Dear Reviever 2,

Thank you very much for the opportunity to improve the manuscript.

Below are presented detailed responses to all your questions.

Your Sincerely,

Piotr Szymczyk

Reviewer 2

1. Title

Synthetic plant promoters

2. Abstract

In the end, a perspective (usefulness) of the review would be missing.

In general, a well-structured, clear summary with the necessary information. It would only be necessary to address the topic, description, types, etc. more. Of synthetic promoters.

The abstract was modified according to suggestions. Following fragment was added in the final Abstract section:

The broader application of novel, synthetic promoters reduces the frequency of homology-based gene silencing or improves the stability of transgenes.

Among them are presented orthogonal systems based on transcription activator-like effectors (dTALEs), synthetic dTALE activated promoters (STAPs) and dCas9-dependent artificial *trans*-factors (ATFs).

Synthetic plant promoters are a valuable tools to provide the precise spatio-temporal regulation and introduction logic gates into complex genetic traits, that is important for basic research studies and their applications in crops plant development. Exactly regulated metabolic routes are less prone to undesirable feed-back regulation and energy waste, improving the efficiency of transgenic crops.

3. Keywords

Excess keywords

The excess of keywords is removed. Following keywords were left: plant synthetic promoter, orthogonal systems, bidirectional promoters

1. Introduction

I. Lines 29-32 are 10 quotes to support that idea?

The number of citations was reduced to 7.

II. Lines 32-34 Not only through transgenesis is plant resistance to biotic or abiotic factors achieved.

Following sentence was added with proper citations: Plant genetic modifications support other methods increasing plant resistanc to biotic and abotic stresses as crops breeding and priming [24,25].

III. In general, the introduction contains more information on the disadvantages found when using synthetic promoters than the advantages.

Advantages of plant synthetic promoters were provided in the final part of Introduction, following fragment was added:

Despite these limitations, studies on plant synthetic promoters provide valuable information of regulatory mechanism controlling gene expression in complex traits, enabling the regulation of metabolic pathways activity [28,50,78]. Moreover, of particular importance is research on plant bidirectional and orthogonal expression systems, providing solutions to problems associated with coordinated and controlled expression of stacked genes as well as decreasing the exposition to homologous DNA sequences [28,78,79]. Besides the putative practical applications, studies on plant synthetic promoters are often performed in the whole genome scale, supported by advanced machine and deep learning algorithms. These studies supply essential and novel information concerning regulatory functions of promoter segments or *cis*-active motifs in context of DNA or chromatin structure modifications [35,44,46,50,78].

2. Vegetable Promoter Sections

I. Lines 114-125 is the description of the same work, Amack et al. (2023). It would be better to synthesize the information (address more the results than the methods).

Results are now addressed more than methods. The fragment is corrected to the following one: The importance of the TATA-box sequence for proper gene expression regulation was confirmed by Amack et al. [89]. The authors used PCR and specific primers to introduce single nucleotide substitutions to the TATA-box of the CaMV35S promoter. 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 [89].

II. This entire section is based on the description of studies related to plant promoters. However, a review that can contribute something different to those already on the network should mention the possible the possible causes of these phenomena based on the available literature (see the example in lines 174-177). Several sentences- marked in red were addend to show mechanisms of observed phenomena.

This could be explained by the relative austerity of *cis*-active motifs, and lower space required to gather them into promoters which reply to limited stimuli.

Putative mechanism and reasons of presented results were presented. Following fragments were added to section 2.1 to address putative mechanisms of presented results:

The common lack of TATA-box in plant core promoters suggests its putative functional substitution by other core promoter elements as Inr or Y patch, supporting correct DNA-protein interaction during Polymerase II preinitiation complex formation [85,90,96].

The fraction of Y-Patches in plant promoters with distribution peaks around TSS is directionally oriented and methylation sensitive [96]. Moreover, plant promoters with Y-Patch and Inr sequences are also generally stronger than those lacking them, suggesting their putative function in a proper organization of Polymerase II preinitiation complex [85]. Some elements of core promoters observed in humans and *D. melanogaster* were not found in *A. thaliana*, such as the Sp1 binding sites or CpG and BRE elements, referring to putative specificity of DNA-protein interactions between plants and animals [96, 98].

The putative functional role of these differences in DNA sequences could be explained by X-ray crystallography studies providing details of DNA-protein interactions in both types of core promoters.

Putatively, the transcription initiation in these promoters is controlled by specific and abundant *cis*-regulatory elements as well as the open nucleosomal status.

Probably plants GA elements play a role similar to mammalian CpG islands, however GA lements were not methylated [101]. Until now, the significance of presented differences in context of a proper transcription initiation was not studied experimentally.

III. Lines 183-185 it is not appropriate to contrast a previous study with the same study.

It is corrected to not contrast results of the same study. The fragment was corrected as follows:

The association of TATA-box promoters with conditional, regulated gene expression was recently tested [103]. The Coreless promoters indicated a bias towards uniform expression regulation in dicots but not in monocots. A comparison of different plant species found they tended to demonstrate similar expression patterns for a particular promoter. However, the orthologs of the uniformly-expressed genes could be found more easily than those of conditional genes. Moreover, none of the screened core promoter types was consistently associated with changes in gene expression patterns. Therefore, a correlation only occurs between the promoter architecture and expression parameters [103].

IV. Line 214 More significantly?

These words were exchanged by the word “Furthermore”. The sentence was corrected as follows: Furthermore, 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].

Moreover, following fragment was added to the final part of section 2.2 to explain more mechanistically the functional role of trans-factor complexes organized within proximal promoter:

Transcription factors complexes organized within proximal promoter interacts with Mediator subunits looped towards the RNA Polymerase II preinitiation complex, to affect its activity, represented by gene transcription rate [34]. Among examples of such interactions is MED25 subunit physically associating with the basic helix-loop-helix transcription factor MYC2 in promoter regions of MYC2 target genes to positively affects the MYC2-regulated gene expression. However, the binding of MED25 to the basic Leu zipper transcription factor ABA-INSENSITIVE5 (ABI5) in promoter regions of ABI5 target genes shows a negative effect on ABI5-regulated gene transcription [120]. Moreover, MED18 subunit interacts with SUPPRESSOR OF FRIGIDA4 and ABA INSENSITIVE 4 to regulate flowering time and abscisic acid responses, respectively [121].

V. Lines 245-260 only describe previous studies without trying to give a scientific explanation.

Following fragment was added to give scientific explanation:

Therefore, the activation of enhancer and maintenance of its active state is a coordinated, multistep process, precisely regulated through the recruitment of particular proteins, that are sensitive to molecular signals, modifying their performance. Therefore, the activity of Mediator subunits, affecting the functions of the entire enhancer is regulated by crucial plant phytohormones. Amont them are jasmonie acid (JA) regulating MED25, abscisic acid (ABA) controlling the MED18 or general plant defence processes affecting MED21, MED19a and CKD8 activity [34].

5. Orthogonal expression systems

Line 572-577 cite the developer of the new technique

Citation [28] was added .

6. Support for machine learning and deep learning for synthetic promoter preparation

Line 688, so many appointments are necessary

Two references among five were removed.

7. Discussion

Lines 725-726 If there are already several studies on promoter synthesis, how is this review different? Just the year of completion? I think this is the opportunity to justify the reason for the revision.

Following sentence was added to explain putative differences: Therefore, presentation of the novel research results, combined with individual perspective, could add interesting features to presented studies.

Lines 726-727 For what purpose?

Following fragment was added to explain better the purposes of synthetic promoter development:

Presented processes increase demand for genetic modification of plant’s complex metabolic routes, which could be supported by synthetic promoters, enabling precise spatio-temporal regulation of multiple transgenes [153-155,178-180].

New line 730? In what sense. Could it be with outstanding agronomic characteristics?

Yes. The sentence was modified as follows: Plant genetic modification by synthetic promoters is pivotal to introducing metabolic switches, and for enabling the development of novel, more efficient, or stress-resistant crop variants of outstanding characteristics and increased biosynthesis rate of valuable metabolites [1,4,5,9,10].

In general, a good section addressed what was raised in the review. However, the pre-conclusion is reached that although the topic of the synthesis of synthetic promoters has advanced excessively in recent years, we still do not fully understand the phenomenon. This is indicated at the beginning of the review, therefore the contribution of this review is not seen.

Discussion was rewritten to show achievements in the field.

8. Conclusions and future directions

With what purpose?

Section was rewritten to show purposes of research.

The manuscript addresses a current topic of global interest. However, gathering information from a large number of previous studies does not produce a higher quality review than one that contains few references. The quality of the reviews is based on trying to explain with scientific support the why of the phenomena that are studied? (clearly based on the literature). The manuscript cannot be accepted in its current form, minor corrections must be addressed.