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Characterization of the *Xerophyta humilis* desiccation induced-1 (*Xhdsi-1^{VOC}*) gene; a member of the Vicinal Oxygen Chelate (VOC) metalloenzyme superfamily upregulated in *X. humilis* (BAK) DUR and SCHINZ during desiccation

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ABSTRACT

Desiccation tolerance in vascular plants is common in seeds and pollen. However, there are only 350 species (0.2% of total flora) known as resurrection plants that have the ability to acquire desiccation tolerance in their vegetative tissue. They are able to survive a loss of 95% of their relative water content and resume biological functions upon rehydration. The resurrection plant, *Xerophyta humilis* is used as a model system to identify and characterise genes which play an important role in conferring desiccation tolerance in plants. In this study, the expression of a novel gene named *desiccation induced-1 (dsi-1^{VOC})* during desiccation in *X. humilis* and desiccation-sensitive plants is characterised. Transgenic studies show that ectopic expression of *dsi-1^{VOC}* is able to confer tolerance to abiotic stress in transgenic *Arabidopsis thaliana* plants.

Dsi^{VOC} was originally identified as being up-regulated during desiccation in *X. humilis* leaves in a small-scale microarray study. *Xhdsi-1^{VOC}* has high sequence similarity to the *A. thaliana* (*At1g07645*) gene annotated as a glyoxalase I-like family protein. Although *At1g07645* was annotated as a glyoxalase I, both *Xhdsi-1^{VOC}* and its orthologue lack the glutathione and zinc binding sites required for glyoxalase I activity. Furthermore, the overexpression of *Xhdsi-1^{VOC}* did not complement yeast glyoxalase I mutant. However, *Xhdsi-1^{VOC}* and *At1g07645* share a conserved $\beta\alpha\beta\beta\beta$ structural fold which is common to all members of the vicinal oxygen chelate (VOC) superfamily, which includes glyoxalase I. *At1g07645* and other plant specific orthologues were present only in seed derived EST libraries. In addition, RT-PCR showed that *At1g07645* transcripts were absent in *A. thaliana* vegetative tissue exposed to dehydration, NaCl and mannitol but were present in mature dry seeds. In contrast, *Xhdsi-1^{VOC}* mRNA transcripts and protein levels in *X. humilis* increased significantly in leaves and roots during desiccation, in addition to being expressed at high levels in mature dry seeds. To investigate the role of *Xhdsi-1^{VOC}* or *At1g07645* in planta, transgenic *A. thaliana* plants were generated.

At1g07645-deficient *A. thaliana* seeds generated by both posttranscriptional silencing and T-DNA *At1g07645* mutagenesis did not show an obvious phenotype. Similarly, transgenic *A. thaliana* plants expressing the *Xhdsi-1^{VOC}* or *At1g07645* transgene under

the control of a constitutive promoter did not show significant differences at seed germination and at the two-week-old stages relative to wild type when exposed to osmotic stress (NaCl and mannitol). In contrast, adult plants constitutively expressing *Xhdsi-1^{VOC}* or *At1g07645*, showed higher growth rates, less photo-oxidative damage and lipid peroxidation compared to control plants when exposed to NaCl and mannitol. However, whilst adult transgenic plants were able to recover after mannitol stress, these transgenic lines were unable to recover when exposed to NaCl. Therefore, while ectopic expression of *Xhdsi-1^{VOC}* and *At1g07645* was able to protect transgenic plants against osmotic stress, these genes were not able to confer tolerance against ion toxicity, an additional stress caused when plants are exposed to NaCl. Furthermore, there was no phenotypic difference between adult transgenic plants expressing *Xhdsi-1^{VOC}* compared to *At1g07645*, suggesting that these orthologues function in the same manner. The overall growth characteristics of these transgenic plants did not differ from wild type plants in the absence of stress. *Dsi-1^{VOC}* shows great promise as an endogenous gene that is able to confer tolerance to abiotic stress if it could be activated in vegetative tissues in desiccation-sensitive plants.

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LIST OF ABBREVIATIONS

Bp	base pairs
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
g	times gravity
KLH	Keyhole limpet hemocyanin
LB	Luria bertani media
LiCl	lithium chloride
M	Molar
MDA	malondialdehyde
MOPS	4-morpholine propanesulfuric acid
mRNA	messenger RNA
MS media	Murashinge and Skoog basal media
Mw	molecular weight
mM	millimolar
NaCl	sodium chloride
nm	nanomolar
OD	optical density
PCR	polymerase chain reaction
RNAi	RNA interference
RNase	ribonuclease
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SE	standard error
T-DNA	transfer DNA

Chapter 1

Introduction

Desiccation tolerance has been reported in the prokaryote, animal, fungi and plant kingdoms (Wood and Jenks, 2007; Alpert, 2006; Hoekstra *et al.*, 2001). In the animal kingdom, desiccation tolerance is found only in small animals that are less than 5 mm in size and do not have a skeletal structure (Alpert, 2006). These desiccation tolerant animals include nematodes, rotifers, Tardigrades, and the larva of arthropods. In the plant kingdom, vegetative desiccation tolerance is frequently found in lower order plants such as algae, liverworts, hornworts, lichens, bryophytes and Selaginella (Alpert, 2006; Bernacchia and Furini, 2004; Dickie *et al.*, 2002; Hoekstra *et al.*, 2001; Oliver *et al.*, 2000). In vascular plants (higher order plants) desiccation tolerance is common in orthodox seeds, spores and pollen (Alpert, 2006). However, desiccation tolerance is rare in the vegetative tissue of higher plants, such as ferns and angiosperms, and absent from the gymnosperms (Alpert, 2006; Proctor and Tuba, 2002; Ingram and Bartels, 1996). There are only 350 angiosperm species (0.2% of total flora) that have the ability to survive desiccation of their vegetative tissues (Michael *et al.*, 2002). These desiccation tolerant angiosperm species are known as resurrection plants (Gaff, 1971; Gaff, 1977; Ramanjalu and Bartels, 2002).

In angiosperms, desiccation tolerance is found in both monocotyledonous and eudicotyledonous plant species (Bernacchia and Furini, 2004). These plants survive desiccation by upregulating protective mechanisms during drying (Bernacchia and Furini, 2004). The cellular components protected during water loss include cell walls, membranes, proteins and genetic material (Bohnert, 2000; Bewley, 1979). In addition, desiccation tolerant plants are able to repair damage caused during desiccation or the subsequent introduction of water upon rehydration. In the lower order plants such as the moss, *Tortula ruralis*, desiccation is so rapid that there is little time to switch on protective mechanisms (Wood and Jenks, 2007; Oliver *et al.*, 2000; Proctor and Tuba, 2002). These plants tend to have some constitutive protection, but

rely more heavily on repair of damage upon rehydration (Oliver *et al.*, 2000; Wood and Jenks, 2007; Biutink and Leprince, 2004). Higher order plants such as ferns and angiosperms can only survive slow drying because they rely on protective mechanisms laid down during desiccation (Wood and Jenks, 2007; Oliver *et al.*, 2000).

Unlike resurrection plants that activate protective mechanisms when exposed to periods of severe water loss, desiccation tolerance in orthodox seeds is a pre-programmed mechanism that occurs during seed development (Kermode and Finch-Savage, 2002; Vertucci and Farrant, 1995).

1.1. Desiccation tolerance in orthodox seeds

Orthodox seeds acquire desiccation tolerance during the late maturation phase of seed development (Weber *et al.*, 2005; Dickie and Pritchard, 2002). Seed development can be divided into two broad stages: morphogenesis and maturation (Gutierrez *et al.*, 2007; Weber *et al.*, 2005; Kermode and Finch-Savage, 2002). The maturation phase can further be subdivided into the embryo growth/seed filling (early maturation) and the desiccation/dormancy (late maturation) stages (Gutierrez *et al.*, 2007). During the morphogenesis phase, the fertilised ovum undergoes cell division and subsequently differentiates into the embryo (Weber *et al.*, 2005; Kermode and Finch-Savage, 2002). In the early maturation stage, cell division is halted and embryonic growth occurs (Weber *et al.*, 2005; Kermode and Finch-Savage, 2002). In addition to embryo growth, the seeds accumulate proteins, lipids and carbohydrate in storage vacuoles (reserve accumulation) (Weber *et al.*, 2005; Kermode and Finch-Savage, 2002). In both the morphogenesis and early maturation stages, orthodox seeds are desiccation sensitive (Kermode and Finch-Savage, 2002). During the late maturation stage, orthodox seeds lose the bulk of their water and metabolism is halted (Kermode and Finch-Savage, 2002). They become desiccation tolerant during the late maturation phase and can survive the desiccated state for long periods of time (Weber *et al.*, 2005; Dickie and Pritchard, 2002; Kermode and Finch-Savage, 2002; Vertucci and Farrant, 1995). Similarities in mechanisms involved in desiccation tolerance between resurrection plants and orthodox seeds have been reported (Oliver, 2007; Illing *et al.*, 2005; Oliver *et al.*, 2000). In contrast to orthodox seeds, desiccation sensitive seeds

(recalcitrant seeds) do not undergo severe water loss during development and are unable to remain viable when subjected to long-term storage (Dickie and Pritchard, 2002; Kermode and Finch-Savage, 2002). Upon germination of the orthodox seeds, desiccation tolerance is maintained in the vegetative tissue of only 0.2% of total flora of angiosperm species (see above)(Michael *et al.*, 2002) and lost in most of the angiosperm species (Vertucci and Farrant, 1995).

1.2. The distinction between desiccation sensitive and desiccation tolerant plants

Plants can be divided into two broad categories, namely, desiccation sensitive and desiccation tolerant, depending on their ability to endure water loss (Le and McQueen-Mason, 2006). The desiccation sensitive plants can further be subdivided into drought sensitive and drought tolerant, depending on their ability to retain water during drought (Oliver, 2007; Le and McQueen-Mason, 2006; Alpert and Oliver, 2002). A critical water content associated with irreversible damage in drought sensitive and tolerant plants is not clearly defined in the literature. For example, it is generally reported that relative water content (RWC) in the majority of desiccation sensitive plants can drop to between 99-80% for only a limited time before causing irreversible damage (Moore *et al.*, 2008). However, Gigon and colleagues (2004) reported that in the drought sensitive plant *Arabidopsis thaliana* (ecotype Columbia), the ability of the plant to maintain cell homeostasis is lost below 73.2% RWC, and at 47.5% RWC, the damage to cell membranes was irreversible upon rehydration (Gigon *et al.*, 2004). In addition, a review by Farrant (2007) reports that three desiccation sensitive grasses *Eragrostis curvula*, *Eragrostis teff*, and *Eragrostis capensis* can survive RWC of 45 %, 50 % and 65 % RWC, respectively. However, below these RWCs, the plants die. Although no critical water content distinguishing drought sensitive and drought tolerant plants has been elucidated, drought sensitive and tolerant plants can be distinguished by their ability to avoid water loss during drought (Moore *et al.*, 2008; Oliver, 2007).

Most plants that grow in arid areas, such as deserts, and in semi-arid areas where water is scarce for a limited period during the year survive by avoiding water loss (Moore *et al.*, 2008; Oliver, 2007; Cushman, 2001). Examples of plants that avoid water loss in arid and semi-arid areas are the drought tolerant succulents, which

include cacti, agave, aloe, elephant trees and some euphorbias plants (Alpert and Tuba, 2000). Succulents rapidly absorb and store high quantities of water in modified leaf and stem tissues so that water is available for use during the periods of drought (Moore *et al.*, 2008; Oliver, 2007; Cushman, 2001). In addition to storing high amounts of water, succulents produce a waxy coating at the surface of their leaves and stems to avoid water loss by transpiration (Alpert and Tuba, 2000). Furthermore, these plants conserve water loss by using a method of photosynthesis called Crassulacean Acid Metabolism (CAM) (Luttge, 2004). During CAM photosynthesis, the stomata open during the night to allow the absorption of carbon dioxide (CO₂) which is fixed by the enzyme phosphoenolpyruvate carboxylase (PEPC) in the cytosol of chloroplasts-containing cells (Luttge, 2004; Cushman, 2001). The CO₂ in the form of organic acids is stored in large vacuoles that occupy approximately 98% of the cells (Luttge, 2004). The stomata close during the day and the stored CO₂ is then used for photosynthesis. Other plants, such as eucalypts, maintain cell homeostasis by developing long roots extending to the water underground (Moore *et al.*, 2008; Luttge, 2004).

By contrast, desiccation tolerant plants are able to withstand the loss of 80-90% of their water and upon rehydration, resume biochemical functions (Alpert, 2006; Oliver *et al.*, 2000). Rather than avoid water loss, desiccation tolerant plants have evolved mechanisms to prevent damage during the loss of intracellular water, the duration of the dry state and/or during recovery following the administration of water (Bohnert, 2000; Bewley, 1979).

Resurrection plants sense water loss by an unknown mechanism. Upon water loss, transcription of dehydration responsive genes are upregulated either through the abscisic acid (ABA)-dependent or ABA-independent pathways (Bernacchia and Furini, 2004). Resurrection plants undergo structural morphological changes, such as increased flexibility of the cell walls and increased fluidity of the plasma membranes, to prevent mechanical damage caused by the loss of turgor due to water loss (Moore *et al.*, 2006; Walters *et al.*, 2002; Farrant, 2000). These plants shut down metabolic activities, and activate several mechanisms that prevent the denaturation/aggregation of proteins and the disruption of lipid membranes. They also prevent or repair oxidative damage caused by reactive oxygen species (ROS) (Bernacchia and Furini,

2004; Hoekstra *et al.*, 2001; Smirnov, 1993). The mechanisms involved during desiccation of resurrection plants are summarised in Figure 1.1. These mechanisms are described in the following sections.

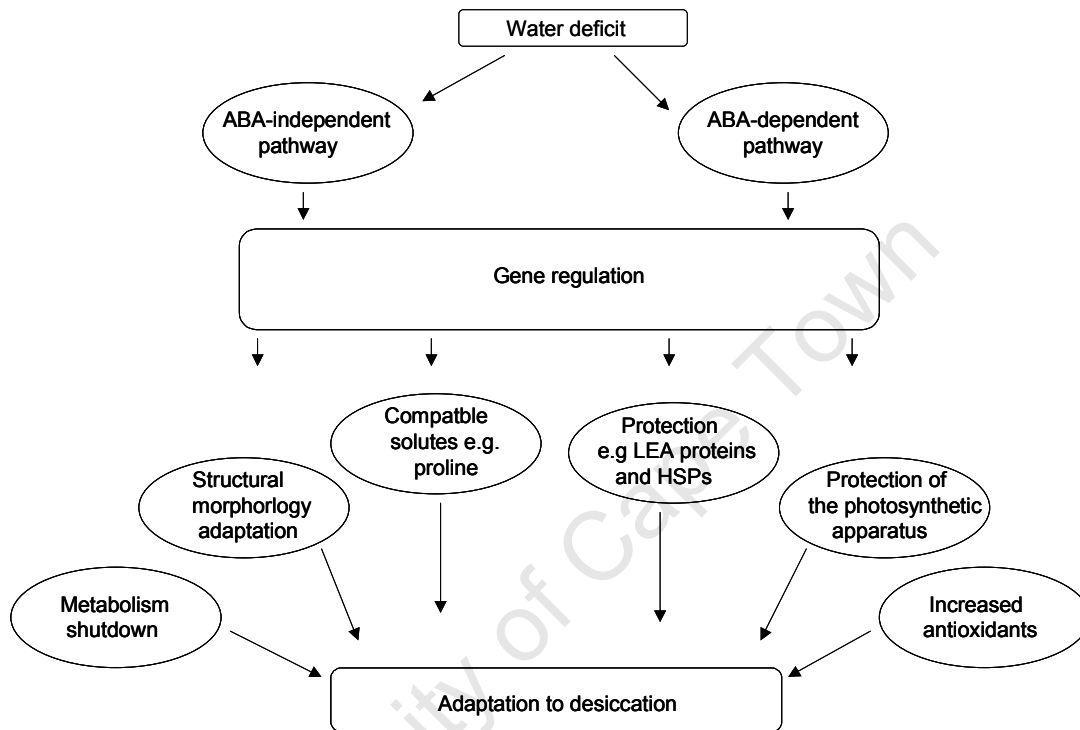


Figure 1.1 Structural and biochemical changes in resurrection plants during desiccation (Ramanjulu and Bartels, 2002)

1.3. Structural morphology adaptation of desiccation tolerant plants during water loss

The decrease of water in plants results in reduced turgor causing cells to shrink (Walters *et al.*, 2002). In desiccation sensitive plants, the loss of water results in plasmolysis, the mechanism where by the cell membrane detaches from the cell wall (Munns, 2002). This mechanism causes the cell membrane to rupture. Resurrection plants contain several methods to avoid the effects of mechanical damage, such as plasmolysis. In some resurrection plants, cell wall folding has been observed (Moore *et al.*, 2006; Vire *et al.*, 2004; Farrant, 2000). Electron microscopy images of desiccated *Myrothamnus flabellifolius* and *Craterostigma wilmsii* leaf tissue (5%

RWC) illustrated that the volume of mesophyll cells decreased as a result of cell wall folding (Moore *et al.*, 2006; Vicre *et al.*, 2004; Farrant, 2000). Other resurrection plants with the ability to fold their cell walls during dehydration are *Craterostigma plantagineum* and *Eragrostis nindensis* (Le and McQueen-Mason, 2006; Vander Willigen *et al.*, 2004; Jones and McQueen-Mason, 2004). The rigid cell wall of these resurrection plants is able to fold during desiccation due to the change in their cell wall composition (Jones and McQueen-Mason, 2004).

Jones and McQueen-Mason (2004) have shown that in *C. plantagineum* leaf tissue, an mRNA transcript that encodes for expansin *Cp1Exp1*, a protein that promotes cell wall flexibility, increased by a 200 fold. The protein levels of *Cp1Exp1* also increased. Interestingly, Jones and McQueen-Mason (2004) reported that mRNA transcripts of a *Cp1Exp1* orthologue in *A. thaliana* are upregulated when mature dry *A. thaliana* seeds are incubated in water. It is speculated that the high levels of expansin reduce cell wall rigidity during water loss. Cell wall flexibility not only prevents mechanical damage during water loss, but also prevents cell damage during rehydration when large amounts of water enter the cells (Jones and McQueen-Mason, 2004). In addition to folding of cell walls to prevent mechanical damage, resurrection plants induce increased flexibility in plasma membranes (Le and McQueen-Mason, 2006). For example, levels of polyunsaturated lipids have been shown to increase during desiccation within the membranes of the resurrection plants, *Boea hygroscopea* and *Sporobolus stapfianus* to increase membrane fluidity, (Le and McQueen-Mason, 2006).

Although cell wall folding occurs in the mesophyll cells of the resurrection plants such as *E. nindensis*, *C. wilmsii* and *M. flabellifolius*), cell wall folding is absent in the bundle sheath cells of their leaf tissue (Vander Willigen *et al.*, 2004; Farrant *et al.*, 2000). Instead of cell wall folding, the cells in the bundle sheath contain numerous vacuoles located at the border of the cells that are filled with substances that replace water (Vander Willigen *et al.*, 2004; Farrant *et al.*, 2000). In resurrection plants such as *Xerophyta humilis* and *Xerophyta viscosa*, cell wall folding is absent in both the mesophyll and the bundle of sheath cells (Vander Willigen *et al.*, 2004; Farrant, 2000). Upon desiccation, *X. humilis* and *X. viscosa* develop numerous large vacuoles that fill the cells (Vander Willigen *et al.*, 2004; Farrant, 2000). The composition of

the substances that fill the vacuoles are not known, however, high concentrations of polyphenols were observed in the vacuoles of desiccated *Myrothamnus flabellifolius* leaf tissue (Moore *et al.*, 2005).

Vacuoles have also been observed in mature dry seeds (Farrant *et al.*, 2000). In mature seeds, vacuoles are filled primarily with protein (Farrant *et al.*, 2000; Vitale and Bollini, 1995). It is believed that the vacuoles within the cells replace water during desiccation to maintain cell turgor and so prevent mechanical damage during dehydration. In addition, vacuoles absorb large amounts of water when seeds imbibe water upon germination (Li *et al.*, 2008; Maurel *et al.*, 1995). The absorption of water occurs through aquaporins located on the membranes of the vacuoles (Maurel *et al.*, 2008; Maurel *et al.*, 1995). A seed-specific aquaporin found on the membranes of vacuoles (α -TIP) ectopically expressed in *Xenopus* oocytes resulted in increased permeability of the membranes by 4 to 8 fold (Maurel *et al.*, 1995). This finding strongly supports the hypothesis that aquaporins on the membrane of vacuoles increase permeability to water. Interestingly, the membranes of vacuoles in the bundle of sheath in *E. nindensis* contain aquaporins TIP (3:1) (Vander Willigen *et al.*, 2004). The presence of aquaporins on the membrane of the vacuoles increases the permeability of membranes to water during rehydration in resurrection plants (Vander Willigen *et al.*, 2004).

1.4. Maintenance of cell wall, membranes and proteins

In plant cells, water is required for optimal metabolism. It maintains the integrity of membranes and the biochemical function of proteins, including enzymes (Alpert, 2006; Hoekstra *et al.*, 2001). To survive severe water loss, desiccation tolerant plants need to maintain the integrity of membranes, avoid aggregation of macromolecules, and stop metabolism to prevent harmful interactions between proteins and molecules in the cytoplasm (Alpert, 2006; Hoekstra *et al.*, 2001). During severe dehydration, the activity of various antioxidant enzymes such as ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase remain active in resurrection plants, suggesting that enzymes are protected from denaturation (Illing *et al.*, 2005; Farrant, 2007; Farrant *et al.*, 2008). The majority of desiccation tolerant plants increase the

levels of intracellular compounds known as compatible solutes and non-reducing sugars upon water loss (Alpert, 2006). The compatible solutes that increase during dehydration in desiccation tolerant plants are proline, glutamate, glycine, betaine, mannitol, sorbitol and D-ononitol (Hoekstra *et al.*, 2001; Nuccio *et al.*, 1999). The accumulated compatible solutes function by preventing disruption of membranes, denaturation of proteins, and interaction of molecules in the cytoplasm (Alpert, 2006; Hoekstra *et al.*, 2001). As mentioned above, in addition to compatible solutes, non-reducing sugars also accumulate in resurrection plants during desiccation.

Non-reducing sugars such as sucrose, trehalose and members of the raffinose oligosaccharides (RFOs) family have been reported to increase in desiccation tolerant organisms (Alpert, 2006; Nuccio *et al.*, 1999). Sucrose and oligosaccharides (raffinose, stachyose and verbascose) are present at high levels at 50% RWC, and are maintained at 5% RWC in leaf tissue of *X. viscosa* (Peters *et al.*, 2007). Increased levels of sucrose have also been reported in *E. nindensis* (Illing *et al.*, 2005), *S. stapfianus* (Martinella, 2008), *C. wilmsii* and *M. flabellifolius* (reviewed by Farrant, 2007; Cooper and Farrant, 2002) during water loss. *M. flabellifolius* also accumulates trehalose (a non-reducing disaccharide) in its leaf tissue during severe water loss (Moore *et al.*, 2007). Seeds accumulate oligosaccharides and non-reducing sugars in the late stages of seed development at the same time as they acquire desiccation tolerance (Koster and Leopold, 1988; Peterbauer *et al.*, 2001). These levels of non-reducing sugars decrease upon germination when desiccation tolerance is lost. For example, in pea and soybean seeds, sucrose, stachyose and verbascose levels decrease when the seeds are imbibed in water (Koster and Leopold, 1988). The abundance of transcripts that encode enzymes responsible for the synthesis of non-reducing sugars increase during desiccation in both resurrection plants and seeds (Peters *et al.*, 2007; Collett *et al.*, 2004; Downie *et al.*, 2003).

The enzyme galactinol synthase (GOLS), which is involved in the biosynthesis of RFOs from UDP galactose, has been shown to increase upon dehydration in *X. humilis* (Collett *et al.*, 2004), *X. viscosa* (Peters *et al.*, 2007) and in tomato seeds during maturation (Downie *et al.*, 2003). In *X. humilis*, three isoforms of *GOLS* have been identified (Collett *et al.*, 2004). In dehydrated leaf tissue of *X. humilis* at 30 % RWC, one isoform was downregulated and the other two isoforms were upregulated.

Presumably, the two upregulated GOLS isoforms are involved in the synthesis of RFOs that accumulate in *X. humilis* during severe water loss. The evidence strongly suggests a common mechanism in acquiring desiccation tolerance between seeds and desiccation tolerant angiosperms (Bernacchia and Furini, 2004).

Non-reducing sugars not only function in the same way as compatible solutes by preventing the disruption of membranes and the aggregation of denatured proteins by replacing water. They are also involved in the formation of a glass complex in desiccated tissues (Alpert, 2006; Goyal *et al.*, 2005; Leprince and Buitink, 2004). The formation of a glass complex has also been reported in seeds containing only 10% water content (Alpert, 2006; Buitink and Leprince, 2004). This glass matrix is highly viscous. It prevents the interaction of molecules, thereby preventing the crystallization of solutes in the cytoplasm and chemical interactions between the cell components (Leprince and Buitink, 2004). Furthermore, Late Embryogenesis Abundant (LEA) proteins and heat shock proteins also prevent the aggregation and denaturation of proteins, including enzymes, during severe water loss (Alpert, 2006).

It has been speculated that LEA proteins contribute to the formation of the glass matrix and also prevent the denaturation of proteins by functioning as chaperones, replacing the lost water and sequestering ions (Alpert, 2006; Buitink and Leprince, 2004). LEA proteins were categorized into six groups based on their amino acid similarity (reviewed by Bernacchia and Furini, 2004; Wise and Tunnacliffe, 2004). Illing and colleagues (2005) further divided LEA proteins into ten groups based on their expression profiles in vegetative tissues of *X. humilis*, *X. viscosa*, *C. plantagineum*, *T. ruralis*, *A. thaliana* and mature seeds from *A. thaliana*. In desiccation tolerant angiosperms, LEA proteins increase upon water deficit (Bernacchia and Furini, 2004). Transcripts encoding LEA proteins upregulated during desiccation have been identified in *C. plantagineum*, *X. viscosa*, and *X. humilis* (Illing *et al.*, 2005; Collett *et al.*, 2005). Thirteen LEA proteins identified from *X. humilis* where upregulated at approximately 48% RWC, and most transcripts were still present at 5% RWC (Illing *et al.*, 2005). One of the LEA proteins upregulated in the vegetative tissue of *X. humilis* is an orthologue of a seed-specific LEA in *A. thaliana* (Illing *et al.*, 2005). In orthodox seeds, LEA proteins accumulate in the late stages of seeds development during the acquisition of desiccation tolerance (Illing *et*

al., 2005; Bernacchia and Furini, 2004). LEA proteins are 4% of the total protein in orthodox seeds (Wise and Tunnacliffe, 2004). Interestingly, a group 1 LEA protein from wheat and a group 3 LEA protein from nematode were able to preserve the activity of citrate synthase and lactate dehydrogenase *in vitro* when subjected to severe dehydration (Goyal *et al.*, 2005). Other experiments performed by Chakrabartee and colleagues (2007) showed that overexpression of LEA 1 and LEA 3 proteins could prevent protein aggregation *in vivo* in nematodes and mammalian cells. These studies support the hypothesis that the increase in the number of LEA proteins in desiccation tolerant organisms during desiccation protects cellular proteins from denaturation and aggregation.

Another group of proteins that prevent the denaturation of functional proteins are the heat shock proteins (HSPs) (Berjak *et al.*, 2007). HSPs are believed to function as chaperones to proteins during water deficit and high temperatures and are involved in the renaturation of denatured proteins upon rehydration (Berjak *et al.*, 2007; Oliver, 2007). HSPs are divided into categories, depending on the organelles or part of the cell in which they are expressed, such as chloroplasts, mitochondria, endoplasmic reticulum and the cytosol (Walters *et al.*, 2002). In seeds, HSPs protein levels increase during seed maturation, and in the resurrection plant *C. plantagineum*, HSPs are present in hydrated tissue and increase upon water loss. In addition to preventing protein aggregation, resurrection plants have evolved mechanisms to prevent oxidative damage of proteins, lipids and genetic material during severe water loss caused by high levels of reactive oxygen species (Ramanjulu and Bartels, 2002).

1.5. Prevention of reactive oxygen species damage

Reactive oxygen species (ROS) produced include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\cdot) (Noctor and Foyer, 1998; Dutilleul *et al.*, 2003). Electrons from the disrupted electron transport chains cause the reduction of molecular oxygen, resulting in the formation of O_2^- (see Equation 1 below) (Smirnoff, 1993). Subsequently, O_2^- is rapidly converted to H_2O_2 by the enzyme superoxide dismutase (SOD) (Equation 2) (Ivanov and Khorobrykh, 2003; Smirnoff, 1993). Furthermore, the reduction of H_2O_2 results in the formation of OH^\cdot the most reactive of the ROS (Equation 3) (Pospisil *et al.*, 2004). Alternatively, in the

presence of reduced transition metal ions such as copper (Cu^{2+}), iron (Fe^{2+}) and manganese (Mn^{2+}), H_2O_2 is reduced, forming OH^\cdot via the Fenton reaction (Equation 4) (Pospisil *et al.*, 2004; Asada, 1999). ROS cause irreversible damage to membranes, proteins and genetic material (Smirnov, 1993; Fridovich, 1986).

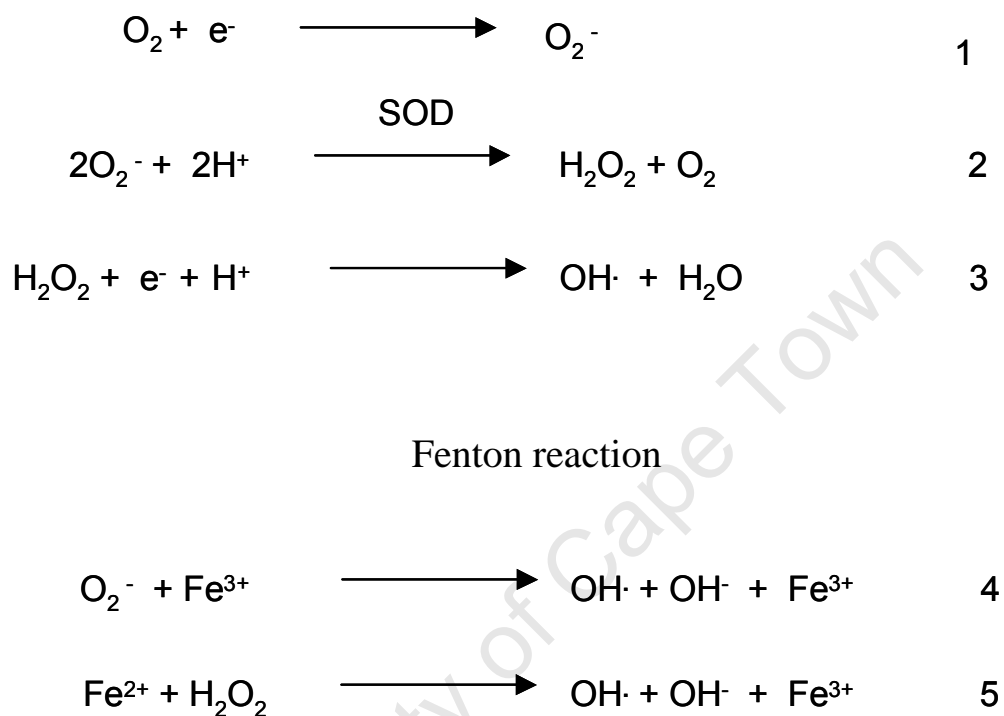


Figure 1.2 Equations 1 to 4 show the formation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\cdot)

Under normal (unstressed) conditions, ROS are produced as by-product of photosynthesis and respiration, but the plants have the ability to quench the ROS produced (Farrant, 2007). Upon dehydration stress, ROS production increases due to the disruption in the electron transport chain in the chloroplasts (Farrant, 2007; Smirnov, 1993). Resurrection plants prevent cellular oxidative damage caused by minimising the generation of ROS and synthesising of *de novo* antioxidants that detoxify ROS (Illing *et al.*, 2005; Bernacchia G and Furini, 2004; Collett *et al.*, 2003; Farrant *et al.*, 2000). Resurrection plants inhibit photosynthesis during severe water loss to minimise the generation of ROS. In *M. flabellifolius*, *X. humilis* and *C. wilmsii* plants, the rate of photosynthesis begins to drop between 80% and 75% RWC (Farrant *et al.*, 2000). Upon further water loss, photosynthesis is completely halted at 55%, 47% and 40% RWC in *M. flabellifolius*, *C. wilmsii* and *X. humilis*, respectively

(Farrant *et al.*, 2000). Furthermore, at 95% RWC, the photochemical efficiency of the photosystem II was reduced by 96% in the resurrection plant *Haberlea rhodopenss*, (Georgieva *et al.*, 2007). In addition to reducing ROS production by inhibiting photosynthesis, resurrection plants further inhibit the generation of ROS and protect the photosynthetic apparatus by either dismantling or by shielding the photosynthetic apparatus (Oliver, 2007; Bernacchia G and Furini, 2004; Collett *et al.*, 2003; Alpert and Oliver, 2002; Farrant *et al.*, 2000).

Resurrection plants that avoid photochemical damage by dismantling their photosynthetic apparatus and degrading chlorophyll are known as poikilochlorophyllous (PDT) (Bernacchia G and Furini, 2004; Collett *et al.*, 2003; Farrant *et al.*, 2000). Resurrection plants known as homoiochlorophyllous (HDT) retain their photosynthetic apparatus during desiccation, but they prevent light-chlorophyll interactions in various ways (Bernacchia G and Furini, 2004; Farrant *et al.*, 2000). Upon severe water loss, PDT plants degrade their chlorophyll and dismantle the thylakoids, which are stored in vesicles (Proctor and Tuba, 2002; Tuba *et al.*, 1998). PDT plants that have been studied include *Xerophyta scabrida*, *X. humilis* and *X. viscosa* (Collett *et al.*, 2003; Tuba *et al.*, 1998). The expression of proteins associated with the photosystem II (PsbA, PsbP and PsbR) are significantly reduced in *X. humilis* (Collett *et al.*, 2003) and (PsbO, PsbP and PSII stability factor) in *X. viscosa* (Ingle *et al.*, 2007). Photosynthesis is inhibited in PDT resurrection plants, such as *X. humilis*, at the same relative water content at which the chlorophyll is degraded the photosynthetic apparatus dismantled (Farrant *et al.*, 2000). Upon rehydration, the photosynthetic apparatus is assembled, and chlorophyll synthesised. In contrast, HDT plants such as *M. flabellifolius* and *C. wilmsii* retain their chlorophyll and do not dismantle their photosynthetic apparatus. However, they curl their leaves so that only the abaxial surfaces of the outer leaves are exposed to light during the loss of water (Moore *et al.*, 2007; Moore *et al.*, 2006; Le and McQueen, 2006; Bernacchia G and Furini, 2004; Vicre *et al.*, 2004). The abaxial surfaces of the outer leaves contain high levels of anthocyanin pigments. Anthocyanins shield the leaves from sunlight, resulting in the prevention of oxidative damage (Moore *et al.*, 2007; Moore *et al.*, 2006; Le and McQueen, 2006). The critical point at which photosynthesis stops in the HDT plants such as *M. flabellifolius*, and *C. wilmsii* correlates with when the leaves of these plants become completely folded (Farrant *et*

al., 2000). To further avoid oxidative damage, resurrection plants also upregulate antioxidants to remove ROS formed during desiccation (Farrant, 2007).

ROS are detoxified by non-enzymatic antioxidants and/or enzymatic antioxidant (Mittova *et al.*, 2004). Non-enzymatic antioxidants that scavenge O_2^- produced in the leaves of plants include pigments such as alpha-tocopherols and beta-carotene (Farrant, 2007; Kranner *et al.*, 2002) and anthocyanins (Moore *et al.*, 2007b; Moore *et al.*, 2006; Le and McQueen, 2006). Ascorbate and glutathione function as non-enzymatic antioxidants and are used as cofactors for several antioxidant enzymes (Noctor and Foyer, 1998; Kranner *et al.*, 2002; Noctor, 1989). In *M. flabellifolius*, the polyphenols that accumulate in vacuoles as mentioned are believed to function as non-enzymatic antioxidants (Farrant, 2007).

During water loss, resurrection plants such as *C. plantagineum*, *X. humilis* and *X. viscosa* upregulate antioxidant enzymes already expressed in hydrated tissue to detoxify ROS. These antioxidants are known as house keeping antioxidant enzymes (Franca *et al.*, 2005; Illing *et al.*, 2005; Mundree *et al.*, 2000). Housekeeping antioxidant enzymes include glutathione reductase, catalase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and glutathione-S-transferase (Oliver, 2007; Mittova *et al.*, 2004; Noctor and Foyer, 1998). When superoxide dismutase is coupled with the superoxide radical, for example, it produces H_2O_2 (Mittova *et al.*, 2004). In turn, H_2O_2 is hydrolysed to water by ascorbate peroxidase, glutathione peroxidase and catalase (Franca *et al.*, 2005; Asada, 1999). The levels and activity of superoxide dismutase and aldose reductase have been shown to increase in *X. viscosa* (Farrant *et al.*, 2000; Mundree *et al.*, 2000). In addition, the transcripts of ascorbate peroxidase have been reported to increase in *X. viscosa*, *X. humilis* and *C. wilmsii* (Farrant *et al.*, 2000; Illing *et al.*, 2005). In *X. viscosa*, the ascorbate peroxidase and 2-cys-peroxiredoxin proteins have been shown to increase during desiccation in a proteomics study (Ingle *et al.*, 2007). In *S. staphianus*, activity of glutathione reductase increases during water loss (Farrant *et al.*, 2000).

In addition to the protection of housekeeping antioxidants, specialist anti-oxidant enzymes are expressed during desiccation (Illing *et al.*, 2005). For example, a seed-specific antioxidant 1-cys peroxiredoxin (Aalen, 1999) is upregulated in the

desiccation tolerant vegetative tissues of *X viscosa* (Mowla *et al.*, 2002) during desiccation. The levels and activity of 1-cys peroxiredoxins also increase during drying in *X. viscosa* (Farrant *et al.*, 2000). By contrast, 1-cys peroxiredoxin is expressed in *T. ruralis* during rehydration (Oliver *et al.*, 1996). The differences in the regulation of antioxidants, such as 1-cys peroxiredoxin, and the differences in the activation of osmoprotectants and LEA proteins between angiosperm resurrection plants and the moss, *T. ruralis*, suggest that desiccation tolerant plants have evolved (slightly) different protective mechanisms.

1.6. Evolution of desiccation tolerance

As mentioned above, lower order plants do not accumulate protective proteins and non-reducing sugars during drying. However, they contain some protectants, such as sucrose and LEA proteins, which are constitutively produced or expressed (Oliver *et al.*, 2004; Oliver *et al.*, 2000). In addition, transcripts believed to be important for repair during rehydration are also transcribed (Oliver *et al.*, 2004). Based on these observations, Oliver *et al.*, (2005) proposed that these lower order plants contain primitive mechanisms for desiccation tolerance that were present in the first plants to populate land. It is speculated that desiccation tolerance was lost during the development of higher plants, although maintained in seeds and pollen. The preservation of desiccation tolerance in seeds meant that the genetic information needed to acquire desiccation tolerance was present in adult plants, although the ability to activate the genes was lost (Alpert, 2006). A change in the genetic regulation in the vegetative tissue of desiccation tolerant plants (Bernacchia and Furini, 2004), based on the mechanisms in seed (Bohnert, 2000), has caused desiccation tolerance to remerge within the angiosperms ten times, independently (Oliver, 2007). This accounts for the differences in the mechanisms used to cope with desiccation within the angiosperm subfamily. Desiccation tolerant angiosperm plants and seeds are said to have 'acquired desiccation tolerance mechanisms' (Oliver, 2007; Oliver *et al.*, 2000). As reported previously, sugars, oligosaccharides and LEA protein levels increase only at the onset of water stress (Peters *et al.*, 2007 Illing *et al.*, 2005; Bernacchia and Furini, 2004; Buitink and Leprince, 2004). Furthermore, the hormone abscisic acid (ABA) is involved the activation of genes that confer desiccation tolerance in seeds and higher order plants (Bernacchia and Furini, 2004).

However, ABA is not involved in the regulation of desiccation tolerant genes in lower order plants, such as bryophytes (Oliver *et al.*, 2000). The role of the hormone abscisic acid (ABA) during dehydration in the vegetative tissues of desiccation sensitive, desiccation tolerant plants and orthodox seeds has been extensively researched (Gutierrez *et al.*, 2007; Alpert, 2006; Yamaguchi-shinozaki and Shinozaki, 2006; Bernacchia and Furini, 2004; Oliver *et al.*, 2000).

1.6.1. Regulation of desiccation response in seeds and higher order desiccation tolerant angiosperm plants

Endogenous abscisic acid (ABA) levels have been reported to increase during the late maturation stage of seed development (Gutierrez *et al.*, 2007). In orthodox seeds, high ABA levels increase the expression of Abscisic Acid Insensitive 3 (ABI3), FUSCA3 (FUS3), Leafy cotyledon 1 (LEC1) and Leafy cotyledon 2 (LEC 2) regulatory genes (Gutierrez *et al.*, 2007). The well studied of these regulatory genes is the transcription factor ABI3 (Bernacchia and Furini, 2004). The role of ABA in activating genes important for the acquisition of desiccation tolerance and dormancy in orthodox seeds has been extensively studied by the analysis of ABA-deficient (*aba*) and -insensitive (*abi*) *A. thaliana* mutants during seed development (Meurs *et al.*, 1991). In ABA deficient/insensitive *A. thaliana* double mutant seeds (*abi3/aba*), the accumulation of storage proteins during the late maturation phase of seed development is impaired (Meurs *et al.*, 1991). These double mutants were unable to breakdown chlorophyll and did not undergo dormancy. In addition, the seeds of these *abi3/aba* double mutants never acquire desiccation tolerance (Meurs *et al.*, 1991). When subjected to desiccation, the seeds were unable to germinate. However, *abi3/aba* double mutant seeds that were treated with exogenous ABA were able to accumulate storage proteins. These seeds acquired desiccation and dormancy and were able to germinate (Meurs *et al.*, 1991).

An ABI3 orthologue from yellow cedar, *CnABI3*, was also involved the promotion of storage protein accumulation during the late maturation stage of yellow cedar seeds (Zeng *et al.*, 2003). ABI3 *A. thaliana* mutant seeds ectopically expressing CnABI3 were able to accumulate storage proteins and acquired desiccation tolerance upon drying (Zeng and Kermode, 2004). Interestingly, the expression of these seed-

specific transcription factors, AB13 and CnABI3, when activated by ABA in the vegetative tissues of *A. thaliana* were able to activate seed-specific genes (Bernacchia and Furini, 2004; Zeng *et al.*, 2003). These results suggest that ABA is important in the acquisition of desiccation tolerance, dormancy and the inhibition of premature germination (Zeng *et al.*, 2003; Hoekstra *et al.*, 2001; Meurs *et al.*, 1991).

The role of ABA during water stress in vegetative tissues of desiccation sensitive plants and resurrection plants has also been extensively researched (Guitierrez *et al.*, 2007; Alpert, 2006; Yamaguchi-shinozaki and Shinozaki, 2006; Bernacchia and Furini, 2004; Oliver *et al.*, 2000). Although ABA is involved in the activation of many genes during water loss in the vegetative tissues of desiccation sensitive plants, the scope of this section is restricted to ABA regulation during severe water loss (desiccation) in orthodox seeds and in the vegetative tissues of resurrection plants.

ABA levels have been reported to increase in vegetative tissues of resurrection plants during desiccation. For example, in the leaves of *M. flabellifolius* and *Borya nitida*, ABA levels increase 3 to 7 fold (reviewed by Bernacchia and Furini, 2004). The role of ABA during desiccation tolerance has been extensively studied in *C. plantagineum* (Bernacchia and Furini, 2004). Piatkowski *et al.*, (1990) reported that three genes of unknown function (pcC6-19, pcC27-04 and pcC3-06) were upregulated in *C. plantagineum* callus treated with ABA. Interestingly, these genes of unknown function share similarity to genes expressed during embryo development. In addition, transcripts of two LEA proteins were induced by ABA in *C. plantagineum* (Ditzer and Bartels, 2006; Rodrigo *et al.*, 2004). A *C. plantagineum* early response dehydration induced gene, *CpEDi-9* which encodes a protein that shares high physiological and biochemical properties with group 2 LEA proteins, was upregulated in roots and leaves of dehydrated or by ABA treated of *C. plantagineum* (Rodrigo *et al.*, 2004). Furthermore, the transcripts of a gene coding for a protein belonging to the group 4 of the LEA proteins accumulated upon dehydration or ABA treatment (Ditzer and Bartels). In addition, ABA-induced transcription factors belonging to the MYC and MYB transcription factor families have been identified in both desiccation sensitive and tolerant plants and seeds (Villalobos *et al.*, 2004; Kizis *et al.*, 2001). For example, a member of the MYB transcription factor family, the CpMYB10 transcription factor from *C. plantagineum* is induced by ABA. Many other

transcription factors activated by increased ABA concentrations have been identified, and these include members of the homeodomain leucine zipper genes in *C. plantagineum* (Deng *et al.*, 2002) and in *A. thaliana* seeds (Bensmihen *et al.*, 2005). In addition, ABA-responsive regulatory elements (ABREs) have been identified within the promoters of ABA-induced genes; these ABREs are recognised by ABA-induced transcription factors (Kizis *et al.*, 2001). The promoter of the *C. plantagineum* CpEDi-9 gene, for example, contains ABRE core G-box elements and a myb-like regulatory element (Rodrigo *et al.*, 2004). Another *C. plantagineum* protein, PCC2, contains ABRE-ABA-responsive elements and a basic region leucine-zipper (bZip) within its promoter (Ditzer and Bartels, 2006).

Certain genes are upregulated during dehydration in the absence of ABA, indicating the presence of ABA-independent pathways for dehydration in plants and seeds. Gene regulation through the ABA-independent pathway has been extensively researched in desiccation sensitive plants (Yamaguchi-shinozaki and Shinozaki, 2006). AP2/EREBP transcription factors that recognise the DNA-damage responsive element 1 regulatory elements, for example, are believed to function in the ABA-independent pathway (Kizis *et al.*, 2001). This pathway is not well documented in the literature for resurrection plants. The identification and study of genes upregulated during desiccation through both the will ABA-dependent and ABA-independent will contribute understanding the acquisition of desiccation tolerance of resurrection plants.

1.7. Investigating the role of genes upregulated during water loss

Microarray technology is a high throughput tool that has enabled the identification of genes involved in dehydration in drought sensitive plants such as *A. thaliana* (Umezawa *et al.*, 2006; Seki *et al.*, 2003), *Oryza sativa* (Rabbani *et al.*, 2003;) and *Physcomitrella patens* (Cuming *et al.*, 2007), in a moderate drought tolerant plant such as barley (Umezawa *et al.*, 2006; Ozturk *et al.*, 2002) and in the desiccation tolerant plant, *X. humilis* (Collett *et al.*, 2004). In addition, genes upregulated during rehydration, following dehydration stress, have been identified in *A. thaliana* (Oono *et al.*, 2003) and *T. ruralis* (Oliver *et al.*, 2004). The genes identified can be divided into two categories: regulatory genes, such as transcriptional factors, and functional genes,

which are activated by regulatory genes, and directly contribute to the protection of the plant against damage caused by water deficit (Bhatnagar-Mather *et al.*, 2008; Umezawa *et al.*, 2006; Rabbani *et al.*, 2003) (see figure 1.1).

Several regulatory and functional genes have been identified that are upregulated in both desiccation sensitive and tolerant plants. It is speculated that during the initial stages of water loss, desiccation sensitive and desiccation tolerant plants share common mechanisms to prevent damage (Illing *et al.*, 2005; Bartels *et al.*, 2007). However, during severe dehydration (desiccation), which desiccation sensitive plants do not survive, resurrection plants upregulate unique genes that contribute to the protection of cells and tissues. For example, desiccation sensitive plants do not have the ability of upregulate osmoprotectants such as trehalose (Bhatnagar-Mathur *et al.*, 2008). Also, some genes are only switched on at low water contents (The point at which the bulk protoplasmic water is lost (Ramanjulu and Bartels, 2002)). For example, the levels of thirteen *X. humilis* LEA protein transcripts only start to increase at 48 % RWC (Illing *et al.*, 2005). In *X. humilis*, many genes that were upregulated during severe dehydration in a small scale microarray experiment were absent in the vegetative tissues of desiccation sensitive plants, but present in orthodox seeds (Collett *et al.*, 2004). These findings further strengthen the hypothesis that desiccation tolerant plants share a common mechanism in combating dying during water deficit with seeds. The challenge is to determine the function of these regulatory and functional genes during desiccation tolerance in order to understand the acquisition of desiccation tolerance.

One of the strategies to understand how regulatory genes and functional genes contribute to drought tolerance in plants has been to engineer transgenic plants (Umezawa *et al.*, 2006; Rabbani *et al.*, 2003). Table 1.1 shows a summary of genes that confer tolerance to transgenic plants exposed to water deficit stress.

Generation of transgenic plants either by overexpressing or the generation of a transgenic plant deficient in the gene of interest have contributed to the understanding of the mechanisms involved during water stress. For example, desiccation sensitive plants do not accumulate trehalose or high concentrations of polyamine in their vegetative tissues during osmotic stress (Garg *et al.*, 2002). Interestingly, desiccation

sensitive transgenic plants constitutively overexpressing trehalose and polyamines are tolerant to dehydration relative to the control plants (Bhatnager-Mathur *et al.*, 2008, Karim *et al.*, 2007; Garg *et al.*, 2002). Furthermore, Goyal and co-workers (2005) reported that the sugar trehalose was able to preserve the activity of lactate dehydrogenase during desiccation *in vitro*. These results support the hypothesis that the increase of compatible solutes and non-reducing sugars, either during the late-maturation of orthodox seeds or upon desiccation in vegetative tissues of resurrection plants, contributes to the acquisition of desiccation tolerance (see section 1.4). However, as a side effect, overexpression of trehalose was reported to interfere with the normal development of the transgenic tobacco and *A. thaliana* plants, which had a dwarf phenotype (Bhatnager-Mathur *et al.*, 2008; Karim *et al.*, 2007). These findings strongly suggest that the accumulation of sugars, such as trehalose, has to be tightly controlled. This explains either the absence of sugars, or their presence in very low levels, in angiosperm desiccation tolerant plants (Martinella, 2008; Peters *et al.*, 2007; Moore *et al.*, 2007).

Table 1.1. A summary of genes that confer tolerance to transgenic plants exposed to water deficit stress

Classification	Gene name	Transgenic plant	Origin of gene	Purpose	References
Regulatory Genes					
MYB	<i>CpMYB10</i>	<i>A. thaliana</i>	<i>C. plantagineum</i>	Overexpression	Villalobos <i>et al.</i> , 2004
Other	<i>HaHSfA9</i>	Tobacco	Sunflower seed	Overexpression	Prieto-Dapena <i>et al.</i> , 2008
Compatible solutes					
Fructan	<i>SacB</i>	Tobacco	<i>Bacillus subtilis</i>	Overexpression	Pilon-Smits <i>et al.</i> , 1995
Glycine betaine	<i>codA</i>	Potato	<i>Arthrobacter globiformis</i>	Overexpression	Ahmad <i>et al.</i> , 2008
	<i>betA</i>	Maize	<i>Escherichia coli</i>	Overexpression	Quan <i>et al.</i> , 2004
Proline	<i>HvProT</i>	<i>A. thaliana</i>	Barley	Overexpression	Ueda <i>et al.</i> , 2008
	<i>P5CS</i>	Wheat	<i>Vigna aconitifolia</i>	Overexpression	Vendruscolo <i>et al.</i> , 2007
	<i>P5CS</i>	Tobacco	Mothbean	Overexpression	Kishor <i>et al.</i> , 1995
Mannitol	<i>mtlD</i>	Wheat	<i>Escherichia coli</i>	Overexpression	Abebe <i>et al.</i> , 2003
	<i>mtlD</i>	Tobacco	<i>Escherichia coli</i>	Overexpression	Tarczynski <i>et al.</i> , 1992
Non-reducing sugars					
D-ononitol	<i>IMT1</i>	Ice plant	Tobacco	Overexpression	Sheveleva <i>et al.</i> , 1997
Galactinol	<i>GolS 1</i> and <i>GlolS 2</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	Overexpression	Nishizawa <i>et al.</i> , 2008

Classification	Gene name	Transgenic plant	Origin of gene	Purpose	References
Raffinose	<i>OsUGE-1</i>	<i>A. thaliana</i>	Rice	Overexpression	Liu <i>et al.</i> , 2007
Trehalose	<i>OtsA</i> and <i>OtsB</i>	Rice	<i>Escherichia coli</i>	Overexpression	Garg <i>et al.</i> , 2002
Protective proteins					
LEA 2	<i>DHN-5</i>	<i>A. thaliana</i>	Wheat	Overexpression	Brini <i>et al.</i> , 2007
LEA3	<i>HVA1</i>	Mulberry	Barley	Overexpression	Lal <i>et al.</i> , 2008
	<i>HVA1</i>	Rice	Barley	Overexpression	Xu <i>et al.</i> , 1996
LEA	<i>D113</i>	Tobacco	Cotton (mature seed)	Overexpression	Luo <i>et al.</i> , 2008
	<i>OsLEA3-1</i>	Rice	Rice	Overexpression	Xiao <i>et al.</i> , 2007
Antioxidants					
Aldehyde dehydrogenase	<i>ALDH7B4</i> and <i>ALDH3I1</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	Overexpression	Kotchoni <i>et al.</i> , 2006
	<i>ZmALDH22A1</i>	Tomato	Maize	Overexpression	Huang <i>et al.</i> , 2008
Aldose/aldehyde reductase	<i>ALR</i>	Tobacco	Alfalfa	Overexpression	Oberschall <i>et al.</i> , 2000
Ascorbate peroxidase	<i>APX</i>	<i>Festuca arundinacea</i>	Pea	Overexpression	Lee <i>et al.</i> , 2007
	<i>APX</i>	Tobacco	<i>A. thaliana</i>	Overexpression	Badawi <i>et al.</i> , 2004

Classification	Gene name	Transgenic plant	Origin of gene	Purpose	References
Superoxide dismutase	<i>CuSOD (Sod1)</i>	Rice	<i>Avicennia marina</i>	Overexpression	Prashanth <i>et al.</i> , 2008
	<i>CuZnSOD</i>	<i>Festuca arundinacea</i>	Pea	Overexpression	Lee <i>et al.</i> , 2007
	<i>MnSOD</i>	Rice	Pea	Overexpression	Wang <i>et al.</i> , 2005
ABA biosynthesis					
	AtNCED3	<i>A. thaliana</i>	<i>A. thaliana</i>	Overexpression	Luchi <i>et al.</i> , 2001
Zeaxanthin epoxidase	<i>ZEP</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	Overexpression	Park <i>et al.</i> , 2008
ABA catabolism					
	<i>CYP707A3</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	T-DNA knockout	Umezawa <i>et al.</i> , 2006
Unknown					
	<i>CDT1</i>	<i>C. plantagineum</i>	<i>C. plantagineum</i>	T-DNA knockout	Smith-Espinoza <i>et al.</i> , 2005

APX, Ascorbate peroxidase; *ALR*, Aldose/aldehyde reductase; *BetA*, Choline dehydrogenase; *CuZnSOD*, Copper superoxide dismutase; *codA*, choline oxidase; *GolS*, galactinol synthase; *HvProT*, proline transporter; *IMT*, myo-inositol O-methyltransferase; *MnSOD*, manganese superoxide dismutase; *mtlD*, mannitol-1-phosphate dehydrogenase; *NCED*, 9-cis epoxy-carotenoid dioxygenase; *OtsA*, Trehalose-6-phosphate synthase; *OtsB*, trehalose-6-phosphate phosphatase; *P5CS*, Delta- (1)- pyrroline-5 carbonyl synthase; *UGE*, UDP-glucose 4-epimerase; *SacB*, levansucrase.

1.8. Characterisation of *X. humilis desiccation induced-1* (*Xhdsi-1*^{VOC})

The aim of this PhD was to characterise a gene, initially named *HC205*, which had high similarity to a seed-specific *A. thaliana* gene, *At1g07645*, which was annotated as a putative glyoxalase I. *HC205* was identified as dehydration-induced gene in a small-scale microarray analysis using 424 annotated cDNAs from a *X.humilis* dehydration and rehydration cDNA library. Bioinformatics analysis showed that the predicted *HC205* and *At1g07645* amino acid sequences did not contain conserved amino acids that form the binding sites for zinc and glutathione in glyoxalase I. In addition, the functional analysis showed that *HC205* did not function as a glyoxalase I. Structural analysis, however, showed that the protein encoded by *HC205* and its plant orthologues belonged to the Vicinal Oxygen Chelate (VOC) superfamily. Based on these findings, *HC205* was named *X. humilis desiccation induced-1* (*Xhdsi-1*^{VOC}). The role of *Xhdsi-1*^{VOC} during water loss was investigated by generating *A. thaliana* transgenic plants constitutively overexpressing *Xhdsi-1*^{VOC} and its orthologue, *At1g07645*. In addition, loss-of-function was investigated by RNAi-mediated silencing of *At1g07645* and by using *At1g07645* T-DNA knockout mutants in *A. thaliana* seeds.

Chapter 2

Xhdsi-1^{VOC}*, a novel member of the Vicinal Oxygen Chelate (VOC) metalloenzyme superfamily, is upregulated in the vegetative tissues of *X. humilis

2.1 Introduction

The acquisition of desiccation tolerance in resurrection plants involves the activation of many genes that encode proteins involved in the protection of desiccating cells. For example enzymes involved in carbohydrate metabolism and antioxidants involved in the detoxification of reactive oxygen species (ROS) (Farrant, 2007; Bartels and Salamini, 2001). During desiccation, transcripts down- and upregulated in roots and leaves of *X. humilis* have been identified by microarray analysis in our research group (Shen, unpublished; Walford, unpublished; Collett *et al.*, 2004; Collett *et al.*, 2003). The desiccation-upregulated genes identified in *X. humilis* microarray studies either have high sequence similarity to genes of known function or are of unknown function. The orthologues of *X. humilis* desiccation upregulated genes are either expressed in vegetative tissues of desiccation-sensitive plants during abiotic stress or are expressed in orthodox seeds. However, detailed comparisons of the expression profiles of these genes during the desiccation-rehydration cycle in *X. humilis* to orthologues in other resurrection plants, desiccation sensitive plants and orthodox seeds will elucidate similarities or differences in gene regulation. Information on the expression patterns of the desiccation tolerant upregulated genes and the knowledge of their function will contribute to understanding the mechanisms involved during the acquisition of desiccation tolerance due to changes in environmental factors (Bartels and Salamini, 2001).

Changes in environmental factors such as water, temperature and light lead to abiotic stress which perturb various metabolic pathways in plants for example photosynthesis

and respiration (Mittler *et al.*, 2004; Udemá and Uchimiya, 1994). These developments lead to an increase in ROS and glycating agents (Fig. 2.1) (Mittler *et al.*, 2004; Thornalley, 2003; Noctor and Foyer, 1998). ROS include superoxide (O_2^-), hydroxyl radicals (OH^\cdot) and hydrogen peroxide (H_2O_2) (Dutilleul *et al.*, 2003; Noctor and Foyer, 1998). Oxidative stress caused by increased ROS levels causes an increase in cellular damage such as lipid peroxidation, protein degradation/modification and damage of DNA (Fridovich, 1986). Abiotic stress also results in increased production of glycation agents. Glycation agents include oxoaldehydes such as glyoxal formed by lipid peroxidase and glyoxalate oxidation (Freire *et al.*, 2003) and methylglyoxal, a triosephosphate by-product of glycolysis and the breakdown of threonine and acetone (Yadav *et al.*, 2008; Freire *et al.*, 2003; Thornalley, 2003). Glycation agents modify proteins, nucleic acids and basic phospholipids producing advanced glycation end products (AGEs) (Thornalley, 2003). AGEs further cause damage by reacting with cellular components, for example the increase of AGEs in humans exacerbates chronic complications in patients with diabetes mellitus and also causes kidney failure.

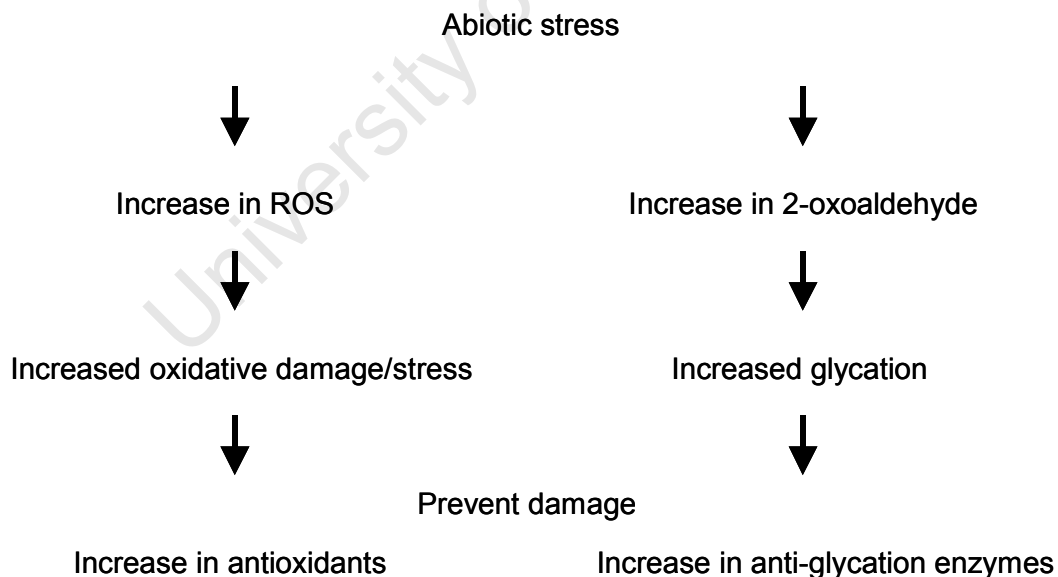


Fig. 2.1. Abiotic stress leads to an increase in ROS and glycating agents such as 2-oxoaldehyde. This results in increased oxidative damage and glycation. Plants increase the levels of antioxidants and anti-glycating enzymes to prevent damage caused by ROS and glycating agents.

Thus, to prevent damage to cellular components, it is crucial that methylglyoxal is removed from the cells. Glyoxalase I is part of the glyoxalase system found in animals, plants and bacteria that detoxifies 2-oxoaldehydes to hydroxycarboxylic acids. It has an affinity for a particular oxoaldehyde, methylglyoxal (Thornalley, 2003). Methylglyoxal and the reduced glutathione react spontaneously to form a hemithioacetal (Fig. 2.2). The hemithioacetal is subsequently recognised by glyoxalase I, which is also known as lactoylglutathione lyase and is then isomerised to S-D- lactoylglutathione. In the presence of an H₂O molecule, the S-D- lactoylglutathione is converted into D-lactic acid in a reaction catalysed by glyoxalase II (hydroxy-lacylglutathione hydrolase), and the reduced glutathione is recycled (Yadav *et al.*, 2008; Thornalley, 2003; Martins *et al.*, 2001).

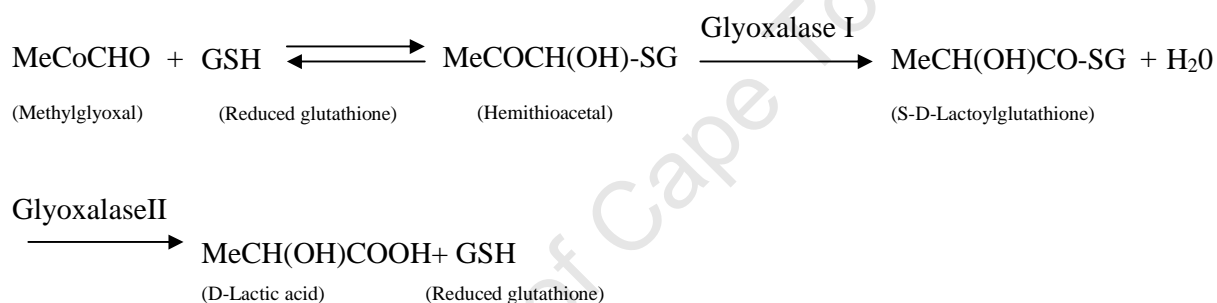


Figure 2.2. The detoxification of methylglyoxal by the glyoxalase system (Veena *et al.*, 1999). Methylglyoxal spontaneously reacts with reduced glutathione to form a hemithioacetal, which is recognised and isomerised to S-D- lactoylglutathione by glyoxalase I. In the presence of water, S-D- lactoylglutathione is catalysed by glyoxalase II into D-lactic acid and the reduced glutathione is released.

Glyoxalase I has been shown to be upregulated in plants exposed to environmental stress (Singla-Parrek *et al.*, 2003; Martins *et al.*, 2001; Veena *et al.*, 1999). For example, glyoxalase I mRNA transcripts and protein levels increase in *Brassica juncea* (Veena *et al.*, 1999) and tomato (Espartero *et al.*, 1995) when the plants are exposed to salt stress, osmotic stress or heavy metals. Overexpression of the *B. juncea* glyoxalase I in tobacco transgenic lines led to a significant increase in tolerance compared to wild type after treatment with methylglyoxal, NaCl (Veena *et al.*, 1999) and zinc (Singla-Pareek *et al.*, 2006). In addition, overexpression of either glyoxalase I or II in transgenic tobacco plants resulted in a higher tolerance to high NaCl and methylglyoxal relative to wild type (Singla-Parrek *et al.*, 2003). Interestingly, when both glyoxalase I and II are overexpressed in the same plant, there

is a greater increase in tolerance than when the two enzymes are expressed separately (Parrek *et al.*, 2003). It is speculated that glyoxalase I is upregulated in plants during environmental stresses such as high salt due to an increase in glycolysis activity causing a high demand for ATP (Veena *et al.*, 1999). However, this gene has been identified in a resurrection species, *Sporobolous stapfianus* (Clugston *et al.*, 1998). To date there is no evidence that glycolysis increases during desiccation in resurrection plants. Thus, there is a possibility of an alternative mechanism for the formation of methylglyoxal during desiccation in resurrection plants.

Glyoxalase I is a member of the vicinal oxygen chelate (VOC) superfamily (Table 2.1). The VOC superfamily consists of proteins that have a common $\beta\alpha\beta\beta$ structure fold (Armstrong, 2000; Rife *et al.*, 2002). Several three-dimensional structures belonging to the VOC superfamily have been studied by X-ray crystallography. These proteins are classified as metalloenzymes, and the similarities in structure fold provides a cavity within the structure containing similar metal coordination sites (Armstrong, 2000). The name, VOC, originates from the belief that interaction between the substrate and the metals are via the vicinal oxygen atoms of the substrate (Armstrong, 2000). However, these metalloenzymes catalyze diverse types of reactions (Martin *et al.*, 2002; McCarthy *et al.*, 2001; Armstrong, 2000; Kita *et al.*, 1999; Bernat *et al.*, 1997; Gatignol *et al.*, 1988). The bleomycin resistant protein is different from the other members of the VOC family; it is not an enzyme and does not contain metal binding sites, but instead contains a hydrophobic cavity for bleomycin adhesion. (Armstrong, 2000). The bleomycin resistant protein inhibits damage induced by bleomycin, which is a glycopeptide antibiotic from *Streptomyces verticillus* (Kumagai *et al.*, 1999). It is suggested that the conserved $\beta\alpha\beta\beta$ structure derives from a common ancestor and that due to gene duplication and fusion, the family contains enzymes with divergent function (McCarthy *et al.*, 2001; Armstrong, 2000; Bergdoll *et al.*, 1998). To date, glyoxalase I is the only member of the VOC superfamily reported to be upregulated in the resurrection plant *Sporobulus stapfianus* (Clugston *et al.*, 1998).

Table 2.1. Characteristics of members of the VOC Superfamily

Name	Abbreviation	Organism	Metal ion	Co-enzyme	Function	Functional Unit
Bleomycin resistance protein	BRP	Fungi & eubacteria	No		Sequesters bleomycin & related compounds (no degradation or transformation)	Homodimer ^{*1}
2,3-Dihydroxy-biphenyl 1,2-dioxygenase (estradiol dioxygenase)	DHBD	Eubacteria	Fe ²⁺		Microbial degradation of aromatic compounds e.g. degrades biphenyl and polychlorinated biphenyls	Monomer ^{*1}
Glyoxalase I (small)	GLO	Eubacteria, plants & animals	Zn ²⁺	glutathione	Isomerisation reaction: glutathione-dependent inactivation of toxic methylglyoxal	Homodimer ^{*1}
Glyoxalase I (large)	GLO	Fungi	Zn ²⁺	glutathione	Isomerisation reaction: glutathione-dependent inactivation of toxic methylglyoxal	Monomer ^{*2}
Fosfomycin resistance protein	FosA	Eubacteria	Mn ²⁺	glutathione	Inactivation of the antibiotic fosfomycin by nucleophilic opening of epoxide ring	Homodimer ^{*3}
Methylmalonyl-CoA epimerase	MMCE	Eubacteria, Archea & animals	Co ²⁺		Epimerization reaction: catalyses conversion of (2R)-methylmalonyl-CoA to (2S)-methylmalonyl-CoA. Methylmalonyl-CoA is a metabolic intermediate in several degradation pathways (e.g. lipids and branched amino acids) and biosynthetic pathways (e.g. important polyketide antibiotics)	Homodimer ^{*4}

*1 Bergdoll *et al.*, 1998; *2 Thornalley, 2003; *3 Rigsby *et al.*, 2007; *4 McCarthy *et al.*, 2001; Summarised by Nicola Illing (from Mulako *et al.*, 2008).

A small-scale microarray analysis using 424 annotated cDNA clones randomly selected from a 10 900 cDNA library from *X. humilis* leaf and root dehydration and rehydration was performed in our lab (Collett *et al.*, 2004). The microarray experiments identified 55 genes that were upregulated during dehydration in leaf tissue (30% RWC) when compared to hydrated leaf tissue (100% RWC). Several of these cDNAs were orthologues of genes that are already known to be expressed in the desiccated tissue of other resurrection plants. These included enzymes that synthesize osmoprotectants such as aldose reductase and galactinol synthase, as well as protective proteins such as LEAs and dehydrins (Collett *et al.*, 2004). In addition, there were many novel cDNAs which showed significant similarity to *A. thaliana* genes that were annotated as genes of unknown function. The *X. humilis* HC205 desiccation upregulated gene, which formed the study of this thesis, showed similarity to the *A. thaliana* gene *At1g07645*, annotated as a glyoxalase-1 like protein in the database, and was named *X. humilis* desiccation induced-1^{VOC} (*Xhdsi-1^{VOC}*).

This chapter describes the expression profile of *Xhdsi-1^{VOC}* in *X. humilis* during different stages of desiccation and rehydration as well as the expression profile of the *A. thaliana* orthologue. *Xhdsi-1^{VOC}* was tested for glyoxalase I activity by growing *Escherichia coli* expressing the *Xhdsi-1^{VOC}* protein in the presence of different concentrations of methylglyoxal and by yeast complementation studies.

2.2. Materials and Methods

2.2.1. Plant material and stress conditions

X. humilis plants collected from Barakalalo National Park in Limpopo Province, South Africa were maintained in trays in a glasshouse at the University of Cape Town with no supplemental lighting, shading or temperature control. Plants were desiccated by withholding water, allowing the plants to dry naturally to an air-dry state ($\leq 5\%$ RWC). The plants were desiccated under ambient conditions in the glasshouse in a cycle lasting from 30th January 2007 to 12th February 2007. Plants were kept dry for two weeks before rehydration by irrigation of the soil. During this sampling period, relative humidity ranged from 35% to 75% on a daily basis, temperature ranged from 15°C to 30°C, and daylight intensity ranged from 200-1000 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Leaves and roots were harvested at different stages in the desiccation and rehydration time course. Twenty leaves were harvested from different plants within a tray. Each leaf was split in half, one half was weighed, immediately frozen in liquid nitrogen and stored at -70°C until further analyses were performed as described below. The other half was used to determine the leaf's RWC as described by Dace *et al.*, (1998). Half leaves with the same RWC were pooled to obtain 0.1 g tissue for RNA extraction. 0.2 g root material was also collected from different plants within the tray at the different stages of the dehydration – rehydration time course. The soil from the harvested roots was removed by gently rubbing the roots with paper towel. Root tissue was cut into 2 cm segments. 0.1 g root tissue was frozen in liquid nitrogen and stored at -70°C while 0.1 g was used to determine the RWC.

Mature *X. humilis* seeds were collected from flowers that developed regularly in response to a drop in ambient temperature, followed by high light conditions (Dr. Laura Roden, personal communication). Flowers were hand pollinated. Seeds were shaken onto white paper for collection following flower senescence and natural pod desiccation. These mature dry seeds were stored at 4°C until required. The seeds used in the experiments were collected over a period of 2 years.

A. thaliana was used as a model of a desiccation sensitive plant. 0.5 g of wild type *A. thaliana* seeds were surface sterilised in 70% ethanol (v/v) for 7 min followed by 10% bleach containing 0.02% Triton (v/v) for 15 min. Seeds were thoroughly rinsed five times in sterile distilled water and resuspended in 0.1% sterile agar (v/w). The sterilized seeds were stratified at 4°C for 3 days. The stratified seeds were plated on plant nutrient agar (Haughn and Somerville, 1996) in sealed Petri dishes and incubated in a 16 h light (100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) / 8 h dark cycle at 25°C. Two weeks after germination, plants were used for stress treatments. For osmotic stress, they were transferred to Petri dishes containing 150 mM NaCl or 300 mM mannitol. As controls, the plants were transferred to Petri dishes containing water. For dehydration stress, plants were left in plant nutrient agar but lids were removed. The control for this experiment was maintenance of sealed growth conditions. Roots and leaves were harvested from 0.1 g of plantlet tissue after 4 hours of each treatment. The samples were frozen immediately in liquid nitrogen and stored at -70°C. A biological repeat was performed a few weeks later in the same manner.

2.2.2. Isolation of total RNA

Total RNA from leaves and roots of *X. humilis* was isolated using 1 ml of TriReagent per 150 mg tissue (Molecular Research Centre Inc, Cincinnati, U.S.A) following manufacturer's recommendations with the modification of adding 0.01 g of polyvinylpolypyrrolidone (PVPP) per 1 ml of TriReagent to inactivate polyphenolics. The phenol-chloroform phase was stored at -20°C for protein extraction. Total RNA from vegetative tissue of *A. thaliana* was also isolated using TriReagent with the exception that PVPP was omitted in the extraction. Total RNA from batches of pooled seed of both *A. thaliana* and *X. humilis* was extracted using a method described by Wan and Wilkins (1994).

cDNA synthesis was obtained from 2.5 μg of total RNA by using 200 units of Superscript III reverse transcriptase enzyme (Life Technologies, USA) following manufacturer's instructions. The cDNA yield was checked by using the NanoDrop^R ND-1000 spectrophotometer (NanoDrop Technologies, Inc, DE) and integrity of the

RNA was checked on a 1% agarose formaldehyde denaturing gel (Ausubel *et al.*, 1995).

2.2.3. Northern blot analysis

Twenty micrograms of total RNA isolated from roots and leaves at different stages of desiccation and rehydration were run on a 1% agarose formaldehyde denaturing gel (Ausubel *et al.*, 1995) then transferred to HybondTM-N⁺ membrane (Amersham Biosciences, UK) by capillary blotting, using 20 X SSC. The membrane was dried at 80°C for 10 min and fixed by UV cross linking (Amersham LIFE SCIENCE, UK). To check the success of the RNA transfer, the membrane was stained in methylene blue (0.2% (w/v) in 0.3 M sodium acetate (pH 5.5)).

The membrane was prehybridized at 42°C for 16 h in hybridization buffer containing 0.1% SDS, 50% formamide, 5 X SSC, 50 mM NaPO₄, pH 6.8, 0.1% sodium pyrophosphate, 5 X Denhardt's solution, and 50 µg.ml⁻¹ heat-denatured salmon sperm DNA. α-³²P labelled probe was added and incubated for a further 16 h at 42°C. The probe consisted of *Xhdsi-1^{VOC}* digested with *EcoRI* and *XhoI* to release the cDNA from the pBluescript SK +/- plasmid. The *Xhdsi-1^{VOC}* was labelled with α-³²P using the MegaprimeTM DNA labelling kit (Amersham Biosciences, UK) and purified using SigmaSpinTM Post-Reaction clean up columns (Sigma, Germany) following manufacturer's instructions.

Conditions for washes were: 2 X SSC, 0.1% SDS for 30 min at room temperature; 1 X SSC, 0.1% SDS for 30 min at room temperature and 0.2 X SSC, 0.1% SDS for 30 min at 55°C. The membrane was then wrapped in plastic and exposed to a HyperfilmTM-βmax, high performance autoradiography film (Amersham, UK).

2.2.4. Primer design

For real time PCR (RT-PCR) expression analysis, forward (Dsi-1^{VOC} F) and reverse (Dsi-1^{VOC} R) degenerate primers were designed to the conserved regions of the *X. humilis Xhdsi-1^{VOC}* gene (Genbank Accession number AY570978) and the *A. thaliana* orthologue (*At1g07645*). 18S rRNA primers (18S F and 18S R) were designed to

nucleotide sequences conserved between the *X. humilis* and *A. thaliana* 18S rRNA genes. As a positive control for abiotic stress treatment, primers were designed to the *A. thaliana* LEA2 gene, *At1g76180*, (LEA2 F and LEA2 R), which is known to be activated by salt, mannitol and dehydration stress. Primers were also designed to one of the *A. thaliana* ubiquitin genes, *At4g05320*, (Ubq F and Ubq R) as a positive control to check the integrity of RNA. Primer sequences are shown in Table 1. All the primers were synthesised at the Molecular and Cell Biology Department, University of Cape Town.

2.2.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The number of PCR cycles for amplification of each of the products was based on identifying the point at which the transcripts were amplified in a linear range (Table 1). This was established by removing a 10 µl aliquot from the PCR reactions at two cycle intervals from 16 cycles to 35 cycles. 250 ng of synthesized cDNA was used for the PCR reaction, which included 0.5 µM of each degenerate primer, 0.2 mM dNTP, 0.5 u Supertherm Taq (Hoffman-La Roche, USA) and 2.4 mM MgCl₂. The following PCR conditions were standard for all the primers and only the annealing temperature was different for each set of primers (summarised in Table 1). Initial denaturation temperature of 94°C (1 min), followed by denaturation at 94°C (30 s), annealing time of 1 min and extension time at 72°C (1 min). These experiments were repeated twice on independent biological samples.

Table 1. A summary of primers with their annealing temperatures used in all PCR reactions

Name	Forward primer		Reverse primer	Anneal. Temp.	N° cycles
Dsi-1 ^{VOC} F	5'-ACAGGTGGGG(AG)GAGCT(AG)GAG-3'	Dsi-1 ^{VOC} R	5'-ATTAACGTGGCTTCCGA(TG)GCG-3'	58°C	25
18S F	5'-CAGGCGCGCAAATTACCCAA-3'	18S R	5'-GCGACCATACTCCCCCGG-3'	54°C	20
LEA2 F	5'-GTACAAAGAGCGTGAAATCCGC-3'	LEA2 R	5'-CTTCTCCTCTACGGACC-3'	56°C	30
Ubq F	5'-CAATTCTCTCTACCGTGA-3'	Ubq R	5'-CCATCTTCAAGTTGCTTTCCG-3'	55°C	32
Gly F	5' AGG <u>GGA TCC</u> ATG GCG AAT CT 3' <i>BamHI</i>	Gly R	5' <u>GAA TTC</u> ACA GCT ACA TAC ACA T 3' <i>EcoRI</i>	55°C	35

2.2.6. Expression of Xhdsi-1^{VOC} recombinant protein in *E. coli*

The coding region of the *Xhdsi-1*^{VOC} cDNA was amplified by PCR using forward (Gly F) and reverse (Gly R) primers containing the restriction enzymes sites *Xho* I and *Bam*H I, respectively. The amplified product was first cloned into pGEM^R-T Easy (Promega, U.S.A) and then cloned as a *Bam*HI and *Eco*RI restriction fragment into the pGEX-3X (Amersham Pharmacia Biotech, Sweden) expression vector. *E. coli* XL-1Blue transformed with the pGEX-3X:: *Xhdsi-1*^{VOC} construct was grown at 37°C in Luria broth medium (1% tryptone (w/v), 0.5% yeast extract (w/v) and 0.5% sodium chloride (NaCl) (w/v)) containing 100 µg ml⁻¹ ampicillin until the cell density reached 0.9 at OD₆₀₀. Expression of recombinant Xhdsi-1^{VOC} was induced by adding 0.5 mM IPTG (Roche, USA) to the culture media for a period of 2 h at 30°C. The culture was then spun down at 7000 g at 4°C in a bench-top centrifuge for 10 min and the resulting pellet was resuspended in PBS (147 mM NaCl, 2.7 mM KCl, 1.8 mM KH₂PO₄, 4.3 mM Na₂HPO₄·2H₂O at pH 7.2). A 500 µl aliquot of culture was transferred to a centrifuge tube and the bacteria were lysed by the addition of 1 µg lysozyme. The cells were incubated for 30 min at 4°C before centrifuging for 10 min at 7000 g (4°C) to remove cellular debris. Five microlitre of the recovered supernatant was used to determine protein concentration. The protein concentration of the supernatant was measured by the Bradford assay method using bovine serum albumin as a standard at 600 nm following manufacture's specifications (Bradford, 1976; Bio-Rad, Germany).

2.2.7. Generation of anti- Xhdsi-1^{VOC} / At1g07645 antibodies

A peptide (RRVDNSNRWGELESGE) that is conserved between the N-terminal domain of Xhdsi-1^{VOC} and the *A. thaliana* orthologue At1g07645 was synthesized and coupled to a keyhole limpet hemocyanin (KLH) protein by Princeton BioMolecules Cooperation (U.S.A). The peptide was used to raise anti-Xhdsi-1^{VOC} antibodies in rabbits (South African Vaccine producers Ltd). During the course of the antibody generation, serum was collected from the rabbits prior to immunisation (pre-bleed), on day 52 (first bleed), day 66 (second bleed) and day 70 (final bleed). In the subsequent experiments, only the pre-bleed (negative control) and the final bleed were used. The titre of the anti-Xhdsi-1^{VOC} antibodies was determined using an Enzyme-

Linked ImmunoSorbent Assay (ELISA). Initially, the lyophilised peptide (not conjugated to the KLH protein) was reconstituted in PBS (147 mM NaCl, 2.7 mM KCl, 1.8 mM KH₂PO₄, 4.3 mM Na₂HPO₄·2H₂O at pH 7.2) and 100 µl of (0.5 µg) of the reconstituted peptide was added to wells of the ELISA plates were then incubated with, the plates were covered with plastic wrap and were incubated overnight at 4°C. Subsequently, the wells of the ELISA plates were three times with 200 µl of TBS (50 mM Tris-Cl, 150 mM NaCl pH 7.6) containing 0.1% Tween-20 (v/v) (Sigma, USA). The wells were then incubated with 200 µl of TBS containing 3% bovine serum albumin (BSA) (Bio-Rad, Germany) for an hour at room temperature. After one hour incubation, the 3% BSA solution was decanted and the wells were again washed three times with 200 µl of TBS containing 0.1% Tween-20 (v/v) (Sigma, USA). The serial dilutions of anti-Xhdsi-1^{VOC} serum (from concentrated to 10⁻³) diluted in TBS were added to the wells (100 µl) and the ELISA plates were incubated for an hour at room temperature. Serum collected from the rabbits before administering the antigen was used as a negative control. After one hour incubation, the anti-Xhdsi-1^{VOC} serum was decanted and the wells were again washed three times with 200 µl of TBS containing 0.1% Tween-20 (v/v) (Sigma, USA).

A secondary the goat anti-rabbit alkaline phosphatase conjugated antibody (Sigma, USA) was diluted in TBS (1/10000) and 100 µl was added to the wells. The ELISA plate was again incubated for an hour at room temperature. After an hour incubation, the secondary antibody was decanted and the wells were again washed three times with 200 µl of TBS containing 0.1% Tween-20 (v/v) (Sigma, USA). In addition, the wells were washed with 200 µl of 10% Diethanolamine pH 9.8 (v/v) (Merck, USA) containing 0.5 mM MgCl₂. Finally 100 µl of substrate (10 mg of 4-nitrophenyl disodium orthophosphate (Merck, USA) reconstituted in 10 ml of 10% Diethanolamine pH 9.8 (v/v) (Merck, USA) containing 0.5 mM MgCl₂ was added to the wells. The ELISA plate was incubated in the dark at room temperature for 30 min. The absorbance readings were obtained using the 405 nm filter of the Titretrek multiskan^R plus microplate reader (Labsystems ICN Biomedicals, Canada).

Furthermore, the specificity of the Xhdsi-1^{VOC} antibodies was checked by western blot analysis of crude *E. coli* cell extract containing recombinant Xhdsi-1^{VOC} protein. Twenty micrograms of crude extracts prepared from either *E. coli* transformed with

expression vector pGEX-3X only, or uninduced culture containing *E. coli* pGEX3-3X::*Xhdsi-1*^{VOC} were used as a negative controls. The extraction of total protein is described in section 2.2.6 and the western blot protocol is described below.

2.2.8. Western blot analysis

To investigate expression profiles of *Xhdsi-1*^{VOC} during desiccation and the subsequent rehydration, total protein from *X. humilis* leaves and roots was extracted from the phenolic layer recovered from the RNA extraction in TriReagent, according to manufacturer's recommendations (Molecular Research Centre Inc, Cincinnati, OH). The protein concentration was measured by the Bradford assay method using bovine serum albumin as a standard at 600 nm following manufacturer's specifications (Bradford, 1976; Bio-Rad, Germany). Twenty micrograms of total protein isolated from different stages of desiccation and rehydration in both roots and leaves were run on a 12% SDS-PAGE gel as described by Ausubel and colleagues (1995) then electro-blotted onto PROTRAN^R nitrocellulose membrane (Schleicher and Schuell Biosciences GmbH, Germany). The nitrocellulose membrane was stained with Ponceau S (0.1% (v/v) in 5% acetic acid) to confirm equal loading and transfer of protein samples.

For western blot analysis, the membrane was incubated in blocking buffer, TBS (50 mM Tris-Cl at pH 7.6; 150 mM NaCl) containing 5% fat free milk powder at 4°C for 16 h. The membrane was then transferred to TBS containing 2% fat free milk powder and the *Xhdsi-1*^{VOC} anti-serum at a dilution of 1/1000 at 4°C for 16 h. The membrane was then washed with 1 X TBS containing 0.1% Tween-20 (Sigma, USA) and incubated at room temperature for 1 h with the goat anti-rabbit-horseradish peroxidase (HRP) conjugated secondary antibody (Sigma, USA) at a dilution of 1/10000. Detection was carried out using a LumiGlo^R Reserve western blot kit (KPL, USA) and exposing the membrane to high performance autoradiography film (HyperfilmTM-βmax Amersham, UK) for 1 min.

2.2.9. Methylglyoxal resistance studies in *E. coli*

For the methylglyoxal viability/resistance assay, an *E. coli* culture containing the pGEX-3X::Xhdsi-1^{VOC} plasmid was grown for 16 h at 37°C and the turbidity/density of the culture was adjusted to an OD₆₀₀ reading of 0.9. Two microlitre of the culture were serially diluted (concentrated to 10⁻⁶) and these dilutions were spotted on Luria agar plates containing different concentrations of methylglyoxal (0 mM -10 mM), 100 µg ml⁻¹ ampicillin, and 0.5 mM of IPTG (Roche, USA) to activate the promoter (modified from Veena *et al.*, 1999). The plates were incubated at 37°C overnight, and the tolerance to the different concentrations of methylglyoxal was measured by growth of *E. coli* on the agar plates.

2.2.10. Complementation studies in yeast

A *Saccharomyces. cerevisiae* glyoxalase I mutant (*glo1Δ*) together with its isogenic wild type strain (YPH250) were donated by Dr Yoshiharu Inoue (University of Kyoto, Japan). The genotype of the strains is as follows:

YPH250: MATa *Trp1-Δ1 his-Δ200 leu2- Δ1 lys2-801 ade2-101 ura3-5*

YPH250 (*glo1Δ*): MATa *Trp1-Δ1 his-Δ200 leu2- Δ1 lys2-801 ade2-101 ura3-5, glo1 Δ::HIS3*.

The ability of Xhdsi-1^{VOC} to complement the yeast glyoxalase I mutant was tested. The Xhdsi-1^{VOC} coding region from pGEM-T-Easy was cloned into the *Bam*HI and *Eco*RI cloning site of the pYES2 yeast expression vector (Invitrogen, life technologies, U.S.A). The pYES2::Xhdsi-1^{VOC} plasmid was purified from *E. coli* XL-1 Blue using the High Pure Plasmid Isolation Kit (Roche, USA) and was then transformed into *glo1Δ* yeast cells by electroporation as described by Adams *et al.* (1997).

The transformed yeast cells were grown in Synthetic Minimal Medium containing 6.7 g nitrogen base without amino acids (DIFCO Laboratories, INC, U.S.A) and 0.77 g complete synthetic medium without uracil (BIO 101 systems, USA) per litre with 2% glucose and incubated at 30°C for three days. A single colony was inoculated into 10 ml Synthetic Minimal Medium and grown until exponential phase. Three microlitres of the cell culture was spotted in duplicate on YPG (1% yeast extract, 2% peptone and 2% agar) plates containing different concentration of methylglyoxal (0 mM, 1 mM, 2

mM, 5 mM and 10 mM) and 2% galactose (v/v) to induce the promoter. The glyoxalase I mutant and wild type *S. cerevisiae* transformed with the parental vector were used as controls.

2.2.11. Bioinformatics

The Genbank non-redundant protein sequences (nr), est databases and the TIGR Gene Indices database (<http://compbio.dfci.harvard.edu/tgi/>) were searched using the blastp, blastn and tblastx algorithms to identify Xhdsi-1^{VOC} orthologues. The predicted amino acid sequences of Xhdsi-1^{VOC} and its orthologues were aligned by ClustalW in MEGA4 (Tamura *et al.*, 2007). Protein structural motifs in the predicted Xhdsi-1^{VOC} and At1g07645 amino acid sequences were identified using PROSITE (Bairoch *et al.*, 1997) submitted via the PredictProtein server (<http://www.predictprotein.org>) (Rost *et al.*, 2004). This server was also used to submit sequences to PROF, a secondary structure prediction tool (Rost and Sander, 1993). A cut-off of reliability index value > 4 was used to predict the presence of β sheets and α helices in Xhdsi-1^{VOC} and At1g07645. The PSIPRED server (<http://bioinf.cs.ucl.ac.uk/psipred/>) was used to submit the Xhdsi-1^{VOC} and At1g07645 amino acid sequences to mGenTHREADER, a fold recognition tool that aligns a protein sequence against a known three-dimensional structure from the Protein Data Base (PDB) (Bryson *et al.*, 2005; McGuffin and Jones, 2003).

2.3. Results

2.3.1. *X. humilis* *Xhdsi-1^{VOC}* is a member of a novel gene family in plants

The *X. humilis* *Xhdsi-1^{VOC}* cDNA was isolated as a desiccation-upregulated gene via microarray analysis (Collett *et al.*, 2004; Genbank accession number AY570978) and encodes a 140 amino acid protein with a predicted molecular weight of 15.6 kDa. A general Blastp search of the non-redundant protein sequences (nr) database (NCBI) indicated that *Xhdsi-1^{VOC}* has high similarity to *Oryza sativa* NP_001049720 (Os03g0277500) (E-value $1e^{-45}$) and *A. thaliana* NP_973779 (At1g07645) (E-value $2e^{-45}$). The *At1g07645* gene is annotated as a glyoxalase family I protein and/or a lactylglutathione lyase family protein in Genbank. A multiple protein sequence alignment with known glyoxalase I enzymes shows that *Xhdsi-1^{VOC}* and At1g07645 do not share high sequence similarities with glyoxalase (12 – 16%) (Fig. 2.3). Moreover, *Xhdsi-1^{VOC}* and its orthologue At1g07645 do not contain the two zinc-binding sites and a glutathione binding site in glyoxalase enzymes important for glyoxalase I activity (Fig. 2.3) (Cameron *et al.*, 1997).

A Blastn search against the TIGR gene indices database (<http://www.tigr.org>) identified six plant EST sequences with very high similarity to the *X. humilis* *Xhdsi-1^{VOC}* gene (Table 2.2). The search for distribution of expression of these orthologues in the TIGR EST libraries (<http://compbio.dfci.harvard.edu/tgi/>) show that these genes are not expressed in vegetative tissue during abiotic stress. Interestingly, all these ESTs were originally identified in libraries constructed from seed or seed-related tissues. An amino acid multiple alignment of *Xhdsi-1^{VOC}* and its plant orthologues (Fig. 2.4) show two putative/conserved phosphorylation sites, namely a protein kinase C phosphorylation site and a casein kinase II phosphorylation site. A single cysteine, which is predicted to form a disulphide bond by DISULFIND (Vullo and Frasconi, 2004), is conserved in all the plant orthologues (Fig. 2.3). *Dsi-1^{VOC}* appears to have evolved early in the plant lineage because a clear orthologue (CN203139, E-value = $5e^{-30}$) was identified in the *Tortula ruralis* EST library (Table 2.3). The gene probably evolved from ancestral eubacteria, since the closest homologues to this gene family are found in bacteria. For example the *Roseobacter* sp CCS2 glyoxalase family

protein (ZP_01750924) shares 58% similarity with *X. humilis* Xhdsi-1^{VOC} (E-value= $2e^{-23}$).

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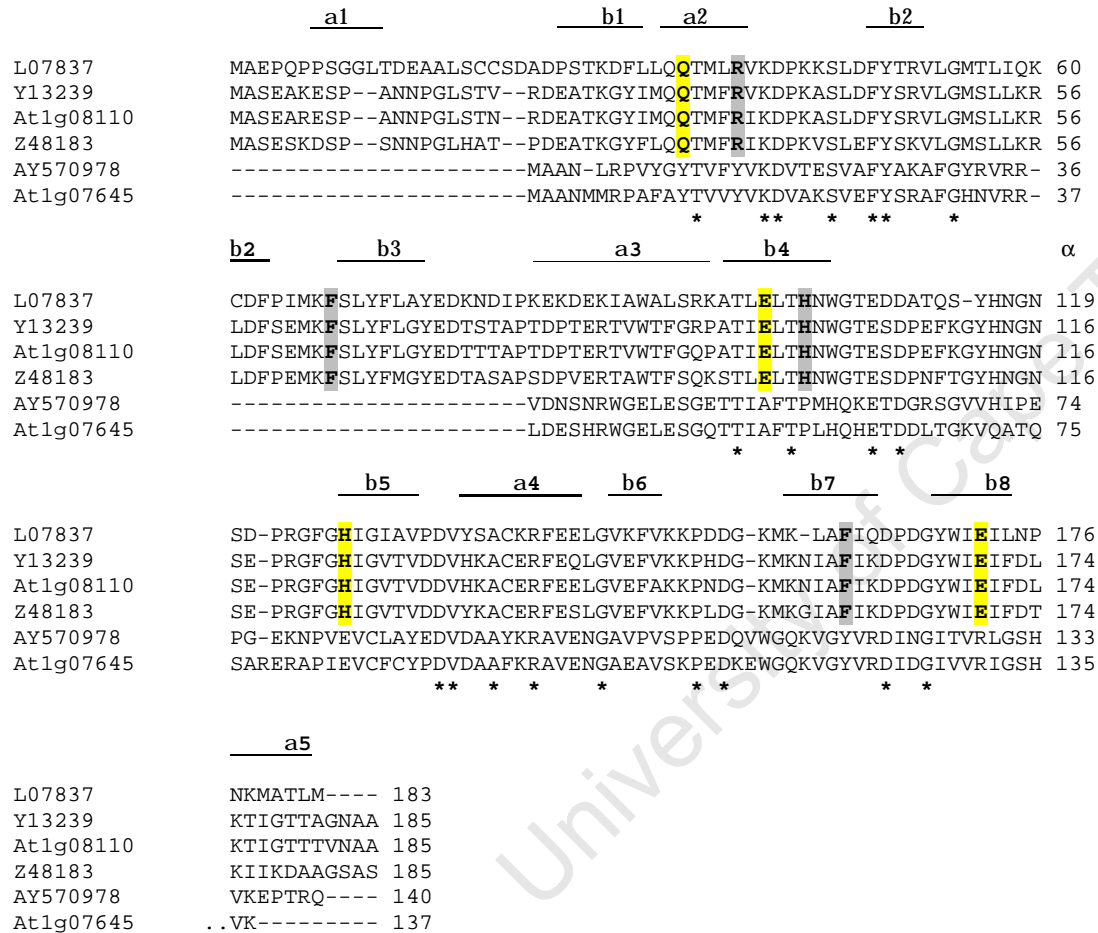


Figure 2.3. ClustalW alignment of amino acid sequences of *X. humilis* Xhdsi-1^{VOC} (AY570978) and the *A. thaliana* orthologue (At1g07645), with known glyoxalase I genes, including L07837 (*Homo sapiens*), Y13239 (*Brassica juncea*), At1g08110 (*A. thaliana*), and Z48183 (*Lycopersicon esculentum*). The asterisks represent amino acids that are identical in all of the six sequences. The $\beta\alpha\beta\beta$ structural repeat determined in the X-ray crystal structure of the human glyoxalase I protein is indicated. This structural repeat is represented by $\beta_1\alpha_2\beta_2\beta_3\beta_4$ in the first domain ($\alpha_1-\beta_4$), and by $\beta_5\alpha_4\beta_6\beta_7\beta_8$ in the second domain ($\beta_5-\alpha_6$) (Cameron *et al.*, 1997). Conserved amino acids that constitute the glutathione binding sites of glyoxalase I are highlighted in grey. Conserved amino acids, which form the zinc binding sites, are highlighted in yellow.

Table 2.2 Percentage similarity and E-values for tblastx search of full-length orthologues of Xhdsi-1^{VOC} present in the plant gene indices (TC) in TIGR gene indices database (<http://compbio.dfci.harvard.edu/tgi/>). The tissue origins of libraries in which these ESTs are present are summarised. At1g07645 is represented by TC302237 in the TIGR *A. thaliana* EST gene index.

Plant species	Gene Index	Similarity	Expect value	Tissue Origin for EST libraries
<i>Triticum aestivum</i>	TC262761	81%	2.2e ⁻⁴⁴	Wheat dormant embryo; Y. Ogihara unpublished cDNA library, Wh_SL; G468,550,608; wdk3c;4c;5c
<i>Glycine max</i>	TC211205	76%	3.0e ⁻⁴⁴	Endoperm tissue in developing seeds
<i>Oryza sativa</i>	TC307859	82%	3.7e ⁻⁴⁴	Panicles mixture of one, two, three weeks after flowering; germinating seeds; 30 days after pollination pistil; UVC irradiated shoot; callus 100ppm ZnSO4 for 1week; 10 days after anthesis); supermix
<i>Brassica napus</i>	TC20284	79%	5.0e ⁻⁴⁴	<i>Brassica napus</i> seed library
<i>A. thaliana</i>	TC302237	80%	5.1e ⁻⁴⁴	RAFL16, RAFL19, Perp-dry-seedA
<i>Brassica napus</i>	TC17768	78%	5.8e ⁻⁴⁴	Not informative
<i>Hordeum vulgare</i>	TC132259	80%	1.0e ⁻⁴³	<i>H vulgare</i> 5-45 DAP spike EST library; S00008; S00011; germination shoots; testa/pericarp EST library; Barley EBem06/EBem08/EBem09/EBem10 libraries; HF; HD; HT; HM
<i>Lycopersicon esculentum</i>	TC180705	79%	7.3e ⁻⁴³	Maturing fruit
<i>Triticum aestivum</i>	TC262846	79%	2.4e ⁻⁴²	Wheat dormant embryo; Y. Ogihara unpublished cDNA library

<i>Picea glauca</i> (<i>spruce</i>)	TC24554	74%	6.0e ⁻³⁹	Immature somatic embryos, mature somatic embryos; Male strobili developmental sequence
<i>Picea glauca</i>	DR585676	74%	6.7e ⁻³⁹	Somatic embryo tissue Somatic embryo tissue was harvested at the callus stage, and after 2 weeks, 4 weeks and 6 weeks of growth on media supplemented with the growth regulators abscisic acid (ABA) and indole-3-butyric acid (IBA).
<i>Zea mays</i>	TC339998	74%	2.4e ⁻³⁷	Endosperm, early embryo
<i>Zea mays</i>	TC327708	74%	2.4e ⁻³⁷	Zmrww005;zmrww00; early embryo; endosperm
<i>Pinus taeda</i>	TC77599	73%	3.1e ⁻³⁷	Embryo

Table 2.3. Percentage similarity and E-values for tblastx search of Xhdsi-1^{VOC} full-length orthologues in the Genbank EST_other database. The tissue origins of libraries in which these ESTs are present are summarised.

Plant species	Gene Index	Similarity	E-value	Tissue Origin for EST libraries
<i>Juglans regia</i>	CV195407	84%	2e ⁻⁵⁶	Seed coat from mid-season walnut embryos
<i>Centaurea maculosa</i>	CV195407	76%	9e ⁻⁵⁶	Mixed sources
<i>Vitis vinifera</i>	EC948701	76%	4e ⁻⁵⁵	Flower, leaf and root
<i>Phaseolus coccineus</i>	CA898774	72%	1e ⁻⁵⁴	Embryo-proper regions were micro-dissected from globular-stage embryos six days after pollination
<i>Glycine max</i>	EV275991	75%	2e ⁻⁵⁴	Mixed sources (Drought stressed and control stem, tissue culture suspension, salt stressed, drought stressed, and <i>Pseudomonas</i> infected leaves, etiolated seedlings)
<i>Cyamopsis tetragonoloba</i>	EG979999	73%	3e ⁻⁵³	Seeds harvested 15, 20, 25, 30, 35, and 40 days after flowering (DAF)
<i>Oryza sativa</i>	CI253561	88%	1e ⁻⁵²	Panicles mixture of one, two, three weeks after flowering
<i>Triticum aestivum</i>	CD906048	87%	1e ⁻⁵²	Grain (468 degrees per day after pollination)
<i>Triticum aestivum</i>	CD490536	87%	1e ⁻⁵²	Plants were grown to seed maturity under conditions favoring seed dormancy. Embryos were cut from mature dormant seed (Doherty). Total RNA was prepared from these embryos
<i>Pinus taeda</i>	DT625190	72%	6e ⁻⁴⁹	Pooled RNA from zygotic embryos, megagametophytes, and somatic embryos
<i>Tortula ruralis</i>	CN203139	61%	5e ⁻³⁰	Gametophyte rehydration library

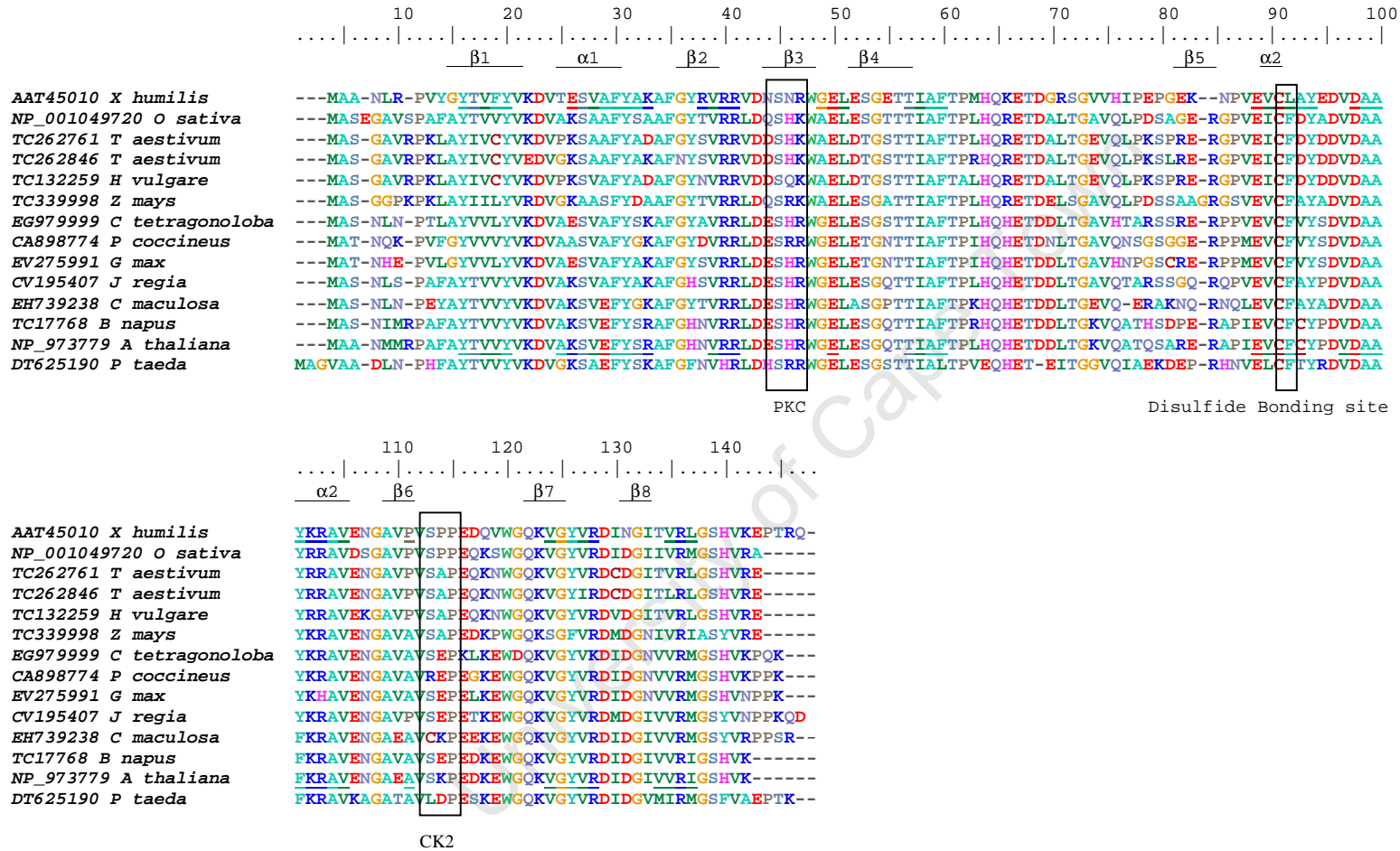


Figure 2.4. ClustalW alignment of predicted amino acid sequences of plant *dsi-1^{VOC}* orthologues identified in the Genbank and TIGR EST databases, including *X. humilis*, rice (*Oryza sativa*), wheat, (*Triticum aestivum*), barley (*Hordeum vulgare*), maize (*Zea mays*), Guar (*Cyamopsis tetragonoloba*), runner bean (*Phaseolus coccineus*), soybean (*Glycine max*), walnut (*Juglans regia*), spotted knapweed (*Centaurea maculosa*), oilseed rape (*Brassica napus*), *Arabidopsis thaliana*, and loblolly pine (*Pinus taeda*). Boxed amino acids demarcate conserved phosphorylation sites for protein kinase C (PKC) and protein casein kinase II (CK2) identified by PROSITE, and a potential disulfide bonding site predicted by DISULFIND. Amino acids in the *X. humilis* and *A. thaliana dsi-1^{VOC}* orthologues which are predicted to fold into the $\beta\alpha\beta\beta$ structural repeats characteristic of the VOC 46 superfamily, are underlined.

2.3.2. Xhdsi-1^{VOC} is a novel member of the VOC metalloenzyme superfamily

In an effort to determine the function of the Xhdsi-1^{VOC} gene, the secondary structure of the encoded protein was investigated using the GenTHREADER secondary structure recognition tool. A $\beta\alpha\beta\beta\beta$ fold was identified in Xhdsi-1^{VOC}, which is a feature of members of the VOC superfamily (Table 2.4). The members of the VOC superfamily include divergent enzymes from *A. thaliana*, human glyoxalase I, and a number of bacterial antibiotic resistance proteins and enzymes. Although Xhdsi-1^{VOC} had very low sequence identity to these distant homologues (ranging from 12.8%-21.9%), they had significant structural homology (P value < 10e⁻⁰⁹). Proteins encoded by Xhdsi-1^{VOC} and its *A.thaliana* orthologue, At1g07645, were predicted by PROF (Rost and Sander, 1993) to fold into two distinct $\beta\alpha\beta\beta\beta$ domains, which are a common structural feature of members of the VOC superfamily (Fig. 2.2).

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Table 2.4. Structural homologues of Xhdsi-1^{VOC} identified by mGenTHREADER recognition software via the PredictProtein server, ranked according to their best net scores.

Net Score	p-value	Pdb ID*	Species	Gene Name	Metal ions	Sequence identity
0.860	1e ⁻⁰⁹	1xy7	<i>Arabidopsis thaliana</i>	At5g48480, unknown protein	none	21.5%
0.841	2e ⁻⁰⁹	1ecs	<i>Klebsiella pneumoniae</i>	Bleomycin resistance protein	none	20.8% (A chain)
0.838	3e ⁻⁰⁹	1qip	<i>Homo sapiens</i>	Glyoxalase I	Zn ²⁺	19.3%
0.829	3e ⁻⁰⁹	2i7r	<i>Streptococcus pneumoniae</i>	unknown function	none	17.4%
0.828	4e ⁻⁰⁹	1kl1	<i>Streptomyces lavendulae</i>	Mitomycin resistance protein	none	20.3%
0.826	4e ⁻⁰⁹	1sqd	<i>Arabidopsis thaliana</i>	At1g06570; Oxidoreductase; Dihydroxybiphenyl dioxygenase	Fe ²⁺	20.0%
0.825	4e ⁻⁰⁹	1nki	<i>Pseudomonas aeruginosa</i>	Fosfomycin resistance protein	K ⁺ , Mn ²⁺	13.4%
0.809	7e ⁻⁰⁹	1r9c	<i>Mesorhizobium loti</i>	Fosfomycin resistance protein	Mn ²⁺	14.4%
0.801	9e ⁻⁰⁹	1mpy	<i>Pseudomonas putida</i>	Catechol 2,3 dioxygenase	Fe ²⁺	12.9%

* Pdb ID, Identity name in the Protein Data Base (<http://www.rcsb.org/pdb/home/home.do>)

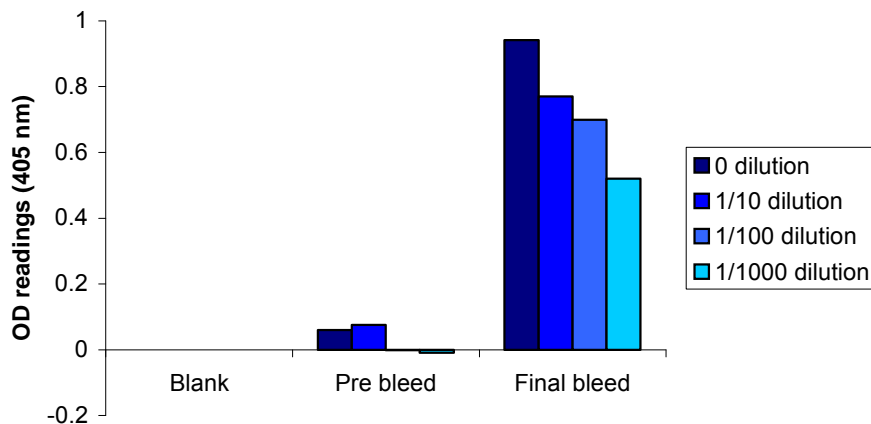
2.3.3. Optimisation of Xhdsi-1^{VOC} antibody

The titre and specificity of the anti-Xhdsi-1^{VOC} anti-sera was determined by the immunodetection of serial dilutions of anti-Xhdsi-1^{VOC} serum against the synthesised peptide (not conjugated to the KLH protein). Results showed that the serum collected before administering the peptide to the rabbits (pre bleed) did not recognise the Xhdsi-1^{VOC} peptide and the serum containing the anti- Xhdsi-1^{VOC} IgG recognised the synthesised peptide (Fig. 2.5A; Fig. 2.5B). In addition, western blot results of crude protein from *E.coli* overexpressing the pGEX-3X::*Xhdsi-1^{VOC}* construct and pGEX-3X (parental vector) shows that the Xhdsi-1^{VOC} anti-serum at a dilution of 1/1000 is specific to Xhdsi-1^{VOC} (Fig. 2.5C). Furthermore, 1/1000 dilution of anti-Xhdsi-1^{VOC} anti-sera recognized a single band of 15 kDa protein in total protein extracts from *X. humilis* desiccating leaves (Fig. 2.5D) which agrees with the predicted size of Xhdsi-1^{VOC}. The Xhdsi-1^{VOC} was absent at 100% RWC and present in desiccating tissue (56% and 5% RWC).

A

Blank	Pre bleed	Final bleed	Xhdsi-1 ^{VOC} anti-serum dilutions
0.000	0.060	0.942	0
0.000	0.076	0.770	1/10
0.000	-0.002	0.700	1/100
0.000	-0.009	0.521	1/1000

B



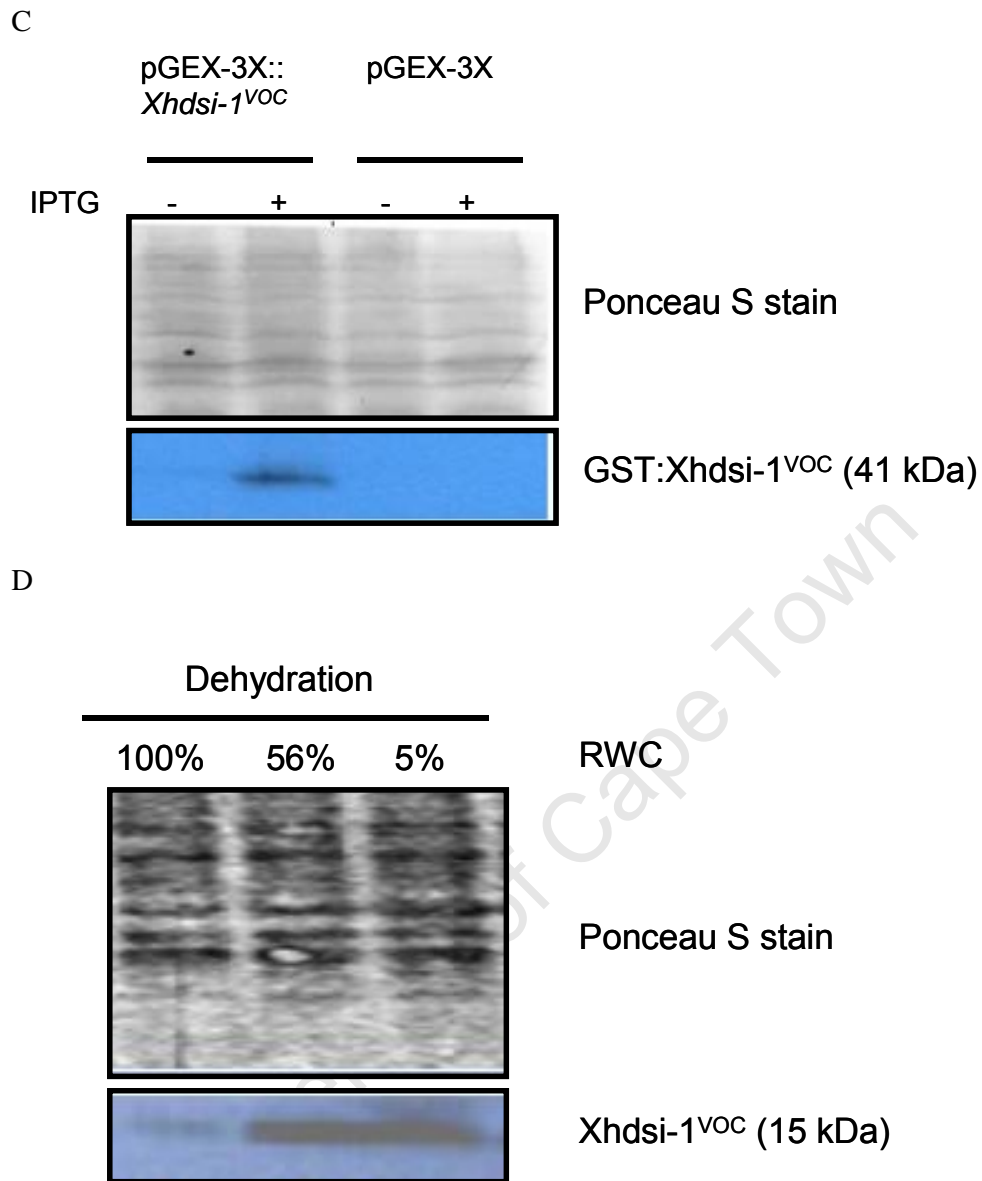


Fig. 2.5. A and B. ELISA results indicating antibody activity against the synthesised peptide lacking the KLH protein. The blank column contains no serum, the pre bleed column contains serum before immunization and the final bleed column indicates serum collected at 70 days after immunization. A serial dilution of the serum was performed. C. Western blot of overexpressed pGEX-3X: *Xhdsi-1*^{VOC} and pGEX-3X in *E.coli*. -IPTG and +IPTG indicates protein from uninduced and induced cultures, respectively. D. Western blot analysis of total protein extracts from *X. humilis* leaves at 100%, 56% and 5% RWC. The upper panels show the Ponceau S stained nitrocellulose membrane confirming equal loading and transfer of each experimental sample. The lower panel shows the western blot result following the probing of these membranes with anti-*Xhdsi-1*^{VOC} anti-sera.

2.3.4. Xhdsi-1^{VOC} confers low levels of methylglyoxal tolerance in *E. coli*

Xhdsi-1^{VOC} has high secondary structural homology to human glyoxalase I, therefore, Xhdsi-1^{VOC} was tested for glyoxalase activity via a methylglyoxal assay. The assay investigated whether overexpression of Xhdsi-1^{VOC} in *E. coli* would increase tolerance to methylglyoxal. The expression of recombinant GST::Xhdsi-1^{VOC} in crude extracts of *E. coli* was first confirmed by western blot analysis (Fig 2.5B). Figure 2.6 shows that in the presence of 1 to 2 mM methylglyoxal, *E. coli* cells transformed with pGEX-3X::Xhdsi-1^{VOC} were viable at 1000 fold more than the control strain. At higher concentrations (up to 7.5 mM methylglyoxal), *E. coli* (pGEX-3X: Xhdsi-1^{VOC}) was consistently more viable (10 to 100 fold) than the control strain. At 10 mM methylglyoxal, *E. coli* cells containing both the experimental and control constructs are unable to grow (data not shown). Recombinant expression of Xhdsi-1^{VOC} therefore confers low-level methylglyoxal tolerance in *E. coli* (up to 7.5 mM concentrations).

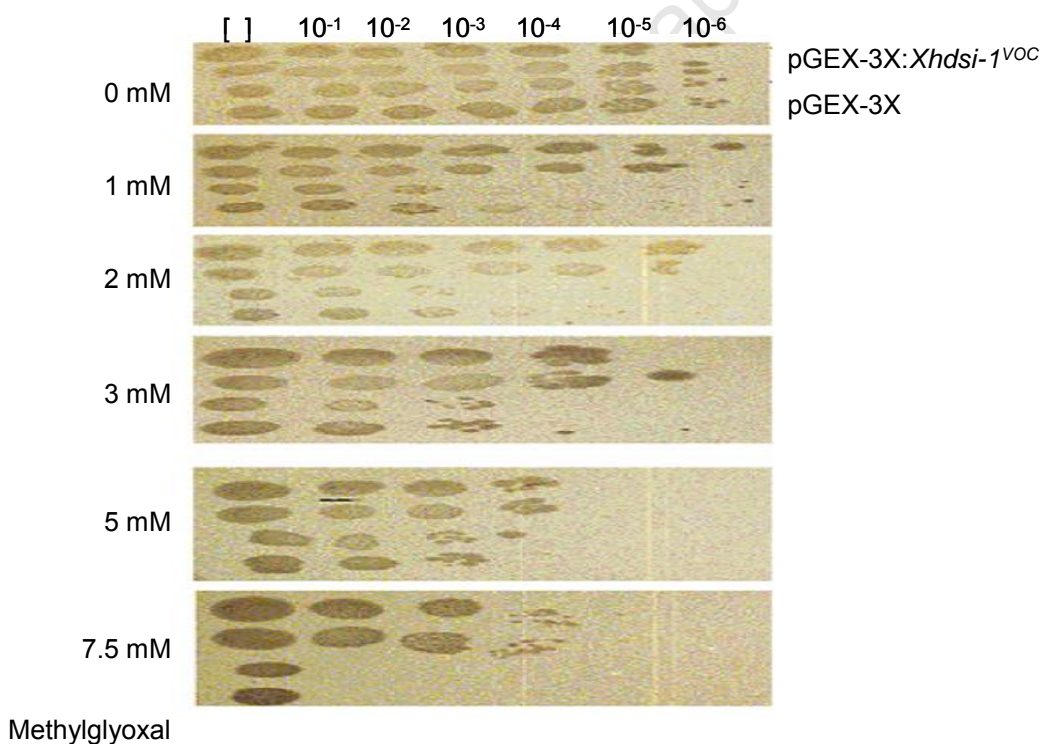


Figure 2.6. *E. coli* methylglyoxal resistance assay in which serial dilutions of the *E. coli* transformed with either pGEX-3X: Xhdsi-1^{VOC} or pGEX-3X were spotted in duplicate on IPTG plates containing different concentrations of methylglyoxal (0-7.5 mM).

2.3.5. Ectopic expression of Xhdsi-1^{VOC} is lethal in yeast

Xhdsi-1^{VOC} was also tested for its ability to complement a *S. cerevisiae* glyoxalase I mutant in the presence of galactose, which induces promoter activity. The yeast wild type tolerates concentrations of up to 10 mM methylglyoxal, whereas growth of the mutant is inhibited by concentrations above 2 mM methylglyoxal (Fig. 2.7). However, the mutant strain containing the Xhdsi-1^{VOC} construct fails to grow in the presence of galactose. This suggests that expression of Xhdsi-1^{VOC} produces a lethal phenotype in yeast.

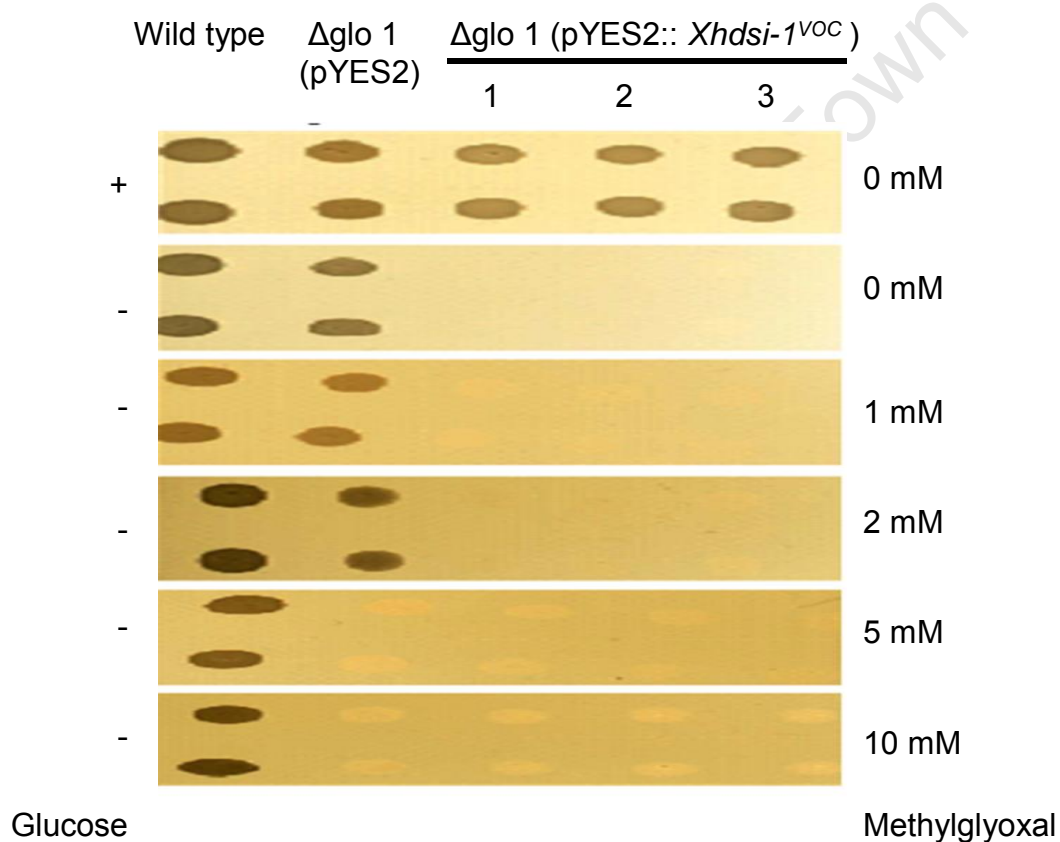
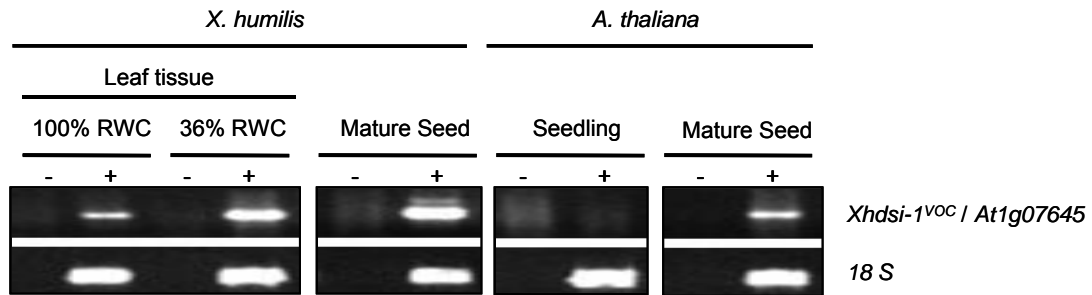


Figure 2.7. Methylglyoxal viability assays in *S. cerevisiae* wild type, the glyoxalase I mutant transformed with the pYES2 vector ($\Delta glo 1(pYES2)$), and in the glyoxalase I mutant transformed with the pYES2: Xhdsi-1^{VOC} recombinant vector ($\Delta glo 1(pYES2:: Xhdsi-1^{VOC})$), grown in triplicate (1-3). The addition (+) or absence (-) of glucose which suppresses expression from the pYES2 vector is indicated in the left hand side panel. Galactose was added to the medium in the absence of glucose to activate expression of the pYES2 vector. Concentration of methylglyoxal added to the growth media is indicated in the right hand side panel.

2.3.6. A comparison of *Xhdsi-1^{VOC}* and *At1g07645* expression in *X. humilis* and *A. thaliana*

The search for distribution of expression of the *Xhdsi-1^{VOC}* orthologues in the TIGR EST libraries indicated that these genes are not expressed in vegetative tissue of desiccation-sensitive plants during abiotic stress. However, the EST sequences for these genes were represented in seed, embryo, endosperm, callus and germinating shoot libraries (Table 2.2). These findings were confirmed in a RT-PCR study in which the expression of *Xhdsi-1^{VOC}* in *X. humilis* and *At1g07645* in *A. thaliana* were compared. Figure 2.8A shows that *X. humilis* *Xhdsi-1^{VOC}* transcripts are present at low levels in hydrated leaf tissue and dry seed and increase during dehydration (36% RWC). On the other hand, the orthologue transcript is absent in unstressed *A. thaliana* three-week-old seedlings (vegetative tissue), but present in dry mature seed. Furthermore, Figure 2.8B shows that *Xhdsi-1^{VOC}* orthologue transcripts were not detected in two-week old *A. thaliana* seedlings exposed to mannitol, salt and dehydration stress, whereas the expression of *LEA 2* mRNA transcripts was induced. *LEA-2* transcripts have been reported to be induced by abiotic stress (Illing *et al.*, 2005). The results strongly suggest that *Xhdsi-1^{VOC}* orthologue transcripts are present in *A. thaliana* seed, but absent in stressed vegetative tissue.

A



B

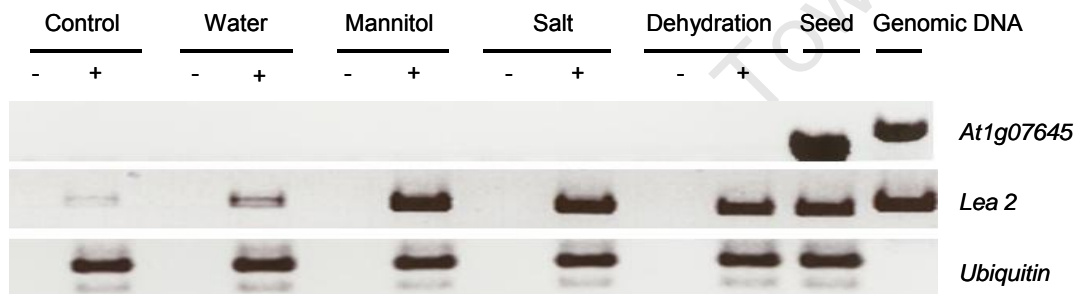


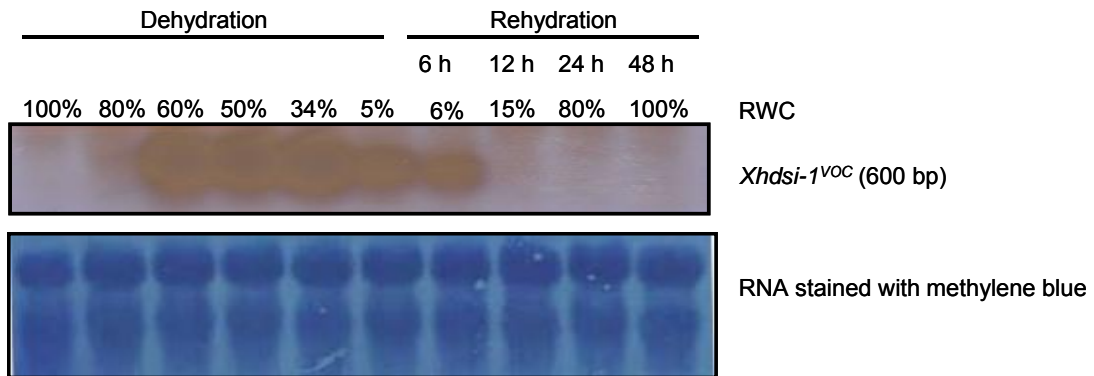
Figure 2.8. A. RT-PCR analysis of *Xhdsi-1^{VOC} / At1g07645* mRNA transcript abundance in seed and leaf tissue of *X. humilis* and *A. thaliana*. Amplification was performed on *X. humilis* leaf tissue (100% and 36% RWC) and mature dry seeds, and *A. thaliana* 3-week old seedlings and mature dry seeds as indicated. Amplification of 18S rRNA transcripts are shown as a control for relative abundance of sample RNA. B. Amplification of *At1g07645*, *LEA2* and ubiquitin mRNA transcripts in mature seeds and two-week old *A. thaliana* seedlings exposed to mannitol, salt and dehydration stress for 4 h. Controls include *A. thaliana* seedlings prior to stress treatments. Amplification of *LEA 2* mRNA transcripts was included as a positive control to show activation of the abiotic stress response. Ubiquitin mRNA transcripts are expected to be expressed at similar levels in all samples, and were included to control for starting sample RNA concentrations. For each RT-PCR reaction, + indicates the presence, and - the absence, of reverse transcriptase in the cDNA synthesis reactions.

2.3.7. Expression profile of *Xhdsi-1^{VOC}* in *X. humilis* vegetative tissue during desiccation and rehydration

Xhdsi-1^{VOC} was upregulated during desiccation in microarray experiments (Collett *et al.*, 2004), therefore its expression in leaves and roots of *X. humilis* in response to water loss and subsequent rehydration was investigated. Northern blot analysis of total RNA extracted from *X. humilis* vegetative tissue shows that *Xhdsi-1^{VOC}* mRNA transcripts were not detected in hydrated leaves or roots (Fig. 2.9). The 600 bp mRNA transcripts encoded by *Xhdsi-1^{VOC}* were first detected at low levels in leaf tissue at 80% RWC and their levels increased substantially between 80% and 60% RWC, their expression appeared to remain constant up to 34% RWC. Transcript abundance declined slightly in fully desiccated leaves (5% RWC) (Fig. 2.9A). *Xhdsi-1^{VOC}* transcripts are not present in hydration tissue in northern blots but are detected in RT-PCR experiment (Fig. 2.8A), this is due to RT-PCR being more sensitive than northern blot analysis. In roots, *Xhdsi-1^{VOC}* mRNA transcripts were also absent in hydrated tissue but were abundant in 76% RWC root tissue and appeared to reach a steady-state thereafter until full desiccation (<5% RWC). Roots rehydrate more rapidly than leaves, and after 6 h their RWC had reached 56%, compared to 6% in leaves. The rate of disappearance of *Xhdsi-1^{VOC}* mRNA transcripts in leaves and roots after watering followed the same trend (Fig 2.9B). After 12 h of rehydration, *Xhdsi-1^{VOC}* mRNA transcripts were absent in both rehydrated leaves and roots. *Xhdsi-1^{VOC}* transcripts are induced in response to desiccation in both leaves and roots, the mRNA transcripts are stored in desiccated tissue and rapidly removed during rehydration.

Western blot analysis showed that *Xhdsi-1^{VOC}* was strongly expressed in roots and leaves following desiccation. The immunoreactive protein bands were clearly visible in leaf and root samples of 50% and 76% RWC, respectively. Unlike mRNA transcripts, which were rapidly turned over following rehydration, *Xhdsi-1^{VOC}* protein was stably expressed throughout the desiccation and rehydration cycle (Fig. 2.10).

A



B

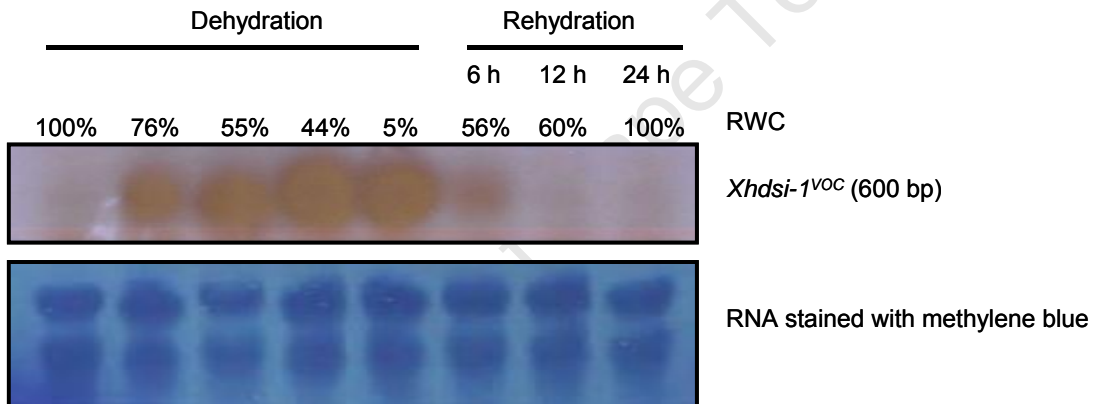
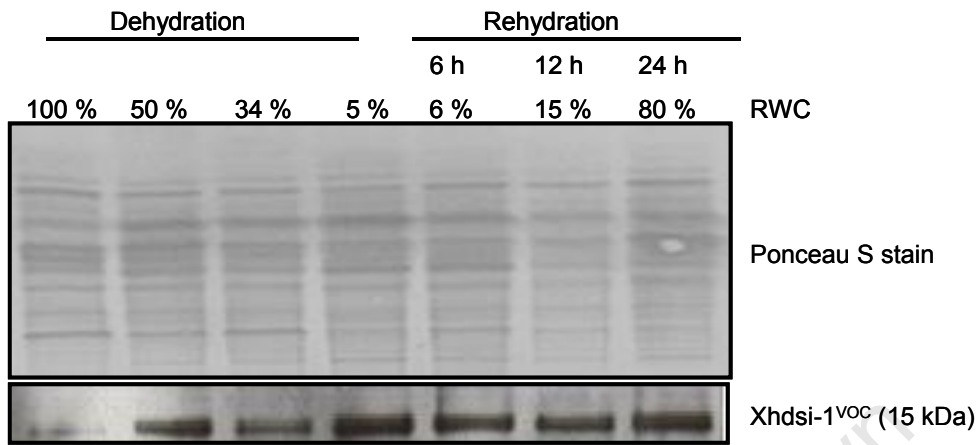


Figure 2.9. Northern blot analysis of *Xhdsi-1^{VOC}* at different stages of desiccation and rehydration in leaves (A) and roots (B) of *X. humilis*. The time scale of rehydration is shown relative to the change in RWC in leaves and roots. Equal loading and transfer of total RNA in each sample is shown by methylene blue staining of the membranes.

A



B

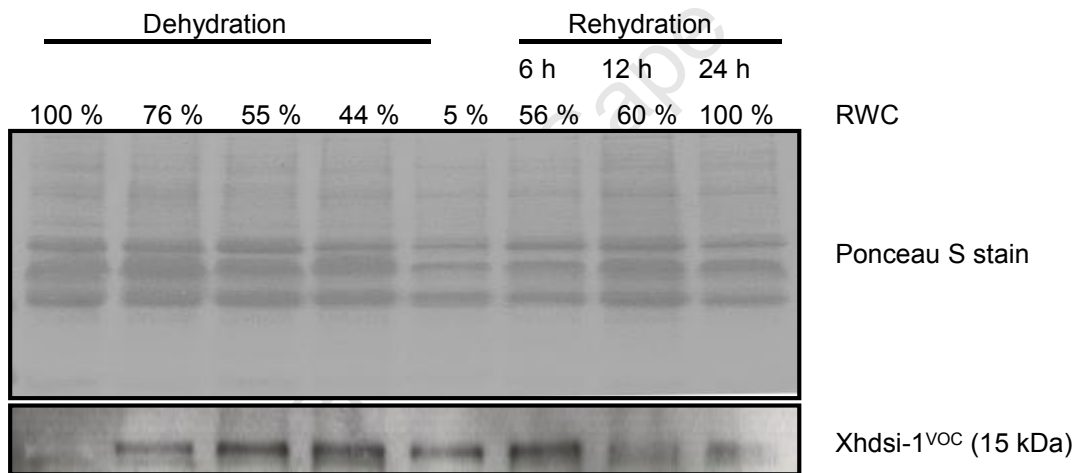


Figure 2.10. Western blot analysis of total protein extracts from *X. humilis* leaves (A) and roots (B) at different stages of desiccation and rehydration. The upper panels show the Ponceau S stained nitrocellulose membrane confirming equal loading and transfer of each experimental sample. The lower panel shows the western blot result following the probing of these membranes with anti-Xhdsi-1^{VOC} anti-sera.

2.4. Discussion

The *X. humilis* *Xhdsi-1^{VOC}* is a novel member of the VOC superfamily (Table 2.4). Members of VOC superfamily share very low sequence identity and have diverse functions, but they all contain a conserved $\beta\alpha\beta\beta$ structural fold annotated as a Glyoxalase/bleomycin resistance protein/dioxygenase InterPro domain (IPR004360). The *Xhdsi-1^{VOC}* gene and its *A. thaliana* orthologue, *At1g07645*, share high structural homology with the bleomycin resistance protein and the human glyoxalase I, which are both members of this VOC superfamily. The former does not function as an enzyme and does not contain metal binding sites, but it contains a hydrophobic cavity used for sequestering the antibiotic bleomycin (Kumagai *et al.*, 1999). The latter is part of the glyoxalase system found in animals, plants and bacteria that detoxifies 2-oxoaldehydes to hydroxycarboxylic acids and has an affinity for a particular oxoaldehyde, methylglyoxal (Thornalley, 2003). Methylglyoxal is the by-product of three pathways, namely the breakdown of triose phosphates in glycolysis, the catabolism of threonine, and the catabolism of acetone. In human cells, particularly rapidly dividing cancer cells, most of the methylglyoxal that is produced is believed to derive from the removal of a phosphate from the enediol intermediate due to the increase in glycolysis (Thornalley, 2003). In plants it has been suggested that methylglyoxal produced during environmental stresses such as high salt is due to an increase in glycolysis activity caused by high demand for ATP (Veena *et al.*, 1999). There is no evidence that glycolysis increases during desiccation in resurrection plants. However glyoxalase I gene has been identified in a resurrection species, *Sporobolous stafianus* (Clugston *et al.*, 1998). Thus, it can be speculated that during desiccation, there is an alternative mechanism for the formation of methylglyoxal during desiccation in resurrection plants. For example, methylglyoxal could be produced via the breakdown of threonine and acetone (Thornalley, 2003).

As *Xhdsi-1^{VOC}* shares high structural homology to the human glyoxalase I protein, the hypothesis that *XhDsi-1^{VOC}* detoxifies glyoxalase I was tested. When overexpressed in *E. coli*, *X. humilis* *XhDsi-1^{VOC}* conferred low-level resistance to methylglyoxal (7.5 mM). This is approximately three times lower than the levels of tolerance (25 mM) conferred by the *B. juncea* glyoxalase I in *E. coli* (Veena *et al.*, 1999). Thus, although

XhDsi-1^{VOC} does increase tolerance to methylglyoxal in *E. coli* to a certain extent, it may not function as a glyoxalase I. Complementation studies using the yeast glyoxalase I mutant failed to help characterise the function of the *X. humilis* protein since XhDsi-1^{VOC} proved to be lethal in yeast. These results, together with the lack of conserved glutathione and metal binding sites important for the activity of glyoxalase I strongly suggest that XhDsi-1^{VOC} does not function as a glyoxalase I.

According to EST representation, *XhDsi-1^{VOC}* orthologue expression is confined to seeds or seed-related tissues in desiccation-sensitive angiosperm plants, such as *A. thaliana*, *T. aestivum* (wheat) and *Zea mays* (maize), and in gymnosperm plants such as *P. glauca* (spruce) and *P. taeda* (pine). The orthologues were not present in stress libraries generated from vegetative tissues. The sizes of stress libraries are significantly smaller than those of seed libraries i.e. not as well represented. As a result the presence of *Atlg07645* transcript abundance, an orthologue of *XhDsi-1^{VOC}* was investigated in vegetative tissue of *A. thaliana*. A comparative RT-PCR study demonstrated that while *XhDsi-1^{VOC}* is expressed in vegetative tissue and seed in the desiccation-tolerant *X. humilis* plant, and upregulated in vegetative tissue during dehydration, in *A. thaliana* it is indeed expressed only in seeds, and not upregulated by stress (Fig. 2.8). In *X. humilis*, *XhDsi-1^{VOC}* transcripts are upregulated in both roots and leaves in response to desiccation (Fig. 2.9). *Xhdsi-1^{VOC}* mRNA transcripts are upregulated early in the desiccation cycle in vegetative tissue and are abundant in *X. humilis* roots at 76% RWC. Transcriptional activation of *Xhdsi-1^{VOC}* in response to water loss may be slower in leaves, with lower levels in leaves at 80% RWC, but with abundant expression by 60% RWC. In both leaves and roots, mRNA transcripts remain abundant at 5% RWC and rapidly decline upon rehydration. A striking difference between *Xhdsi-1^{VOC}* mRNA expression in roots and leaves is that transcripts decline rapidly in roots within the first 6 h of rehydration. However, *X. humilis* roots rehydrate at a much faster rate than leaves (Collett *et al*, unpublished manuscript) and it is possible that the *Xhdsi-1^{VOC}* transcripts decline once a critical water RWC is obtained upon rehydration. Norwood *et al.* (2003) have shown similar rapid root rehydration, with concomitant earlier initiation of metabolism in roots than in leaves of *C. plantagineum*.

The protein expression profile of the Xhdsi-1^{VOC} protein during desiccation of roots and leaves correlates with the mRNA profiles (Fig. 2.9; Fig. 2.10). In contrast to the mRNA levels, XhDsi-1^{VOC} protein levels do not decline immediately upon rehydration. However, similar to the pattern of the mRNA transcripts, the protein levels decline earlier in roots than leaves, 24 h after rehydration. The results suggest that in *X. humilis* XhDsi-1^{VOC} might be important during desiccation and rehydration in vegetative tissue (roots and leaves). The expression patterns of *XhDsi-1^{VOC}* and its orthologues differ dramatically between desiccation sensitive and desiccation tolerant plants. *XhDsi-1^{VOC}* orthologues from desiccation sensitive plants such as *A. thaliana* are expressed in their seeds that are able to acquire desiccation tolerance. Unlike resurrection plants that activate protective mechanisms when exposed to periods of severe water loss, desiccation tolerance in orthodox seeds is a pre-programmed mechanism that occurs during seed development (Kermode and Finch-Savage, 2002; Vertucci and Farrant, 1995). Thus XhDsi-1^{VOC} orthologues from desiccation sensitive plants such as *A. thaliana* could be important during seed maturation (i.e. acquisition of desiccation) and/or during germination. The results strongly suggest that *X. humilis* has acquired the ability to activate this “seed-specific” gene in vegetative tissue (roots and leaves) during the desiccation process.

It is also likely that Xhdsi-1^{VOC} is regulated at the post-translational level. Maeda *et al.*, (2004) have shown that the protein of a Xhdsi-1^{VOC} orthologue in barley (referred to as “glyoxalase-like”) is reduced by thioredoxin h isoforms in barley seed. Reduction of disulphide bonds by thioredoxin is known to modulate protein activity. XhDsi-1^{VOC} may be similarly inactivated by endogenous thioredoxins in leaves and roots during rehydration in *X. humilis*. In addition, two putative phosphorylation sites, a protein kinase C and a protein casein kinase II phosphorylation site are conserved in both angiosperm and gymnosperm *Dsi-1^{VOC}* orthologues (Fig. 2.4).

As mentioned, XhDsi-1^{VOC} does not contain any known metal binding sites reported in other members of the VOC superfamily. The speculation is that XhDsi-1^{VOC} and its orthologues could function similarly to the Bleomycin resistance protein and sequester a metabolite that is toxic in high concentrations in the absence of water (i.e. during desiccation). Ectopic expression of Xhdsi-1^{VOC} in yeast may be lethal because it

sequesters the metabolite and because the metabolite is essential for cellular activity under normal growth conditions.

Based on the results presented in this chapter, it can be concluded that *X. humilis* has acquired the ability to activate this “seed-specific” gene in vegetative tissue (roots and leaves) during the desiccation process. Evidence for this phenomenon is provided by Illing *et al.*, (2005) in an *A. thaliana* microarray data-mining study that examines the seed-specific versus stress-specific expression of a number of *LEA* and anti-oxidant genes, some of which are upregulated in *X. humilis* during desiccation. Mowla *et al.*, (2002) have similarly reported the characterisation of an antioxidant gene, 1-cys peroxiredoxin that is activated in the leaves of the closely related resurrection plant, *Xerophyta viscosa* during abiotic stress. Furthermore, Maeda *et al.*, (2004) report that amongst others, the glyoxalase and XhDsi-1^{VOC} proteins (the latter designated “glyoxalase-like”) are specific to the barley seed proteome i.e. they are abundant in mature barley seeds and their levels decrease in germinating seeds. Seeds and resurrection plants therefore share some mechanisms to protect against metabolic damage that usually occurs during desiccation and rehydration (Bernacchia *et al.*, 1996).

Chapter 3

Using transgenic plants to investigate the function of Xhdsi-1^{VOC} and its orthologue At1g07645 in *A. thaliana*

3.1 Introduction

Microarray technology is a high throughput tool that has enabled the identification of genes involved in dehydration in drought sensitive plants such as *A. thaliana* (Umezawa *et al.*, 2006; Seki *et al.*, 2002), *Oryza sativa* (Rabbani *et al.*, 2003;) and *Physcomitrella patens* (Cuming *et al.*, 2007), in a moderate drought tolerant plant such as barley (Umezawa *et al.*, 2006; Ozturk *et al.*, 2002) and in the desiccation tolerant plant, *X. humilis* (Collett *et al.*, 2004). In addition, genes upregulated in rehydration following dehydration stress have been identified in *A. thaliana* (Oono *et al.*, 2003). The function of some of these genes has been assigned on the basis of their sequence similarity to genes of known function and subsequently confirmed by the appropriate biochemical assays (Helliwell and Waterhouse, 2003). However, there are a large number of genes which do not contain known functional domains and are annotated as genes of unknown function. The challenge is to determine the function of these genes. Several reverse genetic strategies that include insertional mutagenesis (An *et al.*, 2005), RNA interference (post-transcriptional silencing) (Rossi, 2005; Kusaba, 2004; Helliwell and Waterhouse, 2003) and overexpression of the gene of interest (Birch, 1997) are commonly used.

The widely used insertional mutagenesis methods for studying the loss-of-function of unknown genes in plants are transposons and T-DNA knockout mutants (An *et al.*, 2005; Krysan *et al.*, 1999; Aziroz-Leehan and Feldmann, 1997). Insertional mutagenesis methods rely on the physical disruption of a gene sequence within the genome (An *et al.*, 2005). For example, insertional mutagenesis by T-DNA, a binary vector containing the T-DNA region is transformed into *A. tumefaciens* (Hellens and Mullinaux, 2000; Bevan, 1984). Plants are infected with *A. tumefaciens* containing

the binary vector and subsequently, the T-DNA region is inserted into the plant genome (Figure 3.1). The advantage of T-DNA knockout mutants is that they are stable insertions and thus are inherited by the next progeny. In addition, the insertion of T-DNA into the genome is random, therefore saturation mutagenesis of a genome can be obtained (Tinland, 1996). Growing the plants in the presence of a selectable marker initially screens for the integration of the T-DNA within the genome. The disrupted gene (adjoining the T-DNA) is then identified by inverse PCR and subsequently, the disruption of the gene is further confirmed by sequencing using a primer designed at the borders of the T-DNA insert (Azizroz-Leehan and Feldmann KA, 1997). To investigate the phenotype of the gene of interest by T-DNA knockout mutants, it is important to work with at least two independent knockouts to make sure that the mutation is due to the disruption of the gene of interest (An *et al.*, 2005). Alternatively, the T-DNA mutant line could be complemented with the wild type gene. In *A. thaliana*, T-DNA mutants have been generated for most, if not all, of the genes within the genome (Joen *et al.*, 2000). Recently, this method of generating mutants has been employed in rice and more than 300, 000 genes have been disrupted (An *et al.*, 2005). The large numbers of disrupted genes by T-DNA in *A. thaliana* are available at public stock centres and T-DNA knockouts in rice are available commercially (Jeon *et al.*, 2000; Alonso *et al.*, 2003).

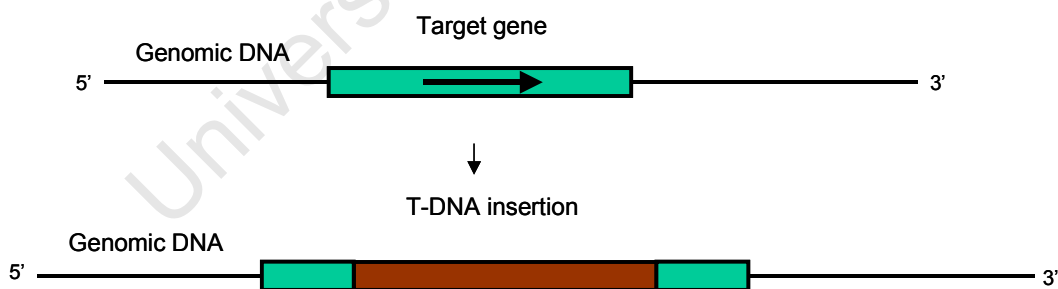


Figure 3.1. T-DNA insertion within a plant genome, disrupting the gene of interest.

Although successful T-DNA mutants have been generated in *A. thaliana* and rice, identification of a phenotype of many disrupted genes is frequently difficult to obtain by using insertional mutagenesis (An *et al.*, 2005). This is due to redundancy of many genes in plant genomes due to either duplication of chromosomal segments or the

duplication of individual genes (Briggs *et al.*, 2006; An *et al.*, 2005). Alternatively, the gene disrupted could be important during the developmental stages of the plant; therefore, the disruption of such a gene results in a lethal phenotype (An *et al.*, 2005). In the instance where the investigation in loss-of-function by T-DNA knockout is difficult, alternative methods for example post-transcriptional silencing by RNA interference (RNAi) are useful (Rossi, 2005; Kusaba, 2004).

Post-transcriptional silencing by RNAi requires the construction of a double stranded RNA (dsRNA), identical to a segment of the gene of interest (Fu *et al.*, 2007; Saumet and Lecellier, 2006). The dsRNA is then introduced into the plant and is subsequently recognised and cleaved by an RNase III Dicer enzyme into small interfering RNAs (siRNAs) of 21-23 nucleotides (Fig. 3.2) (Fu *et al.*, 2007; Saumet and Lecellier, 2006; Sharp, 2001). The siRNAs form a complex with the RNA-induced silencing complex (RISC) and an Argonaute protein leading to the degradation of the target transcript (Saumet and Lecellier, 2006; Tijsterman and Plasterk, 2004; Sharp, 2001; Tuschl *et al.*, 1999). In plants, the process of recognising foreign dsRNA is believed to be a defence mechanism against viral infection (Saumet and Lecellier, 2006; Tijsterman and Plasterk, 2004; Waterhouse *et al.*, 2001; Tuschl *et al.*, 1999). Expression of the RNAi construct can be driven by a range of promoters, including the constitutive 35 S cauliflower mosaic virus (Ca MV 35S), which is the most frequently used (Kusaba, 2004; Vain *et al.*, 2003; Hellens *et al.*, 2000; Hellens and Mullinaux, 2000). However, in the case where disruption of the gene of interest by insertional mutagenesis and constitutive expression of the RNAi construct results in a lethal phenotype or perturbs the development of the transgenic plants, expression of the RNAi construct can be driven by inducible promoters (Gatz and Lenk, 1998).

Inducible promoters only activate transcription in the presence of a specific chemical (Bhatnager-Mathur *et al.*, 2008; Gatz and Lenk, 1998, Zuo *et al.*, 2000). Inducible promoters reported to date include a copper response element, a tetracycline repressor site, a glucocorticoid receptor which is activated by glucocorticoids, estradiol receptors that are activated by estradiol and a lac operone regulatory element activated by isopropyl- β -D-thiogalactopyranoside (IPTG) (Moore *et al.*, 2006; Guo *et al.*, 2003; Zuo *et al.*, 2000; Gatz and Lenk, 1998). Recently, the uses of endogenous promoters induced upon stress are being used for the expression of transgenes, for example the

drought/ABA-regulated promoter *AtRA318* from *A. thaliana* (Karim *et al.*, 2007) and a heat shock protein promoter, *HSP18.2* activated at activated at temperature of 37 °C (Masclaux *et al.*, 2004).

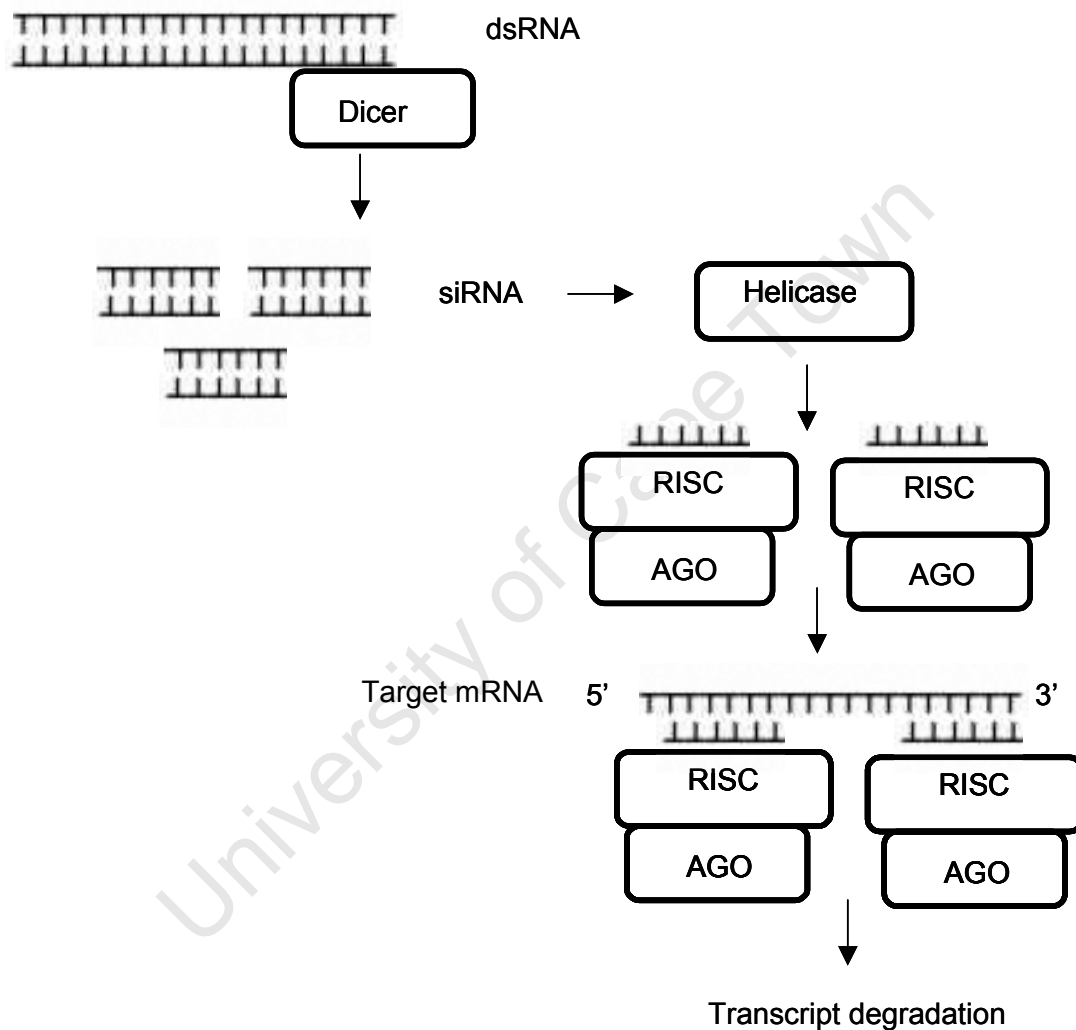


Figure 3.2. Generation of small interfering RNAs (siRNA) from exogenous dsRNA. dsRNA is cleaved by an RNase III Dicer enzyme into siRNA of 21-23 nucleotides. The enzyme Helicase unwinds the cleaved fragments. The RNA-induced silencing complex (RISC) and Argonaute (Ago) bind to the single-stranded fragments. The complex binds and degrades the target mRNA.

In addition to RNAi technology being a useful tool for controlled gene silencing by the use of inducible promoters, it is also a useful tool in silencing multiple redundant genes, a common phenomena within plant genomes, particularly in polyploid species such as wheat which contains three copies of each gene (Fu *et al.*, 2007). In addition to RNAi being specific to a targeted sequence, it has been reported that dsRNA can also silence gene families with highly conserved regions leading to simultaneous silencing of genes in a subfamily (Miki *et al.*, 2005; Kusaba, 2004).

Although posttranscriptional silencing by RNAi overcomes the problems featured by insertional mutagenesis such as redundancy, the construction and generation of transgenic plants expressing the RNAi construct is very time consuming. As a result, it is not suitable for high throughput analysis (Henikoff *et al.*, 2004). In recent years, TILLING (Targeted Induced Local Lesions in Genomes) is an alternative reverse genetics strategy used for high throughput mutagenesis analysis. Initially, seeds are treated with a chemical with the ability to form mutations within the genetic material for example the ethyl methanesulfonate (EMS) (De-Kai *et al.*, 2006; Henikoff *et al.*, 2004). Pooled DNA samples from the second generation progeny are used as templates for PCR using primers specific to the gene of interest (Fig. 3.3). One primer is labelled with 700 nm and the other with 800 nm fluorescent dyes (De-Kai *et al.*, 2006). The PCR products are denatured and subsequently allowed to renature, as a result strands with mutations will form mismatched duplexes with the wild type strands (De-Kai *et al.*, 2006). The PCR products are then digested with CEL 1 endonuclease which cleaves mismatched pairings between wild type and mutant PCR strands (Henikoff *et al.*, 2004). The digested products are then subjected to electrophoresis using the LI-COR-gel analysis system and subsequently analysed by an image processor program (Henikoff *et al.*, 2004). When a mutation is identified within the pooled DNA, DNA from the individual lines within the pooled DNA is screened to identify the lines containing the gene mutation (Henikoff *et al.*, 2004).

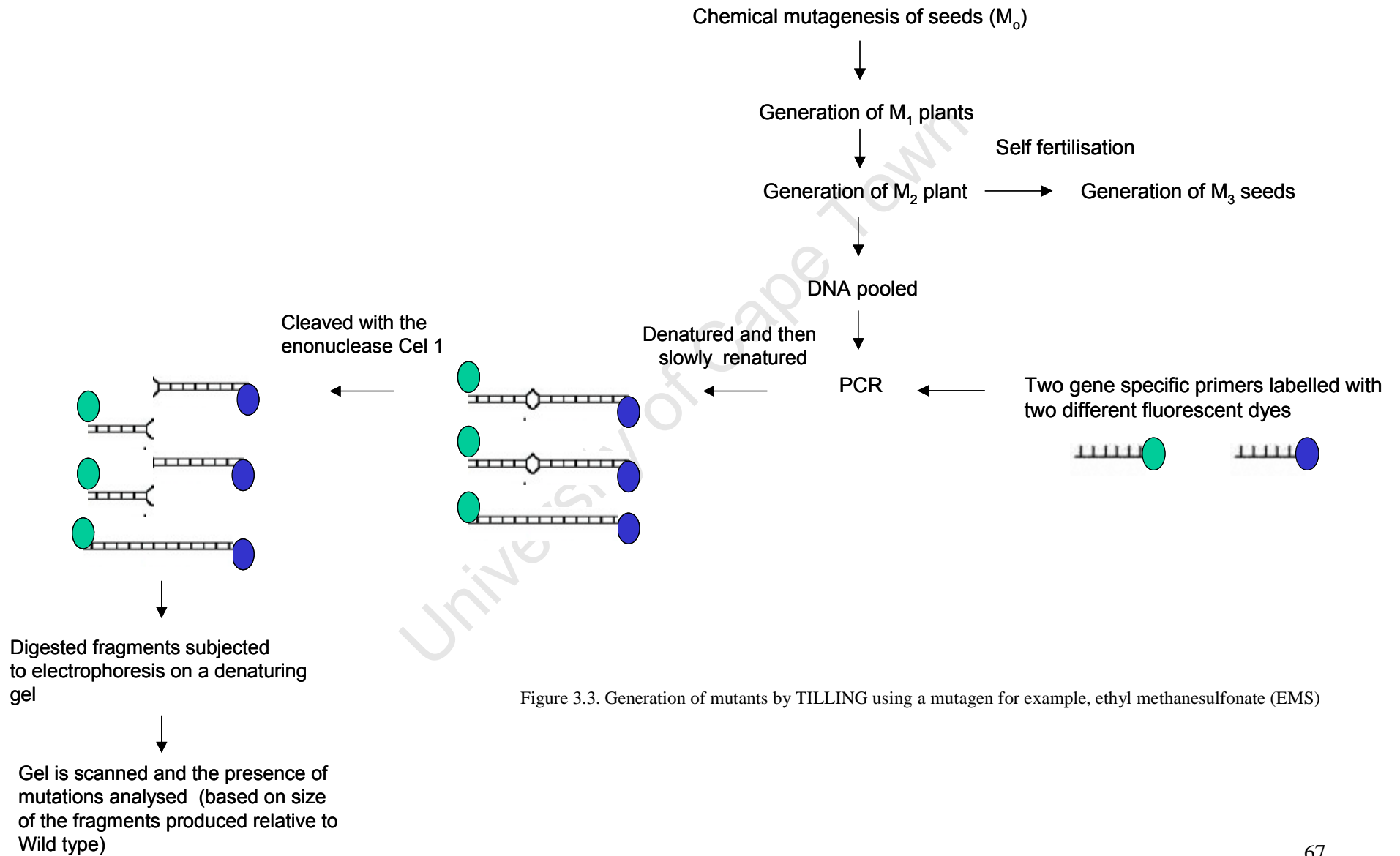


Figure 3.3. Generation of mutants by TILLING using a mutagen for example, ethyl methanesulfonate (EMS)

Mutants generated by TILLING in *A. thaliana* are available in the *Arabidopsis* information centre database (<http://Arabidopsis.org>). When the mutant of the gene of interest is absent, the mutation by TILLING can be ordered at the *Arabidopsis* TILLING project (ATP) database (<http://tilling.fhcrc.9366>) (Henikoff *et al.*, 2004). TILLING has been applied to other plants such as rice, maize (De-Kai *et al.*, 2006), soybean (Cooper *et al.*, 2008), barley, wheat (Silme and Çagırgan, 2007) and tomato (Emmanuel and Levy, 2002). High throughput generation of mutants via TILLING is a powerful tool. The other advantage is that TILLING generates point mutations; as a result there is usually a range of allelic variances, resulting a range of phenotypes (from weak to strong) (Slade and Knauf, 2005). This is useful in the instances where complete knockout is lethal. In addition, TILLING does not require complicated manipulations compared to RNAi technology.

In addition to investigating the function of a gene/protein by the loss-of function-phenotype, gain-of-function analysis by overexpressing a gene of interest can be an alternative or a complementary approach to insertional mutagenesis or posttranscriptional silencing by RNAi to assign a function to a gene of unknown (Zhang, 2003). Similarly to RNAi technology, overexpression of the gene of interest can be under a constitutive promoter for example the frequently used Ca MV 35S (Kusaba, 2004; Vain *et al.*, 2003; Hellens *et al.*, 2000; Hellens and Mullinaux, 2000). In instances where the constitutive overexpression of the gene of interest is lethal or affects normal plant development, as described for posttranscriptional silencing, inducible promoters are the best option (Moore *et al.*, 2006). In addition overexpression of the gene of interest overcomes redundancy, a problem face when investigating loss-of-function phenotype by mechanisms such as insertional mutagenesis (Zhang, 2003).

Given that Xhdsi-1^{VOC} and its *A. thaliana* orthologue, At1g07645 are unlikely to function as glyoxalase I enzymes (chapter 2), this chapter reports three strategies which were used to investigate the function of Xhdsi-1^{VOC} and At1g07645 during osmotic stress. This chapter describes the construction of transgenic plants overexpressing Xhdsi-1^{VOC} or At1g07645, as well as plants in which RNAi knockdown endogenous expression of At1g07645. In addition, two lines of *A.*

thaliana plants with a T-DNA insertion that disrupted the *At1g07645* gene in the genome were identified.

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3.2. Materials and Methods

3.2.1. Isolation of total RNA and cDNA transcription

Total RNA was extracted from mature dry seeds of *X. humilis* and *A. thaliana* as described by Wan and colleagues (1994). The isolated mRNA from seed was further purified with the RNeasy^R Plant mini kit (Qiagen, USA) following the manufacturer's instruction. In addition, the RNeasy^R Plant mini kit was used to extract *A. thaliana* leaf tissue according to the manufacturer's instruction. RNA from both seeds and leaf tissue was treated with DNase I during the purification step of the RNeasy^R Plant mini kit as described by the manufacturers. The concentration of the RNA was obtained using the NanoDrop^R ND-1000 spectrophotometer (NanoDrop Technologies, Inc, DE) and integrity of the RNA was checked on a 1% agarose formaldehyde denaturing gel (Ausubel *et al.*, 1995).

cDNA was obtained from synthesis of 0.5 µg –1 µg of total RNA by using 200 units of Superscript II reverse transcriptase enzyme (Life Technologies, USA) following manufacturers instructions. In all PCR reactions, 250 ng of synthesized cDNA was used for the PCR reaction, which included 0.5 µM of each primer, 0.2 mM dNTP, 0.5 u Supertherm Taq (Hoffman-La Roche, USA) and 2.4 mM MgCl₂. The PCR parameters were the same for all PCR/primer reactions with exception of the annealing temperature summarised in Table 3.1. The PCR parameters were as follows; initial denaturation temperature for one min at 94 °C, 30 s at 94 °C, annealing temperature (Table 3.1) for 1 min and an extension step of 72 °C for 1 min repeated for 35 cycles prior to a final extension step of 5 min at 72 °C (if different, PCR parameters will be indicated).

Table 3.1. A summary of all primer pairs with their annealing temperatures used in PCR reactions

Name	Forward primer	Name	Reverse primer	Anneal. Temp.	Purpose
Aperpfgf	5' GGCTCGAGATGGCAGCTAATA T 3' <i>XhoI</i>	ApFOR	5' GGGGAT CCTAAATATATTCATCAAGA 3' <i>BamHI</i>	57°C	Ectopic expression
Aperpfgf	5' GGCTCGAGATGGCAGCTAATA T 3' <i>XhoI</i>	ApOR	5' GGA CTAGTGGACTAGGCTGGACAC 3' <i>SpeI</i>	60°C	Ectopic expression
Atg22	5' TTC GAA AAT GTG ACA AAT CGA G 3'	Atg23	5' GGA CAT CGC ACC AAT GA ATC 3'	58 °C	T-DNA
Atg22	5' TTC GAA AAT GTG ACA AAT CGA G 3'	Lba1	5' TGG TTC ACG TAG TGG GCC ATC G 3'	55 °C	T-DNA
Atg24	5' CCC TTT CCT CTT CCT GAC AT C 3'	Atg25	5' TAC AAA CCT GTG GGA TTC GTC 3'	58 °C	T-DNA
Atg24	5' CCC TTT CCT CTT CCT GAC AT C 3'	Lba1	5' TGG TTC ACG TAG TGG GCC ATC G 3'	55 °C	T-DNA
CHSAI	5' GCC ATC GCGATA AAG GAAAGGCTA 3	Rfgr1	5' GGCCCGGGTGAACCCGGCGTT T 3' <i>XmaI</i>	66 °C	RNAi
F-ACTIN	5' CCCGCTGGAGAAATCAAT TA 3'	R-ACTIN	5' GGCATTTCAAGCAAAT 3'	60°C	Real-time PCR
FAT	5' ATCGGATCGAAATCAGAATCAT 3'	RAT	5' ACGTCCTTCACGTACACAG 3'	60°C	Real-time PCR
2HANBF	5' GG GGATCC CTCGAGATGAGACCGG CGTT 3' <i>BamHI XhoI</i>	3HANBR	5' GG AAGCTT GGT ACC CAC GTA TCC AAC <i>KpnI HindIII</i> CTTCT 3'	63 °C	RNAi
PDKI	5' GGAAGT TCATTT CAT TTG GAGAG 3'	2HANBF	5' GG GGATCC CTCGAGATGAGACCGG <i>BamHI XhoI</i> CGTT 3'	57 °C	RNAi
PDKI	5' GGAAGT TCATTT CAT TTG GAGAG 3'	PER8R	5' ATC ATA GGC GTC TCG CAT ATC TC 3'	55 °C	RNAi

Name	Forward primer	Name	Reverse primer	Anneal. Temp.	Purpose
PER8F	5' GCCATGTAATATGCTCGACTCA 3'	PER8R	5' ATC ATA GGC GTC TCG CAT ATC TC 3'	55 °C	RNAi
pFGC-2F	5' GAGAGGACACGCTCGAG 3'	pFGC-R	5' TTTACAAGCTGCACAACAGA 3'	55.7 °C	RNAi
Pfgf1	5' GGCTC GAGTGAGACCGGCGT TT 3' <i>XhoI</i>	Rfgr1	5' GGCCATGGCGATGCGTACAACAATG 3' <i>NcoI</i>	61°C	RNAi
Pfgf2b	5' GGCCCGGGTGAACCCGGGCGTT T 3' <i>XmaI</i>	Rfgr2	5' GGCCTAGGCGATGCGTACAACAATG <i>AvrII</i>	56°C	RNAi
Xperpfgf	5' GGCTCGAGATGGCGGCGA 3' <i>XhoI</i>	XpFOR	GGGGATCCCACATCAAACCAGAAA <i>BamHI</i>	57°C	Ectopic expression
Xperpfgf	5' GGCTCGAGATGGCGGCGA 3' <i>XhoI</i>	XpOR	5' GGA CTAGT CACATCATCA AACCGAAA T 3' <i>SpeI</i>	69°C	Ectopic expression

3.2.2. DNA extraction

Genomic DNA was extracted from *A. thaliana* by grinding one leaf in a 1.5 ml centrifuge tube in the presence 500 µl of extraction buffer containing 200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8 and 0.5% SDS. The homogenate was incubated at 60°C for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1) (v/v) was added and centrifuged for 10 min at 8000 g at room temperature in a bench top centrifuge. The supernatant was collected into a new centrifuge tube, 500 µl of chloroform: isoamyl (24:1) (v/v) was added and the sample was subjected to 10 min centrifugation. This step was repeated twice. DNA was precipitated by adding two volumes of ethanol and 1/10 volume of 3M sodium acetate pH 5 and incubated at -20°C overnight. The samples were centrifuged for 10 min, the supernatant discarded and the DNA pellet washed with 70% ethanol and air-dried for 10 min. The pellet was reconstituted into 50 µl sterile water. The DNA concentrations were determined by NanoDrop^R ND-1000 spectrophotometer (NanoDrop Technologies, Inc, DE) and stored at -20°C.

3.2.3. Protein extraction

Protein was extracted from 0.008 g of *A. thaliana* seeds by homogenising the seeds in a centrifuge tube containing one 3 mm stainless steel ball-bearing (Qiagen GmbH, Germany) by a Mixmill tissuelyser (Qiagen, Germany). 200 µl of extraction buffer (2% SDS, 5 mM EDTA pH 8 and 50 mM Tris-HCL pH 6.8) was added to the homogenised tissue. The samples were heated at 100°C for five min and centrifuged at 15 000 g for five min. The upper phase was transferred to a new centrifuge tube and centrifuged at 15 000 g for a further five min. The supernatant was transferred to a fresh centrifuge tube and stored at -20°C to be used for western blot analysis.

Protein was extracted from *A. thaliana* leaf tissue (50 mg of tissue). The leaf tissue was homogenised in liquid nitrogen and 150 µl of extraction buffer was added to the homogenised tissue (2 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM Benzamide, 10 mM ε amino caproic acid, 1 mM EDTA pH 7.5 and 1 X TBS (50 mM Tris-Cl at pH 7.6 and 150 mM of NaCl)). The extraction buffer containing the

homogenised tissue was centrifuged for five min at 12 000 rpm at 4°C. The supernatant was transferred to a new centrifuge tube. 20 µl of the supernatant was removed for protein quantification. The remaining supernatant was stored at -20°C for western blot analysis. The protein concentration was determined by Bradford assay, following manufacturer's specification (Bradford, 1976; Bio-Rad, USA).

3.2.4. Real-time PCR on RNAi mutants

The software known as primer3 (<http://frodo.wi.mit.edu/>) was used to design primers for real-time PCR summarised in Table 3.1. FAT and RAT primers were designed against *At1g07645* (outside of the target sequence fragment used for RNAi construction) and F-actin and R-actin primers were designed against F-actin (*At3g05520*). Prior to performing real-time PCR, a conventional PCR was used to optimise PCR conditions. For both sets of primers, 1 µl of seed cDNA was used in the PCR reaction. The PCR reaction included 0.5 µM of each primer, 0.2 mM dNTP, 1 µl of polymerase (5 units/µl) containing 1.5 mM MgCl₂ (Roche diagnostics, Germany) and 1 X PCR buffer (Roche diagnostics, Germany). The following PCR conditions were used: initial denaturation for one min at 94 °C, 30 sec at 94 °C, 30 sec, annealing step of 60 °C for 30 sec and an extension step of 72 °C for 1 min, repeated for 35 cycles prior to a final extension step of 5 min at 72 °C.

The cDNA used in real-time PCR was diluted 1 in 100. In a 384 well plate, each PCR reaction and control (without template added) was performed in triplicate. The following components were included in the PCR reaction: 1 X SYBR^R Green PCR master mix (including SYBR green I dye, DNA polymerase, dNTPs and 1.5 mM MgCl (Applied Biosystems, USA)), 0.5 µM of each primer and 0.5 µl of the diluted DNA. The real-time PCR machine used was the Taqman ABI prism 7900HT sequence detection (Applied Biosystems, USA). The PCR cycling conditions were: initial denaturation temperature of 94 °C for 10 min, followed by denaturation at 94 °C (15 s), annealing temperature of 60 °C (one min) and extension time of 72 °C (five min) for 40 cycles. At the end of the run, the dissociation curve, standard curves and data analysed by Prism SDS 2.1 (Applied biosystems, USA) were obtained. The reference sample used to create standard curves was cDNA from mature dry wild type

A. thaliana seeds. Standard curves of five-fold dilutions were prepared for both F – actin and At1g07645 reactions.

3.2.5. Optimisation of the primary and secondary antibodies against Dsi-1^{voc}

Five microgram of protein extracted from *A. thaliana* and *X. humilis* dry seed was spotted on eight nitrocellulose membranes (Schleicher and Schuell Biosciences GmbH, Germany). The membranes were incubated with 1 X TBS (50 mM Tris-Cl at pH 7.6 and 150 mM of NaCl) containing 5% fat free milk powder at 4 °C for 16 h. After 16 h, the membranes were incubated overnight in 1 X TBS containing 2% fat free milk powder and 1/500, 1/1000 or 1/3000 of primary anti-sera dilutions. The membranes were then washed with 1 X TBS containing 0.1% Tween-20 (Sigma, USA) three times for five min and incubated at room temperature for 1 h with 1/3000 or 1/5000 dilutions of anti-rabbit/goat horseradish peroxidase (HRP) conjugated secondary antibody (BIO RAD Laboratories GmbH, Munchen).

The membranes were washed four times for five min with 1 X TBS containing 0.1% Tween 20. The chemiluminescence substrate solutions used to detect the secondary antibodies are listed in Table 3.2. The membranes were incubated for five minutes with an equal mixture of Solution 1 and 2. The membrane was exposed to a high performance autoradiography film (HyperfilmTM-βmax Amersham, UK) for one min.

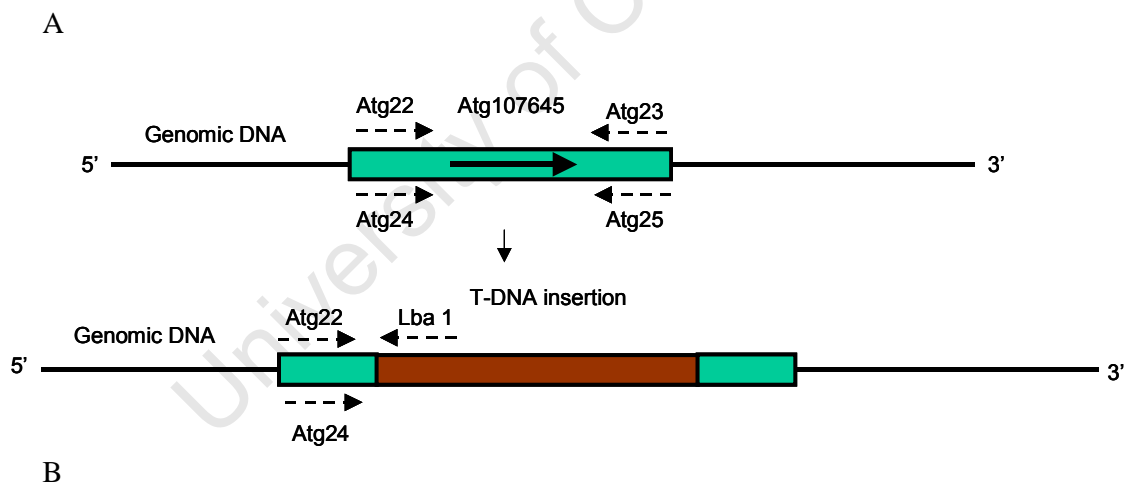
Table 3.2. Chemiluminescence substrates used for the detection of the secondary antibody in western blot analysis

Solution	Description
Solution A	90 mM P-coumaric acid (Sigma, USA) dissolved in DMSO*
Solution B	250 mM 3- Aminophalhydrazin (Sigma, USA) dissolved in DMSO
Solution 1	3 ml of 100 mM Tris-Cl (pH 8.5), 13.3 µl of solution A and 1.66 µl of 30% hydrogen peroxide
Solution 2	3 ml of 100 mM Tris-Cl (pH 8.0) and 30 µl of solution B

* DMSO; Dimethyl sulfoxide

3.2.6. Identification of a T-DNA homozygous line disrupting *At1g07645* gene

Two T-DNA insertion lines disrupting *At1g07645* (NASC ID, N549529 and N597861) were obtained from the Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Gene specific primers were used in a PCR reaction using 100-150 ng of genomic DNA as a template to identify homozygous lines. In addition, the T-DNA specific forward primer (Lba 1) and the gene specific reverse primers were used in a PCR reaction to identify the position of the T-DNA insertion. The gene specific primers used for N549529 were Atg22 and Atg23 (Fig. 3.4; Table 3.1). The primers for the N597861 line were Atg24 and Atg25 (Fig. 3.4; Table 3.1). Primer pairs Lba 1 and Atg22, Lba 1 and Atg24 were used to identify a homozygous line in the disrupted *At1g07645* gene in lines N549529 and N597861, respectively (Fig. 3.4). Reduction of *At1g07645* protein expression in these lines was determined by western blot analysis using total protein isolated from mature seeds.



PCR Product (primers)	Wildtype (+/+)	Heterozygous (+/-)	Homozygous (-/-)
Atg22 + Atg23 or Atg24 + Atg25	Yes	Yes	No
Atg22 + Lba 1 or Atg24 + Lba 1	No	Yes	No

Figure 3.4. (A) T-DNA disruption of the *At1g07645* genes. The disruption is confirmed by PCR by using a T-DNA specific primer (Lba1) and *At1g07645* specific primers, either Atg22 or Atg24 (Table 3.1). (B) PCR analysis. A PCR reaction with gene specific forward and reverse primers will result in a fragment amplified in wild type (+/+) and heterozygous (+/-) lines. However, the product will not be amplified in homozygous lines (-/-) as the T-DNA insert is too large to amplify. A T-DNA specific primer (Lba 1) and *At1g07645* specific primer (either Atg22 or Atg24) will result in the amplification of a fragment in heterozygous (+/-) and homozygous (-/-) lines and not in the wild type.

3.2.7. Binary vectors used for the generation of transgenic plants

The binary vectors pFGC5941 (GenBank Accession AY310901) and pER8 (GenBank Accession AF309825; Zuo and Chua, 2000) were used to create transgenes, either by overexpressing or knocking down the expression of a targeted plant gene (Fig. 3.5). pFGC5941 contains the constitutive promoter, Ca MV 35S, and can be selected by kanamycin in bacteria or phosphinotricin (Basta) in plants. The pER8 is a chemically induced vector that contains a chimeric XVE construct which consists of a DNA binding domain, LexA, a transcription activator domain and the C-terminal of the human estradiol receptor constitutively expressed by the promoter P_{G10-90} (Zuo *et al.*, 2000). In the presence of estradiol, the XVE-estradiol complex binds to the LexA operator sequence, resulting in the transcription of the inserted gene of interest. In addition, pER8 has selectable markers, namely spectinomycin in bacteria and hygromycin phosphotransferase II (HPT II) in plants.

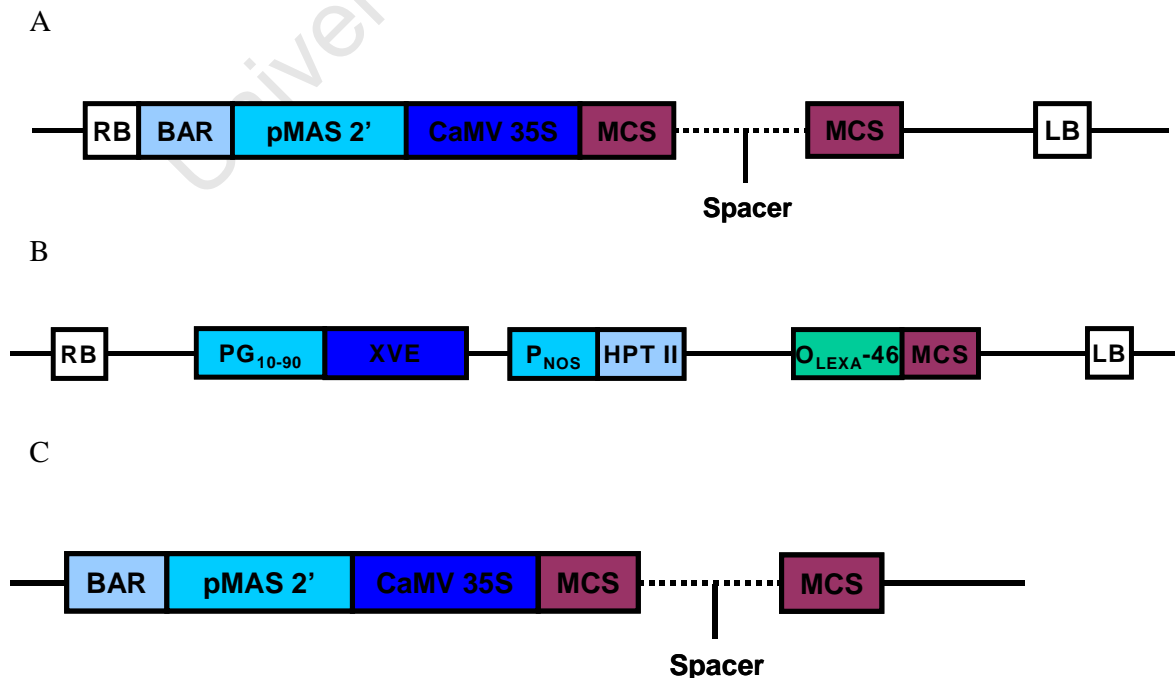


Figure. 3.5. Schematic diagrams of T-DNA regions of binary vectors pFGC5941 (A), pER8 (B) and non-binary cloning vector pKANNIBAL (C). The pFGC5941 contains a Basta (phosphinotricin) (BAR) resistance gene driven by a pMAS2' promoter. The transcription of a transgene is driven under the CaMV 35S promoter. The pER8 vector is a chemically induced vector that contains a chimeric XVE construct, which consists of a DNA binding domain (LexA), a transcription activator domain and the C-terminal of the human estradiol receptor constitutively expressed by the synthetic promoter P_{G10-90} (Zuo *et al.*, 2000). LexA⁴⁶ represents eight copies of the LexA operator sequence. pER8 contains a gene encoding a hygromycin phosphotransferase II resistant protein (HPT II) driven by the nopaline synthase promoter (P_{NOS}). MCS stands for multiple cloning site. pFGC5941 and pKANNIBAL contain two multiple cloning sites separated by a spacer. These two vectors can be used for directional cloning of fragments in a sense and anti-sense orientation to form a hairpin construct, a mechanism used in RNAi interference. In the vector pFGC5941, the spacer is removed when cloning a transgene for overexpression. RB and LB represent the right and left border, respectively.

3.2.8. Amplification of PCR products and the subsequent cloning

All amplified PCR products were initially ligated into the pGEM^R-T Easy plasmid (Promega, U.S.A). The constructs were then transformed into *E.coli* XL-1Blue strain. The transformed *E. coli* was grown on LB agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar in 1L) in the presence of 100 µg ml⁻¹ of ampicillin at 37°C, overnight. Positive (white) colonies were inoculated into Luria broth (10 g Tryptone, 5 g yeast extract, 5 g NaCl in 1L) and incubated overnight at 37°C with constant shaking. The plasmid was purified from *E. coli* XL-1 Blue using the High Pure Plasmid Isolation Kit (Roche, USA). The PCR products ligated into the pGEM^R-T Easy plasmid were then digested with the appropriate restriction digests and cloned into the appropriate vectors.

3.2.9. Generation of RNAi constructs: cloning of an inverted repeat sequence fragment into the pFGC5941 and pER8 vectors

Primers were designed to amplify a 380 bp fragment matching the regions between 53 bp and 433 bp of the *At1g07645* cDNA from *A. thaliana* seed. This region was used in the construction of an RNAi inverted repeat by cloning the fragment into a sense and anti-sense orientation into the pFGC5941 vector (Fig. 3.6). The fragment ligated into the sense orientation was amplified by using Pfgf1 and Rfgr1 primers containing *XhoI* and *NcoI* restriction enzymes sites, respectively (Fig. 3.6). The fragment ligated into the antisense orientation was amplified using Pfgf2b and Rfgr2 containing *XmaI* and *AvrII* restriction enzymes, respectively.

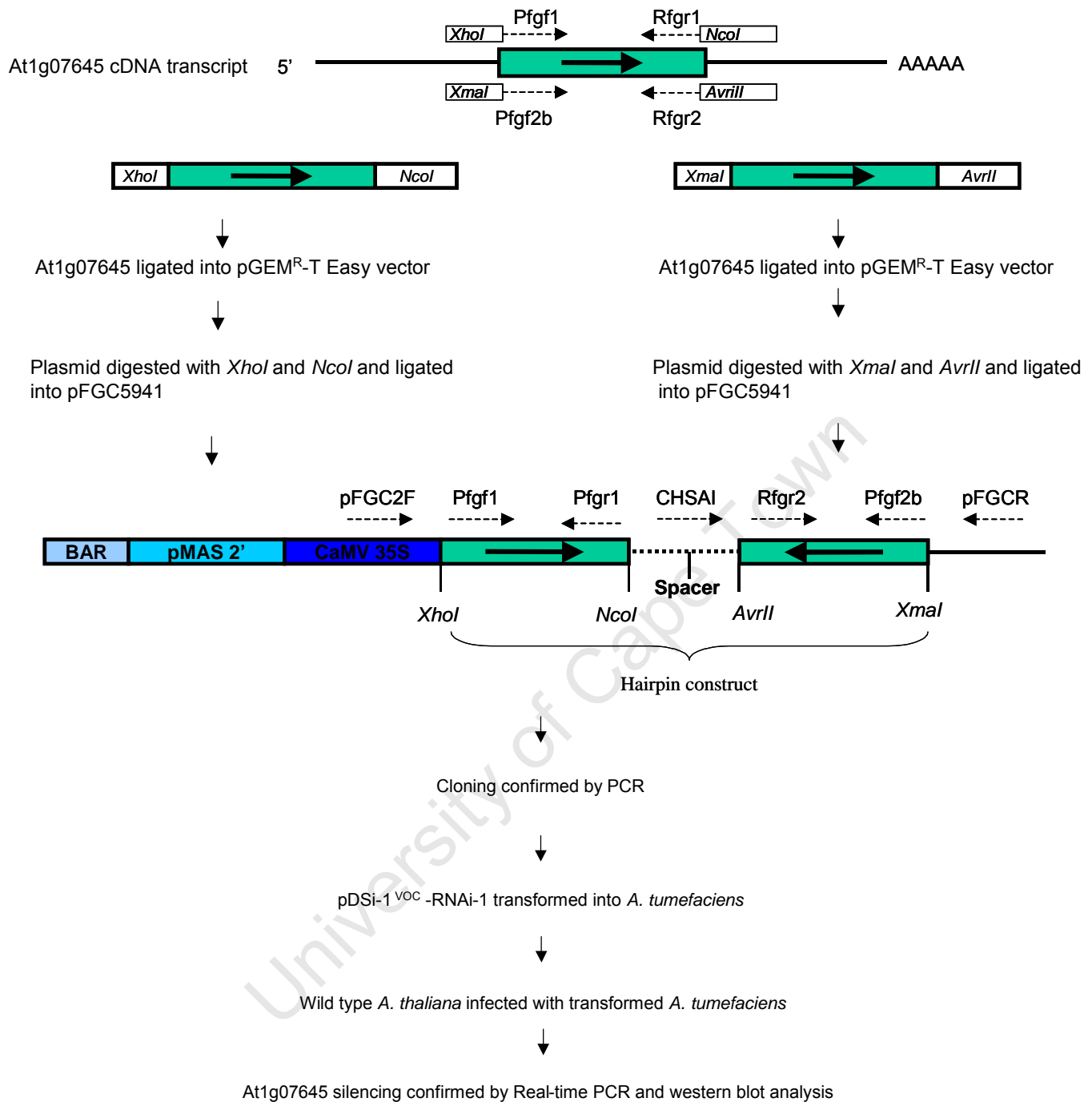


Figure 3.6. Flow diagram showing the construction of a hairpin in the pFGC5941 vector for posttranscriptional silencing of At1g07645 in *A. thaliana*. The PCR products are amplified using primers with engineered restriction enzyme sites (Table 3.1). The products were ligated into pGEM^R-T EASY vector and digested with the appropriate restriction enzymes. The fragment digested with are *XhoI* and *NcoI* was ligated into the sense orientation into the first cloning site of pFGC5941. The fragment digested with *XmaI* and *AvrII* was ligated into the antisense orientation in the second cloning site. The broken arrows show the position of the primers and the solid arrows indicate the 5' to 3' direction.

The sense fragment was ligated and the successful cloning was verified by PCR using fragment specific primers Pfgf1 and Rfgr1 (Fig. 3.6). The fragment in the opposite orientation was subsequently ligated and the cloning was checked by amplifying a PCR product using pFGC5941 specific primer that flank the inverted repeat sequence, pFGC2F and pFGCR. The presence and orientation of both fragments was confirmed by sequencing using Pfgf1 and CHSAI primer specific to the pFGC5941 spacer.

The binary vector pER8 does not contain multiple cloning sites separated by a spacer. As a result, a hairpin RNAi construct targeting *At1g07645* was constructed in the pKANNIBAL vector and then sub-cloned into the pER8 binary vector. A 351 bp fragment was amplified corresponding to 52 bp and 403 bp of the *At1g07645* cDNA transcript using the 2HANBF and 3HANBR primers (Fig. 3.7). The 2HANBF primer contains restriction enzymes *BamHI* and *XhoI* at the 5' end and 3HANBR contains the restriction enzymes *KpnI* and *HindIII* at the 5' end. When the fragment was digested with *XhoI* and *KpnI*, and ligated into the sense orientation in the first cloning site of pKANNIBAL. Confirmation of the successful ligation was verified by amplifying the fragment from the plasmid using the fragment specific primers, 2HANBF and 3HANBR (Fig. 3.7). The anti-sense orientation was obtained by digesting the fragment with *BamHI* and *HindIII* restriction enzymes. One primer specific to the spacer (PDK I) and another specific to the insert (2HANBF) were used to verify the *At1g07645* insert ligated into the anti-sense orientation in pKANNIBAL (Fig. 3.7).

This inverted repeat (hairpin) constructed in pKANNIBAL was excised by digesting the plasmid with *StuI* and *SpeI* restriction enzymes. The digested fragment was subcloned into corresponding restriction sites in the pER8 vector (Fig. 3.7). The successful ligation of the construct into the pER8 (pDSi-1^{VOC}-RNAi-2) was verified using a primer specific to the spacer (PDK I) and a primer specific to the pER8 vector (pER8 R). The presence and quality of both fragments was confirmed by sequencing using primers specific to the pER8 vector (pER8F and pER8). All primers are summarised in Table 1.

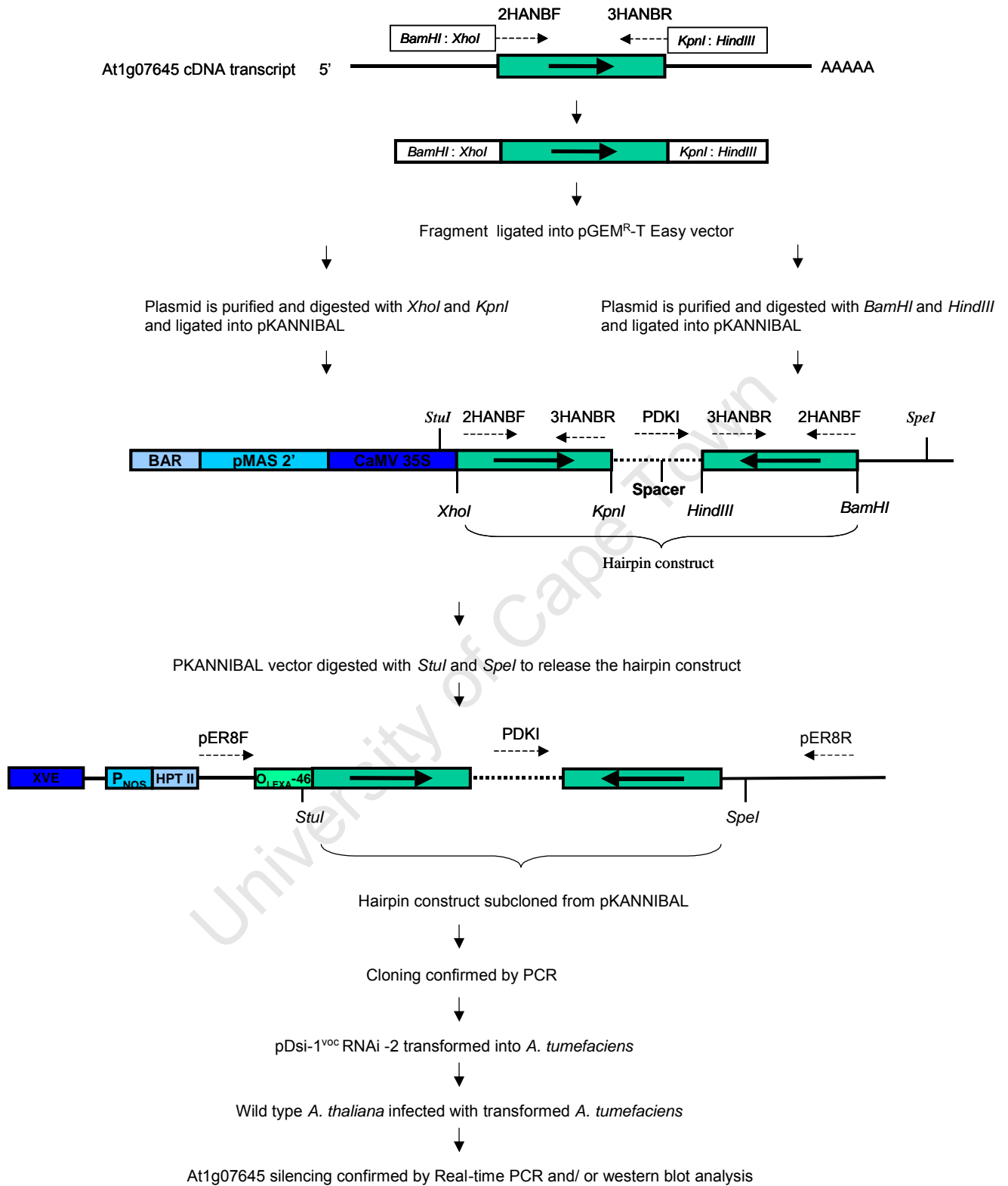


Figure 3.7. Flow diagram showing the construction of a hairpin in the pER8 vector for posttranscriptional silencing of *At1g07645* in *A. thaliana*. The PCR product was amplified using primers with engineered restriction enzyme sites (2HANBF and 3HANBR) (Table 3.1). The amplified PCR product was ligated into pGEM^R-T EASY vector and digested with the appropriate restriction enzymes. When digested with *XhoI* and *KpnI*, the fragment was ligated into the sense orientation and when digested with *BamHI* and *HindIII*, the fragment was ligated with into the antisense orientation. The construct was subcloned into pER8 vector (pDSi-1^{VOC}RNAi-2). The broken arrows show the position of the primers and the solid arrows indicate the 5' to 3' direction.

3.2.10. Construction of binary vectors containing the open reading frames of *At1g07645* and *Xhdsi-1^{VOC}*

The open reading frame of *Xhdsi-1^{VOC}* from *X. humilis* and its orthologue *At1g07645* from *A. thaliana* were amplified from *X. humilis* and *A. thaliana* cDNA using primer pairs containing engineered restriction enzymes sites shown in Figure 3.8 and Figure 3.9 and listed in Table 3.1. The PCR products were ligated into pGEM^R-T Easy vector (Promega, U.S.A). The pGEM^R-T Easy vector containing the coding region of *Xhdsi-1^{VOC}* and *At1g07645* were digested with appropriate restriction enzymes and ligated into the pFGC5941 vector. The PCR product was digested with the restriction enzymes sites *XhoI* and *BamHI* and ligated into the pFGC5941 vector; the construct was named 35S::*Xhdsi-1^{VOC}* (Fig. 3.8 A). For the ectopic expression of *Xhdsi-1^{VOC}* under an inducible (XVE) promoter, the coding sequence was ligated into the *XhoI* and *SpeI*, restriction sites of the pER8 vector and was named XVE::*Xhdsi-1^{VOC}* (Fig. 3.8B). In addition, the coding region of *At1g07645* was ligated into pFGC5941 vector for the constitutive expression (Fig 3.9A). This construct was termed 35S::*At1g07645*. Finally, *At1g07645* was ligated into *XhoI* and *SpeI* restriction sites in pER8 vector, the construct was named XVE::*At1g07645* (Fig 3.9B). PCR primer pairs summarised in Fig. 3.8 and 3.9 were used to confirm the cloned constructs.

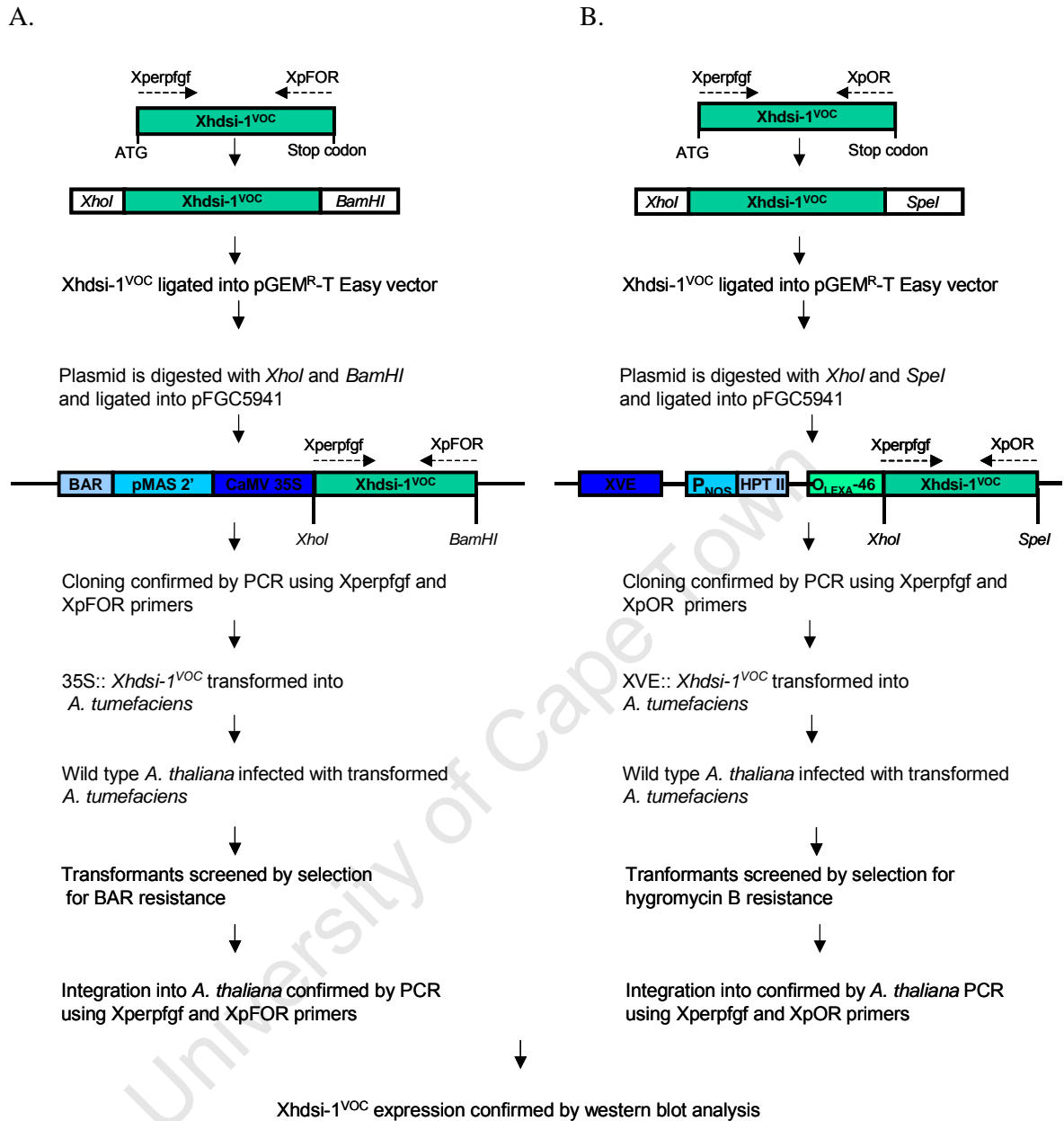


Figure 3.8. Flow diagram showing the cloning of the open reading frame of *Xhdsi-1^{VOG}* into pFGC5941 (A) and pER8 (B). The PCR products are amplified using primers with engineered restriction enzyme sites (Table 3.1). The products were ligated into pGEMR-T EASY vector and digested with the appropriate restriction enzymes. Subsequent to the digestion, the fragments were cloned into pFGC5941 and pER8. The successful cloning was verified by amplification of the *Xhdsi-1^{VOG}* sequence using gene specific primers. The vectors termed 35S::*Xhdsi-1^{VOG}* and XVE::*Xhdsi-1^{VOG}* were transformed into *A. tumefaciens*. Wild type *A. thaliana* plants were infected with *A. tumefaciens*. Antibiotic resistance and PCR confirmed the integration of *Xhdsi-1^{VOG}* into the *A. thaliana* genome. Expression of *Xhdsi-1^{VOG}* in vegetative tissue of the transgenic lines was confirmed by western blot analysis. The broken arrows show the position of the primers.

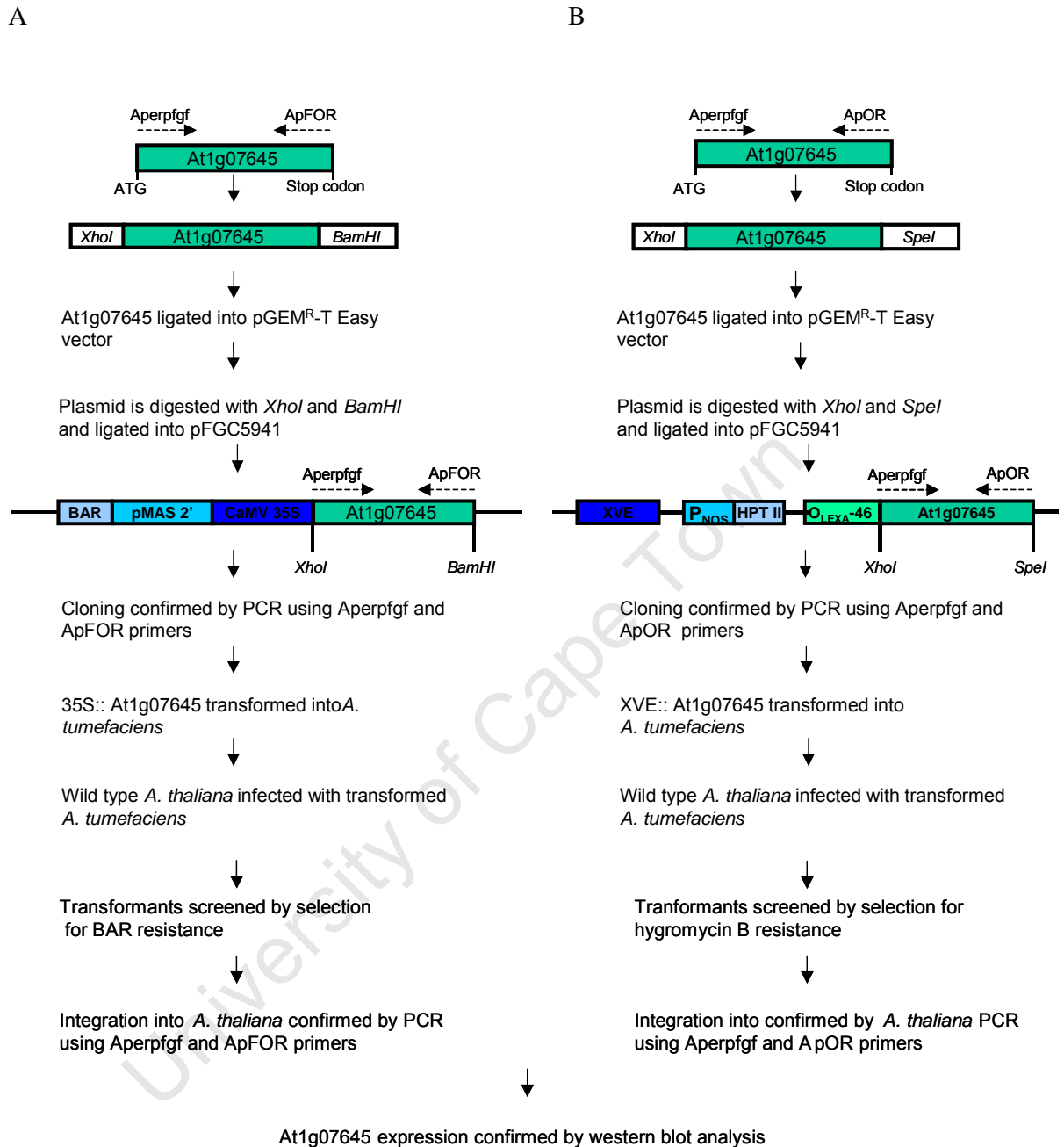


Figure 3.9. Flow diagram showing the cloning of the open reading frame of *At1g07645* into pFGC5941 (A) and pER8 (B). The PCR products are amplified using primers with engineered restriction enzyme sites (Table 3.1). The products were ligated into pGEM^R-T EASY vector and digested with the appropriate restriction enzymes. Subsequent to the digestion, the fragments were cloned into pFGC5941 and pER8. The successful cloning was verified by amplification of the *At1g07645* sequence using gene specific primers. The vectors termed 35S:: *At1g07645* and XVE:: *At1g07645* were transformed into *A. tumefaciens*. Wild type *A. thaliana* plants were infected with *A. tumefaciens*. Antibiotic resistance and PCR confirmed the integration of *At1g07645* into the *A. thaliana* genome. Expression of *At1g07645* in vegetative tissue of the transgenic lines was confirmed by western blot analysis. The broken arrows show the position of the primers.

3.2.11. Transformation of *Agrobacterium tumefaciens*

The binary vectors containing the constructs were transformed into *A. tumefaciens* GV3101. The GV3101 strain is resistant to rifampicin and gentamicin and was streaked on LB plates from a glycerol stock in the presence of 150 µg/ml rifampicin and 15 µg/ml of gentamicin. The plates were incubated for two d at 28°C. A single colony was inoculated into 10 ml LB containing 150 µg/ml rifampicin and 15 µg/ml of gentamicin. The Luria broth was incubated overnight at 28°C with mild shaking. Cells were harvested when absorbance readings at 600 nm reached an OD reading between 0.5 and 1.0. The culture was centrifuged at 3500 g for 10 min at 4°C. The supernatant was decanted and the pellet resuspended in 0.5 ml ice-cold 20 mM CaCl₂. One microgram of plasmid was added to a 100 µl of the *A. tumefaciens* and placed on ice. 900 µl of Luria broth was added to the centrifuge tube and incubated for 3 h at 28°C with gentle shaking. After the incubation, the culture was centrifuged at 3500 g for 3 min. 900 µl of the supernatant was discarded and the pellet was resuspended in the remaining 100 µl LB. The transformed *A. tumefaciens* was then plated on LB agar plates containing 150 µg/ml rifampicin and 15 µg/ml of gentamicin, and either 75 µg / ml kanamycin (for selection of the pFGC5941 vector) or 100 µg /ml spectinomycin (for selection of the pER8 vector).

3.2.12. Introduction of constructs into *A. thaliana*

A. thaliana was infected with *A. tumefaciens* containing the binary vectors by the floral dip method (Clough and Bent, 1998). Wild type seeds from *A. thaliana* (*Columbia ecotype*) were germinated in soil and were allowed to grow. The first sets of bolts were cut off to encourage additional side shoots and bolts to grow. A single colony of *A. tumefaciens* transformed with either pFGC5941 or pER8 vectors (summarised in Table 3.3) was inoculated into a 5 ml Luria broth containing the appropriate antibiotics and incubated at 28°C for 3 d. The culture was diluted into 500 ml in a 2L flask and incubated at 28 °C for 24 hours. The bacteria were pelleted by centrifugation for 15 min at 3 500 g at room temperature. The bacterial pellet was resuspended in 500 ml of 5% sucrose solution containing 0.05% of the surfactant Silwet L-77 (VAC-IN-STUFF, USA). The 500 ml solution was transferred into a

small beaker and the bolts and flowers on the plants were dipped into the beaker for a couple of 15 sec. To generate controls, plants were dipped in *A. tumefaciens* that did not contain the vectors. The plants were placed on their side (horizontally) and covered with Saran wrap overnight. After 24 hours the saran wrap was removed and the plants were placed upright. The plants were allowed to set dry, mature seed and the seed (T₁ generation) was collected.

Table 3.3. A summary of constructs

Name	Vectors used	Purpose
35S:: <i>Xhdsi-1</i> ^{VOC}	pFGC5941	Overexpression
35S:: <i>At1g07645</i>	pFGC5941	Overexpression
XVE:: <i>Xhdsi-1</i> ^{VOC}	pER8	Overexpression
XVE:: <i>At1g07645</i>	pER8	Overexpression
pDSi-1 ^{VOC} -RNAi-1	pFGC5941	RNAi
pDSi-1 ^{VOC} -RNAi-2	pKANNIBAL and pER8	RNAi

3.2.13. Selection of transgenic lines

The selection of transgenic plants containing the integrated fragments driven by the XVE inducible promoter (pER8) was performed by sterilising and stratifying seeds as described in chapter two give section and plating them on plant nutrient agar (Haughn and Somerville, 1996) in sealed Petri dishes in the presence of 20 µg/ml of hygromycin. The seeds were germinated under 16 h light (100 µmol m⁻² s⁻¹)/8 h dark cycle at 25°C. Two weeks after germination, seedlings showing resistance to hygromycin were transplanted to soil (T₁ generation). The seeds derived from plants transformed with the pFGC5941 constructs (under the 35S CaMV promoter) were sown directly on soil. Two weeks after germination, the seedlings were sprayed with 0.03% Basta (phosphinotricin). The transgenic plants were allowed to set dry mature seed and the T₂ generation was collected. This procedure was repeated for transgenic lines containing either the pER8 or pFGC5941 vectors until the lines were homozygous in the T₃ generation.

3.2.14. Initial characterisation of transgenic lines

Levels of ectopic expression of Xhdsi-1^{VOC} and At1g07645 were determined in four-week-old transgenic plants from the T₃ generation by western blot analysis. Total protein extracted from leaf tissue was used for western blot analysis. Prior to harvesting leaves from transgenic lines expressing Xhdsi-1^{VOC} and At1g07645 under the inducible promoter, the transgenic plants were sprayed with 25 µM β-estradiol containing 0.05% silwet every 24 hours for fourteen days.

The levels of *At1g07645* in the T₃ generation of *A. thaliana* transgenic plants expressing RNAi constructs, pDSi-1^{VOC}-RNAi-1 or pDSi-1^{VOC}-RNAi-2 and *At1g07645* T-DNA knockout plants was investigated by quantitative PCR and/or western blot analysis. Transgenic plants containing the RNAi construct under the inducible promoter (pDSi-1^{VOC}-RNAi-2) were sprayed with 25 µM β-estradiol containing 0.05% silwet (VAC-IN-STUFF, USA) every 24 hours. After fourteen days of spraying, green siliques were harvested and RNA and protein extracted. In addition, protein was extracted from mature dry seeds.

3.3 Results

3.3.1. Determining optimal dilution of Dsi-1^{VOC} anti-sera

Optimal dilution of the Dsi-1^{VOC} anti-sera (described in Chapter 2) was determined by dot blot analysis (Fig. 3.10) prior to screening *A. thaliana* transgenic lines. Although endogenous Xhdsi-1^{VOC} was easily detected with the lowest dilution (1/3000) of primary and secondary antibodies, a dilution of 1:500 (primary antibody) and 1:3000 (secondary antibody) show the strongest detection of endogenous At1g07645 protein extracted from mature dry seed (Fig 3.10A).

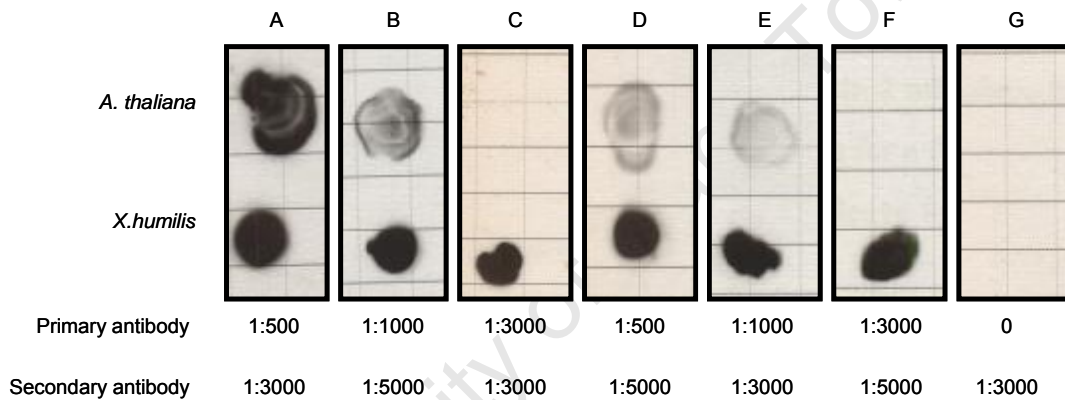


Figure 10. 5 µg of total protein from *A. thaliana* and *X. humilis* dry mature seed was dotted on 7 strips of nitrocellulose membrane (A-G). Membranes were incubated with various concentrations of the primary and secondary antibodies as indicated. There was no detection in both the *A. thaliana* and *X. humilis* total protein in the strip not incubated with the primary antibody (G).

3.3.2. Identification of two T-DNA *At1g07645* homozygous knockout line

Insertional T-DNA mutagenesis was one of the methods used in this study to investigate the loss-of-function phenotype of the *Xhdsi-1^{VOC}* orthologue, *At1g07645* in *A. thaliana*. The advantage of using T-DNA mutants is that homozygous mutants result in complete gene/protein knockouts. Segregating T₃ seed of two T-DNA lines with T-DNA insertions disrupting the *At1g07645* gene (NASC ID, N549529 and N597861) were obtained from the Salk Institute Genomic Analysis Laboratory. The disruption was confirmed by PCR by using a T-DNA specific primer (Lba1) and *At1g07645* specific primers, either *Atg22* (for N549529 plants) or *Atg24* (for N597861 plants) (Table 3.1; Fig. 3.4). Two plants from N549529 contained a T-DNA insertion within the *At1g07645* gene (Fig. 3.11A) and twelve plants from the N597861 line (Fig. 3.11B). In addition, gene specific primers were used to determine which plants were heterozygous for the insertion and which were homozygous. The gene specific primers, *At22* and *Atg23* primers (Fig. 3.4) were used for the N549529 line and *Atg24* and *Atg25* primers (Fig. 3.4) for the N597861 line. The homozygous plants identified were: 1, 11 (Fig. 3.11C) from the N549529 line and 12 (Fig. 3.11D) from the N597861 line. T₄ seeds from plants 1 and 12 were selected for western blot analysis. Western blot analysis showed that *At1g07645* protein was undetectable in T-DNA homozygous plants 1 and 12 (Fig. 3.12) confirming the homozygous nature of the T-DNA insertion. Both lines 1 and 12 were selected for further phenotypic analysis.

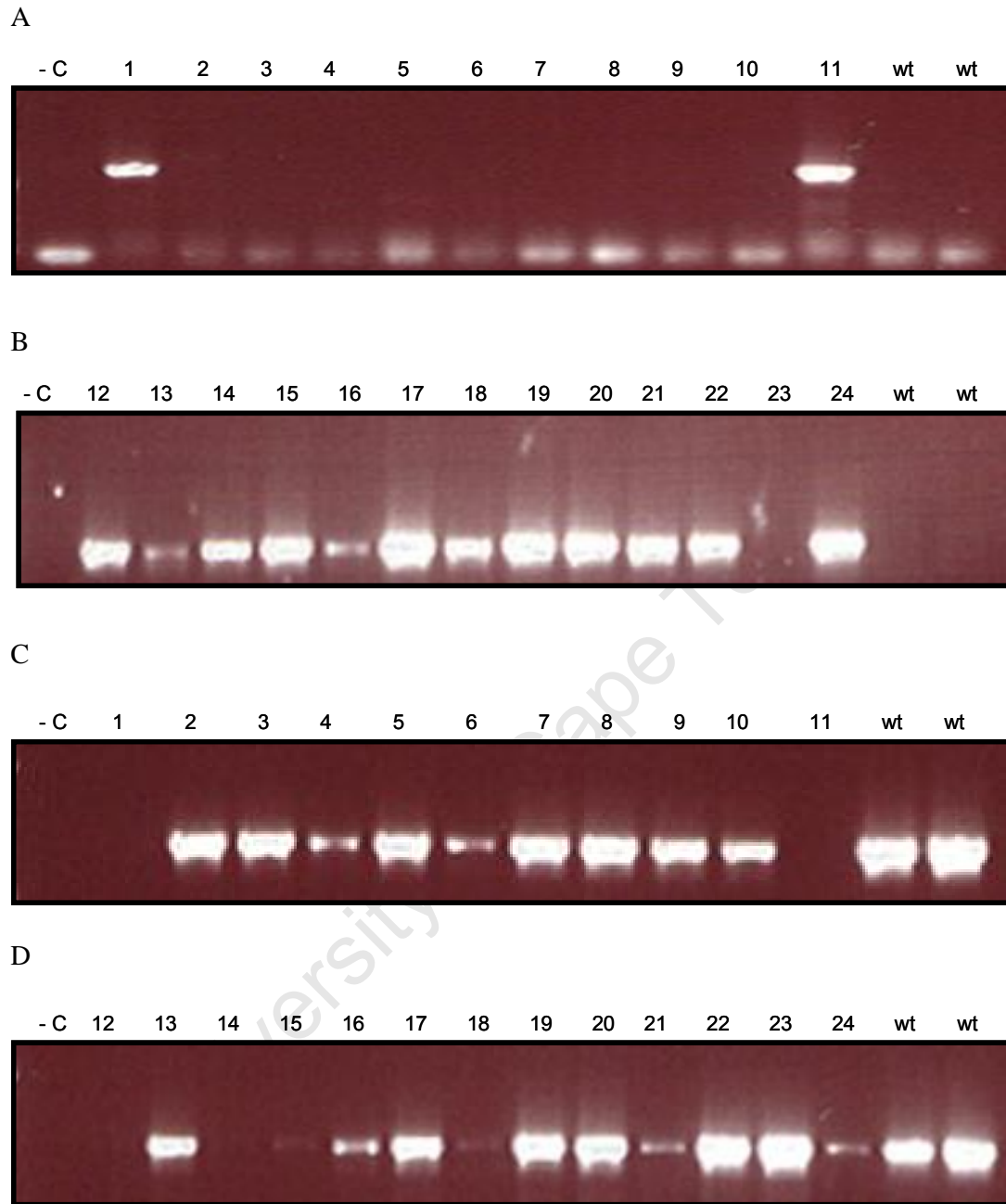


Figure 3.11. The correct insertion of the T-DNA insertion in the *AtIg07645* knockout lines N549529 (A) and N597861 (B) was confirmed by PCR using forward *AtIg07645* specific primers Atg22 and Atg24, respectively, and a T-DNA specific reverse primer (Lba 1) (Table 3.1; Fig. 3.4) on genomic DNA extracted from original T₃ *AtIg07645* knockout lines obtained from NASC. Homozygous *AtIg07645* T-DNA disrupted lines were identified by PCR using Atg22 and Atg23 primers (Fig. 3.4) for the N549529 line (C) and Atg24 and Atg25 primers (Fig. 3.4) for the N597861 line (D). Lane -C contains the negative control, which is no template added to the PCR reaction. The lane labelled wt contains genomic DNA from wild type *A. thaliana* and the other lines represent individual knockout lines.

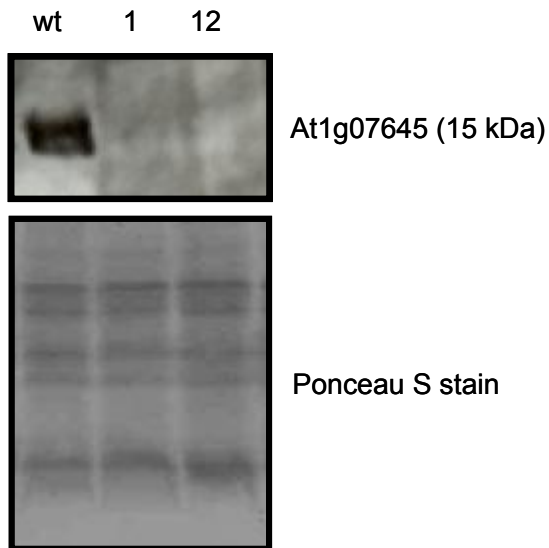


Figure 3.12. Western blot of total protein extracted from dry seeds of wild type (wt) or homozygous plants from line N549529 (1) or N597861 (12), probed with anti-Dsi-1^{VOC} antibody (upper panel). The lower panel shows total protein stained by Ponceau S staining of the membrane.

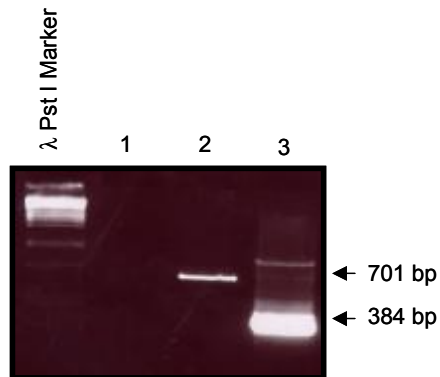
3.3.3. Generation of RNAi construct for silencing At1g07645 in *A. thaliana*

Post-transcriptional silencing by RNA interference (RNAi) was used as a complementary approach to T-DNA At1g07645 knockout mutants for the investigation of the loss-of-function phenotype. The aim was to generate RNAi constructs under a constitutive promoter and an inducible promoter.

3.3.3.1 Cloning of the inverted repeat construct into pFGC5941

A 384 bp fragment *At1g07645* was amplified from *A. thaliana* seed cDNA with engineered restriction enzymes for cloning into the pFGC5941 vector in the sense and antisense orientation (Fig. 3.13). The fragment cloned into the sense orientation into pFGC5941 was amplified using Pfgf1 and Rfgr1 primers (Fig. 3.6). The fragment for cloning into pFGC5941 was amplified using Pfgf2b and Rfgr2 primers (Fig. 3.6).

A



B

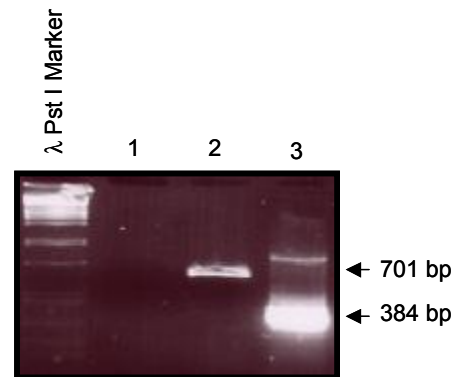
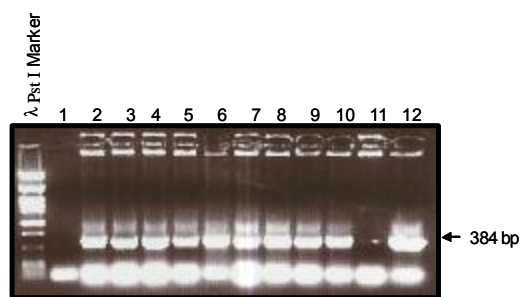


Figure 3.13. Amplification of 384 bp PCR products with different restriction enzymes for cloning into pFGC5941 in the sense-orientation (A) and the antisense-orientation (B) which produces an inverted repeat sequence. Lane 1 negative control with no template added to the PCR reaction. Lane 2 contains the 701 bp product from *A. thaliana* genomic DNA (a positive control). Lane 3 contains the 384 bp PCR product amplified from *A. thaliana* seed cDNA. DNA marker used is lambda DNA digested with the *Pst*I restriction enzyme.

Initially, the At1g07645 PCR product was cloned into the sense orientation in the pFGC5941 and gene specific primers Pfgf1 and Rfgr1 (Table 3.1) were used to confirm the successful cloning (Fig. 3.14A). From the 10 clones checked for the integration of the sense fragment, only one did not contain the fragment. Clone 8 was chosen for further use (Fig. 3.14A). Primers that flank the inverted repeat sequence, pFGC2F and pFGC-R (Table 3.1) were used to screen for the correct insertion of the second 384 bp PCR fragment. Eight plasmids were screened and two contained the predicted 2270 bp fragment (fig. 3.14B). Sequencing results of the two plasmids (pDsi-1-RNA-1) confirmed that the 384 bp fragments from At1g07645 were inserted in the opposite orientation in the pFGC5941 vector.

A



B

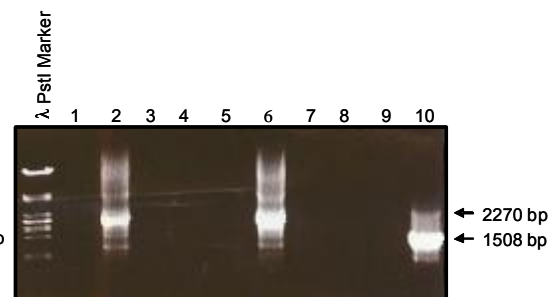


Figure 3.14. Screening of pFGC5941 transformants to check for correct insertion of the 384 bp PCR product fragment of *At1g07645* in the sense and antisense orientations for creating an inverted repeat. A. Screening for 384 bp PCR products in the sense-orientation with Pfgf1 and Rfgr1 primers. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-11, contain the 384 bp PCR product amplified from pFGC5941 vector. Lane 12 contains a 384 bp PCR product amplified from *A. thaliana* seed cDNA (positive control). B. Screening for a 384 bp PCR products in the antisense-orientation to create a 2270 bp inverted repeat. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-9 contain the 2270 bp PCR product that represents the inverted repeat (fragments ligated into the sense and antisense-orientation). Lane 10 contains an amplified PCR product from parental vector lacking the inverted repeat sequence (negative control). DNA marker used is lambda DNA digested with the *Pst* I restriction enzyme.

3.3.3.2. Identifying transgenic lines containing pDsi-1 RNAi-1

A. thaliana flower heads were infected with *A. tumefaciens* containing pDsi-1 RNAi-1 from lane 6 (Fig. 3.14). Twenty-five of the T₁ generation plants transformed with pDsi-RNA-1 showed tolerance when treated with the herbicide Basta. These transgenic plants were independently transformed lines. These twenty-five independent lines were screened to check for the stable integration of pDsi-RNAi-1 using primers specific to the *At1g07645* sequence fragment and a primer specific to the pFGC5941 spacer, named Rfgr1 and CHSAI primers (Fig. 3.6). Of the 25 plants screened, 17 contained the integrated RNAi construct targeting *At1g07645* in its nuclear DNA (Fig. 3.15). Fourteen independent lines were randomly selected for further analysis, and were grown to the T₃ generation to obtain homozygous lines before being screened for the degree of knockdown of *At1g07645* by the pDsi-1 RNAi-1 construct.

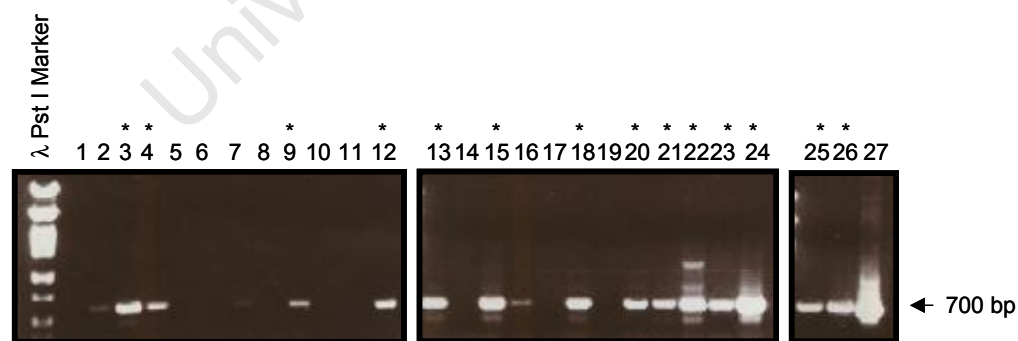


Figure 3.15. Screening of T₁ plants for the successful integration of pDSi-1RNA-1 using a primer specific to pFGC5941 and a gene specific primer. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-26 represent PCR products amplified from genomic DNA extracted from 25 independent transgenic plants. Lane 27 contains a PCR product from a positive control, pDsi-RNAi-1. The DNA marker is lambda DNA (λ DNA) digested with the *Pst* I restriction enzyme. Asterisks indicate lines selected for further studies.

Western blot analysis confirmed that the At1g07645 protein was expressed at high levels in *A. thaliana* mature dry seed and that expression of At1g07645 was absent in *A. thaliana* leaves (Fig. 3.16). The T₃ generation of the different transgenic lines transformed with pDSi-1RNAi-1 showed varying degrees of reduction of the At1g07645 protein compared to wild type. The At1g07645 protein was lowest in lines 2, 3, 8 and 13. The At1g07645 protein levels were not altered in lines 6, 7 and 14. The lines that showed reduction of At1g07645 protein (2, 3, 4, 5, 10, 11, 13) were selected for quantitative real-time PCR.

As expected, mRNA transcription abundance was suppressed in all lines tested, with lines 2, 3, 5 and 13 showing the greatest amount of suppression (Fig. 3.17). The relative quantity (fold change) of At1g07645 transcript in the transgenic lines ranged from 1.6 - 0.36 compared to the wild type value of 2.8. Lines 2, 5 and 13 (termed 35S 2, 35S 5 and 35S 13, respectively) were selected for phenotypic studies.

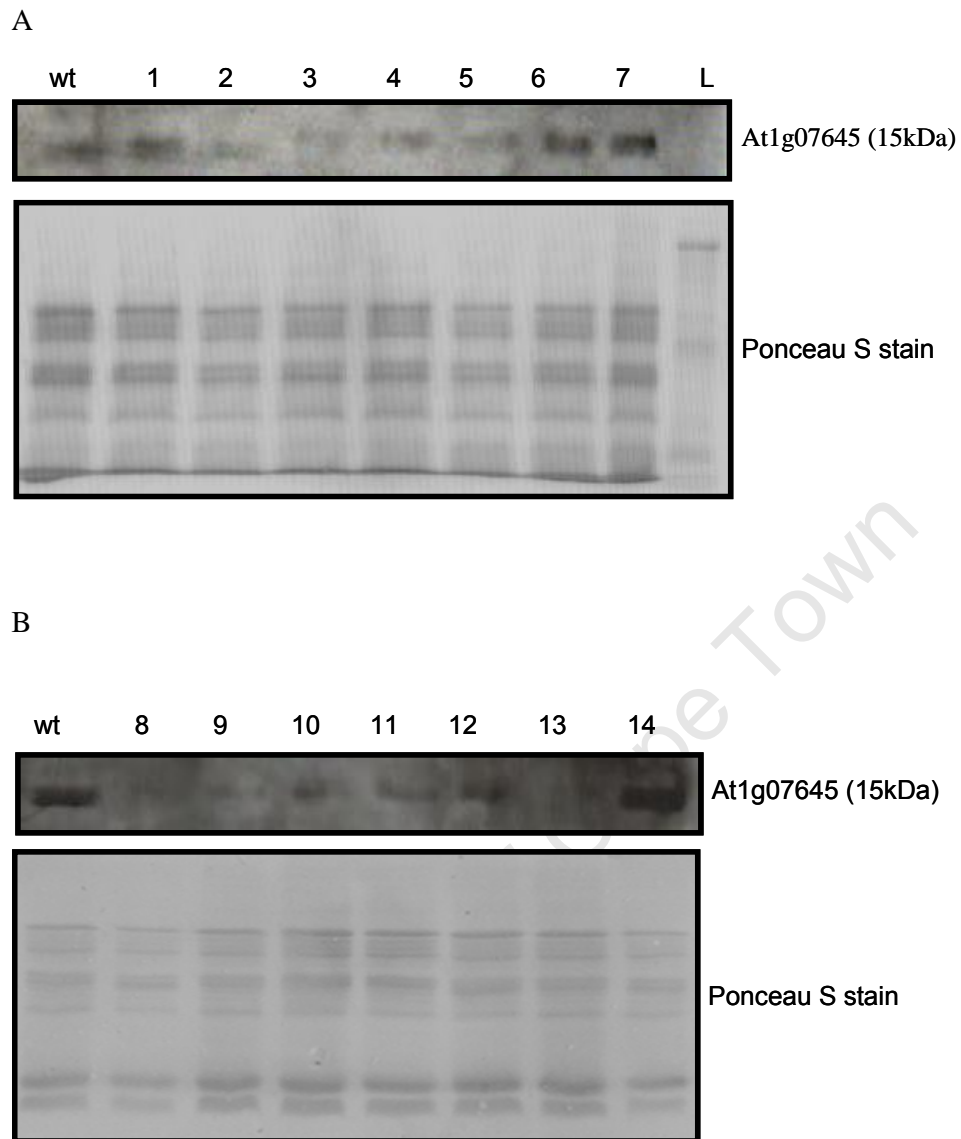


Figure 3.16. Western blot analysis of total protein extracted from mature dry seed of 14 T_3 homozygous lines transformed with pDSi-1^{VOC}-RNAi-1 (lanes 1-14). The lane labelled wt contains total protein extracted from seeds of wild type *A. thaliana* plants and the lane labelled L contains total protein extracted from wildtype *A. thaliana* leaves. The lower panels show the nitrocellulose membranes stained with ponceau S.

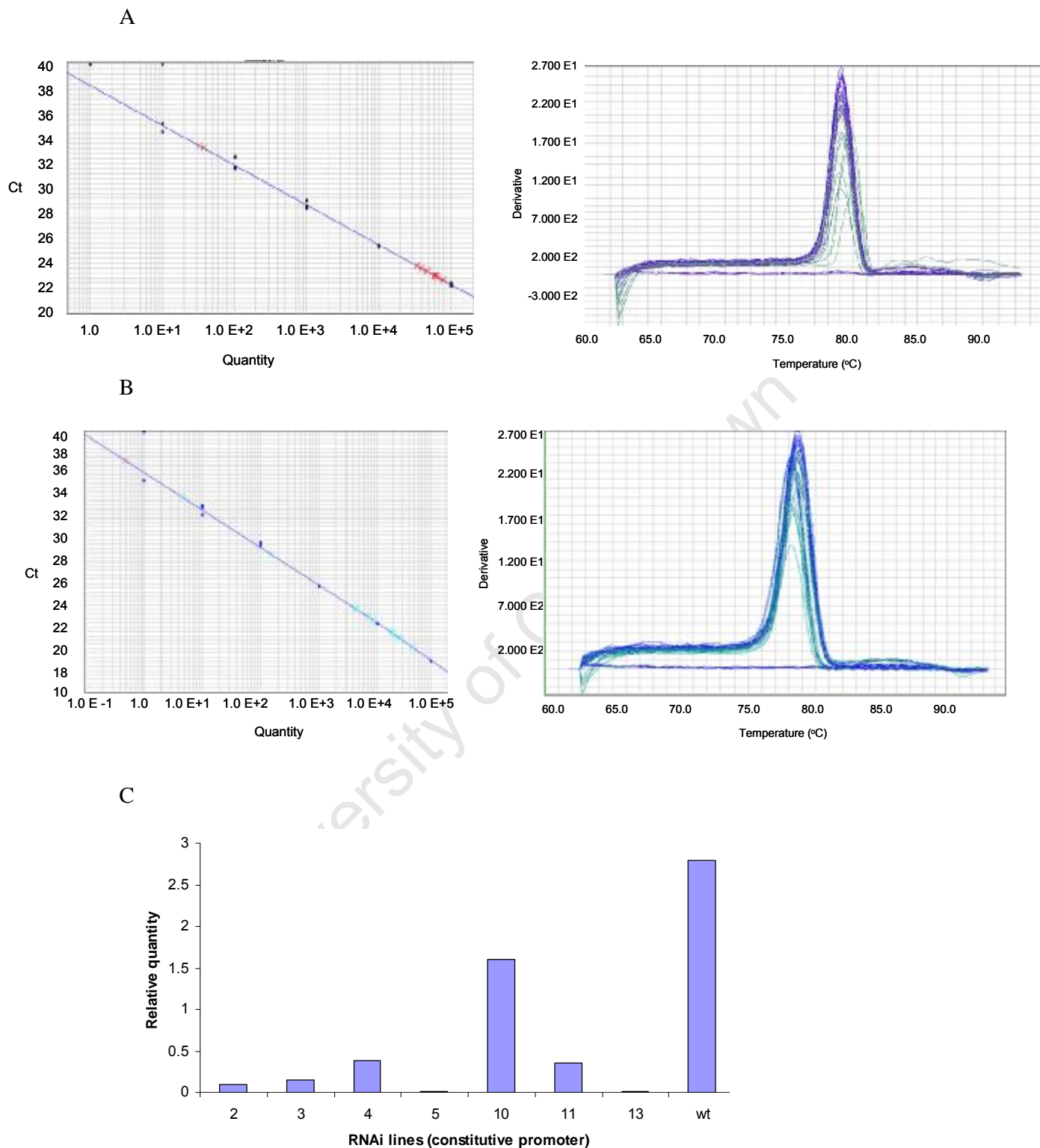


Figure 3.17. Quantification of At1g07645 mRNA transcript abundance from mature dry seeds of seven T_3 homozygous lines expressing pDSi-1^{VOC}-RNAi-1. A and B represent the standard and dissociation curves of F-actin and At1g07645, respectively. F-actin was used as the housekeeping gene. C. Represents the relative quantity (At1g07645 / F-actin). Lanes, 2,3,4,5,10,11 and 13 are lines that showed reduced At1g07645 protein levels (Fig. 3.16). The lane labelled wt contains total protein extracted wildtype *A. thaliana* seeds.

3.3.3.3. Cloning of the inverted repeat construct into pER8 vector

In the instance where the disruption of the *At1g07645* by T-DNA knockouts and posttranscriptional silencing by constitutively expressing the *pDSi-1^{VOC}-RNAi-1* (section 3.3.3.1.) would have resulted in a lethal phenotype, an RNAi construct against *At1g07645* under an inducible promoter was constructed by using the pER8 vector (Fig. 3.5B). The pER8 vector contains a chimeric XVE construct activated by β -estradiol (Zuo *et al.*, 2000).

A 342 bp PCR product from *At1g07645* was amplified from *A. thaliana* seed cDNA using 2HANBF and 3HANBR with (*Bam*HI: *Xho*I) and (*Kpn*I: *Hind*III) engineered restriction sites, respectively, for cloning into pKANNIBAL vector (Table 3.1; Fig. 3.18). The fragment digested with *Xho*I and *Kpn*I was ligated into the sense orientation in the first multiple cloning site of pKANNIBAL (Fig. 3.7).

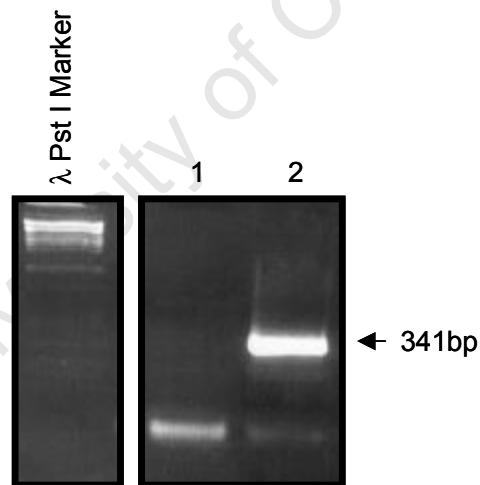


Figure 3.18. PCR amplification of a 341 bp fragment with *Bam*HI: *Xho*I and *Kpn*I: *Hind*III restriction enzyme sites on the 5' end and 3' end of the sequence, respectively for cloning into pKANNIBAL. Lane 1 contains the negative control with no template added to the PCR reaction. Lane 2 contains the 341 bp PCR product amplified from *A. thaliana* seed cDNA. The molecular weight marker is lambda DNA digested with the Pst I restriction enzyme.

Gene specific primers were used to confirm the successful cloning of the At1g07645 fragment (Fig. 3.19A). All five vectors checked contained the sequence fragment ligated into the sense orientation. The clone in lane 2 was chosen for the subcloning of the 342 bp PCR fragment into the antisense orientation into the *HindIII* and *BamHI* cloning site. Recombinant clones were screened using PDK1 and 2HANBF primers (Fig. 3.7). A 515 bp PCR product was amplified in thirteen of the fourteen vectors screened (Fig. 3.19B). The inverted repeat construct from the pKANNIBAL vector was subsequently subcloned into the *StuI* and *SpeI* of the pER8 vector (Fig. 3.7). The successful integration of the construct into pER8 (pDSi-1^{VOC}-RNAi-2) was checked by the amplification of the fragment cloned into the antisense-orientation using PDK and pER8R primers (Fig. 3.7). The expected size of 515 bp was successfully amplified in all of the eleven transformants screened (Fig. 3.19C). Wild type *A. thaliana* plants were transformed with the pER8 vector containing the hairpin construct (lane 2).

3.3.3.4. The isolation of six independent lines transformed with pDSi-RNAi-2

Ten plants from T₁ generation of plants transformed with pDsi-1RNA-2 construct showed tolerance to hygromycin B. Genomic DNA was extracted from the ten T₁ plants and checked for stable integration of pDsi-RNAi-2 using primers specific to At1g07645 and a primer specific to the spacer, 2HANBF and PDK 1 (Fig. 3.7). Of the ten plants screened, six contained the integrated inverted repeat sequence targeting At1g07645 in its nuclear DNA (Fig. 19D). These six positive transgenic plants are recognized as independently transformed lines and all six were selected for further analysis.

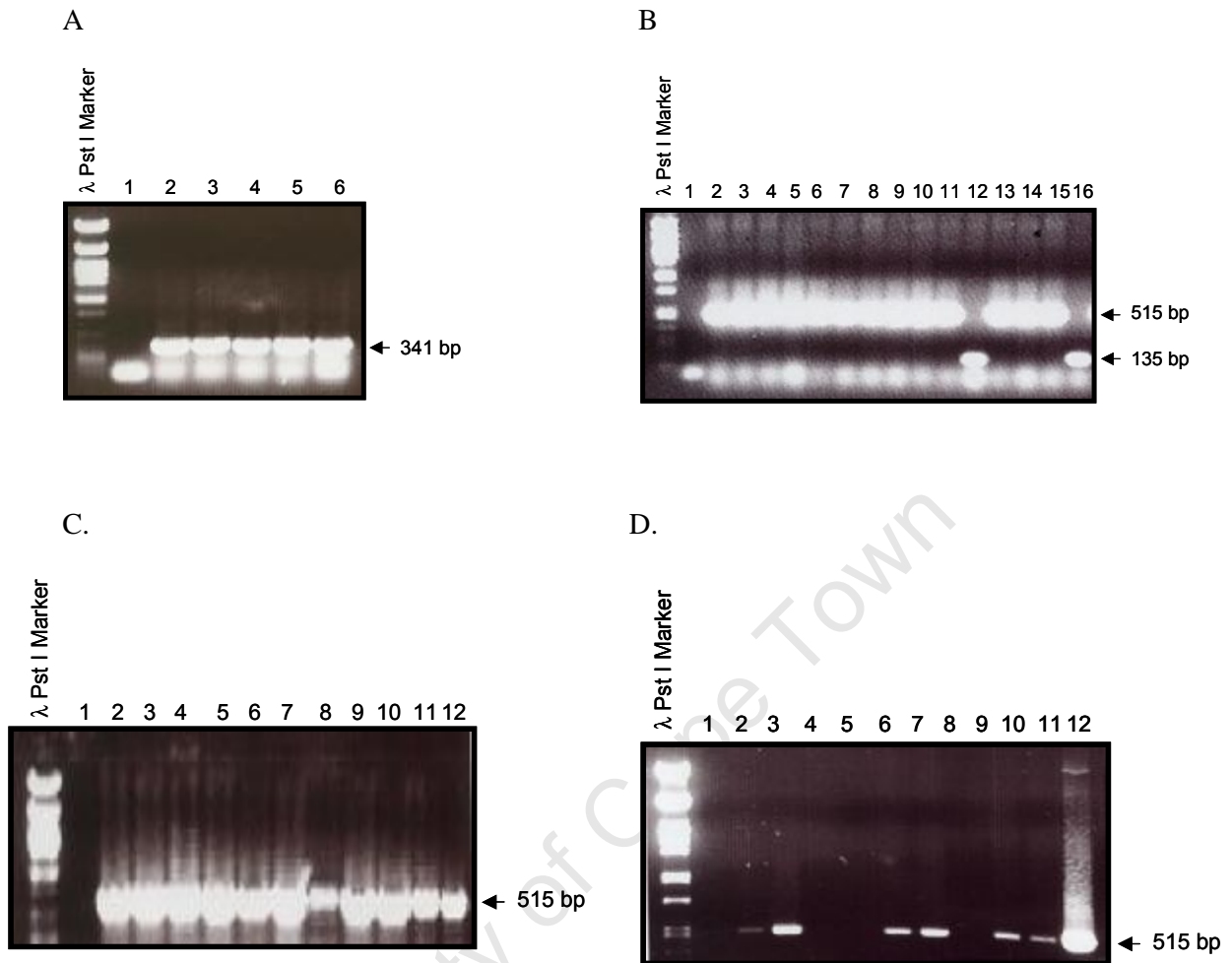
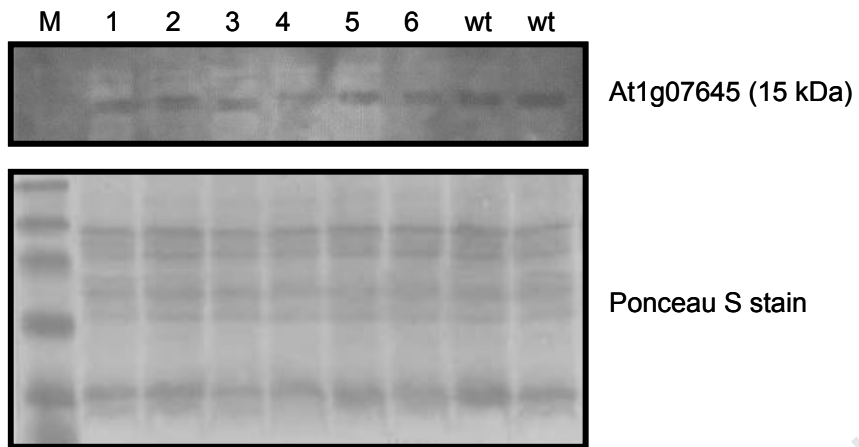


Figure 3.19. A. Confirmation of the cloning of a 341 bp PCR product from *At1g07645* ligated into the sense orientation in the pKANNIBAL vector. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-6 contain a 341 bp PCR product amplified from the pKANNIBAL vector. B. A 515 bp PCR product confirming the successful cloning of the antisense orientation fragment into pKANNIBAL. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-15 contain a 515 bp PCR product amplified from the pKANNIBAL vector. Lane 16 represents a PCR product amplified from the parental vector (negative control). C. Confirmation of the inverted repeat sequence subcloned into pER8 vector. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-12 contain a 515 bp PCR product amplified from the pER8 vector (pDSi-1^{VOC}-RNAi-2). D. Screening of T₁ plants for the successful integration of pDSi-1^{VOC}-RNAi-2 using a primer specific to the spacer and a gene specific primer. Lane 1 contains the negative control with no template added to the PCR reaction. Lanes 2-11 contain PCR products amplified from genomic DNA extracted from ten independently transformed transgenic plants. Lane 12 contains a PCR product from a positive control, pDSi-1^{VOC}-RNAi-2 construct. The DNA marker is lambda DNA digested with the Pst I restriction enzyme.

At1g07645 protein is normally expressed in seed of *A. thaliana*, therefore in order to determine whether the expression of pDSi-1^{VOC}-RNAi-2 construct would induce At1g07645 silencing, the expression of the pDSi-1^{VOC}-RNAi-2 construct was induced during seed development. Flowering *A. thaliana* plants containing the pDSi-1^{VOC}-RNAi-2 construct were sprayed with 25 µM of estradiol every 24 h, the spraying of the plants continued until siliques turned brown. *A. thaliana* plants sprayed with estradiol dried approximately 3 weeks later than the control plants (data not shown). Wild type plants were also sprayed with 25 µM of estradiol. In addition, control plants (wild type and transgenic plants containing the pDSi-1 RNA 2 construct) were sprayed with water. The results suggest that estradiol causes a delay in *A. thaliana* seed maturation and senescence. Silencing of At1g07645 in transgenic *A. thaliana* lines transformed with inducible pDSi-1^{VOC}-RNAi-2 was not as effective as the constitutively expressed construct pDSi-1 RNAi-1. Western blot analysis of the total protein extracted from green siliques (Fig. 3.20A) and mature dry seed (Fig. 3.20B) showed a slight down-regulation of the At1g07645 protein compared to wild type seed. Real-time PCR was used to quantify the effects of pDSi-RNAi-2 on *Atg107645* mRNA abundance extracted from *A. thaliana* developing green siliques. As expected, *At1g07645* mRNA transcript levels were reduced (Fig. 3.21), but not as effectively as the lines constitutively expressing pDSi-1 RNAi-1.

A.



B.

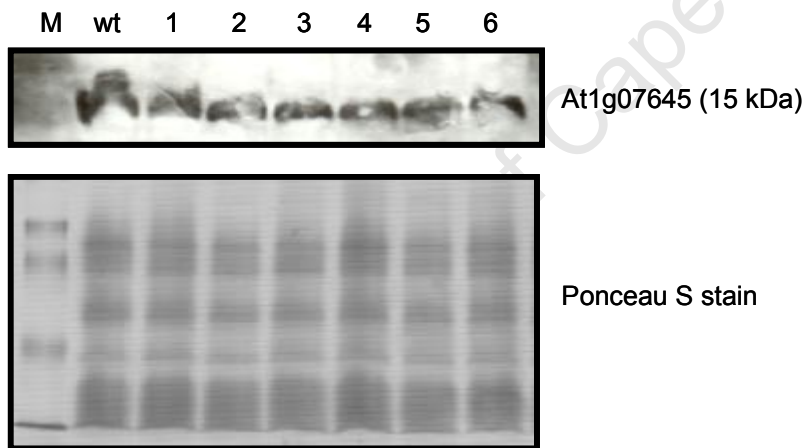


Figure 3.20. Western blot analysis of total protein extracted from developing green siliques (A) and mature dry seeds (B) of six T_3 homozygous lines transformed with pDsi-RNAi-2 (lanes 1- 6). The lane labelled wt contains total protein extracted from developing green siliques of *A. thaliana* plants. The lower panels show the nitrocellulose membranes stained with ponceau S.

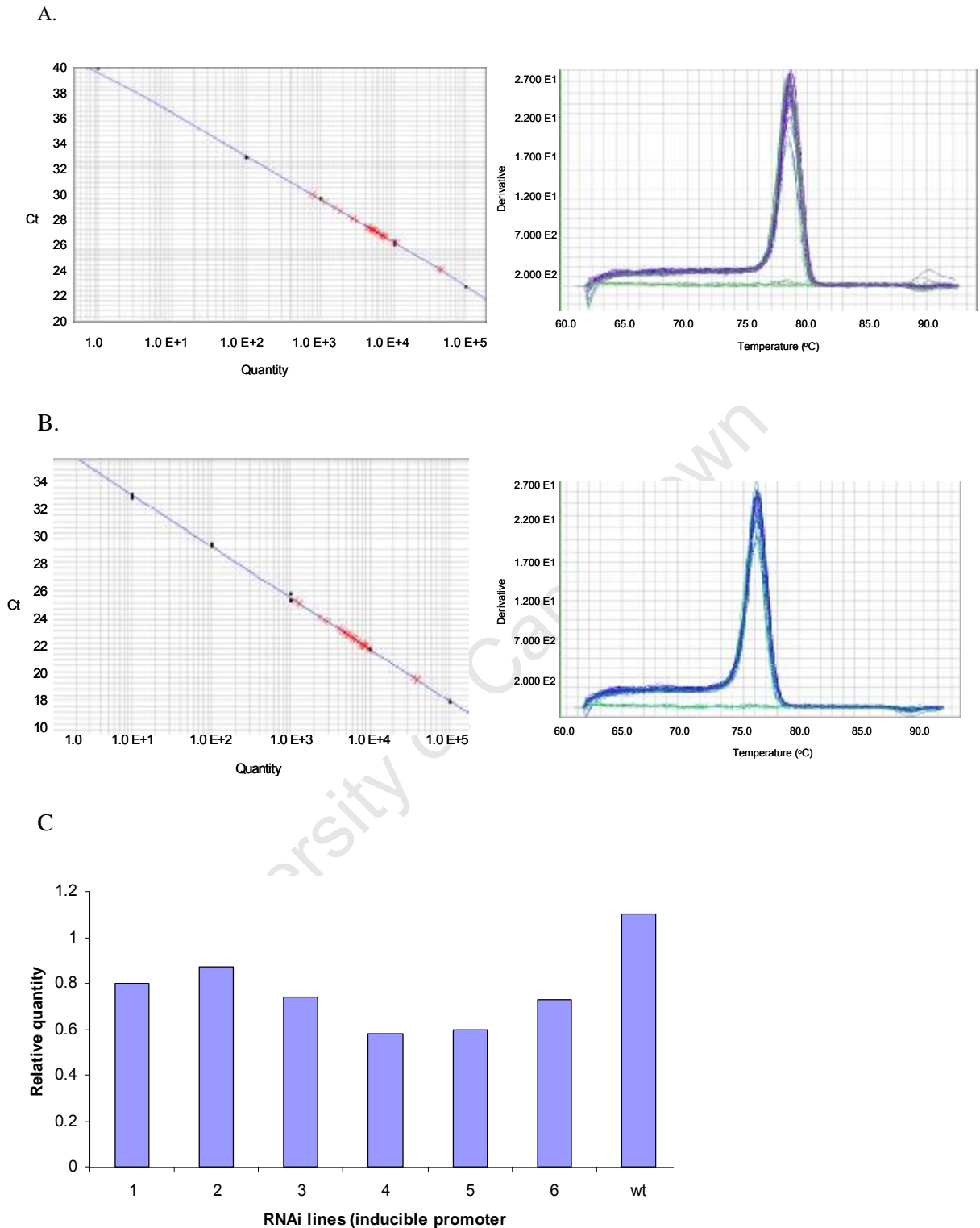


Figure 3.21. Quantification of *At1g07645* mRNA transcript abundance from developing green siliques of six T_3 homozygous lines expressing pDSi-1 RNA2. A and B represent the standard and dissociation curves of *F-actin* and *At1g07645*, respectively. *F-actin* was used as the housekeeping gene. C. Represents the relative quantity (*At1g07645* / *F-actin*). Lanes, 1 to 6 represent the six T_3 homozygous lines transformed with pDsi-RNAi-2. The lane labelled wt contains total RNA extracted from wildtype *A. thaliana* seeds.

3.3.4. Investigating the gain-of- function phenotype

The gain-of-function phenotype by ectopically expressing *Xhdsi-1^{VOC}* and *At1g07645* in the vegetative tissues of *A. thaliana* was the third method used to determine the function of the two orthologues. This section reports the cloning of the coding regions of *Xhdsi-1^{VOC}* and *At1g07645* into the two binary vectors, pFGC5941 and pER8 used for the construction of RNA constructs and the transformation of the constructs into *A. thaliana*.

3.3.4.1. Cloning the coding region of *Xhdsi-1^{VOC}* and *At1g07645* into pFGC5941 and pER8

The open reading frames of *Xhdsi-1^{VOC}* and *At1g07645* were amplified from *X. humilis* and *A. thaliana* seed cDNA with engineered restriction cloning sites (Fig. 3.8, Fig. 3.9; Fig. 3.22). A 528 bp and a 543 bp product corresponding to the coding regions of *At1g07645* and *Xhdsi-1^{VOC}*, respectively, were amplified for ligation into the pFGC5941 vector (Fig. 3.22). The 580 bp and 530 bp products corresponding to the coding region of *At1g07645* and *Xhdsi-1^{VOC}*, respectively, were amplified for ligation into the pER8 vector (Fig. 3.22).

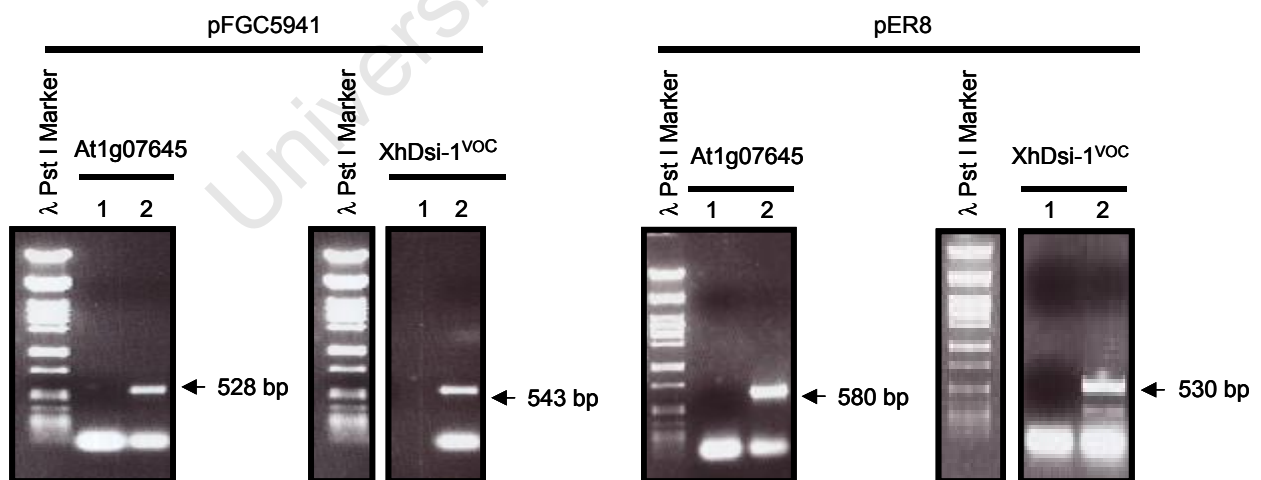


Figure 3.22. *At1g07645* and *Xhdsi-1^{VOC}* PCR products amplified with primers containing engineered restriction sites for cloning into pFGC5941 and pER8. Lane 1 is the negative control with no template added to PCR reaction. Lane 2 contains the amplified products: the 528 bp *At1g07645* product and the 543 bp *Xhdsi-1^{VOC}* product for cloning into pFGC5941 and the 580 bp *At1g07645* product and 530 bp *Xhdsi-1^{VOC}* product for cloning into pER8 vector. DNA marker used is lambda DNA (λ digested with the *Pst*I restriction enzyme).

Nine colonies were identified by PCR amplification with Xperpfgf and XpFOR primers (Fig. 3.8A) as containing the *Xhdsi-1^{VOC}* insert in pFGC5941 (Fig. 3.23A). Using the primers Aperpfgf and ApOR (Fig. 3.8B), the presence of *Xhdsi-1^{VOC}* was confirmed in 6 of the 14 pER8 colonies screened (Fig. 3.23B). Fourteen colonies were screened for the presence of the *At1g07645* insert into pFGC5941 by using Aperpfgf and ApFOR primers (Fig. 3.9A); five pFGC5941 plasmids contained the *At1g07645* coding sequence (Fig. 3.23C). Using Aperpfgf and ApOR primers (Fig. 3.9B), ten colonies screened contained the *At1g07645* insert in the pER8 vector (Fig. 3.23D). Two pFGC5941 vectors containing *Xhdsi-1^{VOC}* (35S::*Xhdsi-1^{VOC}*) and *At1g07645* (35S::*At1g07645*) and two pER8 vectors containing *Xhdsi-1^{VOC}* (XVE::*Xhdsi-1^{VOC}*) and *At1g07645* (XVE::*At1g07645*) were sent for sequencing and a blastx search in NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) showed no mutations within the sequences (data not shown).

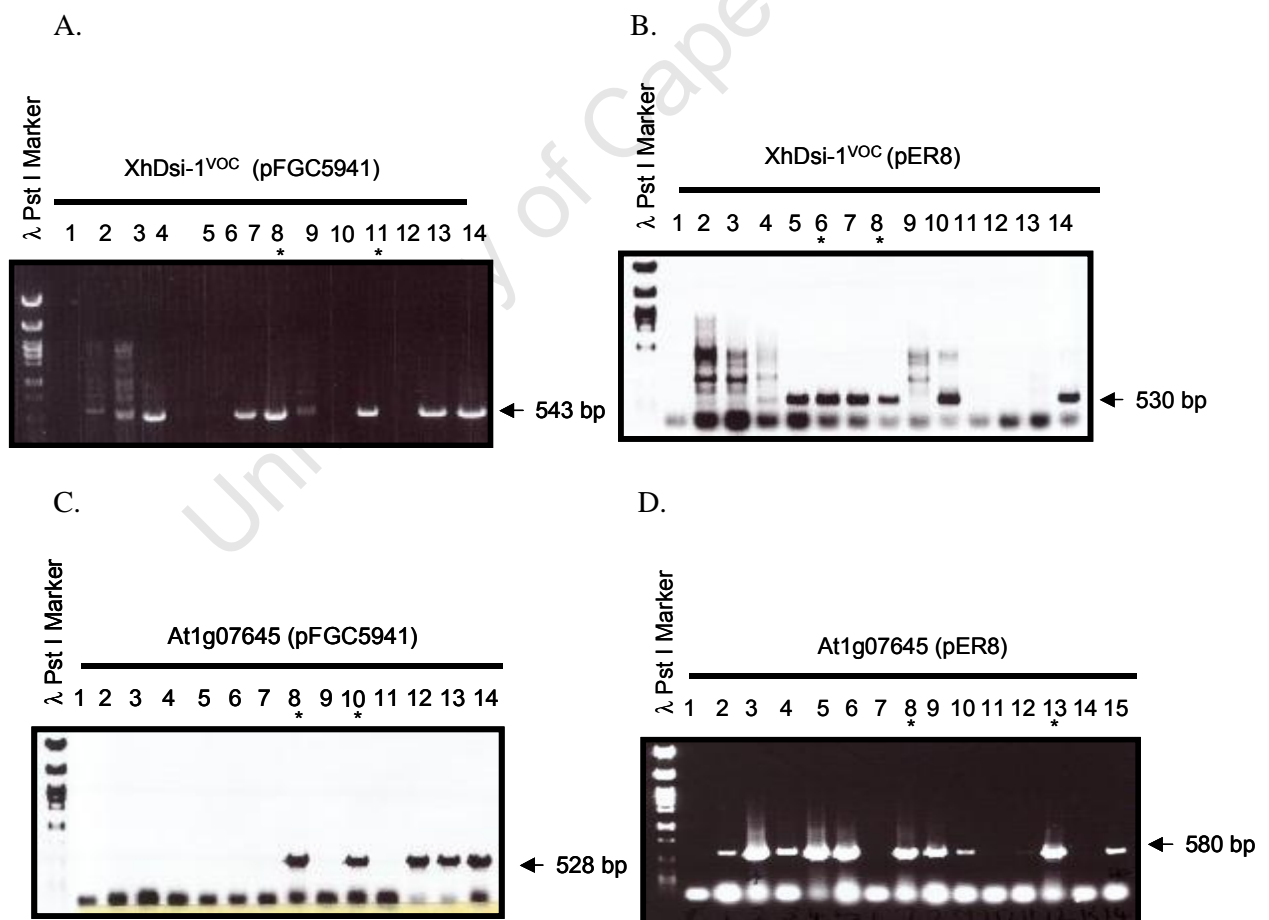


Figure 3.23. The confirmation of *Xhdsi-1^{VOC}* ligated into pFGC5941 (A) and pER8 (B) and *At1g07645* ligated into pFGC5941 (C) and pER8 (D) by PCR amplification. Lane 1; negative control with no template added to PCR reaction. Lanes 2 – 14/15 shows the amplified products. DNA marker used is lambda DNA (λ DNA) digested with the Pst I restriction enzyme. Asterisks indicate plasmids sent for sequencing.

3.3.4.2. Generation of transgenic lines expressing Xhdsi-1^{VOC} and At1g07645

A. thaliana flower heads were infected with *A. tumefaciens* containing either 35S::*Xhdsi-1*^{VOC}, 35S::*At1g07645*, XVE::*Xhdsi-1*^{VOC} or XVE::*At1g07645*. Ten (T₁) independent lines were identified from T₀ seeds transformed with 35S::*Xhdsi-1*^{VOC} and sixteen (T₁) lines containing 35S::*At1g07645*, respectively (Table 3.4). Six (T₁) independent lines contained XVE::*Xhdsi-1*^{VOC} and seven (T₁) lines containing the XVE::*At1g07645* construct (Table 3.4). All the lines were grown to the T₃ generation before and several lines indicated in Table 3.4 were selected for the characterisation of levels of ectopic expression of Xhdsi-1^{VOC} and At1g07645.

Table 3.4. Transgenic lines generated

Transgenic <i>A. thaliana</i> lines			
35S:: <i>Xhdsi-1</i> ^{VOC}	35S:: <i>At1g07645</i>	XVE:: <i>Xhdsi-1</i> ^{VOC}	XVE:: <i>At1g07645</i>
XhA1	AtA1*	XV1*	AV1*
XhA2	AtA2*	XV2*	AV2*
XhA3*	AtA3*	XV3*	AV3*
XhB4	AtB4*	XV4*	AV4*
XhB5*	AtB5*	XV5*	AV5*
XhB7*	AtB6	XV6	AV6*
XhC8	AtB7		AV7
XhC9*	AtB8		
XhC10*	AtC9*		
	AtC10		
	AtC11		
	AtC12		
	AtC13		
	AtC14		
	AtC15		
	AtC16*		

* transgenic plants selected for further analysis

Western blot analysis was performed on the T₃ homozygous transgenic plants to determine expression of Xhdsi-1^{VOC} and At1g07645 in the transgenic *A. thaliana* plants. Ectopic expression of Xhdsi-1^{VOC} in leaves of four transgenic lines (XhA3, XhB5, XhC9 and XhC10) transformed with the 35S::Xhdsi-1^{VOC} plasmid was clearly visible by western blot analysis (Fig. 3.24A). Transgenic line XhA3 had the lowest detectable expression level and expression of Xhdsi-1^{VOC} was not detected in line XhC7. All seven transgenic plants transformed with the 35S::At1g07645 construct ectopically expressed the At1g07645 protein in leaves (Fig. 3.24B). Upon visual inspection of the results, the levels of expression between the independent lines differ. There was no expression of either Xhdsi-1^{VOC} or At1g07645 protein transformed with the XVE::At1g07645 or XVE::Xhdsi-1^{VOC} constructs (Fig. 3.24C and Fig. 3.24D).

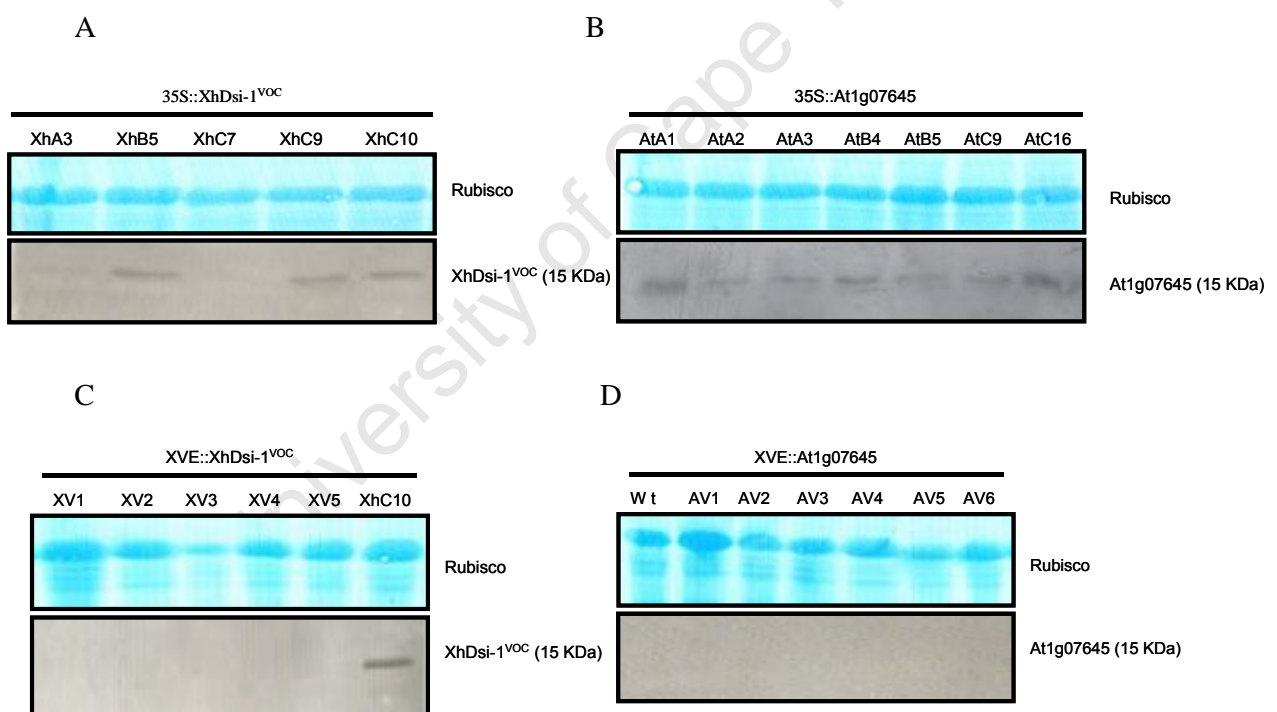


Fig. 3.24. Western blot analysis of total protein extracted from leaf tissue of different *A. thaliana* transgenic lines transformed with either 35S::Xhdsi-1^{VOC} (A), 35S::At1g07645 (B), XVE::Xhdsi-1^{VOC} (C) or XVE::At1g07645 (D). The upper panels show nitrocellulose membrane stained with MemCode™ Reverse protein stain kit (Pierce, USA). The lower panel shows the western blot result following the probing of the membranes with anti-dsi-1^{VOC} antibodies.

3.4. Discussion

The ability to introduce genes into plants for overexpression and to create mutants has made it possible to investigate the phenotype of genes with unknown function (Birch, 1997). Because methods of transformation have not yet been developed for *X. humilis* plants, the desiccation-sensitive model plant *A. thaliana* was chosen for our phenotypic experiments. The *Xhdsi-1^{VOC}* orthologue, *At1g07645*, is expressed in seeds of *A. thaliana*, and therefore, by creating *At1g07645*-deficient seeds, the phenotype of *At1g07645* can be investigated. Moreover, the results described in Chapter 2 showed that *At1g07645* is not expressed in the vegetative tissues of *A. thaliana*. Therefore, the ectopic expression of *Xhdsi-1^{VOC}* and *At1g07645* in *A. thaliana* vegetative tissues would enable us to determine whether these orthologues can confer tolerance to abiotic stress in a desiccation sensitive plant. This chapter outlines the successful generation of *At1g07645* T-DNA knockout mutants. This chapter also reports the successful generation of *A. thaliana* transgenic plants overexpressing *Xhdsi-1^{VOC}* or *At1g07645*, as well as plants in which RNAi knockdown endogenous expression of *At1g07645*. Both the transgenic plants overexpressing the two orthologues and the RNAi constructs are either under a constitutive or an inducible promoter.

To induce post-transcriptional silencing against *At1g07645*, pFGC5941 and pKANNIBAL vectors (Helliwell and Waterhouse, 2003) were used to design a hairpin RNAi construct. The RNA hairpin construct has been reported to be the most effective post-transcriptional silencing method in plants (Waterhouse *et al.*, 2003; Helliwell and Waterhouse, 2003; Wesley *et al.*, 2001). The RNAi hairpin consists of two inverted sequences, which are separated by a spacer to aide successful directional cloning (Waterhouse *et al.*, 2003; Wesley *et al.*, 2001). As pKANNIBAL is not a binary vector, the construct was subcloned into the pER8 binary vector (Zuo *et al.*, 2000) under a promoter induced by 17- β estradiol. The *At1g07645* fragment size for RNAi construction was chosen following recommendations reported in the literature. Tuschl and colleagues (1999) have shown that a 49 bp dsRNA and a 149 bp dsRNA had no *in vitro* silencing effect on two luciferase genes *Renilla reniformis* (sea pansy) and *Photinus pyralis* (firefly), whereas 505 bp and 997 bp dsRNA fragments had a significant silencing effect. Helliwell and Waterhouse (2003) propose that the RNAi target sequence of a gene should be between 300 and 600 bp to obtain optimum

silencing. The *At1g07645* fragments cloned into pFGC5941 and pER8 were 384 bp and 341 bp in size, respectively.

In transgenic lines constitutively expressing the RNAi construct (pDSi-1^{VOC}-RNAi-1), the decrease in transcript levels correlates with the decrease in protein levels (Fig. 3.16; Fig. 3.17C). Transcript and protein levels ranged in these lines from a 2-fold decrease to almost complete silencing of the target gene. A similar distribution of silencing in different transgenic lines has been reported (Helliwell and Waterhouse, 2003). The difference in gene silencing of the different independent lines is due to several allelic variances which result in silencing ranging from partial knockdown of the transcripts to almost complete silencing (Small, 2007). The variation in gene silencing leads to various phenotypes of different severity. The varying levels of gene silencing are useful in instances where the complete knockout of the gene of interest would result in a lethal phenotype or affect the normal development of the transgenic plant. However, one of the disadvantages of RNAi mediated silencing is that complete silencing of a gene is difficult to obtain. Furthermore, to prevent cross silencing, it is important for the fragment to have very low sequence similarity to other known genes. For example, Miki *et al.*, (2005) have shown that in rice a 470 bp fragment designed for RNAi construction against a highly conserved sequence of an OsRac gene family was able to silence multiple genes in this gene family. A blast search of the 384 bp and 341 bp fragments against the NCBI database showed that these regions did not have high sequence similarity to other genes in the *A. thaliana* genome (data not shown). Thus, there is a high possibility that the RNAi silencing of *At1g07645* is specific.

Unlike the transgenic plants constitutively expressing the RNAi construct, transgenic lines containing the RNAi construct driven by the inducible promoter (pDSi-1^{VOC}-RNAi-2) showed a marginal decrease in transcript abundance (Fig 3.21C). These results are mirrored by the detection of a marginal decrease in protein levels when the expression of the RNAi construct is activated in green siliques (Fig 3.20A). Therefore, silencing using the construct under the inducible promoter was not as effective as in those lines constitutively expressing the RNAi construct (pDSi-1^{VOC}-RNAi-1). In dry mature seeds containing the induced RNAi construct, there was no decrease of protein observed when compared to the wild type (fig. 3.20B). There are

a number of reasons why the induction of pDSi-1^{VOC}-RNAi-2 does not efficiently result in the silencing of *At1g07645*. It is well documented that gene silencing is acquired when the transcription of the RNAi inverted repeat construct reach a critical threshold (Schubert *et al.*, 2004). The threshold levels vary between different genes. Thus it is possible that the induced (pDSi-1^{VOC}-RNAi-2) construct may not have been expressed as highly as the constitutively expressed RNAi construct (pDSi-1^{VOC}-RNAi-1), therefore, threshold levels were not obtained for *At1g07645* silencing to occur. The alternative explanation would be that since the time point at which the *At1g07645* transcripts start to accumulate is not known, it is possible that transcript are present in the developing wild type flower head. As a result, *At1g07645* protein might already be present before spraying the developing siliques with estradiol to activate the transcription of pDSi-1^{VOC}-RNAi-2. Furthermore, the spraying of the estradiol inducer must be stopped during the late stages of seed maturation to allow the seeds to dry. As a result, the RNAi construct becomes inactivated and *At1g07645* mRNA is transcribed and translated to protein.

Due to the reasons given above, XVE inducible promoter was not a suitable choice for silencing *At1g07645* in developing seeds. Alternative promoters that could have been used include a Cre/lox P (CLX) promoter, which is activated by 17- β estradiol (Guo *et al.*, 2003). Cre/lox P (CLX) is an inducible promoter, however, once induced, its expression becomes constitutive. The advantage of using this promoter is that once activated, it would remain active when seeds are allowed to dry and acquire dormancy. Alternatively, a seed-specific promoter such as the USP from *Vicia faba L* could be useful (Ziakharov *et al.*, 2004). Induced RNAi-mediated silencing was generated as an alternative to investigate the loss-of-function phenotype if the constitutive RNA-mediated silencing or the T-DNA knockouts resulted in a lethal phenotype. However, the constitutive expression of pDSi-1^{VOC}-RNAi-1 successfully knocked down *At1g07645* protein levels and complete knockdown was obtained in T-DNA *At1g07645* knockout mutants. In addition, the constitutive knockdown and knockout of *At1g07645* did not result in lethal phenotype. Therefore the transgenic plants expressing the pDSi-1^{VOC}-RNAi-1 and *At1g07645* T-DNA were selected for further analysis (chapter 4).

In addition to investigating the loss-of-function phenotype, *Xhdsi-1^{VOC}* and *At1g07645* were ectopically expressed in *A. thaliana* vegetative tissues. The expression of the two orthologues was either under a constitutive promoter or an inducible promoter. In the transgenic *A. thaliana* plants protein expression was detected in leaf tissue of both the 35S::*Xhdsi-1^{VOC}* and 35S::*At1g07645* constitutive promoter constructs (Fig 3.24). The transgenic plants used for further phenotypic analysis (chapter 4). Unfortunately, no protein was detected in the leaf tissue of the transgenic plants that contained the XVE::*Xhdsi-1^{VOC}* and XVE::*At1g07645* inducible promoter constructs (Fig 3.24). The promoter might have not been strong enough to induce detectable levels of the two orthologues.

This chapter reports the successful generation of transgenic lines with altered levels of *At1g07645* protein and ectopically expressing the *Xhdsi-1^{VOC}* orthologue. The loss-of-function and the gain-of-function are useful strategies to investigate the function proteins of unknown function.

Chapter 4

Phenotypic function of At1g07645 and Xhdsi-1^{VOC} in transgenic *A. thaliana* plants

4.1 Introduction

The term abiotic stress refers to various environmental stress conditions such as drought and salt (NaCl) that affect plant growth and viability (Mitler, 2006). Drought and high NaCl causes osmotic stress in plants due to a decrease in the surrounding water content (Mitler, 2006; Wang *et al.*, 2003; Walters *et al.*, 2002). In plant cells, water is required for optimal metabolism and maintains the integrity of biochemical function (Alpert, 2006; Hoekstra *et al.*, 2001). Thus loss of water within cells disrupts metabolic pathways, such as photosynthesis, and the optimal function of proteins and enzymes, such as antioxidant enzymes (Mitler, 2006; Nocter and Foyer, 1998). Osmotic stress causes the closure of stomata to reduce further water loss via transpiration (Chaves and Oliveira, 2004). As a consequence, CO₂ uptake is blocked, which results in the inhibition of photosynthesis. Photosynthetic inhibition during osmotic stress is also due to the reduced efficiency of components of the photosynthetic metabolic pathway. These include proteins such as ribulose 1, 5-bisphosphate carboxylase/ oxygenase (Rubisco) and components of the electron transport chain.

During photosynthesis, chloroplasts produce a substantial amount of reactive oxygen species (ROS) in plants (Navrot *et al.*, 2007; Moller, 2001; Nocter and Foyer, 1998). The increased production of ROS is a secondary effect of osmotic stress and leads to irreversible damage of cellular components such as proteins and membranes (Wang *et al.*, 2003; Walters *et al.*, 2002). Under normal conditions, ROS are detoxified by antioxidants. Upon osmotic stress, as mentioned above, the function of the photosynthetic apparatus is impaired. This results in increased ROS production, which is exacerbated by antioxidant enzymes that are unable to function optimally

(Botella *et al.*, 2005; Cohen *et al.*, 2006; Moller, 2001). As a result, the plants are unable to detoxify the high concentrations of ROS produced. For example, high concentrations of ROS cause damage to lipid membranes by oxidation of the polyunsaturated fatty acids (lipid peroxidation), forming malondialdehydes (Hodges *et al.*, 1999).

In order to avoid the detrimental effects caused by osmotic stress, plants upregulate the expression of regulatory genes, such as transcription factors, which in turn activate the transcription of functional genes that directly protect the plants against damage caused by osmotic stress (Bhatnager-Mathar *et al.*, 2008; Umezawa *et al.*, 2006). For example functional genes such as antioxidants, LEA proteins and osmoprotectants, as well as genes encoding proteins of yet unknown function are upregulated during osmotic stress (Bhatnager-Mathar *et al.*, 2008; Cuming *et al.*, 2007; Umezawa *et al.*, 2006; Rabbani *et al.*, 2003; Ozturk *et al.*, 2002; Seki *et al.*, 2002). These transcription factors and functional genes upregulated during water loss have been identified in both desiccation sensitive and tolerant plants which include, *X. humilis* and orthodox seeds. Similar genes have been shown to be upregulated during water loss between desiccation sensitive and tolerant plants (Bartels *et al.*, 2007). However, upon severe water loss, where desiccation sensitive plants die, desiccation tolerant plants up regulate unique genes that contribute to the protection of cells against severe water loss (Bhatnager-Mathur *et al.*, 2008). For example, unlike resurrection plants, desiccation sensitive plants do not have the ability of upregulate genes that encode enzymes involved in the synthesis of osmoprotectants such as trehalose raffinose, stachyose and verbascos (Bhatnager-Mathur *et al.*, 2008; Peters *et al.*, 2007). Similarities in mechanisms involved in desiccation tolerance between resurrection plants and orthodox seeds that are also desiccation tolerant have been reported (Oliver, 2007; Illing *et al.*, 2005; Oliver *et al.*, 2000). However, resurrection plants activate protective mechanisms in response to environmental cues when exposed to periods of severe water loss, desiccation tolerance in orthodox seeds is a pre-programmed mechanism that occurs during the late maturation stages of seed development (Kermode and Finch-Savage, 2002; Vertucci and Farrant, 1995).

To help elucidate the role of dehydration-induced genes in response to water loss, the gain-of-function phenotype (by overexpression) and the loss-of function phenotype

(gene silencing or by mutagenesis) of many of these genes have been investigated in model plants such as rice, tobacco and *A. thaliana* (Bhatnager-Mathur *et al.*, 2008; Witcombe *et al.*, 2008). Improved tolerance to abiotic stress in transgenic plants overexpressing regulatory genes, osmoprotectants, antioxidants and proteins of unknown function has been reported. (Umezawa *et al.*, 2006; Rabbani *et al.*, 2003)

Regulatory genes upregulated during osmotic stress have the ability to activate expression of genes that are important in the protection of plants against the damage caused by water loss. Transgenic tobacco constitutively overexpressing a seed-specific transcription factor, HaHSFA9 (*Helianthus annuus* heat-stress factor A9), in vegetative tissues showed increased tolerance to severe dehydration (Prieto-Dapena *et al.*, 2008). HaHSFA9 activates the transcription of heat shock proteins (HSPs) that protect proteins during desiccation and help the refolding of proteins into their native conformation during rehydration in desiccation tolerant seeds (Prieto-Dapena *et al.*, 2008). Another transcription factor, the MYB10 transcription factor (CpMYB10), isolated from a desiccation tolerant plant *C. plantagineum* ectopically expressed in *A. thaliana* increased the tolerance of these plants to salt and sorbitol stress (Umezawa *et al.*, 2006). A regulatory 14-3-3 protein (TFT7) from tomato, which binds several transcription factors involved in the stress response was constitutively overexpressed in *A. thaliana* exposed to high salinity stress (Xu and Shi, 2007). The transgenic lines had a higher tolerance to salt relative to the wildtype. Interestingly, transgenic plants expressing both OsNAC6, a member of the rice NAC transcription factor family, and CARAVI from pepper conferred tolerance to dehydration and high salinity, as well as biotic stress in transgenic rice and *A. thaliana* transgenic plants, respectively (Nakashima *et al.*, 2007; Sohn *et al.*, 2006). The fact that expression of OsNAC6 and CARAVI confer tolerance to rice and *A. thaliana* transgenic plants, respectively suggest that plants have common mechanisms in response to both biotic and abiotic stresses (Nakashima *et al.*, 2007; Sohn *et al.*, 2006).

Various osmoprotectants have been reported to be involved in the reduction of mechanical damage and the protection of macromolecules in desiccation tolerant plants (Alpert, 2006; Hoekstra *et al.*, 2001). UDP-D-galactose is a substrate for the synthesis of various carbohydrates. Overexpression of the rice UDP-glucose 4-epimerase, which is involved in the synthesis of UDP-D-galactose, increased the

tolerance of *A. thaliana* transgenic plants to abiotic stress by increasing the levels of raffinose (Liu *et al.*, 2007). In addition, transgenic plants overexpressing mannitol-1-phosphate dehydrogenase accumulated mannitol and showed an increased tolerance to osmotic stress (Abebe *et al.*, 2003). The upregulation of the sugars such as raffinose and mannitol are believed to be involved in the protection of cellular components in some desiccation tolerant species by preventing disruption of membranes, denaturation of proteins, and interaction of molecules in the cytoplasm during water loss (Alpert, 2006; Hoekstra *et al.*, 2001). Indeed, the overexpression of both raffinose and mannitol conferred tolerance to transgenic plants.

Other types of functional genes upregulated during water loss are the genes that encode LEA proteins (Alpert, 2006; Buitink and Leprince, 2004; Collett *et al.*, 2004). LEA proteins are believed to prevent (*inter alia*) the denaturation of proteins by functioning as chaperones, replacing the lost water and sequestering ions (Alpert, 2006; Buitink and Leprince, 2004). Overexpression of the *Triticum durum* DHN-5 LEA 2 conferred tolerance to transgenic *A. thaliana* plants during NaCl and water deficit stress (Brini *et al.*, 2007). The CASAR82A gene of unknown function from pepper has been shown to increase resistance of transgenic lines exposed to salt, dehydration and *B. cinera* stresses.

Several non-enzymatic and enzymatic antioxidants that detoxify high levels of ROS during water loss have been extensively researched (Ramanjulu and Bartels, 2002; Nocter and Foyer, 1998). For example transgenic tobacco overexpressing homogentisate phytyltransferase and homogentisate geranylgeranyl transferase genes, which are involved in the synthesis of the non-enzymatic antioxidant α -tocopherol, showed low levels of oxidative damage in the presence of abiotic stress compared to wildtype (Abbasai *et al.*, 2007). In addition, plants deficient in α -tocopherol and ascorbate showed increased susceptibility when exposed to osmotic stress (Abbasai *et al.*, 2007; Huang *et al.*, 2005). Enzymatic antioxidants such as the aldole/aldehyde reductase gene from alfalfa overexpressed in tobacco have been shown to reduce the levels of lipid peroxidation during abiotic stress, possibly by detoxification of the aldehydes, 4-hydroxynon-2-enal and methylglyoxal (Oberschall *et al.*, 2000). In addition, overexpression of other enzymes that are believed to detoxify aldehydes, such as the aldehyde dehydrogenase genes ALDH3I1 and ALDH3I1 from *A. thaliana*,

conferred tolerance to *A. thaliana* transgenic plants in the presence of drought and high salinity stress when overexpressed in transgenic plants (Kotchoni *et al.*, 2006).

The results above report improved tolerance to abiotic stress in transgenic plants overexpressing regulatory genes, osmoprotectants and antioxidants (Umezawa *et al.*, 2006). Therefore the generation of transgenic plants to investigate the role of genes upregulated during water loss is a useful strategy (Birch, 1997). However, it is critical that accurate ways of assessing water deficit tolerance in transgenic plants are utilised (Birch, 1997)

Several parameters are used to assess how transgenic plants cope in the absence and presence of osmotic stress. Water loss inhibits growth and causes death of drought sensitive plants (Barnabas *et al.*, 2008). Therefore, changes in growth, along with the rates of photosynthesis and lipid peroxidation are routinely used as indicators of stress in plants (Brini *et al.*, 2007; Xu and Shi, 2007; Prieto-Dapena *et al.*, 2008). The photosynthetic light absorbing pigments found in chloroplasts are chlorophyll A, B and carotenoids (Lichtenthaler, 1987). In the absence of stress, chlorophyll A is the most abundant of the photosynthetic pigments; chlorophyll B and carotenoids are known as accessory pigments. The damage to the photosynthetic apparatus during osmotic stress results in the reduction of photosynthetic pigments and maximum photosynthetic efficiency (Chaerle *et al.*, 2007). Transgenic plants expressing genes that confer tolerance during osmotic stress would have a higher level of photosynthetic pigments and higher photosynthetic efficiency relative to wildtype plants. Furthermore, the levels of damage to lipid membranes by ROS are quantified by measuring the amount of malondialdehydes present, as a result of lipid peroxidation (Hodges *et al.*, 1999).

The aim of this study is to investigate the role of *Xhdsi-1^{voc}* and *At1g07645* during water loss. In the desiccation tolerant plants *X. humilis*, *XhDsi-1^{VOC}* transcripts and protein levels are upregulated in both roots and leaves in response to desiccation (reported in chapter 2). *XhDsi-1^{VOC}* transcripts are also present in dry mature seeds. The *A. thaliana* orthologue *At1g07645* transcripts are absent in the vegetative tissues but only present in its desiccation tolerant seeds (reported in chapter 2). The upregulation of *XhDsi-1^{VOC}* in *X. humilis* during desiccation and the expression of its

orthologue in *A. thaliana* seeds suggest that *XhDsi-1^{VOC}* may play a role in the acquisition of desiccation tolerance. However, it is not possible to alter levels of *XhDsi-1^{VOC}* in *X. humilis* because methods of transformation have not yet been developed for *X. humilis* plants. Therefore, in order to test the hypothesis that *Xhdsi-1^{VOC}* contributes to the protection of the plant during water loss, the desiccation-sensitive model plant *A. thaliana* was chosen for our phenotypic experiments.

This chapter reports strategies used to investigate the role of *Xhdsi-1^{VOC}* and its *A. thaliana* orthologue, *At1g07645*. The gain-of-function phenotype by constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* in the vegetative tissue of transgenic *A. thaliana* plants (*At1g07645* is not normally expressed in vegetative tissue of *A. thaliana*) in the absence and presence of water deficit. The phenotypic observations of transgenic plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* were performed on plants at germination, seedling and adult stages, treated with various osmotic stresses. In addition *A. thaliana* plants constitutively expressing the two orthologues were subjected to NaCl and biotic stress. There is increasing evidence that although plants upregulate different genes in response to different biotic and abiotic stresses there is a significant overlap of genes upregulated in response to different types of stresses (Fujita *et al.*, 2006). Therefore, the aim is to investigate whether the constitutive expression of *Xhdsi-1^{VOC}* and *At1g07645* confer tolerance to both abiotic and biotic stress. In addition, *Xhdsi-1^{VOC}* orthologue, *At1g07645*, is expressed in seeds of *A. thaliana*, and therefore, by creating *At1g07645*-deficient seeds, the phenotype of *At1g07645* can be investigated. *At1g07645* knockdown seeds constitutively expressing the pDsi-RNAi-1 and *At1g07645* T-DNA knockout mutant seeds were used to investigate the loss-of-function phenotype of *At1g07645* during seed development and germination.

4.2. Materials and methods

Three independently transformed lines (XhA3, XhC9 and XhC10) constitutively expressing *Xhdsi-1^{VOC}*, and three independently transformed lines (AtA2, AtB5 and AtC9) constitutively expressing *At1g07645* in *A. thaliana* vegetative tissue under the control of the CaMV 35S promoter were chosen for phenotypic analysis. Three independently transformed lines constitutively expressing the RNAi construct pDSi-RNAi-1 (35S 2, 35S 5 and 35S 13) that showed reduced *At1g07645* transcript and protein expression were selected for further loss-of-function phenotypic analysis. In addition, homozygous lines (TDNA1 and TDNA 12) selected from two independent T-DNA *At1g07645* knockout mutants showing the absence of the *At1g07645* protein in seeds were selected for further phenotypic experiments.

4.2.1. Growth conditions

Soil-grown *A. thaliana* were grown in 7cm x 7cm top x 7cm high pots in a glasshouse at Warwick HRI, University of Warwick in the United Kingdom. The soil contained a mixture of Scotts Levingtons F2s compost, silica sand and fine grade vermiculite (6: 1: 1) (Horticultural services, Warwick HRI, UK). The phenotypic experiments were performed under ambient conditions in the glasshouse with supplemented light from September 2007 to January 2008. *A. thaliana* seeds were germinated and grown on half-strength MS media (Sigma-Aldrich inc., St Louis USA) in sealed petri dishes under 16 h light (100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)/ 8 h dark at 22-25 °C.

4.2.2. Biomass and germination tests

Several assays were performed to determine the effects of constitutive expression of *Xhdsi-1^{VOC}* and *At1g07645* and either the knockdown of *At1g07645* or mutation of *At1g07645* under normal conditions and in the presence of increasing concentrations of NaCl and mannitol. Thirty sterilised and stratified seeds (as described in Chapter two) were sown on half strength MS media (Sigma-Aldrich inc., St Louis USA) supplemented with various concentrations of NaCl (0, 50, 100 and 200 mM) and mannitol (0, 100, 200, and 400 mM). Each experiment was performed in triplicate.

Wild type seed was the same age as the transgenic or mutant seeds and was germinated on the same plates as the transgenic lines in order to reduce variation.

Germination score was calculated as the percentage of seeds germinated per total number of seeds sown. Two weeks after germination, pictures were taken of the plates. The seedlings were harvested and the total fresh weight per plate was recorded. Wild type and transgenic seedlings either constitutively expressing *Xhdsi-1^{VOC}* or *At1g07645* were further separated into two batches and each batch was weighed again; these two batches were used to determine chlorophyll content and lipid peroxidation levels. The samples were labeled, frozen in liquid nitrogen and stored at -80°C .

4.2.3. Root length assays

Three seeds from wildtype and each transgenic line constitutively expressing either *Xhdsi-1^{VOC}* or *At1g07645* proteins were sown singly on a horizontal line on half-strength MS plates containing varying concentrations of NaCl and mannitol. As above, the plates were placed vertically in a growth room. Two weeks after germination, pictures were taken and the lengths of the roots were measured using Image J 1.36 downloaded from (<http://rsb.info.nih.gov/ij/download.html>).

4.2.4. Osmotic stress treatment on four -week -old plants grown on soil

Wild type and homozygous T₃ transgenic seeds constitutively expressing either *Xhdsi-1^{VOC}* or *At1g07645* were sown in 7cm x 7cm top x 7cm high pots containing the same amount of soil (approximately 95 g of soil). The soil contained a mixture of Scotts Levingtons F2s compost, silica sand and fine grade vermiculite (6: 1: 1) (Horticultural services, Warwick HRI, UK).

For each stress, three pots of wildtype and each transgenic line containing five plants were used for the experiment. Each pot containing five transgenic plants from each line was matched with a control pot of five wildtype plants (Fig. 4.1). These plants were grown in a greenhouse for four weeks. Four-week-old plants were subjected to salt stress by watering with 200 mM NaCl for four weeks and to mannitol stress by

watering with 400 mM mannitol for one week. Plants were placed on horizontal trays and watered from the bottom to ensure that all the plants were receiving equal amounts of the solutions. Subsequent to the stresses, the plants were allowed to recover by irrigation with water for four weeks. In addition, plants were subjected to drought stress by withholding water for 14 days prior to rehydrating the plants for four weeks. Control plants were not subjected to any of the stresses.

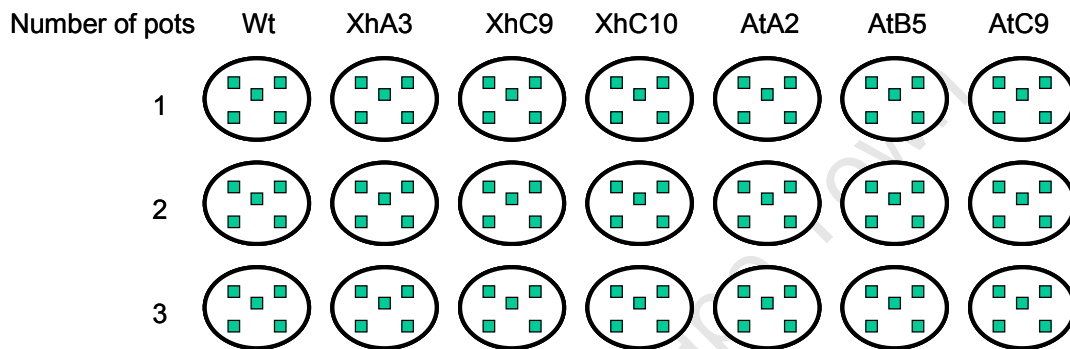


Figure 4.1. For each stress (NaCl, mannitol and dehydration), three pots, each containing five plants for wild type and each transgenic line constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) were used for the experiment. The solid back circle represents the pots and the green boxes within the circle represent the five plants.

Photographs of the four-week-old plants were taken before administering the stress, at the end of each stress and every week for two or four weeks after the plants were rescued by irrigation of water. Rosette leaves 7, 8, and 9 were tagged at four to five weeks after germination by marking the stems of the leaves with permanent markers containing three different colours. Each colour represented each rosette leaf number.

4.2.4.1. Harvesting leaf tissue for chlorophyll and lipid peroxidation assays

Rosette leaves eight and nine were excised from wild type or transgenic plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* in the absence of stress (four weeks after germination) and after application of either NaCl (after four weeks), mannitol (after one week) or dehydration (after 14 d). The samples were immediately weighed and placed in centrifuge tubes; the tubes were labeled, frozen in liquid

nitrogen and stored at -80°C for chlorophyll assays and lipid peroxidation assays, respectively. The samples were harvested in triplicate per line.

4.2.3.2 Relative water content

Rosette leaf number seven was removed from wild type and transgenic plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645*. Leaf seven was weighed prior to and after soaking in distilled water overnight at 4°C . The leaf was dried in a 70°C oven for 24 hours and weighed again. The equation to calculate relative water content was as follows: (fresh weight-dry weight)/(rehydrated weight-dry weight) (Brini *et al.*, 2007).

4.2.5. Quantification of chlorophyll, carotenoids and total soluble protein

Leaves from two-week-old seedlings or rosette leaf eight were homogenised in a centrifuge tube containing one 3 mm stainless steel ball-bearing (Qiagen GmbH, Germany) by a Mixmill tissuelyser (Qiagen, Germany). 500 μl of LE buffer containing 50 mM lithium phosphate pH 7.2, 1 mM monoiodoacetic acid, 120 mM mercaptoethanol, 5% (v/v) glycerol, 1 mM PMSF and 2% (w/v) SDS was added to the homogenised tissue and mixed by inverting the centrifuge tube several times. 100 μl of the extract was removed for chlorophyll A, chlorophyll B and carotenoid content analysis. The balance of the extract was boiled for 45 s. Soluble protein concentration was measured by the Bradford assay method using bovine serum albumin as a standard at 600 nm following manufacturer's specifications (Bio-Rad, Germany).

900 μl of 80% (v/v) acetone was added to the 100 μl samples of total extract in LE buffer. The samples were mixed and placed at -20°C for 1 h. The samples were centrifuged for 3 min at maximum speed on a benchtop centrifuge (9 000 g) and the supernatant was collected. The absorbance of the samples was measured at 663 nm, 646 nm and 470nm, for chlorophyll A (chl a), chlorophyll B (chl b) and carotenoids, respectively. 900 μl of 80% (v/v) acetone containing 100 μl LE buffer was used as a blank at all three wavelengths. The assay was performed in triplicate per line.

The following equations giving pigment concentration ($\mu\text{g}/100\mu\text{l}$) were used to calculate chl, chl b and carotenoids:

$$\text{Chl a} = (13.19 * \lambda_{663}) - (2.57 * \lambda_{646})$$

$$\text{Chl b} = (22.1 * \lambda_{646}) - (5.26 * \lambda_{663})$$

$$\text{Carotenoids} = (1000 * \lambda_{470} * \lambda_{663} - 104 * \lambda_{646}) / 229$$

chl a, chl b and carotenoids pigments were presented as concentration mg/total protein, the equations used were:

$$\text{Chl A} = (\text{Chl A } \mu\text{g} / 100\mu\text{l} * 5.5) / \text{total protein} / 1000$$

$$\text{Chl B} = (\text{Chl B } \mu\text{g} / 100\mu\text{l} * 5.5) / \text{total protein} / 1000$$

$$\text{Carotenoids} = (\text{Carotenoids } \mu\text{g} / 100\mu\text{l} * 5.5) / \text{total protein} / 1000$$

4.2.6. Fluorescence measurements

Photosynthetic efficiency of photosystem II (PSII) was measured with a FluorImager (Technologica Ltd, Colchester, UK). The FluorImager contains a 5 X GA + CCD progressive scan camera that captures images and data is analysed by FluoroImager two. Individual plants grown in pots were placed in the FluorImager, the plants were kept in the dark for 20 min before measurements were performed. The minimal fluorescence (F_o) was obtained by exposing the dark-adapted plants to a low beam of $0.8 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. Maximum fluorescence (F_m) was obtained by saturating the plant with a pulse of $6259 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 800 ms. The variable fluorescence (F_v) was obtained by subtracting the F_o value from the F_m value. The ratio F_v/F_m represents maximum photochemical efficiency of PSII (Barbagallo *et al.*, 2003; Baker *et al.*, 2001).

4.2.7. Quantification of lipid peroxidation and soluble protein

Leaves from two-week-old seedlings or rosette leaf eight were homogenised in a centrifuge tube containing one 3 mm stainless steel ball bearing (Qiagen GmbH, Germany) by a Mixmill tissuelyser (Qiagen GmbH, Germany). 200 μl of water was added to homogenised tissue. 50 μl of the samples were used for protein quantification as previously described. 650 μl of 2% (w/v) 2-thiobarbituric acid (TBA) in 20% (v/v) TCA was added to the remaining 150 μl sample. The samples were heated at 95°C for 30 minutes and immediately cooled on ice for 5 min. The samples were subjected to centrifugation at maximum speed in a benchtop centrifuge (8,000 g) for 10 min. Endogenous malondialdehyde (MDA) levels were assayed

spectrophotometrically, by measuring the supernatant at a specific absorbance of 532 nm and non-specific absorbance at 600 nm. The extraction coefficient used was 155 (nmol/l)⁻¹.cm⁻¹ (Shalata and Tal, 1998) and the following equation was used to calculate MDA levels: $MDA = ((\lambda_{532} - \lambda_{600}) / 155) * \text{volume of extract} / \text{total protein}$. The units were (nmol MDA)/(mg protein).

4.2.8. *Botrytis cinerea* inoculation

A necrotrophic fungal pathogen, *Botrytis cinerea* strain isolated from peppers was used for biotic stress experiments (Denby *et al.*, 2004). *B. cinerea* was grown on sugar-free sterile tinned apricot halves in sterile petri-dishes. The cultures are maintained by sub-culturing the spores onto fresh apricots every three weeks.

Fourteen-day spores were harvested by adding three ml of sterile water to the plate containing infected apricots. The spores were rubbed off the apricots and gently dispersed into the water with a sterile loop. The concentration of the spores was counted in a haemocytometer. Spores were diluted to 5 x 10⁴ of spores / ml in 50% grape juice for infection. Fourteen leaves from four-week-old wild type and from the six independent lines expressing *Xhdsi-1*^{VOC} and *At1g07645* were excised and placed into trays containing 0.8% (w/v) agar. A 10 µl drop of the spore suspension was inoculated on the surface of the leaves. Leaves were inoculated with 10 µl of 50% (v/v) grape juice as a negative control. The trays were placed in a growth cabinet (Sanyo Gallenkemp, Leicester, UK) and maintained at a constant temperature of 23°C, 90% humidity under fluorescent tungsten lamps with an irradiation of approximately 100 µE m⁻² S⁻¹ (16 hour light - 8 hour dark cycle). All the leaves were photographed at 24, 48 and 72 h post infection. The circumference of the developing lesions caused by *B. cinerea* was measured by Image J 1.36 (<http://rsb.info.nih.gov/ij/download.html>).

4.2.9. Seed development in T-DNA and RNAi lines (pDsi-RNAi-1)

Green and brown siliques from wild type, *At1g07645* T-DNA mutants, or plants constitutively expressing the RNAi construct pDsi-RNAi-1 were harvested from flowering plants grown at the same time. Ten green and ten brown siliques were

excised from the inflorescence intact and placed into a labelled centrifuge tube. The siliques were placed under a dissecting microscope and the morphology of the siliques and the pattern of the seeds within the siliques were observed. Subsequently, the seeds were removed from the siliques and observed under a dissecting microscope. The siliques and seeds of wild type and T-DNA 1 (*At1g07645* T-DNA homozygous mutant) plants were photographed.

4.2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 3 (Motulsky, 1999). The data was analysed using one-way anova using the post-hoc test, the Dunnett's test, which compared all transgenic lines to the control plants (wild type). The Bonferroni test was performed to determine whether there was a difference between transgenic plants expressing *Xhdsi-1^{VOC}* and *At1g07645*. P-Value < 0.05 was used as significant for both tests.

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4.3. Results

In order to gain insight into the role of *Dsi-1*^{VOC} in abiotic stress tolerance, seed development and germination, the gain-of-function and loss-of-function phenotypes in *A. thaliana* lines were analysed. The phenotypes of three independent transgenic lines constitutively expressing *Xhdsi-1*^{VOC} (XhA3, XhC9 and XhC10) and three independent transgenic lines constitutively expressing *At1g07645* (AtA2, AtB5 and AtC9) were characterised by analysing the effects of abiotic stress on germination and two-week-old seedlings and adult plants. The effect of loss-of-function on seed development and germination in two *At1g07645* T-DNA mutant lines (T-DNA 1 and T-DNA 12) and three lines in which *At1g07645* is knocked down by RNAi (35S 2, 35S 5 and 35S 13) was characterised.

4.3.1. Constitutive expression of *Xhdsi-1*^{VOC} and *At1g07645* does not increase tolerance to NaCl and mannitol during germination and seedling growth

Although seed germination of both wild type and transgenic lines overexpressing *XhDsi-1*^{VOC} and *At1g07645* was delayed as the concentration of NaCl and mannitol increased, there was no difference in the timing of seed germination between wild type and transgenic lines. In the absence and the presence of 50 mM NaCl, sown seeds from both wild type and transgenic lines germinated after three days. In the presence of 100 and 200 mM NaCl, the seed germinated after four and six days, respectively. In addition, there was no significant difference in the percentage of seed germinated on the different concentrations of NaCl between wild type and the transgenic plants (Fig. 4.2A). On half strength MS supplemented with 100, 200 and 400 mM mannitol, the seeds germinated after four, five and six days, respectively. There was no significant difference in the germination success in the presence of 100 mM and 200 mM mannitol (Fig.4.2B). In the presence of 400 mM mannitol, the percentage of seed germination in transgenic lines XhA3 and XhC9 constitutively expressing *XhDsi-1*^{VOC} was significantly lower than the wild type. There was no significant difference in root inhibition of wild type or transgenic seedlings constitutively expressing *XhDsi-1*^{VOC} and *At1g07645* grown in the absence or presence of NaCl (Fig. 4.2C; Fig. 4.3A) and mannitol (Fig. 4.2D; Fig. 4.3B). 50 and 100 mM concentration of NaCl severely inhibited root growth in both the wildtype and

transgenic lines. Growth rate of roots was not significantly reduced between the wild type and transgenic lines in the presence of 100 mM mannitol. However, 200 and 400 mM concentrations of mannitol severely inhibit the growth of the roots, in both the wild type and transgenic seedlings.

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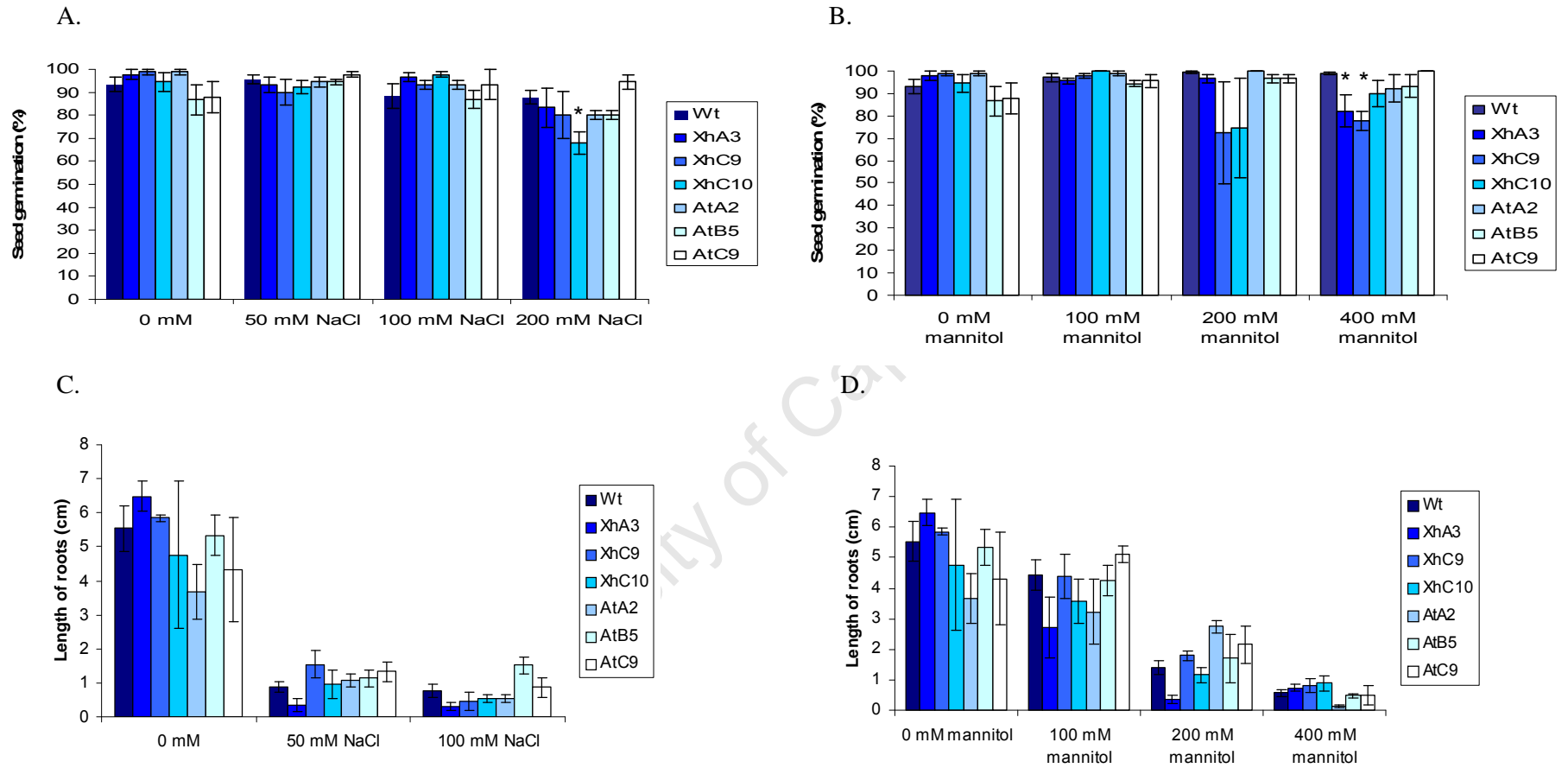


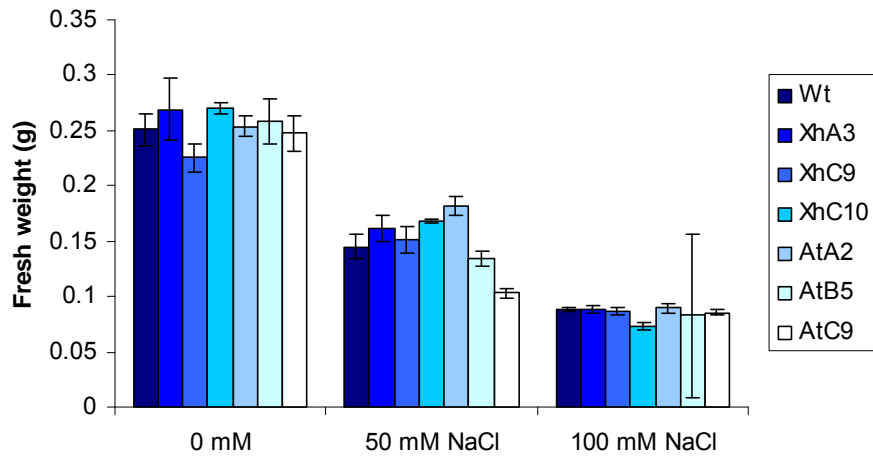
Figure 4.2. Percentage germination of wild type (Wt) and transgenic seeds constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) in the presence of various concentrations of NaCl (A) and mannitol (B). Root length of wild type and transgenic plant measured two weeks after germination in the presence of indicated concentrations of NaCl (C) and mannitol (D). Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value < 0.05) of transgenic plants relative to wildtype.

Figure 4.3A. Comparison of root growth of two-week-old wild type (wt) and transgenic seedling constitutively expressing Xhdsi-1^{voc} (Xh) and A1g07645 (At) grown in the presence of various concentrations of NaCl.

Figure 4.3B. Comparison of root growth of two-week-old wild type (wt) and transgenic seedling constitutively expressing Xhdsi-1^{voc} (Xh) and A1g07645 (At) grown in the presence of various concentrations of mannitol.

The growth rate of seedlings in the absence and presence of increasing amounts of NaCl or mannitol was measured by total fresh weight (Fig. 4.4) and visual inspection of two-week-old seedlings (Fig. 4.5). Increasing concentrations of NaCl and mannitol correlated with the decrease in fresh weight, therefore the increasing concentrations of NaCl and mannitol inhibited seedling growth. However, there was no significant difference observed in the growth of seedlings measured by total fresh weight or visual inspection of two-week-old seedlings grown in the absence or presence of NaCl and mannitol the wild type and transgenic plants (Fig. 4.4A; Fig. 4.5A). The majority of wild type and transgenic seedlings constitutively expressing *Xhdsi-1^{voc}* and *Atlg07645* grown in the presence of salt were chlorotic two weeks after germination (Fig. 4.5A). Although the seeds germinated (with emergence of the radicle) in the presence of 200 mM NaCl, the seedlings were completely bleached a day after germination and died (Fig. 4.5A). There was no difference in fresh weight between seedlings grown in the absence of stress and at 100 mM mannitol (Fig. 4.4B; Fig. 4.5B). In the presence of 200 mM and 400 mM, the mass of wild type and transgenic seedlings was severely reduced relative to seedlings not subjected to osmotic stress. However there was no significant difference between wild type and the transgenic seedlings (Fig. 4.4B).

A



B

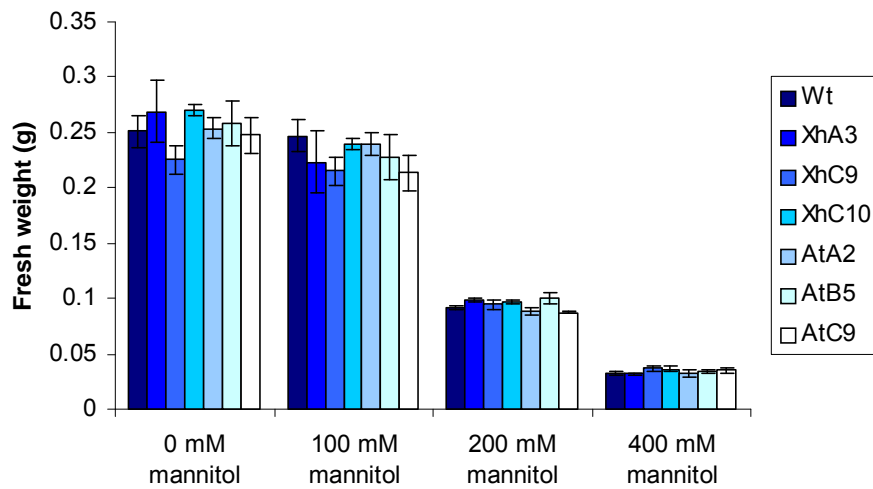


Figure 4.4. Fresh weight (g) of seedlings harvested two-weeks after germination from wild type and transgenic lines constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) grown in the presence of increasing concentrations of NaCl (A) and mannitol (B). Error bars represent \pm SE (n=3).

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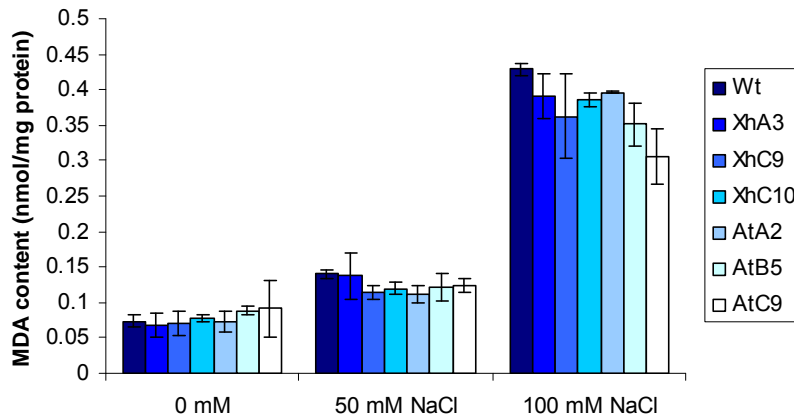
Figure 4.5A. Images of two-week old transgenic wild type (wt) or transgenic seedlings constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) and germinated on half-strength MS containing 0, 50, 100 and 200 mM NaCl concentrations. Thirty seeds from wild type and thirty from each transgenic line were germinated.

Figure 4.5B. Images of two-week old transgenic wild type (Wt) or transgenic seedlings constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) and germinated on half-strength MS containing 0, 100, 200 and 400 mM mannitol. Thirty seeds from wild type and thirty from each transgenic line were germinated.

4.3.1.1. Wild type and transgenic seedlings suffer similar levels of damage after NaCl and mannitol treatment

The ability of the *Dsi-1^{VOC}* to protect seedlings from oxidative damage caused by NaCl and mannitol treatments was assayed in the transgenic lines. MDA levels increased with increasing concentration of NaCl in two-week-old wild type and transgenic seedlings (Fig 4.6A). However, there was no significant difference in MDA levels between wild type and transgenic seedlings. Although seedlings did not show an increase in MDA levels in the presence of 100 mM mannitol compared to wild type. All the transgenic seedlings constitutively expressing *XhDsi-1^{voc}* and *At1g07645* exposed to 200 mM mannitol contained significantly lower MDA levels relative to wild type (Fig. 4.6B).

A



B

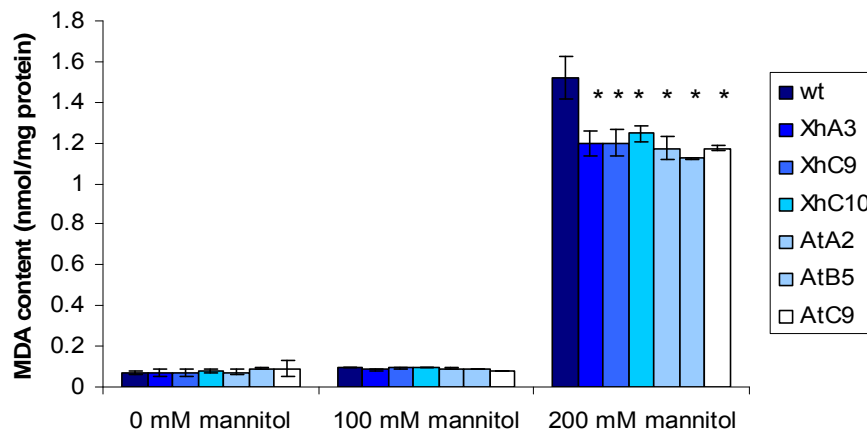
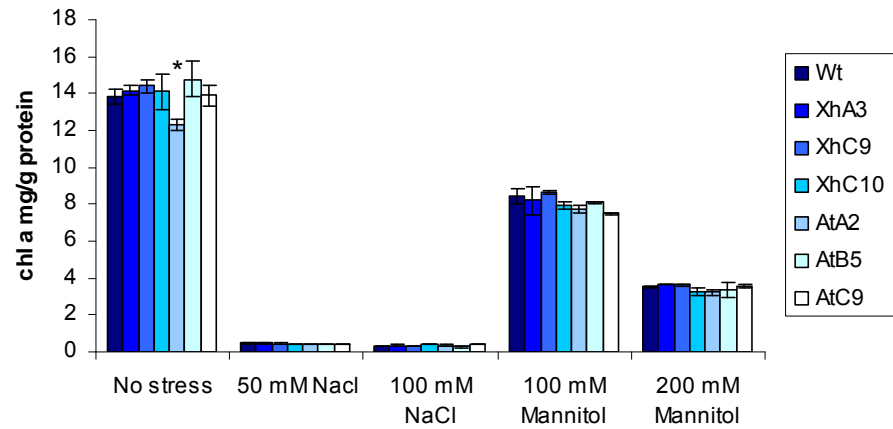


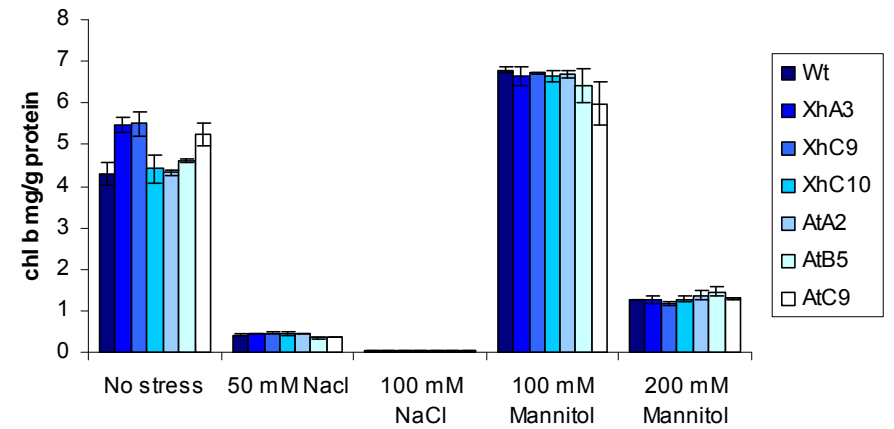
Figure 4.6. Measurement of MDA levels on two-weeks-old seedlings of wild type (Wt) and transgenic lines constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) grown in the presence of increasing concentrations of NaCl (A) and mannitol (B). Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value <0.05) of transgenic plants relative to wildtype.

The level of stress in wild type and transgenic two-week-old seedlings exposed to NaCl and mannitol treatments were assessed by measuring the content of photosynthetic pigments namely chlorophyll a (chl_a), chlorophyll b (chl_b) and carotenoids (Fig. 4.7). In the absence of stress, there was no significant difference in chlorophyll A levels, with exception of the AtA2 line constitutively expressing *At1g07645* (Fig. 4.7A). Chl_a content decreased with the increasing concentrations of NaCl and mannitol. Seedlings exposed to NaCl contained lower chl_a levels than seedlings grown in the presence of mannitol. Chl_b content decreased with increasing concentrations of NaCl, but there was no significant difference between the seedlings. Wild type and transgenic seedlings exposed to 100 mM mannitol contained higher chl_b content than the control seedlings and the levels decreased dramatically in the presence of 200 mM mannitol. However, there was no significant difference between wildtype and transgenic seedlings in the presence of NaCl and mannitol. In addition, the carotenoid content decreased with increasing concentration of NaCl and mannitol (Fig. 4.7C). Again there were no significant differences between wild type and transgenic seedlings in the absence or presence of NaCl and mannitol.

A



B



C

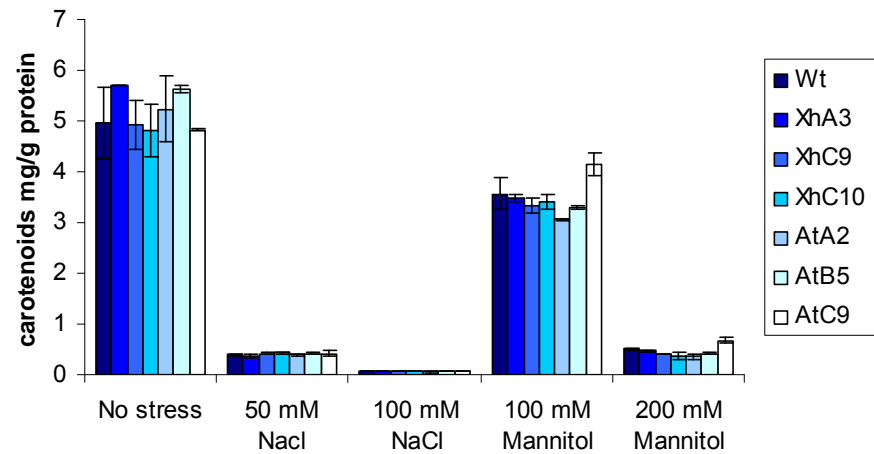


Figure 4.7. Measurement of A. chlorophyll A (chl a) , B. Chlorophyll B (chl b) and C. carotenoids on two-weeks-old seedlings of wild type (Wt) and transgenic lines constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) grown in the presence of increasing concentrations of NaCl and mannitol. Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value < 0.05) of transgenic plants relative to wild type.

4.3.2. Phenotypic analysis of wildtype and transgenic mature plants

As described in Chapter 2, overexpression of *Xhdsi-1^{VOC}* in yeast resulted in a lethal phenotype. The effect of constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* in vegetative tissues of *A. thaliana* was investigated. Constitutive expression of either *Xhdsi-1^{VOC}* or *At1g07645* in vegetative tissues of *A. thaliana* did not interfere with normal plant development. There were no gross morphological or growth differences between controls (wild type) plants and transgenic plants. The height of the transgenic plants was identical to wild type (Fig. 4.8). In addition, both wild type and transgenic plants started to produce flowers at the same time and the siliques began to turn brown (mature) at the same time.

Photochemical efficiency (F_v/F_m) and the relative water content were also not significantly different between wild type and transgenic lines (Fig. 4.9, no stress conditions). F_v/F_m values for both transgenic and wild type plants were close to the expected normal value of 0.8. The measurements of F_v/F_m were supported by the visual images where the maximum F_v/F_m of all rosette leaves on each plant are represented by an orange colour and accompanied by colour histograms generated by CCD progressive camera from the FluorImager which shows that both wild type and transgenic plants had a similar distribution (Fig. 4.8).

Figure 4.8. Comparison of wild type (Wt) or transgenic plants constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) in the absence of stress. A. shows photographs taken eight weeks after germination. B. represents F_v/F_m images of rosette leaves four weeks after germination. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

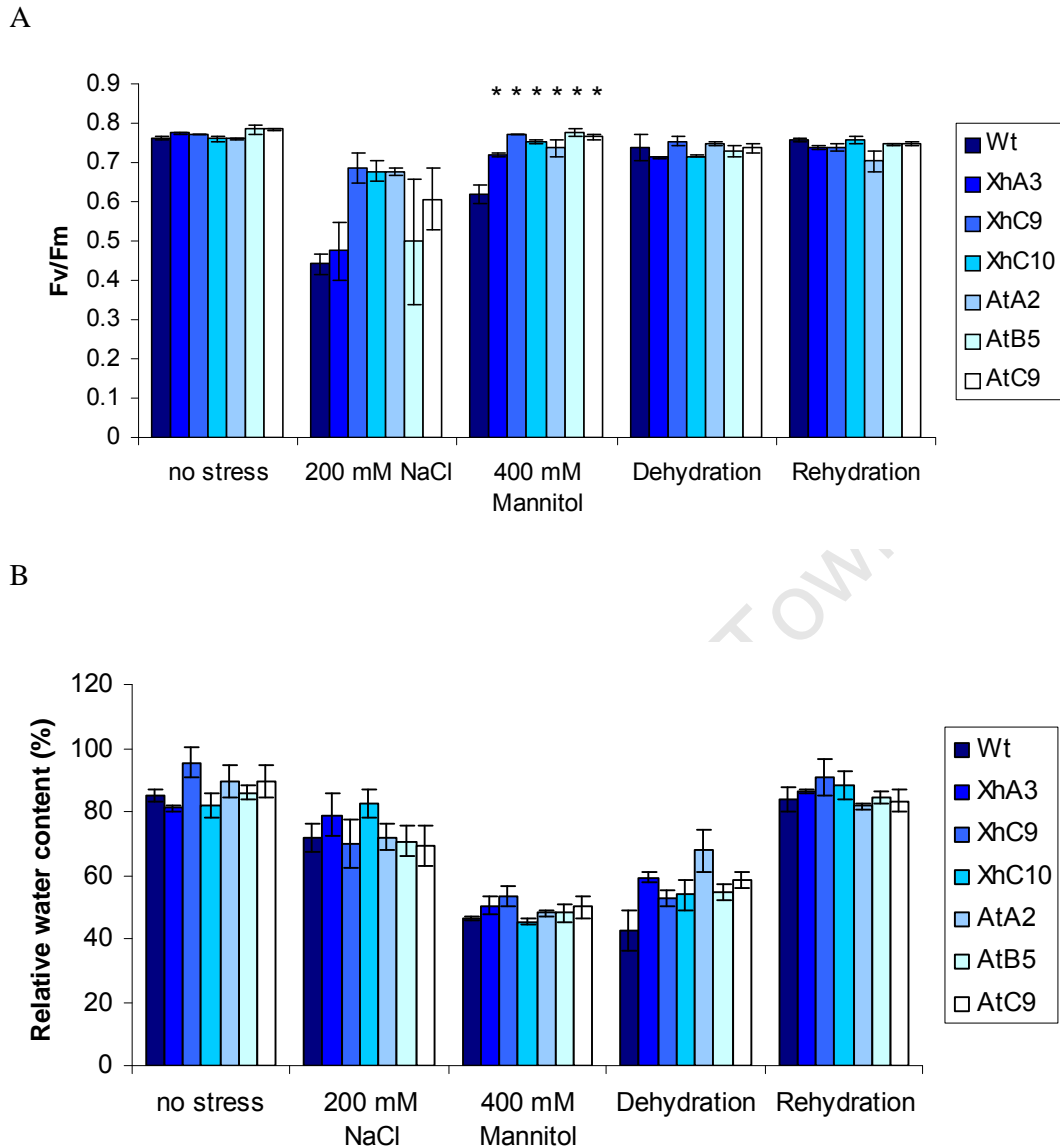


Fig 4.9. Measurement of F_v/F_m (A) and relative water content (B) on four-week-old wild type (Wt) and transgenic plants constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) in the absence of stress or exposed 200 mM NaCl, 400 mM mannitol, dehydration and the subsequent rehydration. Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value <0.05) of transgenic plants relative to wildtype.

4.3.2.1. A pilot study to establish conditions for abiotic stress

Prior to performing phenotypic studies on transgenic plants constitutively expressing the two *X. humilis* and *A. thaliana* orthologues in the presence of various stresses, a pilot study was performed using wildtype plants to determine the optimal number of days required to perform NaCl, mannitol, dehydration and the subsequent rehydration

experiments. The aim was to determine the number of days when plants showed abiotic stress by visual inspection. Wild type plants watered with 200 mM NaCl only started to show signs of chlorosis and folding of leaves four weeks after treatment (data not shown). Wild type plants watered with 400 mM mannitol were severely stressed after one week (data not shown). The length of time for NaCl and mannitol stress chosen for phenotypic studies of the transgenic plants was therefore four weeks and one week, respectively. Obtaining an optimal number of days for dehydration was extremely difficult. Plants dehydrated between one and fourteen days did not show severe dehydration stress and these plants could be rescued (data not shown). However, plants dehydrated between fifteen and eighteen days showed signs of dehydration stress, but failed to rehydrate (data not shown). Therefore, the optimal length of time chosen for dehydration stress (mild dehydration stress) was 14 days to investigate in the rate of recovery between wild type and transgenic plants.

4.3.2.2. Transgenic plants exposed to abiotic stress

The ability of the Dsi-1^{VOC} protein to protect adult transgenic plants exposed to various abiotic stresses was investigated based on the pilot study. The plants were subjected to NaCl, mannitol and dehydration stress and the subsequent rehydration (Fig. 4.10). Four-week-old wild type and transgenic plants were subjected to NaCl stress for four weeks (Fig. 4.10A). Four weeks after NaCl treatments, various physiological and biochemical parameters were used to assess the levels of stress in the plants. The plants were rescued by irrigation of the soil with water and photographed two weeks later. Four-week-old plants were subjected to one-week mannitol stress (Fig. 4.10B). The level of stress on the plants was assessed after one week of treatment with mannitol. Subsequently, the plants were irrigated with water and photographs were taken two, three and four weeks after irrigation with water. In addition to NaCl and mannitol stress, four-week-old plants were subjected to dehydration by withholding water for fourteen days (Fig. 4.10C). After fourteen days of dehydration, the level of stress was assessed. The plants were rescued by irrigation with water and the level of recovery was assessed two weeks after rehydration.

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Figure 4.10. Diagrams illustrating the treatment of four-week-old wildtype and transgenic plants either with NaCl (A), mannitol (B) or dehydration (C). The biochemical and physiological parameters used to assess the level of abiotic stress and damage are indicated.

4.3.2.2. The response of transgenic plants to abiotic stress

Four weeks after treatment of wild type and transgenic plants with 200 mM NaCl, the leaves of the plants were folded and appeared coarse and pale green in colour (Fig. 4.11). The transgenic plants constitutively expressing *Xhdsi-1^{voc}* and *At1g07645* varied in height. However, they were all taller than the wild type plants. The graphs showing F_v/F_m data between wild type and transgenic plants constitutively expressing *Xhdsi-1^{voc}* and *At1g07645* are not statistically significant due to high degrees of variation in XhA3 and AtB5 (Fig. 4.9A). F_v/F_m images and histograms on whole rosette leaves show that transgenic plants XhA3 constitutively expressing *Xhdsi-1^{voc}* and AtB5 constitutively expressing *At1g07645* have a similar profile to wild type (Fig. 4.11). The F_v/F_m images show that transgenic plants XhC9, XhC10, AtA2 and ATC9 have higher photochemical efficiency than wild type plants. In addition, RWC measurements showed no significant difference between wild type and transgenic plants (Fig. 4.9B). Consistent with no significant differences between the lines, both transgenic lines and wild type lines did not recover after salt stress when irrigated with water.

One week of 400 mM mannitol treatment caused severe bleaching of leaves of wild type when compared to the transgenic lines (Fig. 4.12A). Wild type plants could not be rescued after mannitol stress. However, transgenic lines constitutively expressing *Xhdsi-1^{voc}* and *At1g07645* recovered (Fig. 4.12B; Fig. 4.12C). The old leaves of the transgenic lines did not recover; nevertheless the transgenic plants generated new leaves that resembled regenerating leaves from callus. The recovered plants were able to flower and set seed. Consistent with recovery of the transgenic lines, the F_v/F_m values of transgenic lines constitutively expressing *Xhdsi-1^{voc}* and *At1g07645* 1 week after exposure to 400 mM mannitol were significantly higher than wildtype (Fig. 4.9A; Fig. 4.12A). The average F_v/F_m values for the transgenic lines were 0.71 ± 0.003 (XhA3), 0.005 ± 0.04 (XhC9), 0.75 ± 0.005 (XhC10), 0.74 ± 0.02 (AtA2), 0.78 ± 0.01 (AtB5), 0.76 ± 0.007 (AtC9) and the average value for wild type was 0.62 ± 0.02 . There was no significant difference between transgenic lines expressing the two orthologues. Relative water content was measured and showed no significant differences between the wild type and transgenic plants (Fig 4.9B).

Figure 4.11. Comparison of wildtype (Wt) and transgenic plants constitutively expressing Xhdsi-1voc (Xh) and At1g07645 (At) exposed to 200 mM NaCl for four weeks. A. shows photographs taken four weeks after NaCl treatment. B. represents F_v/F_m images of rosette leaves four weeks after germination. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange). D. Wildtype and transgenic plants did not recover upon irrigation with water (2 weeks after recovery)

Figure 4.12A. Photographs of wildtype (Wt) or transgenic lines constitutively expressing Xhdsi-1voc (Xh) and At1g07645 (At) exposed to 400 mM mannitol for one week. A. shows photographs taken, in the photograph, the upper row of plants was irrigated with water and the lower row were plants subjected to mannitol stress. B. represents F_v/F_m images of rosette leaves four weeks after germination. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

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Figure 4.12B. Photographs showing rescued wildtype (Wt) and transgenic plants constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) two weeks after irrigating the plants with water following a one-week exposure to mannitol stress.

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Figure 4.12C. Photographs showing rescued wildtype (Wt) and transgenic plants constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) four weeks after irrigating the plants with water following a one-week exposure to mannitol stress.

Although leaves of wild type and transgenic plants exposed to mild dehydration stress of fourteen days wilted upon visual inspection when compared to control plants, there were no physiological differences (Fig. 4.13A). There was no significant difference in F_v/F_m values (Fig. 4.9A). However, the images and histograms showing F_v/F_m values on all rosette leaves showed a difference in profile between wild type and transgenic plants (Fig. 4.13A). In contrast to all rosette leaves from transgenic plants, wild type images show that the F_v/F_m profile is distributed from low (blue) to high (orange). There was no significant difference in the RWC between wild type and transgenic plants (Fig. 4.9B). Wild type and transgenic plants rehydrated following the fourteen-day dehydration showed no significant differences in F_v/F_m values (Fig. 4.9A; Fig. 4.13B) and relative water content (Fig. 4.9B).

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Figure 4.13A. Photographs of wild type (Wt) and transgenic plants constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) exposed to a fourteen-day dehydration period. A. shows photographs taken, the upper rows of plants represent plant irrigated with water and the lower row are plants subjected to dehydration. A. shows photographs taken four weeks after NaCl treatment. B. represents F_v/F_m images of rosette leaves four weeks after dehydration. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

Figure 4.13B. Photographs of wild type (Wt) and transgenic plants constitutively expressing Xhdsi-1^{voc} (Xh) and At1g07645 (At) two weeks after rehydration following a fourteen day rehydration period. A. upper row of plants represent plants irrigated with water and the lower row are plants subjected to dehydration. B. represents F_v/F_m images of rosette leaves. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

4.3.2.2.1. Changes in lipid peroxidation and photosynthetic pigments of wild type and transgenic plants during abiotic stress

In addition to the measurements of F_v/F_m , the levels of lipid peroxidation as well as chlorophyll and carotenoid content of adult plants was measured to assay whether constitutive expression of *Xhdsi-1^{voc}* or *At1g07645* led to protection against ROS caused by abiotic stress. The levels of malondialdehydes (MDA) in both wild type and transgenic plants increased when subjected to NaCl, mannitol and dehydration-rehydration when compared to plants not subjected to abiotic stress, as expected (Fig. 4.14). There was no significant difference on MDA levels between wild type and transgenic plants in the absence of abiotic stress. However, wild type plants treated with 200 mM NaCl had significantly higher MDA levels (4.1 ± 0.14 nmol/ mg protein) than transgenic lines constitutively expressing *Xhdsi-1^{voc}* and *At1g07645* (1.9-3.5 nmol/ mg protein). The lowest levels of MDA were in lines XhC9 and AtC9. XhA3 had the highest MDA levels among the transgenic plants (Fig. 4.14). Transgenic plants subjected to 400 mM mannitol had significantly lower MDA levels (1.16-2.89 nmol/ mg protein) than wild type (4.06 ± 0.07 nmol/mg protein). Once again transgenic line XhA3, constitutively expressing *Xhdsi-1^{voc}* had the highest levels of MDA. There was an increase in MDA levels in wild type plants after fourteen days of dehydration, as well as in the rehydrated plants (Fig. 4.14). However, although MDA levels were lower in transgenic plants, there was no significant difference.

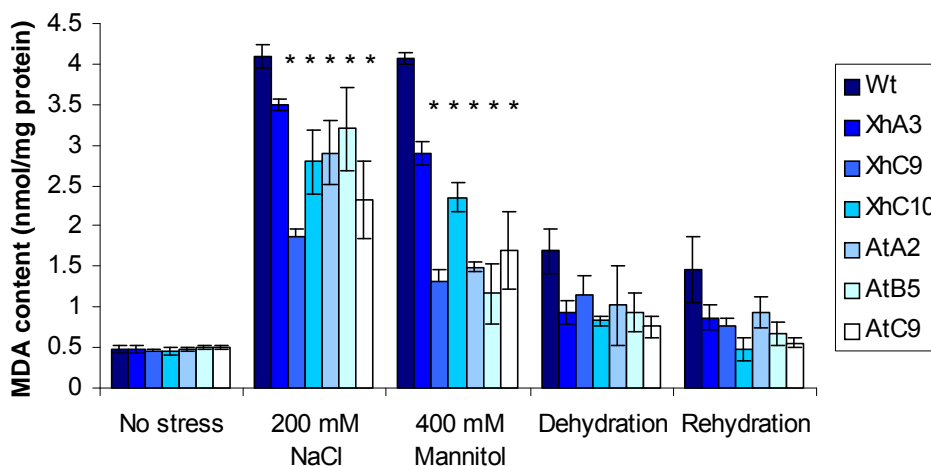


Figure 4.14. Measurement of MDA levels on two-weeks-old seedlings of wild type (Wt) and transgenic plants constitutively expressing *Xhdsi-1^{voc}* (Xh) and *At1g07645* (At) in the absence of stress or exposed 200 mM NaCl, 400 mM mannitol, dehydration and the subsequent rehydration. Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value <0.05) of transgenic plants relative to wildtype.

Plants exposed to the various abiotic stresses had a lower chl_a content relative to plants not exposed to stress. In the absence of stress, wild type and transgenic plants contained similar chl_a levels (Fig. 4.15A). Plants exposed to NaCl had very low chl_a content (Fig. 4.15A). Chl_a levels were significantly higher in transgenic plants than wild type, with the exception of AtA2, an independent line constitutively expressing At1g07645 when exposed to 400 mM mannitol stress (Fig. 4.15A). There was no significant difference between wild type and the six transgenic plants dehydrated for fourteen days and the subsequent rehydration (Fig. 4.15A).

There was a high level of variation in the chl_b content between the transgenic plants. However, in the absence of abiotic stress, there was no significant difference between the transgenic plants and wild type (Fig. 4.15B). As with chl_a levels, chl_b levels in both the wild type and transgenic plants subjected to NaCl were very low (Fig. 4.15B). Although chl_b content appears to be higher in transgenic plants relative to wild type when exposed to mannitol stress, the difference was not significant (Fig. 4.15B). In addition, the measurements of chl_b during dehydration and the subsequent rehydration show no significant difference between wild type and transgenic plants constitutively expressing *Xhdsi-1^{voc}* and At1g07645 (Fig. 4.15B)

Interestingly, in contrast to chl_a and chl_b measurements, carotenoid levels in the absence of abiotic stress in wild type (an average of 15.57 mg/g protein) were significantly lower than the transgenic lines (25.52-36.86 mg/g protein) (Fig. 4.15C). These high carotenoid levels found in transgenic plants decreased when plants were exposed to osmotic stress. Similar to the measurements of chl_a and chl_b in plants exposed to NaCl, carotenoid content was very low (Fig. 4.15C). Measurement of carotenoid content in plants exposed to mannitol stress showed that carotenoid content was significantly higher in transgenic plants relative to wild type, with the exception of the AtA2 line constitutively expressing At1g07645 (Fig. 4.15C). AtA2 had similar carotenoid contents as the wild type. There was no significant difference in carotenoid content between wild type and the transgenic plants dehydrated for fourteen days and the subsequent rehydration (Fig. 4.15C).

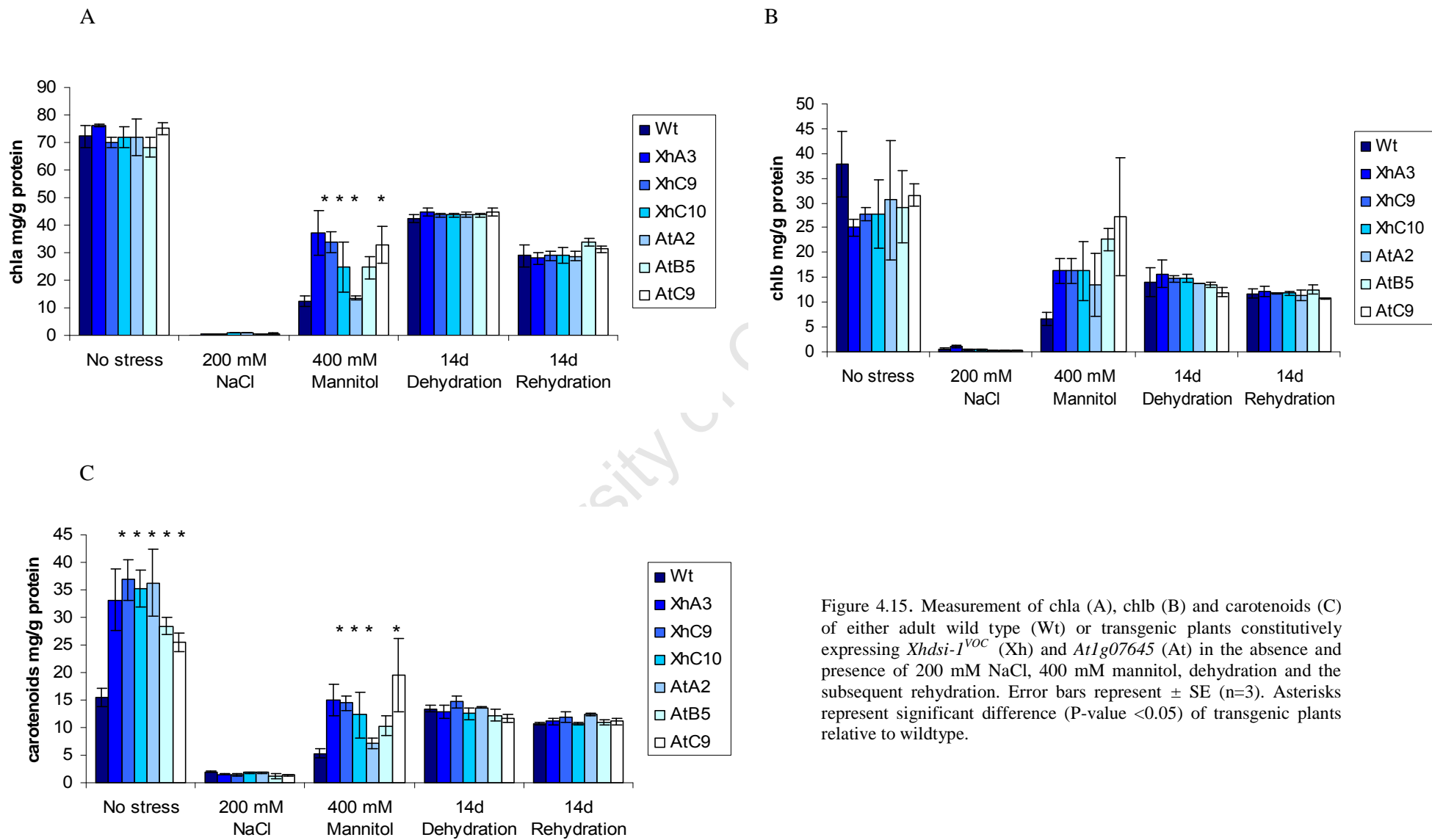


Figure 4.15. Measurement of chl a (A), chl b (B) and carotenoids (C) of either adult wild type (Wt) or transgenic plants constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *Atlg07645* (At) in the absence and presence of 200 mM NaCl, 400 mM mannitol, dehydration and the subsequent rehydration. Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value <0.05) of transgenic plants relative to wildtype.

4.3.3 Wildtype and transgenic plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* show similar susceptibility to *B. cinerea*.

Transgenic plants were infected with *B. cinerea*, a necrotrophic fungal pathogen to determine whether *Xhdsi-1^{VOC}* and *At1g07645* would contribute to the protection of the plants during biotic stress. Lesion development has been used as an indication to susceptibility to *B. cinerea*, this measurement correlates with whole plant susceptibility assays (Denby et al., 2004). Lesions began to appear 48 h post infection with *B. cinerea* on both detached wild type and transgenic leaves constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* (Fig. 4.16; Fig. 4.17). There was no significant difference in lesion size at 48h between wild type and transgenic leaves. Transgenic leaves from XhA3 and XhC10 had smaller lesion sizes than wild type 72h post infection. The other transgenic lines were not significantly different relative to wild type.

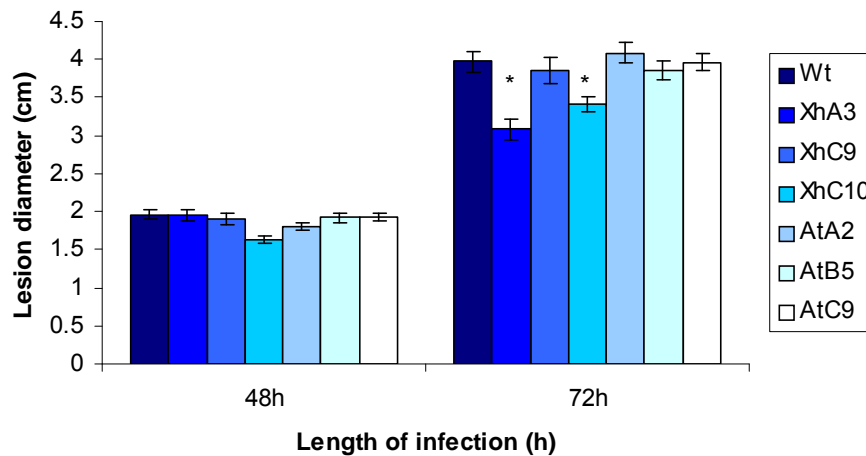


Figure 4.16. The measurement of the infected area representing infected lesions formed on the leaves of wild type (Wt) and transgenic lines constitutively expressing *Xhdsi-1^{VOC}* (Xh) and *At1g07645* (At) inoculated with *B. cinerea*. Error bars represent \pm SE (n=15). Asterisks represent significant difference (P-value < 0.05) of transgenic plants relative to wild type.

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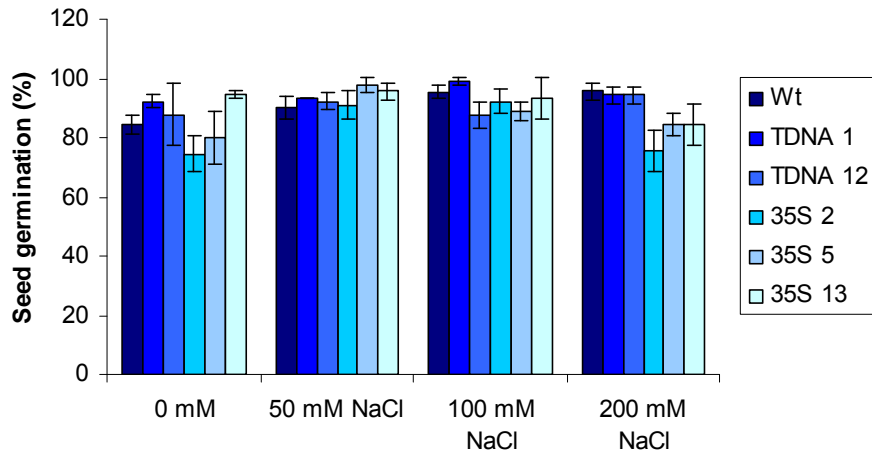
Figure 4.17. Detached leaves from wildtype (Wt) and transgenic lines expressing Xhdsi-1voc (Xh) and At1g07645 (At), inoculated with *B. cinera*. Photographs were taken 24 hours (A), 48 hours (B) and 72 (C) hours post inoculation.

4.3.4. There is no difference in seed germination and development between *A.thaliana At1g07645*-deficient and wild type seeds

The effect of loss-of-function of *At1g07645* in *A. thaliana* seeds by RNAi knock down and T-DNA knockout was investigated during seed germination and development. There was no difference in the percentage or rate of germination in seeds (upon emergence of the radicle) in which expression was knocked down by RNAi or in the seeds of T-DNA mutants compared to wild type (Fig. 4.18). In the absence of abiotic stress, both wild type and transgenic seeds lacking *At1g07645* germinated three days after being sown. Similarly, wild type and experimental seeds germinated after three days when grown in the presence of 50 mM NaCl. In the presence of 100 mM and 200 mM NaCl, the seed germinated after four and six days, respectively. The wild type and *At1g07645* deficient transgenic seeds germinated after four, five and six days on half-MS supplemented with 100 mM, 200 mM and 400 mM mannitol, respectively.

The photographs show that in the absence of stress, two-week-old seedlings were green in colour (Fig. 4.19A). In the presence of 50 mM NaCl, some of the seedlings had bleached leaves. At 100 mM sodium chloride, most of the seedlings were chlorotic two weeks after germination. At 200 mM sodium chloride, seeds germinated, but the seedlings did not grow. The growth of two-week-old seedlings grown on 400 mM mannitol was severely inhibited (Fig. 4.19B). However, there was no significant difference between wild type and the *At1g07645* deficient seeds.

A



B

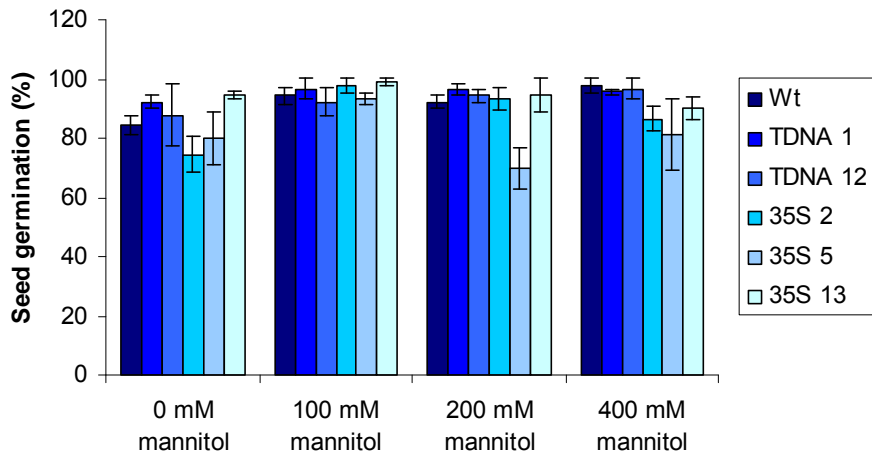


Fig. 4.18. Seed germination of wild type (Wt) or *At1g07645* T-DNA knockouts (TDNA 1, TDNA12), and RNAi lines silencing *At1g07645* constitutively expressing the pDSi-RNAi-1 construct (35 S 2, 35 S 5, 35 S 13) germinated on half-strength MS supplemented with various concentrations of NaCl (A) and mannitol (B). Error bars represent \pm SE (n=3). Asterisks represent significant difference (P-value < 0.05) of transgenic plants relative to wildtype.

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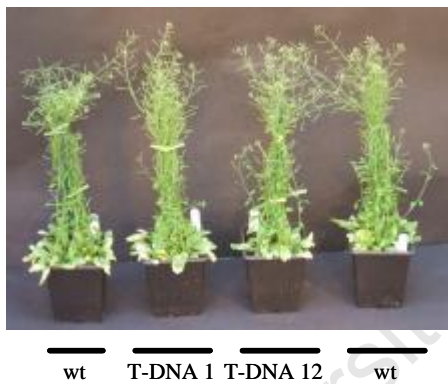
Figure 4.19A. Photographs of two-week-old seedlings of wildtype (Wt) or At1g07645 T-DNA knockouts (TDNA 1, TDNA12) and RNAi lines silencing At1g07645 (35 S 2, 35 S 5, 35 S 13) germinated on indicated NaCl concentrations.

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Figure 4.19B. Photographs of two-week-old seedlings of wildtype (Wt) or At1g07645 T-DNA knockouts (TDNA 1, TDNA12) and RNAi lines silencing At1g07645 (35 S 2, 35 S 5, 35 S 13) germinated on indicated mannitol concentrations.

There were no significant differences in silique and seed development in wild type and *A. thaliana* seeds deficient in *At1g07645*. Visual observations showed that wild type and transgenic lines exhibiting *At1g07645* knockdown began to flower at the same time as mature dry plants and looked identical (Fig. 4.20). Immature (green) and mature (brown) siliques were observed under the microscope. There were no morphological differences between wild type siliques or siliques where expression of *At1g07645* was knocked down by RNAi or knocked down by T-DNA insertion. In addition, the seeds removed from the siliques and observed did not show any significant differences in morphology or size between *At1g07645*-deficient seeds and wild type. Figure 4.21 shows photographs of green and brown siliques of wildtype and *At1g07645* T-DNA1 line.

A



B

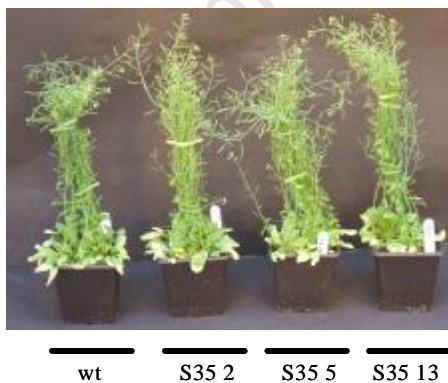


Figure 4.20. Transgenic plants of *At1g07645* T-DNA knockouts (TDNA 1, TDNA12), RNAi lines silencing *At1g07645* (35 S 2, 35 S 5, 35 S 13) and wild type (Wt).

Figure 4.21A. A comparison of green (immature) siliques and seeds of wild type (Wt) and At1g07645 T-DNA1 knockout line.

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Figure 4.21B. A comparison of brown (mature) siliques and seeds of wildtype (wt) and At1g07645 T-DNA1 knockout line.

4.4 Discussion

The first aim of the work described in this chapter was to determine whether constitutively expressing *Xhdsi-1^{VOC}* and its seed-specific *A. thaliana* orthologue, *At1g07645* in vegetative tissues altered plant tolerance to abiotic stress. The second aim was to determine whether lack of the *At1g07645* gene affected seed development. The gain-of-function phenotype was investigated by constitutively expressing *Xhdsi-1^{VOC}* (XhA3, XhC9 and XhC10) and its seed-specific *A. thaliana* orthologue, *At1g07645* (AtA2, AtB5 and AtC9), in the vegetative tissue of *A. thaliana* in the absence and presence of various osmotic stresses. The loss-of-function phenotype was investigated on seed development and germination using two *At1g07645* T-DNA mutant lines (T-DNA 1 and T-DNA 12) and three lines in which *At1g07645* is knocked down by RNAi (35S 2, 35S 5 and 35S 13). To assess the levels of tolerance of transgenic plants constitutively expressing either *Xhdsi-1^{VOC}* or *At1g07645* several parameters, such as germination, plant growth assays, photosynthetic pigments and lipid peroxidation, were measured.

4.4.1 Osmotic stress tolerance of transgenic plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645*

Constitutive expression of many genes such as those involved in the increase of trehalose and polyamines have been reported to cause growth retardation in transgenic plants, the overexpression of these genes interferes with the normal development of transgenic plants (Bhatnagar *et al.*, 2008; Karim *et al.*, 2007). Based on this information, and the observation that the induced expression of *Xhdsi-1^{VOC}* in yeast resulted in a lethal phenotype (reported in Chapter Two), the effects of constitutively expressing either *Xhdsi-1^{VOC}* or *At1g07645* on *A. thaliana* transgenic plant development was investigated. The results showed that the constitutive expression of *Xhdsi-1^{VOC}* and *At1g07645* in the vegetative tissues of *A. thaliana* did not interfere with the normal plant and seed development in the absence of stress. No signs of growth retardation were observed in adult plants (Fig. 4.8). In addition, photosynthetic efficiency of PSII (F_v/F_m) (Fig. 4.9A), lipid peroxidation levels (Fig. 4.14) and chl a and chl b contents (Fig. 4.15) were similar in wild type and all six transgenic plants.

Interestingly, the carotenoid content in the adult transgenic plants was higher than that of wild type plants. The results show that constitutive expression of either *Xhdsi-1^{voc}* or *At1g07645* could activate the synthesis of carotenoid enzymes.

Constitutive expression of *Xhdsi-1^{voc}* or *At1g07645* did not confer tolerance to abiotic stress in seedlings, as there was no significant difference in fresh weight, root length and photosynthetic pigments when compared to wild type although MDA levels were lower than wild type after treatment with 200 mM mannitol. However, mature plants constitutively expressing both orthologues showed increased tolerance to mannitol stress when compared to wild type. The transgenic adult plants had significantly lower MDA levels and retained significantly higher chl_a and carotenoids levels than the wild type. Upon irrigating with water, all the transgenic plants subjected to mannitol stress were able to recover and set seed. However, wild type plants did not recover.

Interestingly, the constitutive expression of either *Xhdsi-1^{voc}* or *At1g07645* did not confer the same level of tolerance to seedlings as in adult plants. Similar findings were reported by Brini and colleagues (2007) who showed that a dehydrin *DH-5* from *Triticum durum*, a member of the LEA 2 group did not confer tolerance to seedlings during mannitol stress when overexpressed in *A. thaliana*. However, the seedlings were able to recover once transferred to MS plates without mannitol. The adult plants overexpressing *DH-5* had a higher tolerance to ten days of dehydration than the wild type plants. The researchers concluded that the *DH-5* gene was important for the prevention of damage caused by osmotic stress, as well as for recovery. It is possible that *Xhdsi-1^{voc}* or *At1g07645* protect the transgenic plants during dehydration by limiting severe irreversible damage and are involved during rehydration. To confirm whether *Xhdsi-1^{voc}* and *At1g07645* play a role during recovery in seedlings, further studies investigating the recovery of transgenic seedlings exposed to mannitol need to be performed. Although transgenic line XhA3 constitutively expressing *Xhdsi-1^{voc}* showed tolerance to mannitol stress relative to wild type, MDA levels were significantly higher than the other five transgenic plants (Fig. 4.14). The *Xhdsi-1^{voc}* protein levels might be too low to confer the same levels of tolerance to XhC3, as in the other transgenic lines. The results suggest that for the optimal protection of the plant, a certain amount of *Xhdsi-1^{voc}* and *Atg107645* are needed. This speculation is

supported by a similar trend which is observed when transgenic plants are exposed to NaCl stress (see below).

Line XhA3 constitutively expressing *Xhdsi-1^{VOC}* had a growth rate similar to the wild type when exposed to NaCl (Fig. 4.11). The transgenic line XhA3 had similar photochemical efficiency to wild type when exposed to NaCl. The low levels of protein expression in this transgenic line as mentioned above, can explain the low tolerance in line XhA3 in the presence of both NaCl and Mannitol.

Although NaCl reduced the rate of growth of plants, adult transgenic plants with exception of XhA3 showed a higher growth rate than the wild type plants. Transgenic plants expressing the two orthologues showed lower levels of lipid peroxidation relative to the wild type when exposed to NaCl (Fig 4.14). Interestingly, photosynthetic pigments were severely reduced in plants exposed to NaCl when compared to plants exposed to mannitol stress (Fig. 4.15). Unlike transgenic plants exposed to mannitol stress, transgenic plants exposed to NaCl, together with the wild type plants, did not recover upon irrigation with water. NaCl causes osmotic stress in plants and also leads to the accumulation of Na^+ and Cl^- that cause ionic toxicity (Wang *et al.*, 2003). Salt tolerant plants avoid ionic toxicity by storing high ionic concentrations in the central vacuole mediated by Na^+/H^+ antiporters located on membranes of vacuoles (Wang *et al.*, 2003). Salt sensitive plants such as *A. thaliana* avoid toxicity mainly by inhibiting ionic uptake into the roots. However, at high ionic concentrations, this mechanism does not function, resulting in irreversible damage to plants (Yamaguchi and Blumwald, 2005). Overexpression of Na^+/H^+ antiporters, such as the SOS1 a Na^+/H^+ antiporter located on the plasma membrane and the AtNHX Na^+/H^+ antiporter located on the vacuole membrane have been reported to confer tolerance in transgenic plants exposed to salt stress (Yamaguchi and Blumwald, 2005; Shi *et al.*, 2003). It can be speculated that *Xhdsi-1^{VOC}* and At1g07645 proteins contribute to the protection of vegetative tissues of the transgenic *A. thaliana* plants from osmotic stress but not ionic stress. Similar findings have been reported in literature. Research by Brini *et al.*, (2007) showed that the overexpression of the dehydrin DH-5 conferred tolerance to the initial osmotic stress caused by salt. However, the dehydrin DH-5 did not protect the plant from ionic toxicity. Therefore, to prove the hypothesis that *Xhdsi-1^{VOC}* and At1g07645

specifically protect those transgenic plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* from osmotic stress, the plants should be subjected to ionic stress, such as LiCl. Unlike NaCl which causes both osmotic stress in plants and ionic toxicity (Wang *et al.*, 2003), LiCl only causes ionic toxicity in plants (Brini *et al.*, 2007), therefore *Xhdsi-1^{VOC}* and *At1g07645* would not confer tolerance to transgenic plants LiCl.

In addition, Xu and Shi, (2007) reported that constitutive overexpression of a regulatory protein 14-3-3 (TFT7), which was induced during stress in tomato, increased tolerance to transgenic plants during salt stress. Lipid peroxidation levels were significantly higher in wild type plants than in transgenic plants. The researchers reported that 14-3-3 (TFT7) did not reduce ionic levels (Na^+ , K^+ , Ca^{2+} and Mg^{2+}) in the transgenic plants relative to wild type plants. However, the 14-3-3 (TFT7) showed increased levels of the antioxidant, ascorbate peroxidase (APX). They concluded that the tolerance of the transgenic lines was due to the detoxification of ROS by APX. Interestingly, it appears that the constitutive expression of *Xhdsi-1^{VOC}* and *At1g07645* induce carotenoid synthesis in the adult transgenic plants (Fig. 4.15). Although adult transgenic plants, which were exposed to mannitol stress, contained lower carotenoid levels when compared to transgenic plant grown in the absence of stress, transgenic plants contained higher carotenoid levels than the wild type. It can be speculated that the high levels of ROS produced during osmotic stress (Navrot *et al.*, 2007; Moller, 2001; Nocter and Foyer, 1998) are detoxified by the carotenoids, which protect the cellular components thus preventing lipid peroxidation.

In addition, to investigate whether the constitutive expression of *Xhdsi-1^{VOC}* and those expressing *At1g07645* contributes to reducing water loss during osmotic stress, relative water content (RWC) was measured. Relative water content decreased during osmotic stress in both the wild type and transgenic plants (Fig. 4.9B). However, the difference in the relative water content between the wild type and transgenic plants was not significant. The results suggest that *Xhdsi-1^{VOC}* and *At1g07645* did not protect the plants by reducing water loss.

Interestingly, in this study the constitutive expression of *Xhdsi-1^{VOC}* or *At1g07645* had the same effect on *A. thaliana* transgenic plants in the absence and presence of stress.

These observations strongly suggest that *Xhdsi-1^{VOC}* and *At1g07645* function in same manner during desiccation in *X. humilis* vegetative tissues and *A. thaliana* seeds. As mentioned in Chapter 2, *X. humilis* plants are able to activate seed-specific genes during desiccation, and so this finding supports the suggestion that seeds and resurrection plants share some mechanisms to protect against the metabolic damage that usually occurs during desiccation and rehydration (Bernacchia *et al.*, 1996).

Adult transgenic plants constitutively expressing *Xhdsi-1^{VOC}* or *At1g07645* were also subjected to dehydration by withholding of water. However, unlike transgenic plants exposed to mannitol and NaCl stress, there were no significant differences between the wild type and the transgenic lines exposed to dehydration and the subsequent rehydration. The effects of dehydration by the withholding of water were difficult to investigate. Plants dehydrated for one to fourteen days could be rescued by irrigation. Between fifteen and eighteen days, the plants could not be rescued. Between the fourteenth and the fifteenth day, moisture in the soil drops dramatically. This type of rapid drying of the soil has been observed in our research lab when investigating expression profile of genes such as *Xhdsi-1^{VOC}* in *X. humilis* at different stages of desiccation and rehydration. Dehydration experiments by withholding of water are a very severe stress for a desiccation sensitive plant such as *A. thaliana*. Desiccation tolerance of resurrection plants such as *X. humilis* is caused by the activation of multiple genes (Bhatnagar-Mathur *et al.*, 2008; Vinocur and Altman, 2005; Collett *et al.*, 2004). Dehydration triggers the activation of transcription factors, and these transcription factors in turn effect transcription of proteins and metabolites that directly protect the plants from the damages caused by dehydration (Ramanjulu and Bartels, 2002; Nuccio *et al.*, 1999). Compatible solutes, such as trehalose, mannitol and proline, contribute the reduction of damage to the cell membrane and enzymes during osmotic stress (Nuccio *et al.*, 1999). In addition, structural changes of the desiccation tolerant plants contribute to the reduction of damage to the plants (Ramanjulu and Bartels, 2002). As a result, the overexpression of a single gene, such as *Xhdsi-1^{VOC}* or its orthologue *At1g07645*, is not sufficient to confer tolerance during severe dehydration in a drought sensitive plant such as *A.thaliana*.

4.4.2. Xhdsi-1^{voc} and At1g07645 did not confer tolerance to transgenic plants infected with *B. cinerea*

Several genes upregulated by both biotic and abiotic stresses in *A. thaliana*, *Oryza sativa*, barley and *X. humilis* have been identified in microarray experiments (Cumming *et al.*, 2007; Nakashima *et al.*, 2007; Collett *et al.*, 2004; Rabbani *et al.*, 2003; Ozturk *et al.*, 2002; Seki *et al.*, 2002). Phenotypic studies have illustrated that some dehydration-induced transcription factors and functional genes confer tolerance to transgenic plants during various stresses (Nakashima *et al.*, 2007; Sohn *et al.*, 2006). For example, a seed specific transcription factor from sunflower, HaHSFA9, when transformed into *A. thaliana*, was able to function by the activation of genes required to protect the plant from dehydration-induced damage. There is no evidence, however, that the gene contributed to biotic stress (Priet-Dapena *et al.*, 2008). In contrast, transgenic plants overexpressing OsNAC6, a member of the rice NAC transcription factor family, showed tolerance to dehydration, high salinity and biotic stress (Nakashima *et al.*, 2007; Sohn *et al.*, 2006). Single functional transgenes have also been reported to confer multiple resistance to various abiotic and biotic stresses (Lee *et al.*, 2006). For example, the CASAR82A gene from pepper increased the resistance of transgenic lines exposed to high salinity, dehydration and *B. cinerea*. The results reported in this chapter show that there was no significant difference in *A. thaliana* wildtype and transgenic leaves constitutively expressing *Xhdsi-1^{voc}* and *At1g07645* inoculated with *B. cinerea* (Fig. 4.16; Fig. 4.17). The results show that *Xhdsi-1^{voc}* and *At1g07645* are specific to protecting plants during water loss.

4.4.3. *At1g07645*-deficient *A. thaliana* seeds

There was no significant difference in the development or germination of *A. thaliana* seeds deficient in *At1g07645*, relative to the wild type (Fig. 4.18; Fig. 4.19; Fig. 4.20; Fig. 4.21). A loss-of-phenotype was not observed. Perhaps knockdown of the *At1g07645* protein does not show a phenotype because there may be other proteins with the same function in seeds. Interestingly, two members of the VOC superfamily showed high structural similarity to *Xhdsi-1^{voc}*. The first structure orthologue was At5g48480, which is a gene of unknown function. The second structure orthologue was At1g06570, which is annotated as a putative oxidoreductase dihydroxybiphenyl/-

dioxygenase. It is possible that At5g48480 and At1g06570 could share a similar function to At1g07645 *in vivo*. Successful phenotypes in seed mutants have been reported in the literature. For example, T-DNA knockout of GSH1, an essential enzyme in the biosynthesis of glutathione, resulted in bleaching of the seeds during seed development (Carins *et al.*, 2006) and T-DNA disruption of ATEM6, a seed-specific protein belonging to LEA 1 group in *A. thaliana*, resulted in premature drying of the seeds within siliques (Manfre *et al.*, 2006). The genes are important in the normal development of seeds.

4.4.4. Summary

Xhdsi-1^{VOC} is expressed in the vegetative tissue of the resurrection plant, *X. humilis* and its orthologue is expressed in orthodox seeds belonging to drought sensitive plants. It appears that *X. humilis* has the ability to activate this seed-specific gene during desiccation. During high salinity and mannitol stress, adult plants constitutively expressing *Xhdsi-1^{VOC}* and *At1g07645* showed increased tolerance relative to wildtype plants. Transgenic plants subjected to mannitol stress showed a higher tolerance than when subjected to NaCl. The former causes osmotic stress in plants and the latter causes both osmotic stress and ionic toxicity. This phenotype was observed in most of the independent transgenic lines, strongly suggesting that the phenotype is due to the expression of *Xhdsi-1^{VOC}* and *At1g07645* gene. Furthermore, these genes can play a positive role in protecting against water deficit. The experiments to test the role of *At1g07645* in seed desiccation yielded surprising results. *A. thaliana* seeds deficient in *At1g07645* did not differ from wild type seeds, however, it is possible that there are other proteins present in seeds with the same function.

Chapter 5

Conclusion

5.1. General discussion

Abiotic stress such as drought is detrimental to plants worldwide (Thompson, 2008; Mitra, 2001). Thus the generation of drought tolerant crops is essential for improved productivity in the agriculture field (Reynolds and Tuberosa, 2008; Inze, 2005; Mitra, 2001). One of the strategies that will contribute to generating drought tolerant crops is the understanding of molecular mechanisms involved in desiccation tolerant plants (Reynolds and Tuberosa, 2008). Desiccation tolerant angiosperm species known as resurrection plants are able to survive a loss of 95% of their relative water content and resume biological function upon rehydration (Alpert, 2006; Oliver *et al.*, 2000). Resurrection plants survive desiccation by upregulating protective mechanisms during drying (Bohnert, 2000; Bewley, 1979).

The resurrection plant, *Xerophyta humilis* is used as a model system to identify and characterise genes which play an important role in conferring desiccation tolerance in vegetative tissue in plants. Microarray analysis using cDNA libraries prepared from dehydration and rehydration *X. humilis* leaf and root tissues has identified genes upregulated during water loss that encode proteins such as antioxidants, enzymes involved in sugar metabolism, LEAs, as well as proteins of unknown function (Collett *et al.*, 2004; Shen, unpublished; Walford, unpublished). Interestingly, a substantial number of the genes that were upregulated in desiccated leaf and root tissue of *X. humilis* were not expressed in the vegetative tissues of desiccation sensitive plants but are expressed in orthodox seeds (Illing *et al.*, 2005; Shen, unpublished; Walford, unpublished). The expression of seed specific genes in the vegetative tissues of other resurrection plants has been reported (Illing *et al.*, 2005; Mowla *et al.*, 2002). Seeds and resurrection plants therefore share some mechanisms to protect against metabolic damage that usually occurs during desiccation and rehydration (Bernacchia *et al.*,

1996). The challenge is to determine the function of the genes identified as desiccation-upregulated in the microarray studies during water deficit. In this study, I investigated the role of *X. humilis* desiccation induced-1^{VOC} (*Xhdsi-1^{VOC}*) upregulated during desiccation in the vegetative tissues of *X. humilis* and its orthologues in desiccation sensitive plants.

Xhdsi-1^{VOC} had significant sequence similarity to genes from desiccation sensitive plants including *A. thaliana*, *T. aestivum*, *G. max*, *B. napus* and *H. vulgare* and a desiccation tolerant bryophyte, *T. ruralis*. Interestingly, an analysis of representation showed that the cDNAs of these orthologues were derived from seeds or seed-related EST libraries. Furthermore, RT-PCR showed that the *A. thaliana* orthologue At1g07645 was absent in vegetative tissue of *A. thaliana* exposed to osmotic stress but was present in mature dry seed. Based on these results, it can be concluded that *XhDsi-1^{VOC}* and its orthologues are expressed in tissues capable of acquiring desiccation tolerance. However to demonstrate whether the ectopic expression of *XhDsi-1^{VOC}* and its *A. thaliana* orthologue, At1g07645 had a role in desiccation tolerance, phenotypic analysis in planta was investigated

Genetic manipulation and methods of transformation have not yet been developed for non-model plants such as *X. humilis*. Thus it is not possible to directly investigate the role of genes such as *Xhdsi-1^{VOC}* in *X. humilis* by genetic methods. As an alternative, a desiccation-sensitive model plant *A. thaliana* was chosen for our phenotypic experiments. *A. thaliana* is routinely used by molecular biologists as a suitable plant to perform phenotypic studies (Reynolds and Tuberosa, 2008; Somerville and Knorrneef, 2002). *A. thaliana* has a small genome, thus easy to manipulate. Furthermore, foreign genes can be introduced by simply inoculating the flowers with *Agrobacterium tumefaciens*. In addition, large numbers of disrupted genes by T-DNA and mutants generated by TILLING in *A. thaliana* are available at public stock centres commercially (Alonso *et al.*, 2003; Henikoff *et al.*, 2004).

In this study, two reverse genetic strategies were used to determine the function of *Xhdsi-1^{VOC}* and At1g07645 from *A. thaliana* in planta. The first strategy was to investigate the loss-of-function phenotype (i) by RNAi mediated knockdown of endogenous At1g07645 and (ii) *A. thaliana* plants with a T-DNA insertion that

disrupted the *At1g07645* gene within the genome. There was no significant difference in the development or germination of *A.thaliana* seeds deficient in *At1g07645*, relative to wildtype. No clear loss-of-phenotype was observed. Perhaps knockdown of the *At1g07645* protein does not show a phenotype because there may be other proteins with the same function in seeds.

The second strategy was to investigate whether the ectopic expression of *Xhdsi-1^{VOC}* and *At1g07645* confer tolerance to the vegetative tissues of transgenic *A. thaliana* plants exposed to various osmotic stresses. The results showed that the constitutive expression of *Xhdsi-1^{VOC}* or *At1g07645* did not confer tolerance to two-week-old *A. thaliana* seedlings exposed to osmotic stress. In contrast, adult transgenic plants constitutively expressing both orthologues showed increased tolerance to both salt and mannitol stress when compared to wildtype. However, the transgenic plants showed higher tolerance to mannitol than salt stress. Upon irrigating with water, all the transgenic plants subjected to mannitol stress were able to recover and set seed, however, all the transgenic plants exposed to 200 mM NaCl did not recover. Unlike transgenic plants exposed to mannitol stress which only causes osmotic stress, transgenic plants exposed to NaCl undergo osmotic stress in addition to being exposed to ionic toxicity caused by the accumulation of Na⁺ and Cl⁻ (Wang *et al.*, 2003).

Although a loss-of-phenotype was not observed, the gain -of-function phenotype and the expression profile data suggest that *Xhdsi-1^{VOC}* and *At1g07645* contribute to the protection of desiccation tolerant tissues during water deficit. In addition, *Xhdsi-1^{VOC}* and *At1g07645* function in the same manner, as there were no phenotypic differences between transgenic plants expressing *Xhdsi-1^{VOC}* or *At1g07645* exposed to abiotic stress. *Dsi-1^{VOC}* shows great promise as an endogenous gene that is able to confer tolerance to abiotic stress in desiccation sensitive plants if it could be activated in vegetative tissues. Further work is required to understand the role of *Xhdsi-1^{VOC}* and *At1g07645* during water loss, for example, by comparing the molecular mechanisms leading to the up-regulation in vegetative tissues and seed.

5.2. Future work

5.21. Investigating the transcriptional regulation of *Xhdsi-1^{voc}* and *At1g07645*

The transcripts and protein levels of *Xhdsi-1^{voc}* increase during desiccation in leaves and roots in *X. humilis*. In drought sensitive plants, the orthologues of *Xhdsi-1^{voc}* are absent in vegetative tissue, but are expressed in seeds of these plants. Based on these results, it can be hypothesised that the *Xhdsi-1^{voc}* promoter is activated in both seed and vegetative tissue (roots and leaves) during desiccation in *X. humilis* whereas the *A. thaliana* promoter responsible for the activation of *At1g07645* only functions in seeds in *A. thaliana*. Two mechanisms could have evolved to explain this hypothesis. Firstly, a seed-specific transcription factor is activated in leaves and roots in response to desiccation in *X. humilis*, and this transcription factor binds to the promoters of seed-specific genes leading to their expression in vegetative tissue. In this case, the *Xhdsi-1^{voc}* promoter would be similar to the promoters of *dsi-1^{voc}* in desiccation-sensitive plants, and would have recognizable response elements for the seed-specific transcription factor. Alternatively, the *Xhdsi-1^{voc}* promoter has acquired new binding sites for a vegetative tissue transcription factor, which is activated in response to desiccation stress. In this scenario, it can be predicted that the *Xhdsi-1^{voc}* promoter would have a seed-specific response element (for expression in seeds) and a desiccation-stress vegetative response element (for expression in vegetative tissue), which would bind two different transcription factors.

These two cases will be investigated by the identification of regulatory elements in the promoters of *Xhdsi-1^{voc}* its orthologues. It is speculated that a change in the genetic regulation in the vegetative tissue of desiccation tolerant plants (Bernacchia and Furini, 2004), based on the mechanisms in seed (Bohnert, 2000), has caused desiccation tolerance to remerge within the angiosperms ten times, independently (Oliver, 2007). Therefore, understanding transcriptional regulation of transcripts involved in the acquisition of desiccation tolerance plants will allow us to plan strategies to manipulate the expression of endogenous genes in plants and contribute to the generation of drought tolerant commercial crops such as maize and rice.

Chapter 6

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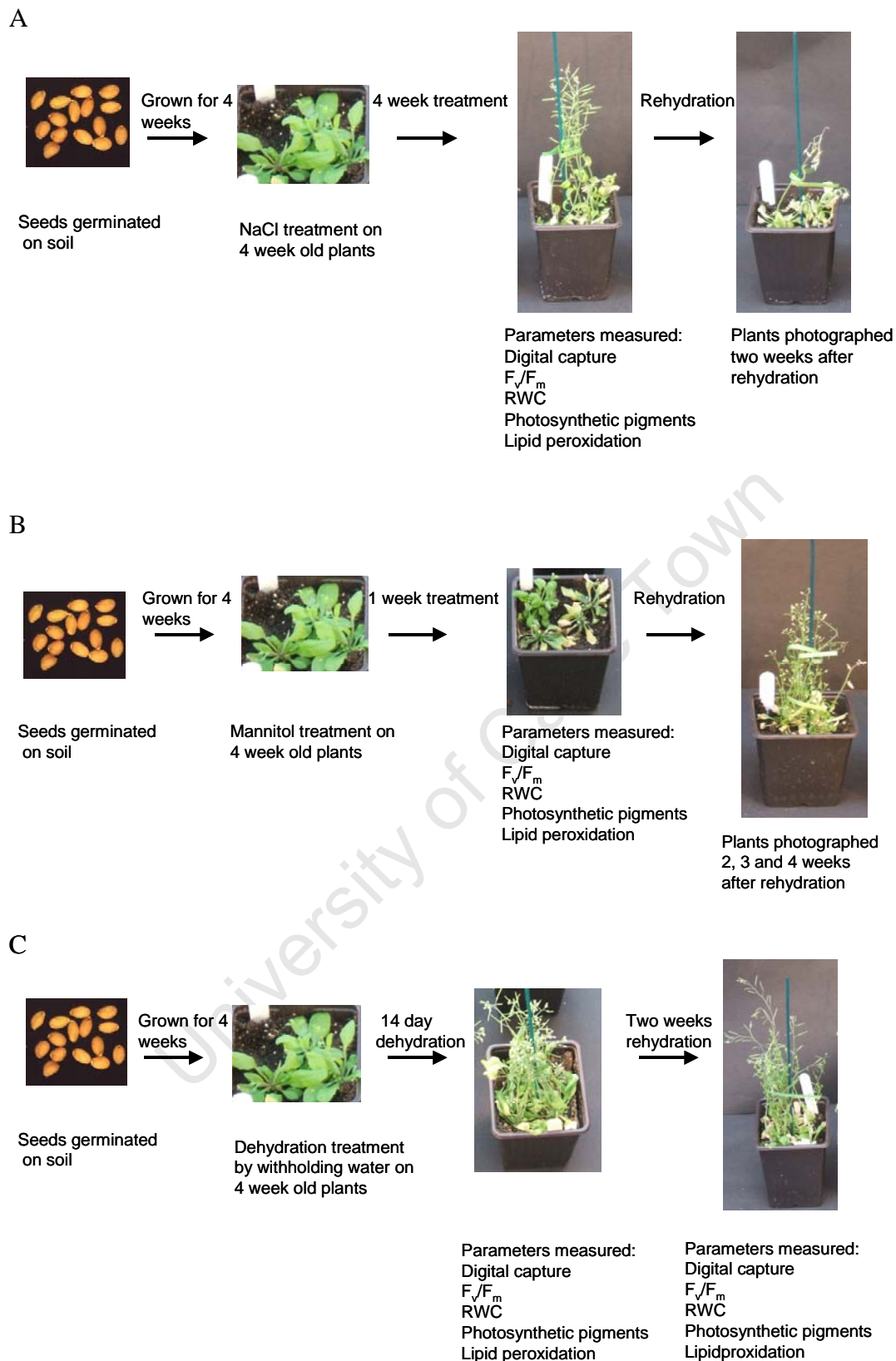


Figure 4.10. Diagrams illustrating treatment of wild type and transgenic plants with either NaCl (A), mannitol (B) or dehydration (C). The biochemical and physiological parameters used to assess the level of abiotic stress and damage are indicated.

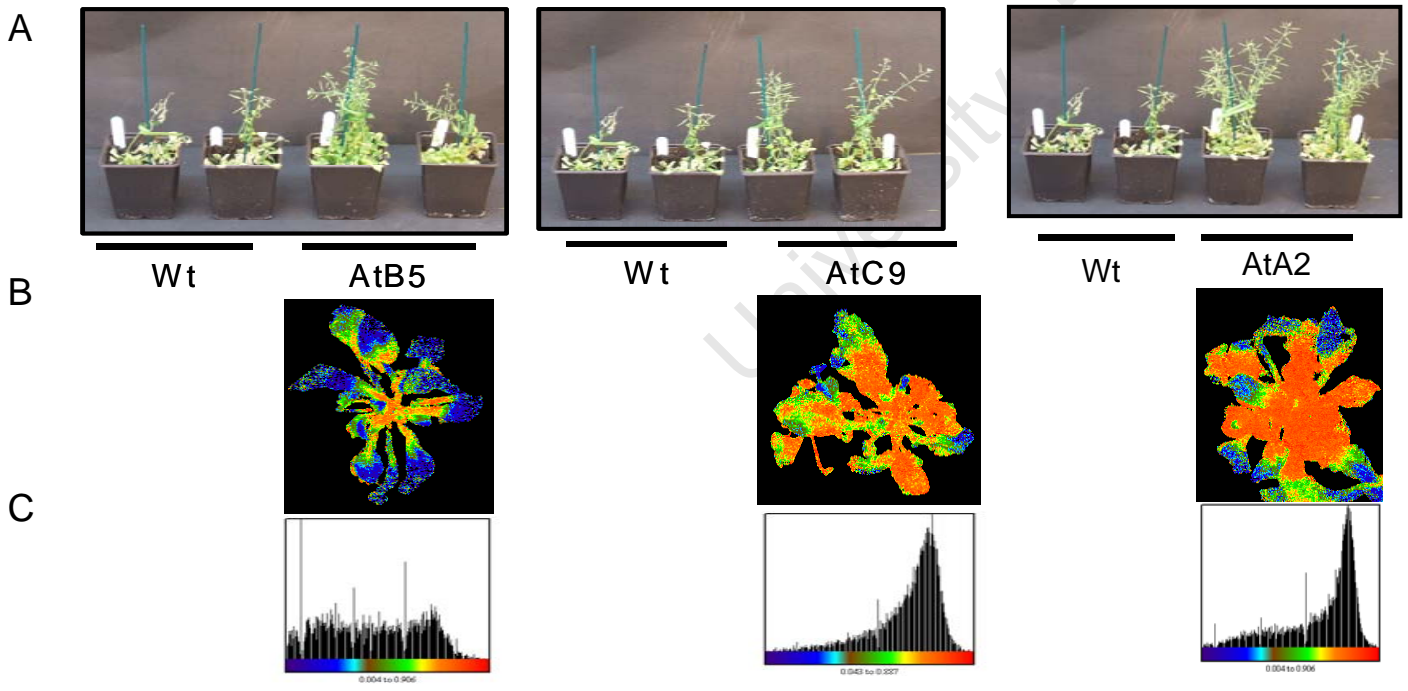
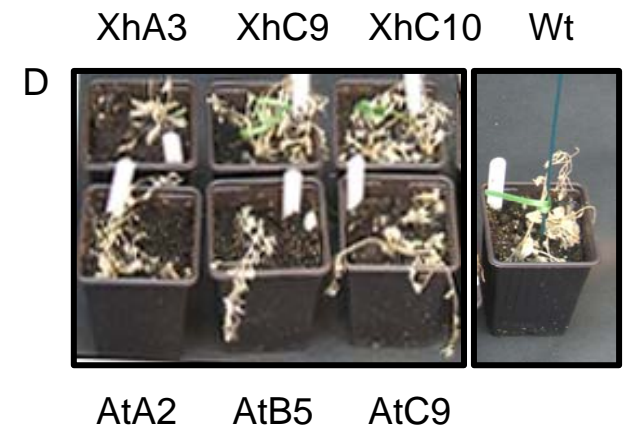
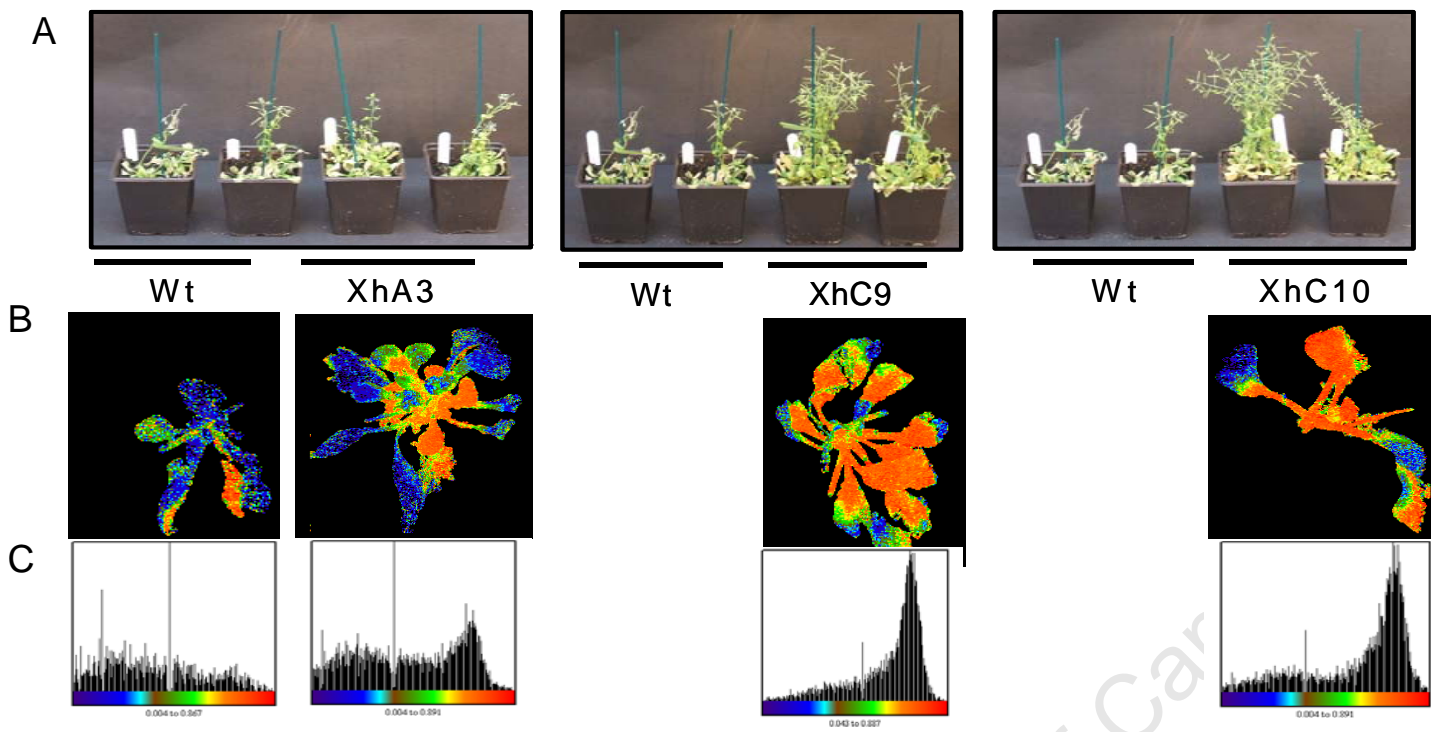


Figure 4.11. Comparison of wild type (Wt) and transgenic plants constitutively expressing Xhdsi-1voc (Xh) or At1g07645 (At) exposed to 200 mM NaCl for four weeks. A. shows photographs taken. B. represents F_v/F_m images of rosette leaves. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange). D. wild type and transgenic plants did not recover upon irrigation with water (2 weeks after recovery)

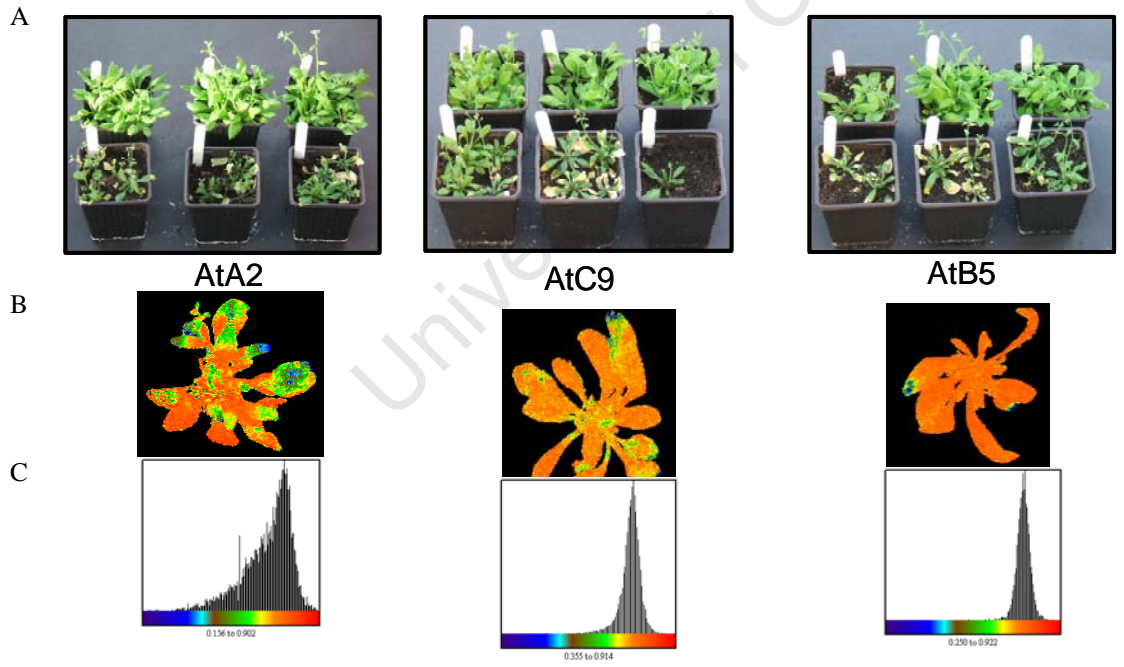
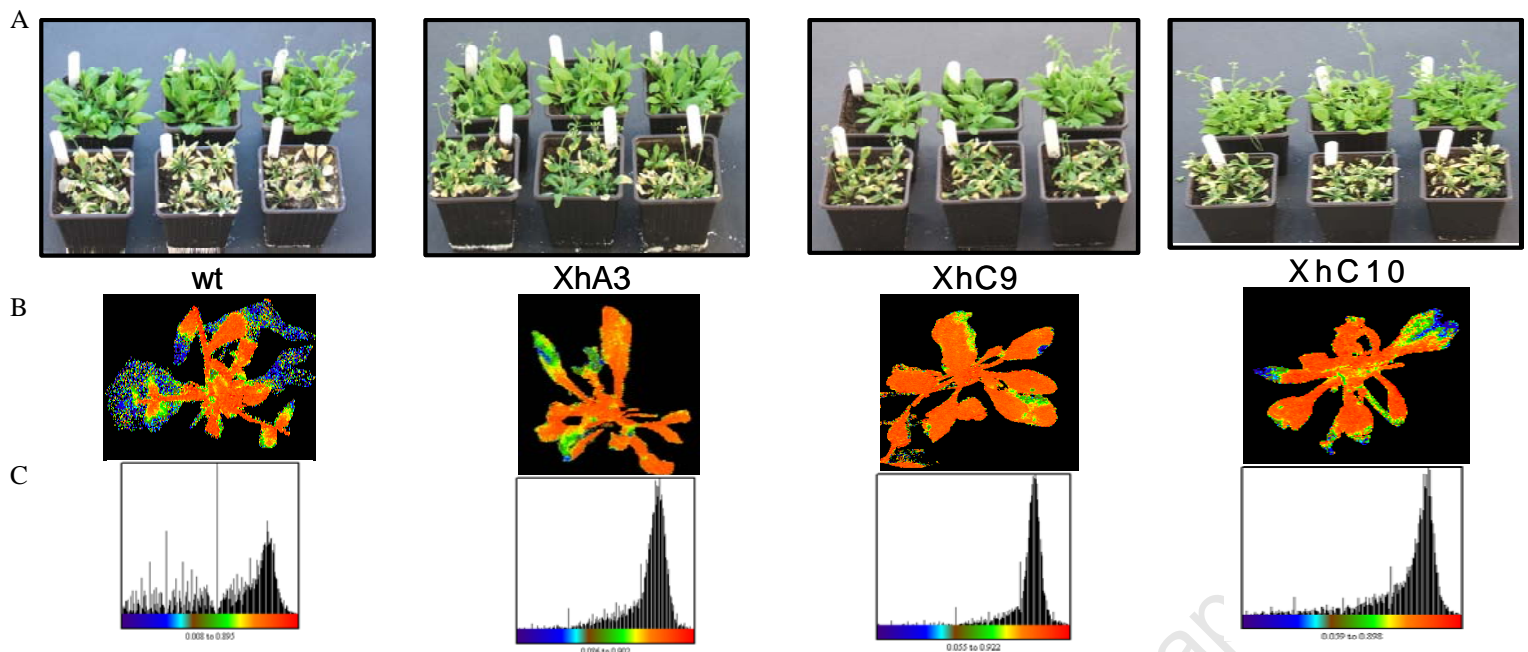


Figure 4.12A. Photographs of wildtype (Wt) or transgenic lines constitutively expressing Xhdsi-1voc (Xh) and At1g07645 (At) exposed to 400 mM mannitol for one week. A. the upper row shows plants irrigated with water and the lower row shows plants subjected to mannitol stress. B. represents F_v/F_m images of rosette leaves four weeks after mannitol stress. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).



Wt XhA3



Wt XhC9



Wt XhC10



Wt AtA2



Wt AtB5



Wt AtC9

Figure 4.12B. Photographs showing rescued wildtype (Wt) and transgenic plants constitutively expressing *Xhdsi-1^{voc}* (Xh) and *At1g07645* (At) two weeks after irrigating the plants with water following a one-week exposure to mannitol stress.

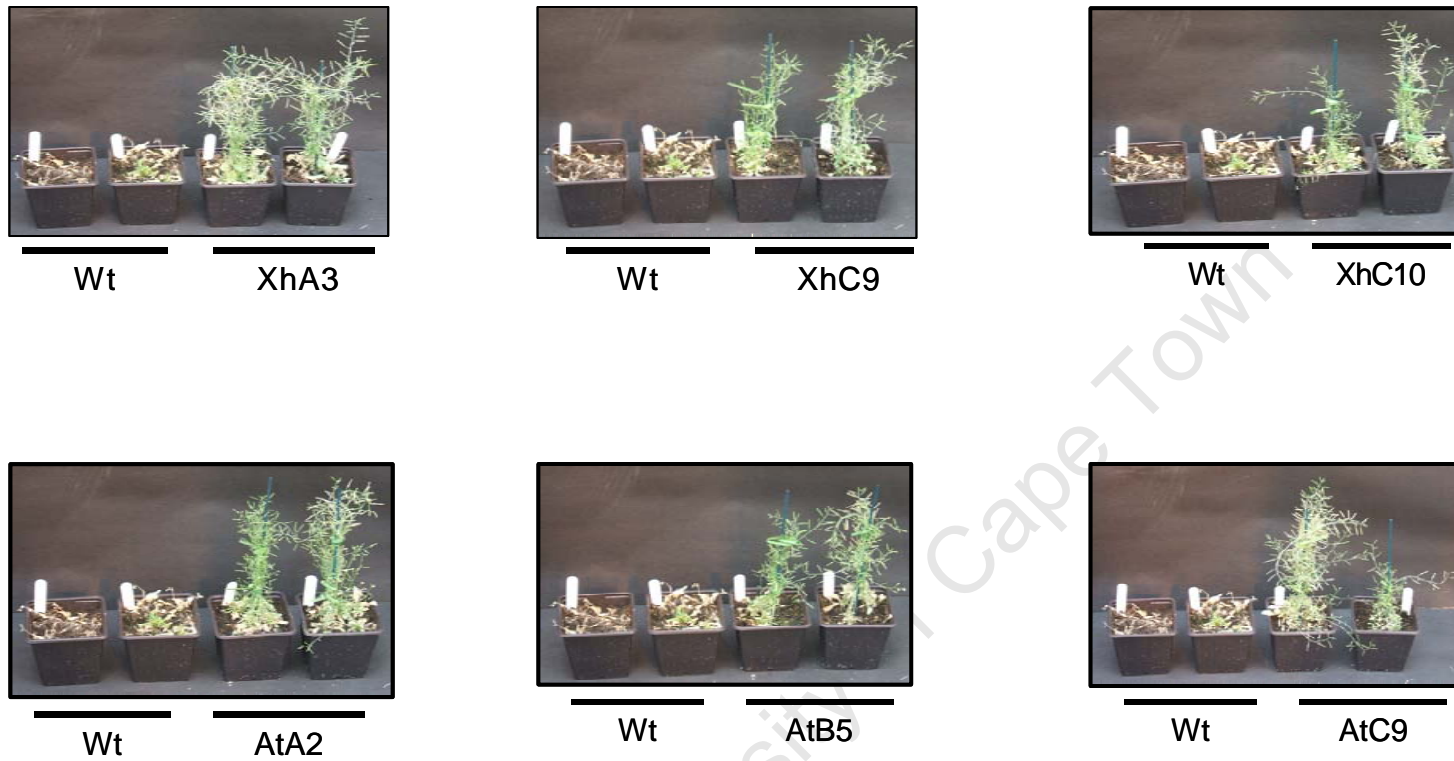


Figure 4.12C. Photographs showing rescued wildtype (Wt) and transgenic plants constitutively expressing *Xhdsi-1^{voc}* (Xh) and *At1g07645* (At) four weeks after irrigating the plants with water following a one-week exposure to mannitol stress.

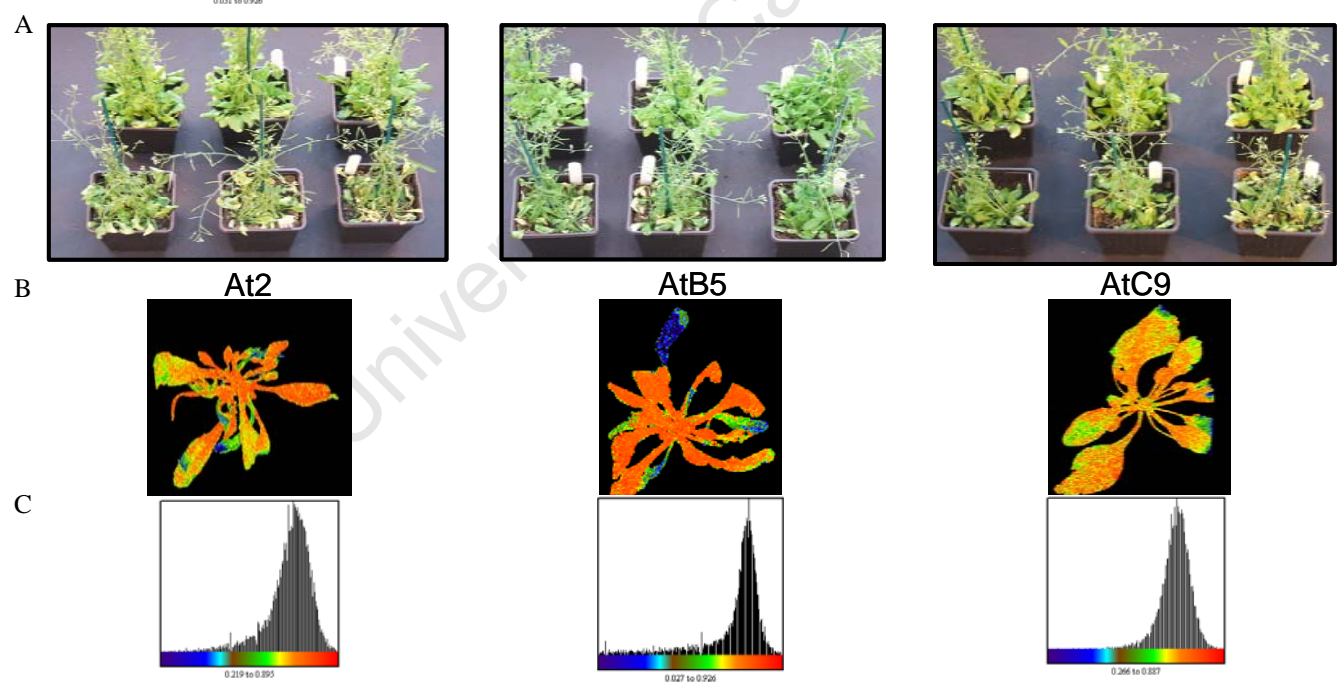
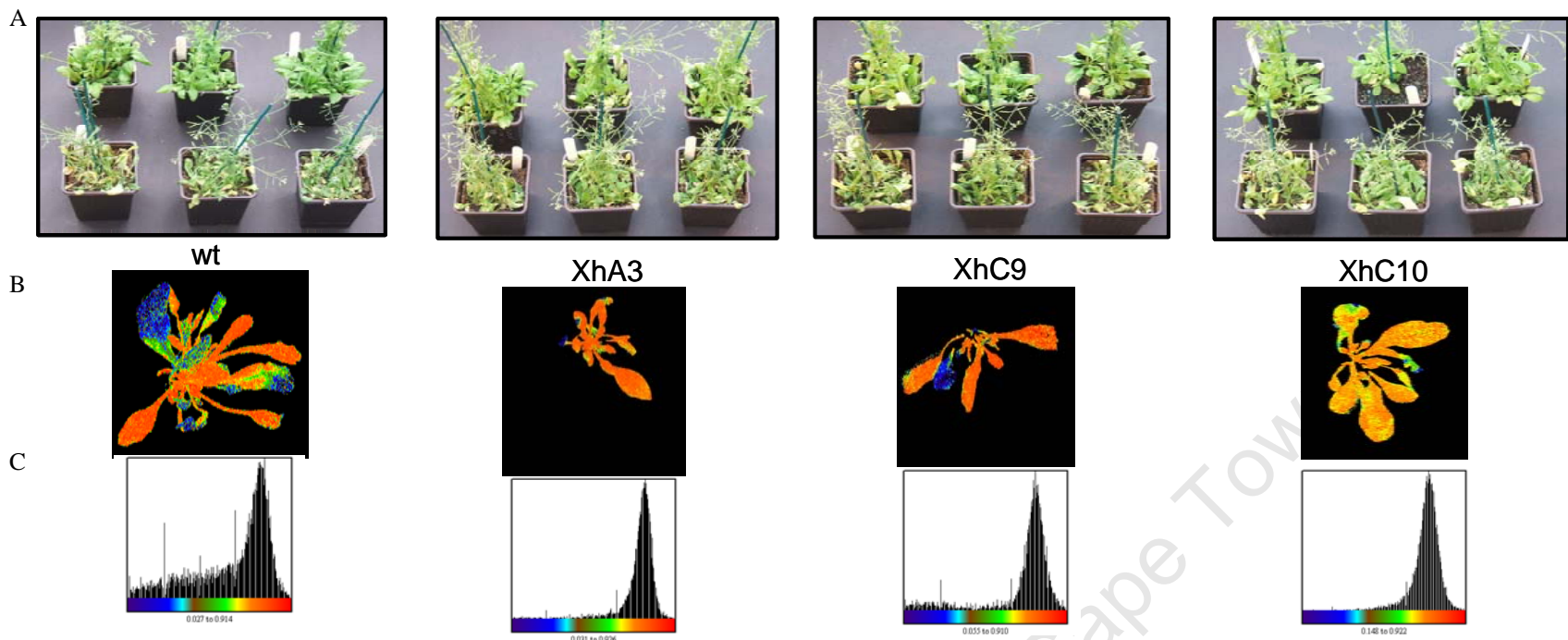


Figure 4.13A. Photographs of wild type (Wt) and transgenic plants constitutively expressing $Xhdsi-1^{voc}$ (Xh) and *At1g07645* (At) exposed to a fourteen-day dehydration period. A. the upper rows of plants represent plant irrigated with water and the lower row are plants subjected to dehydration. B. represents F_v/F_m images of rosette leaves fourteen days after dehydration. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

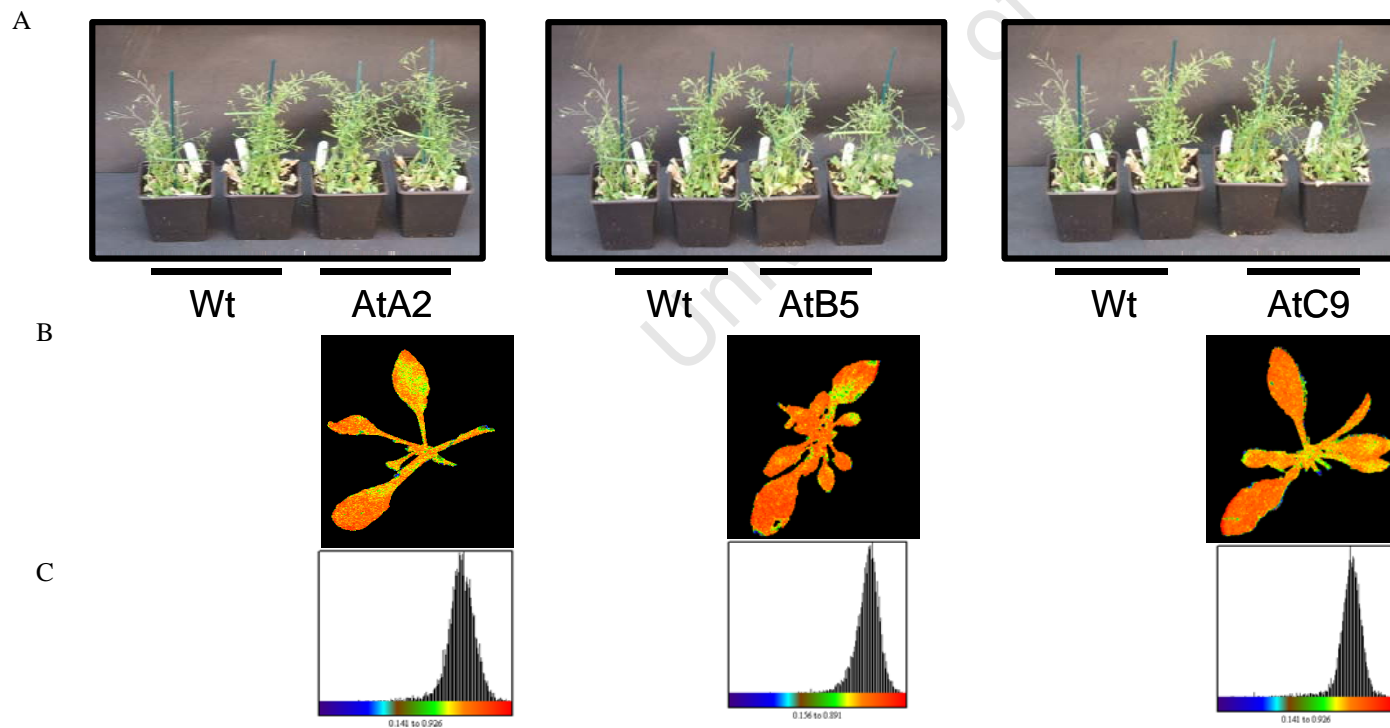
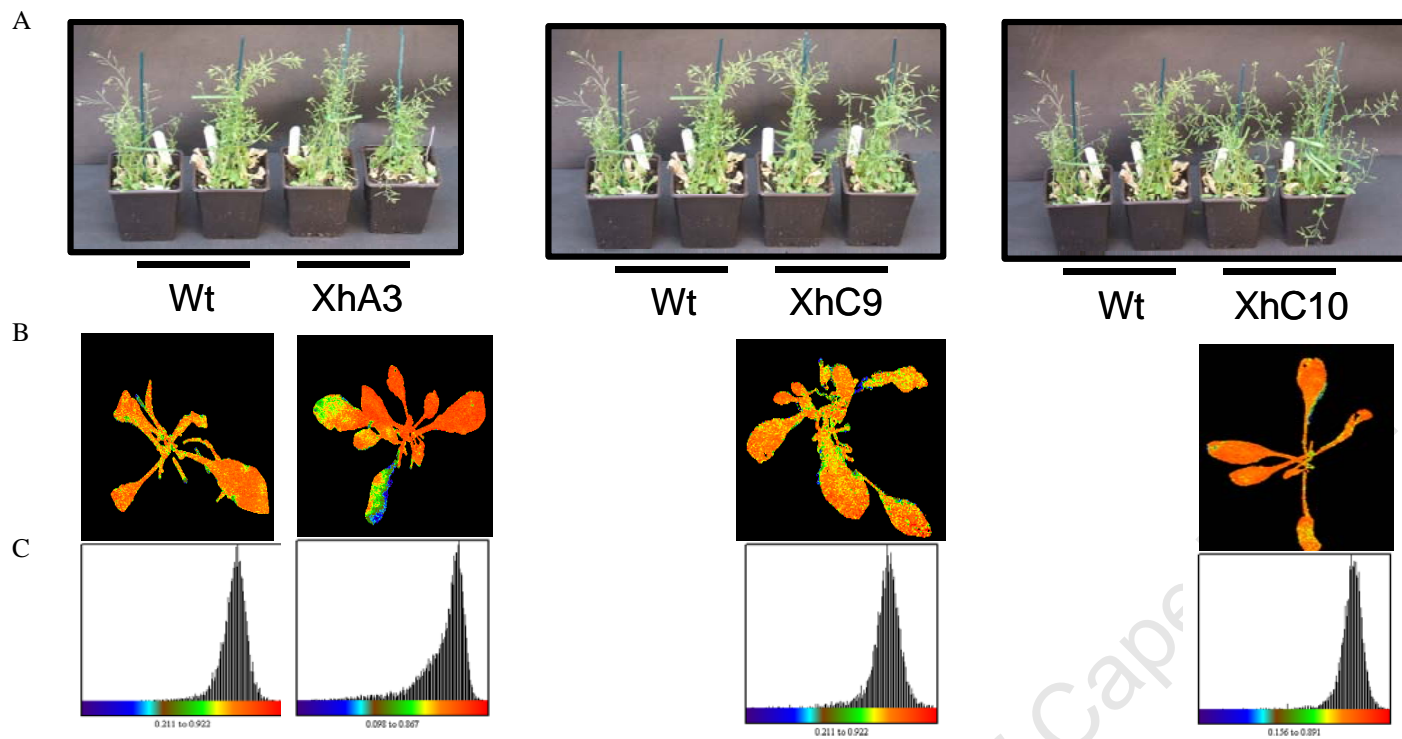
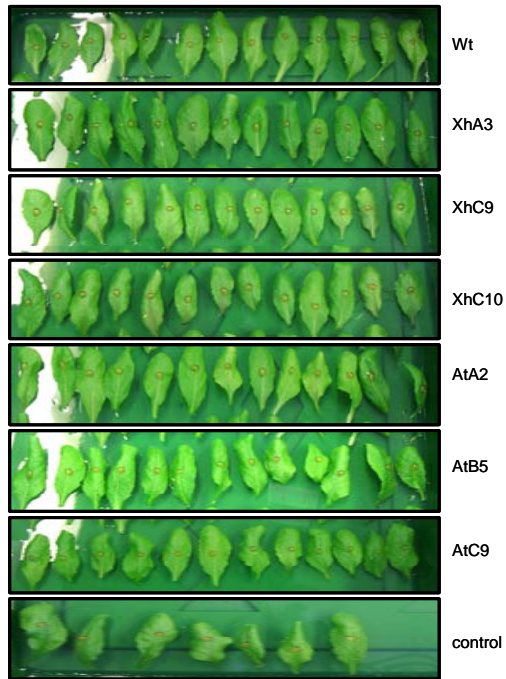
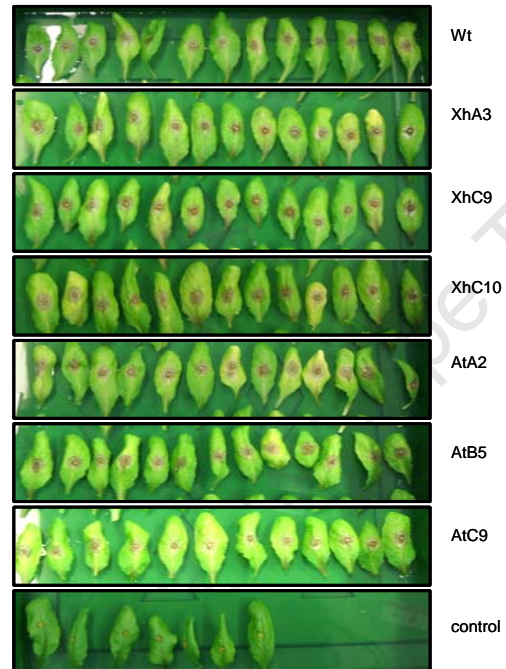


Figure 4.13B. A. Photographs of wild type (Wt) and transgenic plants constitutively expressing *Xhdsi-1^{voc}* (Xh) and *At1g07645* (At) two weeks after rehydration following a fourteen day rehydration period. B. represents F_v/F_m images of rosette leaves. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

A



B



C

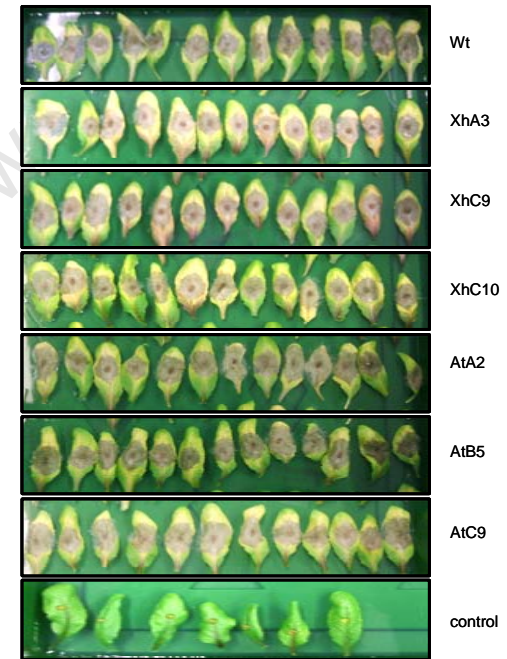


Figure 4.17. Detached leaves from wildtype (wt) and transgenic lines expressing Xhdsi-1voc (Xh) and At1g07645 (At), inoculated with *B. cinera*. Photographs were taken 24 hours (A), 48 hours (B) and 72 (C) hours post inoculation.

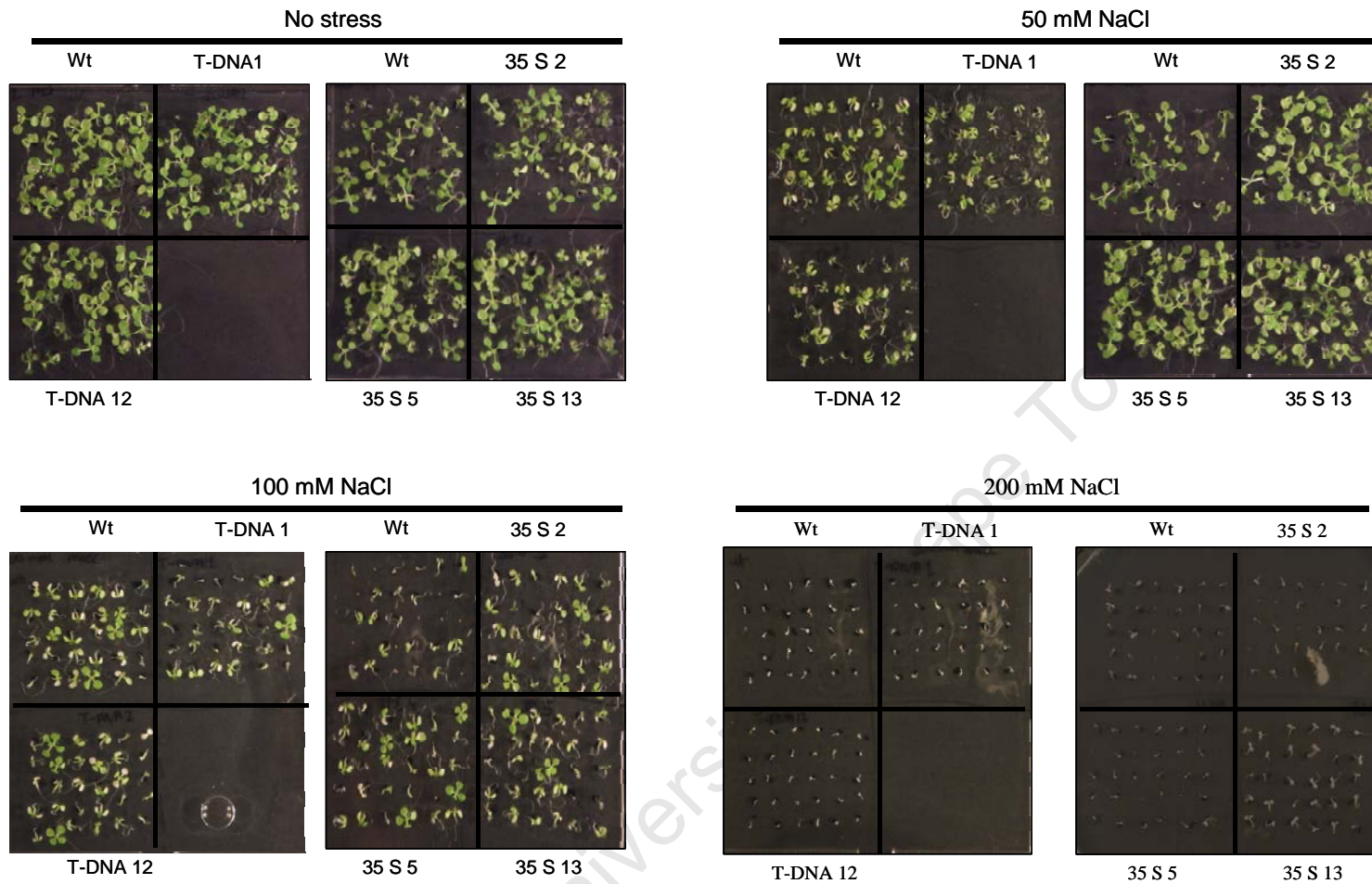


Figure 4.19A. Photographs of two-week-old seedlings of wildtype or At1g07645 T-DNA knockouts (TDNA 1, TDNA12) and RNAi lines silencing At1g07645 (35 S 2, 35 S 5, 35 S 13) germinated on indicated NaCl concentrations.

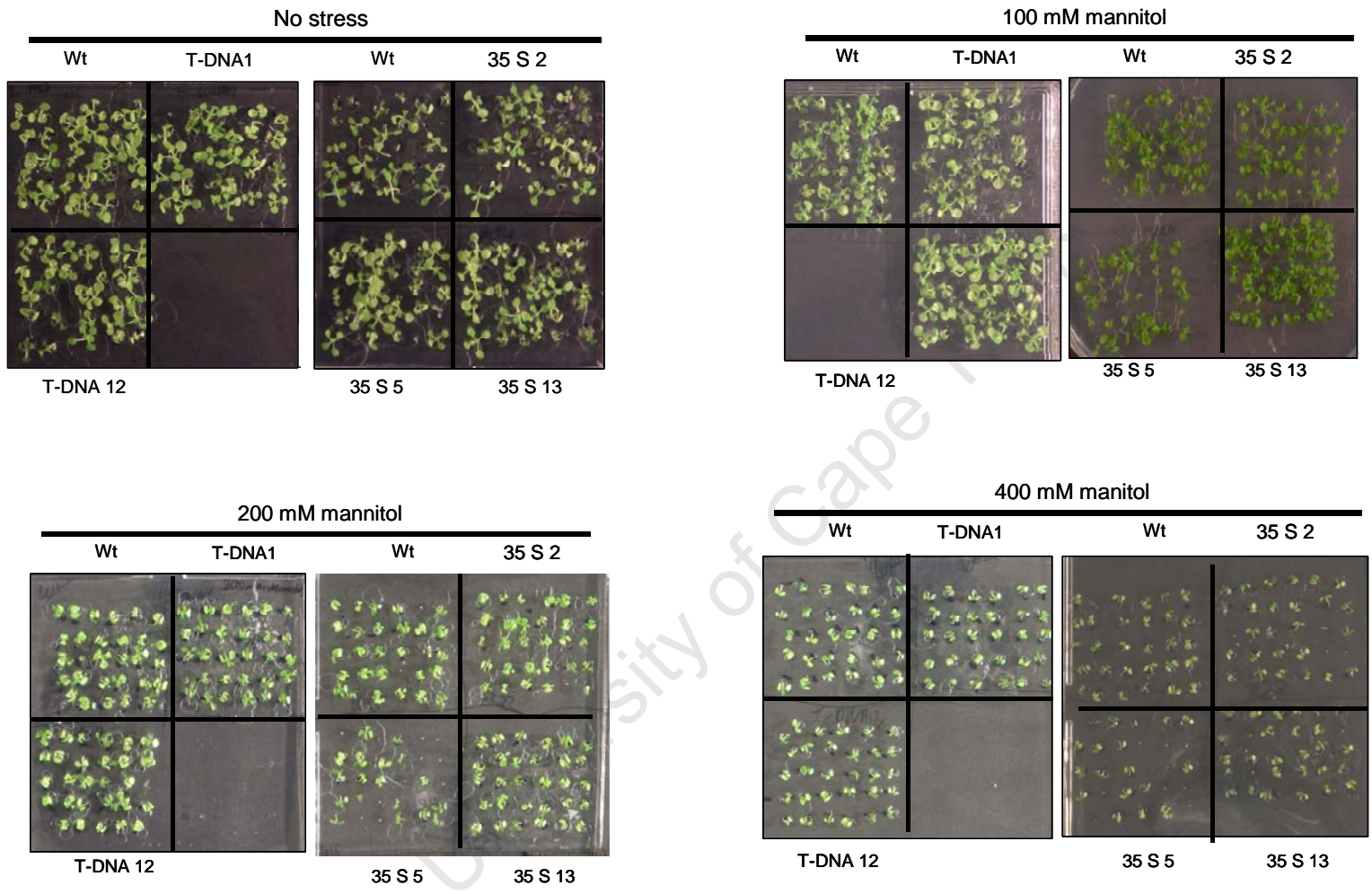


Figure 4.19B. Photographs of two-week-old seedlings of wildtype or At1g07645 T-DNA knockouts (TDNA 1, TDNA12) and RNAi lines silencing At1g07645 (35 S 2, 35 S 5, 35 S 13) germinated on indicated mannitol concentrations.

Wt.



T-DNA 1.



Figure 4.21A. A comparison of green (immature) siliques and seeds of wildtype (wt) and At1g07645 T-DNA1 knockout line.

Wt



T-DNA1



Figure 4.21B. A comparison of brown (mature) siliques and seeds of wildtype (wt) and At1g07645 T-DNA1 knockout line.

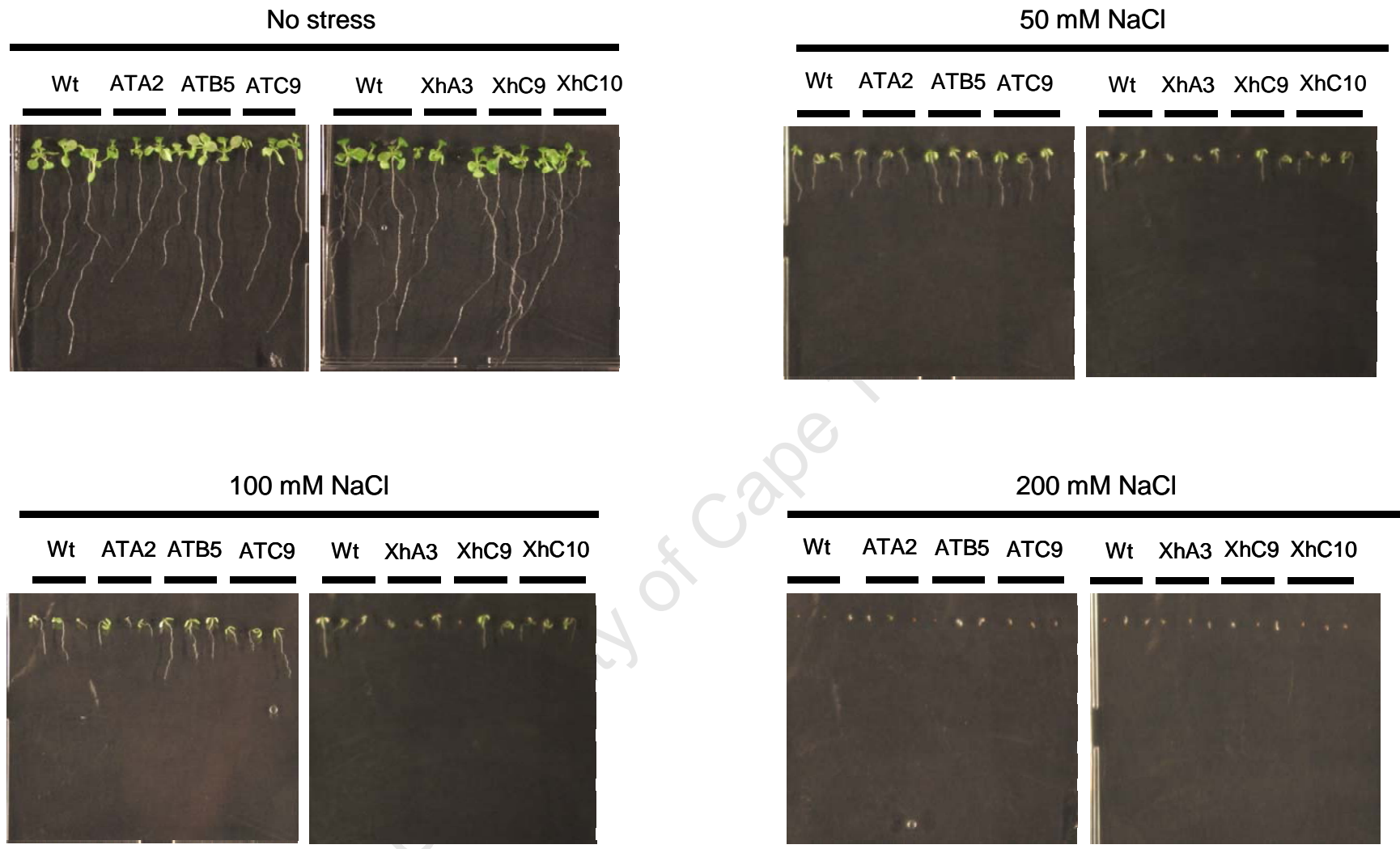


Figure 4.3A. Comparison of root growth of two-week-old wildtype (wt) and transgenic seedlings constitutively expressing Xhdsi-1^{voc} (Xh) and A1g07645 (At) grown in the presence of various concentrations of NaCl.

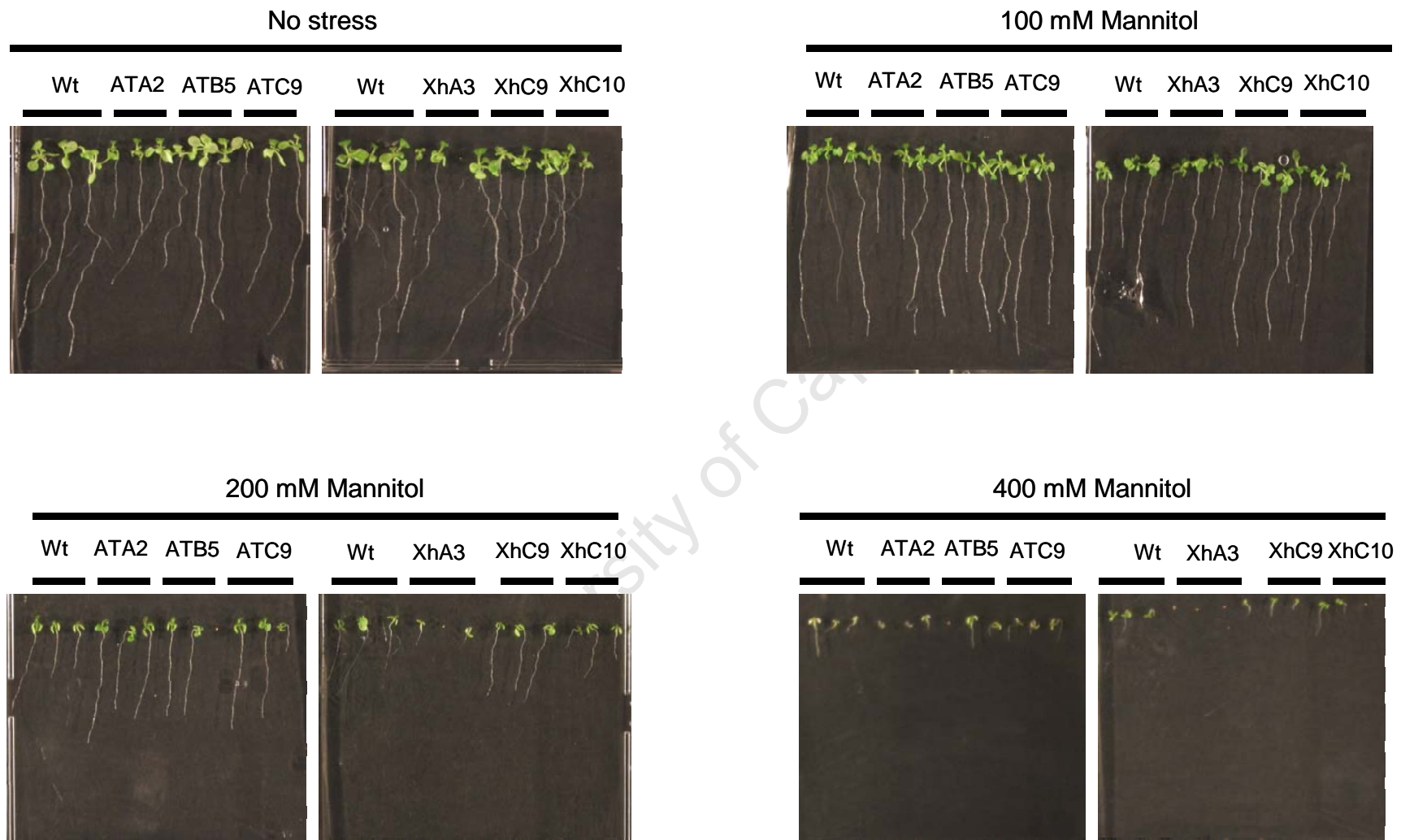


Figure 4.3B. Comparison of root growth of two-week-old wildtype (wt) and transgenic seedling constitutively expressing Xhdsi-1^{voc} (Xh) and A1g07645 (At) grown in the presence of various concentrations of mannitol.

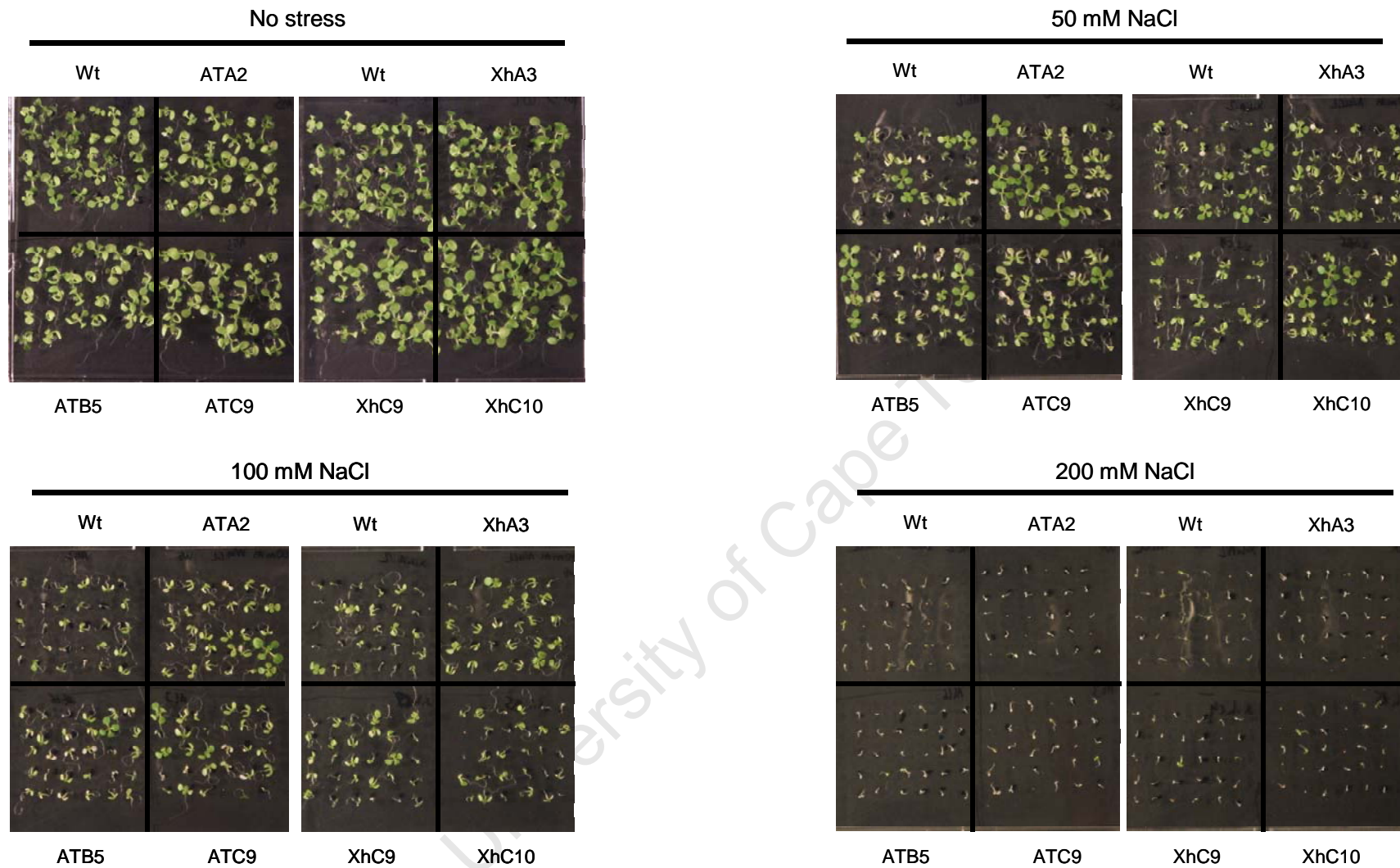


Figure 4.5A. Images of two-week old transgenic wildtype (wt) or transgenic seedlings constitutively expressing *Xhdsi-1^{voc}* (Xh) and *At1g07645* (At) and germinated on half-strength MS containing 0, 50, 100 and 200 mM NaCl concentrations. Thirty seeds from wildtype and thirty from each transgenic line were germinated.

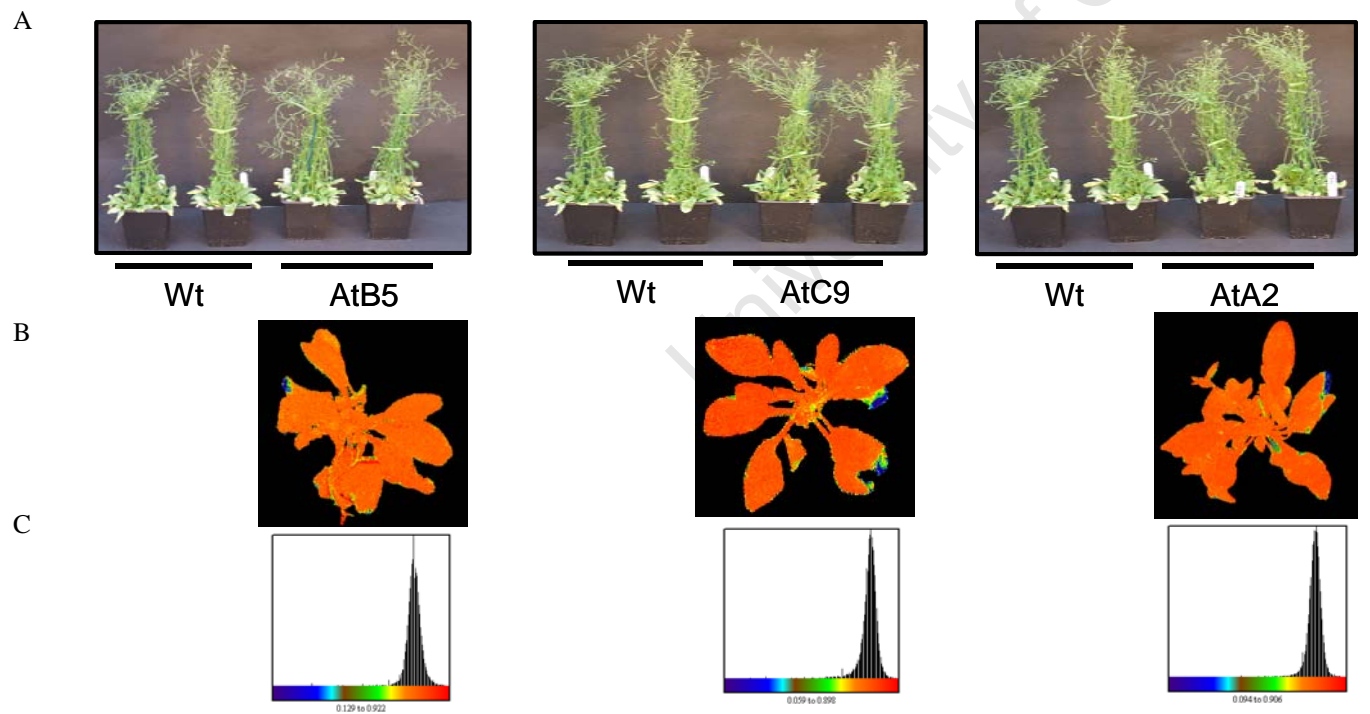
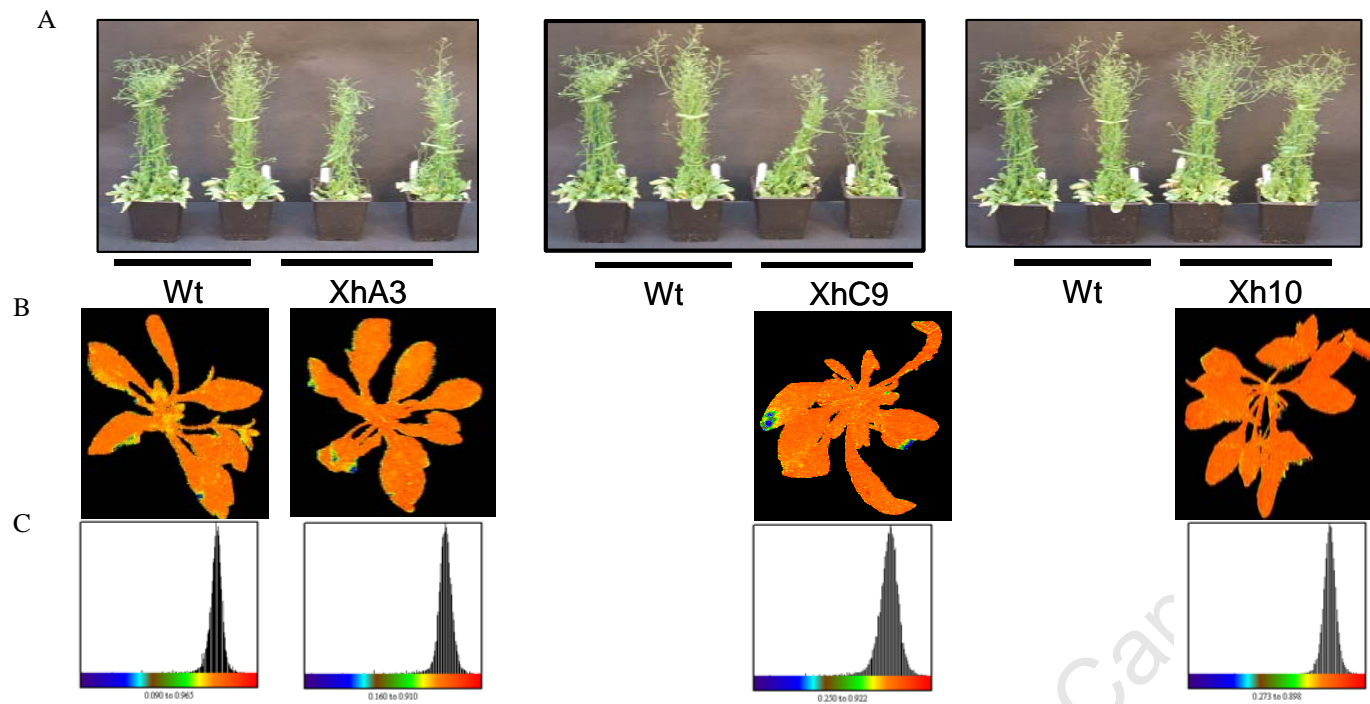


Figure 4.8. Comparison of wild type (Wt) or transgenic plants constitutively expressing $Xhdsi-1^{voc}$ (Xh) and At1g07645 (At) in the absence of stress. A. shows photographs taken eight weeks after germination. B. represents F_v/F_m images of rosette leaves four weeks after germination. C. colour histograms showing F_v/F_m ranging from low (blue) to high (orange).

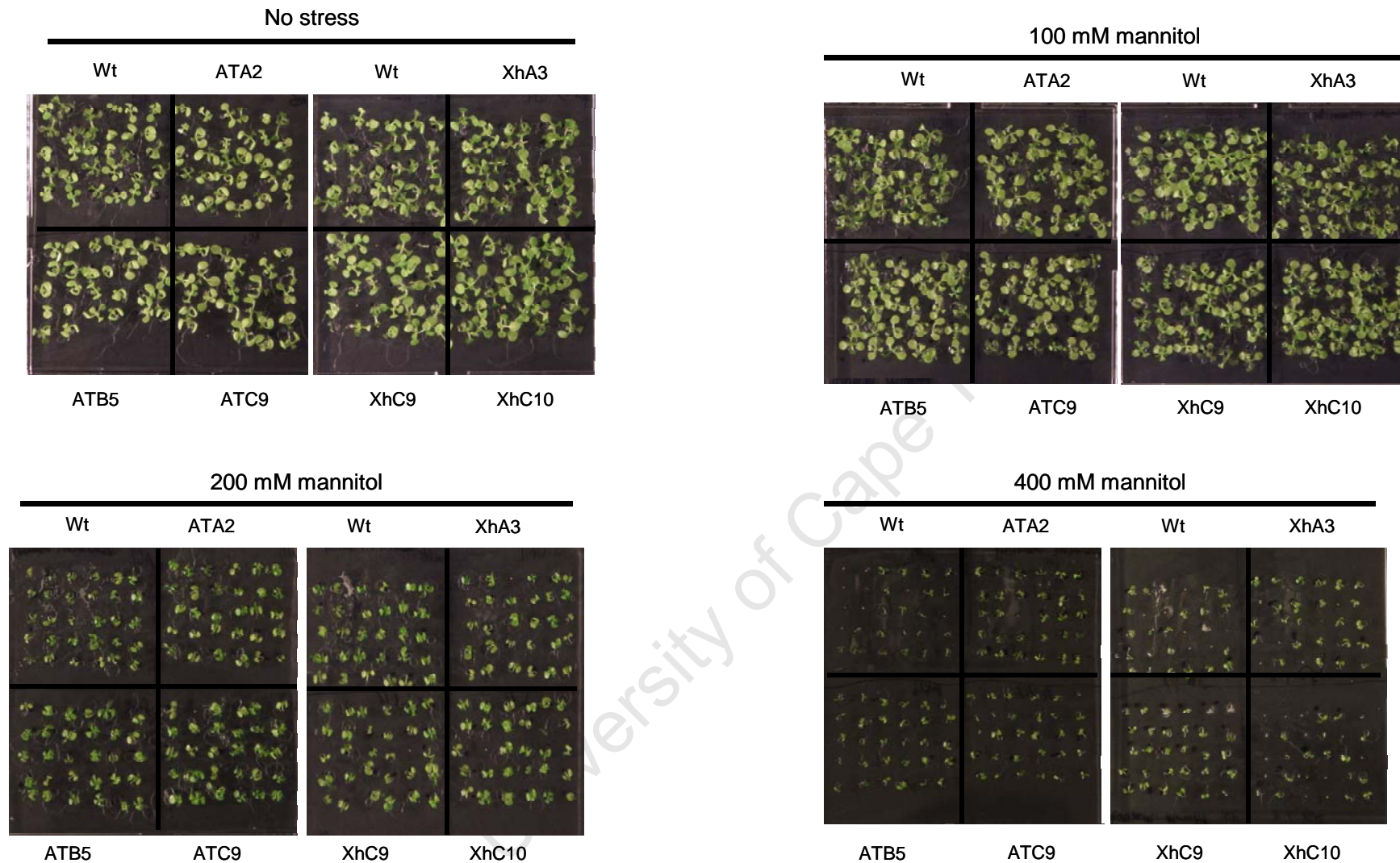


Figure 4.5B. Images of two-week old transgenic wildtype (wt) or transgenic seedlings constitutively expressing *Xhdsi-1^{voc}* (Xh) and *At1g07645* (At) and germinated on half-strength MS containing 0, 100, 200 and 400 mM mannitol. Thirty seeds from wildtype and thirty from each transgenic line were germinated.