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Research Article
Functional Analysis of SBPase Gene Promoter in Transgenic Wheat under Abiotic Stresses

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Abstract

Background and Objective: This study focused on evaluation of SBPase promoter in driving transgenes under drought, high light intensity and salinity as major abiotic stresses limiting plant growth and production in various areas of the world. Wheat production has remained constant despite the rapidly growing population around the world. **Materials and Methods:** This had made it necessary to develop other varieties with higher yield in order to satisfy the future demand. When elite wheat varieties' photosynthesis is genetically manipulated, it gives a high possibility for yield increase. Lack of a properly defined molecular tool-box promoter for driving gene expression for manipulation of photosynthesis, hinders the progression of this field. Gus histochemical staining and quantitative PCR were used to examine the function of *Brachypodium distachyon* sedoheptulose-1, 7-bisphosphatase (SBPase) promoter to drive the expression of GUS in the stably transformed wheat plants under various stresses conditions. **Results:** Results revealed that *B. distachyon* SBPase promoter fully drive the GUS expression in the stable wheat transformants (cv. Cadenza) in the leaf tissues under normal and high light conditions. It also drove GUS gene expression under drought and salinity stress conditions. In addition, the promoter responds to light/dark/light alternation as light responsive genes. **Conclusion:** The *Bd* SBPase promoter provides innovative molecular tool to drive genes in wheat leaves under natural, drought, high light intensity and salinity conditions as well as to allow for multigene photosynthetic manipulation for yield increase under various abiotic conditions.

Key words: SBPase promoter, tissue specific, photosynthesis, reporter gene, gus expression

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Wheat is one of the main staple crops that provides more than 20% of the daily ingested calories[1]. Recently, wheat production has stagnated, whereas it is estimated that an increase of 70% of global wheat supply shall be required by the year 2050 in order to meet the demands of the rising global population[2-4]. Developing higher yield varieties has become necessary to meet the predicted global rising in food demand[4,5]. This has also been exacerbated by global climate change where the average global temperature has increased by 1.8°C since 1880 and the level of carbon dioxide is expected to rise from 408-550 ppm over the next 40 years[6,7]. The global climate changes increase the impact of abiotic stresses, drought, high light intensity, salinity, on plants, but producing crop varieties tolerant to these stresses could significantly increase crop yield in the affected areas[8].

Genetic manipulations involving photorespiration[9,10], Calvin-Benson cycle[11-15] and electron transport[16,17] showed significant increase in yield. In addition, multigene stacking approach for improving yield in tobacco[14] and Arabidopsis[17] was investigated. In wheat, over-expression of multiple genes is not well established due to the lack of a well-defined molecular toolbox. When constitutive promoters, such as figwort mosaic virus (FMV)[18] and cauliflower mosaic virus[19] (CaMV) 35S were used in driving transgenes in plants resulted in gene silencing through co-suppression[20] or ectopic expression[21]. This led to the identification and characterization of plant tissue specific promoters. This included leaf-specific and light-regulated *S. tuberosum* ST-LS1[21], tomato fruit specific promoters[22], seed endosperm specific promoters[23-27], guard cell specific promoters[28] and a variety of tobacco and Arabidopsis photosynthetic tissue specific promoters[15]. Currently, rice actin 1 and the maize ubiquitin 1 constitutive promoters are frequently used to direct transgene expression in wheat[29,30]. In addition, genes within the aerial regions of wheat plants are expressed by semi-constitutive rice tungro virus[31,32]. Recently, the Rubisco gene promoter has successfully been employed in transient expression tests to direct expression in tobacco leaves and immature wheat embryos[33]. Sparks *et al.*[34] carried out a research in maize and rice using wheat rubisco small subunit promoters that aimed at down-regulating gene expression in photo respiration. Using multigene approach to enhance a multigene character requires additional promoters because using repetitious components in transgenic constructs negatively affect the expression and stability of introduced genes[35]. Therefore, it is very important to establish a robust tool-kit of several promoters for future studies to facilitate multigene modification of wheat traits as well as its response to various biotic and abiotic stresses.

Promoters from dicotyledons have been used in wheat, but their use in leaf tissue has been unsuccessful[34]. The availability of *Brachypodium distachyon* annotated genome and its closeness to wheat led researches to use its promoters[36,37]. Recently, it was clearly demonstrated that SBPase and the fructose 1,6-bisphosphate aldolase (FBPA) gene promoters from *B. distachyon* are fully functional in the green leaf tissue of transgenic wheat[38]. Although SBPase promoter was confirmed to drive gene expression in wheat leaf tissue, its function under abiotic stresses, such as drought, high light intensity and salinity has not been investigated.

This study focused on functional analysis of SBPase gene promoter from *B. distachyon* in transgenic wheat (T2) in leaf tissue under abiotic stresses including drought, various light intensity and salinity which represent a limiting factor for plant growth and production in various parts of the world. This could contribute to the application of transgene and exogenous promoter in wheat under stress conditions.

MATERIALS AND METHODS

Plant growth conditions: This study was carried out in the greenhouse at Taif University, Taif, Saudi Arabia during the period from October, 2017-July, 2018. Seeds of wild type (WT) and three independent T2 transgenic wheat lines (R2P2, R5P2, R9P2) were germinated in the greenhouse. Plantlets were randomly distributed into four groups each group contained three plants of each line (WT, R2P2, R5P2, R9P2). Plants of the control group were grown under normal greenhouse light (1900 μmol m^{-2} sec^{-1}) and watering (800 mL/week) conditions. For light treatment, plants were kept in the dark for 12, 24 and 3 h in the greenhouse condition after dark treatment, greenhouse light condition (1900 μmol m^{-2} sec^{-1}) or full light intensity outside of the greenhouse (4250 μmol m^{-2} sec^{-1}). In the full light outside of the green house, samples were collected at three time points at 9:30 am (light intensity 3290 μmol m^{-2} sec^{-1}), at 12:30 pm (light intensity 4970 μmol m^{-2} sec^{-1}) and at 3:30 (light intensity 2575μmol m^{-2} sec^{-1}). For drought treatment, plants were supplied with half water supply (400 mL/week) of the control group (800 mL/week). Watering was adjusted to keep plants alive during drought treatment. For salinity treatment, plants were treated with 250 mM NaCl for 2 weeks. Locations of all wheat plants were rotated in regular to minimize the variation of the growth conditions.

DNA isolation: Flag leaves of the main stem of the plants were lyophilized at -58°C for 36 h, ground to fine powder and used for DNA extraction. DNA was extracted from the control or treated plants using CTAB method[39] after optimization of the protocol for lyophilized ground tissues. Optimization included using 0.5 mL of warm (65°C) CTAB and 5 mg of lyophilized ground leaf powder. The DNA pellet was washed with 70% ethanol, air dried, dissolved in 50 µL DH2O and kept at -20°C until used.

Detection of SBPase-GUS construct: The transgenic plants carrying the pRRes:pSBPase:: GUS construct was confirmed by detection of either the SBPase promoter construct or GUS reporter gene sequence using specific primers (Table 1). The amplification reactions included 30 cycles of 1 min at 94°C, 30 sec at 57°C and 45 sec at 72°C. The PCR reactions were carried in 25 µL containing 12.5 µL of 2X Master mix (Promega, Wisconsin, USA), 10 ng of genomic DNA, 10 pmole of each forward and reverse primers. Amplified PCR products were run and separated on 1% agarose gel.

RNA isolation, cDNA synthesis and qPCR: Flag leaf tissues (0.2 g fresh weight) were collected and ground on dry ice. Then, the total RNA was isolated using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Fisher Scientific, Loughborough, UK). The cDNAs were synthesized using the Fermentas Revert Aid Reverse Transcriptase kit as per manufacturer's instructions (Fermentas Life Sciences, Paisley, UK). Expression of the GUS gene was determined by qPCR. The reactions of qPCR were carried out using SensiFast SYBR No-ROX mix (Bioline Reagents Ltd., London, UK) as stated by manufacturer using the primers in Table 1. The reactions included 45 cycles of 5 sec at 94°C, 10 sec at 60°C and 5 sec at 72°C and determined from three different technical reps per lines. Fold of expressions were determined as stated by Pfaffl[40].

Table 1: Sequence and information of primers used in this study

Primers	Sequence 5'→3'
PCR-pSBPase-F	TCGACGTCCATATGGCCCA
PCR-pSBPase-R	TGCTGCGATGCGAGCTGC
PCR-GUS-F	ACTACGGGAAAGGACTGGAA
PCR-GUS-R	GTCACAACCGAGATGTCCTC
qPCR-GUS-F	GTCATCCTCTGGGAACCACT
qPCR-GUS-R	CGAACGGCTCTTCATAGACA

Histochemical gus assays: Histochemical activity of GUS gene *in situ* was analyzed and assayed as explained in[41]. Tissues from the wild type plants (WT) were used as controls[38].

Statistical analysis: All data in this study are acquired at least from three independent biological replicates. All statistical analyses were done using one-way variance (ANOVA) with a post hoc test at $p \leq 0.5$ significance level.

RESULTS

Detection of pSBPase-GUS construct in wheat T2 plants: The presence of the recombinant plasmid pRRes:pSBPase::GUS (Fig. 1a) was confirmed by PCR amplification. Most tested plants were identified positive and carrying the construct as they amplified DNA diagnostic fragment of approximately 2 kb which indicated the presence of the SBPase promoter of the recombinant plasmids pRRes:pSBPase::GUS compared to the WT line with no detected bands (Fig. 1b). The presence of the GUS gene was also confirmed by the detection of the 500 bp fragments (Fig. 1c). Detection of both fragments confirmed the presence and integrity of the pSBPase::GUS construct.

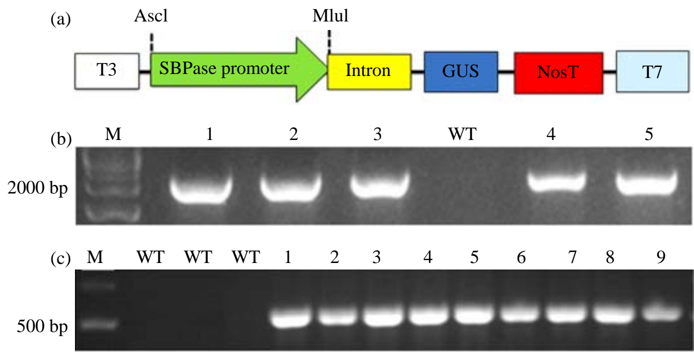


Fig. 1(a-c): Detection of SBPase promoter and GUS gene using PCR into transgenic T2 wheat (a) pSBPase::GUS construct used for biolistic transformation of transgenic lines, (b) PCR detection of SBPase promoter sequences in T2 plants, 1-3, 5-6: Representative transgenic plants and (c) PCR detection of GUS gene in T2 transgenic wheat plants 1-9: representative of T2 transgenic plants. M: DNA Marker, WT: Wild type

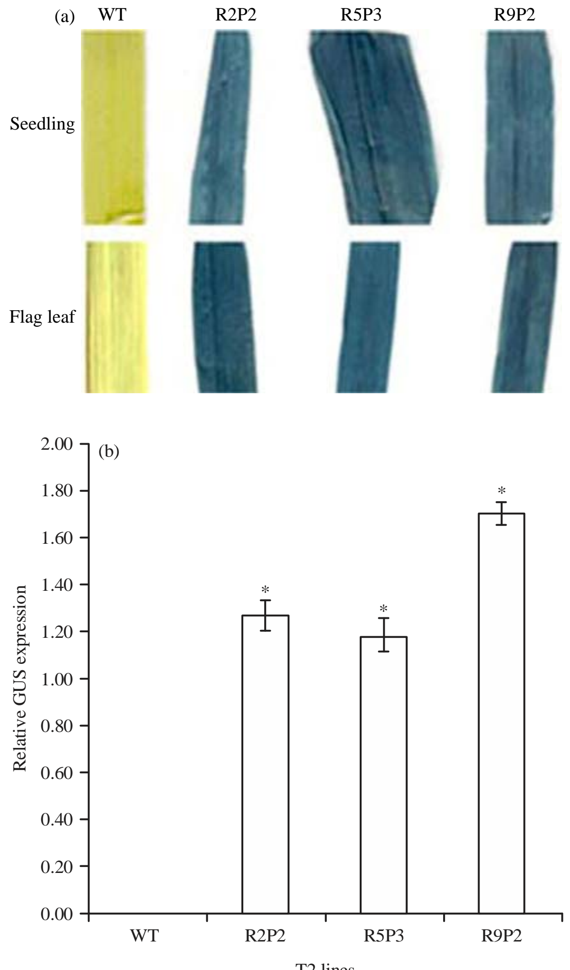


Fig. 2(a-b): (a) Histochemical staining of GUS and (b) Quantitative PCR estimation of GUS gene expression in WT and transgenic lines under normal conditions

Analysis of stable GUS expression: Transgenic wheat plants carrying the pRRes:pSBPase::GUS construct were used to validate and test the function of SBPase promoter and its suitability to express transgenes in wheat under different abiotic stresses. Analysis included WT, R2P2, R5P3 and R9P2 independent SBPase promoter transgenic lines (Fig. 2a). The WT samples showed no staining as expected, whereas, leaf stripes from transgenic lines R2P2, R5P3, R9P2 exhibited intense GUS staining. No difference in the intensity of the blue coloration was observed in the two leaf growth stages indicating that the SBPase promoter is functioning under normal growth conditions. Quantitatively, qPCR results showed that GUS was expressed in all lines with little variations among the three transgenic lines. The R9P2 showed the highest GUS expression compared to R2P2 and R5P3,

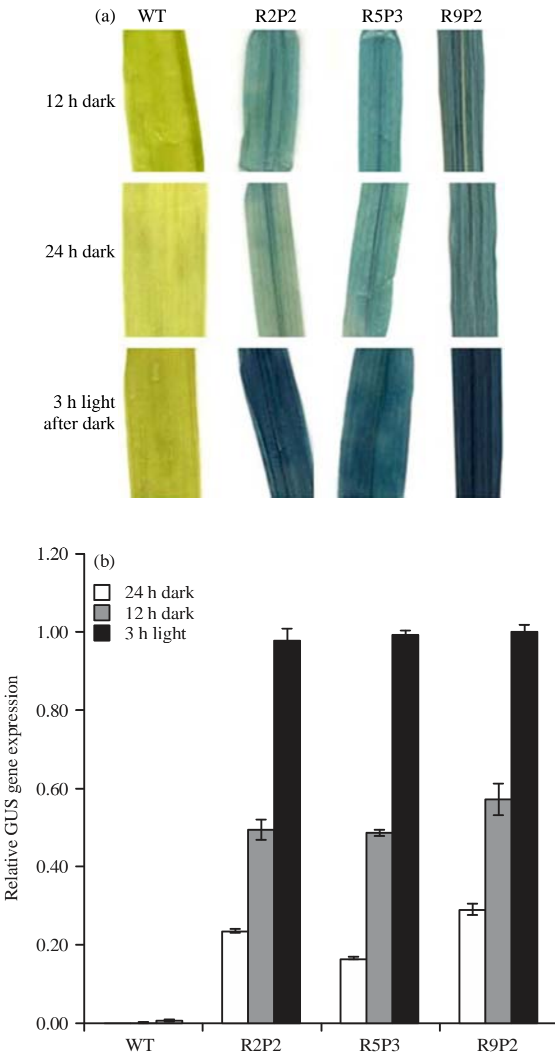
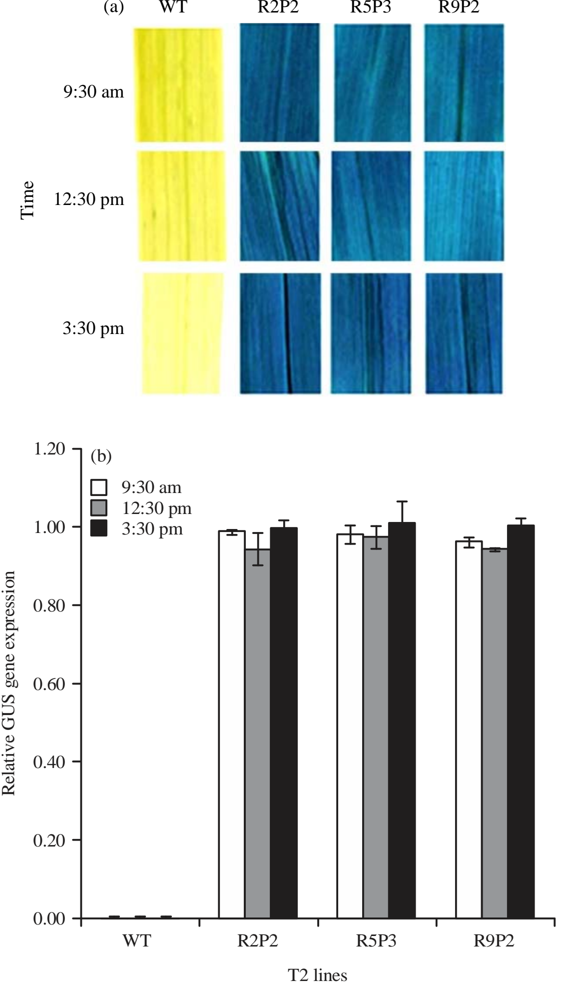


Fig. 3(a-b): (a) Histochemical staining of GUS and (b) Quantitative estimation of GUS expression in WT and transgenic wheat lines in response to dark (12 and 24 h)/light after dark treatment

which showed very close level of GUS expression. The WT plants showed no GUS expression as anticipated (Fig. 2b).

Functional analysis of pSBPase-GUS in light/dark: Light intensity could have an impact on gene expression and may be associated with heat stress. Under dark/ light conditions, transgenic wheat plants (T2) carrying the pRRes:pSBPase::GUS construct grown in the dark for 12 and 24 h showed drastic decrease in pSBPase promoter activity indicated by the activity of GUS viewed by histochemical staining (Fig. 3a). The normal activity of the pSBPase promoter was re-constituted 3 h after plants were moved back to light. It seems that keeping plants



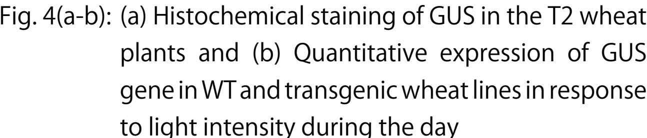
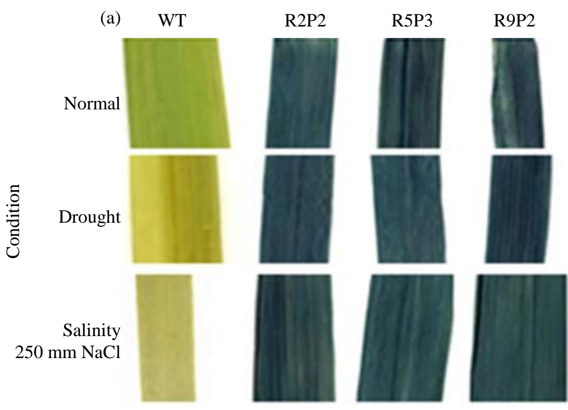


Fig. 4(a-b): (a) Histochemical staining of GUS in the T2 wheat plants and (b) Quantitative expression of GUS gene in WT and transgenic wheat lines in response to light intensity during the day



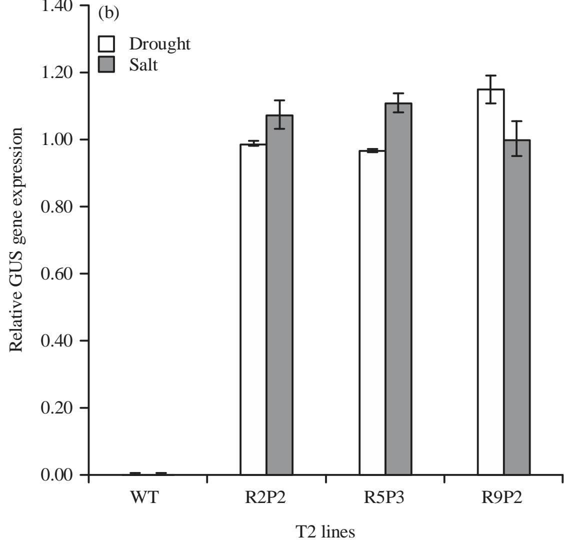


Fig. 5(a-b): (a) Histochemical staining of GUS in the T2 wheat plants and (b) Quantitative expression of GUS gene in WT and transgenic wheat lines in response to drought and salinity

in the dark for 12 or 24 h did not show a recognizable difference in the promoter activity. Interestingly, it can gain its function upon returning plants back to light (Fig. 3a). Quantitative expression of GUS gene using qPCR showed perfect association with GUS activity by histochemical staining (Fig. 3b).

Fluctuation in day light intensity does not affect pSBPase-GUS activity: Light intensity differs during the day being highest at the middle of the day and lower before and after that peak. At 9:30 am light intensity was 3290 μmol m^{-2} sec^{-1} at 12:30 intensity was 4970 μmol m^{-2} sec^{-1} and at 3:30 it was 2575 μmol m^{-2} sec^{-1}.

The impact of light intensity on the activity of the SBPase promoter by was investigated by estimation of GUS expression. Histochemical staining of leaf segments of the WT and the transgenic lines at the three time points (9:30 am, 12:30 pm, 3:30 pm) revealed that the tested promoter drove the expression of GUS gene at the same level indicating that the SBPase promoter is fully functional at various light intensities that ranged from 2575-4970 μmol m^{-2} sec^{-1} (Fig. 4a). Quantitative GUS expression showed very similar level of expression at the three light intensity levels during the day (Fig. 4b). This confirmed the ability of the SBPase promoter to drive GUS expression under a wide range of light intensity.

Activity of pSBPase-GUS under drought and salinity conditions: The SBPase promoter activity was estimated under drought and salinity stresses. The GUS staining of leaf stripes of WT and the transgenic lines showed that the promoter worked in a similar fashion to that of normal conditions. Exposure of plants to 250 mM NaCl (average salinity stress) or half water quantity supplied to the control group did not show an impact on GUS histochemical detection (Fig. 5a). Quantitative expression of GUS derived by the pSBPase promoter presented very close expression level under drought (half water quantity) as well as under 250 mM NaCl in the three transgenic lines (Fig. 5b).

DISCUSSION

Three transgenic lines named R2P2, R5P3 and R9P2 containing the pSBPase-GUS construct along with the wild type were employed in this study to evaluate the functionality of *B. distachyon* SBPase promoter in transgenic wheat lines under normal as well as abiotic stresses. A two-kilobase genomic region was isolated and characterized showing a number of the cis-acting regulatory elements implicated in light and stress-regulation[38]. The region was found to be located directly upstream of the ATG for the *B. distachyon* SBPase gene promoter. Very little information has been published on the regulation of Calvin-benson cycle genes in wheat. On the other hand, adequate literature that demonstrates the function of light in the regulation of various Calvin-benson cycle gene expressions has been published[42-46]. The SBPase promoter also contained regulatory light responsive with motifs that resemble these regulatory sequences[38], which had previously been identified in a number of plant species[26,47-52].

It has been difficult to achieve high levels of introduced transgene expression in the leaves of wheat plants. Attempts have been made using dicot promoters with little or no success being recorded. The promoters have either been ineffective or failing. The common promoters used in monocot plants are maize *Ubi*l and rice *Act*1 promoters and they exhibit efficient function to drive a constitutive expression in almost all plant tissues. However, their activity is shown to be only high in young plant tissues and has a tendency to decrease during mature stages of plant growth. Additionally, the activity of these promoters has not been examined under abiotic stresses[53,54]. Drought and salinity are common abiotic stresses on plants. A promoter optimized to drive exogenous gene expression is an advantage and essential molecular tool for the development and applications of plant biotechnology. Therefore, in this study, the *B. distachyon* SBPase promoter was examined in wheat leaves to drive GUS expression under natural as well as abiotic stresses, dark/light, drought and salinity.

The *B. distachyon* SBPase promoter showed high activity in wheat under normal environmental conditions. Also, the promoter responded to the transition from light/dark/light. In addition, it functioned at the same level in response to different day light intensities. Moreover, it drove the GUS expression under the main two types of abiotic stresses, drought and salinity. Therefore, the results indicated that the promoter has high potential to be used as driver of GUS (or other transgenes) gene expression in mesophyll tissues under natural conditions, drought, salinity and different light intensity stresses. However, it showed less activity under dark conditions. Differences in expression level were clearly detected in independent transgenic lines under 24 and 12 h dark environments. Alotaibi *et al.*[38] has established that *B. distachyon* promoter is normally active in wheat under normal conditions. This provides an indispensable tool for tissue-specific expression of transgenes and an alternative functional promoter for the currently used promoters for wheat transformation and biotechnology applications. Also, results obtained from the same study showed that the *B. distachyon* promoter was active in *N. benthamiana* before they are introduced into wheat[38]. This matches with latest results, which indicated the functionality of Rubisco wheat promoter in tobacco[33].

Variations in transcript levels were observed in individual independent T2 progenies in plants expressing GUS gene under the control of SBPase promoter and grown under dark as well as light conditions. The highest level of the introduced gene expression reached more than 50% of the GUS gene transcript in the plants grown in the dark for 24 h. This could be interpreted as the SBPase activity was increased in the light by more than 10-fold as a consequence of the thioredoxin system through light-modulated activation as it was reported before[55], therefore, the SBPase promoter is expected to be more active in the light compared to in the dark. As GUS staining offers a visual result that is convenient for tissue distribution and protein accumulation, under drought and salinity stresses, a strong positive blue coloration was observed in the flag leaf growth stage when the plants were grown under drought, salinity and different light intensities. This significantly indicated that the functionality of SBPase promoter to drive the GUS gene expression is intriguingly high despite of these stresses. This new features of SBPase promoter will contribute to biotechnology applications in wheat to drive transgene expression under abiotic stresses.

CONCLUSION

Results of this study conclude that the *B. distachyon* SBPase promoter was able to drive transgene expression in wheat leaves under normal, drought, high light intensity and salinity conditions. The SBPase promoter could be used in transgenic wheat production under abiotic stresses which can contribute to increasing wheat yield in areas challenged with such abiotic stresses.

SIGNIFICANCE STATEMENT

This study investigated the functionality of SBPase promoter in wheat leaf tissue under abiotic stresses including drought, high light intensity and salinity that can be beneficial for enhancing the efficiency of photosynthesis in wheat. This study will help researchers to employ the SBPase promoter in producing transgenic wheat to be planted in areas with similar abiotic stresses. With the new reported features of SBPase promoter, transgenes can be highly expressed under abiotic stresses to enhance wheat biotechnology application for high yield.

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