THE ENGINEERING OF ORNAMENTALS AND CROP PLANTS BY GENE TRANSFER TECHNIQUES: AN OVERVIEW

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RESUMEN. El aislamiento y caracterización de genes que codifican caracteres útiles, el desarrollo de técnicas para modificar estos genes *in vitro* y la transferencia de los construidos dentro de plantas blanco, todos juntos han revolucionado la biología de plantas. La transferencia de genes ha generado numerosas plantas transgénicas con nuevos o mejores caracteres, tales como la habilidad para el retrazo de la maduración del fruto, para la síntesis de componentes de interés farmacéutico, para resistencia a virus, bacterias, hongos, nemátodos y varios factores de estrés abióticos. Aunque los aspectos hortícolas eran sólo de un interés marginal en la ingeniería genética de plantas, nosotros a pesar de esto comenzamos con la descripción de los logros en este campo en particular, para que el lector pueda compararlos con los avances en otras áreas de la ingeniería genética de plantas.

PALABRAS CLAVE: Transferencia de genes, mejoramiento genético molecular de flores, ingeniería genética, plantas transgénicas.

SUMMARY. The isolation and characterization of genes encoding useful traits, and the development of techniques to modify these genes *in vitro* and to transfer the constructs into target plants have altogether revolutionized plant biology. The transfer of genes has generated numerous transgenic plants with new or improved traits such as the ability for delayed fruit ripening, for the synthesis of pharmaceutically interesting components, for resistance to virus, bacteria, fungi, nematodes and various abiotic stress factors. Though horticultural aspects were only of marginal interest in plant genetic engineering, we nevertheless start out with portraying the achievements on this particular field, so that the reader can compare them with the advancements in other areas of plant genetic engeneering.

KEY WORDS: Gene transfer, molecular flower breeding, genetic engineering, transgenic plants.

INTRODUCTION

Classical breeding technology has generated a plethora of plants with improved qualities, including yield as well as resistance towards abiotic and biotic stresses and increased contents of metabolites or polymers. Horticulturists have developed many ornamentals with changed characteristics, e.g. flower colour or shape. In spite of all these achievements, plant pathogens and the taste of the consumers change so fast, that breeding efforts certainly have to continue in order to meet the requirements of plant protection and marketing.

Since the advent of genetic engineering of plants and the availability of gene transfer techniques, the advances in tissue culture and plant regeneration technologies and the unprecedented perfection of molecular biology methods, another approach to improve plants has come to the fore: molecular breeding. It does not substitute the classical breeders work, but adds another instrumentarium to precisely and relatively quickly improve plants that proved to be recalcitrant to breeders efforts in the past.

During the last decade so much progress has been made in the genetic manipulation of crop and ornamental plants, that it is difficult to cover all aspects in detail. This overview therefore focusses on selected topics from molecular flower breeding to gene farming.

MOLECULAR FLOWER BREEDING

Molecular flower breeding, i. e. the use of recombinant techniques to support traditional floriculture,

expands the spectrum of colours, shapes, aestethical properties (e.g. fragrance quality), and the post-harvest life of flowers, fruits and foliage. This comparably new discipline has been triggered as early as 1987, when the maize gene for dihydroflavonol-4-reductase (DFR) was transferred to *Petunia*, inducing the synthesis of pelargonidin and a novel pigmentation, namely brick-red flowers (Meyer *et al.* 1992). Since then, driven by an ever expanding floricultural industry, especially in The Netherlands, and the consumers demand for novelty, molecular flower breeding became established and has left its experimental stage by now. Basically, progress in this area falls into three main categories: flower colours, flower morphology, and - to a lesser extent - flower lifetime. First steps are being made to probe into fragrance composition as well.

Engineering flower colour

Almost all of the insect-pollinated plants have large, mostly brightly coloured petals to attract pollinating animals. The colours are produced by the synthesis of flavonoids, to which class the red or purple anthocyanins and the yellow aurones and chalcones belong. In addition, many plants accumulate flavonols or flavonones in their petals, which by themselves are colourless, but change the colour of flowers by complexing anthocyanins or metal ions ("co-pigmentation"). Once pollination occurred, quick senescence is triggered in the petals, finally leading to their decay and abscission (see e.g. Koes *et al.*, 1994).

Since the biochemistry and genetics of anthocyanin synthesis has been explored in some detail, its engineering was among the prime goals of molecular flower breeders. The transfer of the maize dihydroflavonol-4-reductase-gene (dfr) into an elite *Petunia* variety created plants with orange, pelargonidin-containing petals. Instability of flower colours - a consequence of epigenetic effects - led to transformants with pale brick-red flowers (Meyer *et al.*, 1987). More recently, petals with bright orange colour have been engineered stably in *Petunia*, again introgressing the maize dfr gene (Oud *et al.*, 1995).

Alternatively, colour changes in petals can also be brought about by introducing and expressing pigmentation genes in their inverse orientation (antisense genes). Transcription of the antisense gene leads to the accumulation of an antisense RNA strand that interacts with its complementary sense RNA to form an inactive duplex. Since this duplex cannot drive translation, its presence mimics a mutation. Since van der Krol and colleagues (1988) first expressed an antisense version of the chalcone synthase (CHS) gene (encoding a key enzyme of the flavonoid pathway) in *Petunia* with dramatically changed colours, many novel pigmentation patterns were generated by antisense technology (e.g. van der Krol *et al.*, 1990; van Blokland *et al.*, 1994). A critical reappraisal of the antisense effect (e.g. cosuppression or trans-inactivation) has been published (Kooter & Mol, 1993)

Introgression of a single dominant gene encoding a rate-limiting enzyme of the anthocyanin pathway, with the result of flower colour change, certainly is relatively easy to engineer. Polygenic traits, for example a blue colour in rose, carnation or tulip petals, are more difficult to achieve. Engineering a blue flower in these ornamentals involves the synthesis of 3` 5` hydroxylated anthocyanins (delphinidins), flavonol co-pigments, and a high vacuolar pH (Holton and Tanaka, 1994; Yoshida *et al.*, 1995). None of the above plants contained delphinidins, but genes encoding flavonoid 3` 5` hydroxylase and flavonol synthase have already been isolated from *Petunia* and may be used to transform target plants (Holton *et al.*, 1993a, b; Chuck *et al.*, 1993). Engineering a high pH in vacuoles, however, may prove to be difficult. In *Petunia*, at least six genes control intracellular pH in flower petals, coined pH 1 to pH 6 genes (de Vlaming *et al.*, 1983). Additionally, other genes are involved in the regulation of pH-controlling genes (an1, an2, an11; J.N.M. Mol, pers. comm.). Though pH 6 from *Petunia* has already been cloned by transposon tagging (Chuck *et al.*, 1993), and pH 3 and pH 4 have been identified by *Petunia* transposons, and moreover, several an 1, an 2 and an 11 regulated genes ("dif genes") were isolated using differential cDNA screening (Kroon *et al.*, 1994), it is obviously a long way from these achievements to the marketing of, say, a blue rose.

Engineering flower morphology.

Flower architecture is determind by basically two cellular differentiation processes, one taking place in floral meristems, and the other in the primordia originating from these meristems. Largely unknown signals trigger meristematic activities at the positions of the floral axis, where floral organ primordia are determined to appear. Other processes, partly influenced by plant growth regulators, specify the identity of the different primordia (petals vs. sepals vs. stamen vs. pericarps). Organ identity is a consequence of the concerted action of a small set of socalled homeotic (organ identity) genes, mostly encoding transcription factors that regulate the activity of genes through binding to address sites in promotors (Weigeland & Meyerowitz, 1994). Now, flower morphology can be engineered by two routes, the overexpression or repression of such homeotic genes. Ectopic expression (Mandel *et al.*, 1992; Mizukami & Ma, 1992; Tsuchimoto *et al.* 1993; Halfter *et al.*; 1994) and suppression by antisense genes (cosuppression) or antisense RNA of organ

result, the fruits did not soften, were more resistant to mechanical stress as occurs during harvesting, packaging and transport, but other processes such as lycopene or ethylene production or pulpa formation were not affected (Smith *et al.*, 1990). Therefore the tomatoes could be left on the plant to develop their full flavor, and have been coined "Flav'r Sav'r" tomatoes.

IMPROVING NUTRITIONAL QUALITY

Genetic engineering improves the otherwise deficient content of essential amino acids of plants. Two approaches were designed. A gene has been synthesized 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). The synthetic gene route should, however, be more effective by overexpression of the synthetic transgene. Second, an increase of the low methionine content of seed proteins can also be achieved through the insertion of a gene from Brazil nut (Bertholletia excelsa) that encodes a methionine-rich protein, into target tobacco. If expressed to high levels in seeds, it improved the methionine content of the seed protein by some 30% (Altenbach *et al.*, 1989; 1990). Though initially of practical value for fodder improvement only, this strategy could also improve the methionine content in crops, and balance other essential amino acids like lysine and tryptophan.

IMPROVED ABIOTIC STRESS RESISTANCE

Although abiotic stress such as cold, heat, drought and salinity stress limits the development of a crop's potential worldwide, the genetic engineering of anti-stress capacities into plants has been cumbersome, and certainly is still in its infancy. The reasons are manifold, and one major obstacle is our ignorance about the plant's stress management. As a consequence, only a limited number of genes are available that could confer abiotic stress resistance into plants. Nevertheless there are some very encouraging reports. For example, a gene from E. coli encoding mannitol 1-phosphate dehydrogenase was transferred and constitutively expressed in tobacco. This enzyme reversibly interconverses fructose-6-phosphate and mannitol-1-phosphate. The transgenic plants accumulated the otherwise absent mannitol in leaves and roots and were highly tolerant 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.*, 1992a, b). These results support a classical concept, that in response to drought, high salinity, or low temperature, many plants accumulate osmolytes (osmoprotectants), low-molecular weight compounds such as proline, glycine-betaine, or sugar alcohols such as mannitol. These osmolytes apparently increase salt or drought tolerance. At least for mannitol, this property has been proven. Since the genetic engineering of other stress tolerances is already on its way (a gene from an arctic flounder confers cold tolerance into tomato plants, drought tolerance is tackled by abscisic acid - regulated gene engineering, and heat tolerance by heat shock gene design), we expect major breakthroughs in this area in engineering plant stress tolerance soon.

IMPROVED INSECT RESISTANCE

The present concept of insect control uses externally applied, unspecific, hazardous or potentially hazardous organochemicals (which additionally have a negative ecological impact). The expression of insecticidal compounds in transgenic plants would clearly be superior. Therefore, much effort has been invested to confer insect resistance into commercially important crop plants. Resistance could be successfully engineered via two routes: the expression of insecticidal delta-endotoxin proteins from Bacillus thuringiensis, and the ereuse of proteins interfering either with the insect's metabolism or its development.

Bacillus thuringiensis endotoxins

More than 600 different strains of the Gram-positive soil bacterium *Bacillus thuringiensis* exist worldwide. Each strain harbors a plasmid, and probably each plasmid encodes a specific protein, a delta-endotoxin protein, that is used for the construction of spore walls during endospore formation. Non-used delta endotoxin proteins are 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, and generate highly specific and toxic compounds. The fact that endotoxins from specific strains are toxic only in specific insects and close relatives can be explained by specific high-affinity receptor proteins on the brush border membrane in the insect's midgut. The extreme toxicity derives from 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). This specificity has attracted many researchers. For example, about a dozen endotoxin proteins with slight differences in their aminoacid sequences were isolated, that are toxic only to Lepidoptera. Of these toxins, the socalled cry IA (b) and cry IB (for crystal) are both toxic to *Pieris brassicae* larvae, whereas cry YIA (b) kills *Manduca sexta*. This specificity resides in different, species-specific recep-

tors that each bind only one or several related endotoxin proteins with high affinity.

First reports on the isolation, modification and transfer of B. *thuringiensis* endotoxin genes into target plants were published in 1987 (Vaeck *et al.*, 1987; Fischhoff et al., 1987; Barton *et al.*, 1987). The insecticidal protein gene from B. thuringiensis var. kurstaki conferred almost complete resistance to larvae of certain Lepidoptera species (*Manduca sexta*, Heliothis *virescens* and *H. zea*). Also, a series of other delta-endotoxin genes from other *Bacillus thuringiensis* strains have been used with the same positive result. For example, the endotoxin of B. t. strain *tenebrionis* is active mainly against the Colorado Beetle (*Leptinotarsa decemlineata*), so that transgenic plants expressing the corresponding endotoxin gene were highly resistant (Brunke & Meeusen, 1991). The endotoxin strategy has been proven successful in many cases of host plant-insect interactions (e.g. tobacco: Barton *et al.*, 1987; Vaeck *et al.*, 1987; tomato: Fischhoff *et al.*, 1987; cotton: Perlak *et al.*, 1990; potato: Chen *et al.*, 1992). The delta-endotoxin-mediated insect resistance was also effective under field conditions (Delannay *et al.*, 1989), and holds promise for the control of nematodes, trematodes, mites, and protozoa as well (Feitelson *et al.*, 1992).

Usually the expression of endotoxin genes in plants is low, but can be improved by tailoring the genes, such as trimming of the coding regions to remove plant polyadenylation signals, intron/exon splice sites and polymerase II termination signals. These mutations generally booster the expression of endotoxin genes in plants, and consequently their level of resistance (Perlak *et al.*, 1990; 1991; Koziel *et al.*, 1993). Two technical improvements have added to the effectiveness of the B. t. toxin strategy. First, translational fusions e.g. between cry IA and cry IC genes are superior to wild-type B. t. genes. Thus it is recommended to engineer translational fusions into target plants. Second, fully synthetic endotoxin genes, appropriately designed, confer better resistance on crops than their wild-type counterparts, and are also effective in the field, at no expense of the non-transgenic plants (Koziel *et al.*, 1993).

Inspite of all this progress, there remains concern. First, the insect population may develop resistance against the toxin by mutations in the receptor protein genes. In fact, several important pests adapted to B. t. toxins in the laboratory (e.g. *Heliothis virescens*, *Leptinotarsa decemlineata*, *Plodia interpunctella*, *Plutella xylostella*), 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 to cope with two rather than only one endotoxin protein. Second, the presence of relatively high amounts of delta-endotoxin proteins in crop plants may stress the plant's energy balance. One way to overcome this problem could involve promoters linked to the endotoxin gene that are normally silent but become active after the insect starts eating (e.g. wound-inducible promoters).

Protease inhibitors and other proteins.

About eight non-related protease inhibitor families are present in plants and serve to inhibit serine-, cysteine-, aspartic acid-and metallo-proteases. They reach especially high concentrations in seeds and tubers. Since they are less or not active on endogenous plant proteases, they are probably involved in pest and pathogen defence mechanisms. Their specific function in this process would be an inhibition of insect proteases and with an antinutritional effect. There is some evidence in favor of this hypothesis (see e.g. Sanchez-Serrano *et al.*, 1986; Peña-Cortes *et al.*, 1989; Pearce *et al.*, 1991). Genetic engineering of insect resistance could then 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 slight resistance against the tobacco budworm, *Heliothis virescens* (Hilder *et al.*, 1987; 1990). Basically the same strategy has been applied to engineer *Manduca* sexta resistance into tobacco, using potato serine protease inhibitor PI-II (Johnson *et al.*, 1989). However, only high levels of inhibitor show effect. On the other hand, protease inhibitors act unspecifically, so that they could protect against a broad spectrum of insects.

Basically the same strategy has been used to engineer insect resistance into target plants with the help of alpha-amylase inhibitor, or bifunctional alpha-amylase/serine protease inhibitor genes. Moreover, other genes encoding other antinutritional proteins were transformed into plants and there expressed, and conferred at least weak insect resistance (e.g. genes encoding neuropeptides, for example proctolin, that interferes with insect development; or the gene for tryptophan decarboxylase, which converts tryptophan to tryptamine, a potent serotonin precursor, whose presence hampers mating, feeding or development of insects). This incomplete list demonstrates the potential of this approach in plant improvement.

IRPMOVED FUNGUS RESISTANCE

Engineering of traits that are encoded by two or more genes (QTLs; quantitative trait loci), is a more demanding challenge for plant gene technology. The resistance of plants towards pathogenic fungi was originally thought to be encoded by oligogenes. Ho-

wever, in some cases it needs only one gene to confer appreciable tolerance into susceptible host plants. Genetic engineering of resistance to pathogenic fungi follows several promising routes, and some are portrayed below.

The phytoalexin route

Phytoalexins are low-molecular weight organic compounds synthesized rapidly in plant cells that are infected by a fungus. Depending on their concentration, the different phytoalexins exhibit fungistatic or fungicidal activities, and are part of the plant's defence machinery (see e.g. Bailey, 1987). Any change in the composition of the phytoalexins would add to the defence potential of the host plant. The transfer of a single gene encoding stilbene synthase from grapevine 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 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*, as e.g. thionins (Bohlmann & Apel, 1991), osmotins (Vigers *et al.*, 1991; Woloshuk *et al.*, 1991) or zeamatins (Roberts & Selitrennikoff, 1990) are potential intracellular fungicides, but have not yet been tested in transgenic plants.

Chitinases and ß-1.3-glucanases are constituents of most plant cells and belong to the socalled pathogenesis-related proteins (PR proteins), because their synthesis is increased dramatically after attack by phytopathogens (Bol *et al.*, 1990). Both enzymes together destroy fungal cell walls containing ß-1.3-glu-cans and chitin and thereby inhibit fungal growth (Schlumbaum *et al.*, 1986). Of the various classes of chitinases and ß-1.3-glucanases only vacuolar class I hydrolases are potent fungal inhibitors (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993; Cornelissen & Melchers, 1993). The improvement of resident chitinase and/or ß-1.3-glucanase gene expression by strong constitutive promoters has therefore been tried with limited success (Lund *et al.*, 1989; Neuhaus *et al.*, 1991; Broglie *et al.*, 1991). Though the over-expression of a chitinase gene from bean in transgenic tobacco conferred a distinct resistance against *Rhizoctonia* solani, probably due to the hydrolysis of newly formed chitin in growing infection hyphae, a substantial resistance can most likely be engineered only by the combination of several genes (gene pyramiding). Therefore the simultaneous expression of class I or class V chitinase, class I ß-1.3-glucanase and additional genes (e.g. chitin-binding protein [CBP] gene, ribosome-inhibiting protein [RIP] gene, or others) promises a better level of resistance than has been achieved till now. Moreover, the extracellular (apo-plastic) space is probably the first site of encounter between pathogen and host, and fungitoxic hydrolases should therefore be targeted to this compartment. First targeting experiments were successful. Cbalass I hydrolases from tocco were modified and correctly excreted to 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 certainly be developped in 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 enzymes of fungi and probably protect the host plants.

Artificial cell death

Race-specific resistance of potato cultivars against *Phytophthora infestans* is mediated by a programmed cell death at the infection site, which precludes 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 denfense reaction occurs, so that 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 (this 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 suicidal effects of the ba-RNase that might occur in non-affected plant cells by leaky promoters, were minimized by the simultaneous transfer and constitutive expression of barstar genes (encoding a highly specific barnase protein inhibitor; Hartley, 1989). In transgenic potato plants indeed a localized cell death has been observed with concomitant increase in resistance to Phytophthora infestans (Taylor *et al.*, 1990; Martini *et al.*, 1993; Strittmatter & Wegener, 1993).

VIRUS RESISTANCE

Engineering virus resistance into host plants follow several routes: coat protein-mediated resistance, protection through expression of satellite RNA or replicase sequences, interference by defective RNA or DNA sequences, and an antisense RNA-mediated protection. Of these, coat protein-mediated protection has been the first and the most successful way to generate virus-resistant plants.

Coat protein-mediated virus resistance

The concept of coat protein-mediated protection derives from cross protection mechanism: 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. Though known for many years (see Sequeira, 1984), the mechanism of cross protection is far from being clear. One possible explanation of this phenomenon rests on the accumulation of excessive amounts of coat proteins (cps) in the infected cell, that are not bound to viral RNA. These coat proteins 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. This has been found in more than 20 transgenic plants, that were transformed with the sense coat protein gene, including crop plants such as: tomato (Tumer *et al.*,1987; Nelson *et al.*, 1987), potato (Hoekema *et al.*, 1989; van den Elzen *et al.*, 1989; Kawchuk *et al.*, 1990; Kaniewski *et al.*, 1990; MacKenzie & Tremaine, 1990; van der Wilk *et al.*, 1991), alfalfa (Hill *et al.*, 1991) and rice (Hayakawa *et al.*, 1992). Most experiments have been performed with tobacco (Powell-Abel *et al.*, 1986; Tumer *et al.*, 1987; Loesch-Fries *et al.*, 1987; Nelson *et al.*, 1987; van Dun *et al.*, 1987; Hemenway *et al.*, 1988; *Cuozzo et al.*, 1988; van Dun & Bol, 1988; Anderson *et al.*, 1989; Gielen *et al.*, 1991; Lindbo and Dougherty, 1992; de Haan *et al.*, 1992; Brault *et al.*, 1993; for a recent review see Strittmatter & Wegener, 1993, and Willmitzer, 1993). In each case, resistance was strongly correlated with the amount of intact coat protein in transgenic plants (Loesch-Fries *et al.*, 1987; Hemenway *et al.*, 1988).

For virus infection, not only the sense coat protein gene could confer resistance into plants, but also its antisense counterpart. 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 inoculums. Also, defective cp genes have successfully been used, e.g. a cp cistron of potato virus Y from which the translational start signal was deleted (van der Vlugt *et al.*, 1992). Transferring coat protein genes of tobacco etch virus, whose products were untranslatable, into tobacco plants, conferred a limited tolerance to viral infection (Lindbo and Dougherty, 1992).

Coat protein-mediated protection is not specific. In some cases, the transgenic plant is not only protected against the virus from which the coat protein gene originates, but also against other serologically unrelated viruses (Stark and Beachy, 1989; Nejidat and Beachy, 1990; Ling *et al.*, 1991).

The coat protein strategy is also effective in the field (Nelson *et al.*, 1988; Beachy et al. 1990; Kaniewski et al., 1990) and against mixed infections by two different viruses (Lawson *et al.*, 1990). Though the molecular mechanism of cp-dependent protection is unknown, and additional mechanisms may add to this phenomenon (Golemboski *et al.*, 1990; MacKenzie *et al.*, 1990), this strategy has great potential for genetic engineering of plants (see Baulcombe 1994).

Satellite RNA strategy

Satellite RNAs are extragenomic small components of some RNA virus, that require an intact genome of a helper virus for their replication and propagation within a plant. Such satellite RNAs can modulate the disease symptoms caused by the corresponding helper virus. Therefore, the genomes of the satellite RNAs from cucumber mosaic virus and tobacco ringspot virus were introduced into target plants, and expressed under the control of a strong constitutive promoter (Baulcombe *et al.*, 1986; Gerlach *et al.*, 1987; Harrison *et al.*, 1987). As a consequence, a delay of disease symptom development was observed after a challenge with the corresponding virus. However, a risk exists in that a mutation could convert a benign satellite RNA molecule into a virulent one.

Replicase

The introduction of full-length coding sequences for non-structural proteins such as proteases or replicases (RNA-dependent RNA polymerase) also allows to engineer plants for virus resistance. For example, transgenic tobacco expressing a truncated replicase gene from a specific tobacco mosaic virus strain, was highly tolerant to TMV and closely related strains (Golembowski *et al.*, 1990). Also, the corresponding sequence of pea early browning virus replicase conferred resistance to this virus and two related strains (MacFarlane and Davies, 1992). There are also exceptions; the expression of functional alfalfa mosaic virus replicase did not lead to resistance in transgenic tobacco (Taschner *et al.*, 1991) nor were barley protoplasts immune against brome mosaic virus 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

Partial or complete resistance towards the original or related virus can be achieved by the introduction of various antisense sequences of viral genes into target plants. Expression of antisense RNA from the al gene encoding a replication protein leads to protection of the plants against Tomato Golden Mosaic Virus (Day *et al.*, 1991). The presence of antisense coat protein genes mediated considerable resistance of potato to Potato Leafroll Virus (Kawchuk *et al.*, 1990), and an antisense transcript of Brome Mosaic Virus RNA 3 intercistronic region blocked viral RNA replication (Huntley and Hall, 1993). Only weak protection, however, has been achieved with antisense technologies (Cuozzo *et al.*, 1988; Hemenway *et al.*, 1988; Rezian *et al.*, 1988; Powell *et al.*, 1989).

Alternative strategies

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

Moreover, immunoprotection of plants may be possible in future, if antibodies directed towards essential viral components (e.g. coat proteins, repli-cases) are synthesized by transgenic plants. Transgenic plants can produce complete antibodies (Hiatt *et al.*, 1989; Düring *et al.*, 1990).

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

BACTERIAL RESISTANCE

As opposed to the progress in engineering virus resistance into plants, there are only few reports on the successful generation of bacteria-resistant transgenic plants. Basically, three approa-ches are used. First: bacterial genes encoding enzymes degrading bacterial cell walls have been introduced into plants. For example, lysozyme genes from hen egg white or bacteriophage T4 were transferred to tobacco and potato plants. High-level expression and secretion of the lysozyme into the intercellular spaces seem to protect e.g. potato tuber slices of transgenic plants from heavy infection by the pathogenic *Erwinia carotovora* spp. *atroseptica* (Trudel *et al.*, 1992; Düring *et al.*, 1993). Second: genes encoding anti-;nicrobial, cysteine-rich thionins (Bohlmann and Apel, 1991) were transferred into tobacco. High-level expression of thionin genes driven by CaMV 35S promotors reduced disease symptoms and severity caused by *Pseudomonas syringae pv. tabaci* or *P. syringae pv. syringae* (Carmona *et al.*, 1993). Third: conferring the ability to detoxify bacterial toxins onto plants, seems to be the most promising system. For example, Pseudomonas syringae pv. tabaci produces the phytotoxic dipeptide tabtoxin, which induces the chlorotic wildfire disease in tobacco, probably by inhibiting the host's glutamine synthetase, leading to the accumulation of toxic ammonia. The bacterial gene ttr, that encodes an acetylase inactivating tabtoxin, has been transferred into tobacco and constitutively expressed. The wildfire disease symptoms on the transgenic plants were less pronounced (Anzai *et al.*, 1989). A similar strategy was used by Herrera-Estrella and coworkers, who targeted a bacterial gene encoding a toxin-resistant target enzyme, in this case ornithine carbamoyl transferase, into plastids of plants. The presence of a toxin-insensitive enzyme in the host made it resistant to phaseolotoxin of *Pseudomonas syringae pv. phaseolicola* (de la Fuente-Martinez *et al.*, 1992).

GENE FARMING

Major domains of conventional agriculture such as the production of food, feed, fiber and fuel have been taken over by the petrochemical industry. However, the petrochemical resources are definitely limited and non-renewe, and petrochemicals and their derivatives frequently are hazardous to man and his environment. Therefore many activities began to exploit plants as factories to produce fuel or other renewable products. And the genetic engine-ering of plants to highly productive, relatively cheap and easy-to-handle bioreactors indeed is one of the prime goals of many companies and institutions worldwide. A few examples illustrate the versatility of this approach.

Production of peptides and proteins

Pharmaceutical peptides have already been produced in transgenic plants. One of the first bioreactors, the oilseed rape, synthesized the pentapeptide opiate leuenkephalin, after being engineered with a fusion from 2S albumin seed protein gene from Arabidopsis thaliana and leuen-kephalin gene sequences. The fusion product 2S albu-min-leuenkephalin accumulated in the producer plants to relatively high levels (10-200 g per hectare), and leuenkephalin could

be recovered from rape seed extracts after protease treatment and HPLC purification (Vanderkerckhoeve et al., 1989; Krebbers and Vanderkerckhoeve, 1990).

The production of high-molecular weight proteins in plants is also feasible. Human serum albumin was 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 are now being produced in planta (e.g. antigenic proteins for vaccine production; Mason *et al.*, 1992). The gene farming of pharmaceutical peptides and proteins ("gene pharming") is about to begin (e.g. Swain, 1991).

Production of oils and carbohydrates

Higher plants contain about 200 different fatty acids, most of which are non-edible and interesting only for industrial purposes (Murphy, 1992). In major crops, fatty acids of acyl chain lengths from C_{16} to C_{22} are bound to glycerol in the form of triacylglycerols. Generally, the value of such fatty acids would be higher, if certain functional groups could be introduced, or if their degree of unsaturation would be changed. Since some of the responsible desaturases have been cloned (Shanklin and Somerville, 1991; Cahoon *et al.*, 1992 and Arondel *et al.*, 1992), gene transfer techniques could be involved. Seed-specific expression of a stearoylacyl carrier protein (ACP) desaturase anti-sense gene lead to a decrease in desaturase levels and a concomitant accumulation of stearate in rapeseed embryos (Knutzon *et al.*, 1992). This achievement lend to the expectation that the production of plant oils with practically any degree of unsaturation will be possible in future. Also, the chain length of fatty acids could be engineered (Voelker *et al.*, 1992), and the production of complex wax esters becomes a possibility (Kishore and Somerville, 1993).

Plants normally produce a whole series of carbohydrates with various degrees of complexity (e.g. sucrose, ß-1.3,1.4-glucans, hemicelluloses, pectins, cellulose, and starch). Engineering the carbohydrate content and composition is a long-standing goal of food and chemical companies and involved gene technology recently. 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), therefore is a potential target for engineers. A maize SPS cDNA has been expressed in transgenic tomato plants driven by the small subunit 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 will therefore remain a target for genetic engineering (Worrell *et al.*, 1991).

Starch synthesis is another target for genetic engineers. 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 to reduce the oil content of potato chips. Also, transgenic potatoes expressing the antisense ADPGPP-B gene accumulated only minute amounts of starch, but developped more, though smaller tubers than controls (Muller-Rober *et al.*, 1992).

Not only starch content, but also its composition has 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), and both components add to the properties of starch. These properties can be changed by modifying the relative proportions of both constituents, which was recently achieved in transgenic potatoes expressing an antisense counterpart of the granule-bound starch synthase (GBSS) gene. These plants had little or no amylose as compared to wild-type potatoes (Visser *et al.*,1991).

In future these manipulations may turn out to be starting points for the production of environ-mentally safer biodegradable polymers, that may either be mixtures of starch and synthetic plastics, or the products of foreign gene expression within the bioreactor plant (e.g. a gene from *Alcaligenes eutrophus* encodes an enzyme producing polyhy-droxybutyrate polyesters in transgenic *Arabidopsis thaliana*, resembling plastic in its properties, but being degradable; see also Byrom, 1991).

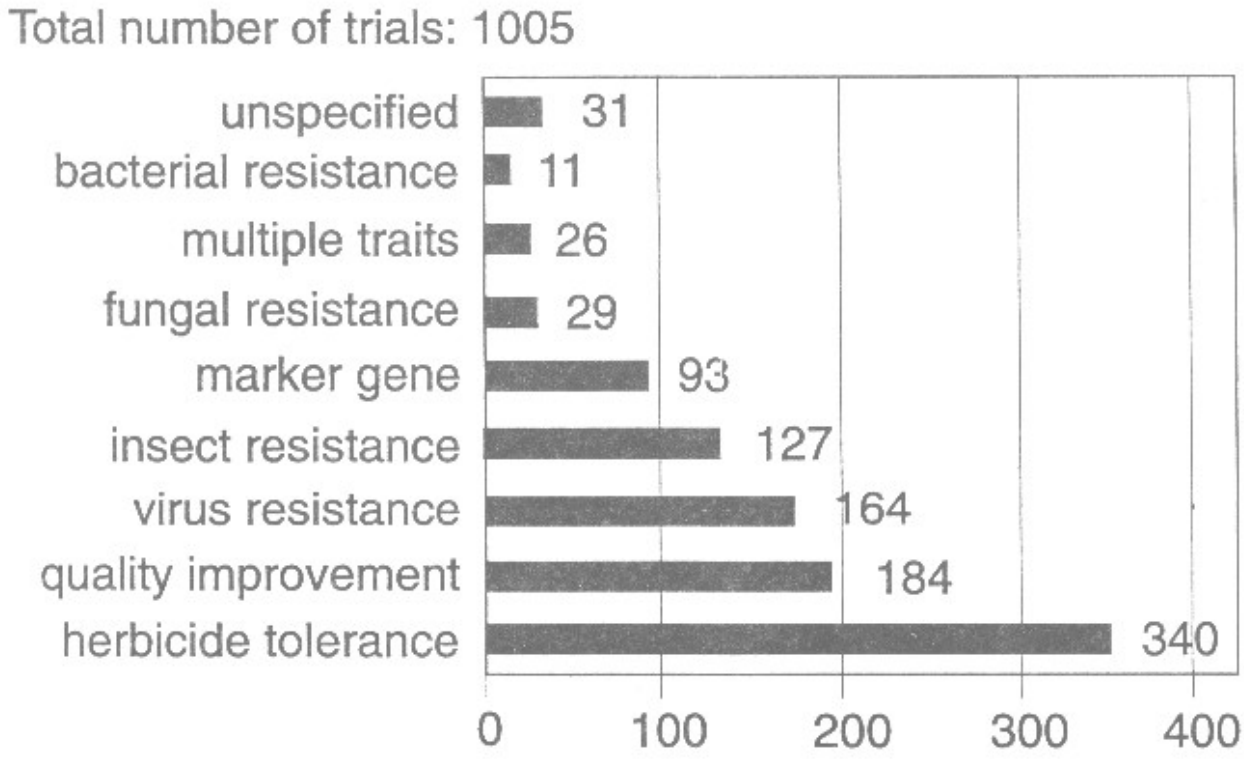
HEAVY METAL SCAVENGERS

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

FIELD TRIALS OF GENETICALLY ENGINEERED PLANTS

The increasing number of field trials worldwide reflects an ever growing commercial interest in genetically

Fig. 1. Field trials of genetically modified plants: world distribution (1986-1995).



Fif. 2. Field trials of genetically modified plants: number of trials per trait (1986-1993).

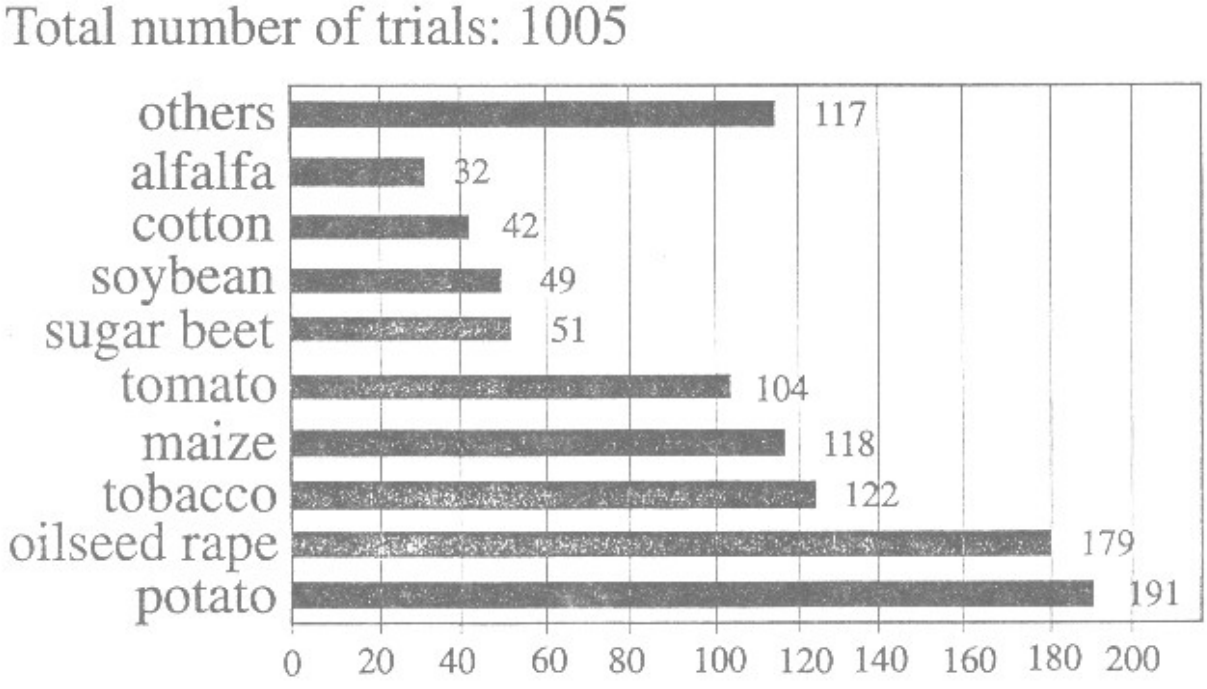


Fig. 3. Field trials of genetically modified plants: number of trials per crop (1986- 1993).

modified plants. World leader in this respect is the USA accounting for more than 50 % of all trials, followed by Western Europe with 40 %. Central and South America, the Pacific Rim and the Middle East together contributed about 10 %. As can be extracted from Fig. 1., the distribution of trials among different countries does not only reflect their agricultural interests but, as exemplified by numbers for Germany, also public opinion influenced by strong opposition of environmentalists who blocked and still hinder the agricultural use of genetically modified plants. Public opinion is slowly changing in Western countries as it becomes obvious that the advantages of genetically engineered crops outnumber potential risks by orders of magnitude. These changes in public perception lead to more practical and less restrictive laws, that allow to release genetically engineered crops to the field more easily.

In the past commercial interests mostly focussed on herbicide tolerance where global players in agricultural industry try to market integrated concepts for plant protection together with their herbicides. Other agricultural aspects like quality improvement and resistances against various pests certainly will have a growing impact in future because the respective resistance genes are coming into reach of researchers. For example, mapped-based cloning and analysis of resistance genes (for a recent review see Winter and Kahl 1995) foster the understanding of natural defense mechanisms. These genes will be the subject of intense laboratory and field studies in the next decade which will help to replace environmentally problematic pesticides by genetically engineered resistant varieties.

Starting with-easy-to-manipulate model plants like tobacco, interests will focus on agricultural important crops like potato, oilseed rape, maize, tomato, sugar beet, soybean, cotton and alfalfa in near future. The fact that no important cereal like wheat, barley or, even more important - rice - shows up in fig. 3 is not because there is no commercial interest in the genetic engineering of these crops but is caused by the relatively late development of reliable and effective transformation and regeneration systems for these crops. These problems have now been solved (Vasil, 1990; Datta *et al.*, 1990; Vasil *et al.*, 1993; Wan and Lemiaux, 1994), and field trials of genetically improved cereals may be expected in due time. The number of field trials on ornamentals like *Petunia*, Chrysanthemum or Gerbera will certainly increase in future when genetic flower breeding becomes common place and engineered traits become marketing factors.

Outlook

The whole spectrum of gene technology is now routinely and successfully applied to a wide range of problems in plant biology, pathology, breeding and, generally, plant improvement. We predict that the coming decade will be confronted with an ever intensified research on this field involving an increasing number of institutions and researchers, both in the developed but also the developing world.

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TABLE 1. Engineering of Ornamentals and Crop Plants by Gene Transfer Techniques.

Engineered Trait	Technique
1. Molecular flower breeding	
Flower colour change	Gene insertion strategy
	Anti-sense strategy
Engineering flower morphology	Homeotic gene modulation
Engineering flower vase life	Anti-sense strategy
	Ethylene suppression strategy
2. Resistance	
Virus resistance	Coat protein strategy
	Satellite RNA strategy
	Replicase strategy
	Anti-sense technology
	Immunoprotection
	Movement protein strategy
	Ribosome-inactivating protein strategy
Bacterial resistance	Lysozyme strategy
	Thionin strategy
	Detoxification
Fungal resistance	Phytoalexin gene insertion strategy
	Immunoprotection
	Programmed cell death
	Chitinase/β-1.3-glucanase strategy
	Antifungal protein (AFP) strategy
Insect resistance	*Bacillus thuringiensis* endotoxin strategy
	Protease inhibitor gene insertion
	a-Amylase inhibitor gene strategy
3. Abiotic stress tolerance	
Salt resistance	Mannitol-l-phosphate dehydrogenase gene transfer
Cold tolerance	Arctic flounder gene transfer
Drought tolerance	Abscisic acid-regulated gene engineering
Heat tolerance	Heat shock gene engineering
4. Fruit quality	
Ripening	Interference with ethylene biosynthesis
	Anti-sense strategy
Softening	Anti-sense strategy
5. Nutritional quality	
Balanced amino acid content	Synthetic gene strategy
	Amino acid complementation strategy
6. Gene farming ("gene pharming")	
Peptide and protein production	Gene insertion strategy

Engineered Trait	Technique
Oil and carbohydrate production	Anti-sense strategy
	Gene insertion strategy
	Gene amplification
Production of degradable polymers	Gene insertion strategy
	Gene amplification
Production of biochemicals	Gene insertion strategy
	(mannitol, cyclodextrin)
Heavy metal trapping	Gene insertion strategy