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Original article

Developing specific leaf promoters tools for genetic use in transgenic plants towards food security

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ABSTRACT

Significant yields enrichments are necessitated for meeting the rapid global growth population together with the expected demanding for food, particularly major crops. Photosynthesis improvement is an unexploited opportunity in research on improving crop yields. However, the lack of sufficient molecular promoters tools leads to the need to explore and analyze native leaf-specified promoters for manipulating photosynthesis activities in plants. Two *B. distachyon* promoters, sedoheptulose-1, 7-bisphosphatase (SBPase) and fructose-1, 6-bisphosphate aldolase (FBPA), were isolated and cloned into an expression vector upstream of the eYFP reporter gene. The results demonstrate that both promoters actively function in *N. benthamiana* leaves in both agro-transiently assays, successfully regulating expression specifically to leaf-tissues. Exploring these active promoters could potentially provide new well genetic tools for any transgene expression in plants or leaves to genetically manipulate photosynthesis for yield improvement.

1. Introduction

Demand for foods has risen with the rising global population, and new native biotechnological tools and practices must be developed for major crop improvement, including rice and wheat. Wheat is one of the foremost valuable agricultural food crops, providing about 20% of the consumed calories in a day (Braun et al., 2010). An increase in yields of around 70% would globally be required by 2050 to meet the increased demand population; conversely, its yields have recently been shown as stagnated (Tilman et al., 2011; Ray et al., 2013; Godfray, 2014).

Such gene manipulation studies have been employed in wheat (Driever et al. 2017), Arabidopsis (Simkin et al., 2017), where a sufficient increase in photosynthesis and biomass yields was observed. However, the simultaneous over-expression of multiple genes is more problematic in wheat because it lacks a well-defined molecular toolbox, such as sufficient promoters, which is critical in molecular plants to select the appropriate promoters for efficient gene transformation (Dale et al., 2002; Chen et al., 2006; Peremarti et al., 2010).

Constitutive promoters are typically used in plant studies to express targeted genes. Several constitutive promoters have been identified in viruses, including the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985) and the figwort mosaic virus (FMV) promoter (Richins et al., 1987; Snowden et al., 2005). Constitutive over-expression of transgenes can probably result in gene silencing due to co-suppression (De Borne Dorlhac et al., 1994; Simkin et al., 2004) or, as an alternative, have a poor effect on plant improvement because of ectopic expression of the brought gene. Thus, identification and characterization of native capable promoters would be valuable to direct transgene expression in specific tissues. Previously, many studies have identified some promoters, such as light-regulated and leaf-specific *Solanum tuberosum ST-LS1* promoter, various Arabidopsis and tobacco photosynthetic specific tissue promoters (Dupree et al., 1991; Xu et al., 2008), tomato fruit promoters (Kuntz et al., 1998; Simkin et al., 2007), guard cell specific promoters (Muller-Rober et al., 1995; Kelly et al., 2013) and specific promoters to seed endosperm (Baumlein et al., 1992; Thilmony et al., 2014). However, there has particularly been a shortage of promoters for wheat where the expression is restricted to green tissues.

Recent studies in wheat have frequently used maize ubiquitin 1 (Christensen et al., 1992) and rice actin 1 constitutive promoters (Mcelroy et al., 1990) to direct transgenic expression. Furthermore, the semi-constitutive rice tungro virus promoter

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(Bhattacharyyapakrasi et al., 1993; Mathur & Dasgupta, 2007) was utilized to express genes in the wheat crop (Driever et al., 2017). Additionally, the rubisco small subunit (*RbcS*) promoter from wheat more recently has shown to have sufficient capability to direct transgene expression in young wheat embryos and also in the leaves of tobacco plants in transient assays (Mukherjee et al., 2015), whereas further studies are required to validate this promoter capability in leaf tissues in stable transgenic wheat crops. The rubisco small subunit promoters from rice and maize plants are insufficient in transgenic wheat to down-regulate the expression of genes (Sparks et al., 2001). Moreover, additional capable promoters would be needed if multigene approaches were to be undertaken to avoid any undesirable influence on the transgenic genes stability and expression (Peremarti et al., 2010). Therefore, a robust toolkit that includes several well-characterized and effective promoters must be established for use in dicot and monocot plant manipulation studies. The aim of this research is to test and analyze new promoters for driving gene expression in *Nicotiana benthamiana* leaves. The genes encoding the Calvin–Benson cycle enzymes, sedoheptulose 1,7-bisphosphatase (SBPase) and fructose 1,6-bisphosphate aldolase (FBPA), are both known to be expressed in plants' green tissues (Uematsu et al., 2012 and Driever et al., 2017). Both gene sequences were chosen from the monocot plant *Brachypodium distachyon* (*B. distachyon*) because of the availability of its annotated genome (Draper et al., 2001).

2. Materials and methods

2.1. Bioinformatics analysis

To compare the *B. distachyon* SBPase promoter (Gene ID: Bradi2g55150) and FBPA promoter (Gene ID: Bradi4g24367), the sequences of both promoters were obtained from a region 2 kb upstream of the particular start-codons (taken from Phytozome database: https://phytozome.jgi.doe.gov/pz/portal.html). The isolated promoter sequences were analysed using PlantCare software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify and characterise the elements present in the promoters.

2.2. Construct generation for transient expression analysis in N. benthamiana leaves

To isolate the DNA fragments of the SBPase promoter and FBPA promoter from the *B. distachyon* genome, particular primers (Table 1) were designed from a section 2 kb upstream of the particular start-codons. The subsequent amplified 2 kb products were then cloned into pENTR/D according to the manufacturer's instructions (Invitrogen, Paisley, UK). The full-length 2 kb sequence of the promoter was introduced into the pGWB40 gateway vector (Nakagawa et al., 2007) by recombining to make the expression clones of pSBPase::eYFP and pFBPA::eYFP.

Table 1
Primers used in this study.

Primer	Sequence
pSBPase fwd	CACC TCGACGTCCATATGGCCCA
pSBPase rev.	TGCTGCGATGCGAGCTGC
pFBPA fwd	CACC TCATTGGACGTGTTGATGTGC
pFBPA rev.	TGTTTCTGGCTCCAAAGG

2.3. Agrobacterium-mediated transient expression in nicotiana benthamiana leaves

Transient expression was carried out using 4- to 5-week-old *N. benthamiana* leaves as per (Goodin et al., 2008). *Agrobacterium tumefaciens* cultures (strain GV1301) carrying the promoter::eYFP binary vectors and silencing suppressor (P19) plasmids were incubated at 28 °C overnight in Luria Broth media with the appropriate selection antibiotics. Cultures were centrifuged at 4500 *g* for 15 min at room temperature and re-suspended gently in an infiltration buffer (5 mM MES, 5 mM $MgSO_4$, pH 5.7, 100 mM acetosyringone) to an optical density of OD_{600} = 0.6. Then, suspensions of *A. tumefaciens* carrying the binary plasmids that included the *eYFP* gene were mixed, before infiltration, in a ratio of 1:1 with the *A. tumefaciens* suspension harboring the viral P19 in the binary plasmid pBIN19-p19 (Lakatos et al., 2004). The final mixtures of *A. tumefaciens* cells were infiltrated into the underside of two leaves from *N. benthamiana* plants. For comparison, controlled leaves were infiltrated with *A. tumefaciens* carrying only the P19 binary plasmid. Treated infiltrated plants were accommodated in a 24 °C growth-room (12 h/12 h light–dark cycle) for three days (Wydro et al., 2006).

2.4. Plant growth conditions

N. benthamiana seeds were germinated, and seedlings were grown in compost (Levington F2S, Fisons, Ipswich, UK) in a climate-controlled room for six weeks (22 °C, 12 h photoperiod). All plants were regularly watered and relocated to minimize spatial variations of growth conditions.

2.5. Microscopy observation

To test the functions of both promoters to drive fusion eYFP gene expression, infiltrated plant leaves were imaged after three days of infiltration, operating a confocal microscope with a 63X oil immersion objective as per Wei and Wang (2008). YFP was excited at 488–514 nm, and the emitted light was captured at 525–650 nm. Images were digitally captured and processed using Leica LCS software.

3. Results

For *in vivo* examining the promoters expression of the Calvin–Benson cycle gene to drive expression in the leaves of *Nicotiana benthamiana*, the two *Brachypodium* promoters 2 kb region upstream of the particular start-codons the genes' SBPase and FBPaldolase were utilized in the chimeric design of expression eYFP clones.

3.1. Analysis of B. distachyon SBPase and FBPA promoters

Towards identifying the promoter sequences from the *B. distachyon* SBPase and FBPA genes, the phytozome databank was operated. A region of two-kilobase upstream of the start codon (ATG) of both genes was separated. The isolated promoter sequences were analyzed using PlantCare software for identification and characterization purposes of the regulatory elements present in the promoters. Therefore, both promoters include and share some functional motifs such as Box G, I box and TATA box (Table2).

3.2. Gene constructions for transient expression analysis

To build the clone constructs for analysing transient expression in *N. benthamiana* leaves, regions upstream of the start codon of

Table 2
Identifications of motifs boxes in *B. distachyon* SBPase & FBPA promoters using PlantCare software.

Motifs name	Sequence	Function
Box I	TTTCAAA CCACGTAA	Light responsive element
G-box	CACATGG TACGTG TTTTA	*cis*-acting regulatory element involved in light responsiveness
TATA-box	TATAA, TATA tcTATAAAta TACAAAA	Core promoter element around −30 of transcription start

both isolated SBPase and FBPA promoter's genes were amplified and cloned intor the binary vector pGWB40 starting from introduction to pENTR/D to the expression gateway vector (eYFP) (Fig. 1). To confirm the success of cloning procedures, colony PCRs analyse were confirmed the success of cloned constructs of SBPase::eYFP and FBPA::eYFP into *Agrobacterium* cells (Fig. 2b and c).

3.3. Transient expression analysis in Nicotiana benthamiana leaves

For function and subcellular examinations of the eYFP fusion gene driven by the 2 kb promoters of *Brachypodium* SBPase and FBPaldolase in the leaves of *N. benthamiana*, transient transformations have been efficiently performed in leaf tissues with the constructs through agro-infiltration assays. Consequently, as shown in Fig. 3, the eYFP signals driven by the SBPase and FBPA promoters were clearly detected in the mesophyll cells compared to both the WT and the infiltrated leaves with p19 plasmid only-with no eYFP signals.

4. Discussion

The predictable demands for food worldwide need significant yield enhancement and different genetic approaches to improve leaf photosynthesis in crops. Yet there is a shortage of some molecular tools, such as promoters, that have efficient capability to direct strong expression levels in mesophyll cells in plants. Hence, there is an urgent need to continue the search, exploration and design of new native genetic tools, such as promoters with the preferred characteristics, to sufficiently direct the desirable expression level,

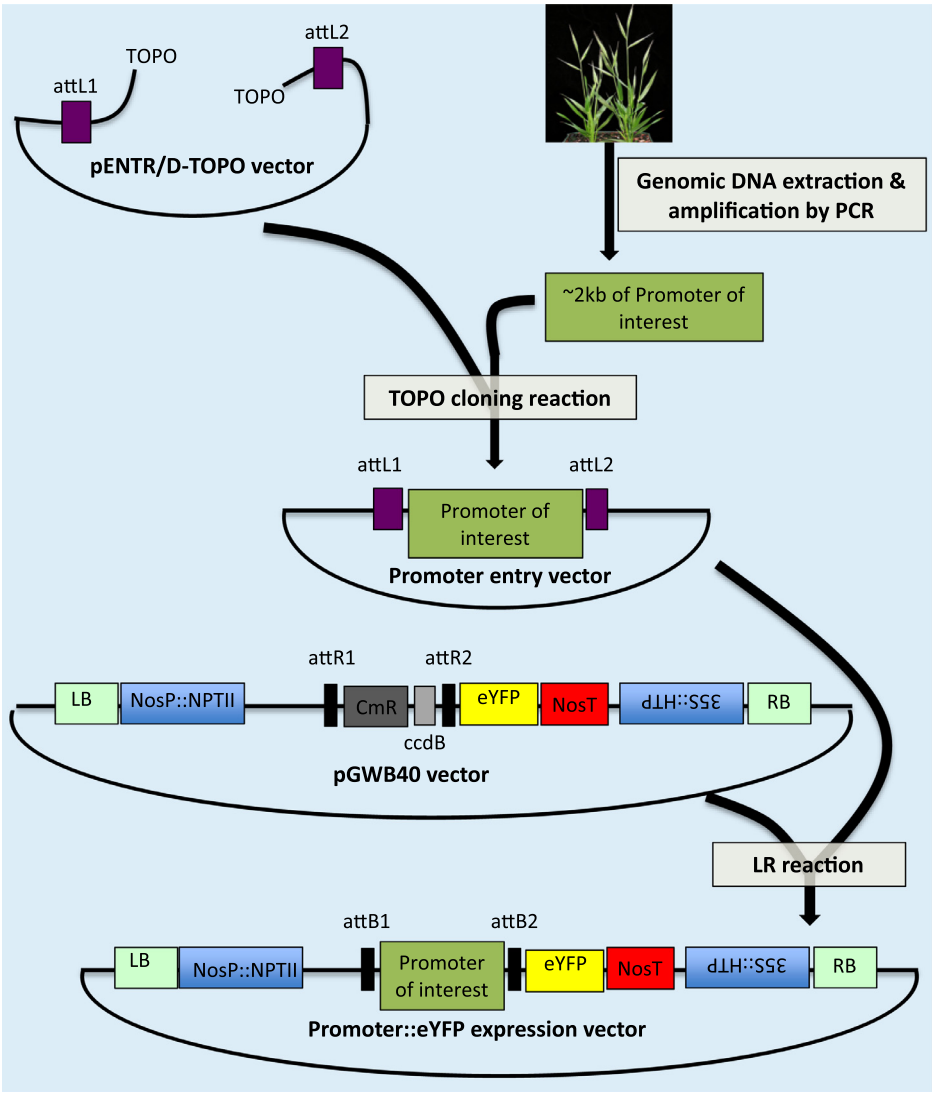

Fig. 1. Assembling transcriptional fusions of *B. distachyon* promoters map; for analysing the enhanced yellow florescence protein (eYFP) to drive gene expression into N. *benthamiana* leaves.

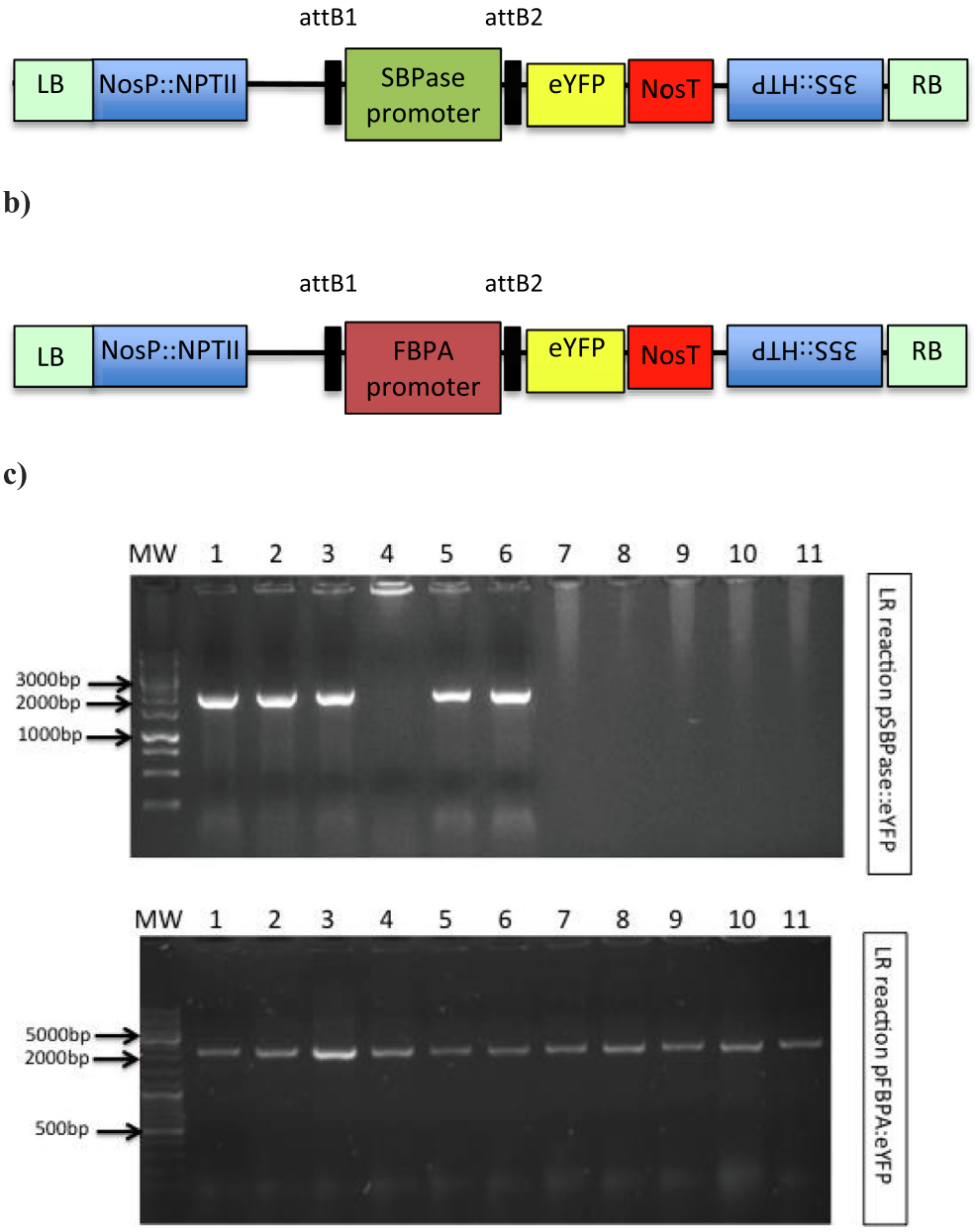

Fig. 2. Analysing and verifying transcriptional fusions of *B. distachyon* SBPase and FBPA::eYFP constructs. The promoter fragment was cloned into pENTR/D-TOPO vector before sub-cloned into pGWB40 vector (eYFP) (a) pSBPase and (b) FBPA. Colony PCR of LR reactions for SBPase::eYFP and FBPA::eYFP constructs into *Agrobacterium cells* (c).

particularly in plant mesophyll cells (Tao et al., 2002; Abdul et al., 2010; Park et al., 2010). Both enzymes of the Calvin-Bensen cycle SBPase and FBPA are detected as significant targets demonstrating high flux control throughout leaf photosynthesis. Some studies have confirmed their location in the leaf tissues of plants also to the high expression of their proteins in chloroplasts (Raines et al., 1999; Lefebvre et al., 2005). For that reason, opportunities for their promoters to drive robust expression, in particular in mesophyll cells, would be high.

Several characterized promoters have widely been obtainable recently. For instance, the constitutive promoter CaMV 35S that originated from the cauliflower mosaic virus (Odell et al., 1985) has been frequently exploited to facilitate a high level of expression in transgenic dicotyledonous plants (Battraw and Hall, 1990; Benfey et al., 1990). However, low activities have also been observed in monocotyledonous crops. Moreover, various promoters facilitate effective levels of genetic expression in monocots, as *Ubi*l from maize (*Zea mays*) and some rice-originated promoters: *Act*1, OsCc1, RUBQ1, and *Rbc*S. The promoters *Ubi*l and rice *Act*1 are mostly employed in monocot crops because of their efficiency in directing strong expression in just about all tissues of plants, while their activity is restricted in undeveloped tissues only (Jang et al., 2002; Park et al., 2010). In this study, conversely, both genes (SBPase and FBPA) were preferred from the *B. distachyon* due to its annotated genome being available (Draper et al., 2001). Hence, bioinformatics analysis revealed that targeted promoters share the similar some motifs such as Box G, I box and TATA box (Table2). The two G and I cis-elements motifs have significantly been known to function effectively in light regulation of gene expression as well as inducing the gene expression to which that comparable to the driven expression that by CaMV35S and *RbcS* promoters (Giuliano et al., 1988; Donald and Cashmore, 1990; Song et al., 2000; Mukherjee et al, 2015).

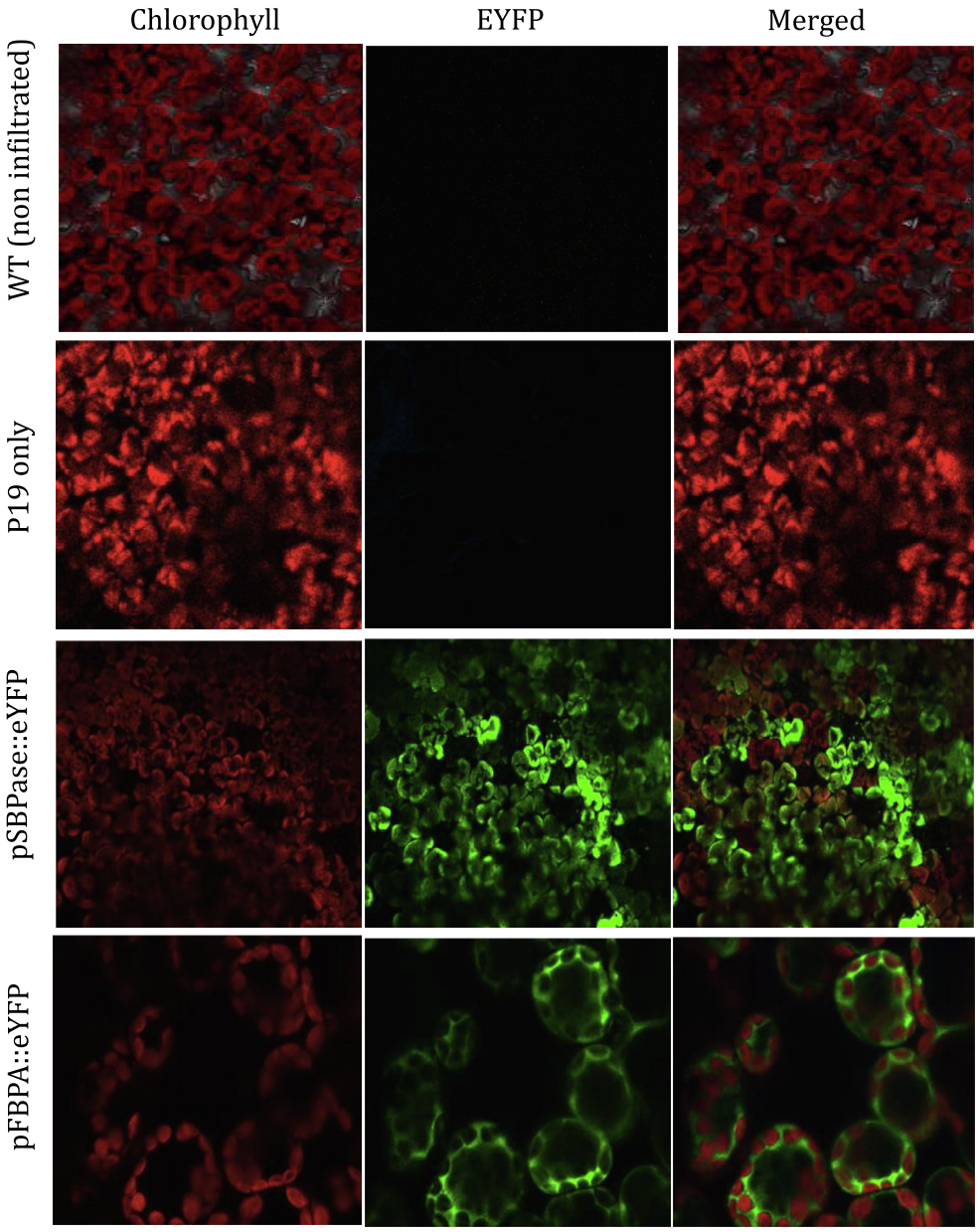

Fig. 3. Confocal Fluorescent images of eYFP-agro-infiltrated leaves of N. *benthamiana* plants.

To test the functionality of these two promoters in dicot plants, constructs were built up with the eYFP fusion gene, and agro-transiently were transformed into *N. benthamiana* leaves. Consequently, the subcellular localization of the eYFP expression signals was detected and observed by confocal microscopy directed by each SBPase or FBPaldolase promoter in eYFP-agro-infiltrated leaves. Interestingly, the eYFP signals were clearly observed, particularly in the cytosolic mesophyll cells of the *N. benthamiana* leaves in comparison to the WT and P19-agrotransformed leaves. Taking into account the different fusion genes, all is similar to the results explained in Mukherjee *et al.*, in which the rubisco small subunit gene promoter was isolated from wheat and hence, drove excellent GUS expression in the green tobacco tissues, beyond roots, conversely, its activity was not studied through various stages of growth (Mukherjee et al., 2015). Moreover, the obtained results agree with Alotaibi et al.'s (2018 and 2019) studies, as the same promoters of both the *B. distachyon* SBPase and FBPA genes were efficient in facilitating GUS transgene expression in wheat leaves. In conclusion, these two *Brachypodium* promoters of the SBPase and FBPA enzymes were validated as robust genetic tools of choice in plant genetic engineering towards guiding the expression of the transgenic of interest, specifically in mesophyll cells. The function of these tested promoters needs to be studied further under various stresses and conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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