The functional characterisation of the XhABFA transcription factor from the resurrection plant *Xerophyta humilis.*

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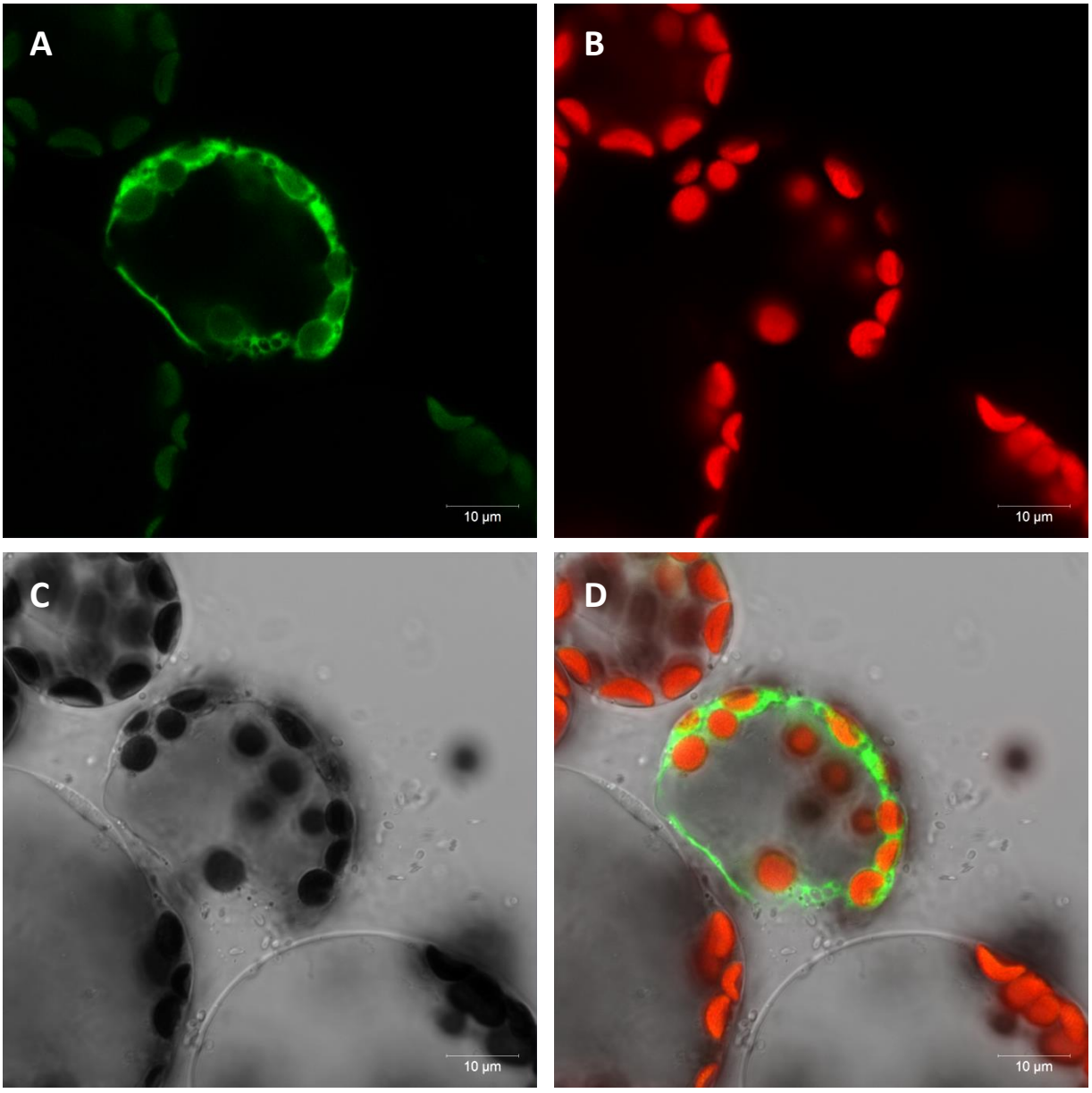
For Kate, who kept me safe.



***Arabidopsis thaliana* protoplast transfected with the pUC19-35S:XhABFA-35S:GFP vector** (A) GFP signal, (B) Chlorophyll autofluorescence, (C) Bright field, (D) Overlay of images A to C.

Plagiarism Declaration

I, Jessica Proctor, know the meaning of plagiarism and declare that all of the work within this thesis is my own, unless otherwise acknowledged.

Abstract

Vegetative desiccation tolerance (VDT), the ability to survive loss of up to 95% of cellular water in leaves and roots, is rare amongst vascular plants. However, the trait has evolved multiple times in a small, diverse group of angiosperms collectively known as “resurrection plants”. The physiological, morphological and metabolic changes that take place during VDT have been well characterised. However, in stark contrast, the underlying regulatory mechanisms that activate the VDT programme are not well understood.

A widely held view is that VDT in resurrection plants may have arisen by a genetic reprogramming of the seed maturation pathways common to the vast majority of angiosperms. In *Arabidopsis thaliana*, the activation of seed maturation genes is regulated by the canonical LAFL (LEC1, ABI3, FUS3 and LEC2) network of transcription factors (TFs). However, thus far there is limited evidence to indicate that the LAFL network itself regulates VDT in resurrection plants, though downstream components of this network (such as the ABI3 regulon) are active. Recently, in a transcriptomic study of the resurrection plant *Xerophyta humilis*, it was found that the LAFL TFs are induced during seed maturation but not during vegetative desiccation. Instead, members of the ABF family of TFs, which are associated with the vegetative abiotic stress response, were strongly upregulated, particularly *XhABFA.* This finding supports an alternative hypothesis: namely that the activation of VDT in *X. humilis* has evolved by the rewiring of the transcriptional network that controls the abiotic drought stress response in desiccation sensitive plants.

The aim of this study was to investigate whether XhABFA is able to bind to the promoters of and activate the expression of three genes, *XhPER1*, *XhECP63* and *XhDSI-1VOC,* which are seed-specific in desiccation sensitive plant species but are upregulated in *X. humilis* leaves as they desiccate. Two experimental approaches were taken in order to determine this: transient expression of XhABFA in *A. thaliana* protoplasts transfected with promoter:firefly luciferase reporter constructs, and a Yeast One-Hybrid analysis. *A. thaliana* protoplasts expressing XhABFA displayed significantly greater firefly luciferase activity than protoplasts transfected with the empty vector, indicating that XhABFA can drive transcription from the promoters of these three canonical seed genes. This is the first evidence of activation of seed-specific genes in desiccating leaves by a “vegetative” abiotic stress TF and suggests that components of the drought stress response may be important in activating VDT in *X. humilis*. This finding may help shed light on our understanding of the regulatory networks underpinning VDT in angiosperm resurrection plants.

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Chapter 1: Hypotheses on the evolution of vegetative desiccation tolerance in higher plants

1.1 An overview of desiccation tolerance

For more than a decade, NASA's strategy for searching for extra-terrestrial life has been to "follow the water" (Bell, 2016). This is because without water, life as we know it would be impossible. But unlike animals, plants are sessile and unable to migrate to areas where water is readily available (Gechev and Hille, 2012). This has resulted in the evolution of mechanisms that plants adopt in response to the environmental water deficits associated with a period of drought. These mechanisms can be broadly categorised into four strategies: drought escape, drought avoidance, drought tolerance and desiccation tolerance (Ludlow, 1989; Kooyers, 2015). To escape, or bypass, drought conditions, plants coordinate their life cycles to occur only when water is available. Examples of this are annual plants, which grow from seed, bloom, produce seeds and die within one growing season; and bulbous plants which regenerate from dormant storage tissue (Kigel et al., 2009; Franks, 2011). Drought avoidance strategies involve the conservation of water and prevention of dehydration. Morphological features which aid in this regard include waxy leaf coatings and the accumulation of large stores of water in fleshy tissues, such as that seen in succulents (Black and Pritchard, 2002). Drought tolerance is the ability of plants to endure low tissue water content through adaptive traits such as stomatal closure and reduction of leaves to spines (Boyer, 1996). The fourth strategy is to employ molecular protective and cellular repair mechanisms to tolerate an almost complete loss of water for extended periods of time (Bewley and Krochko, 1982).

Desiccation tolerance (DT) has been described as the ability of an organism to dry down to a state of equilibrium with the surrounding ambient air and then recover unharmed after rehydration (Jenks and Wood, 2008). This is distinct from drought tolerance. Plants which are drought tolerant are only able to maintain metabolic functions down to relative water contents (RWCs) of about 30%; any lower than this is fatal (Black and Pritchard, 2002). In contrast, DT allows up to 95% of cellular water to be lost (Proctor and Pence, 2002). This trait is commonly seen in basal plants: the liverworts, hornworts and mosses (bryophytes). In angiosperms, flowering plants, DT is typically restricted to the reproductive structures, such as spores, pollen and seeds (Oliver et al., 2000). Most angiosperms do not survive when the RWC of their vegetative tissues (stems, roots and leaves) is below 60% (Giarola et al., 2017). However, a small group of taxonomically diverse plants known as resurrection plants are the exception and are able to tolerate desiccation of their vegetative tissues for prolonged periods without suffering permanent damage (Moore et al., 2008).

1.2 Evolutionary aspects of desiccation tolerance

The land plants that we are familiar with today can be traced back to a common ancestor from a freshwater origin which is thought to have been a type of green algae (Wodniok et al., 2011). The colonisation of the terrestrial habitat occurred about 500 million years ago and was an extremely significant event of plant evolution (Lewis and McCourt, 2004). The transition from freshwater to the vacant land niche required many adaptations in order to survive. Of these, adapting to the lack of

consistently available water was the most crucial and plants had to evolve a multitude of physiological strategies to tolerate desiccation (Lüttge et al., 2011).

The mechanism for DT in the plants which first colonized land is thought to have resembled that seen in modern day bryophytes which is based upon constitutive cellular protection combined with a rehydration-induced recovery process involving cellular repair (Oliver et al., 2007). However, this primitive form of vegetative desiccation tolerance (VDT) is thought to come at a huge metabolic cost and results in slow growth. In addition, it is structurally demanding and therefore requires anatomical simplicity (Lüttge et al., 2011).

As plants became more complex, vascular tissue was developed resulting in the evolution of tracheophytes. These plants acquired efficient mechanisms for internally transporting and conserving water. The early forms of VDT were discarded in favour of increased growth rates and size that these water retention and transport mechanisms afforded (Jenks and Wood, 2008). Even though DT was progressively lost in the vegetative tissues it was retained in the reproductive structures like spores, pollen and seeds (Oliver et al., 2000).

1.3 Vegetative desiccation tolerance

The ability to tolerate desiccation in the vegetative tissues of angiosperms re-emerged independently, multiple times and in a more advanced form. However this trait is scarce and only 135 species (0.04%) out of an estimated 350 000 angiosperm species have evolved this ability (Gaff and Oliver, 2013). This rare group of plants has been termed “resurrection plants”. This originates from the observation that when these plants dry down and reach a desiccated state they appear to be dead (Rhee et al., 2016). The leaves tend to shrivel up due to the loss of cellular water and they lose their green colour as photosynthesis ceases. However, about 24 hours after receiving water the leaves regain turgor and their green appearance, which makes the term “resurrection” appropriate (Lambers et al., 2008). Incredibly, some species of resurrection plants are able to remain desiccated for up to 5 years and still recover (Gaff, 1977).

This feat of tolerating vegetative desiccation is accomplished by the induction of osmoprotectant molecules followed by a quiescent state in which the metabolic activity of the plant appears to cease (Dinakar and Bartels, 2013). This reprogramming of metabolism is systematic and occurs in distinct stages. During the early stages of dehydration responses associated with phytohormones such as abscisic acid (ABA) become prominent (Le et al., 2007; Farrant et al., 2015). There is a controlled shut down of photosynthesis and a range of antioxidant systems are employed to minimise the damage caused by reactive oxygen species (Farrant, 2000). This is accompanied with an increased production in sucrose and oligosaccharides which aid the stabilisation of membranes and macromolecules (Alpert, 2000). The late stages of desiccation are associated with the expression of genes encoding classical stress-associated proteins such as early light-inducible (ELIPs), Late Embryogenesis Abundant (LEAs) and Heat Shock Proteins (HSPs) (Bechtold et al., 2018).

Even though the physiological, morphological and metabolic changes that take place during VDT are relatively well understood, the underlying regulatory mechanisms which control the response remain a mystery. It is likely that the regulation is complex and involves an extensive signalling

cascade of hormones, secondary messengers, kinases, chromatin remodelling and an intricate network of transcription factors (TFs) which ultimately leads to the expression of thousands of desiccation response genes (Bartels et al., 2007; Hilhorst et al., 2018; Neeragunda et al., 2018). However, the key TFs which activate this response are yet to be identified. The prevailing hypothesis to explain the evolution of VDT in angiosperms suggests a co-option of the gene regulatory network responsible for the acquisition of DT in seeds (Illing et al., 2005; Gaff and Oliver, 2013).

1.4 Co-option of the seed maturation network

With the evolution of more complex plants, DT was progressively lost in the vegetative tissues in favour of DT in the reproductive tissues (Oliver et al., 2000). This phenomenon is found in over 95% of vascular plants (Gaff and Oliver, 2013). These seeds, termed "orthodox", which contain DT embryos, can remain viable in the desiccated state for extended periods of time. A remarkable example of this was the germination of a seed of the "Sacred Lotus" which was radiocarbon dated to be 1300 years old (Shen-Miller et al., 1995).

Seed development is an intricate process and can be divided into three major phases (Baud et al., 2002). The first stage is embryogenesis which occurs upon the fertilization of an ovule. During this initial phase cells divide and differentiate to form shoot and root meristems, the embryo axis and cotyledons. The second stage is maturation which is characterized by the synthesis and accumulation of seed storage compounds. The third stage is the acquisition of DT, suppression of precocious germination, and the induction of dormancy (Ni et al., 2016). The ability of seeds to tolerate desiccation involves the expression of a suite of protective molecules. The most noteworthy of these molecules include sugars, antioxidants, heat shock proteins, oleosins and LEA proteins (Farrant and Moore, 2011; Asami et al., 2018).

Many studies which have been done on the mechanisms of DT in angiosperm resurrection plants have found a significant overlap between seed-specific desiccation proteins and those which accumulate during the desiccation response in vegetative tissues of angiosperm resurrection plants (For review see Costa et al., 2017a). These findings have led to the proposition that VDT in resurrection plants arose by a genetic reprogramming of the seed maturation pathways seen in desiccation sensitive (DS) plants (Illing et al., 2005; Shen, 2014). If this is the case then it is important to understand these mechanisms as well as the underlying signalling network which controls these processes.

1.5 The LAFL network: Master regulators of seed development

Seed development is tightly regulated by a set of TFs which together form a complex and intricate network controlling the spatiotemporal expression of hundreds of genes (Lepiniec et al., 2018) In the model plant *Arabidopsis thaliana*, a group of "master" regulators have been identified which include the LEAFY COTYLEDON 1 (LEC1), LEAFY COTYLEDON-Like1 (L1L), ABSCISIC ACID INSENSTIVE 3 (ABI3), FUSCA 3 (FUS3), and LEC2 (Jia et al., 2014). Together these genes comprise the "LAFL" network of TFs.

LEC1 and L1L

LEC1 was one of the first characterized regulators of seed development (West et al., 1994). It is a member of the NUCLEAR FACTOR-Y (NF-Y) protein family. Studies have shown that NF-Y complexes are ubiquitous in eukaryotes and are known to synergistically interact with multitude of distinct TFs to regulate target gene expression (Dolfini et al., 2012). Analyses of interactions among the LAFL TFs suggest that LEC1 acts at the highest level in the regulatory hierarchy controlling seed maturation (Pelletier et al., 2017). LEC1-LIKE (L1L) is the most closely related paralog to LEC1 and in *A. thaliana* it can complement the *lec1* mutation when ectopically expressed under the control of the *LEC1* promoter. However, monogenic loss-of-function mutations in either LEC1 or L1L cause defects in embryo development. These defects have different phenotypes indicating that the endogenous genes cannot substitute one another (Kwong et al., 2003). Although it has been found that the genes have partial overlaps in function, their expression patterns differ (Yamamoto et al., 2009). In *A. thaliana* LEC1 is expressed during the first period of embryonic development whereas L1L is strongly expressed during the maturation phase (Figure 1.1; Boulard et al., 2017).

AFL-B3

ABI3, FUS3, and LEC2, collectively called AFL, belong to the conserved, plant specific, family of B3 domain TFs. Among the 118 B3 domain-containing proteins in *A. thaliana*, the AFL proteins have the most conserved B3 domain and are more similar to each other than to any other members of the superfamily (Swaminathan et al., 2008). This structural similarity can account for the partial functional redundancy existing among the AFL genes and suggests that these factors may have arisen from a common ancestor unique to plants (Roscoe et al., 2015). AFL have several conserved protein domains designated A, B1, B2, and B3 (Han et al., 2017). The A domain is a functional acidic activation domain found at the N-terminus. The B1 domain is involved in the physical interaction with bZIP TFs such as ABSCISIC ACID INSENSITIVE5 (ABI5). The B2 domain has been shown to be important for activating seed maturation genes via the ABA-RESPONSE ELEMENT (ABRE). However this is a weak interaction which most likely requires additional protein-protein interactions. (Ezcurra et al., 2000). The B3 domain acts as the DNA binding domain which recognizes the RY element which is highly represented in the promoters of most seed maturation genes (Guerriero et al., 2009). In terms of temporal expression, LEC2 is predominantly expressed during embryogenesis, while ABI3 and FUS3 peak during the later stages of maturation (Figure 1.1). Analyses have shown that the expression of FUS3 and ABI3 is controlled by a network of partially redundant regulations that involves LEC1 and LEC2. ABI3 and FUS3 also regulate their own transcription through positive regulatory feedback loops (Boulard et al., 2017). In a study by Mönke et al., 2012, the targets of the ABI3 TF were identified in *A. thaliana*. These 98 genes, which included other seed maturation TFs such as FUS3 and LEC2, were termed the “ABI3 regulon”.

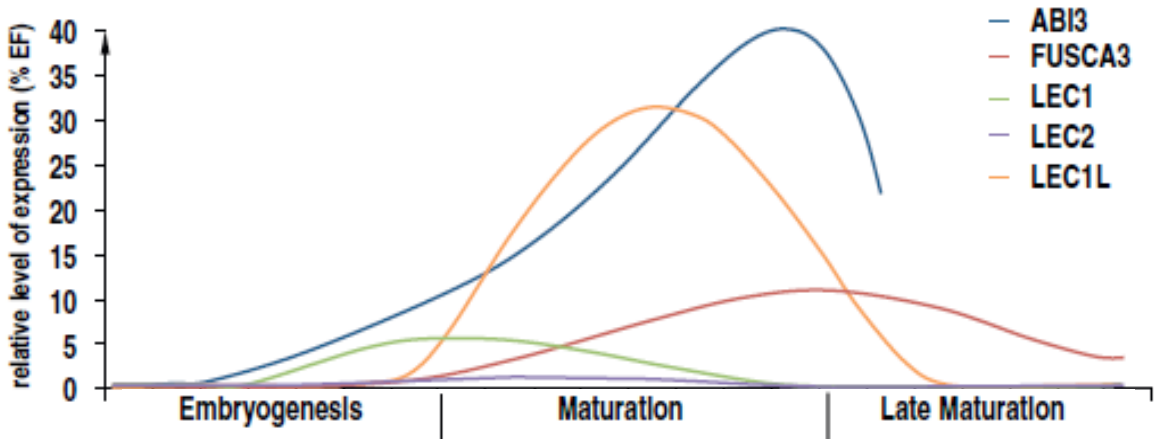


Figure 1.1: Expression of the LAFL transcription factors during seed development of *Arabidopsis thaliana* (Adapted from Boulard et al., 2017).

Functional redundancy, synergy and specificity

Genetic analyses have revealed that the LAFL proteins show similar and overlapping functions which could be explained by synergism and redundancy. For example, the initiation of storage product synthesis and the establishment of embryo morphology were effectively complemented by ectopic expression of LEC1, FUS3, or ABI3 in single or double mutant backgrounds of the other two regulators (To et al., 2006). However, this complementation was not seen in the establishment of DT suggesting that the combined synergy of all three regulators is required for the seed to acquire DT (González-Morales et al., 2016). Although the role of these master regulators during seed maturation is globally similar, some of their functions are very specific. For example ABI3 prevents chlorophyll accumulation, FUS3 prevents anthocyanin expression and LEC1 and LEC2 control the initiation of somatic embryogenesis (Braybook and Harada, 2008; Delmas et al., 2013).

Secondary regulators as targets of the LAFL network

The LAFL network activates the expression of many functional genes important for seed maturation, including seed storage proteins (e.g. cruciferins and napins), proteins involved in oil storage (e.g. oleosins) and proteins which confer DT (e.g. LEAs) (Figure 1.2; Fatihi et al., 2016). However, as master regulators, downstream targets also include other TFs. Among the important secondary regulatory targets are WRINKLED 1 (WRI1) and ABSCISIC ACID INSENSITIVE 5 (ABI5) (Figure 1.2). WRI1 belongs to the AP2 family of TFs and is a direct target of LEC2. It has been demonstrated that WRI1 is necessary for the regulation of oil biosynthesis by LEC2 (Baud et al., 2007). ABI3 regulates ABI5 which as a bZIP TF whose expression is in turn essential for the expression of the LEA genes *AtEm1* and *AtEm6* as well as the desiccation-responsive *rd29A* (Lopez-Molina et al., 2002; Nakashima et al., 2006). In addition to WRI and ABI5 there are other TFs involved in the seed development network which act as protein partners or direct target genes of the LAFL TFs. These include members of the MYB, MADS, DOF, HD-ZIP and AP2 families (Fatihi et al., 2016).

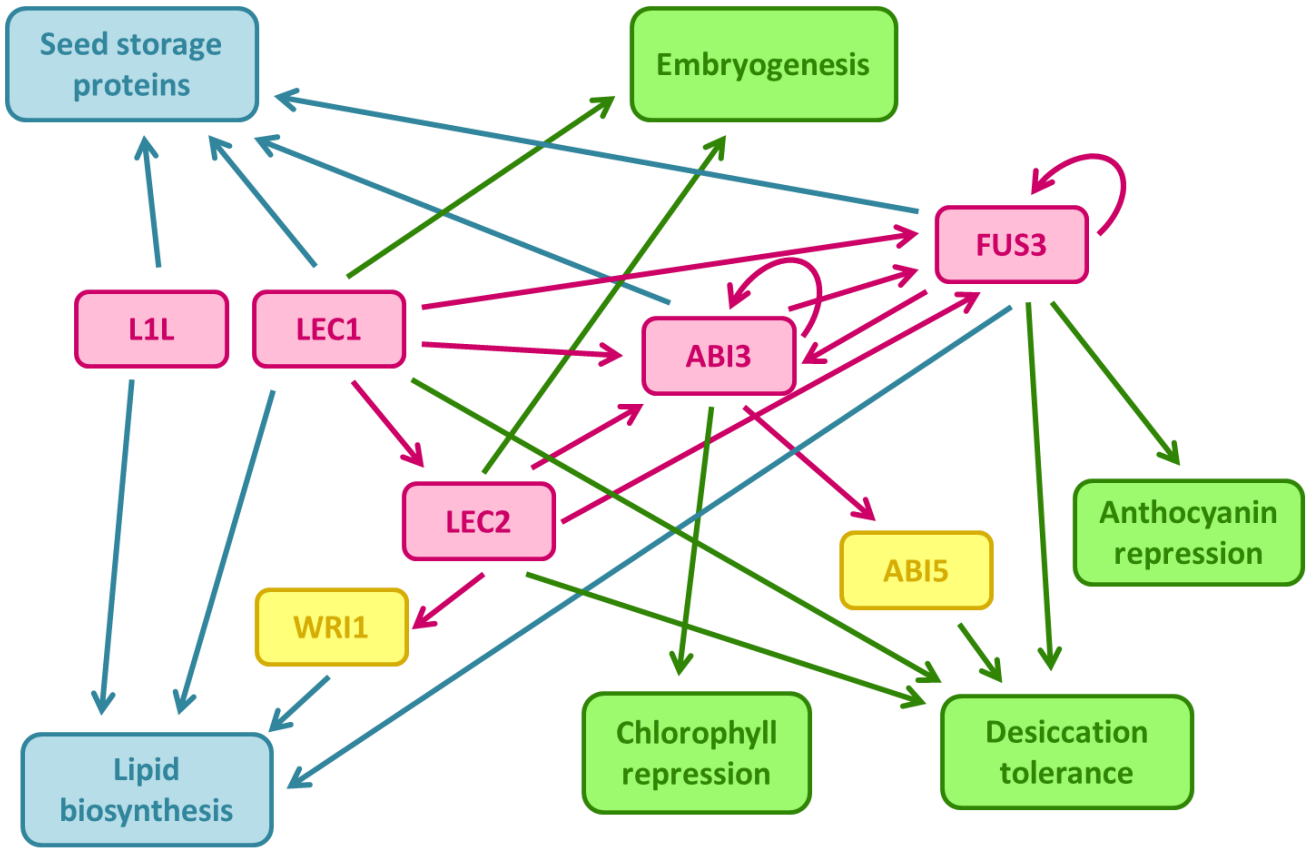


Figure 1.2: Schematic representation of the gene regulatory network controlling seed maturation. Core LAFL network represented in pink; downstream target TFs represented in yellow; reserve stocks represented in blue; seed development processes represented in green. (Adapted from Santos-Mendoza et al., 2008; Fatihi et al., 2016; Boulard et al., 2017)

1.6 A window of desiccation tolerance in germinating seedlings

The switch from embryogenesis to seed maturation is induced by the plant hormone abscisic acid (ABA). When seed maturation is complete and conditions are favourable for growth, genes involved in ABA biosynthesis are repressed and genes involved in the biosynthesis of gibberellic acid (GA) are upregulated. The balance between these two hormones dictates the transition into germination (Karssen and Lacka, 1986). When GA increases and ABA decreases, a massive reprogramming of the transcriptome occurs resulting in the induction of germination. This transition requires the LAFL network to be completely repressed (Jia et al., 2014). However, the LAFL network can be reactivated for a brief period if the embryo is stressed. Following germination, a narrow developmental window exists, during which plants monitor the environmental osmotic status before initiating vegetative growth. If a deficit of water occurs during this window, the germinated embryo enters a quiescent state where it re-acquires DT. This process requires the ABI3-mediated re-activation of ABI5 (Lopez-Molina et al., 2001). Together they initiate the upregulation of a similar set of genes to those activated during seed maturation (Dekkers et al., 2015).

In DS plants, this window is transient. When vegetative growth is resumed, the genes required to establish VDT are permanently silenced and the plant is no longer able to tolerate desiccation in non-seed tissues (Maia et al., 2014). A possible mechanism by which VDT evolved, is that this silencing does not occur in angiosperm resurrection plant seedlings. A study on the resurrection plant *Xerophyta viscosa* investigated whether seedlings at different stages of germination could survive desiccation. While seedlings displayed a transient decline in DT during germination, at no point was the ability to re-establish DT completely lost. This suggests that the window of DT does not close and the ability to induce VDT is retained indefinitely post germination (Lyall et al., 2014).

1.7 Investigations into whether VDT is regulated by the seed maturation transcriptional network

It is becoming increasingly apparent that we are moving into the "omics" era. "Omic" level studies include genomics, transcriptomics, proteomics and metabolomics. Respectively, they provide insights into the genes, transcripts, proteins and metabolites that form part of any given response. "Omic" technologies offer high throughout methods which will allow us to understand the global level changes associated with the VDT response. Thus far, 21 omic studies on angiosperm resurrection plants have been published (Figure 1.3). A number of these studies have found evidence that transcripts of many seed maturation genes accumulate at high levels during vegetative desiccation in resurrection plants, including *X. viscosa, X. humilis, Oropetium thomaeu* and *Lindernia brevidens.*

In a recent comparative study of the DT *Lindernia brevidens* and its DS sister species *Lindernia subracemosa,* the authors explored genomic and transcriptomic differences associated with VDT between the two *Lindernia* species (VanBuren et al., 2018). In *L. brevidens,* gene families associated with the desiccation response, in particular ELIPs, displayed a dramatic expansion compared to *L. subracemosa* . The authors analysed the transcriptomes of both species over a time course of dehydration, desiccation and rehydration. They found that many LEAs exhibited significantly higher expression in *L. brevidens* compared with their orthologous in *L. subracemosa*.

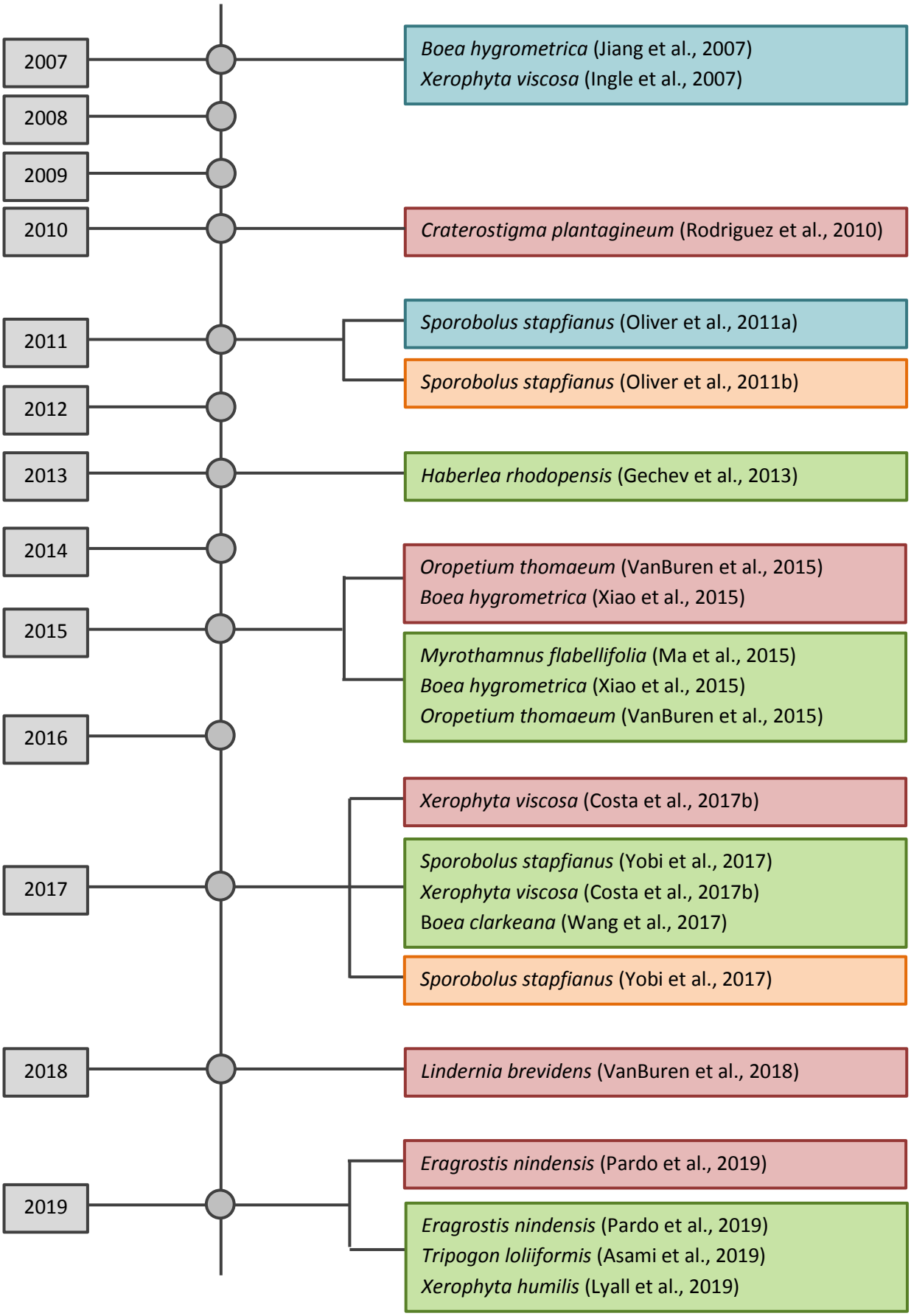


Figure 1.3: Timeline of "omic" studies on resurrection plants. Over the last 13 years there have been 21 omic studies that have been published on 12 different angiosperm resurrection plants. Of these studies, 6 have been genomic (red), 10 transcriptomic (green), 3 proteomic (blue) and 2 metabolomic (orange).

A genomic and transcriptomic study on *X. viscosa* identified that homologues of the majority of the ABI3 regulon were expressed in desiccating leaves (Costa et al., 2017b). The *X. viscosa* genome contained two homologues of ABI3 itself, however they were not upregulated during desiccation. In the resurrection grass *Oropetium thomaeum,* two ABI3 homologues were found to be expressed during the vegetative desiccation response, however they were only upregulated 2-fold (Table 1.1). In contrast, homologues of other seed-specific TFs exhibited far higher levels of transcript accumulation. These TFs included DELAY OF GERMINATION 1 (DOG1), REDUCED DORMANCY 1 (RDO1), EMBRYO DEFECTIVE 1 (EMB1) and GEM-RELATED 5 (GER5) but crucially not the LAFL TFs (VanBuren et al., 2017).

***O. thomaeum* gene**	***A. thaliana* homologue**	**RPKM**	
		Well-watered	**Desiccated**
Oro_25777	ABI3	0.551236	1.06822
Oro_13163	ABI3	0.172961	0.379247
Oro_12581	DOG1	0	1.31681
Oro_07175	RDO1	1.28443	14.9074
Oro_00118	EMB1	0.0874373	11.9757
Oro_26573	GER5	6.49105	1196.36

Table 1.1: Expression patterns of seed-specific TFs during vegetative desiccation in *O. thomaeum.* Homologues of ABI3 had a much lower fold change in expression compared to other seed-specific TFs such as RDO1, EMB1 and GER5. (Adapted from VanBuren et al., 2017).

In addition to the limited evidence which indicates that the LAFL network is upregulated during VDT in angiosperm resurrection plants, knowledge regarding the regulation of seed maturation in resurrection plants is minimal. It has been assumed that the canonical pathways which control seed maturation in DS plants have been conserved in DT plants. And that it is this canonical LAFL network that is responsible for regulating the vegetative desiccation response. Therefor further enquiry into the hypothesis that VDT is regulated by the LAFL network requires investigations into both seeds as well as the vegetative tissue of resurrection plants.

As a step towards investigating the hypothesis that VDT evolved from the activation of seed maturation genes by the LAFL TFs, the transcriptome of the resurrection plant *Xerophyta humilis* was assembled from RNA-Seq expression data collected during both seed maturation and vegetative desiccation (Lyall et al., 2019). Of the *X. humilis* genes that were differentially expressed, 46% were expressed in drying leaves only, 25% in maturing seeds only and 29% during both processes (Figure 1.4).

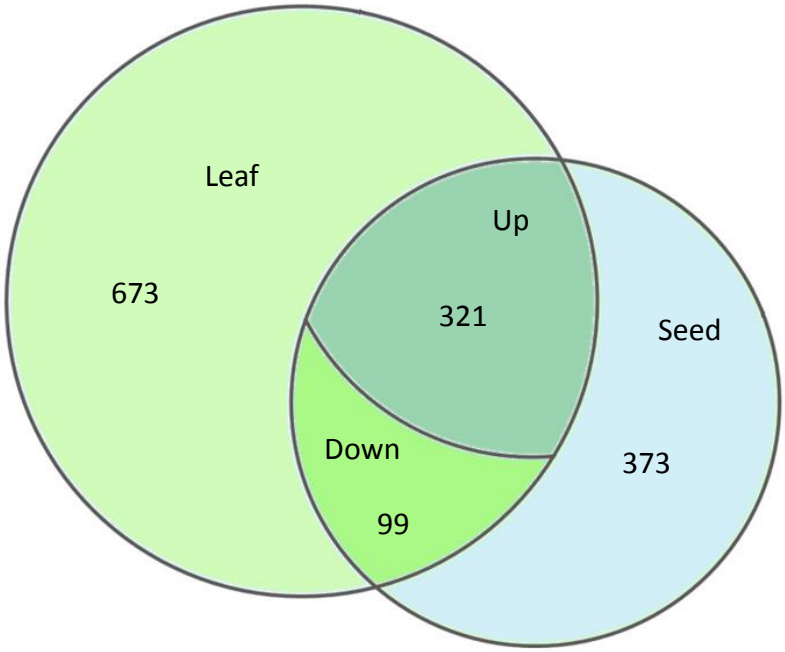


Figure 1.4: Number and overlap of genes differentially expressed during VDT and seed maturation. A total of 14 678 *X. humilis* genes were differentially expressed. Gene sets that were differentially expressed in both leaf and seed tissues are shown in darker shades of green and blue. Genes are grouped according to the shared direction of regulation during progressive vegetative desiccation and seed maturation (Adapted from Lyall et al., 2019).

Of the genes that were differentially expressed in both seed and leaf, 76% showed the same direction of expression: either upregulated in both tissues or downregulated in both. Further analyses of these genes supported previous observations that seed-specific genes are expressed during VDT. Of the 289 *A. thaliana* genes that have been identified as being seed-specific (Le et al., 2010), 204 homologues were differentially expressed in *X. humilis*, with 27% common to both seed and leaf. In addition to this, many *X. humilis* homologues to the ABI3 regulon, the set of genes ABI3 is known to regulate, were identified (Mönke et al., 2012). 84% were upregulated in seeds and nearly 60% were upregulated in the leaves. This overlap suggests that common pathways may exist in the transcriptional reprogramming that occurs during VDT and seed maturation in *X. humilis.*

However, enquiry into whether these common pathways are regulated by the LAFL network found conflicting results. One or more co-orthologues to each of the LAFL TFs, as well as the downstream ABI5, were identified. These orthologues were active during seed maturation however they were barely expressed during VDT. These results indicate that the role of the LAFL network during seed maturation in *X. humilis* is consistent with its role in DS plants. However its absence during VDT suggests that an alternative regulatory network controls this process.

Four ABI3 orthologues were identified in both *X. viscosa* and *X. humilis* as seen in Figure 1.5. *XhABI3A* contained all four conserved protein domains, while the other three (*XhABI3B-XhABI3D*) lacked the conserved B3 DNA binding domain. While *XhABI3A* was expressed at high levels during seed maturation, only transcripts for *XhABI3B* were significantly upregulated during vegetative desiccation. This was an interesting discovery because ABI3 typically activates its target genes via the

binding of its B3 domain to the RY element in the promoter of the target gene. However, since *XhABI3* lacked a B3 domain it would be unable to bind to a RY element.

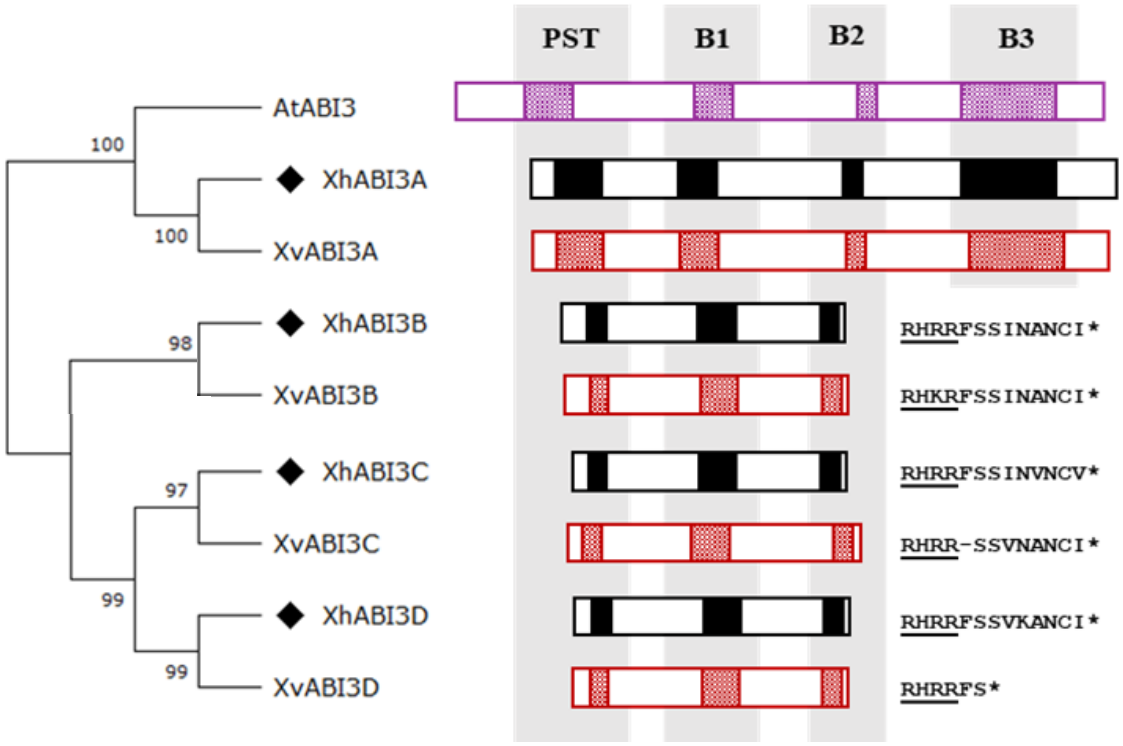


Figure 1.5: Duplication of *ABI3* in *Xerophyta* and loss of the B3 domain. Maximum-likelihood phylogenetic tree showing the relationship between *AtABI3* and the predicted *ABI3* orthologues in *X. humilis* and *X. viscosa.* Positions of the conserved Pro-, Ser- and Thr-rich (PST) region, B1, B2 and B3 domains are given by shaded segments. The conserved stop codon sequences for the *Xerophyta*-specific *ABI3B, ABI3C* and *ABI3D* genes are shown on the right, with the terminal region of the B2 domain underlined. Protein alignments and phylogenetic analysis performed using Clustal Omega and RAxML. (Reproduced from Lyall et al., 2019).

TF binding site enrichment analyses were performed on the ABI3 regulon promoters from *A. thaliana* and *Xerophyta viscosa* (a sister species of *X. humilis* which has a published genome assembly). It was found that in *A. thaliana* the promoters of these genes were enriched for the RY as well as the ABRE element. However the *Xerophyta* promoters displayed enrichment for the ABRE but not the RY element. In addition to being linked to promoters that are activate during seed maturation, the ABRE elements play a central role in co-ordinating the response of DS plants to abiotic stress. An alternative hypothesis to the evolution of VDT, is that the abiotic stress TFs have been rewired to activate seed maturation genes in leaves in *Xerophyta* in response to desiccation.

1.8 Adaptation of the signalling network which regulates the abiotic stress response

As an initial step to determining the validity of this hypothesis, a comprehensive understanding of the regulatory mechanisms underlying abiotic stress is required. In DS plants, the propagation of the abiotic stress response can be broadly classified into pathways that are either ABA dependent or independent (Yoshida et al., 2014).

ABA dependent pathways

ABA is the predominant phytohormone involved in the induction of stress-responsive genes (Matsui et al., 2008). However, before ABA dependent transcriptional pathways are activated, ABA interacts with various proteins which initiate the signalling transduction. ABA signalling involves five core components: ABA receptors, negative regulators, positive regulators, ABA-responsive TFs and ABA-responsive genes (Hauser et al., 2011). Three main ABA receptors have been identified which together form a complex: Pyrabactin Resistance Protein1 (PYR1), PYR1-Like proteins (PYLs) and Regulatory Components of ABA Receptor (RCARs). Negative regulation is carried out by the dephosphorylating activity of type 2C protein phosphatases (PP2Cs). In contrast, the phosphorylation activity of sucrose non-fermenting 1 (SNF1)-related protein kinases 2 (SnRK2s) results in the positive regulation of various downstream effectors (Umezawa et al., 2013). These targets include ion channel proteins (Brandt et al., 2012), NADPH oxidases (Sirichandra et al., 2009), a chromatin-remodelling factor (Peirats-Llobet et al., 2016) and the basic leucine zipper class of TFs (Helander and Cutler, 2018). Figure 1.6 illustrates the interactions between the core components involved in ABA signalling. This core pathway is highly conserved in land plants and seems to have emerged with the evolutionary transition of plants from water to land (Hauser et al. 2011).

ABI5 and ABF transcription factors

Among the best known positive effectors of ABA signalling and the key targets of SnRK2s are members from the basic region/leucine zipper (bZIP) family of TFs (Antoni et al., 2011). *A. thaliana* currently has 78 members that have been subcategorised into 13 groups (Dröge-Laser et al., 2018). Group A bZIPs recognize the ABA-responsive element (ABRE) in the promoters of ABA-inducible genes (Choi et al., 2000). ABI5 is a member of group A and, as mentioned previously, plays a crucial role during the late stages of seed maturation and the early stages of germination (Bensmihen et al., 2005). The ABRE binding factors (ABFs), also known as ABRE Binding Proteins (AREBs), are the prominent subgroup which implement adaptive responses to counteract water deficits in the vegetative tissues of DS plants (Nakashima and Yamaguchi-Shinozaki, 2013). Thus far, nine ABF TFs have been identified, of which four (ABF1-4) are induced by osmotic stress (Yoshida et al., 2015). Overexpression of ABF2, ABF3 and ABF4 in transgenic *A. thaliana* plants showed ABA hypersensitivity and enhanced drought tolerance (Kang et al., 2002). Loss-of-function analyses have shown that the *abf1/abf2/abf3/abf4* quadruple mutant exhibited increased ABA insensitivity and decreased tolerance to drought stress when compared with single, double and triple ABF knockout mutants (Yoshida et al., 2015). Transcriptome analysis revealed that downstream genes of these ABF TFs overlap and include many classic drought response genes such as LEA proteins (Yoshida et al.,

2010). These independent analyses imply that these four ABFs are master transcriptional activators that cooperatively regulate ABRE-dependent gene expression in ABA signalling under drought stress conditions.

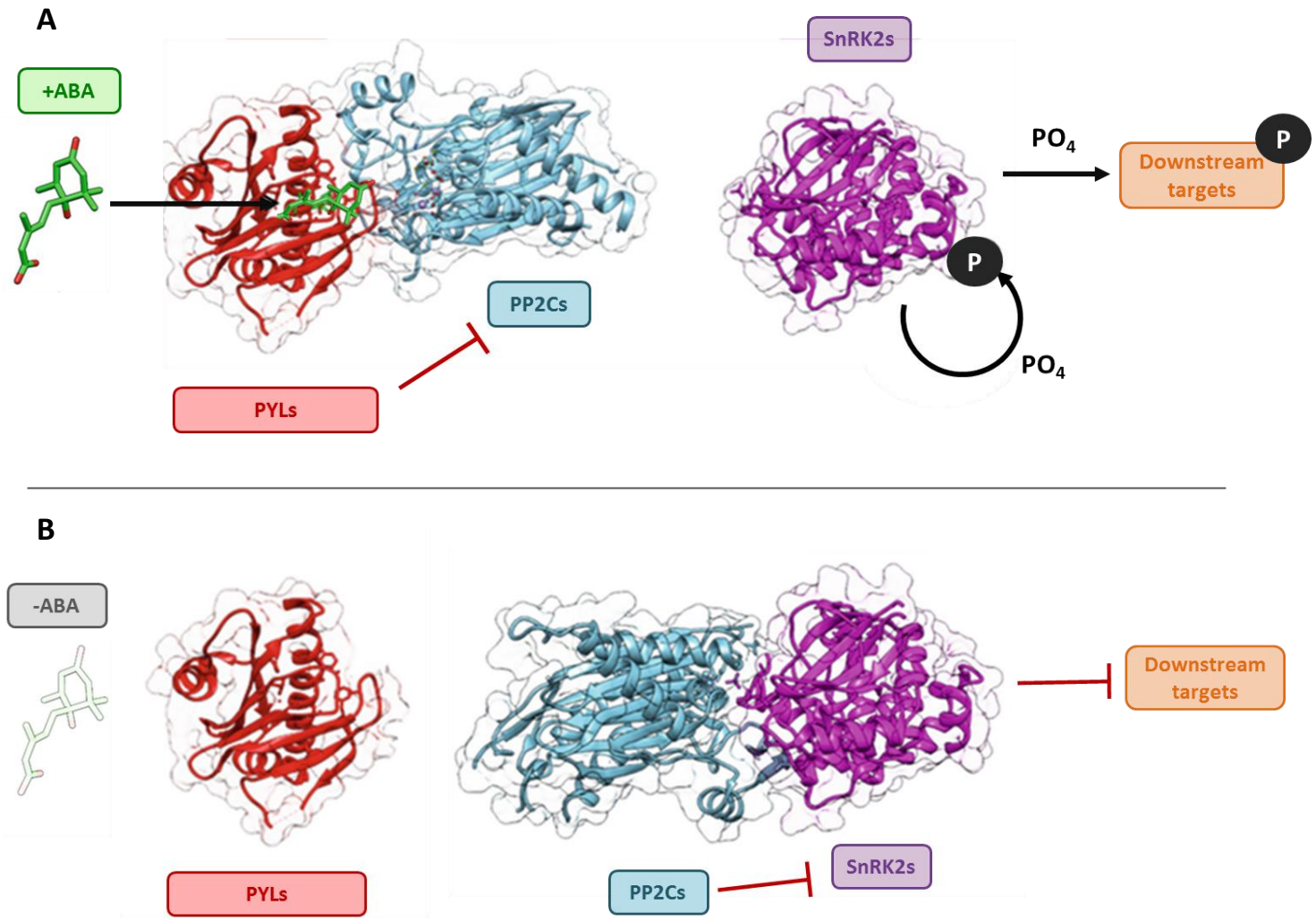


Figure 1.6: Illustration of the core ABA signalling pathway. Panel A – In the presence of ABA, a complex is formed between ABA, PYLs and PP2Cs which supresses the dephosphorylating activity of PP2Cs. This relieves the inhibition of SnRK2s. The released SnRK2s are activated by autophosphorylation allowing them to phosphorylate downstream targets resulting in the induction of the ABA-responsive pathway. Panel B – In the absence of ABA, PP2Cs bind directly to and dephosphorylate SnRK2s, suppressing their kinase activity and therefore blocking ABA signalling. (Adapted from Helander and Cutler, 2018)

Conservation

They are also highly conserved across plant species. In addition to *A. thaliana*, homologues of ABF and ABI5 subfamily bZIP TFs have been identified and analysed in a multitude of monocot plants including rice (Xiang et al., 2008), tobacco (Sano and Nagata, 2002), maize (Yan et al., 2012), wheat (Kobayashi et al., 2008), barley (Schoonheim et al., 2007) and soybean (Gao et al., 2011) and dicot plants including tomato (Orellana et al,. 2010), orange (Huang et al., 2010) and sugar beet (Schmidt et al., 2008). In these studies overexpression of these TFs conferred increased drought tolerance in many of the transgenic plants. This high conservation of functionality highlights how crucial bZIP TFs are in the response to water deficits in higher plants. The regulatory roles of bZIP TFs are complex and involve synergy with TF families as well as crosstalk with ABA-independent pathways (Skubacz et al., 2016).

ABA independent pathways

In addition to ABA, phytohormones such as jasmonic acid (JA), cytokinins, brassinosteroids and salicylic acid are also involved in the drought stress response (Wani et al., 2016). Genes which respond independently of ABA have been found to also play a crucial role during drought stress (Shinozaki et al., 2000). Proteins which belong to the APETALA2 (AP2) family of TFs are the primary regulators of ABA independent genes during drought stress (Roychoudhery et al., 2013). The promoters of these genes contain the dehydration response element (DRE) which is activated (bound) by the DEHYDRATION-RESPONSIVE ELEMENT BINDING (DREB) proteins (Yamaguchi-Shinozaki and Shinozaki, 1994). Of the DREB protein family, DREB2A is a pertinent role player in the drought response (Nakashami et al., 2000). The TF has been shown to activate genes encoding LEA proteins, sugar metabolism enzymes and heat-shock proteins (Mizoi et al., 2012). Interestingly DREB2A also targets other TFs which are members of a group of AP2-type repressors. This suggests the existence of a negative feedback loop downstream of DREB2A (Kuromori et al., 2015). To date, DREBs have been isolated from numerous plants, including eudicots such as tomato (Islam and Wang, 2009), monocots such as maize (Qin et al., 2007) even from a moss (Liu et al., 2007). The existence of these TFs in phylogenetically divergent species indicates the importance of ABA independent pathways in the stress response of land plants (Mizoi et al., 2012).

Crosstalk between ABA dependent pathways and ABA independent pathways

Recent studies have revealed that the transcriptional network activated by drought stress does not only involve distinct ABA dependant and ABA independent responses, but is also composed of elaborate crosstalk between these two major types of pathways (Figure 1.7; Yoshida et al., 2014). This crosstalk occurs at various different levels starting from the initial hormonal responses. Components include hormones other than ABA, secondary messengers, elements of the core ABA signalling network and TFs (Knight and Knight, 2001).

It has been proposed that SnRK2s may also function as a convergence point between ABA dependent and ABA independent pathways (Fujita et al., 2013). An initial study found hyperosmotic stress activated nine *A. thaliana* SnRK2s of which only five of them were also activated by ABA

(Boudsocq et al., 2004). Further studies demonstrated that SnRK2s were activated by osmotic stress in ABA-deficient and ABA-insensitive mutants (Boudsocq et al., 2007).

The presence of both ABRE and DRE binding elements in the promoters of some drought-induced genes, such as RD29, suggests that there are shared components that can interact with either ABA dependent or ABA independent pathways (Roychoudhury et al., 2013). A member of the WRKY family, WRKY40, has been shown to bind to promoters of DREB2 TFs, which are associated with ABA independence, as well as MYB2 and bZIPs which are associated with ABA dependence (Chen et al., 2012).

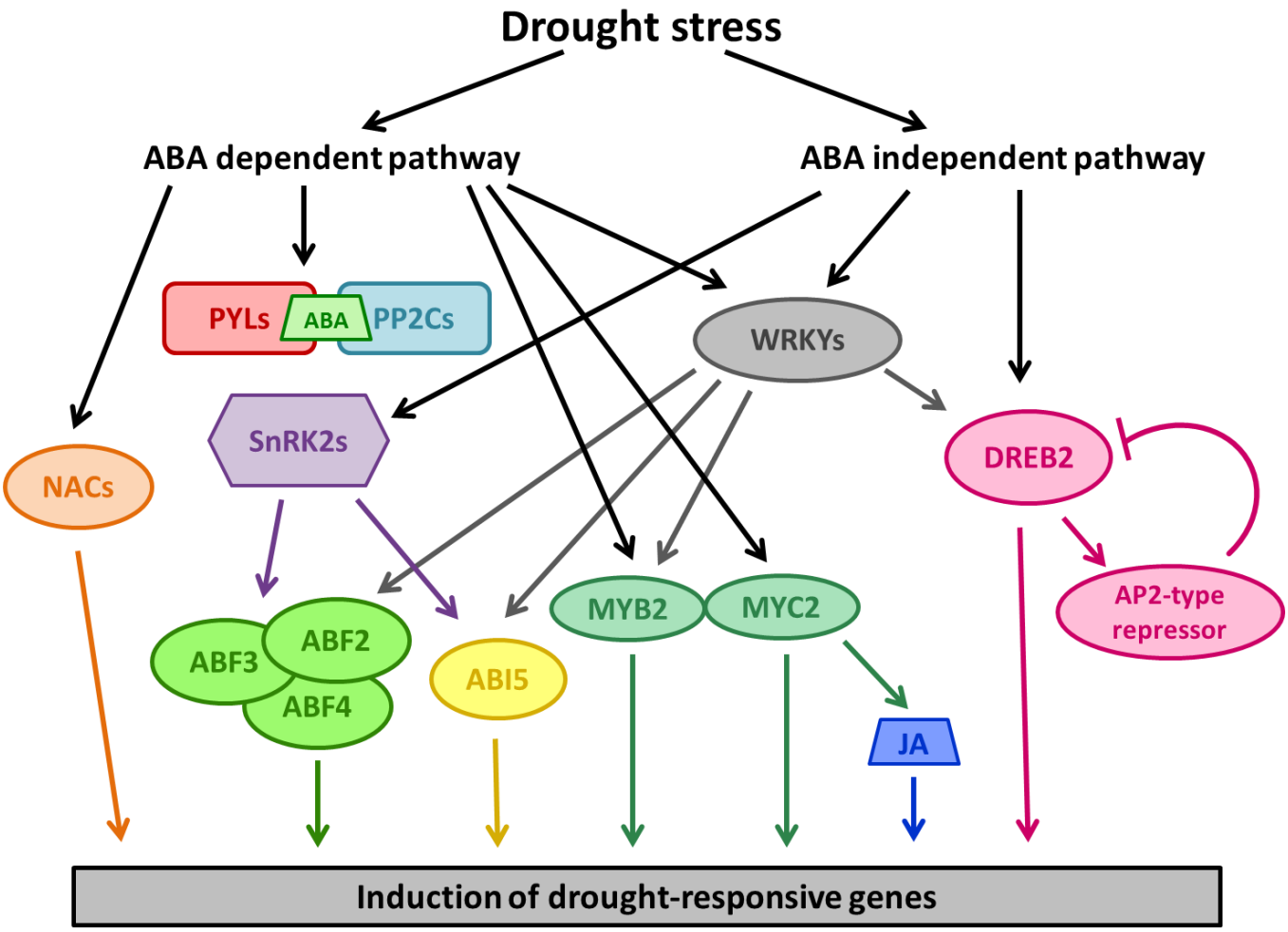


Figure 1.7: Major transcriptional regulatory networks involved in the drought-stress response. Drought signal perception leads to activation of both ABA dependent and ABA independent pathways. In the ABA dependent pathway, accumulation of ABA leads to activation of SnRK2s. Downstream targets of SnRK2s are the ABF and ABI5 TFs. In addition to this, ABA also modulates the activity of MYB/MYC and NAC TFs. MYC2 proteins are also involved in JA signalling. In the ABA independent pathway, DREB2 TFs play the most predominant role. They participate in a negative feedback loop with an AP2-type repressor. WRKY proteins are implicated in both the ABA dependent and ABA independent pathway.

1.9 Expression of ABF transcription factors during VDT in *Xerophyta humilis*

Many of the ABA-metabolism, -signalling and -responsive TFs are activated during desiccation in the leaves of *X. humilis* (Lyall, 2016). Orthologues of the ABA biosynthesis genes ABA4 and NCED as well as the ABA catabolism gene CYP707A were upregulated throughout desiccation, particularly at 80% and 40% RW. In line with the expression of the ABA metabolic transcripts, orthologues of the ABA receptors and positive regulators, PYLs and SnRK2s respectively, were upregulated. Three orthologues in the ABF sub-family were upregulated which, like ABI5, are also bZIPs. Of particular interest was *XhABFA*, which was strongly induced during both the early and late desiccation response. The expression pattern of *XhABFA* matched that of many desiccation-induced genes, including most LEAs and seed storage proteins. This could suggest that components of the drought stress response, rather than seed maturation factors, might be able to regulate the expression of seed-specific genes containing ABREs during VDT in *X. humilis*.

1.10 Aim of the current study

As a first step in testing this hypothesis, this study functionally characterises XhABFA by investigating whether XhABFA is able to bind directly to the promoters, and potentially activate the expression, of three genes which are seed-specific in desiccation sensitive plant species but are upregulated in leaves during VDT in *X. humilis*. These interactions were tested in both a plant and yeast system by transient transfection of protoplasts and a Yeast One-Hybrid assay respectively. This would be the first evidence of activation of seed-specific genes in desiccating leaves by a vegetative abiotic stress TF. Therefore this study will further our understanding of the networks regulating VDT in angiosperm resurrection plants.

Chapter 2: Cloning *XhABFA* and the promoters of four *Xerophyta humilis* genes

2.1 Introduction

This chapter describes the cloning of *XhABFA* and the promoters of four *X. humilis* genes into the vectors required to perform a Yeast One-Hybrid assay and the transient transfection of *Arabidopsis thaliana* protoplasts.

2.1.1 *Xerophyta humilis*: The study species

Xerophyta is a genus of monocotyledonous plants in the family Velloziaceae (Behnke et al., 2013). So far all *Xerophyta* species have been classified as resurrection plants due to their extreme tolerance to desiccation. *Xerophyta humilis* is the smallest species of *Xerophyta* and is found in various regions of Southern Africa (Foden and Potter, 2005; Figure 2.1). *X. humilis* is poikilochlorophyllous meaning that the leaves lose their green colour during desiccation due to a shutdown of photosynthetic machinery, resulting in the loss of chlorophyll (Figure 2.2).

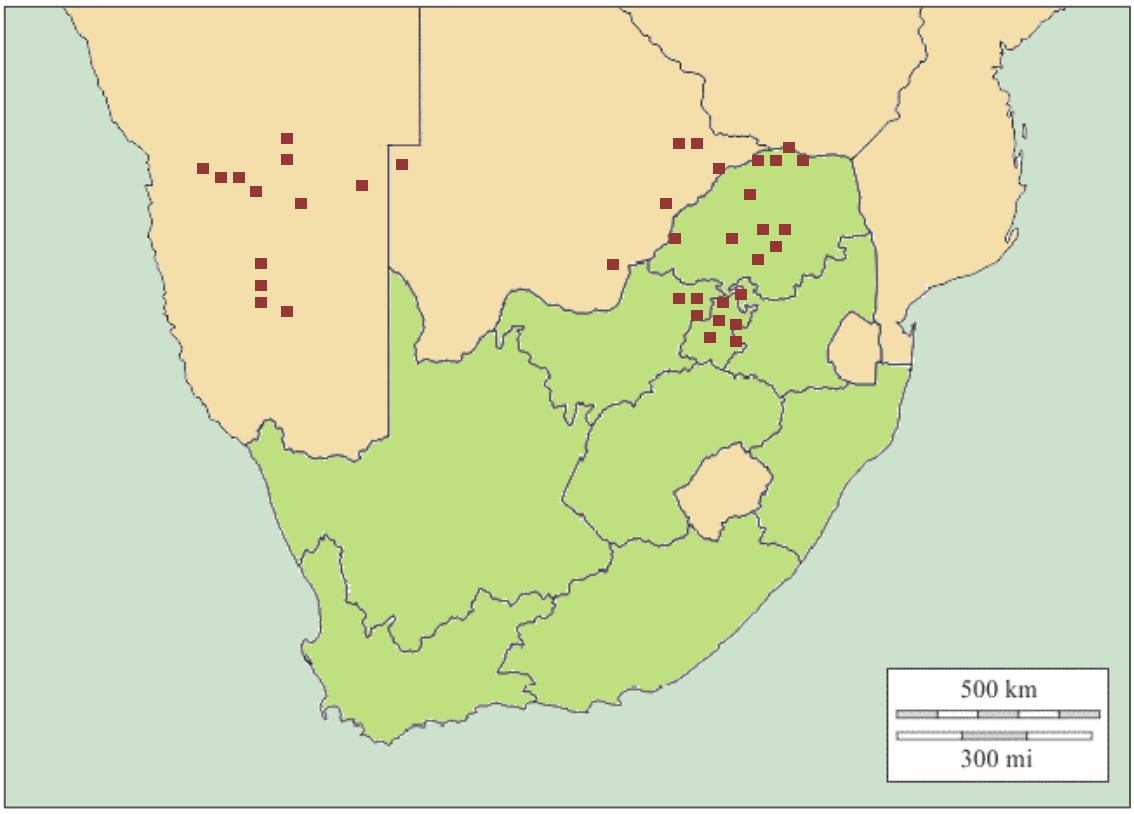


Figure 2.1: Sites where *X. humilis* plants have been recorded. (Adapted from Foden and Potter, 2005).

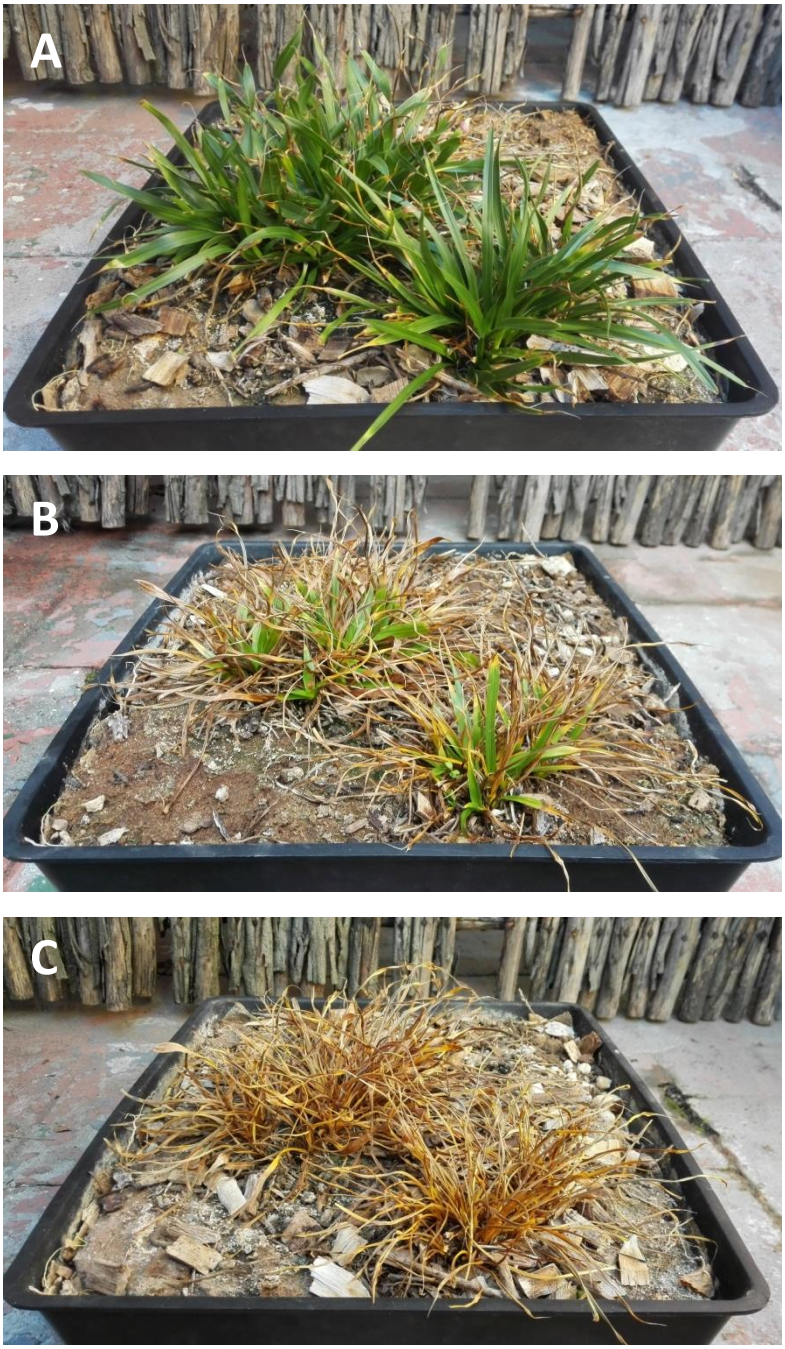


Figure 2.2: The vegetative phenotype of *X. humilis* when (A) hydrated, (B) undergoing dehydration and (C) desiccated. When *X. humilis* undergoes desiccation its leaves contract from the loss of water and turn brown from the loss of chlorophyll.

2.1.2 The four *Xerophyta humilis* genes used in the current study

The promoters of *XhPER1, XhDSI-1VOC, XhECP63* and *XhAHL23* were selected to test the hypothesis that the XhABFA TF could activate seed maturation genes.

XhPER1

1-CYSTEINE PEROXIREDOXIN (PER1; At1g48130) is a member of the thiol peroxidase family of enzymes. These enzymes have been found to catalyse the detoxification of peroxides which are a form of reactive oxygen species (ROS) (Dietz et al., 2002). ROS are derived from normal physiological and metabolic processes that are essential to the cell. At low concentrations, ROS function as signal transduction molecules that regulate various cellular processes, however at high concentrations they oxidise cellular molecules and become detrimental to the cell (Yu, 1994). Antioxidants, such as PER1, are part of the protective mechanism which counters ROS damage by neutralising the oxidative effects of oxygen and its reactive metabolites (Choudry et al., 2017). In addition to its antioxidant role, PER1 acts as a signalling molecule and has been shown to inhibit seed germination by suppressing ABA catabolism and GA biosynthesis (Chen et al., 2019). In DS plants, PER1 exhibits a seed-specific expression pattern and has been classified as being one of the genes regulated by ABI3 (Haslekås et al., 2003; Mönke et al., 2012). Furthermore, PER1 is ectopically expressed in germinating *A. thaliana* seedlings which lack a functional PRC2 required for the epigenetic silencing of seed maturation genes during the embryo to seedling transition (Bouyer et al., 2011). In resurrection plants, homologues of PER1 have been shown to be differentially expressed in the desiccating vegetative tissues as well as mature seeds. These plants include *Xerophyta viscosa, Haberlea rhodopensis, Xerophyta humilis* and *Sporobolus stapfianus,* of which the latter two it was identified as the most highly expressed antioxidant during vegetative desiccation (Mowla et al., 2002; Collett et al., 2004; Illing et al., 2005; Gechev et al., 2013; Yobi et al., 2017).

In this study *XhPER1* was chosen as one of the candidate genes due to its exclusive expression in the mature seeds of DS plants, its high differential expression in the desiccating vegetative tissues of some resurrection plants and being a member of the ABI3 regulon. *XhPER1* exhibits a biphasic expression pattern, peaking at 80% and 40% RWC (Figure 2.3). The promoter of *XhPER1* contains four ABRE elements and one RY element (Figure 2.4).

XhDSI-1VOC

The vicinal oxygen chelate (VOC) proteins are members of a metalloenzyme superfamily (Armstrong, 2000). VOC proteins generally don't share sequence similarity however they display a conserved βαβββ structural fold and protein-chelating residues that secure and localize a metal ion through vicinal oxygen atoms (He and Moran, 2011). The displacement of metals from metalloenzymes or metabolites is linked to heavy metal toxicity in the cell which is one of the products of drought stress (Palma et al., 2013). A member of the VOC family, DESICCATION INDUCED-1VOC (DSI-1VOC; At1g07645), has been shown to be up-regulated during seed maturation and in the vegetative tissues of *X. humilis* during desiccation (Collett et al., 2004). Orthologues of *XhDSI-1VOC* in *A.*

thaliana are present in mature seeds but not in vegetative tissues even when exposed to drought stress (Mulako et al., 2008). In addition to *A. thaliana, DSI-1VOC* has been shown to accumulate during the dehydration process of seed maturation in other DS plants and has been proposed to protect the embryonic developmental process from harm under drought conditions (Gan et al., 2013; Liang et al., 2017). Like PER1, DSI-1VOC is ectopically expressed in *A. thaliana* PRC2 mutants (Bouyer et al., 2011).

In this study, *XhDSI-1VOC* was chosen on the basis of its seed-specific expression in DS plants and its differential upregulation in the desiccating leaves of *X. humilis*. It exhibits a similar biphasic expression pattern to *XhPER1,* where it peaks at 80% and 40% RWC (Figure 2.3). The promoter of *XhDSI-1VOC* contains two ABRE elements and lacks any RY elements (Figure 2.4).

XhECP63

EMBRYONIC CELL PROTEIN 63 (ECP63; At2g36640) is a member of the LEA superfamily (Costa et al., 2015). LEA proteins were first discovered as proteins which were highly expressed during the later stages of embryogenesis in which the seed matures and acquires DT (Dure et al., 1989). Since these initial reports, LEA proteins have also been identified in vegetative plant tissues following dehydration which may be induced by saline conditions, heat, freezing or drought (Bartels, 2005). They are widely distributed in the plant kingdom, from algae, moss, ferns, gymnosperms and angiosperms (Amara et al., 2014). Interestingly, LEA proteins are not restricted to the plant kingdom, and have been documented in bacteria, fungi, protozoa, rotifers, nematodes, insects, crustaceans and other organisms with anhydrobiotic traits (Hand et al., 2011). This ubiquitous nature of LEAs may suggest a common mechanism of DT across distinct life forms (Artur et al., 2019b). Even though it is well established that LEAs are associated with adaptations to water deficits, their physiological and biochemical functions are diverse and largely unknown (Hundertmark and Hincha, 2008). LEAs exhibit intrinsically disordered regions (IDRs) and it has been proposed that this is one of the properties that have contributed to the diversity of LEA proteins (Covarrubias et al., 2017). IDRs are associated with molecular plasticity and proteins with IDRs often exhibit “moonlight activity”, that is, the ability to perform more than one function (Tompa et al., 2005). The versatile functions of LEA proteins include, but are not limited to, the stabilisation of membrane integrity, the prevention of cellular constituents from crystallising, the reduction of mobility, antioxidant activity, molecular chaperoning, transcriptional regulation, metal ion and calcium-binding activity, modulation of glucose content, the slowing down of metabolism and the prevention of protein inactivation, denaturation and aggregation (Goyal et al., 2005; Leprince and Buitink, 2007; Covarrubias et al., 2017).

ECP63, the LEA protein used in this study, was originally identified as being highly expressed during seed maturation in *A. thaliana* (Yang et al., 1997), and was later shown to be a target of ABI3 (Mönke et al., 2012). In *X. humilis,* the orthologue of ECP63 is highly induced in desiccating vegetative tissues and follows a similar expression pattern to *XhABFA* (Figure 2.3). The promoter of *XhECP63* contains four ABRE elements and lacks any RY elements (Figure 2.4).

XhAHL23

In addition to the three candidate promoters, a fourth promoter was included in the experiment in order to demonstrate that any potential transactivation of *XhABFA* is sequence specific. The orthologue of AT-HOOK MOTIF NUCLEAR-LOCALISED PROTEIN 23 (AHL23; At4g17800) was chosen as it is not expressed in the leaves of *X. humilis* during desiccation and its promoter lacks any ABRE or RY elements (Figure 2.4). In *A. thaliana* it has been shown that the AHL23 protein binds AT-rich DNA sequences related to nuclear matrix attachment regions (Fujimoto et al., 2004).

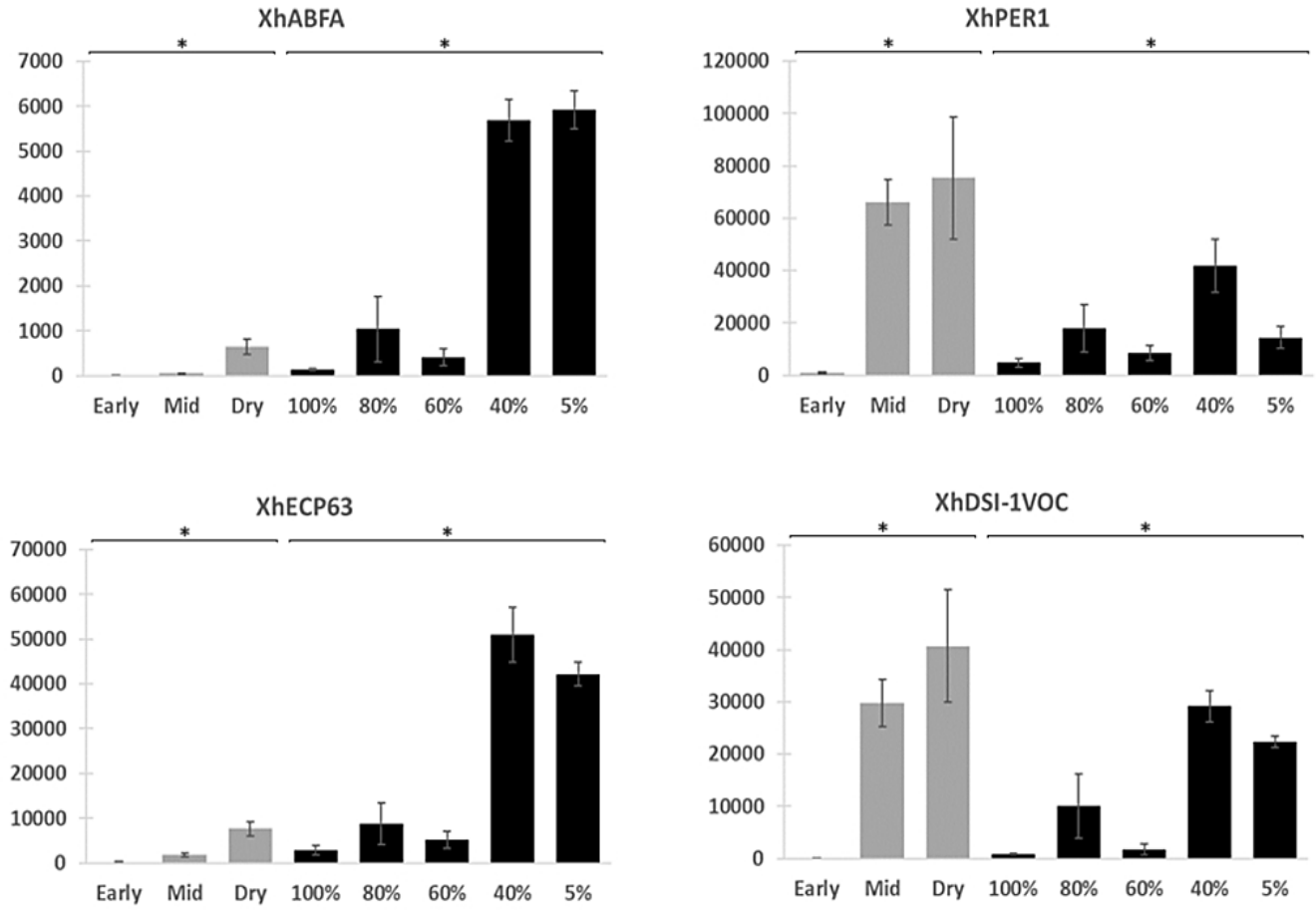


Figure 2.3: Expression of *XhABFA, XhPER1, XhECP63, and XhDSI-1VOC*. DESeq2 normalised counts for these genes during seed maturation (left, grey) and VDT (right, black). Counts from both tissues are displayed on the same axis but are derived from different experiments. Error bars are standard error. (Adapted from Lyall at al., 2019)

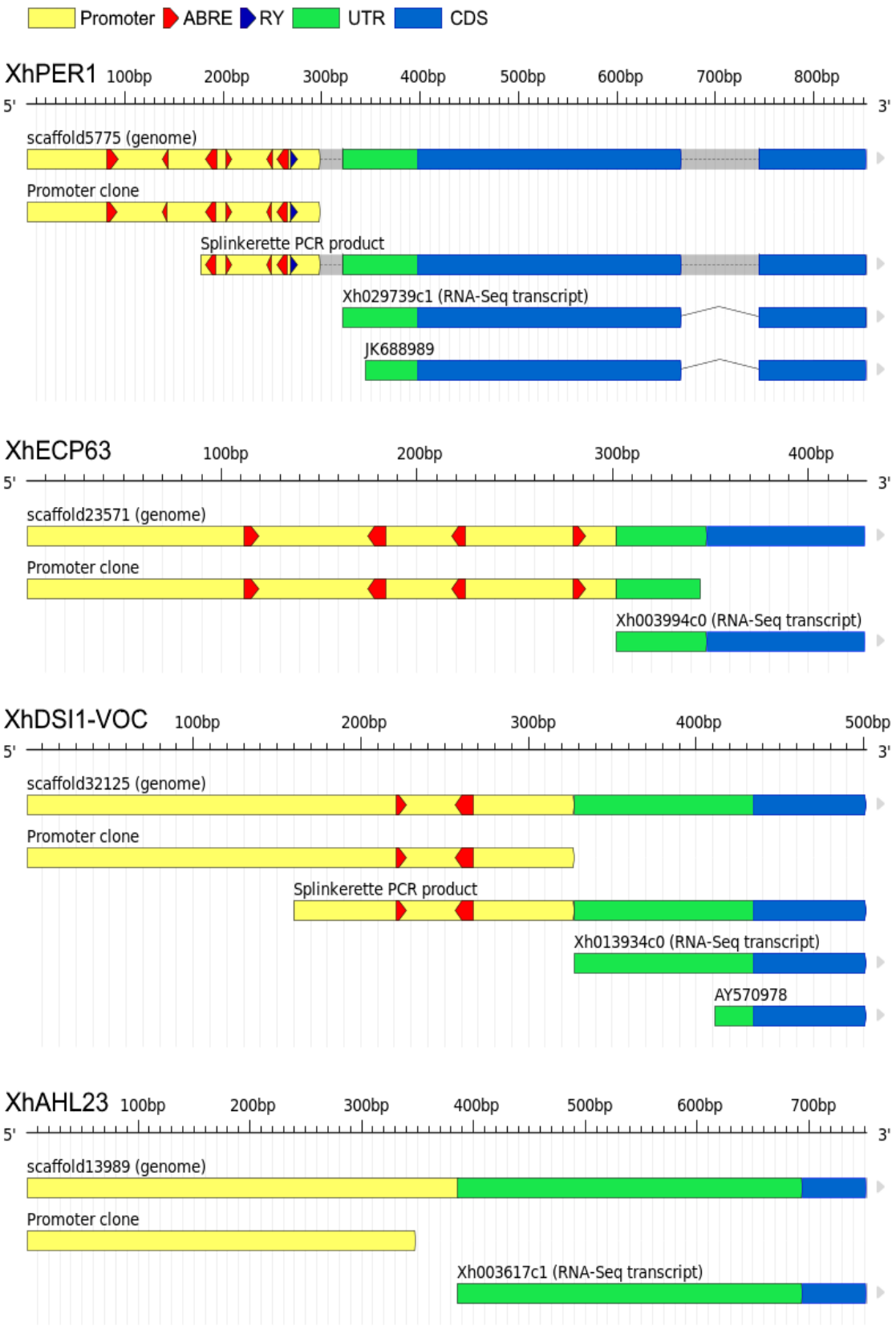


Figure 2.4: **Summary of promoter regions.** Promoters for *XhPER1*, *XhECP63*, *XhDSI-1VOC* and *XhAHL23* were aligned to the *X. humilis* genome assembly scaffolds. Their positions relative to start sites for RNA-seq transcripts are summarized. Conserved ABRE and RY elements are indicated. (Reproduced from Lyall et al., 2019).

2.2 Materials and methods

2.2.1 Vectors used for the transient transfection of *Arabidopsis thaliana* protoplasts

pENTR1A

The pENTR1A Dual Selection Vector (Invitrogen) is a Gateway® compatible entry vector. It is designed for recombination with a Gateway® compatible destination vector to create an expression clone. It is resistant to kanamycin as well as Chloramphenicol which makes it capable of dual selection in *E. coli.* It contains the *ccdB* gene located between the two *attL* sites for counter selection. pENTR1A was propagated from laboratory maintained *E. coli* glycerol stocks.

pGWL7

The pGWL7 plasmid is a Gateway® compatible destination vector. It is a reporter vector as it is able to express firefly luciferase if activated by a promoter cloned upstream of the firefly luciferase gene *LucF* (Figure 2.5 A). It is commonly used in dual luciferase reporter assays. It contains an ampicillin resistance gene for selection in *E. coli*.

pUC19-35S-Rfa-35S-sGFP

pUC19-35S-Rfa-35S-sGFP is a Gateway® compatible destination vector. It contains an ampicillin resistance gene for selection in *E. coli*. Constitutive transcription of a transgene cloned into the Reading Frame Cassette A (Rfa) as well as sGFP are driven by independent CaMV 35S promoters (Figure 2.5 B). This type of destination vector is considered an "effector" construct when a TF or other putative signalling component has been cloned into the Rfa (Pitzschke and Persak, 2012). The vector was donated by Professor Steven Hussey (University of Pretoria).

pUC19-35S-NOS-35S-sGFP

An "empty vector" (EV) control was created by removing the Rfa sequence of the pUC19-35S-Rfa-35S-sGFP through LR recombination with a self-ligated pCR8®/GW/TOPO® vector (Figure 2.5 C). A CaMV 35S promoter constitutively expresses sGFP. This vector was also donated by Professor Steven Hussey.

pBS-35S-Ala-LucR

The pBS-35S-Ala-LucR vector constitutively expresses LucR from the *Renilla* luciferase gene *LucR* which is driven by the CaMV 35S promoter (Figure 2.5 D). It contains an ampicillin resistance gene

for selection in *E. coli*. This was used as a control vector for transfection efficiency. This vector was donated by Professor Albrecht von Arnim (University of Tennessee).

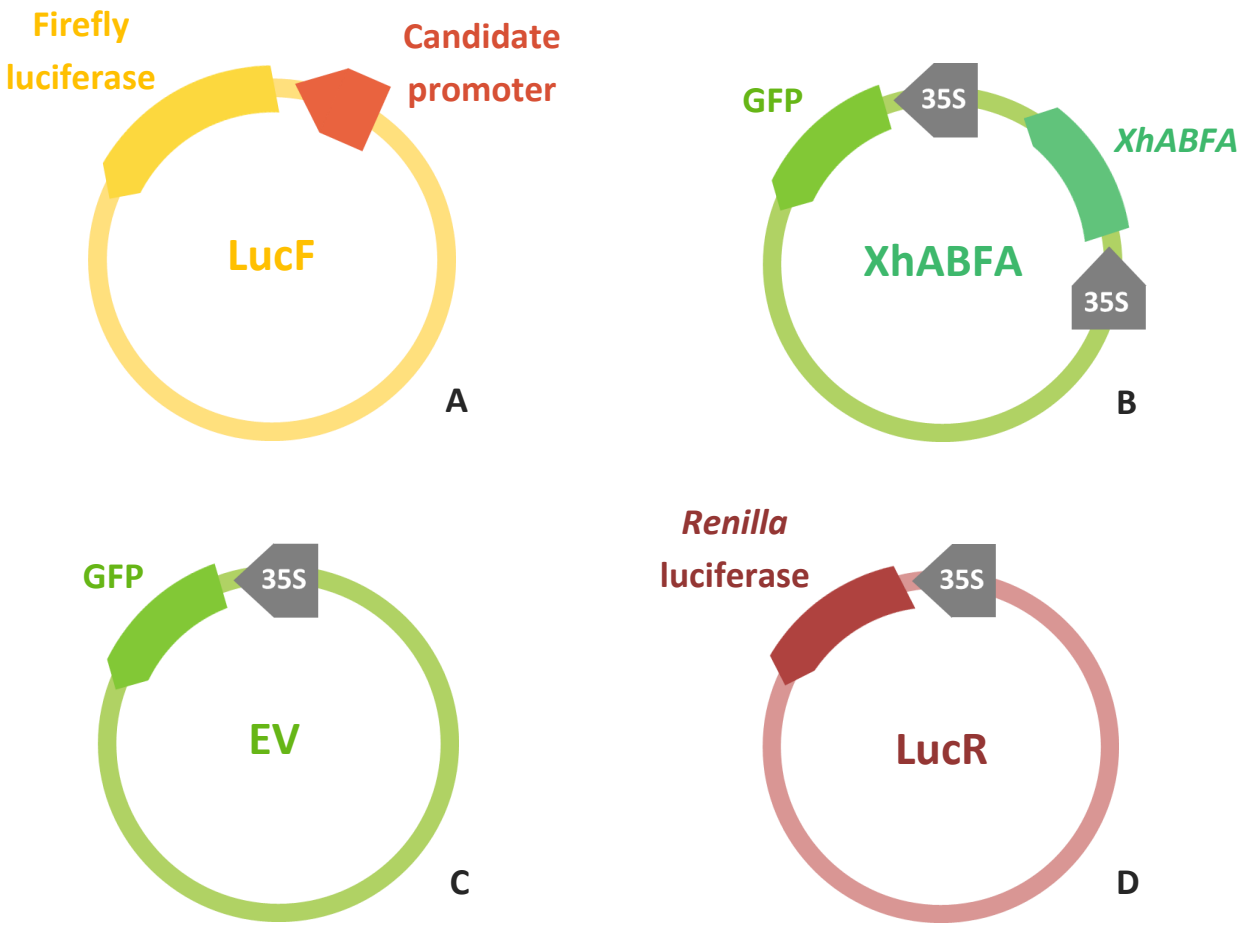


Figure 2.5: Schematic representation of the four vectors used in the transfection of *A. thaliana* protoplasts. (A) Reporter vector containing one of four promoter elements coupled to LucF. (B) Vector which constitutively expresses both GFP and *XhABFA*. (C) An "empty vector" which constitutively expresses GFP but lacks the *XhABFA* gene. (D) Vector which constitutively expresses LucR. "35S" indicates the position of the constitutive promoter CaMV 35S.

2.2.2 Vectors used for the Yeast One-Hybrid assay

All vectors used in the Y1H protocol were supplied in the Clontech MATCHMAKER® One-Hybrid System kit (K1603-1). Basic vector maps are depicted in Figure 2.6.

Reporter Vectors

pHISi

A reporter vector, which expresses histidine, can be generated by inserting a target promoter into the multiple cloning site (MCS) of the pHISi vector. The vector contains the *HIS3* reporter gene, which is located downstream of the MCS and the minimal promoter of the *HIS3* locus (P_{minHIS}). Without activation by a target promoter, constitutive *HIS3* expression from P_{minHIS} is very low in yeast but allows enough growth to select for integration when constructing *HIS3* reporter strains. During the reporter assay, the leaky expression of *HIS3* is controlled by adding 3AT to the media. The yeast *HIS3* and *URA3* genes are used as selectable markers for integration into the non-functional *his3* and *ura3* loci, respectively, of the YM4271 host strain. pHISi cannot replicate autonomously in yeast. It contains an ampicillin resistance gene for selection in *E. coli*.

pHISi-1

A reporter vector, which expresses histidine, can be generated by inserting a target promoter into the MCS of the pHISi-1 vector. Clontech constructed pHISi-1 by transferring the *HIS3* reporter gene from pHISi to the EcoRI/BamHI sites of pBR322. Leaky *HIS3* expression in pHISi-1 is generally lower than that in pHISi according to the Clontech One-Hybrid System user manual. pHISi-1 can be used, together with pLacZi, to construct a dual *HIS3/lacZ* reporter strain. pHISi-1 cannot replicate autonomously in yeast. It contains an ampicillin resistance gene for selection in *E. coli*.

pLacZi

A reporter vector, which expresses β-galactosidase (*lacZ*), can be generated by inserting a target promoter into the MCS of the pLacZi vector. The vector contains the *lacZ* reporter gene, which is located downstream of the MCS and the minimal promoter of the yeast iso-1-cytochrome C gene (P_{CYC1}). Without activation by a target promoter, *lacZ* expression is very low when the vector is integrated into the yeast genome. The yeast *URA3* gene is used as a selectable marker for integration into the non-functional *ura3* locus of the YM4271 host strain after linearising the vector. pLacZi can be used, together with pHISi-1, to construct a dual *HIS3/lacZ* reporter strain. pLacZi cannot replicate autonomously in yeast. It contains an ampicillin resistance gene for selection in *E. coli.*

Activation domain vectors

pGAD424

The pGAD424 vector encodes the AD of the yeast GAL4 transcriptional activator. The MCS is located at the 3' end of the open reading frame for the GAL4 AD sequence. A GAL4 AD fusion protein can be generated by inserting a gene encoding a protein of interest into the MCS in the correct orientation and reading frame. The fusion protein is expressed at high levels in yeast host cells from the constitutive *ADH1* promoter. pGAD424 carries the *LEU2* gene for selection in *leu-* auxotrophic yeast strains, such as YM4271. pGAD424 is also used as a negative control AD vector in the positive control experiment.

pGAD53m

pGAD53m is an AD vector containing the mouse *p53* gene in frame with the GAL4 AD.

Positive control vectors

p53HIS

p53HIS is a reporter vector containing three tandem copies of the consensus *p53* binding site inserted into the MCS of pHISi.

p53BLUE

p53BLUE is a reporter vector containing three tandem copies of the consensus *p53* binding site inserted into the MCS of pLacZi

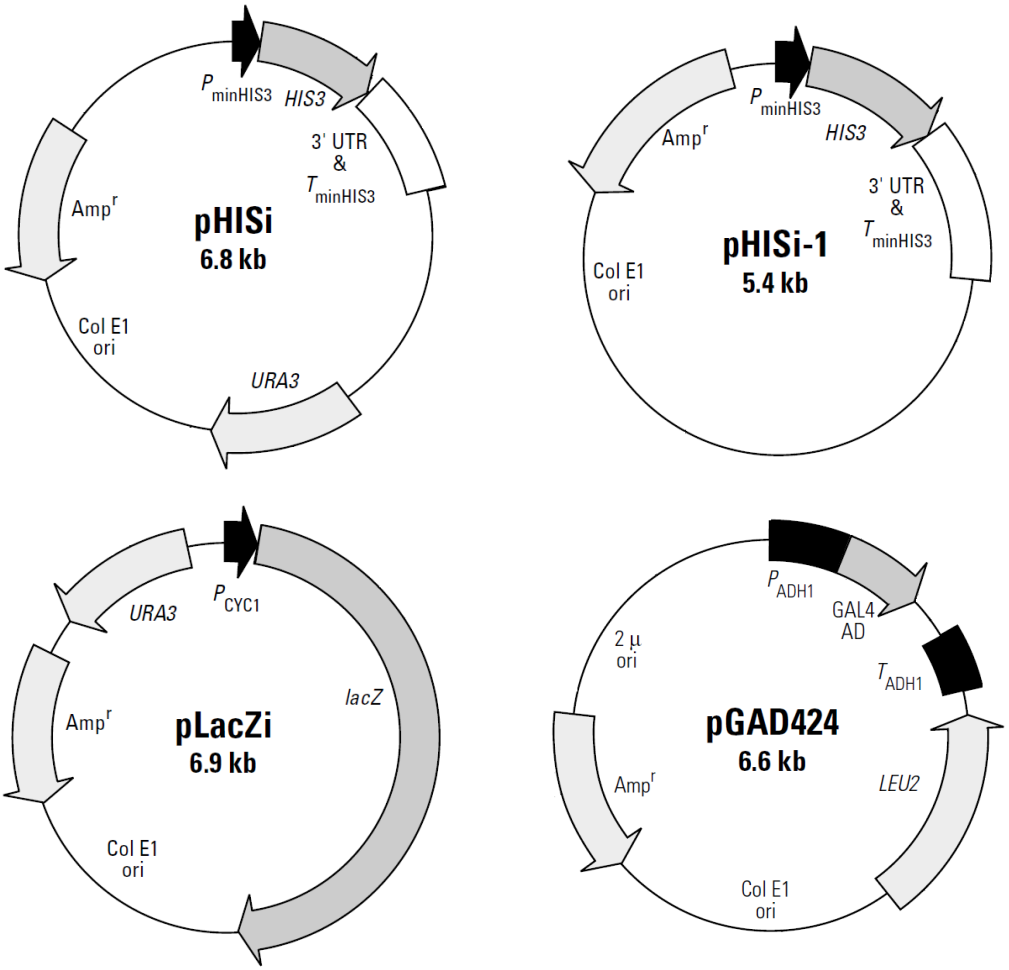


Figure 2.6: Basic vector maps of the constructs supplied by the Clontech Yeast One-Hybrid kit. Vector maps for p53HIS, p53BLUE and pGAD53m are unavailable.

2.2.3 Microbial strains

DH5-Alpha

DH5-Alpha is an *Escherichia coli* strain used for routine cloning applications. They are defined by three mutations: *recA1*, which increases insert stability, *endA1*, which improves the yield and quality of DNA and *lac*ZΔM15 which allows for selection using blue/white screening.

One Shot®

One Shot® Chemically Competent Cells are an *E. coli* strain designed for use with the Gateway® Vector System. In addition to the *recA1, endA1* and *lacZ*ΔM15 mutations they are also resistant to the *ccdB* gene and T1/T5 phages.

Yeast

The YM4271 yeast strain was used in this assay. Genotype is *MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-D512, gal80-D538, ade5::hisG*.

2.2.4 Plant collection and tissue harvesting

Xerophyta humilis plants were originally collected from the Borakalalo Game Reserve in the North West Province (North West Provincial Government Permit 062 NW-12; Cape Nature Permit AAA007-01733). The plants were maintained in a greenhouse under natural light conditions. Roughly two months prior to harvesting, the plants were transferred to a climate-controlled plant growth chamber (Conviron Adaptis A350) to acclimatise to the experimental conditions: 16h long-day, temperature setting of 22°C, an average luminosity of 250 µmol $m^{-2}s^{-1}$ and watering three times per week.

Leaf tissue for genomic DNA extraction was harvested from *Xerophyta humilis* plants which were well hydrated. Leaf tissue for RNA extraction was harvested from plants which had not been watered for two weeks. These plants had just undergone desiccation which is the stage at which *XhABFA* is differentially expressed.

2.2.5 Nucleic acid extractions

RNA extraction

The reagents, concentrations and pH of the solutions used in this protocol to extract and analyse the quality of *X. humilis* RNA are summarised in Supplementary Table 1. Total RNA was extracted from approximately 400 mg of *X. humilis* leaf tissue using a modified version of the Rio et al., 2010 protocol. Leaf tissue was sampled and crushed into a fine powder with liquid nitrogen using a pestle and mortar. The tissue powder was equally divided into four 1.5 ml microcentrifuge tubes each containing 400 µl of RNA extraction buffer. 400 µl of phenol : chloroform : isoamyl alcohol (Sigma Aldritch) was added to each tube. Samples were vortexed for 30 seconds and incubated on ice for 5 minutes. Chilled samples were centrifuged at 12 000 g for 15 minutes at 4°C to pellet debris and separate the supernatant. All further centrifugation steps were performed under these conditions. 400 µl of chloroform was added to each sample and then centrifuged. The upper aqueous layer was transferred to a 1.5 ml microcentrifuge tube containing 1 ml of Trizol. Samples were vortexed for 30 seconds and incubated at room temperature for 5 minutes. 200 µl of chloroform was added and samples were vortexed for 30 seconds and incubated at room temperature for 5 minutes. Samples were centrifuged and the upper aqueous layer was transferred to a 1.5 ml microcentrifuge tube containing 250 µl isopropanol and 250 µl of HSPB. Samples were mixed by inversion and incubated at room temperature for 5 minutes. The samples were centrifuged and the supernatant was removed without disturbing the RNA pellet. The pellets were washed with 1 ml of 75% ethanol which was added to each tube and then centrifuged. The ethanol was removed and the samples were washed a second time. The remaining ethanol was removed and the samples were allowed to

air dry for 10 minutes. 30 µl of DEPC water was added to each tube. Samples were incubated at 55°C for 10 minutes or until the RNA pellet had resuspended. When fully resuspended, the samples were centrifuged at 6000 g for 5 minutes at room temperature, after which the RNA-containing supernatant was transferred to a clean tube. 5 µl aliquots were taken from each sample for diagnostic purposes and the remaining RNA was stored at -80°C until further use.

Analysis of RNA quality

The RNA concentration for each sample was determined using a NanoDrop® ND-1000 spectrophotometer. The absorbance profile of each sample was visualised between 220 nm and 320 nm and the A260/A280 ratio was used to evaluate the purity of the RNA. The integrity of the RNA samples was visually analysed on a formaldehyde-based RNA denaturing agarose gel. 2 µg of RNA from each sample was denatured at 60°C for 5 minutes in 2 x volume of RNA loading buffer. A 1x MOPS buffer solution was used as the running buffer. The samples were electrophoresed at 100V for 30 minutes and viewed using a UV Transilluminator.

cDNA synthesis

The RNA sample was treated with DNase to remove genomic contamination. First strand cDNA synthesis was performed using 500 ng of RNA, 1 µl of oligo (dT) primers and 1 µl of dNTP mix in 13 µl of distilled water. The mixture was incubated at 65°C for 5 minutes and then incubated on ice for 1 minute. 4 µl of 5x First-Strand Buffer, 1 µl of DTT, 1 µl RNase inhibitor and 1 µl SuperScript™ III Reverse Transcriptase (Invitrogen) was added to the mixture. The sample was incubated at 50°C for 45 minutes and then at 70°C for 15 minutes to heat inactivate the Reverse Transcriptase enzyme.

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from *X. humilis* leaf tissue using a modified version of the Dellaporta protocol (Dellaporta et al., 1983). The reagents, concentrations and pH of the solutions used in this gDNA extraction protocol are summarised in Supplementary Table 2. Approximately 100 mg of leaf tissue was sampled and crushed into a fine powder with liquid nitrogen using a pestle and mortar. The tissue powder was transferred into 2 ml microcentrifuge tubes containing 1.4 ml of DNA extraction buffer. Samples were incubated at 65°C for 10 minutes. 500µl of 5M potassium acetate was added to each tube. The samples were vortexed for 30 seconds, incubated at 4°C for 20 minutes and then centrifuged for 20 minutes at 12 000 g. The supernatant was transferred to a 2 ml microcentrifuge tubes containing 1 ml of isopropanol. Samples were mixed by inversion and incubated at -20°C for 30 minutes. DNA was pelleted by centrifugation at 12 000 g for 15 minutes and the supernatant was removed. The samples were allowed to air dry for 5 minutes and then the pellets were resuspended in 70 µl of Tris-EDTA buffer. RNA in the samples was degraded with the addition of 1 µl of a 10 µg/ml stock of RNase A to each sample which were then incubated at 37°C for 30 minutes. 7.5 µl of 3M sodium acetate and 50 µl of isopropanol were added. Samples were mixed by inversion and incubated on ice for 15 minutes. DNA was pelleted by centrifugation for 10

minutes at 12 000 g. The supernatant was removed and 1 ml of 75% ethanol was added to each tube to wash the DNA. The samples were centrifuged for 5 minutes at 12 000 g and the supernatant was removed. The samples were allowed to air dry for 10 minutes and the DNA was resuspended in 30 µl of distilled water. 5 µl aliquots were taken from each sample for diagnostic purposes and the remaining DNA was stored at -20°C until further use. The DNA concentration for each sample was measured using a NanoDrop. The A260/A280 and A260/A230 ratios were used to evaluate the purity of the DNA. The sample with ratios closest to 1.8 and 2 respectively was used for PCR amplification.

2.2.6 Primer design

The coding sequence of *XhABFA* was provided by Dr Rafe Lyall from the assembled transcriptome of *X. humilis* (Lyall et al., 2019). The promoter sequences of *XhPER1, XhECP63, XhDSI-1VOC and XhAHL23* were obtained by aligning the transcripts of these genes to the draft genome assembly of *X. humilis* (Schlebusch, 2018). These promoter sequences and the coding sequence of *XhABFA* are collectively referred to as the "insert DNA". Primers used to amplify these sequences were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Melting temperatures as well as the potential to form secondary structures, such as dimers or hairpins, were predicted using OligoAnalyzer® (https://eu.idtdna.com/calc/analyzer/). Primer pairs with similar melting temperatures and more positive ΔG values were selected. Restriction enzymes were chosen based on compatibility with either pENTR1A, pHISi-1, pLacZi or pGAD424, as well as being absent in the sequences of the insert DNA. Primers are listed in Supplementary Tables 3, 4, 5 and 6

2.2.7 PCR amplification

The cDNA and gDNA previously described were used as the templates to amplify *XhABFA* and the promoter sequences respectively for entry into pENTR1A. PCR amplification was performed using the high-fidelity Accupol DNA polymerase (A211102). A master mix was prepared as per the manufacturer's instructions with the addition of the appropriate primers.

2 ng of either cDNA (*XhABFA)* or gDNA (promoters) was used as the template for pENTR1A. 2 ng of from dilutions made from midi-preps of the pUC19-35S-*XhABFA*-35S-sGFP vector (*XhABFA*) and the pGWL7 firefly reporter vectors (promoters) were used as the template for the Y1H vectors. A "no template" control which excluded DNA was used as a negative control. The PCR cycling parameters were followed according to Supplementary Tables 7 and 8.

2.2.8 Preparation of the insert and vector DNA

Purification of the insert DNA

The PCR products were electrophoresed on a 1% agarose gel and viewed using a UV Transilluminator. DNA bands of the predicted length were excised from the gel using a scalpel blade. The DNA from these gel samples was purified using the Wizard® SV Gel and PCR Clean-Up system (Promega) as per the manufacturer's instructions and eluted into 30 µl of nuclease-free water. The concentrations of the purified samples were determined using a NanoDrop® ND-1000 Spectrophotometer.

Preparation of the vectors

The assays required larger quantities of the vectors than the original stocks provided. One Shot ® TOP10 competent cells and kanamycin were used for the propagation and selection of the entry vector pENTR1A. DH5-Alpha competent cells and ampicillin were used for the propagation and selection of the destination and control vectors of the protoplast transfections and for all of the vectors used in the Y1H assay.

Competent cells were removed from storage at -80°C and thawed on ice in a microcentrifuge tube. 2 µl of the vector DNA was added to 100 µl of competent cells, mixed gently and incubated on ice for 30 minutes. The cells were heat shocked in a water bath for 30 seconds at 42°C and incubated on ice for 3 minutes. 1 ml of pre-warmed (37°C) LB medium was added. The tube was capped tightly and incubated at 37°C for 1 hour in a shaking incubator (225 rpm). 100 µl was spread onto a pre-warmed (37°C) LB agar selection plate which contained 100 µg/ml of the selective antibiotic. The remaining 900 µl was centrifuged for 1 minute at 300 rpm to pellet the rest of the cells. 800 µl of the supernatant was removed and the cells were gently resuspended in the remaining LB medium. 100 µl of these concentrated cells were plated onto another selection plate. The plates were inverted and incubated at 37°C overnight. A single colony was chosen from the selection plates and used to inoculate 5 ml of LB which was incubated at 37°C for 8 hours in a shaking incubator. The 5 ml sample was used to inoculate 150 ml of LB and then incubated at 37°C overnight in a shaking incubator. The PureYield™ Plasmid Midiprep System (Promega) was used to extract purified plasmid DNA from the LB culture as per the manufacturer's instructions. The purity and concentrations of the samples were determined using a NanoDrop.

Restriction enzyme digests

Prior to cloning, restriction enzyme digests were performed on the purified PCR products (insert DNA) and vector DNA. For cloning into pENTR1A, six separate double digests were performed on the five inserts and pENTR1A with the restriction enzymes specified in Supplementary Table 3. For cloning into the Y1H vectors a total of 22 restriction enzyme digests were performed: 11 on the insert DNA and 11 on the vector DNA. For each promoter, two separate double digests were performed with either the restriction enzymes required for cloning into the pHISi-1 vector

(Supplementary Table 4) or those required for the pLacZi vector (Supplementary Table 5). For *XhABFA,* one double digest was performed with the restriction enzymes required for cloning into the pGAD424 vector (Supplementary Table 6). The pHISi-1, pLacZi and pGAD424 vectors were then digested with restriction enzymes corresponding to those used in the insert DNA digests. For all digests, 1 µg of insert DNA was combined with 1 µl of each of the appropriate restriction enzymes and 5 µl of Cutsmart® Buffer (New England Biolabs) in 50 µl of nuclease free water. The samples were incubated at 37°C for 1 hour and then at 65°C for 20 minutes to inactivate enzyme activity. The samples were purified using the Wizard® SV Gel and PCR Clean-Up system (Promega) as per the manufacturer's instructions and eluted into 30 µl of nuclease-free water. The concentrations of the purified samples were determined using a NanoDrop.

In order to confirm their identity, restriction enzyme diagnostics were performed on all of the vectors used in the study except for pUC19-35S-Rfa-35S-sGFP and the "EV" control as the full vector maps are unavailable. 500 ng of each vector was combined with 0.5 µl of each of the restriction enzymes specified in Supplementary Table 9 and 5 µl of Cutsmart® Buffer (New England Biolabs) in 50 µl of nuclease free water. The samples were incubated at 37°C for 1 hour and then 10 µl of each sample was electrophoresis on a 1% agarose gel and viewed using a UV Transilluminator. A "no RE" control which excluded restriction enzymes was used as a negative control.

2.2.9 Cloning

Ligation

T4 DNA Ligase (Promega) was used to ligate: pENTR1A with *XhABFA* and the four promoter inserts; pHISi-1 with each of the four promoter inserts; pLacZi with each of the four promoter inserts; and pGAD424 with *XhABFA,* as per the manufacturer's instructions. A 3:1 molar ratio of insert:vector and a total of 100 ng of DNA was used. The reactions were incubated at room temperature for 2 hours and then at 70°C for 10 minutes to deactivate the ligase activity. The transformation protocol previously specified was used to transform One Shot cells with 2 µl of each pENTR1A ligation reaction and DH5-Alpha cells with 2 µl of each Y1H ligation reaction. The selection plates contained kanamycin and ampicillin respectively and were incubated overnight at 37°C.

Colony PCRs

Positive transformants were screened using colony PCRs and gene-specific primers. Ampliqon Taq DNA Polymerase 2x Master Mix RED (A180303) was prepared as per the manufacturer's instructions with the addition of the appropriate gene-specific primers. A small quantity of bacteria from a distinct colony was collected with the tip of a toothpick and added to the master mix. A "no template" control which excluded bacteria was used as a negative control. The PCR cycling parameters were followed according to Supplementary Tables 7 and 8. PCR products were electrophoresed and visualised to confirm the presence of the insert DNA.

Plasmid isolation and purification

Bacterial colonies with positive transformants were used to inoculate 5 ml samples of LB which were then incubated at 37°C overnight in a shaking incubator. The PureYield™ Plasmid Miniprep System (Promega) was used to extract purified plasmids from the LB culture as per the manufacturer's instructions.

Further diagnostics and sequencing

Restriction enzyme diagnostics were performed to confirm the presence of the insert DNA. Purified samples were sent to the Central Analytical Facilities at the University of Stellenbosch and sequenced by Sanger sequencing. Sequences obtained were aligned against the consensus sequence generated from RNA-Seq or genome data to confirm identity and orientation of the insert DNA in pENTR1A. Sequence data was analysed using MEGA7.

Gateway cloning

The insert DNA was transferred from pENTR1A (entry vector) into pUC19 or pGWL7 (destination vectors) using the Gateway cloning strategy. An LR recombination reaction between the entry vector and destination vectors was performed. 2 µl of LR Clonase™ II Enzyme Mix was added to tube containing 150 ng of the entry vector and 150 ng of the destination vector in 8 µl of TE buffer. Samples were incubated at 25°C for 1 hour. 1 µL of Proteinase K was added to each sample and incubated at 37°C for 10 minutes to terminate the reaction. The reaction mixture was transformed into DH5-Alpha competent cells.

Large scale plasmid isolation and purification

A colony was chosen from each of the selection plates and used to inoculate 5ml samples of LB. These were incubated at 37°C for 8 hours in a shaking incubator. These samples were used to inoculate 300 ml of LB and then incubated at 37°C overnight in a shaking incubator. The NucleoBond® Xtra Maxi kit (Macherey-Nagel) was used to extract purified plasmids from the LB culture as per the manufacturer's instructions. The purity and concentrations of the samples were determined using a NanoDrop.

2.3 Results

2.3.1 Validating that *XhABFA* and the target promoters were successfully cloned into the vectors required for protoplast transfection assays

XhABFA and the promoters of seed maturation genes were cloned into specific vectors required to perform the protoplast assay. The initial step of this process was to amplify *XhABFA* and the target promoters from cDNA and genomic DNA extracted from *X. humilis.*

Good quality RNA was extracted from dry *X. humilis* leaves (Fig 2.7). Samples 2 and 3 were pooled and used for first strand cDNA synthesis. *XhABFA* was successfully amplified by PCR from this cDNA (Figure 2.8).

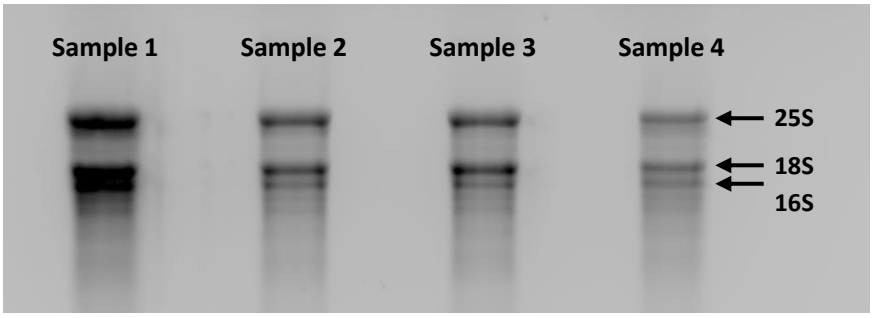


Figure 2.7: The integrity of the *X. humilis* RNA. In order to evaluate the integrity of the RNA isolated from four dry *X. humilis* leaves (samples 1-4) was visually analysed on a formaldehyde-based RNA denaturing agarose gel. Ribosomal bands are indicated (25S, 18S, 16S).

Genomic DNA (gDNA) was extracted from hydrated leaf tissue and used to amplify the four promoter regions of interest. The observation of a single clear band of the predicted length confirmed successful amplification (Figure 2.8).

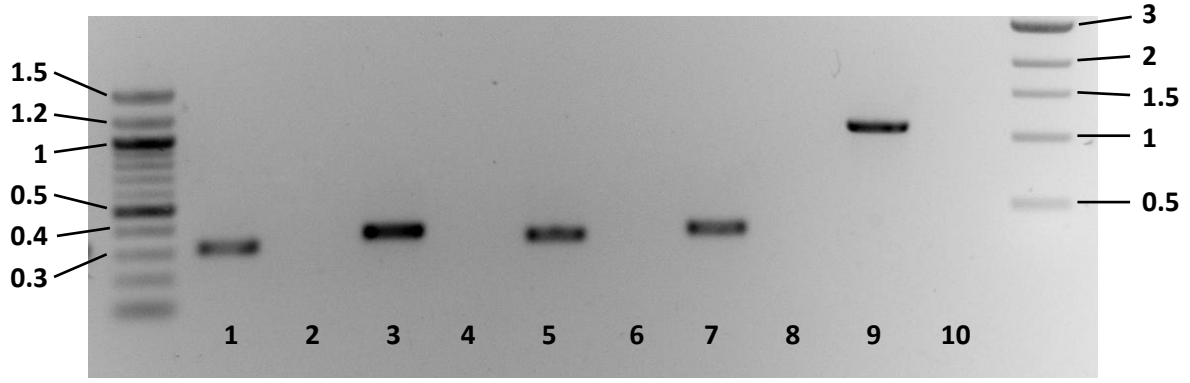


Figure 2.8: PCR amplification of the target promoters and *XhABFA*. Successful amplification of the PCR products was confirmed on an agarose gel. Lanes 1, 3, 5, 7 and 9: *XhPER1, XhECP63, XhDSI-1VOC, XhAHL23* and *XhABFA* with expected product sizes of 322, 368, 352, 371 and 1077 bp respectively. Lanes 2, 4, 6, 8 and 10: respective "no template" controls. All ladder sizes are in kb.

Confirming the identity of the vectors used in the protoplast assays

Following the successful amplification of the promoters and *XhABFA,* the various vectors required for the study were propagated from original stocks and then purified. Restriction enzyme diagnostics were performed on the purified vectors to confirm their identity and integrity prior to performing the cloning procedures (Figure 2.9).

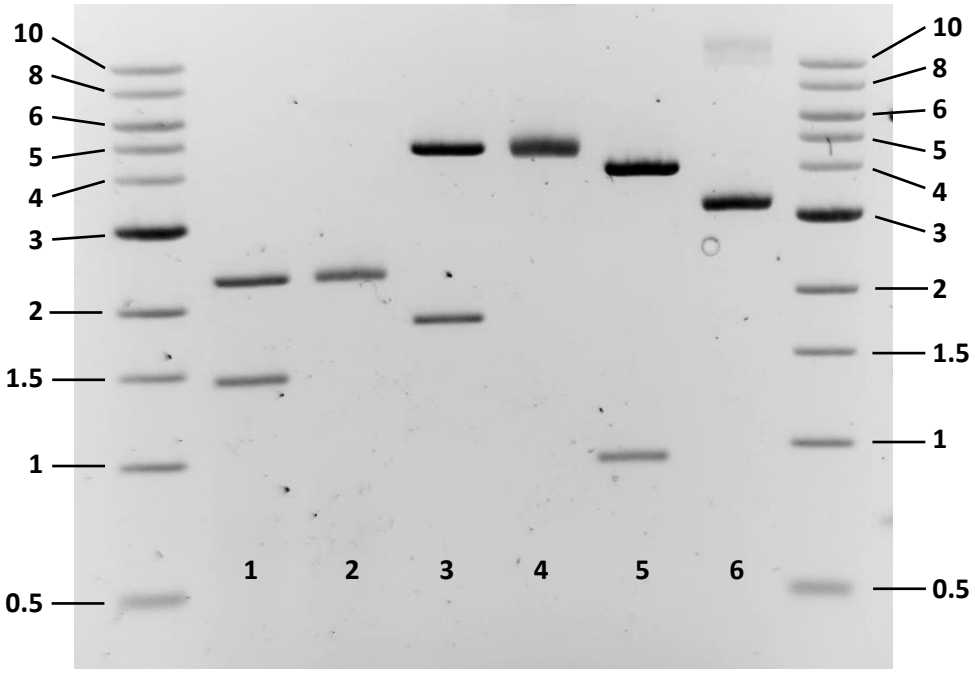


Figure 2.9: Restriction enzyme digests of pENTR1A, pGWL7 and pBS-35S-Ala-LucR. A restriction enzyme (RE) diagnostic was used to confirm the identity of the vectors. Lane 1: pENTR1A digested with KpnI and NotI, expected band sizes of 1460 and 2340 bp. Lane 2: pENTR1A no RE control. Lane 3: pGWL7 digested with EcoRI, expected band sizes of 1855 and 4274 bp. Lane 4: pGWL7 no RE control. Lane 5: pBS-35S-Ala-LucR digested with KpnI and NotI, expected band sizes of 953 and 4098 bp. Lane 6: pBS-35S-Ala-LucR no RE control. All ladder sizes are in kb.

Confirming that *XhABFA* and the target promoters were successfully cloned into the protoplast vectors

Once the identification of the vectors was confirmed, the purified PCR products were cloned into the entry vector pENTR1A. Colony PCRs and sequencing were used to confirm that the promoters and *XhABFA* were successful cloned into the entry vector. The insert DNA was then transferred from the entry vector into the destination vectors using the Gateway cloning strategy. The promoters were transferred into pGWL7 and XhABFA was transferred into the pUC19-GFP vector**.** A final PCR diagnostic was performed to confirm the presence of the promoters in pGWL7 (Figures 2.10) and *XhABFA* in the pUC19-GFP vector (Figure 2.11).

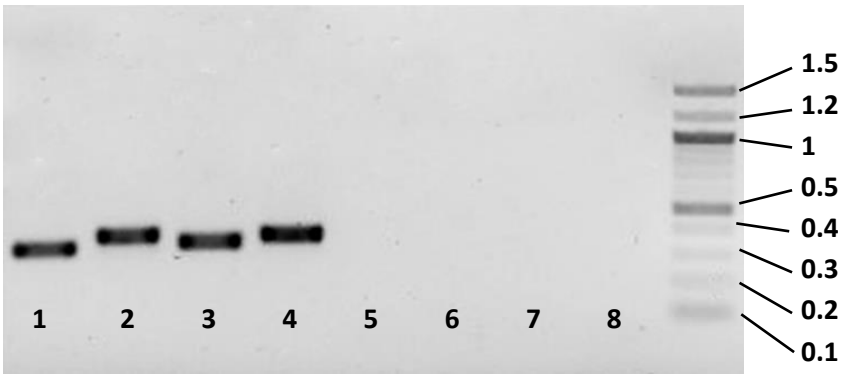


Figure 2.10: PCR amplification of the target promoters in pGWL7 using gene-specific primers. Lane 1: *XhPER1.* Lane 2: *XhECP63.* Lane 3: *XhDSI-1VOC.* Lane 4: *XhAHL23.* Expected product sizes of 322, 368, 352 and 371 bp respectively. Lanes 5-8: Respective no template controls. Ladder sizes are in kb.

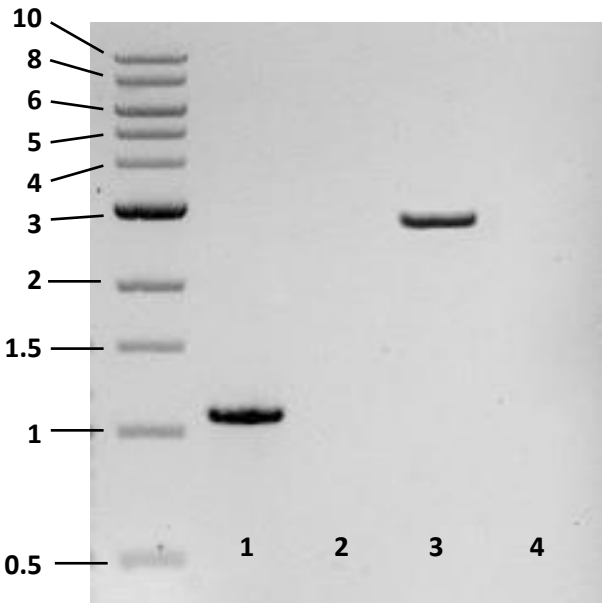


Figure 2.11: PCR amplification of *XhABFA* in the pUC19-GFP vector. Lanes 1 and 3: Gene-specific primers and *XhABFA* forward and GFP reverse primers gave the expected sizes of ~1.1 and 2.9 kb respectively. Lanes 2 and 4: No template control with gene-specific primers and *XhABFA* forward and GFP reverse primers respectively. All ladder sizes are in kb.

The EV control vector and the vector containing *XhABFA* have the same backbone. To confirm that the size of the vectors differed in the length of *XhABFA* a restriction enzyme diagnostic was performed (Figure 2.12).

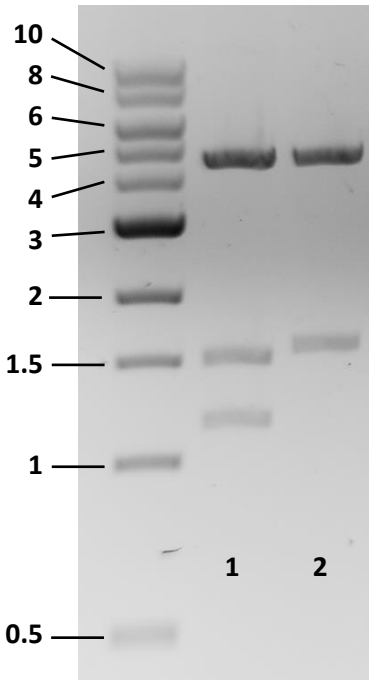


Figure 2.12: The vector containing *XhABFA* and the EV control were digested with XbaI. Lane 1: The digestion of the vector containing *XhABFA* resulted in bands estimated to be 4.6, 1.5 and 1.2 kb resulting in an overall size of 7.3 kb. Lane 2: The digestion of the EV resulted in 4.6 and 1.6 kb bands which estimates the EV to be 6.2 kb in size. Therefore the size difference is 1.1 kb which is roughly the length of *XhABFA* (1077 bp). All ladder sizes are in kb.

2.3.2 Validating that *XhABFA* and the target promoters were successfully cloned into the vectors required for the Y1H assays

Confirming that *XhABFA* and the target promoters were successfully amplified from the vectors used in the protoplast assay

Instead of using *X. humilis* RNA and cDNA as the templates for amplifying *XhABFA* and the target promoters, the vectors they had been successfully cloned into in the protoplast assay were used. Primers with restriction enzyme sites required to clone into the Y1H vectors were used to amplify *XhABFA* and the promoters from dilutions of the pUC19-35S-*XhABFA*-35S-sGFP vector and the pGWL7 firefly reporter vectors. Purified PCR products were visualised on a gel to confirm successful amplification (Figure 2.13).

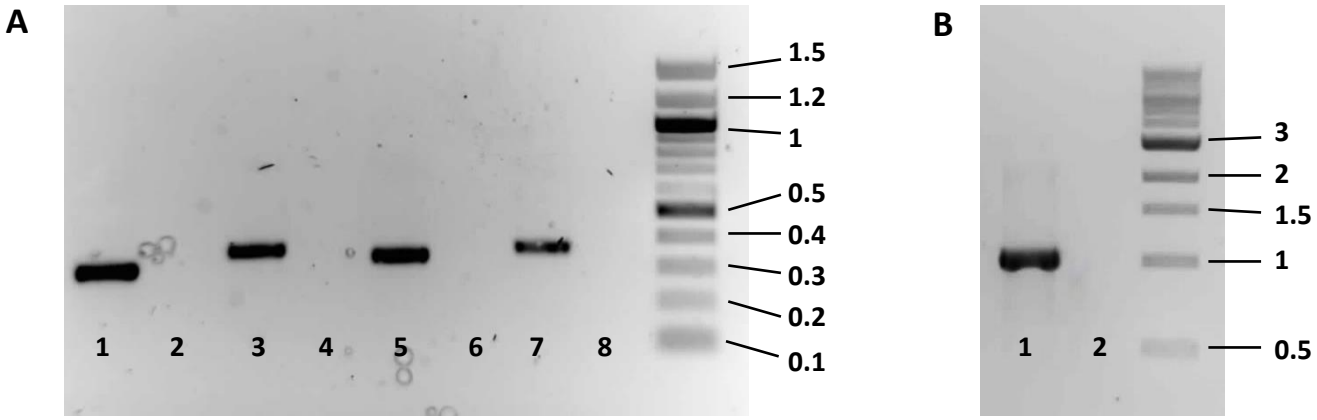


Figure 2.13: PCR amplification of *XhABFA* and the target promoters. (A) Successful amplification of the target promoters from the pGWL7 vector templates. Lanes 1, 3, 5 and 7: *XhPER1, XhECP63, XhDSI-1VOC* and *XhAHL23* with expected product sizes of 322, 368, 352 and 371 bp respectively. Lanes 2, 4, 6 and 8: Respective no template controls. (B) Successful amplification of *XhABFA* from the pUC19-35S-*XhABFA*-35S-GFP vector template. Lane 1: *XhABFA* with expected product size of 1077 bp Lane 2: No template control. All ladder sizes are in kb.

Confirming the identity of the vectors supplied by Clontech in the Y1H kit

The Clontech MATCHMAKER One-Hybrid System kit (K1603-1) provides 50 µl the vectors required for the assay at a concentration of 100 µg/µl. In order to obtain larger volumes with higher concentrations, each of the seven vectors specified in section 3.2.2 were transformed into *E. coli,* grown in LB cultures and purified with the PureYield™ Plasmid Midiprep System (Promega). A restriction enzyme diagnostic was performed to confirm the identity of the purified vectors (Figure 2.14) prior to performing the cloning procedures.

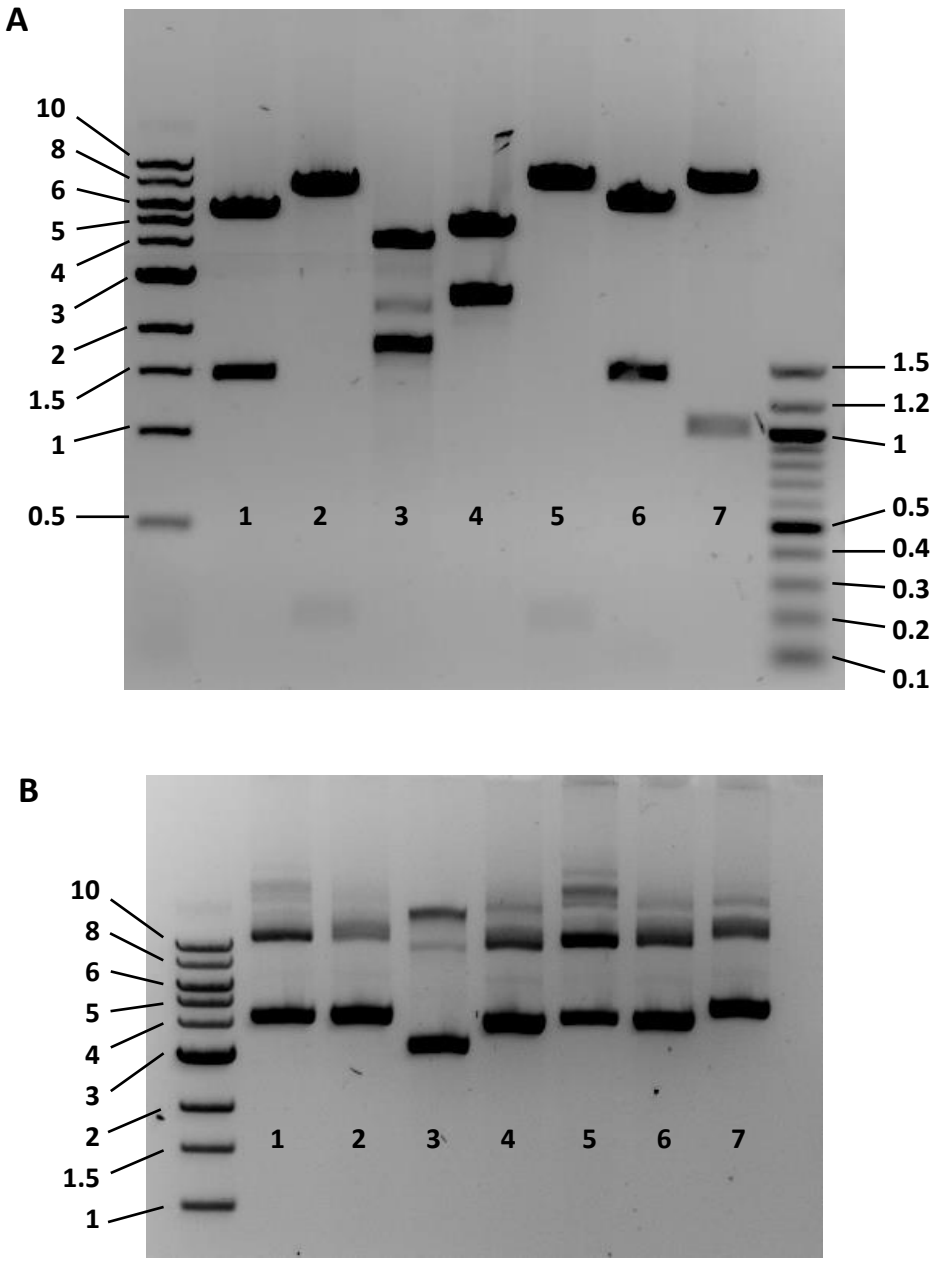


Figure 2.14: Restriction enzyme digests of vectors used in the Y1H assay. (A) A restriction enzyme diagnostic was used to confirm the identity of the vectors. The restriction enzymes used to digest each vector are specified in Supplementary Table 3.6. Lane 1: pHISi, expected band sizes of 5354 bp and 1446 bp. Lane 2: pLacZi, expected band sizes of 6720 bp and 180 bp. Lane 3: pHISi-1, expected band sizes of 3711 bp and 1689 bp. Lane 4: pGAD424, expected band sizes of 4197 bp and 2403 bp. Lane 5: p53BLUE, expected band sizes of 6520 bp and 180 bp. Lane 6: p53HIS, expected band sizes of 5354 bp and 1246 bp. Lane 7: pGAD53m, expected band sizes of 6700 bp and 1100 bp. (B) No restriction enzyme controls of vectors used in the Y1H assay. Lane 1: pHISi. Lane 2: pLacZi. Lane 3: pHISi-1. Lane 4: pGAD424. Lane 5: p53BLUE. Lane 6: p53HIS. Lane 7: pGAD53m. All ladder sizes are in kb.

Confirming that *XhABFA* and the target promoters were successfully cloned into the Y1H vectors

PCRs using gene-specific (Figure 2.15) as well as vector-specific primers (Figures 2.16 and 2.17) were used to confirm the presence of the inserts.

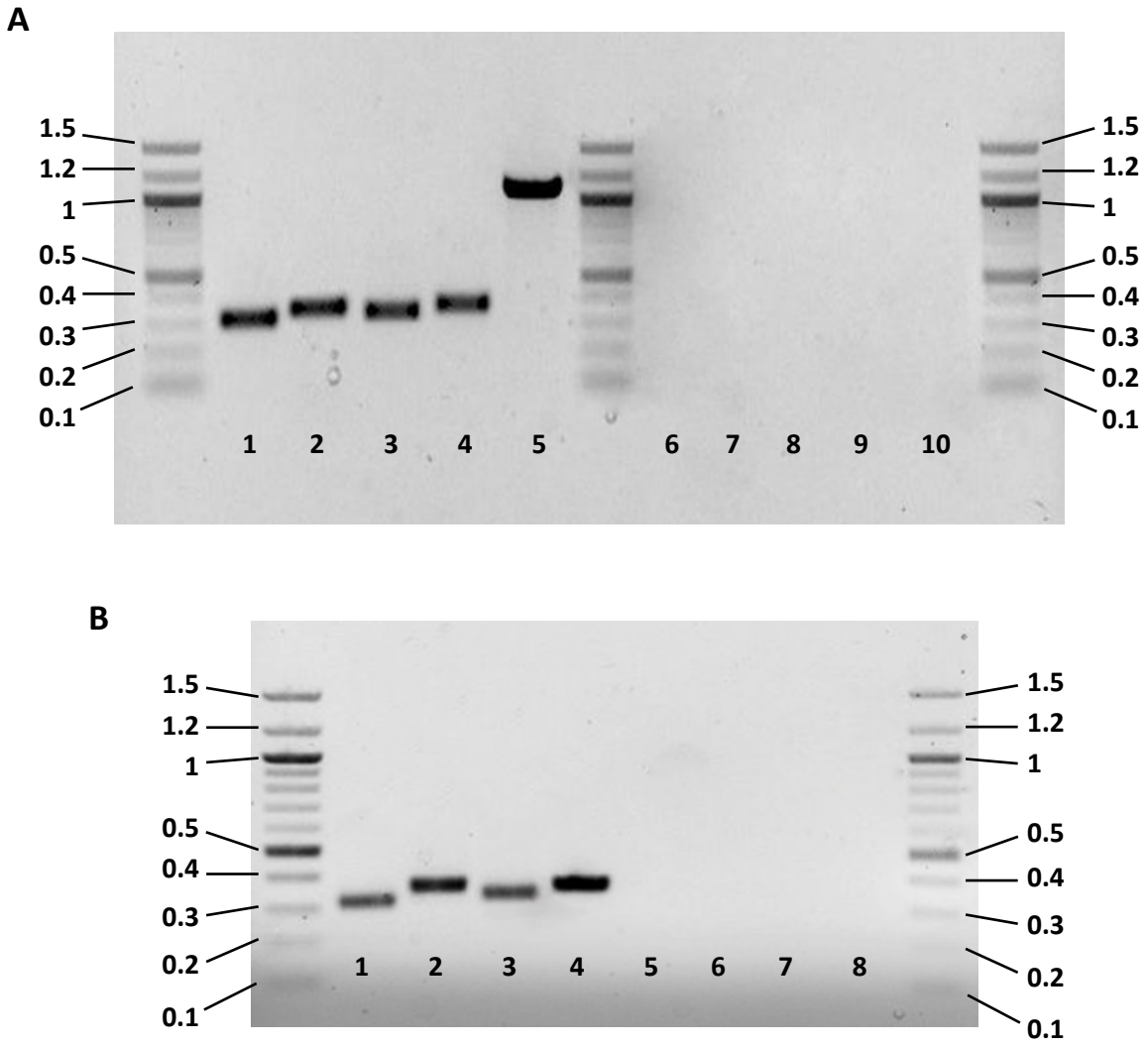


Figure 2.15: PCR amplification of *XhABFA* and the target promoters using gene-specific primers. (A) Amplification of the target promoters in pHISi-1 and *XhABFA* in pGAD424. Lane 1: *XhPER1.* Lane 2: *XhECP63.* Lane 3: *XhDSI-1VOC.* Lane 4: *XhAHL23.* Lane 5: *XhABFA.* Lanes 6-10: Respective no template controls (B) Amplification of the target promoters in pLacZi. Lane 1: *XhPER1.* Lane 2: *XhECP63.* Lane 3: *XhDSI-1VOC.* Lane 4: *XhAHL23.* Lanes 5-8: Respective no template controls All ladder sizes are in kb.

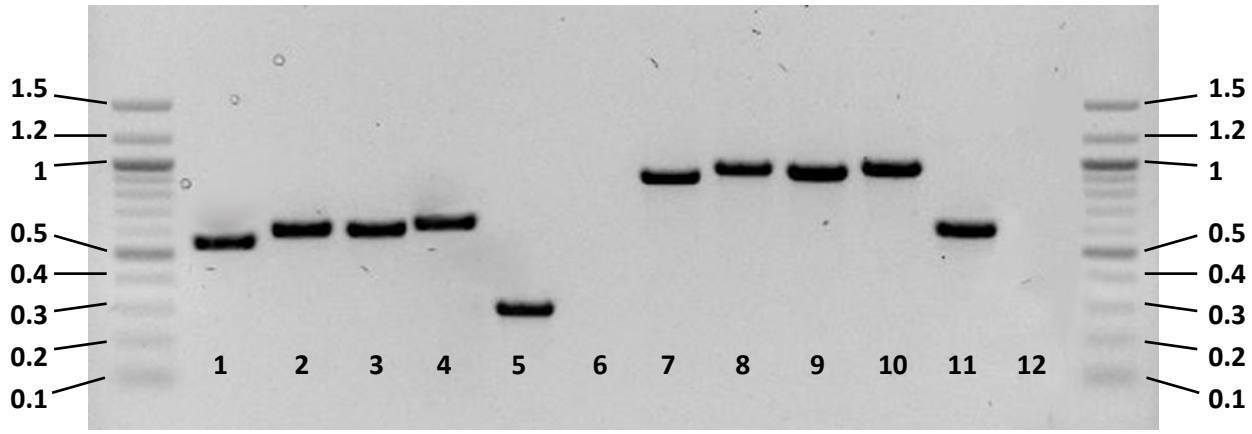


Figure 2.16: PCR amplification of the target promoters using vector-specific primers. The pHISi-1 forward and reverse primers were used in lanes 1-6. The pLacZi forward and reverse primers were used in lanes 7-12. Lane 1: *XhPER1.* Lane 2: *XhECP63.* Lane 3: *XhDSI-1VOC.* Lane 4: *XhAHL23.* Lane 5: pHISi-1 vector. Lane 6: No template control. Lane 7: *XhPER1.* Lane 8: *XhECP63.* Lane 9: *XhDSI-1VOC.* Lane 10: *XhAHL23.* Lane 11: pLacZi vector. Lane 12: No template control. All ladder sizes are in kb.

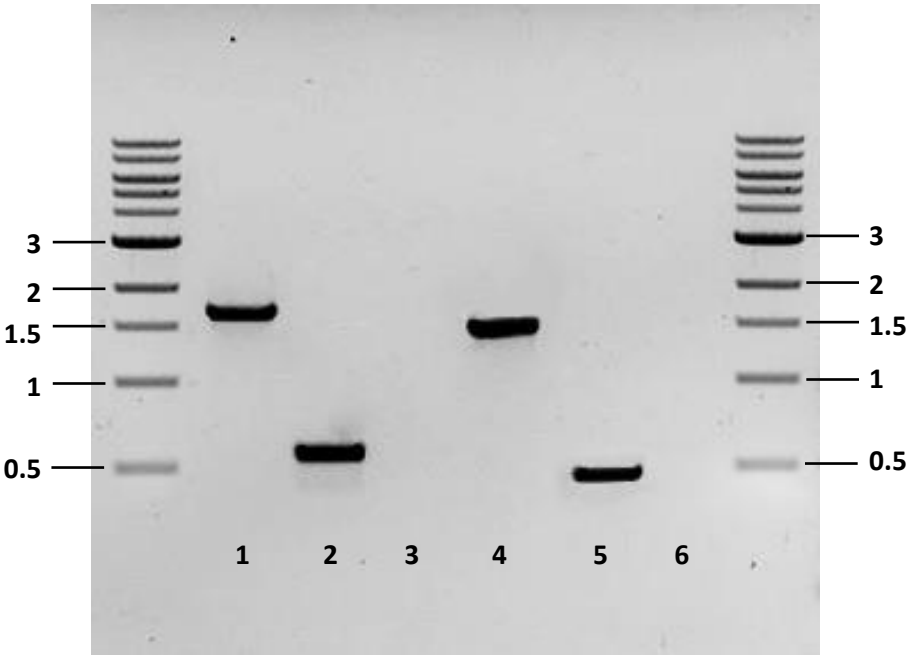


Figure 2.17: PCR amplification of *XhABFA* in pGAD424 using vector-specific primers. The GAL4 forward primer and pHybLex reverse primer were used in lanes 1-3. The GAL4 forward primer and pJG4-5 reverse primer were used in lanes 4-6. Lane 1: pGAD424-*XhABFA.* Lane 2: pGAD424. Lane 3: No template control. Lane 4: pGAD424-*XhABFA.* Lane 5: pGAD424. Lane 6: No template control. All ladder sizes are in kb.

Following the successful cloning of *XhABFA* and the promoters, the appropriate vector constructs were used to perform a Y1H assay (Chapter 3) and the transient transfection of *A. thaliana* protoplasts (Chapter 4).

Chapter 3: A Yeast One-Hybrid system to test XhABFA interactions with candidate seed maturation genes

3.1 Introduction

The current knowledge of the gene regulatory networks which control vegetative desiccation tolerance in angiosperms is limited. Research investigating the relationships between TFs and the genes they interact with during desiccation will provide a deeper understanding of these networks. XhABFA has been previously identified as a candidate TF which could potentially be a master regulator of the vegetative desiccation response in *X. humilis* (Lyall et al., 2019).

3.1.1 Functional binding assays

One method to investigate transcriptional networks is to perform an RNA-seq experiment where the transcriptome of the organism is sequenced and gene expression is quantified. In this type of study, genes which are expressed at a given moment can be identified. An example, in the context of desiccation tolerance, is the sequencing of RNA at intervals during progressive dehydration of a plant. This allows correlations to be made between the expression of genes and different relative water contents. When investigating regulation, the expression of TFs can be correlated with the expression of genes which confer tolerance during desiccation. This allows potential regulators to be identified. However correlation on its own cannot prove that a particular TF directly regulates expression; this requires experimental evidence.

One method to determine possible causation is to perform a functional binding assay. This type of experiment involves testing whether two or more molecules interact with each other (Pollard, 2010). Binding assays can be used to determine whether a TF of interest binds to a *cis*-regulatory element (CRE), which is located in the vicinity of the target gene (McClean, 1998). Some assays simply test binding ability whereas others test activation or repression of a target gene.

Initially, biochemistry-based methods were developed in order to identify protein-DNA interactions between TFs and CREs. These techniques included gel shift, DNAse I foot printing and chromatin-immunoprecipitation (Deplancke et al., 2004). However these are *in vitro* techniques which are performed in a controlled environment outside of a living organism and do not replicate cellular conditions (Kinnberg, 2003). In comparison, *in vivo* assays are performed in the context of the cellular environment or the whole organism. This is advantageous as conditions that are unique to the cell are present, such as post-translational modifications and interactions with additional proteins or signalling molecules (Sheen, 2001). The development of the Y1H system offered an *in vivo* method to detect protein-DNA interactions in the context of a cellular environment.

3.1.2 The Yeast One-Hybrid Assay

While there is a substantial amount of structural and functional variety in what constitutes an activating TF, two features remain constant: an activation domain (AD) and a DNA-binding domain

(DBD). TFs bind to specific sites in the promoters of genes via their DBD; while the AD mediates the recruitment of RNA polymerase II, either directly or through co-activators (Lee and Young, 2000).

Many properties of transcriptional regulation, such as the modular nature of the DBD and AD, were first demonstrated with the yeast GAL4 protein (Ma and Ptashne, 1987**;** Traven et al., 2006). The observations that the AD of the GAL4 TF is separable from its DBD, and that these functional domains can be fused to heterologous proteins, formed the basis of the Yeast One-Hybrid Assay (Sadowski and Ptashne, 1989).

The Y1H assay consists of two main components: (1) a reporter vector with a target CRE cloned upstream of a reporter gene; and (2) an activation domain vector with a TF of interest fused to the AD of the yeast GAL4 TF (Reece-Hoyes and Walhout, 2012a). Both constructs are introduced into a yeast strain and if the hybrid AD-TF binds to the CRE, the expression of the reporter gene will be induced (Figure 3.1). Notably, the transcription of the reporter gene will be activated regardless of whether the TF is an activator or repressor. Therefore the assay only measures physical interactions between a TF and CRE (Bass et al., 2016).

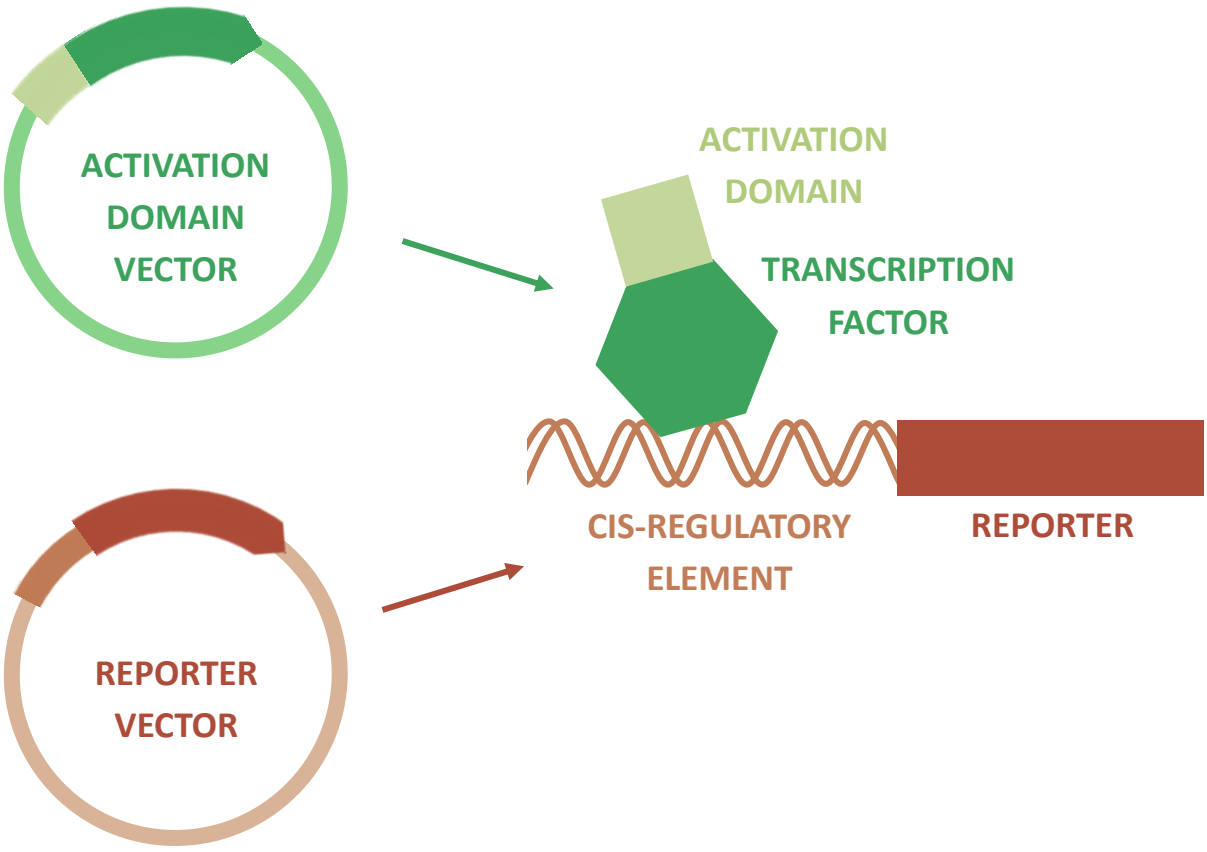


Figure 3.1: An illustration of the basic components of a Yeast One-Hybrid assay. The two main constructs involved in the assay are a vector which expresses a hybrid protein consisting of a transcription factor of interest (dark green) fused in frame to the yeast GAL4 activation domain (light green); and a vector which has a *cis*-regulatory element (light red) cloned upstream of a reporter gene (dark red). If there is a positive interaction between the two components it results in the expression of the reporter gene.

3.1.3 Assaying for reporter gene expression in a Y1H system

Putative interactions between a TF and CRE can be measured using different reporter assays. Two of the most common assays in the Y1H system are the use of auxotrophic mutants and colourimetric markers (Bass et al., 2016). Auxotrophic yeast strains lack a functional copy of a gene which is required for growth. These genes often encode enzymes in metabolic pathways which are necessary for the synthesis of essential monomers, such as amino acids, which are used in biosynthesis. In the Y1H assay, reporter vectors contain a wild type allele of the affected gene. Therefore auxotrophic yeast strains which are complemented with integrated reporters can be selected when grown on media lacking the vital enzyme (Pronk, 2002). Commonly used auxotrophic marker genes include *URA3, HIS3 and LEU2* which encode essential enzymes for the synthesis of uracil, histidine and leucine respectively.

Reporter gene expression resulting from a positive interaction between a TF and CRE can also be measured with a colourimetric assay. The colony-lift filter assay is commonly used in yeast to measure the activity of *lacZ* which acts as a reporter gene. The *lacZ* gene encodes β-galactosidase (β-gal), an enzyme whose catalytic activity cleaves β-glycosidic bonds. In order to determine whether *lacZ* expression has been activated, the presence of β-gal is tested. The compound bromo-chloro-indolyl-galactopyranoside, or X-gal, is a substrate for β-gal that produces a dark blue precipitate when cleaved by β-gal. Therefore, when X-gal is introduced to yeast cells, the presence or absence of a blue pigment can be correlated to the expression of the *lacZ* reporter gene (Schenborn and Groskreutz, 1999).

By using two reporter systems, such as *lacZ* and *HIS3,* the uncertainty of the results from one particular assay can be minimised. Reporter gene expression in the absence of a TF is more likely to be identified as a false positive arising from autoactivity if two independent measurements are used.

3.1.4 Screening for background expression

In the Y1H assay, the *HIS3* and *lacZ* reporter genes both have upstream minimal promoters. This is required in order to select for yeast strains with integrated reporter constructs. However different integrant strains arising from the same transformation can exhibit varying levels of autoactivity which results in background expression. This could be due to differing numbers of reporters integrated into each strain or at each locus within each strain (Deplancke et al., 2004). Therefore it is necessary to screen integrants for background expression as this could result in false positives. *HIS3* integrants with low background expression are selected by performing a titration with 3-amino-1,2,4-triazole (3AT). 3AT is a competitive inhibitor of the HIS3 protein. During the final assay, the leaky expression of *HIS3* is quenched by adding a certain concentration of 3AT, predetermined by the titration, to the media. Therefore yeast which grow on media containing 3AT must produce a significantly greater concentration of the HIS3 protein to produce enough histidine. In terms of *lacZ* autoactivity, strains which produce the lowest intensity of blue are selected prior to the final assay. All integrants should generate some level of blue compound in the assay. The intensity of the blue can be correlated to the strength of the promotor activation by the TF (Figure 3.2).

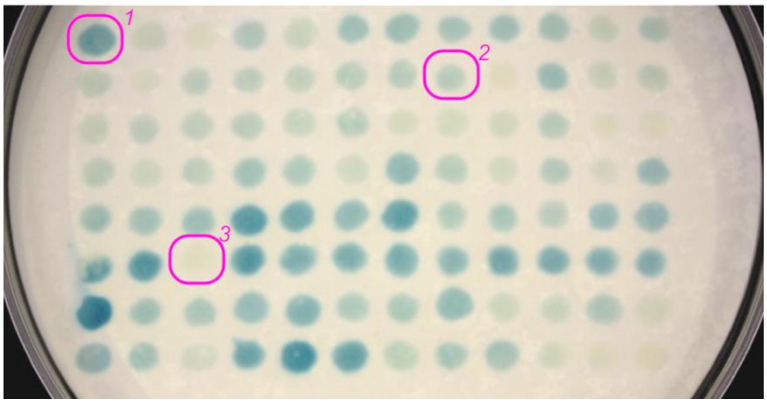


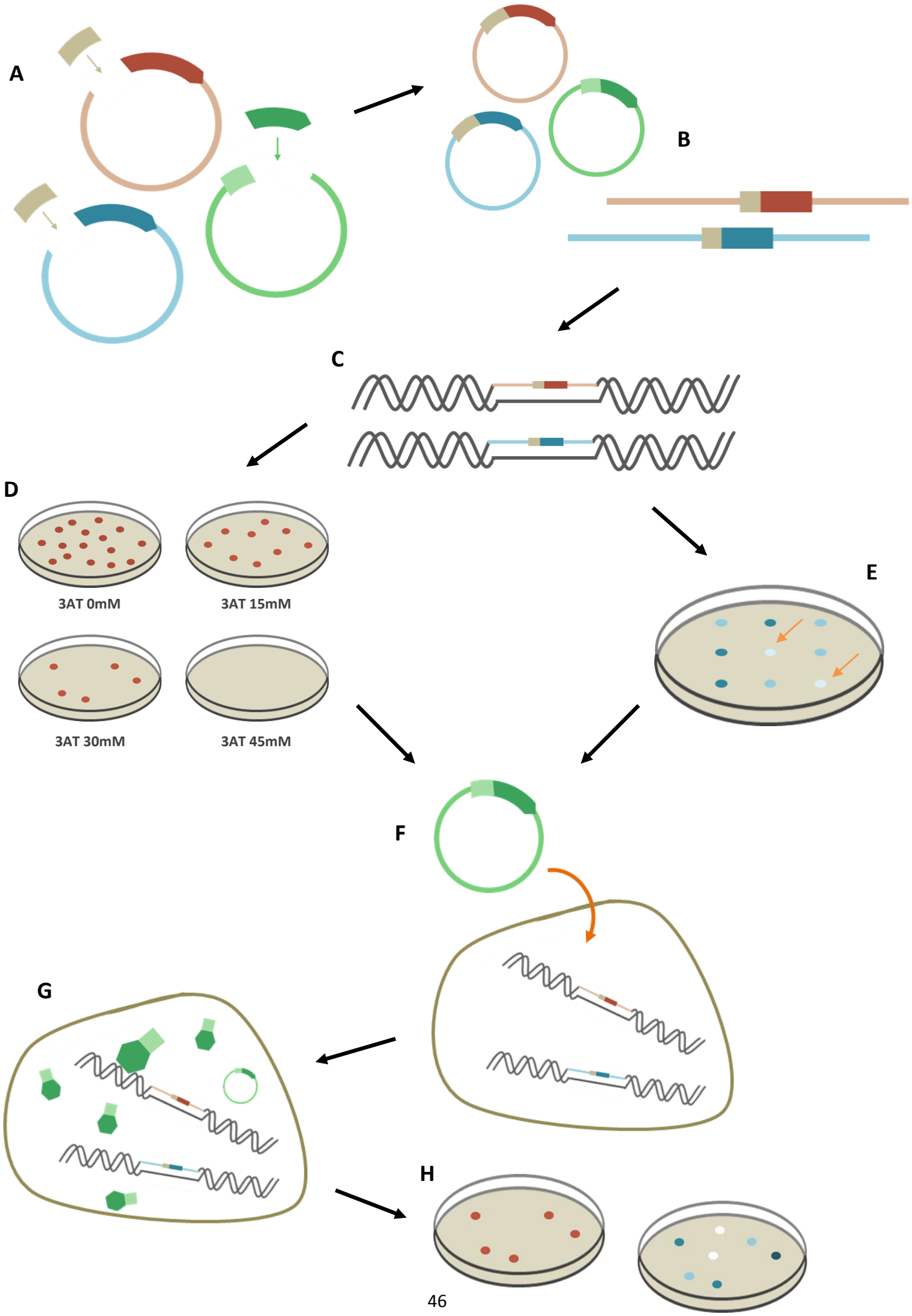
Figure 3.2: An example of a 96-spot *lacZ* assay. (1) strong Y1H positive; (2) weak Y1H positive; (3) Y1H negative. Reproduced from Reece-Hoyes and Walhout, 2012b.

3.1.5 Experimental workflow

The Yeast One-Hybrid (Y1H) assay was used to determine whether the transcription factor XhABFA interacts with the promoters of candidate seed maturation genes. A chief advantage of the Y1H assay is that it offers an experimental system that is set in the context of a chromatin environment. Promoter-reporter constructs are integrated into the yeast genome. This means that eukaryotic TFs are presented with regulatory elements in a form that better reflects endogenous conditions compared to ‘‘naked’’ DNA (Reece-Hoyes and Walhout, 2012a).

Each promoter, which acted as independent target CREs, was cloned into both the pHISi-1 and pLacZi reporter vectors. *XhABFA* was cloned into the pGAD424 activation domain vector to express a hybrid GAL4AD::*XhABFA* fusion protein. A positive control experiment was performed in tandem with the experimental assay. In the positive control experiment, the mouse *p53* protein acted as the TF and three tandem copies of the consensus *p53* binding site acted as the target CRE. An outline of the workflow of a general Y1H assay is illustrated in Figure 3.3.

Figure 3.3: A schematic representation of the experimental workflow of a Yeast One-Hybrid assay. (A) A target CRE (grey) and TF of interest (dark green) are cloned into reporter vectors, such as pHISi-1 (red) and pLacZi (blue), and an activation domain vector respectively. (B) The reporter vectors are linearised. (C) The reporters are transformed into yeast cells and integrated into the genome of the yeast strain by homologous recombination: pLacZi at URA3 and pHISi-1 at HIS3. (D) The autoactivity of the integrants are tested. Background *HIS3* expression is measured by a titration with 3AT where independent integrant strains are each plated onto media containing a series of 3AT concentrations. (E) Background *lacZ* expression is measured by a colony-lift filter assay where the intensity of the blue compound is compared amongst independent integrant strains. (F) Strains with low background are transformed with the AD vector. (G) The AD-TF fusion protein is produced in the cells and interacts with the target CREs if able. (H) Putative interactions between the AD-TF fusion protein and target CREs are identified by measuring *HIS3* expression and *lacZ* activity. Positive interactions result in growth on -his selection plates and the presence of a blue compound from a colony-lift filter assay respectively.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3AT 0mM
3AT 15mM
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3.2 Materials and Methods

3.2.1 Integration of target reporters into the yeast genome

Linearisation of target reporter vectors

The target reporter vectors were linearised with restriction enzymes in order for them to integrate into the genome of YM4271. The four pHISi-1 vectors and p53HIS were digested with XhoI. The four pLacZi vectors and p53BLUE were digested with NcoI. A total of 4 µg of each vector was digested. The 4 µg were divided into four separate digests. Each reaction contained 1 µg of the vector, 1 µl of either XhoI or NcoI and 5 µl of Cutsmart® Buffer (New England Biolabs) in 50 µl of nuclease free water. The samples were incubated at 37°C for 2 hours and then either at 65°C, the pHISi-1 vectors, or 80°C, the pLacZi vectors for 20 minutes. The four reactions of each target reporter were combined and then purified using the Wizard® SV Gel and PCR Clean-Up system (Promega) as per the manufacturer's instructions and eluted into 30 µl of nuclease-free water. The concentrations of the purified samples were determined using a NanoDrop. In order to confirm complete linearisation of the vectors, 2 µl of each sample was electrophoresis on a 1% agarose gel and viewed using a UV Transilluminator.

LiAc-mediated dual transformation of YM4271 with target reporter vectors

YM4271 was transformed with the target reporter vectors using a modified version of the LiAc-mediated yeast transformation according to the Yeast Protocols Handbook (Clontech). The reagents, concentrations and pH of the solutions used are summarised in Supplementary Table 10.

Three days prior to the transformation, YM4271 was streaked onto YPD plates from a glycerol stock kept at -80°C. The yeast was grown for two days or until distinct colonies were 2-3 mm in diameter. 1 ml of YPD was inoculated with several colonies and mixed by pipetting to disperse any clumps. The 1 ml was transferred into a flask containing 50 ml of YPD and incubated overnight at 30°C in a shaking incubator (225 rpm) until stationary phase was reached (OD_{600} > 1.5). Aliquots of the overnight culture were transferred to a flask containing 300 ml of YPD until the OD_{600} reached 0.2 - 0.3. The culture was incubated at 30°C in a shaking incubator (225 rpm) for 3 hours or until the OD_{600} reached 0.4 - 0.6. The culture was aliquoted into 50 ml falcon tubes and centrifuged at 700 g for 5 minutes to pellet the yeast cells. The supernatants were discarded and the cell pellets resuspended in sterile TE buffer. The cells were pooled into one falcon tube (final volume 40–50 ml) and centrifuged at 700 g for 5 minutes. The supernatant was discarded and the cells were washed a second time with 40 ml of sterile TE buffer and centrifuged at 700 g for 5 minutes. The supernatant was discared and the cells were resuspended in 1.5 ml of freshly prepared, sterile 1X TE/LiAc solution. 3 µg of each of the linearised target reporter vectors and 100 µg of single-stranded salmon testes carrier DNA (D7656 SIGMA) were added to 2 ml tubes and mixed by pipetting.

A dual transformation was performed which meant each tube contained two vectors: a target promoter in the pHISi-1 vector and the corresponding promoter in the pLacZi vector; the positive

control contained p53HIS and p53BLUE. There were 6 tubes in total: the 4 target promoters; the positive control vectors; and a control which excluded any plasmid DNA. 100 µl of the yeast cell suspension was added to each tube and mixed by pipetting. 600 µl of sterile PEG/LiAc solution was added to each tube and mixed by inversion. The tubes were incubated at 30°C in a shaking incubator (225 rpm) for 30 minutes. 70 µl of DMSO was added and mixed by gentle inversion. The transformation reactions were heat shocked in a 42°C water bath for 15 minutes and then chilled on ice for 2 minutes. The tubes were centrifuged at 14 000 rpm/g. The supernatant was removed and the cells were resuspended in 500 µl of sterile TE buffer. 100 µl of each transformation was plated on 3 plates each with different selection media: SD/-his plates (selection of pHISi-1 integrants), SD/-ura plates (selection of pLacZi integrants) and SD/-his-ura plates (selection of pHISi-1 and pLacZi integrants). The selection plates were inverted and incubated at 30°C for 3-6 days or until yeast colonies appear.

Colony PCRs

The YM4271 yeast strain requires the digestion of the cell wall to break open the yeast cells in order for plasmids or DNA fragments from the genome to be amplified. Prior to performing the PCR, a whole single yeast colony was collected on a 200 µl pipette tip and added to 20 µl of 40 mM NaOH solution. The cells were resuspended and then incubated at 95°C for 15 minutes. The digested yeast cells were centrifuged briefly and 2 µl of the supernatant was used as the template DNA for the PCR reactions. Gene-specific primers as well as vector-specific primers (pHISi-1 or pLacZi) were used to screen for positive transformants. Ampliqon 2x Master Mix RED (A180303) was prepared as per the manufacturer's instructions with the addition of the template DNA and appropriate primers. A "no template" control which excluded the supernatant of the digestion reactions was used as a negative control. The PCR cycling parameters were followed according to Supplementary Tables 7 and 8. PCR products were electrophoresed and visualised to confirm the presence of the insert DNA.

3.2.2 Screening integrants for background expression

Dual pLacZi/pHISi-1 YM4271 strains with integrated target promoter-reporter vectors were screened for background expression. 3AT titrations and colony-lift filter assays were used to screen for *HIS3* and *lacZ* autoactivity respectively.

3AT titrations

3AT titrations were performed on the YM4271 reporter strains in order to select pHISi-1 integrants with the lowest *HIS3* background expression. Nine distinct transformants (colonies) were sampled from each of the SD/-his-ura selection plates. A small quantity of each colony was collected with a sterile toothpick and suspended in 1 ml of TE buffer. 5 µl of each suspension was spotted on SD/-his plates with a series of 3AT concentrations: O, 5, 15, 30 and 45 mM. The plates were incubated at 30°C for 2 days.

Colony-lift filter assays

The colony-lift filter assay was used to measure β-galactosidase (*lacZ*) expression using the colourless compound X-gal, which turns blue in the presence of *lacZ.* The reagents, concentrations and pH of the solutions used are summarised in Supplementary Table 11.

The nine transformants chosen to be screened for *HIS3* background expression were also screened for *lacZ* background expression. 5 µl of each of the suspensions made for the 3AT titrations were spotted on SD/-ura plates. The plates were incubated at 30°C for 2 days. A modified version of the colony-lift filter assay according to the Yeast Protocols Handbook (Clontech) was performed. For each SD/-ura plate, two Whatman No. 5 filter papers were soaked in 5 ml of Z buffer/X-gal solution in a 90 mm petri dish. Using forceps, a nitrocellulose filter paper was placed over the surface of the SD/-ura plate and gently rubbed with the side of the forceps to help colonies cling to the filter. The periphery of both the Whatman filters and the nitrocellulose filter had been pre-marked in asymmetrical locations to orient them to the colonies on the plate and one another. The nitrocellulose filter was lifted off the SD/-ura plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen. The filter was completely submerged for 10 seconds. The frozen filter paper was removed from the liquid nitrogen and allowed to thaw at room temperature. Once it had thawed, it was placed colony side up on the Whatman filter papers soaked in Z buffer/X-gal solution. Care was taken to avoid trapping air bubbles under or between the filters. The plates were incubated at 30°C and checked periodically for the appearance of blue colonies.

Four transformants of each of the promoter-reporter strains which had the lowest *HIS3* and *lacZ* background expression were selected to be transformed with the pGAD424-*XhABFA* vector; or the pGAD53m AD vector for the positive control experiment.

3.2.3 Testing the transactivation of seed maturation genes by XhABFA

LiAc-mediated transformation of the YM4271 strains with the AD vectors

Four YM4271 yeast strains of each target promoter were transformed with the pGAD424-*XhABFA* vector in a similar manner to the transformations of the reporter vectors (section 3.2.8). The positive control experiment was performed in tandem with these transformations where p53HIS, p53BLUE and pGAD53m were used instead of a target promoter-pHISi-1 reporter vector, a target promoter-pLacZi reporter vector and the pGAD424-*XhABFA* AD vector respectively. For each transformation, a negative control was also performed where a pGAD424 vector excluding *XhABFA* was used instead of the pGAD424-*XhABFA* or pGAD53m vectors. In total, 16 transformations with pGAD424-*XhABFA,* 4 transformations with pGAD53m and 20 transformations with pGAD424 were performed.

Determining positive interactions via selective media and colony-lift filter assays

100 µl of each transformation was plated on four SD/-his-ura-leu plates each of which had a different concentration of 3AT: 0, 5, 30 and 45 mM. The selection plates were inverted and incubated at 30°C for 3-6 days or until yeast colonies appear. Colony-lift filter assays were performed on the transformants which grew on the plates that lacked 3AT (0 mM). Photographs of the plates were taken two hours after incubation.

3.3 Results

3.3.1 Confirming that the reporter constructs were successfully integrated into the yeast genome

Confirming the linearisation of the target reporter vectors

Prior to transformation, the target reporter vectors were linearised at specific sites in order for them to integrate into the genome of the YM4271 yeast strain. The digested vectors were visualised on a gel to confirm that they had been completely linearised and that there was no uncut plasmid still present (Figures 3.4 and 3.5)

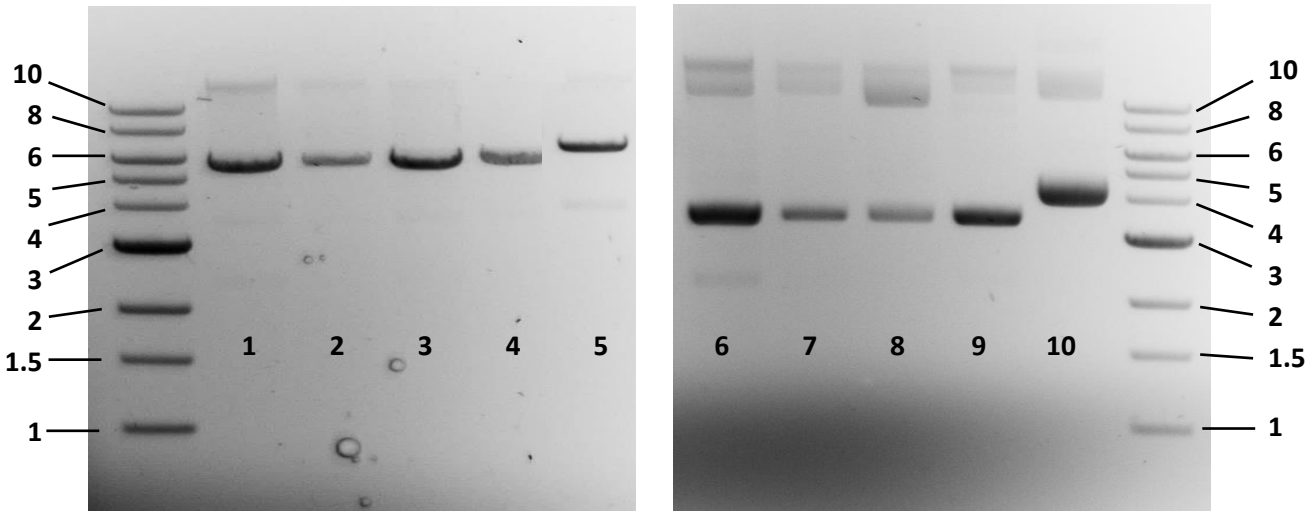


Figure 3.4: Restriction enzyme digests of each of the four promoters in pHISi-1 and the p53HIS vector. The vectors were digested with XhoI and 2ul was run on a gel to confirm sufficient linearisation. Lanes 1-4: pHISi-1 containing *XhPER1, XhECP63, XhDSI-1VOC* and *XhAHL23* respectively. Expected band sizes of 5.7 kb. Lane 5: p53HIS, expected band size of 6.6 kb Lanes 6-10: Respective "no RE" control. All ladder sizes are in kb.

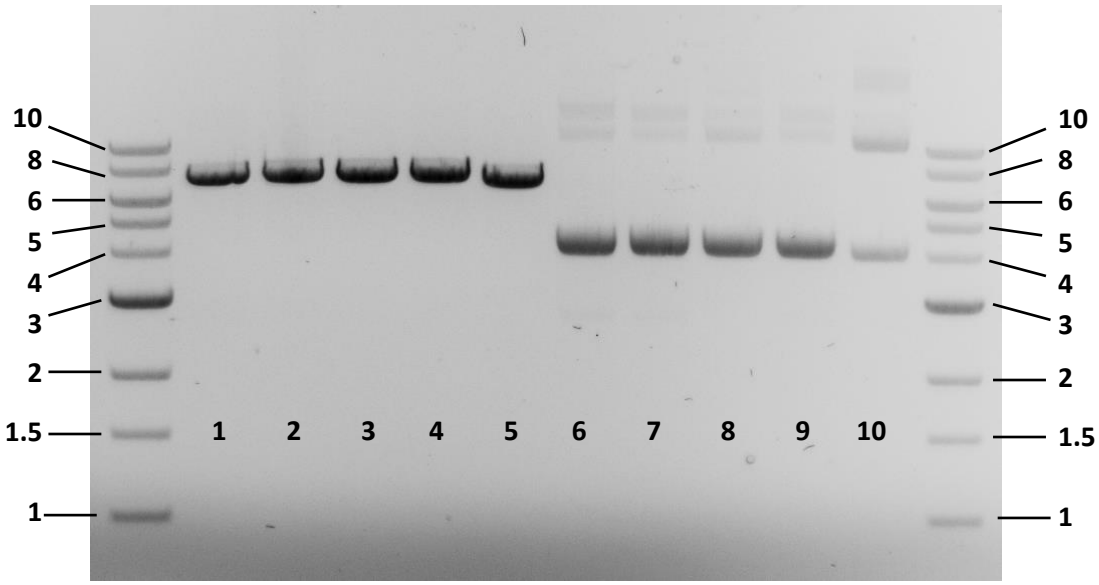


Figure 3.5: Restriction enzyme digests of each of the four promoters in pLacZi and the p53BLUE vector. The vectors were digested with NcoI and 2ul was run on a gel to confirm sufficient linearisation. Lanes 1-4: pLacZi containing *XhPER1, XhECP63, XhDSI-1VOC* and *XhAHL23* respectively. Expected band sizes of 7.2 kb. Lane 5: p53BLUE, expected band size of 6.7 kb Lanes 6-10: Respective "no RE" control. All ladder sizes are in kb.

3.3.2 Testing the transactivation of seed maturation genes by XhABFA

Four dual transformants of each of the promoter-reporter strains which had the lowest *HIS3* and *lacZ* background expression were selected to be transformed with the pGAD424-*XhABFA* vector; or the pGAD53m AD vector for the positive control experiment.

Growth on selective media

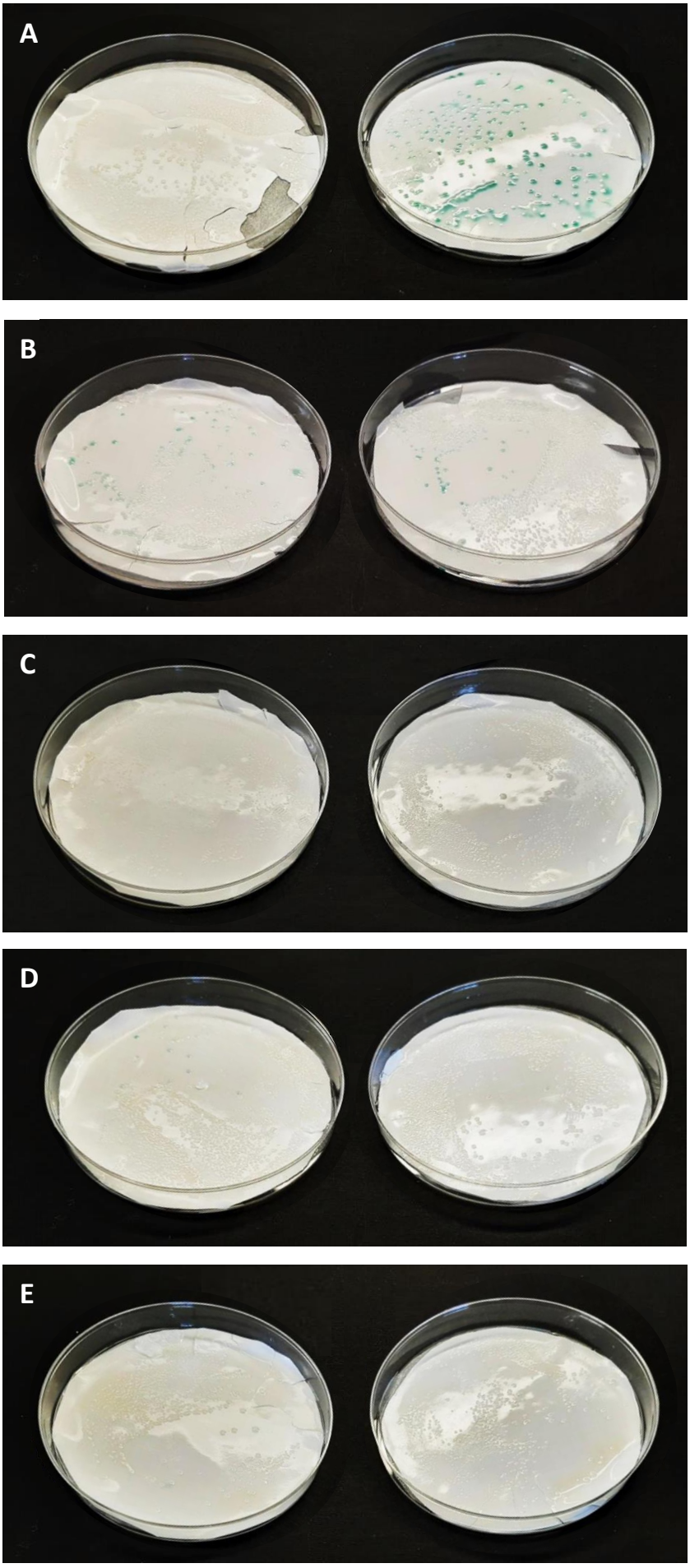
This experiment was performed in tandem with a positive control experiment which included a known interaction of a promoter with a TF: p53HIS (has 3 copies of the *p53* binding site) and pGAD53m (contains the mouse *p53* gene in frame with the GAL4 AD). The results of the growth are summarised in Table 3.1. It is apparent that the only assay where yeast colonies grew on the SD/-his-ura-leu selections plates which contained 3AT was the yeast strain with an integrated positive control promoter (p53HIS), transformed with the positive control AD vector pGAD53m.

Promoter	AD vector	0 mM	5 mM	30 mM	45 mM
XhPER1	pGAD424	✓	x	x	x
	pGAD424-*XhABFA*	✓	x	x	x
XhECP53	pGAD424	✓	x	x	x
	pGAD424-*XhABFA*	✓	x	x	x
XhDSI-1VOC	pGAD424	✓	x	x	x
	pGAD424-*XhABFA*	✓	x	x	x
XhAHL23	pGAD424	✓	x	x	x
	pGAD424-*XhABFA*	✓	x	x	x
p53	pGAD424	✓	x	x	x
	pGAD53m	✓	✓	✓	✓

Table 3.1: Summary of the colony growth after the transformation of pGAD424, pGAD424-*XhABFA* and pGAD53m on selection plates containing various concentrations of 3AT. Colonies were present on all the SD/-his-ura-leu selection plates indicating that both reporter vectors (promoter-pHISi-1 and promoter-pLacZi) as well as the AD vectors were successfully transformed. The only colonies present on the plates containing 3AT (5, 30 and 45 mM) were the positive control transformation (p53HIS and pGAD53m).

Colony-lift filter assays

Colony-lift filter assays were performed on the transformants which grew on the SD/-his-ura-leu (0 mM 3AT) plates. Two hours after incubation, positive (blue) colonies were present on the yeast strain with the positive control *p53* promoter which had been transformed with pGAD53m (Figure 6). The corresponding negative control which was transformed with pGAD424 only had white colonies. Blue colonies were present on the yeast strain with *XhECP63.* However both the negative control (pGAD424) and the yeast transformed with pGAD424-*XhABFA* had blue colonies indicating a high background expression of *lacZ* in the yeast strain with *XhECP63.* Yeast strains with the *XhPER1, XhDSI-1VOC* and *XhAHL23* promoters did not exhibit any white colonies even after 24 hours.


A
B
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D
E

Figure 3.6: Colony-lift filter assays were used to measure promoter-TF interactions. The expression of the *lacZ* reporter gene results in the production of the β-galactosidase enzyme. In the presence of β-gal the colourless compound X-gal turns blue indicating a positive interaction between the promoter and TF. Photographs of the colony lifts were taken two hours after incubation. Plates on the left are the yeast strains transformed with the negative control pGAD424. Plates on the right are those transformed with the positive control pGAD53m (A) and the pGAD424-*XhABFA* vector (B-E). The yeast strains in each photograph have the following promoters integrated into their genome: (A) p53. (B) *XhECP63.* (C) *XhPER1.* (D) *XhDSI-1VOC.* (E) *XhAHL23.*

Colony PCRs

Colony PCRs were performed in order to determine whether the promoter-reporter constructs and the AD-TF construct were present in the yeast cells post transfection. However, the results were unreliable. Some colonies sampled from the transformation of the positive control experiment indicated the presence of a PCR product while others did not, even though all samples should have amplified a product. PCRs were performed using pLacZi forward and reverse primers to test for the presence of the p53BLUE construct; Gal4 forward and pHybLex reverse primers were used to test for the presence of pGAD424 or pGAD53m. Figure 3.7 and 3.8 demonstrate the inconsistent results obtained from the PCRs. In figure 3.7, there should be a 0.6kb band for all lanes except lane 10 which is a no template control. In figure 3.8 there should be a 0.56kb band for lanes 1-4 (pGAD424) and a 1.5kb band for lanes 5-8 (pGAD53m). The colonies used as templates for lanes 5-8 (both figure 3.7 and 3.8) gave strong blue positive colonies in the colony-lift filter assay however according to the gel images, the p53BLUE vector was only present in colonies 1 and 4 (Lane 5 and 8) and the pGAD53m vector was only present in colony 4 (Lane 8). This indicates the inconsistency of the colony PCRs which makes them an unreliable diagnostic tool in this context.

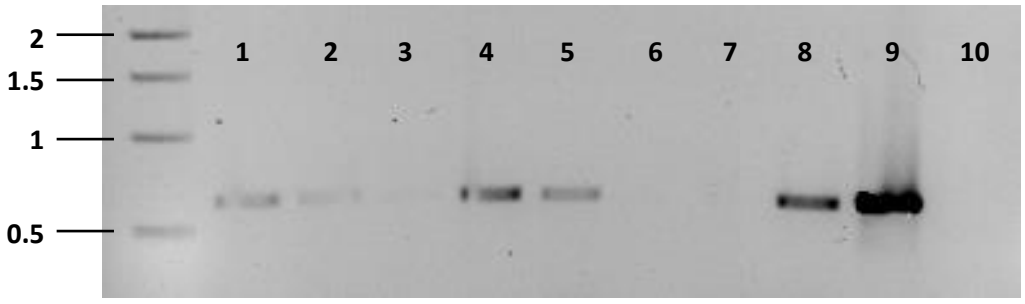


Figure 3.7: PCR amplification of the p53BLUE vector target promoters using pLacZi forward and reverse primers. Lanes 1-4: Colonies 1-4 from the transformation of the p53BLUE yeast strain with pGAD424 (negative control). Lanes 5-8: Colonies 1-4 from the transformation of the p53BLUE yeast strain with pGAD53m (positive control). Lane 9: dilution of p53BLUE midi prep. Lane 10: No template control. All ladder sizes are in kb.

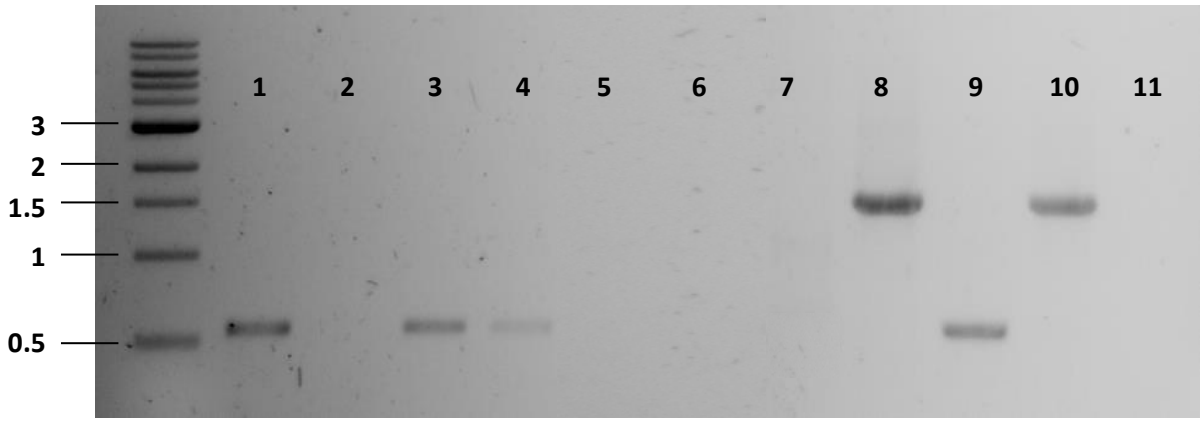


Figure 3.8: PCR amplification of the pGAD424 and pGAD53m vectors using the Gal4 forward and pHybLex reverse primers. Lanes 1-4: Colonies 1-4 from the transformation of the p53BLUE yeast strain with pGAD424 (negative control). Lanes 5-8: Colonies 1-4 from the transformation of the p53BLUE yeast strain with pGAD53m (positive control). Lane 9: dilution of pGAD424 midi prep. Lane 10: dilution of pGAD53m midi prep. Lane 11: No template control. All ladder sizes are in kb.

3.4 Discussion

The Yeast One-Hybrid assay was used to test whether the transcription factor XhABFA is able to bind to the promoters of three canonically seed-specific genes that are upregulated during VDT in the resurrection plant *X. humilis.* In this Y1H assay, putative interactions between XhABFA and the promoters were measured using two different reporter strategies: complementation of the HIS3 auxotrophic mutant and a *lacZ* colourimetric assay. Both involved cloning the candidate promoters upstream of reporter genes, *HIS3* and *lacZ* respectively. *XhABFA* was fused in frame to the yeast GAL4 activation domain. If there were positive interactions between XhABFA and the promoters it would have resulted in the expression of the reporter genes which allows selection on media lacking histidine (in the presence of 3AT) and the appearance of blue colonies in a colony-lift filter assay with blue/white selection. However, this was not observed.

The Y1H assay includes a positive control experiment as a proof of methods. This assay gave a strong positive result for both reporter systems. This indicates that the basic methods used in these assays were robust and the negative result obtained from the experimental Y1H assay was due to other factors. These could either be that the XhABFA TF does not interact with the *X. humilis* promoters in the Y1H context, or that there are errors in the construction of the vectors or yeast strains with integrated vectors

The sequences of all the promoter constructs in the reporter vectors were confirmed. However, sequencing only confirmed that the in-frame fusion of the GAL4AD with *XhABF*A in pGAD424 was correct. The full length GAL4AD from the start codon was not sequenced and therefore it is possible that a mutation might have occurred in the GAL4AD coding region upstream of the cloning site, during the cloning protocol. It would thus be important to confirm the full length sequence of the

GAL4AD::XhABFA fusion protein, as well as the expression of the GAL4AD::XhABFA fusion protein in yeast by Western blotting. Whilst antibodies are not available for XhABFA, GAL4AD specific monoclonal antibodies could be used to confirm the expression of GAL4AD in the control yeast strains, and the GAL4AD::XhABFA fusion protein in the experimental strains.

Another possible reason is that, even though the promoters were successfully cloned into the yeast vectors, they might not have integrated into the genome of the YM4271 yeast strain. However, this is unlikely as the transformed yeast grew on media lacking essential amino acids which are only present in the reporter vectors. This integration can be further validated using colony PCR. Colony PCRs were performed however the results were found to be inconsistent. This is not uncommon as yeast cells have a cell wall that must be broken down prior to the PCR, however the cell wall is proteinaceous making it difficult to lyse. Harsh treatments are required and the use of reagents that can damage the DNA (Dudaite et al., 2015). To overcome these difficulties, some studies perform DNA extractions prior to the PCR, however yeast DNA extraction methods are not ideally suited to extensive screening of colonies due to being lengthy, laborious or yielding poor quality DNA (Blount et al., 2016). Therefore the presence of growth on selective media was used as an indicator of successful integration.

An alternative reason is that in order to bind to the test promoters, XhABFA requires post-translational modifications that do not occur in yeast. The *A. thaliana* orthologue of XhABFA, AREB1/ABF2, is only able to activate expression of its target promoters when phosphorylated in response to ABA-signalling (Furihata et al., 2006). In order to ascertain whether this is also the case for XhABFA, a mutated form of GAL4::XhABFA that mimics the constitutively active ABF2 could be tested for binding activity. It might also be possible that XhABFA is only able to bind to its target promoters in the presence of cofactors. This is one of the disadvantages of using the Y1H to screen for interactions between plant TFs and promoters, as performing a reporter assay in yeast cells as opposed to plant cells may decrease the likelihood that the results obtained mirror the physiological response elicited by whole plants.

Unfortunately the results of the Y1H assays were inconclusive as I was unable to determine whether the lack of a positive signal from the XhABFA-promoter assays was due to: the promoters not integrating into the yeast genome; the lack of expression of the GAL4AD::XhABFA fusion protein in the yeast cells; the inability for GAL4AD::XhABFA to bind to the promoters in yeast cells; or because XhABFA does not interact with these promoters no matter what the test environment is.

Even though the Y1H assay offers a chromatin environment to investigate potential TF-promoter interactions, it does not provide cellular factors that are unique to plant cells. Thus, in the next chapter, I investigated whether XhABFA can activate expression of the same set of *X. humilis* promoters using a system which measures transient gene expression by transfection of plant cells, specifically *A. thaliana* protoplasts.

Chapter 4: Transient transfection of *Arabidopsis thaliana* protoplasts to test XhABFA activation of candidate seed maturation genes

4.1 Introduction

In this study, the transient transfection of *Arabidopsis thaliana* protoplasts was used as an alternative functional assay to study potential interactions between XhABFA and seed maturation genes which are upregulated in the leaves of *X. humilis* during desiccation. Functional experiments for transcription factor-promoter interactions in plants come with their own challenges.

4.1.1 Transient vs stable transfections in plants

In order to perform *in vivo* studies in plants which investigate the interactions of TFs with target genes, DNA must be introduced into cells via transfection. The transfection of DNA can be generally grouped into two approaches – the generation of stable transgenic lines or the use of transient assay systems (Bent, 2000). Stable transformations involve the integration of DNA into the organism's genome. This results in a stable transgenic cell line where descendants of the transfected cells also express the exogenous gene (Chilton et al., 1977). However this approach can be time consuming, particularly if the organism has a long life cycle. In contrast, transient assays offer the advantage of measuring gene expression shortly after transfection (Abel and Theologis, 1994). However the drawback is that transiently transfected cells express the gene for a finite period of time as it has not been stably integrated into the genome. Eventually the gene is lost through cell division or other factors (Smith, 2003).

4.1.2 Reporter gene assays in plants

In the past the most widely used reporter genes for plant systems were Chloramphenicol acetyltransferase (CAT), β-Galactosidase (β-gal), β-glucuronidase (GUS) and neomycin phosphotransferase (Luehrsen et al., 1995; De Ruijter et al., 2003). However these reporters are limited in sensitivity; the speed at which they can be quantified; and the range of their linear response (Sherf et al., 1996). Recently the luciferase protein has been gaining popularity in its use as a reporter gene because it is not as hindered by these limitations. The most commonly used luciferases proteins for reporter gene assays are the firefly luciferase (LucF) from *Photinus pyralis* and the renilla luciferase (LucR) from the sea pansy *Renilla reniformis* (McNabb et al., 2005).

Although both LucF and LucR are bioluminescent reporters, they have distinct evolutionary origins and therefore have different enzyme structures and substrate requirements (Sherf et al., 1996). The dissimilarity in the substrates of the two luciferase proteins makes it possible to selectively distinguish between the luminescent reactions for each enzyme (McNabb et al., 2005). The Dual-Luciferase Reporter Assay System (Promega) exploits this by measuring the luminescence of LucF upon the addition of the luciferin reagent and then subsequently quenching the reaction with a reagent which simultaneously activates the luminescence of LucR. Thus, one can sequentially measure the luminescence of both reporters from the same sample in a single reaction tube.

LucR has been found to be less sensitive than LucF as it exhibits a higher degree of autoluminescence. For this reason LucR is mainly used as an internal control reporter (Matsuo et al, 2001). In the Dual-Luciferase Reporter Assay the LucF gene is coupled to a candidate promoter and on a separate vector the LucR gene is coupled to a constitutive promoter. The assay is based on two assumptions. The first is that if the LucR vector has been successfully transfected into a cell then the LucF vector has also entered that cell (McNabb et al., 2005). This allows LucR activity to act as an internal control with which to normalise the LucF reporter data. To normalise the results, LucF activity is divided by LucR activity (Sherf et al., 1996). The second assumption of the assay is that the expression of LucR is unaffected by co-transfected vectors or experimental treatments (Ho and Strauss, 2004).

The primary purpose of normalisation is to account for variability between samples that are caused by factors other than those being tested in the experiment (Schagat et al., 2007). By accounting for inherent variabilities that can undermine experimental accuracy, it allows data comparisons to be made with greater confidence (McNabb et al., 2005).

These factors include experimental variation such as pipetting inconsistencies and differences in the total number of cells per sample as well as transfection efficiency. Transfection efficiency refers to the proportion of cells that take up the vector DNA, which directly affects the level of expression in the population of cells being measured. Factors which influence transfection efficiency include: the target cell type; the health of the cells; the type of transfection method used to deliver the DNA to the cells (chemical, mechanical or viral); the size of the DNA vector; the quality of the DNA; and the amount of vector applied to the cells (Walker et al., 2004).

4.1.3 The transient transfection of *Arabidopsis thaliana* protoplasts as a system to test transactivation of candidate seed maturation genes by XhABFA

The transient gene expression system based on the transfection of protoplasts has become a powerful tool for rapid gene functional analysis *in vivo* (Chen et al., 2006). Protoplasts are plant cells which have had their cell wall removed by enzymatic digestion. This enables macromolecules such as DNA, RNA and proteins to be delivered efficiently into the cell (Yoo et al., 2007). There are various approaches to induce DNA uptake including *Agrobacterium*-mediated gene delivery, particle bombardment and treatment of protoplast-plasmid mixtures with polyethylene glycol or electroporation. The latter two techniques are most commonly used as they are less time consuming and the efficiency is less variable (Davey et al., 2005). Despite enzymatic treatment, protoplasts maintain many of the same physiological responses and cellular activities as intact plants and therefore conserved aspects of plant signalling mechanisms can be established using these cells (Sheen, 2001).

Although it would be ideal to use *X. humilis* to generate protoplasts, it would be a lengthy process requiring a considerable amount of optimisation. *X. humilis* has a long life cycle, taking over a year to flower from seed. In contrast, model plants such as *A. thaliana* have a six week life cycle. Furthermore, *X. humilis* has a thick waxy coat covering its leaves which would inhibit the release of protoplasts from the leaf tissue. Therefore *A. thaliana* protoplasts were used as a heterologous

assay system as the plant is easily available, it has a relatively short life cycle and a protocol to prepare and transform the protoplasts has been well established (Yoo et al., 2007).

In this study, the transient transfection of *A. thaliana* protoplasts was used in conjunction with the Dual-Luciferase Reporter Assay to investigate whether the XhABFA transcription factor could activate the promoters of three seed maturation genes (*XhPER1, XhDSI-1VOC and XhECP63*).

4.2 Materials and Methods

Following the successful cloning of *XhABFA* and the promoters (Chapter 2), the protocol for protoplast preparation and transfection was performed. An overview of a typical protoplast transfection assay is depicted in Figure 4.1.

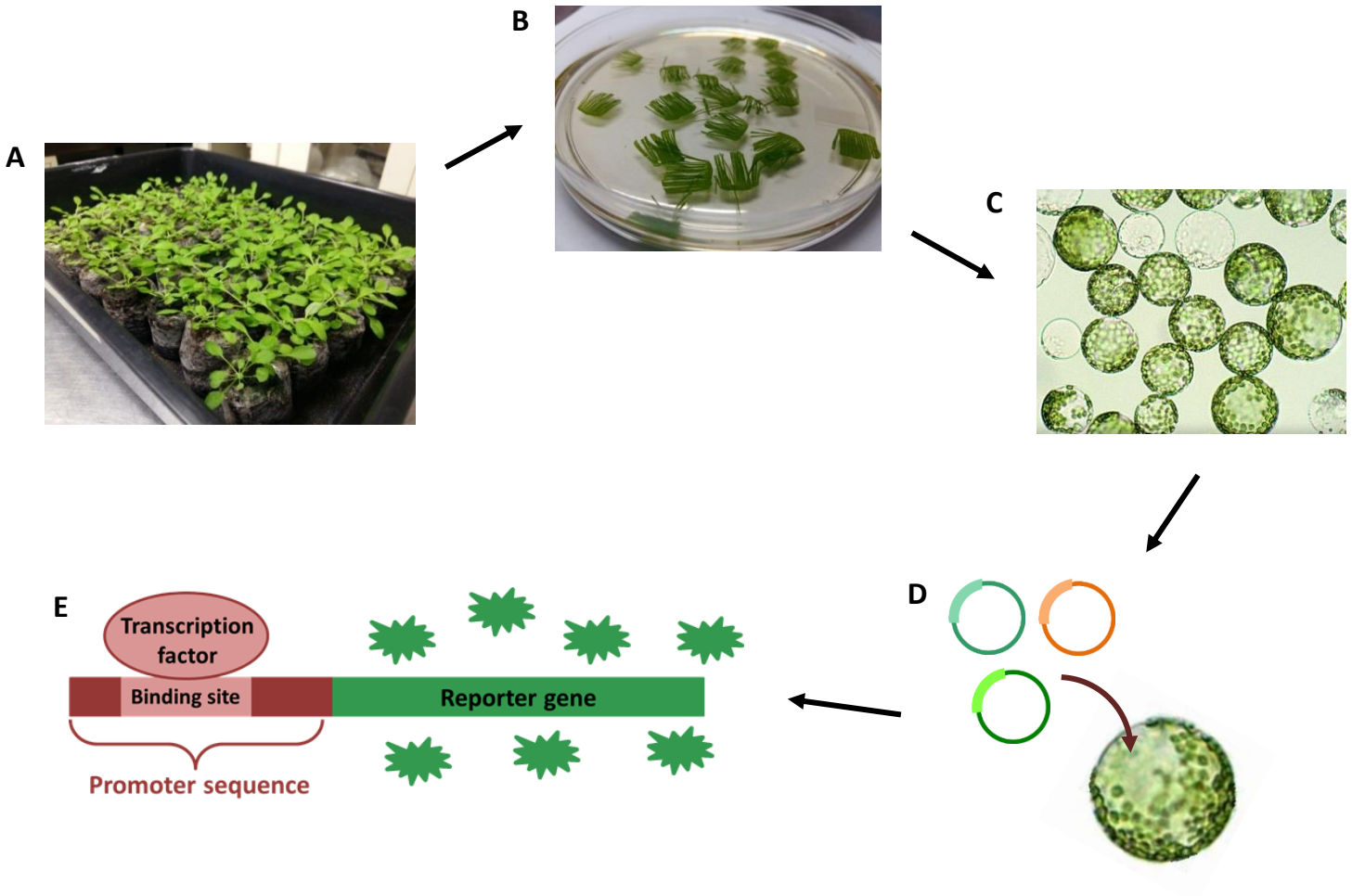


Figure 4.1: Schematic overview of the protoplast transfection assay as a system to study gene expression. A) Leaves are selected from healthy plants which are three to four weeks old. B) The leaves are cut into strips and transferred into a petri dish containing a prepared enzyme solution. C) The leaves are digested allowing protoplasts to be released, washed and collected. D) PEG–calcium mediated transfection is used to deliver DNA constructs into the protoplasts. E) Binding activity of a candidate TF to a target *cis*-regulatory element is measured by fluorescence or luminescence of a selected reporter gene.

4.2.1 Isolation and transfection of protoplasts

Sowing seeds and growing plants

A. thaliana ecotype Columbia (Col-0) seeds were sown on Jiffy soil mix with vermiculite (3:1). The plants were grown for 4 weeks at 22°C under a relatively short photoperiod of 10 hour light and 14 hour dark.

Mesophyll protoplast isolation

Protoplasts were isolated from *A. thaliana* leaves following an optimised version of the protocol previously described by Yoo et al., 2007. The reagents, concentrations and pH of the solutions used in this protocol are summarised in Supplementary Table 10.

Digestion of leaves

Leaves from four-week-old plants were chosen based on expansion – optimally the fifth, sixth or seventh leaf (Figure 4.2). The laminas of the leaves were cut into 1 mm strips using a sharp razor blade. Leaves with wounded or crushed tissue were discarded. Cut leaves were immediately transferred into a petri dish containing freshly prepared enzyme solution and submerged. Between 15 and 20 leaves were used per petri dish. The petri dish was placed in a vacuum infiltrator and incubated in the dark at room temperature for 30 minutes. The petri dish was removed from the vacuum infiltrator and incubated in the dark at room temperature for approximately two hours. The efficient release of protoplasts was indicated by the enzyme solution turning green.

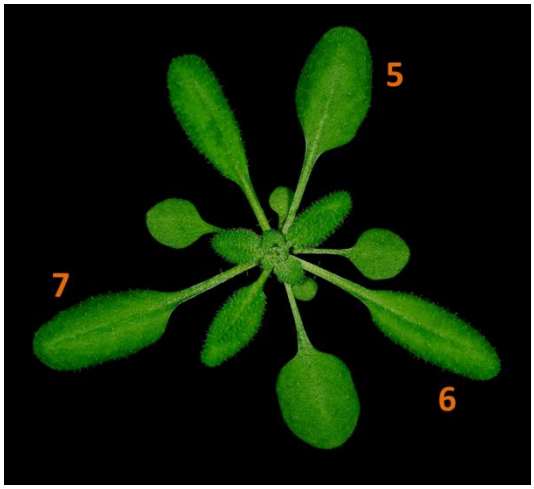


Figure 4.2: A rosette of a four-week-old *Arabidopsis thaliana* plant. The fifth, sixth and seventh leaf are used when sampling leaves for protoplast isolation. (Image adapted from http://www.cashadvance6online.com/leaves-wallpapers/2222742348.html)

Collection and purification of protoplasts

The enzyme solution containing the protoplasts was diluted with an equal volume of W5 solution and filtrated through a 75 µm nylon mesh into a 30 ml round bottom tube to remove cell debris. The tube was centrifuged at 100 g for 2 minutes, the supernatant was removed and the protoplasts were resuspended in W5 solution. This step was repeated in order to wash the protoplasts and remove as much of the enzyme solution as possible.

Counting and dilution of protoplasts

10 µl of the W5-protoplast solution was aliquoted onto a haemocytometer. The number of protoplasts was counted under a bright field microscope in order to calculate their concentration. W5 solution was added until the protoplasts reached a concentration of 4×10^5 ml $^{-1}$. The protoplasts were incubated on ice for 30 minutes in the dark. They were then centrifuged at 100 g for 2 minutes and as much of the W5 supernatant was removed without disturbing the protoplast pellet. The volume removed was recorded and the same volume of MMG solution was added to the tube.

PEG-calcium transfection

10 µl containing a total of 20 µg of plasmid DNA was added to a 2 ml microcentrifuge tube. 100 µl of the protoplast-MMG solution and 100 µl of the PEG-calcium solution was added to each tube and mixed by gentle inversion. The transfection mixture was incubated at room temperature for 15 minutes. 440 µl of W5 solution was added to each tube and mixed by inversion in order to stop the transfection reaction. The tubes were centrifuged at 200 g for 2 minutes, the supernatant was removed and 500 µl of W1 solution was added. Each sample was added to a well in a 12-well plate. Prior to the addition of the protoplasts, each well was thinly coated with 5% (vol/vol) sterile fetal calf serum. The protoplasts were incubated overnight at room temperature in the dark.

4.2.2 Microscopy

Fluorescent microscopy was used in order to evaluate protoplast viability and transfection efficiency simultaneously. The number of protoplasts with intact membranes expressing GFP (from the pUC19-GFP::XhABFA expression vector), were counted under a Nikon Ti-E Inverted fluorescent microscope.

Time course experiment

A time course experiment was performed in order to determine the optimal incubation time before harvesting the protoplasts and measuring gene expression. The protoplasts were sampled every two hours between 16 and 24 hours post transfection. At each time point, 10 µl was sampled and mounted onto a microscope slide. A cover slip was added and sealed with clear nail polish. Transfected protoplasts were visualised and five sets of photographs were taken representing five random fields of the slide. Each set consisted of three photographs: Bright field, GFP and chlorophyll

autofluorescence. Bright field was used to determine the percentage of intact protoplasts. Protoplasts were considered intact if their cell membrane had not ruptured (Figure 2.4). The GFP filter had an excitation and emission spectra of 488 nm and 519 nm respectively. This image was used to measure the number of protoplasts expressing GFP. The percentage of fluorescing protoplasts was calculated as a fraction of intact protoplasts only and did not include protoplasts which had burst. The second fluorescent filter had an excitation and emission spectra of 595 nm and 615 nm respectively. This image was used to measure endogenous chlorophyll fluorescence emitted from the chloroplasts in the protoplasts. One-way ANOVA tests were used to determine significant differences in the percentage of intact protoplasts as well as the percentage of GFP fluorescing protoplasts over the time course.

4.2.3 Testing the transactivation of seed maturation genes by *XhABFA*

Transfection

The 20 µg of plasmid DNA used to test XhABFA transactivation potential consisted of the regulatory effector, promoter reporter and transfection control at a ratio of 5:4:1 respectively. This transfection strategy is illustrated in Figure 4.4. “Protoplast only” control samples which lacked DNA were included in the experiment in order to measure background luminescence and fluorescence. The PEG-calcium transfection protocol was performed. The protoplasts were incubated for 22 hours at room temperature in the dark. Before harvesting the cells to measure gene expression the viability of the protoplasts was checked by fluorescent microscopy.

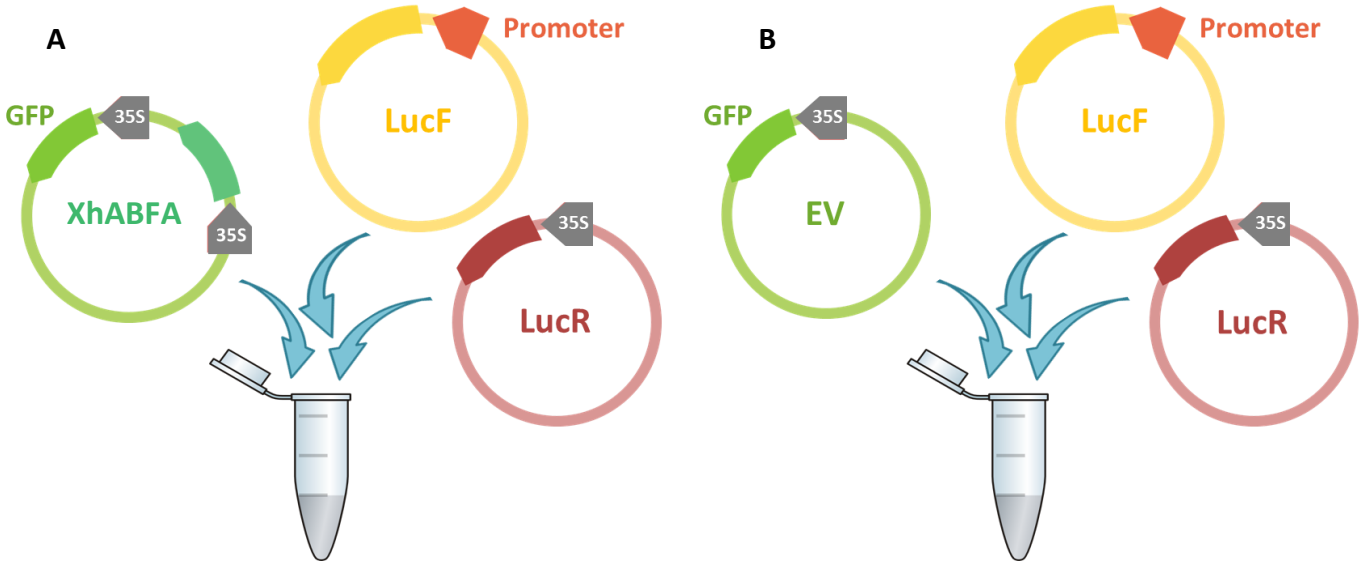


Figure 4.3: Overview of the transfection strategy. Prior to transfection 10 µl, containing a total of 20 µg of plasmid DNA, was added to each tube. The DNA in each tube was at a ratio of 5:4:1 of XhABFA (A) or EV (B) : LucF : LucR. The firefly luciferase reporter contained one of four promoter elements: *XhPER1; XhECP63; XhDSI-1VOC* or *XhAHL23*.

Harvesting of protoplasts and cell lysis

The remaining W1-protoplast solution in each well was added to a 2 ml microcentrifuge tube. Tubes were centrifuged at 100 g for 2 minutes or until the protoplasts pelleted. The W1 supernatant was removed and 250 µl of 1 x Pa was added to each 2 ml microcentrifuge tube. The tubes were vortexed for 30 seconds and incubated at room temperature for 20 minutes. The tubes were centrifuged at top speed for 2 minutes. The lysate from each tube was transferred to new 2 ml tubes, of which 20 µl and 100 µl of each sample was used to perform a Dual-Luciferase Reporter Assay (Promega) and a GFP fluorescence assay respectively.

Dual-Luciferase Reporter Assay

The constituents of the solutions used in this protocol are summarised in Supplementary Table 11. Luciferase activity was measured using the luminescence cartridge of the SpectraMax® Paradigm Microplate Reader (Molecular Devices). Aliquots of 20 µl were transferred from each lysate sample into separate wells of an opaque white 96-well plate. 100 µl of LAR II was dispensed into each well and the LucF activity of each sample was measured using an integration time of 5 seconds. 100 µl of Stop & Glo Reagent was dispensed into each well which simultaneously quenched LucF and initiated the LucR reaction. LucR activity of each sample was measured using an integration time of 5 seconds.

GFP fluorescence assay

Aliquots of 100 µl were transferred from each lysate sample into separate wells of a black 96-well plate. GFP expression was measured using the Fluorescence Intensity cartridge of the SpectraMax® Paradigm Microplate Reader (Molecular Devices). Measurements were taken using excitation and emission spectra of 488 nm and 520 nm respectively.

Protoplast cells contain chloroplasts which have an endogenous fluorescence that has similar excitation / emission wavelengths to the GFP expressed from the vectors used in this experiment. To account for this endogenous expression, samples which only contained protoplasts and no DNA, but still underwent the transfection process, were used. This background GFP expression was deducted from the GFP readings for all the experimental transfections.

Statistical analysis

Two independent transfection experiments were performed with four biological replicates in the first experiment, except *XhECP63* which had three, and five replicates in the second experiment. Therefore there were a total of nine replicates except *XhECP63* which had eight and *XhAHL23* which had five as it was only included in the second experiment due to experimental constraints. There were eight distinct sets of transfection combinations in total (Table 4.1).

Promoter	Effector construct	Set	Exp. 1 reps	Exp. 2 reps	Total # of reps
XhPER1	XhABFA	1	4	5	9
	EV	2			
XhECP63	XhABFA	3	3	5	8
	EV	4			
XhDSI-1VOC	XhABFA	5	4	5	9
	EV	6			
XhAHL23	XhABFA	7	0	5	5
	EV	8			

Table 4.1: The eight distinct sets of transfection combinations. Each set of transfections consisted of a combination of three vectors: The LucF reporter containing one of four *X. humilis* promoter elements; the effector construct containing GFP and either *XhABFA* or the EV control; all sets contained the vector expressing LucR. The number of biological replicates in each of the two independent experiments is indicated as well as the total number of replicates

For each experiment, the average background expression of LucF, LucR and GFP from five "protoplast only" replicates were deducted from all samples in that experiment. The absolute values between the two experiments were scaled in order to combine them. This was done by dividing each sample by the mean value of all the samples in that experiment.

A Pearson correlation between LucF, LucR and GFP was performed in a pairwise manner. Before the correlation was calculated, each replicate was divided by the mean of the replicates for that set of transfections. This means that the average of each set was equal to 1.

All measurements taken from samples containing the LucF reporter were normalised to the geometric mean value of LucR and GFP signal of the same sample. The geometric mean was calculated by multiplying the LucR and GFP values and then taking the square root. The LucF signal was normalised by dividing it by the geometric mean value of the same sample.

A two-tailed T test was used to determine statistically significant differences between protoplast cells transfected with the vector containing *XhABFA* or the EV control. A Bonferroni correction was performed and differences were considered significant if $p < 0.01$.

4.3 Results

Following the successful cloning of *XhABFA* and the promoters, the protocol for protoplast preparation and transfection was optimised.

4.3.1 Optimising protoplast preparation and viability

Various conditions in the *A. thaliana* protoplast protocol were adjusted to test whether changing certain variables extended cell viability. Table 4.2 lists the conditions that were tested, the adjustments that were made and whether these changes resulted in a longer period of time before the membranes of the cells ruptured (Figure 4.4). It was found that the incubation period between transfecting the cells and harvesting made the biggest contribution to keeping cell membranes intact and therefore maintaining viable cells.

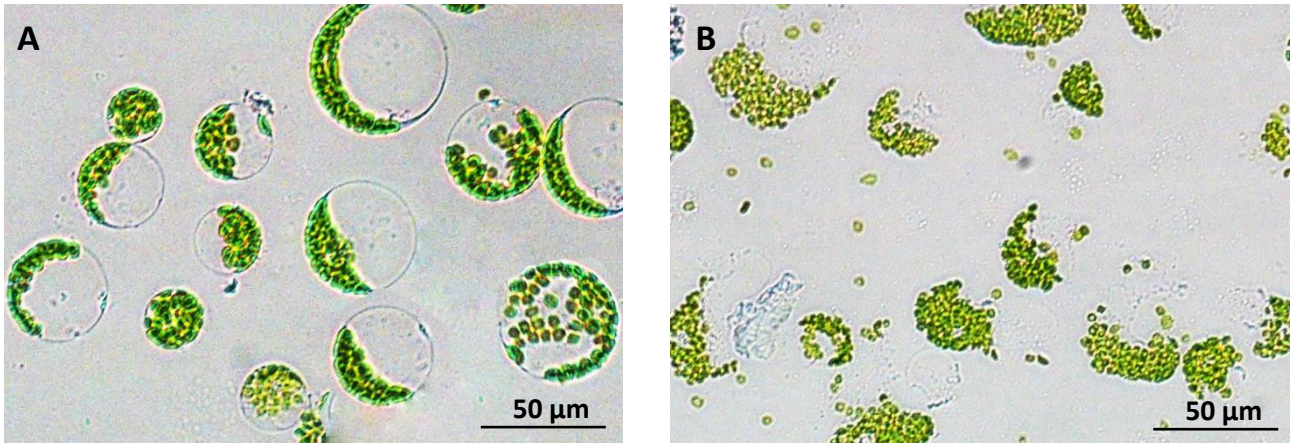


Figure 4.4: Rupturing of cell membranes. Protoplasts were considered viable and therefore capable of reporting an activation of the Xerophyta promoters by XhABFA if their cell membrane was intact. (A) Protoplasts with intact cell membranes. (B) Protoplasts with ruptured cell membranes.

Variable	Conditions tested	Results
Plant growth	Photoperiod	Decreasing the photoperiod to shorter light hours (8 h) than the protocol recommended resulted in premature flowering. Plants grown under a longer photoperiod (14 h light) resulted in dark green leaves.
	Temperature; light intensity; humidity; seed source	The plants were grown in various growth rooms which had moderately differing temperatures, light intensities and humidity. These changes did not influence cell viability.
Age at harvesting	3 weeks; 3 ½ weeks; 4 weeks	The protocol suggests that the plant should be between three and four weeks old when leaf tissue is harvested. Three ages were tested and no significant difference in cell viability was observed. The expansion of the leaves at 4 weeks was most similar to the photographs in the protocol and therefore this age was chosen.
Enzymatic digestion of plant tissue	2 hours; 3 hours	The lowest recommended digestion time of two hours was used in order to minimise potential cell membrane rupture. In order to account for the reduced number of protoplasts released from each leaf, the amount of leaf tissue was increased (from 50 leaves to 80 leaves).
PEG-calcium incubation	10, 15 and 20 minutes	The cell viability between different incubation periods during the PEG-calcium transfection not differ from one another.
Incubation period post transfection	16 h; 18 h; 20 h; 22 h; 24 h	A time course experiment was performed where protoplasts were harvested after different incubation periods post transfection. Significant differences in cell viability were observed.

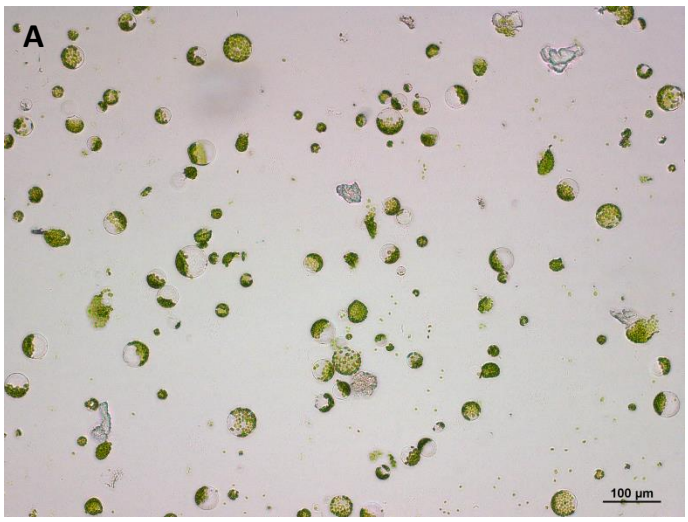
Table 4.2: Variables in the protocol which were systematically changed in order to optimise cell viability. Different incubation periods between transfection and harvesting of protoplasts significantly changed cell viability. Potential damage caused by the enzymes was minimised however other adjustments made to the various growth conditions of the plants had no clear effect on cell viability.

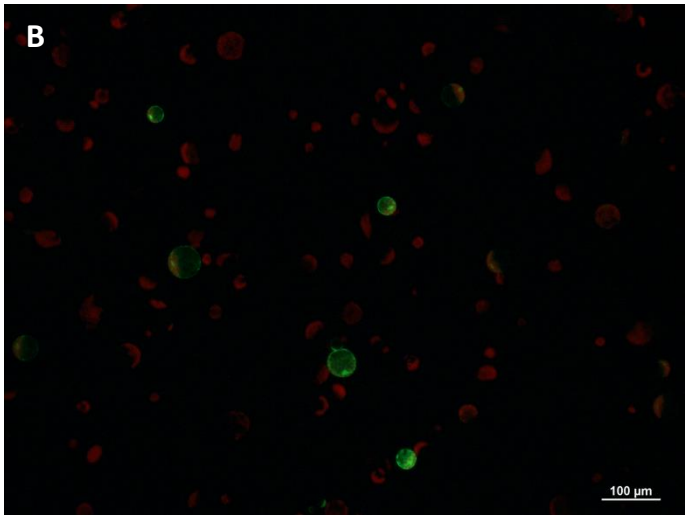
4.3.2 Determining the optimal incubation time post transfection

A time course experiment was performed in order to determine the optimal incubation period between transfecting and harvesting the cells. Protoplasts were sampled every two hours between 16 and 24 hours post transfection and visualised under a fluorescent microscope to determine the percentage of intact cells and the percentage of fluorescing cells over time (Figure 4.5).

Bright field **GFP / Chlorophyll autofluorescence**

16





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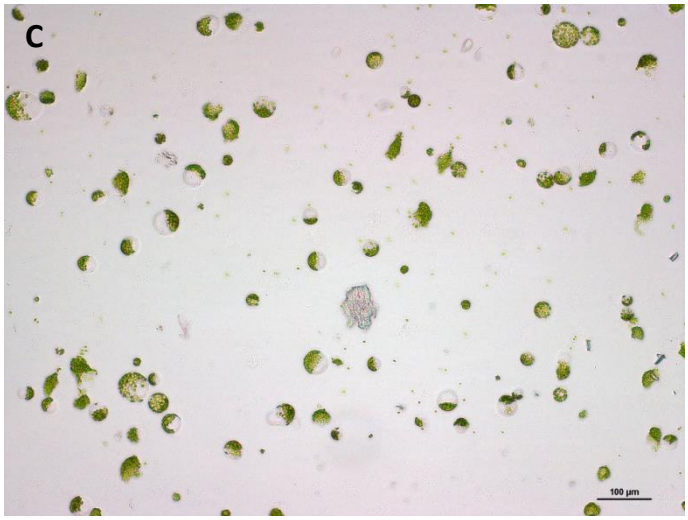

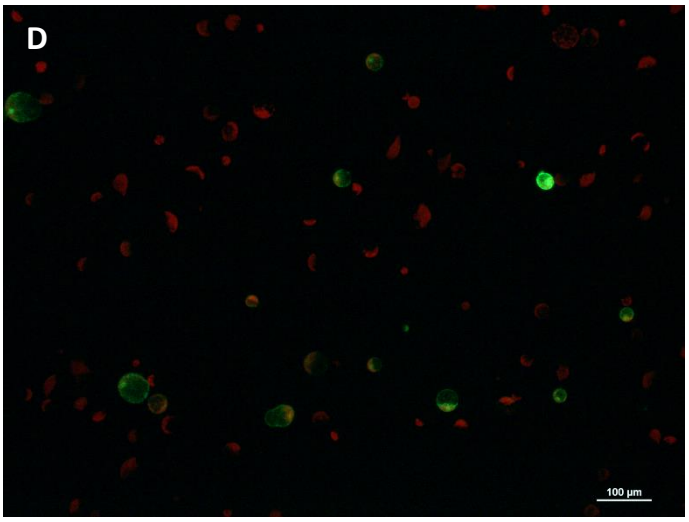

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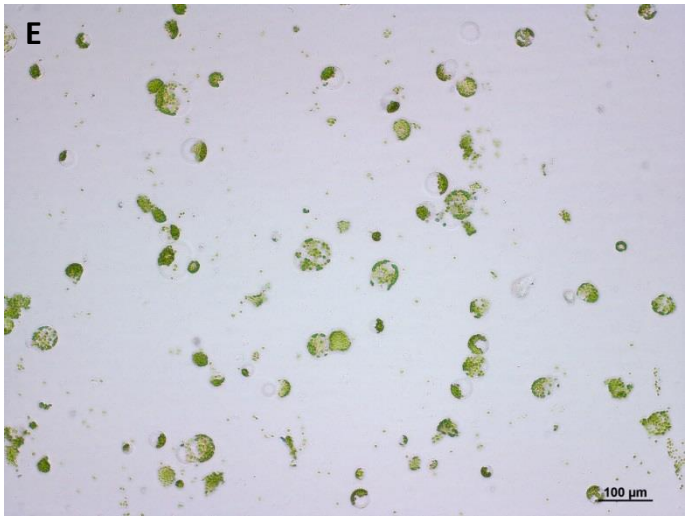
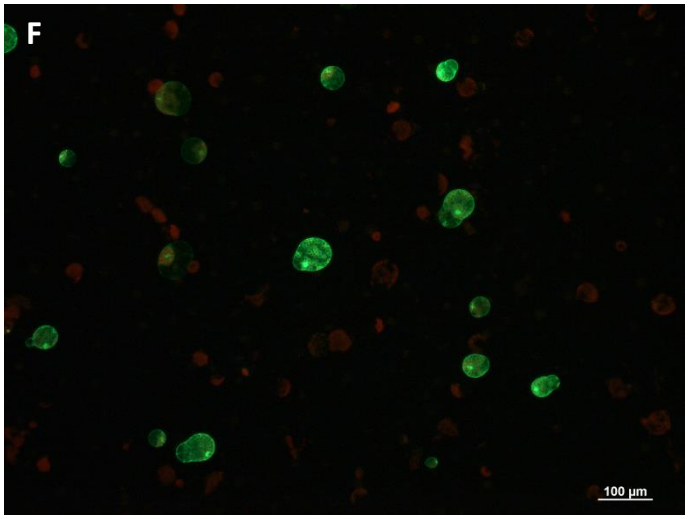




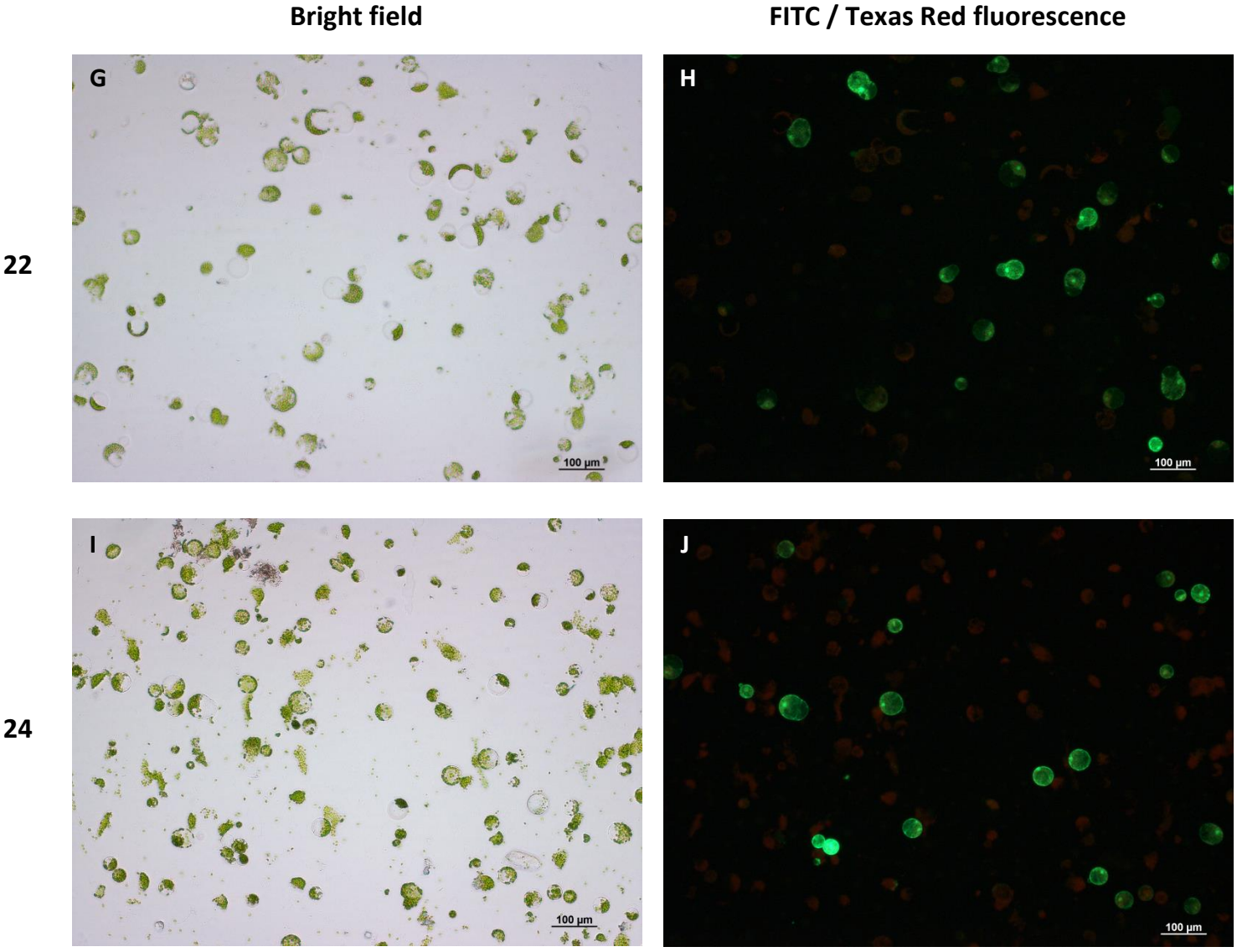


Figure 4.5: Representative photographs used to determine the percentage of intact protoplasts and the percentage of protoplasts fluorescing GFP. *A. thaliana* protoplasts were transfected with the XhABFA effector vector which constitutively expresses GFP. A time course of photographs were taken 16 (A and B), 18 (C and D), 20 (E and F), 22 (G and H) and 24 (I and J) hours post transfection. Images on the left are bright field photographs of the protoplasts. Bright field allows the identification of intact cell membranes. Images on the right are an overlay of two fluorescent filters indicating the distinction of the GFP expressing construct and the background fluorescence of native chloroplasts. These photographs illustrate the relationship of cell viability and transfection efficiency with time.

The number of protoplasts expressing GFP were quantified for each time point (Figure 4.6). Altough protoplast viability decreases over time, the proportion of viable protoplasts expressing GFP increases. One-way ANOVA tests confirmed that these changes are significantly different over time. In terms of intact protoplasts, the F-statistic from a one-way ANOVA test was equal to 8.81 with a corresponding p-value of 2.86×10^{-4}. In terms of protoplasts expressing GFP, the F-statistic was equal to 42.49 and the corresponding p-value was 1.66×10^{-9}. Both p-values are lower than 0.05, suggesting that one or more time points are significantly different from each other.

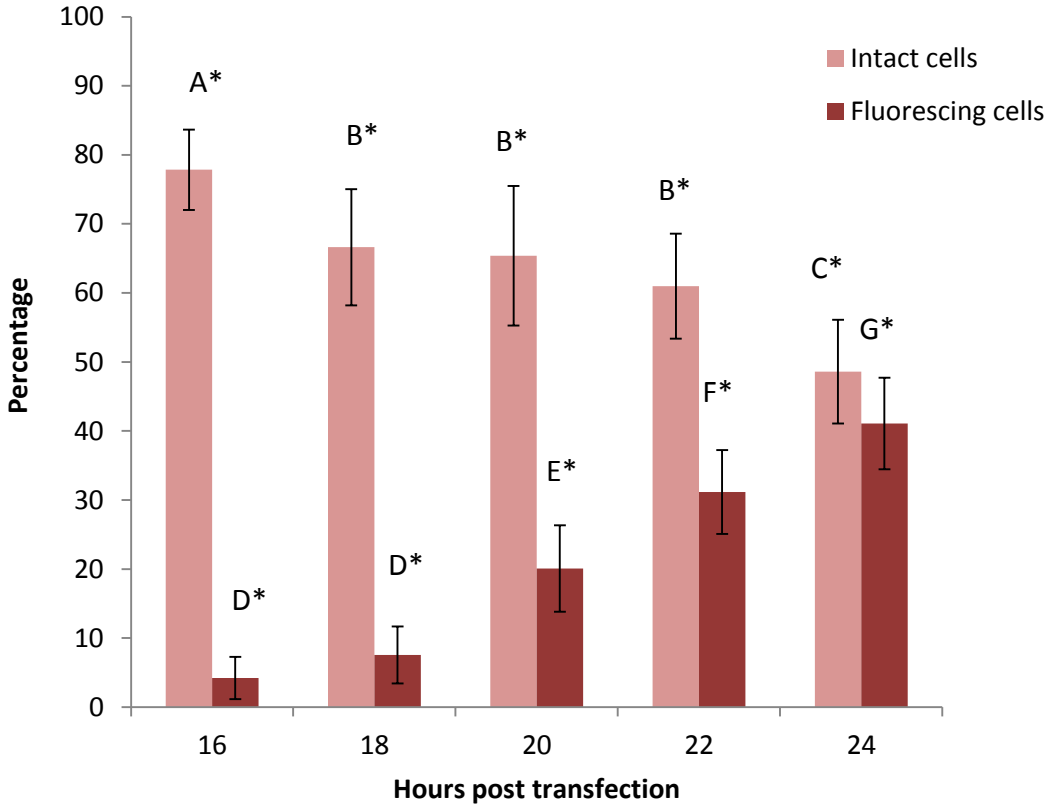


Figure 4.6: Time course of the percentage of intact protoplasts and protoplasts expressing GFP. Protoplasts were sampled every two hours between 16 and 24 hours post transfection. The percentage of intact protoplasts was calculated as a fraction of total protoplasts. The percentage of protoplasts expressing GFP was calculated as a fraction of intact protoplasts only. According to one-way ANOVA tests the p-values for both intact and fluorescing were significant at $p < 0.05$. *Mean values with the same letter are not significantly different from each other at $p < 0.05$ according to Fisher's LSD test

From the analysis, it is apparent that the reduction in protoplast viability significantly decreased between 16 and 18 hours and then again between 22 and 24 hours. The percentage of protoplasts expressing GFP significantly increased between each time point after 18 hours. As a compromise, 22 hours was chosen as the time point post transfection at which to harvest the cells and measure activation of the reporters constructs.

4.3.3 Determining the more suitable way to normalise firefly luciferase activity

In order to determine whether XhABFA activates the expression of candidate seed maturation genes, *A. thaliana* protoplasts were transfected with three vectors: a vector containing one of four *X. humilis* promoter elements coupled to a reporter gene; a vector to normalise the reporter gene expression; and a vector which either contained the effector gene (*XhABFA)* or was "empty" (EV). Respectively, the presence of these vectors was measured by the activity of LucF, LucR and GFP.

Raw LucF data can be seen in Supplementary Figure 1. However the raw data of LucF in a protoplast transfection assay is generally regarded as an inadequate representation of the true relative expression between samples. Therefore this assay requires a form of normalisation to account for experimental variation, such as transfection efficiency, between samples. Traditionally, this is done by dividing the LucF signal by the LucR signal of the same sample (Schagat et al., 2007). However, when LucR expression was measured, a systemic pattern was observed: the samples containing the EV tended to have a higher LucR signal than the samples containing *XhABFA* (Figure 4.7). This means that normalising to LucR expression may skew the data as a result of dividing the LucF signals by the consistently higher LucR expression in EV samples compared to *XhABFA* samples. By doing this, the relative LucF values will be consistently lower in EV samples. This has the potential to bias the results towards producing false positives.

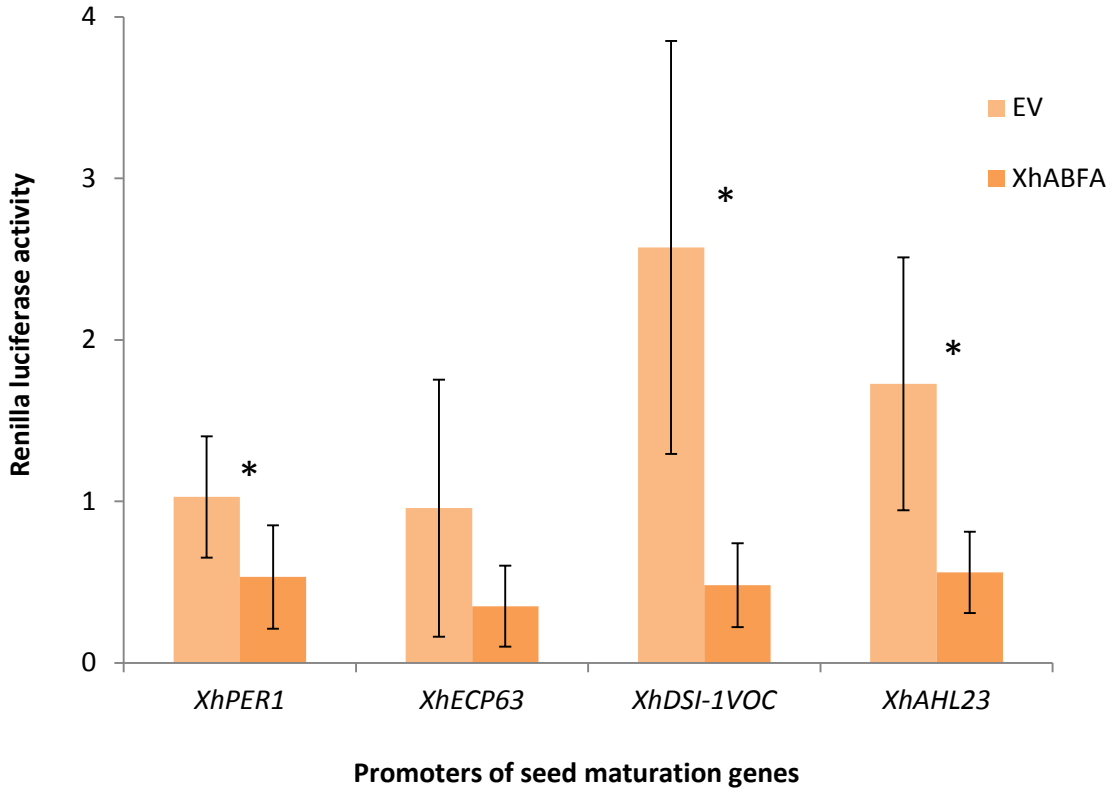


Figure 4.7: Expression of *Renilla* luciferase in samples with different promoter-reporter constructs. A consistent pattern is observed where the LucR signal is higher in the samples containing the EV control compared to the samples containing the effector *XhABFA.* On average this difference is three

times higher. *LucR signals of EV samples and *XhABFA* samples are significantly different from each other at $p < 0.05$ according to a two tailed t-test.

All samples were transfected with a vector constitutively expressing GFP and this offered an alternative method for normalisation. GFP expression was measured and the distinct systemic pattern observed in LucR expression was not apparent in GFP expression (Figure 4.8).

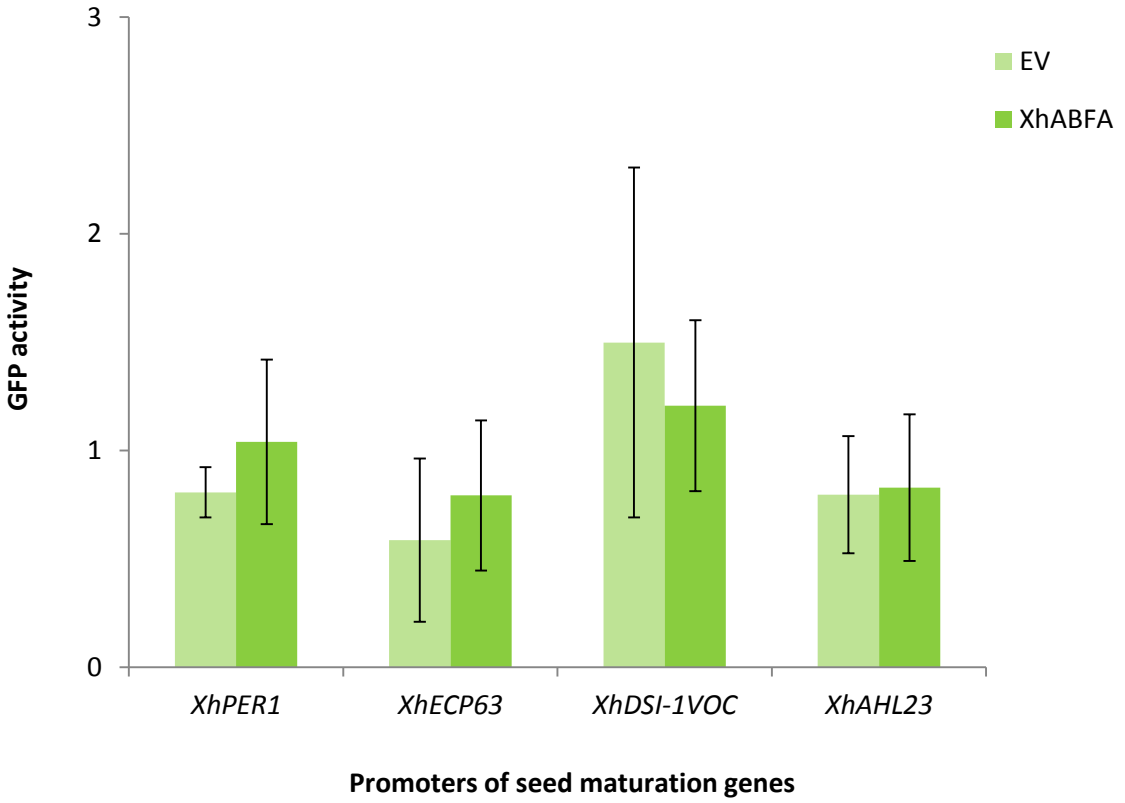


Figure 4.8: Expression of Green Fluorescent Protein in samples with different promoter-reporter constructs. GFP expression is not significantly different between samples containing the EV control and samples containing the effector *XhABFA.* A pronounced pattern in GFP expression is not observed.

Considering the apparent systemic bias in LucR expression observed in this study, GFP expression may be a better estimate of transfection efficiency. If this is the case, levels of GFP should have a better correlation with levels of LucF. In order to evaluate whether LucR or GFP was more suitable as a normalisation of LucF, a Pearson correlation between the three measurement techniques was performed in a pairwise manner. This was done in order to compare the contribution of each measurement technique to the total variation within a set of samples.

Before the correlation was calculated, each replicate was divided by the mean of the replicates for that set of transfections. This means that the average of each set was equal to 1. This was done in order to eliminate the variation caused by potential promoter-TF interactions, or any other interactions amongst the vectors. Therefore the only sources of variation should be transfection efficiency and experimental error.

Since each sample was measured using all three methods, each of the three measurements from the sample will have the same transfection efficiency associated with it. This means that a better transfection efficiency should increase all three measurements proportionately. Any deviation from this variation would have to be a result of experimental error. Therefore if the correlation between the three measurement methods is calculated, the experimental error associated with each one can be indirectly estimated based on the R^2 values.

As seen in Figure 4.9, it appears that LucR has the least error associated with it as it is the common variable of the two lowest R^2 values. Despite the systemic pattern that LucR expression exhibits, according to the Pearson correlation GFP expression contains more experimental error. As it was not clear which measurement is more suitable, a compromise was made where LucF was normalised to the geometric mean of LucR and GFP. Supplementary Figure 2 shows the alternative LucF expression if it had been normalised to either LucR or GFP.

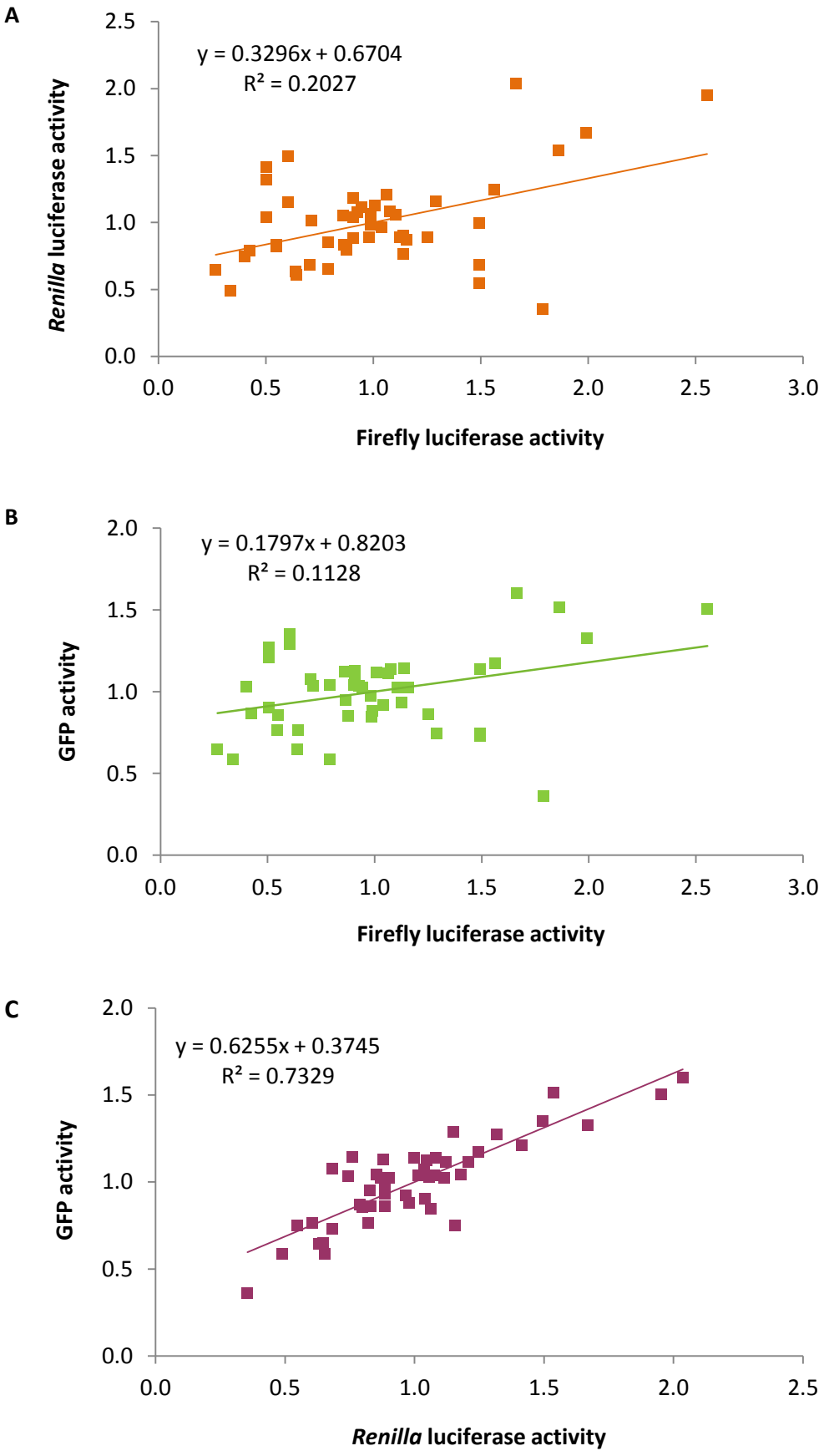


Figure 4.9: Scatter plots of firefly luciferase and *Renilla* luciferase (A); firefly luciferase and GFP (B); *Renilla* luciferase and GFP (C). The graphs indicate the relationships LucF has with LucR and GFP. The equations of the line of best fit and the R^2 values from Pearson correlations are shown.

4.3.4 Using the geometric mean of *Renilla* luciferase and GFP to normalise firefly luciferase activity

The geometric mean is a measure of central tendency which uses the product of a set of values as opposed to the arithmetic mean which uses their sum. The geometric mean is useful when calculating the central tendency of data sets which have different numeric ranges. It is calculated by taking the n^{th} root of the product of n values. In the context of this experiment, the geometric mean of LucR and GFP would be equal to the square root of the product of the LucR and GFP signals of that sample.

Figure 4.10 shows that the geometric mean values do not exhibit the apparent systemic pattern observed in LucR expression. These values also have less experimental error which was associated with GFP expression according to the Pearson correlations that were performed (Figure 4.9).

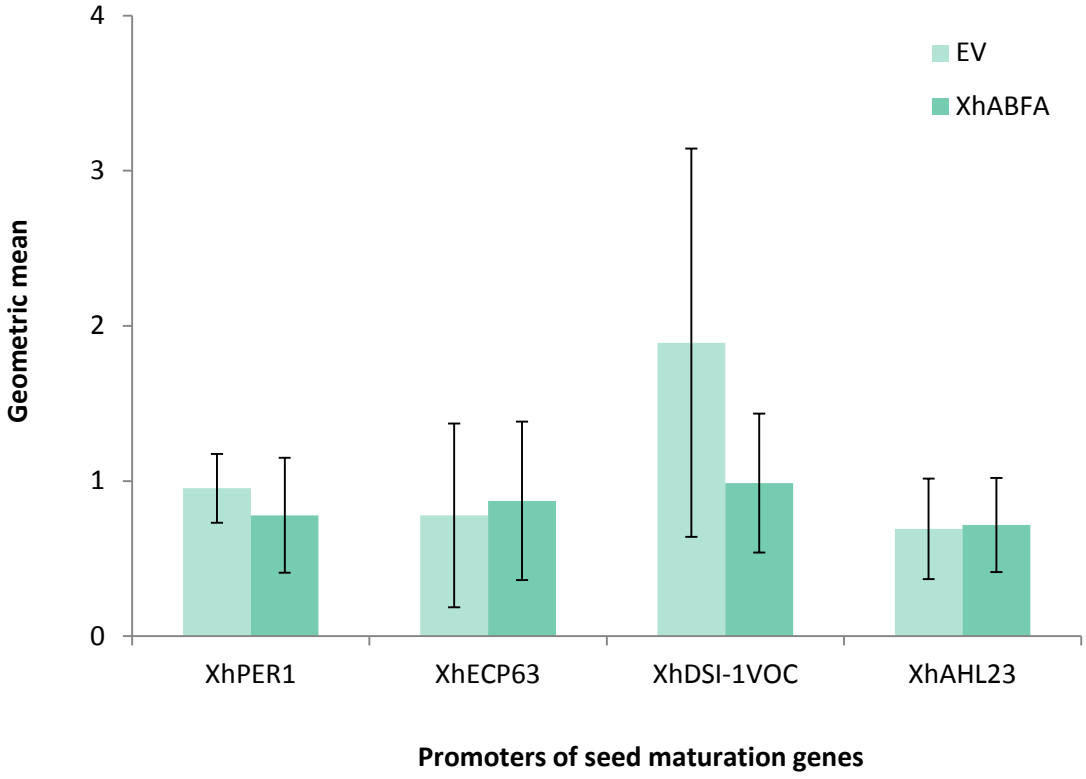


Figure 4.10: The geometric mean of *Renilla* luciferase and GFP. There is no apparent pattern in the geometric mean values of LucR and GFP between samples containing the EV control and samples containing *XhABFA.*

4.3.5 Investigating the transcriptional regulation of seed maturation genes by XhABFA

Prior to harvesting the protoplast cells in order to measure gene expression, microscopy was used to confirm that the protoplasts had been successfully transfected. Representative images are shown in Figure 4.11.

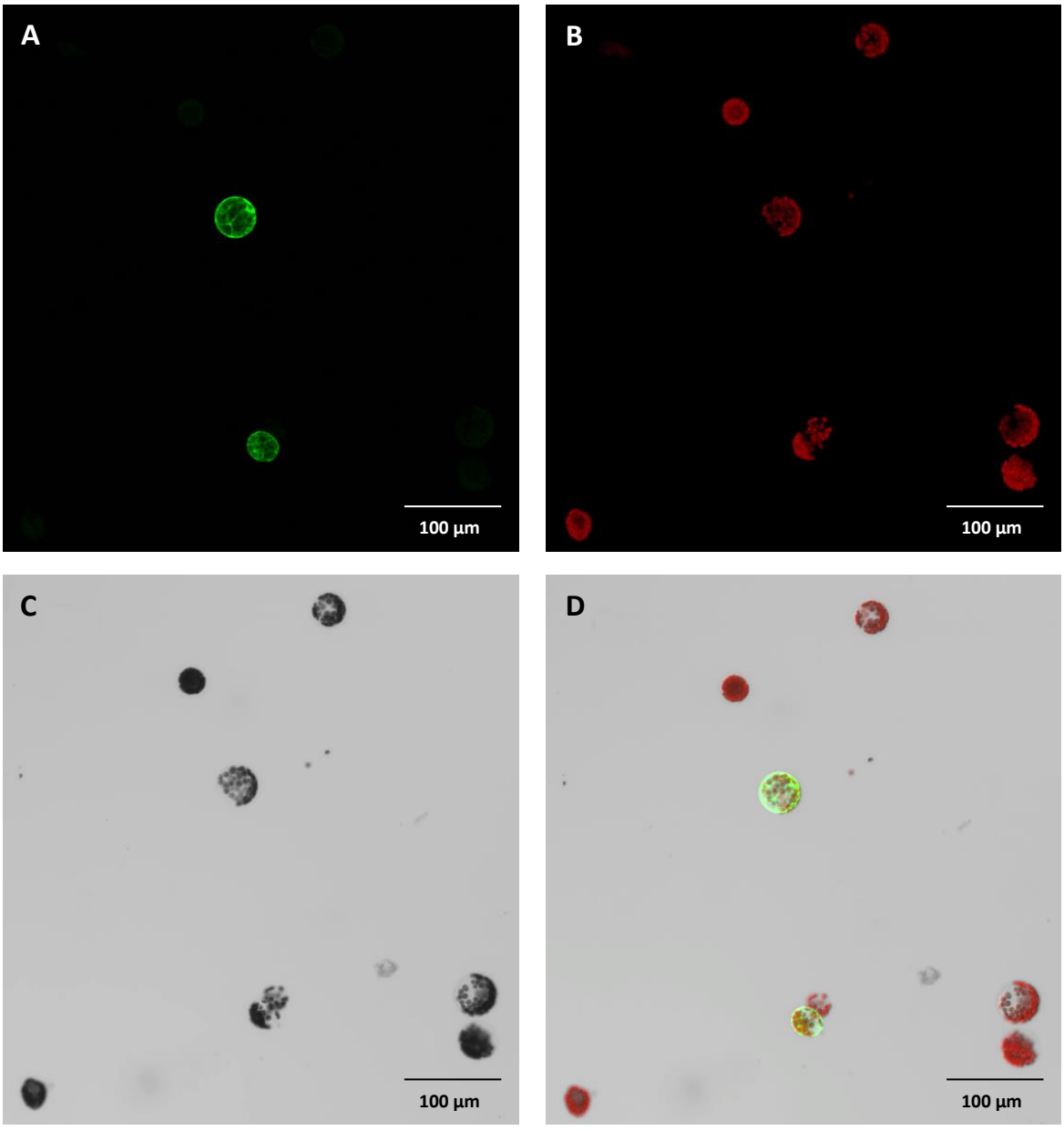


Figure 4.11: *Arabidopsis thaliana* protoplasts expressing GFP. Representative images of successfully transfected protoplasts. (A) Cells expressing GFP from the effector vector; (B) Chlorophyll autofluorescence; (C) Bright field; (D) Overlay of images A-C.

When using the geometric mean of LucR and GFP to normalise LucF activity, it is evident that XhABFA has the potential to transactivate the expression of three seed maturation genes (Figure 4.12). LucF activity under the control of the *XhPER1, XhECP63* and *XhDSI-1VOC* promoters is significantly higher in samples transfected with the *XhABFA* effector vector when compared to samples transfected with the EV control. The inclusion of the EV control demonstrates that this expression is specifically due transactivation by *XhABFA* and not from endogenous TFs.

A fourth promoter was included which encodes *XhAHL23.* This gene is not expressed in the leaves of *X. humilis* during desiccation and is very lowly expressed during seed maturation. In addition to this, the *XhAHL23* promoter lacks ABRE and RY binding motifs which are associated with ABF and B3-domain TFs. A significant difference in the expression of the LucF reporter containing the *XhAHL23* promoter was not observed between protoplasts transfected with the *XhABFA* effector vector and the EV control. This demonstrates that transactivation by *XhABFA* is sequence selective and specific promoter sequences are required.

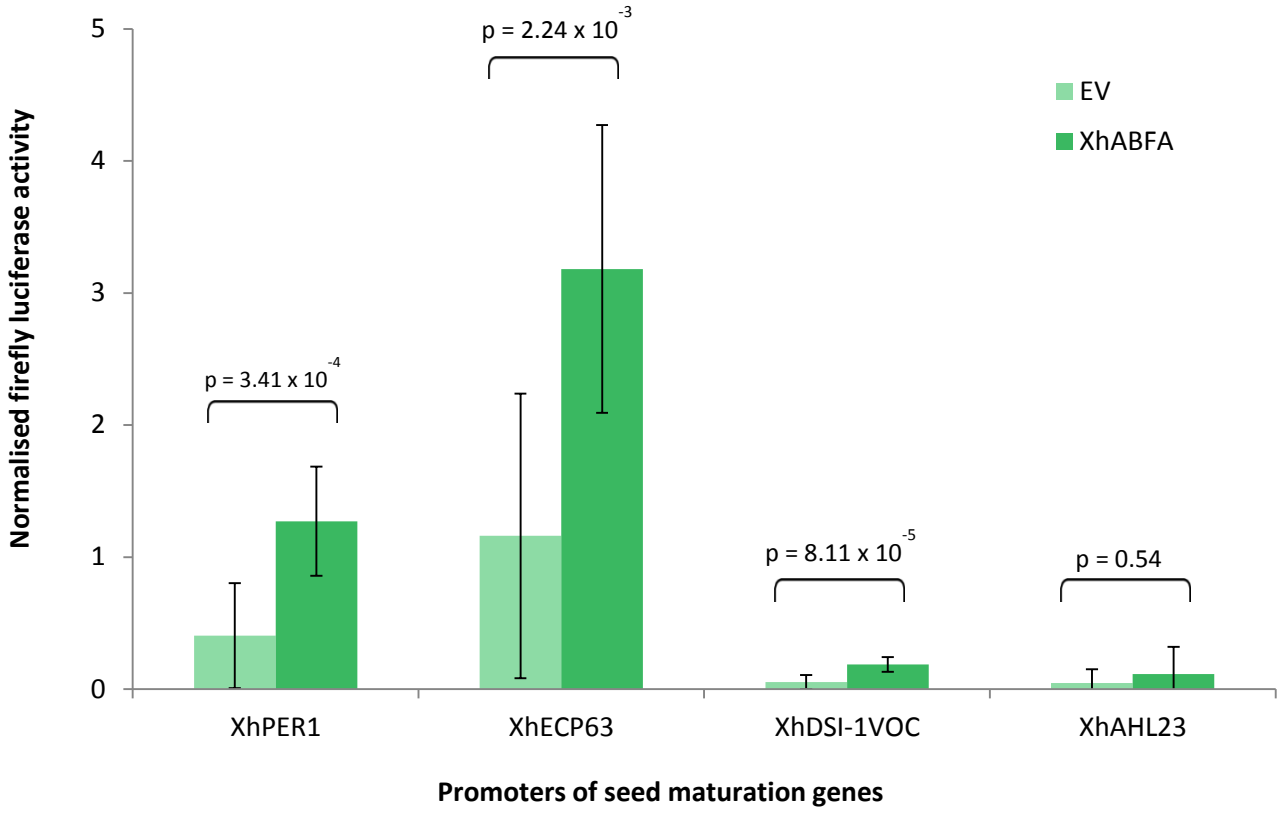


Figure 4.12: Expression of firefly luciferase representing the transactivation of seed maturation genes by XhABFA. *A. thaliana* protoplasts were co-transfected with three vectors: a LucF reporter vector containing one of four *X. humilis* promoter elements; a vector constitutively expressing LucR ; and a vector which constitutively expresses both GFP and *XhABFA* or an "empty vector" which expresses GFP but lacks an effector gene. LucF activity was measured 22 hours after transfection and normalised to the geometric mean value of the LucR and GFP signals for each transfection. Values shown are mean LucF activities ± SD from nine biological repeats (except *XhECP63* where n = 8 and *XhAHL23* where n = 5). The p-values shown are from a two tailed t-test with Bonferroni correction (significant difference at $p < 0.01$).

4.4 Discussion

4.4.1 XhABFA is able to activate the transcription of seed maturation genes

In this study, the transient transfection of protoplasts was used in conjunction with a reporter assay system to demonstrate that XhABFA is able to activate transcription from the promoters of three genes expressed during seed maturation and leaf desiccation in *X. humilis*. Furthermore, this transactivation by XhABFA is not observed in the expression of the control gene, *XhAHL23*. Therefore the transactivity of XhABFA is specific to the promoters of particular genes.

In Figure 4.12 it can be seen that the transactivation of the *XhDSI-1VOC* promoter by XhABFA displayed the highest significant difference compared to the EV control. This is interesting as it appears that the strength of activation does not necessarily correlate with the number of ABRE binding sites in a promoter. *XhDSI-1VOC* contains the lowest number of ABRE elements in its promoter, two, compared to *XhPER1* and *XhECP63* which both have four (Figure 2.4 in Chapter 2). This is also interesting as *AtPER1* and *AtECP63* have been reporter to be direct targets of *AtABI3*, one of the master regulators of seed maturation in desiccation sensitive plants, whereas *AtDSI-1VOC* was not.

4.4.2 Maintaining cell viability until gene expression is measured

Whilst the transient transfection of *A. thaliana* protoplasts was successful, the method did require extensive optimisation. Microscopic analysis of the prepared protoplasts identified that maintaining protoplast integrity was a critical point to the success of the technique. Identifying the primary sources which lead to membrane rupture and cell death was challenging. Numerous variables in the protocol were systematically changed. However it became apparent that, other than differences in the incubation period post transfection, adjusting these variables did not result in a clear prolonging of cell viability. This suggests that the period of time between transfecting cells and harvesting them is an important variable to optimise in a transient transfection assay. If this incubation period is too short the genes will not have had enough time to be expressed as detectable proteins. However if the incubation period is too long then the protoplasts will get stressed. This results in the rupturing of the cell membrane causing the cells to die (Davey et al., 2005). This loss of viability means that the production of new proteins ceases and any existing proteins the cells have made diffuse into the media making them undetectable. This means that there is a trade-off between the percentage of intact cells and the percentage of fluorescing cells over time. Therefore in order to yield the maximum amount of cells which are able to express proteins, a compromise must be made between the peak of protein expression and the point at which cells begin to significantly decrease in viability.

According to the protocol followed in this study, it is recommended that gene expression should be measured between 2-24 hours post transfection (Yoo et al., 2007). This suggested window period is relatively imprecise due to the numerous variables which influence the rate of gene expression. These factors include the health of the cells, the amount of DNA used in the transfection, the quality and purity of the DNA, the method of transfection, the type of reporter gene that is used, the promoter activity of the gene, the duration of post-translation modifications, whether a stimulus is

applied during the incubation period and whether the expression of the gene is measured by RNA or protein accumulation (Karimi et al., 2009). Previous studies which used luciferase reporter genes in their protoplast transfections measured luciferase expression between 12 and 24 hours post transfection (Frey et al., 2001; Bart et al., 2006; Wehner et al., 2011, Bohrer et al., 2015). Studies which transfected with a GFP construct measured GFP expression between 10 and 24 hours post transfection (Chiu et al., 1996; Chen et al., 2006; Takai et al., 2007; Zhang et al., 2011; Bohrer et al., 2015). According to these studies, the optimal incubation period post transfection varies between 12-24 hours for luciferase and 10-24 hours for GFP. My results showed that 22 hours after transfection was the optimum time to measure gene expression. However, if cell viability had been adequately maintained proceeding 22 hours, a higher percentage of transfection efficiency could have been reached and the optimum time would be extended. This highlights the importance of cellular integrity.

4.4.3 The use of a second reporter vector as an internal normalisation may introduce a bias

The other important parameter that was optimised in the transient transfection protocol was how to normalise LucF activity to account for differences in transfection efficiency. Of late, using LucR activity is one of the most common methods to normalise LucF activity. However in this study, LucR expression displayed a systemic pattern where the samples with the control effector (EV) had higher LucR activity than the samples with the experimental effector (*XhABFA*).

This difference might be real, as a consequence of a higher efficiency of transformation of the EV which is smaller than the vector containing *XhABFA*. Alternately, it might be spurious in that there is no consistent difference in the transfection efficiency, but that the vector containing *XhABFA* has an effect on suppressing expression of the LucR reporter. In this case, use of LucR as an internal normalisation may introduce a bias whereby the ability of XhABFA to activate expression of the reporter constructs is overestimated.

The activity of an ideal internal standard should be independent of the experimental reporter and its activity should not interfere, or be dependent on, co-transfected vectors (Huszar et al., 2001). However there is experimental evidence from mammalian cell culture, showing that the co-transfection of different vectors can influence the expression of each other (Bergeron etal., 1995). There have also been reports of aberrant activation or repression of reporters used for normalisation by the effector constructs which express a TF or other regulatory protein (Farr and Roman, 1992; O'Mahoney and Adams, 1994; Huszar et al., 2001). Some of these studies have observed spurious regulation of LucR when using the Dual-Luciferase Reporter Assay. In a study by Sims et al., 2003 it was shown that the LucR internal control vector was responsive to transcriptional co-activators and androgenic compounds. Shifera and Hardin, 2010 review multiple studies which observed suppression or induction of LucR by one or more of the experimental factors. In a study by Vesuna et al., 2005 it was found that the effector construct downregulated LucR activity by 252-fold. The authors tried using two different constitutive promoters for the LucR reporter and also found there to be a downregulation by an average of 3- and 24-fold (SV40, CaMV 35S, and TK promoters respectively). Ho and Strauss, 2004 reported that members of the GATA TF family modulated the expression of two different LucR reporter vectors when measured with the Dual-Luciferase Reporter Assay system.

These studies, which have reported aberrant activity of the internal control vector, were all performed in the mammalian cellular system. There are many published studies which test promoter activity using protoplasts and the Dual-Luciferase Reporter Assay to measure gene expression but don't report spurious expression patterns observed in the internal control (Iwata et al., 2011; Wehner et al., 2011; Díaz-Triviño et al., 2017). However none of these studies report the raw values of LucR expression, making it difficult to ascertain whether this observation of a systemic pattern such as observed in this study is common.

One study was done which addressed the issue of non-specific regulation of non-target genes when performing transient expression analyses using protoplasts (Stege et al., 2002). This study focused on testing various regulatory effectors for non-specific repression or activation. They also created various reporter constructs with binding sites which differed in position relative to the transcriptional start site. They observed that when the binding site was placed downstream of the transcriptional start site, expression was inhibited. The authors suggest that the binding of effector molecules in this location may interfere with the activity of the RNA polymerase complex and subsequently reduce the expression of the reporter gene. This offers a potential explanation for the pattern I observed as there is an ABRE binding site in the LucR vector downstream of the transcriptional start site (Supplementary Figure 2). Therefore *XhABFA* could be binding to this ABRE site and consequently interfering with transcriptional activation of LucR.

Another hypothesis of the systemic pattern observed in LucR expression could be due to the difference in size of the two vectors (Figure 2.8). The vector containing XhABFA is 1 kb larger than the EV. There have been multiple accounts in the literature where the effect of DNA size in transient transfection assays has been investigated. Kreiss et al., 1999 and Yin et al., 2005 inserted various sizes of stuffer DNA into the parental luciferase reporter vector and found an inverse correlation between plasmid size and luciferase activity. However these studies observed a relationship between the size of a vector and the expression of the same vector.

A study by Walker et al., 2004 investigated whether there was a relationship between the size of a vector and the expression of co-transfected vectors. They found that the effects of size were only *cis*-acting and are not observed in *trans* upon co-transfected DNA. They concluded that the size of a vector should not compromise the efficiency of the entire transfection process. Therefore according to these findings, the systemic pattern observed in LucR expression is unlikely to have occurred due to the difference in size of the EV and *XhABFA* vectors. In addition to this, if DNA size of the co-transfected plasmids was indeed causing a bias in the data, then theoretically GFP expression levels of the *XhABFA* vector should be consistently lower than those of the EV. This was not observed.

There have been reports that experimental artefacts can arise from artificially introduced high levels of a potent transcriptional activator causing non-specific transcriptional suppression (Lin et al., 2007). This phenomenon is referred to as transcriptional "squelching" (Prywes and Zhu, 1992). It is thought to be a result from the titration or sequestration of components required for transcriptional activation away from the promoter of the affected gene (Cahill et al., 1994). If this is the case for the observations in this study, *XhABFA* activation of the *X. humilis* promoters would be competing with the constitutive promoter driving LucR for limiting components in the cell which are required for transcription. Seeley et al., 1997 suggests these components could be coactivators or the general TFs which are part of the basal transcriptional machinery.

In this experiment, GFP offered an alternative method to normalise LucF activity as all samples contained a vector, either *XhABFA* or EV, which constitutively expresses GFP. Normalising to GFP is not common practice however there have been several studies in mammalian cellular systems which have used GFP and even regard it as a superior normalisation option to LucR in transient reporter assays (Sims et al., 2003; Vesuna et al., 2005). Dandekar et al., 2005 directly compared GFP to LucR as internal controls. They found that GFP was reliable and reduced the cost and time the assay required compared to LucR. Two studies, where gene expression in protoplasts was measured with the Dual-Luciferase Reporter Assay, used GFP expression to estimate transfection efficiency. However both studies still normalised LucF activity to LucR and did not report the raw LucR values (Takai et al., 2007; Zhang et al., 2011). In the current study it was not clear which source of normalisation would be a more accurate representation of the true transfection efficiency. Therefore, as a compromise, the geometric of GFP and LucR was used.

4.4.4 Recommendations if the experiment were to be repeated

Considering the systemic pattern seen in LucR expression, if this experiment were to be repeated there are various controls which could be included to investigate theories behind the occurrence of this pattern. One potential explanation for this pattern is that there is a correlation between the size of a vector and the transfection efficiency of a co-transfected vector. To test this theory one could clone increasing lengths of stuffer DNA sequence into the cloning site of the *pUC19-Rfa-GFP* vector, separately co-transfect these vectors with the LucR vector and compare the expression levels of LucR.

Another potential source of the pattern in LucR expression could be due to *XhABFA* itself. To test whether this is the case, *XhABFA* could be cloned in reverse orientation. This would keep the vector the same length but it would no longer express the *XhABFA* protein. Therefore any activity of *XhABFA,* which could be contributing to the LucR expression patterns, would be abolished.

An approach which may circumvent the difficulties experienced with normalisation would be to reduce the number of vectors that are used in the transfection protocol by combining 35S-LucR and promoter-LucF on the same vector. LucR expression would then directly reflect the transfection efficiency of the LucF reporter. The transient transfection of a vector carrying both LucF and LucR has been shown to be successful in mammalian promoter-reporter assays (Park, 2001; Malo et al., 2003). However, even if only two vectors are used (GFP-effector and LucF-reporter-LucR-control), there is still a problem that not all protoplasts are transformed with both vectors. A way to assess this could be to use another fluorescent marker instead of LucR such as YFP. One could then visualise the cells under a microscope and estimate the frequency that cells successfully integrate both fluorescent markers.

An alternative option is to remove the necessity of the LucR vector. A transgenic line of *A. thaliana* plants which has a stably integrated promoter-reporter construct could be generated. Protoplasts isolated from these plants could be used to perform a transient transfection of the *XhABFA* effector vector. GFP expression can be measured to determine the transfection efficiency of the effector. However the major drawback of taking this approach is that it is time consuming to generate future generations with a stably integrated promoter-reporter.

4.4.5 Alternative transient expression assays

The transient transfection of protoplasts is a useful tool to analyse gene expression (Yoo et al., 2007). However considering the optimisation required, exploring alternative transient assays is compelling. Presently, transient transfection of leaves mediated by the bacterium *Agrobacterium tumefaciens* is widely used as a rapid and easy way to analyse gene function in plants (Li et al., 2018). *A. tumefaciens* transfection protocols have been well established in numerous plant species and various reporter systems including the Dual-Luciferase Assay and GFP reporter genes (Li et al., 2009; Liu et al., 2015; Hu et al., 2019; Mortensen et al., 2019). In addition to bacteria, viruses are also used to introduce DNA into cells in transient gene expression systems (Mendel and Hänsch, 2017). Direct DNA transfer methods have also been employed such as electroporation and particle bombardment which allow numerous constructs to be introduced into cells consistently and efficiently (Gunadi et al., 2018).

Chapter 5: Conclusion

The current study set out to investigate the regulatory networks underlying VDT in angiosperm resurrection plants. Recently, the transcriptome of *X. humilis* during seed maturation and vegetative desiccation was assembled and analysed (Lyall et al., 2019). It was observed that while the LAFL network was upregulated during seed maturation, only a truncated *ABI3* transcript and none of the canonical LAFL TFs were expressed during vegetative desiccation. Further investigations revealed that XhABFA*,* a vegetative abiotic stress TF, was highly upregulated during VDT and its expression pattern matched that of many of the seed-specific genes expressed during VDT.

In order to investigate whether XhABFA plays a role in regulating the seed-specific genes during VDT, functional binding assays were used to test for potential interactions between XhABFA and the promoters of three of these genes: *XhPER1, XhECP63,* and *XhDSI-1VOC.* Two methods were used to functionally characterize the XhABFA-promoter interactions: a Yeast One-Hybrid assay and the transient transfection of *A. thaliana* protoplasts. The advantage of the Y1H assay is that it offers an experimental system that is set in the context of a chromatin environment. However, unlike the protoplast system, it does not provide cellular factors that are unique to plant cells.

In the Y1H assay, positive interactions between XhABFA and the three *X. humilis* promoters were not observed. This result is not necessarily indicative that XhABFA is unable to interact with the *X. humilis* promoters; there are various alternative explanations for this observation. One possible reason is that the GAL4AD::XhABFA fusion protein was not expressed in the yeast cells. A western blot with GAL4AD-specific antibodies would be able to confirm whether this is the case. If the fusion protein is expressed, then it is possible that in order for XhABFA to bind to the promoters, it requires a co-factor which is not present and/or post-translational modification that does not occur in yeast. A study on the *A. thaliana* orthologue of XhABFA, AREB1/ABF2, showed that it is only able to activate expression of its target promoters when phosphorylated (Furihata et al., 2006). In order to determine whether this is also the case for XhABFA, a mutated form of GAL4::XhABFA that mimics the constitutively active ABF2 could be tested for binding activity.

In contrast, in the experimental system of transiently transfecting *A. thaliana* protoplasts, it was demonstrated that XhABFA is able to activate the expression of the *XhPER1, XhECP63, and XhDSI-1VOC* genes. Protoplasts maintain many of the same physiological responses and cellular activities as intact plants and therefore conserved aspects of plant signalling mechanisms can be established using these cells (Sheen, 2001). Therefore the positive interaction observed in the protoplast system is more likely to be indicative of a real interaction.

This result provides evidence that a vegetative abiotic stress TF is able to activate the expression of three canonically seed-specific genes that are upregulated during VDT in *X. humilis.* This is surprising as it contradicts the hypothesis that historically has been dominant: that VDT in angiosperm resurrection plants is regulated by the transcriptional network which controls DT in the seeds of desiccation sensitive plants, specifically ABI5 and the LAFL TFs. This has been the prevailing theory as there is a large body of research which has shown that seed maturation genes are activated in the leaves of diverse resurrection plant species when they desiccate (Oliver et al., 2000; Bartels 2005; Illing et al., 2005; Farrant and Moore, 2011; Gaff and Oliver, 2013; Costa et al., 2017a; Costa et al.,

2017b; Giarola et al., 2017; VanBuren et al., 2017; Oliver et al., 2020). It seems more parsimonious that the activation of the normally seed-specific TFs in leaves would turn on seed maturation genes and protect leaves from desiccation. However, it should be noted that there is no experimental evidence to date supporting the role of the LAFL TFs in VDT. Furthermore, the only study which compared seed maturation and VDT in a resurrection plant demonstrated that the canonical LAFL TFs were induced in seeds but not in drying leaf tissues (Lyall et al. 2019). This raises the question of which TFs activate the expression of seed maturation genes in *Xerophyta* leaves as they desiccate?

In this study, I have shown that XhABFA, an orthologue of the *A. thaliana* ABF2 abiotic stress TF that is expressed at high levels in desiccating *X. humilis* leaves, is able to activate expression of seed maturation promoters in Arabidopsis protoplasts. This finding is in line with the hypothesis that VDT in angiosperms is regulated by the transcriptional network underpinning the vegetative drought response (Lyall et al., 2019).

The findings of the current study are especially interesting in the context of how VDT evolved in angiosperms. The phenomenon occurs in at least 13 angiosperm families. The families are largely unrelated and are not in a linear phylogenetic sequence to one another, i.e. they do not progress through continuous lines, from early-evolved families to late-evolved families. This implies that VDT has arisen separately at least 13 times during angiosperm evolution (Oliver at al., 2000). The low occurrence of VDT in angiosperms is potentially due to two main requirements: belonging to a family which is predisposed to VDT, but presumably only a small proportion of the species in the family; as well as whose responses to drought become inadequate in a habitat that undergoes extremely xeric episodes and therefore experience selective pressure to improve drought tolerant mechanisms to the extreme until they can tolerate desiccation (Gaff and Oliver, 2013).

Consistent with the phylogenetic independence of the 13 lineages are phenotypic differences in how the trait is displayed. For example, some species retain their photosynthetic apparatus and chlorophyll during desiccation whereas other species dismantle their photosynthetic apparatus and chlorophyll is lost during desiccation (Oliver et al., 2000). Furthermore, genomic studies on some resurrection plants indicate that different genetic architectures underlie the VDT phenotype. For example when comparing the genomes of *Oropetium thomaeum, Boea hygrometrica* and *Xerophyta viscosa, c*haracteristics which have been found to be dissimilar include the percentage of GC content, the proportion of transposable elements, as well the percentage of genes without known homologues (VanBuren et al., 2015; Xiao et al., 2015; Costa et al., 2017b). Therefore it is possible that the underlying regulatory networks may differ amongst the various resurrection plants. If this is the case then the regulatory networks involved in *X. humilis* may not necessarily be representative of VDT in general. However, there is no evidence of *ABI5* and full length *ABI3* transcripts being differentially expressed in *Xerophyta viscosa* and thus it is likely that all *Xerophyta* species have a similar mechanism of activation of seed maturation genes in leaves during desiccation.

Future work

This study has established methods to functionally characterise *X. humilis* TFs in *A. thaliana* protoplasts. This paves the way for further investigations into other TFs of interest and potential promoter targets. A TF which would be next in line to investigate is the *XhABI3B* transcript which was upregulated during vegetative desiccation (Lyall et al 2019). In *A. thaliana,* ABI3 has been shown to bind to bZIPs via its B2 domain. XhABFA is a bZIP TF and therefore it would be interesting to see whether XhABFA and XhABI3B are able to physically interact with each other, suggesting an important role of co-activators during vegetative desiccation. *In vitro* assays such as co-immunoprecipitation and *in vivo* assays such as the Yeast Two-Hybrid would be useful assays to identify potential interactions. A transient transfection in protoplasts could also test this and if both TFs are cloned into a single vector it could overcome potential issues of low transfection efficiencies due to the co-transfection of multiple vectors. It would be interesting to see whether the addition of XhABI3B increases transcriptional activity compared to a transfection with only XhABFA.

In the current study, neither of the cellular environments that were used to test for interactions were based in resurrection plants. The definitive experiment to determine whether XhABFA can indeed bind to the promoters of many seed-specific genes would be to raise an antibody to this protein and use it for chromatin immunoprecipitation (ChIP). Binding of XhABFA to the *XhPER1, XhECP63,* and *XhDSI-1VOC* promoters could be confirmed by ChIP-qPCR and additional targets identified by ChIP-Seq. However, this study has successfully pioneered the use of functional binding assays to test interactions of TFs and potential target promoters of a resurrection plant. A base has established, onto which subsequent studies are able to build on the current understanding of the network responsible for regulating VDT in angiosperm resurrection plants.

References

Abel, S. and Theologis, A., 1994. Transient transformation of Arabidopsis leaf protoplasts: a versatile experimental system to study gene expression. *The Plant Journal*, *5*(3), pp.421-427.

Aleman, F., Yazaki, J., Lee, M., Takahashi, Y., Kim, A.Y., Li, Z., Kinoshita, T., Ecker, J.R. and Schroeder, J.I., 2016. An ABA-increased interaction of the PYL6 ABA receptor with MYC2 transcription factor: a putative link of ABA and JA signaling. *Scientific reports*, *6*(1), pp.1-10.

Alpert, P., 2000. The discovery, scope, and puzzle of desiccation tolerance in plants. *Plant Ecology*, *151*(1), pp.5-17.

Amara, I., Zaidi, I., Masmoudi, K., Ludevid, M.D., Pagès, M., Goday, A. and Brini, F., 2014. Insights into late embryogenesis abundant (LEA) proteins in plants: from structure to the functions. *American journal of plant sciences*, *5*(22), p.3440.

Antoni, R., Rodriguez, L., Gonzalez-Guzman, M., Pizzio, G.A. and Rodriguez, P.L., 2011. News on ABA transport, protein degradation, and ABFs/WRKYs in ABA signaling. *Current opinion in plant biology*, *14*(5), pp.547-553.

Armstrong, R.N., 2000. Mechanistic diversity in a metalloenzyme superfamily. *Biochemistry*, *39*(45), pp.13625-13632.

Artur, M.A.S., Costa, M.C.D., Farrant, J.M. and Hilhorst, H.W., 2019a. Genome-level responses to the environment: plant desiccation tolerance. *Emerging Topics in Life Sciences*, *3*(2), pp.153-163.

Artur, M.A.S., Zhao, T., Ligterink, W., Schranz, E. and Hilhorst, H.W., 2019b. Dissecting the genomic diversification of late embryogenesis abundant (LEA) protein gene families in plants. *Genome biology and evolution*, *11*(2), pp.459-471.

Asami, P., Mundree, S. and Williams, B., 2018. Saving for a rainy day: Control of energy needs in resurrection plants. *Plant science*, *271*, pp.62-66.

Asami, P., Rupasinghe, T., Moghaddam, L., Njaci, I., Roessner, U., Mundree, S. and Williams, B., 2019. Roots of the Resurrection Plant *Tripogon loliiformis* Survive Desiccation Without the Activation of Autophagy Pathways by Maintaining Energy Reserves. *Frontiers in plant science*, 10:459.

Bartels, D., 2005. Desiccation tolerance studied in the resurrection plant Craterostigma plantagineum. *Integrative and Comparative Biology*, *45*(5), pp.696-701.

Bartels, D., Phillips, J. and Chandler, J., 2007. Desiccation tolerance: gene expression, pathways, and regulation of gene expression. *Plant desiccation tolerance*, *35*, pp.115-137

Bass, J.I.F., Reece-Hoyes, J.S. and Walhout, A.J., 2016. Gene-centered yeast one-hybrid assays. *Cold Spring Harbor Protocols*, *2016*(12), pp.pdb-top077669.

Baud, S., Boutin, J.P., Miquel, M., Lepiniec, L. and Rochat, C., 2002. An integrated overview of seed development in Arabidopsis thaliana ecotype WS. *Plant Physiology and Biochemistry*, *40*(2), pp.151-160.

Baud, S., Mendoza, M.S., To, A., Harscoët, E., Lepiniec, L. and Dubreucq, B., 2007. WRINKLED1 specifies the regulatory action of LEAFY COTYLEDON2 towards fatty acid metabolism during seed maturation in Arabidopsis. *The Plant Journal*, *50*(5), pp.825-838.

Bechtold, U., 2018. Plant life in extreme environments: how do you improve drought tolerance? *Frontiers in plant science*, *9*, p.543.

Behnke, H.D., Hummel, E., Hillmer, S., Sauer-Gürth, H., Gonzalez, J. and Wink, M., 2013. A revision of African Velloziaceae based on leaf anatomy characters and rbcL nucleotide sequences. *Botanical Journal of the Linnean Society*, *172*(1), pp.22-94.

Bell, T., 2016, Would We Know Alien Life If We Saw It? *Air and Space Mag,* viewed 11 June 2019, <https://www.airspacemag.com/space/life-in-universe-special-what-is-life-180958432/>

Bensmihen, S., Giraudat, J. and Parcy, F., 2005. Characterization of three homologous basic leucine zipper transcription factors (bZIP) of the ABI5 family during Arabidopsis thaliana embryo maturation. *Journal of experimental botany*, *56*(412), pp.597-603.

Bent, A.F., 2000. Arabidopsis in planta transformation. Uses, mechanisms, and prospects for transformation of other species. *Plant physiology*, *124*(4), pp.1540-1547.

Bergeron, D., Barbeau, B., Léger, C. and Rassart, E., 1995. Experimental bias in the evaluation of the cellular transient expression in DNA co-transfection experiments. *Cellular & molecular biology research*, *41*(3), pp.155-159.

Bergh, N.G., 1998. *Aspects of the prevention of light damage during drying and rehydration of the desiccation-tolerant grass Eragrostis nindensis* (Doctoral dissertation, University of Cape Town).

Bewley, J.D. and Krochko, J.E., 1982. Desiccation-tolerance. In *Physiological plant ecology II* (pp. 325-378). Springer, Berlin, Heidelberg.

Black, M. and Pritchard, H.W. eds., 2002. *Desiccation and survival in plants: drying without dying*. Cabi.

Blomstedt, C.K., Griffiths, C.A., Gaff, D.F., Hamill, J.D. and Neale, A.D., 2018. Plant desiccation tolerance and its regulation in the foliage of resurrection “flowering-plant” species. *Agronomy*, *8*(8), p.146.

Blount, B.A., Driessen, M.R. and Ellis, T., 2016. GC preps: fast and easy extraction of stable yeast genomic DNA. *Scientific reports*, *6*, p.26863

Bohrer, A.S., Yoshimoto, N., Sekiguchi, A., Rykulski, N., Saito, K. and Takahashi, H., 2015. Alternative translational initiation of ATP sulfurylase underlying dual localization of sulfate assimilation pathways in plastids and cytosol in A. thaliana thaliana. *Frontiers in plant science*, *5*, p.750.

Boudsocq, M., Barbier-Brygoo, H. and Laurière, C., 2004. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. *Journal of Biological Chemistry*, *279*(40), pp.41758-41766.

Boudsocq, M., Droillard, M.J., Barbier-Brygoo, H. and Laurière, C., 2007. Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant molecular biology*, *63*(4), pp.491-503.

Boulard, C., Fatihi, A., Lepiniec, L. and Dubreucq, B., 2017. Regulation and evolution of the interaction of the seed B3 transcription factors with NF-Y subunits. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1860*(10), pp.1069-1078.

Bouyer, D., Roudier, F., Heese, M., Andersen, E.D., Gey, D., Nowack, M.K., Goodrich, J., Renou, J.P., Grini, P.E., Colot, V. and Schnittger, A., 2011. Polycomb repressive complex 2 controls the embryo-to-seedling phase transition. *PLoS genetics*, *7*(3).

Boyer, S.J., 1996. Advances in drought tolerance in plants. *Adv. Agron.*, *56*, pp.187-218.

Brandt, B., Brodsky, D.E., Xue, S., Negi, J., Iba, K., Kangasjärvi, J., Ghassemian, M., Stephan, A.B., Hu, H. and Schroeder, J.I., 2012. Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proceedings of the National Academy of Sciences*, *109*(26), pp.10593-10598.

Braybrook, S.A. and Harada, J.J., 2008. LECs go crazy in embryo development. *Trends in plant science*, *13*(12), pp.624-630. Challabathula, D. and Bartels, D., 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. *Frontiers in Plant Science*, *4*, p.482.

Cahill, M.A., Ernst, W.H., Janknecht, R. and Nordheim, A., 1994. Regulatory squelching. *FEBS letters*, *344*(2-3), pp.105-108.

Challabathula, D. and Bartels, D., 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. *Frontiers in Plant Science*, *4*, p.482.

Chen, S., Tao, L., Zeng, L., Vega-Sanchez, M.E., Umemura, K. and Wang, G.L., 2006. A highly efficient transient protoplast system for analyzing defence gene expression and protein–protein interactions in rice. *Molecular plant pathology*, *7*(5), pp.417-427.

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C. and Yu, D., 2012. The role of WRKY transcription factors in plant abiotic stresses. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1819*(2), pp.120-128.

Chen, H., Ruan, J., Chu, P., Fu, W., Liang, Z., Li, Y., Tong, J., Xiao, L., Liu, J., Li, C. and Huang, S., 2019. AtPER1 enhances primary seed dormancy and reduces seed germination by suppressing the ABA catabolism and GA biosynthesis in Arabidopsis seeds. *The Plant Journal*.

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. and Nester, E.W., 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell*, *11*(2), pp.263-271.

Chiu, W.L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J., 1996. Engineered GFP as a vital reporter in plants. *Current Biology*, *6*(3), pp.325-330.

Choudhury, F.K., Rivero, R.M., Blumwald, E. and Mittler, R., 2017. Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal*, *90*(5), pp.856-867.

Collett, H., Shen, A., Gardner, M., Farrant, J.M., Denby, K.J. and Illing, N., 2004. Towards transcript profiling of desiccation tolerance in Xerophyta humilis: construction of a normalized 11 k X. humilis cDNA set and microarray expression analysis of 424 cDNAs in response to dehydration. *Physiologia Plantarum*, *122*(1), pp.39-53.

Costa, M.C.D., Righetti, K., Nijveen, H., Yazdanpanah, F., Ligterink, W., Buitink, J. and Hilhorst, H.W., 2015. A gene co-expression network predicts functional genes controlling the re-establishment of desiccation tolerance in germinated Arabidopsis thaliana seeds. *Planta*, *242*(2), pp.435-449.

Costa, M.C.D., Cooper, K., Hilhorst, H.W. and Farrant, J.M., 2017a. Orthodox seeds and resurrection plants: Two of a kind?. *Plant physiology*, pp.pp-00760.

Costa, M.C.D., Artur, M.A., Maia, J., Jonkheer, E., Derks, M.F., Nijveen, H., Williams, B., Mundree, S.G., Jiménez-Gómez, J.M., Hesselink, T. and Schijlen, E.G., 2017b. A footprint of desiccation tolerance in the genome of Xerophyta viscosa. *Nature plants*, *3*(4), p.17038.

Covarrubias, A.A., Cuevas-Velazquez, C.L., Romero-Pérez, P.S., Rendón-Luna, D.F. and Chater, C.C., 2017. Structural disorder in plant proteins: where plasticity meets sessility. *Cellular and Molecular Life Sciences*, *74*(17), pp.3119-3147.

Dandekar, D.H., Kumar, M., Ladha, J.S., Ganesh, K.N. and Mitra, D., 2005. A quantitative method for normalization of transfection efficiency using enhanced green fluorescent protein. *Analytical biochemistry*, *342*(2), pp.341-344.

Davey, M.R., Anthony, P., Power, J.B. and Lowe, K.C., 2005. Plant protoplasts: status and biotechnological perspectives. *Biotechnology advances*, *23*(2), pp.131-171.

De Ruijter, N.C.A., Verhees, J., Van Leeuwen, W. and Van Der Krol, A.R., 2003. Evaluation and comparison of the GUS, LUC and GFP reporter system for gene expression studies in plants. *Plant Biology*, *5*(02), pp.103-115.

Deeba, F. and Pandey, V., 2017. Adaptive Mechanisms of Desiccation Tolerance in Resurrection Plants. In *Plant Adaptation Strategies in Changing Environment* (pp. 29-75). Springer, Singapore.

Dekkers, B.J., Costa, M.C.D., Maia, J., Bentsink, L., Ligterink, W. and Hilhorst, H.W., 2015. Acquisition and loss of desiccation tolerance in seeds: from experimental model to biological relevance. *Planta*, *241*(3), pp.563-577.

Dellaporta, S.L., Wood, J. and Hicks, J.B., 1983. A plant DNA minipreparation: version II. *Plant molecular biology reporter*, *1*(4), pp.19-21.

Delmas, F., Sankaranarayanan, S., Deb, S., Widdup, E., Bournonville, C., Bollier, N., Northey, J.G., McCourt, P. and Samuel, M.A., 2013. ABI3 controls embryo degreening through Mendel's I locus. *Proceedings of the National Academy of Sciences*, *110*(40), pp.E3888-E3894.Dietz, K.J., Horling, F., Konig, J. and Baier, M., 2002. The function of the chloroplast 2-cysteine peroxiredoxin in peroxide detoxification and its regulation. *Journal of experimental botany*, *53*(372), pp.1321-1329.

Deplancke, B., Dupuy, D., Vidal, M. and Walhout, A.J., 2004. A gateway-compatible yeast one-hybrid system. *Genome research*, *14*(10b), pp.2093-2101.

Díaz-Triviño, S., Long, Y., Scheres, B. and Blilou, I., 2017. Analysis of a plant transcriptional regulatory network using transient expression systems. In *Plant Gene Regulatory Networks* (pp. 83-103). Humana Press, New York, NY.

Dinakar, C. and Bartels, D., 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. *Frontiers in Plant Science*, *4*, p.482.Dolfini, D., Gatta, R. and Mantovani, R., 2012. NF-Y and the transcriptional activation of CCAAT promoters. *Critical reviews in biochemistry and molecular biology*, *47*(1), pp.29-49.

Dröge-Laser, W., Snoek, B.L., Snel, B. and Weiste, C., 2018. The Arabidopsis bZIP transcription factor family—an update. *Current opinion in plant biology*, *45*, pp.36-49.

Dudaite, N., Navickaite, M. and Dinarina, A., 2015. Direct PCR from yeast cells. *Application Note; Thermo Fisher Scientific: Vantaa, Finland*.

Dure, L., Crouch, M., Harada, J., Ho, T.H.D., Mundy, J., Quatrano, R., Thomas, T. and Sung, Z.R., 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant molecular biology*, *12*(5), pp.475-486.

Ezcurra, I., Wycliffe, P., Nehlin, L., Ellerström, M. and Rask, L., 2000. Transactivation of the Brassica napus napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. *The Plant Journal*, *24*(1), pp.57-66.

Farr, A. and Roman, A., 1992. A pitfall of using a second plasmid to determine transfection efficiency. *Nucleic Acids Research*, *20*(4), p.920.

Farrant, J.M. and Moore, J.P., 2011. Programming desiccation-tolerance: from plants to seeds to resurrection plants. *Current opinion in plant biology*, *14*(3), pp.340-345.

Farrant, J.M., 2000. A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. *Plant Ecology*, *151*(1), pp.29-39.

Farrant, J.M., Cooper, K., Hilgart, A., Abdalla, K.O., Bentley, J., Thomson, J.A., Dace, H.J., Peton, N., Mundree, S.G. and Rafudeen, M.S., 2015. A molecular physiological review of vegetative desiccation tolerance in the resurrection plant Xerophyta viscosa (Baker). *Planta*, *242*(2), pp.407-426.

Fatihi, A., Boulard, C., Bouyer, D., Baud, S., Dubreucq, B. and Lepiniec, L., 2016. Deciphering and modifying LAFL transcriptional regulatory network in seed for improving yield and quality of storage compounds. *Plant Science*, *250*, pp.198-204.

Foden, W. & Potter, L. 2005. Xerophyta humilis (Baker), *T. Durand & Schinz. National Assessment: Red List of South African Plants version* 2017.1., viewed 21 July 2019, <http://redlist.sanbi.org/species.php?species=4051-3>.

Franks, S.J., 2011. Plasticity and evolution in drought avoidance and escape in the annual plant Brassica rapa. *New Phytologist*, *190*(1), pp.249-257.

Frey, P.M., Schärer-Hernández, N.G., Fütterer, J., Potrykus, I. and Puonti-Kaerlas, J., 2001. Simultaneous analysis of the bidirectional African cassava mosaic virus promoter activity using two different luciferase genes. *Virus Genes*, *22*(2), pp.231-242.

Fujimoto, S., Matsunaga, S., Yonemura, M., Uchiyama, S., Azuma, T. and Fukui, K., 2004. Identification of a novel plant MAR DNA binding protein localized on chromosomal surfaces. *Plant molecular biology*, *56*(2), pp.225-239.

Fujita, Y., Fujita, M., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2011. ABA-mediated transcriptional regulation in response to osmotic stress in plants. *Journal of plant research*, *124*(4), pp.509-525.

Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2006. Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proceedings of the National Academy of Sciences*, *103*(6), pp.1988-1993.

Gaff, D.F., 1977. Desiccation tolerant vascular plants of Southern Africa. *Oecologia*, *31*(1), pp.95-109.

Gaff, D.F. and Oliver, M., 2013. The evolution of desiccation tolerance in angiosperm plants: a rare yet common phenomenon. *Functional Plant Biology*, *40*(4), pp.315-328.

Gan, L., Zhang, C.Y., Wang, X.D., Wang, H., Long, Y., Yin, Y.T., Li, D.R., Tian, J.H., Li, Z.Y., Lin, Z.W. and Yu, L.J., 2013. Proteomic and comparative genomic analysis of two Brassica napus lines differing in oil content. *Journal of proteome research*, *12*(11), pp.4965-4978.

Gao, S.Q., Chen, M., Xu, Z.S., Zhao, C.P., Li, L., Xu, H.J., Tang, Y.M., Zhao, X. and Ma, Y.Z., 2011. The soybean GmbZIP1 transcription factor enhances multiple abiotic stress tolerances in transgenic plants. *Plant molecular biology*, *75*(6), pp.537-553.

Gechev, T.S. and Hille, J., 2012. Molecular basis of plant stress. *Cellular and Molecular Life Sciences*, *69*(19), pp.3161-3163.

Gechev, T.S., Benina, M., Obata, T., Tohge, T., Sujeeth, N., Minkov, I., Hille, J., Temanni, M.R., Marriott, A.S., Bergström, E. and Thomas-Oates, J., 2013. Molecular mechanisms of desiccation tolerance in the resurrection glacial relic Haberlea rhodopensis. *Cellular and Molecular Life Sciences*, *70*(4), pp.689-709.

Giarola, V., Hou, Q. and Bartels, D., 2017. Angiosperm plant desiccation tolerance: hints from transcriptomics and genome sequencing. *Trends in Plant Science*, *22*(8), pp.705-717.

González-Morales, S.I., Chávez-Montes, R.A., Hayano-Kanashiro, C., Alejo-Jacuinde, G., Rico-Cambron, T.Y., de Folter, S. and Herrera-Estrella, L., 2016. Regulatory network analysis reveals novel regulators of seed desiccation tolerance in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *113*(35), pp.E5232-E5241.

Goyal, K., Walton, L.J. and Tunnacliffe, A., 2005. LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal*, *388*(1), pp.151-157.

Guerriero, G., Martin, N., Golovko, A., Sundström, J.F., Rask, L. and Ezcurra, I., 2009. The RY/Sph element mediates transcriptional repression of maturation genes from late maturation to early seedling growth. *New Phytologist*, *184*(3), pp.552-565.

Gunadi, A., Dean, E.A. and Finer, J.J., 2019. Transient Transformation Using Particle Bombardment for Gene Expression Analysis. In *Transgenic Plants* (pp. 67-79). Humana Press, New York, NY.

Han, J.D., Li, X., Jiang, C.K., Wong, G.K.S., Rothfels, C.J. and Rao, G.Y., 2017. Evolutionary analysis of the LAFL genes involved in the land plant seed maturation program. *Frontiers in plant science*, *8*, p.439.

Hand, S.C., Menze, M.A., Toner, M., Boswell, L. and Moore, D., 2011. LEA proteins during water stress: not just for plants anymore. *Annual review of physiology*, *73*, pp.115-134.

Haslekås, C., Viken, M.K., Grini, P.E., Nygaard, V., Nordgard, S.H., Meza, T.J. and Aalen, R.B., 2003. Seed 1-cysteine peroxiredoxin antioxidants are not involved in dormancy, but contribute to inhibition of germination during stress. *Plant physiology*, *133*(3), pp.1148-1157.

Hauser, F., Waadt, R. and Schroeder, J.I., 2011. Evolution of abscisic acid synthesis and signaling mechanisms. *Current Biology*, *21*(9), pp.R346-R355.

He, P. and Moran, G.R., 2011. Structural and mechanistic comparisons of the metal-binding members of the vicinal oxygen chelate (VOC) superfamily. *Journal of inorganic biochemistry*, *105*(10), pp.1259-1272.

Hecker, M., Lambeck, S., Toepfer, S., Van Someren, E. and Guthke, R., 2009. Gene regulatory network inference: data integration in dynamic models—a review. *Biosystems*, *96*(1), pp.86-103.

Helander, J.D. and Cutler, S.R., 2018. Abscisic acid signaling and biosynthesis: Protein structures and molecular probes. In *Plant Structural Biology: Hormonal Regulations* (pp. 113-146). Springer, Cham.

Hilhorst, H.W., Costa, M.C.D. and Farrant, J.M., 2018. A Footprint of Plant Desiccation Tolerance. Does It Exist?. *Molecular plant*, *11*(8), pp.1003-1005.

Ho, C.K. and Strauss, J.F., 2004. Activation of the control reporter plasmids pRL-TK and pRL-SV40 by multiple GATA transcription factors can lead to aberrant normalization of transfection efficiency. *BMC biotechnology*, *4*(1), p.10.

Hobert, O., 2008. Gene regulation by transcription factors and microRNAs. *Science*, *319*(5871), pp.1785-1786.

Hu, D., Bent, A.F., Hou, X. and Li, Y., 2019. Agrobacterium-mediated vacuum infiltration and floral dip transformation of rapid-cycling Brassica rapa. *BMC plant biology*, *19*(1), p.246.

Huang, A.H., 1992. Oil bodies and oleosins in seeds. *Annual review of plant biology*, *43*(1), pp.177-200.

Hundertmark, M. and Hincha, D.K., 2008. LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. *BMC genomics*, *9*(1), p.118.

Huszár, T., Mucsi, I., Terebessy, T., Masszi, A., Adamkó, S., Jeney, C. and Rosivall, L., 2001. The use of a second reporter plasmid as an internal standard to normalize luciferase activity in transient transfection experiments may lead to a systematic error. *Journal of biotechnology*, *88*(3), pp.251-258.

Hyde, M.A., Wursten, B.T., Ballings, P. and Coates Palgrave, M., 2019. Flora of Zimbabwe: Species information: *Xerophyta humilis. Zimbabwe Flora,* viewed 21 April 2019, <https://www.zimbabweflora.co.zw/speciesdata/species.php?species_id=115280 >.

Illing, N., Denby, K.J., Collett, H., Shen, A. and Farrant, J.M., 2005. The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. *Integrative and Comparative Biology*, *45*(5), pp.771-787.

Ingle, R.A., Schmidt, U.G., Farrant, J.M., Thomson, J.A. and Mundree, S.G., 2007. Proteomic analysis of leaf proteins during dehydration of the resurrection plant Xerophyta viscosa. *Plant, cell & environment*, *30*(4), pp.435-446.

Islam, M.S. and Wang, M.H., 2009. Expression of dehydration responsive element-binding protein-3 (DREB3) under different abiotic stresses in tomato. *BMB reports*, *42*(9), pp.611-616.

Ivanov, I.V., Qian, X. and Pal, R. eds., 2016. *Emerging Research in the Analysis and Modeling of Gene Regulatory Networks*. IGI Global.

Iwata, Y., Lee, M.H. and Koizumi, N., 2011. Analysis of a transcription factor using transient assay in Arabidopsis protoplasts. In *Plant Transcription Factors* (pp. 107-117). Humana Press.

Jenks, M.A. and Wood, A.J. eds., 2008. *Plant desiccation tolerance*. John Wiley & Sons.

Jia, H., Suzuki, M. and McCarty, D.R., 2014. Regulation of the seed to seedling developmental phase transition by the LAFL and VAL transcription factor networks. *Wiley Interdisciplinary Reviews: Developmental Biology*, *3*(1), pp.135-145.

Jiang, G., Wang, Z., Shang, H., Yang, W., Hu, Z., Phillips, J. and Deng, X., 2007. Proteome analysis of leaves from the resurrection plant Boea hygrometrica in response to dehydration and rehydration. *Planta*, *225*(6), p.1405.

Johnson, I., 2008. Xerophyta retinervis (Baker), *SANBI,* viewed 21 April 2019, <http://pza.sanbi.org/xerophyta-retinervis>.

Jung, H.I., Yan, J., Zhai, Z. and Vatamaniuk, O.K., 2015. Gene functional analysis using protoplast transient assays. In *Plant Functional Genomics* (pp. 433-452). Humana Press, New York, NY.

Kang, J.Y., Choi, H.I., Im, M.Y. and Kim, S.Y., 2002. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *The Plant Cell*, *14*(2), pp.343-357.

Karimi, M., Goldie, L.C., Cruickshank, M.N., Moses, E.K. and Abraham, L.J., 2009. A critical assessment of the factors affecting reporter gene assays for promoter SNP function: a reassessment of− 308 TNF polymorphism function using a novel integrated reporter system. *European journal of human genetics*, *17*(11), p.1454.

Karssen, C.M. and Lacka, E., 1986. A revision of the hormone balance theory of seed dormancy: studies on gibberellin and/or abscisic acid-deficient mutants of Arabidopsis thaliana. In *Plant growth substances 1985* (pp. 315-323). Springer, Berlin, Heidelberg.

Kigel, J., Ofir, M., Kon, A., Malinowski, D.P. and Pinchak, W.E., 2009. Physiology of induction and relaxation of summer dormancy in perennial grasses. In *Proceedings from the Workshop on Summer Dormancy in Grasses, The Samuel Roberts Noble Foundation, Ardmore*.

Kim, S.Y., Paeng, S.K., Nawkar, G.M., Maibam, P., Lee, E.S., Kim, K.S., Lee, D.H., Park, D.J., Kang, S.B., Kim, M.R. and Lee, J.H., 2011. The 1-Cys peroxiredoxin, a regulator of seed dormancy, functions as a molecular chaperone under oxidative stress conditions. *Plant science*, *181*(2), pp.119-124.

Kim, N., Moon, S.J., Min, M.K., Choi, E.H., Kim, J., Koh, E.Y., Yoon, I., Byun, M.O., Yoo, S.D. and Kim, B.G., 2015. Functional characterization and reconstitution of ABA signaling components using transient gene expression in rice protoplasts. *Frontiers in plant science*, *6*, p.614.

Kinnberg, K., 2003. Evaluation of in vitro assays for determination of estrogenic activity in the environment. *Danish EPA*, viewed 23 December 2019, <https://www2.mst.dk/udgiv/publications/2003/87-7972-922-3/pdf/87-7972-923-1.pdf>.

Knight, H. and Knight, M.R., 2001. Abiotic stress signalling pathways: specificity and cross-talk. *Trends in plant science*, *6*(6), pp.262-267.

Kobayashi, F., Maeta, E., Terashima, A. and Takumi, S., 2008. Positive role of a wheat HvABI5 ortholog in abiotic stress response of seedlings. *Physiologia Plantarum*, *134*(1), pp.74-86.

Kooyers, N.J., 2015. The evolution of drought escape and avoidance in natural herbaceous populations. *Plant Science*, *234*, pp.155-162.

Kranner, I., Beckett, R.P., Wornik, S., Zorn, M. and Pfeifhofer, H.W., 2002. Revival of a resurrection plant correlates with its antioxidant status. *The Plant Journal*, *31*(1), pp.13-24.

Kreiss, P., Mailhe, P., Scherman, D., Pitard, B., Cameron, B., Rangara, R., Aguerre-Charriol, O., Airiau, M. and Crouzet, J., 1999. Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. *Nucleic acids research*, *27*(19), pp.3792-3798.

Kuromori, T., Mizoi, J., Umezawa, T., Yamaguchi-Shinozaki, K. and Shinozaki, K., 2015. Stress signaling networks: drought stress. Molecular Biology, pp.1-23.

Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., Hirai, N., Koshiba, T., Kamiya, Y. and Nambara, E., 2004. The Arabidopsis cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. *The EMBO journal*, *23*(7), pp.1647-1656.

Kwong, R.W., Bui, A.Q., Lee, H., Kwong, L.W., Fischer, R.L., Goldberg, R.B. and Harada, J.J., 2003. LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development. *The Plant Cell*, *15*(1), pp.5-18.

Lambers, H., Chapin III, F.S. and Pons, T.L., 2008. *Plant physiological ecology*. Springer Science & Business Media.

Landy, A., 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annual review of biochemistry*, *58*(1), pp.913-941.

Latchman, D.S., 1997. Transcription factors: an overview. *The international journal of biochemistry & cell biology*, *29*(12), pp.1305-1312.

Le, T.N., Blomstedt, C.K., Kuang, J., Tenlen, J., Gaff, D.F., Hamill, J.D. and Neale, A.D., 2007. Desiccation-tolerance specific gene expression in leaf tissue of the resurrection plant Sporobolus stapfianus. *Functional Plant Biology*, *34*(7), pp.589-600.

Le, B.H., Cheng, C., Bui, A.Q., Wagmaister, J.A., Henry, K.F., Pelletier, J., Kwong, L., Belmonte, M., Kirkbride, R., Horvath, S. and Drews, G.N., 2010. Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors. *Proceedings of the National Academy of Sciences*, *107*(18), pp.8063-8070.

Lee, T.I. and Young, R.A., 2000. Transcription of eukaryotic protein-coding genes. *Annual review of genetics*, *34*(1), pp.77-137.

Lei, R., Qiao, W., Hu, F., Jiang, H. and Zhu, S., 2015. A simple and effective method to encapsulate tobacco mesophyll protoplasts to maintain cell viability. *MethodsX*, *2*, pp.24-32

Lepiniec, L., Devic, M., Roscoe, T.J., Bouyer, D., Zhou, D.X., Boulard, C., Baud, S. and Dubreucq, B., 2018. Molecular and epigenetic regulations and functions of the LAFL transcriptional regulators that control seed development. *Plant reproduction*, *31*(3), pp.291-307.

Leprince, O. and Buitink, J., 2007. 7 The Glassy State in Dry Seeds and Pollen. *Plant desiccation tolerance*, p.193.

Lewis, L.A. and McCourt, R.M., 2004. Green algae and the origin of land plants. *American journal of botany*, *91*(10), pp.1535-1556.

Li, J.F., Park, E., von Arnim, A.G. and Nebenführ, A., 2009. The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species. *Plant methods*, *5*(1), p.6.

Li, H., Li, K., Guo, Y., Guo, J., Miao, K., Botella, J.R., Song, C.P. and Miao, Y., 2018. A transient transformation system for gene characterization in upland cotton (Gossypium hirsutum). *Plant methods*, *14*(1), p.50.

Liang, Y., Wan, N., Cheng, Z., Mo, Y., Liu, B., Liu, H., Raboanatahiry, N., Yin, Y. and Li, M., 2017. Whole-Genome identification and expression pattern of the vicinal oxygen chelate family in rapeseed (Brassica napus L.). *Frontiers in plant science*, *8*, p.745.

Lin, H., McGrath, J., Wang, P. and Lee, T., 2006. Cellular toxicity induced by SRF-mediated transcriptional squelching. *Toxicological sciences*, *96*(1), pp.83-91.

Liu, N., Zhong, N.Q., Wang, G.L., Li, L.J., Liu, X.L., He, Y.K. and Xia, G.X., 2007. Cloning and functional characterization of PpDBF1 gene encoding a DRE-binding transcription factor from Physcomitrella patens. *Planta*, *226*(4), pp.827-838.

Liu, Q. and Axtell, M.J., 2015. Quantitating plant microRNA-mediated target repression using a dual-luciferase transient expression system. In *Plant Functional Genomics* (pp. 287-303). Humana Press, New York, NY.

Liu, J., Moyankova, D., Lin, C.T., Mladenov, P., Sun, R.Z., Djilianov, D. and Deng, X., 2018. Transcriptome reprogramming during severe dehydration contributes to physiological and metabolic changes in the resurrection plant Haberlea rhodopensis. *BMC plant biology*, *18*(1), p.351.

Lopez-Molina, L., Mongrand, S. and Chua, N.H., 2001. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proceedings of the National Academy of Sciences*, *98*(8), pp.4782-4787.

Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T. and Chua, N.H., 2002. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *The Plant Journal*, *32*(3), pp.317-328.

Ludlow, M.M., 1989. Strategies of response to water stress. In *Kreeb HK, Richter H, Hinckley TM (eds) Structural and functional responses to environmental stresses: water shortage.* SPB Academic, The Hague, pp 269–281

Luehrsen, K.R., DE WET, J.R. and Walbot, V., 1995. Transient expression analysis in plants using firefly luciferase reporter gene. In *Recombinant DNA Methodology II* (pp. 139-156). Academic Press.

Lüttge, U., Beck, E. and Bartels, D. eds., 2011. *Plant desiccation tolerance* (Vol. 215). Springer Science & Business Media.

Lyall, R., Ingle, R.A. and Illing, N., 2014. The window of desiccation tolerance shown by early-stage germinating seedlings remains open in the resurrection plant, Xerophyta viscosa. *PLoS one*, *9*(3).

Lyall, R., 2016. *Regulation of desiccation tolerance in Xerophyta seedlings and leaves* (Doctoral dissertation, University of Cape Town).

Lyall, R., Schlebusch, S.A., Proctor, J., Prag, M., Hussey, S.G. and Ingle, R.A., 2019. Vegetative desiccation tolerance in the resurrection plant Xerophyta humilis has not evolved through reactivation of the seed canonical LAFL regulatory network. *The Plant Journal*.

Lyall, R. and Gechev, T. (2020). Multi-Omics Insights into the Evolution of Angiosperm Resurrection Plants. In Annual Plant Reviews online, J.A. Roberts (Ed.).

Ma, J. and Ptashne, M., 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell*, *48*(5), pp.847-853.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A. and Grill, E., 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science*, *324*(5930), pp.1064-1068.

Ma, C., Wang, H., Macnish, A.J., Estrada-Melo, A.C., Lin, J., Chang, Y., Reid, M.S. and Jiang, C.Z., 2015. Transcriptomic analysis reveals numerous diverse protein kinases and transcription factors involved in desiccation tolerance in the resurrection plant Myrothamnus flabellifolia. *Horticulture research*, *2*, p.15034.

Maia, J., Dekkers, B.J., Dolle, M.J., Ligterink, W. and Hilhorst, H.W., 2014. Abscisic acid (ABA) sensitivity regulates desiccation tolerance in germinated A rabidopsis seeds. *New Phytologist*, *203*(1), pp.81-93.

Malo, M.S., Abedrapo, M., Chen, A., Mozumder, M., Pushpakaran, P., Alkhoury, F., Zhang, W., Fleming, E. and Hodin, R.A., 2003. Improved eukaryotic promoter-detection vector carrying two luciferase reporter genes. *Biotechniques*, *35*(6), pp.1150-1154.

Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M. and Satou, M., 2008. Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant and Cell Physiology*, *49*(8), pp.1135-1149.

Matsuo, N., Minami, M., Maeda, T. and Hiratsuka, K., 2001. Dual luciferase assay for monitoring transient gene expression in higher plants. *Plant biotechnology*, *18*(1), pp.71-75.

McClean, P., 1998. Cis-acting Elements and Trans-acting Factors, *Ndsu.edu*, viewed 21 November 2019, < https://www.ndsu.edu/pubweb/~mcclean/plsc731/cis-trans/cis-trans6.htm >

McNabb, D.S., Reed, R. and Marciniak, R.A., 2005. Dual luciferase assay system for rapid assessment of gene expression in Saccharomyces cerevisiae. *Eukaryotic cell*, *4*(9), pp.1539-1549.

Mendel, R.R. and Hänsch, R., 2017. Gene transfer to higher plants. *Molecular Methods in Plant Pathology*, pp.189-202

Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2012. AP2/ERF family transcription factors in plant abiotic stress responses. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1819*(2), pp.86-96.

Mönke, G., Seifert, M., Keilwagen, J., Mohr, M., Grosse, I., Hähnel, U., Junker, A., Weisshaar, B., Conrad, U., Bäumlein, H. and Altschmied, L., 2012. Toward the identification and regulation of the A. thaliana thaliana ABI3 regulon. *Nucleic acids research*, *40*(17), pp.8240-8254.

Moore, J.P., Westall, K.L., Ravenscroft, N., Farrant, J.M., Lindsey, G.G. and Brandt, W.F., 2005. The predominant polyphenol in the leaves of the resurrection plant Myrothamnus flabellifolius, 3, 4, 5 tri-O-galloylquinic acid, protects membranes against desiccation and free radical-induced oxidation. *Biochemical Journal*, *385*(1), pp.301-308.

Mortensen, S., Bernal-Franco, D., Cole, L.F., Sathitloetsakun, S., Cram, E.J. and Lee-Parsons, C.W., 2019. EASI transformation: An efficient transient expression method for analyzing gene function in Catharanthus roseus seedlings. *Frontiers in plant science*, *10*, p.755.

Mowla, S.B., Thomson, J.A., Farrant, J.M. and Mundree, S.G., 2002. A novel stress-inducible antioxidant enzyme identified from the resurrection plant Xerophyta viscosa Baker. *Planta*, *215*(5), pp.716-726.

Mulako, I., Farrant, J.M., Collett, H. and Illing, N., 2008. Expression of Xhdsi-1VOC, a novel member of the vicinal oxygen chelate (VOC) metalloenzyme superfamily, is up-regulated in leaves and roots during desiccation in the resurrection plant Xerophyta humilis (Bak) Dur and Schinz. *Journal of experimental botany*, *59*(14), pp.3885-3901.

Nakashima, K., Shinwari, Z.K., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2000. Organization and expression of two Arabidopsis DREB2 genes encoding DRE-binding proteins involved in dehydration-and high-salinity-responsive gene expression. *Plant molecular biology*, *42*(4), pp.657-665.

Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2006. Transcriptional regulation of ABI3-and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of Arabidopsis. *Plant molecular biology*, *60*(1), pp.51-68.

Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., Maruyama, K., Yoshida, T., Ishiyama, K., Kobayashi, M. and Shinozaki, K., 2009. Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2. 2, SRK2E/SnRK2. 6/OST1 and SRK2I/SnRK2. 3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant and Cell Physiology*, *50*(7), pp.1345-1363.

Nakashima, K. and Yamaguchi-Shinozaki, K., 2013. ABA signaling in stress-response and seed development. *Plant cell reports*, *32*(7), pp.959-970.

Neeragunda Shivaraj, Y., Barbara, P., Gugi, B., Vicré-Gibouin, M., Driouich, A., Ramasandra Govind, S., Devaraja, A. and Kambalagere, Y., 2018. Perspectives on Structural, Physiological, Cellular, and Molecular Responses to Desiccation in Resurrection Plants. *Scientifica*, *2018*.

Ni, Y., Aghamirzaie, D., Elmarakeby, H., Collakova, E., Li, S., Grene, R. and Heath, L.S., 2016. A machine learning approach to predict gene regulatory networks in seed development in Arabidopsis. *Frontiers in plant science*, *7*, p.1936.

Oliver, M.J., Tuba, Z. and Mishler, B.D., 2000. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology*, *151*(1), pp.85-100.

Oliver, M.J., 2007. Lessons on dehydration tolerance from desiccation tolerant plants. *Plant Desiccation Tolerance*, pp.11-50.

Oliver, M.J., Jain, R., Balbuena, T.S., Agrawal, G., Gasulla, F. and Thelen, J.J., 2011a. Proteome analysis of leaves of the desiccation-tolerant grass, Sporobolus stapfianus, in response to dehydration. *Phytochemistry*, *72*(10), pp.1273-1284.

Oliver, M.J., Guo, L., Alexander, D.C., Ryals, J.A., Wone, B.W. and Cushman, J.C., 2011b. A sister group contrast using untargeted global metabolomic analysis delineates the biochemical regulation underlying desiccation tolerance in Sporobolus stapfianus. *The Plant Cell*, *23*(4), pp.1231-1248.

Oliver, M.J., Farrant, J.M., Hilhorst, H.W., Mundree, S., Williams, B. and Bewley, J.D., 2020. Desiccation Tolerance: Avoiding Cellular Damage During Drying and Rehydration. *Annual Review of Plant Biology*, *71*

O'Mahoney, J.V. and Adams, T.E., 1994. Optimization of experimental variables influencing reporter gene expression in hepatoma cells following calcium phosphate transfection. *DNA and cell biology*, *13*(12), pp.1227-1232.

Orellana, S., Yanez, M., Espinoza, A., Verdugo, I., Gonzalez, E., RUIZ-LARA, S.I.M.Ó.N. and Casaretto, J.A., 2010. The transcription factor SlAREB1 confers drought, salt stress tolerance and regulates biotic and abiotic stress-related genes in tomato. *Plant, cell & environment*, *33*(12), pp.2191-2208.

Palma, J.M., Gupta, D.K. and Corpas, F.J., 2013. Metalloenzymes involved in the metabolism of reactive oxygen species and heavy metal stress. In *Heavy Metal Stress in Plants* (pp. 1-17). Springer, Berlin, Heidelberg.

Pardo, J., Wai, C.M., Chay, H., Madden, C.F., Hilhorst, H.W., Farrant, J.M. and VanBuren, R., 2019. Intertwined signatures of desiccation and drought tolerance in grasses. *bioRxiv*, p.662379.

Park, J.B., 2001. Concurrent measurement of promoter activity and transfection efficiency using a new reporter vector containing both *Photinus pyralis* and *Renilla reniformis* luciferase genes. *Analytical biochemistry*. *291*:162-166.

Peirats-Llobet, M., Han, S.K., Gonzalez-Guzman, M., Jeong, C.W., Rodriguez, L., Belda-Palazon, B., Wagner, D. and Rodriguez, P.L., 2016. A direct link between abscisic acid sensing and the chromatin-remodeling ATPase BRAHMA via core ABA signaling pathway components. *Molecular plant*, *9*(1), pp.136-147.

Pelletier, J.M., Kwong, R.W., Park, S., Le, B.H., Baden, R., Cagliari, A., Hashimoto, M., Munoz, M.D., Fischer, R.L., Goldberg, R.B. and Harada, J.J., 2017. LEC1 sequentially regulates the transcription of genes involved in diverse developmental processes during seed development. *Proceedings of the National Academy of Sciences*, *114*(32), pp.E6710-E6719.

Pollard, T.D., 2010. A guide to simple and informative binding assays. *Molecular biology of the cell*, *21*(23), pp.4061-4067.

Proctor, M.C. and Pence, V.C., 2002. Vegetative tissues: bryophytes, vascular resurrection plants and vegetative propagules. *Desiccation and plant survival. Wallingford: CABI Publishing*, pp.207-237.Qin et al., 2007)

Pronk, J.T., 2002. Auxotrophic yeast strains in fundamental and applied research. *Appl. Environ. Microbiol.*, *68*(5), pp.2095-2100.

Prywes, R. and Zhu, H., 1992. In vitro squelching of activated transcription by serum response factor: evidence for a common coactivator used by muliple transcriptional activators. *Nucleic acids research*, *20*(3), pp.513-520.

Reece-Hoyes, J.S. and Walhout, A.M., 2012a. Yeast one-hybrid assays: a historical and technical perspective. *Methods*, *57*(4), pp.441-447.

Reece-Hoyes, J.S. and Walhout, A.J., 2012b. Gene-centered yeast one-hybrid assays. In *Two Hybrid Technologies* (pp. 189-208). Humana Press, Totowa, NJ.

Rhee, S.Y., Parker, J.E. and Mockler, T.C., 2016. A glimpse into the future of genome-enabled plant biology from the shores of Cold Spring Harbor.

Rio, D.C., Ares, M., Hannon, G.J. and Nilsen, T.W., 2010. Purification of RNA by SDS solubilization and phenol extraction. *Cold Spring Harbor Protocols*, *2010*(6), pp.pdb-prot5438.

Rodriguez, M.C.S., Edsgärd, D., Hussain, S.S., Alquezar, D., Rasmussen, M., Gilbert, T., Nielsen, B.H., Bartels, D. and Mundy, J., 2010. Transcriptomes of the desiccation-tolerant resurrection plant Craterostigma plantagineum. *The Plant Journal*, *63*(2), pp.212-228.

Roscoe, T.T., Guilleminot, J., Bessoule, J.J., Berger, F. and Devic, M., 2015. Complementation of seed maturation phenotypes by ectopic expression of ABSCISIC ACID INSENSITIVE3, FUSCA3 and LEAFY COTYLEDON2 in Arabidopsis. *Plant and Cell Physiology*, *56*(6), pp.1215-1228.

Roychoudhury, A., Paul, S. and Basu, S., 2013. Cross-talk between abscisic acid-dependent and abscisic acid-independent pathways during abiotic stress. *Plant cell reports*, *32*(7), pp.985-1006.

Sadowski, I. and Ptashne, M., 1989. A vector for expressing GAL4 (1-147) fusions in mammalian cells. *Nucleic Acids Research*, *17*(18), p.7539

Sano, T. and Nagata, T., 2002. The possible involvement of a phosphate-induced transcription factor encoded by Phi-2 gene from tobacco in ABA-signaling pathways. *Plant and cell physiology*, *43*(1), pp.12-20.

Santos-Mendoza, M., Dubreucq, B., Baud, S., Parcy, F., Caboche, M. and Lepiniec, L., 2008. Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. *The Plant Journal*, *54*(4), pp.608-620.

Schagat, T., Paguio, A. and Kopish, K., 2007. Normalizing genetic reporter assays: approaches and considerations for increasing consistency and statistical significance. *Cell Notes*, *17*, pp.9-12.

Schenborn, E. and Groskreutz, D., 1999. Reporter gene vectors and assays. *Molecular biotechnology*, *13*(1), pp.29-44.

Schlebusch, S.A., 2018. *Events that shape genomes* (Doctoral dissertation, Faculty of Science, University of Cape Town).

Schmidt, K., Pflugmacher, M., Klages, S., Mäser, A., Mock, A. and Stahl, D.J., 2008. Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus Cercospora beticola supports the role of ABA as a repressor of plant defence in sugar beet. *Molecular plant pathology*, *9*(5), pp.661-673.

Schoonheim, P.J., Veiga, H., da Costa Pereira, D., Friso, G., van Wijk, K.J. and de Boer, A.H., 2007. A comprehensive analysis of the 14-3-3 interactome in barley leaves using a complementary proteomics and two-hybrid approach. *Plant Physiology*, *143*(2), pp.670-683.

Seeley, R.J., Yagaloff, K.A., Fisher, S.L., Burn, P., Thiele, T.E., van Dijk, G., Baskin, D.G. and Schwartz, M.W., 1997. Melanocortin receptors in leptin effects. *Nature*, *390*(6658), p.349.

Sheen, J., 2001. Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiology*, *127*(4), pp.1466-1475.

Shen, A.Y.H., 2014. *The transcriptome response of leaves of the resurrection plant, Xerophyta humilis to desiccation* (Doctoral dissertation, University of Cape Town).

Shen-Miller, J., Mudgett, M.B., Schopf, J.W., Clarke, S. and Berger, R., 1995. Exceptional seed longevity and robust growth: ancient sacred lotus from China. *American Journal of Botany*, *82*(11), pp.1367-1380.

Sherf, B.A., Navarro, S.L., Hannah, R.R. and Wood, K.V., 1996. Dual-luciferase reporter assay: an advanced co-reporter technology integrating firefly and Renilla luciferase assays. *Promega Notes*, *57*(2).

Shifera, A.S. and Hardin, J.A., 2010. Factors modulating expression of Renilla luciferase from control plasmids used in luciferase reporter gene assays. *Analytical biochemistry*, *396*(2), p.167.

Shinozaki, K. and Yamaguchi-Shinozaki, K., 2000. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current opinion in plant biology*, *3*(3), pp.217-223.

Sims III, R.J., Liss, A.S. and Gottlieb, P.D., 2003. Normalization of luciferase reporter assays under conditions that alter internal controls. *Biotechniques*, *34*(5), pp.938-940.

Sirichandra, C., Gu, D., Hu, H.C., Davanture, M., Lee, S., Djaoui, M., Valot, B., Zivy, M., Leung, J., Merlot, S. and Kwak, J.M., 2009. Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1 protein kinase. *Febs Letters*, *583*(18), pp.2982-2986.

Skubacz, A., Daszkowska-Golec, A. and Szarejko, I., 2016. The role and regulation of ABI5 (ABA-Insensitive 5) in plant development, abiotic stress responses and phytohormone crosstalk. *Frontiers in plant science*, *7*, p.1884.

Smith, C. 2013, Stable vs. Transient Transfection of Eukaryotic Cells, *Biocompare,* viewed 23 August 2019, <https://www.biocompare.com/editorial-articles/126324-transfection>.

Stege, J.T., Guan, X., Ho, T., Beachy, R.N. and Barbas III, C.F., 2002. Controlling gene expression in plants using synthetic zinc finger transcription factors. *The Plant Journal*, *32*(6), pp.1077-1086.

Swaminathan, K., Peterson, K. and Jack, T., 2008. The plant B3 superfamily. *Trends in plant science*, *13*(12), pp.647-655.

Takai, R., Kaneda, T., Isogai, A., Takayama, S. and Che, F.S., 2007. A new method of defense response analysis using a transient expression system in rice protoplasts. *Bioscience, biotechnology, and biochemistry*, *71*(2), pp.590-593.

Tan, B.C., Joseph, L.M., Deng, W.T., Liu, L., Li, Q.B., Cline, K. and McCarty, D.R., 2003. Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. *The Plant Journal*, *35*(1), pp.44-56.

To, A., Valon, C., Savino, G., Guilleminot, J., Devic, M., Giraudat, J. and Parcy, F., 2006. A network of local and redundant gene regulation governs Arabidopsis seed maturation. *The Plant Cell*, *18*(7), pp.1642-1651.

Tompa, P., Szász, C. and Buday, L., 2005. Structural disorder throws new light on moonlighting. *Trends in biochemical sciences*, *30*(9), pp.484-489.

Traven, A., Jelicic, B. and Sopta, M., 2006. Yeast Gal4: a transcriptional paradigm revisited. *EMBO reports*, *7*(5), pp.496-499.

Umezawa, T., Sugiyama, N., Takahashi, F., Anderson, J.C., Ishihama, Y., Peck, S.C. and Shinozaki, K., 2013. Genetics and phosphoproteomics reveal a protein phosphorylation network in the abscisic acid signaling pathway in Arabidopsis thaliana. *Science signaling*, *6*(270), pp.rs8-rs8.

VanBuren, R., Bryant, D., Edger, P.P., Tang, H., Burgess, D., Challabathula, D., Spittle, K., Hall, R., Gu, J., Lyons, E. and Freeling, M., 2015. Single-molecule sequencing of the desiccation-tolerant grass Oropetium thomaeum. *Nature*, *527*(7579), p.508.

VanBuren, R., Wai, C.M., Zhang, Q., Song, X., Edger, P.P., Bryant, D., Michael, T.P., Mockler, T.C. and Bartels, D., 2017. Seed desiccation mechanisms co-opted for vegetative desiccation in the resurrection grass Oropetium thomaeum

VanBuren, R., Wai, C.M., Pardo, J., Giarola, V., Ambrosini, S., Song, X. and Bartels, D., 2018a. Desiccation tolerance evolved through gene duplication and network rewiring in Lindernia. *The Plant Cell*, *30*(12), pp.2943-2958.

VanBuren, R., Pardo, J., Wai, C.M., Evans, S. and Bartels, D., 2019. Massive tandem proliferation of ELIPs supports convergent evolution of desiccation tolerance across land plants. *Plant physiology*, *179*(3), pp.1040-1049.

Vesuna, F., Winnard Jr, P. and Raman, V., 2005. Enhanced green fluorescent protein as an alternative control reporter to Renilla luciferase. *Analytical biochemistry*, *342*(2), p.345.

Walker, W.E., Porteous, D.J. and Boyd, A.C., 2004. The effects of plasmid copy number and sequence context upon transfection efficiency. *Journal of controlled release*, *94*(1), pp.245-252.

Wang, Y., Liu, K., Bi, D., Zhou, S. and Shao, J., 2017. Characterization of the transcriptome and EST-SSR development in *Boea clarkeana*, a desiccation-tolerant plant endemic to China. *PeerJ*, *5*, p.e3422.

Wani, S.H., Kumar, V., Shriram, V. and Sah, S.K., 2016. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal*, *4*(3), pp.162-176.

Wehner, N., Hartmann, L., Ehlert, A., Böttner, S., Oñate-Sánchez, L. and Dröge-Laser, W., 2011. High-throughput protoplast transactivation (PTA) system for the analysis of Arabidopsis transcription factor function. *The Plant Journal*, *68*(3), pp.560-569.

West, M.A., Yee, K.M., Danao, J., Zimmerman, J.L., Fischer, R.L., Goldberg, R.B. and Harada, J.J., 1994. LEAFY COTYLEDON1 is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. *The Plant Cell*, *6*(12), pp.1731-1745.

Wodniok, S., Brinkmann, H., Glöckner, G., Heidel, A.J., Philippe, H., Melkonian, M. and Becker, B., 2011. Origin of land plants: do conjugating green algae hold the key?. *BMC Evolutionary Biology*, *11*(1), p.104.

Xiang, Y., Tang, N., Du, H., Ye, H. and Xiong, L., 2008. Characterization of OsbZIP23 as a key player of bZIP transcription factor family for conferring ABA sensitivity and salinity and drought tolerance in rice. *Plant Physiology*.

Xiao, L., Yang, G., Zhang, L., Yang, X., Zhao, S., Ji, Z., Zhou, Q., Hu, M., Wang, Y., Chen, M. and Xu, Y., 2015. The resurrection genome of Boea hygrometrica: A blueprint for survival of dehydration. *Proceedings of the National Academy of Sciences*, *112*(18), pp.5833-5837.

Yamaguchi-Shinozaki, K. and Shinozaki, K., 1994. A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *The Plant Cell*, *6*(2), pp.251-264.

Yamamoto, A., Kagaya, Y., Toyoshima, R., Kagaya, M., Takeda, S. and Hattori, T., 2009. Arabidopsis NF-YB subunits LEC1 and LEC1-LIKE activate transcription by interacting with seed-specific ABRE-binding factors. *The Plant Journal*, *58*(5), pp.843-856.

Yang, H., Saitou, T., Komeda, Y., Harada, H. and Kamada, H., 1997. Arabidopsis thaliana ECP63 encoding a LEA protein is located in chromosome 4. *Gene*, *184*(1), pp.82-88.

Yan, F., Deng, W., Wang, X., Yang, C. and Li, Z., 2012. Maize (Zea mays L.) homologue of ABA-insensitive (ABI) 5 gene plays a negative regulatory role in abiotic stresses response. *Plant growth regulation*, *68*(3), pp.383-393.

Yin, W., Xiang, P. and Li, Q., 2005. Investigations of the effect of DNA size in transient transfection assay using dual luciferase system. *Analytical biochemistry*, *346*(2), pp.289-294.

Yobi, A., Schlauch, K.A., Tillett, R.L., Yim, W.C., Espinoza, C., Wone, B.W., Cushman, J.C. and Oliver, M.J., 2017. Sporobolus stapfianus: insights into desiccation tolerance in the resurrection grasses from linking transcriptomics to metabolomics. *BMC plant biology*, *17*(1), p.67.

Yoo, S.D., Cho, Y.H. and Sheen, J., 2007. A. thaliana mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature protocols*, *2*(7), p.1565

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2010. AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *The Plant Journal*, *61*(4), pp.672-685.

Yoshida, T., Mogami, J. and Yamaguchi-Shinozaki, K., 2014. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Current opinion in plant biology*, *21*, pp.133-139.

Yoshida, T., Fujita, Y., Maruyama, K., Mogami, J., Todaka, D., Shinozaki, K. and Yamaguchi-Shinozaki, K.A.Z.U.K.O., 2015. Four A rabidopsis AREB/ABF transcription factors function predominantly in gene expression downstream of SnRK2 kinases in abscisic acid signalling in response to osmotic stress. *Plant, cell & environment*, *38*(1), pp.35-49.

Yu, B.P., 1994. Cellular defenses against damage from reactive oxygen species. *Physiological reviews*, *74*(1), pp.139-162.

Yu, C., Wang, L., Chen, C., He, C., Hu, J., Zhu, Y. and Huang, W., 2014. Protoplast: a more efficient system to study nucleo-cytoplasmic interactions. *Biochemical and biophysical research communications*, *450*(4), pp.1575-1580.

Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., Wang, P., Li, Y., Liu, B., Feng, D. and Wang, J., 2011. A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant methods*, *7*(1), p.30.

Zhang, W., Abdelrahman, M., Jiu, S., Guan, L., Han, J., Zheng, T., Jia, H., Song, C., Fang, J. and Wang, C., 2019. VvmiR160s/VvARFs interaction and their spatio-temporal expression/cleavage products during GA-induced grape parthenocarpy. *BMC plant biology*, *19*(1), p.111.

Supplementary data

Solution	Reagents	Final concentration
10x MOPS buffer (pH 7)	MOPS	200mM
	Sodium acetate	50mM
	EDTA	10mM
RNA extraction buffer (pH 8)	TRIS HCl	50mM
	Lithium chloride	150mM
	EDTA	5mM
	SDS	1%
High salt precipitation buffer (HSPB)	Sodium Citrate	800mM
	Sodium Chloride	1.2M
RNA loading buffer	Formaldehyde	22% (v/v)
	Formamide	64% (v/v)
	10x MOPS	13% (v/v)
	Ethidium bromide	1% (v/v)
RNA denaturing agarose gel	Agarose	1%
	10x MOPS	9%
	Formaldehyde	16%

Supplementary Table 1: Solutions used for RNA extraction and analysis. All solutions were made with DEPC-treated water.

Solution	Reagents	Final concentration
DNA extraction buffer (pH 8)	TRIS HCl	100mM
	Sodium chloride	500mM
	EDTA	50mM
	β-mercaptoethanol	10mM
	SDS	1.25%
Tris-EDTA buffer (pH 8)	TRIS HCl	10mM
	EDTA	1mM

Supplementary Table 2: Solutions used for gDNA extraction. All solutions were made using sterile Milli-Q purified water.

Name	Sequence (5' to 3')	T_M (°C) excl. RE	T_M (°C) incl. RE	5' flanking RE site	Amplicon length (bp)
XhABFA for	GCGCC**GGTACC**AAGATGAATTCGAAAACTAACATGC	52.4	64.5	KpnI	1081
XhABFA rev	AAATAT**GCGGCCG**CTGAATTTCAGTAACAGGAAGATG	50.8	63.9	NotI	
XhPER1 for	GCGCC**GGTACC**AGAGAAAATAAACAGGATAAACCAC	54.1	64	KpnI	322
XhPER1 rev	AAATAT**GCGGCCG**CTGCTTAAGAAGGCGAGAGAG	54.1	66.7	NotI	
XhECP63 for	GCGCC**GGTACC**GACTTGTGCGTCGATATAGTATG	53	66.2	KpnI	368
XhECP63 rev	AAATAT**GCGGCCG**CGATGAACTCTCGTAATCTGCTC	52.8	66	NotI	
XhDSI-1VOC for	GCGCC**GGTACC**GAGCATAAAAATCAGCTCCTCAG	53.7	66.9	KpnI	352
XhDSI-1VOC rev	AAATAT**GCGGCCG**CGAATCTAGCGAATAAAGTTGCAGC	54.5	66.4	NotI	
XhAHL23 for	GCGCC**GGTACC**AGCTGCATGAATCTGTTGC	53.7	67.7	KpnI	371
XhAHL23 rev	AAATAT**GCGGCCG**CTAAAGGAGAGGTTGAGGAGC	53.7	66.6	NotI	

Supplementary Table 3: A summary of the primers used for cloning into pENTR1A. Sequences, melting temperatures (T_M) and the amplicon length associated with each primer are specified. Forward and reverse primers indicated by (f) and (r) respectively. Restriction enzyme sites are in bold.

Name	Sequence (5' to 3')	T_M (°C) excl. RE	T_M (°C) incl. RE	5' flanking RE site	Amplicon length (kb)
XhPER1 for	AATA**GAGCTC**GAAAATAAACAGGATAAACCAC	47.6	57.2	SacI	316
XhPER1 rev	AATA**TCTAGA**TGCTTAAGAAGGCGAGAGAG	54.1	57.3	XbaI	
XhECP63 for	AATA**GAGCTC**ACTTGTGCGTCGATATAGTATG	52.1	59.1	SacI	362
XhECP63 rev	AATA**TCTAGA**GATGAACTCTCGTAATCTGCTC	52.8	57	XbaI	
XhDSI-1VOC for	AATA**GAATTC**AGCATAAAAATCAGCTCCTCAG	52.9	57.3	EcoRI	346
XhDSI-1VOC rev	AATA**GAGCTC**GAATCTAGCGAATAAAGTTGC	49.4	58.4	SacI	
XhAHL23 for	AATA**GAATTC**AGCTGCATGAATCTGTTGC	53.7	58	EcoRI	368
XhAHL23 rev	AACG**CCCGGG**TTTAAAGGAGAGGTTGAG	45.8	63.8	SmaI	

Supplementary Table 4: A summary of all primer pairs used for cloning into pHISi-1. Primer sequences, melting temperatures (T_M), restriction enzymes and the amplicon length associated with each primer are specified. Restriction enzyme sites are in bold.

Name	Sequence (5' to 3')	T_M (°C) excl. RE	T_M (°C) incl. RE	5' flanking RE site	Amplicon length (kb)
XhPER1 for	AGAC**GGTACC**GAAAATAAACAGGATAAACCAC	47.6	59.9	KpnI	314
XhPER1 rev	AGAC**CTCGAG**CTTAAGAAGGCGAGAGAG	49.2	62.4	XhoI	
XhECP63 for	ATAC**AAGCT**TACTTGTGCGTCGATATAGTATG	51.9	57.9	HindIII	362
XhECP63 rev	ATAA**GGTACC**ATGAACTCTCGTAATCTGCTC	42.9	59	KpnI	
XhDSI-1VOC for	ATAC**GGTACC**AGCATAAAAATCAGCTCCTC	50.2	59.9	KpnI	344
XhDSI-1VOC rev	ATCT**GAATTC**ATCTAGCGAATAAAGTTGCAGC	54.4	59	EcoRI	
XhAHL23 for	ATAC**AAGCTT**AGCTGCATGAATCTGTTGC	53.7	59.4	HindIII	366
XhAHL23 rev	ATAC**GAATTC**TAAAGGAGAGGTTGAGGAGC	53.7	59.3	EcoRI	

Supplementary Table 5: A summary of all primer pairs used for cloning into pLacZi. Primer sequences, melting temperatures (T_M), restriction enzymes and the amplicon length associated with each primer are specified. Restriction enzyme sites are in bold.

Name	Sequence (5' to 3')	T_M (°C) excl. RE	T_M (°C) incl. RE	5' flanking RE site	Amplicon length (kb)
XhABFA for	AATAT**CCCGGG**GAAGATGAATTCGAAAACTAACATGC	53.2	62.4	SmaI	1078
XhABFA rev	GCGCC**AGATCT**TGAATTTCAGTAACAGGAAGATG	50.8	61.8	BglII	

Supplementary Table 6: A summary of all primer pairs used for cloning into pGAD424. Primer sequences, melting temperatures (T_M), restriction enzymes and the amplicon length associated with each primer are specified. Restriction enzyme sites are in bold.

Cycle	Duration	Temperature (°C)	Number of cycles
Initial denaturation	2 mins	95	1
Denaturation	40 secs	95	5
Annealing	30 secs	50	
Extension	2 mins 40 secs	72	
Denaturation	40 secs	95	25
Annealing	30 secs	58	
Extension	2 mins 40 secs	72	
Final extension	5 mins	72	1

Supplementary Table 7: PCR conditions for *XhABFA* amplification. After the initial denaturation, the annealing temperature of the first 5 cycles corresponded to the T_m of the *XhABFA* primers excluding restriction enzyme sites. The annealing temperature of the next 25 cycles corresponded to the T_m of the primers when restriction enzyme sites are included.

Cycle	Duration	Temperature (°C)	Number of cycles
Initial denaturation	2 mins	95	1
Denaturation	40 secs	95	5
Annealing	30 secs	52	
Extension	1 min 20 secs	72	
Denaturation	40 secs	95	25
Annealing	30 secs	60	
Extension	1 min 20 secs	72	
Final extension	5 mins	72	1

Supplementary Table 8: PCR conditions for amplification of *XhPER1*, *XhDSI-1VOC*, *XhECP63* and *XhAHL23* promoters. After the initial denaturation, the annealing temperature of the first 5 cycles corresponded to the T_m of the promoter primers excluding restriction enzyme sites. The annealing temperature of the next 25 cycles corresponded to the T_m of the primers when restriction enzyme sites are included.

Vector	Restriction enzymes
pENTR1A	KpnI and NotI
pGWL7	EcoRI
pBS-35S-Ala-LucR	KpnI and NotI
pHISi	EcoRI and BamHI
pLacZi	EcoRI and BamHI
pHISi-1	PvuII and BamHI
pGAD424	PvuII and BamHI
p53BLUE	EcoRI and BamHI
p53HIS	EcoRI and BamHI
pGAD53m	EcoRI and BamHI

Supplementary Table 9: Restriction enzyme diagnostic of the vectors used in this study. In order to confirm their identity, the vectors were digested with certain restriction enzymes.

Solution	Reagents	Final concentration
10 x TE buffer (pH 7.5)	Tris-HCl	100 mM
	EDTA	10 mM
10 x LiAc (pH 7.5)	Lithium acetate	1000 mM
PEG/LiAc	Polyethylene glycol 4000 (50 %)	40%
	10 x TE buffer	1 x
	10 x LiAc	1 x

Supplementary Table 10: Solutions used for yeast transformations. All solutions were made using sterile Milli-Q purified water.

Solution	Reagents	Final concentration
Z buffer (pH 7)	Sodium phosphate dibasic heptahydrate	60 mM
	Sodium phosphate monobasic monohydrate	40 mM
	Potassium chloride	10 mM
	Magnesium sulfate heptahydrate	1 mM
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in DMF	20 mg/ml
Z buffer / X-gal	Z buffer	98%
	β-mercaptoethanol	0.4%
	X-gal	1.6%

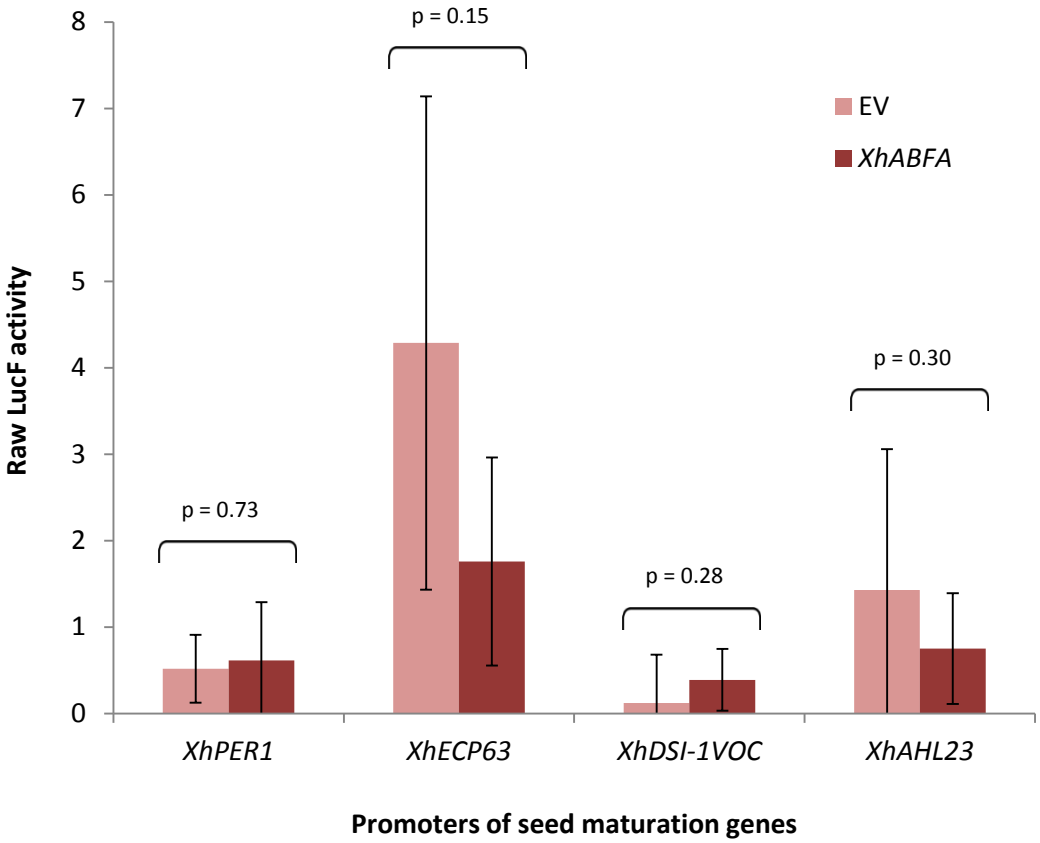
Supplementary Table 11: Colony-lift filter assays. All solutions were made using sterile Milli-Q purified water.

Solution	Reagents	Final concentration
Enzymes	MES pH5.7	20mM
	Cellulase R10	1.5% (wt/vol)
	Macerozyme R10	0.4% (wt/vol)
	Mannitol	400mM
	KCl	20mM
	$CaCl_2$	10mM
	BSA	0.1%
W1	MES pH5.7	4mM
	Mannitol	500mM
	KCl	20mM
W5	MES pH5.7	2mM
	NaCl	154mM
	$CaCl_2$	125mM
	KCl	5mM
MMG	MES pH5.7	4mM
	Mannitol	400mM
	$MgCl_2$	15mM
PEG–calcium	PEG4000	40% (wt/vol)
	Mannitol	200mM
	$CaCl_2$	100mM

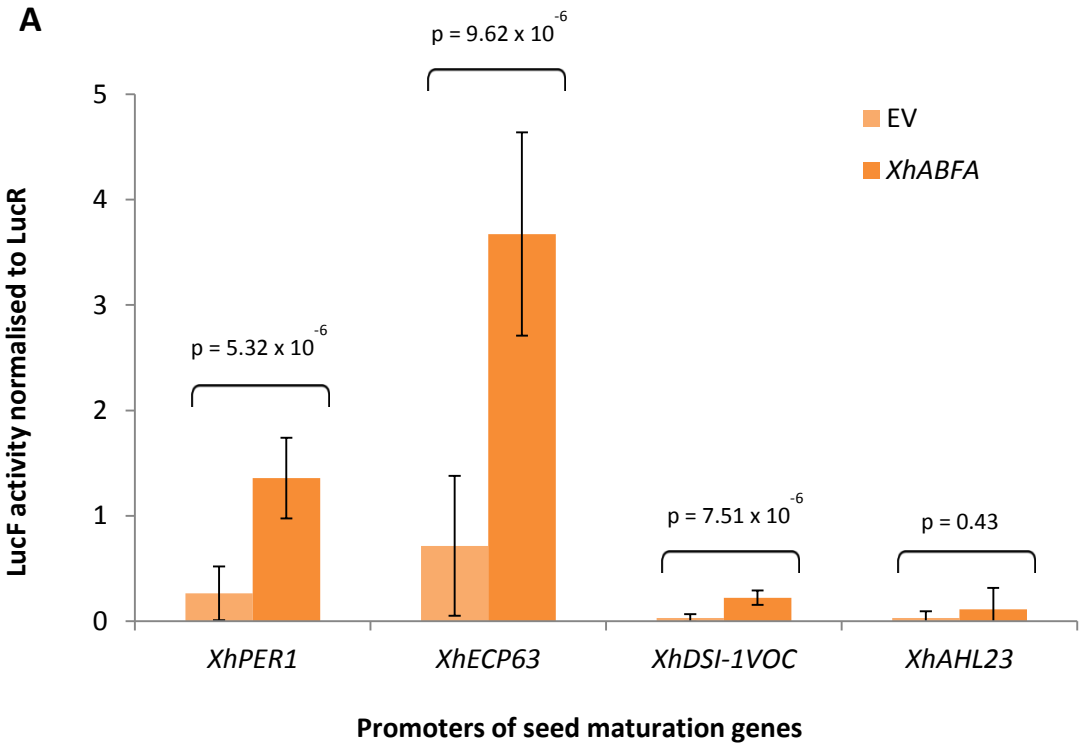
Supplementary Table 12: Solutions used for protoplast isolation and transfection. All solutions were made using sterile Milli-Q purified water.

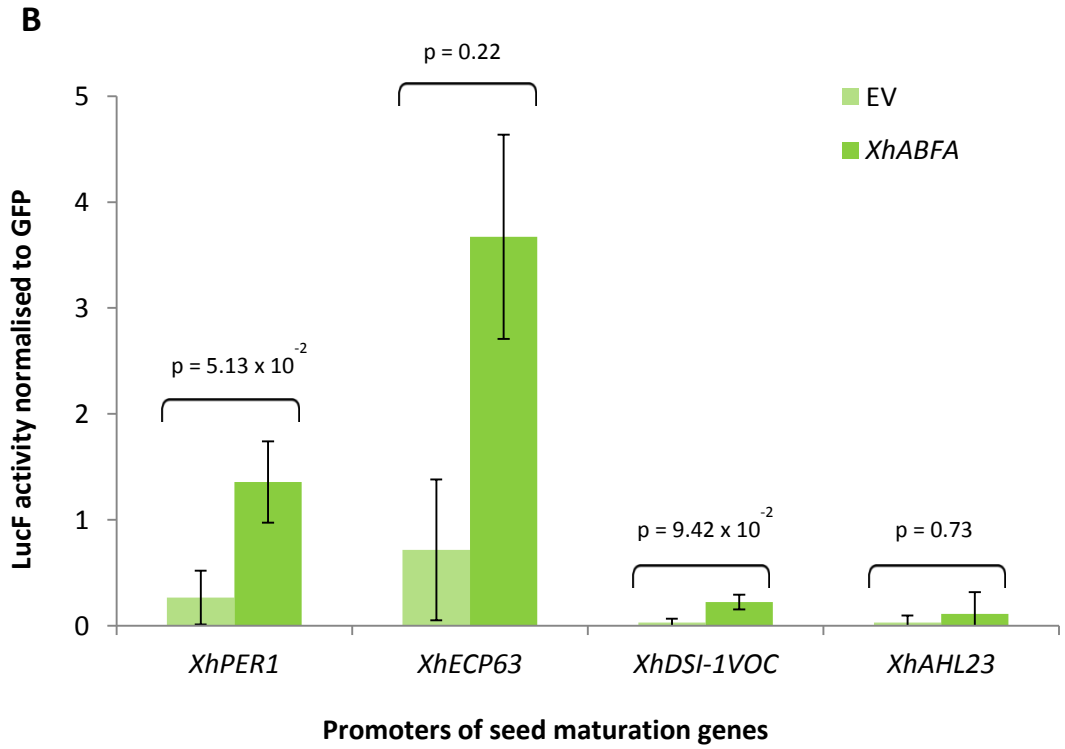
Solution	Reagents	Volume
1 x Passive Lysis Buffer (PLB)	5 x PLB	2 ml
	Distilled water	8 ml
Luciferase Assay Reagent II (LAR II)	Luciferase Assay Substrate	Lyophilized
	Luciferase Assay Buffer II	10 ml
Stop & Glo® Reagent	50 x Stop & Glo® Substrate	2.1 ml
	Stop & Glo® Buffer	105 ml

Supplementary Table 13: Solutions used in the Dual-Luciferase Reporter Assay. The reagents are supplied in the Dual-Luciferase Reporter Assay kit (Promega).

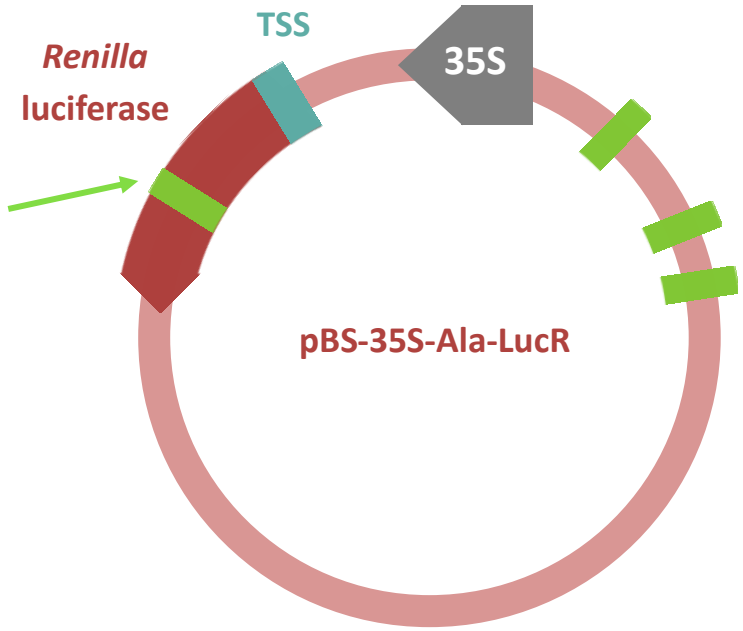


Supplementary Figure 1: Raw expression of firefly luciferase from the promoters of seed maturation genes by XhABFA transactivation. *A. thaliana* protoplasts were co-transfected with three vectors: a LucF reporter vector containing one of four *X. humilis* promoter elements; a vector constitutively expressing LucR ; and a vector which constitutively expresses both GFP and *XhABFA* or an "empty vector" which expresses GFP but lacks an effector gene. LucF activity was measured 22 hours after transfection. Values shown are raw mean LucF activities ± SD from nine biological repeats (except *XhECP63* where n = 8 and *XhAHL23* where n = 5). The p-values shown are from a two tailed t-test with Bonferroni correction (significant difference at $p < 0.01$).


A
p = 9.62 x 10-6
p = 5.32 x 10-6
p = 7.51 x 10-6
p = 0.43
EV
XhABFA
LucF activity normalised to LucR
0
1
2
3
4
5
XhPER1
XhECP63
XhDSI-1VOC
XhAHL23
Promoters of seed maturation genes


B
p = 0.22
p = 5.13 x 10-2
p = 9.42 x 10-2
p = 0.73
EV
XhABFA
LucF activity normalised to GFP
0
1
2
3
4
5
XhPER1
XhECP63
XhDSI-1VOC
XhAHL23
Promoters of seed maturation genes

Supplementary Figure 2: Expression of firefly luciferase representing the transactivation of seed maturation genes by XhABFA. *A. thaliana* protoplasts were co-transfected with three vectors: a LucF reporter vector containing one of four *X. humilis* promoter elements; a vector constitutively expressing LucR ; and a vector which constitutively expresses both GFP and *XhABFA* or an "empty vector" which expresses GFP but lacks an effector gene. LucF activity was measured 22 hours after transfection and normalised to either the (A) LucR or (B) GFP signals for each transfection. Values shown are mean LucF activities ± SD from nine biological repeats (except *XhECP63* where n = 8 and *XhAHL23* where n = 5). The p-values shown are from a two tailed t-test with Bonferroni correction (significant difference at $p < 0.01$).



Supplementary Figure 3: ABRE elements within the pBS-35S-Ala-LucR vector. The pBS-35S-Ala-LucR vector was included as a putative source to normalise LucF activity, however LucR displayed a systemic pattern of expression. It was observed that the samples containing *XhABFA* tended to have a lower LucR signal than the samples containing the EV control. A possible explanation for this is that *XhABFA* may be binding to ABRE elements (green) located within the LucR vector. The green arrow indicates the presence of an ABRE site downstream of the transcriptional start site (blue). *XhABFA* could be binding to this ABRE site and consequently interfering with the transcription of the LucR gene.