Metabolic Engineering 4, 67–79 (2002)
doi:10.1006/mben.2001.0210, available online at http://www.idealibrary.com on IDEAL®

Manipulating Gene Expression for the Metabolic Engineering of Plants

Philip A. Lessard,* Harikrishna Kulaveerasingam,*,† Gregory M. York,*
Amie Strong, and Anthony J. Sinskey*,[1]

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and †Genome Center, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

Received September 17, 2001; accepted September 26, 2001

Introducing and expressing foreign genes in plants present many technical challenges that are not encountered with microbial systems. This review addresses the variety of issues that must be considered and the variety of options that are available, in terms of choosing transformation systems and designing recombinant transgenes to ensure appropriate expression in plant cells. Tissue specificity and proper developmental regulation, as well as proper subcellular localization of products, must be dealt with for successful metabolic engineering in plants. © 2002 Elsevier Science

INTRODUCTION

Metabolic engineering of plants promises to create new opportunities in agriculture, environmental applications, production of chemicals, and even medicine. However, introducing and expressing foreign genes in plants also presents many technical challenges that are not encountered with microbial systems. Unlike bacteria, plants cannot normally express genes from polycistronic messages, meaning that great care must be taken to coordinate the expression of complex traits involving multiple transgenes. Plant cells have numerous organelles, including several that are not found in mammalian or yeast cells, that complicate issues of compartmentalization of resources as well as targeting of gene products. Beyond the perspective of isolated cells, plants have numerous specialized and differentiated organs in which physiological processes and gene expression may differ dramatically. In addition, temporal and developmental processes can profoundly influence whether and when transgenes are active, and should the engineered plants be propagated as crops, environmental effects may cause an additional level of variability and unpredictability that is not encountered in fermentor-based systems.

Taking on a project involving the metabolic engineering of plants, experimentalists must consider several other factors that will influence the design of the engineering strategy. For example, is the planned modification intended to impact some native physiological process or the synthesis of a natural plant metabolite? Rather, is the intention to overproduce new products or proteins? Such considerations dictate the level of expression that will be desired from the transgene. Some experimental strategies might call for only the transient expression of a foreign gene, for example in studying the interaction between different genes and gene products, while other strategies might require the generation of fully transgenic, whole plants that express a new gene product stably for many years. Each of these considerations invokes a different strategy for introducing and controlling the expression of foreign genes. Here, we summarize some of the major themes that have emerged in controlling gene expression in transgenic plants.

OVERVIEW OF TRANSFORMATION METHODS

General strategies and considerations. Rapid progress has been made over the past few years that has resulted in the development of facile plant transformation methodologies that work with a range of agronomically useful species (Table 1). However, while it has been relatively simple to obtain high levels of transient gene expression in plants, it has been considerably more difficult to obtain stably transformed plants. Transient expression can be easily obtained at very high levels using whole-tissue electroporation and particle bombardment (biolistic) with most plant species. The transient expression of gene constructs may be desirable in some cases in which long-term expression is not required. This approach has been used to test the effectiveness of various designs of gene constructs prior to stable transformation. While transient expression in

[1] To whom correspondence should be addressed at the Department of Biology, Massachusetts Institute of Technology, Bldg. 68-370, 77 Massachusetts Avenue, Cambridge, MA 02139. Fax: (617) 253-8550. E-mail: asinskey@mit.edu.

1096-7176/02 $35.00
© 2002 Elsevier Science
All rights reserved.

cultured cells is a useful tool for studying metabolic or gene expression networks in plant cells and even for trouble shooting designs for the transgene, it is important to recognize that identical genes may behave differently in transient versus stably transformed plant cells (Ingelbrecht *et al.,* 1989). It has also proved to be very useful to study the effects of the tissue-specific expression of transgene constructs in mature target tissues such as flowers or fruits. In this manner a rapid assessment can be made of the suitability of the engineering strategy. The production of mature flowers and fruits through a stable transformation approach is more time consuming and, in the case of transgenic perennial trees, may take many years.

Agrobacterium-mediated transformation. Stable transformation is dependent on several factors, the most important being the plant species to be transformed and the transformation protocol used. An attenuated soil-borne pathogen, *Agrobacterium tumefaciens,* is the most commonly used vector to transform numerous dicotyledonous (broad leaf) plants, including familiar fruits and vegetables such as tomatoes, mustards, and beans (Zupan *et al.,* 2000). This method takes advantage of the "natural" plant genetic transformation system that evolved in *Agrobacterium.* Wild-type *Agrobacterium* transfers a segment of DNA (called the T-DNA) from its large tumor-inducing (Ti) plasmid through the plant membranes and incorporates it into the genomic DNA of plant cells adjacent to a wound site. The T-DNA is bounded by 25-bp direct repeats called border sequences, and it contains genes that encode enzymes that direct the commandeered plant cells to produce peculiar amino acids called opines that cannot be catabolized by the plants themselves, but that can be used as primary sources of carbon and nitrogen by the cohabiting bacteria. The T-DNA also includes genes that direct the plant cells to produce plant hormones such as cytokinins, which promote cell division and tumor formation, providing a steadily increasing supply of nutrients for the bacteria. To enable technologically useful plant transformation, the *Agrobacterium* oncogenic hormone biosynthetic genes in the T-DNA have been removed from attenuated bacteria and replaced with multicloning sites where genes of interest as well as dominant selectable markers can be integrated. *Agrobacterium* harboring such recombinant Ti plasmids can then be introduced onto wounded tissues (e.g., leaf explants in culture) or even directly onto mature plant organs (see below) and the bacterium will transfer the modified T-DNA to some of the cells of the host plant.

The wild-type Ti plasmid is very large (200 kb) and is difficult to manipulate. Its utility has been improved by the development of binary vectors (Bevan, 1984). In such a system, the Ti plasmid of *Agrobacterium* has been disarmed, i.e., the T-DNA has been removed but the *vir* regions have been left intact. A separate plasmid that can replicate in both *Escherichia coli* and *Agrobacterium* (hence the term "binary vector") is then used. The binary vector carries an origin of replication that is compatible with the Ti plasmid in *Agrobacterium.* This plasmid also carries an artificial T-DNA region into which different transgenes may be introduced. Thus, when the binary vector is introduced into *Agrobacterium* the *vir* genes from the disarmed Ti plasmid will act *in trans* to transfer the recombinant T-DNA from the binary vector to the plant cell. As the binary vectors are smaller and much easier to manipulate than intact Ti plasmids, this tool makes *Agrobacterium*-mediated transformation much more straightforward.

TABLE I

Three Common Strategies for Introducing Foreign Genes into Plant Cells

Transformation procedure	Advantage	Disadvantage
Agrobacterium mediated	Very effective, cheap and simple to use and can be used in germ-line transformation. The copy number of DNA insertions is often low.	Requires the use of a tissue culture regeneration procedure. Host range may be limited by the plant hypersensitive response.
Particle bombardment	Very effective especially for transient expression. Has been used to produce transplastomic plants. No plant host range problems.	Requires the use of a tissue culture regeneration procedure. Copy number of DNA insertions can be high and lead to gene silencing/cosuppression.
Electroporation	Very effective for transient expression. No plant host range problems. High DNA delivery rate.	Requires the use of a tissue culture regeneration procedure. Copy number of DNA insertions can be high and lead to gene silencing/cosuppression.

Metabolic Engineering **4**, 67–79 (2002)
doi:10.1006/mben.2001.0210

In general, with *Agrobacterium*-mediated transformation it is necessary to select and propagate transformed plant cells containing the integrated *Agrobacterium* T-DNA from those few initially transformed cells (Zupan *et al.,* 2000). If the experiment requires cell cultures then this process is relatively straightforward with many dicot plants. However, if complete regenerated plants are required then the process is complicated by the need for tissue culture-mediated plant regeneration. Despite considerable effort, plant regeneration remains difficult, problematic, and time consuming. In some cases, unwanted somaclonal variation has been introduced through the tissue culture regeneration system.

Until about 5–8 years ago it was thought that *Agrobacterium* was incapable of infecting monocotyledonous plants, which include lilies, palms, and grains. This has led to the development of other transformation systems such as particle bombardment (Klein *et al.,* 1987) and electroporation (Newell, 2000) as a means to transform these plants. However, over the past few years there have been numerous successes with the transformation of monocot plants using *Agrobacterium* (Hansen, 2000; Hernalsteens *et al.,* 1984; Schafer *et al.,* 1987). Strains containing supervirulent plasmids have facilitated transformation of some recalcitrant monocot plants. It is believed that the factor that limits transformation success in monocot plants is not transfer and integration of T-DNA into the plant genome but plant regeneration. Often the regeneration rates are poor with monocot plants and this is further reduced under selection during transformation. As the T-DNA is integrated into the genome at random sites, regions flanking the T-DNA exert a strong influence on expression levels, necessitating the recovery of several independent transgenic lines to account for this variability. Recent advances in using more regenerable starting material have led to several successes with monocot plants such as rice, maize, and sorghum. The poor regeneration in monocotyledon species has also led to the development of germ-line transformation strategies discussed below.

Electroporation and particle bombardment. Electroporation of whole tissues is another transformation method that has been used for the transformation of monocot plants (Newell, 2000; Sorokin *et al.,* 2000; Terzaghi and Cashmore, 1997), although there are fewer reports of the use of this procedure in the literature compared to *Agrobacterium*-mediated transformation. Other considerations when using this method include problems with plant regeneration and the tendency of the technique to insert multiple copies of the transgene(s) into the plant genome. Particle gun-mediated transformation, often called biolistic transformation, is a commonly used procedure that has its advantages (Klein *et al.,* 1987; Maliga, 2001; Ye *et al.,* 1990). This is the most frequently used procedure for transient transformation of tissues and is based on bombarding tissues with microscopic, DNA-coated tungsten or gold particles. As with electroporation, one of the disadvantages of this method is that multiple insertions of the transgene occur and can result in gene silencing and instability of the transgene (Hansen and Chilton, 1996) (see below). However, this method is not limited by the species or type of tissues bombarded and is frequently used for transformation of monocot species. Another advantage of this procedure is that its high transformation frequency has facilitated the successful transformation of plastids in tobacco and tomato (Maliga, 2001). Transforming the plastid genome instead of the nuclear plant genome may be very advantageous for bioengineering reasons (Daniell *et al.,* 1990). For example, this enables targeting of the gene product to the specific organelle in which it is intended to act. Because the plastid genome is often duplicated severalfold within a single plastid and the plastids are themselves present in high copy numbers within many cell types, plastid transformation can lead to substantial gene amplification. This technique also has the added advantage of not transferring transgenes via pollen as plastids are maternally inherited, which makes dispersal of the transgene easier to control.

However, all the methods described above require the use of plant tissue culture procedures to be able to regenerate transgenic plants. Germ-line transformation has been touted as a means to overcome this limitation by directly transforming germ-line cells (Tague, 2001). Its success has been reported for *Arabidopsis thaliana* and some close relatives, of which flowers were dipped into solutions containing *Agrobacterium* in the presence of surfactants (Tague, 2001). Transgenic seeds were produced directly but at low frequency from these dipped flowers without need for tissue culture.

SELECTABLE MARKERS

Antibiotics. Resistance to antibiotics is perhaps the most commonly used trait for selecting genetically transformed plant tissues. Inhibitors of ribosome function such as kanamycin and hygromycin that prevent the growth of bacterial cells are often also effective in preventing the growth of plant cells. By extension of this observation, the enzymes that confer resistance to such compounds in bacteria (such as neomycin phosphotransferase and hygromycin phosphotransferase, respectively) can be employed to confer antibiotic resistance in plant cells. Of course, proper expression of these enzymes requires that the genes encoding them be modified to resemble plant genes (proper promoters, polyadenylation signals, etc., see below). While kanamycin is perhaps the most widely used antibiotic for

selection of transgenic plant tissues (Nap *et al.,* 1992), resistance markers for antibiotics such as hygromycin, streptomycin, gentamicin, and chloramphenicol have also been routinely employed (Dale and Ow, 1991; Gossele *et al.,* 1994; Maliga *et al.,* 1988; Pietrzak *et al.,* 1986; Rogers *et al.,* 1983). The majority of these markers operate by chemically modifying the antibiotic itself, for example via phosphorylation or acetylation, rendering the compound inactive.

Herbicides. Another strategy for selection of transgenic plant cells has been to employ herbicide resistance markers. Many of the herbicides that are used for these purposes act by inhibiting processes such as synthesis of branched-chain or aromatic amino acids, processes that are critical in autotrophic plant cells. For the most part, herbicide resistance can be conferred through three broad strategies. The first of these is to simply overexpress the wild-type enzyme whose activity is impacted by the herbicide (Widholm *et al.,* 2001). The second strategy is to express in plants a modified (mutant) form of the target enzyme that is less sensitive to the herbicide, often resulting from one or a few amino acid substitutions (Lee *et al.,* 1999; Stalker *et al.,* 1985). The third strategy for introducing resistance to herbicides is to express enzymes that modify or metabolize the herbicidal compounds themselves (Shinabarger and Braymer, 1986; Spencer *et al.,* 1992). Extension of this latter strategy has made it possible to generate transgenic plants with increased tolerance of fungicides as well (Tamura *et al.,* 1995).

Alternative strategies. Collectively, antibiotic resistance and herbicide resistance are often called negative selection strategies for isolating transgenic plant cells because the selective agents that are used kill untransformed tissues rather than promote the growth of transformed tissues. Recently a number of new strategies have emerged to change this paradigm. Transgenes that permit plant cells to utilize new carbon sources are good examples of these positive selection strategies (Haldrup *et al.,* 1998; Zhang *et al.,* 2000). While photosynthetic green tissues, even those in actively regenerating cultures, can fix carbon from the air, cultured plant cells and those in the earliest stages of regeneration must have carbon supplied to them, typically in the form of sucrose (the form in which most carbon is translocated in whole plants). Plant cells cannot assimilate the variety of carbon sources that bacteria and yeasts use. Enzymes that convert unusable carbon sources into sugars that are more easily recognized by the plants would enable plant cells to grow on new carbon sources. Thus, transgenic plants have been selected and regenerated on media containing xylose (Haldrup *et al.,* 1998) or mannose (Zhang *et al.,* 2000) carbon sources. Such selection can proceed without the use of any antibiotic or herbicide resistance markers, and additional genes (encoding desirable but nonselective traits) can be successfully introduced alongside the selective markers.

Another spin on the positive selection strategy involves the introduction into plant cells of genes encoding enzymes involved in hormone biosynthesis (Ebinuma *et al.,* 1997; Kunkel *et al.,* 1999). Cytokinins are hormones that promote cell division in plants and are involved in a number of morphogenic processes. Among these are the generation of adventitious shoots from cultured tissues. Taking advantage of this, cytokinin biosynthesis can be stimulated in transformed cells by introducing a gene encoding isopentenyl transferase, a key enzyme in cytokinin biosynthesis. When DNAs including the isopentenyl transferase gene were transformed into plant cells, transgenic tissues were selected without the need for other markers (Ebinuma *et al.,* 1997; Kunkel *et al.,* 1999). Overproduction of a plant hormone might be expected to cause morphological abnormalities in the resulting plants, but in at least one example transgenic tobacco and lettuce generated using an inducible isopentenyl transferase gene were morphologically normal (Kunkel *et al.,* 1999). Data regarding long-term effects on fertility and other measures of robustness will determine whether this strategy can be applied in agronomic applications. Nonetheless, such strategies provide special promise for work with plant species such as trees that have particularly long generation times (Ebinuma *et al.,* 1997).

Removal of selectable markers following transformation and selection. Being able to remove resistance markers such as those described above would ease public concerns relating to the transfer of resistance markers to nontarget species such as weeds or microbes. Moreover, it would obviate the need for different selectable markers in subsequent rounds of gene transfer into the same host (Dale and Ow, 1991). These challenges have motivated researchers to develop strategies for eliminating selectable markers once they had been introduced into the plant cells. Once the transgenic plant has been established there is no further need for the marker gene in most applications, so a mechanism for removing the trait would be welcomed. To accommodate this need, several techniques have been employed (DeBlock and DeBrouwer, 1991). The first of these involves the simultaneous but independent "cotransformation" of plant cells with the marker gene on one DNA molecule and the desired second trait on a separate DNA molecule (DeBlock and DeBrouwer, 1991). At some frequency, these molecules will each integrate into the plant's chromosomal DNA at genetically distinct loci. In such cases, it may be possible to remove the selectable marker from whole plants via normal chromosomal segregation (DeBlock and DeBrouwer, 1991). In practice, however, this approach has proven cumbersome.

Metabolic Engineering **4**, 67–79 (2002)
doi:10.1006/mben.2001.0210

Another strategy has employed the sequence-specific DNA excision functions of plant transposable elements (Yoder and Goldsbrough, 1994). With the Ac/Ds transposon system from maize the Ac element encodes a functional transposase, the enzyme that recognizes the transposon's inverted terminal repeat structures, cleaving the element from one location in the genome and inserting it into a separate locus. The Ds element is similar to Ac, except that it lacks a functional transposase, having only the terminal repeats. If a transgene such as an antibiotic resistance marker embedded within a Ds element is introduced into plants, subsequent crossing of that plant with a line bearing Ac will lead to excision and transposition of the Ds element away from its original locus. If a second transgene had been introduced along with the selectable marker but had not been embedded within the Ds element, this transgene would not be affected by the presence of Ac. Assuming the Ds-embedded marker gene has transposed to a separate chromosome or a locus sufficiently far away, it would then be possible to remove the marker gene via genetic segregation (Yoder and Goldsbrough, 1994). However, because this approach also depends upon multiple rounds of genetic crossing and evaluation, it may be too cumbersome for certain applications.

A third strategy that does not require genetic segregation to separate the marker gene from other introduced traits involves the use of the bacteriophage P1 Cre/*lox* recombination system (Dale and Ow, 1991; Wanggen *et al.,* 2001). In this system, Cre recombinase recognizes *lox* excision-site DNA sequences and precisely clips them from the genome. When a selectable marker is introduced sandwiched between two *lox* sites, the subsequent introduction of Cre recombinase, either by activation from an inducible promoter (Wanggen *et al.,* 2001) or by a single genetic cross with a separate Cre-expressing transgenic line (Dale and Ow, 1991), will cause excision of the gene(s) between the *lox* elements. The excised element is unstable and subsequently lost from all progeny cells.

COMPONENTS OF THE TRANSGENE

Promoters. Several considerations need to be made pertaining to the choice of promoter used and these are dependent on the aims of the engineering process. There are numerous types of promoters that regulate different types of expression ranging from constitutive to inducible (Table 2). Each of the inducible promoter systems has characteristic features that take advantage of either agricultural practices (e.g., safener inducible) or cell culture conditions (e.g., pristinamycin-responsive promoter) to facilitate controlled gene expression. These inducible systems (Zuo and Chua, 2000) are especially useful when examining the consequences of transgene expression in complex biochemical pathways. Safeners are "herbicide antidotes" that are used as a seed treatment to permit the use of herbicides in weed control measures during planting. Exposure to the safener will result in activation of the In2-2 promoter in root and shoot tissues and this may be desirable in the case in which conversion of the seed storage material into the bioengineered product is desired or in cases in which expression of the transgene is desired for only a short period following germination. Pristinamycin is a polyketide antibiotic not normally found in plant cells. In the case of bioengineering of cell cultures, the use of a pristinamycin-responsive promoter driving transgene expression has the advantage of controlling bacterial contamination as well as allowing ease of induction of expression within a bioreactor.

However, if transgenic plants are being generated simply to produce large quantities of the transgene product in all tissues then numerous constitutive promoters are available that have been used successfully. The most commonly used constitutive promoters are of viral origin such as the cauliflower mosaic virus 35S promoter that drives very high levels of transcription in most tissues of the plant (Benfey *et al.,* 1990). Although the constitutive promoters of viral origin can drive very high levels of expression, in some cases deleterious effects such as gene silencing via cosuppression (see below) have been reported (Koosha *et al.,* 1989). This phenomenon may be less common in the case of constitutive promoters of plant origin such as the ubiquitin and actin promoters. Rather than overexpressing a transgene in all tissues, another option is to target expression of the transgene to specific organs rich in precursors for metabolic engineering such as leaves, tubers, or fruits. Promoters that drive expression primarily in these tissues have been identified and used and are well characterized. A further advantage of this option is that it reduces the impact of transgene expression on the normal growth and development of the plant while enabling the production of the desired product in easily harvested tissues. There are other strategies such as the use of a leaf-specific promoter that enable the nonharvested biomass of the plant to be converted into a value-added commodity. Also, by selecting an appropriate native, regulated promoter to control transgene expression it is possible to direct levels of expression that approximate the needs of the metabolic engineer. Thus, while high-level production of a specific protein might require a particularly strong promoter, a more subtle, tightly regulated promoter might be preferred for applications in which a specific physiological pathway is to be affected.

Polyadenylation signals. In addition to the promoters, a further factor to be considered in design of a plant expression cassette relates to the type of polyadenylation signal or

TABLE II

Examples of Different Types of Plant Promoters Used for Construction of Transgenes

Type of promoter	Name	Comments	Reference
Constitutive	35S	Viral origin, very well described and commonly used. High expression in vascular tissue but significantly lower expression in meristem tissue.	(Benfey *et al.*, 1990)
Constitutive	Ubiquitin	Plant origin promoter that drives high-level constitutive expression but expression level during development may vary.	(Plesse *et al.*, 2001)
Constitutive	Actin	Plant origin promoter that drives high-level constitutive expression in most tissues but expression level between tissues and during development may vary. It is a member of a multigene family and hence the choice of the promoter from the correct family member is important.	(An *et al.*, 1996a) (An *et al.*, 1996b) (Becker *et al.*, 1994)
Tissue specific, embryo	*β*-Conglycinin promoter	A well-characterized promoter that directs embryo-specific expression.	(Chen *et al.*, 1986)
Tissue specific, endosperm	Opaque-2 promoter, ZmZ27 promoter, osGT1 promoter	These promoters show developmental regulation and the expression levels may vary accordingly.	(Rossi *et al.*, 1997) (Russell and Fromm, 1997)
Tissue specific, fruit	2A11	2A11 is a fruit-specific promoter derived from tomato.	(Van Haaren and Houck, 1993)
Tissue specific, tuber	Patatin/StMCPI	The patatin promoter drives high-level expression in tubers and in sucrose-treated leaves, StMCPI is a tuber-specific promoter that is regulated independent of environmental or hormonal signals.	(Liu *et al.*, 1990) (Molnar *et al.*, 2001)
Tissue specific, leaf	Lhcb3 promoter	Lhcb3 promoter is a light-regulated leaf-specific promoter from *Arabidopsis*.	(Ali and Taylor, 2001)
Pollen specific	lat52 promoter	Developmentally regulated but drives high expression during pollen maturation.	(Bate and Twell, 1998)
Inducible	Pristinamycin-responsive promoter	An inducible promoter system based on a recombinant transcription factor fusion between Pip (repressor of pristinamycin operon) and VP16 transactivating domain of the herpes simplex virus.	(Frey *et al.*, 2001)
Inducible	Safener-inducible promoter, In2-2 promoter	The maize In2-2 promoter is activated by benzenesulfonamide herbicide safeners.	(De Veylder *et al.*, 1997)
Inducible	Glucocorticoid-inducible promoter, ethanol-inducible promoter, ecdysone-inducible promoter	These systems are based on the interaction between the chemical inducer and a specifically designed transcription factor that results in the transactivation of a synthetic promoter.	(Aoyama and Chua, 1997) (Caddick *et al.*, 1998) (Martinez *et al.*, 1999)
Inducible	APase promoter	This promoter drives phosphate-inducible expression in roots. However, the rate of induction is slow.	(Haran *et al.*, 2000)

transcription terminator to be used. The most commonly used transcription terminator/poly(A) signals are derived from the nopaline synthase gene from *A. tumefaciens*. This has been used successfully in a wide variety of species that include dicot and monocot plants (Gleave, 1992). However, there are indications that there are plant-derived transcription terminators that are more effective such as the 3′ noncoding regions of the Me1 gene (Ali and Taylor, 2001) and wheat histone H3 gene (Ohtsubo and Iwabuchi, 1994). The use of the Me1 3′ noncoding region has led to a generalized enhancement of severalfold in gene expression from promoters of different classes (Ali and Taylor, 2001) without any alteration in the expression pattern of the promoters concerned. There is evidence that the transcription-enhancing effects of the 3′ coding sequence are related to its effectiveness at terminating transcription (Ali and Taylor, 2001). However, this experiment has not been replicated in monocot plants and it is unclear how widely applicable this strategy will be especially since monocot plants form the bulk of crops grown worldwide.

Metabolic Engineering **4**, 67–79 (2002)
doi:10.1006/mben.2001.0210

Introns. Although the mechanisms underlying the phenomenon are not entirely clear, incorporating introns into transgenes has an enhancing effect on gene expression (Koziel *et al.,* 1996). Introns are normally spliced out of eukaryotic messages as the newly transcribed pre-mRNAs are processed in the nucleus. It may be that this RNA processing itself encourages the efficient translocation of the RNA into the cytosol, where it can be translated. A variety of plant introns have been co-opted from their native contexts and used to drive higher expression of transgenes. Examples of such introns include intron 1 of the rice actin gene (McElroy *et al.,* 1991), intron 1 of the maize ubiquitin gene (Christensen and Quail, 1996), intron 1 of the maize sucrose synthase gene (Vasil *et al.,* 1989), and intron 1 of the maize alcohol dehydrogenase gene (Rathus *et al.,* 1993). Nonetheless, not all introns are created equally. In systematic studies in which multiple introns from maize *Adh1* were tested for the ability to enhance expression of a test gene, not all introns were as effective in enhancing expression (Callis *et al.,* 1987). Furthermore, these studies demonstrated that the introns must be within the transcribed portion of the transgene, preferably within the 5′ untranslated leader sequence (Bourdon *et al.,* 2001; Callis *et al.,* 1987; Mascarenhas *et al.,* 1990). Placing the introns upstream of the promoter did not have any effect on expression, indicating that the introns were not acting via a mechanism of transcriptional enhancement. Introns do not perform equally well when introduced into monocots and dicots, suggesting that the requirements for proper splicing may be different between these two major taxa (Goodall and Filipowicz, 1991). Other factors that influence intron-mediated enhancement of gene expression include the strength of the promoter driving transcription, the cell type, and the sequences bordering the intron itself (Chaubet-Gigot *et al.,* 2001; de Boer *et al.,* 1999; Koziel *et al.,* 1996). In some cases the introns play an important role in determining the tissue-specificity of gene expression as well (Deyholos and Sieburth, 2000; Fu *et al.,* 1995; Jeon *et al.,* 2000; Plesse *et al.,* 2001).

5′ Untranslated leaders and start codon context. In addition to introns, other sequences have been found to enhance expression when incorporated into transgene mRNA. 5′ Untranslated leaders (5′ UTLs) from viral mRNAs such as the tobacco mosaic virus omega sequence and the alfalfa mosaic virus 5′ UTL have been used to enhance expression in tobacco cells (Koziel *et al.,* 1996). 5′ UTLs from the maize glutelin and PEP-carboxylase genes have been found to increase expression in maize cells. However, there appears to be a strong loyalty among 5′ UTLs in that those from dicot genes appear to work better in dicot hosts and those from monocot sources work best in monocots (Koziel *et al.,* 1996).

As has been demonstrated in mammalian systems (Kozak, 1986), the context of the start codon can have a strong effect on the level of expression of a transgene, although the specific context differs between plants and animals (Lutcke *et al.,* 1987). Recently, it has been demonstrated that conserved nucleotides downstream of the start codon could augment posttranscriptional effects on gene expression (Sawant *et al.,* 2001). In this work, they describe how inserting the sequence 5′-GCTNCC(T/A)CN-3′ immediately downstream of the start codon strongly increased gene expression while having no noticeable effect on mRNA stability. One such sequence contains the codons for the three amino acids alanine-serine-serine. Introducing this sequence between the initiator methionine and the remainder of a reporter enzyme doubled the stability of the reporter protein while increasing overall expression of the transgene by 30- to 40-fold relative to controls, apparently via a posttranscriptional mechanism. The increase could not be attributed solely to enzyme stability, however, as substituting synonymous codons for the second serine in this sequence actually decreased expression of the transgene.

Nucleotide composition of the translated region. Many of the transgenes that are introduced into plants come from widely divergent species such as bacteria and fungi. Not surprisingly, nucleotide biases among these disparate taxa are often very different in terms of both codon usage and G+C content (Murray *et al.,* 1989). This has prompted the development and testing of many modified or entirely synthetic gene homologues for high-level expression in plants. It is often tempting to attribute the success of such codon bias changes to correctly matching the tRNA complement of the new host, and indeed this may be the case in some instances (Batard *et al.,* 2000; Koziel *et al.,* 1983). However, other mechanisms are likely to play a significant role in such phenomena. Having evolved in completely separate genomic environments there is no selection to prevent a bacterial gene, for example, from accumulating sequence motifs that would be recognized as premature polyadenylation signals or cryptic intron splice sites in plants. Similarly, mRNA secondary structures that might hinder expression or RNA stability in the transgenic host might have no effect on expression in the native host. Mindful attention to such cryptic features can lead to the development of modified transgenes whose expression increases hundreds- or even thousandsfold (Iannacone *et al.,* 1997; Koziel *et al.,* 1983; Perlak *et al.,* 1990, 1991; Rouwendal *et al.,* 1997).

Organelle targeting. In some cases, it is desirable to target recombinant proteins to a particular type of organelle, depending on the particular application. Organelles

are discrete structures within plant cells, each with a distinct biological function and some with their own distinct genetic material (Burgess, 1985). The nucleus is the location of nuclear chromosomal DNA and is the site of transcription of this DNA. The endoplasmic reticulum (ER) and Golgi are where proteins are modified and targeted for transfer to the cell membrane or for secretion. The apoplast is the region between the cell membrane and the cell wall. The mitochondria are the sites of oxidative phosphorylation for energy production. The plastids, including chloroplasts and amyloplasts, are the sites of photosynthesis and starch storage. Finally, the microbodies, including liposomes and peroxisomes, are the sites of oxidation of fatty acids and the glyoxylate cycle. Along with the nucleus, the mitochondria and plastids contain their own distinct genetic material, which encodes many but not all proteins that function within the organelles. Targeting of proteins to organelles can be a useful component of metabolic engineering efforts. For example, it might be desirable to target a transcription factor to the nucleus, while in contrast it might be desirable to target proteins involved in synthesis of carbohydrates or polyesters to plastids.

Specific mechanisms have evolved for recognizing and appropriately targeting proteins to each organelle (Keegstra, 1989; Olsen *et al.,* 1993; Teasdale and Jackson, 1996). Although proteins encoded by the nuclear DNA are translated in the cytoplasm, subsets of these proteins are transported into each type of organelle. Thus, in metabolic engineering applications one mechanism for targeting recombinant proteins to organelles is to express them such that they include organelle targeting signals. Plants can then express the transgene from the nuclear DNA, and the resulting recombinant proteins will be targeted to the appropriate organelles. Specific amino acid sequences required for targeting of proteins to particular organelles and for retention of proteins in organelles have been identified. For example, for targeting of proteins to the ER, an H/KDEL motif near the carboxy terminus specifies that a protein should remain in the ER (Teasdale and Jackson, 1996). In contrast, dilysine or diarginine motifs specify ultimate targeting to cell membranes (Teasdale and Jackson, 1996). For targeting of proteins to plastids, addition of the first 23 amino acids of the pea *Rubisco* small subunit protein at the N-terminus of recombinant proteins is sufficient to direct these proteins to plastids (Keegstra, 1989; Nawrath *et al.,* 1994a,b). Following a similar theme, addition of the last 5 to 34 amino acids of plant isocitrate lyase to the C-terminus of recombinant proteins can direct those proteins to microbodies (Mittendorf *et al.,* 1998; Olsen *et al.,* 1993). Further basic research on organelle targeting will provide more versatility in accomplishing targeting of recombinant proteins.

Considering that mitochondria and plastids contain their own genetic material and can express this material within their own boundaries, another mechanism for targeting proteins to these particular organelles is to transform the genetic material of these organelles directly. Much progress has been made with regard to plastid transformation in particular (Daniell *et al.,* 1990; Hajdukiewicz *et al.,* 2001; Heifetz, 2000; Ruf *et al.,* 2001; van Bel *et al.,* 2001). Given the evolutionary relationship between plastids and bacteria, it seems that many of the advantages of conducting molecular biology in bacteria also apply in plastids. Benefits of plastid transformation include the ability to introduce genes by homologous recombination, the absence of silencing, and the ability to express polycistronic genes. Furthermore, as mentioned above, genes in plastids are present in high numbers, transformed plastids cannot be transmitted in pollen, and selectable markers can be removed from transformed plastids if desired (Hajdukiewicz *et al.,* 2001; Heifetz, 2000), all of which are factors that make plastid transformation particularly attractive for generation of transgenic crops. Thus far, plastid transformation has been used successfully to produce insecticidal agents, herbicide resistance proteins, and proteins involved in synthesis of biopolymers (van Bel *et al.,* 2001). Currently, the main limitation for this technology seems to be generation of fertile plants carrying transformed plastids.

GENE SILENCING

Silencing phenomena are important to consider in transgene experiments. Two types of silencing occur in plants as well as in other eukaryotes: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) (Carthew, 2001; Waterhouse *et al.,* 2001a,b). TGS is based on methylation of promoters and coding sequences of genes, which blocks transcription. PTGS is based on sequence-specific, targeted degradation of particular mRNAs. Interestingly, although there was no reason *a priori* to think that the two systems would be mechanistically related, a growing body of evidence suggests that they are. TGS and PTGS can be thought of as problems or opportunities, depending on whether the goal of a particular application is to accomplish expression of a transgene or silencing of an endogenous gene.

Silencing was first observed with regard to transgene expression in plants about 10 years ago, with a report that transformation of petunia with extra copies of the chalcone synthase gene could result in a block in expression of both the transgene and the corresponding endogenous gene (Napoli *et al.,* 1990). This phenomenon was termed cosuppression (Napoli *et al.,* 1990). Since that time, it has become apparent that there are mechanistic similarities

Metabolic Engineering **4**, 67–79 (2002)
doi:10.1006/mben.2001.0210

between cosuppression and other methods for blocking gene expression, such as antisense RNA technology, in which expression of the antisense version of a gene blocks expression of the corresponding sense version of the gene, and RNAi, in which injection of double-stranded RNA blocks expression of a gene (Carthew, 2001; Waterhouse *et al.,* 2001a,b).

Silencing has the frustrating effect of blocking transgene expression in plants in a manner that is not entirely predictable, which obviously can be a great problem for metabolic engineering in plants. Silencing can occur both for foreign genes and for extra copies of endogenous genes, resulting even in silencing of the normal, nontransgenic copies of endogenous genes (Carthew, 2001; Waterhouse *et al.,* 2001b). There is some predictability to silencing. Counterintuitively, an increasing copy number of a transgene can correlate with an increased risk of silencing. However, determining whether silencing will occur in particular transgenic plants is still largely an empirical problem, requiring testing of transgene expression in the individual plants. Two general approaches can be used to avoid problems with silencing. First, the use of gene delivery methods, such as *Agrobacterium*-mediated transformation, that result in integration of relatively few copies of a transgene into the genome, can minimize problems with silencing (Dai *et al.,* 2001). Second, the use of constructs in which matrix attachment regions (or scaffold attachment regions) flank the transgene may also minimize silencing (Spiker and Thompson, 1996). It is thought that these elements are involved with chromatin structure and act by binding to components of the chromosome scaffold, thereby isolating the intervening DNA from the rest of the chromosome and preventing interference from genes in adjacent regions. While the use of matrix attachment regions has proven beneficial in some instances it is not clear to what extent the absence of silencing may be due to the prevention of transcription of the antisense version of the transgene (from adjacent endogenous plant promoters) as opposed to the prevention of packaging of the transgene as heterochromatin (Gallie, 1998).

While TGS and PTGS were first studied for the problems that they cause in expression of transgenes, it was quickly realized that silencing presents an excellent opportunity to block expression of endogenous genes (Kooter *et al.,* 1999; Napoli *et al.,* 1990). Knocking out the expression of one or more endogenous genes is a desirable outcome in some metabolic engineering applications. Unlike the case for bacteria and yeast, and despite great efforts and recent progress (Beetham *et al.,* 1999; Chiurazzi *et al.,* 1996; Wang *et al.,* 2001), no simple, efficient method has been developed to introduce DNA into the nuclear chromosome of higher plants by homologous recombination. Thus, knocking out gene expression in plants by generating simple deletions in nuclear DNA is not practical. However, expression of endogenous genes can be knocked out by introduction, for example, of transgenes expressing antisense RNA, self-complementary RNA, or perhaps even just high levels of RNA. This is a simple, powerful way to knock out gene expression without need for homologous recombination.

Understanding the mechanism underlying PTGS and TGS may provide insights into avoiding or achieving silencing in a more predictable manner. These mechanisms are not well understood, but much progress has been made recently and the picture that is emerging is fascinating. PTGS is based on monitoring of RNA in cells, such that RNA recognized as foreign (or perhaps as overexpressed) is targeted for degradation by the host's viral defense mechanisms (Carthew, 2001; Waterhouse *et al.,* 2001b). The presence of double-stranded RNA in particular seems to be a crucial factor in triggering PTGS. According to the emerging model for PTGS, double-stranded RNA is degraded to yield short fragments of single-stranded RNA (21 to 25 nucleotides in size). These fragments may then hybridize to homologous single-stranded RNA molecules and target these for degradation. Interestingly, PTGS can spread through plants as a sequence-specific, diffusible signal, although the precise nature of the signal remains to be determined (Miller *et al.,* 2001). The fact that many plant viruses use double-stranded RNA as an intermediate in replication suggests a compelling biological reason for double-stranded RNA to be recognized and destroyed, namely as a means of defense against plant viruses. The observation that integration of transgenes at a high copy number is often associated with the presence of inverted repeats of the transgene also provides a simple explanation for silencing of transgenes, namely that transcription of genes present as inverted repeats would lead to production of self-complementary RNA. This proposed model suggests a strong mechanistic link between PTGS, antisense RNA, and RNAi, in that each system involves a double-stranded RNA species that is targeted for degradation.

TGS is based on sequence-specific methylation of promoter sequences and coding sequences of genes, which serves to block transcription of genes (Carthew, 2001; Waterhouse *et al.,* 2001b). In a mechanism analogous to that proposed for PTGS, transcription of genes present as inverted repeats will yield self-complementary RNA, which is targeted for degradation to short fragments. These fragments may then be recognized by a DNA methylation complex that targets promoters and coding sequences of genes for methylation. The observation that many transposable elements rely on RNA/DNA hybrids for replication and may also yield double-stranded or self-complementary RNA from transcription provides a compelling

biological explanation for why plants would exhibit TGS, namely to limit the spread of transposable elements within their genomes. Current points of contention are the precise roles of short RNAs in triggering TGS and whether partial RNA transcripts have the ability to induce methylation of only the corresponding partial sequence of a gene or of the entire gene (Miller *et al.,* 2001).

PROTEIN OVEREXPRESSION

In addition to modifying the physiology of plants through metabolic engineering there has long been an interest in using transgenic plants as low-cost production factories for high-value proteins such as pharmaceuticals and vaccines. While it can be argued that overproduction of such proteins does not meet the strictest definitions of metabolic engineering per se, this subject is worthy of some mention in the context of this review. As described above, since the earliest days of transgenic plant technology many different groups have used strong constitutive promoters to drive expression of foreign proteins in plants. While these strategies have proven useful in many applications, in others expression of the foreign proteins has proven to be too much of a burden on the host plant, causing a metabolic drain and/or impacting the plant's agronomic viability. Production of foreign proteins in specific organs such as leaves or seeds might often be preferable to generalized overexpression. Two particular strategies for producing heterologous proteins in plants that have met recent commercial success are worthy of note here.

The first of these is a method developed at the University of Calgary (Alberta, Canada). In this system foreign proteins are expressed in the seeds of transgenic oilseed plants as fusions to the plants' native oleosins, which are proteins that coat oil bodies (Parmenter *et al.,* 1995). Because of their association with the oleosin, the heterologous proteins accumulate on the surface of oil bodies that form as seeds mature. Following seed harvest, oil bodies can be easily separated from the remainder of the plant material, yielding a fraction that is almost exclusively oil and recombinant fusion protein. The heterologous protein is then cleaved from the oleosin carrier protein via cleavage of the enzymatically sensitive linker peptide that joins the two species and the protein is then isolated. Proteins expressed in such a manner can accumulate to respectably high levels. In Canada, this system is currently in use for the commercial production of hirudin, an anti-coagulant biopharmaceutical protein (Giddings *et al.,* 2000).

The second class of protein production systems worthy of note here involves the use of viral vectors to express foreign proteins in plants. In such a case, the host plant need not be transgenic. Rather, recombinant plant viruses bearing transgenes embedded within their own genomes can be inoculated onto wild-type hosts and the plants cultivated until titers of recombinant proteins reach a suitable maximum. Using such strategies, cowpea mosaic viruses have been used to produce vaccine proteins in plants (Giddings *et al.,* 2000), and tobacco mosaic viruses have been used to produce a whole range of recombinant proteins (Kumagai *et al.,* 1993; Lindbo *et al.,* 2001) some of which have progressed to clinical testing. Not to be overlooked, though, is the possibility of using such versatile viral vectors for testing transient gene expression and the high-throughput probing of the metabolic controls that operate in plant cells.

CONCLUSION

The variety of permutations that have been used to introduce and control gene expression in transgenic plants is truly dizzying. Metabolic engineers must not only understand the fundamental physiology of the process to be impacted, but also the level, timing, subcellular location, and tissue or organ specificity that will be required from a transgene to ensure successful manipulation of that physiology. Gene expression can be modulated by numerous transcriptional and posttranscriptional processes. Correctly choreographing these many variables is the challenge that makes metabolic engineering in plants so exciting.

ACKNOWLEDGMENT

The authors acknowledge the support of the Malaysia–MIT Biotechnology Partnership Programme.

REFERENCES

Ali, S., and Taylor, W. C. (2001). The 3′ non-coding region of a C4 photosynthesis gene increases transgene expression when combined with heterologous promoters. *Plant Mol. Biol.* **46**, 325–333.

An, Y. Q., Huang, S., McDowell, J. M., McKinney, E. C., and Meagher, R. B. (1996a). Conserved expression of the *Arabidopsis* ACT1 and ACT 3 actin subclass in organ primordia and mature pollen. *Plant Cell* **8**, 15–30.

An, Y. Q., McDowell, J. M., Huang, S., McKinney, E. C., Chambliss, S., and Meagher, R. B. (1996b). Strong, constitutive expression of the *Arabidopsis* ACT2/ACT8 actin subclass in vegetative tissues. *Plant J.* **10**, 107–121.

Aoyama, T., and Chua, N. H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* **11,** 605–612.

Batard, Y., Hehn, A., Nedelkina, S., Schalk, M., Pallett, K., Schaller, H., and Werck-Reichhart, D. (2000). Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems. *Arch. Biochem. Biophys.* **379**, 161–169.

Bate, N., and Twell, D. (1998). Functional architecture of a late pollen promoter: Pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant Mol. Biol.* **37**, 859–869.

Becker, D., Brettschneider, R., and Lorz, H. (1994). Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *Plant J.* **5**, 299–307.

Metabolic Engineering **4**, 67–79 (2002)
doi:10.1006/mben.2001.0210

Beetham, P. R., Kipp, P. B., Sawycky, X. L., Arntzen, C. J., and May, G. D. (1999). A tool for functional plant genomics: Chimeric RNA/DNA oligonucleotides cause *in vivo* gene-specific mutations. *Proc. Natl. Acad. Sci. USA* **96**, 8774–8778.

Benfey, P. N., Ren, L., and Chua, N. H. (1990). Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. *EMBO J.* **9**, 1677–1684.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* **12**, 8711–8721.

Bourdon, V., Harvey, A., and Lonsdale, D. M. (2001). Introns and their positions affect the translational activity of mRNA in plant cells. *EMBO Rep.* **2,** 394–398.

Burgess, J. (1985). "An Introduction to Plant Cell Development," Cambridge Univ. Press, New York.

Caddick, M. X., Greenland, A. J., Jepson, I., Krause, K. P., Qu, N., Riddell, K. V., Salter, M. G., Schuch, W., Sonnewald, U., and Tomsett, A. B. (1998). An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nat. Biotechnol.* **16**, 177–180.

Callis, J., Fromm, M., and Walbot, V. (1987). Introns increase gene expression in cultured maize cells. *Genes Dev.* **1**, 1183–1200.

Carthew, R. W. (2001). Gene silencing by double-stranded RNA. *Curr. Opin. Cell Biol.* **13**, 244–248.

Chaubet-Gigot, N., Kapros, T., Flenet, M., Kahn, K., Gigot, C., and Waterborg, J. H. (2001). Tissue-dependent enhancement of transgene expression by introns of replacement histone H3 genes of *Arabidopsis*. *Plant Mol. Biol.* **45**, 17–30.

Chen, Z. L., Schuler, M. A., and Beachy, R. N. (1986). Functional analysis of regulatory elements in a plant embryo-specific gene. *Proc. Natl. Acad. Sci. USA* **83**, 8560–8564.

Chiurazzi, M., Ray, A., Viret, J. F., Perera, R., Wang, X. H., Lloyd, A. M., and Signer, E. R. (1996). Enhancement of somatic intrachromosomal homologous recombination in *Arabidopsis* by the HO endonuclease. *Plant Cell* **8**, 2057–2066.

Christensen, A. H., and Quail, P. H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* **5**, 213–218.

Dai, S. H., Zheng, P., Marmey, P., Zhang, S. P., Tian, W. Z., Chen, S. Y., Beachy, R. N., and Fauquet, C. (2001). Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Mol. Breeding* **7**, 25–33.

Dale, E. C., and Ow, D. W. (1991). Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* **88**, 10558–10562.

Daniell, H., Vivekananda, J., Nielsen, B. L., Ye, G. N., Tewari, K. K., and Sanford, J. C. (1990). Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc. Natl. Acad. Sci. USA* **87**, 88–92.

de Boer, G. J., Testerink, C., Pielage, G., Nijkamp, H. J. J., and Stuitje, A. R. (1999). Sequences surrounding the transcription initiation site of the *Arabidopsis* enoyl-acyl carrier protein reductase gene control seed expression in transgenic tobacco. *Plant Mol. Biol.* **39**, 1197–1207.

De Veylder, L., Van Montagu, M., and Inze, D. (1997). Herbicide safener-inducible gene expression in *Arabidopsis thaliana*. *Plant Cell Physiol.* **38**, 568–577.

DeBlock, M., and DeBrouwer, D. (1991). 2 T-DNAs co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. *Theor. Appl. Genet.* **82**, 257–263.

Deyholos, M. K., and Sieburth, L. E. (2000). Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. *Plant Cell* **12**, 1799–1810.

Ebinuma, H., Sugita, K., Matsunaga, E., and Yamakado, M. (1997). Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proc. Natl. Acad. Sci. USA* **94**, 2117–2121.

Frey, A. D., Rimann, M., Bailey, J. E., Kallio, P. T., Thompson, C. J., and Fussenegger, M. (2001). Novel pristinamycin-responsive expression systems for plant cells. *Biotechnol. Bioeng.* **74**, 154–163.

Fu, H., Kim, S. Y., and Park, W. D. (1995). High-level tuber expression and sucrose inducibility of a potato Sus4 sucrose synthase gene require 5′ and 3′ flanking sequences and the leader intron. *Plant Cell* **7**, 1387–1394.

Gallie, D. R. (1998). Controlling gene expression in transgenics. *Curr. Opin. Plant Biol.* **1**, 166–172.

Giddings, G., Allison, G., Brooks, D., and Carter, A. (2000). Transgenic plants as factories for biopharmaceuticals. *Nat. Biotechnol.* **18**, 1151–1155.

Gleave, A. P. (1992). A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203–1207.

Goodall, G. J., and Filipowicz, W. (1991). Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. *EMBO J.* **10**, 2635–2644.

Gossele, V., van Aarssen, R., and Cornelissen, M. (1994). A 6′ gentamicin acetyltransferase gene allows effective selection of tobacco transformants using kanamycin as a substrate. *Plant Mol. Biol.* **26**, 2009–2012.

Hajdukiewicz, P. T., Gilbertson, L., and Staub, J. M. (2001). Multiple pathways for Cre/*lox*-mediated recombination in plastids. *Plant J.* **27**, 161–170.

Haldrup, A., Petersen, S. G., and Okkels, F. T. (1998). The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. *Plant Mol. Biol.* **37**, 287–296.

Hansen, G. (2000). Evidence for *Agrobacterium*-induced apoptosis in maize cells. *Mol. Plant–Microbe Interact.* **13**, 649–657.

Hansen, G., and Chilton, M. D. (1996). "Agrolistic" transformation of plant cells: Integration of T-strands generated *in planta*. *Proc. Natl. Acad. Sci. USA* **93**, 14978–14983.

Haran, S., Logendra, S., Seskar, M., Bratanova, M., and Raskin, I. (2000). Characterization of *Arabidopsis* acid phosphatase promoter and regulation of acid phosphatase expression. *Plant Physiol.* **124**, 615–626.

Heifetz, P. B. (2000). Genetic engineering of the chloroplast. *Biochimie* **82**, 655–666.

Hernalsteens, J. P., Thiatoong, L., Schell, J., and VanMontagu, M. (1984). An *Agrobacterium* transformed cell-culture from the monocot *Asparagus officinalis*. *EMBO J.* **3**, 3039–3041.

Iannacone, R., Grieco, P. D., and Cellini, F. (1997). Specific sequence modifications of a cry3B endotoxin gene result in high levels of expression and insect resistance. *Plant Mol. Biol.* **34**, 485–496.

Ingelbrecht, I. L., Herman, L. M., Dekeyser, R. A., Van Montagu, M. C., and Depicker, A. G. (1989). Different 3′ end regions strongly influence the level of gene expression in plant cells. *Plant Cell* **1**, 671–680.

Jeon, J. S., Lee, S., Jung, K. H., Jun, S. H., Kim, C., and An, G. (2000). Tissue-preferential expression of a rice alpha-tubulin gene, OsTubA1, mediated by the first intron. *Plant Physiol.* **123**, 1005–1014.

Keegstra, K. (1989). Transport and routing of proteins into chloroplasts. *Cell* **56,** 247–53.

Klein, T. M., Wolf, E. D., Wu, R., and Sanford, J. C. (1987). High-velocity microprojectiles for delivering nucleic-acids into living cells. *Nature* **327**, 70–73.

Koosha, F., Muller, R. H., and Davis, S. S. (1989). Polyhydroxybutyrate as a drug carrier. *Crit. Rev. Ther. Drug Carrier Syst.* **6**, 117–130.

Kooter, J. M., Matzke, M. A., and Meyer, P. (1999). Listening to the silent genes: Transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.* **4,** 340–347.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292.

Koziel, M. G., Beland, G. L., Bowman, C., Carozzi, N. B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M. R., Merlin, E., Rhodes, R., Warren, G. W., Wright, M., and Evola, S. V. (1983). Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio-Technology* **11**, 194–200.

Koziel, M. G., Carozzi, N. B., and Desai, N. (1996). Optimizing expression of transgenes with an emphasis on post-transcriptional events. *Plant Mol. Biol.* **32**, 393–405.

Kumagai, M. H., Turpen, T. H., Weinzettl, N., Dellacioppa, G., Turpen, A. M., Donson, J., Hilf, M. E., Grantham, G. L., Dawson, W. O., Chow, T. P., Piatak, M., and Grill, L. K. (1993). Rapid, high-level expression of biologically-active alpha-trichosanthin in transfected plants by an RNA viral vector. *Proc. Natl. Acad. Sci. USA* **90**, 427–430.

Kunkel, T., Niu, Q. W., Chan, Y. S., and Chua, N. H. (1999). Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. *Nat. Biotechnol.* **17**, 916–919.

Lee, Y. T., Chang, A. K., and Duggleby, R. G. (1999). Effect of mutagenesis at serine 653 of *Arabidopsis thaliana* acetohydroxyacid synthase on the sensitivity to imidazolinone and sulfonylurea herbicides. *FEBS Lett.* **452**, 341–345.

Lindbo, J. A., Fitzmaurice, W. P., and della-Cioppa, G. (2001). Virus-mediated reprogramming of gene expression in plants. *Curr. Opin. Plant Biol.* **4**, 181–185.

Liu, X. J., Prat, S., Willmitzer, L., and Frommer, W. B. (1990). cis regulatory elements directing tuber-specific and sucrose-inducible expression of a chimeric class I patatin promoter/GUS-gene fusion. *Mol. Gen. Genet.* **223**, 401–406.

Lutcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987). Selection of AUG initiation codons differs in plants and animals. *EMBO J.* **6**, 43–48.

Maliga, P. (2001). Plastid engineering bears fruit. *Nat. Biotechnol.* **19**, 826–827.

Maliga, P., Svab, Z., Harper, E. C., and Jones, J. D. (1988). Improved expression of streptomycin resistance in plants due to a deletion in the streptomycin phosphotransferase coding sequence. *Mol. Gen. Genet.* **214**, 456–459.

Martinez, A., Sparks, C., Hart, C. A., Thompson, J., and Jepson, I. (1999). Ecdysone agonist inducible transcription in transgenic tobacco plants. *Plant J.* **19**, 97–106.

Mascarenhas, D., Mettler, I. J., Pierce, D. A., and Lowe, H. W. (1990). Intron-mediated enhancement of heterologous gene expression in maize. *Plant Mol. Biol.* **15**, 913–920.

McElroy, D., Blowers, A. D., Jenes, B., and Wu, R. (1991). Construction of expression vectors based on the rice actin 1 (Act1) 5′ region for use in monocot transformation. *Mol. Gen. Genet.* **231**, 150–160.

Miller, W. A., Waterhouse, P. M., Brown, J. W. S., and Browning, K. S. (2001). The RNA world in plants: Post-transcriptional control. III. *Plant Cell* **13**, 1710–1717.

Mittendorf, V. V., Robertson, E. J., Leech, R. M., Kruger, N., Steinbüchel, A., and Poirier, Y. (1998). Synthesis of medium-chain-length polyhydroxyalkanoates in *Arabidopsis thaliana* using intermediates of peroxisomal fatty acid beta-oxidation. *Proc. Natl. Acad. Sci. USA* **95**, 13397–13402.

Molnar, A., Lovas, A., Banfalvi, Z., Lakatos, L., Polgar, Z., and Horvath, S. (2001). Tissue-specific signal(s) activate the promoter of a metallocarboxypeptidase inhibitor gene family in potato tuber and berry. *Plant Mol. Biol.* **46**, 301–311.

Murray, E. E., Lotzer, J., and Eberle, M. (1989). Codon usage in plant genes. *Nucleic Acids Res.* **17**, 477–498.

Nap, J. P., Bijvoet, J., and Stiekema, W. J. (1992). Biosafety of kanamycin-resistant transgenic plants. *Transgenic Res.* **1**, 239–249.

Napoli, C., Lemieux, C., and Jorgensen, R. A. (1990). Introduction of a chimeric chalcone synthase gene in petunia results in reversible cosuppression of homologous genes *in trans*. *Plant Cell* **2**, 279–289.

Nawrath, C., Poirier, Y., and Somerville, C. (1994a). Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation. *Proc. Natl. Acad. Sci. USA* **91**, 12760–12764.

Nawrath, C., Poirier, Y., and Somerville, C. R. (1994b). Plastid targeting of the enzymes required for the production of polyhydroxybutyrate in higher plants. *In* "Biodegradable Plastics and Polymers: Proceedings of the Third International Scientific Workshop on Biodegradable Plastics and Polymers, Osaka, Japan, November 9–11, 1993" (Y. Doi and K. Fukuda, Eds.), pp. 136–149, Elsevier, New York.

Newell, C. A. (2000). Plant transformation technology. Developments and applications. *Mol. Biotechnol.* **16**, 53–65.

Ohtsubo, N., and Iwabuchi, M. (1994). The conserved 3′-flanking sequence, AATGGAAATG, of the wheat histone H3 gene is necessary for the accurate 3′-end formation of mRNA. *Nucleic Acids Res.* **22**, 1052–1058.

Olsen, L. J., Ettinger, W. F., Damsz, B., Matsudaira, K., Webb, M. A., and Harada, J. J. (1993). Targeting of glyoxysomal proteins to peroxisomes in leaves and roots of a higher plant. *Plant Cell* **5**, 941–952.

Parmenter, D. L., Boothe, J. G., van Rooijen, G. J., Yeung, E. C., and Moloney, M. M. (1995). Production of biologically active hirudin in plant seeds using oleosin partitioning. *Plant Mol. Biol.* **29**, 1167–1180.

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T., and Fischhoff, D. A. (1990). Insect resistant cotton plants. *Biotechnology (New York)* **8**, 939–943.

Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L., and Fischhoff, D. A. (1991). Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc. Natl. Acad. Sci. USA* **88**, 3324–3328.

Pietrzak, M., Shillito, R. D., Hohn, T., and Potrykus, I. (1986). Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. *Nucleic Acids Res.* **14**, 5857–5868.

Plesse, B., Criqui, M. C., Durr, A., Parmentier, Y., Fleck, J., and Genschik, P. (2001). Effects of the polyubiquitin gene Ubi U4 leader intron and first ubiquitin monomer on reporter gene expression in *Nicotiana tabacum*. *Plant Mol. Biol.* **45**, 655–667.

Rathus, C., Bower, R., and Birch, R. G. (1993). Effects of promoter, intron and enhancer elements on transient gene expression in sugar-cane and carrot protoplasts. *Plant Mol. Biol.* **23**, 613–618.

Rogers, S. G., Brand, L. A., Holder, S. B., Sharps, E. S., and Brackin, M. J. (1983). Amplification of the *aroA* gene from *Escherichia coli* results in tolerance to the herbicide glyphosate. *Appl. Environ. Microbiol.* **46**, 37–43.

Rossi, V., Motto, M., and Pellegrini, L. (1997). Analysis of the methylation pattern of the maize opaque-2 (O2) promoter and *in vitro* binding studies indicate that the O2 B-Zip protein and other endosperm factors can bind to methylated target sequences. *J. Biol. Chem.* **272**, 13758–13765.

Rouwendal, G. J. A., Mendes, O., Wolbert, E. J. H., and deBoer, A. D. (1997). Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. *Plant Mol. Biol.* **33**, 989–999.

Ruf, S., Hermann, M., Berger, I. J., Carrer, H., and Bock, R. (2001). Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat. Biotechnol.* **19**, 870–875.

Russell, D. A., and Fromm, M. E. (1997). Tissue-specific expression in transgenic maize of four endosperm promoters from maize and rice. *Transgenic Res.* **6**, 157–168.

Metabolic Engineering **4**, 67–79 (2002)
doi:10.1006/mben.2001.0210

Sawant, S. V., Kiran, K., Singh, P. K., and Tuli, R. (2001). Sequence architecture downstream of the initiator codon enhances gene expression and protein stability in plants. *Plant Physiol.* **126**, 1630–1636.

Schafer, W., Gorz, A., and Kahl, G. (1987). T-DNA integration and expression in a monocot crop plant after induction of *Agrobacterium*. *Nature* **327**, 529–532.

Shinabarger, D. L., and Braymer, H. D. (1986). Glyphosate catabolism by *Pseudomonas* sp. strain PG2982. *J. Bacteriol.* **168**, 702–707.

Sorokin, A. P., Ke, X. Y., Chen, D. F., and Elliott, M. C. (2000). Production of fertile transgenic wheat plants via tissue electroporation. *Plant Sci.* **156**, 227–233.

Spencer, T. M., O'Brien, J. V., Start, W. G., Adams, T. R., Gordon-Kamm, W. J., and Lemaux, P. G. (1992). Segregation of transgenes in maize. *Plant Mol. Biol.* **18**, 201–210.

Spiker, S., and Thompson, W. F. (1996). Nuclear matrix attachment regions and transgene expression in plants. *Plant Physiol.* **110**, 15–21.

Stalker, D. M., Hiatt, W. R., and Comai, L. (1985). A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. *J. Biol. Chem.* **260**, 4724–4728.

Tague, B. W. (2001). Germ-line transformation of *Arabidopsis lasiocarpa*. *Transgenic Res.* **10**, 259–267.

Tamura, K., Kimura, M., and Yamaguchi, I. (1995). Blasticidin S deaminase gene (BSD): A new selection marker gene for transformation of *Arabidopsis thaliana* and *Nicotiana tabacum*. *Biosci. Biotechnol. Biochem.* **59**, 2336–2338.

Teasdale, R. D., and Jackson, M. R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* **12**, 27–54.

Terzaghi, W. B., and Cashmore, A. R. (1997). Plant cell transfection by electroporation. *Methods Mol. Biol.* **62**, 453–462.

van Bel, A. J., Hibberd, J., Prufer, D., and Knoblauch, M. (2001). Novel approach in plastid transformation. *Curr. Opin. Biotechnol.* **12**, 144–149.

Van Haaren, M. J., and Houck, C. M. (1993). A functional map of the fruit-specific promoter of the tomato 2A11 gene. *Plant Mol. Biol.* **21**, 625–640.

Vasil, V., Clancy, M., Ferl, R. J., Vasil, I. K., and Hannah, L. C. (1989). Increased gene-expression by the first intron of maize shrunken-1 locus in grass species. *Plant Physiol.* **91**, 1575–1579.

Wang, X. H. W., Viret, J. F., Eldridge, A., Perera, R., Signer, E. R., and Chiurazzi, M. (2001). Positive-negative selection for homologous recombination in *Arabidopsis*. *Gene* **272**, 249–255.

Wanggen, Z., Subbarao, S., Rince, A., Shen, A., Armstrong, C., Peschke, V., and Gilbertson, L. (2001). Cre/*lox* mediated marker gene excision in transgenic corn plants. *In* "Plant Biology 2001," Am. Soc. Plant Physiol., Providence, RI.

Waterhouse, P. M., Wang, M., and Finnegan, E. J. (2001a). Role of short RNAs in gene silencing. *Trends Plant Sci.* **6**, 297–301.

Waterhouse, P. M., Wang, M. B., and Lough, T. (2001b). Gene silencing as an adaptive defence against viruses. *Nature* **411**, 834–842.

Widholm, J. M., Chinnala, A. R., Ryu, J. H., Song, H. S., Eggett, T., and Brotherton, J. E. (2001). Glyphosate selection of gene amplification in suspension cultures of 3 plant species. *Physiol. Plant* **112**, 540–545.

Ye, G. N., Daniell, H., and Sanford, J. C. (1990). Optimization of delivery of foreign DNA into higher-plant chloroplasts. *Plant Mol. Biol.* **15**, 809–819.

Yoder, J. I., and Goldsbrough, A. P. (1994). Transformation systems for generating marker-free transgenic plants. *Bio-Technology* **12**, 263–267.

Zhang, P., Potrykus, I., and Puonti-Kaerlas, J. (2000). Efficient production of transgenic cassava using negative and positive selection. *Transgenic Res.* **9**, 405–415.

Zuo, J., and Chua, N. H. (2000). Chemical-inducible systems for regulated expression of plant genes. *Curr. Opin. Biotechnol.* **11**, 146–151.

Zupan, J., Muth, T. R., Draper, O., and Zambryski, P. (2000). The transfer of DNA from *Agrobacterium tumefaciens* into plants: A feast of fundamental insights. *Plant J.* **23**, 11–28.