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Overview of Gene Availability, Identification, and Regulation

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In 1981, the first transgenic plant capable of Mendelian transmission of an introduced gene was reported (Otten et al., 1981). In 1994, barely more than a dozen years later, the first genetically engineered major crop, bromoxynil-resistant cotton (*Gossypium hirsutum* L.), was deregulated by the U.S. Dept. of Agriculture for commercial production. Shortly thereafter, the first genetically engineered food product, the slow-softening Flavr-Savr tomato (*Lycopersicon esculentum* Mill.), was similarly approved for marketing. These historic milestones foretell a future in which gene transfer will be used increasingly for the introduction of important agronomic traits into crops. In planning for this future, it is useful to consider what genes are currently available for transfer, how new genes may be identified, and what elements of gene design affect expression of the transgene.

GENE AVAILABILITY

Table 1 lists more than three dozen genes that, while not exhaustive, indicate the range of genes that have been used to produce phenotypes of potential agronomic importance.

Crop quality or yield

The introduction of antisense genes for specific enzymes has been used to impart such diverse phenotypes as slow-softening tomatoes, which permit longer vine-ripening while retaining good shipping qualities (Hamilton et al., 1990; Oeller et al., 1991; Sheehy et al., 1988; Smith et al., 1988), and highly saturated canola (*Brassica napus* L.) oil, which provides a noncatalytically hydrogenated substitute for margarine (Knutzon et al., 1992; also see Ohlrogge, 1994). The use of sense constructs of other genes have increased the level of polyunsaturated fat in canola oil (Arondel et al., 1992; Okuley et al., 1994), produced sweeter tomatoes (Penarrubia et al., 1992), and altered photosynthate partitioning—owing to diminished regulation of transgene enzyme products in heterologous hosts (Micallef et al., 1993; Stark et al., 1992). Male-sterile plants, useful for hybrid seed production (Mariana et al., 1990), and novel flower colors (Meyer et al., 1992) also have been produced with sense constructs.

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Specialty products

Genes from various sources have been used to produce specialty products. Poly- β -hydroxybutyrate, a thermoplastic feedstock (Poirier et al., 1992), and cyclodextrin, a complexing agent (Oakes et al., 1991), have been synthesized by plants carrying engineered bacterial genes. Engineered human genes have been used to cause production of human serum albumin (Sijmons et al., 1990) and the endorphin leu-enkephalin (Vandekerckhove et al., 1989) in plants. Likewise, a plant lauroyl-ACP thioesterase gene placed under control of a highly active promoter was used to substantially shift the oil content of canola oil to lauric acid, an ingredient of soaps and cosmetics (see Ohlrogge, 1994; Voelker et al., 1992).

Environmental stress tolerance

Several transgenes were used to enhance tolerance to environmental stress. Production of the osmolyte mannitol imparted salt tolerance to tobacco (*Nicotiana tabacum* L.) (Tarczynski et al., 1993), while expression of a glycerol-3-phosphate acyltransferase gene from cold-tolerant *Arabidopsis thaliana* (L.) Heynh. increased the proportion of *cis*-unsaturated fatty acids in membrane phosphatidylglycerol of cold-sensitive tobacco—thereby increasing its tolerance to chilling (Murata et al., 1992). Freezing tolerance was enhanced by transgenes for either fish antifreeze protein (Hightower et al., 1991) or superoxide dismutase (McKersie et al., 1993; Van Camp et al., 1994). The latter enzyme destroys active-oxygen species generated in plants by freeze-thaw cycles or by certain herbicides, and confers herbicide and ozone tolerance, as well.

Herbicide tolerance

Engineered resistance to rapidly degraded herbicides—part of a strategy for environmentally safer weed control—has been achieved in two ways: by introducing bacterial genes that either degrade or detoxify the chemicals (2,4-dichlorophenoxy)acetic acid (2,4-D monooxygenase) (Streber and Willmitzer, 1989) and (bromoxynil-specific nitrilase) (Stalker et al., 1988), respectively, or, alternatively, by introducing a gene encoding a structurally altered target enzyme, 5-enolpyruvylshikimate-3-P (EPSP) synthase (Comai et al., 1985) or acetolactate synthase, that no longer binds the herbicide (Haughn et al., 1987).

Pest resistance

Transgenes conferring disease and insect resistance (Table 1, last category) is the largest and fastest growing category. There are numerous examples in which introduction of specific viral coat protein (see Beachy et al., 1990) or replicase genes (Donson et al., 1993; Golemboski et al., 1990) or viral-specific antisense constructs (see Agrawal, 1992) provided protection against groups of closely related

viruses. A broader range of protection has been achieved by introducing the ribosome-inhibiting protein gene from pokeweed (*Phytolacca americana* L.) (Lodge et al., 1993).

Two approaches have been successful in conferring insect resistance. The first used genes whose products are toxic to feeding insects—Bt insecticidal protein (Perlak et al., 1991; Vaeck et al., 1987) and the cowpea [*Vigna unguiculata* (L.) Walp.] trypsin-inhibitor protein (Hilder et al., 1987). The second used a cytokinin-synthesis

Table 1. Genes introduced into crops and the phenotypes conferred.

Transgene	Trait conferred	Crop modified	Reference ^a
Crop quality or yield			
Antisense polygalacturonase	Slow-softening fruit	Tomato	Sheehy et al., 1988; Smith et al., 1988
Antisense ACC synthase	Delayed fruit ripening	Tomato	Oeller et al., 1991
Antisense ACC oxidase	Delayed fruit ripening	Tomato	Hamilton et al., 1990
Antisense stearoyl-ACP desaturase	High stearic (18:0) acid	Rapeseed, turnip rape	Knutzon et al., 1992; also see Ohlrogge, 1994
Omega-3 desaturase	High linolenic (18:3) acid	<i>Arabidopsis</i>	Arondel et al., 1992; Okuley et al., 1994
Monellin	Sweet protein	Tomato	Penarrubia et al., 1992
Maize sucrose-P synthase	High fruit yield	Tomato	Micallef et al., 1993
<i>Escherichia coli</i> ADP glucose pyrophosphorylase	High starch/dry weight	Potato	Stark et al., 1992
Ribonuclease	Male sterility	Tobacco, rapeseed	Mariani et al., 1990
Dihydroflavonol reductase	Flower color	Petunia (<i>Petunia hybrida</i> Vilm.)	Meyer et al., 1992
Specialty products			
Acetoacetyl-CoA reductase and PHB synthase	Plastic (polyhydroxybutyrate) production	Aradopsis	Poirier et al., 1992
Cyclodextrin glucosyl transferase	Cyclodextrin production	Potato	Oakes et al., 1991
Human serum albumin	Serum albumin production	Potato	Sijmons et al., 1990
Enkephalin pentapeptide	Leu-enkephalin endorphin production	Rapeseed	Vandekerckhove et al., 1989
Lauroyl-ACP thioesterase	High lauric (12:0) acid	Rapeseed	See Ohlrogge, 1994; Voelker et al., 1992
Environmental stress tolerance			
Mannitol 1-P dehydrogenase	Salt tolerance	Tobacco	Tarczyński et al., 1993
Glycerol-3-P acyltransferase	Cold tolerance	Tobacco	Murata et al., 1992
Fish antifreeze protein	Freezing tolerance	Tobacco, tomato	Hightower et al., 1991
Superoxide dismutase	Freezing, herbicide and ozone tolerance	Alfalfa (<i>Medicago sativa</i> L.), tobacco	McKersie et al., 1993; Van Camp et al., 1994
Herbicide tolerance			
2,4-D monooxygenase	2,4-D resistance	Tobacco	Streber and Willmitzer, 1989
Bromoxynil-specific nitrilase	Bromoxynil resistance	Tobacco	Stalker et al., 1988
Phosphinothricin acetyltransferase	Glufosinate (Basta) resistance	Potato, tomato	De Block et al., 1987
Mutated EPSP synthase	Glyphosate (Roundup) resistance	Tobacco	Comai et al., 1985
Mutated acetolactate synthase	Sulfonylurea (Glean, Oust) resistance	Tobacco	Haughn et al., 1987
Pest resistance			
Specific virus coat proteins	Resistance to related viruses	Various	See Beachy et al., 1990
Viral replicase	Resistance to related viruses	Tobacco	Donson et al., 1993; Golemboski et al., 1990
Viral-specific antisense	Virus resistance	Various	See Agrawal, 1992
Pokeweed ribosome-inhibiting protein	Broad-spectrum virus resistance	Tobacco, potato	Lodge et al., 1993
<i>Bacillus thuringiensis</i> (Bt) insecticidal protein	Resistance to lepidopteran insects	Tobacco, tomato	Perlak et al., 1991; Vaeck et al., 1987
Cowpea trypsin inhibitor protein	Resistance to various insects	Tobacco	Hilder et al., 1987
Isopentenyl transferase (<i>ipt</i>)	Resistance to various insects	<i>Nicotiana glumbaginifolia</i> Viv.	Smigocki et al., 1993
Barley ribosome-inhibiting protein	Resistance to <i>Rhizoctonia solani</i>	Tobacco	Logemann et al., 1992
Bean chitinase	Resistance to <i>R. solani</i>	Tobacco, rapeseed	Brogie et al., 1991
Bacterial chitinase	Resistance to <i>Alternaria longipes</i>	Tobacco	Jones et al., 1988
Tobacco osmotin	Resistance to <i>Phytophthora infestans</i>	Potato	Liu et al., 1994
Bacteriophage lysozyme	Resistance to <i>Erwinia carotovora</i> subsp. <i>atropsetica</i>	Potato	During et al., 1993
Tomato Pto (a protein kinase)	Resistance to <i>Pseudomonas syringae</i> pv. tomato	Tomato	Martin et al., 1993
Barley α -thionin	Resistance to <i>P. syringae</i> pv. <i>syringae</i>	Tobacco	Carmona et al., 1993
Insect cecropin	Resistance to <i>E. carotovora</i> and <i>P. solanacearum</i>	Potato, tobacco	Hassan et al., 1993; Jaynes et al., 1993

^aGenerally, only the earliest reference for the introduction of a particular gene is cited.

gene (*ipt*), under control of a wound-inducible promoter (Smigocki et al., 1993), to indirectly induce synthesis of one or more insecticidal metabolites (A. Smigocki, unpublished).

Resistance to certain fungal pathogens has been obtained by introducing a barley (*Hordeum vulgare* L.) gene for ribosome-inhibiting protein (Logemann et al., 1992) and by genes encoding chitinase (Broglie et al., 1991; Jones et al., 1988), osmotin (Liu et al., 1994), and lysozyme (Doring et al., 1993). Bacteria-resistance genes are less well known; however, a tomato *Pto* gene conferred resistance to *Pseudomonas syringae* pv. *tomato* when transferred to a susceptible isoline (Martin et al., 1993). Likewise, the introduction of the bactericidal polypeptide genes, α -thionin (Carmona et al., 1993) and cecropin (Hassan et al., 1993; Jaynes et al., 1993), imparted resistance to certain bacterial pathogens.

GENE IDENTIFICATION

For identification and cloning purposes, genes may be classified into two groups: those for which a product is known and those for which there is no known product associated with the phenotype. All but two of the genes listed in Table 1 fall into the first group. Most mapped traits fall into the second.

Traditional methods of gene cloning, based on antibodies to purified proteins or knowledge of the protein's amino acid sequence, cannot be used with the second class. Consequently, two approaches have been used mainly for this class—gene tagging and map-based cloning.

Cloning tagged genes

In the gene-tagging approach, mutants are created by random insertion of a DNA tag—either modified T-DNA (transfer-DNA) (for example, see Okuley et al., 1994) or a transposon (for example, see Chuck et al., 1993). From these mutants, the tag, along with flanking plant DNA from the gene associated with the altered phenotype, is cloned by T-DNA/plasmid rescue or by hybridization-based screening using the tag as a probe. The main difficulties with gene tagging lie in producing many tag-transgenic plants with recognizable mutations and in efficiently screening plants for the desired mutant.

Map-based cloning

Map-based, or positional, cloning offers a direct approach to cloning mapped traits. As an example, cloning the tomato *Pto* gene, which confers resistance to races of *P. syringae* pv. *tomato* carrying the avirulence gene *avrPto* (Martin et al., 1993), is summarized in Fig. 1. Briefly, progeny of crosses between resistant (*Pto/Pto*) and near-isogenic susceptible (*pto/pto*) lines of tomato were scored for restriction fragment length polymorphisms (RFLP) and resistance to infec-

Fig. 1. Map-based cloning of the *Pto* gene from tomato (adapted from Martin et al., 1993).

tion—the frequency of cosegregation reflecting their proximity on the chromosome. One RFLP marker cosegregated with the *Pto* locus and was used to screen a tomato yeast artificial chromosome (YAC) genomic library and identify a 400-kb clone. To establish that the clone in fact spanned the *Pto* locus, end-specific probes were prepared to the left and right ends by inverse polymerase chain reaction (PCR) amplification and mapped. Having established that the right-end specific probe cosegregated with the *Pto* locus and the accompanying RFLP, the 400-kb DNA fragment was isolated and used to probe a tomato leaf cDNA library. A cDNA clone, so identified, cosegregated with *Pto* and, when transferred into the susceptible isoline, conferred resistance to the pathogen.

The power of this approach was illustrated by their findings that the *Pto* gene encodes a protein kinase-like protein and that DNA homologs were present in all other species examined. The latter finding points to the possibility that other species contain similar genes with similar functions and may, therefore, be easily cloned with the probes developed for tomato.

GENE EXPRESSION AND REGULATION

In constructing genes for use in plants, it is useful to consider whether high expression levels will be needed to impart the desired phenotype. Low-level expression of genes encoding novel enzymes, for example, may be sufficient to achieve the desired end, whereas higher expression of genes encoding pesticidal toxins may be required to provide adequate control of the target pest.

Fig. 2. Schematic representation of gene expression.

Fig. 3. Nucleotide sequence of the 5' UTRs of seven highly expressed, dicot nuclear or viral genes and of the 35S-GUS construct pBI121. The 5' UTR sequences listed are from cDNAs of tobacco mosaic virus Ω sequence (Gallie and Walbot, 1992) and alfalfa mosaic virus RNA 4 (Jobling and Gehrke, 1987), and from genes encoding Rubisco ss from *Arabidopsis thaliana* (Wong et al., 1992), Class I patatin from potato (Rocha-Sosa et al., 1989), the pathogenesis-related (PR) proteins osmotin (Raghothama et al., 1993) and PR-S (van Kan et al., 1989) from tobacco, and proteinase inhibitor II protein *Pin2* from potato (Keil et al., 1986). Repeated sequences in TMV Ω cDNA are indicated by arrows and underlining. The dicot consensus for ATG initiation codon context (Cavener and Ray, 1991) is shaded.

The level of gene-product accumulation will reflect not only the rates of transcription and translation, but also the rates of mRNA and product degradation (Fig. 2). Gene-construct features that affect each of these processes will be considered.

Transcription

The highly active 35S promoter from cauliflower mosaic virus (Odell et al., 1985) has been used widely to provide high constitutive transcriptional rates. Where high transient expression is called for, examples of promoters that have been used include those from proteinase inhibitor II (*pin2*) (Lorberth et al., 1992), osmotin (Xu et al., 1994), or PR-S (van Kan et al., 1989). These provide high, but temporal, transcription rates when induced by certain environmental stimuli, such as wounding or infection by specific pathogens. The *pin2* promoter is also constitutively expressed in potato (*Solanum tuberosum* L.) tubers.

Translation

Translational efficiency is affected by the composition of the 5' untranslated region (5' UTR) and AUG context of the mRNA transcript. An analysis of cDNA sequences of the 5' UTRs of seven highly expressed dicot nuclear or viral genes (Fig. 3, boxed) reveals that all are A- or T-rich (average of 73% A+T), as compared to a random sample of 79 plant 5' UTRs analyzed by Joshi (1987). By way of contrast, a commonly used construct of the 35S promoter from cauliflower mosaic virus, pBI121 (Clontech, Palo Alto, Calif.), uses a less-than-optimal 5' UTR. It consists of only five nucleotides from the 35S 5' UTR (Fig. 3, underlined), a synthetic 22-nucleotide multicloning site and an 8-nucleotide sequence, and ATG translational start signal from the *uidA* gene encoding β -glucuronidase (GUS) from *Escherichia coli*. This 5' UTR is relatively AT-poor (37% A+T). Further, the ATG context of the pBI121 construct (CTT ATG TT) differs considerably from the dicot consensus A₄A/C ATG GC (Fig. 3, shaded) (Cavener and Ray, 1991) and is probably suboptimal for translational efficiency (Taylor et al., 1987).

Stem-and-loop secondary structures in 5' UTRs may, if sufficiently stable, reduce translational efficiency (Kozak, 1986). Ingersoll et al. (1994) observed a 50% decrease in GUS expression when a *pin2* promoter and 5' UTR, joined to the GUS coding region, was compared with a similar construct in which a 12-nucleotide, AT-rich sequence (Fig. 3, underlined) was replaced with an 8-nucleotide sequence that was both AT-poor and capable of forming a hairpin. The hairpin structure was speculated to have caused a part of the diminished expression.

Translational efficiency also is affected by the makeup of the coding region. For example, Perlak et al. (1991) were able to greatly increase the translational efficiency of mRNA encoding the insecticidal protein from *Bacillus thuringiensis* by completely synthesizing/modifying the coding region according to certain rules. The coding sequence was altered to use plant-preferred codons (Murray et al., 1989) and to avoid repeats of the mRNA instability sequence ATTTA (Green, 1993) and potential polyadenylation signal sequences AATAAA and AATAAT. With these modifications, the levels of insecticidal protein in transgenic plants carrying the modified gene were 100-fold higher than in plants carrying the wild-type gene.

Protein stability

Finally, the level of gene-product accumulation will be affected by stability of the product to degradative enzymes present in the target locale. We have studied the stability of the bactericidal polypeptide cecropin B (Boman and Hultmark, 1987) and a synthetic analog MB39 (Fig. 4) in intercellular fluids extracted from leaves of 10 crops (Mills et al., 1994; Owens and Heutte, 1994). Protein stabilities were markedly affected by the crop source of intercellular fluid and the polypeptide sequence. The half-life of cecropin B ranged from 3 min in potato to 25.5 h in rice (*Oryza sativa* L.) intercellular fluid, whereas that of MB39 ranged from 17 min in potato to 10.3 h in maize (*Zea mays* L.) fluid. MB39 was more stable in intercellular fluids from nine of the 10

FIG. 4. LINE ART
SHOOT at 100%

DROP KEYLINE

Fig. 4. Amino acid sequence of cecropin B (Boman and Hultmark, 1987) and a synthetic homolog MB39 of similar bioactivity (unpublished). Shading indicates structural differences.

species examined—its half-life averaging 2.5 times that of cecropin B. Clearly, small differences in structure (Fig. 4) can confer substantial increases in stability. This effect suggests the possibility of modifying protein genes to enhance product stability.

CONCLUSION

Increasingly in the future, gene transfer will be the method of choice for introducing important, value-adding genes into elite lines of horticultural crops. Although other classes of organisms will continue to be an important source of genes for transfer to plants, techniques for cloning plant genes currently known only by phenotype will provide an ever-growing new source of genes for this purpose. Knowledge of the molecular biology of gene transcription and translation now enable modification of transgenes to optimize their expression in the target plant or tissue. Further knowledge of the relationship between protein structure and susceptibility to protease action may guide the engineering of genes to enhance protein accumulation levels.

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