

Chapter 5

Levels and Stability of Expression of Transgenes

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5.1 Introduction

It is well known that in a given cell, at a particular time, only a fraction of the entire genome is expressed. Expression of a gene, nuclear, or organellar starts with the onset of transcription and ends in the synthesis of the functional protein. The regulation of gene expression is a complex process that requires the coordinated activity of different proteins and nucleic acids that ultimately determine whether a gene is transcribed, and if transcribed, whether it results in the production of a protein that develops a phenotype. The same also holds true for transgenic crops, which lie at the very core of insert design.

There are multiple checkpoints at which the expression of a gene can be regulated and controlled. Much of the emphasis of studies related to gene expression has been on regulation of gene transcription, and a number of methods are used to effect the control of gene expression. Controlling transgene expression for a commercially valuable trait is necessary to capture its value. Many gene functions are either lethal or produce severe deformity (resulting in loss of value) if over-expressed. Thus, expression of a transgene at a particular site or in response to a particular elicitor is always desirable.

Usually, the regions responsible for the initiation of transcription lie within the 5' region, upstream to the coding sequence of the gene. These are the promoter regions, defined as *cis*-acting (as they are on the same DNA strand that codes for the gene) nontranscribed elements, which provide sequences for the binding of various transcription initiation factors and RNA polymerase.

One can use a promoter that has known regulatory characteristics; for example, a promoter that is expressed throughout the plant tissue or only in vascular tissues, in

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the leaf epidermis, seed endosperm or embryo, and so on. One can mix and match fragments of DNA and transcription factors to develop chimeric promoters that have the desired patterns and levels of gene expression.

In this chapter, we will discuss the basic aspects of designing genes for insertion and quantifying transgene expression, followed by the different types of promoters and their use in transgenic crops. Also, several factors responsible for high-level expression and stability in transgenic plants/crops will be discussed.

5.2 Gene Design for Insertion

Once a gene of choice has been targeted and cloned, it has to undergo several modifications before it can be effectively inserted into a plant. A promoter sequence is added for the gene to be expressed. Most promoters used for transgenic crop varieties have been “constitutive,” i.e., causing gene expression throughout the life cycle of the plant in most of the tissues. The most commonly used constitutive promoter is CaMV 35S, from the cauliflower mosaic virus, which generally results in a high level of expression in most plants. Some promoters are more specific and are discussed in detail in Sect. 5.4.

Genes of interest are sometimes modified to achieve high level of expression. As plants prefer G-C rich regions, as compared to A-T rich bacterial genes, in order to overexpress bacterial genes in plants, A-T rich regions are to be substituted by G-C rich regions in such a way that the amino acid sequence of the protein remains unaltered (Evans et al. 2003). A selectable marker gene is inserted into the construct so as to identify the cells or tissues that have been successfully transformed (as discussed in Chap. 4). In some cases (e.g., resistance to pesticides), the transgene itself acts as a selectable marker. In other instances, a reporter gene is also inserted in the construct. A reporter gene is a coding sequence that upon expression in the transgenic plant provides conclusive evidence of genetic transformation. These reporter genes are very useful for transient expression experiments where the spatial and temporal activity of a promoter can be elucidated. The genes naturally exhibit an enzyme activity that does not exist in the host plant. Most common reporter genes are from bacteria, insects, or jellyfish as these organisms are so unrelated to angiosperms that their *cis*-regulatory elements are not functional in plants. Thus, when cloned into plant transformation vectors, a terminator sequence should also be fused downstream to the gene. Commonly used reporter genes are *CAT* (*E. coli*), β -*GUS* (*E. coli*), luciferase (firefly), green fluorescence protein (jellyfish), etc.

5.3 Quantification of Transgene Expression

It is necessary to know how a transgene is expressing in order to evaluate its effectiveness and level of expression in transgenic plants. The transgene copy number can greatly influence the expression level and genetic stability in the

plant, and therefore, estimation of the transgene number is of prime importance (Bhat and Srinivasan 2002). Previously, Southern and Northern analyses were used for this purpose (Sabelli and Shewry 1995a, b). But, with time, other methods such as comparative genomic hybridization, fluorescence *in situ* hybridization, multiplex amplifiable probe hybridization, and microarray had been employed to determine the transgene copy number. All these methods are time consuming, laborious, and require large quantities of DNA. Moreover, nucleic acid hybridization-based techniques often involve the application of hazardous radioisotopes.

Recently, quantitative real-time PCR (qPCR) has proved to be an efficient method for transgene expression studies in plants. In qPCR, expression level is monitored per cycle of the reaction, comparing the fluorescent signal generated by the DNA or mRNA sample proportional to its initial quantity (Page and Minocha 2004). It has proved to be a more sensitive and rapid method, providing stringent evaluation through the use of SYBR Green I Fluorescent intercalating dye, which has the ability to detect a single gene among a number of genes in combination with highly specific gene primers. Till now, qPCR has been extensively applied to several transgenic crops such as maize (Ingham et al. 2001; Song et al. 2002; Shou et al. 2004; Assem and Hassan 2008), wheat (Li et al. 2004), rice (Yang et al. 2005), potato (Toplak et al. 2004), rapeseed (Weng et al. 2004), tomato (Mason et al. 2001), tobacco (Miyamoto et al. 2000), cassava (Beltrán et al. 2009), and strawberry (Schaart et al. 2002) for analyzing transgene expression.

5.4 Promoters

As discussed earlier, the 5' upstream regions of a gene are not transcribed but provide sites for attachment of transcription initiation factors. The promoter itself contains many elements (short regions of a defined DNA sequence) for initiation factor attachment. The very basic of these elements is the TATA box, which is present about 25–30 bp upstream of the transcription start site and is primarily responsible for the correct positioning of RNA polymerase II. Many genes contain multiple operational TATA boxes, for example, three for *inrpk1* gene in *Ipomoea nil* (Bassett et al. 2004) and three for *phas* gene in *Phaseolus vulgaris* (Grace et al. 2004). It was thought earlier that the absence of TATA box is associated with constitutively expressing housekeeping genes, but recently TATA box was found to be absent in some inducible genes as well. Apart from TATA box, CAAT and GC boxes are also found to be present upstream; they too enhance the activity of RNA polymerase. Sequence elements like TATA boxes are also referred to as minimal or core promoter elements.

Along with the core promoter elements, other sequence elements are also found that provide sites for attachment of specific transcription factors or enhancer binding protein that trigger transcription of the gene (Alberts et al. 2002). These sequence elements are also called regulatory elements, enhancer binding elements, or simply enhancers. Enhancers are consensus DNA sequence motifs and are

associated with levels, place, and timing of expression in response to internal or external (biotic or abiotic) factors. Enhancers can be located upstream, downstream, within coding regions or even in the intron sequences. One of the chief factors responsible for control of gene expression at the transcription level is the activation of enhancer sequences. A few *cis*-elements have the ability to silence or repress expression of the gene; these are called silencers. The activities of some of the plant promoters are summarized in Table 5.1. The table was generated using TGP, PlantCARE, NCBI, and Plant-Promoter databases for different genes specifically expressed in plants under the influence of suitable reporters/inducers, resulting in higher expression.

5.4.1 Types of Promoters and Their Applications in Transgenic Crops

5.4.1.1 Constitutive Promoters

Constitutive promoters maintain a constant level of activity. The cauliflower mosaic virus (CaMV) 35S promoter (derived from a DNA viral genome) is probably the most widely used plant promoter (Odell et al. 1985). Although “constitutive,” many show differences in the level of expression in different tissues. Apart from delivering very high levels of expression in virtually all regions of the transgenic plant, the CaMV 35S promoter is easily obtainable for research purposes as plant transformation vector cassettes that allow for easy subcloning of the insert transgene of interest.

High levels of transgene expression can be achieved by the CaMV 35S promoter in both monocot and dicot plants (Benfey et al. 1990; Battraw and Hall 1990). The original full-size promoter (−941 to +9) has no significant difference in activity when compared to a −243 bp fragment. Interaction between the *cis*-acting elements within 343 bp upstream of the promoter results in high constitutive expression (Fang et al. 1989). However, tissue-specific individual elements have also been found (Benfey and Chua 1989). For the control of expression in specific tissues, two domains “Domain A” (−90 to +8) and “Domain B” (−343 to −90) are very important. Domain A is involved in expression in roots (Lam et al. 1989), while Domain B contains a conserved GATA motif, very much similar to the light responsive *cis* elements of light inducible promoters (Potenza et al. 2004). Even though CaMV 35S is a very strong promoter, it is strongly down-regulated in plant parasitic nematode feeding sites (Urwin et al. 1997). With the success of CaMV 35S promoter, other viral promoters have also been developed. They include the cassava vein mosaic virus (CsVMV; Verdaguer et al. 1996, 1998; Li et al. 2001), Australian banana streak virus (BSV; Schenk et al. 2001), mirabilis mosaic virus (MMV; Dey and Maiti 1999), and figwort mosaic virus (FMV; Maiti et al. 1997) promoters.

Table 5.1 Promoters used for transgene expression in plants

Promoter	Gene	Specificity	Reporter/inducer	Comment	References
At:SAG12_P1, P2	<i>sag12</i> , senescence-associated gene	transgenic <i>Arabidopsis</i> old leaves	mRNA, GUS activity/auxin, cytokinin, sugar	Essential for senescence-specific regulation of <i>sag12</i>	Noh and Amasino (1999)
Ms:PEPC7_P1, P2	<i>pepc7</i> , <i>pepc</i>	transgenic alfalfa plants	GUS activity/Rhizobium	very high levels of GUS activity	Pathirana et al. (1997)
Nt:COMTII_P1, P2, P3	<i>comtII</i>	transgenic tobacco leaves	GUS activity/chitin, glucan, TMV, pectin, wounding, MeJa	two to seven-fold increase in GUS activity	Toquin et al. (2003)
Ib:BMV1_P1, P2	<i>bmy1</i> , <i>anyb</i> , <i>beta-amy</i>	transgenic tobacco plants	GUS activity/sucrose (10%)	20–57-fold higher GUS activity	Maeo et al. (2001)
Dc:HCBT2_P1 P2, P3, P4	<i>hcbt2</i>	transgenic parsley protoplasts	GUS activity/elicitor	3.7–5.5-fold higher GUS activity	Yang et al. (1998)
Le:E4_P1, P2	<i>e4</i>	transgenic tomato plants	GUS activity/Ethylene	Increased 10–22-fold in unripe and 1,000-fold in ripened fruit	Montgomery et al. (1993a)
Zm:SBE1_P1, P2	<i>sbe1</i>	transgenic maize suspension endosperm cells	LUC activity/sucrose (9%)	two-fold greater LUC activity	Kim and Guiltman (1999)
Vf:GRP3_P1, P2, P3	<i>grp3</i> , <i>glycine-rich early nodulin</i>	transgenic <i>Vicia hirsute</i> (5 week-old) nodules	GUS activity/Rhizobium	Essential for full promoter activity	Kuster et al. (1995)
Nt:CHN48_P1, P2	<i>chn48</i>	transgenic tobacco calli	LUC activity/elicitor	10–40-fold higher activity	Yamamoto et al. (1999)
At:P5CSA_P1, P2	<i>P5CSA_P1</i>	transgenic <i>Arabidopsis</i> plants	GUS activity/dehydration	five-fold increase in activity	Yoshihira et al. (1999)
Ib:SPOA1_P1, P2	<i>spoa1</i> , <i>gspsa-1</i>	transgenic tobacco plants	GUS activity/sucrose (3%)	tissue specific GUS staining	Ohta et al. (1991)
Pc:CMPPG1b_P1	<i>cmppg1</i> , <i>eli17</i>	transgenic parsley suspension cells	GUS activity/elicitor	44-fold higher activity	Kirsch et al. (2001)
Pc:PR2_P1, P2	<i>pr2</i>	transgenic parsley protoplasts	GUS activity/elicitor	three- to eight-fold higher activity	van de Löcht et al. (1990)

(continued)

Table 5.1 (continued)

Promoter	Gene	Specificity	Reporter/inducer	Comment	References
Pc:PR1.1_P1, P2, P3	<i>pr1.1</i>	transgenic parsley suspension cells	GUS activity/elicitor	7.5–12.1-fold higher activity	Rushton et al. (1996)
Pc:PR1.2_P1, P2	<i>pr1.2</i>	transgenic parsley suspension cells	GUS activity/elicitor	5.2–15.2-fold higher activity	Rushton et al. (1996)
Ps:PSL_P1, P2, P3	<i>psl</i>	transgenic tobacco seeds	GUS activity, protein	High level of expression	de Pater et al. (1996)
Lg:LHCB2_P1, P2, P3, P4, P5	<i>lhcb2, cabAB19</i>	transgenic swollen duckweed seedlings	LUC activity/red light (2 min)	2–14-fold higher activity	Kehoe et al. (1994)
Np:LHCB1.2_P1, P2, P3, P4, P5	<i>lhcb1.2</i>	transgenic tobacco seedlings, transgenic <i>Arabidopsis</i> seedlings	GUS activity/far-red light (cont.), red light (pulses 3 min)	Medium 8–20-fold higher expression level	Cerdan et al. (2000)
Os:Amy1A_P1, P2, P3, P4	<i>amy1A</i>	transgenic rice embryos	GUS activity/Glucose, gibberellin A3	two- to six-fold lower expression with glucose, higher with gibberellin A3	Morita et al. (1998)
Os:Amy3D_P1	<i>amy3D</i>	transgenic rice embryos	GUS activity/glucose	six-fold lower Expression	Morita et al. (1998)
Zm:SH1_P1	<i>sh1</i>	transgenic tobacco plants	GUS activity/anaerobic conditions	High level of expression	Yang and Russell (1990)
At:CAB3_P1, P2, P3, P4	<i>cab3</i>	transgenic tobacco shoots (3–4 week-old), suspension cells	CAT activity/white light	Medium to high level of expression	Mitra et al. (1989)
Pc:WRKY1_P1, P2, P3	<i>wrky1</i>	transgenic parsley protoplasts	GUS activity/elicitor	2–50-fold higher activity	Eulgem et al. (1999)
Hv:LOXA_P1, P2, P3	<i>loxA</i>	transgenic barley leaves	GUS activity/methyl jasmonate (MeJA)	11.9–19.4-fold higher expression	Rouster et al. (1997)
Cr:CPR_P1	<i>crp</i>	transgenic tobacco plants	GUS activity/elicitor	Essential for controlled expression	Cardoso et al. (1997)
Nt:BGLUCANASE_P1, P2	<i>gln2, gg/b50</i>	transgenic tobacco plants, transgenic tobacco plants (1 month-old)	GUS activity/tobacco mosaic virus, salicylic acid, ethephon (ethylene), water	2–10-fold higher activity	van de Rhee et al. (1993), Livne et al. (1997)

Nt:PR2D_P1, P2, P3	<i>pr2d</i>	transgenic tobacco plants	GUS activity/tobacco mosaic virus, salicylic acid, water	2–18-fold higher activity	Hennig et al. (1993)
Nt:PR2D_P2	<i>pr2b</i>	transgenic tobacco plants	GUS activity/tobacco mosaic virus, salicylic acid, water	five- to nine-fold higher activity	van de Rhee et al. (1993)
Np:GN1_P1	<i>gn1</i>	transgenic tobacco plants	GUS activity/SA, ethylene, water, elicitor, wounding	1.7–21-fold higher activity	Castresana et al. (1990)
At:CEL5_P1	<i>At1g22880</i>	transgenic <i>Arabidopsis</i> seedlings	GUS activity/auxin, ABA	Effective GUS staining activity	del Campillo et al. (2004)
As:PHYA3_P1, P2	<i>phyA3</i>	transgenic etiolated rice seedlings (2 day-old)	CAT activity/far-red light	five-fold higher activity	Bruce and Quail (1990)
St:GLUB_P1	<i>gluB8-1-3</i>	transgenic potato plants, transgenic tobacco plants	GUS activity/pathogen, tobacco mosaic virus, elicitor	2–12-fold higher activity	Mac et al. (2004)
Hv:GHII_P1	<i>GIII</i>	transgenic rice plants, transgenic rice calli	GUS activity/salicylic acid	Fragment length dependent GUS activity	Li et al. (2005)
Nt:PR1A_P1, P2	<i>pr1A</i>	transgenic tobacco plants	GUS activity/salicylic acid, tobacco mosaic virus	58–110-fold higher activity	Strompen et al. (1998), Uknes et al. (1993)
At:CYP85A1_P1	<i>cyp85A1</i>	transgenic <i>Arabidopsis</i> seedlings	GUS activity/Brassinolide	weak activity	Castle et al. (2005)
At:APX1_P1, P2, P3	<i>apx1</i>	transgenic <i>Arabidopsis</i> seedlings (2 week-old), (10 day-old)	GUS mRNA, GUS activity/ heat shock, methyl viologen, iron	Effective GUS staining	Storozhenko et al. (1998), Fourcroy et al. (2004)
At:APX2_P1	<i>apx2</i>	transgenic <i>Arabidopsis</i> plants	LUC activity/hydrogen peroxide (H ₂ O ₂), high light	ten-fold higher activity	Kimura et al. (2001)

(continued)

Table 5.1 (continued)

Promoter	Gene	Specificity	Reporter/inducer	Comment	References
At:APX2_P2	<i>apx1b</i>	transgenic tobacco mesophyll protoplasts	GUS activity/Heat Stress transcription Factor (HsfA2)	ten-fold higher activity	Schramm et al. (2006)
At:ATHB6_P1	<i>athb6</i>	transgenic <i>Arabidopsis</i> seedlings, transgenic <i>Arabidopsis</i> plants	GUS activity/drought, abscisic acid (ABA), salt	increased GUS staining with high level expression	Söderman et al. (1999)
At:CYP85A2_P1	<i>cyp85A2</i>	transgenic <i>Arabidopsis</i> seedlings	GUS activity/brassinolide	promoter activity down-regulated by brassinolide	Castle et al. (2005)
At:ELIP2_P1	<i>elip2</i>	transgenic <i>Arabidopsis</i> seedlings (10–14 day-old)	LUC activity/high light	100-fold increased expression	Kimura et al. (2001)
At:Fer1_P1	<i>fer1</i>	transgenic <i>Arabidopsis</i> plants, transgenic <i>Arabidopsis</i> cells	GUS activity/iron, senescence	17-fold derepression in response to 0.5 mM iron citrate	Tarantino et al. (2003)
At:Fer1_P2	<i>fer1</i>	transgenic <i>Arabidopsis</i> cells	GUS activity/iron	six-fold higher activity in response to 0.5 mM iron citrate	Petit et al. (2001)
Ca:Chi2_P1, P2, P3	<i>chi2</i>	transgenic tobacco	GUS activity/infection, mannitol, salt, NaCl, salicylic acid	1.5–4.5-fold higher activity	Hong and Hwang (2006)
Gm:Fer_P1, P2	<i>fer</i>	transgenic soybean leaves	LUC activity, GUS activity/iron	iron dependent GUS activity	Wei and Theil (2000)
Hv:BLT101.1_P1, P2, P3	<i>blt101.1</i>	transgenic barley	GUS activity/low temperature	2.5-fold higher expression at low temperature	Brown et al. (2001)
Hv:BLT4.9_P1, P2, P3	<i>blt4.9</i>	transgenic barley	GUS activity/low temperature	2.5–6-fold higher expression at low temperature	Dunn et al. (1998)
POPLA:PAL1_P1	<i>pal1</i>	transgenic tobacco	GUS activity	Differential GUS activity	Gray-Mitsumune et al. (1999)

POPLA:PAL2_P1	<i>pal2</i>	transgenic tobacco, transgenic poplar	GUS activity	Differential GUS activity	Gray-Mitsumune et al. (1999)
Ta:GERMIN_P1	<i>gf-2.8</i>	transgenic tobacco seedlings, transgenic tobacco plants	GUS activity/heavy metal, cadmium, copper, cobalt, wounding, TMV	Gus activity detected after induction	Berna and Bernier (1999)
Zm:Fer1_P1	<i>fer1</i>	transgenic maize cells (BMS, Black Mexican Sweet)	GUS activity/iron	2.5–8-fold increase in activity	Petit et al. (2001)
At:FPS2_P1	<i>fps2</i>	transgenic <i>Arabidopsis</i> plants, transgenic <i>Arabidopsis</i> protoplasts	GUS activity	Differential GUS activity	Cumillera et al. (2000)
At:PAL1_P1, P2	<i>pal1</i>	transgenic <i>Arabidopsis</i> plants (3 week-old), transgenic tobacco plants (6 leaf stage)	GUS activity, GUS mRNA, PAL mRNA/wounding, HgCl ₂ , white light, elicitor, H ₂ O ₂ , mitomycin C (MMC)	30% increase in GUS activity	Ohl et al. (1990)
At:PDF1.2_P1	<i>pdf1.2</i>	transgenic <i>Arabidopsis</i> seedlings (10 day-old), transgenic <i>Arabidopsis</i> plants (4 week-old), transgenic tobacco seedlings (2 week-old), (6 week-old)	GUS activity/jasmonic acid (JA), pathogen, elicitor, methyl jasmonic acid (MeJA), paraquat, methyl viologen, rose bengal, TMV, ethylene	9–25-fold higher activity	Manners et al. (1998)
At:RAD54_P1	<i>atrad54</i>	transgenic <i>Arabidopsis</i> seedlings (1 week-old)	GUS mRNA/gamma irradiation	GUS activity detected after gamma irradiation	Osakabe et al. (2006)
At:THI1_P1, P2	<i>thi1</i>	transgenic <i>Arabidopsis</i> plants (14 day-old)	GUS activity/white light, flooding, salt, sugar deprivation	two to six-fold higher activity	Ribeiro et al. (2005)

(continued)

Table 5.1 (continued)

Promoter	Gene	Specificity	Reporter/inducer	Comment	References
Gm:SCAM4_P1	<i>cam-4</i>	transgenic <i>Arabidopsis</i> seedlings (2–4 day-old), transgenic <i>Arabidopsis</i> leaf protoplasts, transgenic <i>Arabidopsis</i> plants (4 week-old)	GUS mRNA, GUS activity/ salt, glycol chitin, Ca ²⁺ -ionophore A23187, elicitor, pathogen	3–15-fold higher activity	Park et al. (2004)
Le:LAT52_P1	<i>lat52</i>	transgenic tobacco mature pollen	GUS activity, LUC activity	Essential for full promoter activity	Bate and Twell (1998)
Zm:GAPC4_P1	<i>gapc4</i> , <i>gpc4</i>	transgenic tobacco leaves	GUS activity/anaerobic conditions	seven-fold higher activity	Geffers et al. (2001)
Nt:SAR8.2B_P1, P2, P3	<i>sar8.2b</i>	transgenic <i>Arabidopsis</i> plants (3 week-old)	GUS activity/Salicylic acid	4–31-fold higher GUS activity	Song et al. (2002)
Nt:PMT1A_P1	<i>pmt1a</i>	transgenic tobacco suspension cells	GUS activity/ (MeJA), ethephon	10–15-fold higher activity	Xu and Timko (2004)
Nt:G10_P1	<i>g10</i> , tobacco late pollen gene <i>g10</i>	transgenic tobacco pollen and leaves	GUS activity	Essential for full promoter activity	Rogers et al. (2001)
Ps:DRR206D_P1	<i>drr206-d</i> , <i>pl206</i>	transgenic tobacco plants (6-leaf stage)	GUS activity/Mitomycin C, actinomycin D, etoposide, H ₂ O ₂ , elicitor	Promoter only induced by natural tobacco Pathogen	Choi et al. (2001)
Nt:AP24_P1, P2	<i>ap24</i>	transgenic tobacco seedlings, transgenic tobacco plants	GUS activity/ethylene, NaCl, abscisic acid	2.5–13-fold higher Activity	Raghothama et al (1997)
At:OPR1_P1	<i>opr1</i>	transgenic <i>Arabidopsis</i> seedlings (2-week old)	GUS activity/Methyl jasmonate (MeJa), senescence	2–3.5-fold higher activity	He and Gan (2001)
Sc:OSML13_P1 Sc:OSML81_P1	<i>osml13</i> <i>osml81</i>	transgenic potato plants	GUS activity/ABA, NaCl, SA, wounding, fungal infection, cold	Differential GUS activity	Zhu et al. (1995)
Sc:C121A_P1, P2	<i>c121a</i>	transgenic potato plants	GUS activity/low temperature (cold)	1.7–7-fold higher activity	Schneider et al. (1997)

There are a few limitations in the use of virus-derived promoters: first, the potential risk to human health from the genes of infective plant viruses (Hodgson 2000), and second, the ability of plant cells to recognize the inserted sequences of nonplant origin and inactivate them via “transcriptional gene silencing.” Silencing is less common in promoters of plant origins.

Strong constitutive promoters of plant origin have also been isolated and used for the development of transgenic plants. Actin, a fundamental cytoskeleton component of the cell, is expressed in almost all the cells of a plant. The Act2 promoter, developed from *Arabidopsis* showed strong expression in all other parts except the seed coat, hypocotyl, ovary, and pollen sac (An et al. 1996). Similarly, rice Act1 promoter has also been developed (Zhang et al. 1991). Ubiquitins, a highly conserved protein family, are linked to many important cellular functions like chromatin structure and DNA repair. Maize ubiquitin 1 promoter (pUbi) has been successfully used for plant transformation of monocots (Weeks et al. 1993; Gupta et al. 2001) and has shown high levels of expression in actively dividing cells. Transgenic plants developed using Ubi.U4 promoter from *Nicotiana sylvestris* showed a three-fold higher activity when compared to the CaMV-based promoters. The ubiquitin-derived promoters perform very well in metabolically and mitotically active cells.

The constitutive action of a promoter has many drawbacks. Expression (or overexpression) of the transgene at a place where it is not expressed or expressed at a wrong time can have severe consequences on the growth and development of the plant. It can lead to enhanced susceptibility to some pathogens (Berrocal-Lobo et al. 2002) or decreased growth (Bowling et al. 1997). Another concern is the development of resistance by target insects against overexpressed toxins like, e.g., *Bt* toxin (Huang et al. 1999). For these reasons, it is ideal to strategically develop promoters that are “switched on” precisely when they are needed.

5.4.1.2 Nonconstitutive (Tissue-Enhanced) Promoters

Development of a new trait or value-addition of a previously existing one by genetic engineering requires the development of transgenes that are under control and expressed in a tissue-specific, developmental, or inducible manner. This will conserve energy and circumvent the drawbacks associated with constitutive expression to a large extent. It is more realistic to call these promoters tissue enhanced rather than tissue specific as their expression may not be confined to a specific tissue or plant part. Tissue-enhanced gene expression pattern is achieved as a result of several factors executed at various levels of gene control. Also, the more distant 5'-*cis* acting enhancer element may be eliminated during isolation of the promoter, and there may not be effective functional interaction between the promoter *cis*-elements with the heterologous *trans*-acting factors present in the transgenic host plant. Thus, the development of such promoters can be very complex and difficult. Because of these complexities, it is preferable to use promoters from homologous or closely related plant taxa, and knowledge about

the functionality of both homologous and heterologous promoters in target crop plants is essential.

Roots

These promoters are of high interest as they can be used for multiple applications. Expression of proteins responsible for resistance against drought and salt tolerance, resistance to bacterial (or fungal) pathogens or nematodes (Atkinson et al. 2003), and phytoremediation (Grichko et al. 2000) can improve crop yield. Although root-specific promoters have been isolated in plants (Yamamoto et al. 1991; Liu and Ekramoddoullah 2003), other stress-related studies have identified many candidate genes and their promoters. In maize, bacterial promoters are more popular (Qing et al. 2009). *Agrobacterium rhizogenes* causes hairy root disease in dicots. The promoters for rooting loci genes (*rol*) present in the root-inducing (Ri) plasmids are largely studied because of their root-mediated transformation and expression of the transgenes. Most important of the *rol* promoters is the *rolD* promoter, which has been much utilized (Stearns et al. 2005; Jayaraj et al. 2008) and extensively used in nitrogen assimilation studies (Fraisier et al. 2000; Fei et al. 2003). Very high levels of *rolD* promoter activity have been reported earlier (Elmayan and Tepfer 1995), but recently, in a comparative study, it was reported that *Arabidopsis* ubiquitin promoter (UBQ3) has the highest expression in roots (Wally et al. 2008). The domain A (−90 bp upstream) of CaMV 35S also shows root-specific activity (Benfey and Chua 1989; Benfey et al. 1990).

TobRB7, a putative membrane channel aquaporin, is another valuable plant based root-specific promoter isolated from tobacco (Yamamoto et al. 1991). Root-specific activity of this promoter was observed within 2 days of germination. Recently, a novel gene has been isolated from tomato, having very high expression levels in roots (*SIREO*); the 2.4-kb region representing the *SIREO* promoter sequence showed strict root specificity (Jones et al. 2008).

Root Nodules

Root nodules are formed as a result of an endosymbiotic association between *Rhizobium* and other species of bacteria with leguminous host plants. Within the nodule, the bacteroids fix atmospheric nitrogen that is used by the plant and in return, receive carbon substrates from the plant. This type of symbiosis is well studied. Leghemoglobin is an oxygen-binding protein synthesized in the nodule. The expression of leghemoglobin coincides with the nitrogen fixation in the nodule. Thus, this promoter can be used in nodules to increase nitrogen assimilation. The leghemoglobin promoter *glb3* from *Sesbania rostrata* was expressed in *Lotus corniculatus* and tobacco-harboring chimeric *glb3-uidA* (*gus*) gene fusions (Szabados et al. 1990; Szczyglowski et al. 1996). A 1.9-kb fragment of the *glb3* 5'-upstream

region was found to direct high level of nodule-specific β -glucuronidase (GUS) activity in *L. corniculatus* that is restricted to the *Rhizobium*-infected cells of the nodules. In tobacco (a nonleguminous plant), the activity was restricted primarily to the roots and to phloem cells of the stem and petiole vascular system. A deletion analysis revealed that the region between -429 and -48 bp relative to the ATG was effective for nodule-specific expression.

Tubers

Tubers are storage organs in roots and are staple food source in many countries of the world. Improvement of tuber nutrition value, resistance toward infectious disease and pesticides can be manifested by using tuber-specific expression of transgenes. Patatins are glycoproteins that are one of the major products found in potato tuber. These are tuber-specific and can be induced by sucrose (Jefferson et al. 1990). The patatin promoters *Pat1* and *Pat2* were used to overexpress transgenes from the minipathway, of bacterial origin, to drive the synthesis of β -carotene (Provitamin A) in vitamin-A-deficient tubers of potato (Diretto et al. 2007). To enhance the metabolism of the environmental contaminants in tubers the rat P450 monooxygenase gene (*CYP1A1*) was overexpressed in tubers of potato, also under the control of patatin promoter (Yamada et al. 2002). High transgene expression was seen in developing tubers, and the amount of residual herbicides was much lower than that in nontransgenic plants, indicating that the transgenic plant metabolized and detoxified the herbicides. The processing quality of potato products (fries and chips) was increased by overexpressing transgenes in potato tuber under the control of TSSR (tuber-specific and sucrose-responsive) sequence from potato class I patatin promoter (Zhu et al. 2008). It was also demonstrated that tuber-specific expression of the native and slightly modified *MYB* transcription factor gene *StMtf1(M)* activates the phenylpropanoid biosynthetic pathway. The transgenic potato tubers contained four-fold increased levels of caffeoylquinates, including chlorogenic acid while also accumulating various flavonols and anthocyanins (Rommens et al. 2008). An 800-bp 5' upstream sequence of the granule-bound starch synthase (*GBSS*) gene from potato was highly expressed in stolons and tubers (Visser et al. 1991), where the activities of the transgene in these two organs were 3–25-fold higher than the expression of the CaMV-*GUS* gene. The *GBSS* gene promoter was also used to obtain tuber-specific high expression of *AmA1*, a nonallergenic seed albumin gene from *Amaranthus hypochondriacus* in potato (Chakraborty et al. 2000). Two promoters, sporamin and β -amylase, have been well characterized in sweet potato (Maeo et al. 2001). The sweet potato sporamin promoter was found to control the expression of the *E. coli* *appA* gene in transgenic potato, which encoded a bifunctional enzyme exhibiting both acid phosphatase and phytase activities (Hong et al. 2008). Phytase expression levels in transgenic potato tubers were stable over several cycles of propagation. The study demonstrated that the sporamin promoter can effectively direct high-level recombinant protein expression in potato tubers. Moreover,

overexpression of phytase in transgenic potato offers an ideal feed additive for improving phytate-Phosphorous digestibility in monogastric animals along with improvement of tuber yield, enhanced Phosphorous acquisition from organic fertilizers, and has a potential for phytoremediation.

Leaves

The light received in the environment can be roughly categorized as UV, visible, and far red. Three classes of photoreceptors have been identified in higher plants: red light and far-red light absorbing phytochromes (PHYs), blue-light receptors, and UV-light receptors. In *Arabidopsis*, five members compose the PHY family of photoreceptors (PHY A-E) and at least three different blue light photoreceptors have been identified [cryptochromes (CRYs), NPH1, and NPL1] (Martínez-Hernández et al. 2002). These photoreceptors, with association of other molecular systems (transcription factors), control the expression of many genes at the transcriptional and post-transcriptional level. Two important transcription factors are basic Leucine zipper factor HY5 (Oyama et al. 1997) and bHLH factor PIF3 (Martínez-García et al. 2000).

The photosynthesis-associated nuclear genes (*PhANGS*), like the chlorophyll a/b-binding proteins (Cab) and the small subunit of Rubisco (RbcS), contain a number of *cis*-acting elements, the transcription of which is controlled by light. Some of the motifs like G, I, and GTI boxes are found in the promoter regions of many light-regulated genes (Giuliano et al. 1988; Green et al. 1988; Menkens et al. 1995). The LS5-LS7 region from the *Lemna gibba Cab19* gene (Kehoe et al. 1994) and the CGF-1 factor-binding site from the *Arabidopsis CAB2* gene (Anderson and Kay 1995) contain the GATA and GT-1 sequences; still these two regions are unable to activate transcription, thus suggesting that additional regulatory elements are involved. This has led to the general hypothesis that light-responsive elements (LREs) are formed by the aggregation of different transcription factors. It has also been shown that artificial sequences composed of paired combinations of tetrameric repeats of G- and GATA boxes or GT1- and GATA-boxes, but not multimers of a single motif, function as LREs (Puente et al. 1996). Monocot *rbcS* promoters have different *cis*-acting elements and have different patterns of spatial expression than dicots. The C3 *rbcS* is specifically expressed in mesophyll cells, while the C4 *rbcS* is expressed in bundle sheath cells, and not in mesophyll cells (Nomura et al. 2000; Patel and Berry 2008). Overexpression of *Arabidopsis* phytochrome A (PHYA), under the control of *rbcS* promoter, in commercially important rice varieties produced an increased number of panicles per plant (Garg et al. 2006). In an attempt to obtain high-level production of intact *Acidotherrmus cellulolyticus* endoglucanase (E1) in transgenic tobacco plants using the constitutive (Mac) as well as light-inducible tomato Rubisco small subunit promoter (*RbcS-3C*), it was observed that *RbcS-3* promoter was more favorable for E1 expression in transgenic plants than the Mac promoter (Dai et al. 2005). Moreover, by replacing *RbcS-3C* UTL with AMV RNA4 UTL, E1 production was enhanced more than two-fold. In a

comparative study of the expression pattern of heterologous RbcS, RbcS3CP (0.8 kbp) from tomato, SRS1P (1.5 kbp) from soybean, and CaMV 35S in apple, it was found that the activity of SRS1P promoter was strictly dependent on light, whereas that of the RbcS-3C promoter appeared not to be so (Gittins et al. 2000). Later rolCP and CoYMVP were used for expression in vegetative tissues of apple; the CoYMV promoter was slightly more active than the rolC promoter, although expression was at a lower level than the CaMV 35S promoter (Gittins et al. 2003). The results indicated that both promoters could be suitable to drive the expression of transgenes to combat pests and diseases of apple that are dependent on interaction with the phloem.

The Cab proteins are highly expressed in green tissues and are often associated with other proteins to form the light-harvesting complex (Lhc). The expression pattern of the *Cab* gene in plants is different from that of the RbcS under certain physiological conditions as response to light quality and diurnal rhythm is different between these two genes (Ha and An 1988). Upon analysis of regulatory elements of *Cab-E* gene from *Nicotiana plumbaginifolia*, three positive and one negative *cis*-acting elements that influence photoregulation were found and of the three positive promoters two (PRE1 and PRE2) confer maximum level of photoregulation (Castresana et al. 1988). Tobacco plants when transformed with a chimeric gene encoding the A1 subunit of cholera toxin regulated by wheat Cab-1 promoter greatly reduced susceptibility to the bacterial pathogen *Pseudomonas tabaci* (Beffa et al. 1995).

Both RbcS and Cab are members of a multigene family and are expressed at very high level in green tissues (especially leaves), but many genes within the family contribute to the total protein content. Thus, the level of transgene expression is potentially dependent on the gene promoter used, so a strong green tissue-specific promoter from a single gene family will be most valuable.

Flowers

A substantial economic market has developed for cut flowers. Floral-specific promoters are therefore important for use in engineering transgenic flower varieties that may enhance vase life, visually appealing character of the flowers (reviewed by Mol et al. 1999) along with fragrance of interest and resistance to pests (Dolgov et al. 1995). The UEP1 promoter from Chrysanthemum when fused with a reporter gene (*GUS*) and transformed back into Chrysanthemum showed very high levels of expression in ray florets and three-fold lower expression in disk florets (Annadana et al. 2002). The activity of UEP1 promoter in ray florets is limited to petal tissues and does not extend into the tube of the petal or the sexual whorls of the floret. The promoter had 50-fold higher expression when compared with double CaMV-based promoters in petal tissues of ray florets (Annadana et al. 2002). This study also showed that CER6 promoter, associated with the wax biosynthesis pathway, had very high expression in ray florets, but the expression was much variable when compared to the UEP1 promoter.

Flavonoids are common color pigments in flowers and also perform many other functions including signaling and UV-protection. Engineering of the flavonoid biosynthetic pathway has led to the development of blue carnations (Holton 1995) and blue roses (Katsumoto et al. 2007). Chalcone synthase (CHS) genes as well as promoters have been studied extensively. The French bean CHS15 promoter showed expression in flowers and root tips of transformed tobacco plants (Faktor et al. 1996). In flowers, expression was confined to the pigmented part of petals and was induced in a transient fashion. Floral and root-specific expression required two conserved motifs, G-box and H-box, located near the TATA box. To evaluate the tissue-specific role of these motifs, a 39-bp DNA fragment containing the two motifs was prepared and fused with minimal promoters of CHS15 and CaMV 35S along with a marker gene (*GUS*). Tobacco plants were transformed and it was observed that the 39-bp polymer confers, upon both minimal promoters, a high level of expression that follows the typical tissue-specific expression pattern (Faktor et al. 1997). A chromoplast-specific carotenoid-associated gene (*OgCHRC*) and its promoter (*Pchrc*) was isolated from an orchid species (*Oncidium*), which showed very high and had flower-specific expression (Chiou et al. 2008).

Pistils

Pistil comprises the female part of the flower and includes stigma, style, and ovary. Identification of ovule-specific promoters is useful for the genetic engineering of crops with a variety of desirable traits, such as genetically engineered parthenocarp, female sterility, or seedless fruits. The *SK2* gene from *Solanum tuberosum* encodes a pistil-specific endochitinase; the promoter from this gene was fused with a reporter (*GUS*) and when transformed back into potato, high-level expression specific to pistil was observed (Ficker et al. 1997). The 2.4-kb 5'-flanking region of the pistil-specific thaumatin gene (*PsTL1*) from Japanese pear, when transformed in tobacco, showed high expression in pistil, low in anther, and no detectable expression in the floral organs or the leaves. The promoter for *Arabidopsis AGL11* gene, when transformed back into *Arabidopsis*, showed high expression in the center of the young ovary, while expression was not seen in vegetative plant tissues, sepals, petals, or androecium (Nain et al. 2008).

Pollen/Anther

Anther as well as pollen-specific expression can be classified into "early" and "late" phases. The "early" phase comprises genes that are expressed during anther development and sporophytic tissue formation, while "late" phase involves expression during gametophyte generation and pollen formation/maturation. The 122-bp 5' region of a tapetum-specific gene (*TA29*) isolated from tobacco programmed

tapetum-specific expression as seen by fusing this promoter with a reporter (Koltunow et al. 1990). The expression increased in the developing anther and decreased as the microspores began to mature into pollen. The *TA29* promoter, fused with RNase (*barnase*), has been used to develop nuclear male-sterile plants (Mariani et al. 1990). In a comparative analysis, expression patterns of Bp4 promoter from rapeseed and the NTM19 promoter from tobacco were studied in transgenic tobacco (Custers et al. 1997). The Bp4 promoter became active only after the first pollen mitosis and not in the microspores, while the NTM19 promoter turned out to be highly microspore specific and directed very high level of GUS expression to the unicellular microspores; more importantly both the promoters were expressed only in the male germline (Custers et al. 1997). In *indica* rice, promoter of OSIPA was active during the late stages of pollen development and remained active till anthesis, whereas OSIPK promoter was active at a low level in developing anther till the pollen matured. OSIPK promoter activity diminished before anthesis. Both the promoters showed a potential to target expression of the genes of interest in developmental stage-specific manner and could help engineer pollen-specific traits in transgenic crops (Gupta et al. 2007). The anther- and tapetum-specific gene *TomA108* was present in as single copy per haploid genome of tomato. The fusion of β -glucuronidase to the *TomA108* promoter demonstrated that the promoter was highly active from early meiosis to free microspores production in tapetum of tobacco (Xu et al. 2006).

Recently, a gene from pea, *PsEND1*, showed very high and early expression in anther primordium cells. Later *PsEND1* expression became restricted to the epidermis, connective, endothecium, and middle layer, but it was never observed in tapetal cells or microsporocytes. On fusion of the *PsEND1* promoter region to the cytotoxic *barnase* gene to induce specific ablation of the cell layers, where the *PsEND1* was expressed it produced male-sterile plants in tobacco and tomato (Roque et al. 2007). The *PsEND1-barnase* gene is quite different from other chimeric genes previously used to obtain male-sterile plants. The tapetum-specific promoter produces the ablation of specific cell lines during the initial steps of the anther development, but this chimeric construct (*PsEND1-barnase*) arrests the microsporogenesis before differentiation of the microspore mother cells and so, no viable pollen grains are produced. This strategy represents an excellent alternative to generate genetically engineered male-sterile plants. The *PsEND1* promoter has high potential to prevent undesirable horizontal gene flow in many plant species (Roque et al. 2007). Two anther-specific cDNAs (designated *GhACS1* and *GhACS2*) encoding acyl-CoA synthetases (ACs) isolated from cotton flower cDNA library were seen to accumulate in developing anthers. *GUS* expression controlled under the *GhACS1* promoter showed high and specific expression in primary sporogenous cells, pollen mother cells, microspores, and tapetal cells (Wang and Li 2009).

Compared to “early” phase genes a few “late” phase genes have also been characterized. The promoter of tomato *Lat52* gene showed pollen-specific activity

when transformed to tomato, tobacco, and *Arabidopsis* plants. Its expression was also correlated with the onset of microspore mitosis and increased progressively until anthesis (Twell et al. 1990). The elements necessary for expression in transgenics were present within 600 bp of the 5' flanking region. The promoter sequence of *BAN215-6* gene from Chinese cabbage (*Brassica campestris*) showed high similarity with the *Lat52* gene (Kim et al. 1997). Expression studies, by *Agrobacterium*-mediated transformation of tobacco plants, revealed that 383 bp of the *BAN215-6* promoter region was sufficient for the anther-specific expression. The expression level was increased during anther development, reaching highest levels in mature pollens (Kim et al. 1997). The promoter of a maize pectin methytransferase gene (*ZmC5*) was found to be expressed specifically in late pollen development when transformed to tobacco plants (Wakeley et al. 1998). By genome walking PCR, a novel β -mannase gene (*LeMAN5*) was discovered in tomato, which is involved in cell wall disassembly and degrading mannan polymers. The 5'-upstream region of this endo- β -mannanase gene contained four copies of the pollen-specific *cis*-acting elements POLLEN1LELAT52 (AGAAA). The expression of the putative *LeMAN5* promoter region (-543 to +38) in transgenic *Arabidopsis* was detected in mature pollen, sporangia, discharged pollen, and elongating pollen tubes (Filichkin et al. 2004).

Fruit

Fruits are one of the best delivery vehicles for value-added nutrients and other characters like increasing shelf-life, development of oral vaccines, etc. and there has always been a need for fruit-enhanced gene expression. The promoters of fruit-specific genes, especially fruit ripening genes, have been sought after. The ACC (1-aminocyclopropane-1-carboxylate) oxidase gene, the *E8* gene, and polygalacturonase (*PG*) genes are all fruit-ripening-specific promoters and have been characterized from apple and tomato (Montgomery et al. 1993a,b; Nicholass et al. 1995; Atkinson et al. 1998). The ACC oxidase gene is induced by application of ethylene, and fragments of 1,966 and 1,159 bp of the 5' region showed both fruit and ripening specificity, whereas for the *PG* gene promoter, fragments of 1,460 and 532 bp conferred ripening-specific expression in transgenic tomato fruit (Atkinson et al. 1998). The promoter of the *E8* gene of tomato is by far the most important fruit-ripening-specific promoter. It has been successfully applied in a number of instances including enhancement of aroma of tomato by expressing *Clarkia breweri* S-linalool synthase gene (*LIS*) (Lewinsohn et al. 2001), fruit-specific expression of viral proteins (Sandhu et al. 2000), and cholera toxin gene (*CTB*) in an effort to make edible vaccines (He et al. 2008). The tomato *PG* gene is also associated with fruit ripening and its promoter was successfully employed to overexpress a bacterial phytoene synthase gene resulting in increased carotenoid content (Fraser et al. 2002) and a lemon basil α -zingiberene synthase gene (*ZIS*) in tomato fruit to increase both mono- and sesqui-terpene contents (Davidovich-Rikanati et al. 2008).

Seeds

Like fruits, seeds are also an excellent vehicle to pack transgenic products. Seed-specific transgenic technology can be used to enhance nutrient quality, production of pharmaceutical compounds, edible vaccines, etc. The genes expressed at very high level in the seeds are seed storage proteins and these have become the target of choice. Promoters for dicots as well as monocots have been extensively studied and several seed-specific elements have been characterized. The promoter region of soybean β -conglycinin was expressed in the embryo during the mid to late stages of seed development (Chen et al. 1989). The 2.4-kb upstream region of the sunflower *Helianthinin* gene (*HaG3-A*) also conferred high embryo-specific expression in transgenic *Arabidopsis* (Nunberg et al. 1994). The 0.8-kb fragment of the 5' β -phaseolin gene of French bean (*Phaseolus vulgaris*) showed strong, temporally regulated, and embryo-specific expression in transgenic tobacco plants (Bustos et al. 1989). The expression pattern of the promoter fragment (1,108 bp) of the α -globulin gene in cotton was studied in transgenic cotton, *Arabidopsis*, and tobacco. Expression was initiated during the torpedo stage of seed development in tobacco, *Arabidopsis*, and during cotyledon expansion stage in cotton. The activity increased sharply until embryo maturation in all the three species. Expression was not detected in stem, leaf, root, pollen, or floral bud of transgenic cotton, thus confirming the high seed specificity of the promoter (Sunilkumar et al. 2002).

For monocots, several seed-specific promoters have been used successfully to incorporate many traits. The promoter region of the endosperm-specific protein hordein (D and B hordein) from barley has been well characterized in transgenic rice, barley, and wheat (Furtado et al. 2008, 2009). Six promoters (GluA-1, GluA-2, GluA-3, GluB-3, GluB-5, GluC) of seed storage glutenin genes were isolated from rice and their expression potential was checked in transgenic rice. The GluA-1, GluA-2, and GluA-3 promoters directed expression in the outer portion of the endosperm, while GluB-5 and GluC promoters directed expression in the whole endosperm. The GluB-3 promoter directed expression solely in aleurone and sub-aleurone layers, while maximum activity was pertained to the GluC promoter (Qu et al. 2008). Recently, edible vaccines are being made in transgenic rice against house dust mite allergy (Yang et al. 2008) and Japanese cedar pollen allergen (Yang et al. 2007) under the control of GluB-1 promoter and cholera toxin B subunit under the control of wheat Bx17 promoter containing an intron of the rice *act1* (Oszvald et al. 2008). The zein promoters from maize have been used for many applications. Transgenic maize with enhanced provitamin A content in the kernel was developed by endosperm-specific expression of the bacterial genes (*crtB* and *crtI*) under the control of a "super γ -zein promoter" (Aluru et al. 2008). Increase of total carotenoids was up to 34-fold with a preferential accumulation of β -carotene in the maize endosperm. The *phyA2* from *Aspergillus niger* was successfully expressed in maize seeds using the maize embryo specific globulin-1 promoter. The transgenic seeds showed a 50-fold increase in phytase activity (Chen et al. 2008). The developed maize hybrids had improved phosphorus availability for pig and poultry feed.

5.5 Factors Affecting Stability and Level of Transgene Expression

5.5.1 SAR/MAR Effect on Transgene Expression

Transgenic plants often display the chromosomal position effect, which results because of transgene integration events taking place within euchromatin, producing irregular and mixed expression. The pre-existing chromatin structure at the site of integration ultimately determines the expression level, acting either as an enhancer or as a silencer (Taddei et al. 2004). The chromosomal position effect can be prevented if the transgene is flanked by matrix attachment regions (MARs) also known as scaffold attachment regions (SARs) which are DNA elements that bind to the nuclear matrix (Mirkovitch et al. 1984; Allen et al. 2000). The location of MARs within transcription regulatory elements suggests that MARs may serve to bring these DNA sequences in proximity to the scaffold, thereby promoting enhancer and promoter activity by facilitating interaction with transcription factors (Nardoza et al. 1996). This inference is supported by loop domain model studies in which different expression profiles were observed on comparative analysis of transgenes that were flanked by MARs and those lacking it. Transgenes lacking MARs are influenced much by the surrounding chromatin structure; their expression levels are also dependent on local chromatin state. Transgenes that are flanked by MARs act independent of local chromatin state; thus, multiple copies of MAR-flanked transgene insertion might proportionally increase expression (Gasser and Laemmli 1986; Stief et al. 1989).

During gene activation chromatin structure becomes relaxed and the DNA is more accessible to DNase I. MARs that flank the chromatin loop domain function as the boundaries to differentiate active from inactive chromatin (Martienssen 2003). Later, on the basis of this inference, comparative study of the higher order chromatin structure and their accessibility to DNase I was performed in *Arabidopsis* and maize nuclei resulting in 45-Kb and 25-Kb domains, respectively, (Paul and Ferl 1998). It was reported that transgenic plants containing the synthetic MAR (sMAR) sequences derived from the MAR 3' end of the immunoglobulin heavy chain (IgH) enhancer, exhibited high levels of expression compared to transgenic plants that lacked the sMARs (Nowak et al. 2001). A diversity of promoters and MAR sequences has been used to analyze transgene expression. Mankin et al. (2003) analyzed the effects of a MAR, from the tobacco *RB7* gene on transgene expression from six different promoters in stably transformed tobacco cell cultures. The presence of MARs flanking the transgene increased expression of constructs based on the constitutive CaMV 35S, NOS (nopaline synthase 5' region), and OCS (octopine synthase 5' region) promoters (Mankin et al. 2003). Expression from a heat-shock induced promoter also increased five- to nine-folds, and MARs did not cause expression in the absence of heat shock (Schöffl et al. 1993). The effect of MAR fragments from tobacco gene transformed to two hybrid poplar

clones and in tobacco plants was analyzed and found that MARs increased expression approximately ten- and two-fold, respectively, 1 month after cocultivation with *Agrobacterium*. Apart from gene expression, increased frequency of kanamycin resistance was also reported in poplar shoots (Han et al. 1997).

Different studies on MAR function in plant transgene expression provided interesting conclusions. The effect of MAR on transgene expression is analyzed only after the integration of transgene construct within plant genome. Enhancement of transgene expression has been reported in MARs containing stably transformed plant cell lines of soybean Gmhsp 17.6.L (Schöffl et al. 1993), yeast ARS1 (Allen et al. 1993; Vain et al. 1999), tobacco and rice Rb7 (Allen et al. 1996), tomato HSC80 (Chinn and Comai 1996), bean phaseolin (van der Geest et al. 1994), maize Adh1, Mha1 (Brouwer et al. 2002), and *Arabidopsis* ARS (Liu and Tabe 1998).

In a nutshell, MARs are not highly conserved but possess AT-rich DNA motifs of 100–3,000 bp containing binding sites for DNA topoisomerase II, DNA helicase, and DNA polymerase and thus are involved in structural organization of the genome. The loops created by MARs are topologically independent units of gene regulation and were found to facilitate the transcription of genes by changing topology along with less-condensed chromatin structure. The transgene constructs containing MARS are observed to create its own chromatin domain favorable for transcription; thus, MARS can reduce variability of transgene expression and increase level of expression.

5.5.2 Effect of 5' and 3' UTR Regions

The use of a specific promoter, with or without one or more enhancers, does not necessarily guarantee the desired level of gene expression in plants. In addition to the desired transcription levels, other factors such as improper splicing, polyadenylation, and nuclear export can affect accumulation of both mRNA and the protein of interest. Therefore, methods of increasing RNA stability and translational efficiency through mechanisms of post-transcriptional regulation are needed in the transgenic approach.

With regard to post-transcriptional regulation, it has been demonstrated that certain 5' and 3' untranslated regions (UTRs) of eukaryotic mRNAs play a major role in translational efficiency and RNA stability. For example, the 5' and 3' UTRs of tobacco mosaic virus (TMV) and alfalfa mosaic virus (AMV) coat protein mRNAs can enhance gene expression 5.4-fold and three-fold, respectively, in tobacco plants (Zeyenko et al. 1994). The 5' and 3' UTRs of the maize alcohol dehydrogenase-1 gene (*adh1*) are required for efficient translation in hypoxic protoplasts (Bailey-Serres and Dawe 1996; Hulzink et al. 2002).

Experiments with various 5' UTR leader sequences demonstrate that various structural features of a 5' UTR can be correlated with levels of translational efficiency. It was reported that 5' UTR elements are required for the high-level expression of pollen *ACT1* gene in *Arabidopsis* (Vitale et al. 2003). During the

process of initiation of translation 40S ribosomal subunit enters at 5' end of the mRNA and moves linearly until it reaches the first AUG codon, whereupon a 60S ribosomal subunit attaches and the first peptide bond is formed. Certain 5' UTR contain AUG codons in mRNA, which interact with 40S ribosomal subunit resulting in a weak context in terms of initiation codon, thus decreasing the rate of translation (Kozak 1991; Lee et al. 2009; Luttermann and Meyers 2009). Additionally, the 5' UTR nucleotide sequences flanking the AUG initiation site on the mRNA have an impact on translational efficiency. If the framework of the flanking 5' UTR is not favorable, part of the 40S ribosomal subunit fails to recognize the translation start site such that the rate of polypeptide synthesis will be slowed down (Kozak 1991; Pain 1996). Secondary structures of 5' UTRs (e.g., hairpin formation) also obstruct the movement of 40S ribosomal subunits during their scanning process and therefore negatively impact the efficiency of translation (Kozak 1986; Sonenberg and Pelletier 1988). The relative GC content of a 5' UTR sequence was shown to be the stability indicator of the potential secondary structure, high GC content indicated instability (Kozak 1991), and long UTRs exhibit a large number of inhibitory secondary structures. The translational efficiency of any given 5' UTR is highly dependent upon its particular structure and optimization of the leader sequence, which has been shown to increase gene expression as a direct result of improved translation initiation efficiency. Furthermore, significant increase in gene expression has been produced by addition of leader sequences from plant viruses or heat-shock genes (Datla et al. 1993).

In addition to 5' UTR sequences, 3' UTR sequences of mRNAs also influence in gene expression and known to control nuclear export, polyadenylation status, subcellular targeting, and rates of translation and degradation of mRNA from RNases. In particular, 3' UTRs contain one or more inverted repeats that can fold into stem-loop structures, which act as a barrier to exoribonucleases, and interact with RNA-binding proteins known to promote RNA stability (Gutiérrez et al. 1999). However, certain elements found within 3' UTR were reported to be RNA destabilizing, one such example occurring in plants is the DST element which can be found in small auxin up RNAs (SAURs) (Gil and Green 1996). A further destabilizing feature of some 3' UTRs is the presence of AUUUA pentamers (Ohme-Takagi et al. 1993).

The 3' UTRs were demonstrated to play a significant role in gene expression of several maize genes. Specifically, a 200-bp 3' sequence is responsible for suppression of light induction of maize small m3 subunit of the ribulose-1, 5-biphosphate carboxylase gene (*rbc/m3*) in mesophyll cells (Viret et al. 1994). Monde et al. (2000) observed that the *pet D3'*-UTR stem loop secondary structure was not able to form RNA-protein complex, essential for translational activity and thus acted as weak terminator required for RNA maturation. One 3' UTR frequently used in genetic engineering of plants is derived from nopaline synthase gene (3' *nos*) (Wyatt et al. 1993).

In certain plant viruses, such as alfalfa mosaic virus (AMV) and tobacco mosaic virus (TMV), the highly structured 3' UTRs are essential for replication and can be folded into either a linear array of stem-loop structures, which contain several high-

affinity coat protein binding sites or a tRNA-like site recognized by RNA-dependent RNA polymerases (Olsthoorn et al. 1999).

5.5.3 Effect of Introns

Introns are the intragenic regions that are not translated into proteins. These noncoding portions are present in pre-mRNA and further removed by splicing to yield mature RNA. Introns contain acceptor and donor sites at either end as well as a branch point site, which is required for proper splicing by the spliceosome. The number and the length of introns vary widely among species and among genes within the same species. Introns with alternative splicing may introduce greater variability in protein sequences translated from a single gene. Introns also enhance the level of transgene expression in plants (Callis et al. 1987).

Recent studies provided several examples of introns, whose impact on expression is larger than that of the promoter from the same gene. Many genes with fully functional promoter are not essentially expressed at all but require an intron for their expression. A study in *Arabidopsis* showed that *PRF2* intron is required for full expression of a *PRF2* promoter and the β -glucuronidase (*GUS*) and also to convert *PFR5:GUS* fusion from a reproductive to vegetative pattern (Jeong et al. 2006). Introns can increase the expression level through their enhancer element, an alternative promoter activity, or it can be independent of their conventional enhancer elements, i.e., intron-mediated enhancement (IME). The second intron of *Arabidopsis* agamous gene (*AG*) is a well-characterized enhancer-containing intron that can function in both orientations to force the expression of a reporter from a minimal promoter. The *Arabidopsis* *AG*, *STK*, *FLC* introns and wheat *VRN-1* intron act as enhancers. All these introns are large in size providing sufficient room for controlling elements and allow the establishment of stable chromatin conformation required for appropriate expression (Rose 2008).

The studies conducted by Morello et al. (2002, 2006) revealed the role of intron as an alternative promoter in rice. Presences of introns in promoterless genes drive weak expression; these introns are considered to contain promoters that are responsible for expression. The first intron acts as the alternative promoter as observed in *Ostub16* and *OsCDPK2* in rice, *PpAct1* and *PpAct5* in *Physcomitrella patens* and sesame, and *FAD2* in *Arabidopsis* (Kim et al. 2006; Weise et al. 2006).

IME of gene expression in plants indicates that the insertion of one or more introns in a gene construct results in increased accumulation of mRNA and protein relative to similar fusions that lack introns (Mascarenhas et al. 1990). The deletion studies of different introns such as maize *Adh1*, *Sh1* first intron, rice *Ostub A1* first intron, *Arabidopsis* *TRP1* first intron and *PRF2* intron1 revealed that no specific sequences were absolutely required and no conserved motif was found between

enhancing introns (Rose 2008). Sequence analog studies showed enhancement can be restored by substituting the U/GC-rich region of intron with similar sequence analog from another part of intron (Rose 2002). The mutation studies in *Arabidopsis TRP1* intron1 and maize *Sh1* intron1 revealed that IME is destroyed by simultaneous elimination of branch-points and the 5' splice site, further indicating that splicing machinery is required for IME (Rose 2002). Additionally, the positions of introns also influence IME on gene expression. The most prominent is the location of the intron within the gene, i.e., the introns present in 5' UTR of the rice *rub13* gene was shown to enhance expression (Lu et al. 2008). The other significant position of intron is near the starting of the gene (Rose 2004; Chung et al. 2006). Presently, there are several examples of introns (e.g., first intron of *OsTua2*, *OsTua3*, *OsTub4*, and *OsTub6*) that can greatly influence both the amount and the actual size of the expression, attributing different patterns of expression to the different intron iso-types, thus generating the intron-dependent spatial expression (IDSE) profile (Gianì

Table 5.2 Introns affecting transgene expression in different plants

Intron	Specificity	Remark	Reference
<i>COX 5c-1</i> <i>COX 5c-2</i>	<i>Arabidopsis</i>	Increased GUS expression level	Curi et al. (2005)
<i>Ubi7</i>	Potato	Ten-fold higher expression	Garbarino et al. (1995)
<i>Adh1</i>	Maize	40–100-fold increase in expression	Callis et al. (1987)
<i>Act1</i>	<i>Arabidopsis</i>	High level of reproductive tissue expression	Vitale et al. (2003)
<i>RBCS2</i>	<i>Chlamydomonas reinhardtii</i>	Stable high-level expression	Lumbreras et al. (1998)
<i>reg A3</i> <i>regA5</i> <i>STK</i>	<i>Volvox carteri</i>	Required for <i>regA</i> expression	Stark et al. (2001)
<i>VRN-1</i>	Wheat	Essential for promoter activity	Fu et al. (2005)
<i>Ostub 16</i>	Rice	Required for maximum promoter activity	Morello et al. (2002)
<i>OsCDPK 2</i>	Rice	Required for promoter activity	Morello et al. (2006)
<i>PpAct 1</i> <i>PpAct 5</i> <i>PpAct 7</i>	<i>Physcomitrella patens</i>	11–18-fold higher expression	Weise et al. (2006)
<i>Sh1</i>	Maize	10–1,000-fold enhanced expression	Maas et al. (1990, 1991)
<i>Gap A1</i>	Maize	Required for full promoter activity	Donath et al. (1995)
<i>Actin 3rd intron</i>	Maize	IME	Luehrsen and Walbot (1991)
<i>Hsp81</i>	Maize	IME	Sinibaldi and Mettler (1992)
<i>Act 1</i>	Rice	IME	McElroy et al. (1990)
<i>tpi</i>	Rice	Required for promoter activity	Xu et al. (1994)

et al. 2009). The specificity of introns acts as enhancer, and alternative promoter, or mediates enhancement on the basis of their numbers and position in a gene construct. Some of the introns with defined specificity have been summarized in [Table 5.2](#).

5.5.4 Role of Transcription Factors

Transcription factors are sequence-specific DNA-binding proteins that interact with the promoter regions of the target genes and modulate the rate of initiation of mRNA synthesis by RNA polymerase II (Gantet and Memelink 2002). The role of transcription factors in transgene expression is studied by overexpression and antisense technology. For highly conserved transcription factors such as MADS-box or the Myb-like transcription factors, generation of antisense plant is difficult since the target requires an antisense RNA homology of over 50 bp, which is not preferred. Also, due to the presence of highly conserved regions, the specificity of antisense RNA is significantly reduced (Cannon et al. 1990). High-level expression of a transcription factor in a transgenic plant cell might favor the binding of the transcription factor to low affinity binding sites and result in activation of gene expression from noncognate promoters.

To study the effect of transcription factor on transgene, the steroid-binding domain of the glucocorticoid receptor is fused to a plant transcription factor. The absence of ligand represses nuclear localization and DNA-binding activities of transcription factor. After induction, repression is relieved and active protein can rapidly enter the nucleus and exert its transcription factor function. A glucocorticoid-responsive GAL4-VP16 fusion protein has been used to induce the activation of a luciferase reporter gene in transgenic *Arabidopsis* and tobacco plants, either by growing the plants on nutrient agar containing dexamethasone or by spraying the plants with the inducing compound (Aoyama and Chua 1997).

The *Arabidopsis* transparent testa glabra (*ttg*) mutant plants are not able to produce trichomes, anthocyanins, and seed coat pigment but generate excess root hairs. Production of trichomes and anthocyanins could be restored by overexpression of the maize transcription factor *R* in a constitutive and inducible manner (Lloyd et al. 1994). An interaction study carried out between transcription factor (*myb305*) and its promoter-binding site in *PAL2* in transgenic tobacco plant revealed that when leaves were inoculated with a PVX-construct expressing *Myb305* reporter gene, expression increased (Sablowski et al. 1995). Thus, the ectopic expression of *Myb305* in infected tissue incites the higher expression of *GUS* reporter gene in transgenic tobacco plant with nonmutant *PAL2* promoter element.

Synthetic transcription factors are an assembly of multiple zinc finger domains designed to achieve better regulation of gene expression. It is estimated that *Arabidopsis* contains 85 genes that encode zinc finger transcription factors (Riechmann and Ratcliffe 2000). Such synthetic zinc finger transcription factors (*TFsZF*) can be custom designed for binding to any DNA sequence (Segal and Barbas 2001).

Furthermore, the addition of herpes simplex virus VP16 activation domain to the polydactyl six-zinc finger protein 2C7 increased the expression more than 450-fold in transgenic plants (Liu et al. 1997). Later, Van Eenennaam et al. (2004) constructed five, three-finger zinc finger protein (ZFP) DNA-binding domains which tightly bound to 9-bp DNA sequences located on either the promoter or the coding region of the *Arabidopsis* *GMT* gene. When these ZFPs were fused to a maize *opaque-2* nuclear localization signal and the maize *CI* activation domain, four out of the five resulting ZFP-TFs were able to up-regulate the expression of the *GMT* gene in leaf protoplast transient assays. The seed-specific expression of these ZFP-TFs was reported to produce heritable increase in seed α -tocopherol level in subsequent generations of transgenic *Arabidopsis*.

The transcription factors, *R* and *CI*, interact to regulate anthocyanin biosynthesis in the maize kernel (Grotewold et al. 2000). In a recent study, it was reported that ectopic expression of a conifer *Abscisic Acid Insensitive 3* (*ABI3*) transcription factor induced high-level synthesis of recombinant human α -L-iduronidase gene in transgenic tobacco leaves (Kermode et al. 2007). Transgenic rice with *DREB 1s/CBF* or *OsDREB 1A/1B* transcription factor interact specifically with *DRE/CRT* or *OsDRE cis*-acting elements and control the expression of many stress-inducible genes (Ito et al. 2005). In continuation, Zhao et al. (2009) reported on the role of transcription factors on abiotic stress where the expression of yeast *YAP1* gene in transgenic *Arabidopsis* resulted in increased salt tolerance. The *YAP1* contains a basic leucine zipper domain similar to that of Jun (Moye-Rowley et al. 1989), which is a component of mammalian *AP-1* transcription factor complexes. Nuclear *YAP1* regulates the expression of up to 70 genes that are related to oxidative stress caused by high salinity (Zhao et al. 2009).

5.5.5 Effect of DNA Acetylation and Methylation

The interaction of histones with DNA plays an important role in chromatin remodeling and consequently the activation or repression of gene expression (Tian et al 2005). Intrinsic histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, HDACs) drive acetylation and deacetylation, respectively, thus providing a mechanism for reversibly modulating chromatin structure and transcriptional regulation (Jenuwein and Allis 2001). Hyperacetylation relaxes chromatin structure and activates gene expression, whereas hypoacetylation induces chromatin compaction and gene repression. Histone acetylation and deacetylation are reversible and therefore play a significant role in transcriptional regulation associated with developmental programs and environmental conditions. These include day-length (Tian et al. 2003), flowering (He et al. 2003), osmotic and oxidative stress (Brunet et al 2004, De Nadal et al 2004), and cell aging (Imai et al 2000).

Acetylation neutralizes the lysine residues on the amino terminal tails of the histones, thereby neutralizing the positive charges of histone tails and decreasing

their affinity to bind DNA. HATs are often associated with proteins forming coactivator complexes, stabilizing the chromatin in an open conformation and transcriptionally active state. These complexes are targeted to promoters by specific transcription factors, allowing the RNApol II holoenzyme to access the promoter DNA sequence, which results in activation of transcription and increased gene expression. Histone deacetylases (HDAC) ameliorate the affinity of histones for DNA as deacetylation of histone tails result in stronger interaction between the basic histone tails and DNA. HDACs are often associated with other proteins that are associated with chromatin condensation and repression of transcription. These corepressor complexes promote heterochromatin formation, blocking access of RNApol II, thus resulting in repression of transcription.

Arabidopsis has 18 members of putative histone deacetylase family (Pandey et al. 2002). Among them *AtHDA6* is responsible for silencing transgenes (Murfett et al. 2001), whereas *AtHDI* is reported to be a global transcriptional regulator throughout the development of *Arabidopsis* (Tian et al. 2003). The analysis of microarray data revealed that gene activation is associated with increased levels of site-specific histone acetylation, whereas gene repression does not correlate with the changes in histone acetylation or histone methylation. Many of the HDACs found in plants are *Rpd3*, *HD2*, *SIR2*, and their homologs (Chen and Tian 2007).

DNA methylation is known to play a role in plant gene silencing (Ng and Bird 1999). Methyl CpG-binding protein (MeCP2) was reported to be involved in the recruitment of HDAC to methylated DNA through a corepressor complex, which results in gene silencing. Earlier studies showed that hemimethylation results in inhibition of transient gene expression, whereas nonmethylated gene expressed normally (Weber et al. 1990). In one of the studies, a mutation isolated via a transgene reactivation screen in *Arabidopsis*, *mom1*, was thought to act downstream of DNA methylation signals in controlling silencing because it did not confer obvious methylation changes (Amedeo et al. 2000). In recent studies, Shibuya et al. (2009) reported that the *pMADS3* gene in petunia, specifically expressed in the stamen and carpels of developing flower, showed ectopic expression after introduction of intron 2. This is known as ect-*pMADS3* phenomenon and is due to transcriptional activation based on RNA-directed DNA methylation (RdDM) occurring in a particular CG in a putative *cis*-element in *pMADS3* intron 2. The CG methylation was maintained over generations, along with *pMADS3* ectopic expression, even in the absence of RNA triggers. Transcriptional or post-transcriptional gene silencing was expected; instead, upregulated gene expression was observed (Shibuya et al. 2009).

Recently, the new Amplicon-plus targeting technology (APTT) has been developed to overcome the problems of post-transcriptional gene silencing and lower accumulation of transgenic protein. This technology uses a novel combination of techniques, i.e., expression of a mutated PTGS suppressor, P1/HC-Pro, with PVX (potato virus X vector) amplicon encoding a highly-labile L1 protein of canine oral papillomavirus (COPV L1). Appreciable amount of protein accumulation was achieved by targeting the L1 to various cellular compartments, by

creating a fusion between the protein of interest and different targeting peptides. Additionally, a scalable “wound-and-agrospray” inoculation method has been developed that allows high-throughput *Agrobacterium* inoculation of *Nicotiana tabacum* to facilitate large-scale application of this technology (Azhakanandam et al. 2007).

5.6 Conclusions

Genetic transformation of crops has opened a new dimension to increase production that benefits both producers and consumers. Its effect can be best utilized in less developed or developing countries where crop yield is severely affected by biotic and abiotic stress. Also, value addition of existing nutrients along with production of novel nutraceuticals will help alleviate nutrition-related deficiencies in famine-stricken countries. Apart from enhancing food value in crop species, transgenic technology can be used to develop visual marker systems to monitor crops and carry out fine scale studies of agricultural crops. Despite the hostility against genetically modified crops in Eastern Europe, many countries in Asia and North America have accepted transgenic crops. In the present scenario, some of the factors responsible for the control of transgene expression at different levels have been summarized in Fig. 5.1. The primary challenge lies with the detailed understanding of the underlying mechanism involved in gene expression, and there is a pressing need to study gene expression, especially its regulation.

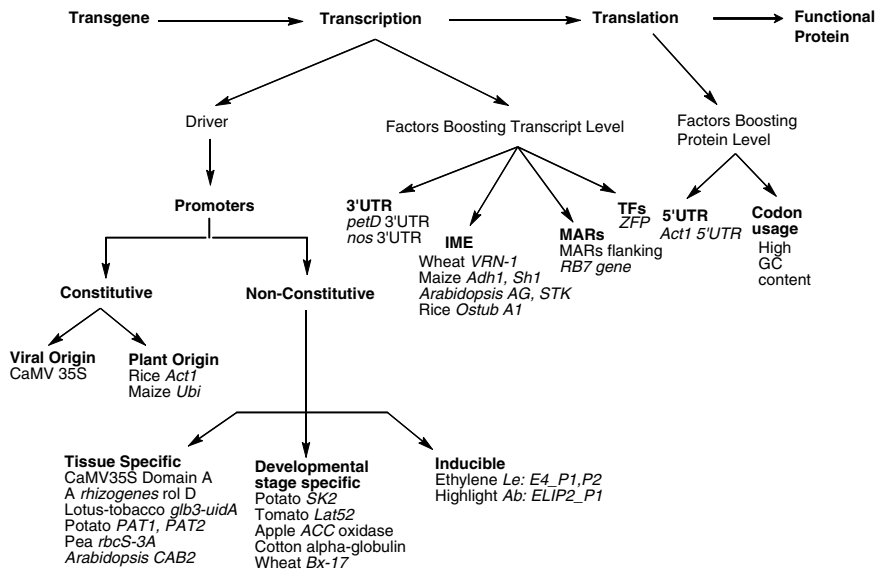


Fig. 5.1 Transgene expression and stability factors

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