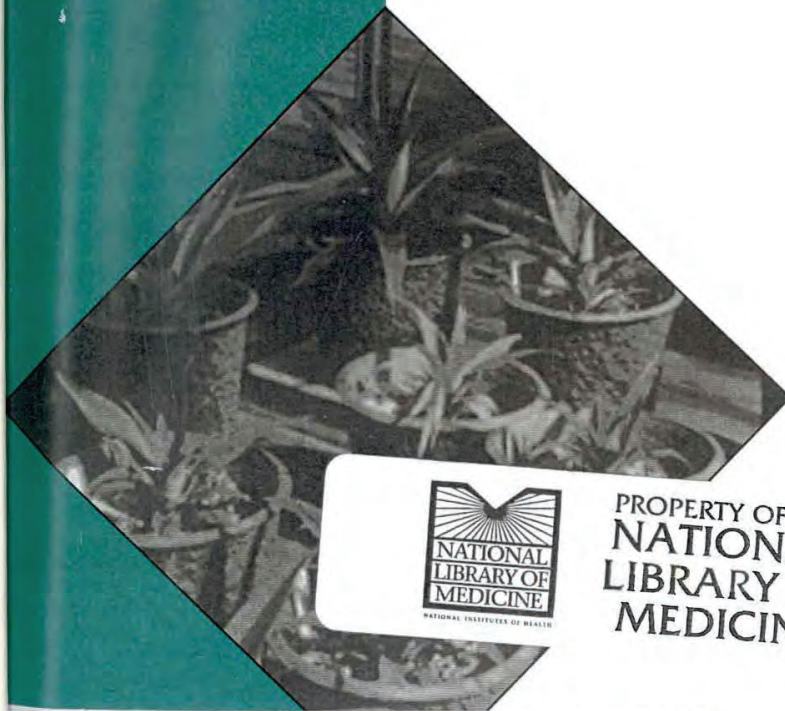


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In Vitro Cellular & Developmental Biology— Plant publishes peer-reviewed original research and reviews concerned with the latest developments and state-of-the-art research in plant cell and tissue culture and biotechnology from around the globe. Four issues cover cellular, molecular and developmental biology research using *in vitro* grown or maintained organs, tissues or cells derived from plants. Two special IAPTC&B issues deal with plant tissue culture, and molecular and cellular aspects of plant biotechnology. The IAPTC&B and SIVB maintain completely separate and independent International Editorial Review boards for their issues. From the start of the 2000 volume *In Vitro Cellular and Developmental Biology — Plant* will be available in print and on the Internet in Acrobat PDF and HTML formats.

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INVITED REVIEW: TARGETING TRANSGENE EXPRESSION IN RESEARCH, AGRICULTURAL, AND ENVIRONMENTAL APPLICATIONS: PROMOTERS USED IN PLANT TRANSFORMATION

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SUMMARY

Plant genetic engineering has contributed substantially to the understanding of gene regulation and plant development, in the generation of transgenic organisms for widespread usage in agriculture, and has increased the potential uses of crops for industrial and pharmaceutical purposes. As the application of genetically engineered plants has widened, so has the need to develop methods to fine-tune control of transgene expression. The availability of a broad spectrum of promoters that differ in their ability to regulate the temporal and spatial expression patterns of the transgene can dramatically increase the successful application of transgenic technology. Indeed, a variety of promoters is necessary at all levels of genetic engineering in plants, from basic research discoveries, concepts and questions, to development of economically viable crops and plant commodities, to addressing legitimate concerns raised about the safety and containment of transgenic plants in the environment. This review covers the characterization and usage of a broad range of promoters employed in plant genetic engineering, including the widespread use of plant promoters with viral and plant origin that drive constitutive expression. Also covered are selected tissue-specific promoters from fruit, seed and grain, tubers, flowers, pistils, anther and pollen, roots and root nodules, and leaves and green tissue. Topics also include organellar promoters, and those found in specific cell types, as well as the development and evaluation of inducible (endogenous and exogenous origin) and synthetic plant promoter systems. Discussions on the relevance and potential pitfalls within specific applications are included.

Key words: transgenic plants; tissue-specific promoters; genetic engineering; constitutive promoters; inducible promoters.

INTRODUCTION

Plant genetic transformation is an incredibly powerful application used to study gene expression in plants. It has contributed to the understanding of gene regulation and plant development. It has allowed for the manipulation and analysis of biochemical processes, and permitted integration and study of genes that cannot be manipulated by classical breeding. It has led to an explosion of value-added crops that have the potential to increase food security in developing countries and create pharmaceutical and nutraceutical products for the improvement of human health worldwide. Despite the prospective socioeconomic and environmental benefits from this technology, plant genetic engineering has also become one of the most divisive topics in agriculture. Scientific peers and a concerned public have raised legitimate questions and challenges about the safety of genetically engineered plants to both the consumer and the environment (Eaglesham et al., 2001; Nelson, 2001). It has also become increasingly clear that to minimize potential adverse effects of transgenically engineered plants on

beneficial or nontargeted organisms, the appropriate level of gene expression must be established in the appropriate parts of the plant, both spatially and temporally. The promoters that drive transgene expression can be a part of the answer to questions about the safety of transgenic organisms. The benefits of directing gene expression in a temporal, spatial, and even in a deliberately controlled manner has been understood by researchers studying regulation and development in transgenic organisms for a number of years, as confirmed by the diversity of promoters developed. This review specifically focuses on targeting plant transgene expression using an array of gene promoters, from viral and plant constitutive promoters, to tissue- and cell-specific promoters, to inducible and synthetic promoters. Whether they are used in a primary research setting, or in the development of plant products for an agricultural or industrial setting, promoter selection has become increasingly important for successful gene transfer and expression of transgenes in plants (Dale et al., 2002).

Regulation can occur at many different stages of gene expression, and can be particularly important during transcription. The promoters that drive transgene expression ensure this control. Promoters are regions of the DNA upstream of a gene's coding region that contain specific sequences recognized by proteins involved in the initiation of transcription (Buchanan et al., 2000).

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For most genes encoding proteins, initiation of transcription includes the binding and activation of RNA polymerase II. Variability in gene expression occurs when other diverse, semiconserved sequence elements are present within regulatory regions of the gene, generally upstream or 5' of the RNA polymerase binding site. These elements bind protein factors that are involved in controlling the level and pattern of a gene's expression. The availability of a broad spectrum of promoters that differ in their ability to regulate the temporal and spatial expression patterns of the transgene can dramatically increase the successful application of transgenic technology.

Over the years, numerous promoters have been isolated from a wide variety of organisms and applied to plant genetic engineering systems. Because promoters affect transcription both quantitatively and qualitatively, the success of gene transfer technologies, varying from basic research to crop improvement to biopharming, depends on their efficacious selection and use. Transgene expression and patterns can be beneficially adjusted by using a promoter suitable for the plant's background and for the type of transgene. For example, the presence of multiple transgenes driven by the same constitutive promoter in a single plant (Beyer et al., 2002; Galili and Hofgen, 2002; Lessard et al., 2002) may result in homology-dependent gene silencing, especially when the promoter is also highly active (Elmayan and Vaucheret, 1996). Variation in the promoters selected to drive these transgenes might reduce gene silencing concerns. Localized and targeted gene expression may also be necessary to satisfy regulations regarding food safety, such as potential allergenicity effects resulting in increased health risk to consumers (Kaiser, 2000; Taylor and Heffe, 2002). Promoters that target transgene expression can be used to avoid potential transgene genetic drift through pollen (Scott and Wilkinson, 1999; Daniell, 2002), or address the hypothetical risk of viral recombination events between host species' DNA and viral promoters heavily utilized in plant genetic engineering (Vaden and Melcher, 1990; Ho et al., 1999; Kohli et al., 1999; Morel and Tepfer, 2000). These problems are all oft-stated concerns of opponents of genetically enhanced crops. Similarly, triggered induction of transgenes at times when expression is needed (induction by pathogens or insects – see Cao et al., 1998) or desired (study of transgene toxicity) is useful both in research and commercial settings. Therefore, the isolation and characterization of promoters suitable for plant genetic engineering is highly desirable.

GENERAL DESCRIPTION OF PROMOTERS

There have been intensive efforts to characterize the molecular structure of genomic elements involved in transcriptional regulation of gene expression because of their importance during initiation of transcription. Initiation of transcription involves the initial interaction of RNA polymerase II with various sequence elements within the genomic DNA. RNA polymerase II binding ultimately allows for the further recruitment of numerous proteins (transcription factors) that facilitate transcription of the protein-coding region of the gene. The binding regions for transcription factors are located primarily upstream or 5' of the coding region of a gene, and the DNA sequence that includes these binding regions is generally referred to as the promoter. Promoters are a set of transcription control modules clustered around the initiation site of RNA polymerase II (Russell, 1996). They are important in the control of

the overall expression profile of a gene, either driving or preventing transcription at appropriate times and places. Generally, the basal or core promoter is located about 40 base pairs upstream of the start of transcription, and the upstream promoter region may extend as many as 200 base pairs farther upstream. Although initiation of transcription is dependent on sequences found in the core and upstream promoter region, many other DNA sequence motifs, which occur within the surrounding DNA, are also involved in the regulation of gene expression.

Indeed, much of the fine-tuning of gene expression is controlled by sequence-targeted transcription factors or enhancer-binding proteins (Alberts et al., 2002). Enhancer-binding proteins bind to sequence motifs or *cis*-elements within the promoter of a gene. These *cis*-elements are also called regulatory elements, enhancer elements or enhancers. Enhancer elements can be generally defined as imperfectly conserved DNA sequence modules, varying in size and showing sequence degeneracy that can make it difficult to recognize them in comparative analyses. A large assortment of enhancers has been discovered using promoter deletion studies, DNA foot-printing, and site-specific sequence mutation. The activities of some well-studied plant enhancers are associated with tissue-specific expression, stress or pathogen-related expression, regulation by light, or by the presence of specific cellular substrates (Table 1).

Enhancers can be located hundreds or thousands of base pairs away from the gene they control. They can be found upstream of the coding region, downstream of the coding region, within coding or intronic sequences, and even in regions of the chromosome structurally adjacent to the promoter. Enhancer elements are also orientation-independent. While many of the well-studied enhancer elements are required for maximal transcriptional activation of a gene, a few *cis*-elements, referred to as silencers, have the ability to repress gene expression. Otherwise, these silencer elements have very similar properties to those of enhancer elements. One accepted model for the mechanism of enhancer-mediated activation is that activation occurs after binding of their specific regulatory proteins. DNA loops form after protein binding such that regulatory proteins interact with transcription factors bound to promoter elements (Fig. 1). Through the interaction of all the proteins, gene transcription is either activated or repressed (Alberts et al., 2002).

The complexity of specific gene regulation at the level of transcription is therefore dependent on at least two distinct factors. The first is the type, number, position, and combination of enhancers and silencers present in and around the coding sequence of a particular gene. The second is the activation of the enhancer sequences, which is dependent on the presence, absence, or activity within the cell of their associated binding proteins.

PROMOTERS USED IN PLANT TRANSFORMATION

The choice of the promoter used within a transgene construct is fundamentally dependent on the objectives of the engineering project. There are a large number of well-characterized promoters in the literature and a select number are presented in this review (Fig. 2). While different classes of promoters can confer tissue-specific or temporal expression, plant promoters that drive high, constitutive expression have become a valuable tool in plant genetic engineering.

TABLE 1
EXAMPLES OF SEVERAL PLANT PROMOTER ELEMENTS THAT CONFER DIFFERENT ENHANCED GENE EXPRESSIONS

Organism/Element	Gene/Accession no.	Sequence ID	Method	Function	Reference
<i>Arabidopsis thaliana</i> AT ~ as1	rd29B and rd29A D13044	TCACGTC	Sequence homology to activator sequence (<i>as1</i>) ^b	<i>cis</i> -Acting regulatory element involved in root-specific expression	Yamaguchi-Shinozaki and Shinozaki, 1994
<i>Nicotiana tabacum</i> NT ~ TC-rich repeats	Anionic peroxidase L02124	ATTCCTCAACATTTTCCTT CAGTTTCCTTACATTTCTCCCA	<i>Agrobacterium</i> -mediated transformation; promoter deletions	<i>cis</i> -Acting element involved in defense and stress responsiveness	Klotz and Lagermini, 1996
<i>Peroledinium crispum</i> PC ~ Box-W1	Pathogenesis-related protein PR1-2, U48963	TTGACC	Gel mobility shift assays; promoter deletions and mutations	Fungal elicitor responsive element WRKY1 (U48931) protein binding site	Rushion et al., 1996
<i>Pisum sativum</i> PS ~ Box II	Ribulose-1,5-bisphosphate carboxylase W21356	GTCAGCTAATAT ^a	Gel retardation; DNase I footprinting; methylation interference	Involved in light responsiveness	Green et al., 1987
<i>Lycopersicon esculentum</i> LE ~ circadian	Leghemoglobin Lbc b1-1 M14445	CAANNNNATC	<i>Agrobacterium</i> -mediated transformation; promoter deletions	<i>cis</i> -Acting regulatory element involved in circadian control	Piechulla et al., 1998
<i>Arabidopsis thaliana</i> GATA-motif	Ferredoxin Fd-2 X51370	AAGATAAGATT ^a	Sequence alignment with other Fd genes ^b	Part of a light-responsive element	Arguello-Astorga and Herrera-Estrella, 1996
<i>Zea mays</i> DNA-binding with one finger	C4-type phosphoenolpyruvate carboxylase X14579	(T/A)AAAG	Direct gel shift	Associated with carbon metabolism in maize	Yanagisawa and Schmidt, 1999

The table was generated using PlantCARE (Lescot et al., 2002), Transfact (Wingender et al., 2000), and NCBI databases (Altschul et al., 1997). The promoter regulatory elements are classified by the organism from which they were isolated, the gene name and its accession number, the sequence identification (ID), the method of identification, function, and reference.

^a *cis*-Elements work synergistically with other promoter elements to confer a given function and appear to be loosely conserved from species to species.

^b Comparative analysis *in silico* is the initial step in identifying promoter elements, but is not as definitive as functional *in vivo* analysis.

PROMOTERS THAT DRIVE CONSTITUTIVE EXPRESSION: VIRAL ORIGIN

The backbone of proof-of-concept plant genetic engineering research has been the creation of transgenic plants that are generated simply to overexpress the gene and ultimately the protein of interest within the system. The most common promoters used for constitutive overexpression are derived from plant virus sources, such as the cauliflower mosaic (CaMV) 35S promoter (Odell et al., 1985). This promoter, like similar virally derived promoters used in plant systems, is harvested from double-stranded DNA viral genomes, which use host nuclear RNA polymerase and do not appear to depend on any *trans*-acting viral gene products. In particular, the CaMV 35S promoter is valuable not only because it delivers high expression in virtually all regions of the transgenic plant, but also because it is readily obtainable in research and academic settings, and available in plant transformation vector cassettes that allow for easy subcloning of the transgene of interest.

The CaMV 35S promoter can drive high levels of transgene expression in both dicots and monocots (Battraw and Hall, 1990; Benfey et al., 1990a). The original full-sized 35S promoter (−941 to +9 bp) (Odell et al., 1985) has no significant difference in activity when compared to a −343 bp fragment. Dissection of *cis*-acting elements within 343 bp upstream of the promoter suggests that interaction between elements results in the high constitutive expression seen in plants (Fang et al., 1989). However, individual elements have been found that are involved in tissue-specific expression (Benfey and Chua, 1989). Two particular regions designated 'Domain A' and 'Domain B' are important for control of gene expression in specific tissues. In Domain A (−90 to +8 bp), an activating sequence repeat motif at −82 to −64 bp binds a tobacco transcription factor, ASF-1, and is involved in root expression (Poulsen and Chua, 1988; Lam et al., 1989). Domain B (−343 to −90 bp) contains a conserved GATA motif located at −85 to −105 bp. This 35S GATA motif is similar to *cis*-elements found to be light responsive in plant light-inducible promoters, and interacts with a transcription factor from tobacco leaf tissue, ASF-2 (Lam and Chua, 1989). Interestingly, a duplication of the −343 to −90 bp fragment enhances transcriptional activity of the promoter by 10-fold (Kay et al., 1987). Limitations have been found, including results that show the CaMV 35S promoter is strongly down-regulated in plant parasitic nematode feeding sites (Goddijn et al., 1993; Urwin et al., 1997). However, it will continue to be one of the most common nonplant-derived promoters in use.

Because of the success of the CaMV 35S promoter, other viral promoters have been developed or are being developed for use. Many of these new virally derived promoters perform similarly or better than the CaMV 35S promoter, and drive high expression in both dicots and monocots. They include the cassava vein mosaic virus (CsVMV) promoter (Verdaguer et al., 1996, 1998; Li et al., 2001), Australian banana streak virus (BSV) promoters (Schenk et al., 2001), mirabilis mosaic virus (MMV) promoter (Dey and Maiti, 1999), and figwort mosaic virus (FMV) promoter (Sanger et al., 1990; Maiti et al., 1997).

There are a number of limitations in the use of virally derived promoters. Although there is little scientific data to support it, one is the perception of risk to human health with the use of transgenes made with genes of infective (albeit plant) viruses (Ho et al., 1999; Hodgson, 2000; Hull et al., 2000; Morel and Tepfer, 2000). Another appears to be the ability of a cell to recognize these sequences as

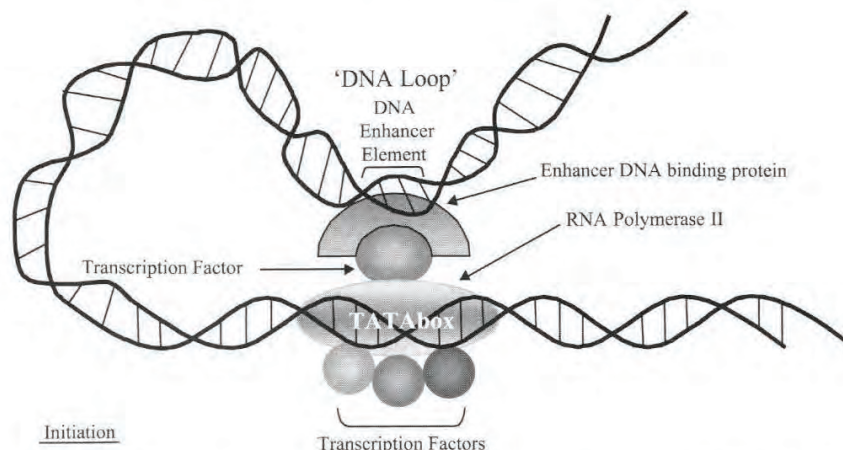


FIG. 1. Transcription initiation; enhancer mechanism model. During transcriptional initiation, transcription factors and RNA polymerase II bind to the core promoter DNA elements to initiate transcription (initiation complex). Enhancer DNA binding proteins, bound to enhancer elements within or near the initiation complex are believed to interact because of DNA 'looping' to bring the enhancer complex within close proximity of the initiation complex.

foreign and inactivate them (Elmayan and Vaucheret, 1996). Falling under the broad heading of 'transcriptional gene silencing' (TGS), mechanisms include methylation-dependent and methylation-independent inactivation of the transgene and promoter (Scheid et al., 2002), chromatin remodeling and excision of foreign DNA (reviewed in Kumpatla et al., 1998), and homologous or ectopic pairing when multiple copies of the transgene integrate into the genome (Meyer and Saedler, 1996). Transgene silencing is unpredictable, but when it occurs, it can lead to the stable shutdown of gene activity throughout the plant or affect the specificity of a promoter (Kloti et al., 2002). Silencing, however, may be less common when constitutive promoters of plant origin are used.

PROMOTERS THAT DRIVE CONSTITUTIVE EXPRESSION: PLANT ORIGIN

Endogenous plant promoters are used regularly to drive high constitutive levels of transgene expression (Gupta et al., 2001; Dhankher et al., 2002). A number of these strong constitutive promoters are derived from actin and ubiquitin genes. Actin is a fundamental cytoskeletal component that is expressed in nearly every plant cell. The *Act2* promoter was developed from the actin gene family in *Arabidopsis* (An et al., 1996). When transformed back into *Arabidopsis*, the 1.5 kb 5' upstream region of the *Act2* gene drives strong expression of a reporter gene in virtually all regions of the plant throughout development with the exception of the seed coat, the hypocotyls, the ovary, and the pollen sac. The rice actin 1 gene promoter has also been developed for use in cereal systems (McElroy et al., 1991; Zhang et al., 1991) and drives expression of β -glucuronidase (GUS) in virtually all tissues except xylem when transformed back into rice. In an applied use, Xu et al. (1996) reported that the 5' region of the rice actin gene (including the 1st intron) drives high levels of *HVA1* expression in both leaf and root tissue of rice. *HVA1* encodes for a late embryogenesis abundant (LEA) protein from barley, and confers salt and water deficit stress tolerance when overexpressed in rice.

Ubiquitin is one of the most highly conserved proteins known. It has been linked to many vital cellular processes including protein

turnover, chromatin structure, and DNA repair. It is highly abundant in the cytoplasm of most every cell in the plant. Some are constitutively expressed (Kawalleck et al., 1993) while others also respond to stress (Christensen et al., 1992; Cornejo et al., 1993). One of the most popular ubiquitin promoters used for transformation is the maize ubiquitin 1 promoter (pUbi). Initial studies showed that pUbi-1:CAT (chloramphenicol acetyltransferase) expression was 10-fold higher in maize protoplasts than that of 35S:CAT, but was 10-fold lower in tobacco protoplasts than 35S:CAT, limiting its potential utility to cereal monocots (Christensen et al., 1992; Weeks et al., 1993; Gupta et al., 2001). The maize *Ubi1* promoter:GUS fusion was used in rice to characterize expression and activity in the whole plant (Cornejo et al., 1993). In stably transformed rice plants, *Ubi1* promoter activity was highest in young roots, strong in lateral roots, and was also observed in root hairs. Activity decreased in older roots. In leaves, the stomata and the vascular system exhibited activity but again, activity decreased with aging. Decrease in activity was associated with lack of cellular division.

The *Ubi.U4* gene promoter (-263 bp 5' flanking) from *Nicotiana sylvestris* contains two putative *cis*-elements that appear to be critical for gene expression. Mutation in either significantly decreased transgene expression (Plesse et al., 2001). It was also shown previously that the presence of both the leader intron and ubiquitin gene as a translation fusion increased transgene expression dramatically (Garbarino et al., 1995). GUS activity in transgenic plants carrying the -263:GUS construct showed 3-fold higher activity when compared to the CaMV 35S:GUS construct in tissues where expression overlapped. Most organs contained tissue or cells expressing GUS, but the *Ubi.U4* promoter drove exceptionally high GUS expression in metabolically and mitotically active cells.

Constitutive expression can be problematic for several reasons. If a specific transgene is overexpressed at the wrong time in development, in tissues where it is not normally expressed, or at very high levels, it can have unexpected consequences on plant growth and development, and, potentially, the environment. For instance, the constitutive expression of signal-transduction

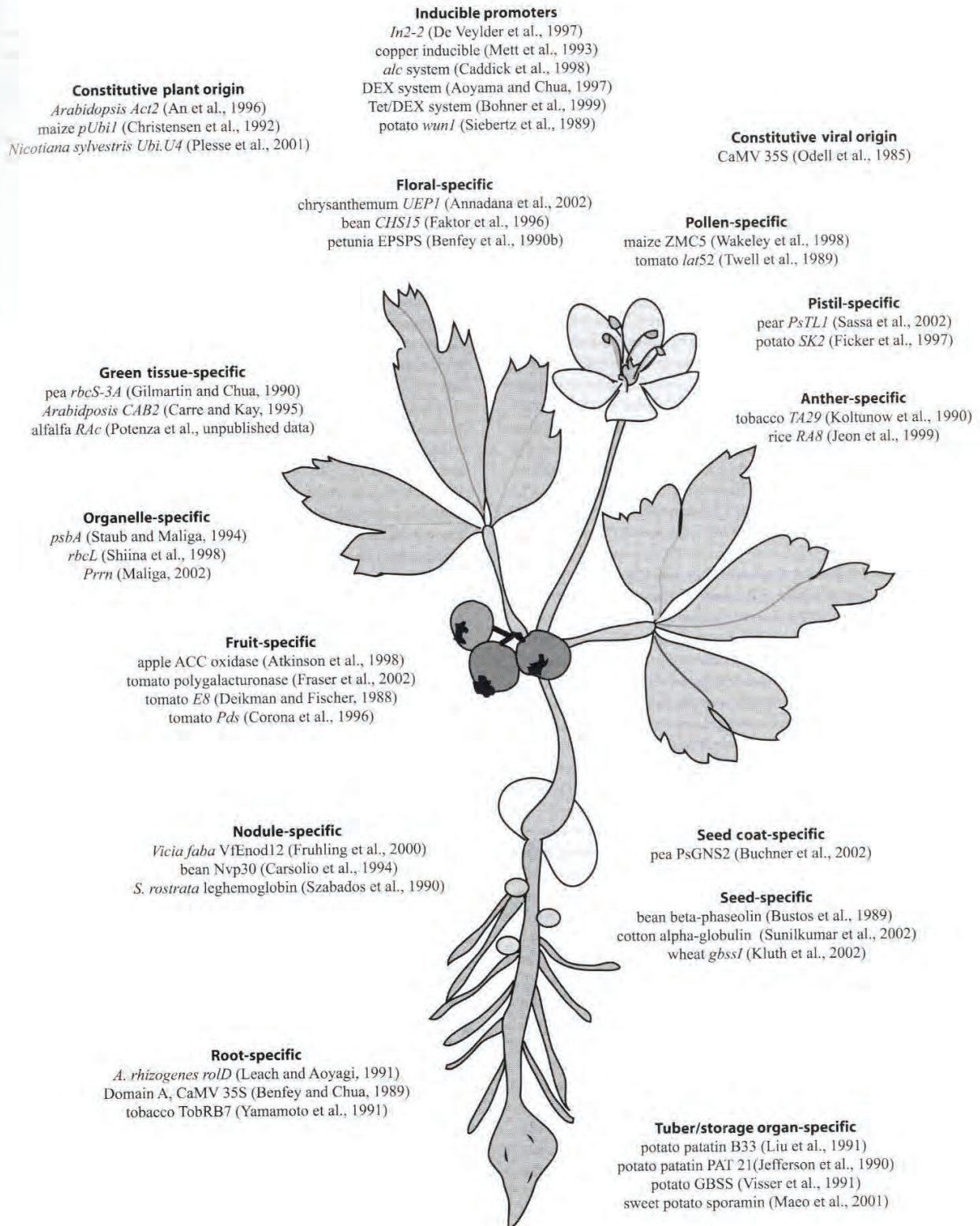


FIG. 2. Composite plant with specific promoters clustered near the relevant tissue.

'master-switches' for pathogen resistance can lead to decreased growth (Bowling et al., 1994, 1997), or enhanced susceptibility to other pathogens (Stuiver and Custers, 2001; Berrocal-Lobo et al., 2002). Concerns that constitutive overexpression of *Bacillus thuringiensis* insect toxins in commodity crop plants will increase targeted insect resistance (Huang et al., 1999) have led the Environmental Protection Agency (EPA) to announce rules for resistance management by planting refuges of conventional crops (http://www.epa.gov/osepmon/sap/2000/october/brad4_irm.pdf). Plant promoters that are activated precisely when and where they are needed would be ideal for genetic engineering strategies for enhanced disease resistance.

TISSUE-SPECIFIC/ENHANCED PROMOTERS

Constitutive overexpression of transgenes that interfere with normal processes in a plant underscore the need for refinement of transgene expression. The development of tissue-specific promoters to drive transgene expression has helped fulfill that need. Targeted expression has become particularly important for the future development of value-added crops because the public may be more likely to accept 'less intrusive' expression of the transgene. For example, confinement of an insecticidal transgene product to tissue besieged by insect pests instead of harvestable material could have potentially defused the Starlink corn fiasco (Bucchini and Goldman, 2002).

The development of tissue-specific promoters for genetic engineering can be complex. The size of the promoter fragment isolated can have an effect on expression, as more distant 5' *cis*-acting enhancer elements can be eliminated during promoter isolation. Potential *trans* interactions that occur based on chromatin structure or location within the genome could also be lost by random integration of the transgene into the host genome. There may not be effective interaction of promoter *cis*-elements with the heterologous *trans*-acting factors present in the transgenic host plant species. Additional knowledge on the functionality of both homologous and heterologous promoters in target host plants is essential in efforts to improve the efficiency of transgene expression.

The tissue-specific promoters presented in this section are only a sample of the promoters characterized in the literature. Many of the selected promoters have been used as tools to drive targeted transgene expression in numerous publications and are fairly well characterized. A few of the promoters are more recent additions to transgenic research. It is also more truthful to call many of the promoters *tissue-enhanced*, because their expression may not be completely confined to a single tissue or plant part.

Fruit. Use of fruit as an edible delivery vehicle for micronutrients and oral vaccines has dictated the need for fruit-specific/enhanced gene expression. For example, although vaccines to prevent dozens of different infectious diseases have been or are being developed (Katz, 1997), in many instances lack of public-health infrastructure in the countries that need them most prevents their deployment. Facilities that provide refrigeration and sterile conditions necessary for safe vaccinations are just not available. Recombinant antigens expressed in the tissues of edible plants would become naturally bioencapsulated vaccines.

The promoters of fruit ripening-related genes have been targeted for their potential use in fruit-specific expression of transgenes. Promoters from the 1-aminocyclopropane-1-carboxylate (ACC)

oxidase gene, the *E8* gene, and polygalacturonase (PG) genes have been characterized from apple (Atkinson et al., 1998) and tomato (Montgomery et al., 1993; Nicholass et al., 1995; Deikman and Fischer, 1988). The ACC oxidase gene is induced by application of ethylene, and analysis of the promoter region shows the presence of ethylene-responsive elements (Atkinson et al., 1998). Analysis of the apple ACC oxidase in transgenic tomato shows that promoter fragments ranging from -1966 to -1159bp from the start of transcription were able to drive ripening-specific expression of a reporter gene in the tomato fruit, with no activity present in other tissues (roots, leaves, flowers) or in immature or mature green fruits.

The product of the *E8* gene appears to participate in the feedback regulation of ethylene biosynthesis during fruit ripening (Kneissl and Deikman, 1996). The promoter of the tomato *E8* gene has been used successfully in a number of instances to target transgene expression to fruit, including increasing the distinctive tomato 'aroma' through metabolic engineering (Lewinsohn et al., 2001), and directing expression of the virus-F protein as an oral vaccine to induce systemic immunity in mice (Sandhu et al., 2000). Although it also drives expression of the transgene in flowers (mainly anthers), its uniform expression throughout the ripening fruit makes it very useful.

Tomato is a useful system for characterization of fruit-specific expression because it is relatively easy to transform and produces large fruits with well-defined ripening characteristics. But the micronutrient and vitamin content of the ripe tomato fruit also make it an attractive target for transgenic research (Hadley et al., 2002; Rao, 2002). The promoter of the tomato polygalacturonase gene (PG gene product accumulates during ripening and is associated with fruit softening) has been used to drive expression of a bacterial phytoene synthase to increase the total fruit carotenoid content (Fraser et al., 2002). In tomato, a single gene encodes PG, and analysis of a 1.4kb promoter fragment shows that it also directs ripening-specific expression (Montgomery et al., 1993). Phytoene desaturase (*Pds*) is the second dedicated enzyme in carotenoid biosynthesis and is also encoded by a single gene in tomato (Giuliano et al., 1993). Because carotenoids accumulate in the chloroplasts and chromoplasts, the tomato *Pds* promoter (2.0kb from start of translation) drives high levels of expression in organs and developing tissues where chromoplasts are found (fruits, petals, anthers) (Corona et al., 1996).

Seed/grain. Seeds are a convenient package for the production of transgenic products. Seed-specific transgene expression has been used for a number of genetic engineering applications, including improvement of seed nutrient quality (Keeler et al., 1997; Shintani and DellaPenna, 1998; Goto et al., 1999; Ye et al., 2000), functional qualities of milled grain (Barro et al., 1997), and production of industrial or pharmaceutical compounds (Cahoon and Shanklin, 2000). However, the production of industrial or consumer products has really benefited from research focused on fundamental processes of plant growth and development, especially as it pertains to the regulation of seed development.

Seed storage proteins are expressed at high levels during seed development, and the promoters of these genes have become an attractive target for use in genetic engineering technologies. Dicot seed-specific promoters have been extensively characterized and several, well-conserved, seed-specific *cis*-elements have been characterized. Dicot seed-specific promoters used to drive seed-specific expression include those from the soybean β -conglycinin

genes, where expression is restricted to the embryo during the mid- to late maturation phase (Chen et al., 1989; Chamberland et al., 1992; Lessard et al., 1993), and the sunflower helianthinin genes (Nunberg et al., 1994), where a full-length promoter (−2379 to +24 bp) drives high, seed-specific expression in tobacco. One of the best-characterized and most commonly used seed-specific promoters is the French bean β -phaseolin gene (Bustos et al., 1989; van der Geest and Hall, 1997). Strong, spatial expression of the β -phaseolin promoter is usually limited to the developing seed in transgenic tobacco (Sengupta-Gopalan et al., 1985). Specifically, the −295 bp region showed high expression in both the endosperm and embryo (van der Geest and Hall, 1996). Extensive *in vivo* footprint analysis showed that over 20 *cis*-elements are present that bind *trans*-acting factors in seeds (Chandrasekharan et al., 2003), and that the complex binding of factors within the seed represents module-specific binding that mediates expression in various regions of the embryo. Matrix attachment regions found flanking the β -phaseolin gene also act to enhance transcription when included in transgenic constructs (van der Geest et al., 1994; van der Geest and Hall, 1997).

More recent work has focused on a cotton α -globulin promoter (Sunilkumar et al., 2002). The cotton α -globulin gene promoter has been functionally characterized in three plants: cotton, *Arabidopsis*, and tobacco. The promoter drove reporter gene expression restricted to the seed, starting 12 and 15 d post-anthesis in *Arabidopsis*, tobacco, and cotton, and expression increased throughout seed maturation, which suggests that gene expression is temporally confined to mid- to late stages of embryo development. In cotton, expression started during the cotyledon expansion stage. However, activity of the cotton α -globulin promoter in tobacco and *Arabidopsis* was substantially lower, showing the necessity for the development of endogenous tissue-specific promoters for plant transformation.

In monocots, several promoters of storage proteins have been studied in detail, including the endosperm-specific hordein promoters in barley (Forde et al., 1985), glutenin promoters from wheat (Lamacchia et al., 2001), and zein promoters in maize (Marzabal et al., 1998). Interest has also increased in promoters that drive expression of genes involved in synthesis of storage starch, the most significant source of carbon reserve in the cereal grain. Expression of the granule-bound starch synthase 1 (*gbss1*) gene in wheat is observed throughout the grain-filling process, peaking at 10–15 d after pollination. This makes its promoter a good candidate for the study of grain development and to drive transgene expression to produce seeds of increased nutrient value. Indeed, a 4.0 kb promoter fragment from *gbss1* drives expression restricted to endosperm and pericarp tissues of the developing kernel (Kluth et al., 2002), equivalent to endogenous *gbss1* transcription.

Seed coat-specific gene expression has implications in the study of seed development, transfer of nutrients into the developing cotyledons, and in its potential for visually marking seeds produced by transgenic plants. A pea β -1,3-glucanase isoform, PsGNS2, is temporally and spatially regulated within the seed coat, although it is also expressed in floral tissue and seed (Buchner et al., 2002). Because transgenic seed does not look any different than nontransgenic seed, seed and seed coat promoters driving genes for coloration effects might be valuable as visual markers for differentiating seeds, and decreasing incidents of seed mixing, like the one that occurred with Starlink corn.

Tubers/root storage organs. Tubers and tuberous root storage organs, including potatoes, sweet potatoes, yams, and cassava are staple food sources in numerous developing nations because of their relatively dense caloric content. In Africa, 200 million people rely on cassava, while in South America, a large variety of potatoes are grown and propagated by small farmers for their own consumption as well as for market. Tuber improvement through genetic engineering has focused on combating common insect and fungal diseases (Mohammed et al., 2000), improving the nutritive quality (Chakraborty et al., 2000), as well as studying the tuber to understand its growth and development (Kuhn et al., 2003). The best-characterized, tuber/storage organ-specific promoters are from genes involved in the massive deposition of starch and the storage of highly abundant glycoproteins.

Patatins are glycoproteins that account for approximately 40% of the total soluble protein found in the tuber (Paiva et al., 1983). The potato class I patatin family members, B33 and PAT 21, are highly expressed at early stages of tuber development in the vascular tissues, and, in later stages of development, in both parenchyma and vascular tissues (Jefferson et al., 1990; Liu et al., 1991). They are tuber-specific, but can be induced in the leaves by sucrose. Indeed, the presence of *cis*-acting sucrose-responsive elements within the class I patatin promoters allows for continued gene expression because of sucrose uploading into the tuber during growth and development (Liu et al., 1990). There is substantial sequence homology between the B33 and PAT 21 promoters. In reporter gene studies, 1.5 kb of 5' flanking of the B33 promoter and 2164 bp of the PAT 21 promoter appeared to contain all the elements necessary for tuber-specific expression when transformed back into potato. In a novel use of the patatin promoter, the cytochrome P450 gene from rat (*CYP1A1*) was introduced into potatoes to enhance the detoxification of residual herbicides in the soil (Yamada et al., 2002). Developing tubers showed high levels of the *CYP1A1* mRNA and protein and the concentrations of specific herbicides were much less than that of nontransformed tubers. Generation of herbicide-detoxifying tubers may be useful for phytoremediation of soil chemical pollutants.

Tubers contain vast quantities of starch, the main polysaccharide reserve found in most higher plants. Starch in potato tubers consists of up to 25% amylase, and granule-bound starch synthase (GBSS) is the key enzyme in amylase biosynthesis. A 5' flanking potato GBSS promoter fragment of 800 bp was sufficient to cause high levels of reporter gene expression in both stolons and tubers, with little to no activity in leaves (Visser et al., 1991). Like patatin promoters, sugars can induce the GBSS promoter's expression in leaves, but not to levels as high as that of the patatin promoters.

Sweet potato has produced two well-characterized promoters. These promoters drive expression of two of the most abundant proteins in the tuberous storage roots of sweet potato, sporamin and β -amylase (Maeo et al., 2001). Sporamin makes up 60–80% of total soluble proteins in the sweet potato storage organ. It is composed of two multigene subfamilies, A and B, which contain approximately 10 total members. Analysis indicates that sporamin was expressed almost exclusively in the storage tuber, with a small amount of expression in stems (1–4.5% soluble protein) (Maeshima et al., 1985). When approximately 1000 bp (−970 to +41) of the *gSPO-A1* promoter was fused to the reporter gene, CAT, and expressed in transgenic tobacco, it was found that various sugars caused induction of CAT to high levels in the stem. Induction was retained

when a deletion was made to -305bp, but abolished with a deletion to -94bp (Hattori et al., 1990). The *gSPO-A1* promoter shares two conserved sequence elements with another sporamin promoter that appear to act independently of each other. One directs expression to the phloem (-305 and -237bp), and the other to the pith parenchyma (-192 to -94bp) (Ohta et al., 1991).

Similar deletion experiments were also performed with the sweet potato β -amylase promoter fused to the GUS reporter gene. β -Amylase is developmentally regulated and highly expressed in the storage root where it is involved in starch synthesis. A -1267bp promoter fragment drove high GUS expression in the stems of tobacco when induced by sugar, but deletion to -786bp completely abolished the induction. Continued deletion analysis found a carbohydrate metabolic signal-responsible element-1 (CMSRE-1) comparable in sequence to an element found in the sporamin promoter (Maeo et al., 2001).

Florets/flowers. Color is one of the most visually appealing aspects of flowers and one that has helped create a substantial economic market for cut flowers. This market has also helped drive the search for floral-specific promoters for use in transgenic production of flowers with increased market value. Specifically, qualities impacting resistance to pests (Dolgov et al., 1995), increased vase life, and modification of color (Holton et al., 1993; de Vetten et al., 1999; Mol et al., 1999) and fragrance are of interest in the production of fresh flowers for the consumer.

Chrysanthemums (*Dendranthema grandiflora*) are the second largest cut flower produced next to roses, and problems with insect pests that reduce flower quality has led to a search for floral-specific promoters. A chrysanthemum endogenous *ubiquitin extension protein* (*UEPI*) was fused to the GUS reporter gene and measured for tissue-specific expression. When transformed back into chrysanthemum, the 1.45 kb promoter for *UEPI* drove high levels of expression in a floral-enhanced manner, both in ray and disc florets, although 9-fold lower GUS expression could be detected in leaves (Annadana et al., 2002). When compared to the CaMV 35S, the *UEPI* promoter expression showed a 40-85-fold higher expression in the flower. A second, heterologous promoter also showed high levels of gene expression in chrysanthemum petals. The *Arabidopsis thaliana eceriferum* gene *CER6* is involved in wax production in flower petals (Hannoufa et al., 1996), and, when transformed into chrysanthemum, the promoter for this gene drove high, specific expression in flower petals. However, compared to the *UEPI* promoter, its expression had higher variability among primary transformants. This could be a property of the *CER6* promoter within the chrysanthemum system or an indication that position effect could play a greater role in the ability of the *CER6* promoter to direct consistently strong expression (Hannoufa et al., 1996).

Flavonoids serve as UV protectants, signal molecules, and flower color pigments. Indeed, it has been the ability to control specific cytochrome P450 genes (Holton et al., 1993; de Vetten et al., 1999) within the flavonoid secondary metabolic pathway that has led to the newly marketed 'blue' carnations (Holton, 1995) and could lead to blue roses. Chalcone synthase genes are also involved in flavonoid production, and their promoters have been extensively studied and their *cis*-acting elements dissected. The bean chalcone synthase 15 (*CHS15*) gene promoter:GUS fusion showed that expression was activated in the inner epidermal (pigmented) cells of petals, in root tips and in lateral-root meristems (Faktor et al., 1996). Promoter

dissection studies in tobacco showed that the presence of two *cis*-acting elements in close proximity to the TATA box were essential for high, petal-specific expression of the *CHS15* promoter. Point mutations in an H-box element (CCTACC) and a G-box element (CACGTG) dramatically decreased *CHS15*-GUS fusion gene expression in floral tissue. However, a series of constructs using a 39 bp synthetic *cis*-element containing these two boxes showed that cooperative action of both of these elements is essential for floral-specific expression, and expression is influenced by the distance between the two elements (Faktor et al., 1997).

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) catalyzes an essential step in the biosynthesis of aromatic amino acids, and is the target for the broad-spectrum herbicide glyphosate, 'Roundup'. EPSPS mRNA is also expressed at very high levels in petals of petunia flowers and faintly in petunia leaves (Gasser et al., 1988). In a stunning visual series of experimental photographs, Benfey and Chua (1989) and Benfey et al. (1990b) examined the tissue specificity of a deletion series of petunia EPSPS promoter:GUS fusions. Deletion analysis showed that sequences between -1800 and -800bp were sufficient for petal-specific expression in petunia in nearly all cell types in the limb of the flower and restricted to cells adjacent to and in the upper epidermis in the upper portion of the floral tube. Activity of the promoter also increases dramatically as the flower opens. A construct consisting of -1752 to -282bp fused to the minimal CaMV 35S promoter (-46 to +8bp) showed GUS staining in epidermal layers as well as in vascular and mesophyll cells, while there was a gradient of expression in the floral tube, with high expression near the limb and tapering to nondetectable levels at the base of the tube (Benfey et al., 1990a). Interestingly, the petunia EPSPS promoter construct showed very low expression in both petals and leaves of tobacco, also a member of the *Solanaceae* family (Benfey and Chua, 1989).

Pistils. The term pistil is used to describe the female parts of a flower, which commonly include the stigma, where pollen grains bind, the style in which the pollen tube grows, and the ovary at the base of the pistil, which differentiates into the fruit following fertilization. Characterization of genes expressed in the pistil structures could be helpful in understanding incompatible and compatible pollination and fertilization, as well as understanding pistil development. The two pistil-specific promoters and their genes described here appear to be defense-related, but could also play an unknown role in compatible pollination.

The *SK2* gene in potato encodes a pistil-specific basic endochitinase, distinct from wound-induced endochitinases. A deletion analysis of the *SK2* promoter fragment starting 1.0kb 5' from the start of transcription and deleting down to -230bp showed that all of the elements necessary for pistil-specific expression in transgenic potato were contained within the -230bp promoter fragment (Ficker et al., 1997). Expression increases steadily during development of the pistil. Long-term GUS staining also showed weak expression in the anther wall.

A second group of pistil-specific genes are the PR5-like protein, or thaumatin-like proteins, which are believed to be involved in antifungal activity and hydrolysis of β -1,3-glucans (Wang et al., 1996; Grenier et al., 1999). The 2.4kb 5' flanking region of the Japanese pear *PsTLL1* gene was fused to the GUS coding region and transformed into tobacco (Sassa et al., 2002). This promoter fragment directed high GUS expression in pistils, comparable to that

of the CaMV 35S promoter. No GUS activity was detected in any other tissue except for weak expression in anther and pollen grains.

Anther/pollen. Interest in the molecular processes underlying the development of the male gametophyte and the self-incompatibility response in plants has driven an explosion of anther- and pollen-specific gene characterizations in both monocots and dicots (Goldberg et al., 1993; Taylor and Helper, 1997; Shiba et al., 2001). Anther- and pollen-specific expression can be divided into two general phases. In the first or 'early' phase, genes with anther-specific promoters are expressed during anther development and the formation of sporophytic tissue and cells. Phase two or 'late' gene expression involves both anther- and pollen-specific genes expressed during gametophyte generation and pollen formation and maturation.

Promoters for 'early' gene expression are generally considered anther-specific. These genes are expressed as the morphology of the anther is established, cells within the anther are differentiating, and microspore mother cells are undergoing meiosis to produce microspores. The promoter of an anther-specific cDNA isolated from tobacco, *TA29*, was fused with a reporter gene to define cellular expression (Koltunow et al., 1990). *TA29*, a glycine-rich protein similar to those found in cell walls, is expressed in tapetum, a nutritive tissue that lines pollen sacs. Its expression increases in the developing anther but decreases after the microspores begin to mature into pollen. A *cis*-element localized in the *TA29* promoter between -207 and -85 bp is responsible for strong tapetum-specific spatial and temporal expression. A *TA29* promoter-*RNase* (*barnase*) chimeric construct has since been used to produce nuclear male sterile plants, both in tobacco and oilseed rape (Mariani et al., 1990).

A 2.5 kb 5' region of the *RA8* gene (unknown function) from rice appears to contain all the elements necessary for anther-specific expression in transgenic rice. The *RA8*:GUS fusion shows that expression starts in the anthers at the tetrad stage of microspore development, about the time *TA29* expression declines, and reaches its highest levels at the vacuolated pollen stage. However, just before dehiscence, activity decreases abruptly (Jeon et al., 1999). No GUS staining was observed in the pollen, making this promoter active late in the 'early' phase of anther development.

Two different late-phase promoters described direct expression in pollen. The tomato *lat52* gene, which encodes a cysteine-rich protein, is expressed abundantly in mature pollen (Twell et al., 1989), with only low levels of expression detected in anther walls and petals (Ursin et al., 1989). Expression studies using the tomato *lat52* promoter showed that the elements necessary for strong pollen-specific expression in tomato, tobacco, and *Arabidopsis* were present within about 600 bp (-492 to +110 bp) of the 5' flanking region (Twell et al., 1990). The promoter of a pectin methylesterase gene from maize (*ZmC5*) shows expression similar to that of *lat52* when transformed as a promoter:GUS fusion into tobacco. Approximately 2.0 kb of the maize *ZmC5* promoter, a 'late' pollen-specific gene, was shown to drive strong expression of a reporter gene in transgenic tobacco pollen (Wakeley et al., 1998).

The genes found in the self-incompatibility locus (S-locus) in plants prevent self-fertilization and promote out-crossing in certain plants. Interest in understanding incompatible and compatible pollination responses have led to the characterization of the S-locus genes and their promoters. When a promoter from a *Brassica* S-locus glycoprotein was transformed into *A. thaliana*, two cells types

showed reporter gene expression: cells in the stigma and the tapetal cell layer in anthers (Toriyama et al., 1991). Cell ablation studies were also done using this promoter fused to the diphtheria toxin A chain gene, to study developmental processes in plant reproductive tissues (Thorsness et al., 1991). The *Brassica SP11* gene of the S-locus has characteristics of a pollen coat family of proteins (Takayama et al., 2000) and is expressed at high levels in both anthers and pollen (Shiba et al., 2001). Deletions of a 522 bp *SP11* 5' flanking region down to -192 bp did not affect promoter function, and a further deletion to -143 bp promoter resulted in loss of anther tapetum expression and directed only pollen expression.

Development of male sterile plants has been a controversial consequence of targeted transgenic expression in pollen. It can be argued that Genetic Use Restriction Technology (GURT) or 'terminator' technologies were originally developed to substantially decrease the specter of transgene 'pollution' by pollen drift. However, environmental activists opposed to GEOs (Genetically-Engineered Organisms) and agribusinesses that were developing transgenic plants saw terminator technologies as a way of controlling seed production. If a plant could not produce seed, people would have to purchase new seed every year from companies controlling the transgenic technologies. While in the developed nations, the purchase of new seed each year is common practice because of high-yield hybrids, in the developing world, saving seeds for the next season is much more common among small farmers. There were (and are) fears that farmers from developing nations would again be dependent on large agribusinesses, as they had been with chemical additives and pesticides, and end up in a cycle of debt (Shiva, 2000). A study commissioned by the Food and Agricultural Organization of the United Nations (FAO) on the effect of GURTs on agricultural practices and agrobiodiversity is available at <http://ext-ftp.fao.org/ag/cgrfa/BSP/bsp15e> (2000).

Legume-rhizobium-associated root nodules. Rhizobial endosymbiont associations with leguminous host plants are the best-studied type of nitrogen-fixing symbiosis. Legume hosts form unique, root-associated structures (nodules) in the presence of specific rhizobial soil bacteria. Rhizobial bacteria differentiate into bacteroids within the nodule and begin to fix atmospheric nitrogen for the plant in exchange for carbon substrates. The promoters of plant genes specifically expressed during nodule organogenesis and involved in nitrogen assimilation events have been exploited to study the dynamics of this unique and valuable interaction. They have also been used in attempts to understand and improve nitrogen assimilation within the nodule (Schoenbeck et al., 2000).

Nodule-specific plant genes are classified as nodulins. Genes expressed early in nodule organogenesis are called early nodulins (ENOD genes) (Lauridsen et al., 1993; Vijn et al., 1995; Fang and Hirsch, 1998; Hohnjec et al., 2000), while genes expressed after nitrogen fixation begins are late nodulins (Carsolio et al., 1994; MacKnight et al., 1995). Many late nodulin promoters contain the common nodulin consensus motifs, AAAGAT and CTCTT (Sandal et al., 1987; Stougaard et al., 1987), which are present in a *cis*-acting, organ-specific element (OSE) within the promoter (Lauridsen et al., 1993; Ramlov et al., 1993). An AT-rich promoter motif (consensus TATTWAT) also appears to be important in nodule-specific expression (Carsolio et al., 1994; Fruhling et al., 2000). A majority of the work done using nodulin promoters has been directed at the study of the nodule, when and where nodulin

genes are expressed during nodule development and the subsequent nitrogen fixation phase, and the function of the nodulins. For example, a promoter fragment (−692 to −41 bp) of the *Vicia faba* L. early nodulin Vfenod12 mediated reporter gene expression in root cortical cells, nodule primordia, and the prefixing zone II of transgenic nodules (Fruhling et al., 2000), while the *Phaseolus vulgaris* late nodulin Npv30 promoter (−868 to −67 bp) showed GUS activity only in infected cells in nodules of transgenic plants (Carsolio et al., 1994).

Leghemoglobin, a plant oxygen-binding protein, plays a major role in maintaining low, free-oxygen conditions within the nodule. The expression of leghemoglobin coincides with nodule nitrogen fixation and leghemoglobin promoters drive expression to high levels within the nodule. Leghemoglobin promoters have become useful tools for nodule targeting of genes with the potential to increase nitrogen assimilation and maximize the efficient use of the nitrogen fixed in the nodule. Promoters from a number of different legumes have been functionally analyzed (Stougaard et al., 1986; Szabados et al., 1990; Strozycski et al., 2000). The *Sesbania rostrata* leghemoglobin *glb3* promoter has been studied in both transgenic *Lotus corniculatus* and tobacco (Szabados et al., 1990; Szczyglowski et al., 1996). The *glb3* promoter (−1914 to −48 bp) drove high GUS activity in a nodule-specific manner in transgenic *Lotus*. In transgenic tobacco (a non-nodulating plant), GUS activity was approximately 40% of that seen in *Lotus*, with major expression in roots and detectable expression in stems.

Roots. Root-specific promoters are of particular interest because they hold promise for an extensive array of applications. Recombinant proteins can be expressed for almost anything that is related to the root–soil interface, including genetic engineering for bioremediation of soil contaminants, protection against drought, increasing tolerance to salt, macro- and micronutrient uptake, or increasing resistance to root pathogens. In general, root-specific mRNAs appear to represent only a small percentage of total root mRNAs (Evans et al., 1988; Conkling et al., 1990), and while root-specific plant promoters have been isolated and functionally characterized (Yamamoto et al., 1991), nonplant promoters have become some of the most utilized when targeting transgene expression to the root.

The soil bacteria *Agrobacterium rhizogenes* causes hairy root disease in infected dicotyledonous plants. The promoters of the rooting loci (*rol*) genes found in the Ri (root-inducing) plasmid of *A. rhizogenes* are popular targets of study because of the root-directed transformation and expression of their gene products. Specifically, the *rolD* promoter has been analyzed by a series of promoter deletion:GUS fusion constructs and has been found useful. However, results have been conflicting when looking at the intact *rolD* promoter in transgenic tobacco. In one study, the *rolD* promoter (1222 bp 5' flanking) was found to direct high levels of transgene expression in both leaves and roots of young tobacco, up to five times higher than the CaMV 35S promoter (Leach and Aoyagi, 1991). However, a second study showed that a similar-sized construct (1271 bp 5' flanking *rolD*) showed a nearly 30-fold higher expression of GUS in roots of mature tobacco transgenic plants (Elmayan and Tepfer, 1995). Differences between the studies included age of the plants during analysis (young versus mature), and differences in the construct fusions within the transcriptional start site region. In both studies, intermediate-sized *rolD* promoter constructs (373 and 426 bp 5' flanking) showed root-specificity and high levels of

expression. Sequence analysis of the *rolD* promoter does demonstrate motifs similar to root-specific elements (RSEs) found in plant root-specific promoters (Keller and Baumgartner, 1991; Elmayan and Tepfer, 1995). The *rolD* promoter has been used extensively for plant nitrogen assimilation studies, including root-directed glutamine synthetase overexpression (Fei et al., 2003) and overexpression of a high-affinity nitrate transporter (Fraisier et al., 2000).

A second important, nonplant promoter used for root-specific expression is Domain A (−90 bp upstream) of the CaMV 35S promoter (Benfey and Chua, 1989). In a direct comparison to the *rolD* promoter, expression from the CaMV 35S Domain A promoter:GUS fusion was 5–15-fold less than that of the *rolD*:GUS fusion constructs, and was localized to a significant level in root tips (Elmayan and Tepfer, 1995).

A valuable root-specific promoter that has been used for many genetic engineering objectives is the TobRB7 promoter from tobacco (Yamamoto et al., 1991). TobRB7 is a putative membrane channel aquaporin, which is expressed in a root-specific manner. A promoter:GUS deletion series showed that the highest activity was directed by the Δ0.6 promoter, which included 636 bp 5' flanking (Yamamoto et al., 1991). Root-specific activity was shown as early as 2 d post-germination in tobacco transgenics and was strongest in the meristem and central cylinder. The Δ0.3 promoter:GUS construct of TobRB7 (299 bp 5' flanking) is responsive to root-knot nematode-directed expression (Opperman et al., 1994), although it appears to express at some basal level in root tissue. Using the Δ0.3 promoter construct, Shen et al. (2000) fused the *hmrA* gene from *Pseudomonas syringae* to convert a compatible plant–pathogen interaction into an incompatible interaction. The transgenic tobacco in this study also displayed high levels of resistance to multiple other pathogens, including viral, fungal, and bacterial (Shen et al., 2000). These results suggest that expression of bacterial *avr* genes using controlled low-level expression in the roots could generate broad-spectrum resistance to any type of bacterial, fungal, or viral root pathogen.

Leaves/green tissue. Chlorophyll-containing tissue, like the leaf, supports the expression of a number of well-characterized, light-inducible genes. The best-characterized light-inducible genes in plants are members of the *rbcS* multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. Promoters from *rbcS* genes contain an intricate assortment of positive and negative regulatory elements that are able to confer light-inducible and tissue-specific expression in transgenic plants (Gilmartin and Chua, 1990). Deletion analysis of a model *rbcS* promoter, the pea *rbcS*-3A, revealed redundant light-specific response elements (LREs), which, when removed, greatly reduced light-induced expression (Kuhlemeier et al., 1987). The LRE located between −169 and −112 bp contained two binding sites for the transcription factor GT-1 (Green et al., 1987). These binding sites, labeled box II (−151 to −136 bp) and box III (−125 to −114 bp), are both required for transcriptional activation by light (Kuhlemeier et al., 1988). Regions in the *rbcS*-3A promoter responsible for light induction are also associated with tissue-specific expression, again emphasizing redundancy in the expression system of the *rbcS* genes. Only 410 bp 5' flanking of the pea *rbcS*-3A gene was found to be sufficient for strong light and tissue-specific expression (Kuhlemeier et al., 1988). An alfalfa *rbcS* promoter of 608 bp drives strong, light-inducible and tissue-specific expression of a GUS fusion gene in tobacco (Potenza et al., unpublished data). Although many of the

rbcS promoters isolated and characterized from dicots appear to contain highly conserved *cis*-elements that maintain similar spatial parameters and expression patterns, monocot promoters may have different *cis*-activating sequences than those of dicots (Schaffner and Sheen, 1991). Furthermore, an *rbcS* promoter from C3 (rice) has a different pattern of expression than the endogenous maize *rbcS* when transformed into a C4 (maize) plant (Nomura et al., 2000), telling a cautionary tale, once again, about heterologous plant promoters used in plant genetic engineering. Even the Rubisco promoter, one of the best-characterized plant tissue-specific promoters available, gives surprising results when transformed into a heterologous system.

A second, highly expressed, green tissue gene family is the chlorophyll *a/b*-binding (*Cab*) protein genes. *Cab* proteins are associated with the light-harvesting complex proteins to form the light-harvesting complex (Lhc). Like the *rbcS* proteins, it is also one of the most abundant proteins found in the leaves of all green plants. However, the expression pattern of the *Cab* genes in plants is different from that of the *rbcS* genes. *Cab* promoters respond to light and diurnal or circadian rhythms (Ha and An, 1988). Carre and Kay (1995) have demonstrated that the region between -111 to -38 bp of the *Arabidopsis CAB2* promoter sequence confers circadian regulation to a luciferase reporter gene. This region contains a CAT-box and three I-boxes or GATA motifs, which all play a role in tissue-specific and light-mediated regulation (Anderson and Kay, 1995). Sequence alignment of specific promoter regions also reveal a novel motif (CAANNNNATC) conserved in 5' upstream regions of circadian-controlled tomato *Lhc* genes. This element overlaps with a sequence relevant in light-mediated gene expression, which supports the belief that light perception and the biological clock are closely related (Piechulla et al., 1998).

A problem that might arise with the use of *rbcS* and *Cab* gene promoters could be related to their membership in multigene families. *Cab* and *rbcS* proteins are very abundant in green tissue, especially leaves, but many genes within the families could contribute to the final total pool of protein. High expression levels of a transgene could potentially be dependent on which gene family member promoter is isolated and characterized. For this reason, a strong, green tissue-specific promoter from a small or single gene family might be valuable (Aleman, 2001). Rubisco activase (RAC) is a soluble chloroplast protein required for the activation of *rbc* in an ATP-dependent manner (Portis, 1992), and appears to be a single gene in alfalfa (Aleman, 2001). Comparison of the *RAC* and *rbcS* promoter sequences revealed that the 5'-upstream region of the spinach *RAC* gene contains sequences that are homologous to *cis*-elements conserved among *rbcS* (Orozco and Ogren, 1993). An alfalfa 1532 bp *RAC* promoter:GUS fusion construct used in transgenic tobacco confers strong, light-regulated expression in green tissue (Potenza et al., unpublished data).

CELL TYPE-SPECIFIC PROMOTERS

A number of cell-specific or cell type-specific plant promoters are available or can be isolated for specific cells or cell types. A few of these from recent publications will be reviewed.

Trichome-specific. Trichomes are modified epidermal structures that appear on the surface of many flowering plants (Szymanski et al., 2000), and act as the first line of defense against insect and pathogen attack. Glandular trichomes, small protruding stalks with

a stem and slightly bulbous head, secrete compounds that contribute to this defense, as well as natural products that are of interest because they are harvested as essential oils, flavors, and aromas (McCaskill and Croteau, 1999). Cytochrome P450 promoters from tobacco (Clark et al., 1997) and a cytochrome P450 gene from catmint (*Nepeta racemosa*) (Wang et al., 2002) have been isolated that are specific for trichome expression. The promoters have the potential to enhance natural resistance to plant pests and contribute to molecular 'pharming' of commercially useful natural products (Lange and Croteau, 1999). The tobacco *CYP71D16* promoter comprised of 1852 bp upstream of the translation start site and including +111 bp of the *CYP71D16* gene was fused to GUS to determine spatial and temporal expression patterns. Strong trichome-specific expression was found as early as the rooting stage and continued in the leaves, stems, and flower petal trichomes at all stages of development (Wang et al., 2002). No root expression was detected. Intense GUS staining was observed in the leaf trichome glands and deletion analysis showed that the region from -223 to +111 bp was found to be sufficient for trichome-specific expression, but not strong gland-specific expression (Wang et al., 2002). The cytochrome P450 monooxygenase gene (*CYP71A5*) identified in catmint is from the same subfamily as that of tobacco and shows expression exclusively in trichomes (Clark et al., 1997).

Guard cell/stomata. Guard cells are highly specialized epidermal cells that are an important part of the stomatal complex. They are present in the leaves, stems, and reproductive structures of many plants and control the stoma pore size, which in turn allows gas and vapor exchange between the plant and the environment (Alberts et al., 2002). Controlling stomatal opening and closure could have broad implications in controlling plant reaction to environmental changes, including drought and heat. In *Arabidopsis*, genes encoding functional myrosinase enzymes were expressed in guard cells and very specific vascular cells in the phloem. The promoter of the thioglucoside glucohydrolase 1 (TGG1) myrosinase gene was fused to GUS and green fluorescent protein (GFP) and transformed back into *Arabidopsis*. Expression studies showed that a 2.5 kb promoter fragment directed expression in guard cells and specialized phloem cells at all stages and tissues (Husebye et al., 2002). A second guard cell-specific gene, the *KST1* gene, encodes a K⁺ influx channel in the guard cells of potato. It is thought to be involved in the influx of K⁺ ions after guard cell stimulation by light or hormones. Deletion of the *KST1* promoter showed that -1086 bp to -236 bp 5' flanking directed strong activity of a reporter gene in guard cells, but a deletion to -191 bp showed very little to no expression. Sections of the *KST1* promoter were fused to the -56 bp CaMV 35S promoter to try to find a guard cell-specific element. Studies showed that a zinc-finger transcription factor Dof protein bound to the sequence motif (5'-TAAAG-3'), and binding was highly correlated with guard cell-specific expression (Plesch et al., 2001).

Root hair. Root hairs are made up of single cells and have an important function in water and ion uptake. They are also involved in the initiation of nodulation and formation of the infection thread in rhizobium-host interactions (Buchanan et al., 2000; Frayse et al., 2003). Interest in gene expression to address root hair development and function (Cosgrove et al., 2002) has led to the study of a number of genes, including ion channel genes (Downey et al., 2000) and genes involved in symbiosis (Cattoria et al., 2001). Simplified methods for cloning root hair-specific cDNA (Jones and

Grierson, 2003) is leading toward the isolation and characterization of root hair-specific promoters that will allow targeted study of this unique root cell.

Companion cell. Sucrose transporters catalyze the uptake of sucrose in phloem loading for long-distance transport in plants. A number of sucrose transporters have been localized to the phloem (Gahrtz et al., 1994; Riesmeier et al., 1994; Truernit and Sauer, 1995), and molecular and immunocytochemical studies narrowed down expression specifically to the companion cell (Stadler et al., 1995; Stadler and Sauer, 1996). A 945 bp promoter including the entire 5' untranslated region of the *A. thaliana* *AtSUC2* sucrose-H⁺ symporter gene fused to GFP has been used to study long-distance trafficking and symplastic uploading of GFP in phloem (Imlau et al., 1999), and to study vein maturation in developing tobacco leaves (Wright et al., 2003). Further study of the *AtSUC2* promoter showed that it is expressed in the plant parasitic, cyst nematode-induced syncytia (Juergensen et al., 2003), and could be used in a strategy to interfere with syncytia formation and disrupt the nematode's life cycle.

ORGANELLE-SPECIFIC PLANT PROMOTERS

The generation of transgenic organisms and their subsequent release and widespread usage in agriculture has raised valid concerns about their safety and containment. While many scientists behind the development of genetically enhanced organisms believe they understand their potential and limitations, their release has led them into the path of public 'peer review' concerning the methods, genes, and expression patterns of these genes in transgenic organisms. The advent of many of these challenges has been beneficial, for example, leading to the development and potential use of promoters that target transgene products so as to help allay fears. But questions still remain. One of the most publicized concerns involves transgenic 'pollution', or the spread of transgenes from cultivated crops into wild relatives by means of pollen drift. Recent studies have shown that, indeed, transgenes can introgress into wild relatives with low probability (Scott and Wilkinson, 1999), but in areas where wild populations are known to exist, restrictions on growth and planting are applied. However, GEO opponents have recently begun to challenge the success of transgenic crops using racial and cultural arguments that enflame passions and fears. This is especially true of recent reports that traditional maize landraces in Mexico have been 'contaminated' by transgenic corn pollen (Quist and Chapela, 2001). Although these reports have been refuted with great spirit by the scientific community (Christou, 2002; Kaplinsky et al., 2002; Mann, 2002; Metz and Fütterer, 2002), steps are being taken to diffuse this type of argument by placing transgenes in plastids under the control of plastid promoters as a means of reducing or eliminating transgenic pollen drift.

Plastid transformation by biolistics was first done in *Chlamydomonas* chloroplasts (Boynton et al., 1988), showing through complementation that photosynthetic mutants could be restored to photosynthetic growth. Wild-type genes replaced mutant genes via homologous recombination. Plant chloroplast recombination followed in 1990 (Svab et al., 1990). Plastid transformation has multiple advantages when compared to nuclear transformation. These organelles are prokaryotically derived and have relatively small circular genomes, allowing for homologous

recombination, which would eliminate gene placement effects. There is also no gene silencing like that seen with nuclear transfer and, because polycistronic expression of genes occurs in plastids (Staub and Maliga, 1995), there is the possibility of expressing multiple genes in a single transformation vector. Genomes in plastids are present in very high copy number within the organelle (up to 10 000 per single cell) and these genomes have highly active inter- and intramolecular exchange. This would allow for the propagation of the transgene throughout the plastid genome pool very quickly (Goulding et al., 1996), and result in high transgene expression. Finally, a majority of cultivated crop plants inherit their plastid genomes uniparentally through the maternal parent (Corriveau and Coleman, 1988; Nagata et al., 1999). Plastid transformation would therefore effectively reduce or eliminate transgene transfer through pollen (although all the other genes present could still spread to native wild relatives). As an added plus, Monarch butterflies would no longer fear death through pollen ingestion (Losey et al., 1999) because there is no chloroplast DNA in the pollen of most crops and transgenic insecticidal proteins would not be produced in pollen. There is a recent report that studied the transfer rate of chloroplastic DNA into the nucleus (Huang et al., 2003a), but questions have been raised about the high rate of transfer and whether the original chloroplast transgenic lines selected also contained the transgene integrated into the nuclear DNA (Daniell and Parkinson, 2003; Huang et al., 2003b).

Promoters used to control expression of transgenes are mostly derived from those endogenous to the specific plant to be transformed. Chloroplast transformation vectors most often include a light-responsive promoter *psbA*, a photosystem II promoter (Staub and Maliga, 1994), and *rbcL*, the promoter for the ribulose-1,5-bisphosphate carboxylase large subunit gene (Shiina et al., 1998), and the 16S rRNA (*Prrn*), a strong and constitutive plastid operon promoter (Maliga, 2002). Non-endogenous plastid promoters have also been used successfully (Sidorov et al., 1999; Ruf et al., 2001), and a 'universal' vector that integrates into a highly conserved region of the chloroplast genome has recently been developed (Daniell et al., 1998). Another report details a dual plastid/nuclear genome system that places the phage T7 RNA polymerase with an *rbcS* chloroplast transit peptide, driven by the CaMV 35S promoter, into the nucleus of tobacco plants. The T7 RNA polymerase controls expression of a T7 promoter/reporter gene stably transformed into the plastids (McBride et al., 1994). Expression of the reporter gene was extremely high in mature leaves and lower in stems and roots. With modification of the promoter driving the T7 RNA polymerase, more selective expression could be possible.

Other systems that are being developed involve the selective use of plastid polymerases. Plastid polymerases are encoded by both plastid genes (plastid-encoded polymerases; PEP), and by nuclear-encoded polymerases (NEP), which are imported post-transcriptionally into the organelle (Hajdukiewicz et al., 1997). PEP and NEP have been shown to have different expression patterns and recognize different promoter sequences (Hajdukiewicz et al., 1997). Using plastid promoters that are specific for one or the other polymerase may be useful for regulated expression of transgenes in selected plastid types.

Successful use of plastid transformation systems includes high levels of expression of the insecticidal Bt toxin Cry1Ac (McBride et al., 1995), and Cry2Aa2 (Kota et al., 1999), both using the *Prrn*

promoter. Expression of the Bt Cry2Aa2 protein was at such high levels that it killed both susceptible and Bt-resistant insects. Ye et al. (2001) showed that high (10 000-fold higher than nuclear expression) plastid expression of EPSPS, the plant enzyme targeted by glyphosate herbicides ('Roundup'), showed a high level of tolerance to herbicide. Because pollen does not carry the transgene in most crops, the possibility of the creation of 'superweeds' through pollen pollution is substantially decreased. Finally, extremely high expression and concentration of a recombinant human therapeutic hormone (somatotropin) in tobacco chloroplasts (Staub et al., 2000), and transformation and high expression of a transgene in tomato fruit (Ruf et al., 2001), gives promise for the expression of therapeutic drugs and oral vaccines in the chloroplastic transgenic system.

INDUCIBLE PLANT PROMOTERS

Targeting transgene expression using tissue-specific promoters is a powerful technique used in the control of gene expression. However, that control is still dependent on the plant and the expression pattern of endogenous *trans*-activating factors. Furthermore, although numerous, well-characterized tissue-specific promoters are available, there are potential pitfalls when using them. The most obvious is that a characterized heterologous promoter might act differently when placed in a different genus, species, or even cultivar of a plant. Inducible (and repressible) promoter systems using both endogenous and exogenous components have several potential advantages over some tissue-specific systems.

Stress/wound-inducible promoters. In plants, various types of stresses, both biotic and abiotic, induce a large number of genes. The stresses can include a wide range of environmental changes like cold, heat, UV light, high salt or minerals/heavy metals in the soil, and water deficit or flooding, or by herbivore and pathogen attack that causes localized wounding in plant tissues. When a plant is wounded, genes are mobilized for defense and repair, enzymes are induced that produce deterrent chemicals, and proteins and other molecules are made to signal the plant systemically that an attack has occurred. Genes induced by stress and defense have produced a number of well-characterized and useful promoters. Many of these promoters are valuable because they direct specifically targeted expression directly at the point of wounding or infection. These characteristics make wound- and stress-inducible promoters potentially useful for the engineering of resistance genes or insecticidal proteins that are turned on only at the time of attack, whereas global or systemic induction systems have the potential to protect the whole plant from the applied stress only when needed.

Some of the best-characterized wound-inducible promoters were first studied over 10 years ago. The potato *wun1* and proteinase inhibitor II (*pin2*) gene promoters both direct high wound- and pathogen-inducible expression, but have little to no constitutive expression without stimulus (Logemann et al., 1989; Keil et al., 1990; Xu et al., 1993). The 1.2 kb 5' flanking region of the *wun1* gene fused to reporter genes showed very high levels of expression at the site of wounding and pathogen attack (Siebertz et al., 1989). Most of this inducible expression was lost with a deletion to -571 bp. Reporter gene induction was maximal at 10 h, but leveled off between 10 and 12 h, and expression was highest in epidermal cells of leaf and stem. The promoter for the potato proteinase

inhibitor II gene also directed low constitutive expression, but was induced to high levels by wounding and methyl jasmonate when transformed into rice (Xu et al., 1993). However, expression was systemic and was maximal after 24 h (Keil et al., 1990).

Many genes induced by abiotic stresses contain distinct two *cis*-acting elements within their promoters. They are a dehydration-responsive element, or DRE (TACCGACAT) (Yamaguchi-Shinozaki and Shinozaki, 1994), and an abscisic acid (ABA)-responsive element, or ABRE (ACGTGG/TC) (Bonetta and McCourt, 1998). The *Arabidopsis rd29A* and *rd29B* are stress-responsive genes, but are differentially induced under abiotic stress conditions. The *rd29A* promoter includes both DRE and ABRE elements and the gene is induced by dehydration, high salinity, low temperature, and abscisic acid (Yamaguchi-Shinozaki and Shinozaki, 1994), while the *rd29B* promoter includes only ABREs and induction is ABA-dependent (Uno et al., 2000). Systematic site-specific mutation of the DREs and ABREs present in 120 bp of the *rd29A* 5' flanking region (-174 to -55) showed that the two types of elements interact under stress conditions (Narusaka et al., 2003).

Exogenous inducible promoter systems. Although endogenous plant promoters can be used for inducible transgene expression, most inducible systems available for use in plant genetic engineering employ a combination of exogenous regulatory control elements, both *trans*-acting and *cis*-acting, and take advantage of the transcriptional system of plants. They respond to signals, or inducers, not normally seen in plants, ones that can be selected and applied at the discretion of the researcher to turn the transgene(s) on or off. Ideally, the inducible promoter should not show any basal level of gene expression in the absence of inducing agent, and expression should be reversible and dose-dependent. The inducer should be inexpensive, easy to apply, and nontoxic. This is especially true if the system were to be used in field or commercial situations (Gatz and Lenk, 1998).

Why might such a system be useful for transgene expression? To answer specific questions, it might be necessary to develop promoter systems where precise temporal regulation of transgene expression is required. Examples include situations where inappropriate gene expression is harmful or lethal, especially during the development of the plant (Guo et al., 2003). Inducible expression of the desired gene at specific stages of development can contribute to the understanding of the correlation of phenotype to specific gene expression. Metabolite flow in plants with altered expression of enzymes could be studied and monitored with greater precision (Caddick et al., 1998). Switching transcription off with specific promoters would allow analysis of RNA or protein turnover, effectively replacing use of actinomycin D or cyclohexamide. Regulated gene expression systems would also be valuable in biotechnological applications such as conditional expression of 'pesticide' genes (i.e., *Bacillus thuringiensis cry* genes), targeted expression of herbicide resistance genes, or induction of synchronous flowering or ripening. All of these examples point to the need for inducible promoter systems useful in the production of genetically engineered plants.

Herbicide safeners are herbicide antidotes. When applied, they cause increased herbicide resistance in crops, which can be useful for selective weed control. Their mode of activation is believed to be through the induction of detoxifying enzymes, which quickly inactivate applied herbicides (Persans et al., 2001; DeRidder et al., 2002). Differential screening in maize exposed to substituted

benzenesulfonamide safeners (Hershey and Stoner, 1991) led to the isolation of the safener-induced gene *In2-2* and its promoter. Use of the *In2-2* promoter fused to a herbicide-detoxifying enzyme could increase herbicide resistance only when herbicide application becomes necessary. Transformation with the *In2-2* promoter-reporter gene construct showed reporter gene induction in root and shoot apical meristem on plants grown in media or hydroponics containing safener, while foliar application did not show any reporter gene induction (De Veylder et al., 1997). Otherwise, expression was strictly correlated with the presence of safeners. However, this system is unsuitable for prolonged exposure to safeners due to inhibition of plant growth.

Most inducible/repressible promoter systems are two-component systems with molecular 'on-off' switches that vary in complexity. They include a transcription factor gene whose product, when selectively expressed, binds to a 'target' promoter that contains the transcription factor's *cis*-acting element. The target promoter is fused to the transgene of interest and the binding of the transcription factor modulates its expression. A copper-induced model uses a metallo-activated yeast transcription factor gene (*Ace1*) under the control of the CaMV 35S promoter, so that it is expressed constitutively. However, the ACE1 protein is only activated in the presence of copper or silver (Mett et al., 1993). When activated, it induces expression throughout the plant of the second target promoter:transgene whose chimeric promoter is composed of the CaMV 35S Domain A and the ACE1 transcription factor binding site. Activity of the GUS reporter gene in the leaves of transgenic tobacco increased up to 50-fold with the application of 0.5–50 μ M CuSO₄ to the plants. A copper system that allows for tissue-specific expression (nodule-specific) has also been developed (Mett et al., 1996). While long-term exposure of the plants to copper causes toxicity effects, it is suitable for transient expression studies (Granger and Cyr, 2001).

A second two-component system is the ethanol-inducible system based on a well-characterized alcohol regulon of the fungus *Aspergillus nidulans* (Felenbok et al., 1988). It is composed of the promoter of the alcohol dehydrogenase gene, *alcA*, and an alcohol-activated transcription factor gene (*alcR*) driven by the CaMV 35S promoter. The transcription factor (ALCR) binds in the presence of ethanol to specific sites in a chimeric *alcA*:CaMV 35S minimal promoter (TATA box region), activating transcription of a target gene (Caddick et al., 1998; Salter et al., 1998; Roslan et al., 2001). The *alc* system is extremely sensitive to ethanol and is induced rapidly and in a dose-dependent manner (Salter et al., 1998; Roslan et al., 2001). The target gene is induced by soil root drenching (0.5% v/v ethanol), hydroponics (0.1% v/v ethanol), and foliar spraying of mature plants (5% v/v ethanol). It has low to no basal expression of the target gene, and it has the advantage of an inducer that is inexpensive and nontoxic at the effective concentrations. Induction of target gene expression was similar to that of genes driven by the CaMV 35S promoter, but was reversible (Roslan et al., 2001). One drawback is that under anaerobic conditions, plants that are known to express alcohol dehydrogenase are likely to activate this system with the production of ethanol, a fermentation metabolite. However, this system appears to be one of the most promising for use under field and commercial conditions.

Tetracycline and hormone-inducible systems are also composed of two components. Hormone-derived systems take advantage

of the regulatory mechanisms of well-characterized vertebrate steroid receptors, and include estrogen (Zuo et al., 2000), ecdysone (Martinez et al., 1999), and glucocorticoid-based systems. Target gene promoters include tandem copies of *cis*-acting elements responsive to the co-transformed *trans*-activating factor, linked to minimal or strong promoters. The *trans*-activating factors are chimeric and can allow both inducible activation and repression. For example, in an inducible system developed by Aoyama and Chua (1997), the transcription factor includes the *trans*-activating domain of the *Herpes simplex* VP16 protein, the regulatory domain or hormone-binding domain of the glucocorticoid receptor, and the DNA-binding and *trans*-activating domains from the yeast transcription factor, GAL4. The expressed transcription factor binds to a target gene promoter composed of six copies of the GAL4 upstream activating sequences fused to a minimal (–46 bp) CaMV 35S promoter. It is activated by the presence of as little as 0.1 μ M dexamethasone, a strong synthetic glucocorticoid steroid.

A tetracycline-responsive gene expression system developed by Weinmann et al. (1994) is a promoter-repressing system. Its chimeric transcription factor is composed of the Tn10 encoded Tet repressor (TetR) and the VP16 activation domain. In the absence of tetracycline, the transcription factor binds to and activates an artificial promoter composed of 7 *tet* operators and a TATA box. However, the DNA-binding affinity of the TetR-VP16 transcription factor is eliminated in the presence of tetracycline, and transgene expression is repressed.

The glucocorticoid-inducible and tetracycline-repressible systems have also been merged, permitting greater control over gene expression (Bohner et al., 1999). While valuable for laboratory research applications, the hormone- and tetracycline-mediated systems will not be acceptable for commercial purposes because they include a gene from *Herpes simplex* and use antibiotics and human-associated steroid hormones as inducers. All of these components would be red-flag items for the anti-GEO movement.

Most published reports focus on the expression of a known target gene(s) and its effect on the plant system. In an elegant usage of an estradiol-inducible system, Bruce et al. (2000) were able to profile and characterize genes induced in the flavonoid pathway of maize. Activation of the anthocyanin pathway in maize is controlled by the Myb transcription factor homolog *C1* and DNA binding factors *R* or *B*. Genes within another flavonoid pathway that directs phlobaphene synthesis are activated by the single Myb homolog, *P*. These transcription factors trigger expression of specific flavonoid genes by binding to *cis*-acting elements in the genes' promoters. Cell suspension lines were developed from the maize inbred Black Mexican Sweet (BMS) and transformed with a constitutively expressed chimeric transcription factor made up of the estrogen receptor/maize Myb *C1* activator domain. These lines were then cotransformed with the Myb*C1*:*R* binding factor gene fusion or the Myb homolog *P* gene, under the control of a promoter containing four repeats of the estrogen receptor binding sites upstream of the CaMV 35S core promoter (–59 bp). While neither cell line showed any detectable amount of flavonoids before induction, they were detected within 12–24 h after addition of estradiol. RNA was isolated from control and induced lines and, using the GeneCalling process (Shimkets et al., 1999), unknown and known sequences associated with flavonoid expression were identified. The inducible activation of endogenous transcription factors is a powerful

technique for identification of genes involved in metabolic pathways and metabolite cascades.

SYNTHETIC OR ARTIFICIAL PROMOTERS

The continued study of individual enhancer elements and their involvement in the specifics of gene expression leads to an important question: Can one artificially select the specialized combinations of *cis*-acting elements within the promoter to allow greater control of transgene expression? The construction and use of synthetic or chimeric promoters can potentially give the researcher some known level of control. Construction of synthetic promoters takes advantage of the modular arrangement of *cis*-acting elements found within the enhancer regions of promoters although, without functional analysis, in most cases it is not known if individual *cis*-acting elements will retain their activity away from their native promoter. Synthetic promoters that have been created generally couple a basal or core promoter with composite, *cis*-acting elements that have the ability to function independently from their natural promoter. A popular basal promoter for use in the creation of synthetic plant promoters is the CaMV 35S promoter (Ulmasov et al., 1997; Vernhettes et al., 1997).

Pathogen-induced promoters are some of the best-studied promoters in plants. There is a large number of known pathogen-induced genes, and many similar *cis*-acting elements are found between their sequences. The W box ([T]TGAC[C/T]) is part of a major class of *cis*-elements responsible for the pathogen induction of multiple plant genes (Du and Chen, 2000). The GCC box (AGCCGCC) is also found in the promoters of numerous defense genes (Ohme-Takagi and Shinshi, 1995). An element similar to the GCC box, called an S box (AGCCACC), has been found in promoters induced by fungal elicitors (Kirsch et al., 2000). Using modules of these and other pathogen- and stress-related *cis*-elements, Rushton et al. (2002) have made and tested a number of synthetic pathogen-inducible promoters by fusing up to eight copies of each alone or in combination with other elements in front of the minimal CaMV 35S promoter and GUS reporter gene, and transforming *Arabidopsis*. The results shed light on how number, spacing, and combination of these elements affect the promoter's response to wounding, fungal penetration, and bacterial challenge, with encouraging results. Interestingly, none of the elements used in this study originated in *Arabidopsis*, which suggests that defense signaling is well conserved across species.

Transposable elements are believed to provide *cis*-acting regulatory elements that lead to changes in gene expression patterns, thus contributing to the evolution of genes and genomes (McDonald, 1990; Kidwell and Lisch, 1997). Because regulatory sequences in transposons and retrotransposons are selected to be adaptive in their host genomes, they are highly condensed and optimized in their activities and can be very useful in the construction of potential artificial promoters. The 13-base pair motif of the tobacco *Tto1* LTR retrotransposon promoter is a *cis*-acting regulatory element that confers temporal and spatial responsiveness to tissue culture, wounding, and methyl jasmonate when multiple copies were fused in front of the CaMV 35S (–46) minimal promoter (Takeda et al., 1999). Point mutations resulting in loss of function in the *Tto1* element demonstrated its regulatory abilities.

In contrast, highly active synthetic auxin response elements were created by point mutation in a natural composite AuxRE (auxin-responsive element) found in the soybean GH3 promoter (Ulmasov et al., 1997). A single base pair change resulted in a 25–50-fold induction by auxin compared to the native AuxRE. And while many enhancer elements have been associated with light induction, DE1, a 12-base pair *cis*-regulatory element fused to the minimal CaMV 35S (–46) promoter, was sufficient to confer dark induction when transformed into pea (Inaba et al., 2000).

Finally, a plant synthetic promoter would not be complete without the tobacco mosaic virus (TMV) omega sequence (Gallic and Walbot, 1992). The TMV omega 'enhancer' element is a 68bp leader sequence that contains numerous functionally redundant motifs and enhances translation of the transgene in both eukaryotes and prokaryotes. It does not appear to alter organ-specificity when fused to the CaMV 35S promoter (Holtorf et al., 1995).

CONCLUSION

The promoters covered in this review are but the tip of the genomic iceberg. New technologies, from transcriptomics to proteomics to genome sequencing projects will open up new vistas in the isolation of promoters tailored to answer specific questions in research or create new transgenic crops and products to feed and help the people of the world. Indeed, transcriptomics has the potential to rapidly increase our knowledge of spatial and temporal gene expression and lead to new promoters for research and development. Microarray analysis is a method for assessing plants, responses to any manner of manipulation (i.e., drought, salt, pathogen infection, cold, xenobiotic exposure...) by measuring global changes in RNA levels. Monitoring the expression profiles of thousands of genes in a single experiment is a powerful tool to search for genes responsive to the selected stimuli and to capture the promoters that drive the response of these genes. For example, microarrays have been used in the development of metal-specific promoters that have the potential to be used in the development of metal-specific biomonitors (Krizek et al., 2003). The power of microarrays coupled with plant genomic sequencing projects is that not only can stimuli- or tissue- or cell-specific promoters be discovered, but that promoters of similarly regulated genes can be mined for noncoding sequences that have been conserved during evolution due to functional constraints. These sequences will help us to identify candidate regulatory *cis*-acting elements and patterns of promoter evolution (Guo and Moose, 2003), indeed allowing researchers to become genetic or genomic archeologists.

The expanding field of bioinformatics will lead to computational analysis of primary structure and function of single promoters, helping define enhancer function and relationships with other conserved motifs in the genome *in silico*. Because only a small portion of an organism's genome is actually composed of transcribed sequences, there is a large, untapped resource of promoter sequences to be studied that can help focus on global views of the evolutionary relationship within and across species.

There are still untapped gene families that show potential for the development of new promoters because of their diversity of function and evolutionary conservation. The cytochrome P450 enzymes are a superfamily of more than 400 known members found in both eukaryotes and prokaryotes (<http://www.icgeb.trieste.it/~p450srv/new/p450.html>), and the glutathione *S*-transferase genes are a

multifunctional family of enzymes present both in plants and animals. Both of these gene families play a major role in the metabolism of numerous physiological substrates, as well as a wide array of xenobiotics, including drugs, chemical carcinogens, insecticides and herbicides, petroleum products, and other environmental pollutants. The promoters that drive these genes are induced by a wide array of natural chemicals and xenobiotics and can be valuable tools in the improvement of plants as environmental indicators, for phytoremediation, or for the metabolic engineering of medicinal products (Yun et al., 1992; Dixon and Steele, 1999). Their one drawback is their tendency to be regulated by the products of their metabolism, but this feature is one that should promote caution when developing any promoter for a specialized system.

The use and development of transgenic organisms for agricultural and industrial purposes has brought a unique focus to potential problems that could arise with public and environmental exposure. Questions have been raised and addressed about antigenic effects of transgene products, pollen drift and the transfer of transgenes to related wild species, and toxic or unintentional effects on nontarget organisms. Scientists have been challenged in finding solutions for these potential problems to calm public doubts and fears. The advent of many of these challenges has been beneficial, for example, leading to new methods of selection, greater testing of transgene products, and the development and potential use of promoters that target transgene products in such a way as to help allay fears. The intelligent use of this technology will include the development of carefully crafted transgenic organisms, which should include the selection of specialized promoters for the best expression of the transgene.

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