Evaluation of Drought Stress-Inducible *Wsi*18 Promoter in *Brachypodium distachyon*

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Abstract

The rice *Wsi*18 promoter confers drought-inducible gene expression. This property makes it a useful candidate to drive relevant genes for developing drought resistant traits for different monocot crops. In this study, we showed that the *Bradi2G*47700 gene, the closest homologue to rice *Wsi*18, was upregulated in *Brachypodium distachyon* plants exposed to ABA and mannitol. *Wsi*18: *uidA* transgenic *B. distachyon* plants were produced and then subjected to ABA or mannitol treatment. The expression of *uidA* in three transgenic lines (line 10, 18 and 37) was significantly upregulated in plants exposed to ABA (fold increases of 5.61 ± 0.98, 2.88 ± 0.75 and 9.13 ± 1.96, respectively) compared to the same transgenic plant lines without treatment. The expression of *uidA* in two transgenic lines (lines 18 and 37) also showed upregulation when treated with mannitol (fold increases of 4.43 ± 1.07 and 8.47 ± 2.90, respectively) compared to the same transgenic plant lines without mannitol treatment. Moreover, GUS histochemical assay showed increased *Wsi*18 promoter activity in the leaves and stems of transgenic lines upon treatment with ABA or mannitol. This is the first report of the drought inducible rice *Wsi*18 promoter being active in *B. distachyon* which is a model plant for molecular biology research of various monocot plants. Taken together, the results indicate that the *Wsi*18 promoter and its homologue may be explored as a useful tool for drought stress-inducible gene expression in different monocot crops.

Keywords

1. Introduction

Abiotic stresses are a seriously threats to crop production. Each year, more than 50% of the global major crop yield is lost due to abiotic stress, with water deficit stresses such as drought and high salinity being major contributors [1]. It is estimated that by the year 2050, the global demand for crop derived calories will have increased by 100%, and thus developing crop varieties with greater tolerance to abiotic stresses will be essential to meeting these needs [2].

The introduction of relevant genes to crops via genetic engineering is an important approach for developing stress tolerance for plants. Several constitutive promoters have been previously used for such purposes, such as *Ubi*1 from maize, *Act*1, *Rbcs* and *OsCc*1 from rice [3] [4] [5] [6]. However, transgenic plants with strong constitutive promoters often suffer undesirable phenotypes. For example, transgenic rice constitutively over-expressing *Ubi1: OsNAC*6 suffers from growth retardation and low reproductive yields [7]. Transgenic tobacco plants constitutively over-expressing 35*S: TPS*1 have greater drought tolerance, but suffer stunted growth [8]. This could be due to the cost of the resources needed to constantly overexpress the transgene, or negative interactions with normal cell metabolism [9]. Stress-inducible promoters offer the advantage of expressing the gene only under stress conditions. Thus, plants with stress-inducible promoters could eliminate undesirable phenotypes induced by constitutive transgene expression [10] [11].

Only a few drought inducible promoters have been identified in plants so far. *Wsi*18 is a drought-inducible rice gene from the group 3 late embryogenesis abundant (*LEA*3) family [12]. The promoter has been analyzed in transgenic rice [13] [14] [15] and under normal conditions, the basal expression of *Wsi*18 was low in transgenic rice tissues, but following drought, NaCl or ABA treatment, the expression of genes driven by *Wsi*18 was induced in the whole plant body [15]. Following drought conditions or exposure to ABA, *Wsi*18 had a level of transient expression comparable to that displayed by the strong constitutive promoter *Act*1 [16]. Despite the research conducted on *Wsi*18 in rice, its stable expression profile has never been studied in other plant species. Furthermore, it is not clear if the drought-inducible property can function in other monocot crops, and more importantly, be used to drive expression of suitable genes to develop drought tolerance in different monocot crops.

The family of Poaceae consists of grasses in three subfamilies, Ehrhartoideae, Panicoideae, and Pooideae. The Ehrhartoideae includes rice, the Panicoideae includes maize and sugar cane, and the Pooideae includes Purple false brome (*Brachypodium distachyon*) and the Triticeae tribe which includes many important crops such as wheat, rye, and barley [17]. Rice is distantly related to Pooideae and does not share some important agricultural traits with Pooideae members. Thus, rice is less suitable as research model plant for Poodeae members. *B. distachyon* is an emerging model for monocot plants and is more pertinent to many cereal crops [17].

In this study, we identified the *Bradi*2*G*47700 gene which is the closest homologue to the rice *Wsi*18 gene and found this gene was upregulated in *B. distachyon* plants upon exposure to ABA or mannitol. Furthermore, we show that the *Wsi*18 promoter drives high levels of *uidA* expression in transgenic *B. distachyon* plants under ABA and mannitol treatments. Our results demonstrate that the *Wsi*18 promoter has drought inducible activity in transgenic *B. distachyon* plants, which is a model research plant closely related to many important monocot crops, and that the *Wsi*18 promoter might be an effective promoter to drive relevant genes for drought resistance in different monocot crops.

2. Materials and Methods

2.1. Vector Construction

The *Wsi*18 promoter region was amplified by PCR using the pWsi18 plasmid as the template (obtained from Ju-Kon Kim at Seoul National University) and the primers of FWsi18 and RWsi18 (Table 1). The 2 × *CaMV*35*S* promoter region was amplified by PCR using the pMDC32 plasmid [18] and the primers of F2xCaMV35S and R2xCaMV35S with additional attB sites (Table 1). The PCR products of the *Wsi*18 promoter and the 2 × *CaMV*35*S* promoter were separately inserted into the pMDC163 plasmid using Gateway® Technology (Thermo Fisher Scientific) [19], generating the constructs of *Wsi*18:: *uidA* and 2 × *CaMV*35*S*:: *uidA* respectively. The *Wsi*18:: *uidA* and 2 × *CaMV*35*S*:: *uidA* plasmids were separately introduced into *Agrobacterium tumefaciens* strain AGL1 via electroporation.

Table 1. Primers employed in this study.

Primer name	Primer use	Primer sequence 5' → 3'
FWsi18	*Wsi*18 gateway cloning	GGGGACAAGTTTGTACAAAAAAGCAGGCT GGCTCTAGAGGATCCTGAGA
RWsi18		GGGGACCACTTTGTACAAGAAAGCTGGGTC CATGGCGCAAACTTGGCTG
F2xCaMV35S	2*xCaMV*35*S* gateway cloning	GGGGACAAGTTTGTACAAAAAAGCAGGCT CGACGGCCAGTGCCAAGC
R2xCaMV35S		GGGGACCACTTTGTACAAGAAAGCTGGGTC TCCTCTCCAAATGAAATGAAC TTC
FWsi18/GUS	PCR genotyping	CCCGTTTCTCTGTCTTTTGC
RWsi18/GUS		GACCCACACTTTGCCGTAAT
FGUSQ	RT-qPCR*uidA*cDNA	TACCGTACCTCGCATTACCC
RGUSQ		GAGGTTAAAGCCGACAGCAG
FBradi2G47700Q	RT-qPCR *Bradi*2*G*47700 cDNA	GGCAAGGACAAGACAGGAAG
RBradi2G47700Q		CCATTCCGATGGTGTTCATC
FSamDCQ	RT-qPCR *SamDC* cDNA	TGCTAATCTGCTCCAATGGC
RSamDCQ		GACGCAGCTGACCACCTAGA

2.2. Development of Transgenic *B. distachyon* Plants

Brachypodium distachyon ecotype Bd21 seeds were obtained from the United State Department of Agriculture (USDA). Seeds were germinated on medium containing 2.17 g/L Murashige & Skoog (MS) basal salt mixture (Murashige and Skoog, 1962) (Phyto Technology Laboratories), 10 g/L sucrose, and 12 g/L of the gelling agent Phytagel™ (Sigma-Aldrich) in double distilled water (ddH$_2$O). After germination, plants were grown in a growth room with a 20-hour photoperiod, light intensity of 172 μmol·m^{-2}·s^{-1} and temperature of 22˚C. *B. distachyon* callus induction and plant transformation were conducted according to the protocol of Alves *et al.* [20]. Briefly, immature seeds that had just begun desiccation were collected from plants. The embryo was removed from the seed, plated on solid MS medium and cultured in the dark at 25˚C. Once calli had developed, compact embryogenic calli (CEC) were separated from any soft wet friable calli, and transferred to fresh medium. CEC were then divided into smaller fragments and subjected to transformation by co-cultivation with *Agrobacterium* AGL1 containing the respective transformation vector. Calli infected by *Agrobacterium* were grown on MS medium containing 100 μg/ml hygromycin B (Sigma-Aldrich) to regenerate transgenic plants. Our kill curve study indicated that this level of hygromycin selection could essentially inhibit the growth of non-transgenic tissues. Shoots developed from selection medium were picked and transferred to fresh medium for plant development. T1 seeds (seeds from T0 plants) were transferred to MS medium containing 115 mg/L hygromycin B for germination.

DNA was extracted from plants developed from hygromycin selection. Leaves of T0 plants were flash-frozen in liquid nitrogen and homogenized with a Tissue Lyser II machine (Qiagen). The forward primer FWsi18/GUS, which binds to the *Wsi*18 promoter, and the reverse primer RWsi18/GUS, which binds to the *uidA* gene (Table 1), were used to amplify the *Wsi*18: *uidA.* GoTaq® Flexi DNA Polymerase (Promega) was used according to the product instructions for a PCR reaction with an annealing temperature of 60˚C and an extension time of 60 seconds. Transformed plants were expected to produce a 628 bp PCR product. To further confirm transformation, T1 seeds collected from T0 plants were germinated on seed germination medium containing 115 mg/L hygromycin B. Only the plants that were positive for PCR analysis and successfully germinated on 115 mg/L hygromycin medium were used to evaluate Wsi18 promoter expression.

2.3. ABA and Drought Stress Treatments

B. distachyon plant seeds were placed on MS medium in Petri dishes for germination. Successfully germinated seedlings were transferred to the same MS medium in Magenta™ GA-7 Plant Tissue Culture Boxes (Sigma-Aldrich). Plants were grown in the growth room with a 20 hour photoperiod, light intensity of 172 μmol·m^{-2}·s^{-1} and temperature of 22˚C for 14 days. At this time, plants were

well established and an extended photoperiod (20 hrs) was used to help to promote plant growth. For ABA treatment, plants were grown in hydroponic growth medium containing 100 μM ABA (Sigma-Aldrich) for 8 hours. For mannitol treatment, plants were grown in hydroponic growth medium containing 400 mM mannitol (Sigma-Aldrich) for 18 hours, which was previously used as an effective and typical mannitol-mediated drought treatment [12]. Hydroponic growth medium was composed of 49 mg/L H_3PO_4, 250 mg/L $CaCl_2$, 185 mg/L $MgSO_4 \cdot 7H_2O$, 179 mg/L KCl, 58 mg/L NaCl, 241 mg/L NH_4Cl, 454 mg/L KNO_3, 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 220 mg/L $ZnSO_4 \cdot 7H_2O$, 51 mg/L $CUSO_4$, and 120 mg/L $NaMoO_4 \cdot 2H_2O$ in ddH_2O.

2.4. Relative Water Content

Relative water content (RWC) was measured for each experimental group to gauge the amount of water loss created by each stress treatment [21]. All of the leaves from two non-transformed plants were collected, and weighed immediately after plants were removed from hydroponic growth medium to obtain the fresh weight (Wfresh). The leaves were floated on ddH_2O in petri dishes for 6 hours, blotted dry, and reweighed to give the turgid weight (Wturgid). Leaves were then placed in an oven at 50˚C for 48 hours and reweighed to give the dry weight (Wdry). RWC was calculated using the equation: RWC = (Wfresh − Wdry)/(Wturgid-Wdry) × 100.

2.5. RNA Extraction and Quantitative Real-Time PCR

A sample used for expression analysis consisted of the entire shoot portion including the leaves of a single 3-week-old plant. Samples were collected in RNase-free microcentrifuge tubes, immediately flash frozen in liquid nitrogen, and stored at −80˚C until RNA extraction was performed. For RNA extraction, frozen samples were homogenized with a Tissue Lyser II machine, and 1 mL of TRIzol® reagent (Thermo Fisher Scientific) was added to homogenized samples, followed by 200 μl chloroform. The RNA concentration of each sample was quantified using a Nanodrop™ 1000 spectrophotometer. DNase 1 treatment (Thermo Fisher Scientific) of 1 μg of RNA was performed as per the product's instructions to remove residue DNA, and heat deactivation was used for DNase 1 deactivation. Complementary DNA (cDNA) conversion of the DNase 1 treated RNA samples was performed using iScript™ Reverse Transcription Supermix (BIO-RAD) as per the product instructions. cDNA was stored at −20˚C until use for quantitative PCR (qPCR) reactions. Quantitative PCR (qPCR) was performed on the cDNA templates using qPCR reaction mix SsoFast™ EVAGreen® supermix (BIO-RAD) as per the products instructions in a CX96™ Real Time system—C1000 touch thermal cycler. The primers used for qPCR are listed in Table 1. Reactions had an annealing temperature of 60°C and an extension time of 30 seconds. The comparative Ct (Ct) method (Livak and Schmittgen, 2001) was used to calculate the relative fold change between stress-treated plants and

unstressed control plants within each transgenic line used. For each sample, at least three qPCR reactions were performed for the gene of interest as well as for the internal control gene. The technical replicates were averaged for each gene to obtain the cycle threshold (Ct) values. *S-adenosylmethionine decarboxylase* (*SamDC*) was used as the internal control gene to normalize expression levels between different samples, because it has been shown to have stable expression under water deficit conditions [22]. The Ct value of the internal control gene was subtracted from the Ct value of the gene of interest (*uidA*, or *Bradi2G*47700) to obtain the Ct value of each sample. Each transgenic line analyzed had 3 stress-treated samples, and 3 unstressed control samples. The Ct values of stress-treated plants and unstressed control plants were subtracted from the mean Ct value of the unstressed control plants within each line to obtain the Ct values. Fold change was calculated to express the difference in expression between stress-treated groups and unstressed control groups within each line.

2.6. GUS Histochemical Analysis

GUS histochemical staining was performed as described by Jefferson *et al.* [23]. Briefly, shoots with leaves of plants in each treatment group were cut off and immediately submerged in GUS staining solution (2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 0.1 M $NaPO_4$ (pH 7.0), 10 mM EDTA, 1% triton X-100, and 1 mM K_3 $Fe(CN)_6$). X-gluc is a substrate of the β-glucuronidase enzyme, the product of the *uidA* gene. Each sample was vacuum infiltrated for 30 minutes, then incubated overnight at 37˚C. Samples were submerged in 95% ethanol for 48 hours to remove chlorophyll and make the staining easier to observe.

2.7. Statistical Analysis

All statistical analyses were performed using the program "R" version 3.1.3 Copyright© 2015 (The R Foundation for Statistical Computing). RWC measurements were expressed as mean ± standard error of 9 - 10 biological replicates for each stress-treated and unstressed control group. The statistical difference between each stress and its corresponding unstressed control group was assessed using a Welch's two sample t-test. Significance was established at $p < 0.05$. All RT-qPCR results were expressed as the mean ± standard error of 3 biological replicates in each stress treatment group, and 3 biological replicates in each unstressed control group of each transgenic line. The statistical difference between the fold change of each stress treatment group and their corresponding unstressed control group was assessed using a Welch's two sample t-test. Significance was established at $p < 0.05$.

3. Results and Discussion

3.1. RWC Decreases in *B. distachyon* Plants Subjected to Mannitol Treatment

In order to determine the effectiveness of drought treatment, plant relative water

content (RWC) was measured after plants were subjected to ABA and mannitol treatment. Plants treated with ABA did not show a significant difference in RWC compared to control plants, with RWCs of 93.44% ± 2.75% and 90.41% ± 2.80% respectively (**Figure 1(a)**). This was predictable as ABA itself should not cause drought condition, rather, ABA only functions to mediate plant response to drought. On the other hand, the plants grown in hydroponic growth medium containing mannitol had a RWC of 64.81% ± 4.63%, a significant decrease compared to control plants which had a RWC of 90.99% ± 3.87% (**Figure 1(b)**). Thus, mannitol treatment was effective to induce water deficit condition in *B. distachyon* as observed in some other plants such as *Sesuvium portulacastrum* [24], tobacco [25] and wheat [26].

3.2. *Bradi*2*G*47700 Gene Expression Is Upregulated in *B. distachyon* Plants Under Drought Stress

The plant genome database http://www.phytozome.net/ was used to identify possible *Wsi*18 homologous genes native to *B. distachyon*. Putative homologues were identified based on amino acid sequence similarity to that of the *Wsi*18 gene of rice rather than the promoter DNA sequence because coding regions are usually more conserved than non-coding regions [27]. The *Bradi*2*G*47700 amino acid sequence was identified as the most similar protein to *Wsi*18 in *B. distachyon* and shares 74% amino acid sequence similarity with *Wsi*18 (**Figure 2**). To investigate the expression of the *Bradi*2*G*47700 gene in *B. distachyon* under drought stresses, the non-transformed *B. distachyon* plants were subjected to ABA and mannitol treatments, and the *Bradi*2*G*47700 gene expression was analyzed via RT-qPCR at 8 hours and 18 hours after treatment, respectively. The *SamDC* gene, which has been shown to express stably under water deficit conditions [22], was used as a reference gene for RT-qPCR to normalize *Bradi*2*G*47700 transcript levels between samples. The normalized level of *Bradi*2*G*47700 transcripts in stress-treated plants were compared to the transcript level of unstressed
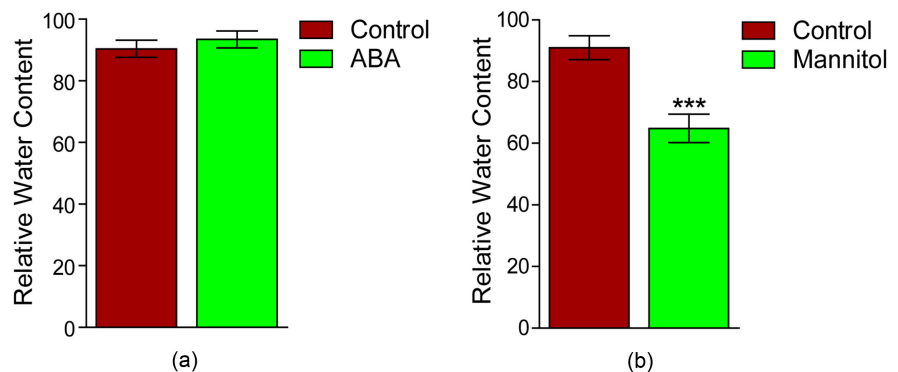
Figure 1. RWC decreases significantly following mannitol but not ABA treatment. Relative water content was measured to assess the difference in water status between stress treated plants (ABA and mannitol) and control plants. Measurements of control groups were obtained at the same time as their respective stress treatment group. Data are mean ± se (n = 10), ***$p < 0.001$ versus control, Student *t*-test, two-tailed.

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Bradi2G47700    MASRQDQASYRAGEAKARTEEKTGHVMGAAQEKAREAKDTASDAAGRAMGRGGDAKEATK
Wsi18           MASQQERASYHAGETKARAEEKTGRMMGTAQEKAREAKDTASDAAGRAMGRGHGAKEATK
                ***:*:.:***:***:***:***:*****::**:********************  *****

Bradi2G47700    E----------KAYEAKDMASDATGRAMNKGRDAAEVTKEKGLEAKDKTAE--------
Wsi18           EKAYETKDATKEKAYEAKDAASDATGRAMDKGRGAAGATRDKAYDAKDRAADTAQSAADR
                *          ****** *********:*** ** .*::*. :***::*:

Bradi2G47700    ---TAQQTGSYIGQTAEAAKQKAAGAAQYAMDTVVAGKDKTGSVLVQAGETVKSAAVGAK
Wsi18           ARDGAGQTGSYIGQTAEAAKQKAAGAAQYAKETAIAGKDKTGAVLQQAGEQVKSVAVGAK
                   * ********************** :*.:*******:** **** ***.*****

Bradi2G47700    DAVMNTIGMGADNNGSSTNVPGKDTSTYKPGKGY*---
Wsi18           DAVMYTLGMSGDNKN--NAAAGKDTSTYKPGTGSDYQ*
                **** *:**..**:  . . **********.*
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Figure 2. The *Bradi2G*47700 amino acid sequence analysis. The *Bradi2G*47700 amino acid sequence of *B. distachyon* was aligned with rice *Wsi*18 amino acid sequence from rice. An asterisk (*) indicates a position with a single, fully conserved residue. A colon (:) indicates conservation between groups with strongly similar chemical properties. A period (.) indicates conservation between groups with weakly similar chemical properties. *Bradi2G*47700 was identified as a putative *Wsi*18 homologue using www.phytozome.net [30], and the sequence alignment was generated using Clustal Omega [31] [32] [33]. *Bradi2G*47700 and *Wsi*18 proteins share 74% sequence similarity.

control plants to calculate the relative fold change. The *Bradi2G*47700 gene expression in ABA treated plants was upregulated 794.14 ± 504.91 (P = 0.01914) fold on average compared to the non-stressed control group (**Figure 3(a)**). The *Bradi2G*47700gene expression in mannitol treated plants resulted in 101.85 ± 8.17 (P < 0.01) fold increase on average as compared to the non-stressed control group (**Figure 3(b)**). Yi *et al.* [15] reported that *Wsi*18 mRNA abundance increased in leaf tissues or roots exposure to ABA. Interestingly, Joshee *et al.* [12] reported that the *Wsi*18 gene can be induced by water stress conditions such as mannitol and NaCl, but not by ABA. Probably Joshee *et al.* [12] used low dose of ABA (20 M). The dose of ABA as Yi *et al.* reported (100 M) was five times higher. Taken together, the upregulation of the *Bradi2G*47700 gene after ABA and mannitol treatments indicates that the *Bradi2G*47700 gene in *B. distachyon* is water stress inducible similar to *Wsi*18. As *Bradi2G*47700 shares a high level of similarity with *Wsi*18 and is also inducible for expression under drought treatments, *Bradi2G*47700 is probably the true homologue of *Wsi*18. The results indicate that *Wsi*18 homologues in other monocot crops may be explored for drought stress tolerance development for respective and specific situations.

3.3. *Wsi*18 Expression Was Upregulated in *Wsi*18: *UidA* transgenic *B. distachyon* Subjected to ABA and Mannitol Treatments

Transgenic *B. distachyon* plants were developed to evaluate the expression of the *Wsi*18 promoter under water deficit condition and in response to ABA. PCR was first performed to evaluate plant transformation. Bands of 628 bp, which was the expected size of the PCR product, were detected in 21 of the 32 regenerated plants (**Supplemental Figure S1**). In addition to PCR genotyping, T1 seeds (seeds from T0 plants) were transferred to MS medium containing 115 mg/L hygromycin B for germination. This level of selection was found to be sufficient
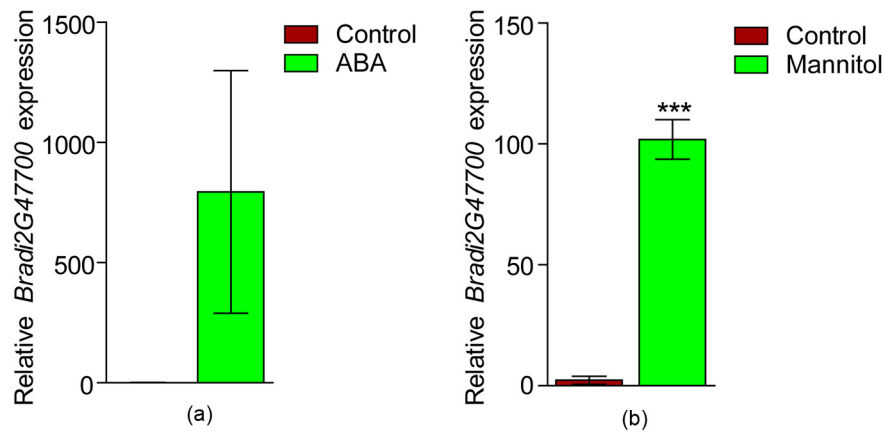
Figure 3. Expression of *Bradi2G47700* gene in *B. distachyon* following ABA and mannitol treatments. The expression of *Bradi2G47700* in wild type *B. distachyon* was analyzed following exposure to ABA (a) and mannitol (b). Real-time qPCR was used to measure mRNA levels of the *Bradi2G47700* gene and the internal control gene *SamDC* within each sample. Results were normalized to *SamDC* expression in the same sample and then normalized to the control. Data are mean ± se (n = 3), ***$p < 0.001$ versus control, Student *t*-test, two-tailed.

to kill non-transgenic plants (Supplemental Figure S2). All the lines which were PCR positive germinated normally on medium containing 115 mg/L hygromycin. On the other hand, the plants which did not produce the correct 628 bp product in PCR genotyping either did not germinate in the presence of hygromycin B, or produced only black roots (not shown). PCR genotyping and hygromycin B seed selection provided two levels of plant transformation analyses and only the plants that were PCR positive and showed normal germination and growth under a high level (115 mg/L) of hygromycin B selection were used to evaluate *Wsi*18 expression.

Real-time PCR analysis was used to examine the expression of the *uidA* reporter gene, which was driven by the *Wsi*18 promoter, in transgenic lines of *Wsi*18: *uidA* following ABA or mannitol treatment. The *SamDC* gene was used as a reference gene for RT-qPCR to normalize *uidA* gene expression between samples. Normalized *uidA* expression levels within each line were compared to the expression level of non-drought-treated control plants of the same transgenic line to determine their relative fold change. Plants that were grown in fresh hydroponic growth medium for the same duration as their respective stress treatment were used as a control. Of the five transgenic lines, the *uidA* expression in three lines (lines 10, 18 and 37) was significantly upregulated after ABA treatment. Lines 10, 18 and 37 showed fold increases of 5.61 ± 0.98 (P < 0.01), 2.88 ± 0.75 (P < 0.05) and 9.13 ± 1.96 (P < 0.05), respectively (Figure 4(a)). The *uidA* expression in the other two lines (lines 3 and 12) did not display a significant increase in expression (Figure 4(a)). For mannitol treatment, the *uidA* expression in two lines (lines 18 and 37) was significantly upregulated. The *uidA* expression in lines 18 and 37 displayed fold increases of 4.43 ± 1.07 (P < 0.05) and 8.47 ± 2.90 (P < 0.05) respectively. Although *uidA* expression in lines 11, 15
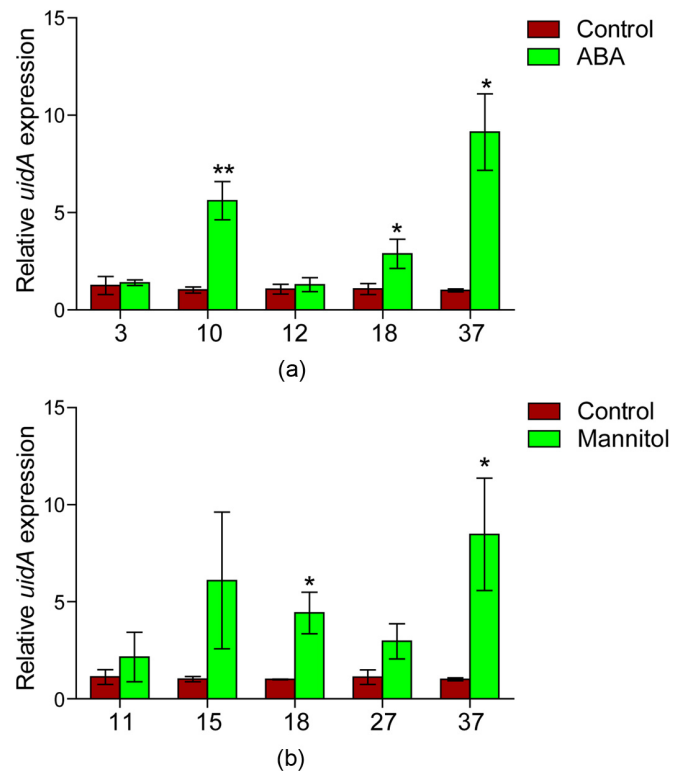
Figure 4. *Wsi*18 derived *uidA* gene expression is upregulated in *Wsi*18: *uidA* transgenic *B. distachyon* subjected to ABA and mannitol using RT-PCR. The expression of *uidA* driven by the *Wsi*18 promoter is responsive to ABA and mannitol exposure in *B. distachyon*. RT-qPCR was used to analyze the level of *uidA* expression in control and treated plants following exposure to ABA (a) and mannitol (b). *UidA* expression levels in each sample were normalized to *SamDC* and then normalized to the control. Data are mean ± se (n = 3), *$p < 0.05$, **$p < 0.01$ versus control, Student *t*-test, one-tailed.

and 27 showed an increased upregulation of 2.16 ± 1.27 (P = 0.2298), 6.10 ± 3.52 (P = 0.0938) and 2.96 ± 0.90 (P = 0.0659) fold respectively, there was no significant difference between the control and treated groups (**Figure 4(b)**). Variation of transgene expression in different transgenic plants has been frequently reported for both constitutive and inducible promoters before [14] [28]. One explanation for the observed differences in *uidA* expression between the transgenic lines in this study could be the differences in the site of transgene integration into the *B. distachyon* genome, known as positional effects. Transcriptional regulatory sequences such as enhancers and inhibitors found around the site of integration, as well as the chromatin structure around the site of integration, could all influence the expression of the transgene. Integration at highly expressing loci will produce higher expressing transgenic lines than integration at loci with lower transcriptional activity [34]. Additionally, copy number has been shown to influence transgene expression levels. Integration of multiple transgene copies often produces low levels of expression due to homology-dependent gene silencing

[29]. Homology-dependent gene silencing can be the result of transcriptional gene silencing or post-transcriptional gene silencing. Transcriptional gene silencing includes processes such as promoter methylation, which blocks transcription from occurring from the promoter, while post-transcriptional gene silencing causes transcripts to be degraded before they can be translated into protein. If *Wsi*18 was not water deficit-inducible in *B. distachyon*, no difference in *uidA* expression would be expected between the stress-treated and unstressed control plants of any transgenic lines. The mannitol and ABA treatment did show a significant increase in *Wsi*18 driven *uidA* expression in certain transgenic lines, suggesting *Wsi*18 does have water deficit inducible activity in *B. distachyon*.

3.4. Qualitative Analysis of *Wsi*18 Activity in *Wsi*18: *UidA* Transgenic *B. distachyon* Using the GUS Histochemical Assay

In addition to Real-time PCR analysis, GUS histochemical analysis was used to analyze and visualize the expression mediated by the *Wsi*18 promoter following mannitol and ABA treatments. The GUS histochemical assay can provide a further level of evaluation of Wsi18 protein expression. The *CaMV*35*S*: *uidA* transgenic *B. distachyon* plants showed a greater amount of GUS activity in both the stem and leaves compared to wild type *B. distachyon* without drought treatment (**Figure 5(a)**). Three transgenic lines in which the *uidA* reporter gene was driven by the *Wsi*18 promoter were used for each analysis; plants were 3 weeks old at the time of analysis. Plants in the ABA-treated group were grown for 8 hours in hydroponic growth medium containing 100 μM ABA, plants grown in the mannitol-treated group were grown for 18 hours in hydroponic growth medium containing 400 mM mannitol. Unstressed control plants were of the same transgenic lines used for the stress treatments, and were grown in fresh hydroponic growth medium for the same duration as their respective stress treatment. The three *Wsi*18: *uidA* transgenic lines 10, 12 and 37 showed a greater amount of GUS activity in both the stem and leaves of plants which had undergone the ABA treatment, compared to control plants (**Figure 5(b)**). The three *Wsi*18: *uidA* transgenic lines 11, 18 and 24 which underwent the mannitol treatment also showed a greater degree of GUS staining compared to control plants, with no detectable GUS activity in any of the control plants (**Figure 5(c)**). There was variation in the level of GUS activity between the different transgenic lines, however, GUS activity was more prevalent following stress treatments than in unstressed control plants for each stress treatment. Some unstressed control plants did show small areas of GUS activity, but always to a lesser degree than their stress-treated counterparts. Low level *Wsi*18 promoter activity in unstressed control plants is consistent with the observations of *Wsi*18 activity in transgenic rice reported by Yi *et al*. [15]. The low levels of expression driven by *Wsi*18 in unstressed control plants may be due to less stringent regulation of gene expression in transgenes than genes in their native context. Non-transformed plants acted as a negative control to ensure there was no endogenous GUS
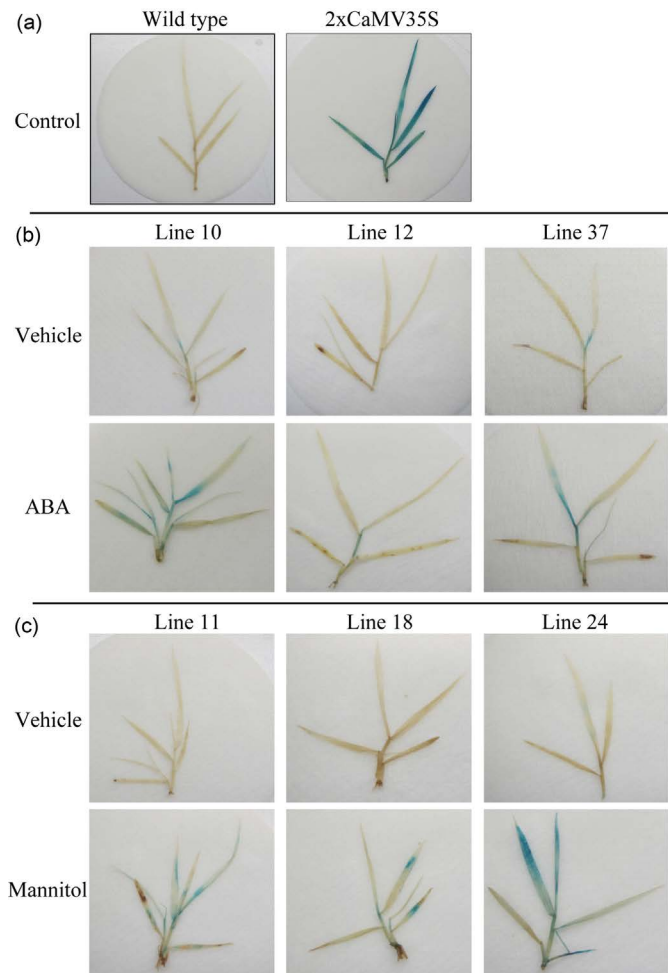
Figure 5. Qualitative analysis of *Wsi*18 activity in *Wsi*18: *uidA* transgenic *B. distachyon* using the GUS histochemical assay. Histochemical GUS assays were carried out to observe the pattern of GUS expression following ABA and mannitol treatments. (a) Wild type *B. distachyon* plant was as a negative control, transgenic 2 × *CaMV*35*S*: *uidA B. distachyon* plants was as a positive control; (b) GUS expression of transgenic *Wsi*18: *uidA B. distachyon* plants grown in hydroponic growth medium containing 100 μM ABA for 8 hours, or control condition; (c) GUS expression of transgenic *Wsi*18: *uidA B. distachyon* plants grown in hydroponic growth medium containing 400 mM mannitol for 18 hours or control condition.

expression in *B. distachyon*, or contamination of the staining solutions.

Our results showed that the *Bradi*2*G*47700 gene, the closest homologue to rice *Wsi*18, was upregulated in *B. distachyon* plants exposed to ABA and mannitol. Further, we showed that the *Wsi*18 promoter can be induced to express by ABA and the water deficit conditions created by mannitol. Response to ABA suggests that Wsi18 is probably induced through the ABA dependant signaling pathway to mediate plants for drought stress. The research indicates *Wsi*18 homologues may be used to drive relevant genes for drought stress resistance in different

monocot crops.

Acknowledgements

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplemental

(a)

(b)
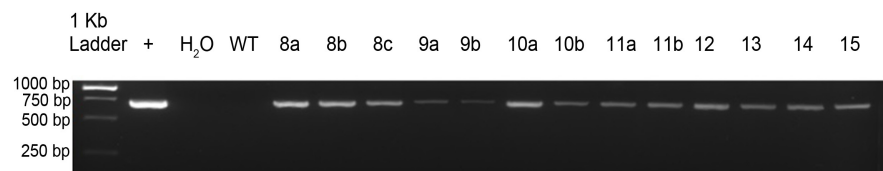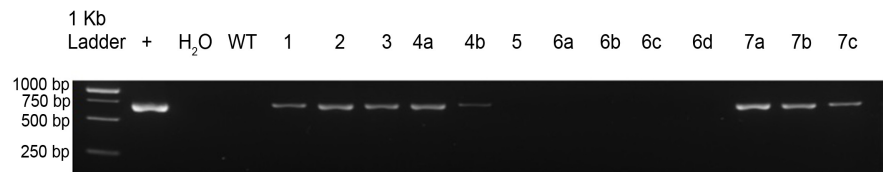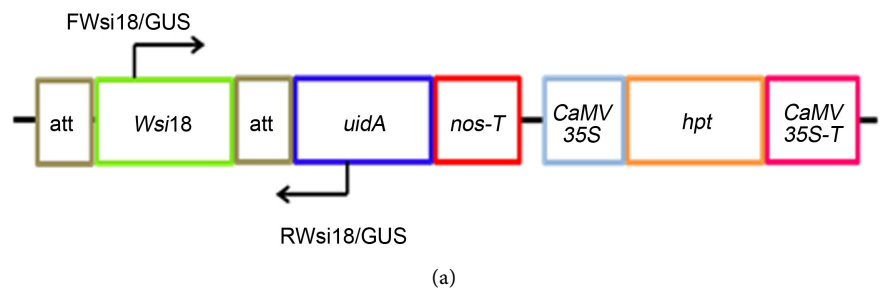
Figure S1. PCR genotyping of *B. distachyon* plants regenerated from pMDC163-*Wsi*18 transformed calli. In PCR genotyping the forward primer FWsi18/GUS bound to the *Wsi*18 promoter 331 bp upstream of the *uidA* gene and the reverse primer RWsi18/GUS bound to the *uidA* gene 297 bp downstream of the start codon. The *hpt* gene conferred hygromycin B resistance to all plants which had successfully incorporated the T-DNA region into their genome, and was used as a selectable marker for the germination of T1 seeds on seed germination medium containing 115 mg/L hygromycin B. Results of PCR genotyping of *B. distachyon* plants regenerated from calli that were inoculated with *A. tumefaciens* containing the pMDC163-*Wsi*18 plasmid. PCRs using the DNA of transgenic plants produced a 628 bp sized band when run on an agarose gel. The pMDC163-*Wsi*18 plasmid was used as template DNA for positive control PCR reactions (+). Wild-type *B. distachyon* DNA (WT), and sterile water (H_2O) in place of DNA, were used as negative control PCR reactions.
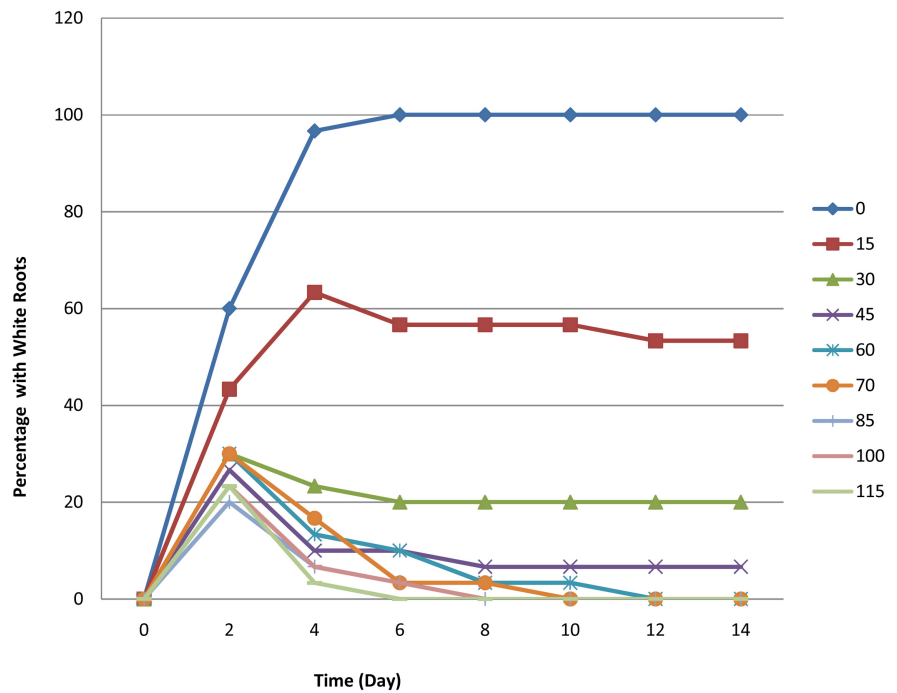
Figure S2. Hygromycin kill curve. Wild-type *B. distachyon* seeds were sown on seed germination medium containing various concentrations of hygromycin B for 14 days. Hygromycin B caused the seedlings either to not germinate, or to exhibit black roots and retarded growth. Germination for 12 days on medium containing 60 mg/L hygromycin B was sufficient to disrupt the growth of all wild-type seeds. Higher concentrations of hygromycin B enabled the identification of hygromycin B sensitive seeds over a shorter period of time.