Review

Plant Synthetic Promoters: Advancement and Prospective

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Abstract: Native/endogenous promoters have several fundamental limitations in terms of their size, Cis-elements distribution/patterning, and mode of induction, which is ultimately reflected in their insufficient transcriptional activity. Several customized synthetic promoters were designed and tested in plants during the past decade to circumvent such constraints. Such synthetic promoters have a built-in capacity to drive the expression of the foreign genes at their maximum amplitude in plant orthologous systems. The basic structure and function of the promoter has been discussed in this review, with emphasis on the role of the Cis-element in regulating gene expression. In addition to this, the necessity of synthetic promoters in the arena of plant biology has been highlighted. This review also provides explicit information on the two major approaches for developing plant-based synthetic promoters: the conventional approach (by utilizing the basic knowledge of promoter structure and Cis-trans interaction) and the advancement in gene editing technology. The success of plant genetic manipulation relies on the promoter efficiency and the expression level of the transgene. Therefore, advancements in the field of synthetic promoters has enormous potential in genetic engineering-mediated crop improvement.

Keywords: synthetic promoter; CRISPR; Cis-engineering; transcription factors; gene expression

1. Introduction

Genetic engineering has emerged as a promising molecular discipline that can efficiently resolve the existing agricultural problems related to the escalating global population and can boost the agricultural productivity. Assuming that the success of transgenics depends on the expression level of the transgene, researchers in the field of plant synthetic promoter engineering are striving towards the synthesis of robust, constitutive, inducible, and bidirectional synthetic promoters. These promoters will enhance the transcriptional regulation of transgene expression in model plants and agricultural crops [1–6].

The Cauliflower Mosaic Virus 35S (CaMV35S) promoter has been extensively employed in plant molecular biology-based research ever since it was discovered [7–16]; still, it is regarded as a ‘dark horse’ in this field. With the gradual expansion of transgenic research, there has been an increased demand for a versatile promoter with a higher transcriptional activity. Diverse members of the family Caulimoviridae were reported to contain useful native promoters, such as the mirabilis mosaic virus (MMV) [17], figwort mosaic virus (FMV) [18], soybean chlorotic mottle soymovirus (SbCMV) [19], dahlia mosaic virus (DMV) [20], rice tungro spherical virus (RTSV) [21], tobacco vein clearing virus, (TVCV) [22], horseradish latent virus (HRLV) [23], petunia vein clearing viruses
(PVCV) [24], and cassava vein mosaic virus (CVMV) [25]. Nonetheless, the available number of native promoters is insufficient to meet the increasing demand in plant biotechnology. Moreover, native promoters are neither constitutive/tissue-specific/inducible nor bidirectional in nature [26,27].

Owing to the above limitations of native promoters, researchers are engaged in designing and testing synthetic promoters capable of pursuing gene transcription in a controlled fashion, depending on the environmental stimuli. In the last two decades, redesigning promoter structure by “Cis-rearrangement” and “shuffling of sub-domains” has acquired importance in maintaining robust gene expression in plants against abiotic and biotic stresses. [10,28–37]. Such a genetically manipulated transcriptional unit is constructed by employing several state-of-the-art molecular strategies, including promoter DNA shuffling, hybridization, domain exchange, domain duplication, site-directed mutagenesis, etc. [9,10,38]. The overall purpose of these approaches is to manipulate the architecture of the Cis-element present in the promoter DNA’s backbone, in order to generate a synthetic module with altered Cis-clouding that mainly facilitates their interaction with the cognate transcription factor (TF), leading to a different module with specific functionality. Such recombinant/chimeric promoters are gaining high popularity in the plant biotechnology arena, for boosting the gene expression in plants [9].

However, the main lacuna associated with conventional approaches is the transfer of promoter cassette coupled to a marker gene into the host genome of transgenic plant/organism, which raises complications for the acceptance of GMOs at present. This undesirable consequence can now be evaded owing to the development of CRISPR (clustered regularly interspaced short palindromic repeats)-based gene-editing technology, which enables in vivo promoter manipulation [39]. The advent of the CRISPR- system improved the alterations in endogenous promoters’ genetic architecture in vivo through ‘enhancer evolution’, where the native endogenous domain can be substituted by a foreign enhancer domain or by site-specific ‘base editing’ using an advanced CRISPR-based editing system. Such approaches can induce modifications in the endogenous promoter sequence to make it more competent for achieving the desired effect.

In this review, we discuss the basic promoter structure and associated functions, the concept of generating synthetic promoters, using Cis-engineering to enhance the transcriptional activity of synthetic promoters, and conventional and advanced approaches for designing efficient and useful synthetic promoters. We anticipate that this review with enriched information on advancement in synthetic promoters will significantly benefit researchers in the field of plant biotechnology.

2. Promoter Structure and Function

A promoter is the region of genomic DNA located upstream of a gene that initiates the process of transcription under specific cellular conditions. Structurally, it is modular in nature and comprises a core promoter that includes a TATA box and a CAAT box, as well as proximal and distal regions [39] (Figure 1). Functionally, promoters play a central role in regulating gene expression by their combinatorial interaction between various Cis-regulatory elements such as ‘silencers, repressors, insulators, enhancers’, and different TFs, as depicted in Figure 1 [10,40–42].

The core promoter covers nearly 100 bp upstream of the TSS, whereas a proximal promoter extends up to a few hundred bp from the core promoter and contains several TFs binding sites [43]. The core domains consist of a conserved TATA box and motifs such as the CAAT box, GC box, Inr, and DPE. More specifically, the CAAT box recognizes and binds additional co-factors of RNA polymerase II complex such as TFIIB, TFIID, TDIF, and TFIIE, whereas the GC box binds to various trans-activators for transcription initiations, as depicted in Figure 1. The Initiator element (Inr) is the part of the core promoter that directs the transcription initiation in typical conditions when the TATA box is dysfunctional. It has a YYANWYY consensus sequence that facilitates the binding of TF-IID and helps in strengthening the promoter [44]. Other protein fac-
tators bind to the downstream of a gene, such as Inr and regulate gene expressions [10] (Table 1, Figure 1). Several studies have shown that the proximal promoters (~1–2 kb upstream from the transcription start site) contain various important Cis-elements that strongly influence gene expression by recruiting TFs under specific cellular or environmental conditions [45–47]. For instance, the proximal sequence of the DREB2C promoter is sufficient for the tissue-specific spatiotemporal gene expression by heat stress in Arabidopsis [47].

![Figure 1. The basic architecture of plant promoters. The core promoter consists of a CAAT box, TATA box, Initiator element (Inr), and a downstream promoter element (DPE). Protein factors (TF IIA, IIB, IID, IIH, IIE, IIF, etc.) near the TATA box facilitate the binding of RNA Pol II to initiate transcription. The proximal regions (~1000 bp) and the distal promoter regulate the promoter activity by recruiting transcription factors (TF) on discrete Cis-elements (Cis-E).](image)

Similarly, it has been shown that the simultaneous mutation at five protein binding sites (~410–86) in the glutelin gene promoter leads to the elimination of full-length (1.8 kb) promoter activity [48]. Also, the minimal promoter proximal unit (86 to +217) of the tobacco late pollen gene g10 is sufficient for its expression in mature pollen [49]. The above studies show that the proximal regions contain crucial Cis-elements, and modulation of these elements leads to strongly altered promoter activity.

The distal promoter is the distant portion of the promoter ranging several kb upstreams of the proximal promoter and contains additional Cis-regulatory elements that have a weaker influence than the proximal promoter. Additionally, the distal regions mostly contain various Cis-elements and discrete DNA motifs (enhancers and insulators) that specifically recruit different TFs [50]. In particular, the enhancers are short DNA motifs bound with activators and they help the transcription initiation complex for enhanced gene expression. In contrast, insulators recruit repressors protein complexes to minimize or block the enhancer activity [40,51]. The downstream promoter element (DPE) recruits TF-IID at the core promoter with the help of the initiator (Inr) in the absence of the TATA box (Figure 1). Several TFs bind with Cis-motifs along with other protein factors that help the genes in enhancing/suppressing their transcriptional activity by altering the chromatin structure to facilitate/prevent the binding of the RNA polymerase complex [52,53]
Table 1. List of various Cis-motifs and trans-factors in the promoter complex.

<table>
<thead>
<tr>
<th>Promoter Cis-Motifs and Trans-Factors</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAAT Box</td>
<td>Determine the efficiency of the promoter</td>
<td>[50]</td>
</tr>
<tr>
<td>GC Box</td>
<td>Bound by SP1 transactivator and related transcription factors</td>
<td>[54]</td>
</tr>
<tr>
<td>TATA Box</td>
<td>Binding site for RNA polymerase II</td>
<td></td>
</tr>
<tr>
<td>TSS/Inr</td>
<td>Beginning of transcription</td>
<td></td>
</tr>
<tr>
<td>TFIIA</td>
<td>Promote the binding of TBP to the TATA box</td>
<td></td>
</tr>
<tr>
<td>TFIIIB</td>
<td>Couples to TFIID/TFIIA complex and Brings RNA polymerase II to the core promoter.</td>
<td></td>
</tr>
<tr>
<td>TFIID</td>
<td>Adheres to core promoter</td>
<td>[50]</td>
</tr>
<tr>
<td>TFIIIE</td>
<td>Bind to the polymerase/promoter complex</td>
<td></td>
</tr>
<tr>
<td>TFIIIF</td>
<td>Tightly binds to the RNA Pol II</td>
<td></td>
</tr>
<tr>
<td>TFIIH</td>
<td>Bind to the polymerase/promoter complex</td>
<td></td>
</tr>
<tr>
<td>BRE</td>
<td>TFIIB binding sequence</td>
<td></td>
</tr>
<tr>
<td>MTE</td>
<td>Motif Ten element; functions cooperatively with the Inr and is a recognition site for TFIIID</td>
<td>[55]</td>
</tr>
<tr>
<td>DPE</td>
<td>Downstream promoter element; Recognition site for TFIIID in Drosophila</td>
<td>[56]</td>
</tr>
<tr>
<td>TAFs</td>
<td>TBP associated factor, subunits of TFIIID that assist TBP binding to DNA</td>
<td>[57]</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein, subunit of TFIIID</td>
<td>[58]</td>
</tr>
<tr>
<td>Y Patch</td>
<td>Located between the TATA boxes and the TSS</td>
<td></td>
</tr>
<tr>
<td>CpG Island</td>
<td>Sp1 recognition site and simplify the regulation of gene activity by DNA-methylation</td>
<td>[59,60]</td>
</tr>
<tr>
<td>I box</td>
<td>Light-responsive element</td>
<td>[61]</td>
</tr>
<tr>
<td>G-box</td>
<td>Associated with floral and root-specific expression</td>
<td>[60]</td>
</tr>
<tr>
<td>H-box</td>
<td>Associated with floral and root-specific expression</td>
<td>[60]</td>
</tr>
<tr>
<td>W Box</td>
<td>Play a role in systemic acquired resistance</td>
<td>[62]</td>
</tr>
<tr>
<td>S Box</td>
<td>Directs expression by fungal elicitors</td>
<td>[28]</td>
</tr>
<tr>
<td>CCAAT element</td>
<td>Act cooperatively with heat shock promoter elements (HSEs)</td>
<td>[63]</td>
</tr>
<tr>
<td>PB element</td>
<td>Potential overlap with WRKY and TGA binding</td>
<td>[40]</td>
</tr>
<tr>
<td>GCC Box</td>
<td>Regulation of jasmonate-responsive gene expression</td>
<td>[64,65]</td>
</tr>
<tr>
<td>HSRE</td>
<td>Up-regulation during the hypersensitive response</td>
<td>[41]</td>
</tr>
<tr>
<td>PC4</td>
<td>Cofactor that interacts with VP16 TAD</td>
<td></td>
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<tr>
<td>SAGA</td>
<td>Histone acetyltransferases recruited by VP16 TAD</td>
<td>[65]</td>
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<tr>
<td>PCAF</td>
<td>Play distinct roles in transactivation by MyoD</td>
<td></td>
</tr>
<tr>
<td>UAS</td>
<td>Tends to contain primary regulatory elements</td>
<td>[66]</td>
</tr>
</tbody>
</table>

3. Need of Developing Synthetic Plant Promoters

As discussed before, the major lacuna associated with the endogenous/native promoters is the weak transcriptional levels achieved within the host. Synthetic promoters offer significant ways for overcoming this obstruction and facilitating the desired gene expression, thereby allowing the efficient optimization of transgene expression at an appropriate time, place, and level [42,63]. The significant advantage of designing synthetic promoters is that it alters the promoter strength either by enhancing or reducing the promoter activity by rearranging Cis-elements via altering their copy number and spacing, which is ultimately reflected in their temporal and spatial expression pattern [27,53]. Additionally, synthetic promoters can minimize undesirable expression and remove other elements that may give rise to unwanted expression characteristics [53]. Overall, the significant role that is anticipated by synthetic promoters is to increase the target gene expression.
and control the unwanted and leaky expression to improve the precision and strength of the promoter [63,67]. Another objective of designing synthetic promoters is to establish sequence heterogeneity among them, which provides an added advantage for gene stacking/gene pyramiding approaches to achieve the simultaneous expression of multiple genes by avoiding promoter DNA homology-based combinations [10].

Depending upon the need of plant molecular biology, various environmental stimuli-specific synthetic promoters have been developed. [9] We described the different types of synthetic plant promoters as well as the responsible host. Our earlier review [9] detailed the different synthetic plant promoter types. In the present review, we discuss the latest advancement and development of such promoters over the past decade, along with the corresponding Cis-element and its nature; the responsible host is represented in an informative way in Table 2. Advancements made in the field of synthetic promoters are presented below.

3.1. Biotic Stress-Inducible Synthetic Promoters

The main role of a pathogen-inducible promoter is to provide resistance to a wide range of pathogens [68]. Lin et al. developed an 868-bp pathogen-inducible promoter \( P_{LsGRP1} \) from \( L.\), which showed high activity on the pathogen and cold stress. \( LsGRP1 \) is a defense-related leaf-specific gene against a fungal pathogen causing gray mold disease. The 131-bp 3′-end region of \( P_{LsGRP1} \) also showed activity against various biotic, abiotic, and phytohormone exposure [69]. Four pathogen-inducible Cis-regulatory elements (PICEs) were identified in rice, namely AS-1, GCC-box, G-box, and H-box. About 53.5% of PICEs showed up- or down-regulation when exposed to pathogen attack [70].

3.2. Abiotic Stress-Inducible Synthetic Promoters

Crop productivity is majorly affected by various abiotic factors, among which high salinity and drought are the major ones. Moisture deficiency critically checks plant growth and poses constraints to crop production worldwide. This necessitates the construction of salt- and drought-inducible promoters, which can possess an optimum affinity to such stresses and can be used for imparting increased levels of tolerance to either one or both of these two abiotic stress agents. Chen et al. identified the \( OsHAK1 \) promoter sequence of 3037 nt in rice, which was found to activate under drought stress [71]. The stress-responsive gene \( gNAC21 \) was characterized from pearl millet (\( P.\) glaucum) and was reported to induce salinity tolerance in various crops [72].

3.3. Chemical-Responsive Synthetic Promoters

Certain chemicals have also been reported to regulate the transgene expression by enhanced transcription. These chemicals include various antibiotics, ethanol, herbicides, insecticides, etc. Chemically induced synthetic promoters were developed by employing probenazole, a chemical inducer which induces salicylic acid (SA) biosynthesis by inducing (SAR) systemic acquired resistance in plants [73]. A tetracycline-inducible system was developed for tobacco BY-2 suspension cells [74]. Alcohol-inducible gene expression system (AlcR-PalcA) has been successfully used in various plants such as \( A.\) thaliana [75], \( L.\) esculentum (tomato), and \( P.\) sp. [76,77]. AlcR-PalcA has also been reported in microalgae Chlamydomonas reinhardtii. The alcohol-inducible AlcR-PalcA system originates from Aspergillus nidulans, a filamentous fungus [78]. A list of agrochemicals that can be used as chemical inducers is also available [79].

3.4. Development of Hormonal-Responsive Synthetic Promoters

The transcriptional regulation of different promoters is a complex process heavily influenced by several plant hormones. Wu et al. reported that the spatial pattern of abscisic acid (ABA) mediated transcriptional regulation by evaluating an ‘abscisic acid responsive element’ (ABRE) in root tissues [80]. The interaction of the synthetic promoter in association
with functional genes responsible for cytosolic ABA, receptor kinase 1 (CARK1), and regulatory components of ABA receptor 11 (RCAR11) lead to drought stress tolerance [81].

3.5. Constitutive Promoters

Constitutive promoters continuously control the downstream gene expression, irrespective of space and time. To date, many promoters have been manifested with constitutive expression of genes, which have been reported to have low quality and poor yield [69]. The CaMV35S RNA promoter is the most common constitutive promoter used for various plant species, and other promoters include ubiquitin promoters [82].

3.6. Wound-Inducible Promoters

A wound-inducible promoter works exceptionally well to express the insecticidal gene in many crop plants. These promoters usually contain WRKY, W-box, and FORCA Cis-elements that recruit specific TFs for their action. Until now, several wound-inducible promoters have been isolated from different sources, namely OsDof1, Shpx6, win3.12, fib, mpiC, AopR1, and RbpCD1 promoters [83–87]. Some of the above-listed promoters, such as AopR1, mpiC1, and RbpCD1, have been used to derive CryIAc-type proteins with varying success rates [88,89]. Recently, PW220 and pRHA3B promoters have been characterized and have been induced with wound-stress treatment in Arabidopsis [90,91]. These promoters could be used as excellent tools for developing insect-tolerant crop plants.

3.7. Bidirectional Promoters

Bidirectional promoters (BiP) are more pertinent than unidirectional promoters, as they can control the expression of two genes simultaneously, thereby saving time from expression vector construction and piling multiple genes [5]. Moreover, the limited availability of unidirectional promoters with the same expression pattern has also encouraged the application of bidirectional promoters [92]. Bidirectional promoters have been designed in many plant species such as Arabidopsis [93], rice [94], melon [95], and Capsicum annum [96], all of which have been successfully reported. In Zea mays, a novel gene stacking strategy was applied by combining a bidirectional promoter (BDP) with biCistronic approaches. This gene stacking configuration demonstrated the application of a single promoter for the coordinated expression of multiple genes in corn, a crop plant [97]. Recently, a CRISPR-Cas9 approach for genome editing in rice was investigated employing a BiP [39]. A BiP has also been used in microalgae [98].

3.8. Chimeric/Hybrid Promoters

Chimeric/hybrid promoters are developed when a different functional part from one promoter is fused with the homologous/heterologous counterpart taken from another promoter. These Cis-modified promoters are developed for their utility in avoiding unpredictable and unwanted genetic recombination in plants. A new chimeric promoter was developed for controlling vascular pathogen infections by the fusion of the CaMV35S promoter and the xylanogen protein 1 promoter (Px), where it serves as a vital factor in the development of the xylem in Arabidopsis [99].

3.9. Tissue-Specific Synthetic Promoters

Several tissue-specific promoters have been developed for regulating gene expression in a specific organ or tissue, such as root-specific [100], green-tissue specific [101], endosperm-specific, pollen-specific [102], etc. Green tissue-specific promoters were developed in switchgrass (Panicum virgatum L.) to enhance the biofuel production and to reduce cell wall resistance without altering the root phenotypes [103]. Recently, bidirectional green tissue-specific promoters (BiGSSP2, BiGSSP3, BiGSSP6, BiGSSP7) have been constructed [104].
Table 2. List of synthetic promoters.

<table>
<thead>
<tr>
<th>Synthetic Promoter</th>
<th>Cis-Element Involved</th>
<th>Nature of the Promoter</th>
<th>Host Organism Tested</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SynP16</td>
<td>Multiple combinations of soybean ABF, ABRE, ABRE-Like, CBF, E2F-VARIANT, G-box, GCC-Box, MYB1, MYB4, RAV1-A, and RAV1-B. Minimum 35S core promoter</td>
<td>Multiple expression</td>
<td>Glycine max</td>
<td>[105]</td>
</tr>
<tr>
<td>12–10, 12–48, 12–79</td>
<td>Skn-1 motif, HD Zip/WUN, ABRE/boxii/Ace, CGTCA/TGACG-motif, O2 site, caat box, G box, AAGAA-motif, TATA box</td>
<td>Constitutive expression</td>
<td>Physcomitrella patens</td>
<td>[106]</td>
</tr>
<tr>
<td>AZprom (1–21)</td>
<td>Camv35s and Ribulose-1,5-bisphosphate carboxylase small subunit promoter</td>
<td>Constitutive expression</td>
<td>Nicotiana tabacum var Samsun</td>
<td>[107]</td>
</tr>
<tr>
<td>Saps (Sap-11)</td>
<td>GC content, AT and TC rich motifs, POWRS motifs</td>
<td>Constitutive expression</td>
<td>Chlamydomonas reinhardtii</td>
<td>[108]</td>
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<tr>
<td>TGA1 (CmYLCV)</td>
<td>Cis-elements of CmYLCV (cestrum yellow leaf curl virus) at distal promoter region.</td>
<td>Constitutive expression</td>
<td>N. tabacum, Arabidopsis thaliana</td>
<td>[109]</td>
</tr>
<tr>
<td>SynS1, SynS2</td>
<td>Various Cis-elements in the synthetic module (SynS)</td>
<td>Constitutive expression</td>
<td>Saccharum officinarum</td>
<td>[110]</td>
</tr>
<tr>
<td>Ap, Dp, ANDp</td>
<td>The sequences of RD29A and RD29B promoters as promoter sequences and as Cis-elements.</td>
<td>Inducible expression</td>
<td>A. thaliana</td>
<td>[81]</td>
</tr>
<tr>
<td>P_DRE:35S</td>
<td>Core element of camv35s promoter and tmv omega 5′-UTR, 35S core sequence.</td>
<td>Inducible expression</td>
<td>A. thaliana</td>
<td>[110]</td>
</tr>
<tr>
<td>SINC, GmubiSINC</td>
<td>5′ UTR of soybean polyubiquitin promoter, Upstream camv35s</td>
<td>Inducible expression</td>
<td>Glycine max</td>
<td>[111]</td>
</tr>
<tr>
<td>SP-DDEE</td>
<td>e17 elements and minimal promoter, Parsley-D</td>
<td>Inducible expression</td>
<td>Brassica napus</td>
<td>[112]</td>
</tr>
<tr>
<td>GWH</td>
<td>JERE, SARE, GCC, 2x HSRE and 6x W-box</td>
<td>Inducible expression</td>
<td>A. thaliana</td>
<td>[73]</td>
</tr>
<tr>
<td>MAMP</td>
<td>Minimal promoter, uidA reporter</td>
<td>Microbial pathogen attack</td>
<td>Petroselinum crispum</td>
<td>[113]</td>
</tr>
<tr>
<td>Pme, Psec, Pdec and Ptec</td>
<td>TAM(transcription activation module), Pme (minimum cassette expression)</td>
<td>Enhanced expression</td>
<td>Solanum lycopersicum</td>
<td>[16]</td>
</tr>
<tr>
<td>4 x CCTC</td>
<td>Pi transporter 3 (StPT3)</td>
<td>Inducible expression under low Pi condition</td>
<td>Rizhophagus irregularis</td>
<td>[114]</td>
</tr>
<tr>
<td>4 x GCC-box motifs</td>
<td>AtPDF1.2 promoter</td>
<td>Inducible expression Jasmonic acid (JA)</td>
<td>A. thaliana</td>
<td>[115]</td>
</tr>
<tr>
<td>ROSE 1–7</td>
<td>ROSE7/GCC-box, MPK6, ERF6</td>
<td>ROS response during oxidative stress</td>
<td>A. thaliana</td>
<td>[116]</td>
</tr>
<tr>
<td>4 x RSRE</td>
<td>RSRE::LUC reporter, GSR-motif</td>
<td>Rapid stress response</td>
<td>A. thaliana</td>
<td>[117]</td>
</tr>
<tr>
<td>PRSGA, P2RSGA, P2RSPA, PRSGPA, P2RSGPA, PR5SGPA, P2RS5GPA</td>
<td>ACGT and AACA motifs, Skn-1 (S), Prolam in box (P), GCN4 (G), RY repeats (R), Pzmbd1</td>
<td>Seed-specific bidirectional Promoters</td>
<td>Zea mays</td>
<td>[118]</td>
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<tr>
<td>BiGSSP2, BiGSSP3, BiGSSP6, and BiGSSP7</td>
<td>Osactin (1), Ostubulin (6, 6i) previously reported sequences, Posrbscs-550, Posrbscs-62.</td>
<td>Bidirectional expression in green tissues specifically.</td>
<td>Oryza sativa</td>
<td>[104]</td>
</tr>
<tr>
<td>Synthetic Promoter</td>
<td>Cis-Element Involved</td>
<td>Nature of the Promoter</td>
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<td>Reference</td>
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<tr>
<td>SynR2 SynR1</td>
<td>SynR1—Synthetic module at 5′ end</td>
<td>Root specific</td>
<td>N. tabacum</td>
<td>[119]</td>
</tr>
<tr>
<td></td>
<td>SynR2—Synthetic module at 5′ and 3′ end</td>
<td></td>
<td></td>
<td></td>
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<td>GSSP1, GSSP3, GSSP5, GSSP6, GSSP7</td>
<td>First intron in rice (Act1), GT and G box. Regulatory sequences of rice, tobacco and Arabidopsis (PD 500–540, Posrbc-550, 62 and enp3-110)</td>
<td>Green tissue-specific</td>
<td>Oryza sativa</td>
<td>[120]</td>
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<td>MUASMSCP</td>
<td>Mmvflt and mmvsgt</td>
<td>Constitutive expression</td>
<td>N. tabacum, entire plant of Petunia hybrida and protoplasts of A. thaliana</td>
<td>[121]</td>
</tr>
<tr>
<td>F Sgt-P Fit, M Sgt-P Fit, P Fit-UAS-2X</td>
<td>UAS of sub-genomic promoter transcript of MMV and FMV coupling with peanut chlorotic streak virus</td>
<td>Constitutive expression</td>
<td>N. tabacum</td>
<td>[35]</td>
</tr>
<tr>
<td>FSuasFcp, FuasFscp</td>
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4. Cis-Reshuffling and beyond

Cis-regulatory elements (CREs) are unique short DNA sequences that recruit specific TFs and control gene expression under various environmental conditions. Such sequences are typically non-coding sequences that reside adjacent to the gene-coding regions of the DNA [137]. Interestingly, it has been shown that selective changes in CRE sequences directly impact the level of gene expression at various plant developmental stages. The CRE modification also favors the genes involved in multiple pathways or genes that are active in different organs to show the detrimental pleiotropic effect [138].

5. Role of Cis-Element in the Regulation of Gene Expression

Recently, several important CREs have been identified in plants that function with discreet TFs to regulate gene expression. For instance, the intron of rice ELONGATED UPPERMOST INTERNODE1 (Eui1) has a Cis-silencing element (SE1) that has been reported to recruit a minimum of one trans-acting repressor complex to regulate the expression of the Eui1 gene negatively [139]. Similarly, the expression of the Al-activated root citrate transporter “SbMATE” has shown to be affected by the Cis-acting tandemly repeated sequence, which binds to SbWRKY1 and SbZNF1 TF and regulates the expression of SbMATE in sorghum [140].

Furthermore, the release of phytohormones at specific developmental stages activates several TFs that selectively bind to sequence-specific response elements (REs) on the pro-
moter of the targeted gene. These REs are very short in length with a core motif of just three to four base pairs (bp) and are degenerative in nature. To know the functional significance of RE-degeneracy in a biological system, an algorithm was developed to identify regulatory sequence conservation linked with a phytohormones-related RE in 45 angiosperms. Surprisingly, it was observed that the specific RE variants were conserved in the core hormone response genes, which regulate the spatiotemporal distribution of hormonal responses in tomato and Arabidopsis [141]. Likewise, a study showed how a particular Cis-element helped an insect to acquire resistance against toxic allelochemicals produced by plants. To prove this, a CYP305A1 gene promoter was identified and characterized from Aphis gossypii Glover, which showed induction by two plants, allelochemicals 2-tridecanone and gossypol. Furthermore, it has been confirmed that the 5′-end promoter region (−810 to −581 bp) of the CYP305A1 gene promoter comprises an essential allelochemical responsive element and a unique Cis-element (5′-CACACTA-3′) specific for the aryl hydrocarbon receptor binding site [142].

The significance of Cis-elements in the evolution of stomatal mobility and Crassulacean acid metabolism (CAM)-CO₂ related genes have been widely investigated recently [143]. The study implied that the Cis-elements can be a potential target to modify and restructure the transpiration rate as well as the plant metabolome. Another study provided large-scale Cis-regulatory information for Fe-responsive genes and identified over 100 putative CREs, which further advances our understanding of the formulation of Cis-regulatory code for efficient Fe uptake or its transport in crop plants [144]. Interestingly, the shared Cis-elements were found to positively activate transcriptional machinery within giant bean suspensors of the plant embryo. By analyzing the functions of three unique DNA sequences, the G564 suspensor-specific gene transcription was studied (10 bp, Region 2, and the Fifth motifs). Parallelly, this study also justifies the hypothesis that stated the need for these motifs to transcribe the SRB GA 20-oxidase gene, which encodes a central enzyme in the gibberellic acid biosynthesis pathway which was co-expressed with G564 in giant bean suspensors. The deletion analysis further confirmed that two GA 20-oxidase DNA regions which are 5′ UTR (+119 to +205; −341 to −316) are required for the suspensor-specific expression of the GA 20-oxidase and the G564 gene [145].

6. Conventional Approach for Synthetic Promoter

Most conventional approaches are performed in vitro to generate synthetic promoters by reshuffling specific Cis-elements. This process customizes the desired Cis-elements over the native promoter for enhanced gene expression. Most of the native promoters are dynamic in nature. Their activity upon gene expression goes up and down depending upon the environmental or cellular conditions, with all different regulatory promoters containing specific Cis-elements (Figure 2). The transcriptional apparatus of such promoters always generates a basal level of gene expression. The location of such Cis-elements is not defined on the promoter backbone and could be found in proximal or distal regions. Studies have shown that engineering any element at the proximal promoter would be the best option for customized gene expression in planta [9]. One of the conventional ways to generate a synthetic promoter is to distribute all different kinds of Cis-elements at proximal regions and test them in different combinations under various environmental conditions. This could also be implemented in designing the synthetic bidirectional promoter with the combination of different Cis-arrangements, as shown in Figure 2. The active enrolment of Cis-elements to control the gene expression in different physiological and developmental pathways allows us to fine-tune the targeted genes. This can be achieved by specific or sequential Cis-modification through various approaches [9].
The careful selection of Cis-elements in various environmental conditions such as light, hormonal, stress response, and cellular differentiation [10], thereby providing insight for utilizing the specific Cis-elements from various sources on proximal regions effectively produce synthetic promoters with desirable transcriptional activity in different environmental conditions.

A few important points need to be taken into consideration while experimenting with Cis-elements and reshuffling in native promoters in order to generate synthetic promoters. The distribution of Cis-elements greatly differs in different gene promoters and only activates under respective stimuli. The arrangement, distribution, and positioning of Cis-elements has been found to revert the transcriptional activity of the designed promoter. The optimal spacing between Cis-elements is also required and should not change the physical conformation of the TF/Cis-element binding complex [9].

Several studies have been performed to classify the types of Cis-elements that recruit TFs in various environmental conditions such as light, hormonal, stress response, and cellular differentiation [10], thereby providing insight for utilizing the specific Cis-elements to design the complex synthetic promoter. Such approaches involve the computational evaluation of the targeted promoter with an appropriate Cis-element. In order to generate an efficient synthetic promoter, the first approach could be the selective deletion of Cis-elements that are not required and/or negatively impact the transcriptional rate. Similarly, the addition, substitution, or duplication of conditionally inducible Cis-elements could be inserted at an appropriate position to engage the TFs that work as activators or co-activators by interacting with the core promoter protein complex for enhancing the gene expression (Figure 3A). This in vitro assembly of Cis-elements at the proximal promoter allows for generating the strong synthetic promoter, which could ideally be employed in planta transgene expression [9,10].

**Figure 2.** Plant response to different environmental stimuli activates a variety of TFs (shown by different symbols), which binds to specific Cis-elements (shown as respective target symbols) over the targeted promoters. The distribution of Cis-elements greatly differs in different gene promoters and only activates under respective stimuli. The arrangement, distribution, and positioning of Cis-elements from various sources on proximal regions effectively produce synthetic promoters with desirable transcriptional activity in different environmental conditions.
evaluation of the targeted promoter with an appropriate Cis-element. In order to generate an efficient synthetic promoter, the first approach could be the selective deletion of Cis-elements that are not required and/or negatively impact the transcriptional rate. Similarly, the addition, substitution, or duplication of conditionally inducible Cis-elements could be inserted at an appropriate position to engage the TFs that work as activators or co-activators by interacting with the core promoter protein complex for enhancing the gene expression (Figure 3A). This in vitro assembly of Cis-elements at the proximal promoter allows for generating the strong synthetic promoter, which could ideally be employed in planta transgene expression [9,10].

Figure 3. Approaches for generating synthetic promoters employing conventional and advanced technology. (A) In vitro assembly of desired Cis-elements from the Cis-elements pockets over the proximal promoter through deletion (yellow), addition (red), substitution (purple), and duplication (sky blue). The basal level of selected promoter expression is shown as neutral (gray). (B) CRISPR-mediated targeted knock of the core nucleotide from Cis-elements through deletion (gray) and knock-in of the same or different Cis-elements through addition (red), substitution (purple), and duplication (sky blue) in vivo. Alteration in the Cis-elements distribution over the proximal region of synthetic promoter changes the gene expression pattern.

The drawback of the conventional approach for developing synthetic promoters is that the Cis-engineering could only be performed in vitro. This means we might be unable to fine-tune the native promoter within the system in vivo. Also, gene expression under this synthetic promoter is usually associated with marker gene transmission in the transgenic organism, which is undesirable. This lacuna could be solved by opting for the advanced CRISPR tools to modulate the gene expression in vivo under the edited promoter.

7. CRISPR-Mediated Generation of Synthetic Promoter: Advanced Approach

The introduction of CRISPR-mediated genome editing techniques enables the robust and efficient modification of ds-DNA at specified locations throughout the genome. In the last few years, this technique has proven effective modulation of protein-coding sequences, but very few studies have shown the use of CRISPR in reshuffling the regulatory sequence of a gene. The in vivo editing of specific CREs offers an excellent opportunity for precise alteration in targeted genomic loci [9,146]. The recently developed more diverse and sophisticated CRISPR/Cas tools derived from dead (d)-Cas9 will allow even more efficient
editing of regulatory sequences in vivo within the system. Reportedly, CRISPR-Cas9 was found to be successful in troubleshooting Cis-elements present upstream of open reading frames, which restricts the translation of downstream primary ORFs in eukaryotic genes [147].

Nevertheless, it is not always necessary to mutate the ORF genes that may have a negative impact on developmental traits in plants. In such a case, one might consider targeting the regulatory elements of the targeted gene in order to have precise and controlled expression. Likewise, in the case of the rice Xa13 gene, which favors the bacterial blight infection, knocking out this gene causes a developmental defect in another along with suppression of infection [148]. Recently, this has been shown by Li et al. 2020, where they deleted 129 bp of the Xa13 gene promoter region and found improved resistance without affecting the overall anther development [149]. The possible use of CRISPR technology in Cis-modification to achieve a higher tolerance level against abiotic stress characteristics such as drought, salinity, heavy metal toxicity, and temperature was discussed in detail [150].

Targeting specific regulatory regions has become a realistic and long-lasting alternative in recent years due to the introduction of CRISPR technology and its widespread application in single gene editing and global genome modification. This could be accomplished by introducing random deletions and insertions in the promoter area to create allelic series with distinct transcriptional activity. Similarly, targeting TF (transcription factor) binding sites and NHEJ (non-homologous end joints)- or HDR (homology-directed repair)-mediated fragment insertion in the promoter region could also be effective for predictable changes in gene expression. Alternatively, epigenome-editing tools for targeted methylation of DNA in the promoter regions can effectively activate/suppress gene expression in plants [151]. Using a CRISPR-base editor to modify specific nucleotides in plant genomes is a potential tool for advancing biotechnology and crop improvement [152,153]. This enables the targeted nucleotide substitutions, which in turn could ideally change the pattern of Cis-elements distribution over the proximal and distal promoter in vivo. Similarly, the selective disruption of Cis-regulatory regions can impact gene expression levels in a dose-dependent way, either positively or adversely [154]. Recently, this has been demonstrated in the tomato model by introducing random deletions through multiplex editing at the regulatory regions of several yield-related genes, and quantifiable variations in quantitative traits were found [155].

The base editing strategies to create specific changes in the genome allows the targeted Cis-elements mutation to occur at a particular location in the genome. Adenine base editors (ABEs), which convert an A-T to G-C base pair, and cytosine base editors (CBEs), which convert a C-G to T-A base pair, are the most extensively investigated types of DNA base editors. [153]. The basic principle of deamination of exocyclic amine to generate uracil is used to convert a CG base pair (bp) to a TA base pair. The primary approach greeted for the deamination is called base editor 1 (BE1), involving the fusion of APOBEC1 to dead Cas9 [152]. The more updated form of BE1, which is fused with a uracil DNA glycosylase inhibitor (UGI), is referred to as the BE2 system. The upgradation of BE2 into third-generation base editor 3 (BE3) involves the nicking of non-edited DNA strands [153]. Similarly, for the ABEs system, the mutant tRNA adenosine deaminase (TadA*) enzyme was fused to dCas9 (TadA*-dCas9), which converted the targeted deoxyadenosine to a deoxyinosine. This system was again modified into the more specific and developed system (TadA–TadA*–Cas9 nickase) with the wild-type non-catalytic TadA monomer and TadA* monomer fused with Cas9 nickase [153]. Both CBEs and ABEs systems could be effectively employed in mutating targeted nucleotides in the promoter region to obtain the desired Cis-elements for the recruitment of specific TFs. This approach could effectively modulate multiple Cis-elements at a time for more than one gene to regulate multiple gene expressions in vivo.

DNA knock-in and gene replacement via homologous recombination is a robust technique used extensively in many organisms [156,157]. Systematically, the knock-in of a desired DNA segment from the targeted genome is possible due to the advent of
CRISPR technology [156,157]. There has been a recent comprehensive summary of the strategies used to boost the frequency of knock-in in plants via homologous recombination pathways and non-homologous end joining [158]. Similarly, knock-out alleles for the targeted gene could be generated in different plants using the same approach [159–162]. This idea could also mimic re-engineering the regulatory sequence by deleting non-desired nucleotides from the selective Cis-elements. Likewise, inserting additional duplication or similar Cis-elements in the proximal region of the targeted promoter could also be possible via the CRISPR tool to generate a synthetic promoter for efficient gene expression in planta (Figure 3B). The opportunity to create a few nucleotide deletions and/or the insertion in regulatory sequence through a donor template could be adequate for precise and controlled gene regulation in order to produce essential traits in plants.

8. Conclusions

The advancement in biotechnological tools and their implementation has unveiled many genes that control plants’ growth, development, and interaction with the environment [67]. The administration of these plant genes is imperative for ameliorating crop yield and production. In this context, the promoter, the basic unit for gene regulation and transcriptional mechanism, plays a vital role [67,69]. The ‘Cis-trans’ interaction primarily coordinates gene function and regulation due to the interaction between a Cis-regulatory element in the promoter sequence and its corresponding transcription factors (TFs). Native promoters have a few inherited limitations that can be rectified by redesigning their Cis-architecture and by adapting advanced bio-techniques, resulting in a synthetic module with altered Cis-clouding capable of transcribing genes with high efficacy under environmental negatives. Several promoters with biotic and abiotic stress inducibility, chemical and hormonal responsive, constitutive, and bidirectional promoters have been successfully designed and tested in planta. With time, such engineered synthetic promoters may become potential assets for targeted plant modifications. Synthetic versions generated in vitro through conventional approaches may not be appropriate for gene expression, and in many cases, leaky gene expression under the control of such promoters is experienced. Scientists can now transform the promoter Cis-architecture in vivo through ‘knock-in’ or ‘knock-out’ of a given domain, yielding more complicated transcriptional modules with greater specificity and functionality. The CRISPR-edited promoter sequence coupled to the desired gene appears to be an economical and less time-consuming process that avoids the transfer of marker genes in modified plants. This could ensure the high acceptability of plant-made products obtained through CRISPR-system generated synthetic promoter. In future, we assume that in vivo manipulating endogenous promoter sequences employing CRISPR technology has tremendous potential in plant biotechnology. On the other, it has a high potential for promoting ‘green technology’ to keep the environment healthy.

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