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Review

An update and perspectives on the use of promoters in plant genetic engineering

Divya Kummari[1,2], Sudhakar Reddy Palakolanu[1]*, P B Kavi Kishor[3], Pooja Bhatnagar-Mathur[1], Prasanth Singam[2], Vincent Vadez[1] and Kiran K Sharma[1]

[1]*International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502 324, India*

[2]*Department of Genetics, Osmania University, Hyderabad 500 007, India*

[3]*Department of Biotechnology, Vignan's Foundation for Science, Technology and Research Deemed to be University, Vadlamudi, Guntur 522 213, India*

**Corresponding author (Email, p.sudhakarreddy@cgiar.org, palakolanusreddy@gmail.com)*

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Genetically engineered plants have varied applications in agriculture for enhancing the values of food and feed. Genetic engineering aims to introduce selected genetic regions with desirable traits into target plants for both spatial and temporal expressions. Promoters are the key elements responsible for regulating gene expressions by modulating the transcription factors (TFs) through recognition of RNA polymerases. Based on their recognition and expression, RNA polymerases were categorized into *RNA pol II* and *pol III* promoters. Promoter activity and specificity are the two prime parameters in regulating the transgene expression. Since the use of constitutive promoters like *Cauliflower mosaic virus* (*CaMV*) *35S* may lead to adverse effects on non-target organisms or ecosystem, inducible/tissue specific promoters and/or the *RNA pol III* promoters provide myriad opportunities for gene expressions with controlled regulation and with minimum adverse effects. Besides their role in transgene expression, their influence in synthetic biology and genome editing are also discussed. This review provides an update on the importance, current prospects, and insight into the advantages and disadvantages of promoters reported thus far would help to utilize them in the endeavour to develop nutritionally and agronomically improved transgenic crops for commercialization.

Keywords. *CaMV35S* promoter; constitutive promoters; synthetic promoters; genetic engineering; *RNA pol II* promoters; *RNA pol III* promoters; *U3* promoter; *U6* promoter

1. Introduction

Promoters are gene switches located upstream of gene coding regions, which turn on and off the functional activity of genes and contain specific *cis*-acting elements which are binding targets for proteins involved in the initiation and regulation of transcription. Promoters are molecular biological clocks crucial for choice of the targeted gene expression (Potenza *et al.* 2004), that act as key regulatory check points for transcription of genes that are recognized by transcription factors (TFs) (Smale and Kadonaga 2003). TFs bind to specific *cis*-acting elements present on the respective promoter sequences through RNA polymerase and regulate expression of the downstream genes (Hernandez-Garcia and Finer 2014). Promoters of coding genes often contain core, proximal

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and distal regions. Further, proximal and distal regions of the promoter contain different regulatory sequences such as enhancers, silencers, insulators, and *cis*-elements that contribute to fine regulation of gene expression at the transcriptional level (Hernandez-Garcia and Finer 2014). Detailed structural properties of promoters and gene regulatory elements were discussed earlier by Hernandez-Garcia and Finer (2014), Porto *et al.* (2014) and Shah *et al.* (2015). Promoter sequences that regulate the controlled transgene expression in plants are useful for developing the genetically modified (GM) crops with improved agronomical and nutritional traits (Mittler and Blumwald 2010). There are few promoter prediction bioinformatics tools; one among them is TSSPlant, a novel tool that predicts both TATA and TATA-less promoters in sequences of a wide spectrum of plant genomes (Shahmuradov *et al.* 2017). As complete genome sequences of most of the plant species are becoming available, several promoters are being identified, isolated and evaluated, while many more are likely to be elucidated in the near future. Predicted regulatory sequences may or may not be functionally active or necessitating the confirmation of the role of specific elements for promoter activity. Promoters can be isolated through different methods some of which being: genome walking, inverse PCR (IPCR) plasmid rescue, screening of genomic DNA library constructed from mutant plant, the thermal asymmetric interlaced PCR TAIL-PCR (Reddy *et al.* 2008; Liu and Whittier 1995). Quick, efficient, predictable, and high-throughput analysis of gene expressions and their promoters will be crucial for validating the functional regulatory element sequences.

Promoters can be selected to develop transgenic plants based on the type of trait and target tissue to be regulated (Bilas *et al*. 2016a, b). A variety of plant promoters that regulate the degree of expression of a transgene can be obtained from various sources. These can be categorized into *pol II* and *pol III* that are activated upon recognition by the RNA polymerases II and III. *Pol II* promoters can in turn be classified into constitutive, tissue-specific, stress-inducible and synthetic (Bilas *et al*. 2016a, b), whereas *pol III* promoters are U3 and U6 (Marshallsay *et al.* 1992). *CaMV35S* (commonly referred to as 35S) constitutive promoter is the most widely used for gene expressions in transgenic plants and in basic functional genomic studies (Porto *et al.* 2014). Hence, specific or inducible promoters not only provide increased gene expression in a tissue of interest or at specific developmental stage(s), but also provide more predictable gene expression, with minimal or no penalties on final yield. The discovery of proteins with programmable DNA-binding specificities has triggered an array of applications in synthetic biology, regulation of transcription, including genome editing, and epigenetic modifications. Among those, transcription activator-like effectors (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system played high attention due to their in-built function as transcription regulators for the control of gene expression. Recent studies have shown that the expression of Cas9/gRNA with the help of specific promoters is important for highly efficient genome editing in plants (Hyun *et al.* 2015; Wang *et al.* 2015; Yan *et al.* 2015; Mao *et al.* 2016). Here, we attempt to provide a comprehensive overview of promoters in plants, their selection, specificity, cross activity, and how they can be useful in maximizing the transgene expression for potential applications in crop improvement programs.

2. Types of promoters

Based on the type of recognition of RNA polymerases, they are mainly classified into RNA polymerase (*pol II* and *pol III*) promoters. Detailed information and study of these promoters are provided in the subsequent sections of this review.

2.1 *RNA pol II promoters*

RNA *pol II* promoters are the shortest sequences where RNA *pol II* polymerase binds to the DNA to initiate transcription with help of the TATA box, a common component located at -35bp upstream of the transcription start codon. Based on the type of expression, *pol II* promoters are further categorized into constitutive, organ- or tissue-specific, stress-inducible and synthetic (figure 1).

2.1.1 *Constitutive promoters:* Constitutive promoters express constitutively throughout the plant life cycle, irrespective of the external and developmental factors which is beneficial for expression of the industrial enzymes, insect resistance and selectable marker genes (Jiang *et al.* 2018). Based on the source of origin, they are further classified into viral, bacterial and plant constitutive promoters.

2.1.1.1 Viral: In 1980s, Chua and collaborators isolated a promoter, responsible for the transcription of the whole genome of a Cauliflower mosaic virus (CaMV) infecting turnips. Later, this was named as *CaMV35S*

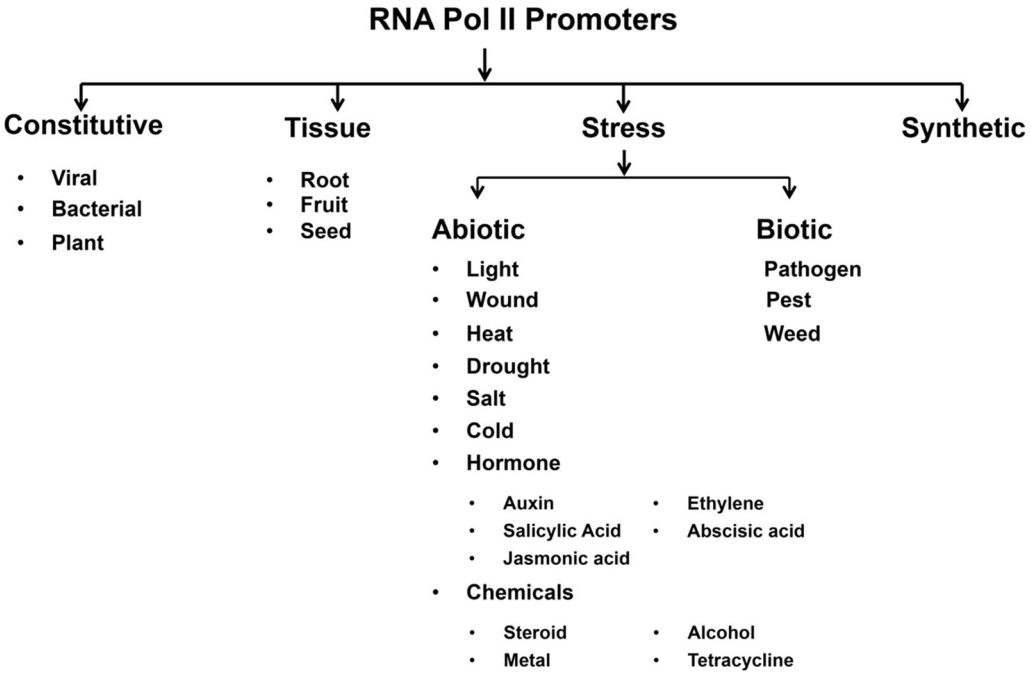

Figure 1. A flow chart showing different types of RNA Pol II promoters categorized based on their mode of expression into four major classes: constitutive, tissue-specific, stress-inducible and synthetic promoters. Each of these promoters are further categorized into different types; constitutive promoters (viral, bacterial, plant), tissue-specific promoters (root, fruit, seed), stress-inducible promoters (abiotic {light, wound, heat, drought, salt, cold, hormone and chemical} and biotic {pathogen, pest, weed}).

promoter due to the sedimentation coefficient of the viral transcript whose expression is driven by *CaMV35S* promoter and used for constitutive overexpression of genes (Odell *et al.*, 1985). There is not much variation in the activities between the full-length *CaMV35S* promoter (−941 to +9 bp) (Odell *et al.* 1985) and −343bp deletion fragment (Fang *et al.* 1989). Although the *CaMV35S* promoter and its derivatives can drive high levels of transgene expression in dicotyledonous plants (Benfey *et al.* 1990a, 1990b), their activities are substantially lower in monocotyledonous plants (Gupta *et al.* 2001). The expression of *uid*A gene under *CaMV35S* promoter was observed in different parts of transgenic tobacco with maximum levels in leaf and root tissues (Malik *et al.* 2002). In contrast, *uid*A gene expression under the *CaMV35S* promoter in transgenic canola and *Arabidopsis* showed expression in all plant tissues (Malik *et al.* 2002). Sharma *et al.* (2006) demonstrated a higher activity of the *cry1Ab* gene under the constitutive *CaMV35S* promoter in flowers of pigenopea, although other parts like leaves, seeds and pod walls also showed expression of the transgenes to varying degrees.

Apart from *CaMV35S*, *peanut chlorotic streak caulimovirus* (*PC1SV*) and *figwort mosaic virus* (*FMV*) have also been shown to be very useful for generating GM plants. *PC1SV* is a strong constitutive promoter and comparative analysis with *FMV* promoter in GM plants showed similar expression pattern. Although functionally analogous, the nucleotide sequence of *PClSV* promoter has limited homology with other caulimovirus promoters (Maiti and Shepherd 1998). The *FMV Sgt* (Figwort mosaic virus sub-genomic transcript) promoter is another constitutive promoter whose expression was observed in all tissues (Bhattacharyya *et al.* 2002). A comparative analysis of the *FMV Sgt* promoter with that of *CaMV35S* promoter showed that it was 2-folds stronger than the latter, and was also less active in monocots like maize where its expression was about 27.5-folds lower than in tobacco (Bhattacharyya *et al.* 2002). Gemini viral promoters are in general located within the intergenic region, though promoters have also been identified within the genes. In the same way, Gemini virus-associated beta satellite harbors a promoter element for driving the expression of its only ORF, which can be useful in the regulation of plant genes (Borah *et al.* 2016). TaB virus-derived promoters may be useful for the high level constitutive expression of transgenes either in monocotyledonous or dicotyledonous species (Yang *et al.* 2003), thus proving its versatility. *Rep* promoter from *cotton leaf curl Burewala virus* (*CLCuBuV*) showed consistent strong transient expression in tobacco and cotton leaves

as compared to the *CaMV35S* promoter, whereas other *CP* promoter showed lower expression (Khan *et al.* 2015). Promoters originating from the *tomato leaf curl virus* (*TLCV*) drive both constitutive as well as tissue-specific expression in transgenic tobacco (Seemanpillai *et al.*, 2003). But it is not widely used and accepted from the research community. Comparative analysis of the *CaMV35S* and the enhanced *CaMV35S (E35S), CsVMV, FMV*, and the *Strawberry vein banding virus (SVBV2)* promoters indicated that the *FMV* promoter facilitated a strong expression of target genes in soybean hairy roots and root nodules (Govindarajulu *et al.* 2008). Transgenic maize with *aryloxyalkanoate dioxygenase* (*aad-1*) expression under the influence of different promoters demonstrated that viral promoters such as *CaMV35S* and *SCBV* produce lower transformation efficiencies, but higher percentages of low copy number events contrary to plant constitutive promoters like *OsAct1* and *ZmUbi1* (Beringer *et al.* 2017). These promoters are recommended for wide use since they promote low copy number transgenic events. Various constitutively expressed viral promoters used in the development of transgenic plants are listed in table 1.

2.1.1.2 Bacterial: Bacterial-origin promoters such as *nopaline synthase* (*nos*), *octopine synthase* (*ocs*) and *mannopine synthase* (*mas*) have been isolated from *Agrobacterium tumefaciens* and used for the control the transgene expression. Although their level of expression and activity can be affected by hormones and wounding, they are repeatedly being used for the transformation of plants. *mas* promoters can be used as enhancers or silencers, since they have the ability to bind with the factors of nuclear protein from different plants (Shah *et al.* 2015). Thus, it appears that both *mas* and *CaMV35S* promoters can be used for the construction of improved plant transformation vectors. Tn5 *neomycin phosphotransferase* (*nptH*) gene was expressed with either of these promoters (Kevin *et al.* 1989). Sequence analysis of the *AV3* promoter from *Ageratum yellow vein virus* (*AYVV*) showed that it might be a remnant of prokaryotic ancestors that could be related to certain promoters of bacteria from marine or freshwater environments (Wang *et al.* 2013). Bacterial promoters show differential expression in transgenic plants, and regulation of *rol* gene expression plays a role in the biological effects that are caused by the *rol A*, *B*, and *C* genes containing the *uid*A reporter gene under the control of *rol A*, *B*, and *C* promoters of *Agrobacterium rhizogenes* (Schmulling *et al.* 1989). However, their use for generating transgenic crop plants is limited.

2.1.1.3 Plant: Promoters derived from plant origin are used for the constitutive transgene expressions in plants (Dhankher *et al.* 2002). Plant constitutive promoters such as rice *actin* (*OsAct1*) (McElroy *et al.* 1991), maize *alcohol dehydrogenase1* (*ZmAdh1*) (Fromm *et al.* 1990), and maize *ubiquitin* (*ZmUbi1*and *ZmUbi2*) (Christensen *et al.* 1992) are the most commonly used in crop plants. *Act2* promoter was obtained from the *actin* gene family, which is the cytoskeletal component and expressed in every plant cell (An *et al.* 1996). Similarly, the *OsActin1* promoter also displayed expression in almost all tissues when transformed back into rice (McElroy *et al.* 1991). The *OsAct* promoter was shown to drive high levels of expression of *HVA1* gene in the leaf and root tissues of rice, thereby conferring salt and water stress tolerance (Xu *et al.* 1996). *ZmAdh* is another constitutive promoter expressed in specific tissues like root, shoot meristems, endosperms and pollen (Kyozuka *et al.* 1991). When *MtHP* promoter from *Medicago* was fused to a *uid*A gene, it showed expression in various plant parts in *Medicago* and *Arabidopsis* with an expression pattern similar to that of the *CaMV35S* promoter (Xiao *et al.* 2005). Expression of *uid*Ab gene driven by the *CaMV35S* and *MpEF1*α promoters were compared throughout plant development. While *CaMV35S* promoter resulted in in adequate expression in the meristematic tissues and a strong expression in the callus, the *MpEF1*α-promoter caused a strong meristematic *uid*A gene expression and was more active in female sexual tissues. It appears therefore, *MpEF1*α- promoter is a better option for obtaining strong and ubiquitous transgene expression compared to the *CaMV35S* promoter as has also been pointed out by Althoff *et al.* 2014.

Expression of *uid*A under the influence of *UBQ1*or *UBQ2* rice ubiquitin promoters were 8 to 35-folds higher in transgenic rice plants, respectively, when compared to *CaMV35S* (Wang and Oard 2003). This indicates that *ubiquitin* promoters are superior for activating the transgenes. Expression of the *gfp* gene mediated by a *GmUbi* showed high levels of constitutive expression in soybean tissues, thereby providing an alternative to viral promoters for driving gene expression in soybean (Hernandez-Garcia *et al.* 2009). The native *ubi1* promoter is a promising genetic element for constitutive expression of any gene in rice tissues (Bhattacharyya *et al.* 2011). *OsCon1* promoter exhibited comparable activity with *OsCc1*, *OsAct1* or *ZmUbi* promoters in most tissues, and even more active than the *CaMV35S* promoter in roots, seeds and calluses indicating that it is a novel constitutive promoter which could potentially be used for developing

Table 1. Constitutively expressed viral promoters used in development of transgenic plants

Promoter	Source	Host	References
SVFLt	*Peanut chlorotic streak caulimo virus (PC1SV)*	Tobacco	Maiti and Shepherd (1998)
Enhanced 35S (E35S)	*Cauliflower mosaic virus 35S*	Soybean	Li *et al.* (2001) and Bhattacharyya *et al.* (2002)
Sgt	*Figwort mosaic virus (FMV)*	Tobacco/maize	Bhattacharyya *et al.* (2002)
CsVMV	*Cassava vein mosaic virus (CsVMV)*	Soybean/grape	Seemanpillai *et al.* (2003)
sgRNA β 1, β 2, and γ	*Barley stripe mosaic virus (BSMV)*	Tobacco	Johnson *et al.* (2003)
SV (antisense)	*Peanut chlorotic streak caulimovirus (PC1SV)*	Tobacco	Bhattacharyya *et al.* (2003)
35S	*Cauliflower mosaic virus 35S*	*Arabidopisis*/Tobacco/Soybean/grape/ *Phaeodactylum*/*Jatropha*	Yang *et al.* (2003)
CLCuBuV Rep/ CLCuBuV CP	*Cotton leaf curl Burewala virus (CLCuBuV)*	Tobacco/cotton	Obertello *et al.* (2005)
FMV	*Figwort mosaic virus (FMV)*	Soybean	Govindarajulu *et al.* (2008)
Pptca1	*Cytomegalo virus (CMV)*	*Phaeodactylum tricornutum*	Sakaue *et al.* (2008)
e35S-4ocs	*Cauliflower mosaic virus 35S*	*Allocasuarina verticillata*	Govindarajulu *et al.* (2008)
AV3	*Ageratum yellow vein virus (AYVV)*	*E. coli*	Wang *et al.* (2013)
TLCV	*Tomato leaf curl virus*	Tobacco	Khan *et al.* (2015)
SVBV2	*Strawberry vein banding virus*	Soybean	–
T500/T600/ T1200	*Taro bacilliform virus (TaBV)*	Banana, tobacco	–

transgenics (Li *et al.* 2014). Incorporation of *AtTCTP* promoter into creeping bent grass showed that it can be used as a plant-derived constitutive promoter for the expression of selectable marker genes, and as an alternative to the *CaMV35S* promoter for developing GM crops (Han *et al.* 2015). The plant-derived *JcUEP* promoter could be another alternative to the *CaMV35S* promoter for directing constitutive transgene expression in *Jatropha* and other plants (Tao *et al.* 2015). Pineapple *SUI1* and *L36* promoters seem to drive *uid*A expression in all tissues of *Arabidopsis* at levels comparable to that of the *CaMV35S* promoter (Koia *et al.* 2013). Studies on rice *APX* promoter show edits constitutive expression in the seed, root, blade, flower and leaf of transgenic rice (Park *et al.* 2010). The inducible activities of *PvUbi1* and *PvUbi2* promoters in switchgrass, rice and tobacco are strong constitutive promoter candidates that can be used in genetic transformation of both monocots and dicots (Mann *et al.* 2011). Promoters such as *pBdEF1α* and *pBdUBI10* are constitutively expressed and highly active in maize, whereas *pBdGLU1* isolated from *Brachypodium* was clearly endosperm-specific, thereby indicating that it is an excellent resource for promoters for transgenic research in heterologous cereal species (Coussens *et al.* 2012). The *KST1* partial promoter was shown to drive constitutive expression in the guard cells of monocots and dicots, as well as in both annual and perennial plants (Kelly *et al.* 2017). Three *Citrus sinensis* constitutive gene promoters confirmed their role in vegetative tissues (Erpen *et al.* 2018), thus enabling the researchers to use it specifically in vegetative tissues. On the other hand, Jiang *et al.* 2018 reported that *AtSCPL30* promoter could be an alternative for the *CaMV35S* in terms of reducing transgene silencing and also a good source for the multigene transformation. *GAPC2* and *EF1* promoter fragments from *Citrus sinensis* were expressed constitutively in the transgenic tobacco plants (Erpen-Dalla Corte *et al.* 2020). Description of constitutively expressed plant promoters used for the development of transgenic plants is listed in the table 2.

2.1.2 *Tissue-specific promoters:* Tissue-specific promoters drive the expression of a targeted gene in a specific tissue(s) or organ at a specific stage(s) of plant growth and development (figure 2b). Since the constitutive promoters cause potential negative effects such as metabolic burden on the transgenics, specific promoters are used to regulate and target gene expressions in an effective manner for improving traits like grain

Table 2. Description of constitutively expressed plant promoters used for the development of transgenic plants

Promoter	Source	Host	References
UBQ1/UBQ2	Rice	Rice	Wang and Oard (2003)
UBQ1	*Arabidopsis*	*Allocasuarina verticillata*	Obertello *et al.* (2005)
VR-ACS1	Mung bean	Tobacco/*Arabidopsis*	Cazzonelli *et al.* (2005)
Ubi	Soybean	Soybean	Hernandez-Garcia *et al.* (2009)
Ubi1	Rice	Rice	Bhattacharyya *et al.* (2011)
Ubi1	Corn	Rice	Bhattacharyya *et al.* (2011)
GAI	Rice	Rice	Bhattacharyya *et al.* (2011)
Ubi1/Ubi2	Switchgrass	Switchgrass/ Rice/ Tobacco	Mann *et al.* (2011)
uceApro2	Cotton	Cotton/*Arabidopsis*	Viana *et al.* (2011)
SUI1	Pineapple	*Arabidopsis*	Koia *et al.* (2013)
L36	Pineapple	*Arabidopsis*	Koia *et al.* (2013)
EF1α	*Marchantia polymorpha*	*Marchantia polymorpha*	Althoff *et al.* (2014)
EF1α/UBI10/GLU1	*Brachypodium distachyon*	Maize	Coussens *et al.* (2012)
Con1	Rice	Rice	Li *et al.* (2014)
TCTP	*Arabidopsis*	*Agrostis stolonifera*	Han *et al.* (2015)
RD29A/RD29B	*Arabidopsis*	Soybean	Bihmidine *et al.* (2013)
UEP	*Jatropha curcas*	*Jatropha curcas, Arabidopsis*	Tao *et al.* (2015)
KST1	*Potato*	*Potato, Tobacco, Cucumber, Grape, Barley*	Kelly *et al.* (2017)
CsCYP,CsGAPC2, CsEF1	*C. sinensis*	*C. sinensi*	Erpen *et al.* (2018)
AtSCPL30	*Arabidopsis*	Tobacco	Jiang *et al.* (2018)
CsGAPC2, CsEF1	*Citrus sinensis*	Tobacco	Erpen-Dalla Corte *et al.* (2020)

nutritional quality. Due to the demand from biosafety regulators for less intrusive transgene expression, targeted expression of transgenes has become more important for the future development of value-added crops (Bucchini and Goldman 2002).

2.1.2.1 Promoters that help in biomass production: *Eucalyptus* transgenics with AtEXP4-pro:CBM2a showing increased plant height, enlargement of xylem, xylem fiber and vessel cells can be an attractive choice for plant biomass improvement (Keadtidumrongkul *et al.* 2017).

2.1.2.2 Promoters specific to guard cells and vascular tissues: A partial promoter of *KST1* from potato is active in a monocot and the first promoter reported to confer guard cell expression in barley and cucumber (Kelly *et al.* 2017). Thus, *KST1* appears to be a cell specific promoter and highly useful for manipulating proteins in the guard cells. The use of phloem specific promoters (*Citrus phloem protein 2-CsPP2*, *Arabidopsis thalianaphloem protein 2-AtPP2*, *A. thaliana sucrose transporter 2-AtSUC2*, and *sucrose synthase 1-SUS1*) might increase the chances of producing more bacterial disease resistant transgenic cultivars in many crop plants (Singer *et al.* 2011; Miyata *et al.* 2012).

2.1.2.3 Root-specific promoters: Root-specific promoters are of special interest since they help in understanding the root architecture and in alleviating drought and salt stress conditions. The *pNtREL1* promoter can be used to direct root-specific expression of target genes to protect the root from different abiotic stresses (Zhang *et al.* 2016a, b). Alternatively, Tau Class *Glutathione-S-Transferase* (*SbGSTU*) gene promoter of *Salicornia brachiata* can be used for both constitutive as well as stress-inducible expression of transgenes (Tiwari *et al.* 2016). More data are needed if such promoters help to mitigate the stress better in comparison with the constitutive promoters with minimum or no yield penalty. A root-preferential promoter *GmPRP2* isolated from soybean has been found useful in developing transgenics of novel soybean cultivars (Chen *et al.* 2014). Rice promoters such as *Os03g01700* and *Os02g37190* are highly active in the root tissues of rice and can be helpful for the root-

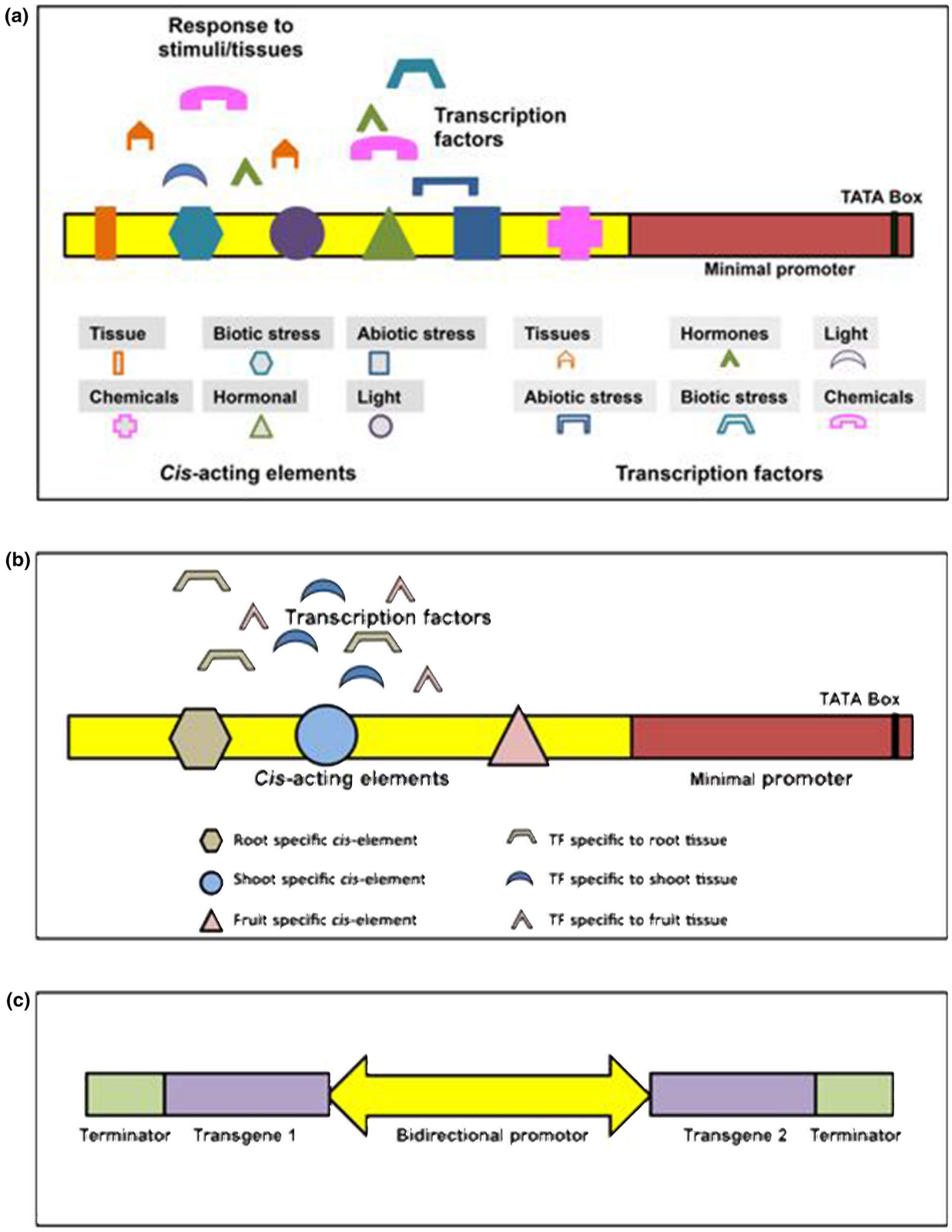

Figure 2. Schematic depiction of different types of promoters. (**a**) Stress-inducible promoters can be activated under any biotic or abiotic stressby binding the corresponding transcription factors and *cis*-acting elements. (**b**) Tissue-specific promoters are expressed in a particular tissue by binding the corresponding transcription factors and *cis*-acting elements. (**c**) Bidirectional promoters drive the transcription of two genes simultaneously that flank the promoter.

specific expression of target gene (Li *et al.* 2013a, b, c, d, e). Novel nematode-responsive root-specific promoter (NRRS) from *Arabidopsis* is seen to be expressed high in gall and root of transgenic Arabidopsis plants in defence to the infection of *Meloidogyne incognita* nematode (Kakrana *et al.* 2017).

2.1.2.4 Flower-specific promoters: High-level expression was recorded in a floral-enhanced manner for 1.45 kb promoter of endogenous ubiquitin extension protein (UEP1), although 9-fold lower *uid*A expression was noticed in the leaves (Annadana *et al.* 2002). Comparison between *CaMV35S* and *UEP1* promoters revealed that the *UEP1* has 40- to 85-fold higher expression in the flower, indicating its high tissue-specificity. The *Arabidopsis CER6* gene when transformed into *Chrysanthemum* resulted in high, specific expression in flower petals due to the *CER6* promoter, but had high variability as compared to the *UEP1* promoter (Hannoufa *et al.* 1996).

2.1.2.5 Fruit-specific promoters: For the fruit-specific expression, promoters related to fruit ripening such as*1-aminocyclopropane-1-carboxylate* (*ACCoxidase*), *E8* and *polygalacturonase* (*PG*) have been characterized from apple (Atkinson *et al.* 1998) and tomato (Nicholass *et al.* 1995). A fruit-ripening-induced promoter 1(*RIP1*) of tomato is perhaps more crucial for controlled manipulation of ripening-related agronomic traits not only in tomato but also in many other fruit crops (Agarwal *et al.* 2017). In transgenic tomato, the promoter *ACC oxidase* was able to drive ripening-specific expression of a reporter gene in the fruit only with no detectable activities in other tissues and mature green fruits (Atkinson *et al.* 1998). Transgenic tomato plants with enhanced sweetness, flavor and nutritive values were developed by expressing the *monellin* gene under the control of *E8* promoter (Reddy *et al.* 2015). For the expression of target genes and directing expression of the virus-F protein as an oral vaccine to induce systemic immunity in mice, the promoter of the tomato *E8* gene was used successfully in a number of instances (Lewinsohn *et al.* 2001). Although this promoter drives the expression of transgenes in flowers (predominantly anthers), its invariable expression throughout the ripening fruit makes it very valuable. The improved quality of ripened tomatoes in terms of vitamins and micronutrients content makes it valuable for use in transgenic research (Hadley *et al.* 2002). For the production of biopharmaceuticals, the use of organ-specific promoters are important so as to express the gene of interest in those organs that are able to produce protein(s) in an appropriate manner (Twyman 2003). Deletion analysis showed improvement in the quality and nutritional value of the fruits when the stearoyl-acyl-carrier-protein desaturase (Des) promoter region located between −590 and +10 was used for transforming oil palm and also dicots (Saed *et al.* 2012). However, comparative studies between constitutive and inducible promoters elaborating the specific merits of inducible promoters especially in the fruits are very meager in the literature. Therefore, such a study is vital to pinpoint the advantages of inducible promoters over that of constitutive (table 3).

2.1.2.6 Seed-specific promoters: Seed-specific transgene expression has been used in many applications including nutritive value improvement, milled grain quality and production of industrial or pharmaceutical compounds (Ye *et al.* 2000; Cahoon and Shanklin 2000). Promoter's specific to seed storage genes is an attractive target for such uses in GM crops. Soybean *β-conglycinin* gene promoter was used for seed-specific expression, where gene expression was restricted to the embryo during the mid-to-late maturation phase (Lessard *et al.* 2002). Likewise, groundnut seed promoter (GSP) can potentially be used for modification of seed phenotypes in agronomically important crops (Sunkara *et al.* 2014). While the sunflower *helianthinin* full-length promoter was used to drive seed-specific expression in tobacco (Nunberg *et al.* 1994), the expression of the *β-phaseolin* promoter in transgenic tobacco plants was restricted to the developing seed only (van der Geest and Hall 1997). Several endosperm-specific promoters like those from *hordein* in barley (Forde *et al.* 1985), *glutenin* in wheat (Lamacchia *et al.* 2001) and *zein* in maize (Marzabal *et al.* 1998) have been studied extensively. The guar *MS* promoter could manifest its usage for directing

Table 3. Bacterial constitutively expressed promoters expressed in different plants

Promoter	Source	Host	References
Rol A/B/C	*Agrobacterium rhizogens*	-	Schmulling *et al.* (1989)
AV3	*Ageratum yellow vein virus (AYVV)*	*E.coli*	Wang *et al.* (2013)
Ocs	*Agrobacterium*	-	Zhou *et al.* (2013)
Mas	*Agrobacterium*	-	Zhou *et al.* (2013)
Nos	*Agrobacterium*	Rice	Zhou *et al.* (2013)

endosperm-specific expression of transgenes in legume species (Naoumkina and Dixon 2011). The expression of a 4kb promoter fragment from *granule-bound starch synthase1* (*gbss1*) gene in wheat is restricted to endosperm and developing kernel pericarp tissues only (Kluth *et al.* 2002).

The *WM403* promoter from watermelon may be useful in driving nucellus-specific gene expression in plants inclusive of candidate genes for important nucellus-specific attributes such as apospory or adventitious embryony (Dwivedi *et al.* 2010). *HaFAD2-1* promoter is as strong as the *CaMV35S* promoter even though it is a tissue-specific promoter and its activity is derived from the embryo, thereby confirming that it can be considered as a strong, highly specific seed promoter useful for genetic manipulation applications in the seed (Zavallo *et al.* 2010). The *Arabidopsis At4g12960* (*AtGILT*) promoter was employed as a canola seed coat outer integument-specific promoter after the production and selection of desired transformants from many transgenic lines (Wu *et al.* 2011). Seed coat-specific promoters can also be used to assess the effects of many pathway enzymes/proteins and cell-wall modifying proteins on mucilage structure as has been pointed out by Dean *et al.* (2017). Maize *BD1*, *Def1* and *Def2* promoters were active and reproduced the expression patterns of both *Def1* and *Def2* genes in transformed immature maize embryos, as well as in developing seeds of transgenic maize (Liu *et al.* 2016). Thus, an array of tissue-specific promoters has been used in diverse taxa with many beneficial effects. Table 4 highlights a list of tissue-specific promoters that have been used so far in plant genetic engineering studies.

2.1.2.7 Underground storage-tissue-specific promoters: Promoters like *pDJ3S* and to a lesser extent *pMe1* from yam and casava respectively drive the high and preferential expression of genes in carrot storage roots (Arango *et al.* 2010). Such promoters help in improving the biomass and nutritional quality of the underground storage tissues.

2.1.3 *Abiotic stress inducible:* Plant performance is not conditioned to endogenous factors alone, but also to environmental factors and external stimuli. Several promoters are induced by hormones, chemicals, and environmental stresses (figure 2a) (Chakravarthi *et al.* 2016). Based on the source and type of cells in which they regulate gene expression, hundreds of inducible promoters have been identified (Singh *et al.* 2002). For induction of promoters under different abiotic stresses, *cis*-acting elements like dehydration responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki 2006), an abscisic acid-responsive element (ABRE) (Bonetta and Mccourt 1998), and heat shock element (HSE) have been identified. The necessity of such a system for transgene expression may be vital to

Table 4. List of tissue-specific promoters expressed in different plant species

Promoter	Source	Host	References
aPAL	*Loblolly pine*	Tobacco	Osakabe *et al.* (2009)
DJ3S	Yam	Carrot	Arango *et al.* (2010)
Me1	Casava	Carrot	Arango *et al.* (2010)
WM403	Watermelon	*Arabidopsis*	Dwivedi *et al.* (2010)
GILT	*Arabidopsis*	Canola	Wu *et al.* (2011)
HaFAD2-1/ HaAP10	Sunflower	*Arabidopsis*	Zavallo *et al.* (2010)
CCR	*Eucalyptus gunnii*	Grapevine	Gago *et al.* (2011)
MS	Guar	*Medicago sativa*	Naoumkina and Dixon (2011)
CsSUS1p-1/ CsSUS1p-2	*Citrus sinensis*	*Arabidopsis*/ tobacco	Singer *et al.* (2011)
Des	Oil palm	Tomato	Saed *et al.* (2012)
DX1	Rice	Rice	Ye *et al.* (2012)
Os03g01700/ Os02g37190	Rice	Rice	Li *et al.* (2013b)
tCUP1	Tobacco	Rice	Zhou *et al.* (2013)
RIP1	Tomato	Tomato/ *Arabidopsis*	Agarwal *et al.* (2017)
PZmBD1/ PZmDef1/ PZmDef2	Maize	Maize	Liu *et al.* (2016)
AtMYB60	Arabidopsis	*Arabidopsis tobacco* tomato rice	Cominelli *et al.* (2011), Meyer *et al.* (2010) Oh *et al.* (2005) and Rusconi *et al.* (2013)
CYP86A2	Arabidopsis	Arabidopsis	Francia *et al.* (2008)
GC1	Arabidopsis	Tobacco	Wang *et al.* (2014)
NRRS	*Arabidopsis*	*Arabidopsis*	Kakrana *et al.* (2017)

develop promoter set-ups where precise temporal regulation of transgene expression is a requisite. Examples include situations where unwanted gene expression is harmful or lethal, especially during the development of the plant (Guo *et al.* 2003). For example, peanut transgenics overexpressing *AtDREB1A* transcription factor under the influence of the *CaMV35S* promoter showed severe stunting that could be overcome by using the *rd29A* stress-inducible promoter to drive the expression of *DREB1A* (Bhatnagar-Mathur *et al.* 2007). Overexpression of a stress-related gene under an inducible promoter may confer better tolerance to stress than when under a constitutive one, while causing minimal or no growth retardation (Nakashima *et al.* 2007; Li *et al.* 2013a, b, c, d, e). The growth and development of *CaMV35S*-TaEXPB23 transgenic tobacco plants were altered under normal conditions, with a faster growth rate at the seedling stage, early flowering and maturation, and a shorter plant height compared to wild-type plants. On the other hand, RD29A-TaEXPB23 transgenic tobacco plants exhibited greater tolerance to water stress than the wild-type. Therefore, the use of stress-inducible promoters, such as*rd29A* may minimize the negative effects of constitutive transgene expression and improve the water-stress tolerance of plants (Shen *et al.* 2003; Bhatnagar-Mathur *et al.* 2007; Li *et al.* 2013a, b, c, d, e). These studies infer that stress-inducible promoters are superior for enhancing plant productivity under stress conditions in comparison with that of the constitutive promoters. However, it must be ensured that the stress-inducible promoter expression is very tightly controlled in response to the desired stress to avoid any unintended effects on plant growth and development.

Rd29X promoters have been demonstrated to be useful in controlling targeted transgenes to mitigate abiotic stress in soybean (Bihmidine *et al.* 2013) and peanut (Bhatnagar-Mathur *et al.* 2007; Rao *et al.* 2017). However, the usefulness of the endogenous inducible promoters is often limited since they are leaky. There may be a possibility for the activation of other endogenous genes by the same inducer. Srivastava *et al.* 2014 reported that *PsSEOF1* promoter from pea could serve as an important candidate for tissue-specific promoter for engineering plants for both biotic and abiotic stress conditions. *HsfB2c* and *PM19* promoters from rice are highly heat-inducible and further characterization and reorganization of *cis*-acting elements in their promoters could lead to the development of highly effective, heat-inducible promoters (Rerksiri *et al.* 2013). It was noticed that *PR10* promoter from *Erianthus* though highly constitutive, was quickly induced upon wounding as well as on treatment with ABA and methyl jasmonate (Chakravarthi *et al.* 2016). The *OsABA2* promoter was shown to drive a low constitutive transgene expression under normal conditions but high induction in response to ABA, salt and drought stresses (Rai *et al.* 2009). The *AlSAP* promoter from the halophyte grass *Aeluropus littoralis* directs a stress-inducible expression pattern in transgenic rice plants making it an interesting candidate for engineering abiotic stress tolerance in cereals (Ben-Saad *et al.* 2015). Bang *et al.* 2012 similarly demonstrated that *OsNCED3* promoter was stress-inducible in whole rice plant except in the aleurones and endosperm and stably active over three generations. Stress-inducible expression of *Hsc70*, *Lea*, *Hsp10*, *Dhn* and *Apx* promoters from *Pennisetum glaucum* have been shown to confer abiotic stress tolerance in different tissues (leaf, stem and root) of the transgenic tobacco plants when exposed todifferent abiotic stresses (Divya *et al.* 2019; 2020, Divya *et al.* unpublished data). Hou *et al.* 2016 demonstrated that maize *Type-II* H^+*-pyrophosphatase* promoter has higher expression under drought and salinity conditions compared to the *CaMV35S* promoter. The *AtUSP* promoter is highly inducible by phytohormones as well as multiple abiotic stresses that can be exploited as a stress-inducible promoter to develop multi-stress tolerant crops with least adverse effects on other important traits (Bhuria *et al.* 2016).

Promoter like the *AhMTP1* having a variety of *cis*-acting elements, particularly the MYB-binding sites are involved in the evolution of zinc tolerance (Fasani *et al.* 2017). The three cadmium-inducible gene promoters such as *OsGSTU5*, *OsGSTU37* and an *OsHsp20* from rice could be potentially used for bio-environmental contamination and improving heavy metal tolerance in crops (Qiu *et al.* 2015). Recent study indicated that *pGAL*-2kb could be a useful in developing droughttolerant cultivars by driving transgene expression (Conforte *et al.* 2017). *CcHyPRP* promoter in *Arabidopsis* has been shown to be regulated by different stress factors which can be deployed for enhancing abiotic stress tolerance in transgenics (Srinath *et al.* 2017). *ZmPIS* and *PZ7* promoter fragments in tobacco would be ideal candidates for overexpression of drought- and salinity-responsive genes to improve crop tolerance (Zhang *et al.* 2016a, b).

Regulated gene expression systems would also be valuable in GE applications such as conditional expression of herbicide, flowering and fruit ripening genes that emphasize the need for developing transgenics by deploying inducible promoter systems (Liu *et al.* 2013). Myb-related protein-like promoter from

rice showed ethanol-inducible promoter activity and could be used to generate transgenic crops with desirable traits as demonstrated by Khanthapok *et al.* (2018). Aforementioned studies indicate that a wide spectrum of stress-inducible promoters exist, however their effectiveness and relative performance under multiple stress conditions in different taxa needs to be investigated. Recent study by Divya *et al.* (2019) it's observed that *PgApx* and *PgDhn* promoters are upregulated in drought, heat, cold and salt stresses and *PgHsc70* promoter is active in heat and drought stress. In another study, *PgHsp10* promoter from same plant i, e., *Pennisetum glaucum* seen to be expressed in heat and drought stress in transgenic tobacco plants (Divya *et al.* 2020). Different stress-inducible promoters used in varied studies so far are listed in table 5.

2.1.4 *Synthetic:* Recent advances in plant promoter engineering are making strides to generate more constitutive, bidirectional or inducible synthetic promoters for a proper transcriptional modulation of transgene expression in plants. Synthetic promoters provide enormous advantages over their counterparts with respect to transgene expression at a specific developmental stage, strength and tissue specificity. Synthetic promoters can be rationally sketched and constructed using specific type, copy number and positioning of motifs upstream of synthetic or native core promoters. To date, most synthetic promoters tested were either hybrids of multiple promoter parts or fusions of specific *cis*-regulatory elements with a core promoter. Typically, synthetic motif sequence is derived from extant sequences that are multiplied or recombined. The selection, copy number and spacing of *cis*-elements ascertain the strength, temporal and spatial expression patterns of synthetic promoters. Selection of motifs with known functions can be conducted with the help of previous studies (Banerjee *et al.* 2015) or from the databases, synthetic motif library screening (Roccaro *et al.* 2013) and bioinformatics based *de novo* motif discovery (Tompa *et al.* 2005). As the deconstructive analysis of plant natural promoters for functional motif discovery has slowly increased and the effectiveness of using systems biology tools for *denovo* motif discovery has been experimentally demonstrated in *Arabidopsis* (Koschmann *et al.* 2012) and soybean (Liu *et al.* 2014). Once motifs of interest have been selected for synthetic promoter construction, copy number and spacing of motif need to be optimized. Usually, motif copy number often correlates with synthetic promoter strength which has been demonstrated in various plant species like rice (Wu *et al.* 1998), tobacco (Sawant *et al.* 2005) and *Arabidopsis* (Sahoo *et al.* 2014). The motif dosage effect in synthetic promoters is not surprising, since congruent

Table 5. List of stress-inducible promoters studied in transgenic plants

Promoter	Source	Host	References
ABA2/ rab16A, HP1	Rice	Rice	Rai *et al.* (2009)
DREBa	*Chrysanthemum dichrum*	*Arabidopsis*	Chen *et al.* (2012)
Rab16A	Rice	Rice	Ganguly *et al.* (2011)
NCED3	Rice	Rice	Bang *et al.* (2012)
HsfB2cp, PM19p, Hsp90p	Rice	Rice	Rerksiri *et al.* (2013)
DXS, GGPPS	*Ginkgo biloba*	-	Xu *et al.* (2013)
hsp82	Rice	Rice	Company *et al.* (2014)
SOS1-AB	*Triticum aestivum*	*Arabidopsis*	Feki *et al.* (2015)
SEOF1	Pea	Tobacco	Srivastava *et al.* (2014)
SEOF1	*Pisum sativum*	Tobacco	Srivastava *et al.* (2014)
PR10	*Erianthus arundinaceus*	Tobacco/ rice/ sugarcane	Chakravarthi *et al.* (2016)
DREB1	Buckwheat	Tobacco	Fang *et al.* (2016)
ERF3	Soybean	Soybean/ tobacco	Hernandez-Garcia and Finer (2016)
UGT71C5	*Arabidopsis*	*Arabidopsis*	Liu *et al.* (2015)
GAPP	Maize	Tobacco	Hou *et al.* (2016)
pGAL	Soybean	Arabidopsis	Conforte *et al.* (2017)
HyPRP	Cajanus cajan	Arabidopsis	Srinath *et al.* (2017)
SnRK2.7	*Triticum aestivum*	Arabidopsis	Wang *et al.* (2018)
Myb-related Protein	*Oryza sativa*	Oryza sativa	Khanthapok *et al.* (2018)
PgAPX	*Pennisetum glaucum*	Tobacco	Divya *et al.* (2019)
PgDhn	*Pennisetum glaucum*	Tobacco	Divya *et al.* (2019)
PgHsc70	*Pennisetum glaucum*	Tobacco	Divya *et al.* (2019)
PgHsp10	*Pennisetum glaucum*	Tobacco	Divya *et al.* (2020)

findings have been observed in native promoters like in *Malus* (Espley *et al.* 2009) and *Arabidopsis* (Cao *et al.* 2014). When many motifs are accommodated in to a single synthetic promoter, spacing among motifs should be proper that is required for the hierarchical arrangement of their corresponding TFs in order to obtain full synergistic interactions with the RNA polymerase II complex (Sawant *et al.* 2005). Interestingly, two copies of the ACGT motif in synthetic promoters was shown to result in salicylic acid inducibility when separated by five nucleotides, but were ABA-inducible when separated by 25 nucleotides (Mehrotra and Mehrotra 2010).

The parsley (*Petroselinum crispum*) protoplast system was used for analyzing MAMP-responsive synthetic promoters which can be used by other plant systems to respond in cases of microbial pathogen attack (Kanofsky *et al.* 2016). Li *et al.* (2013a) reported that the synthetic promoter pCL made from *Arabidopsis* and potato was found optimal use for gene function research in potato tubers in response to low temperature. Altering the structure of CRT/DRE enhanced the CBF-associated transcription complex formation and thus improved the activity of this pCL, synthetic tuber-specific and cold-inducible promoter (Li *et al.* 2015). Comparative studies amongst the three natural promoters from rice (*Rab16A*) and two synthetically designed promoters, viz., 4X ABRE (abscisic acid-responsive element) having four tandem repeats of ABRE, and 2X ABRC (abscisic acid-responsive complex) having two tandem repeats of ABRE and two copies of coupling elements showed that 2XABRC make a better salinity/ABA-inducible promoter. The studies of Ganguly *et al.* (2011) indicated strong *GUS* expression in the whole seed (both embryo and aleurone layer of endosperm) only by 2X ABRC, while it was localized in the embryo for the other two promoters. Wang *et al.* (2015) demonstrated the synthesis of rice tissue-specific promoters and also developed a novel, feasible method for screening as well as for functional characterization of tissue-specific *cis*-acting elements with their flanking sequences at the genome-wide level in rice. This synthetic promoter including the 35S core sequence and two binding sites for cold-inducible CBF transcription factors (PDRE::35S) exhibited transient expression under chilling conditions using synthetic cold-inducible promoter which enhanced the target protein accumulation, and may decrease greenhouse heating expenses (Gerasymenko and Sheludko 2017). Synthetic *SynP16* promoter designed from *cis*-motifs of soybean viz. *ABF, ABRE, ABRE-Like, CBF, E2F-VARIANT, G-box, GCC-Box, MYB1, MYB4, RAV1-A*, and *RAV1-B* (in multiple copies and various combination) with a minimal 35s core promoter and a 222 bp synthetic intron sequence. This synp16 promoter induced GUS expression in stress inducible manner and tissues specifically in transgenic soybean and *Arabidopsis* (Jameel *et al.* 2020). Thus, synthetic promoters that are rationally designed have become highly efficient components for precise regulation of target gene expression. Different synthetic promoters used thus far are shown in table 6.

2.2 *RNA pol III promoters*

RNA polymerase III (pol III) has been used for transcription of structural and catalytic RNAs including 5S rRNA, tRNA and most small nuclear RNAs. Promoters for pol III have diverse structural features including reinitiation property and transcribing of at least four different types of genes, such as tRNA, 5S rRNA, U6 snRNA, 7SL-Sc-RNA, 7SK RNA. Thus *pol III* transcribes housekeeping genes required at all times. Different types of *pol III* promoters such as type 1 (5S RNA), type 2 (tRNA *Leu*) promoters of the *Xenopus laevis*, type 3 promoter of the *Homo sapiens* U6 snRNA gene and the type 4 Sc type of the *Saccharomyces cerevisiae* promoters were noticed. Other *pol III* promoters are *U3* and *U6* which are used in monocots and dicots, respectively (Belhaj *et al.* 2013). *Pol III* promoters are generally used to express small RNAs, short hairpin RNA, and guide RNA in the CRISPR/Cas9 system adapted for genome editing. *Pol III* promoters have not been much characterized excepting in model plants. Also, their relative advantage over that of other promoters need to be explored further.

3. Promoters in genome editing (CRISPR/Cas9 system)

Genome editing is a type of GE where the specific DNA sequence can be inserted, deleted or replaced. This can be accomplished by introducing site-specific double-strand breaks (DSBs) at specific sites in the targeted genome. The induced DSBs are repaired either through non-homologous end joining (NHEJ) or homologous recombination (HR), resulting in specific and targeted mutations. Currently, four families of engineered nucleases are being used: meganucleases (MGNs), zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the clustered regularly interspaced short palindromic

Table 6. Synthetic promoters that have been designed for use in plant systems

Promoter	Type	Source	Host	References
pCL	Tuber-specific and cold-inducible	At cor15a promoter region and potato patatin promoter region	Potato	Li *et al.* (2013a)
Sab/sba	Cold-inducible	At cor15a promoter regions and cor15b promoter regions	Tobacco	Li *et al.* (2013a)
4 x CCTC	Fungal colonization under low-Pi condition-inducible	Potato Piransporter 3 (StPT3) promoter regions; 35S core promoter	Potato/Lotus	Lota *et al.* (2013)
4xRE/B4REA	Hormonal and bacterial pathogen inducible	Hormone-response elements; 35S core promoter	Tobacco, *Arabidopsis*	Liu *et al.* (2014)
FsFfCBD	Bidirectional	FsCP; 35S core promoter; a tri-hybrid enhancer FsEFfECE	Tobacco	Patro *et al.* (2013)
4 x GCC	Jasmonic acid inducible	AtPDF1.2 promoter; 35S core promoter	*Arabidopsis*	Van der Does *et al.* (2013)
4 x ROSE1 ~ 7	ROS-inducible	AtROS-responsive elements; 35S core promoter	*Arabidopsis*	Wang *et al.* (2013)
FSgt-PFlt;MSgt-PFlt;PFlt-UAS-2X	Constitutive	FSgt, Msgt, PFlt-UAS; PFlt core promoter (PFlt)	Tobacco, *Petunia, Arabidopsis,* Tomato, Spinach	Acharya *et al.* (2014)
4 x RSRE	Stress-inducible	*Arabidopsis* rapid stress response elements; NOS core promoter	*Arabidopsis*	Benn *et al.* (2014)
p35S-PCHS-Ω;p35S-Ω;LCHSΩ;pOCS-PCHS-Ω;pOCS-LCHS-Ω	Flower-specific	35S or OCS enhancer; petunia CHSA core promoter; lily CHS core promoter, an omega element	*Toreniafournier*	Du *et al.* (2014)
CL	Tuber-specific and cold-inducible	*Arabidopsis* cor15a promoter region and potato patatin promoter region; 7 nucleotides mutation in the 5'and 3' flanking sequences of CRT/DRE.	Potato	Li *et al.* (2015)
MAMP-responsive synthetic promoter	Microbial pathogen attack	Four copies of a potential MAMP-responsive cis-sequence	*Petroselinum crispum*	Kanofsky *et al.* (2016)
P_DRE::35S	chilling temperatures	two binding sites for CBF transcription factors (CRT/DRE), CaMV35S promoter core sequence and 5-leader sequence of TMV omega	*N. excelsior*	Gerasymenko and Sheludko (2017)
SynP16	Soybean	Soybean *ABF, ABRE, ABRE-Like, CBF, E2F-VARIANT, G-box, GCC-Box, MYB1, MYB4, RAV1-A*, and *RAV1-B* (in multiple copies and various combination) with a minimal 35s core promoter and a 222 bp synthetic intron sequence	Soybean, Arabidopsis	Aysha *et al.* (2020)

repeats (CRISPR). While the MGNs and ZFNs were costly and hard to engineer, TALE nucleases are flexible, specific and relatively complex and CRISPR/Cas9 nucleases are user-friendly and cost-effective (Belhaj *et al.* 2013). The CRISPR/Cas9 system derived from bacterial immune system and has been widely used for targeted genome editing in diverse organisms including mammalian and most of the plant species (Hsu *et al.* 2014; Ma *et al.* 2015; Guo *et al.* 2018). The CRISPR/Cas9 system uses either single or multi-guide RNA system to induce the specific edits and this efficiency depends on the endonuclease activity of the sgRNA/Cas9 complex and selection of the promoters (Guo *et al.* 2018). It has been observed that targeted efficiency of the CRISPR/Cas9 depends on the codon optimization of the Cas9 and the promoters. Targeted

efficiency increases when expressing *Cas9* gene other than *CaMV35S* promoter such as the dividing cell specific *INCURVATA2* (Hyun *et al.* 2015), egg cell-specific promoters (Wang *et al.* 2015), the cell division-specific *YAO* (Yan *et al.* 2015), and the germ-line-specific *SPOROCYTELESS* (Mao *et al.* 2016) and embryo-specific promoter DD45 (Miki *et al.*, 2018). Targeted efficiency increased significantly when *ZmUbi1* promoter was used instead of *CaMV35S* (Feng *et al.* 2018). Feng *et al.* (2018) reported that *Zmdmc1* promoter could be an alternative to the CaMV35S for expressing the CRISPR/Cas9 system in maize for generating highly efficient targeted genome editing.

The *pol III* promoters such as *U3* and *U6* are commonly used to express the small RNAs and guide RNAs of the CRISPR/Cas9 system. Due to their long-term regulation of target genes and their defined sites for transcription initiation and termination, *U3* and *U6* promoters gained special attention over the others. Characterization of *pol III* promoters in many organisms was not properly explored and this becomes difficult in choosing the right promoters for CRISPR/Cas9 gene edited targeted mutagenesis. Hence, in most of the studies, *pol III* promoters such as *U3* or *U6* from *Arabidopsis* or rice or maize or wheat are often chosen to drive gRNAs (Nekrasov *et al.* 2013; Bortesi and Fischer 2015; Feng *et al.* 2018). Maize *ubi1* gene promoter in combination with two rice *U6* promoters performed well in maize with mutation efficiency up to 70% (Char *et al.* 2016). Processing of a pol II transcript into functional gRNA has been found successful using ribozyme or Csy4RNA cleavage systems. Mikami *et al.* (2017) have shown that functional gRNAs can be efficiently processed using SpCas9 protein and plant RNA cleavage systems without any need for a specific RNA processing. Recent studies have shown that *AtU6* promoter from *Arabidopsis* and *Fve U6* promoter of wild strwaberry are equally good for the high-efficiency genome editing (Zhou *et al*. 2013). Hashimoto *et al.* (2018) demonstrated increased efficiency and also improved multiplex genome editing using *Cas9* gene expression with *SlEF1α* promoter. Ordon *et al.* (2020) demonstrated that *DD45* and *RPS5a* promoter-driven Cas9 showed higher mutational frequency. Promoters used for genome editing are shown in the table 5.

4. Bidirectional promoters (BDPs)

A synthetic bidirectional expression module was prepared by placing a computationally designed minimal promoter sequence at the 5′ and 3′ sides of a transcription activation module. BDPs were identified in diverse organisms including plants. Structural features and functional consequences of BDPs received much attention in plant biotechnology due to their effective usage in gene-stacking/pyramiding and targeting the complex traits (Que *et al.* 2010). However, the underlying mechanisms responsible for the bidirectional transcription and co-expression of BDPs has remained poorly understood in plants where they drive the transcription of the two target genes (Patro *et al.* 2013). Such promoters play a pivotal role in the transcription of bidirectional gene pairs where the two genes are positioned head-to-head on opposite strands of DNA (figure 2c). The BDPs efficiently regulate two transgenes simultaneously which can be evaluated in multiple model-plant systems. The BDPs representing *Rep* and *coat protein* (*CP*) genes of CLCuBuV were characterized and their efficacy assayed (Khan *et al.* 2015). They also showed that the strong constitutive *CLCuBuV Rep* promoter could be of use for higher expression of transgenes in a variety of plant cells. Wang *et al*. (2016a, b) combined RNA-seq data and cDNA microarray data to discover the potential BDPs in rice genome. Comparative analysis between *ZmBD1*, *ZmDef1* and *ZmDef2* promoters revealed that *PZmBD1* shared most of the expression characteristics of the two polar promoters, but displayed more stringent embryo specificity, delayed expression initiation, and asymmetric promoter activity (Liu *et al.* 2016).

The initiation of transcription from both the unidirectional and bidirectional promoters made from the same sequence elements were evaluated by using the *uid*A and *gfp* reporter genes. The investigation based on transient and stable transformation of tobacco exhibited that the artificially designed multifactorial activation module activated the transcription simultaneously to proportionate levels in both the directions (Chaturvedi *et al.* 2006). The transcription regulatory module responded to elicitors like salicylic acid (SA), NaCl and IAA in the forward as well as reverse directions. It implied that constitutive and chemically-inducible bidirectional promoters can be deployed for predictable simultaneous regulation of two genes for genetic engineering in plants (Chaturvedi *et al.* 2006). Fang *et al.* 2016 elucidated the unique epigenetic mechanism of BDPs and regulation of the bidirectional gene pairs and eventually used for GE. The bidirectional promoters *At4g35985* (P85) and *At4g35987* (P87) in both orientations display up-regulation under salt stress. Such regulatory elements of BDPs showing spatial and stress-inducible promoter and functioning in heterologous systems might be an important tool for

Table 7. List of the promoters that have been designed for use in CRISPR/Cas9 genome editing

Species	Cas9 codon optimization	Promoters for Cas9	Promoter for gRNA	Target gene	Reference
A. thaliana	Human	*AtUBQ1*	*AtU6*	*GUUS*	Mao *et al.* (2016)
A. thaliana	*Arabidopsis*	*PcUBI4-2*	*AtU6*	*GUUS, UGUS*	Fauser *et al.* (2014)
A. thaliana	*Arabidopsis*	*PcUBI4-2*	*AtU6*	*GUUS, UGUS*	Fauser *et al.* (2014)
A. thaliana	Human	*2xCaMV35S*	*AtU6*	*YFFP*	Feng *et al.* (2013)
A. thaliana	Arabidopsis	*PcUBI4-2*	*AtU6*	*ADH1*	Schiml *et al.* (2014)
N. benthamiana	Arabidopsis	*CaMV35SPDK*	*AtU6*	*NbPDS3*	Li *et al.* (2013a, b, c, d, e)
O. sativa	Rice	*2xCaMV35S*	*OsU3*	*OsPDS, OsBADH2*	Shan *et al.* (2013)
O. sativa	Rice	*ZmUbi*	*OsU3*	*GUUS*	Mao *et al.* (2016)
A. thaliana	Plant	*35S PPDK*	*AtU6*	*AtPDS3, AtFL2, AtRACK1b, AtRACK1c*	Li *et al.* (2013a, b, c, d, e)
A. thaliana	Arabidopsis	*ICU2*	*AtU6*	*AtFT, AtSPL4*	Hyun *et al.* (2015)
N. benthamiana	Human	*CaMV35*	*PEBV*	*NbPDS, NbPCNA*	Ali *et al.* (2015)
G. max	Soybean	*EF1A2*	*GmU6*	*GmDD20, GmDD43*	Li *et al.* (2015)
Z. mays	Human	*ZmDMC1*	*ZmU3*	*Zmzb7*	Feng *et al.* (2018)
Gossypium hirsutum		*2xP35s*	*GhU6.3, AtU6-29*	*GUS*	Long *et al.* (2018)
A. thaliana		*CaMV35s*	*AtU6-26*	*AtM20*	Ma *et al.* (2018)
Tomato, N. benthamiana	Human	*CaMV35s*	*U6-26s*	*CP, Rep*	Tashkandi *et al.* (2018)
Fragaria vesca	Arabidopsis and maize	*AtUBQ10, CaMV35s*	*FveU6-2, AtU6-26*	*TAA1, ARF8*	Zhou *et al.* (2013)
Solanum lycopersicum	Arabidopsis	*SlEF1α, SlP16, Pcubi4, 2xCaMV35S*	*AtU6-26*	*SlNADK2A*	Hashimoto *et al.* (2018)
A. thaliana	Arabidopsis	*DD45*, *RPS5a*		*Lhcb1*	Ordon *et al.* (2020)

plant biotechnology and gene stacking applications (Banerjee *et al.* 2013). Recently, Araceli *et al.* (2017) have shown that BDPs function can be regulated by degree/intensity of the abiotic stresses like Pi availability. *GhZU* promoter from *Gossypium hirsutum* is seen to regulate the expression of *gus* and *gfp* genes in both the directions in transgenic *Arabiposis* plants (Yang *et al.* 2018). From these studies, it can be stated that BDPs have a potential role to play in plant genetic engineering (Table 7).

5. Conclusions and future perspectives

The selection of a promoter suitable for the targeted transgene expression is one of the most important criteria for developing the GM crops. There are some important implications for producing transgenic plants with higher yield without any compromise on environmental and biosafety concerns. Improved expression of transgenes has been carried by hybrids or combined promoters that are from the constitutive promoters. Constitutive promoters are often seen to be beneficial for a high-level expression of selectable marker genes, necessary for efficient selection and generation of transgenics. Constitutively active promoters are not necessarily always desirable for GE plants as the constitutive overexpression of a transgene may compete for energy and building blocks for synthesis of proteins, RNA and others. Transgenic plants constitutively expressing TFs have been shown to exhibit mild/severe growth retardation in the aerial parts, shorter petioles, rounder leaves, delayed flowering and a dwarf phenotype. In these situations, use of tissue-specific or stress-inducible promoters of moderate strength may be more desirable.

Tissue-specific and stress-inducible promoters can be exploited to minimize the unintended effects of adverse environmental conditions such as heat, cold, drought, and salinity. Inducible promoters are very powerful resources for GE plants, since the expression of genes operably linked to them can be regulated to function at certain stages of growth and development of an organism or a particular tissue which help in reduction of energy expenditure of the plant. Further, inducible promoters will be activated only when required, thereby maintaining yield or productivity even under adverse conditions. On the other hand, synthetic

promoters paired with synthetic TFs can be used to provide a coordinated transcriptional control of multiple genes, which would be needed for successful metabolic engineering.

Genome-wide computational prediction and analysis of core promoter elements across plant monocots and dicots will be useful for future work related to genome annotation projects and can inspire research efforts aimed to better understand regulatory mechanisms of transcription. Genome-wide transcriptomic analysis such as RNA-seq will also help in gene regulation as well as in the identification of promoters and *cis*-acting elements. Advancement in the area of systems biology and the development of new tools would be beneficial in the discovery of novel promoters and their *cis*-acting elements. Identification and deployment of promoters that can help under diverse stress conditions are desirable, ultimately for the generation of transgenics with better survival and productivity. CRISPR technology has become popular and an alternative to the plant breeding techniques. Genome editing efficiency can be improved by optimizing the diverse range of the promoters. Hence, crop varieties developed by above mentioned technologies are considered as non-GM in USA and other regions. Relaxing in usage of such crop varieties from the scope of the GMO legislation may have a positive impact on the development of the plant biotechnology and breeding sector for the betterment of humans.

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