remain a target for genetic engineering (Worrell *et al.* 1991).

Starch synthesis has also been modified genetically. The gene for the initial unique enzyme in starch biosynthesis, ADP-glucose pyrophosphorylase (ADPGPP), has been cloned from an *E. coli* mutant, transferred to potato plants and expressed under the control of a patatin promoter. This manipulation increased the starch content of tubers (Stark *et al.* 1992), a desirable character as it reduces the oil content of potato chips. Although transgenic potatoes expressing the anti-sense ADPGPP-B gene accumulated only minute amounts of starch, they developed more, but smaller tubers than controls (Muller-Rober *et al.* 1992).

Starch composition has also been changed recently. Normally, starch is a two-component system of amylopectin (a polymer with 1,6-glycosyl-linked branches) and amylose (a linear polymer), the ratio of which affects the properties of starch. Transgenic potatoes expressing an anti-sense counterpart of the granule-bound starch synthase (GBSS) gene had much less amylose than wild-type plants (Visser *et al.* 1991).

All these manipulations may turn out to be starting points for the production of environmentally safer biodegradable polymers. These may be mixtures of starch and synthetic plastics or the products of foreign gene expression within bioreactor plants. For example, a gene from *Alcaligenes eutrophus* encodes an enzyme producing polyhydroxybutyrate polyesters in transgenic *Arabidopsis thaliana*, resemble plastic in their properties except that they are degradable.

Transgenic Plants as Scavengers and Ornamentals

Gene technology may also help to engineer plants that can clean pollutants from the environment. Expression of a human or mouse metallothionein gene in *Brassica napus* and tobacco conferred tolerance to cadmium (Maiti *et al.* 1989; Misra & Gedamu 1989). Crop plants could also be engineered to sequester heavy metals, such as copper, zinc, mercury or silver in tissues that are not consumed. Such transgenic scavengers, whether they be crops or not, could be exploited to concentrate heavy metals and to remove them from heavily contaminated soils.

The spectrum of colours and possible shapes in ornamental flowers can be expanded by molecular techniques. As early as 1987, the maize gene for dihydroflavonol-4-reductase (DFR) was transferred to *Petunia*, inducing a novel pigmentation, namely brick-red flowers (Meyer *et al.* 1992). Moreover, the anti-sense expression of the chalcone synthase gene, encoding the key enzyme of flavonoid biosynthesis, produced dramatic changes in floral pigment patterns, including novel patterns (Mol *et al.* 1989, 1990; Van Der Krol *et al.* 1990; Kooter & Mol 1993).

Perspectives

As this short and by no means comprehensive review indicates, the whole spectrum of gene technology is now routinely and successfully applied to a wide range of problems in plant biology, pathology, breeding and plant improvement in general. We predict that the coming decade will bring increasingly intense research in this field, in an increasing number of institutions and by an increasing number of researchers. It is our hope that these developments will not only be beneficial and profitable for the developed world but also for the developing world.

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