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Molecular Tools for Engineering Plant Cells

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1. Introduction

The introduction, establishment, and expression of foreign genes in the nuclei of plant cells involves three basic steps: the introduction of DNA into the cell, the identification and propagation of the transformed cell, and the evaluation of expression levels of the gene or genes of interest. This chapter examines the molecular tools required for the successful completion of plant transformation and desired gene expression. The goal is to illustrate some of the ideas and selection criteria that enter into the decision of which of the many choices available may best fit the object of the exercise. Particular components are mentioned to illustrate a specific point, because, as the field advances, the number of options steadily increases. A wide range of published information is available for more in-depth pursuit of a specific topic.

Chapter 10 covers the subject of plant-cell tissue culture in the development of transformation technologies. That chapter discusses some of the molecular components that will be mentioned in greater detail here. It should also be noted that substantial research has been directed toward transformation of plants through the integration of foreign genes into the chloroplast genome, but that topic is discussed separately (Chapter 11).

The categorization of transforming genes is based on two functions. The first category includes genes that produce proteins that confer upon the transformed cell a phenotype that is scored physiologically or visually (selectable or screenable markers, respectively). The second category is represented by genes that produce proteins (or in some instances, RNAs), which confer commercially or scientifically important phenotypes (for example, insect resistance), or have intrinsic value. In other uses, the genes encode enzymes that participate in biochemical steps that lead to commercially relevant compounds.

2. Selectable Marker Genes

Selectable marker genes confer a growth advantage to transformed cells under conditions in which nontransformed cells will not grow. These genes are generally necessary because current plant transformation methods are extremely ineffective when considered at the cell level. Without selection, the identification and isolation of a small population of transformed cells from among an overwhelming and growing population

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of nontransformed material is nearly impossible. Although modern high-throughput methods may allow screening of large numbers of samples to identify the rare events, these methods are time-consuming and expensive. Furthermore, plants regenerated from nonselected tissues are likely to be chimeric (composed of both transformed and nontransformed tissues), and a substantial amount of genetic follow-up work is required to identify purely transgenic progeny.

2.1. Hormone Independence

The earliest plant transformation experiments exploited the ability of pathogenic *Agrobacterium tumefaciens* cells to transfer T-DNA via the Ti plasmid to the plant cell, with eventual integration into the genome (discussed in Chapter 10). Encoded within the T-DNA are genes that encode enzymes which are biochemically responsible for the synthesis of two types of plant hormone—an auxin and a cytokinin *(1)*. Cells of some plant species, when transformed with the wild-type T-DNA, acquire the ability to grow in vitro on defined growth media that lack plant hormones. Nontransformed cells are unable to grow without the exogenous hormones. Although it allows for selection of transformed cells and tissues, the acquisition through the T-DNA of the uncontrolled hormone biosynthesis genes disrupts the ability of the transformed cells to organize into differentiated growth, and the result is the formation of tumor-like callus or teratomas *(2)*. Therefore, selection for hormone-independent growth, although useful in the early days of developing and understanding plant transformation, has been replaced by dominant selectable marker genes that offer the advantage of positive selection of transformed cells on media that has all the components needed to induce regeneration of fertile whole plants.

2.2. Antibiotic Resistance

Bacterially derived antibiotic resistance genes were the first to be used successfully to select for totipotent transformed plant cells. The native bacterial promoters of these genes are not functional in plant cells, so the coding regions of the resistance genes must be cloned under the control of plant expression elements. In most instances, the antibiotic is a protein-synthesis inhibitor, and resistance is mediated through metabolic detoxification of the drug, usually by phosphorylation or acetylation. Genes that encode aminoglycoside phosphotransferases (*aph* genes) have seen the most widespread use for selection of transformed plant cells in the presence of normal inhibitory concentrations of aminoglycoside antibiotics such as kanamycin or G418 *(3)*. The neomycin phosphotransferase II (NptII) coding region (encoded by *aphII*) from bacterial transposon Tn*5* has been used broadly in both commercial and research applications, after being placed under the control of appropriate plant gene-expression elements *(3)*. At least 36 plant genera have been genetically modified and shown to express the NptII protein at levels sufficiently high to confer drug resistance *(4)*.

The coding regions of the *aphI* genes from Tn*601* and Tn*903*, both of which encode neomycin phosphotransferase I (NptI), have also been used to a limited extent *(5)*. Other drug resistance genes have been used as plant selectable marker genes, primarily in experimental settings. Examples are genes that encode chloramphenicol acetyltransferase (from Tn*9*) *(5)*, bleomycin resistance (from Tn*5*) *(6)*, streptomycin phosphotransferase (from Tn*5*) *(7)*, and hygromycin phosphotransferase (*hpt*, from an *Escherichia coli* plas-

mid) *(8)*. Methotrexate resistance is mediated not by a detoxification mechanism but through overexpression of a bacterial (from plasmid R67) *(3)* or mouse *(9)* methotrexate-insensitive dihydrofolate reductase. A mouse gene encoding adenosine deaminase has been expressed in transgenic maize, and confers resistance to 2'deoxyadenosine *(10)*.

Emerging public concerns about the issue of widespread release of transgenic plants harboring antibiotic resistance genes *(4)* has led to the general discontinuance of these genes as plant selectable markers for commercial crops. The basis for these objections primarily revolves around the perceived possibility that the genes will escape into the microbial environment, thereby initiating eventual transfer to human or animal pathogens *(11,12)*. It is a matter of open debate whether this concern is scientifically justified. The risk of an antibiotic-resistance gene escaping from a corn plant to a soil microbe seems inconsequential when compared to the proven interspecific transfer of antibiotic resistance via multidrug-resistance conjugal plasmids that are ubiquitous in bacteria-rich environments such as sewage treatment plants *(13)*.

2.3. Herbicide Resistance

Genes that confer plant resistance to herbicidal compounds have three advantages in the production of commercial transgenic crops. First, the genes are often used for in vitro selection of transformed plant cells during the first stages of transformation and regeneration. Later, during the trait introgression process (i.e., plant breeding) in which transgenes are introgressed into commercially important varieties, the herbicide resistance trait is often used to identify transformants and to cull nontransformed or weakly expressing plants from the segregating population. Finally, the herbicide resistance trait is commercially valuable, as it allows growers to spray a crop with a broad-spectrum chemical to kill susceptible weeds, and causes no damage to the crop plants that express engineered resistance to the chemical.

For many commercial herbicides, naturally selected or mutation-induced resistant plants have been identified. Many published reports have described the identification of the target-site enzyme and elucidation of the modes of resistance to particular compounds or classes of herbicides. Plant herbicide resistance mechanisms generally fall into only a few categories *(14,15)*. Exclusion of the herbicide molecule from the site in the plant where it normally induces the toxic effect is accomplished by inhibiting the uptake/translocation of the compound, by compartmentalization of the compound, or by metabolism of the compound. In other cases, growth-sustaining levels of the target enzyme activity are attained by overproduction of the target enzyme, or by accumulation of site mutations that render the target enzyme resistant to inhibition by the herbicide molecule. A third category of resistance—one that has had substantial commercial success—involves production of metabolic detoxification enzymes. In a few instances, a single gene introduced transgenically into plant cells has been sufficient for both transgenic selection and field-level resistance.

2.3.1. Acetolactate Synthase (ALS) Inhibitors

ALS (sometimes called AHAS; acetohydroxyacid synthase), catalyzes the first committed step in the synthesis of the branched-chain amino acids *(14)*. ALS-inhibitor herbicides comprise four chemical families: the sulfonylureas (products of DuPont Crop Protection), the imidazolinones (products of American Cyanamid/BASF), the

triazolopyrimidine sulfonanilides (products of Dow AgroSciences) and the pyrimidyl-oxy benzoates (products of Kumiai Chemical Industry Company). Naturally selected resistance mutants of several plant species are known. The ALS-encoding genes of several of these plants have been cloned and sequenced, and resistance to particular classes of compounds has been traced to point mutations in the ALS protein. At least 10 mutation sites have been identified—some of which, alone or in combination, confer cross-resistance to more than one compound class. Engineered resistance through overproduction of the mutant ALS enzyme has been successful in several plant species, including crops. This type of resistance is problematic because of the presence of a basal level of susceptible endogenous enzymes in the cell. Generally, this limitation to attaining herbicide resistance is more serious when high levels of endogenous enzymes must be maintained.

From a commercial herbicide safety standpoint, chemical inhibitors of ALS are desirable because mammals lack the target enzyme. Currently, multiple agricultural chemical companies produce ALS inhibitors; many of which have been developed to be crop-selective. Maximum value for herbicides and herbicide-resistant crops is derived from the combination sale of the chemical and the seeds of the resistant crops. Since many of the ALS mutations confer cross-resistance to multiple chemical classes, the deployment of crops that are resistant to one compound class may prompt sales of a herbicide sold by a competitor who has not incurred the expense of developing the resistant crop. If crop-selective ALS inhibitors already exist to control weeds in the crop, there is probably no advantage to be gained by incurring the expenses of producing and registering a herbicide-resistant crop that harbors a mutant ALS gene.

2.3.2. Glutamine Synthetase (GS) Inhibitors

Glutamine synthetase is involved in nitrogen metabolism through the conversion of L-glutamate to L-glutamine. A bacterially derived compound, phosphinothricin (an analog of L-glutamate), and its synthetic version, glufosinate, are both highly effective GS inhibitors *(14)*. A third compound, bialaphos, a bacterial tripeptide precursor to phosphinothricin, is also a GS inhibitor via conversion in plants to the highly toxic phosphinothricin. All three compounds are useful as selective agents for plant transformation. The fermentation product bialaphos, and particularly glufosinate, are successful field-use herbicides. Two *Streptomyces* genes that detoxify these compounds by acetylation are routinely used to confer plant resistance to these compounds. The bar (bialaphos resistance) gene was cloned from *S. hygroscopicus*, the source of bialaphos and hygromycin *(16)*, and the pat (phosphinothricin acetyltransferase) gene was discovered in *S. viridochromogenes (17)*. When placed under the control of the appropriate plant expression control elements, both of these genes have been useful as selectable markers in a wide variety of plant species, including about 20 crops *(14)*. To date, no attempts to attain herbicide resistance through overproduction of herbicide-resistant GS have been reported, although phosphinothricin resistance has been observed in plants in which the native GS copy number has been naturally amplified *(18)*.

2.3.3. EPSP Synthase Inhibition

Glyphosate is a low-residual broad-spectrum herbicide that is a potent inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This enzyme is involved in the synthesis of many aromatic compounds in plants, including the aromatic amino acids

(14), but is not found in animals. Selection of transformed cells and plant-level resistance to glyphosate has been accomplished via transgenic production of various enzymes. Overexpression of chimeric glyphosate-resistant EPSPS-encoding *aroA* genes from bacteria (*Salmonella* or *E. coli*) *(19,20)* requires targeting of the enzyme for transport into the chloroplast for high-level glyphosate resistance *(20)*. Expression of glyphosate-resistant EPSPS-encoding plant genes provides moderate to high levels of resistance *(21)*. In many cases, the resistant EPSPS is less enzymatically efficient than the wild-type, and thus high-level expression is required to support normal plant metabolism. A gene for a glyphosate-resistant EPSPS with relatively high enzymatic efficiency has been isolated from an *Agrobacterium* strain, and alleviates some of the necessity for overproduction *(21)*. In addition, production in plants of glyphosate-degrading enzymes from bacteria such as *Achromobacter* has been an effective method of establishing the glyphosate-resistant trait *(21,22)*. The combination sale of glyphosate-resistant crop seeds and the formulated herbicide has been commercially successful.

2.3.4. Photosystem II Inhibitors

Bromoxynil and the triazines (e.g., atrazine) inhibit electron transport in the photosystem II reaction center in plant chloroplast thylakoid membranes *(14)*. Dicot crops are particularly sensitive to bromoxynil, and resistance to this herbicide could be a valuable trait in crops such as cotton. Although some weed resistance to bromoxynil has emerged, the mechanism of the resistance, and any genes associated with it, have yet to be exploited. So far, engineered resistance to bromoxynil has been accomplished through expression of a chimeric gene for bromoxynil nitrilase isolated from *Klebsiella ozaenae (23)*. In cotton, transgenic lines carrying this gene are resistant to up to 10-fold the field-rate recommendation of formulated bromoxynil *(24)*.

Many plant transformation protocols require incubation of early-stage processed materials in the dark prior to beginning the whole-plant regeneration steps. Because bromoxynil is a photosynthesis inhibitor, it is not as useful as certain other compounds in the selection of early-stage transformants. Selection must be applied later on after photosynthetically competent plants are produced.

The emergence of weeds resistant to atrazine in the field was first reported in 1970 *(25)*, and is usually found to be the result of a single amino-acid mutation in a chloroplast gene that encodes a thylakoid protein of about 32,000 Daltons *(26)*. Engineered atrazine resistance has not been used either as a selectable marker or as a commercial trait.

2.4. Metabolic Inhibitors

Two recent developments in selectable marker systems employ genes that confer resistance to an amino-acid analog or enable sugar utilization. In the first example, *Arabidopsis* plants grown from seeds treated with a mutagen were screened for the ability to grow in inhibitory concentrations of O-methyl-threonine. This trait was named *omr1* (for O-methyl-threonine resistance) and through genetic and molecular studies the *omr1* gene was found to encode a mutated threonine deaminase enzyme. Threonine deaminase (also called threonine dehydratase) catalyzes the first committed step in the biosynthesis of isoleucine. Although it is useful for selection of transformed tissues of some plant species *(27)*, the omr1 gene is not generally as valuable as some other genes.

The second example utilizes the observation that mannose cannot be converted into metabolizable sugars by plant cells, and inhibits seed germination in *Arabidopsis* and

respiration in wheat and tomato *(28)*. Mannose that enters plant cells is converted to mannose-6-phosphate, which accumulates and is believed to be responsible for the growth-inhibitory effects. However, when transformed with a modified *E. coli manA* gene that encodes phosphomannose isomerase, the transformed cells are able to thrive on medium with mannose as the primary carbon source, and thus outgrow nontransformed tissues *(28)*.

3. Screenable Marker Genes

Screenable markers are proteins that do not confer a growth advantage to transformed cells, but are recognized by their ability to cause physical or chemical changes in the transformed cell. In the ideal case, such changes can be detected via a nondestructive, high-throughput assay on putatively transformed samples. Less useful are assays that consume a sample removed from a precious candidate tissue that is often available in limited amounts or only for a short time.

3.1. Opine Synthesis

Early plant-cell transformation protocols utilized oncogenic, teratogenic, or disabled Ti plasmid T-DNA transfers via *Agrobacterium tumefaciens* or *A. rhizogenes*. The T-DNA in these early experiments transferred a small number of genes that produced, among other compounds, modified amino acids (opines) in the transformed plant cells. Transformation by non-oncogenic T-DNAs, which allowed regeneration of whole fertile plants, was detected by paper electrophoretic analysis of opines—(e.g., octopine, nopaline, or mannopine) *(29)* in plant-cell extracts from putatively transformed tissues. **Note:** The octopine synthase (lysopinedehydrogenase) enzyme has been used as a selectable marker to overcome plant-cell toxicity of L-homoarginine. Because homoarginine is not a potent inhibitor of plant cell growth, this selection regime is not routinely useful *(30,31)*.

3.2. Chloramphenicol Acetyltransferase (CAT)

The CAT protein is encoded by the *cmr* (chloramphenicol resistance) gene of bacterial transposon Tn*9*. After modification for plant expression, the CAT coding region is useful as a screenable marker *(32)*. The assays are quite cumbersome, and involve thin-layer chromatography or ethyl acetate extraction to determine the transfer of radiolabeled acetate to chloramphenicol *(33,34)*. Total amounts of the CAT protein can be measured by enzyme-linked immunosorbent assay (ELISA) *(32)*. Because plant-cell growth is not particularly sensitive to inhibition by chloramphenicol, the CAT protein has limited use as a selectable marker *(5)*.

3.3. β-Glucuronidase (GUS)

GUS is encoded by the *E. coli uidA* gene *(35)*. When placed under control of plant-expression elements, production of the GUS protein has proven extremely useful for a variety of experiments beyond the simple identification of transformed tissues. The most commonly used substrates are XGLUC (5-bromo-4-chloro-3-indolyl glucuronide for histochemical assays) and MUG (4-methyl umbelliferyl glucuronide for quantitative fluorescence assays) *(36)*. GUS acts upon the XGLUC substrate by cleaving the glucuronide bond to release an indoxyl derivative, which dimerizes in the presence of

oxygen to form a highly colored indigo blue dye precipitate. This property makes the GUS protein highly useful in the analysis of promoter-expression patterns, as the cells in which the promoter is active are stained blue. The GUS protein is relatively stable, and thus it is possible to overestimate promoter activity because of the accumulation of GUS and the resulting intense blue staining in histochemical assays. This artifact makes GUS expression somewhat less useful than markers such as luciferase or green fluorescent protein (GFP) in comparative analysis of promoter-expression levels (*see* below).

Sensitive fluorometric assay of the level of GUS enzymatic activity in plant-cell extracts is possible through the use of the MUG substrate. MUG is not fluorescent until GUS activity releases 4-methyl umbelliferone, which is fluorescent at pH8-9. Standard curves established with commercially available GUS enzyme allow precise quantitation of GUS activity in multiple samples; therefore, quantitative determination and comparison of chimeric gene expression at various time-points is possible.

3.4. Luciferases

The gene that encodes the luciferase enzyme responsible for light emission from firefly lanterns (*Photinus pyralis*) has been cloned, and the coding region has proven quite useful as a marker gene for promoter expression studies in plants *(37)*. The addition of ATP and luciferol substrate are sufficient to induce light emission that can be captured quantitatively by photon-capture devices in vitro, or qualitatively by standard photography.

It is relatively common in transgenic promoter expression studies to compare the expression level or tissue specificity of a test promoter to a standard reference promoter that is co-transformed with the test gene construct *(38)*. In these cases, the reference promoter may be used to drive expression of the GUS gene, for example, while the test promoter is used to drive expression of the luciferase gene. The relative expression levels of the two promoters can be assayed in vitro in a single extract, and the test promoter activity is normalized to the activity of the reference promoter, which serves as an internal standard. Samples taken from different tissues or on different days can thus be compared. It is not an understatement that these reporter genes, GUS and luciferase, have been the two most valuable enzymes utilized in the advancement of our understanding of plant gene expression.

Another substrate-dependent light-emitting enzymatic activity that has been used to a limited extent in plant gene-expression studies involves the products of the *Vibrio harveyi luxAB* genes *(39)*. The active enzyme is normally comprised of two subunits, but they can be genetically joined into a single protein that retains activity *(40)*. In addition, a gene that encodes the luciferase from *Renilla reniformis* (sea pansy) is functional in plants *(41,42)* and is useful because it emits light of a wavelength different from that of the firefly luciferase. This permits both the firefly and *Renilla* reporter genes to be used in the same experiment *(43)*. Engineered genes for the firefly and *Renilla* luciferases, as well as reagent kits, are commercially available (Promega, Madison, WI).

3.5. Fluorescent Proteins

The first gene that encodes a non-substrate-dependent light-emitting (fluorescent) protein that was used as a reporter gene in plants *(44,45)* was cloned from the jellyfish *Aequoria victoria (46)*. This protein, called the green fluorescent protein (GFP) emits fluorescent light with a peak wavelength of 508–509 nm when excited with ultraviolet light

(360–400 nm) or blue light (440–480 nm) *(45)*. No external substrate is required; the fluorescence is produced from an active chromophore formed by cyclization and oxidation of three amino acids (serine-65, tyrosine-66, and glycine 67) within the peptide. At first, plant expression of the native gene was problematic because of native sequence similarity with known plant introns. Improved expression resulted from re-engineering the gene to reduce the AU (adenosine + uracil) content of the mRNA and the addition of a targeting signal peptide to direct the protein to the endoplasmic reticulum (ER) *(47)*. With these improvements, production of this protein in transgenic plants provides the opportunity to assay gene function in a nondestructive manner. Newer versions of the protein have been engineered to emit more intense green light *(48)*, and other versions have emission peaks at wavelengths different from the native GFP, including cyan, yellow, and red *(49,50)*. These differences in emission spectra allow microscopic imaging of the amounts of the respective proteins *(51)*. Genes that encode fluorescent proteins from other sources are also available—for example, the red-fluorescing DsRed protein from *Discosoma* spp. *(52)*.

3.6. β-Galactosidase

The *lacZ* gene of *E. coli* encodes β-galactosidase and is widely used as a reporter gene in bacterial studies. In contrast, the production of β-galactosidase in plant cells as a screenable marker has had only limited use *(53–55)*.

4. Expression Control Elements

The success of any plant transformation effort, particularly one whose object is the commercialization of crops with an engineered trait, ultimately relies on how well the phenotype produced matches the needs of the market. High level production of a protein that is toxic to corn rootworms, for example, is not likely to confer a market advantage to corn plants that have an average yield that is substantially below competitive varieties ("yield drag"). A market-viable transgenic must produce adequate levels of the insect toxin in the appropriate tissues (the roots), at the appropriate time (essentially the first two-thirds of the growing season), and result in no detrimental side effects such as yield drag, low harvestability, or lowered feed value. Achievement of these goals requires insightful choices of gene-expression control elements and appropriate combinations of the chosen elements. These considerations include: the choice of host plant, which promoter to use, the sequence of the 5' untranslated region, whether or not introns will be included, how the coding region for the desired protein will be configured, and which sequences will be used for the 3' untranslated region that encodes the transcription termination and polyadenylation signals. These decisions for individual genes must be made in the larger context of the element composition of the other transgenes that will be co-transformed (e.g., the selectable marker gene). A brief description of some of the factors to be considered is presented here. This discussion is necessarily superficial because the entire field of plant gene-expression control is advancing so rapidly.

4.1. Promoters

Most transgenic plants destined for commercial production employ promoters derived from plants or plant viruses to control transgene expression. Although some research has examined the plant functionality of promoters from animals or other sources, regulatory approval of commercial transgenics is facilitated when the promoter of the

transgene is of plant (or plant virus) origin, particularly if the promoter was originally derived from the host plant. Examples of commonly used promoters are the maize polyubiquitin1 (*ubi*1) promoter *(56)* and the 35S promoter derived from the Cauliflower Mosaic Virus *(57)*.

Both of these promoters are considered to be constitutive in their expression patterns. Thus, they are usually expressed at some level throughout the tissues of the plants during the lifetime of the plant. However, this designation is not absolute, as the expression level and pattern of an individual promoter is markedly affected by the position into which it integrates in the chromosome during the transformation process. This "position effect" results in expression patterns in individual, independently transformed plants in which some degree of tissue specificity, developmental preference, or inactivity usually occurs *(32,58)*.

Constitutive promoters are useful to drive expression of genes when tissue specificity is not a prerequisite—for example, selectable marker genes. As mentioned previously, herbicide-resistance selectable marker genes often have a triple role. They are used early in the transformation process to select for transformed cells, in the breeding process to follow transgenes in whole plant populations, and finally as a commercially valuable trait in the field to provide crop resistance to weed-control chemicals. A constitutive promoter may also be a good choice to control the expression of an insecticidal protein when the target pest is one that feeds on stems, leaves, and other plant parts.

In contrast to constitutive expression characteristic of cellular housekeeping genes, for example, many plant promoters are highly regulated in terms of when, where, and to what level they are functional. Regulation can take many forms, and plant promoters have been characterized that respond to internal chemistries such as plant hormone levels or osmotic potential, or to external stimuli such as heat, light, (or the absence of light), touch, drought, or stress induced by insect feeding or pathogen attack *(59,60)*. In addition, there are genes controlled by promoters that function only at limited times during development (e.g., seed formation) or only in a very limited number of cells comprising a single cell layer (e.g., seed aleurone). Other promoters are specifically functional in certain tissues that exist throughout the life of the plant, for example, the root or leaf meristems *(59)*. The choice of such a tightly regulated promoter may be mandatory for the commercial success of some transgenic traits such as the modification of oil levels or composition in oil seed crops, in which the oil bodies are present only in the embryo of the seed.

Chimeric promoters have been useful to combine the regulated aspects of one promoter with the high expression characteristics of another. For example, a seed-specific sequence element can be combined with a constitutive, strong promoter (CaMV 35S) to confer seed-specific expression *(61)*. Many other response elements that respond to light, hormone, stress, or other stimuli have been characterized, and continue to be exploited.

In some instances, it would be desirable, or even necessary, that the expression of a particular trait or production of a particular protein be completely under the control of the grower. It may be advantageous that high-level production of a high-value protein be inducible by chemical treatment of the transgenic plant. Chemically regulated plant promoters have been available for some time *(62,63)*, but to date none of the published systems has been developed to the point that it is commercially useful in the field. One

primary barrier to deployment of such systems is the governmental regulatory requirement that the chemical must be registered for environmental release on large acreages. Borrowing from the bacterial world, a tetracycline-controlled promoter has proven functional in plants, and expression (either induction or repression) is controlled by external application of the antibiotic tetracycline *(65)*. This system has obvious limitations for field-level application. Another inducible promoter system employs a mammalian glucocorticoid receptor and is controlled by the application of the steroid dexamethasone *(66)*. An analogous system that employs an estrogen receptor yielded higher constitutive expression *(66)*. Again, it is obvious that spraying large acreages with a steroid hormone poses many problems.

The most useful chemically controlled system is one based on a compound that is already registered as a pest control agent. Two such systems are under development, a safener-induced system and a system based on insect ecdysone receptors. In the first case, promoters were identified by screening for genes that were activated following treatment of plants with chemicals added to a commercial herbicide formulation to help prevent damage to the non-target crop plant (a "safener"). Safeners comprise a group of structurally diverse compounds, and induce expression of genes such as glutathione-S-transferases and cytochrome P450 mixed-function oxygenases *(62,63)*. The inducing chemicals activate a number of stress or defense genes, so these inducible promoter systems can be used only in applications in which the pleiotropic effects can be tolerated.

In the second case, a receptor for the insect molting hormone ecdysone *(67)* was modified and exploited for its ability to respond to synthetic ecdysone agonists, which are registered for use as insecticides *(68)*. Despite the commercial nature of the inducing compounds, there are no reports of the use of either of these systems to control gene expression in the field.

To summarize, one should begin the development of any commercial transgenic project with the end in mind. It is essential to fully understand which phenotype the final transgenic plant will have and then work backwards to the point where one can design the entire gene cassette so that it incorporates as many features as possible to increase the likelihood of attaining the trait. Often, this requires an up-front extensive biochemical and molecular biological characterization of the host plant, to determine which factors must be considered. After laying the groundwork with a full understanding of what the object of the effort is, one can then make the proper choice of which promoter to use.

4.2. Untranslated Flanking Regions and Introns

The rapidly advancing and impressive array of molecular biology tools and genomics technologies now available (*see* Chapter 8) allow the isolation of virtually every promoter in a plant, if one is willing to expend the effort. This process will be even easier in the future, as the entire genome sequences of more plants are determined *(69)*. But the promoter is only one element in the list of choices to be made in assembling a successful gene construct.

Much work has been published regarding the interactions of coding region flanking sequences on gene expression in plants *(70,71)*. It is known that the sources of the 5' and 3' untranslated regions (UTR) can play an important role in the level of gene expression. Particular sequence features of both of these elements are impor-

tant. The 5' UTR must be able to efficiently assemble ribosomes and present the start codon in an appropriate configuration. It is not uncommon to utilize the native 5' UTR associated with a particular plant promoter, since this combination of promoter/ 5' UTR has evolved to function as a unit. However, there are instances in which substitution of a different 5' UTR—for example, from a plant virus gene—has been found to increase expression levels *(72)*. The 5' UTRs from the genomic RNA of tobacco mosaic virus *(73)* and the alfalfa mosaic virus coat protein gene *(74)* have been shown to be particularly effective in enhancing transgene expression. This effect may be the result of the relatively low degree of secondary structure provided by these sequences, which may facilitate easy passage of the initiating ribosomes *(73)*. From a practical standpoint, one may expect that any gene with a product that is required in relatively large quantities such as a viral coat protein may possess sequence elements that have evolved for high expression.

The 3' UTR provides mRNA stability and facilitates polyadenylation of the mRNA. Many gene constructs have utilized the 3' UTR captured from the nopaline synthase (nos) gene of *Agrobacterium* Ti plasmids (C58 or T37) *(75)*. This small unit (about 260 bp) functions well in both dicots and monocots, and is probably the most widely used 3' UTR today. Experiments with advanced constructs, however, have shown that the nos 3' UTR is fairly weak in terminating transcription, and transcriptional readthrough is sometimes seen *(76,77)*. Utilization of plant-derived 3' UTRs often facilitates better chimeric gene expression than seen with the T-DNA derived 3' UTRS *(77,78)*, but some combinations of 3' UTRs and other gene components work better than other combinations *(71,77,79)*. As with the 5' UTR, it can be advantageous to utilize the 3' UTR associated with the plant gene from which the promoter was isolated, but some testing with alternative sources may reveal a better combination of elements.

Although some plant genes (and all plant virus genes) lack introns, the addition of a plant intron to a construct can have a dramatic positive effect on expression level, particularly in monocots *(76,80)*. Intron processing is a stringent process in the plant kingdom, and the recognition/splicing mechanisms are loosely conserved between monocots and dicots. However, the phylogenetic origin of a plant intron does not completely dictate its ability to be spliced in a heterologous plant system *(71)*. Some monocot introns are spliced inefficiently or not at all in dicots, and other monocot introns are spliced in dicots with reasonable efficiency. As a general rule, monocots are able to efficiently process both monocot and dicot introns, but some dicot introns are not spliced in other dicots *(71)*.

When added to a construct, placement of the intron can affect the expression level attained *(76,81)*. The most difficult task is to introduce an intron directly into a protein-coding region. If a heterologous intron sequence is cloned as a restriction fragment into a compatible site within a coding region, the splicing reaction will sometimes leave several bases behind. The effects of these "footprint" sequences range from the addition of a few new amino acids to the protein, which may or may not affect its function, to a reading-frame shift that terminates translation or results in production of a new chimeric protein. Thus, the engineering must be carefully planned, and the spliced product must be tested to determine that no loss in activity or stability of the protein results from the addition of the new bases. If the genomic version of a coding region contains an intron, the simplest solution is to add a native

intron to the cDNA, in the same position that it occurs in the genomic copy. Polymerase chain reaction (PCR) techniques now allow the addition of an intron sequence to occur so that splicing does not leave extraneous bases *(82)*. Looking outside the coding region, it is not uncommon to find native plant genes that have an intron in the 5' UTR *(77,79)*. These introns can be quite long; the maize ubiquitin1 gene has a 1010-basepair (bp) intron in the 5' UTR sequence *(56)*, and the 5' leader/intron of the potato Sus4 gene is 1612 bp *(77)*. It has been well-demonstrated that engineering an intron into the 5' UTR can be beneficial to expression levels *(76,80)*. The degree of expression enhancement is not predictable, because it depends on the plant system used (dicot or monocot), the type of study (stable or transient expression), the source of the intron and promoter, the intron placement, and the assay method (mRNA level/processing efficiency or encoded protein levels) *(71)*.

As previously stated, certain 5' and 3' sequences appear to work best in combination with others, and the effect may be different when the same elements are combined with different coding regions and promoters *(77, 79)*. Thus, it is often necessary to examine the expression activity of several combinations of components to determine the combination that is best suited to the ultimate goal. This is no easy task, although some shortcuts are available. A preliminary understanding of how a particular combination will function can be gained through transient expression systems such as electroporated protoplasts or bombarded tissues. Usually, these calibration experiments substitute the coding region of an easily assayed reporter protein for the ultimate protein of choice. Such experiments require substantial calibration, and must include well-characterized internal control genes, against which expression levels of the test genes can be normalized *(38)*. Because of large variations between individual experiments, many replicates are needed to establish a statistically sound answer. Once a particular combination of promoter and other elements is identified as providing good expression, the experiments can be repeated using the coding region for the desired protein.

Because protoplast experiments do not involve differentiated tissues, these systems are only useful for examinations of element combinations driven by constitutive promoters. Sometimes this is adequate to identify appropriate UTR/coding region combinations, but ultimately the gene must be tested in whole plants.

Transgenic *Arabidopsis* plants are frequently used when large numbers of independently transformed plants are needed *(83)*. The *Agrobacterium*-mediated transformation method results in the production of transformed seeds directly from the treated plant. Seeds are germinated on selective medium, and several hundreds of independent events can be quickly generated. The short seed-to-seed generation time of *Arabidopsis* allows studies of gene expression in multiple tissues and generations, and this system is extremely useful as a next step up from the in vitro systems. However, a growing body of evidence indicates that results obtained with a particular gene in *Arabidopsis* are not necessarily predictive of those seen in other dicots (such as tobacco) *(84)*, and are certainly not always predictive of expression in monocots such as corn and rice. Thus, these model systems are useful in eliminating advancement of genes that have severe expression problems (frameshift errors), but for the final analysis, the gene must be tested in the crop of interest. This is usually an expensive, time-consuming, resource-intensive process that can extend over several years for crops such as corn and cotton. Because the results obtained from a particular combination of elements are often

unpredictable, it is desirable to have an extensive library of promoters and other expression components available, so that the best combination can be assembled.

A recent publication *(85)* nicely illustrates the points that: i) plant virus promoters can be exploited for transgenic expression (banana bunchy-top virus promoters BT1 to 5), ii) promoters from the same virus differ in strength (BT4 and BT5 are stronger than BT1, BT2, or BT3), iii) some coding regions work better with certain promoters than other coding regions (GFP is better than GUS), iv) transgenic promoters can promote tissue-preferential expression (mostly vascular-associated), v) introns increase expression (maize polyubiquitin1 intron), and vi) relatively exotic crops are transformable (banana).

4.3. Other Expression Controls

In addition to the gene components mentioned in **Subheading 4.2.**, other constraints may limit or even prevent the production of acceptable levels of a transgenic protein.

4.3.1. mRNA Stability and Translation

During early attempts at plant genetic engineering, it was frequently observed that only miniscule amounts of the foreign proteins were accumulated. This was particularly true in efforts to produce insect-resistant plants through the introduction of genes that encoded insecticidal crystal proteins of *Bacillus thuringiensis* (Bt). Ultimately, the low levels of expression were found to be the results of messenger RNA instability *(86–88)*. Careful analysis of the sequences of the introduced genes revealed that several sequence motifs associated with plant mRNA instability or processing were present in the native Bt toxin gene sequence. For example, the 3537-bp coding region of the HD73 Cry1Ac delta-endotoxin gene contains 10 motifs similar to plant 5' or 3' consensus intron splice sites, and 32 motifs associated with plant mRNA polyadenylation sites *(89)*. The result of the presence of these extraneous motifs within the toxin-coding mRNA is that the full-length message is highly unstable in plant cells, and thus very little translation of intact protein occurs. These motifs have no effect on expression of the gene in the bacteria, and they arise as a result of the relatively high adenosine-plus-thymidine (AT) composition (and correspondingly low guanosine-plus-cytosine content; GC) of *Bacillus* genomes (approx 60% AT). Many eukaryotic gene-control sequence elements are rich in AT content, so the presence of sequence homologs to the eukaryotic motifs in a high-AT-content genome is favored.

A second consequence of the high-AT genome composition is that average codon usage for the amino acids encoded by redundant codons is different between plants and many bacteria. A comparative analysis of the codon bias statistics of maize genes and the Cry1Ac coding region shows that for amino acids specified by two or more codons, the bacterial gene is comprised of codons that rarely occur in maize genes *(89)*. Furthermore, whereas maize genes usually employ codons with G or C in the third position, the Cry1Ac coding region has a majority of A's or T's in the third codon position. This difference in codon bias, coupled with the presence of sequences deleterious to mRNA stability in plant cells, renders it extremely difficult to attain commercial levels of the Bt protein toxin from the native coding region.

The solution to this problem is to completely synthesize the protein toxin-coding region in vitro, using a sequence design that substitutes plant-preferred codons for rarely used ones *(90–92)*. Since the gene design is *de novo*, all the deleterious sequences

can be eliminated from the coding region, and other desirable features such as strategically placed restriction-enzyme recognition sites can be incorporated into the sequence. This gene-rebuilding process is now a routine step in transgenic plant programs. The abundance of gene sequences from many different crops allows computation of codon usage tables that facilitate the design of genes tailored specifically for expression in a particular crop (e.g., corn vs cotton), or class of plants (dicots vs monocots) if desired.

4.3.2. Position Effects

None of the plant transformation systems available today is capable of directing the integration of transgene DNA into a predetermined site in the genome. Many experiments have attempted to exploit endogenous plant homologous recombination mechanisms by flanking the transgene with large pieces of native plant DNA. All have failed to produce targeted events at high frequency. Consequently, transgenes integrate into the chromosomes at random positions, and in one to many partial or full-length copies. The result is a wide distribution of expression activity, partially a function of the nature of the flanking genomic DNA. Sorting through a population of transformed individuals for the few that exhibit desired expression patterns and heritability is a costly, time-consuming process. Studies on nuclear matrix structure have shown that regions of the chromosome that are populated by highly expressed genes are flanked by sequences designated as matrix attachment regions (MARs) or structural attachment regions (SARs). Substantial work is in progress to examine the effects that the use of MARs or SARs sequences to flank transgenes will have on transgenic expression. It is possible that certain MARs will provide more predictable, and perhaps higher overall—expression levels and patterns *(93,94)* with some genes or transformation systems. It is apparent that different types of MARs, SARs, and other flanking elements exist, and that they can have different effects on gene expression in different systems *(95)*. In one study *(94)*, the use of chicken lysozyme A elements did not reduce variability of NPTII expression in tobacco, but the elements did cause a highly significant reduction in variation in GUS expression. As with other expression-control elements, prediction of the expression characteristics imbued by a new combination of MARs elements is somewhat risky, and experimental determination of the answer often yields surprises and new insight on the control of plant gene expression.

4.3.3. Gene Silencing

In addition to unpredictable expression patterns, another result of the random integration of transgenes into the chromosome is the phenomenon of gene silencing. Broadly defined, this is seen as the diminution and eventual elimination of transgene expression in the first or successive plant generations. Several gene-silencing patterns have been discovered, and more than one mechanism exists *(96,97)*. Transcriptional silencing results in the loss of mRNA synthesis and is mediated at least in part by hypermethylation of the promoter and/or coding region of the transgene. Triggering of this DNA modification may be induced by some mechanism in the nucleus that scans the chromosome for foreign DNA that has, for example, a DNA GC content that is not typical of that particular chromosomal region. A variation of this type of gene silencing is apparently driven to some degree by sequence homology between the transgene and native plant sequences, or between multiple copies of introduced

transgenes *(98)*. Although silencing of single-copy transgenes does occur *(96)* it may be less likely than silencing of multiple copies.

This homology association is not absolute, as demonstrated by two examples. First, the viral 35S promoter seems particularly susceptible to transcriptional silencing in monocots, although no known sequence homologs in monocot genomes are known *(99)*. Others have shown that unlinked sequences as small as 300 bp can detect and inactivate each other in a genome of $>10^9$ bp *(100)*, so unknown monocot homologies responsible for the silencing effect may exist. Second, plant promoters re-introduced back into the native host plant are not always silenced. If large numbers of independently transformed lines are examined, it is usually possible to find suitable candidates for exploitation.

Some studies have shown that the inclusion of flanking MARs or SARs sequences in transgene constructs may insulate the integrated transgene from silencing mechanisms, but more studies are needed to confirm the general utility of this approach *(93,94)*.

Post-transcriptional silencing does not affect the transcription level of nascent mRNAs, but results in the rapid degradation of the targeted mRNA. What induces this process is unknown, but a partial trigger may be the absolute level of a particular mRNA present in the cell. Homology to endogenous RNA is also implicated in some mechanisms (cosuppression) *(97)* and can be used to deliberately downregulate endogenous genes to engineer new traits *(101)*.

Although they are detrimental to the outcome of some research programs, gene downregulation mechanisms can be turned to an advantage. One of the first commercial transgenic products was developed by means of anti-sense RNA mediated downregulation of a tomato polygalacturonase gene, which encodes an enzyme that plays an important role in fruit softening *(102,103)*. Production of this anti-sense mRNA induced silencing of the endogenous gene, resulting in tomatoes that had a longer shelf life. In addition to cosuppression and anti-sense RNA production, other approaches to gene downregulation such as ribozymes *(104)* have been examined in attempts to produce commercial products, or used as fundamental tools to study gene function.

4.3.4. Polycistronic mRNA

Polycistronic mRNAs, which contain the coding regions for more than one protein, are common in bacteria and plant chloroplasts. In contrast, the genes in eukaryotic nuclei are generally expressed as mRNAs that contain the information for only a single protein (for the purposes of this discussion mechanisms such as alternative splicing are not considered). Translation processes in plants are very similar to those in other eukaryotic organisms, and can generally be explained with the scanning model, wherein the ribosomes assemble on 5'-end-capped RNAs and scan down the 5' leader to begin translation at the first ATG start codon *(105)*. Particularly among plant viruses, (e.g., caulimoviruses, badnaviruses, crucifer tobacco mosaic virus) *(106)* unconventional mRNAs are frequent and use modulated translation processes for their expression. Examples include leaky ribosome scanning, translational-stop-codon readthrough or frameshifting, and transactivation by virus-encoded proteins that are used to translate polycistronic mRNAs. In some cases, 5' leader and 3' trailer sequences confer efficient, cap-independent ribosome binding. Although this usually happens via an end-dependent

mechanism, true internal ribosome entry may also occur to initiate translation at internal start codons *(106)*. Translation in plant cells is known to be regulated under conditions of stress and during development, but the underlying molecular mechanisms of regulation are unknown. Thus far, only a small number of nonviral plant mRNAs have been discovered with a structure that suggests that they may require some unusual translation mechanisms *(105)*.

The use of polycistronic mRNAs in plant engineering would simplify the alteration of traits such as pest resistance, which may involve multiple proteins. In a hypothetical case, these proteins would be produced through expression from a single nuclear promoter that drives transcription of the polycistronic mRNA. This would obviate the need for coordinated expression of individual promoters driving genes that encode the individual proteins. An alternative to nuclear transformation is the use of chloroplast transformation (*see* Chapter 11), or infection by engineered viruses *(106)*, but these systems have complications of their own.

The expression of polycistronic messages of caulimovirus (a type of plant pararetrovirus) employs a highly unusual mechanism to express the multiple cistrons of their pregenomic RNA. It involves translation of the polycistronic mRNA utilizing *cis*-acting viral RNA sequences and a transacting virus-encoded protein (P6) *(107)*. In addition to its role in polycistronic translation, the translational trans-activator protein P6 also activates its own expression from a monocistronic subgenomic RNA. The efficient expression of polycistronic and monocistronic caulimovirus mRNAs in plant cells thus requires compatible interactions between the P6 translational trans-activator and its cognate *cis*-element at the 3' end of the mRNA. Exploitation of this type of translational mechanism to express an engineered polycistronic mRNA will probably require co-expression of the P6 protein or an analog.

A variation on the theme of producing multiple proteins from a single mRNA is also seen in some plant viruses, in which a polypeptide primary translation product is self-processed to produce multiple proteins *(108)*.

4.3.5. mRNA Targeting

In addition to protein-targeting mechanisms mediated by specific amino acid sequences, proteins may also be targeted to specific subcellular compartments by localization of their mRNAs *(109,110)*. Studies of mRNA localization are a relatively new area in plant science, and results thus far reveal that mRNA localization is involved in many aspects of expression and cell structure. mRNA localization has been implicated in the assembly of macromolecular structures within the cell, in the formation of endoplasmic reticulum subdomains, in facilitating protein localization in the endomembrane system, and in the control of gene expression. In addition, mRNA localization in plants may be part of a mechanism for controlling intracellular communication, not only between adjacent cells but between cells separated by relatively long distances. Although mRNAs can be localized by various mechanisms, evidence gathered to date implicates the role of a translation initiation codon along with cytoskeletal elements, microfilaments, and microtubules that function in the transport and anchoring of RNAs to specific subcellular locations within the cell *(110)*.

5. Heterologous Recombination Systems

Given the unpredictable outcomes of transformation methods that integrate transgenes randomly into the plant genome, various approaches have been used in an attempt to lessen the variability of transgene expression, or to control or alter the result

of transgene integration. The inclusion of MARS sequences in the transgene construct was mentioned previously. Site-directed integration through homologous recombination between the plant chromosome and plant DNA included on the transgene construct has met with limited success *(111)*. Another approach that holds some promise is the use of sequence-specific recombination systems derived from bacteria or yeast. These systems have two features in common, a recombinase that promotes DNA recombination and a set of specific DNA sequences that comprise 20–30 bp and serve as the recombination substrate for the recombinase *(112)*. Each recombinase recognizes a pair of the specific sequences and catalyzes recombination between them, with a result that depends on the initial physical relationship of the members of the substrate pair. For example, if the individual members of the substrate sequences are on separate plasmids, the recombinant result is a co-integrated plasmid comprised of both starting plasmids, with a copy of the substrate sequence at each junction of the plasmid sequences. These two intramolecular copies of the substrate sequences can act as substrates for a second recombination event that resolves the cointegrant plasmid into the starting molecules. Thus, the outcome an intramolecular event can be predetermined by controlling the orientation of the two substrate sequences relative to one another. The outcome of recombination between directly repeated substrate sequences is deletion of the DNA located between the substrates (plasmid resolution in the previous example). In contrast, the outcome of recombination between substrates in inverted orientation relative to one another is inversion of the intervening DNA.

Several such sequence-specific systems have been engineered to work in plants (reviewed in ref. *111*). The first was the cre/loxP system derived for the bacteriophage P1. By including copies of the substrate sequence (loxP, locus of crossing-over) in the transgene construct, various types of recombination events can be driven in planta by expression of the recombinase causes recombination (cre) protein. Examples include gene activation or inactivation, deletion of antibiotic selectable marker genes, and non-reversible chromosomal rearrangements mediated by mutant lox sites *(113)*.

The development of other recombination systems for plant use has followed the examples set with the cre/lox system. These include the FLP/FRT system from yeast (*Saccharomyces cereviseae*) *(114,115)*, the R/RS system (from *Zygosaccharomyces rouxii*) *(116)*, the Gin/gix system (from *E. coli* bacteriophage Mu) *(117)* and the attP system (from the *E. coli* lambda phage) *(118)*. The combination of an estrogen-induced transactivator system and the cre/lox recombination system allows chemical-inducible, site-specific DNA excision in *Arabidopsis (119)*.

6. Intellectual Property Issues

The scientific field of plant genetic engineering has existed for about twenty years. Although it is not my intent to diminish in any way the huge public-domain contributions made by scientists in academic institutions, it is important to understand that many of the basic enabling technologies in the field were developed and patented by agricultural research companies, or the intellectual property rights to the technologies were licensed or were purchased by them. It is prudent for any research program initiated with an intent to commercialize the products of its research to conduct an analysis of the intellectual property landscape surrounding all aspects of the technology. Many of the initial pioneering patents were filed in the 1980s and early 1990s. Competing

parties sometimes filed patents within a few days or weeks of one another. As a result, many of the patent applications are still being examined in the patent offices, are in interference proceedings, or have been issued but are being challenged in the courts. As these cases are resolved, the intellectual property ownership will sometimes shift from one party to another, with the result that a license to use a technology from one party may be rendered in question by the issuance of a new patent or court decision. Thus, a certain amount of risk is incurred by the decision to use a particular promoter or transformation method in the development of a commercial product. What may seem to be "free and clear" at the inception of the program may become encumbered later on as the intellectual landscape evolves.

In academic or other public institutions, it is common to find that exciting new genes being discovered or technologies being developed are "contaminated" through the use of components that must be licensed for commercial use. Although many industrial research companies will grant free licenses to some of their intellectual property to academic scientists for research purposes only, the line between what constitutes basic research and commercial development research is becoming increasingly blurred. The researcher may begin with good intentions and obtain readily available promoters or selectable marker genes in order to get his or her research program off to a fast start. As the years pass and excitement builds over the progress of the research, these components become so ubiquitous in all the constructs that it becomes extremely time-consuming to go back and re-engineer with different, unencumbered components. Eventually the program may reach a point when an invention is patentable, and the institution's intellectual property licensing office tries to find a royalty-paying commercial partner that will acquire a license, sell a product, and turn money back to the institution. In some cases, this further commercial development is hindered or made impossible because of high licensing and royalty costs owed to third-party companies that own particular pieces of intellectual property essential to practicing the new invention. As more public institutions become more aggressive in filing patents to protect the inventions of their faculty, it might be advisable for them to institute intellectual property workshops or other types of training for their research community. This training could emphasize the importance of decisions made early in the initiation of a research program, and how those decisions may affect future deployment of the products from that research. The results may benefit both the institution and the prospective commercialization partner through the availability of a "cleaner" invention with higher value.

A current example is provided by the development of transgenic "golden rice" *(120)*, which was engineered with the complement of genes needed to create a biosynthesis pathway for beta-carotene, which humans convert into vitamin A. A potential benefit of golden rice (so named because the beta-carotene imparts a yellow color to the grain) is that it could help alleviate a widespread public health problem, vitamin A deficiency. Globally, about 400 million people suffer from this affliction, which can lead to vision impairment and increased disease susceptibility. Although the beta-carotene content of the first sets of transgenic plants is not sufficient to supply the total dietary requirement of vitamin A, widespread inclusion in human diets could provide some benefits. However, two studies have found that between 25 and 70 proprietary techniques and materials were involved in the gene transfers *(121)*, and agreements must be reached with

the affected parties before the transgenic plants can be passed on to third parties, such as rice-breeding institutes.

A second example is provided by the events following the discovery of an ALS gene that contained two point mutations that together confer resistance to all four chemical classes of ALS inhibitors (*see* **Subheading 2.3.1.**) *(122)*. One mutation conferred resistance to sulfonylureas and/or triazolopyrimidine sulfonanilides, and the second mutation mediated resistance to the imidazolinones and pyrimidyl-oxy benzoates. If expressed in a transgenic crop, such a four-way gene may allow the application of a mixture of two different herbicides, one from each group corresponding to the resistances conferred by the individual mutations. It was proposed that these combinations of chemistries could thus delay or eliminate the appearance of weeds resistant to the ALS inhibitors *(123)*, since it seemed extremely unlikely that both point mutations would occur in the ALS genes of a weed in the same generation. When approached by an enthusiastic seeds industry group about commercializing the gene, however, the company that held patents on the ALS gene responded negatively, and ultimately the public research on the gene was terminated.

The lesson to be emphasized is that, as public institutions become more involved in research that lends itself to development of a commercial product, these institutions must gain a much better understanding of the scope and nature of business decisions. United States patent laws, combined with the potential for large profits in the agricultural biotechnology industry, are profoundly changing the nature of "public domain" research.

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