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Evaluation of the Water Stress-Inducible Promoter Wsi18 in the Model Monocot Brachypodium distachyon

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Abstract

Water deficit-inducible promoters that function in multiple species are valuable components for engineering stress-tolerant crops. *Wsi18* is a water deficit-inducible promoter native to *Oryza sativa*. In this study, *Brachypodium distachyon* (*B. distachyon*) was used to determine if *Wsi18* retained its water deficit-inducible characteristics in another monocot. Transgenic *B. distachyon* plants, in which the *Wsi18* promoter drove the expression of the *uidA* reporter gene, were developed and exposed to osmotic stress generated by mannitol, salt stress conditions, and the water deficit-signaling phytohormone abscisic acid (ABA). GUS histochemical assays demonstrated increased *uidA* expression in the leaves and stem of mannitol, NaCl, and ABA-treated plants. RT-qPCR demonstrated maximum expression increases of 8.5-fold following mannitol treatment, and 9.1-fold following ABA treatment, although no change was induced by the NaCl treatment. These findings suggest the *Wsi18* promoter is induced by water deficit in *B. distachyon*, and could be an excellent tool for future crop improvement.

Keywords

Acknowledgments

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List of Abbreviations

ABA	Abscisic acid
ABRE	Abscisic acid response element
AREB/ABF	ABRE binding proteins/ABRE binding factor
ATP	Adenosine triphosphate
attB	Gateway cloning attachment site
B. distachyon	*Brachypodium distachyon*
bHLH	Basic helix-loop-helix
BRE	B recognition element
bZIP	Basic leucine zipper
CaMV35S	Cauliflower mosaic virus 35S
CBF/DREB	C-repeat binding factor/DRE binding protein
cDNA	Complementary DNA
CE	Coupling element
CEC	Compact embryogenic callus
Ct	Cycle threshold
CTAB	Cetyltrimethyl ammonium bromide
ddH$_2$O	Double distilled water
Dicot	Dicotyledonous
DNA	Deoxyribonucleic acid
DPE	Downstream promoter element
DRE/CRT	Dehydration responsive element/cold repeat

GFP	Green fluorescent protein
GUS	β-glucuronidase
Hpt	Hygromycin phosphotransferase
Inr	Initiator element
LB	Lysogeny broth
LEA	Late embryogenesis abundant
Monocot	Monocotyledonous
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
nos-T	Nopaline synthase terminator
Npt II	Neomycin phosphotransferase
PCR	Polymerase chain reaction
PLACE	Plant *cis*-acting regulatory DNA elements
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SamDC	S-adenosylmethionine decarboxylase
SOS	Salt overly sensitive
TBP	TATA binding protein
T-DNA	Transfer DNA
TSS	Transcriptional start site
X-gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronide

Chapter 1: Introduction

1.1   Water deficit stresses

Each year more than 50% of global crop yields are lost due to abiotic stresses (Valliyodan and Nguyen, 2006), with water deficit stresses such as drought and high salinity being major contributors. Globally, water deficit stresses have been estimated to be responsible for over $10 billion in lost crop yield each year (Xu et al., 2014). It is estimated that by the year 2050, the global demand for crop foods will have increased by 100% (Tilman et al., 2011). Taken together with the effects of climate change, developing crop varieties with greater tolerance to water deficit will be essential to maintaining an adequate global food supply (Challinor et al., 2010).

Water is a crucial resource for all plants and plays several key biological roles. The water potential inside of a plant cell pushes outwards creating turgor pressure. Turgor pressure keeps plants rigid and upright and plays an important role in cellular division. In addition, the polar properties of water molecules are essential for maintaining the proper folding of proteins, and the assembly of phospholipids into biological membranes (Hoekstra et al., 2001). Furthermore, water is essential to photosynthesis where it acts as a reactant necessary for carbon fixation. Other functions of water in plants include transporting dissolved nutrients and minerals throughout the plant, regulating the plant's temperature through heat dissipation and transpiration, and acting as a solvent for chemical reactions (Hoekstra et al., 2001; Schilling et al., 2016).

Water deficit conditions result in changes to many of the cellular processes of a plant. Water deficit creates osmotic stress and dehydration in plant cells causing the

cytoplasm to become increasingly viscous. This increases the chance of molecular interactions that can cause protein denaturation and aggregation (Hoekstra et al., 2001). Osmotic stress and dehydration also cause destabilization of cellular membranes due to the insertion of amphiphillic substances into the membrane as their cytoplasmic concentration increases with water loss (Golovina and Hoekstra, 2002). Under water deficit conditions turgor pressure in a plant's guard cells is reduced causing the closure of stomata. Stomata closure decreases the rate of transpiration resulting in less water being lost from the plant. An additional consequence of stoma closure is reduced CO_2 diffusion from the environment which reduces the plant's ability to fix carbon (Chaves et al., 2009). Due to the reduction in photosynthesis, reactive oxygen species (ROS) are produced, resulting in oxidative stress (Osakabe et al., 2014). Perturbation of the mitochondrial membrane caused by water loss can also create oxidative stress due to ROS leakage from the mitochondria into the cytoplasm (Hoekstra et al., 2001).

1.2   Mechanisms of water deficit tolerance

Plants are sessile organisms and consequently cannot escape abiotic stress conditions like water deficit by moving to a more favorable environment. Instead, plants adjust at the structural and cellular levels when stressful conditions are detected. Some ways plants detect water deficit conditions include through osmosensor proteins, changes in ion concentration, and increased cellular ROS (Urao et al., 1999; Knight and Knight, 2001; Miller et al., 2008). Water deficit-induced cellular changes affect the activity of regulatory proteins, which then initiate signaling cascades resulting in the production of transcription factors and phytohormones. Following their production, these transcription

factors and phytohormones induce the expression of functional proteins that increase the water deficit tolerance of the plant (Shinozaki and Yamaguchi-Shinozaki, 2007).

Central to a plant's response to water deficit stress is the phytohormone abscisic acid (ABA). ABA is involved in the regulation of many plant processes, including seed maturation, maintenance of seed dormancy, regulation of cell growth and division during seedling development, pathogen resistance, flowering time, senescence, and abiotic stress response (Finkelstein, 2013). In response to water deficit stress, the level of ABA increases inside the cell, and induces the expression of many water deficit-responsive genes (Rabbani et al., 2003). Additionally, ABA is involved in physical adaptations to limit water loss under water deficit, such as signaling to reduce the turgor pressure of guard cells to close the stomata (Shinozaki and Yamaguchi-Shinozaki, 2007).

1.2.1   Osmotic stress and cellular dehydration

Many genes that are induced in response to water deficit are responsible for the production of molecules that limit the damaging effects of osmotic stress and dehydration of the cell. These include antioxidants and ROS scavenging enzymes which reduce oxidative stress, and compatible solutes which act to balance osmotic pressure and protect cellular components from dehydration (Hoekstra et al., 2001; Chaves et al., 2003). Compatible solutes are protective compounds which do not interfere with normal cell structure or function (Hoekstra et al., 2001). Under water deficit conditions, one function of compatible solutes is to act as hydration buffers. It is thermodynamically unfavourable for the hydration buffers to interact with cellular proteins, and as a result a layer of water separates the proteins from the hydration buffers keeping the proteins preferentially hydrated. This results in the proteins maintaining their folded structure to reduce the

surface area that needs to be protected from the compatible solutes. In this way the native state of proteins can be conserved, and proteins can continue to be active under water deficit conditions (Hoekstra et al., 2001). Upon further water loss, preferential hydration is no longer feasible as there is not enough water left in the cell to hydrate the protein. At this time, molecules which can replace the hydrogen bonding activity of water are accumulated. These molecules often have polar residues which interact with proteins in a way similar to how a water molecule would interact with the protein. The interaction of the polar residues with the cellular proteins helps to maintain proper protein folding and decrease the likelihood of protein aggregate formation (Hoekstra et al., 2001). In addition, sugar molecule-compatible solutes accumulate and increase the viscosity of the cytoplasm, producing glassy state cytoplasm. Glassy state cytoplasm adds stability to the cytoplasm matrix, provides support to the membrane, and reduces the movement of cellular components which decrease the likelihood of protein aggregation (Hoekstra et al., 2001). One notable group of compatible solutes are the late embryogenesis abundant (LEA) proteins which have been found to increase the water deficit stress tolerance of plants (Tunnacliffe and Wise, 2007), and whose promoters have been used as water deficit-inducible promoters to drive the expression of transgenes due to their dehydration responsive nature (Xiao and Xue, 2001).

1.2.1.1   Late embryogenesis abundant proteins

The late embryogenesis abundant (LEA) protein family is expressed during the late stages of embryo development in plants, coinciding with seed desiccation and the synthesis and redistribution of ABA in the plant (Galau et al., 1987). Most LEA genes are ABA responsive and begin to accumulate in the embryo as a result of the increased level

of cellular ABA (Amara et al., 2014). In addition to their role during embryogenesis, LEA proteins are expressed in vegetative tissues under water deficit conditions (Amara et al., 2014).

LEA proteins increase the abiotic stress tolerance of plants, but their function is still largely unknown. Hypothesized functions of LEA proteins include roles in protein stabilization, membrane protection, organic glass formation, as hydration buffers, antioxidants, or ion chelators (Amara et al., 2014). LEA proteins are less susceptible to denaturation during dehydration of the cell due to their hydrophilic and unstructured nature, suggesting they may have a role as hydration buffers (Goyal et al., 2005). In addition, the charged amino acid residues of LEA proteins may sequester ions preventing their interference with macromolecular functions (Dure, 1993). Furthermore, LEA proteins may play a role in ROS scavenging by sequestering metals that could generate ROS (Tunnacliffe and Wise, 2007). LEA proteins may also strengthen hydrogen bonds between sugar molecules to increase the density of glassy state cytoplasm. Denser sugar glasses support the cell/cytoplasm matrix, and balance the osmotic potential within the cell (Iturriaga et al., 2009; Shimizu et al., 2010).

1.2.2   Salt stress

In addition to osmotic stress and cellular dehydration, high salinity conditions also cause hyper-ionic stress. Elevated Na^+ concentrations cause the Na^+/K^+ homeostasis of the cell to be disrupted, creating ion toxicity. Enzymes that require K^+ as a co-factor are particularly sensitive to high Na^+ concentrations (Munns, 2002). In addition to the processes mentioned in Section 1.2.1 to deal with osmotic stress and cellular dehydration, mechanisms that protect against ionic stress are also initiated in the cell. Ionic stress is

dealt with primarily through the salt overly sensitive (SOS) pathway. The SOS pathway has four primary enzymes SOS1, SOS2, SOS3 and SCABP8. SOS3 is expressed in the roots and senses the increased Ca^+ concentrations caused by salt stress. SCABP8 is an SOS3-like protein expressed in shoots. SOS3 and SCABP8 bind to Ca_2^+, enabling them to bind to SOS2. SOS2 is a serine/threonine kinase that when bound by SOS3 or SCABP8 phosphorylates and activates SOS1, a Na^+/H^+ antiporter located in the plasma membrane. SOS1 then expels Na^+ from the cell or into vacuoles for compartmentalization (Huang et al., 2012). By expelling or compartmentalizing Na^+ ions, the cellular components are protected from their effects.

1.3   Promoters

Transcription is the process in which DNA is used as a template to produce a RNA transcript. It is at the transcriptional level that the greatest regulation of gene expression occurs (Hernandez-Garcia and Finer, 2014). Regulation of transcription is primarily controlled by the promoter region of a gene, which is located upstream of the transcriptional start site (TSS). The promoter is often described as consisting of three main sections. These are the core promoter, the proximal promoter region, and the distal promoter region. The core promoter is responsible for transcription initiation, while the proximal and distal promoter regions are mostly responsible for regulating the pattern and level of transcriptional activity (Figure 1). Within the promoter sequence are *cis*-elements which act as binding sites for transcription factors. Transcription factors are proteins that are coded elsewhere in the genome and bind to the promoter to regulate transcription (Hernandez-Garcia and Finer, 2014).

1.3.1   Promoter classification

Promoters can be classified by the pattern of gene expression that they produce. Constitutive promoters drive a constant level of gene expression in all tissues of a plant, at all times (Hernandez-Garcia and Finer, 2014). Most constitutive promoters in plants originate from highly expressed housekeeping genes such as actin or ubiquitin (Zhang et al., 1991; Cornejo et al., 1993), or plant viruses such as the *Cauliflower mosaic virus 35S* promoter (*CaMV35S*) (Odell et al., 1985). Spatio-temporal promoters only express the genes under their control within specific tissues, at certain times, or at specific developmental stages of the plant. Spatio-temporal promoters are a broad category, and sources for these promoters depend on the expression profile of interest (Hernandez-Garcia and Finer, 2014). Cryptic promoters are normally inactive in their native context, but can actively drive the expression of transgenes when inserted elsewhere in the genome. For example the *tCUP* promoter from tobacco is a cryptic promoter that drives strong constitutive expression of transgenes, but is inactive in its native location in the tobacco genome (Tian et al., 2003). Inducible promoters do not initiate transcription of a gene until the plant experiences a specific stimulus. The stimulus can be endogenous signals like plant hormones, external signals such as chemical application, or external physical stimuli such as biotic and abiotic stress. Sources of inducible promoters are usually genes expressed in response to that stimulus (Hernandez-Garcia and Finer, 2014).

Water deficit-inducible promoters are excellent candidates to drive the expression of genes whose products increase the water deficit tolerance of plants. Constitutive promoters have been used previously to express genes in an effort to increase tolerance to water deficit, and in some cases this has been successful. In other examples however,

continuous overproduction of the transgene product has resulted in negative side effects. For example, tobacco plants constitutively over-expressing the *trehalose-6-phosphate synthase* gene had greater drought tolerance, but suffered stunted growth (Romero et al., 1997). These negative side effects could be due to the cost of resources to over-produce the protein, or negative interactions of the gene product with normal cell metabolism (Mahajan and Tuteja, 2005). Inducible promoters offer the advantage of expressing the gene only when necessary. In this way the plant is not inflicted with the burden of continuous production when it is not required.

1.3.2 Transcription initiation at the core promoter

The core promoter is the primary area in which the transcriptional machinery binds to the promoter, and is located in the DNA sequence approximately 40 bp upstream of the TSS (Hernandez-Garcia and Finer, 2014). RNA polymerase II is the enzyme responsible for transcribing the DNA of nuclear protein coding genes of eukaryotic organisms into messenger RNA (mRNA) (Butler and Kadonaga, 2002). RNA polymerase II binds to the core promoter with the help of general transcription factors TBP, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Porto et al., 2014). Together the RNA polymerase II enzyme and the general transcription factors make up the transcription initiation complex (Butler and Kadonaga, 2002).

Some core promoter *cis*-elements are commonly found, but none are universally conserved. Common *cis*-elements include the TATA-box, the initiator element (Inr), the downstream promoter element (DPE), and the B recognition element (BRE) (Porto et al., 2014). Assembly of the transcription initiation complex in promoters with a TATA-box begins with the TATA-box being identified by the TATA-binding protein (TBP) subunit

of the TFIID general transcription factor (Hernandez, 1993). TFIIA then binds to TFIID, followed by TFIIB. TFIIB recruits RNA polymerase II to the promoter and is essential for linking the polymerase to TFIID. RNA polymerase II is bound to TFIIF, which is important for connecting RNA polymerase and TFIIB (Porto et al., 2014). Before transcription can be initiated TFIIE and TFIIH must also bind to the polymerase/transcription factor complex. TFIIE and TFIIH are required for the hydrolysis of ATP in order to phosphorylate RNA polymerase II. Unphosphorylated RNA polymerase II is involved in DNA binding and transcription initiation, whereas phosphorylated RNA polymerase II is involved in mRNA elongation (O'Brien et al., 1994; Ohkuma and Roeder, 1994). In promoters lacking a TATA-box, the Inr element may act as a substitute for the TATA-box, and is similarly recognized by TFIID. In promoters with both a TATA-box and Inr element, the TATA-box and Inr elements can work together to facilitate the binding of TFIID (John Colgan, 1995). Aside from the TATA-box and Inr element, the BRE and DPE are common core promoter elements which can play an integral role in transcription initiation. The BRE element facilitates the incorporation of TFIIB into the transcription initiation complex (Lagrange et al., 1998). The DPE element is usually found in TATA-less promoters and works in sync with the Inr element (Kutach et al., 2000). The locations of elements in the core promoter are essential for positioning the RNA polymerase at the correct site in relation to the TSS (Zawel and Reinberg, 1995). For example, the distance between the Inr and DPE elements has been found to be essential to the binding of TFIID, and even a one nucleotide change in this distance can reduce transcriptional activity (Porto et al., 2014). The CCAAT box and GC-box elements, commonly located 100 bp and 200 bp upstream

of the TSS respectively, are located outside of what is generally considered to be the core promoter, but can also be important for positioning of the transcription initiation complex when they are present (Porto et al., 2014).

1.3.3 Gene expression patterns are established in the proximal and distal promoter regions

Binding RNA polymerase II to the DNA is generally not enough to initiate transcription. Transcription factors which bind to the proximal and distal regions of the promoter are essential to mediating gene transcription, and defining the gene's expression profile. One way that transcription factors bound to the proximal and distal promoter regions affect transcription is by remodeling the chromatin structure at the gene to which they are bound. Transcription factors can recruit co-activator proteins that acetylate histones, decreasing the affinity of their interactions with DNA and giving greater access to the DNA for the transcriptional machinery (Kadonaga, 1998). Furthermore, transcription factor binding can alter gene expression by interacting with the transcription initiation complex. All protein coding genes in the nucleus are competing for the same general transcription factors and transcriptional machinery. Transcription factors that are able to recruit the transcription initiation complex to form at the core promoter of the gene to which they are bound can increase the likelihood of that gene being expressed. Transcription factors that can restrict the transcription initiation complex's access to the DNA can reduce the expression of genes to whose promoters they are bound (Zawel et al., 1995). Transcription factors that increase the level of transcription from the promoters to which they are bound are called activators, while those that decrease expression are called inhibitors (Vaahtera and Broshé, 2011).

The location of *cis*-elements in the promoter sequence can be essential to the proper functioning of transcription factors. The distal regions of the promoter can be located several kbp away from the gene whose expression they are mediating, but are still able to affect expression of that gene due to folding of the DNA which brings them near the transcriptional machinery in 3D space (Vaahtera and Broshé, 2011). Some transcription factors act in unison and require others to bind to the DNA. Due to the helical nature of DNA, a difference of a few nucleotides can place the *cis*-elements to which the transcription factors bind on opposite sides of the DNA helix and affect whether or not two transcription factors are able to interact (Vaahtera and Broshé, 2011). For this reason, spacing between certain elements can be very important.

Distal regions of the promoter contain regulatory elements such as enhancers, and insulators which can greatly affect transcriptional activity. Enhancers are generally 100-200 bp in length and contain many *cis*-elements. They can be located hundreds or thousands of bp away from the TSS, both upstream or downstream, or in the introns of the gene (Porto et al., 2014). The majority of interacting enhancers and promoters are located within 500 kbp of each other. Enhancers do not necessarily interact with their nearest promoter, and can skip over genes to interact with distant promoters. Generally, an active promoter will be influenced by 4-5 different enhancer-like elements (Sanyal et al., 2012; Jin et al., 2013). An enhancer's interaction with a promoter could be the result of biochemical compatibility where the proteins recruited to the promoter and enhancer regions interact to bridge the physical distance separating the promoter and enhancer. (van Arensbergen et al., 2014). Conversely, insulator elements are able to prevent the interaction of promoters and enhancers by altering the chromatin structure to restrict

transcriptional machinery access to the promoter, or by altering the folding of DNA to distance promoters from enhancer elements (van Arensbergen et al., 2014).
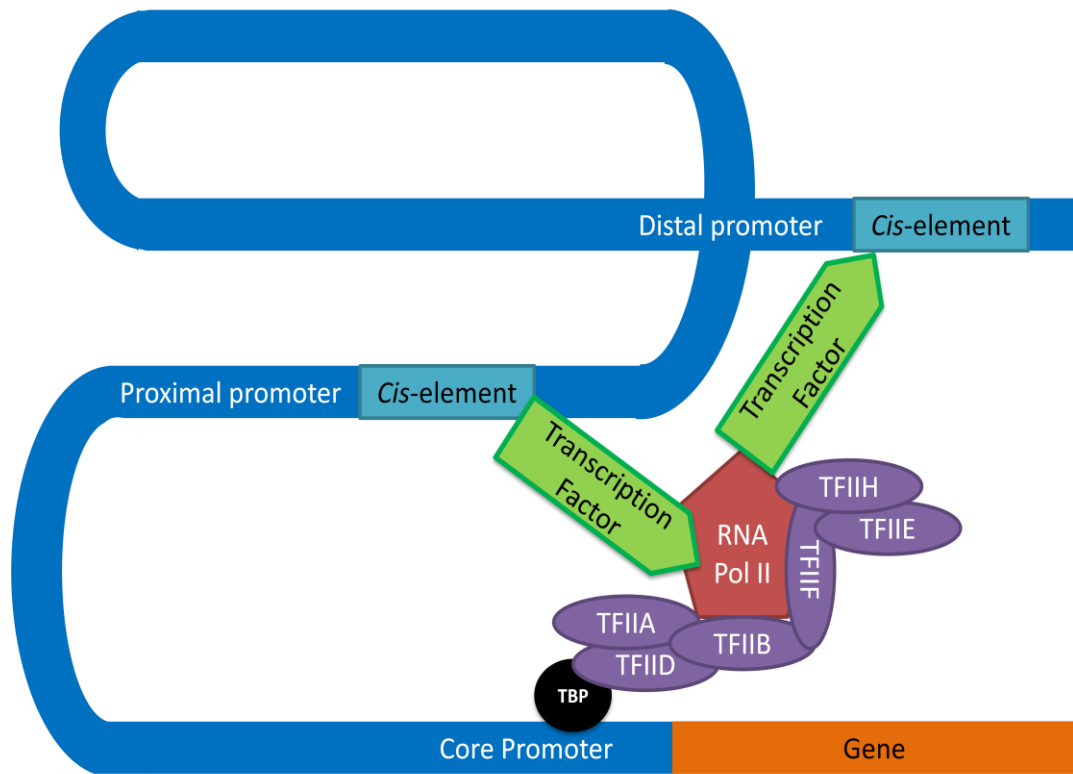
Figure 1: Transcriptional activation at the promoter

RNA polymerase II is responsible for the transcription of nuclear protein-coding genes. It binds to the DNA at the core promoter through associations with general transcription factors to create the transcription initiation complex. *Cis*-elements in the proximal and distal promoter regions are the binding sites for transcription factors, which regulate the activity of the transcription initiation complex to control gene expression. Transcription factors bound to promoter areas far away from the core promoter can still interact with the transcription initiation complex through DNA folding to bring them closer together in 3D space.

1.3.4   Water deficit-inducible promoters

Water deficit-inducible promoters are regulated by signaling cascades that follow both ABA-dependent and ABA-independent pathways (Huang et al., 2012). The main *cis*-element in the ABA-dependent pathway is the abscisic acid response element (ABRE). ABREs are bound by basic leucine zipper (bZIP) transcription factors called ABRE binding proteins/ABRE binding factors (AREB/ABF) which activate ABA-dependent transcription (Huang et al., 2012). AREB/ABF transcription factors require an ABA mediated signal to become activated (Huang et al., 2012). This signal is likely ABA-dependent phosphorylation (Shinozaki and Yamaguchi-Shinozaki, 2007). A single ABRE element is not sufficient to affect transcription and requires either a coupling element (CE) or a second ABRE sequence nearby to function (Hobo et al., 1999). MYB and MYC *cis*-elements have been shown to regulate ABA-dependent gene expression in promoters which lack ABRE elements (Abe et al., 1997). MYB transcription factors which bind to MYB *cis*-elements, and basic helix-loop-helix (bHLH) transcription factors which bind to MYC elements, are thought to be active in the later stages of the stress response (Huang et al., 2012).

The main *cis*-element of the ABA-independent pathway is the dehydration-responsive element/cold repeat (DRE/CRT) *cis*-element (Shinozaki and Yamaguchi-Shinozaki, 2007). This *cis*-element is bound by cold-repeat binding factor/DRE binding protein (CBF/DREB1) and dehydration responsive element binding factor (DREB2) transcription factors. CBF/DREB1 transcription factors are mainly responsive to cold stress, while DREB2 is mainly responsive to drought stress. CBF/DREB1 proteins are constitutively active when expressed. However, DREB2 transcription factors require

post-transcriptional modification, which occurs in response to osmotic stress signaling (Liu et al., 1998).

There is some cross-talk between the ABA-dependent and ABA-independent pathways. The CBF4 gene is a member of the ABA-independent CBF/DREB1 family. Expression of CBF4, however, is induced by ABA (Haake et al., 2002). Therefore CBF4 interacts with *cis*-elements of the ABA-independent pathway in an ABA-dependent fashion. The *Rd29A* promoter from Arabidopsis is another example of cross-talk between the ABA-dependent and ABA-independent signaling pathways. A DRE/CRT element in the *Rd29A* promoter acts in place of the coupling element for the ABRE element so that expression of a transcription factor from the ABA-independent pathway is required for ABA-dependent transcription via the ABRE in this promoter (Narusaka et al., 2003).
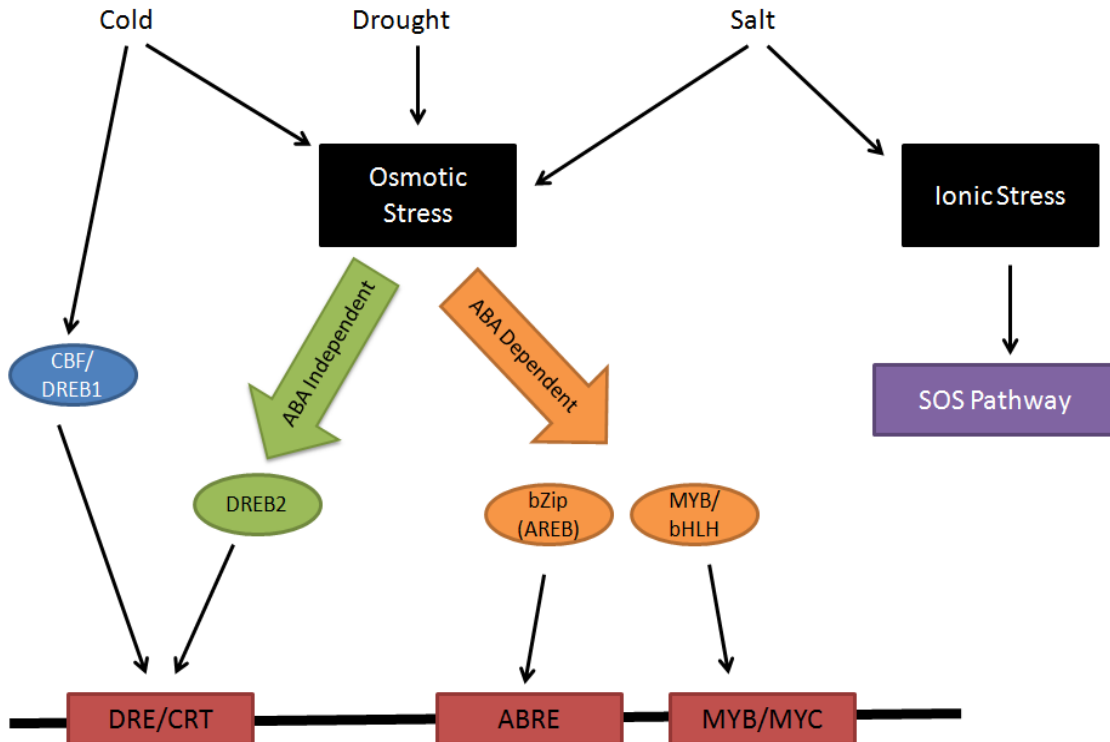
Figure 2: Transcriptional regulatory networks for water deficit response signaling

The two main regulatory pathways of water deficit signaling are the ABA-independent and ABA-dependent pathways. The main *cis*-element involved in ABA-independent signaling is the DRE/CRT element, which is bound by CBF/DREB1 and DREB2 transcription factors. The main *cis*-element in the ABA-dependent signaling pathway is the ABRE element which is bound by bZIP transcription factors such as AREB. MYB and MYC *cis*-elements can also determine ABA-dependent expression. In addition, high salinity conditions create ionic stress, which induces the SOS signaling pathway to restore ion homeostasis. This figure was adapted from Huang et al. (2012).

1.4   The *Wsi18* promoter

Wsi18 is a water deficit-inducible promoter native to rice (*Oryza sativa* L.), from the group 3 LEA family (LEA3) (Joshee et al., 1998). Expression of the *Wsi18* gene was originally characterized as water deficit-inducible by Takahashi et al. (1994). Joshee et al. (1998) then isolated the promoter in the sequence 1.7 kbp upstream of the transcriptional start site, and analyzed the expression profile of genes under the *Wsi18* promoter's control in water deficit conditions. Since then, expression driven by the *Wsi18* promoter has been analyzed in transgenic rice (Yi et al., 2010, 2011; Nakashima et al., 2014), compared to several other water deficit stress-inducible promoters (Xiao and Xue, 2001; Yi et al., 2010; Nakashima et al., 2014), and used to express reporter genes transiently in one other species, barley (Xiao and Xue, 2001). Despite the extensive research conducted on *Wsi18*, its stable expression profile has never been analyzed in a plant species other than rice.

In rice, genes driven by the *Wsi18* promoter have low expression levels under normal conditions, but are considerably induced following water deficit stresses such as drought stress and salt stress, as well as exogenous ABA application (Yi et al., 2011). Under normal conditions, expression driven by *Wsi18* is low in vegetative tissues. Following water deficit conditions, *Wsi18*-driven expression is detectable in rice flowers, the whole body of etiolated seedlings, and in mature leaves and roots at all developmental stages (Yi et al., 2011). In leaves, *Wsi18* drives expression in the mesophyll, vascular bundle sheath cells, and stomata. In roots, expression is observed in the root apex, root cap, root vascular cylinder, and elongating regions (Yi et al., 2010, 2011; Nakashima et al., 2014). Like many LEA3 genes, *Wsi18* has been observed to drive ubiquitous expression in

drying rice seeds. *Wsi18*-driven expression remains elevated throughout seed maturation in the embryo, aleurone layers, and endosperm (Yi et al., 2010, 2011). Several studies have compared *Wsi18* driven expression to that of other promoters, both constitutive and inducible (Xiao and Xue, 2001; Yi et al., 2010, 2011; Nakashima et al., 2014). Xiao and Xue (2001) found that following an air drying treatment or exposure to ABA, *Wsi18* had a similarly high level of transient expression of the *uidA* reporter gene to that of the strong constitutive promoter *Act1*. Joshee et al. (1998) found that rice protoplasts treated with 400 mM mannitol had a 4-7 fold lower expression of the *uidA* reporter gene when *uidA* was driven by *Wsi18* compared to the constitutive promoter *CaMV35S*.

The water deficit-inducible characteristics of *Wsi18* are a product of the *cis*-elements found within its promoter sequence. Joshee et al. (1998) found that the *Wsi18* promoter was not induced by ABA, which is in disagreement with all subsequent studies of the *Wsi18* promoter (Xiao and Xue, 2001; Yi et al., 2010, 2011; Nakashima et al., 2014). The difference in response to ABA was determined to be due to sequence variability between the different cultivars used in these studies (Xiao and Xue, 2001; Yi et al., 2011). The rice cultivar IR36 used by Joshee et al. (1998) is missing a single nucleotide from the inter-element spacer region between the CE and ABRE elements. The additional nucleotide is present in the ABA responsive versions of *Wsi18* found in the rice cultivars Nakdong (Yi et al., 2010, 2011), and Jarrah (Xiao and Xue, 2001). The relationship between ABRE and CE elements has been well studied in the barley promoter *HVA1*, which is a LEA3 promoter like *Wsi18*. The longer spacer region between the CE and ABRE elements was found to be essential for an ABA-dependent response (Ross and Shen, 2006). The nucleotide sequence between the CE and ABRE of *Wsi18* is the same

length in the ABA responsive versions of *Wsi18* as it is in the *HVA1* promoter. Xiao and Xue, (2001) confirmed that this CE-ABRE pair has a role in the ABA responsive nature of *Wsi18* by using base pair substitution mutagenesis to alter two base pairs of the ABRE core sequence. This resulted in a 4-fold reduction in ABA-induced expression. *Wsi18* has been shown to be induced by transcription factors in the ABA-dependent signaling pathway, but not those in the ABA-independent signaling pathways. AREB and ABF3 transcription factors, when constitutively expressed, have been shown to increase *Wsi18* mediated expression. CBF/DREB1 and OsNAC6 transcription factors from the ABA-independent signaling pathway when constitutively expressed did not change *Wsi18* mediated expression (Oh et al., 2005; Nakashima et al., 2014).

1.5 *Brachypodium distachyon*, a model monocot

Angiosperms (flowering plants) can be divided into two groups; monocotyledonous plants (monocots) and dicotyledonous plants (dicots). It is estimated that monocots diverged from dicots 140-150 million years ago (Chaw et al., 2004), and since then many differences have evolved. Dicots for example, have two cotyledons per embryo, a reticulated pattern of leaf venation, vascular tissue arranged in bundles, and a tap root system. Monocots only have one cotyledon per embryo, leaf veins arranged in a parallel pattern, unorganized vascular tissue, and a fibrous root system. The vast majority of molecular biology research in plants has been performed in dicots. Unfortunately, research done in dicots concerning some important agricultural traits, such as root system development, cannot be directly applied to monocots.

The family Poaceae consists of grasses in three subfamilies, Ehrhartoideae, Panicoideae, and Pooideae. The Ehrhartoideae include rice, The Panicoideae include

maize and sugar cane, and the Pooideae include Purple false brome (*Brachypodium distachyon*) and the Triticeae tribe which includes crops such as wheat, rye, and barley (The International Brachypodium Initiative, 2010). Rice has often been used as a model monocot plant, because it has a relatively small genome (441MB), and is an agriculturally important crop. However, in many ways rice is not an ideal model organism for research in the Pooideae subfamily as it does not share some important agricultural traits with Pooideae members. For instance, the root anatomy of rice is not a good model for Pooideae grasses because rice roots normally grow submerged in water, and have adapted to anaerobic conditions (Chochois et al., 2012). Other agriculturally interesting traits not found in rice include freezing tolerance, mycorrhizae symbiosis, and resistance to certain pathogens (Ozdemir et al., 2008).

Brachypodium distachyon (*B. distachyon*) is an emerging model for monocot plants that is more pertinent to the Pooideae subfamily. Phylogenetically *B. distachyon* diverged from its last common ancestor with the other Pooideae much more recently than the Pooideae subfamily's divergence from their last common ancestor with rice. It is estimated that *B. distachyon* diverged from wheat 32-39 million years ago, and from rice 40-53 million years ago (The International Brachypodium Initiative, 2010). As a result of its evolutionary relationship with both rice and wheat, *B. distachyon* has more co-linearity with the Pooideae genomes than they do with rice. This has proven useful in helping to arrange physical genetic maps for the barley genome (The International Barley Genome Sequencing Consortium, 2012), and can be applied to larger, more complex genomes, such as wheat's. *B. distachyon* is a native plant to the Mediterranean and Middle East regions, which are similar to where wheat originated, and has never been

domesticated. This means it still retains much genetic diversity that could be a valuable resource for improving crop traits such as disease resistance (Opanowicz et al., 2008).

B. distachyon has several traits desired of a model plant. It is self-fertilizing, has an established transformation protocol, a short stature of only 30 cm at maturity, a sequenced genome, and a short life cycle of 10-18 weeks. It is a diploid plant with 10 chromosomes (2n), and has a genome size of only 300 MB. This is much smaller than rice, which has 24 chromosomes (2n) and a genome size of 441 MB, and wheat, which has 42 chromosomes (2n) and a genome size of 16,700 MB (Opanowicz et al., 2008). These characteristics make *B. distachyon* a convenient model for molecular biology research.

1.6   Research objective

Water deficit stresses such as drought and high salinity are major causes of agricultural crop loss each year. Increasing the tolerance of crop plants to water deficit stresses would help to mitigate these loses and increase food availability. Tolerance to water deficit can be improved in crop plants by altering the expression of genes that confer increased stress tolerance to the plant, which requires the use of well-characterized promoters. The *Wsi18* promoter from rice is induced by various water deficit conditions such as drought stress, salt stress, and exogenous ABA (Xiao and Xue, 2001; Yi et al., 2010, 2011; Nakashima et al., 2014). A promoter's activity in different plants is not guaranteed to be the same as in its native plant, and it remains unknown whether the *Wsi18* promoter retains its water deficit-inducible characteristics when stably integrated into the genome of another monocot species. I hypothesize that the expression of genes under the control of the *Wsi18* promoter will increase following water deficit stress in the

model monocot species *B. distachyon*. The objective of this research is to evaluate the expression of the *uidA* reporter gene driven by the *Wsi18* promoter, in stably transformed *B. distachyon*, in response to water deficit stresses. Water deficit stress conditions were induced using the phytohormone ABA which is involved in water deficit stress cellular signaling, and water deficit stresses generated by mannitol and NaCl which generate osmotic and salt stresses respectively.

Chapter 2: Materials and Methods

2.1 Preparation of media and solutions

2.1.1 *Brachypodium distachyon* seed germination medium

Brachypodium distachyon (L.) P.Beauv (*B. distachyon*) ecotype Bd21 was used for all experiments. Seeds were germinated on medium containing 2.17 g/L Murashige & Skoog (MS) basal salt mixture (Murashige and Skoog, 1962) (PhytoTechnology Laboratories), 10 g/L sucrose, and 12 g/L of the gelling agent Phytagel™ (Sigma-Aldrich) in double distilled water (ddH$_2$O). For transgenic *B. distachyon* seeds which were hygromycin B-resistant, seed germination medium was supplemented with 115 mg/L hygromycin B (PhytoTechnology Laboratories) after autoclaving.

2.1.2 *B. distachyon* transformation media

All media used for *B. distachyon* transformation (see Sections 2.1.2.1 – 2.1.2.6) were made according to the *B. distachyon* transformation protocol by Alves et al. (2009).

2.1.2.1 M5 vitamin stock solution (100x)

M5 vitamin stock solution was a component of several media used for *B. distachyon* transformation. M5 vitamin solution contained 40 mg/L nicotinic acid, 50 mg/L thiamine-HCL, 4 g/L cysteine, 200 mg/L glycine, and 40 mg/L pyridoxine-HCL in ddH$_2$O.

2.1.2.2 B5 vitamin stock solution (100x)

B5 vitamin stock solution was used in MSR63 medium for root establishment. B5 vitamin solution contained 100 mg/L nicotinic acid, 1 g/L thiamine-HCL, 100 mg/L pyridoxine-HCL, and 10 g/L myo-inositol in ddH$_2$O.

2.1.2.3   MSB3 solid medium for callus culture

Calli were cultured on MSB3 medium composed of 4.33 g/L MS basal salt mixture, 40

mg/L Ethylenediaminetetraacetic acid ferric sodium salt (Fe-EDTA), 30 g/L sucrose, 2.5

mg/L 2,4 dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich), and 2 g/L Phytagel™ in

ddH$_2$O. After autoclaving, 10 ml/L of M5 vitamin stock solution (100x) was added.

MSB3 medium was supplemented with additional components for certain stages of the

transformation process. For callus production, MSB3 medium was supplemented with

600 µg/L CuSO$_4$·5H$_2$O (MSB3 + Cu0.6). Calli and *A. tumefaciens* co-culturing MSB3

medium   (MSB3+AS60)   was   supplemented   with   60   mg/L   3',5'-dimethoxy-4'-

hydroxyacetophenone (acetosyringone) (Sigma-Aldrich). Callus selection MSB3 medium

(MSB3 + H100 + T225) was supplemented with 600 µg/L CuSO$_4$·5H$_2$O before

autoclaving,   as   well   as   100   mg/L   hygromycin   B   and   225   mg/L   timentin

(PhytoTechnology Laboratories) after autoclaving.

2.1.2.4   MSB + AS45 liquid medium for *Agrobacterium tumefaciens* suspension

Agrobacterium tumefaciens (*A. tumefaciens*) strain AGL1 was incubated in MSB +

AS45 liquid medium prior to the inoculation of calli. This medium was composed of 4.33

g/L MS basal salt mixture, 40 mg/L Fe-EDTA, 10 g/L sucrose, and 10 g/L mannitol in

ddH$_2$O. After autoclaving, 45 mg/L acetosyringone was added.

2.1.2.5   MSR26 medium for shoot regeneration

A. tumefaciens-inoculated calli that survived the selection process were cultured on

MSR26 medium to regenerate shoots. MSR26 medium was composed of 4.33 g/L MS

basal salt mixture, 40 mg/L Fe-EDTA, 30 g/L sucrose, and 2 g/L Phytagel™ in ddH$_2$O.

After autoclaving, 10 ml/L M5 vitamin stock solution (100x), 200 µg/L kinetin (Sigma-Aldrich), 20 mg/L hygromycin B, and 225 mg/L timentin were added to the medium.

2.1.2.6   MSR63 medium for root establishment

Shoots regenerated from calli were transferred to MSR63 medium to establish roots prior to transferring to soil. MSR63 medium was composed of 4.33 g/L MS basal salt mixture, 40 mg/L Fe-EDTA, 10 g/L sucrose, 6 g/L agar, 2 g/L Phytagel™, and 7 g/L charcoal in ddH$_2$O. After autoclaving, 10 ml/L B5 vitamin stock solution (100x), and 112 mg/L timentin were added.

2.1.3   *B. distachyon* hydroponic growth medium

All *B. distachyon* plants used for GUS histochemical assays, RT-qPCR analysis, and relative water content measurements were grown hydroponically. Hydroponic growth medium was composed of 49 mg/L H$_3$PO$_4$, 250 mg/L CaCl$_2$, 185 mg/L MgSO4·7H$_2$O, 179 mg/L KCl, 58 mg/L NaCl, 241 mg/L NH$_4$Cl, 454 mg/L KNO$_3$, 2.86 g/L H$_3$BO$_3$, 1.81 g/L MnCl$_2$·4H$_2$O, 220 mg/L ZnSO$_4$·7H$_2$O, 51 mg/L CUSO$_4$, and 120 mg/L NaMoO$_4$·2H$_2$O in ddH$_2$O.

2.1.4   Bacterial culture medium

Escherichia coli (*E. coli*) and *A. tumefaciens* were cultured using lysogeny broth (LB) medium. Liquid LB medium contained 5 g/L Bacto™ yeast extract (Becton, Dickson and Company), 10 g/L Bacto™ tryptone (Becton, Dickson and Company), and 10 g/L sodium chloride in ddH$_2$O. Solid LB medium contained an additional 10 g/L Bacto™ agar (Becton, Dickson and Company). LB were supplemented with antibiotics for selection purposes, which were added after autoclaving. Bacterial cultures hosting the

pWsi18 plasmid were cultured with 100 mg/L ampicillin (Sigma-Aldrich), bacterial cultures hosting the pDONR221 or pMDC163 plasmids were cultured with 50 mg/L kanamycin (Sigma-Aldrich), and all *A. tumefaciens* cultures included 100 mg/L rifampicin (Sigma-Aldrich).

2.1.5   Sterilization of media and pH adjustment

All media were adjusted to pH 5.8, except for MSB + AS45 liquid medium for *A. tumefaciens* suspension, which was adjusted to pH 5.5. All media were sterilized in the autoclave for 20 minutes at 121˚C, and 20 psi. Vitamin stock solutions (M5 and B5) were filter sterilized using AcroVac™ filter units, pore size 0.2 μm (Pall Corporation). Antibiotic stock solutions were filter sterilized using Corning® syringe filters, pore size 0.2 μm (Sigma-Aldrich).

2.2   *In silico* analysis

The promoter sequence of *Wsi18* was obtained from the NCBI GenBank, entry GQ903792.1 (http://www.ncbi.nlm.nih.gov/genbank/).

The PLACE database was used to identify *cis*-elements in the *Wsi18* promoter sequence up to 1734 bp upstream of the *Wsi18* gene coding sequence (Higo et al., 1999).

The site www.phytozome.net was used to identify possible homologues of *Wsi18* native to *B. distachyon*, based on sequence similarity of *B. distachyon* proteins to the *Wsi18* protein sequence (Goodstein et al., 2012). Possible protein homologues were identified by www.phytozome.net, which uses the dual affine Smith-Waterman algorithm to align sequences based on the minimum number of mutational events needed to convert one sequence to the other, while accounting for deletions and insertions (Smith and

Waterman, 1981). Clustal Omega was used to generate a protein sequence alignment of the *Wsi18* and *Bradi2G47700* amino acid sequences, as shown in Figure 6 (Goujon et al., 2010; Sievers et al., 2011; McWilliam et al., 2013).

2.3   Vector construction

An ampicillin-resistant plasmid, pWsi18, containing the *Wsi18* promoter sequence was obtained from Ju-Kon Kim at Seoul National University. *E. coli* DH5α was transformed with the pWsi18 plasmid via electroporation. Transformed DH5α were grown on LB supplemented with 100 mg/L ampicillin at 37°C overnight to select successfully transformed colonies. DH5α containing pWsi18 were grown in LB with 100 mg/L ampicillin at 37°C overnight. The pWsi18 plasmid was extracted from the overnight culture using a QIAprep Spin Miniprep Kit (Qiagen). PCR was performed to amplify the *Wsi18* promoter from the pWsi18 plasmid template using primers FWsi18 and RWsi18 (Table 1). These primers include the Gateway® Technology attachment site sequences attB1 and attB2, on the forward and reverse primer respectively. Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc.) was used according to the product's instructions to amplify the *Wsi18* promoter sequence flanked by attB sites in a PCR reaction with an annealing temperature of 55.7°C and an extension time of 90 seconds. The primers F2xCaMV35S and R2xCaMV35S with additional attB sites (Table 1) were used to amplify the *2xCaMV35S* promoter from the plasmid pMDC32 (Curtis and Grossniklaus, 2003) in a PCR reaction using Phusion® High-Fidelity DNA Polymerase, with an annealing temperature of 57°C and an extension time of 30 seconds. The *2xCaMV35S* promoter is a modified version of the CaMV35S promoter which

contains a tandem duplication of 250 bp upstream of the core promoter and drives strong constitutive expression of genes under its control (Kay et al., 1987).

The *Wsi18* promoter and the *2xCaMV35S* promoter, each flanked by Gateway® attachment site sequences, were inserted into separate pMDC163 plasmids using Gateway® Technology (Hartley et al., 2000). To accomplish this, the promoters were cloned into the kanamycin-resistant pDONR221 plasmid using Gateway® BP Clonase II® Enzyme Mix (Thermo Fischer Scientific) according to the product instructions to generate entry vectors. The entry vectors were introduced into *E. coli* DH5α via electroporation and grown on LB agar with 50 mg/L kanamycin overnight at 37°C. DH5α colonies containing the entry vectors were grown overnight in LB with 50 mg/L kanamycin at 37°C. A Qiaprep Spin Miniprep kit was used to extract the entry vectors from the overnight cultures. Entry vectors were cut using the PvuI restriction enzyme (New England Biolabs Inc.) to cut the kanamycin resistance *neomycin phosphotransferase II* (*npt II*) gene and linearize the plasmid. Linearized entry vectors were run on an agarose gel and extracted using a QIAquick Gel Extraction Kit (QIAgen). This ensured the absence of intact kanamycin-resistant entry vectors, as *npt II* is also the selective marker on the expression vector pMDC163. Gateway® LR clonase II® Enzyme Mix (Thermo Fisher Scientific) was used according to the product instructions to insert the promoters from the linearized pDONR221-*Wsi18* and pDONR221-*2xCaMV35S* plasmids into separate pMDC163 vectors (Curtis and Grossniklaus, 2003). The LR reactions were introduced into DH5α via electroporation and grown overnight in LB with 50 mg/L kanamycin at 37°C. Plasmids were extracted using a QIAprep® Spin Miniprep Kit. The end results were pMDC163 plasmids containing either the *Wsi18* promoter or

the *2xCaMV35S* promoter. The promoters were inserted upstream of the *uidA* reporter gene (Jefferson et al., 1987), and followed by a *nopaline synthase* (*nos)* terminator (Bevan et al., 1983). Other features of this plasmid include the left and right transfer DNA (T-DNA) border sequences for *A. tumefaciens*-mediated transformation. The inserted promoter, the *uidA* gene followed by a *nos* terminator, and a *hygromycin phosphotransferase* (*hpt*) gene for hygromycin B resistance (Elzen et al., 1985) driven by a *CaMV35S* promoter (Odell et al., 1985) and followed by a *CaMV35S* terminator (Pietrzak et al., 1986), are all found within the T-DNA borders. Outside the T-DNA borders is an *npt* II gene for kanamycin resistance (Brzezinska and Davies, 1973) and the bacterial origin of replication pSV1 (Figure 3).
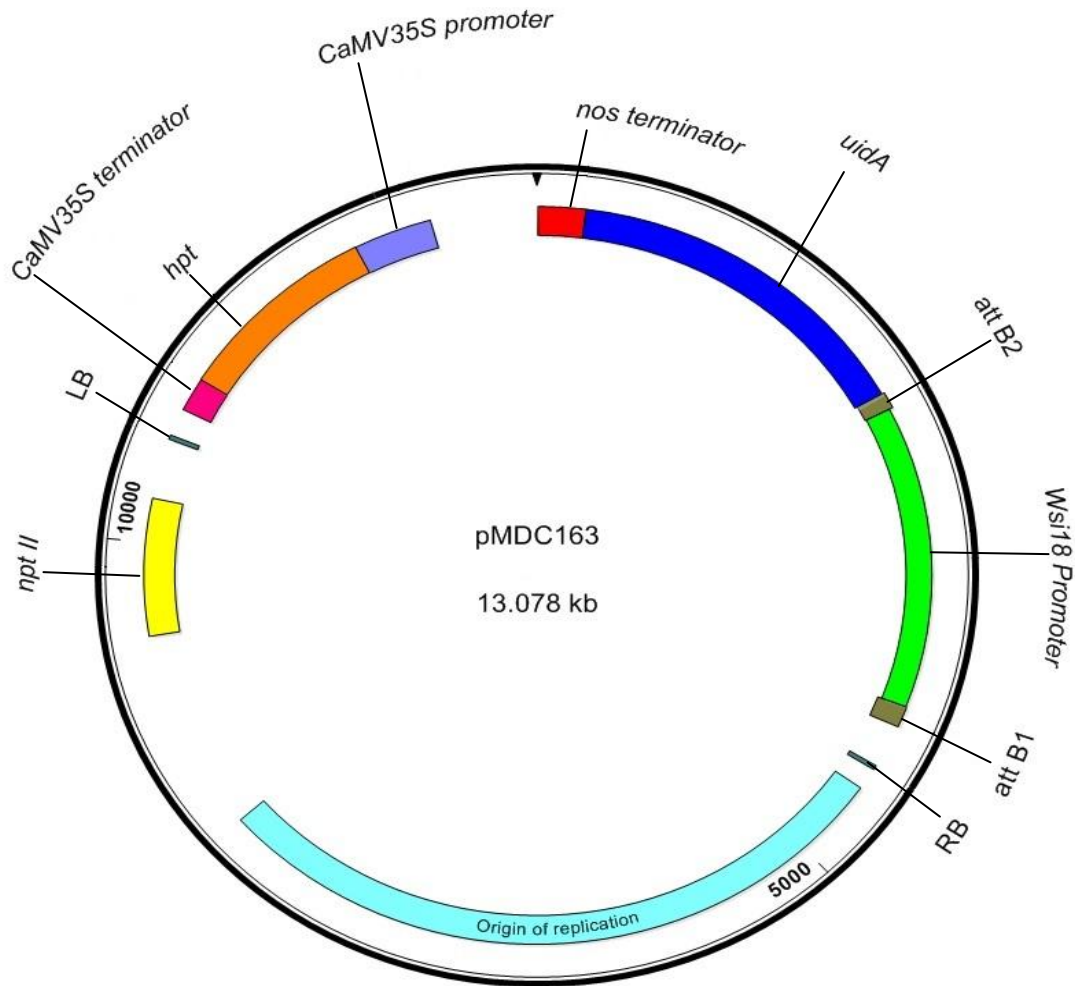
Figure 3: Plasmid map of pMDC163-*Wsi18*

A map of the pMDC163-*Wsi18* plasmid used for *A. tumefaciens*-mediated transformation of *B. distachyon* calli. The region between the left and right transfer DNA borders (LB, RB) is the region transferred into the *B. distachyon* genome during transformation. Within the LB and RB is the *Wsi18* promoter inserted upstream of the *uidA* reporter gene for GUS expression, followed by a *nos* terminator. There is also an *hpt* gene encoding hygromycin B resistance under the control of a *CaMV35S* promoter and followed by a *CaMV35S* terminator. Outside of the T-DNA borders is an *npt II* gene for kanamycin resistance and a bacterial origin of replication pSV1.

2.4   *B. distachyon* growth conditions

B. distachyon seeds were germinated in aseptic conditions. The lemma was removed from the seeds and seeds were soaked in ddH$_2$O for 2 hours at room temperature. For sterilization, seeds were soaked in 70% ethanol for 30 seconds. Ethanol was then drained and seeds were rinsed with sterile ddH$_2$O. Seeds were then soaked in a 1.3% hypochlorite solution for 4 minutes, followed by three washes with ddH$_2$O. Sterilized seeds were sown on solid seed germination medium and placed at 4°C for 4 days in the dark to synchronize germination. To initiate germination, seeds were transferred to a growth room with a 16 hour photoperiod, a light intensity of 75 µmol/m^2/s, and a temperature of 25°C, for 7 days.

Successfully germinated seedlings were transferred to sterilized Magenta™ GA-7 Plant Tissue Culture Boxes (Sigma-Aldrich) containing 125 ml of hydroponic growth medium. Plants were floated on the medium using styrofoam rafts with holes through the centre and strips of sterilized sponge to hold the plants in place. Seedlings were placed through the raft holes so that the roots were fully submerged in the medium (Figure 5B). Six *B. distachyon* plants were grown in each Magenta™ box. Plants were grown in the growth chamber with a 20 hour photoperiod, a light intensity of 172 µmol/m^2/s, a temperature of 22°C, and 25% humidity.

2.5   *B. distachyon* callus culture

B. distachyon calli were cultured according to the protocol of Alves et al. (2009). Immature seeds that had just begun desiccation were collected from *B. distachyon* ecotype Bd21, and sterilized as described in Section 2.4. The embryo was removed from

the seed, plated on solid MSB3 + CU0.6 medium, and cultured in the dark at 25°C. Any roots which grew from the embryos were removed. Once calli had developed, compact embryogenic calli (CEC) were separated from any soft wet friable calli, and transferred to fresh MSB3 + CU0.6 medium. CEC were grown in the dark at 25°C for up to 8 weeks. In that time, CEC were divided into smaller fragments to multiply explants for transformation, and placed on fresh MSB3 + Cu0.6 medium every 2 weeks.

2.6   Transformation of *B. distachyon* calli

B. distachyon calli were transformed as described by Alves et al. (2009). The pMDC163-*Wsi18* expression vector and the pMDC163-*2xCaMV35S* expression vector were introduced into separate cultures of *A. tumefaciens* strain AGL1 via electroporation. AGL1 was rifampicin-resistant. *A. tumefaciens* was grown in LB containing 100 mg/L rifampicin and 50 mg/L kanamycin, at 28°C, until the optical density of the suspension at $\lambda = 600$ nm (OD_{600}) was 0.8. The *A. tumefaciens* liquid culture was centrifuged at 40,695 x g at 4°C for 15 minutes to pellet the bacterium. The supernatant was removed and replaced with an equal volume of AS45 liquid medium. The cells were resuspended and the culture was grown at 28°C for approximately 45 minutes to achieve an OD_{600} of 1. Calli were flooded with *A. tumefaciens* in AS45 medium and left in the laminar flow hood at room temperature for 5 minutes. After the 5-minute inoculation, calli were transferred to sterile filter paper for 7 minutes as a desiccation treatment. Following desiccation, calli were transferred to MSB3 + AS60 medium for co-culturing of *A. tumefaciens* and CEC. Co-culturing continued for 2 days in the dark at 25°C. After co-culturing, CEC were transferred to MSB3 + H100 + T225 solid medium containing 100 mg/L hygromycin B to select for transformed CEC, and 225 mg/L timentin to stop the

growth of *A. tumefaciens*. *A. tumefaciens*-inoculated CEC were cultured in the dark at 25°C for 6 weeks. CEC which survived hygromycin B selection were transferred to MSR26 medium for shoot regeneration. MSR26 plates were cultured at 25°C, with a 16-hour photoperiod and a 75 µmol/m^2/s light intensity. Plant regeneration lasted up to 12 weeks, with calli being transferred to fresh MSR26 medium every 4 weeks. As shoots regenerated, any shoots that appeared robust and healthy were transferred to tubes containing MSR63 medium for root establishment and allowed to grow in MSR63 medium until plants were fully rooted. Fully rooted plants were transferred to pots containing Pro-mix® BX Mycorrhizae growing medium (Premier Tech Horticulture) to continue growing (Figure 4).
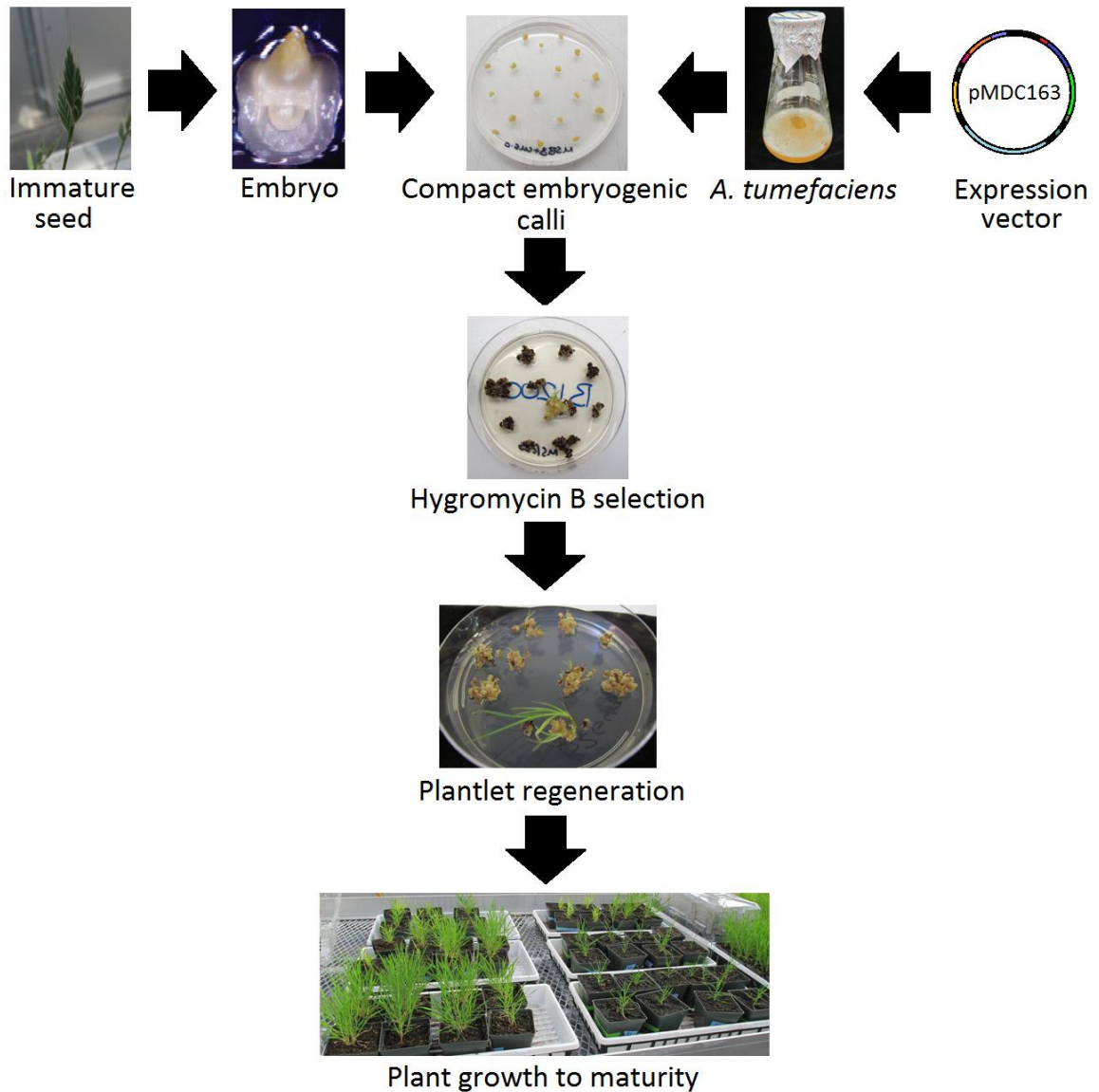
Figure 4: *A. tumefaciens*-**mediated transformation of** *B. distachyon* **calli**

Transgenic *B. distachyon* plants were generated through *A. tumefaciens*-mediated transformation of compact embryogenic calli. Immature embryos were extracted from immature seeds and plated on callus inducing medium to produce CEC. CEC were inoculated with *A. tumefaciens* hosting either the pMDC163-*Wsi18* plasmid, or pMDC-*2xCaMV35S* plasmid. Transgenic calli were selected using hygromycin B, and plants were regenerated from calli that survived selection.

2.7　　Verification of transgenic plants

PCR was performed to genotype plants regenerated from calli (T0 generation) by using DNA extracted from T0 plants to amplify the *Wsi18:uidA* construct. Leaves of T0 plants were flash-frozen in liquid nitrogen and homogenized with a TissueLyser II machine (Qiagen). To each homogenized sample, 500 µL of DNA extraction buffer was added. DNA extraction buffer consisted of 2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris, 20 mM EDTA, and 1.4 M NaCl, in ddH$_2$O at pH 8.0. Subsequently, 500 µL of chloroform was added, and samples were incubated at 50°C for 1 hour. Samples were centrifuged at 18,000 x g for 5 minutes, and the DNA-containing supernatant was transferred to a fresh microcentrifuge tube. To precipitate the DNA, 280 µL of isopropanol was added. Samples were then centrifuged at 18,000 x g for 15 minutes to pellet the DNA, and the supernatant was discarded. The DNA pellet was washed 3 times with 70% ethanol. The DNA was then dissolved in sterile ddH$_2$0. PCR reactions were set up for each putative transgenic line. The forward primer FWsi18/GUS, which binds to the *Wsi18* promoter, and the reverse primer RWsi18/GUS, which binds to the *uidA* gene, were used to amplify the *Wsi18:uidA* construct from the T0 plant DNA (Table 1). 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. The DNA of transformed lines produced a 628 bp PCR product.

Seeds collected from T0 plants were also germinated on seed germination medium containing 115 mg/L hygromycin B for 6 days to confirm they were transgenic. The seeds of successfully transformed lines produced healthy-looking seedlings, while plants which were not transgenic produced seeds which failed to germinate (Figure 5A).
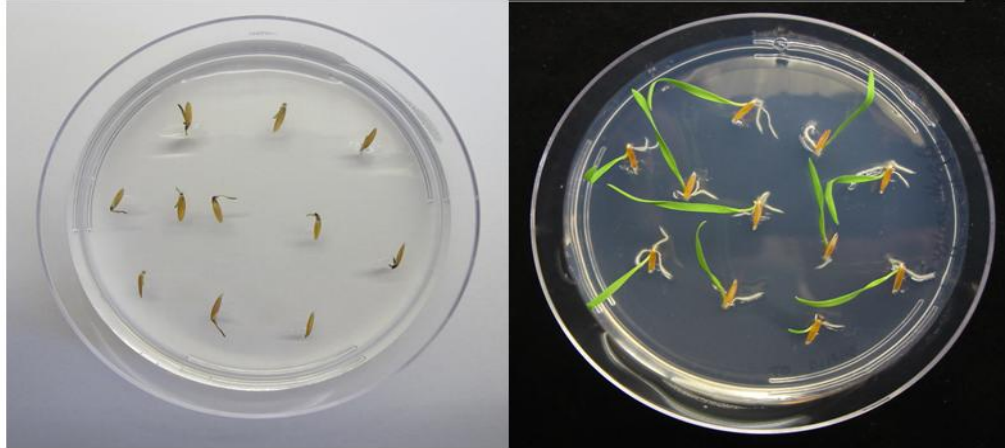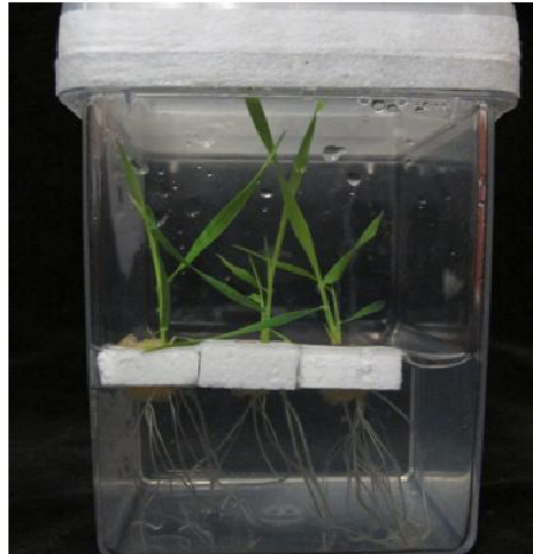
Figure 5: Germination and growth of *B. distachyon* in sterile conditions

A)   *B. distachyon* seeds on seed germination medium containing 115 mg/L hygromycin B after 6 days. On the left are wild-type *B. distachyon* seeds which are not hygromycin B-resistant. On the right are *B. distachyon* seeds collected from T0 plants which were confirmed to contain the T-DNA region of the pMDC163 plasmid using PCR genotyping, and thus are hygromycin B-resistant.

B)   Three-week-old hydroponically-grown *B. distachyon*. Plants at this growth stage were used for stress treatments described in Section 2.8.

2.8   Water deficit stress treatments

Stress treatments consisted of mannitol to produce osmotic stress conditions, NaCl to produce salinity stress, or ABA exposure to initiate the ABA-dependent water deficit signaling pathway. Treatments were performed by transferring 3-week-old, hydroponically grown *B. distachyon* plants of the T1 generation to fresh hydroponic growth medium supplemented with the stressor (Figure 5B). For mannitol treatment, plants were grown in medium containing 400 mM mannitol for 18 hrs. For NaCl treatment, plants were grown in medium containing 300 mM NaCl for 2 hours. For ABA treatment, plants were grown in medium containing 100 μM ABA for 8 hours. Unstressed control plants of the same transgenic lines used in the stress treatments were transferred to fresh hydroponic growth medium for the same duration as their respective stress treatment. These stress treatments were used for the GUS histochemical assay (Section 3.5), RT-qPCR analysis (Sections 3.3, and 3.6), and relative water content measurements (Section 3.7).

2.9   Relative water content

Relative water content (RWC) was measured following mannitol, NaCl, and ABA treatments to gauge the amount of water loss caused by each stress treatment (Smart and Bingham, 1974). Measurements were taken using wild-type plants grown at the same time and under the same conditions as described in Section 2.8 for plants used in the GUS histochemical and RT-qPCR analyses. All of the leaves from two plants were collectively used for one weight measurement. Leaves were collected and weighed immediately after plants were removed from hydroponic growth medium to obtain the fresh weight (W_{fresh}) measurement. The leaves were then floated on ddH_2O in petri

dishes for 6 hours, blotted dry, and reweighed to obtain the turgid weight (W_{turgid}). Leaves were then placed in an oven at 50°C for 48 hours and reweighed to obtain the dry weight (W_{dry}). RWC was calculated using the equation:

$$RWC = (W_{fresh} - W_{dry}) / (W_{turgid} - W_{dry}) * 100$$

RWC is a measure of the water status of a plant, showing the percentage of water in a set of leaves compared to the total amount of water the leaves are able to hold.

2.10   GUS histochemical assay

GUS histochemical assays were carried out as described by Jefferson et al. (1987). Shoots of plants, which had undergone the stress treatments described in Section 2.8, were cut off and immediately submerged in GUS staining solution. The GUS staining solution was composed of 2.0 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 1.0 mM $K_3Fe(CN)_6$ in ddH_2O. X-gluc is a substrate of β-glucuronidase (GUS), which is the product of the *uidA* gene. Each sample was vacuum-infiltrated for 30 minutes and incubated overnight at 37°C. Samples were submerged in 95% ethanol for 48 hours to remove chlorophyll and make the staining easier to observe.

2.11   RNA extraction, and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

A sample used for expression analysis consisted of the entire shoot portion 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 TissueLyser II

machine, and 1 mL of TRIzol® reagent (Thermo Fisher Scientific) was added to homogenized samples, followed by 200 µl chloroform. Samples were vigorously shaken, incubated at room temperature for 2 minutes, and centrifuged at 15,300 x g at 4°C for 15 minutes. The aqueous phase containing the RNA was then transferred to a fresh RNase-free micro-centrifuge tube. RNA was precipitated by adding 500 µl of 100% isopropanol to each sample and incubating at room temperature for 10 minutes. To pellet the precipitated RNA, samples were centrifuged at 15,300 x g for 15 minutes at 4°C. The supernatant was then removed and the pellet was washed three times with 70% ethanol, centrifuging at 2,655 x g for 5 minutes between each wash. When the final ethanol wash was removed, samples were re-suspended in 100 µl of RNase-free ddH₂O. 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, 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 ($\triangle\triangle$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 internal control gene, and also for the gene of interest. 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 (Hong et al., 2008). The Ct value of the internal control gene was subtracted from the Ct value of the gene of interest (*uidA*, or *Bradi2G47700*) to obtain the $\triangle$Ct value of each sample. Each transgenic line analyzed had 3 stress-treated samples, and 3 unstressed control samples. The $\triangle$Ct values of stress-treated plants and unstressed control plants were subtracted from the mean $\triangle$Ct value of the unstressed control plants within each line to obtain the $\triangle\triangle$Ct values. Fold change was calculated to express the difference in expression between stress-treated groups and unstressed control groups within each line using the equation:

$$\text{Fold change} = 2^{(-\triangle\triangle\text{Ct})}$$

2.12   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 $p < 0.05$.

Table 1: Primers used in this study

Primer name	Purpose	Primer sequence 5'→3'
FWsi18	*Wsi18* gateway cloning	GGGGACAAGTTTGTACAAAAAAGCAGG CTGGCTCTAGAGGATCCTGAGA
RWsi18		GGGGACCACTTTGTACAAGAAAGCTGG GTCCATGGCGCAAACTTGGCTG
F2xCaMV35S	*2xCaMV35S* gateway cloning	GGGGACAAGTTTGTACAAAAAAGCAGG CTCGACGGCCAGTGCCAAGC
R2xCaMV35S		GGGGACCACTTTGTACAAGAAAGCTGG GTCTCCTCTCCAAATGAAATGAACTTC
FWsi18/GUS	PCR genotyping	CCCGTTTCTCTGTCTTTTGC
RWsi18/GUS		GACCCACACTTTGCCGTAAT
FGUSQ	qPCR *uidA* cDNA	TACCGTACCTCGCATTACCC
RGUSQ		GAGGTTAAAGCCGACAGCAG
FBradi2G47700Q	qPCR *Bradi2G47700* cDNA	GGCAAGGACAAGACAGGAAG
RBradi2G47700Q		CCATTCCGATGGTGTTCATC
FSamDCQ	qPCR *SamDC* cDNA	TGCTAATCTGCTCCAATGGC
RSamDCQ		GACGCAGCTGACCACCTAGA

Chapter 3: Results

3.1 Identification of water deficit-related *cis*-element consensus sequences in the *Wsi18* promoter sequence

Consensus sequences of water deficit-related *cis*-elements were identified in the *Wsi18* promoter sequence by *in silico* analysis in an effort to determine which transcription factors may be binding to the *Wsi18* promoter to produce its water deficit-inducible expression pattern. The *cis*-element consensus sequences that were identified are not necessarily functional *cis*-elements, but are putative binding sites of transcription factors. Promoter analysis using the PLACE database revealed the consensus sequence of several water deficit-related *cis*-elements within the *Wsi18* promoter. As shown in Table 2, a number of *cis*-elements within both the ABA-dependent and ABA-independent stress signaling pathways were identified. From the ABA-dependent pathway, ABRE (Ross and Shen, 2006), CE (Ross and Shen, 2006), MYB (Abe et al., 1997), MYC (Abe et al., 1997), E-box (Seitz et al., 2010), WRKY (Rushton et al., 2012), and DC3 promoter-binding factor elements (Lopez-Molina and Chua, 2000) were all identified. From the ABA-independent pathway, DRE/CRT (Yamaguchi-Shinozaki and Shinozaki, 1994) and GT-1 box elements (Park et al., 2004) were identified. The core promoter elements identified include the TATA-box, as well as box II elements (Le Gourrierec et al., 1999). The presence of a variety of water deficit-related *cis*-elements in the *Wsi18* promoter sequence is encouraging for *Wsi18* to be water deficit responsive in species other than its native plant, rice, because the transcription factors which bind to these *cis*-elements are well conserved among plant species (Tripathi et al., 2012; Muthamilarasan et al., 2014; Liu and Chu, 2015; Chen et al., 2016)

Table 2: Putative water deficit-related *cis*-elements in the *Wsi18* promoter sequence identified using the PLACE database

Cis-element group	Factor site name	Consensus sequence	Number of occurrences	Related transcription factors
Core promoter	TATABOX2	TATAAAT	1	TATA binding protein
	TATABOX3	TATTAAT	1	
	TATABOX4	TATATAA	1	
	TATABOX5	TTATTT	2	
	Box II	GRWAAW	9	GT-1 factors
ABRE	ABREOSRAB21	ACGTSSSC	1	bZIP AREB/ABF
	ABRERATCAL	MACGYGB	8	
	ABRELATERD1	ACGTG	7	
	ABREATCONSENSUS	YACGTGGC	1	
	ABREA2HVA1	CCTACGTGGC	1	
	ACGTABREMOTIFA2OSEM	ACGTGKC	2	
CE	CE3OSOSEM	AACGCGTGTC	1	
MYB	MYBCOREATCYCB1	AACGG	5	MYB
	MYBCORE	CNGTTR	3	
	MYB2AT	TAACTG	1	
	MYBPZM	CCWACC	1	
	MYBST1	GGATA	6	
	MYB1AT	WAACCA	1	
	MYB2CONSENSUSAT	YAACKG	3	
	TATCCAOSAMY	TATCCA	1	
MYC	MYCATERD1	CATGTG	1	bHLH
	MYCATRD22	CACATG	2	
	MYCCONSENSUSAT	CANNTG	18	
E-box	EBOXBNNAPA	CANNTG	18	
W-box	WBOXHVISO1	TGACT	3	WRKY
	WRKY71OS	TGAC	7	
	WBOXNTCHN48	CTGACY	1	
DC3	DPBFCOREDCDC3	ACACNNG	6	bZIP
DRE/CRT	DRECRTCOREAT	RCCGAC	1	CBF/DREB1 DREB2
	CRTDREHVCBF2	GTCGAC	2	
	DRE1COREZMRAB17	ACCGAGA	1	
	CBFHV	RYCGAC	3	
GT-1 box	GT1GMSCAM4	GAAAAA	3	GT-1-like

3.2   Identification of the putative *Wsi18* homologous gene, *Bradi2G47700*, in *B. distachyon*.

In order to identify possible *Wsi18* homologous genes native to *B. distachyon*, the plant genome database of www.phytozome.net was used. Putative homologues were identified based on amino acid sequence similarity to the protein sequence of the *Wsi18* gene of rice. Protein sequence similarity was used rather than the promoter DNA sequence because coding regions are usually more conserved than non-coding regions (Taher et al., 2011). Using the amino acid sequences rather than the genes' DNA sequences also avoided differences in codon usage bias, which is the different frequency in the genome of different species for a particular DNA codon to encode an amino acid, over that of synonymous codons (Campbell and Gowri, 1990). As demonstrated in Figure 6, the *Bradi2G47700* amino acid sequence was identified as the most similar protein to *Wsi18* in *B. distachyon*. *Bradi2G47700* and *Wsi18* share 74% amino acid sequence similarity.

```
Bradi2G47700    MASRQDQASYRAGEAKARTEEKTGHVMGAAQEKAREAKDTASDAAGRAMGRGGDAKEATK
Wsi18           MASQQERASYHAGETKARAEEKTGRMMGTAQEKAREAKDTASDAAGRAMGRGHGAKEATK
                ***:*::***:***:***:*****::**:********************   ******

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

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

Bradi2G47700    DAVMNTIGMGADNNGSSTNVPGKDTSTYKPGKGY*---
Wsi18           DAVMYTLGMSGDNKN--NAAAGKDTSTYKPGTGSDYQ*
                **** *:**..**:   . . **********.*
```

Figure 6: Amino acid sequence similarity of *Wsi18* to the *B. distachyon* native gene *Bradi2G47700*

The *Bradi2G47700* amino acid sequence from *B. distachyon* aligned with the *Wsi18* 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. *Bradi2G47700* was identified as a putative *Wsi18* homologue using www.phytozome.net (Goodstein et al., 2012), and the sequence alignment was generated using Clustal Omega (Goujon et al., 2010; Sievers et al., 2011; McWilliam et al., 2013). *Bradi2G47700* and *Wsi18* proteins share 74% sequence similarity.

3.3 Quantitative measurement of *Bradi2G47700* gene transcription in *B. distachyon* using RT-qPCR

The level of transcription of the *Bradi2G47700* gene in wild-type *B. distachyon* plants subjected to water deficit conditions was compared to the transcription level of *Bradi2G47700* in unstressed control plants to assess whether *Bradi2G47700* is water deficit-inducible like *Wsi18* is in rice. The existence of a putatively homologous gene with a similar expression pattern as *Wsi18*, in *B. distachyon*, is indicative that the transcriptional regulatory network necessary to regulate *Wsi18* may be conserved between *B. distachyon* and rice.

RT-qPCR analysis was used to examine the transcription level of the *Bradi2G47700* gene in 3-week-old wild-type *B. distachyon* plants. The *SamDC* gene, which has been shown to be stable under water deficit conditions (Hong et al., 2008), was used as a reference gene for RT-qPCR to normalize *Bradi2G47700* transcript levels between samples. The normalized level of *Bradi2G47700* transcripts in stress-treated plants were compared to the transcript level of unstressed control plants to calculate the relative fold change.

As shown in Figure 7, the transcription level of *Bradi2G47700* in *B. distachyon* was significantly higher following ABA and mannitol treatments. Plants treated with ABA were grown for 8 hours in hydroponic growth medium containing 100 µM ABA, which resulted in a 794.1 ± 504.9 (p = 0.01) fold increase in the average *Bradi2G47700* transcript level, as compared to the unstressed control group. Mannitol-treated plants were grown for 18 hours in hydroponic growth medium containing 400 mM mannitol, which resulted in a 101.8 ± 8.1 (p = 0.04) fold increase in the average *Bradi2G47700*

transcript level, as compared to the unstressed control group. NaCl-treated plants were grown for 2 hours in hydroponic growth medium containing 300 mM NaCl. NaCl treatment resulted in a 144.3 ± 114.3 (p = 0.12) fold change in the average *Bradi2G47700* transcript level, as compared to the unstressed control group.
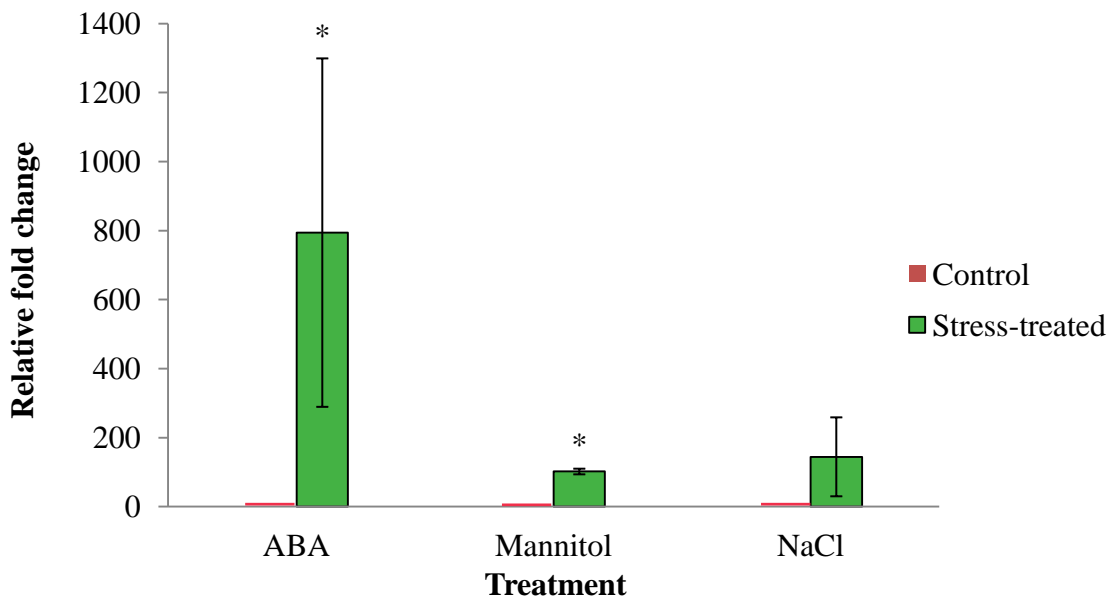
Figure 7: Change in transcript levels of the *Bradi2G47700* gene in *B. distachyon* following ABA, mannitol, and NaCl treatments

The level of transcription of *Bradi2G47700* in wild-type *B. distachyon* was analyzed following exposure to ABA, mannitol, and NaCl. RT-qPCR was used to measure transcript levels of the *Bradi2G47700* gene and the internal control gene *SamDC,* within each sample. *Bradi2G47700* transcript levels were normalized to those of *SamDC* to adjust between samples. The normalized values of each stress-treated group (ABA, mannitol, and NaCl) were compared to those of an unstressed control group to determine the relative fold change. Results are representative of 3 biological replicates in each treatment group, and error bars represent the standard error. Welch's two sample t-tests were used to assess the difference in fold change between stress-treated plants and unstressed control plants. The * symbol indicates a significant difference between stress-treated and unstressed control groups of $p < 0.05$.

3.4 *B. distachyon* transformation

Transgenic *B. distachyon* plants were developed in order to evaluate how the level of expression driven by the *Wsi18* promoter changes under water deficit-stress conditions and in response to ABA, in *B. distachyon*. To accomplish this, a genetic construct in which the *Wsi18* promoter drove the expression of the *uidA* reporter gene responsible for GUS expression (Figure 8A), was assembled and introduced into *B. distachyon* using *A. tumefaciens* mediated transformation of *B. distachyon* calli.

All shoots which regenerated from separate calli were considered independent transgenic lines and labeled with a number denoting the transgenic line (e.g. 1, 2, 3). Multiple shoots that regenerated from the same callus possibly resulted from the same or from separate transformation events, and were also denoted with a letter in order to differentiate between them (e.g. 4a, 4b). It was important not to consider these plants to be of the same line in order to ensure that all plants used to measure *uidA* expression that were considered to be of the same transgenic line, truly represented the same transformation events. When assessing the change in *uidA* expression (Sections 3.5, and 3.6), stress-treated plants were compared to unstressed control plants of the same transgenic line. Plants that resulted from a separate transformation event within one of the treatment groups would lead to a misrepresentation of the change in expression for that line. Conversely, separate plants that regenerated from the same callus could not be considered separate lines because if they were derived from the same transformation event, the similarity of their expression would misrepresent the variation between different transgenic lines. To avoid these issues, only 1 plant regenerated from each callus was used for further experimentation. In total, 884 calli were inoculated with *A.*

tumefaciens containing the expression vector pMDC163-*Wsi18*, and 32 of those inoculated calli regenerated shoots.

Genotyping using PCR amplification was performed to determine whether the transgenes were present in the DNA of T0 plants regenerated from calli after *A. tumefaciens* transformation. A PCR reaction was performed using primers FWsi18/GUS and RWsi18/GUS (Table 1) to amplify a 628 bp band of DNA, which overlapped the *Wsi18* promoter and *uidA* gene, from the DNA of regenerated plants (Figure 8A). As shown in Figure 8B, bands of the correct size (628 bp) were observed in 21 of the 32 regenerated plants. As 21 of the 884 inoculated calli were successfully transformed, the transformation efficiency was calculated to be 2.4%.

In addition to PCR genotyping, seeds of plants that had regenerated from the *A. tumefaciens*-inoculated calli (T1 generation seeds) were transferred to seed germination medium containing 115 mg/L hygromycin B for 6 days (Figure 5A). This level of selection was found to be sufficient to identify non-hygromycin-resistant, and therefore non-transgenic, seeds (Appendix Figure 2). All transgenic lines confirmed by PCR genotyping were able to germinate successfully with no discernible phenotypic difference from wild-type seedlings grown without hygromycin B. All regenerated lines 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 (Figure 5A). PCR genotyping and hygromycin B seed selection provided two forms of confirmation that the transgenic plants used to evaluate *Wsi18* driven expression of *uidA* in *B. distachyon*, had successfully integrated the T-DNA region of the pMDC163-*Wsi18* plasmid (Figure 8A).

A group of calli were also inoculated with *A. tumefaciens* containing the pMDC163-*2xCaMV35S* plasmid. Plants regenerated from these calli were used as a positive control for the GUS histochemical assay (Section 3.5), and were also genotyped using hygromycin B selection of seed germination. From the 803 calli inoculated with *A. tumefaciens* containing pMDC163-*2xCaMV35S*, 34 plants were regenerated. When seeds of those 34 plants were transferred to seed germination medium containing 115 mg/L hygromycin B for 6 days, 25 lines were found to be hygromycin B-resistant, giving a transformation efficiency of 3.11%.

A)
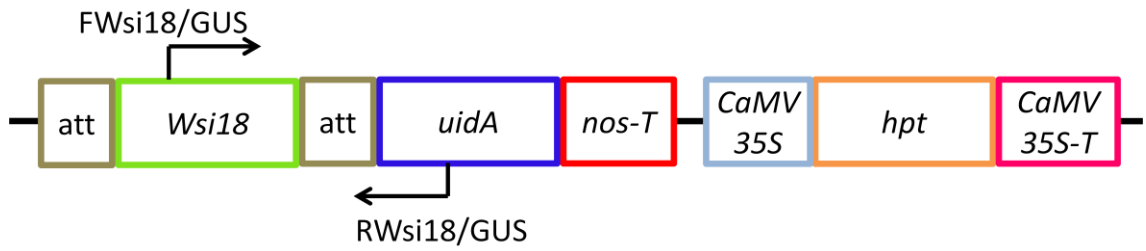
B)
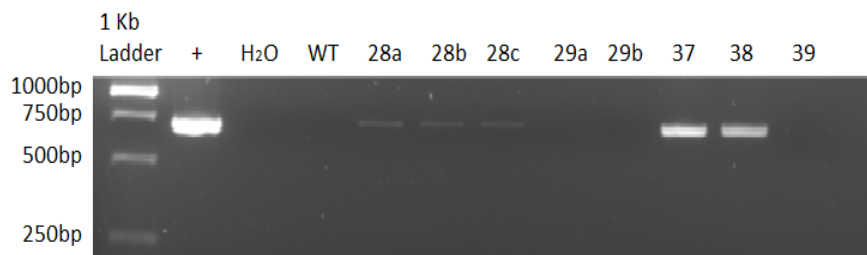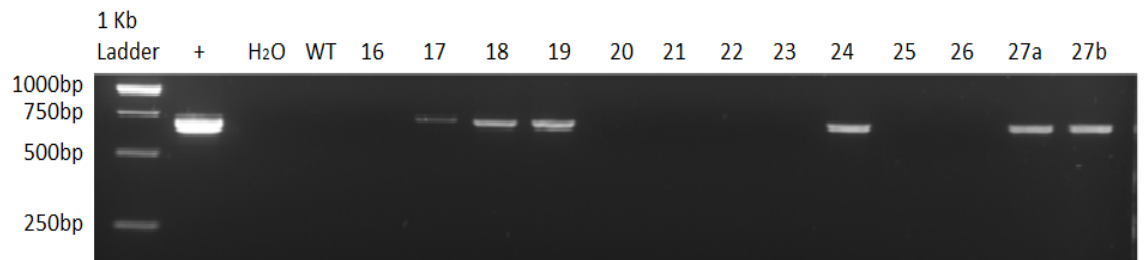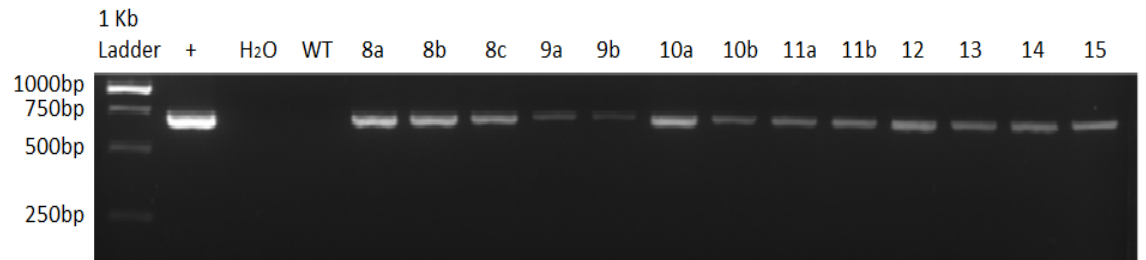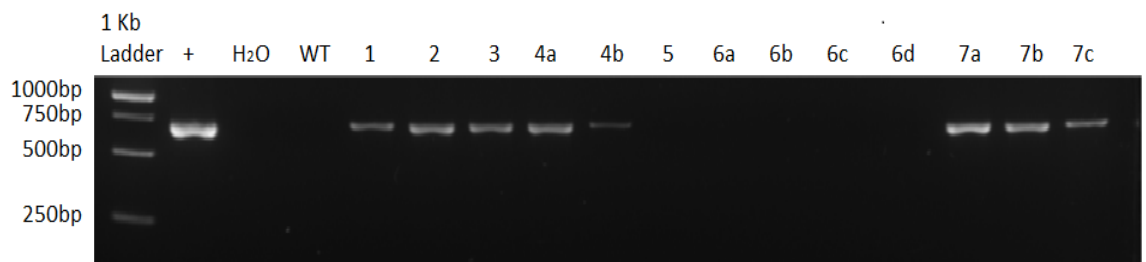
Figure 8: PCR genotyping of *B. distachyon* plants regenerated from pMDC163-*Wsi18* transformed calli

A)   The T-DNA region of pMDC163-*Wsi18*. In PCR genotyping the forward primer FWsi18/GUS bound to the *Wsi18* 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.

B)   Results of PCR genotyping of *B. distachyon* plants regenerated from calli that were inoculated with *A. tumefaciens* containing the pMDC163-*Wsi18* plasmid. PCRs using the DNA of transgenic plants produced a 628 bp sized band when run on an agarose gel. The DNA of plants regenerated from calli which were not transformed but escaped selection still to regenerate a plant, did not produce a band using these primers. The pMDC163-*Wsi18* 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.

3.5 Qualitative analysis of *uidA* expression driven by the *Wsi18* promoter in *B. distachyon* using the GUS histochemical assay

GUS histochemical analysis using X-gluc was used to visualize the pattern of expression mediated by the *Wsi18* promoter following ABA, mannitol, and NaCl treatments. β-Glucuronidase (GUS) is the product of the *uidA* gene. GUS cleaves the indoxyl portion of X-gluc, which then undergoes oxidative dimerization to produce dichloro-dibromoindigo, an insoluble blue dye. This blue dye accumulates in the sample, staining the plant blue in cells that are expressing the *uidA* gene.

Three transgenic lines in which the *uidA* reporter gene was driven by the *Wsi18* 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, and plants grown in the NaCl-treated group were grown for 2 hours in hydroponic growth medium containing 300 mM NaCl. 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.

For each stress-treatment group, GUS activity was observed in both the stem and leaves of shoots. As shown in Figure 9A, ABA-treated lines 10, 12, and 37 all showed a greater area of GUS activity in plants which had undergone the ABA treatment, compared to unstressed control plants. Plants from lines 10 and 37 did show small areas of GUS staining even in unstressed control plants. As shown in Figure 9B, when treated

with mannitol lines 11, 18, and 24 also showed a greater area of GUS staining compared to unstressed control plants, with only a small area of GUS activity detectable in the unstressed control plant of line 24. As shown in Figure 9C, lines 1, 10, and 37, which were grown in medium containing NaCl, also showed a larger area of GUS activity than their respective unstressed control plants. Unstressed control plants in line 10 and 37 again showed some GUS activity. 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.

GUS staining of plants transformed with the pMDC163-*2xCaMV35S* plasmid in which *uidA* is constitutively expressed acted as a positive control to demonstrate that the GUS staining procedure was effective for the entire plant. A wild-type plant acted as a negative control to ensure there was no endogenous GUS expression in *B. distachyon*, or contamination of the staining solutions (Figure 9D).

A)	Line 10	Line 12	Line 37

ABA

Unstressed control

B)	Line 11	Line 18	Line 24

Mannitol

Unstressed control

C)	Line 1	Line 10	Line 37

NaCl

Unstressed control

D)	*2xCaMV35S:uidA* (+ control)	Wild-type (- control)

GUS staining controls
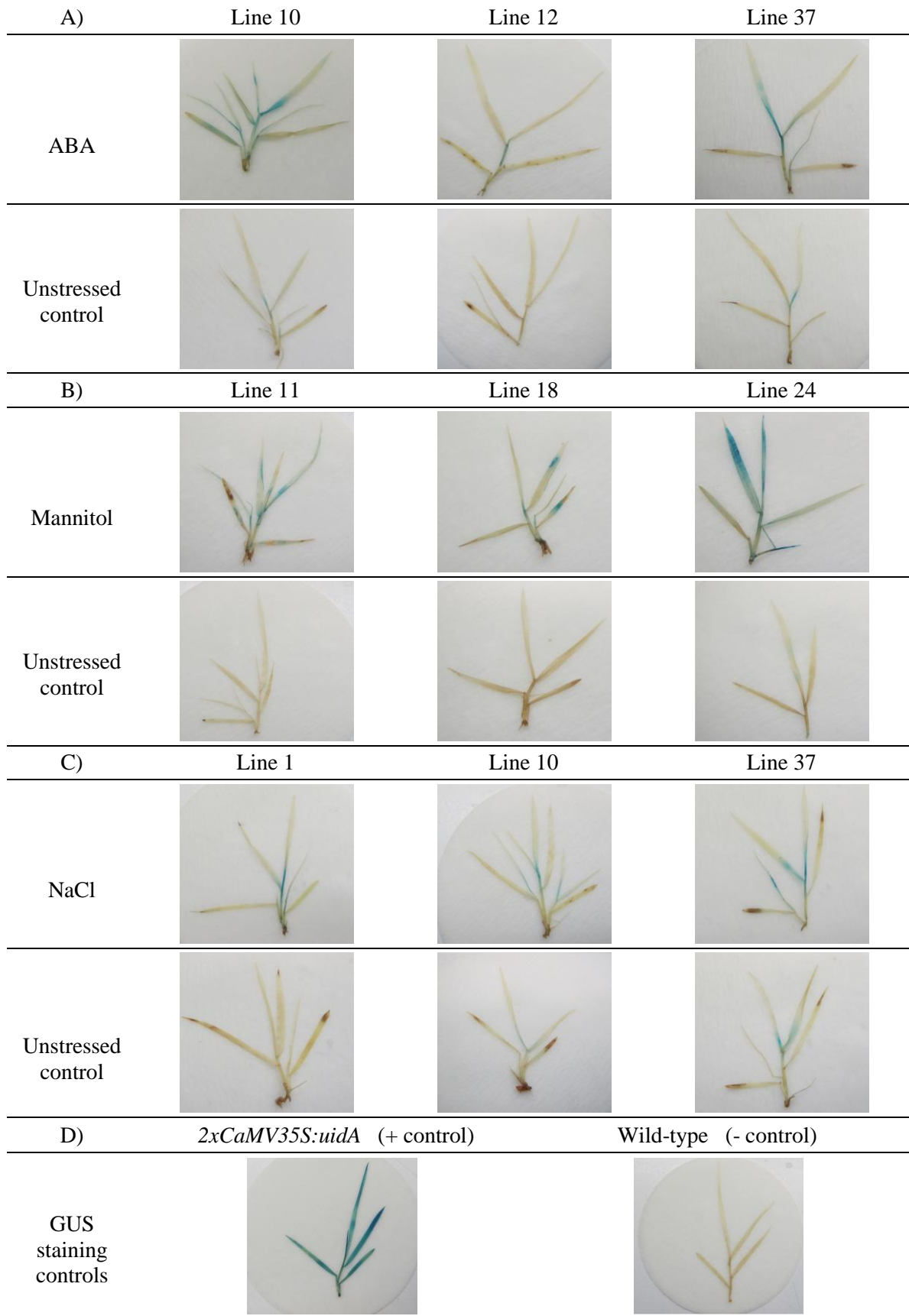
Figure 9: *Wsi18* **drives greater GUS activity in** *B. distachyon* **following ABA, mannitol, and NaCl treatments**

GUS histochemical assays were carried out to test the pattern of GUS expression following ABA, mannitol, and NaCl treatments. GUS activity is indicated by blue staining of the plant tissue. (A) GUS activity of plants grown for 8 hours in hydroponic growth medium containing 100 μM ABA, or unstressed control conditions. (B) GUS activity of plants grown for 18 hours in either hydroponic growth medium containing 400 mM mannitol or unstressed control conditions. (C) GUS activity of plants grown for 2 hours in either hydroponic growth medium containing 300 mM NaCl, or unstressed control conditions. (D) A *B. distachyon* plant in which the *uidA* gene was under the control of the constitutive *2xCaMV35S* promoter as a positive control, and a wild-type *B. distachyon* plant as a negative control.

3.6   Quantitative measurement of *Wsi18* mediated *uidA* transcription in *B. distachyon* using RT-qPCR

RT-qPCR analysis was used to examine the transcription levels of the *uidA* reporter gene driven by the *Wsi18* promoter in multiple transgenic lines of *B. distachyon* following ABA, mannitol, and NaCl treatments. The *SamDC* gene, which has been shown to be stably expressed under water deficit conditions in *B. distachyon* (Hong et al., 2008), 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 unstressed control plants of the same transgenic line to determine their relative fold change. All unstressed control plants were grown in fresh hydroponic growth medium for the same duration as their respective stress treatment. Plants were 3 weeks old at the time of analysis.

Plants in the ABA-treated group were grown for 8 hours in fresh hydroponic growth medium containing 100 µM ABA. As shown in Figure 10A, transgenic lines 10 and 37, had a significant increase in *uidA* transcript levels ($p < 0.05$) compared to unstressed control plants, while lines 3, 12, and 18 did not show a significant difference. Lines 10 and 37 had an increase of 5.6 ± 1.0 ($p = 0.002$) and 9.1 ± 2.0 fold ($p = 0.004$) respectively. Lines 3, 12, and 18 had relative fold changes of 1.4 ± 0.1 ($p = 0.61$), 1.3 ± 0.3 ($p = 0.62$) and 2.9 ± 0.8 ($p = 0.06$) respectively.

Mannitol-treated plants were grown for 18 hours in fresh hydroponic growth medium containing 400 mM mannitol. As shown in Figure 10B, transgenic lines 18 and 37, had a significant increase in *uidA* transcript levels ($p < 0.05$) compared to unstressed control plants, while lines 11, 15, and 27 did not show a significant difference. Lines 18 and 37

had an increase of 4.4 ± 1.1 (p= 0.04) and 8.5 ± 2.9 fold (p = 0.02) respectively. Lines 11, 15, and 27 had fold changes of 2.2 ± 1.3 (p = 0.57), 6.1 ± 3.5 (p = 0.38) and 3.0 ± 0.9 (p = 0.14) respectively.

Plants in the salinity treatment group were grown for 2 hours in fresh hydroponic growth medium containing 300 mM NaCl. As shown in Figure 10C, salinity treatment did not create a significant change in *uidA* transcript levels in any transgenic lines tested. Lines 1, 2, 3, 10, 12, 18 and 37 had fold changes of 0.5 ± 0.3 (p = 0.07), 1.6 ± 0.3 (p = 0.44), 1.5 ± 0.4 (p = 0.69), 1.8 ± 0.9 (p = 0.62), 0.8 ± 0.1 (p = 0.33), 0.9 ± 0.2 (p = 0.74), and 2.7 ± 0.9 (p = 0.24) respectively.

Overall, these results demonstrate that in *B. distachyon*, the *Wsi18* promoter can be induced by the phytohormone ABA and the osmotic stress conditions generated by mannitol. *Wsi18* is either not induced by NaCl in *B. distachyon*, or the intensity of the salinity treatment was not sufficient to induce *Wsi18*.
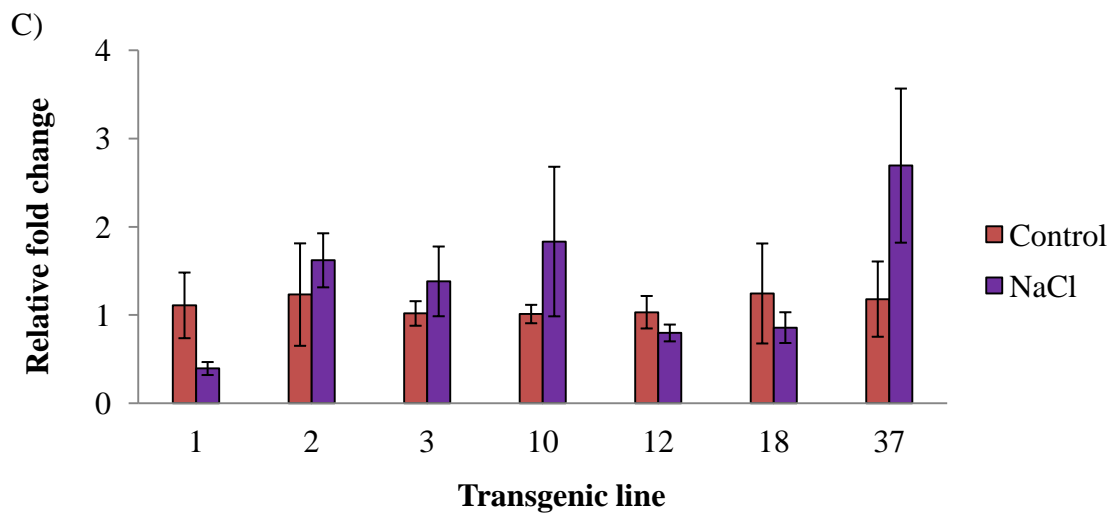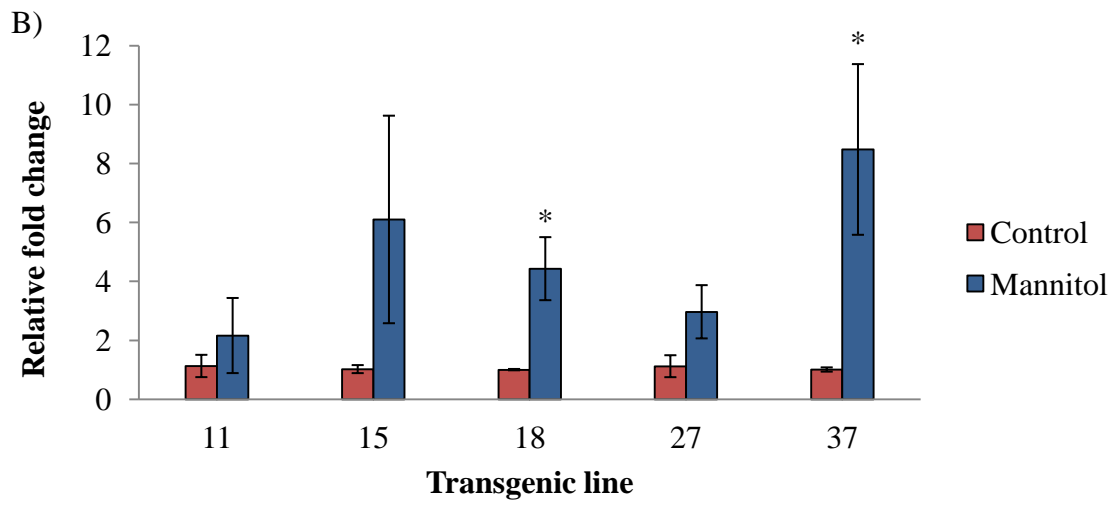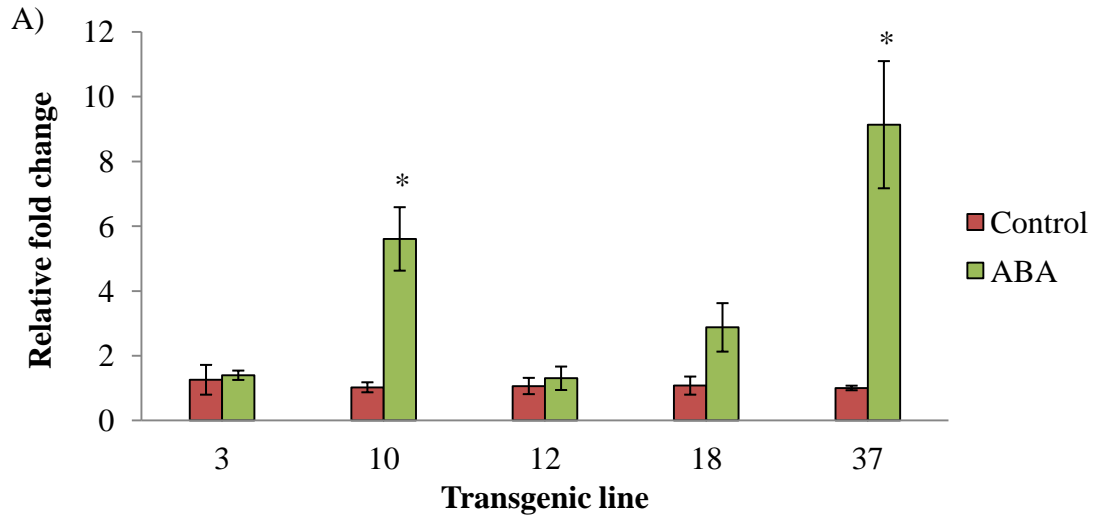
Figure 10: Transcription of the *uidA* gene driven by the *Wsi18* promoter following ABA, mannitol, and NaCl treatments in *B. distachyon*

The expression of *uidA* driven by the *Wsi18* promoter is responsive to ABA and mannitol exposure in *B. distachyon*. RT-qPCR was used to measure the level of *uidA* transcription in plants grown in hydroponic growth medium containing either (A) 100 µM ABA (for 8 hours), (B) 400 mM mannitol (for 18 hours), or (C) 300 mM NaCl (for 2 hours). Unstressed control plants were all grown in fresh hydroponic growth medium for the same duration as their respective stress treatment group. The level of *uidA* transcription in each sample was normalized to that of the internal control gene *SamDC*. The normalized level of *uidA* transcription of stressed plants was shown relative to that of unstressed control plants of the same transgenic line to obtain the relative fold change value. Mean ± standard error values are representative of 3 biological replicates. A Welch's two sample t-test was used to assess the difference in fold change between stress-treated and unstressed control plants of the same transgenic line. The * symbol indicates a statistical significance of $p < 0.05$.

3.7 RWC measurement of plants subjected to mannitol, NaCl, and ABA treatments

In order to confirm that the water deficit stress treatments used in the GUS histochemical assays and RT-qPCR analyses did in fact cause the plants to experience water deficit conditions, relative water content was measured in wild-type *B. distachyon* plants that had undergone the stress treatments. All unstressed control plants were grown in fresh hydroponic growth medium for the same duration as their respective stress treatment. As demonstrated in Figure 11, plants grown for 18 hours in hydroponic growth medium containing 400 mM mannitol had a RWC of 64.8 ± 4.6 %, a significant decrease compared to unstressed control plants, which had a RWC of 90.9 ± 3.9 % ($p = 0.0005$). Plants grown for 2 hours in hydroponic growth medium containing 300 mM NaCl had a RWC of 76.2 ± 4.1 %, a significant decrease compared to unstressed control plants, which had a RWC of 90.9 ± 1.1 % ($p = 0.006$). Plants grown for 8 hours in hydroponic growth medium containing 100 μM ABA did not elicit a significant difference in RWC compared to unstressed control plants, with RWCs of 93.4 ± 2.8 % and 90.4 ± 2.8 % respectively ($p = 0.45$). These results indicate that the mannitol and NaCl treatments were sufficient to reduce the RWC of 3-week-old *B. distachyon*. ABA is a signaling hormone and does not create osmotic stress. For this reason, ABA was not expected to induce a change in RWC.
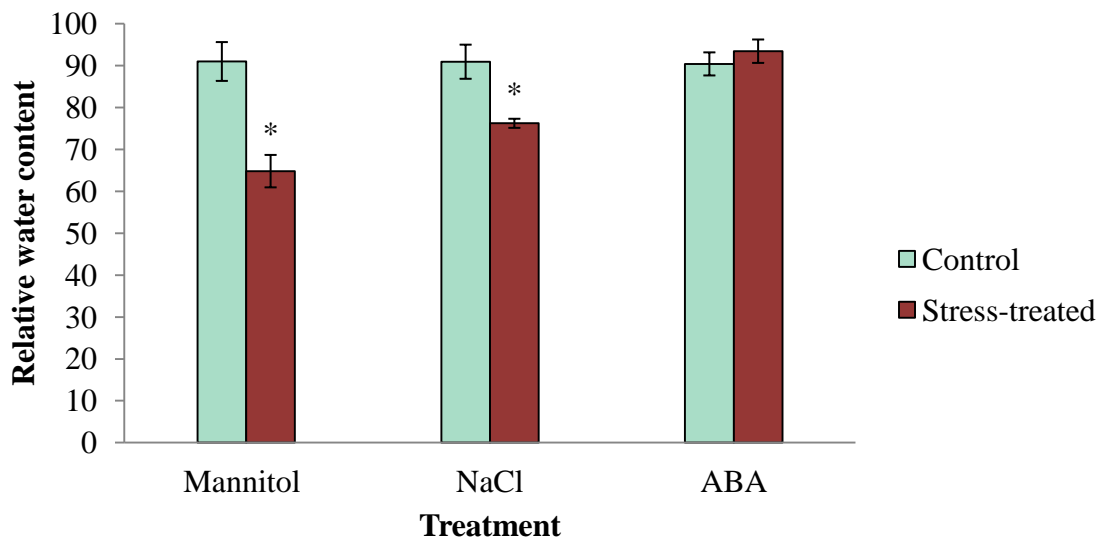
Figure 11: RWC decreases significantly following mannitol and NaCl treatments, but not ABA treatment.

Relative water content was measured in wild-type *B. distachyon* to assess the difference in water status between stress-treated plants, and unstressed control plants. Mannitol-treated plants were grown for 18 hours in hydroponic growth medium containing 400 mM mannitol, NaCl-treated plants were grown for 2 hours in hydroponic growth medium containing 300 mM NaCl, and ABA-treated plants were grown for 8 hours in hydroponic growth medium containing 100 µM ABA. Unstressed control plants were grown in hydroponic growth medium for the same duration as their respective stress treatment. These results are the average of 9-10 biological replicates within each treatment group. Error bars represent standard error. Welch's two sample t-tests were used to assess the difference between each stress treatment group and their respective unstressed control group. The * symbol indicates a significance of $p < 0.05$.

Chapter 4: Discussion

4.1 Evaluating the *Wsi18* promoter's suitability for water deficit-inducible expression in *B. distachyon*

4.1.1 A diverse selection of water deficit stress related *cis*-elements were putatively identified in the *Wsi18* promoter sequence

Promoter analysis using the PLACE database predicted putative *cis*-elements within the *Wsi18* promoter sequence. Several different groups of water deficit stress-related *cis*-elements were identified from both the ABA-dependent and ABA-independent abiotic stress response pathways, as well as core promoter elements (Table 2).

Core promoter element consensus sequences identified include TATA-boxes, as well as general transcription factor binding sites. Many box II *cis*-elements were identified in the *Wsi18* promoter sequence. Box II *cis*-elements are bound by GT-1 transcription factors which play a role in stabilizing the TFIIA-TBP-DNA binding in the transcription initiation complex (Le Gourrierec et al., 1999). These putative *cis*-elements may be involved in transcription initiation at the core promoter, which would make them integral to the proper functioning of the *Wsi18* promoter.

The PLACE database identified within the *Wsi18* promoter sequence several putative ABRE elements which could be contributing to the ABA responsive nature of *Wsi18*. ABRE elements are the main *cis*-elements responsible for ABA-dependent expression, and require either a coupling element (CE) or a second ABRE to be functional (Ross and Shen, 2006). Previously, Xiao and Xue, (2001) have demonstrated the existence of one functional CE-ABRE pairing in the *Wsi18* promoter sequence using mutational base pair substitution analysis. Other *cis*-elements involved in the ABA-dependent stress response

pathway that were identified in the *Wsi18* promoter sequence include MYB elements, MYC elements, W-boxes, DC3 promoter-binding factor elements, and E-boxes. MYB and MYC elements have been found sufficient to elicit an ABA response from promoters which lack any ABRE elements, such as the Arabidopsis *Rd22* promoter (Abe et al., 1997). W-box elements which bind WRKY transcription factors have been shown to regulate many steps of the ABA-dependent response pathway, acting as both activators and repressors. For example, the bZIP transcription factor ABI5 is repressed by the WRKY transcription factor AtWRKY40 in the absence of ABA. Once ABA removes this repression, ABI5 then upregulates the expression of another WRKY transcription factor, AtWRKY63, which activates the transcription of water deficit-responsive genes such as *Rd29A* (Rushton et al., 2012). Furthermore, other ABA-dependent *cis*-elements were identified within the *Wsi18* promoter sequence such as the DC3 promoter binding factor element, which is common in LEA genes (Lopez-Molina and Chua, 2000), and the E-box, which has been found to be important for ABA responsiveness to abiotic stresses such as cold (Seitz et al., 2010). Any of these putative *cis*-elements from the ABA-dependent pathway could play a role in the *Wsi18* promoter's ABA responsiveness. The different ABA-dependent transcription factors that bind these *cis*-elements may be expressed or activated by different concentrations of ABA, at different time points in the plant's response to water deficit stress, or in different cell types of the plant, to generate a finely tuned ABA-dependent expression response from the *Wsi18* promoter.

In addition to ABA-dependent *cis*-elements, the *Wsi18* promoter was found to contain consensus sequences of *cis*-elements from the ABA-independent pathway. Specifically, the DRE and GT-1 box *cis*-element consensus sequences were identified.

The DRE is the primary *cis*-element in the ABA-independent signaling pathway for water deficit response. A single DRE has been shown to be sufficient for water deficit-induced expression (Yamaguchi-Shinozaki and Shinozaki, 1994). The GT-1 box has been found to be involved in NaCl-induced expression in an ABA-independent manner (Park et al., 2004). Since it has been shown that the *Wsi18* promoter is induced by ABA (Xiao and Xue, 2001; Yi et al., 2010, 2011; Nakashima et al., 2014) and that ABA-insensitive varieties of the *Wsi18* promoter are still induced by water deficit (Joshee et al., 1998), putative *cis*-elements in the *Wsi18* promoter sequence from the ABA-independent signaling pathway suggest that *Wsi18* may be regulated by multiple signaling pathways.

The abundance and diversity of putative water deficit stress-related *cis*-elements in the *Wsi18* promoter sequence supports the possibility of *Wsi18* retaining its water deficit-inducible characteristics in other plants, as the transcription factors that bind to these *cis*-elements are well conserved across plant species. Many of these transcription factor families are also present in *B. distachyon*. Basic leucine zipper (bZIP) proteins, which bind to ABRE elements, have 96 members in *B. distachyon*, 75% of which have homologues in rice (Liu and Chu, 2015). MYB proteins, which bind to MYB *cis*-elements, have 98 members in *B. distachyon* (Muthamilarasan et al., 2014), and WRKY transcription factors, which bind to W-box *cis*-elements, have 86 members in *B. distachyon* (Tripathi et al., 2012). The ABA-independent transcription factors are well represented in *B. distachyon* as well. DREB transcription factors, which bind DRE/CRT *cis*-elements, have 65 members in *B. distachyon*, 51% of which have homologues in rice (Chen et al., 2016).

Although the transcription factor families that likely bind to the water deficit related *cis*-elements of the *Wsi18* promoter are available in *B. distachyon*, the transcription factors may not interact with the *cis*-elements in the same way in *B. distachyon* as they do in rice. Many levels of regulation affect transcription, and these regulatory processes may differ between species. Gene expression is regulated by signaling cascades, where one transcription factor initiates the expression of other transcription factors, continuing until the target gene is expressed. The expression of all of the transcription factors in the signaling cascade must be coordinated to produce the expected gene expression. Many regulatory processes can affect the availability of transcription factors in the cell, and interspecies differences in these processes could alter the coordination of signaling cascades in different species. Redox reactions activated by the ROS produced under stress conditions, protein degradation, proteolytic activation, protein interactions, and phosphorylation are all responsible for controlling the activation of transcription factors (Vaahtera and Broshé, 2011). DREB2A in Arabidopsis for example, is continuously degraded by a ubiquitin proteasome system which does not allow it to accumulate to high enough levels to be effective until stress is detected and the degradation signal is removed (Vaahtera and Broshé, 2011). Translation occurs in the cytosol, and therefore restricted access to the nucleus could be responsible for regulation of transcription factor activity. Some bZIP transcription factors in Arabidopsis are tethered to the membrane of the endoplasmic reticulum and cannot enter the nucleus until they are cleaved off in response to stress (Vaahtera and Broshé, 2011). Furthermore, phosphorylation via kinases regulates some WRKY, MYB, and bZIP transcription factors (Vaahtera and Broshé, 2011). The complicated nature of transcriptional regulation limits the knowledge that can

be gained from *cis*-element prediction alone. Therefore, further experimentation is needed to deduce whether the necessary transcriptional regulatory pathways are present in *B. distachyon* for *Wsi18* to retain its water deficit-inducible characteristics.

4.1.2   *B. distachyon* contains a putative *Wsi18* homologous gene, *Bradi2G47700*

The *Bradi2G47700* gene was identified as the protein with the greatest sequence similarity to *Wsi18* in the *B. distachyon* genome, sharing 74% amino acid sequence similarity (Figure 6). Transcription of *Bradi2G47700* was analyzed using RT-qPCR in wild-type *B. distachyon* plants following ABA, mannitol, and NaCl treatments, and was significantly induced by ABA and mannitol treatments. The presence of a putative *Wsi18* homologous gene in *B. distachyon* that has a water deficit-induced expression profile similar to *Wsi18* in rice, supports the possibility that the necessary transcriptional regulatory network for regulating *Wsi18* expression is somewhat conserved between rice and *B. distachyon*.

In response to mannitol and ABA treatments, the fold change of *Bradi2G47700* expression in wild-type *B. distachyon* was much greater than the fold change of *uidA* expression driven by the *Wsi18* promoter in transgenic *B. distachyon*. One reason for this could be that *Bradi2G47700* is in its native context in *B. distachyon*, including chromatin structure and the presence of other regulatory elements such as enhancers (van Arensbergen et al., 2014). The *Wsi18:uidA* construct, however, could be inserted anywhere in the *B. distachyon* genome and would be affected by the local chromatin structure as well as regulatory elements such as enhancers and insulators present in that area of the *B. distachyon* genome. Alternatively, the *Wsi18* promoter may just drive

weaker expression of genes under its control than the *Bradi2G47700* promoter. Experiments to test the level of water deficit-inducible expression from the *Wsi18* and *Bradi2G47700* promoters in their native hosts, *B. distachyon* and rice, as well as other plant species, would help to elucidate the actual difference in expression level between them. The promoter sequence associated with the *Bradi2G47700* gene would be a good target for future research to characterize water deficit stress-inducible and ABA inducible promoters.

4.2   *Wsi18* is induced by ABA and mannitol treatments in *B. distachyon*

In rice, its native plant, the *Wsi18* promoter is induced by water deficit stresses such as drought, NaCl, and ABA. The main source of stresses generated by water deficit are osmotic stress and cellular dehydration, while NaCl also causes hyper-ionic stress (Huang et al., 2012). To investigate the inducibility of *Wsi18* in *B. distachyon*, transgenic plants in which the *uidA* reporter gene was under the control of the *Wsi18* promoter were generated. The plants were treated with ABA to initiate the ABA-dependent water deficit signaling pathway, mannitol to generate osmotic stress, or NaCl to generate salinity stress, and the pattern and level of *uidA* expression was observed.

Qualitative analysis of GUS activity using the GUS histochemical assay showed the pattern of *uidA* gene expression driven by the *Wsi18* promoter in the treated plants. GUS activity was visibly increased in the leaves and stalk of *B. distachyon* following ABA, mannitol, and NaCl treatments, compared to the unstressed control plants (Figure 9). This is similar to the expression pattern observed in rice by Yi et al. (2011), who observed *Wsi18* driven expression of GFP in the whole rice plant body. Some unstressed control

plants did show small areas of GUS activity, but always to a lesser degree than their stress-treated counterparts. Low level *Wsi18* promoter activity in unstressed control plants is consistent with observations of *Wsi18* activity in transgenic rice made by Yi et al. (2011). In wild-type rice plants, the expression of the *Wsi18* gene was only detectable under water deficit conditions, and not under control conditions (Yi et al., 2011). However, in transgenic rice plants in which the *Wsi18* promoter drove the expression of the GFP reporter gene, low levels of GFP expression were detected in the leaves and roots of unstressed control plants (Yi et al., 2011). The low levels of expression driven by *Wsi18* in unstressed control plants may be due to less stringent regulation of gene expression in transgenes than genes in their native context.

RT-qPCR analysis showed that in the ABA-treated and mannitol-treated plants transcription of *uidA* was significantly higher than in unstressed control plants. NaCl treatment, however, did not elicit a significant increase in *uidA* expression for any of the transgenic lines. This is in disagreement with the NaCl treatment results of the GUS histochemical analysis of *uidA* expression, in which there was a greater amount of GUS activity in NaCl-treated plants compared to unstressed control plants. A possible reason for the discrepancy between the results of the NaCl treatment in the GUS histochemical assay and the RT-qPCR analysis could be that the GUS histochemical assay is considered less precise than RT-qPCR. During the GUS histochemical assay, samples were left in the histochemical staining solution overnight. In that time any β-glucuronidase enzyme in the cell could continue to process X-gluc until either the enzyme is denatured or the X-gluc in the solution is fully depleted. The blue dye that results from X-gluc hydrolysis by β-glucuronidase is very stable and accumulates in the tissue without being degraded. β-

Glucuronidase has a half life of ~50 hours in plant cells, and so would still be active by the time observations were made (Jefferson et al., 1987). The overnight incubation could have given enough time for low levels of *uidA* expression in the NaCl-treated samples to produce a low, but sufficient, level of β-glucuronidase for a visible amount of the blue dye to accumulate in the tissue. RT-qPCR measures *uidA* expression more precisely and only at one time point because samples were flash frozen in liquid nitrogen. These low levels of transcription were not high enough to produce a significant fold change in comparison to the unstressed control plants. In a field setting plants respond to water deficit-stresses such as drought over a period of time, rather than all at once. The greater GUS activity observed in the NaCl-treated plants compared to the non-stressed control plants in the GUS histochemical assay shows that the utility of *Wsi18* as a salt inducible promoter warrants further investigation.

The observed level of *Wsi18*-mediated expression in this experiment was lower in *B. distachyon* than had been previously observed in rice by Yi et al. (2010). In transgenic rice in which the *Wsi18* promoter drove the expression of GFP Yi et al. (2010) observed a 53-fold increase in GFP expression in leaves of 20 day old plants following water deficit treatment. In comparison, the highest level of *uidA* expression observed in the transgenic *B. distachyon* plants in this study was 9.1-fold for line 37 following ABA treatment. It is possible that *Wsi18* could drive a comparably high level of expression in *B. distachyon* as has been observed in rice under more severe stress conditions, or perhaps *Wsi18* is not as highly induced in *B. distachyon*. This could be elucidated with further experiments to test different intensities or durations of water deficit stress.

4.3   Variation among transgenic lines

Levels of *Wsi18* promoter activity exhibited variation among different transgenic lines (Figure 10). A similar result was observed by Yi and colleagues (2010) in transgenic rice plants expressing GFP under the control of the *Wsi18* promoter following water deficit stress. This phenomenon is not unique to the *Wsi18* promoter and has been observed in both constitutive and inducible promoters many times before (Peach and Velten, 1991; Yi et al., 2010). It is very common for transgenic lines to exhibit a low level of transgene expression (Butaye et al., 2005). One explanation for the observed differences in *uidA* expression between the transgenic lines 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, will all influence expression of the transgene. Integration at highly expressing loci will produce higher expressing transgenic lines than integration at loci with lower transcriptional activity (Butaye et al., 2005). 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 (Meyer and Saedler, 1996). 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. Post-transcriptional gene silencing includes processes such as RNA silencing, which is a form of plant defense

against viruses, transposons, and other foreign DNA (Butaye et al., 2005). If *Wsi18* 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 *Wsi18* driven *uidA* expression in two transgenic lines each, suggesting *Wsi18* does have water deficit-inducible activity in *B. distachyon*.

Chapter 5: Future Perspectives

The research reported in this thesis demonstrated that the *Wsi18* promoter retains its water deficit-inducible and ABA responsive characteristics in *B. distachyon*. Future areas of work which could be worthwhile include identifying the functional *cis*-elements in the *Wsi18* promoter, evaluating *Wsi18* promoter activity in additional plant species, particularly crops, and using the *Wsi18* promoter to drive the expression of genes that can increase the water deficit tolerance of plants.

5.1   Further characterization of the *Wsi18* promoter

The 1.7 kbp *Wsi18* rice promoter used in this study has proven to be capable of inducing the expression of a reporter gene under water deficit conditions in *B. distachyon*. However, the entire length of the 1.7 kbp sequence used may not be important for water deficit-induced expression. Much shorter promoters such as *OsABA2*, *HPI,* and *rab16A*, which have sequence lengths of 738 bp, 526 bp, and 358 bp respectively, are all capable of water deficit stress-induced expression (Rai et al., 2009). Constructing modified promoters in which segments of the 1.7 kbp *Wsi18* promoter sequence have been removed could be used to examine whether water deficit-induced expression is affected by those segments, and identify regions that contain important water deficit-related *cis*-elements. To determine if individual putative *cis*-elements are involved in water deficit-responsive gene expression, base pair substitution mutagenesis at *cis*-elements, as used by Xiao and Xue, (2001) to identify the CE-ABRE pair in *Wsi18*, could be employed. Combined, constructing modified promoters and base pair substitution mutagenesis experiments could identify the essential *cis*-elements of the *Wsi18* promoter necessary for water deficit-inducible expression. With the knowledge of

which sequence elements make *Wsi18* water deficit-inducible the characteristics of *Wsi18*-induced expression could be improved by removing or inserting *cis*-elements, or changing their location within the promoter sequence. Identifying key elements of the *Wsi18* promoter could be very beneficial for the generation of synthetic promoters. Synthetic promoters are an exciting innovation, as they can be engineered to have precisely the expression profile required of the gene they are mediating without any unwanted activity, which can be difficult to avoid using natural promoters (Liu and Stewart, 2016). Several synthetic promoters driving water deficit-inducible expression have been used to improve the water deficit tolerance of crop plants. Examples include the stress-inducible *AIPC* synthetic promoter used to express the $\triangle^1$-*pyrroline-5-carboxylate synthetase* gene for proline biosynthesis in transgenic wheat (Vendruscolo et al., 2007), and expression of the *HVA1* gene under the control of the stress-inducible synthetic promoter *ABRC321* in transgenic rice (Chen et al., 2015). An increased understanding of how a promoter's expression profile is determined by its sequence will facilitate the engineering of new synthetic promoters in the future. The knowledge acquired from the complete characterization of promoters with unique expression profiles, such as *Wsi18*, will be essential in this endeavor.

5.2 Using the *Wsi18* promoter to improve the water deficit stress tolerance of crops

Ultimately, the most promising use of *Wsi18* as a water deficit-inducible promoter is to improve the water deficit tolerance of agriculturally important crops. Future research in which the *Wsi18* promoter drives the expression of genes that confer water deficit tolerance could be useful to develop hardier crops and increase agricultural yield.

Demand for food increases each year as the global population grows. By the year 2050, demand for food is estimated to increase by 100% (Tilman et al., 2011). Greater crop yields will be necessary to meet these demands. It has been estimated that each year 54-80% of the potential yield of crops is lost due to abiotic stresses such as drought and high salinity (Gill et al., 2014). Developing crops with greater water deficit tolerance can reduce the amount of lost yield, helping to meet future increases in crop demand.

Developing more water deficit tolerant crops could be done by changing the expression of functional proteins or transcription factors (Umezawa et al., 2006). Functional genes which produce molecules that protect the cell under water deficit stress conditions, such as osmoprotectants, compatible solutes, and ROS scavenging enzymes, have successfully been used to increase the stress tolerance of many plant species (Umezawa et al., 2006). The *trehalose-6-phosphate synthase* gene from *E. coli* has been employed to produce trehalose in transgenic rice, resulting in increased tolerance to drought, cold, and salt stress (Jang et al., 2003). Another example is the overexpression of an aldose/aldehyde reductase gene *MsALR* from alfalfa introduced into tobacco, which resulted in decreased production of the ROS lipid peroxide and increased drought survival (Oberschall et al., 2000). Improving crops by altering the expression of transcription factors has been previously used to improve stress tolerance as well. This strategy offers the advantage of also affecting the expression of many of the downstream genes of that transcription factor. In Arabidopsis, overexpression of the DREB1/CBF transcription factor altered the expression of more than 40 genes, and increased tolerance to drought, salinity, and cold stresses (Valliyodan and Nguyen, 2006).

Constitutive expression of stress related genes can have unwanted side effects. Stress related genes are normally only expressed under extreme conditions, and their products can disrupt normal cell function by changing the conditions within the cell. In addition, continuous overexpression can exhaust the cell's supply of resources. Using transcription factors as transgenes to increase stress tolerance can amplify this burden due to the downstream effects their expression has on the expression of other genes. Inducible promoters offer the advantage of expressing the gene under stress conditions when it is useful, but not otherwise when it is unnecessary. Several studies have compared the expression of water deficit stress related genes under the control of water deficit-inducible and constitutive promoters. Su and Wu, (2004) developed transgenic rice plants in which the $\triangle^1$-*pyrroline-5-carboxylate synthetase* gene from mothbean, which is responsible for proline production, was driven by either the constitutive *Act1* promoter, or the stress-inducible *AIPC* promoter. Rajwanshi et al. (2016) developed transgenic *Brassica juncea* plants in which the *Glyoxalase I* gene was either constitutively expressed by the *CaMV35S* promoter, or driven by the water deficit-inducible promoter *Rd29A*. Kasuga et al. (1999) developed transgenic Arabidopsis plants overexpressing the DREB1A transcription factor under the control of either the constitutive *CaMV35S* promoter, or the water deficit-inducible *Rd29A* promoter. In all of these studies it was found that both the constitutive promoter and stress-inducible promoter driving the expression of the transgene resulted in increased drought and salt stress tolerance of the transgenic plants. However, when grown without stress, growth was retarded in plants constitutively expressing the transgene. This growth penalty was not observed with inducible expression of the transgene. In addition, the increase in stress tolerance

resulting from inducible expression of the transgene was greater than the increase in stress tolerance that resulted from constitutive expression in all cases (Kasuga et al., 1999; Su and Wu, 2004; Rajwanshi et al., 2016).

It has been suggested that a stress-induced gene's own inducible promoter would be the ideal promoter to drive the gene's expression in a new host plant, as this would help to maintain the normal spatial and temporal expression pattern of the gene (Qin and Qin, 2016). Members of the LEA3 family, which includes *Wsi18*, have been used to improve the stress tolerance of many plants. Overexpression of *HVA1*, a LEA3 gene from *Hordeum vulgare* (barley), has been very successful in improving the water deficit tolerance of plants such as rice (Xu et al., 1996; Rohila et al., 2002; Chandra Babu et al., 2004; Chen et al., 2015), wheat (Sivamani et al., 2000; Bahieldin et al., 2005), mulberry (Lal et al., 2008; Checker et al., 2012), maize (Nguyen and Sticklen, 2013), common bean (Kwapata et al., 2012), and oats (Oraby et al., 2005). The *Wsi18* protein is a LEA3 protein like *HVA1*, but it has not yet been studied in transgenic plants to see if it can increase the plant's stress tolerance (Ross and Shen, 2006). *Wsi18* gene expression under the control of the *Wsi18* promoter could be an interesting area of future research into functional proteins which could be used to increase the water deficit tolerance of plants. The *Wsi18* promoter's potential as a water deficit-inducible promoter that functions in multiple species, will make it a valuable tool for developing hardier crops in the future.

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Appendices

Appendix 1: The *Wsi18* promoter sequence

5′—GGCTCTAGAGGATCCtGAGATCCGGCTTAATGCTTTTCTTTTGTCACATATACTGCATTGCAACAATT
GCCATATATTCACTTCTGCCATCCCATTATATAGCAACTCAAGAATGGATTGATATATCCCCTATTACTAAT
CTAGACATGTTAAGGCTGAGTTGGGCAGTCCATCTTCCCAACCCACCACCTTCGTTTTTCGCGCACATACT
TTTCAAACTACTAAATGGTGTGTTTTTTAAAAATATTTTCAATACAAAAGTTGCTTTAAAAAATTATATTGA
TCCATTTTTTTAAAAAAAAATAGCTAATACTTAATTAATCACGTGTTAAAAGACCGCTCCGTTTTGCGTGCA
GGAGGGATAGGTTCACATCCTGCATTACCGAACACAGCCTAAATCTTGTTGTCTAGATTCGTAGTACTGG
ATATATTAAATCATGTTCTAAGTTACTATATACTGAGATGAATAGAATAAGTAAAATTAGACCCACCTTAA
GTCTTGATGAAGTTACTACTAGCTGCGTTTGGGAGGACTTCCCAAAAAAAAAAGTATTAGCCATTAGCAC
GTGATTAATTAAGTACTAGTTTAAAAAACTTAAAAAATAAATTAATATGATTCTCTTAAGTAACTCTCCTAT
AGAAAACTTTTACAAAATTACACCGTTTAATAGTTTGGAAAATATGTCAGTAAAAAATAAGAGAGTAGAA
GTTATGAAAGTTAGAAAAAGAATTGTTTTAGTAGTATACAGTTATAAACTATTCCCTCTGTTCTAAAACAT
AAGGGATTATGGATGGATTCGACATGTACCAGTACCATGAATCGAATCCAGACAAGTTTTTTATGCATAT
TTATTCTACTATAATATATCACATCTGCTCTAAATATCTTATATTTCGAGGTGGAGACTGTCGCTATGTTTT
TCTGCCCGTTGCTAAGCACACGCCACCCCCGATGCGGGGACGCCTCTGGCCTTCTTGCCACGATAATTGA
ATGGAACTTCCACATTCAGATTCGATAGGTGACCGTCGACTCCAAGTGCTTTGCACAAAACAACTCCGGC
CTCCCGGCCACCAGTCACACGACTCACGGCACTACCACCCCTGACTCCCTGAGGCGGACCTGCCACTGTT
CTGCATGCGAAGCTATCTAAAATTCTGAAGCAAAGAAAGCACAGCACATGCTCCGGGACACGCGCCACC
CGGCGGAAAAGGGCTCGGTGTGGCGATCTCACAGCCGCATATCGCATTTCACAAGCCGCCCATCTCCACC
GGCTTCACGAGGCTCATCGCGGCACGACCGCGCACGGAACGCACGCGGCCGACCCGCGCGCCTCGATGC
GCGAGCCCATCCGCCGCGTCCTCCCTTTGCCTTTGCCGCTATCCTCTCGGTCGTATCCCGTTTCTCTGTCTT
TTGCTCCCCGGCGCGCGCCAGTTCGGAGTACCAGCGAaACCCGGACACCTGGTACACCTCCGCCGGCCAC
AACGCGTGTCCCCCCTACGTGGCCGCGCAGCACATGCCCATGCGCGACACGTGCACCTCCTCATCCAAAC
TCTCAAGTCTCAACGGTCCTATAAATGCACGGATAGCCTCAAGCTGCTCGTCACAAGGCAAGAGGCAAG
AGGCAAGAGCATCCGTATTAACCAGCCTTTTGAGACTTGAGAGTGTGTGTGACTCGATCCAGCGTAGTTT
CAGTTCGTGTGTTGGTGAGTGATTCCAGCCAAGTTTGCGCCATGG—3′

Appendix 2: Hygromycin B seed germination
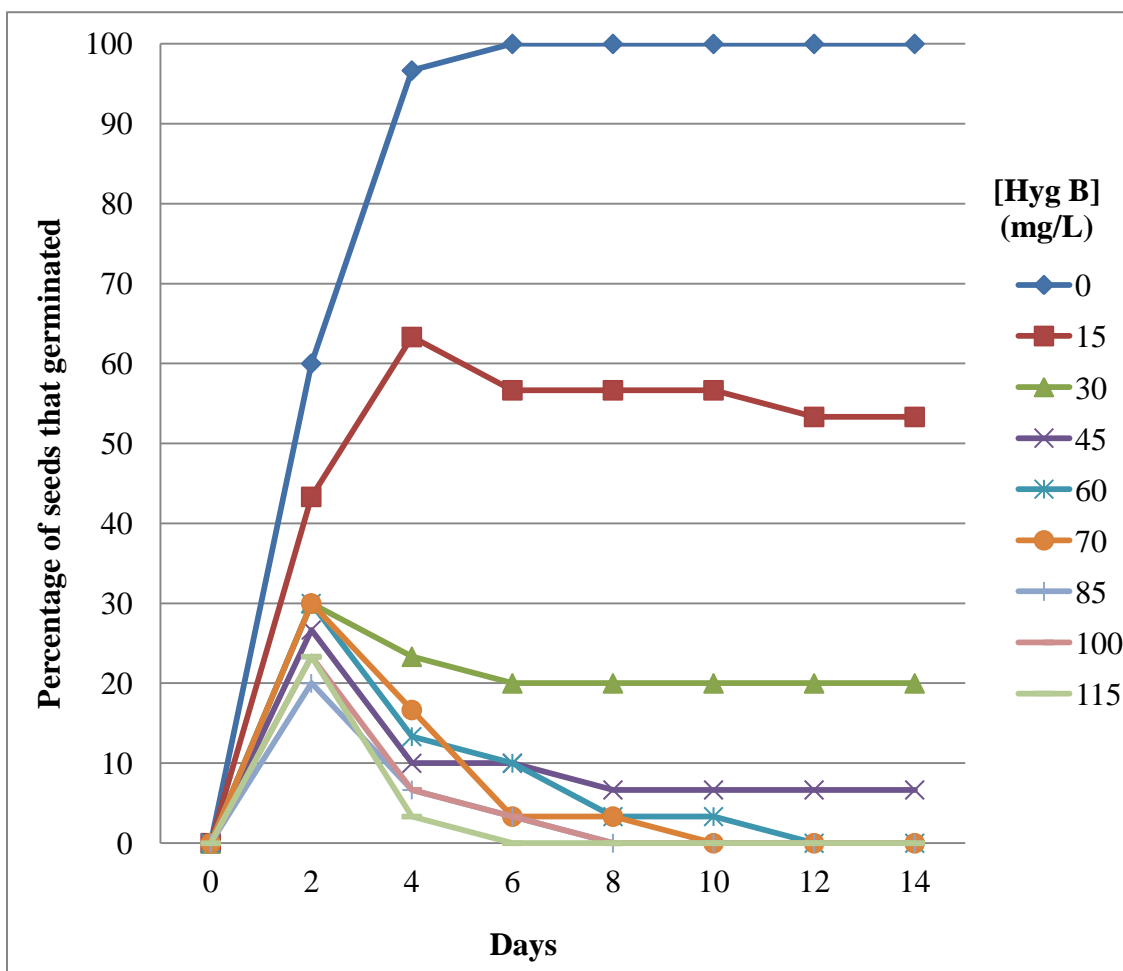
Wild-type *B. distachyon* seeds were sown on seed germination medium containing various concentrations of hygromycin B for 14 days. Hygromycin B caused the seedlings either to not germinate, or to exhibit black roots and retarded growth. Germination for 12 days on medium containing 60 mg/L hygromycin 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.

Curriculum Vitae

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- Plant Biotech 2016 – Kingston, Ontario
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