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Review Article

Regulated promoters applied to plant engineering: an insight over promising soybean promoters under biotic stress and their *cis*-elements

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Highlights

- Although the significant number of reports on plants carrying transgenes conferring specific characteristics, little is known about plant-specific promoters, mainly those that are agronomically relevant, such as cotton and soybean. This review highlights promising plant promoters applied to molecular breeding and conserved *cis*-acting elements from promoters responsive to different biotic stresses in soybean, raising new possibilities for constructing synthetic and optimized promoters.

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Abstract: Promoters are upstream gene regulatory sequences recognized by transcription factors (TFs) involved in controlling transcription initiation and progression. For modern crop improvement, the design of efficient gene constructs relies on promoter efficiency, tissue specificity, and other characteristics that allow the introgression of agronomically relevant traits to overcome biotic and abiotic stresses. Several constitutive viral promoters, such as *pCaMV35S*, remain widely employed in the transgenic plant generation, but their indiscriminate use leads to gene silencing triggering and metabolic penalties impacting plant fitness. The identification and functional characterization of plant-derived promoters can unveil alternatives to commonly used non-homologous promoters; however, knowledge over them remains limited, especially for crops. This review summarizes plant promoters used to drive foreign gene expression in homologous and heterologous systems, focusing on inducible soybean promoters from genes upregulated by different biotic stresses. Analyses of these soybean promoters revealed 22 co-incident *cis*-acting elements that can be used for synthetic engineering promoters responsive to multiple biotic stresses and, therefore, efficiently drive gene expression, conferring desirable traits in transgenic soybean. In addition, we also revisited commercial and protected promoters to provide an update on soybean promoters and gain new insights into superior crops' development.

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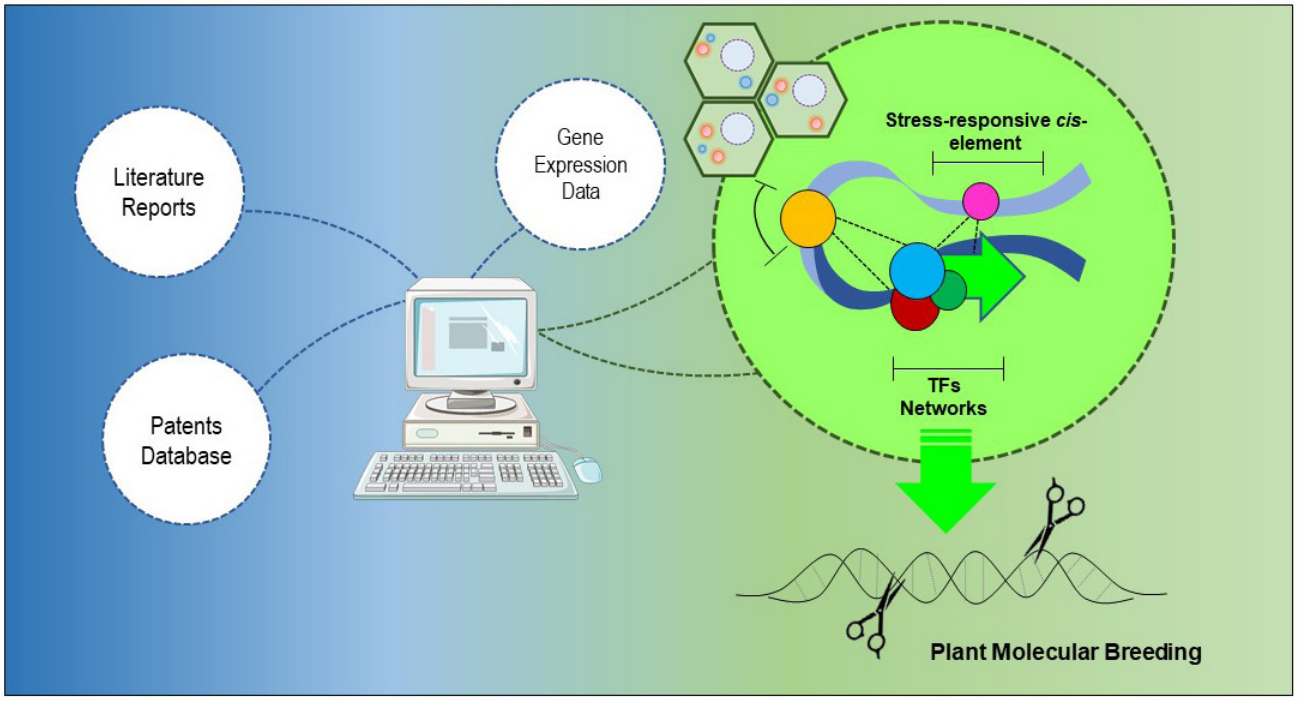
Graphical abstract



Introduction

New approaches in biotechnology have allowed considerable progress in the introduction of beneficial traits to improve crops for higher production under normal and stressful conditions. Despite several transcriptional and translational mechanisms in the control of gene expression because of posterior processes (e.g., splicing, RNA/protein transport, gene silencing, ubiquitination), those involving promoter-driven gene transcription are the most important in plants. This strategy is advantageous when the goal is to incorporate new traits into crops by driving transgene expression (Hernandez-Garcia & Finer, 2014).

Theoretically, promoters are regulatory regions upstream of genes that grant specific transcription rates. They can be classified according to the position of the regulatory sequences relative to the transcription starting site (TSS): core promoters include minimal *cis*-elements, such as TATA and GACA boxes (located at -10 and -35 bp of the TSS, respectively), required for basal transcription in eukaryotic cells. They are widely distributed, and the assembly of pre-initiation of transcription pre-initiation of transcription complex (PIC) complex derives from them (Louder et al., 2016). Proximal promoters are typically located within 1,000 bp upstream of the TSS and encompass key specific regulatory sequences recognized by specific TFs in a cell fate-dependent manner. Finally, distal promoters comprise long-distance regulatory sequences whose chromatin topology imposes physical interactions with the basal transcription machinery, either favorable or unfavorable, enhancing or suppressing gene transcription, depending on the nature of their *cis*-elements.

Transcription regulation is determined by the interaction of several enhancers or silencers in the DNA sequence and specialized proteins, designated transcription factors (TFs), which recognize conserved *cis*-elements and interact simultaneously with the basic transcriptional machinery. Since TFs can be dynamically present or absent in cells, depending on life stage, cell type, physiological conditions and hormone signals, biotic- and abiotic-stresses, transcriptional regulation of gene expression is finely-tuned regulated, resulting in appropriated transcription rates in consonance with all sorts of internal and environmental signals (Melo et al., 2021).

Overall, genes can be classified into two main categories, based on their expression profile: i. constitutive genes (also referred to as housekeeping genes), whose expression is nearly constant across tissues/organs at all developmental stages and under all environmental conditions; or ii. regulated genes, whose expression can increase a thousand-fold in response to specific stimuli. They can also be classified as spatiotemporally regulated (tissue or lifespan specific) or induced promoters (hormone-, abiotic stress-, wounding-, pathogen-inducible expression). Their common characteristic is that they are uniquely transcribed by RNA polymerase II (RNA pol II). So naturally, the promoters of these genes harbor sites for RNA pol II and are collectively designated *pol II* promoters (Biłas et al., 2016; Kummari et al., 2020).

A compelling and universal correlation suggests that constitutive genes are generally driven by CpG promoters (previously referred to as TATA-less), whereas most regulated genes are strongly controlled by TATA-containing promoters (Müller & Tora, 2014). In plants, a comprehensive analysis of thousands of core promoter sequences showed a high incidence of AT base pairs, with particularly strong AT enrichment around −30 bp, suggesting that TATA-like promoters are present in 31% of plant promoters (Hetzel et al., 2016). TATA-like promoters confer highly regulated gene expression, with initiation concentrated at the TSS and nearby regulatory

sites, including canonical CAT-boxes and *Inr* sequences, in addition to the downstream 5′UTR. Some genes exhibit TSS variants, typically in response to tissue differentiation, physiological regulation, and genetic variability (Hetzel et al., 2016), which impose additional levels of transcriptional regulation. A comprehensive mapping of Arabidopsis nascent RNA found "TYA(+1)YYN" and "TYA(+1)GGG" as a consensus *Inr* (Hetzel et al., 2016) that could be predicted using several algorithms. For CAT-boxes, 5′(T/C)(A/G)(A/G) CCAATC(A/G)3′ is considered the consensus sequence and can recruit NFY, which enhances gene activation across TFs through nucleosome replacement (Vernimmen & Bickmore, 2015). Unlike GC-boxes, the CAT box is position-dependent, typically occurring around -60 to -100. TATA-containing genes are generally not involved in essential cellular functions (Bae et al., 2015). Similarly, CAAT boxes are rarely found in constitutively expressed genes in all cell types (Tripathi et al., 2010).

Briefly, CpG promoters display GC-rich regions with several CpG sites (Saxonov et al., 2006), i.e., cytosine bound to guanosine 5′ phosphate, where cytosine may or may not be methylated as an epigenetic mark. When unmethylated, CpG islands (typically exhibiting dispersed SCTs of 50-100 bp) are recognized by TFs that recruit RNA pol II for transcription initiation. In plants, methylation patterns of CpG islands are strikingly uniform, negatively correlating with constitutive expression of housekeeping genes (Song et al., 2013). Another feature of CpG promoters is the presence of additional regulatory core sequences, such as GC-box (KRGGCGKRRY, usually GGGCGG motif - about -40 to -100) and DRE and TCT motifs (Lorberbaum & Barolo, 2015). DRE motifs are predictable TATCGATA sequences in proximal promoters (-200 bp). They positively regulate transcription in different ways, depending on the enhancer function (Lorberbaum & Barolo, 2015), but at different rates with fluctuation up to 50-fold, likely associated with the number of GC-box and proximal enhancers.

Besides the *cis*-elements of the core promoter, the combination of different regulatory sequences recognized by TFs delineates the induction pattern of a gene and, therefore, the promoter's responsiveness. The ACGT core motif, found in G-boxes, C-boxes, A-boxes, and ABRE (ABA binding responsive element), is recognized by bZIPs in many promoters. To date, plant genomes encompass dozens of bZIPs in different species [(75 in Arabidopsis thaliana, 89 in rice, 125 in maize, 131 in soybean, and 69 in tomato (Fassler et al., 2002; Wei et al., 2012; Llorca et al., 2014; Li et al., 2015; Wang et al., 2015)]. The bZIPs are frequently linked to the control of normal plant morphophysiology and specific responses to biotic and abiotic stresses. Similarly, W-box is the WRKY-recognized *cis*-element (consensus: C/TTGACC/T) detected in several gene promoters responsive to multiple developmental stresses and processes, such as senescence (Basu et al., 2014; Llorca et al., 2014; Sheshadri et al., 2016). Along these lines, some TF families integrate hormonal signaling and physiological remodeling. The *cis*-elements recognized by these TFs are valuable sources of multiple-response regulatory sequences for promoter engineering.

AP2/ERF TFs control processes of floral development, seed germination, and yield regulation. Considering the common hormonal branch of these processes and responses to multiple stresses, mainly coordinated by salicylic acid (SA), jasmonic acid (JA), ethylene (ETH), and abscisic acid (ABA), AP2/ERF TFs also respond to environmental signals (Cui et al., 2016; Gu et al., 2017) by recognizing the A/GCCGAC motif in DRE (Basu et al., 2014) and ERFb (Yamada & Sato 2013), and CAACA in RAVb elements (Feng et al., 2014; Moran Lauter et al., 2014). The same is reported for NAC TFs, which recognize NACr elements (ACACGCATGT) (Yamaguchi-Shinozaki & Shinozaki 2005).

Therefore, studies of gene function and global variation in gene expression provide the most abundant reliable information on inducible promoters. From this, useful features on expression kinetics, specific induction or repression profile, and time or tissue dependence can be obtained, which allow retrieving useful promoters for a predictable increase in gene expression, in a specific tissue, under specific conditions, with minimal penalties on plant yield (Porto et al., 2014; Biłas et al., 2016; Kummari et al., 2020). The variety of TFs, the different *cis*-elements they recognize, and the plasticity of the DNA-binding domain in interacting with secondary regulatory sequences (partially divergent from canonical *cis*-elements) make promoters' engineering a promising and enthusing research field in synthetic biology and molecular breeding. Furthermore, the application and use of synthetic promoters have expanded the concern for regulating the expression of different genes of interest in response to pathogen attack or other specific stimuli (Koschmann et al., 2012).

Herein, we have attempted to summarize the progress in the elucidation and functional characterization of plant-specific promoter sequences, primarily regulated promoters, whose impact of conserved *cis*-elements associated with response to multiple stimuli can provide a useful tool for crop engineering. Our analysis focused on soybean genes highly responsive to several biotic stresses and uncovered 22 co-incidental *cis*-acting elements of 50 genes upregulated by viruses, fungi, insects, and nematodes. We also reexamined commercial and patent-protected plant promoters, which revealed that constitutive and regulated promoters have been continuously patented; however, constitutive promoters are five times more characterized and protected annually (213.36 ± 108.84) than regulated ones (46.45 ± 21.89). Overall, all data provide an updated dataset for projecting transgene expression in plants and designing synthetic promoters in soybean, setting new trends in the field in modern agribusiness.

Constitutive plant promoters

Constitutive promoter encompasses transcriptional regulatory regions widely expressed in plant tissues and organs, not regulated by specific conditions or specific transcription factors, universally applied in transgene expression in plants (Jiang et al., 2018; Ali & Kim, 2019; Kummari et al., 2020).

Viral promoters, such as cauliflower mosaic virus *(pCaMV35S*), peanut chlorotic streak virus (*pPC1SV*), and figwort mosaic virus (*pFMV*) promoters have been used in plant transformation over the years and are the most widely

used constitutive promoters in plant engineering. However, in most cases, plant constitutive promoters might be a more suitable option for plant transformation due to their plant origin. Additionally, plant transcriptional regulatory regions harbor *cis*-acting elements compatible with basal and plant cell-specific transcription factors, allowing precise regulation of gene expression compatible with their regulatory machinery (Mittler & Blumwald 2010; Biłas et al., 2016).

Despite its strong *quasi*-universal gene expression capacity, *pCaMV35S* has become the most widely used constitutive viral promoter in transgenic plants. However, its activity is generally low in reproductive tissues, prompting a demand for plant tissue-specific promoters towards the expression of genes whose phenotypical effect is relevant in flower buds, anthers, pollen, and related tissues (Moura et al., 2021). In addition, excessively high transcript levels generated under viral promoter's control can interplay some pleiotropic effects on transgenic plants (Freitas et al., 2019). For example, higher transcriptional ratio is frequently associated with protein accumulation and, primarily in non-target plant tissues, might be energy costly for the plant. Furthermore, simultaneous expression of different transgenes under the control of the same promoter usually triggers post-transcriptional gene silencing mechanisms (Freitas et al., 2019).

Plant constitutive promoters have emerged as a viable solution to these limitations (Porto et al., 2014). These promoters can be specifically applied to regulate plant resistance for pathogen or herbicide-related genes, thus improving plant performance under diverse conditions. Constitutive plant promoters can drive gene expression in most tissues and organs at different stages of development (Bhattacharyya et al., 2012; Jiang et al., 2018). Nonetheless, they can be a strategy for priming the defense of transgenic plants against abiotic or biotic stresses, as the target protein will be continuously produced (Singhal et al., 2016; Kummari et al., 2020).

Another feature to be addressed over plant constitutive promoters relies on the evidence that constitutive promoters of monocots and eudicots are usually more efficient in homologous systems (Wilmink et al., 1995). The most commonly used constitutive promoters in monocot crops are the rice *Actin1* (*pOsAct1*) (McElroy et al., 1990, 1991), the maize *Ubiquitin 1* and *2* (*pZmUbi1* and *pZmUbi2*) (Christensen et al., 1992), and *Alcohol Dehydrogenase1* (*pZmAdh1*) (Kyozuka et al., 1991). As summarized in Table 1, several monocot promoters have been identified and evaluated for application in plant transformation as alternatives to viral options.

The rice *pOsActin1* was used to drive the *Bacillus thuringiensis Cry1A(b)* gene expression in indica and japonica rice-varieties, leading to Cry1A(b) protein levels similar to those driven by *pCaMV35S*. Efficient expression of the gene ensured plant protection against yellow stem borer (*Scirpophaga incertulas*) larvae (Datta et al., 1998).

Other monocot-specific promoters have also been applied to control insects, fungi, viruses, and nematodes. The ubiquitin extension protein (*pUep1*) promoter from the oil palm tree could drive ß-glucuronidase expression (*uidA*; GUS) transiently in different plant tissues, including embryogenic calli, embryoid, immature embryo, young leaf, green leaf, mesocarp, and meristematic tissues (Masura et al., 2010). Interestingly, it has also reported the potential for use in dicot systems, displaying transcriptional regulatory activity in tobacco (Masura et al., 2010). The *pAPX*, *pPGD1,* and *pR1G1B* from rice were also investigated using *GFP* as a reporter gene in the homologous system. All promoters were highly active in the whole plant at vegetative and reproductive stages, and the *pPGD1* showed excellent transcriptional activity as similarly observed for the well-characterized *pZmUbi-1* (Park et al., 2012). The same approach was applied to analyze the *pOsUbi1* promoter driving GUS gene expression in native plants compared to *pZmUbi1* and rice *Gibberellic Acid Insensitive* (*pGAI*) promoters. The expression levels were higher when *pOsUbi1* was employed, followed by *pZmUbi1* and *pGAI*, standing *pOsUbi1* as a promising promoter for synthetic biology, capable of driving constitutive gene expression in rice (Bhattacharyya et al., 2012), as has also been shown for *pOsCon1* (Li et al., 2014).

Expressive progress on promoters' characterization in monocots has been made, but it remains limited to certain species, such as rice (*O. sativa*) and corn *(Z. mays*). Few precedents in the literature report characterization of promoters in other important monocot crops, such as wheat (*Trictum* ssp.), sugarcane (*Sacharum* ssp.), barley (*Hordeum vulgare*), and several others, limiting the expression of transgenes in these plant systems under the control of endogenous promoters. Eudicot characterized promoters are distributed in a slightly wider variety of plants (Table 2); however, most precedents describe promoters from Arabidopsis and tobacco (*Nicotiana* ssp.) while regulatory sequences from important agronomic crops, such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), and cotton (*Gossipium* ssp.), are not widely reported.The *pMtHP* promoter of the *Medicago truncatula* PR-10 related gene drove GUS expression in different tissues and organs of *A. thaliana* at various developmental stages. The *MtHP:::GUS* expression was higher than the expression detected in *CaMV35S:::GUS* plants (Xiao et al., 2005). The *pVR-ACS1* promoter naturally regulates the expression of an auxin-inducible ACC synthase gene (*VR-ACS1*) in *V. radiata L.* (mung bean). In situ assays in tobacco and Arabidopsis showed 4- to 6- fold higher protein levels for both GUS and luciferase compared with *pCaMV35*-constructs (Cazzonelli et al., 2005), highlighting a better performance of plant-specific constitutive promoters compared with viral promoters.

The soybean (*G. max*) *polyubiquitin* promoter (*pGmUbi*) was evaluated by driving GFP expression in stably transformed soybean. Tissues carrying *pGmUbi:::GFP* showed a 2- to 5-fold increase in gene expression compared to constructs containing the *pCaMV35S* promoter (Chiera et al., 2007; Hernandez-Garcia et al., 2009). Recently, the upstream regulatory region of the *GmUBC4* gene was isolated from soybean by TAIL-PCR, uncovering a plant inducible promoter designated *pUceS8.3*, which has been cloned and patented for using in plant expression vectors (Grossi-de-Sa et al., 2013). The core promoter of *pUceS8.3* exhibits a sequence motif pattern, as the presence of the TATA-Box, CAT-Box, initiator element (*Inr*) consensus, and low GC content, characteristic of TATA-containing promoters. *In silico* analysis of *pUceS8.3 cis*-acting elements showed the presence of hundreds of DNA motifs and its ability to drive gene expression was reported in different tissues of *A. thaliana*, including root, stem, leaf, and flower bud (Grossi de Sa et al., 2013).

Table 1. Constitutive promoters of monocot plants used in transgenic plants.

Source	Host	Promoter	Reporter Gene	Comments	Ref.
Oryza sativa (rice)	*O. sativa* (rice)	*pOsActin1*	*GUS*	High levels of reporter gene expression in transformed rice protoplasts.	McElroy et al. (1990)
Zea mays (maize)	*Nicotiana tabacum* (tobacco)	Alcohol dehydrogenase (*pAdh-1*)	*GUS*	Mainly induced in roots after 24h of anaerobic treatment (up to 81-fold).	Kyozuka et al. (1991)
Z. mays (maize)	*Z. mays* (maize) and *N. tabacum* (tobacco)	Ubiquitin (*pZmUbi1* and *pZmUbi2*)	*Chloramphenicol acetyl transferase (CAT)*	CAT assays of protoplasts extracts indicated higher expression in monocot maize (10-fold increase) than in dicot tobacco (one-tenth the level) compared with *pCaMV35S* constructs.	Christensen et al. (1992)
O. sativa (rice)	*O. sativa* (rice)	*pOsActin1*	*CrylA(b)*	Cry1Ab content varied in the tissues and organs studied; protein levels in leaves and stems were similar for *pCaMV35S* and *pOsActin1* promoter plants.	Datta et al. (1998)
O. sativa (rice)	*O. sativa* (rice)	*pOsCc1*	*GFP*	Displayed particular high activity in calli and roots with 3-fold higher potential than the *pOsAct1* promoter and comparable expression to the light-regulated RbcS promoter in leaves.	Jang et al. (2002)
Z. mays (maize)	*Triticum aestivum* (wheat) and *Z. mays* (maize)	*pH2B*	*GUS*	Wheat: stronger expression in floral tissues and in the young part of leaves and roots; Maize: stronger activity in leaves and roots.	Rasco-Gaunt et al. (2003)
O. sativa (rice)	*O. sativa* (rice)	*pOsUbi1* and *pOsUbi2*	*GUS*	Activity levels were 8 to 35-fold higher in transgenic rice, respectively, compared with the *pCaMV35S* construct.	Wang & Oard (2003)
Palm oil	Palm oil and *N. tabacum* (tobacco)	Ubiquitin extension protein (*pUep1*)	*GUS*	Transient expression in all palm oil tissues tested and in tobacco. The highest potential was found for *pZmUbi1*, *pCaMV35S*, and *pUep1* constructs.	Masura et al. (2010)
Panicum virgatum (switchgrass)	*P. virgatum* (switchgrass), *O. sativa* (rice) and *N. tabacum* (tobacco)	*pPvUbi1*/ *pPvUbi2*	*GUS*	Strong constitutive expression in all plants analyzed, being good candidates for monocot and dicot transformation.	Mann et al. (2011)
O. sativa (rice)	*O. sativa* (rice)	*pAPX*, *pPGD1* and *pR1G1B*	*GFP*	High activity in the whole plant at vegetative and reproductive stages, but low activity specifically in ovary and pistil filaments.	Park et al. (2012)
O. sativa (rice), *Z. mays* (maize)	*O. sativa* (rice)	*pOsUbi1* and *pZmUbi1*	*GUS*	Higher expression when using rice *pOsUbi1* promoter than the maize *pZmUbi1*.	Bhattacharyya et al. (2012)
O. sativa (rice)	*O. sativa* (rice)	Gibberellic acid insensitive (*pGAI*)	*GUS*	GUS expression was lower than the rice and maize *pZmUbi1* promoters.	Bhattacharyya et al. (2012)
Brachypodium distachyon	*Z. mays* (maize)	*pEF1a* and *pUB110*	*GUS*	*pUBI10*-GUS plants contained 6- to 14-fold more GUS protein, whereas the *pEF1a*-GUS plants had 3- to 6-fold more protein than the *pCaMV35S* construct.	Coussens et al. (2012)
Ananas comosus (pineapple)	*A. thaliana*	*pSUI1* and *pL36*	*GUS*	GUS expression in all tissues at similar levels of *pCaMV35S*.	Koia et al. (2013)
Marchantia polymorpha	*M. polymorpha*	*pMpEF1a*	*GUS*	Strong meristematic expression and greater activity in female sexual tissues.	Althoff et al. (2014)
O. sativa (rice)	*O. sativa* (rice)	*pOsCon1*	*GUS*	Comparable activity to *pOsCc1*, *pOsAct1*, or *pZmUbi* promoters in most tissues, but more active than the *pCAMV35S* promoter *in* roots, seeds, and calli.	Li et al. (2014)

Table 2. Constitutive promoters of eudicot plants used in transgenic plants.

Source	Host	Promoter	Reporter Gene	Comments	Ref.
N. tabacum (tobacco)	*N. tabacum* (tobacco)	*ptCUP*	*GUS*	Activity detected in all organs studied, being at similar levels in leaves of *ptCUP* and *pCaMV35S* plants.	Foster et al. (1999)
A. thaliana	*N. tabacum* (tobacco)	*pPTSB1 and pPPHYB*	*GUS*	Both promoters showed 50% or more activity compared to *pCaMV35S* promoter	Shirasawa-Seo et al. (2002)
N. tabacum (tobacco)	*N. tabacum* (tobacco)	*Polyubiquitin (Tubi.u4)*	*Chloramphenicol acetyl transferase (CAT)*	*CAT* expression was almost twice as high as *pCaMV35S* in the leaves evaluated.	Kang et al. (2003)
A. thaliana	*Allocasuarina verticillata*	*pUBQ1*	*GUS*	The *pUBQ1* and *pCaMV35S* promoters were minimally active, with *pUBQ1* being inadequate for *A. verticillata*.	Obertello et al. (2005)
M. truncatula	*M. truncatula*, *A. thaliana* and *Trifolium repens* (white clover)	*pMtHP*	*GUS*	Expression detected in all tissues analyzed generally similar to or higher than that of *pCaMV35S*-GUS plants.	Xiao et al. (2005)
Vigna radiata (Mung bean)	*N. tabacum* (tobacco) and *A. thaliana*	*pVR-ACS1*	*GUS/Luciferase*	Protein and expression levels were generally 4-to 6-fold higher for both reporter genes in comparison with plants containing the *pCaMV35S*.	Cazzonelli et al. (2005)
G. max (soybean)	*Phaseolus lunatus* (Lima bean)	*Polyubiquitin (pGmubi) and pGmHSP90L*	*GFP*	*pGmubi* with and without its intronic region displayed 5- and 2- fold higher expression compared with *pCaMV35S*, respectively. The full-length *pGmHSP90L* promoter displayed 4-times increase in activity.	Chiera et al. (2007)
G. max (soybean)	*G. max* (soybean)	*Polyubiquitin (pGmubi)*	*GFP*	*pGmubi*-GFP plants generally had higher GFP expression than *pCaMV35S*-GFP plants.	Hernandez-Garcia et al. (2009)
G. hirsutum (cotton)	*A. thaliana*	*pUceA1.7*	*GUS*	Expression levels were equal to or higher than those of *pCaMV35S*. Activity was 7-fold higher in flowers, 2-fold in roots and similar in leaves and stems.	Viana et al. (2011)
Populus tomentosa	*A. thaliana* and *N. tabacum* (tobacco)	*pPtMCP*	*GUS*	Activity detected in all tissues and organs studied, but at lower levels than in *pCaMV35S*-GUS plants.	Chen et al. (2013)
A. thaliana	*A. thaliana*	*pAtTCTP*	*GUS*	Small (0.3 kb) promoter with high activity in all tissues, representing ~55% of the reporter gene expression in *pCaMV35S*-GUS plants.	Han et al. (2015)
A. thaliana	*Agrostis stolonifera* (creeping bentgrass)	*pTCTP*	*BAR*	High BAR expression in all tissues, corresponding to ~46-86% of that in *pCaMV35S*-BAR plants.	Han et al. (2015)
Jatropha curcas	*J. curcas* and *A. thaliana*	*pJcUEP*	*GUS*	*pJcUEP* and *pCaMV35S* had similar activities in stems, mature leaves and female flowers, but *pCaMV35S* was more effective in young leaves and inflorescences.	Tao et al. (2015)
Chrysanthemum morifolium (White Wing)	*A. thaliana* and *C. morifolium*	*pCmActin*	*GUS*	Higher GUS expression in *C. morifolium* compared to *pCaMV35S* and exhibited similar activity in all *A. thaliana* tissues, not being detected in seeds.	Hong et al. (2016)
G. hirsutum (cotton)	*N. tabacum* (tobacco)	*pGhEF1A1.7*	*GUS*	Activity was higher in leaves and stems and similar in flower and roots using *pCaMV35S* as a comparison.	Sun et al. (2016)

Table 2. Continued...

Source	Host	Promoter	Reporter Gene	Comments	Ref.
Solanum tuberosum (potato)	*A. thaliana*, *S. tuberosum*, *N. tabacum*, *Cucumis sativus* (cucumber), *Vitis vinifera* (grape) and *H. vulgare* (barley), *Citrus sinensis* (sweet orange) and *Solanum lycopersicum* (tomato)	*pKST1*	*GFP*	Exhibited guard cell expression in all species evaluated, being the first dicot-originated guard cell promoter active in monocots.	Kelly et al. (2017)
A. thaliana	*N. benthamiana*	*pAtSCPL30 fragments*	*GUS*	Strong activity in almost all tissues, displaying 2 times more transgene expression than the *pCaMV35S*.	Jiang et al. (2018)
C. sinensis (sweet orange)	*C. sinensis* (sweet orange)	*pCsCYP, pCsGAPC2, pCsEF1*	*GUS*	mRNA levels were up to 60–41.8% of the value obtained for *pCaMV35S* in leaves, stems, and roots.	Erpen et al. (2018)
G. hirsutum (cotton)	*G. hirsutum* (cotton)	*pGhSCFP*	*Expansin (CpEXPA1)*	Activity was higher in cotton fibers than in other parts of the plant, whereas *pCaMV35S*-driven expression was low in fibers but continuous in all tissues.	Yaqoob et al. (2020)
C. sinensis (sweet orange)	*N. benthamiana*	*pCsGAPC2, pCsEF1 and fragments*	*GUS*	*pCsCYP* promoter activity was not affected by any deletion. Truncated fragments of *pCsEF1* had higher GUS expression in leaves.	Corte et al. (2020)

From cotton (*Gossypium hirsutum*), the *pGhEF1A1.7* promoter with its 5'-untranslated region (5'UTR) was transcriptionally fused to the *uidA* reporter gene and evaluated in tobacco. The reported gene activity was remarkably higher in leaves and stems compared *to pCaMV35S::GUS* plants and similar to *pCaMV35S* in flowers and roots (Sun et al., 2016). Likewise, the *pUceA1.7* cotton promoter resulted in 7-fold higher GUS expression in flowers, 2-fold higher expression in roots, and similar expression levels in leaves and stems compared to *pCaMV35S* (Viana et al., 2011; Basso et al., 2020). Higher gene expression levels in flowers are particularly useful in cotton since its floral buds are attacked by the coleopteran *Anthonomus grandis*, the cotton boll weevil (CBW). The coleopteran insect lays eggs into the floral buds, and the larva develops by feeding on their reproductive structures, impairing fiber production (Ribeiro et al., 2021). Recently, Moura et al. (2021) have described two uncharacterized cotton promoters, *pGhERF105* and *pGhNc-HARBI1*, highly responsive to CBW infestation and active in vegetative and reproductive tissues, potentially applied to insect-pest control. A complete list of eudicots constitutively expressed promoters can be found in Table 2.

Inducible/regulated plant promoters responsive to biotic stresses

Plants are constantly exposed to biotic stresses, such as insects, nematodes, fungi, bacteria, and viruses. Insect attack and pathogen infection can induce plant defense mechanisms by activating or inhibiting the expression of different genes that are regulated through the activity of their respective promoters and transcription factors (Figure 1). With the advances in transcriptome sequencing, it has been possible to identify many genes induced by biotic stresses that may have numerous biotechnological applications in basic and applied research (Dong et al., 2018; Shukla et al., 2018; Koch et al., 2020; Ren et al., 2020). Moreover, a detailed study of promoters driving expression and regulation of biotic stress-responsive genes has identified attractive promoters with the potential to be used in the development of efficient transgenic plants resistant to several economically important insect-pests and pathogens that threaten global food production (Baruah et al., 2020). Promoters of defense-related genes induced by biotic stresses are of particular interest to plant improvement research and have been explored for use in genetically engineered plants for regulation of

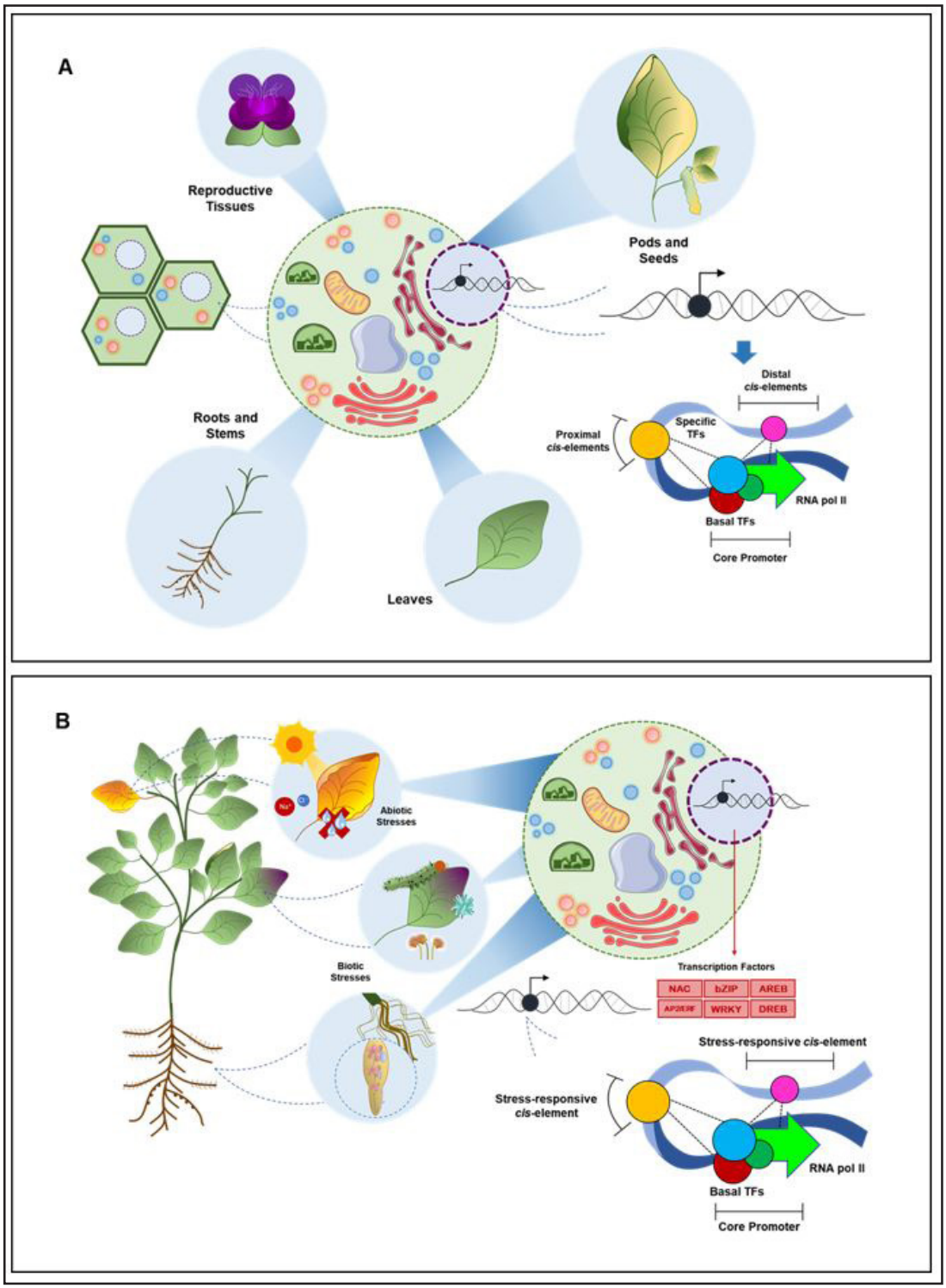


Figure 1. Schematic diagram of gene expression control by constitutive and inducible promoters in plants. (A) Gene expression controlled by constitutive promoters. Basal transcription factors recognize canonical *cis*-elements in core promoters as well as in proximal and distal regulatory regions. By multiple protein-protein and protein-DNA interactions, the regulatory region is sterically adjusted to accommodate the transcription factors and other pre-initiation complexes (PIC) proteins in the transcription bubble. Conceptually, constitutive promoters allow the expression in most plant tissues, independent of environmental conditions, leading to high levels of gene expression and protein accumulation in engineered plants; (B) Stress-inducible promoters respond to multiple environmental signals, usually coordinated by phytohormones. Facing some abiotic or biotic adversity, plants release hormones, such as ABA, JA, SA, and ETH, which stimulate the transcription of stress-responsive TFs. These TFs recognize specific *cis*-actin elements in promoter regions and, collectively, drive gene expression. Mechanistically, the transcription initiation coordinated by constitutive and inducible promoters is the same. The difference between the two stems from the dependence on specific TFs, whose expression varies with plant tissues, developmental stage, and environmental signals.

transgene expression (Gurr & Rushton 2005; Ali & Kim 2019; Kummari et al., 2020).

An ideal promoter should have a strong response against a broad spectrum of pathogens and/or insect-pests and be rapidly induced in response to the biotic stress. The major advantage of the stress-inducible promoters over the constitutive promoters is the suppression of the transgene expression upon repression of biotic stress stimuli to avoid unnecessary energy consumption that is required for other plant physiological processes and, consequently, any fitness penalty in plant growth and development (Kasuga et al., 2004; Nakashima et al., 2007; Quilis et al., 2014; Boni et al., 2018).

Thus, inducible promoters must drive an absence or very low levels of basal background expression in the plant to ensure successful plant protection from insects and pathogens. Although the availability of identified inducible promoters is relatively low, some promoters are increasingly used for transgene expression in plants (Quilis et al., 2014; Liu et al., 2019; Pandey et al., 2019). In addition, characterization of inducible promoters and identification of key pathogen-inducible *cis*-acting elements have enabled the engineering of diverse types of synthetic inducible promoters that have also been employed in the development of elite crops (Ali & Kim, 2019). More importantly, regulated promoters allow for the direction of gene expression only in specific organs, such as roots and flowers, thereby limiting the ectopic protein accumulation at the site of pathogens infection (Singhal et al., 2016). Hence, each application must be evaluated separately to select the best available promoter and decide on the best strategy, tailored to specific pathosystems, resulting in a perfect balance between agronomic and molecular characteristics. Previously described inducible promoters in plants are described in Table 3, and the following sections describe relevant features of promoters responsive to the major biotic stress conditions faced by plants.

Insect-inducible promoters

Several inducible promoters have been explored to engineer insect-resistant transgenic plants. Pandey et al. (2019) demonstrated that Arabidopsis transgenic lines expressing GUS under the control of the rose *RbPCD1* promoter were strongly wound-inducible by *H. armigera* and *M. persicae* as early as 5h after insect infestation. In addition, strong wound-inducible GUS expression was observed in stably transformed chickpea leaves and transiently agroinfiltrated cotton sepals, rose petals, gladiolus tepals, and tobacco leaves, indicating that it is active in a wide range of plant species. Moreover, the *RbPCD1* promoter was evaluated to drive *Cry1Ac* gene expression in Arabidopsis and tomato transgenic plants, leading to a 4.5- to 27- fold increase in *Cry1Ac* expression in the transgenic lines compared with lines under *CaMV35* promoter control. Phenotypically, the transgenic plants exhibited higher insect mortality and a stable protective effect against insect-pest attacks (Pandey et al., 2019).

Similarly, the expression of *GUS* and *GFP* reporter genes under the control of the *S. humilis peroxidase* (*Shpx6b*) gene promoter was induced in transgenic tobacco plants in response to the attack by the chewing and sucking insects *Phthorimaea operculella* and *M. persicae*, indicating that the *Shpx6b* promoter may be useful for the development of transgenic plants resistant to insect-pests (Perera & Jones, 2004). Godard et al. (2007) showed that the potato *proteinase inhibitor II* (*PinII*) promoter was able to drive the wound- and insect-inducible GUS expression in *Picea glauca* (white spruce), Arabidopsis, or tobacco. Li et al. (2020) observed that the rice *hydroperoxide lyase* (*OsHPL2*) gene promoter was significantly upregulated after herbivory of *C. suppressalis*, but not after feeding of *Nilaparvata lugens*, mechanical wounding, abscisic acid, jasmonic acid or salicylic acid treatments. Despite the advantages of inducible promoters over constitutive promoters that have been widely used in the genetic engineering of many plants, the trade-offs between inducibility and strength should be considered for practical biotechnological application.

Nematode-inducible promoters

Inducible promoters have also been explored to improve plant tolerance to phytonematodes. For instance, the promoters of the *NRRS* genes from Arabidopsis, *At1g74770* and *At2g18140*, were used in the development of transgenic Arabidopsis expressing the *GUS* reporter gene. Bioassays with the transgenic plants revealed that both promoters were responsive to the root-knot nematode (RNK) *M. incognita*, regulating GUS activity in roots and galls (Kakrana et al., 2017). Using a similar experimental strategy, Mitchum et al. (2004) evaluated a nematode-inducible promoter that is expressed in roots. Tobacco and Arabidopsis lines harboring the Arabidopsis *endo-1,4-B-glucanase* (*Cel1*) promoter transcriptionally fused to the *uidA* gene were infected with *M. incognita*, *Globodera tabacum* and *H. schachtii*. The authors observed *Cel1*-driven GUS expression in the giant-cells of the tobacco roots infected with *M. incognita* 11–13 days after inoculation, but GUS expression was not detected in syncytia of the tobacco or Arabidopsis roots induced by *G. tabacum* and *H. schachtii*, respectively.

Other promoters from genes related to defensive pathways were also explored to achieve application for nematode tolerance. The *Hahsp17.7G4* gene encodes a small heat shock protein involved in embryogenesis and stress responses. The validation of the sunflower *Hahsp17.7G4* promoter demonstrated that it was responsible for mediating high GUS expression in tobacco galls, particularly in the latter stages of *M. incognita* infection, but not in other root regions. Furthermore, the nematode-inducible promoter regulated GUS activity primarily in giant cells (Escobar et al., 2003). These studies demonstrated preferential or specific induction of promoters in the gall or syncytia structures upon nematode infection by bioassays performed in transgenic plants.

Fungus-inducible promoters

Over the past decade, various plant promoters have been tested to regulate gene expression upon plant-pathogenic fungi. Himmelbach et al. (2010) isolated and functionally analyzed the barley *GER4c* gene promoter. Strong transient expression of GUS under the control of the *GER4c* promoter was observed in barley leaves inoculated with *B. graminis* f. sp. *hordei*. To further evaluate the promoter, the authors stably introduced it into barley transgenic lines. Bioassays

Table 3. Insect/Pathogen-responsive promoters isolated from plants.

Gene (s)	Promoter (s)	Source of the promoter	Insect/Pathogen responsible for the induction	Reporter gene	Ref.
RbPCD1 gene	*RbPCD1* promoter	*Rosa bourboniana*	*Helicoverpa armigera, Myzus persicae*	*GUS*	Pandey et al. (2019)
peroxidase gene (*Shpx6b*)	*Shpx6b* promoter	*Stylosanthes humilis*	*Phthorimaea operculella, M. persicae*	*GUS/GFP*	Perera & Jones (2004)
proteinase inhibitor II (*pinII*)	*pinII* promoter	*S. tuberosum*	*Bradysia spp., Pissodes strobi*	*GUS*	Godard et al. (2007)
hydroperoxide lyase gene (*OsHPL2*)	*OsHPL2* promoter	*O. sativa*	*Chilo suppressalis*	*GUS*	Li et al. (2020)
putative subtilisin/ chymo-trypsin inhibitor	*B1-A04* promoter	*O. sativa*	*C. suppressalis*	*GUS*	Hua et al. (2007)
flavin-containing monooxygenase (*W250*), *speckle-type POZ protein* (*A360*), *early flowering 4* (*A080*)	*W250* promoter, *A360* promoter, *A080* promoter	*A. thaliana*	*Bemisia tabaci* (*W250* promoter), *M. persicae* (*A360* and *A080* promoters)	*GUS*	Dubey et al. (2018)
Hahsp17.7G4 gene	*Hahsp17.7G4* promoter	*Helianthus annuus*	*M. incognita*	*GUS*	Escobar et al. (2003)
AT1G26530 gene	*AT1G26530* promoter	*A. thaliana*	*M. incognita*	*GUS*	Kumar et al. (2016)
Hs1^{pro-1} gene	*Hs1^{pro-1}* promoter	*Beta vulgaris*	*Heterodera schachtii*	*GUS*	Thurau et al. (2003)
CYP97A29, *DFR*, *FLS*, *NIK* and *PMEI* genes	*CYP97A29* promoter, *DFR* promoter, *FLS* promoter, *NIK* promoter, *PMEI* promoter	*S. lycopersicum*	*Globodera rostochiensis*	*GUS*	Wiśniewska et al. (2013)
*endo-*Β*-1,4-glucanase* (*cellulase*) gene	*NtCel7* promoter	*N. tabacum*	*H. glycines, M. incognita, H. schachtii, G. tabacum*	*GUS*	Wang et al. (2007)
pyk20 gene	*pyk20* promoter	*A. thaliana*	*H. schachtii*	*GUS*	Puzio et al. (2000)
cel1 endo 1,4 Β *glucanase* gene (*Atcel1*)	*Atcel1* promoter	*A. thaliana*	*M. incognita*	*GUS*	Sukno et al. (2006)
*Cel1 endo-1,4-*Β *glucanase* gene	*Cel1* promoter	*A. thaliana*	*M. incognita*	*GUS*	Mitchum et al. (2004)
LEMMI9 gene	*LEMMI9* promoter	*S. lycopersicum*	*M. incognita*	*GUS*	Escobar et al. (1999)
wun1 gene	*wun1* promoter	*S. tuberosum*	*G. pallida, M. javanica,*	*GUS*	Hansen et al. (1996)
germin-like GER4 gene	*GER4c* promoter	*H. vulgare*	*Blumeria graminis* f. sp *hordei B. graminis* f. sp *tritici, Rhynchosporium secalis*	*GUS*	Himmelbach et al. (2010)
polyphenol oxidase 12 gene (*GmaPPO12*)	*GmaPPO12* promoter	*G. max*	*Phytophthora sojae, Phytophthora capsici*	*GUS*	Chai et al. (2013)
GRMZM2G174449 gene	*GRMZM2G174449* promoter	*Z. mays*	*Rhizoctonia solani*	*GUS/GFP*	Yang et al. (2017b)
CYP76M7 gene	*CYP76M7* promoter	*O. sativa*	*Magnaporthe oryzae*	*GUS*	Vijayan et al. (2015)

Table 3. Continued...

Gene (s)	Promoter (s)	Source of the promoter	Insect/Pathogen responsible for the induction	Reporter gene	Ref.
senescence-associated gene (*SAG12, SAG13*)	*SAG12* promoter, *SAG13* promoter	*A. thaliana*	*Botrytis cinerea* (*SAG12* and *SAG13* promoters), *Trichoderma harzianum T39S* (*SAG13* promoter)	*GUS*	Swartzberg et al. (2008)
phenylalanine ammonia-lyase 1 gene (*PAL1*)	*PAL1* promoter	*A. thaliana*	*Peronospora parasitica*	*GUS*	Mauch-Mani & Slusarenko (1996)
osmotin-like protein gene (*OSML13, OSML81*)	*OSML13* promoter, *pOSML81* promoter	*Solanum commersonii*	*P. infestans*	*GUS*	Zhu et al. (1995)
puroindoline-a gene (*PinA*)	*PinA* promoter	*T. aestivum*	*Magnaporthe grisea*	*GUS*	Evrard et al. (2007)
OsNAC6 gene	*OsNAC6* promoter	*O. sativa*	*M. grisea*	*GUS*	Nakashima et al. (2007)
prp1-1 gene	*prp1-1* promoter	*S. tuberosum*	*P. infestans*	*GUS*	Hahn & Strittmatter (1994)
Pgst1 gene	*Pgst1* promoter	*S. tuberosum*	*Erwinia amylovora*, *Venturia inaequalis*	*GUS*	Malnoy et al. (2006)
ACC oxidase - LEAC01 gene	*LEAC01* promoter	*S. lycopersicum*	Tobacco mosaic virus (TMV), *Cladosporium fulvum*, Powdery mildew	*GUS*	Blume & Grierson (1997)
plastid lipid-associated protein ChrC gene	*ChrC* promoter	*C. sativus*	*Sphaerotheca fuliginea* (*Oidium* sp.)	*GUS*	Leitner-Dagan et al. (2006)
defensin PDF1.2 gene	*PDF1.2* promoter	*A. thaliana*	*Alternaria brassicicola*, *B. cinerea*	*GUS*	Manners et al. (1998)
anionic peroxidase gene (*tap1, tap2*)	*tap1* promoter, *tap2* promoter	*S. lycopersicum*	*Fusarium solani* f. sp. *pisi*	*GUS*	Mohan et al. (1993)
calmodulin methyl transferase gene (*At4g35987*), *senescence associated* gene (*At4g35985*)	*At4g35987* promoter, *At4g35985* promoter	*A. thaliana*	*Peronospora tabacina*	*GUS/GFP*	Banerjee et al. (2013)
proteinase inhibitor-like gene (*win3.12*)	*win3.12T* promoter	*Hybrid poplar (Populus trichocarpa* × *Populus deltoides)*	*F. solani*	*GUS*	Yevtushenko et al. (2004)
calmodulin isoform-4 gene (*GmCaM-4*)	*GmCaM-4* promoter	*G. max*	*Pseudomonas syringae* pv. *Tabaci*	*GUS*	Park et al. (2009)
acid-O-methyltransferase of class II gene (*COMTII*)	*COMTII* promoter	*N. tabacum*	TMV, *Phytophthora parasitica* var. *nicotianae*	*GUS*	Toquin et al. (2003)
CAPIP2 gene	*CAPIP2* promoter	*Capsicum annuum*	*P. syringae* pv. *tabaci*	*GUS*	Lee et al. (2007)

Table 3. Continued...

Gene (s)	Promoter (s)	Source of the promoter	Insect/Pathogen responsible for the induction	Reporter gene	Ref.
hypersensitivity related gene (*hsr203J*), *sensitivity related gene* (*str246C*)	*hsr203J* promoter, *sgd24* promoter	*N. tabacum*	*E. amylovora* (*str246C* and *hsr203J* promoters), *P. syringae* pv. *tabaci* (*str246C* promoter), *P. syringae* pv. *syringae* (*str246C* and *hsr203J* promoters)	*GUS*	Malnoy et al. (2003)
basic Β-1,3-glucanase gene	*gglb50* promoter	*N. tabacum*	TMV, potato virus Y (PVY), cucumber mosaic virus (CMV)	*GUS*	Livne et al. (1997)
*acidic 1,3-*ß*-glucanase* gene (*gluB*)	*gluB* promoter	*S. tuberosum*	TMV, *P. infestans*	*GUS*	Mac et al. (2004)
acidic ß*-1,3-glucanase* gene *(PR-2*)	*PR-2d* promoter	*N. tabacum*	TMV	*GUS*	Hennig et al. (1993)
Pathogenesis-related protein of group 1 gene (*PR-1*)	*PR-1a* promoter	*N. tabacum*	TMV	*GUS*	Strompen et al. (1998)
gf-2.8 germin gene	*gf-2.8* promoter	*T. aestivum*	TMV	*GUS*	Berna & Bernier (1999)

with *B. graminis* f. sp. *hordei* and *Rhynchosporium secalis* demonstrated robust induction of GUS expression in leaves upon infection. In addition, high induction of GUS expression was observed in transgenic wheat plants that were generated through a transient expression system after inoculation with *B. graminis* f. sp. *tritici* and *hordei* (Himmelbach et al., 2010).

The soybean *polyphenol oxidase 12* gene (*GmPPO12*) promoter is strongly induced by the oomycetes *P. sojae* and *P. capsici*. Transient expression assays in *Nicotiana benthamiana* showed 8.2- and 10.8-fold increase in GUS activity levels upon *P. capsici* infection at 0.5 and 2 h, respectively. Additionally, high GUS activity was demonstrated in stable transgenic soybean hairy roots following *P. sojae* infection (Chai et al., 2013). Likewise, the inducible activity of the poplar *Win3.12T* promoter (Yevtushenko et al., 2004), *A. thaliana PAL1* and *PDF1.2* promoters (Mohan et al., 1993; Mauch-Mani & Slusarenko, 1996), *T. aestivum PinA* promoter (Evrard et al., 2007), *O. sativa OsNAC6* promoter (Nakashima et al., 2007), *Solanum tuberosum Prp1* promoter (Hahn and Strittmatter 1994) and *S. lycopersicum LEAC01* promoter (Blume & Grierson, 1997) under fungal infection has also been reported.

Yang et al. (2017a) reported that engineered rice lines expressing the *GUS* reporter gene under the control of the *GRMZM2G174449* promoter isolated from maize, whose gene responds to *R. solani* infection, exhibited a 3-fold increase in GUS activity in leaves after 8 h of inoculation with the fungus (Yang et al., 2017a). Another study demonstrated that transgenic soybean plants expressing GFP under the control of soybean *chitinase* gene promoter in *Phakopsora pachyrhizi*-challenged plants exhibited GFP fluorescence around fungal appressorium 24 and 72 h post-infection (Cabre et al., 2021).

Genetic constructs harboring the maize *proteinase inhibitor* and the potato *carboxypeptidase inhibitor* genes under the control of the wound- and pathogen-inducible *Mpi* promoter from maize were used to transform rice. Subsequently, evaluation of the transgenic plants indicated increased resistance to the insect-pest *C. suppressalis* and the fungus *M. oryzae* without penalty to the plant phenotype (Quilis et al., 2014). This study exemplifies how inducible promoters hold great potential for the development of insect-pest- and disease-resistant plants.

cis-acting co-incident elements in soybean promoters from genes responsible for broad-spectrum biotic stress conditions: new perspectives for synthetic promoter design

Gene stacking for broad-spectrum resistance is expected to be applied in the development of transgenic plants to improve the agronomic performance of important crops (Guo 2021). However, despite considerable efforts to identify and characterize new promoters for soybean genetic engineering, the availability of effective inducible promoters is far from satisfactory, and it will be worthwhile to continue exploring such promoters for use not only in plant research, but also in commercial transgenic plants.

To identify putative *cis*-regulatory elements (CREs) commonly found in soybean gene promoters related to biotic stress, we selected 50 top-list upregulated genes using six transcriptome-wide expression data in which soybean was

subjected to several pests, including: one virus (Soybean Mosaic Virus – SMV – (Zhang et al., 2019), two caterpillars (*H. armigera* and *Lamprosema indicate* - (Wang et al., 2017; Zeng et al., 2017), two fungi (*Sclerotinia Sclerotiorum* and *Fusarium oxysporum* - (Chang et al., 2019; Ranjan et al., 2019), and one nematode (*M. javanica*) (Beneventi et al., 2013). The gene list and their respective annotations are shown in Supplementary Table S1. The promoter sequence of each biotic stress-related gene was identified and downloaded from the soybean genome (*G. max* - Wm82.a2.v1), available on Phytozome v13 (https://phytozome-next.jgi.doe.gov/). A motif search was performed by the *in silico* tool PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace), taking 2.0 Kb of regulatory sequences upstream of the predicted Transcription Start Site (TSS). A *Venn* analysis (http://bioinformatics.psb.ugent.be/webtools/Venn/) was performed with all putative regulatory motifs identified by the PLACE analyses, in order to identify CREs common across to all evaluated promoter sequences. To identify the over-represented transcription factor (TF) families, considering all 50 soybean promoters, we used the PlantPAN database v3.0 (http://plantpan.itps.ncku.edu.tw/). The list of CREs and respective TFs is presented in Table 4.

We found 22 co-occurring CREs in all the promoters evaluated, including regulatory motifs related to basal or light-regulated expression in plants, such as CAATBOX1 (5'-CAAT-3'), CCAATBOX1 (5'-CCAAT-3'), GATABOX (5'-GATA-3'), IBOX (5'-GATAA-3'), and TATABOX5 (5'-TTATTT-3'). In addition, 15 of the 22 CREs screened in the promoter of the 50 biotic stress-related soybean genes were linked to tissue-specific activity and several stimuli or stress responses, as phytohormonal responses, dehydration response, or fungal elicitor activation (Table 4).

Interestingly, a recent study using 13 genes acting as a protein phosphatase 2A (PP2A)-related hub in *A. thaliana*, co-expressed under several abiotic stress conditions (cold,

Table 4. Relevant *cis*-elements commonly present in the promoter sequences of 50 biotic stress-related genes in soybean.

Motif name	Consensus sequence*	TF family	Activity	References
ARR1AT	NGATT	ARR1	Cytokinin response	Sakai et al. (2000) Ross et al. (2004)
CACTFTPPCA1	YACT	-	Mesophyll specific	Gowik et al. (2004) Wang et al. (2015)
DOFCOREZM	AAAG	Dof	Carbon metabolism and stress responses	Yanagisawa (2000)
TAAAGSTKST1	TAAAG	Dof	Guard-cell specific	Plesch et al. (2001)
EBOXBNNAPA	CANNTG	bHLH/MYB	Seed-specific	Stålberg et al. (1996) Hartmann et al. (2005)
GT1CONSENSUS	GRWAAW	GT1-like	Seed-specific, SA- and light-responses	Terzaghi & Cashmore (1995) Buchel et al. (1999)
GT1GMSCAM4	GAAAAA	GT1-like	Fungal-elicitors and salt responses	Park et al. (2004)
GTGANTG10	GTGA	-	Pollen-specific	Rogers et al. (2001) Bate & Twell (1998)
POLLEN1LELAT52	AGAAA	-	Pollen-specific	Filichkin et al. (2004)
MYCCONSENSUSAT	CANNTG	bHLH	Dehydration and ABA response	Abe et al. (2003)
	ATATT	-	Root-specific	Elmayan & Tepfer (1995)
WBOXATNPR1	TTGAC	WRKY	Salicylic acid response and disease resistance	Yu et al. (2001)
WBOXHVISO1	TGACT	WRKY	Sugar metabolism	Sun et al. (2003)
WBOXNTERF3	TGACY	WRKY	Wounding response	Nishiuchi et al. (2004)
WRKY71OS	TGAC	WRKY	Pathogenesis-related and GA responses	Zhang et al. (2004)

This list does not include CREs involved in basal expression or light-regulated responses. (*) R = G or A; Y = T or C; W = A or T; N = A or T or C or G

drought, heat, osmotic, genotoxic, salt, and wounding) identified 16 CREs co-occurring in the promoters of these 13 genes (Khan et al., 2020). A comparative analysis between 16 CREs found in *A. thaliana*, with the 22 CREs screened by our analyses using the promoter sequences of 50 biotic-stress related soybean genes, revealed the co-occurrence of 14 CREs in both datasets, including tissue-specific activity-related motifs (CACTFTPPCA1, TAAAGSTKST1, EBOXBNNAPA, GT1CONSENSUS, GTGANTG10, POLLEN1LELAT52 and ROOTMOTIFTAPOX1), carbon or sugar metabolisms (DOFCOREZM and WBOXHVISO1), phytohormone response (ARR1AT, MYCCONSENSUSAT, and WRKY71OS), biotic stimulus (GT1GMSCAM4, WBOXATNPR1, and WRKY71OS) or abiotic stimulus (DOFCOREZM, MYCCONSENSUSAT, and WBOXNTERF3). Considering the frequency of CREs in each promoter of the 50 biotic stress-related soybean genes and their specific *trans*-acting TFs, we observed an over-representation of regulatory motifs that act as binding sites for specific TF families, such as MYB, DOF, bHLH, and WRKY, as was found for co-expressed *Arabidopsis* genes (Khan et al., 2020); which may indicate conserved transcriptional regulation and a crosstalk between pathways required for plant responses to multiple stresses. The highest occurrence was observed for WRKY members, the *trans*-acting factor binding to the W-box *cis*-regulatory elements. The WRKY TFs are known regulators involved in plant defense responses to pathogens, such as fungi, insects, and nematodes, as well as to abiotic stresses, such as drought, salinity, wounding, chilling and heat (Yu et al., 2001; Bencke-Malato et al., 2014; Yang et al., 2017a; Dhatterwal et al., 2019; Viana et al., 2021).

The identification of *trans*-acting factors and respective *cis*-regulatory motifs regulating the spatial and temporal activity of a plant promoter sequence is crucial to construct an effective synthetic promoter. Our data evidenced the co-occurrence of 14 CREs in 50 biotic stress-related soybean promoters, also found in 13 co-expressed gene promoters of stress-responsive Arabidopsis (Khan et al., 2020). Hence, we suggested that the 14 CREs identified in this study could be used to construct synthetic promoters with efficient transcriptional activity.

The basic composition of a synthetic promoter is the core, also known as the minimal promoter region, and the different specific *cis*-elements, where they will synergistically regulate transgene expression. When designing a synthetic promoter, the focus should be on the architecture, which means choosing different positions, nucleotide sequences, combinations, and amounts of inducible *cis*-elements (Dey et al., 2015). Typically, these *cis*-elements are chosen from other known sequences, and precedents in the literature, particularly for biotic stress responses, have reported a considerable number of engineered promoters responsive to different pathogens and pathogenesis-related hormones associated to tissue specificity-associated elements.

Elements such as silencers, insulators, and enhancers, as well as their combined transcription factors, play an important role (Spitz & Furlong, 2012). Like native promoters, synthetics still require appropriate transcriptional activators and repressors for precise regulation of transgenes (Liu et al., 2013; Petolino & Davies, 2013). The development of promoter libraries and the use of other computational tools allow the design of different *cis*-regulatory elements for synthetic promoters and could evolve into an effective way to evaluate and find new regulatory sequences. In practice, synthetic promoters not only consent to engineered gene expression regulation, but also shorten and optimize the length of constructs, facilitating genetic modification of target organisms. Most synthetic plant promoters are a precise combination of well-characterized specific *cis*-elements and properly interspaced core motifs (Banerjee et al., 2013; Ali & Kim, 2019; Kummari et al., 2020). Specific CREs related to biotic stresses and/or hormones already employed in synthetic promoters are summarized in Table 5.

Naturally, given the functional and structural conservation of plant TFs, coupled with predictive bioinformatics algorithms (upon *cis*-elements conservation and distribution in endogenous promoters), and wide data from gene expression variation in soybean, it is possible to create an integrated *omics* pipeline to design genetic constructs precisely focused on controlling specific pests.

Commercial and patent-protected plant promoters

Ongoing efforts to explore plant promoters for biotechnology applications have promising implications for developing optimized crops with stable yield and profitable production. The vast knowledge accumulated on promoter regulation, as outlined above, paves the way for precise control of transgene expression in genetically modified (GM) crops, allowing the minimization of undesired effects and the exploitation of new traits to be engineered. Furthermore, the possibility to control transgene expression spatially and temporally can have a notable impact on food biosecurity, since transgene expression can be directed to inedible plant tissues and towards the development of environmentally friendly GM crops with less environmental impact, by aiming to improve insect-pest and disease resistance and reduce the use of chemical defenses.

The potential role of inducible promoters in these challenges is highlighted not only by the growing number of scientific publications, but also by the number of patent applications. A search for patent applications on the World Intellectual Property Organization (WIPO) PatentScope database from 2011 to April 2021, using different combinations of keywords and logical operators adopted, are presented in Supplementary Table S2. This search disclosed 2,347 patent applications related to constitutive promoters in plants, all filed by biotechnology companies in 18 countries (Figure 2A). A similar search for plant promoters induced by biotic stresses revealed 511 patent applications, most of them filed by biotechnology companies, with some contributions from universities, in 11 countries (Figure 2B). Despite the continuous tendency toward the search for novel plant promoters, the scenario of commercial transgenic crops available worldwide seems to go in the opposite direction.

We searched for plant promoters used in commercial GM varieties of cotton and soybean, considered as the two major GM eudicot crops grown worldwide, listed in GM Approval Database of International Service for the Acquisition of Agri-biotech Applications (ISAAA) (https://www.isaaa.

Table 5. CREs employed in synthetic plant promoters responsible for different biotic stresses and hormones related.

Nature of the promoter	CREs	Reference
Pathogen-inducible	W- box ((T)TGAC(C/T))	Rushton et al. (2002) Shokouhifar et al. (2011)
	D- box (GGAACC)	
	GCC box (AGCCGCC)	
	JERE(AGACCGCC)	
	DRE (TACCGACAT)	
	Box S (AGCCACC)	
	Gst-1 box (S and W boxes)	
	PR1-motif (ACGTCATAGATGTGGCGGCA TATATTCTTCAGGACTTTTC)	Mazarei et al. (2008) Liu et al. (2011)
	SARE (TTCGACCTCC)	
	JAR (TTCGACCTCC and ACGTG)	
	ERE (AGCCGCC)	
	UTP boxes (ATAGAAGAAGAGACCC consensus)	Römer et al. (2009)
SA inducible	TGACG motif of *F-Sgt* promoter	Kumar et al. (2012)
Wounding-, JA, and leaf senescence-inducible	JASE1 (CGTCAATGAA) and JASE2 (CATACGTCGTCAA)	Xie et al. (2001)
p-coumaric acid (4-CA)	H-box (CCTACC(N)7CT) and the G-box (CACGTG)	Loake et al. (1992)
SA/ABA inducible	ACGT - Pmec minimal promoter	Mehrotra & Mehrotra (2010)

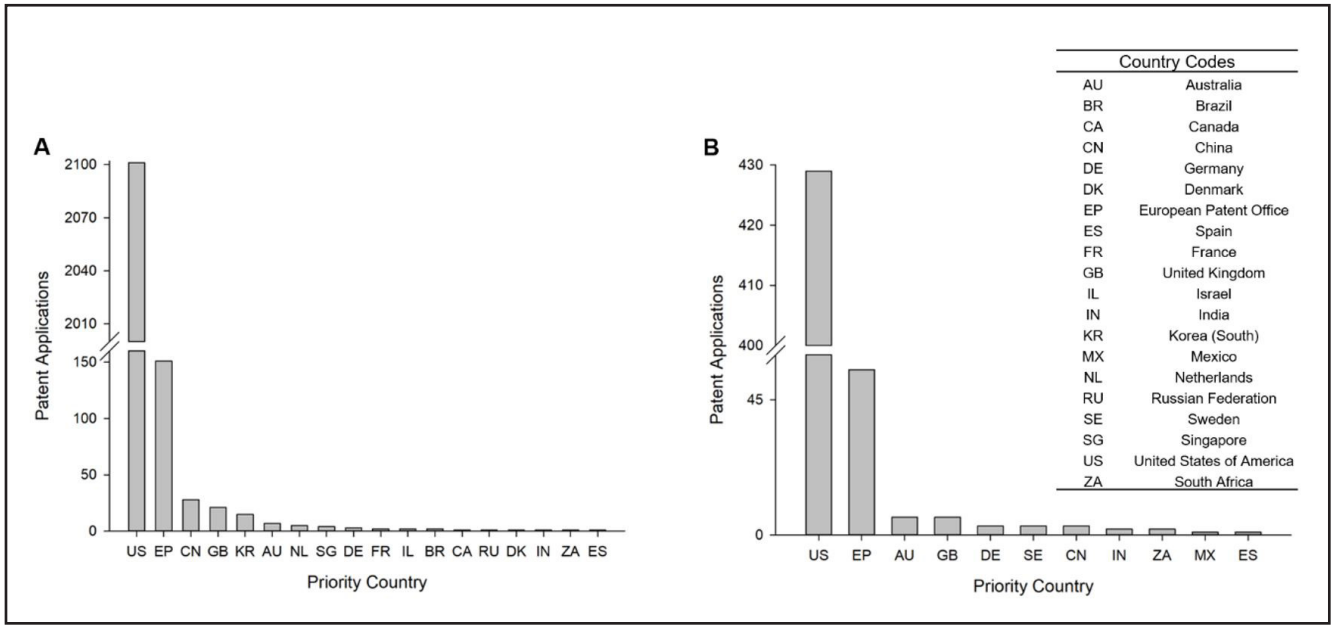


Figure 2. Distribution of patent applications by 1st priority country filed for plant promoters according to WIPO Patentscope, from 2011 to 2021. (A) Data on constitutive plant promoters; (B) Regulated promoters in plants induced by biotic stress.

org/) and in the Biosafety Clearing-House (BCH) database of Living Modified Organisms (LMOs) (http://bch.cbd.int/) (Figure 3). Most of the identified promoters are used for strong constitutive transgene expression, with the exception of seed-specific promoters of the ß-conglycinin α' subunit and *Kunitz trypsin inhibitor* (*KTi3*) genes of soybean, and

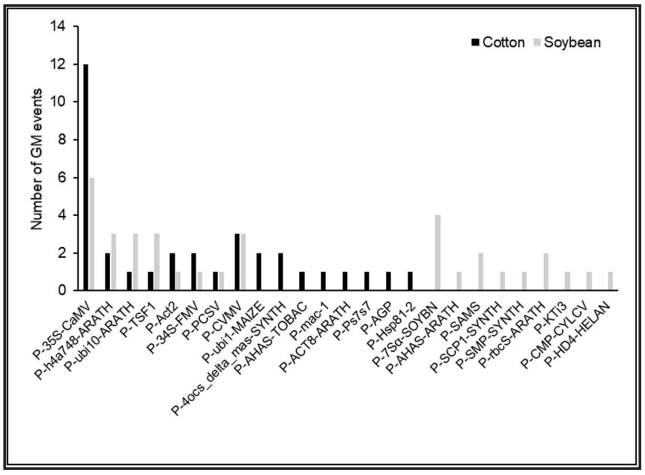


Figure 3. Promoters applied for the development of commercially approved GM soybean and cotton crops listed in the GM Approval Database of the International Service for the Acquisition of Agri-biotech Applications (ISAAA) and in the Biosafety Clearing-House (BCH) database of Living Modified Organisms (LMOs). *P-35S-CaMV*, cauliflower mosaic virus 35S promoter; *P-h4a748-ARATH*, *Arabidopsis thaliana* histone H4 gene promoter; *P-ubi10-ARATH*, *A. thaliana* polyubiquitin10 gene promoter; *P-TSF1*, *A. thaliana* elongation factor EF-1α gene promoter; *P-Act2*, *A. thaliana* actin 2 gene promoter; *P-34S-FMV*, figwort mosaic virus 34S promoter; *P-PCSV*, peanut chlorotic streak virus promoter; *P-CVMV*, cassava vein mosaic virus promoter; *P-ubi1-MAIZE*, *Zea mays* ubiquitin1 gene promoter; *P-4ocs_delta_mas-SYNTH*, *4ocsΔMas2*' synthetic promoter; *P-AHAS-TOBAC*, *N. tabacum* acetohydroxy acid synthase gene promoter; *P-mac-1*, *mac-1* synthetic promoter; *P-ACT8-ARATH*, *A. thaliana* actin 8 gene promoter; *P-Ps7s7*, subterranean clover stunt virus promoter; *P-AGP*, *G. hirsutum* α-globulin B gene promoter; *P-Hsp81-2*, *A. thaliana* heat shock protein 81-2 gene promoter; *P-7Sa-SOYBN*, *G. max* α' subunit of ß-conglycinin gene promoter; *P-AHAS-ARATH*, *A. thaliana* acetohydroxy acid synthase gene promoter; *P-SAMS*, *G. max* S-adenosyl-L-methionine synthetase gene promoter; *P-SCP1-SYNTH*, *SCP1* synthetic promoter, *P-SMP-SYNTH*, synthetic minimal plant promoter; *P-rbcS-ARATH*, *A. thaliana* ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) gene promoter; *P-KTi3*, *G. max* Kunitz trypsin inhibitor gene promoter; *P-CMP-CYLCV*, *Cestrum* yellow leaf curling virus promoter; *P-HD4-HELAN*, *Helianthus annuus* homeodomain-leucine zipper 4 gene promoter. It was considered promoters driven transgenes related to major crop traits. Promoters controlling reporter or selection markers genes and stacked GM events were not considered. Promoters' designation was considered according to the patent register.

the α-globulin B gene of cotton (Jofuku & Goldberg, 1989; Fujiwara & Beachy, 1994; Sunilkumar et al., 2002). There is a predominance of the use of plant virus promoters to maximize gene expression, such as *pCaMV35S*, figwort mosaic virus (FMV), cassava vein mosaic virus promoters, among others. These promoters are sometimes combined with other viral elements, like the 5' leader sequences of tobacco etch virus (TEV) and tobacco mosaic virus (Supplementary Table S3).

In some cases, plant virus promoters are joined to the 5' elements of plant genes, as in the combination of the *pCaMV35S* promoter with the 5' UTR and transit peptide of *Petunia hybrida* chlorophyll a/b-binding protein in GM GHB119 cotton event (Bayer CropScience) or with the 5' UTR of *P. hybrida HSP 70* gene in MON88701 and Bollgard IITM Monsanto cotton events. The 5' UTR of the *O. sativa* tapetum-specific *E1* gene was assembled with *pCaMV35S* and *pPs7s7* promoter of subterranean clover stunt virus in T303-3 (Bayer) and T304-40 (BASF) cotton events, respectively.

The synthetic promoters available are modular combinations of plant virus promoters with enhanced activity, except for the *P-4ocs_delta_mas-SYNTH*, which is a modular combination of mannose synthase gene promoter and *octopine synthase* gene enhancers from Ti plasmid from *Agrobacterium tumefaciens* (Ni et al., 1995). *pmac-1*, for example, is a combination of *pCaMV35S* and Ti plasmid *mannopine synthetase* promoters, showing 3 to 15 times expression of a double *pCaMV35S* (Comai et al., 1990). The plant minimal synthetic promoter (*P-SMP-SYNTH*), found in the SYHT0H2 soybean event (Syngenta), includes core sequences from the Cestrum yellow leaf curling virus and *pCaMV35S* virus promoters associated with CaMV and FMV 35S enhancers.

Most of the plant promoters regulating transgene expression identified in commercial GM soybean and cotton crops are from well-known constitutive genes, such as those encoding *actin*, *ubiquitin*, *histone*, *elongation factor EF-1α*, and *ribulose-1,5-bisphosphate carboxylase*. Some of these promoters were modified with the addition of plant introns, which are used to enhance transgene expression (Laxa, 2017). For example, the *A. thaliana* histone *H4* gene promoter combined with the intron II of the histone *H3* gene was able to increase gene expression up to 70-fold (Chaubet-Gigot et al., 2001). This modified promoter is present in the Enlist E3™ (Dow AgroSciences) and FG72 (Bayer CropScience) commercial GM soybean events and in the GlyTol™ and GHB811 Bayer CropScience cotton events. Similarly, the maize *ubiquitin* intron I, which can enhance up to 5-fold the transgene expression guided by the *ubiquitin* promoter (Pan et al., 2016), was used in the 281-24-236 cotton event (Dow AgroSciences). Plant promoters are also employed in GM crops combined with viral elements, such as FMV and CaMV35S enhancers and TEV 5' leader sequence (Supplementary Table S3). The *Actin 8* promoter, for example, was modified by including an upstream *pCaMV35S* enhancer as well as the actin 8 5' UTR and intron sequences to drive the *5-enolpyruvylshikimate-3-phosphate synthase* gene in Monsanto Roundup Ready™ Flex™ GM cotton event.

The higher number of patents related to constitutive promoters, which is a current trend, and the predominance of GM crops with strong constitutive promoters reflect the GM traits explored to date. The majority of GM soybean and cotton events were developed for insect resistance through the high expression of *B. thurigiensis Cry* genes or tolerance to glyphosate and glufosinate-ammonium herbicides by expressing high levels of *5-enolpyruvylshikimate-3-phosphate synthase* and *phosphinothricin N-acetyltransferase* genes, respectively. Few GM events show improved oilseed quality, such as the altered fatty acid composition in soybean and reduced gossypol levels in cotton, for which seed-specific promoters were applied.

Another issue to analyze is the cost and time for the development and release of a GM event. The entire process, including event selection, large-scale testing in multiple

locations, regulatory dossier, and registration affairs, can take about 10 years with an estimated cost of over US$ 2 billion (Schiek et al., 2016). This means that new trends take a decade to be brought into a biotechnology product. Also, the high costs of releasing a GM crop, exploring new traits, sometimes with low economic value products, are a barrier for non-profit institutions, even though these institutions are hubs where most of the basic exploratory research is performed.

Considering all the accumulated information on plant stress-inducible genes, supported by high-throughput genome analyses, and the molecular tools for promoter *cis*-acting elements identification, some questions can be raised. Why are research advances on this topic not widely applied to the development of GM crops? Why is the availability of effective inducible promoters far from satisfactory? The establishment of a list of effective promoters for the introgression of specific traits in GM plants depends strongly on the knowledge of the reactivity of certain plant genes, their kinetics and their mode of expression in multiple situations. Thus, the first step in genetic engineering relies on the identification of genes whose expression profiles converge on the characteristics that need to be explored in GM plants and whose promoters can be employed in expression cassette design. Functional characterization of genes in crop plants remains featureless, imposing barriers to the wide availability of inducible promoters, which justifies the hallmarked abundance of GM (commercial or not) plants with transgenes driven by viral promoters. In summary, the more gene function is known, the more inducible promoters will be available for molecular plant breeding.

Final remarks

Considerable success has been achieved in the last few decades on promoters' characterization and engineering. They have a direct impact on the molecular selection of plants and promote the release of new crops. However, several issues related to transgene expression must be addressed to avoid compromising crop yield and biosafety. Viral constitutive promoters remain widely employed to drive the expression of genes of interest and have provided high transcripts and protein levels in a large number of plant species. Most patent-protected transgenic crops have transgene expression driven by viral constitutive promoters. Nonetheless, the indiscriminate expression of transgenes in engineered plants can impose energy costs that affect plant fitness. With the advancements in the genome and transcriptome-wide analyses, novel monocot and eudicot constitutive promoters have been characterized and introduced into model and crop plants, demonstrating a new way to partially overcome the typical complications of using viral promoters. Notably, constitutive plant promoters do not interplay a fine-tuned control of gene expression and, for specific conditions, still, impair plant yield. Moreover, most of them do not exhibit significant expression in specific plant tissues and do not represent the best choice for improving specific traits, such as insect-pest and disease controls.

In this light, we reviewed the progress made over the past 10 years on characterizing inducible plant promoters, primarily those involved in biotic stress responses. Several promoters have been described as responsible for insect-pest attack and applied in constructs encompassing Cry-based toxins, followed by other promoters responsible for certain nematodes and fungi species, but more information is still needed for other pests. A promising alternative is the use of synthetic promoters harboring *cis*-acting elements already characterized in a broad spectrum of promoters from genes induced by multiple biotic stresses. By revisiting transcriptome data from soybean subjected to multiple biotic stresses, we have pointed out several co-incident conserved *cis*-elements in promoters of genes responsible to virus, insect, fungi, and phytonematodes, providing a new set of elements suitable for promoter design, consistent with elements already described in previous reports in the literature. Our analyses reinforce the need to study the function of genes, their expression profile and the regulatory networks to which they belong, as an efficient way to obtain basic data for biotechnological selection.

Finally, genome editing has opened a new era in the design of synthetic promoters. The CRISPR activation and CRISPR interference techniques, referred to as CRISPRa/CRISPRi, occupy an unexplored place in the field of gene expression control dispensing with the need for *cis*-element engineering. In this case, by using a dCas9-based strategy, a sgRNA molecule is able to direct transcriptional modulator complexes to a region of a specific promoter, up- or down-regulating target genes (Lowder et al., 2018; Roca Paixão et al., 2019; Melo et al., 2020). Collectively, knowledge over different promoters in different plant systems coupled with the precise design of synthetic promoters and CRISPRa/CRISPRi transcriptional modulator complexes can effectively modernize plant genetic engineering, fueling the biotechnological goal of successfully expressing multiple transgenes in a single superior crop with higher productivity and multiple desirable traits.

Conflict of interests

The authors declare that this work was conceived in absence of interest conflicts.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Table S1. Dataset of soybean biotic stress-related genes. The 50 soybean genes were selected from 06 transcriptomes of several biotic stresses, including a virus, a phytonematode, different caterpillars, and fungi.

Supplementary Table S2. Search strategy and total patent entries for constitutive plant promoters and regulated promoters in plants induced by biotic stress in WIPO PatentScope database.

Supplementary Table S3. Soybean and Cotton promoters and transgenes in commercial plants.

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