Review

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Review

Plant Synthetic Promoters

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Abstract: The article features the structure and functions of plant synthetic promoters frequently exercised to precisely regulate complex regulatory routes. The composition of plant native promoters together with interacting proteins is presented to provide a better understanding of tasks associated with synthetic promoter development. The production of synthetic promoters is conferred on relatively small libraries produced generally by basic molecular or genetic engineering methods such as cis-element shuffling or domain swapping. Moreover, the preparation of large-scale libraries supported by synthetic DNA fragments, directed evolution, and machine or deep learning methodologies is presented. A particularly interesting group of synthetic promoters are bidirectional forms that enable the putative expression of up to 6–8 genes by one regulatory element. The introduction and controlled expression of several genes after one transgenic event strongly decreases the frequency of such problems as complex segregation patterns and random integration of multiple transgenes. These complications are commonly observed during transgenic crop development through traditional, multistep transformation by genetic constructs containing a single gene. Another path to solving problems associated with the low complexity and homology of already tested DNA fragments is through orthogonal expression systems composed of synthetic promoters and trans-factors that do not occur in nature or arise from different species. Their structure, functions, and applications are rendered in the article.

Keywords: plant synthetic promoter; enhancer; *cis*-element; *trans*-factor; orthogonal systems; bidirectional promoters; machine learning; deep learning

1. Introduction

Global climate changes increased the role of sustainable agriculture, resulting in the growing demand for novel plant genotypes or genetically modified plants with increased tolerance to biotic and abiotic stresses or the production of valuable plant secondary metabolites [1–10]. These requirements are met by the expression of foreign genes that mediate the plant's resistance to insects, herbicides, and glyphosphate, or by enabling the engineered carotenes or astaxanthin biosynthesis in rice [11–19]. Usually, these modifications use the constitutive CAMV35S or native plant promoters to drive transgene expression, often resulting in silencing, toxicity, or decreased viability effects in modified plants [20–26]. Gene regulatory problems arise when the simultaneous and precise spatio-temporal or development stage-dependent regulation of numerous transgenes is required to avoid unnecessary feed-back regulations or energy losses [27]. Such a fine-tuning of transgene expression could be addressed by orthogonal synthetic activators, repressors, and promoter systems containing the introduced *cis*-active elements to control the binding of transcription regulators and the gene expression rate [28,29].

In a traditional and general point of view, the promoters could be recognized as 5′-terminal gene fragments that control expression through the coordinated recruitment of particular proteins known as ***trans***-factors, recognizing ***cis***-active oligonucleotide sequences in the DNA (Figure 1) [30–35]. The ***trans***-factor is organized around the promoter through interaction with a particular ***cis***-active element to build up a protein complex with RNA polymerase II, resulting in the creation of the polymerase RNA II preinitiation complex [36–42].

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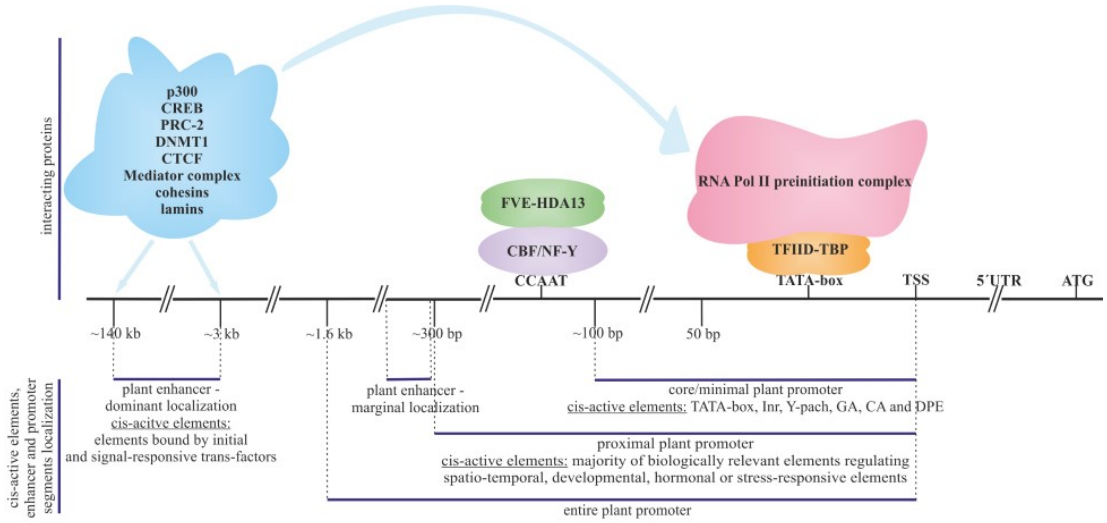

Figure 1. Structure of the plant core, proximal, entire promoter, and enhancer. Localization of representative *cis*-active motifs was provided together with corresponding *trans*-factors.

However, the precise description of the presented gene-regulatory element functions is impaired by the incomplete understanding of such a basic issue as the *cis*-element informative content. Although the position weight matrices (PWM) of *cis*-elements have been characterized, the real, *in vivo*-occurring regulatory functions are usually provided by imperfectly matched, DNA or protein modification-dependent, weak, and cooperative DNA-protein interactions [43–47]. Interactions between *cis*-active elements and *trans*-factors are also strongly regulated by the local 3D shape of DNA [48].

In the broader context, gene expression is regulated by the different coding and non-coding regions encompassing far-localized elements such as enhancers, silencers, insulators, gene-embedded promoters, introns, 3′ and 5′ untranslated regions (UTRs), and terminators, affecting not only the chromatin state or assembly of the polymerase RNA II preinitiation complex but also the 3′-end processing, stability, translation efficiency, and nuclear to cytoplasmic export of mRNAs [49–54].

Assuming that the *trans*-factors could be further regulated through such post-translational modifications as ubiquitination, acetylation, and phosphorylation, these molecular developments connect the gene expression rate with a plethora of signaling events occurring within the plant cell after exposure to biotic or abiotic stresses [43,55–61]. Therefore, the *trans*-factor-dependent changes in promoter activity induce gene expression variability, leaning toward adjustment to current plant cell metabolic demand or external regulatory events [62–64]. Coordinated gene expression, stimulated by numerous signaling routes, is reflected in the organized promoter structure, where the *cis*-active motifs are not distributed statistically. Instead, they typically form the evolution-conserved clusters known as *cis*-regulatory modules (CRM), containing *cis*-active elements assembled closely enough to support the interaction between recognizing *trans*-factors [65–72]. Such promoter structure-dependent dimerization or oligomerization of *trans*-factors could further increase the complexity of the biological response to the particular changes within plant cells [31,67]. According to Blanchette et al. (2006), the CRM could be found as far as over 100 kb upstream from TSS and within 1–10 kb downstream from the 3′ end of the gene [73].

The gene expression regulation mediated by promoter regions and other regulatory DNA sequences as enhancers, silencers, or insulators is recognized as a necessary, but still rough, adjustment of plant metabolic machinery to a particular demand that is further refined through post-translational protein modifications or precise enzyme regulation [72,74–79]. Therefore, the changes in gene expression are not directly associated with modifications of the encoded protein concentration or enzyme activity. Despite these limitations, the presented article will concentrate on advances in synthetic promoter research that are systematically designed and reconstructed to properly reshape the gene expression rate in genetically modified plants.

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2. Sections of Plant Promoters

2.1. Core Promoter

Studies on the A. *thaliana* genome suggest that the plant promoters of complex transcriptional regulation, common in the stress-responsive genes, are approximately longer (1672 bp) as compared to the remaining genes, responding to few signals (1113 bp) [80]. The segment of promoter localized approximately 50–100 bp upstream and downstream from the transcription start site (TSS) is recognized as a core or minimal promoter, where transcription initiation occurs (Figure 1) [81].

The first core promoter component that was identified in the SV40 early region is TATA-box [82]. Such an element is localized approximately 32 bp upstream to the transcription start site in plant genes (Figure 1) [83,84]. The species-specific distribution of TATA-box in several plants showed a peak ~30 bp upstream of the TSS. In the sorghum, the peak was found ~40 bp upstream of the TSS, with an additional lower peak at ~30 bp upstream of the TSS. In the maize genome, two additional peaks of TATA-box localization were observed at ~55 and ~70 bp upstream of the TSS. Core promoters containing a TATA box were up to fourfold stronger as compared to the TATA-less ones, particularly when the TATA box is located within the region from 23 to 59 bp upstream of the TSS. Therefore, increasing the distance from TATA-box to TSS decreases the strength of maize promoters [85].

The consensus sequence of TATA-box in plants is TATAWAW (W = A/T) [86]. However, the computational analyses of 79 plant promoters suggest that the TATA-box consensus sequence is TCACTATATATAG [87]. The TATA-box is bound by a subunit of TFIID known as TATA-box binding protein (TBP), enabling the assembly of the RNA polymerase II preinitiation complex [81,88]. The relative stability of TATA-box localization in relation to TSS, combined with the chromatography of interacting proteins, distinguishes TATA-box among other regulatory DNA sequences in the core promoter.

The importance of the TATA-box sequence for proper gene expression regulation was confirmed by Amack et al. (2023) [89]. The authors used PCR and specific primers to introduce single nucleotide substitutions to the CaMV35S promoter's TATA box. The obtained library of 21 variants was assigned to measure promoter activity using firefly luciferase assays in transgenic protoplasts and the RT-PCR method in transformed *A. thaliana* plants. Two obtained variants, A4T and T5A, showed respectively 1.2 and 1.1-fold higher activity in plant protoplasts as compared to the wild-type TATA-box sequence. Moreover, eight mutants (T1G, A4C, A4G, T5A, A6G, A7C, A7G, and A7T) presented similar or higher activity as the wild-type TATA-box in transgenic *A. thaliana*. Among them, the T5A variant indicated a 2.89-fold larger activity in transgenic plants as compared to the native TATA-box [89]. The TATA box is required not only for accurate transcription initiation but also to determine the level and selectivity of gene expression in plants [90–92]. Molina et al. (2005) found that among the 12749 tested dicot *A. thaliana* genes, only approximately 29% indicated TATA-box [83]. Even a lower 19% proportion of monocot *Oryzae sativa* genes possess the TATA-box element [93]. Moreover, TATA-box genes are less frequently found in genes present in EST databases and have shorter 5'UTRs (108 bp) as compared to TATA-less promoters (138 bp), lacking the TATA-box element [83]. The presence of TATA-box is also common in gene promoters regulated by light or biotic and abiotic stress [92,94].

Nucleotide frequency matrices within TSS in dicot and monocot plants and different promoters were described by Shahmuradov et al. (2003) [95]. Analysis of promoters from 217 unrelated dicot plants revealed the sequence WnT/aC/tA/cw (-4 to +2), while in 70 unrelated monocot plant promoters the TSS sequence was aNnCA (-2 to +3) [95]. In the same way, the 171 TATA-box promoters showed T/cCAnM, while 130 TATA-less promoters indicated T/a/cYA/ca/c/tt/a/g (-2 to +3), where W=A or T, N=any nucleotide of A, C, T, or G, Y=C or T, and M=A or C [95]. A general rule of TSS in *Arabidopsis thaliana* and rice is the localization of Y (C or T) at -1 and R (A or G) at +1 [96].

Besides the TATA-box, an important constituent required for plant transcription initiation is the Inr of the PyTCANTPyPy consensus sequence, usually overlapping the TSS (Figure 1) [90,91]. In plant photosynthesis nuclear genes, the TATA-box is frequently lacking, and its role is played by Inr interacting with the TFIID *trans*-factor [90,96]. This interaction is supported by the evolution-conserved downstream promoter element (DPE) A/GGA/TCGTG (*D. melanogaster*) interacting with subunits of TFIID in the form of the heterotetramer dTAFII60–dTAFII40 [97].

Another regulatory sequence found in plant promoters is the Y Patch, built from repeated pyrimidine CT or TC dimers, alternatively adopting the form of the Y Patch-related motif (TTTCTTCTTC) (Figure 1) [96]. The fraction of Y-Patches in plant promoters, which have distribution

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peaks around TSS, is directionally oriented, contrary tothe fact that CpG elements are methylation-insensitive [96]. Some elements of core promoters observed in humans and *D. melanogaster* were not found in *A. thaliana* as the Sp1 binding sites or CpG and BRE elements [96,98]. Plant promoters with Y-Patch and Inr sequences are also generally stronger than those lacking them [85].

Analysis of both core promoter types, TATA-box and TATA-less containing Inr, suggests that TATA and Inr are not swappable and regulate light-responsive gene expression in a different way. Moreover, the TATA-box core promoters are enriched in AC/CA-augmented motifs, conserved around the TSS and immediately downstream in the 5′-UTR region. However, TATA-less promoters showed a higher percentage of pyrimidine-rich TC/CT motifs around the TSS and immediately downstream in the 5′-UTR region [99].

In *A. thaliana*, three types of core promoters were identified: TATA, GA, and Coreless (Figure 1). Analysis of 10285 gene promoters indicated that the TATA-containing promoters are relatively long and of short distance to TSS or CDS. Moreover, their expression profile is high and regulated. Contrary to this, the coreless promoters do not contain any elements such as TATA, Y Patch, GA, or CA and are relatively short promoters of long distance from TSS or CDS. The expression level of these promoters is low, and they are constitutive in nature. Another GA type of plant core promoter regulates the expression of constitutive genes and is characterized by a short distance from TSS or CDS [100].

Combining the cap-trapper and massively parallel signature sequencing methods (CT-MPSS) enabled us to identify 158 237 *Arabidopsis* transcription start site (TSS) tags corresponding to 38 311 TSS loci [101]. The TATA-type promoters (25.1%) are enriched in Y Patch and the Inr motif and cause a high gene expression rate with sharp-peak TSS clusters. By contrast, the GA type, representing 21.6 percent of *A. thaliana* core promoters, is not associated with Y Patch and the Inr, which contain broad-type TSS clusters [101]. The distribution of TSS and corresponding core promoters in the plant genome should not be seen as a stable process but rather as a dynamic process that could be altered by the introduction of foreign DNA into the genome [102].

The association of TATA-box promoters with conditional, regulated gene expression was confirmed in the recent work of Yang et al. (2023) [103]. Moreover, the Coreless promoters indicated a bias towards uniform expression regulation only in dicots but not in monocot plants. A comparison of different plant species showed a tendency to maintain the expression pattern of a particular promoter. However, the orthologs of the uniformly expressed genes could be found easier as compared to conditional genes. Moreover, none of the screened core promoter types is consistently associated with changes in gene expression patterns. Therefore, only a correlation occurs between the promoter architecture and expression parameters [103].

The last *cis*-active element observed in plant core promoters is the CCAAT motif, which is localized on its borders, predominantly in positions -120 to -40 in dicot and more broadly in monocot promoters within -460 to -140 (Figure 1) [104]. The CCAAT motif is also observed in plant enhancers [105]. The CCAAT *cis*-element is bound by the CCAAT-binding factor (CBF), also known as nuclear transcription factor Y (NF-Y) [106]. The NF-Y transcription factor is composed of three subunits: NF-YA, NF-YB, and NF-YC [107]. Two of them, NF-YB and NF-YC, dimerize through their histone fold domain (HFD), which can bind DNA in a non-sequence-specific fashion. Obtained DNA-protein complexes are bound by NF-YA to build trimeric structures. Upon trimerization, NF-YA specifically recognizes the CCAAT box [105]. In soybean (*Glycine max),* the NFYA interacts with FVE, which is complexed with histone deacetylase HDA13 for putative transcriptional repression by reducing H3K9 acetylation at target loci [108].

An important role in regulation of gene expression is played by 5′UTR regions. Some of *the trans-factor* binding sites, such as those for ERF, SBP, G2-like, GATA, and ARR-B, are concentrated within the region localized 200 bp downstream from TSS in *A. thaliana* [109,110].

2.2. Proximal Promoter

In the proximal promoter, encompassing the region within up to 300 bp upstream from TSS, most of the functional *cis*-active elements required for the proper spatio-temporal or stress-responsive gene expression regulation (Figure 1) [67,81,111–113]. According to Yu et al. (2016), 63% of all *A. thaliana cis*-active elements are concentrated within a region slightly exceeding the proximal promoter, up to 400 bp upstream from TSS [109]. Also in the peach (*Prunus persica), cis*-active motifs are

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concentrated within -500 to +200 in relation to TSS [114]. Moreover, the results of Keilwagen et al. (2011) clearly show the preferred localization of AuxRE (TGTSTSB, where S = C or G and B=C, G, or T) within the proximal promoter, 250 bp upstream from TSS [115]. More significantly, these *cis*-active motifs are gathered within conserved *cis*-regulatory modules (CCRM), enabling interactions between *trans*-factors bound by neighboring, closely-spaced *cis*-active motifs [67,113,116]. Results of Jo et al. (2020) suggest that LEC1 interacts with ABA-Responsive Element Binding Protein3 (AREB3), Basic Leucin Zipper67 (bZIP67), and ABA-Insensitive (ABI3) *trans*-factors to regulate different sets of genes controlling morphogenesis, gibberellin signaling, photosynthesis, and maturation-related processes during soybean seed development [117]. Similarly, the TaNAC100 *trans*-factor could orchestrate the expression of starch synthesis genes in wheat [118]. Also, the responsiveness to auxin is mediated not only by a single AuxRE but also by multiple AuxREs. In the composite AuxRE elements associated with auxin response, ABRE-like and Y-Patch are 5′-flanking or overlapping AuxRE, whereas the AuxRE-like motif is 3′-flanking [110].

An interplay between *trans*-factor concentration, dimerization efficiency, and affinity to *cis*-active elements was studied among *trans*-factors in the CCRM-1 of the Glu gene that are responsible for the biosynthesis of high-molecular-weight glutenin subunits (HMW-GS) [113]. The concentration of the WUSCHEL *trans*-factor could regulate its amount in a monomer or dimer state through the binding of corresponding *cis*-element firstly by monomers and then switches to forming dimers at increasing concentrations [119]. The transition from monomer to dimer WUSCHEL state in *elementsata3* (*Clv3*) gene promoter is correlated with the transcriptional switch from activation to repression of *Clv3* [119].

2.3. Plant Enhancers

Enhancers can regulate gene expression over large distances, independent of orientation [120–123]. Although the plant enhancers stimulate the target gene expression over the distance of up to 140 kb, they could also be found within up to 400 bp from TSS (Figure 1) [124–128]. Moreover, differences between enhancers and promoters are often fuzzy, and their roles are interchangeable, as some promoters (epromoters) could function as enhancers [129].

Activation of enhancers requires the binding of an initial set of *trans*-factors, recognizing their cognate *cis*-elements on DNA wrapped in nucleosomes [130–132]. Then, the signal-dependent *trans*-factors interact with *cis*-elements that are more degenerate at enhacers as compared to promoters to support the protein-protein interactions and strengthen the cooperativity of their binding to DNA [123,133–136]. Together with *trans*-factors are recruited coactivators such as the Mediator complex, lysine acetyltransferases p300, and CREB-binding protein (CBP) to maintain the nucleosome-free chromatin state (Figure 1) [137–141]. As a result, the plant active enhancers pea PetE and maize b1 are enriched in H3/H4ac and H3K9/K14ac, respectively [127,142]. However, the increased level of H3K27ac is a marker of active enhancers in animals but not in plants [143,144]. Another epigenetic modification typical for active enhancers is the monometylation of H3K4 (H3K4me1) [145]. Putatively, H3K4me1 is required for nucleosome removal, while H3K27ac is pivotal for the production of the short, approximately 200-nt-long transcripts known as enhancer RNA (eRNA) [145,146]. Nascent eRNA in active enhancers recruits repressive histone and DNA modificators-polycomb repressive complex 2 (PRC2) and DNA methyltransferase 1 (DNMT1) to further protect the open DNA state from their suppressive influence [147,148].

Enhancers communicate with promoters mostly through looping interactions, engaging also the nuclear matrix components as CTCF or lamins, to bring both gene elements in close proximity in 3D space (Figure 1) [127,149]. Although the relationship between these interactions and gene activity is unclear, several models suggest that the pivotal role in creating linking contact is played by cohesin/CTCF, Mediator/RNA PolII, Polycomb repressive complex, or *trans*-factor dimerization. Moreover, low-affinity interactions or *trans*-factors, transcriptional co-actvators, and other proteins can create protein aggregates, promoting and reinforcing enhancer-promoter association [150,151].

3. Objectives and General Methodology of Synthetic Promoter Creating

Current biotechnology tasks require plant genetic modification of approximately up to 15 genes [152]. Coordinated expression of numerous genes is necessary to engineer complex regulatory networks underlying almost every metabolic and signaling pathway in plants [152–155]. Therefore, sets of different regulatory elements as synthetic promoters are required to facilitate such complex

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multigene engineering. Another reason to develop the novel synthetic promoters are problems observed after repeated application of the same synthetic promoters, such as plasmid instability or homology-based gene silencing [156,157].

Genetically modified plants could produce valuable metabolites, acquire resistance to herbicidal, insect, or fungal infections, and efficiently grow under abiotic stress conditions [155,158]. Although the final outcome of such approaches is the modification of metabolic fluxes adopting sessile plants to external conditions, the starting point is the precise and simultaneous expression control of numerous genes [159–162].

Stacking of numerous genes in transgenic plants requires sequential transformation by single gene vectors or multiple separate gene expression vectors [163,164]. Multiple rounds of crossing of different transgenic events are mandatory for conventional breeding techniques. These traditional strategies suffer from inherent pitfalls such as complex segregation patterns and random integration of multiple transgenes, resulting in non-stoichiometric and variable transgene expression [165,166].

An efficient approach to addressing such an issue is the coordinated expression of multiple transgenes by the minimum number of regulatory elements in a single transgenic event [160,167]. Therefore, stacking is the optimal method of simultaneous gene set expression due to their integration into adjacent regions of the genome and reducing the probability of transgene segregation in the fortcoming generations [168,169]. Multicistronic, versatile plant expression vector systems are developed to realize such a task [170–172].

Particularly helpful for the controlled expression of numerous genes are bidirectional promoter systems. [173–176]. Contrary to tandemly repeated monodirectional expression cassettes, bidirectional promoters offer a solution to identify optimal promoter contributions for transgene co-expression, available in a single round of cloning, expression, and screening experiments. However, a library of synthetic promoters is required to study their different expression levels and regulatory profiles in both expression directions [177]. According to Kumar et al. (2015), the gene stacking approach could potentially be extended to express 6–8 genes with a single bidirectional promoter [173].

Synthetic promoters join different *cis*-regulatory elements with the core promoter to combine tissue specificity with an increased or precisely tuned expression level within the same construct [85,178–181]. *Cis*-active elements within synthetic promoters are introduced accordingly to their positions, copy number, spacing, and orientation to promote optimum spacing among cis-motifs and corresponding *trans*-factors [182–188].

Obtained promoters indicate different tissue-specific, strength, and inducibility properties to enable the co-expression of numerous genes at different levels, as is required by the complex biosynthesis traits or regulatory circuits responding to different environmental conditions, i.e., biotic, abiotic, tissue-specific, light-stress, or hormonal-stimulation conditions [184,185,189–194]. To avoid unwanted recombination or homology-dependent gene silencing, the expression of each gene is regulated by a different promoter [189,195].

A promising tool to increase the diversity of available synthetic promoters as a remedy to decreasing plasmid stability and homology-based gene silencing are orthogonal expression systems based on promoters and *trans*-factors that do not occur in nature or originate from different species (Figure 2) [28,29,157,196–199]. Expression of such an orthogonal system is usually controlled by a tissue-specific or inducible promoter, enabling the biosynthesis of the orthogonal *trans*-factor. Produced *trans*-factors recognize *cis*-elements in promoters that are absent in native gene, to avoid the off-targets effects [28,29,196,199,200]. As an orthogonal *trans-factors, they* could also be adapted as transcription activator-like effectors (TALEs) due to the modular structure of their DNA-binding site (Figure 3). Such DNA-binding domains could be engineered to recognize a DNA sequence that does not occur in the genome [199,201]. Moreover, orthogonal expression systems are also based on artificial transcription factors (ATFs) built from the deactivated form of the Cas9 protein (dCas9) and the transcriptional activator domains VP64 or EDLL, repressor domain SRDX, or DNA epigenetic modifiers [196,202,203].

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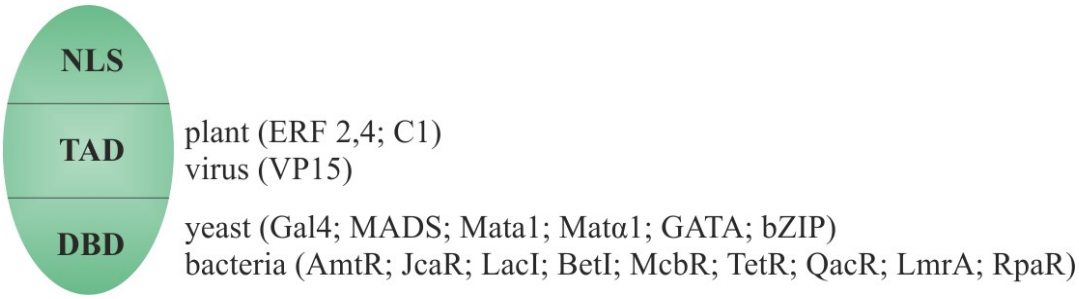

Figure 2. Structure of an orthogonal *trans*-factor. NLS-Nuclear localization signal, TAD-*trans*-activating domain, containing plant- or virus-derived elements, and DBD-DNA-binding domain not existing in plants and originating from the yeast of bacteria.

Figure 3. Structure of synthetic promoters recognized by transcription activator-like effectors (TALEs). The 19-base-long degenerate sequence is followed by the 18-base-long TALE-site, a TATA-box, a 43-base-long degenerate sequence, and the reporter gene. The presence of the 43-base-long degenerate sequence is consistent with the observation that TALE's site localization, approximately within -55 or -40, is optimal to confer TALE-mediated inducibility. .

3.1. Methods of Synthetic Promoter Generating

As a method of choice to produce relatively small synthetic promoter sets, PCR-based techniques [204–209] are exercised. Recently, plant synthetic promoters were prepared by PCR-based deletion mutagenesis and subsequent GUS activity assays. In this way, two core promoters were obtained from the *Stellaria media* Antimicrobial Peptide1 (AMP1) and Antimicrobial Peptide2 (AMP2) genes. Although the shorter versions of the pro-SmAMP1 and pro-SmAMP2 promoters, represented by the −58 and −60 bp fragments, were weak, the longer fragments of 102 and 104 bp were used as core promoters, largely retaining the properties of the original promoters [210]. Another application of primer-driven PCR mutagenesis enabled the obtaining variants of the synthetic promoter pCL suitable to regulate the cold-induced sweetening of potatoes. Several point mutations were introduced into the region encompassing the 5′ and 3′ sides of the C-repeat/dehydration-responsive element (CRT/DRT), localized within -490 to -500 bp from TSS. Moreover, the additional cassette of 27 bp containing the second CRT/DRT element was placed at a distance of 31 bb from the existing CRT/DRE site [211].

Results presented by Zhang et al. (2019) suggest that even discrete point mutations of repeated G-box (CACGTG) and its 2-4 bp long flanking regions could significantly increase the reporter GFP gene expression level, opening the potential for useful and predictable genome editing approaches in the promoter regions [212].

Besides the presented approaches dealing with the mutagenesis of a single promoter, providing a relatively small number of variants, the larger libraries are produced by the stepwise directed evolution of the promoter sequence using error-prone PCR. Perfoming the 83 cycyles of mutation, construction, screening, and property characterization of the 74-bp-long Ptrc promoter, combined with sampling 10–20 variants of the lowest and highest activity after each step, resulted in a library of 3665 clones. Among them, a 454-fold difference was found between the strongest and weakest expression rates [213]. A smaller library containing hundreds of variants is obtained by shuffling the Figwort mosaic virus full-length transcript promoter (F) and the sub-genomic transcript promoter (FS) sequences. Shuffled promoters were weaker as compared to those prepared by hybridization. However, shuffled libraries contained promoters with a broad spectrum of activities, enabling the application of both weaker and stronger variants in metabolic engineering approaches [209]. To significantly increase the complexity of synthetic promoter libraries, a combination of site-directed mutation with degenerate primers, overlap-extension PCR, and Gibson assembly was proposed. Prepared libraries showed a diversity in the order of 10^4–10^7 variants and were screened rapidly by performing fluorescence-activated cell sorting (FACS) of transformed yeast cells [214].

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Construction of larger synthetic promoter libraries composed of over 10^8 elements is possible after avoiding the use of already-known DNA sequences. Random, 80-bp-long DNA oligos are synthesized and used to build such complex libraries in yeast, enabling the putative study of all pairwise interactions between TF (*trans*-factor) binding sites in the context of orientation and specific spacing constraints. Analysis of such large synthetic promoter libraries induces analytical problems as the complexity of the synthetic promoter library (>10^8) exceeds the number of sorted yeast cells (<10^8). Also, the analysis of such large libraries using simplified TF binding models, assuming their position, orientation, and TF-TF interaction independence, explained nearly 93% of the variation in gene expression in tested libraries and proved to be insufficient in relation to native yeast genes, where only ~16% of the variability was demonstrated [46].

3.2. Cis-Elements Juggling and Domain Swapping

As components to build the synthetic promoters, they typically engage the existing oligonuclotide *cis*-elements that are juggled or shuffled. However, larger promoter domains with known regulatory properties could be exchanged in the process of domain swapping [215–220]. Alternatively, completely novel *cis*-elements or domain variants identified by searches of available promoter databases or obtained through artificial intelligence support could be experimentally validated in plant material and then employed to construct synthetic promoters [221]. An example of *cis*-element shuffling is placing the *cis*-elements of known functions in a novel or synthetic stretch of DNA. Optionally, fragments of one promoter could be exchanged with functionally equivalent domains from other heterologous promoters [195]. The last approach is often practiced as ligating the upstream activation sequence (UAS) from one promoter to the TATA box-containing domain of another promoter. It is expected that the transfer of the *cis*-element or upstream DNA sequence from one promoter into a different promoter containing the TATA sequence could provide a novel transcription regulatory mechanism [128,181,222].

Placing the TATA-box of the CAMV35S promoter (TATATAA) in a synthetic stretch of DNA provided the synthetic core promoter, indicating the 85% activity of the native sequence. However, core promoters prepared by domain swapping were significantly weaker, indicating only 20–68% of CAMV35S activity. These relatively weaker synthetic promoters could be important for complex metabolic engineering approaches, where the decreased or precisely tuned load of heterologously expressed genes could be salutary to host primary metabolic routes [195,223–227]. Further applications of the domain hybridization generated many superior plant promoters [128,181,209,222,228–230].

Viral and plant pathogen genomes are exercised as a relatively simple and easily available source of naturally occurring *cis*-active elements to prepare novel synthetic promoters through domain hybridization [181,183,222,230]. These synthetic promoters could indicate the higher activity of CAMV35S. That was seen for two recombinant promoters, S100 and D100, prepared from the Strawberry Vein Banding Virus, demonstrating 1.8 and 2.2 times stronger activities in tobacco (*Nicotiana tabacum*) than that of the CaMV35S promoter. The S100 recombinant promoter (621 bp) was obtained from the 250-bp-long upstream activation sequence (UAS) of the Strawberry Vein Banding Virus (SV10UAS; − 352 to − 102 relative to TSS) and the 371-bp-long TATA-containing core promoter domain (SV10CP; − 352 to +19) of the same gene. Correspondingly, the 726-bp-long D100 promoter was assembled by fusion of the 170-bp-long UAS of the Dahlia *Mosaic* Virus (DaMV14UAS; − 203 to − 33) with its 556-bp-long core promoter domain (DaMV4CP; − 474 to +82) [231].

In a comparable way, a synthetic promoter, FUASCsV8C, was designed by placing the Upstream Activation Sequence (UAS) of Figwort Mosaic Virus (FMV; -249 to -54) at the 5′-end of the Cassava Vein Mosaic Virus (CsVMV) promoter fragment 8 (CsVMV8; -215 to +166). Such a hybridized, synthetic promoter exhibited approximately 2.1 and 2.0 times higher GUS activities as compared to the CaMV35S promoter, as assessed in tobacco protoplasts and in agroinfiltration assays, respectively [222].

The strength of synthetic promoters could be significantly higher compared to the CAMV35, as shown by the construction of two chimeric promoters, MBR3 and FBR3, by fusing the UASs (upstream activation sequences) of *Mirabilis Mosaic Virus* (MUAS; − 297 to + 38; 335 bp) and *Figwort Mosaic Virus* (FUAS; − 249 to + 54; 303 bp), respectively, to the core promoter domain of BR3 (BR3; − 212 to + 160; 372 bp). The activities of MBR3 and FBR3 promoters were comparable to that of the activity of

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the CaMV35S2 promoter and four times stronger than that of the CaMV35S, as confirmed by histochemical and fluorometric GUS assays [232].

In the building of synthetic promoters, novel cis-active elements are also utilized, such as the W-box sequence (GACTTTT), MYB-like (TGGTTT), bZIP-related (TACGTGACG), and TACGTCACG, from the genome of the fungal pathogen *Phytophthora sojae*. These *cis*-active motifs, assembled in tetrameric mode, drove the expression of the CAMV35S core-based synthetic promoter in response to the elicitor peptide Pep25 (N-DVTAGAEVWNQPVRGFKVYEQTEMT-C) from *Phytophthora sojae* [183].

The profuse source of *cis*-active elements or larger promoter domains is complex and not fully characterized in plant genomes. *In silico* analyses of 63 and 183 soybean drought-inducible genes were a starting point to select *cis*-active motifs responsible for drought stress, which were fused to the minimal 35S promoter as concatamers of 4–7 elements [184,185]. Analogously, two *cis*-regulatory motifs M1.1 (TAAAATAAAGTTCTTTAATT) and M2.3 (ATATAATTAAGT) in the soybean (*Glycine max*) genome, reacting to the infection of cyst nematode (SCN), were exploited in the form of four-fold repeats to develop synthetic promoters, which run the GUS expression in roots infected by *Meloidogyne incognito* [189]. Moreover, the simultaneous response to fungal pathogens SA (salicylic acid) and MJ (metyl jasmonate) was achieved in synthetic promoters containing core 35S placed downstream to dimers of both D (31 bp) and F (39 bp) *cis*-active motifs from the *Arabidopsis AtCMPG1* gene [190].

Since *cis*-elements in patogen genes have evolved to utilize the plant's transcriptional machinery as TGACG-motif binding (TGA) of basic-leucine-zipper (bZIP) TFs, they are much stronger activators of synthetic promoters as compared to other TFs found in plant constitutive promoters. However, the combination of three different plant *cis*-active elements recognized by weak and dissimilar activators resulted in the strongly increased reporter gene expression, that is putatively mediated by passive cooperativity and not the direct interaction between *trans-factors,* as suggests the result of distance extension between *cis*-active motifs [186].

Other properties of synthetic promoters not related to patogen resistance could be engineered by the application of different *cis*-active elements. Deployment of a 6-times repeated abscisic acid (ABA) responsive element (6xABRE) upstream to a minimal CAMV35S promoter -90 to -1 produced a system that was responsible for ABA, salt, and mannitol treatment in transformed *A. thaliana* roots [233]. Combining the more complex set of seven *cis*-active elements from three photorespiratory genes in *A. thaliana* (*AtPLGG1*, *AtBASS6*, *AtPGLP*) results in synthetic promoters that could be responsive to elevated temperature, low CO_2, and high irradiance stress. Among the analyzed *cis*-responsive elements, MYB and b-ZIP reduced the transgene expression rate measured by luciferase activity through recruiting transcriptional repressors. However, bHLH and AP2 increased luciferase activity in response to elevated temperatures and high irradiance, respectively [192]. Studies on the role of UTR regions in plants and viruses suggest that plant-derived UTRs outperform viral UTRs under ambient conditions. However, plant-derived UTRs are more sensitive to environmental stress conditions compared to viral UTRs [192].

To construct synthetic promoters, not only *cis*-elements but larger fragments could be assigned as enhancers or entire promoters of selected crop genes, extending the number of available plant synthetic promoters [128,221]. To that end, the publicly available RNA-Seq datasets were searched to find genes indicating a high dynamic range, activity in different plant species, and ubiquitous expression in diverse tissues. Among the genes obtained, those of various mean abundances of transcripts were selected, suggesting differences in their strengths. A prepared set of 15 plant constitutive promoters driving these genes showed expression levels spanning nearly two orders of magnitude [221]. Seven of them were stronger than the NOS, and five are comparable with the CAMV35S promoter [221]. Moreover, Jores et al. (2020) handled the CAMV35S core enhancer (subdomains A1 and B1-3) and enhancers from three plant genes identified by self-transcribing active regulatory region sequencing (STARR-seq) to stimulate the activity of the CAMV35S minimal promoter independently from orientation. These enhancers efficiently stimulate synthetic expression systems only when positioned relatively nearly, within 500 bp from the CAMV35S minimal core promoter and outside of the 3'UTR [128].

Creating synthetic promoters opens the springboard to analyzing the subtle or mutually exclusive metabolic events. Endeavours to study the antagonistic pairs of plant regulators as JA/SA

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or auxin/cytokinin were addressed by the close spacement between *cis*-elments bound by *trans*-factors responding to SA and JA, or alternatively auxin or cytokinin treatment. Obtained synthetic promoters could attenuate the antagonistic effects of the SA/JA and auxin/cytokinin pairs and strengthen responses to these stimuli [193]. Also, the feed-back-regulated catabolism of SA to 2,3-dihydroxybenzoic acid, together with such physiologic effects as SA concentration in plant tissue, growth rate, leaf senescence, and pathogen resistance, was reconstructed in plants by the application of synthetic promoters containing the SA 3-hydroxylase (S3H) gene under the control of the SA-inducible promoter from SA 5-hydroxylase (S5H) [193]. Furthermore, plant synthetic promoters based on the Q system from *Neurospora crassa* could be not only activated by expression QF2 and QF2w *trans*-factors but also constrained by the QS repressor, enabling the precise control of gene expression rate [234,235]. In addition, the precise control of three gene expression levels could modulate photosynthesis efficiency and plant productivity by photorespiratory bypass. Such a synthetic photorespiratory circumvent, known as the GMA bypass, allows the plastidial glycolate to decompose, leading to the release of CO_2 directly into the chloroplasts and an increase in the local CO_2/O_2 ratio. To efficiently execute such a task, two different constitutive promoters, the CaMV35S and the maize UBIQUITIN promoter (*pUbi*), were exercised to avoid gene-silencing effects while driving *Cucurbita maxima* malate synthase (*CmMS*) and *O. sativa* ascorbate peroxidase 7 (*OsAPX7*), respectively. However, the third gene in the GMA bypass, known as *O. sativa* glycolate oxidase 1 (*OsGLO1*), should be controlled by a light-inducible Rubisco small subunit promoter to dynamically adapt the gene expression rate to light condition changes and significantly improve the photosynthetic efficiency [194].

4. Native and Synthetic Bidirectional Promoters

Bidirectional promoters assure the more coordinated expression of several genes compared to constructs containing these genes driven independently by unidirectional promoters [236]. Such a property indicated the bidirectional promoter obtained from the *Zea mays* Ubiquitin-1 (ZMUbi1) gene that was utilized to regulate expression of insect (*cry34Ab1* and *cry35Ab1*) or herbicide (*aad1*) resistance and a phi-yfp reporter gene in corn (*Zea mays*) [173].

A set of green-tissue-specific, bidirectional promoters was built by combining the unidirectional promoter POsrbcs-550 with the inversely-oriented OsTub6 intron to increase both the transcription efficiency and green-tissue expression rate of the GFP in the 5′ direction. Another group of regulatory elements, such as the reverse-oriented core promoter of POsrbcs-550 (POsrbcs-62), or its alternative PD540-544, combined with the reversed first intron of *OsAct* (*OsAct1*) and four-times repated GEAT regulatory motif, were joined to increase the high level of the 3′-directed Gus gene expression [236]. The obtained constructs were tested in the transgenic *O. sativa* to show a generally predominant expression in green tissues such as leaves, stems, panicles, or sheaths and a low expression in roots and endosperm [236].

Some bidirectional promoters exist naturally as the 1156-bp-long fragment in the hot pepper genome localized between two head-to-head-oriented sesquiterpene cyclase (EAS) and a hydroxylase (EAH) genes. Analysis of promoter deletion mutant activity assessed by EGFP and shRLUC reporter genes, expressed in *Nicotiana benthamiana* leaves, showed that a 199-bp fragment containing the GCC-box of EAH and a downstream 226-bp long fragment of EAS bearing four W-boxes are minimal promoters for the pathogen-inducible expression of both genes [237].

The naturally occurring bidirectional histone gene promoters PHTX1, PHHX1, and PHHX2 are much shorter and more compact as compared to monodirectional promoters and drive gene expression in a TATA-box-dependent manner [177]. Promoter compactness, combined with the clear regulatory mechanism in both directions, triggers the application of plant gene expression regulation. Therefore, these bidirectional promoters were used to construct a library of 168 synthetic BDPs in the yeast *Komagataella phaffii* (syn. *Pichia pastoris*) for the rapid screening and optimization of different expression ratios [177]. Prepared synthetic promoters showed not a constitutive but rather a tight cell-cycle-regulated expression. Such tight, temporal expression control of metabolic pathway genes combined with the precise regulation of subsequent gene co-expression rate protects against excessive loads of heterologous proteins or concentrations of toxic metabolites [177]. By truncating and deleting PHHX2 fragments, BDPs of varying strength were produced. Moreover, the inducibility property was added by shortened and bidirectionalized versions of the methanol utilization pathway

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promoters PDAS1-DAS2, which were fused with reversely oriented histone core promoters. Such an inducible approach was efficiently exercised in taxadiene biosynthesis, where the CYP2D6 and CPR genes were controlled by methanol-inducible PDAS1-DAS2, while the *GGPPS* was regulated by a PGAP+CAT1 fusion promoter that is repressed in the presence of glucose, partially derepressed when the glucose is absent, and fully induced by metanol [177]. Moreover, assembly of the four carotenoid pathway biosynthesis genes CrtE, CrtB, CrtI, and CrtY under the control of histone BDPs enables the achievement of a 14.9-fold higher β-carotene yield as compared to the use of a monodirectional PGAP constitutive promoter [177].

A related approach enabled the expression of β-carotene ketolase and hydroxylase genes in the maize seed using a seed-specific bidirectional promoter. Obtained transgenic maize lines accumulated astaxanthin from 47.76 to 111.82 mg/kg DW in seeds, and the maximum level is approximately sixfold higher than those in previous reports (16.2–16.8 mg/kg DW) [238].

5. Orthogonal Expression Systems

Recently developed, orthogonal expression systems built from over 500 elements could be employed to introduce complex, multi-gated logic principles such as „or", „nor" or „kill" into genetic circuits. Such a broad range or regulatory effect was obtained after combining five concatenated *cis*-active elements from yeast (Gal4, MCM1, ata1, Matα2, Gat1, Yap1) with the WUS minimal promoter (Figure 2). The regulatory properties of synthetic *trans*-factors were extended by the repression activity achieved by combining the yeast DBD from Gal4 with the SRDX repression domain (Figures 4 and 5). Expression of synthetic *trans*-factors was controlled by an endosperm-specific At2S3 or phosphate-responsive AtPht1.1. promoter. Interestingly, the MCM1 and Yap1 containing solely DBDs, without the addition of TAD, preserved some *trans*-activity properties, suggesting the inherent activation functions of DBD that may be interrupted by the introduction of TAD [28].

Figure 4. Synthetic *trans*-factors positively regulate the orthogonal expression system.

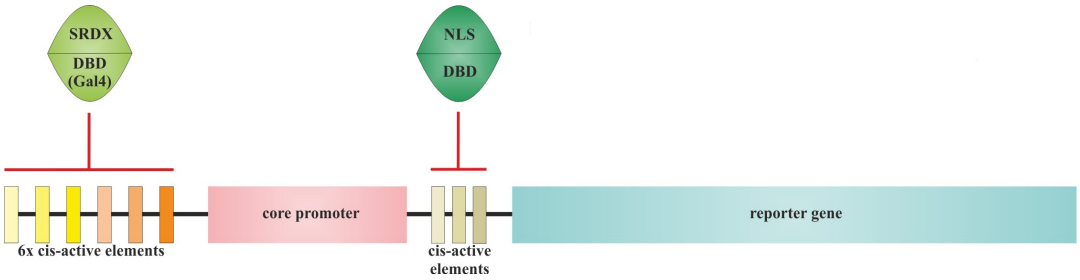

Figure 5. Synthetic *trans*-factors nagatively regulating the orthogonal expression system are produced by combining the yeast DBD from Gal4 with the SRDX repression domain. Alternatively, a STF containing only NLS and DBD could recognize DNA sequence downstream of the core promoter to create a steric hindrance, protecting against RNA polymerae II preinitiation complex formation.

The synthetic transcription regulators were also created from bacterial TF-derived DBD, combined with the TAD domain from VP16 or *Arabidopsis* ETHYLENE RESPONSE FACTOR 2 (ERF2) and the SV40 nuclear localization signal (NLS). The orthogonal set of plant synthetic promoters was created by fusing a core plant promoter, encompassing positions -66 to +18 of the CaMV35S, with the six copies of the DNA sequence (operator) bound by these TFs. The repressor activity was mediated by synthetic regulators composed exclusively of DNA binding domains and NLS sequences, localized 3′ downstream to the core promoter (Figure 5). Obtained direct and layered logic gates are

 doi:10.20944/preprints202404.1084.v1

exercised to qualitatively modify the expression of the root-specific promoters SOMBRERO (proSMB) and PIN-FORMED 4 (proPIN4) [29].

An interesting approach to developing plant synthetic promoters controlled by orthogonal regulators is the application of transcription activator-like effectors (dTALEs) [198]. The prepared synthetic dTALE activated promoters (STAPs) are composed of the 19-base-long degenerate sequence, followed by the 18-base-long TALE-site, a TATA-box, a 43-base-long degenerate sequence, and the ATG start codon (Figure 3). This is consistent with the observation that the TALE's site localization approximately within -55 or -40 is sufficient to confer TALE-mediated inducibility. Forty-three of these promoters were tested in transient assays in *N. benthamiana* using a GUS reporter gene to show their expression strength range from around 5% to almost 100% of the viral CAMV35S promoter activity. Moreover, these STAPs were neatly utilized to transiently express three genes for the production of a plant diterpenoid in *N. benthamiana* [201].

STAPs developed by Brückner et al. (2020) for application in dicot plants were the starting point to assemble related systems STAP1 and STAP2 in a monocot plant, *Oryza sativa* [197,201]. To assure the spatial regulation of the GUS reporter gene, the TALE-dependent expression was driven by the bundle sheath cell-specific *Zoysia japonica* PHOSPHOENOLPYRUVATE CARBOXYKINASE (ZjPCK) promoter (ZjPCKpro) and two PHOSPHOENOLPYRUVATE CARBOXYLASE promoters (PEPCpro), maintaining direct and strong mesophyll-specific gene expression in rice. The tissue gene expression mediated by the STAP1 and STAP2 systems was heritable and scalable enough to efficiently regulate up to four different genes in one genetic construct. Moreover, the relatively low off-target activity of dTALE1 expression was found through a comparison of RNAseq study results performed on wild-type and T2 plants. Only 139 up- and 8 down-regulated genes could be attributable to the dTALE1 expression [197]. TALES could be exercised not only for the direct regulation of STAPS but also indirectly for the control of orthogonal TF expression [186].

DNA sequences recognized and bound by TALEs, known as effector binding elements (EBEs), could be predicted *in silico* and exercised to build repeatedly repeated fragments, controlling the expression of reporter genes of significant biological functions such as the *avrGf2* gene from the bacterial pathogen *Xanthomonas citri*. The gene could induce plant cell death, protecting against the development of the pathogen [239].

To produce an artificial *trans*-factor (ATF), it is broadly utilized in the deactivated form of the Cas9 protein (dCas9) fused to the transcriptional activator domain VP64. The ATF dCas9:VP64 upregulates the expression of reporter genes via specific guide RNAs (gRNAs) that target the promoter region upstream of those genes (Figure 6). The expression of ATF is usually controlled by CAMV35S, while the gRNA transcription rate is regulated by Pol III (U6) [196]. The presented system is known as the first generation of dCas9-dependent ATFs and was further developed to achieve a vast modulation of target gene expression. The novel form, known as the second generation of dCas9-dependent ATFs, contains the modified gRNA, which contains two aptamer loops to allow the attachment of the viral MS2 protein. To the MS2 protein are fused activators (VPR), transcriptional repressors (SRDX), or epigenetic regulators such as the catalytic core domain of *Homo sapiens* p300, H3K27 histone acetyltransferase, H3K9 histone methyltransferase, the SET domain of KRYPTONITE (KYP), or H3K9 methyltransferase from *Arabidopsis* [202,203].

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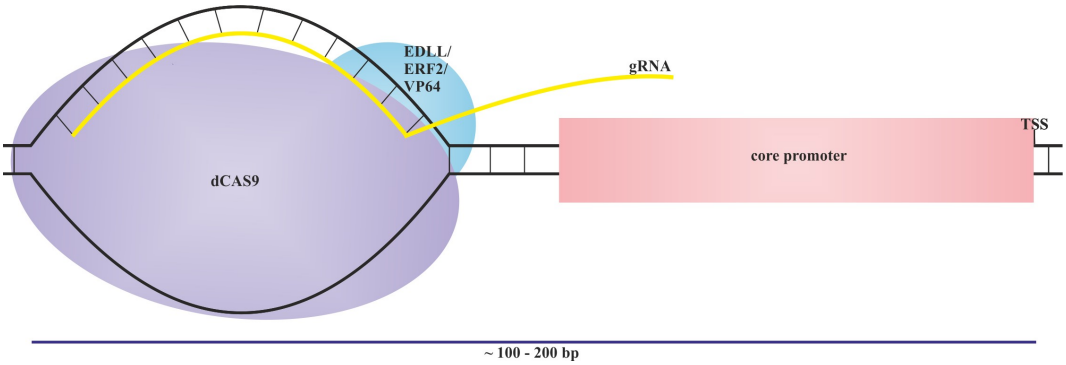

Figure 6. The first generation of dCas9-dependent artificial *trans*-factors (ATFs). The deactivated form of the Cas9 protein (dCas9) is fused to the transcriptional activator domains VP64, ERF2, or EDLL to create ATFs. The ATF upregulates the expression of reporter genes via specific guide RNA (gRNA).

To add another regulatory layer to the orthogonal systems, the expression of dCas9:EDLL and MS2:VPR could be regulated not by constitutive CAMV35S but by the copper-inducible promoter, containing the *cis*-active motif named copper-binding site (CBS), recognized by the copper-responsive factor CUP2, fused to the yeast Gal4 domain [199]. The presented system enabled a 2,600-fold and 245-fold increase in the endogenous *N. benthamiana* DFR and PAL2 gene transcription, respectively, with the trace expression level in the absence of the copper ions [200]. Another approach to introducing inducibility into the orthogonal expression systems was proposed by Lopez-Salmeron et al. (2019) [240]. The authors developed an inducible, tissue-specific expression system based on the chimeric transcription factor LhG4 fused to the ligand binding domain of the rat corticoid receptor (GR), localized under the control of well-characterized, cell-type-specific promoters. In resting conditions, the GR domain is bound by the cytosolic HSP90, handling the transcription factor outside of the nucleus. After the addition of the synthetic ligand dexamethasone (Dex), nuclear translocation is induced, and LhG4 will mediate transcription of expression cassettes under the control of a synthetic pOp-type promoter [240].

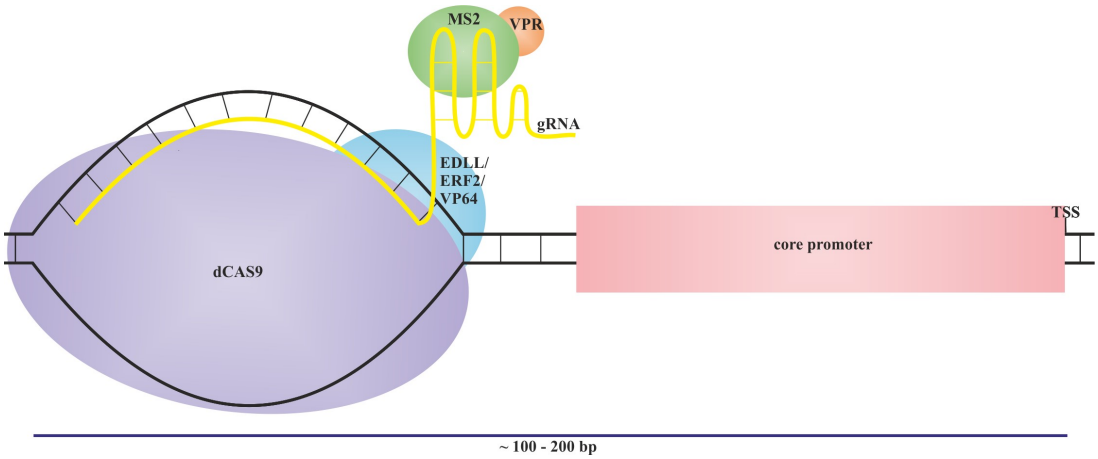

Figure 7. The second generation of dCas9-dependent artificial *trans*-factors (ATFs) contains the modified gRNA with two aptamer loops to allow the attachment of the viral MS2 protein. To the MS2 protein are fused activators (VPR) or other components as repressors or epigenetic regulators to increase the scope of regulatory functions known in the first generation of dCas9-dependent artificial *trans*-factors (ATFs).

6. Machine Learning and Deep Learning Support to Synthetic Promoter Preparing

The fast and accurate prediction of promoter strength remains challenging, resulting in time- and labor-consuming promoter construction and characterization processes. The main reason for the presented problems is the shortfall of suitable high-throughput analytical methodology and a lack of a large promoter library that has broad enough dynamic ranges, gradient strengths, and clear sequence profiles to be implemented to train machine learning (ML) or deep learning (DL) models

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[49]. ML approaches offer models characterizing properties between synthetic promoter structure and functions [46,85,241]. Advanced ML algorithms produce highly accurate models of gene expression, uncovering novel regulatory features in nucleotide sequences involving multiple *cis*-regulatory regions across whole genes and DNA structural properties. These details broaden our understanding of gene regulation and point to new principles to test and adopt in the field of plant science [242]. ML models as linear regression and random forests combine input data as sequences of promoters, distrubution of *cis*-active elements, DNA structural properties, position weight matrices, or K-mer frequencies to obtain output information as the expression patterns of corresponding synthetic promoters [46,48,50,85,242]. To better predict the synthetic promoter strength, the EVMP (Extended Vision Mutant Priority) system was developed. The EVMP uses better mutation information through the equivalent transformation of synthetic promoters into base promoters and corresponding *k*-mer mutations, which are input into BaseEncoder and VarEncoder, respectively. Therefore, the EVMP can significantly improve the prediction accuracy of promoter strength [243].

The ML systems work with the large libraries of synthetic promoter variants and corresponding expression data [46]. The input/output data is then split into training and testing parts for building the model and final validation, respectively. The model's accuracy generally increases when a greater number of promoter sequences and corresponding RNA-seq, ATAC-seq, and self-transcribing active regulatory region sequencing (STARR-seq) data are applied [85,244]. Therefore, the DL models implemented on over 20,000 mRNA datasets in 7 model organisms, from bacteria to humans, provided novel information, suggesting that the gene expression level is controlled not by a single regulatory motif or region but by the entire gene regulatory structure and specific combinations of regulatory elements [50].

Such basic ML approaches cannot properly predict the properties of synthetic promoter sequences in the context of other regulatory elements such as the position of enhancers, 3′ and 5UTR, DNANA methylatio or histone acetylation status [50,242,245–247]. These details should be included in the more advanced form of ML, known as DL, which uses neural networks such as artificial neural networks (ANN) and convolutional neural networks (CNN), offering multiple layers to analyze complex patterns and relationships in data [85,248,249].

Another limitation of ML and DL model development is the intrinsic properties of genome DNA sequences due to their dependency on evolution or relatively low complexity. Available ML and DL models are developed and trained on genomic DNA that is too short and has insufficient sequence diversity to learn all relevant parameters. Therefore, synthetic, random DNA sequences provide the opportunity to test and train a larger sequence space as compared to the genome. Models trained on these synthetic data can predict genomic activity better than genome-trained models [250]. Moreover, the DL algorithms usually neglect evolutionary processes within biological systems, often resulting in false positives and counterfeit interpretations. Among approaches including evolutionary significance in machine learning are gene-family-guided splitting and ortholog contrasts. Analysis of the obtained model weight suggests that the 5′ UTR is more important for large-scale gene expression changes, while the 3′ UTR is more significant for fine-tuning mRNA levels [251].

7. Discussion

The properties of synthetic promoters and their applications were described in several review articles [252–257]. However, research in this field is progressing rapidly, fueled by progressing climate changes, decreasing arable land area, and a fast-growing global population [1]. Plant genetic modication by synthetic promoters controlling the expression of particular genes is pivotal to introducing metabolic switches, enabling the development of novel, more efficient, or stress-resistant crop variants and increased biosynthesis of valuable metabolites [1,4,5,9,10].

Genetic modifications of common crop plants require the application of up to 15 different genes [13,15,16,152,155,158]. However, the repeated use of the same or related DNA promoter sequences, combined with conventional, multi-round breeding techniques, results in homology-based gene silencing, complex segregation patterns, and random integration of multiple transgenes that are expressed at different, hardy controlled levels [156,157,165,166]. Therefore, to cope with the presented problems, transgenes are generally stacked in a single construct controlled by a minimum number of synthetic promoters that should not be homologous to those already used [160,167,170–172]. The

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necessity of gene stacking, combined with the decreasing number of available promoters, could be addressed by a more broad application of bidirectional promoters that are relatively short and compact, enabling the expression of up to 6–8 genes per one synthetic promoter [173–177,238].

However, the demand for novel synthetic promoters is still increasing [152–155]. The standard methods used to obtain them are hybridizations of existing *cis*-active elements with other core promoters or exchange of entire promoters as well as their domains [195,215–222]. Therefore, synthetic promoters combine different domains or *cis*-regulatory elements with the core promoter, joining tissue specificity with an increased or precisely tuned expression level in the same construct [85,178,180,181]. *Cis*-active elements are introduced accordingly to their positions, copy number, spacing, and orientation to promote optimum spacing among *cis*-motifs and corresponding *trans*-factors [182–186,188]. Prepared promoters indicate different tissue-specificity, strength, and inducibility properties to precisely control the co-expression of numerous genes in complex biosynthesis traits [189–194].

Besides the exchange of domains or *cis*-elements of known properties, the novel synthetic promoter variants are prepared by the introduction of genetic changes into existing *cis*-active elements and core promoters [89,214]. Usually, these approaches are based on error-prone PCR [204–212]. Larger libraries of synthetic promoters are obtained after repeated applications of the directed evolution methodology [213]. Further growth of libraries above 10^8 elements requires the application of synthetic DNA fragments and is not related to already known DNA genome sequences [46]. However, analysis of such large libraries obtained by evolution- or synthetic DNA-based methods is usually supported by ML or DL approaches, searching for a relation between DNA sequence properties and promoter activity [46,50,241–243,251,258,259]. These ML and DL approaches should be adopted to cope with their limitations linked to genome DNA properties, such as dependency on evolution or relatively low complexity, through training on synthetic, random DNA fragments to test a larger sequence space. Models trained on these synthetic data can predict genomic activity better than solely genome-trained models [250].

Usually, information obtained from the ML and DL methods is verified experimentally to test the accuracy of model predictions [213,260]. Repeated application of cycles built from ML/DL models and promoter experimental validation lead to their stepwise improvement. However, the scale of the applied libraries results in problems with prompt and accurate analysis of the obtained variant properties. A promising solution could be the more broad application of a fluorescence-inducing laser projector (FLIP) platform equipped with an ultra-low-noise camera, which enabled researchers to discriminate between numerous fluorescent signatures under different stress conditions [261–263].

Besides the production of huge synthetic promoter libraries, the synthetic promoters are also developed on the basis of orthogonal expression systems. The main advantage of these systems is the general lack of similarity with existing DNA sequences, resulting in low homology-based gene silencing. Orthogonal expression systems could be precisely tuned and accustomed to use in complex logic gates to provide a very precise control of complex genetic circuits [28,29].

Although the research on synthetic promoter properties is progressing rapidly, some basic issues, such as the precise deciphering of the information present in the *cis*-active elements in the context of chromatin modification and *trans*-factor dimerization, are far from complete understanding [43,48,67,68,264].

8. Conclusions and Future Directions

The main impediments to synthetic promoter development that should be addressed in future research are the better understanding of *cis*-element informative content and the progress in analytical methodology of large-scale synthetic promoter libraries. Moreover, the development of novel synthetic promoters, which is already supported by ML and DL algorithms, should be less reliant on evolution-dependent and relatively low-complex genomic DNA sequences to provide more decent results. Therefore, more research based on synthetic promoters, which are completely synthetic and independent from genomic DNA, should be initiated to develop and train more reliable ML and DL for OLs. Improved ML and DL systems could further support the expansion and enrichment of artificial promoter libraries. These ML and DL algorithms should be trained on large, synthesized DNA sets to avoid evolution-based biases in genome DNA.Moreover high-throughput methods

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based on fluorescence-inducing laser projector (FLIP) platform equipped with an ultra-low-noise camera, ed vaiants should be broadly applied to synthetic promoter analytical studies.

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