Studies into the link between plant development and abiotic stress tolerance in Arabidopsis thaliana

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Abstract

The links between plant development and responses to stress, both biotic and abiotic, is an area of major interest. With an ever increasing food production requirements, and changes in climate putting strain on a plants intrinsic ability to cope with altering conditions, the potential for modulating stress responses is a research area of great potential.

A major regulator of plant growth and development is a class of proteins called the DELLA proteins, which act as negative regulators of gibberellic acid signalling. Potential interactors of the DELLA proteins were identified as WRKYs transcription factors; a major family of plant transcription factors involved in the regulation of a wide variety of processes. Research conducted showed that WRKY transcription factors and DELLA proteins interact *in planta.* A specific target of interest, *AtWRKY26*, was shown to interact strongly with the *Arabidopsis* DELLA protein, *AtRGA*. Specific protein domains where interaction occurs were also identified via domain mutational techniques, whereby individual domains were baited against full length proteins. The function of *AtWRKY26* was further elucidated after identifying knock out mutants, highlighting decreased tolerances to heat shock in *wrky26* plants. Relative gene expression of *AtWRKY26* was also demonstrated to vary between wild type and DELLA mutants, further highlighting the interaction between these proteins. This highlights the potential for modulating protein levels to alter stress responses.

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Abbreviations

3-AT	3-Amino-1,2,4-triazole
cDNA	complementary DNA
Co-IP	Co-Immunoprecipitation assay
DEPC	diethylpyrocarbonate
dH_2O	deionised water
DMF	Dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DO	drop-out
GAL4 AD	GAL4 activation domain
GAL4 BD	GAL4 binding domain
gDNA	genomic DNA
hrp	horseradish peroxidase
kb	kilo base
LB	Luria-Bertani media
M	molar
mg	milligram
mM	millimolar
MS	Murashige & Skoog medium
ng	nanogram

PCR	polymerase chain reaction
PPIs	protein-protein interactions
RNA	ribonucleic acid
ROS	Reaction Oxygen Species
rpm	revolutions per minute
SD	synthetic defined media
SDS	sodium dodecyl sulphate
SOC	super optimal broth with catabolite repression media
TF	transcription factor
T_m	Melting Temperature
UAS	upstream activating domain
UV	ultraviolet
V	volts
Y-2H	yeast-2 hybrid
YPD	yeast peptone dextrose media
x g	gravitational force
µg	microgram
µl	microlitre
µM	micromolar

This thesis has not been submitted or accepted for any other degree.
All sections of this thesis are the result entirely of my own work.

Statement of copyright:

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CHAPTER I:

Introduction

1.1 Phytohormones and plant development overview

The publication of the full *Arabidopsis thaliana* genome (*Arabidopsis Genome Initiative, 2000*) cemented its role in the analysis of various plant developmental responses. This plant has many benefits of a model organism, such as a short life cycle and easy cultivation, and enables, those who wish, to study a wide array of questions and hypotheses. One such important question is the mechanisms behind how plant development is controlled. Another is what molecular and physiological changes are initiated when a plant is subjected to some form of stress, whether that be biotic or abiotic. How these two fundamental questions can be linked is an interesting area of research, and one that shall be addressed within the following chapters.

A complex web of integrated signals, both environmental and endogenous, controls plant development. The key regulators of these signals are plant hormones, or phytohormones, which regulate various growth and developmental responses. In altering the pattern of synthesis and distribution of these hormones, plants are able to coordinate growth, enabling their response to a diverse spectrum of developmental signals. Additionally, the responses to biotic and abiotic stresses are also under phytohormonal control, which in turn may result in altering growth patterns in order to survive and overcome perceived stresses. This is particularly important when reminded that, as sessile organisms, it is imperative for plants to be able to modulate their growth patterns in order to quickly respond to the particular environmental conditions faced.

Although varied in their specific functions, these regulatory phytohormones can be linked due to their fundamental roles in controlling plant growth and development. There have been numerous phytohormones characterised, such as ethylene, auxin and abscisic acid (ABA), which have roles in fruit ripening, cell cycle control and

maintaining seed dormancy respectively (*Gray, 2004*). However, it is important to note, that although general functions can be assigned, quite often there is cross-talk between phytohormonal functions. In essence, a specific output often cannot be assigned to one particular hormone; development and defence responses result from combinations of hormonal actions (*Weiss & Ori. 2007*). One particular class of hormone, Gibberellins (GA) have been well documented as major regulators of various developmental processes. The role of GA, and how GA signalling is in turn regulated, shall be discussed in further detail within the following sections.

1.2 Gibberellins (GA)

1.2.1 What are Gibberellins?

GA hormones are involved in the regulation of various aspects of plant development, including seed germination, stem and root elongation, flowering time and seed development (*MacMillan, 2002*). However, of the numerous GAs identified, it is thought that the majority do not actually function as bioactive hormones in higher plants; non-bioactive GAs co-occur with their functional cousins, and act as biosynthetic precursors or deactivated metabolites of the bioactive forms. Indeed, these non-bioactive GAs are often present in higher concentrations than the hormones themselves (*Hedden & Thomas, 2012)*. The importance of GAs, and the related regulatory elements associated with plant development, has become increasing obvious over the past few decades. Early research into the field highlighted the role of GA in determining plant stature in agricultural crops, which had immeasurable importance in ensuring food security in the 1960s. The so-called 'Green Revolution' resulted in increased grain

production via the introduction of dwarfing genes into rice and wheat populations; these higher yield crops maintained per capita food supplies, thus maintaining food demands of an ever increasing global population (*Daviere & Achard, 2013*).

Although ubiquitous in higher plants, GAs were originally identified as secondary metabolites of *Gibberella fujikuroi,* a fungus known to cause *Bakanae* in rice plants; a disease resulting in surplus GA biosynthesis. The physical ramifications of this disease were clear, whereby increased GA biosynthesis resulted in excessive growth, particularly of the vegetative structures (*Candau et al. 1992*). The fundamental issues relating to this point were the vast decreases in yield exhibited and, even more importantly, the unviability of crops, often a result of excessive GA production. With these points in mind, it is easy to see why research into GA, especially regulatory elements involved in its signalling, was of major importance, and is a key focus of research to this day.

1.2.2 GA biosynthesis

The GA name actually encompasses a large group of diterpenoid compounds; currently there are 136 GA structures identified from various sources, and are so named in the order of their discovery (GA_1 -GA_n). It is generally believed that the current list of GAs identified is incomplete, and that further examples are yet to be discovered. However, the general low abundance and complex structures of these compounds makes isolation a difficult process (*Hedden & Thomas, 2012*). As previously stated, the number of bioactive GAs is actually relatively few compared with the total numbers of GA identified; many GAs are non-bioactive, acting either as precursors or secondary metabolites. In higher plants, the numbers of GAs identified as possessing biological

activity is low; some of the common forms being GA_1, GA_3 (gibberellic acid) and GA_4 (*Hedden & Thomas, 2012*). The process of synthesising biologically active GAs is a complex one, integrating various components. The primary step, located in the plastid, is the methylerythritol phosphate pathway, whereby a hydrocarbon intermediate is formed from *trans*-geranylgeranyl diphosphate (GGDP) (*Kasahara et al. 2002*). The steps that follow, highlighted in *figure 1.1*, span the endoplasmic reticulum and terminate in the cytosol with the formation of bioactive GA. This final step is catalysed by Gibberellin 3-oxidase (GA3ox) (*Hu et al. 2008*). In Arabidopsis, GA3ox is encoded by a small gene family containing four members (as currently identified); *AtGA3ox1* *AtGA3ox4* (*Hedden et al. 2002*).

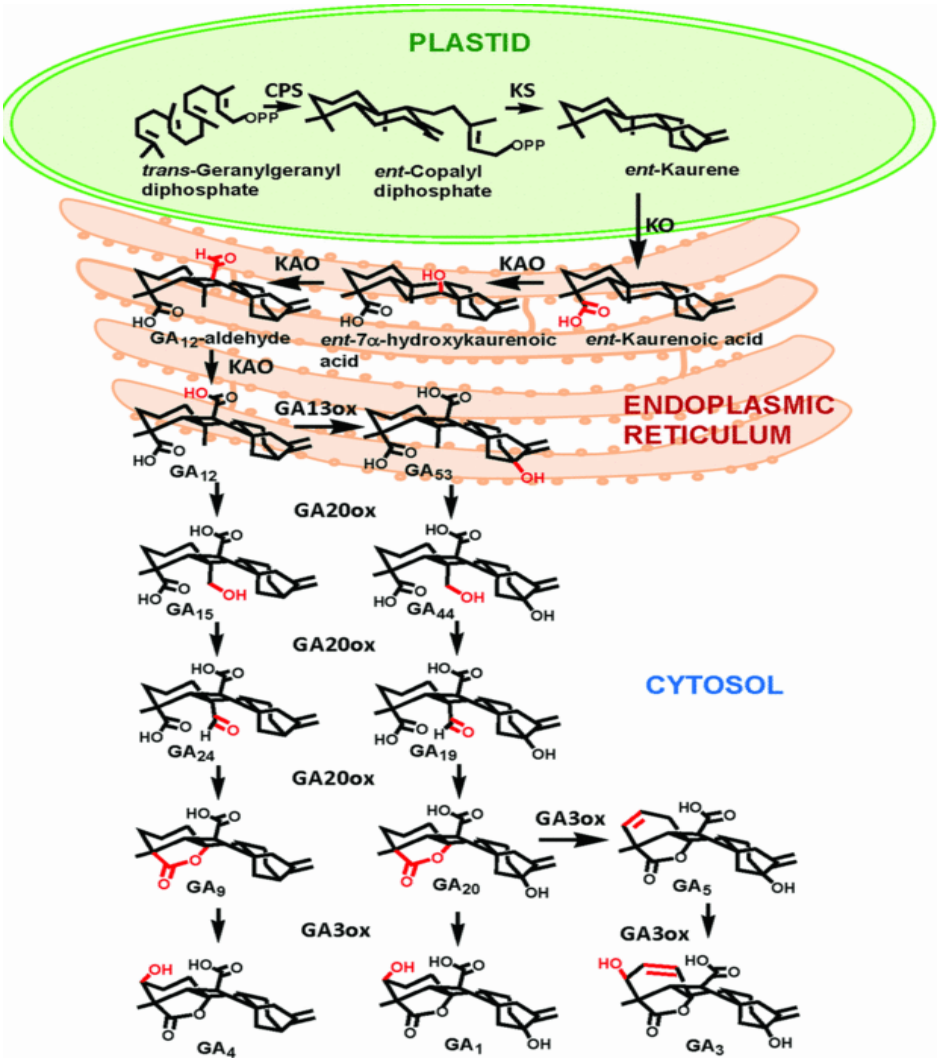


Figure 1.1: Overview of the GA biosynthetic pathway *(Hedden & Thomas, 2012)*

1.2.3 GA-dependent developmental regulation

Analysis of expression profiles, and subsequent quantification of expression levels for GA3ox genes, has enabled the function of these genes to be determined. Also, and one could argue more importantly, it has been possible to highlight in which organs bioactive GAs are being synthesised, thus elucidating the various developmental patterns exhibited. Work conducted in this field has indicated the roles of GA3ox1 and GA3ox2 in the synthesis of bioactive GAs during vegetative growth. Also, it has been shown that GA3ox1 is the only gene to be expressed in all stages of development, whereas GA3ox3 is primarily found during flower and silique development (*Mitchum et al. 2006*); indeed, GA is important in developmental processes from seed-to-seed. Additionally, it is important for accurate balancing of GA concentrations for optimal growth and development; plants with impaired GA biosynthesis exhibit atypical phenotypes, showing low fertility and dwarfism. Conversely overexpression of GA results in excessive growth, specifically taller stems, and increased sterility due to defects in pollen development (*Fleet & Sun, 2005*). This highlights the importance in the regulation of GA signalling, and the fine balance between the differing effects of over- and under-expression of this hormone.

Specific examples of developmental process controlled by GA are abundant, seed germination, for example is one such process. The actual progression of germination results from the amalgamation of various environmental signals, such as light, temperature and nutrient availability (*Seo et al. 2009*). Favourable conditions break ABA maintained dormancy, resulting in GA biosynthesis and hence GA-dependent development to be initiated; the antagonistic relationship between ABA and GA, and specifically the ratio between the endogenous concentrations of these two hormones, has been shown to either break or maintain dormancy. One particular

example of the alternating effects of ABA and GA in controlling seed germination is shown in the transcriptional regulation of α-amylase, an enzyme that induces starch hydrolysis. The hydrolysis of starch, and also proteins by various other hydrolytic enzymes, provides the nutrients required for developing embryos. During germination, the GA signal is perceived in the aleurone cells and α-amylase transcription is initiated. Conversely the presence of ABA inhibits the expression of this gene (*Rogers & Rogers, 1992*), and hence germination is inhibited. The actual involvement of GA in the stimulation of seed germination has been experimentally verified using both GA and ABA mutants. One such example, *ga1-3* null mutants, lack an enzyme required for an early stage of GA biosynthesis and are non-germinating until GA is exogenously supplied (*Ogawa et al. 2003*).

Additionally GA signalling has been shown to be involved in normal floral development where the expression of floral homeotic genes, AP3, PI and AG, are up-regulated upon GA signalling. The actual process behind this appears to be in the GA-dependent suppression of a class of transcriptional regulators, the DELLA proteins, which are involved in regulating GA signalling (*Yu et al. 2004*).

The role of GA signalling in abiotic stress tolerances has also been identified as an important mechanism modulated to withstand such conditions. Mutant lines, with either reduced or inhibited GA signalling, such as the *ga1-3* biosynthetic mutants, have been shown to exhibit increased survival rates, compared to wild type plants, when faced with osmotic stress (*Achard et al. 2006*). Additionally, when faced with cold stress, GA levels decrease. This reduction has been quantified via expression profiles of certain GA metabolic enzymes, such as GA2ox3 and GA2ox6, which are up-regulated in response to cold (*Achard et al. 2008*). This attenuation of the GA signal results in an accumulation of DELLA proteins, which act to negatively regulate the GA signal. The

role that GA plays in controlling plant development is inherently linked to the role that DELLAs play in the regulation of the GA signal; the specific function these proteins play in modulating GA signalling shall be discussed in greater detail within the following sections.

1.3 DELLA proteins negatively regulate GA signalling

1.3.1 What are DELLA proteins?

DELLA proteins are conserved repressors of GA signalling (*Griffiths et al. 2006)* and are subfamily members of the larger GRAS family of transcriptional regulators, of which seven other subfamilies have currently been identified. GRAS proteins show variation in their N-terminals but are highly homologous in their C-terminals (*Pysh et al. 1999*). Over thirty *Arabidopsis* GRAS proteins have currently been identified (*Lee et al. 2008*) and have been implicated in the regulation of various signalling cascades, such as root and shoot development, as well as phytochrome and GA signalling (*Bolle, 2004*).

The DELLA proteins are nuclear localised and are highly conserved within different plant species, including *Oryza spp.* (rice), *Triticum spp.* (wheat) and *Arabidopsis*. There are five DELLAs encoded by the *Arabidopsis* genome; GA-INSENEITIVE (GAI), REPRESSOR OF GAI-3 (RGA), RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2) and RGA-LIKE3 (RGL3), and each of these genes carries out varying, but somewhat overlapping, roles within the repression of GA signalling responses (*Peng et al. 1997*). A phylogenetic tree highlighting the evolutionary relationships between the *Arabidopsis* DELLAs can be seen in *figure 1.2.* The individuals most closely related are represented in the three clades on the diagram, with all proteins related to a single common ancestor.

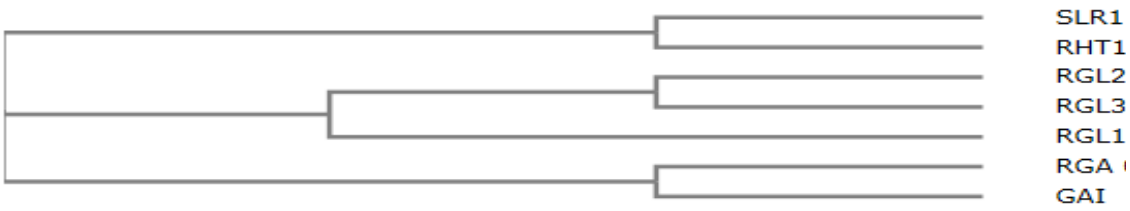


Figure 1.2: Phylogenetic tree depicting the relationship between the Arabidopsis, wheat (RHT1) & rice (SLR1) DELLA proteins. *The DELLAs are highly conserved proteins and can be grouped into distinct clades.*

Produced using the ClustalW2 sequence alignment tool, with protein sequences obtained from TAIR

The only currently DELLAs identified in rice and wheat are SLR1 and RHT respectively, and these function to repress all GA signalling responses (*Fleet & Sun, 2005*). One point of particular interest is the fact that DELLA proteins lack a typical DNA-binding domain (*Zentella et al. 2007*), thus implicating the role of additional transcription factors to aid in controlling the expression of target genes.

All members of the DELLA family have an N-terminal DELLA domain, which contains a conserved 27 amino acid motif, the first five amino acids of which gives (aspartic acid, glutamic acid, leucine, leucine, alanine) this class of proteins their name. The DELLA motif is required for the inactivation of these proteins via GA, which when endogenous concentrations are increased, acts to suppress the inhibitory mechanisms of the DELLAs. It is therefore necessary for a functional DELLA motif for GA signalling to be regulated (*Dill et al. 2001*). The alignment of the five *Arabidopsis* DELLAs, as well as the homology of the DELLA domain, can be seen in *figure 1.3*. The high sequence homology of the DELLAs is highlighted here; an asterisk represents identical amino acids at a particular position, whereas semicolons and full stops represent conserved and semi-conserved replacements respectively.

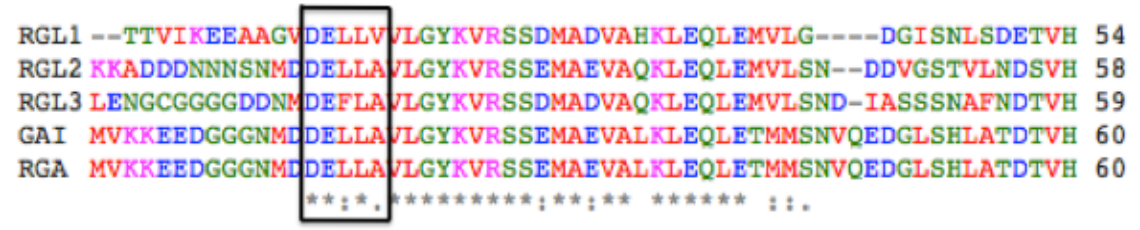


Figure 1.3: Sequence alignment of the five Arabidopsis DELLA proteins. *The sequences shown here highlights the conservation seen in the DELLA motif, found within the DELLA domain. The box depicts the amino acid sequence whereby the protein family name if derived.*

The second domain found within the DELLAs is the C-terminal GRAS domain, which has various roles in the regulation and dimerisation of these proteins (*Bolle, 2004*). Further subdivisions of the GRAS domain can be analysed due to the five distinct sequence motifs found within the domain, namely LHRI, VHIID, LHRII, PFYRE and SAW as shown in *figure 1.4*. Like the DELLA domain, again there is a high level of conservation in these sequence motifs (*Pysc et al. 1999*). The function of these two protein domains shall be addressed in greater detail within the following chapter.

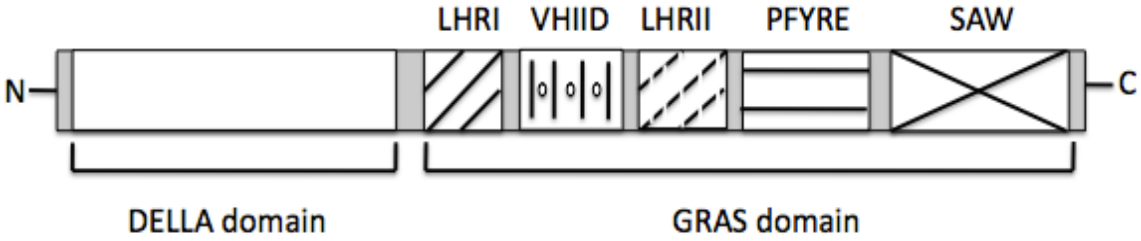


Figure 1.4: Schematic of a generic DELLA protein. *DELLA proteins have two domains, the DELLA and the GRAS domains. The GRAS domain can be subdivided into five distinct sequence motifs, of which each has varied functions.*

1.3.2 DELLA proteins modulate GA signalling

DELLA proteins act immediately downstream of the GA receptor to inhibit various GA-induced growth and developmental responses (*Griffiths et al. 2006*). The role of DELLA proteins in the negative regulation of GA responses has various important

implications; the wild type of the wheat dwarfing genes (*Rht-b1* and *Rht-d1*), utilised in the 'green revolution' to produce shorter crops with increased yield, have since been isolated to encode DELLA proteins, orthologues of the GAI gene in *Arabidopsis*. Point mutations within the DELLA domain caused the introduction of stop codons within the gene, resulting in truncated proteins lacking a functional DELLA domain. Without this domain, DELLA proteins accumulate as they lack the signal, the DELLA motif, which targets their degradation after cellular GA concentrations are increased. These plants are insensitive to exogenously supplied GA and hence are constitutive repressors of GA-regulated growth (*Peng et al. 1999*); dwarf cultivars such as these are less likely to topple in adverse weather conditions and have increased yields compared to wild type plants (*Daviere & Achard, 2013*). The phenotypes exhibited in some of the various dwarfing alleles are shown in *figure 1.5*.

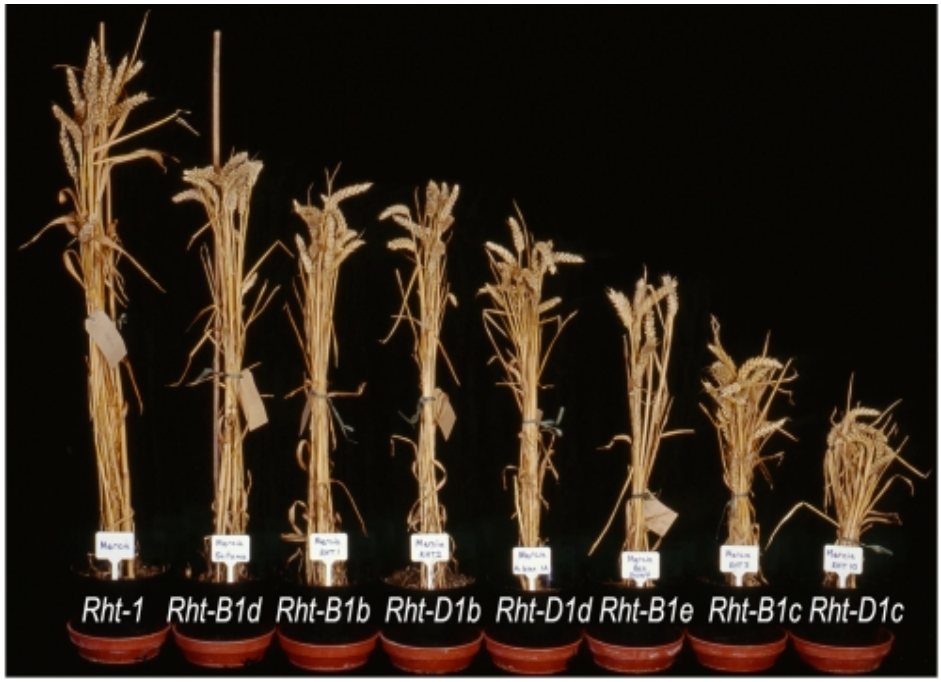


Figure 1.5: Phenotypic variations exhibited in Triticum aestivum. *The introduction of mutations into the protein sequence of the DELLA motif results in the various phenotypes seen in these wheat plants (John Innes Centre archives).*

Specific examples of plants lacking a functional DELLA motif have experimentally proven the importance of this sequence; transgenic Arabidopsis containing mutations within the *AtRGA* DELLA motif result in semi-dwarf phenotypes, which cannot be rescued even when exogenous GA is supplied. This phenotype indicates constitutive repression of GA signalling is a result of this mutation (*Dill et al. 2001*). This work was a continuation of similar studies on *AtGAI* mutants, which possessed a seventeen amino acid deletion within the DELLA motif of the *AtGAI* protein. These mutants, *gai-1*, similarly had a semi-dwarf phenotype and were unresponsive to treatment with GA (*Peng et al. 1997*).

The final size specific plant organs are able to reach is dependent on both the rate of growth, and the duration that growth is maintained. In part this is controlled by the extent of favourable environmental signals received, which in turn modulate internal signalling cascades. At the cellular level, the balance between both cell proliferation and cell expansion determines the rates of growth exhibited, whereby mitotic activity supplies the cells required for subsequent targeted expansion (*Lee et al. 2012*). The role GA plays within both cell expansion and division has been identified within the root meristem, to name one example, whereby GA signalling stimulates the destruction of the DELLAs, thus lifting the inhibitory effects of these proteins (*Ubeda-Tomas et al. 2009*). However, although the role of GA in stimulating the destruction of DELLAs has been well documented, the role that DELLAs play in inhibiting GA-dependent growth had been a somewhat illusive question. Recent advances however have elucidated certain inhibitory mechanisms utilised when cellular DELLA protein concentrations are high, such as the accumulation of certain cell cycle inhibitors, SIAMESE (SIM) and kip-related protein 2 (KPN2). Increased expression of these genes reduces both cell proliferation and expansion (*Achard et al. 2009*), thus inhibiting GA-dependent growth.

Work on *Arabidopsis* DELLAs, in particular *AtRGA* and *AtGAI*, have shown the importance of these proteins in regulating GA- stimulated vegetative growth, such as root growth (*Fu & Harberd, 2003*) and stem elongation (*King et al. 2001*). Additionally these, and similar, studies have shown that *rga gai* double knockouts can counter the vegetative deformities seen in *ga1-3* mutants. However, *AtRGA* and *AtGAI* seemingly have little significant effect in controlling seed germination or flower development. Numerous experimental techniques have been utilised showing this to be the case; *gai* and *rga* mutants cannot rescue the non-germinating phenotype of *ga1-3*. An additional *Arabidopsis* DELLA, *AtRGL2*, has been identified as the major regulator of GA signalling during seed germination (*Tyler et al. 2004*). Transcriptional profiles of RGL2 indicate that gene expression increases following seed imbibition (the uptake of water into the seed), but decreases rapidly as germination progresses. Additionally, *rgl2* null mutants can counter the *ga1-3* phenotype, which usually require GA supplementation for germination to occur (*Lee et al. 2002*). These two points highlight the specific importance of RGL2 in controlling seed germination. Nevertheless, it has been shown that the remaining DELLAs do play minor regulatory roles; analysis of various combinations of null alleles for each *Arabidopsis* DELLA has highlighted the crossover between these genes during regulation of plant development (*Zentella et al. 2007*).

In addition to controlling GA-dependant development, it has also been shown that DELLAs are involved in regulating the metabolism of ABA. The analysis of DELLA targets involved in early GA signalling has shown that DELLA-induced up-regulation of XERICO (a RING E3 protein) gene expression in turn promotes ABA accumulation. ABA, which acts antagonistically with GA, inhibits seed germination (germination is stimulated by GA). Furthermore, ABA has been implicated in the

inhibition of GA signalling downstream of DELLA responses (*Zentella et al. 2007*), thus further highlighting hormonal cross-talk.

1.3.3 Regulation of DELLA proteins

As previously stated, a functional N-terminal DELLA motif is required for the repression of GA signalling; Arabidopsis *AtRGA* and *AtGAI*, which share 82% sequence homology, encode transcriptional regulators that repress GA signalling. It has been demonstrated that *rga* and *gai* mutants, containing amino acid deletions within the DELLA motif, result in dwarf phenotypes in transgenic *Arabidopsis.* Additionally, supplementing the plant with GA has no effect, and constitutive repression of GA signalling occurs. A quantitative measurement of hypocotyl length is one such method utilised to experimentally demonstrate the inability to initiate GA-induced degradation of *AtRGA* and *AtGAI* when a functional DELLA domain is lacking. Both *gai* and *rga* DELLA motif mutants show greatly reduced final heights, additionally there is no statistically significant difference in hypocotyl length, whether GA is supplemented or is not (*Dill et al. 2001*).

The specific role of GA in initiating the degradation of DELLAs has been elucidated, in part, via quantifying the percentage of RGA protein degradation in *ga1-3* mutants, where initial basal GA levels are low. The use of *ga1-3,* rather than wild-type backgrounds, enables the responses between exogenously supplied GA, and the pre-existing endogenous GA levels, to be distinguished. In one such study, supplementing *ga1-3* with GA for 30 minutes was sufficient to render the RGA protein undetectable, thus indicating its degradation (*Zentella et al. 2007*). Additionally, such studies have aimed to identity early GA signalling responses, such as specific changes in gene

expression levels, when exogenous GA is supplied to GA-biosynthetic mutants. Such attempts are of great benefit in trying to elucidate how GA and DELLA proteins co-regulate plant development.

Although the actual signal that initiates increases in GA production has not currently been identified, the pathways that result from its biosynthesis have been described. Before the identification of a receptor for GA, early research suggested that GA-dependant phosphorylation was required for DELLA degradation via interaction with F-box proteins (component of the E3 ligase SCF complex, required for protein ubiquitination) (*Fu et al. 2002*). However, mechanisms behind GA perception and subsequent signalling were greatly elucidated via the discovery of both DELLA and the GA receptor, GID1 (GA INSENSITIVE DWARF1). Following GA binding to GID1, a conformational change to the N-terminal extension of the receptor results in a favourable binding environment, allowing an association between GID1 and DELLA; this forms the GA-GID1-DELLA complex (*Sun, 2010*). A simplified signalling pathway can be seen in *figure 1.6*. Following GA-GID1 binding to the DELLA protein, a coil - to-helix transition of the tertiary structure of the protein is induced in the DELLA domain. This transitional change probably causes conformational alterations within the C-terminal GRAS domain, thus enhancing recognition by the Ubiquitin E3 ligase SCF complex (SLY1 in *Arabidopsis*; GID2 in rice) (*Murase et al. 2008*). Ubiquitin is highly conserved within eukaryotes and serves as a tag for protein degradation via the 26S proteasome (*Hochstrasser, 2000*); degradation of DELLAs results in their inhibitory effects to be lifted, and GA-dependent developmental responses to be 'switched on'.

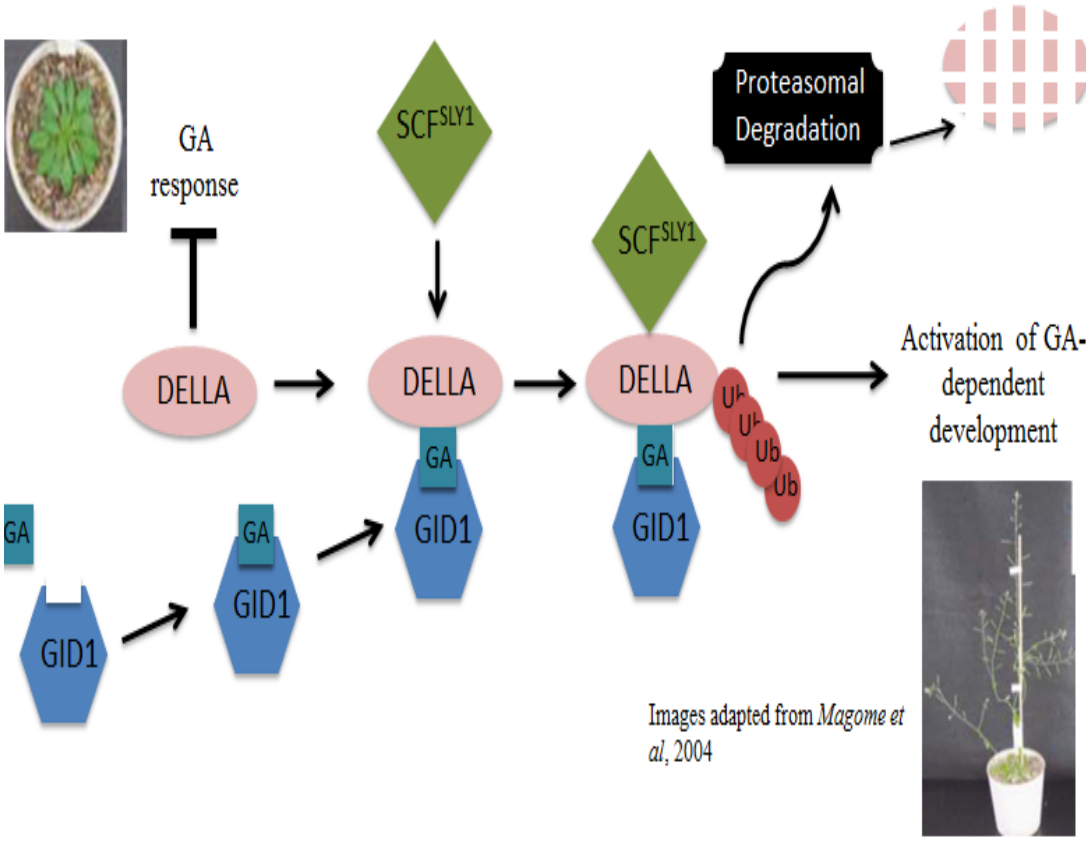


Figure 1.6: Overview of GA-dependent DELLA protein degradation. *GA binding to its receptor, GID1, enables the subsequent binding to DELLA. This forms the GA:GID1:DELLA complex, which recruits the SCF complex, resulting in the poly-ubiquitination of DELLA and hence its degradation via the 26S proteasome.*

Work on GID1 mutants in rice has highlighted the importance of a functional receptor for GA-induced DELLA degradation; a GA-insensitive phenotype is exhibited in rice *gid1* plants, furthermore, SLR1 (the rice DELLA protein) is not degraded in *gid1* mutants (*Ueguchi-Tanaka et al. 2005*). Additionally, the importance of ubiquitination in the degradation of DELLAs has been shown via further investigation into the SCF complexes, specifically the F-box proteins SLY1 and GID2 (found in *Arabidopsis* and rice respectively), which, via yeast two- hybrid and pull-down assays, have been shown to interact direct with DELLA proteins. Further study shows that *sly1* and *gid2* mutants display GA-unresponsive and dwarf phenotypes (*Sasaki et al. 2003*).

1.4 WRKY Transcription Factors

1.4.1 Structure and classification of WRKY transcription factors

An interesting question posed in relation to DELLA regulation is what additional interacting partners are associated with these proteins, and what role these partners may possess. One particular class of transcription factors, the WRKY transcription factors (WRKY TF), have subsequently been identified as proteins of interest when looking for additional targets of the DELLAs. The WRKY TF have become increasingly well characterised since their initial identification (*Ishiguro & Nakamura, 1994*) as members of the WRKY-GCM1 super family of zinc finger transcription factors (*Babu et al. 2006*). Although their presence has been implicated in various additional eukaryotic organisms (*Pan et al. 2009*), they remain a major family of plant transcriptional regulators, with seventy-four members currently identified in *Arabidopsis* alone (*Ulker & Somssich, 2004*).

The name 'WRKY' relates to the conserved WRKYGQK motif, found at the N-termini of the highly conserved 60 amino acid WRKY DNA-binding domain. Another feature of the WRKY domain is the novel zinc-finger motif characterised at the C-termini (*Rushton et al. 1995*). All known WRKY proteins contain either one or two of these DNA-binding domains and hence were originally classified into two groups based on this fact; those with two WRKY domains were found in class I, whereas those with one were found in class II. The two domains found in class I WRKY TF have been shown to be functionally distinct; while a role for the C-terminal domain has been characterised as being the main regulator of DNA binding, the role of the N-terminal domain remains somewhat unclear. Additionally, since their initial classification, a

further class, class III WRKY TF, has been distinguished from the other two classes; although there also is just a single DNA-binding domain, a atypical zinc-finger motif is exhibited. Ubiquitous in higher plants, all currently studied species contain various members of each of the three classes (*Eulgem et al. 2000*), and have been shown to be involved in the transcriptional regulation of various processes, further details of which shall be discussed later.

The DNA-binding ability of the WRKYs is controlled by the WRKY domain which has specific binding affinity for the *cis-acting* element containing the sequence 5'-TTGACC/T-3', which has been aptly named the 'W box'. The conservation of the WRKY domains correlates with the observed conservation of W box elements, of which various experimental techniques have shown that the minimal consensus sequence required for DNA binding is the presence of this W box (*Rushton et al. 1996*). However, to add additional levels of regulation, a recent study on DNA-binding selectivity of various WRKYs has shown that although the core W box is required, neighbouring nucleotide sequences also play a role in determining binding sites (*Ciolkowski et al. 2008*)

1.4.2 WRKY function in planta

Research into identifying plant responses under the control of the WRKY TF has implicated their roles in regulating numerous processes. This stretches from various developmental responses, to modulating the response to both biotic and abiotic stresses. One example is the control of seed development by WRKY TF, which has been demonstrated in various plant species. *Arabidopsis* gene expression profiles have highlighted *AtWRKY10* as a regulator in various stages of seed development.

Additionally, homozygous knockouts for this gene results in reduced seed development (*Luo et al. 2005*). Studies into both seed dormancy and germination have similarly highlighted that WRKY TF are involved in this regulation, which in turn can be linked to the roles of both GA and ABA. As previously mentioned, α-amylase expression is induced upon the GA signal perceived in the aleurone cells, which in turn provides nutrients for the developing embryo via the hydrolysis of starch (*Rogers & Rogers, 1992)*. Early studies indicated that two wild oat WRKYs, ABF1 and ABF2, were able to bind to W box of the α-amylase gene (*Rushton et al. 1995*), thus promoting its expression. A more recent study in rice has shown that different WRKY TF, *OsWRKY51* and *OsWRKY71,* repress α-amylase expression and are key regulators in modulating the responses to GA and ABA signalling (*Zhang et al. 2004*).

There has been much evidence that elucidates the regulatory role of WRKYs relating to various seed developmental and regulatory processes. However, there is a current lack of clarity in the roles that WRKYs play in any additional developmental processes; one exception to this however is the regulation of senescence. Expression profiling has revealed that, in *Arabidopsis*, WRKY TF are the second largest family of transcription factors that regulate cellular senescence (*Guo et al. 2004*). Early evidence into WRKY TF involved in senescence implicated *AtWRKY6* as a key regulator, which is highly up-regulated during senescence (*Robatzek & Somssich, 2002*). Other *At*WRKYs have similarly been implicated; overexpression of *AtWRKY53* results in a senescent phenotype (*Miao et al. 2004*), whereas *AtWRKY70* knockout lines have identified this WRKY as a negative regulator of senescence (*Ulker et al. 2007*).

Although developmental regulation by WRKYs has been in certain areas well studied, a major area of research is the role of WRKY TF in response to biotic stresses,

with a particular emphasis on defence signalling. Numerous WRKY TF have been implicated in plant defence, however, functional redundancy has proven a major obstacle with attempts to identify specific members involved in these regulatory pathways (*Ulker et al 2004*). Nevertheless, certain members can be causally linked to transcriptional reprogramming resulting from pathogen attack. *AtWRKY70*, for example, which as previously mentioned is involved in senescence, has also been shown to mediate the signalling between two additional phytohormones; Jasmonic acid (JA) and Salicylic acid (SA), in response to bacterial pathogen attack (*Li et al. 2006*). Another example is *AtWRKY33,* which has a pivotal role in defences against the necrotic fungus *Botrytis cinerea (Birkenbihl et al. 2012*). The induction of *AtWRKY33* is controlled via its phosphorylation by MPK4 and MPK6, two mitogen-activated protein kinases, and results in phytoalexin production, which is essential for defence against pathogen infection (*Mao et al. 2011*). Additional members in *Arabidopsis,* such as *AtWRKY18*, *AtWRKY40* and *AtWRKY60,* have similarly been shown to have roles in pathogen resistance; however reports have indicated some functional redundancy (*Xu et al. 2006*). A crossover of WRKY function between various species has also been demonstrated; *AtWRKY72* and its orthologues in various plant species have been shown to be involved in basal immunity against root-knot nematodes (RKN). Additionally, *WRKY72*-type transcription factors, found in tomato, has been shown to contribute to basal defence against the bacterial pathogen *Pseudomonas syringae*. This function however has not been isolated in *Arabidopsis* (*Bhattarai al. 2010*). A so-called 'WRKY web' has been defined, and in containing both positive and negative transcriptional regulators (*Eulgem & Somssich, 2007*), results in the modulation of various defence signalling pathways.

One final area for discussion is the regulation of abiotic stress responses via WRKY TF, a field which has received a great deal of emphasis, although arguably less

than that of the biotic stress responses. Various studies have shown WRKY TF playing a role in regulation of abiotic stress, with expression profiling implicating WRKYs in various signalling processes (*Chen et al. 2012*). Drought stress, which is often linked with salinity and osmotic stress as major factors limiting plant growth, are such abiotic stresses shown to be regulated via WRKY TF. One example of this, *OsWRKY11,* which, when over-expressed, enhances drought tolerances as indicated via the increased survival of vegetative structures (*Wu et al. 2009*). Additionally, the expression of two closely related WRKY TF, *AtWRKY25* and *AtWRKY33*, increases after being subjected to both drought and sodium chloride (NaCl) treatment (*Li et al. 2011*) indicating the crossover of regulatory roles within various stress responses. A recent study has also implicated *AtWRKY8* in modulating salinity stress and shows high up-regulation upon NaCl treatment (*Hu et al. 2013*). WRKY TF also participate within nutrient-deficiency signalling pathways; *AtWRKY75* is highly up-regulated upon phosphate deficiency, whereas disrupted expression of this gene enhances susceptibility to phosphate stress (*Devaiah et al. 2007*). Similarly, during reactive oxygen species (ROS) signalling, the expression of various *Arabidopsis* WRKY TF is induced after treatment with hydrogen peroxide (*Vanderauwera et al. 2005*), and WRKY TF are thought to be key mediators in the ROS signalling web (*Davletova, et al. 2005*). Finally, the role of WRKY TF in a plants ability to tolerate heat stress is an important area of research into abiotic stress responses. Indeed, any temperature that fluctuates from an optimal range is detrimental to plant development, and often results in decreased crop yields (*Chen et al. 2011*), hence much of the research from the past few decades has focused upon this area. Various WRKY TF have been implicated in responses to both high and low temperature stresses in various plant species, however much of the current literature does focus on *Arabidopsis* WRKYs. In one microarray analysis, nine out of sixty *At*WRKYs studied

were involved in the heat stress response (*Busch et al. 2004*). A recent study on *AtWRKY25*, *AtWRKY26* and *AtWRKY33* have implicated their role in the regulating resistance to heat stress, where after high temperature treatment, expression of both WRKY25 and WRKY26 was induced whereas WRKY33 expression was repressed. Combinations of single, double or triple mutants for of these three WRKYs resulted in decreased heat tolerances, whereas over-expression lines had enhanced resistance. Additionally, the expression of various heat-responsive genes was altered in these mutant and over-expressing lines, implicating the somewhat redundant role of these WRKYs in the positive regulation of certain heat-responsive genes (*Li et al. 2011*).

1.5 Thesis aims

The role of WRKY TF in regulating the expression of genes involved in various developmental and defence pathways, as previously described, is widespread. However, the identification of additional interacting partners of WRKY TF is a research area that has yet to receive much attention. However, certain publications have highlighted DELLA proteins as possible WRKY TF interactors. One example of this is *AtWRKY27*, which via microarray and transcript analysis studies, has been identified as a direct target of *AtRGA*. Current hypotheses suggest that DELLA-induced At*WRKY27* may play a role in regulating GA signalling (*Zentella et al. 2007*). This is not an entirely novel idea; transiently expressed *WRKY51* and *WRKY71* act as transcriptional repressors of GA signalling in rice (*Xie et al 2006*). With this in mind, an interesting area of study is looking for additional WRKY TF that are involved in regulatory aspects

of GA-dependant development, and if the DELLA proteins play a role in the activation of any of these genes.

The initial objective of this project aimed to identify whether any chosen WRKY TF showed protein-protein interactions with DELLA proteins, specifically *AtRGA* and *AtGAI*. The identification of potential interacting partners was first assed using yeast (*Saccharomyces cerevisiae*) as a host organism. The yeast-2 hybrid (Y-2H) technique allowed for putative interaction to be identified relatively quickly, however subsequent study *in vivo* was required for such evidence to be shown *in planta*. Both DELLA and WRKY proteins were co-expressed transiently in *Nicotiana benthamiana (N.benthamiana)*, followed by co-immunoprecipitation (co-IP) assays, which further elucidated the interaction states of said proteins. The interaction between *AtWRKY26* and *AtRGA* proved to be particularly interesting, with strong interaction highlighted between these proteins. Specific interacting protein domains were also identified, again using Y-2H analysis.

Following on from the identification of *AtWRKY26* and *AtRGA* protein-protein interactions, elucidating the potential function of said interactions was the final aim of work conducted in this project. *AtWRKY26* gene knockouts were obtained from the *Arabidopsis* seed centre; NASC, and subsequent thermotolerance assays were performed on various mutant and wild type lines. The relative expression of *AtWRKY26* was also analysed, with the aim to assess how protein levels may vary between mutants and wild type.

CHAPTER II:

Materials and Methods

2.1 DNA isolation for cloning or genotyping

2.1.1 RNA extraction

Approximately 50 mg of fresh, frozen leaf tissue was homogenised, eluted with 500 µl of TRI reagent (Sigma), then centrifuged at 12,000x g for 10min at 4 °C. RNA was purified using the Direct-zol^{TM} RNA MiniPrep kit (Zymo Research) and suspended in 50µl nuclease-free water. Extracted RNA was stored at -80°C. If required RNA quantification was carried out (*section 2.3.5*)

2.1.2 cDNA synthesis

1.5 µg of RNA was DNase treated, using 1µl DNase (Promega) and 1µl 10x reaction buffer (Promega). The reaction was made to a final volume of 10 µl with DEPC H_2O and then incubated at 37 °C for 30min. The reaction was terminated using 1 µl of RQ1 stop solution (Promega) and incubated for 10min at 65 °C.

1 µl of 500 µg/ml Oligo dT and 1 µl of 10 mM dNTPs (VWR) was added to DNase treated RNA and samples heated to 65 °C for 10m. 4 µl 5x First Strand buffer (Invitrogen), 2 µl 0.1M DTT (Invitrogen) and 1µl RNaseOUT (Invitrogen) was then added and samples heated to 42 °C for 2min. 1µl of Superscript II Reverse Transcriptase (Invitrogen) was added and samples heated to 42 °C for 50min. Reactions were inactived by heating to 70 °C for 15min. Synthesised cDNA was diluted to 100 µl with nuclease-free water and stored at -20 °C. If required, DNA quantification was carried out (*section 2.3.5*)

2.1.3 gDNA extraction

One Arabidopsis leaf (any age) was homogenised in a sterile 1.5 ml eppendorf (Starlab). Tissue was eluted in 150 µl gDNA extraction buffer [200 µl 1M Tris-Cl pH8.5, 50 µl 5M NaCl, 50 µl 0.5M EDTA, 50 µl 10 % SDS, 650 µl dH_2O] and centrifuged at 17,000x g for 5min. 100 µl of supernatant was transferred to a new eppendorf and DNA precipitated using 100 µl of propan-2-ol (Fisher). Samples were left to stand for 5min at room temperature (RT) and then centrifuged at 17,000x g for 10min. Supernatant was discarded and pellet washed with 500 µl 70 % ethanol. Samples were centrifuged for 5min at 17,000x g, pellet removed and then re-centrifuged with any remaining supernatant discarded. Pellet was left to air dry to ensure evaporation of residual ethanol. gDNA was suspended in 50 µl of 10 mM Tris pH8.5 and stored at -20 °C.

2.2 DNA amplification for cloning or analysis

2.2.1 Polymerase Chain Reaction (PCR) for target sequence amplification

For amplification of specific target sequences, PCR incorporating proofreading enzymes was used (Q5®, NEB). In sterile 0.2 ml PCR tubes (Starlab) the following components were added for a standard 50 µl reaction; 10 µl Q5 reaction buffer (NEB), 1 µl 10 mM dNTPs (VWR), 2.5 µl 10 µM 5' primer^, 2.5 µl 10 µM 3' primer, 0.5 µl Q5® DNA-polymerase (NEB), 5 µl ~250 ng template cDNA and nuclease-free water to 50 µl. Reactions were cycled using a Sure Cycler 8800 (Aligent Technologies), at 98 °C for 30s, followed by 25-35 cycles of 98 °C (15s), 50-72 °C* (20s), 72 °C for time

determined by the amplicon length (40 s/kb), followed by a final of extension 2min at 72°C.

^ Gene sequences were obtained using the CDS (TAIR), with the inclusion of a 5' CACC for cloning primers. Primers not for cloning were designed using the Primer3 tool. Primers were supplied by Eurofins MWG Operon. A list can be seen in *table 2.1*.

*Optimal annealing temperatures were calculated using the NEB T_m calculator. Gradient PCR of +/- 5°C of T_m could be carried out to further optimise annealing temperature.

2.2.2 PCR for bacterial colony screening or genotyping

PCR using *Taq* DNA polymerase was used for screening transformed bacterial colonies and plant genotyping (*sections 2.4.7* and *2.1.3*). 20 µl reactions were comprised of 10 µl 2x*MyTaq* Red Mix (Bioline), 7 µl nuclease-free water, 2 µl 10 µM 5' primer, 2 µl 10 µM 3' primer and 2 µl template DNA. Reactions were cycled using a Sure Cycler 8800 (Aligent Technologies), at 95 °C for 2min, followed by 25-35 cycles of 95 °C (30s), 50-72 °C* (30s), 72 °C for time determined by the amplicon length (1 min/kb), followed by a final of extension 10min at 72 °C.

*Annealing temperatures approximated 3°C below the melting temperature (Tm) of primers. Gradient PCR of +/- 5 °C of T_m could be carried out to further optimise annealing temperature.

2.3.3 Quantitative real-time PCR (qPCR)

qPRC using SYBR Green *Taq* ReadyMix (Sigma) was used for the analysis and quantification of gene expression in a given cDNA sample. 20 µl reactions were comprised of 10 µl SYBR Green *Taq* ReadyMix, 6.7 µl nuclease-free water, 1 µl 10 µM 5' primer, 1 µl 10 µM 3' primer, 0.3 µl rox, 1 µl template DNA. Reactions were cycled using a Applied Biosystems® Real-Time PCR machine, at 95°C for 3min, followed by 30 cycles of 95 °C (20s), 60 °C (30s).

Oligoname	**Nucleotide sequence 5'→ 3'**
WRKY72_cloF	CAC CAT GGA GGT TCT TTT GAA ATT ACC C
WRKY72_cloR	GCT TTT CTC TTC CTT GTT CAC GAA
WRKY47_cloF	CAC CAT GGA AGA ACA TAT TCA AGA TCG
WRKY47_cloR	GTT TGT AGA GAA AGT GGT GCA A
RGA_cloF	CAC CAT GAA GAG AGA TCA TCA CCA ATT C
RGA_cloR	GTA CGC CGC CGT CGA GAG TTT
RGA_seq1	AAT TTG ACT CTA GCG GAA GCT CT
RGA_seq2	AAA GTT CTC GGC GTT GTG AAA
WRYK47_seq1	AAA GGA TAT GAA CCA TGA AAC T
WRYK47_seq2	TCA TCA TCC TTC TAC CAT AAC TT
WRYK72_seq1	AAA TTC TGG CTC GGA AGA AGC TT
WRYK72_seq2	TTC ACC CAC CCT TCA CTC CCC ATT A
attB_F	TTT GTA CAA AAA AGC AGG CTC C
attB_R	GCT TTT TTG TAC AAA CTT GT

act_F	TGC TGG ATT CTG GTG ATG GT
act_R	AAT TTC CCG CTC TGC TGT TG
W26_spl1F	ATG GTA ACT CAG CCT TTA CCT CAA
W26_spl1R	GAG GTA AAG GCT GAG TTA CCA T
W26_spl2F	ATG GGC TCT TTT GAT CGC C
WRKY26_spl2R	TTA TGT CTC TGT TTT TCC AA
WRKY26I_splout	AGG AGT TGT GTC TTC ATA TT
W26_309splF	CCC TCT TTC TCC TTC GCC TT
W26_309splR	GGA GAC GGC AAA ATC TGT GA
SALK_geW26	AAA CGT CCG CAA TGT GTT AT
W26_geF	AGA AGG GAA ATG GAC AAA TCA
W26_geR	AAC CCA TCA AAA AAT AGC TGA GT
della_cloF	CAC CAT GAC GAG CTT CTC GCT GTT
della_cloR	TCA AGG AGG AGG ATT AAG CTC A
gras_cloF	CAC CAT GGT TCG TTT AGT CCA CGC
gras_cloR	TTCACCAAGCGGAGGTGGTA
wrky26I_cloF	CAC CAT GTC TGA TGA CGG CTA
wrky26I_cloR	TTA ATG ATT GTG GCT TCC TTT ATA GAC
wrky26II_cloF	CAC CAT GGA CAT TCT TGA CGA TGG
wrky26II_cloR	TTA GTG TTT GTG TTT TCC TTC GTA AG

Table 2.1: Oligonucleotides used for PCR-amplification of target sequences

2.3 DNA isolation, manipulation and quantification

2.3.1 Agrose gel electrophoresis

DNA samples were mixed with blue 10x DNA loading dye (*MyTaq* Red Mix did not require loading buffer) and electrophoresed in 0.8 % agrose (Melford) 1x TAE buffer (Bio Rad) containing 1 µl ethidium bromide (Fisher) per 100 µl TAE. Fragments were run with a 1KB Plus DNA hyperladder (Bioline) between 70-100 v and visualised under UV light.

2.3.2 DNA extraction from agrose gel

Gel containing desired DNA fragment was visualised under a UV light and excised using a clean scalpel. DNA was extracted following the QIUquick gel extraction kit (Qiagen) protocol, and then eluted in nuclease-free water. Extracted DNA was stored at -20 °C.

2.3.3 Restriction digestion of plasmid DNA

Digestion of plasmid DNA was carried out using desired restriction enzymes (NEB) and appropriate buffer (NEB). Restriction enzymes were chosen by comparing gene sequence with restriction sequence; checking that enzyme will cut at the chosen site and an appropriate number of times. Standard reactions comprised of approximately 500 ng DNA, 2 µl 10x reaction buffer, 0.5 µl restriction enzyme and nuclease-free water to 20 µl. Reactions were incubated at 37 °C for a minimum of 1h. When specified, reactions were terminated via heat-inactivation at either 65 °C or 80 °C.

Digested DNA was electrophoresed (*section 2.3.1*) for isolation of desired fragment, and then extracted from agrose gel (*section 2.3.2*)

2.3.5 DNA/RNA quantification

DNA/RNA concentration was quantified using a NanoDrop 1000 (ThermoScientific).

2.3.6 Nucleotide sequencing

DNA sequencing was carried out by the Durham University Biological Sciences department using 6 µl of DNA with custom primers supplied at 3.2 pmol/ µl.

2.4 Gene Cloning and Bacterial Transformation techniques

2.4.1 TOPO® cloning of target sequences

Desired fragments containing the directional specific CACC sequence at the start the 5' primer, derived from PCR amplification (*section 2.2.1*), were cloned into the pENTR™/D-TOPO® vector (Invitrogen). 0.5-4 µl of PCR product was combined with 1 µl salt solution, 1 µl TOPO® vector and made to a final volume of 6 µl with nuclease-free water following the Invitrogen protocol. The whole reaction was incubated for 30min at RT and then transformed into chemically competent TOP10 *E.coli* cells (Invitrogen) (*section 2.4.2*).

2.4.2 Transformation of chemically competent E.coli

Defrosted *E.coli* cells were incubated on ice for 30min. The entire cloned/Gateway vector was then added, with the cells then promptly heat shocked for 30s at 42 °C without shaking. Cells were immediately returned to ice and 250 µl RT sterile SOC medium added, followed by 1h incubation at 37 °C with shaking (220 rpm). Approximately 200 µl of the culture was spread onto LB agar plates containing vector-appropriate antibiotic selection (*table 2.2*) and incubated overnight at 37 °C.

Vector/	**Antibiotic Selection**
d-TOPO	50 µg ml^{-1} Kanamycin
pDEST22	25 µg ml^{-1} Ampicillin
pDEST32	25 µg ml^{-1} Gentamicin
pEG201	50 µg ml^{-1} Kanamycin
pEG104	50 µg ml^{-1} Kanamycin

Table 2.2: Vectors and appropriate antibiotic selection

2.4.3 Conversion from entry clone to destination vector (LR reaction)*

Conversion from entry clone to Gateway® compatible destination vectors for use in various assays, such as Y-2H, was carried out via the LR reaction. 1-7 µl entry clone was combined with 1 µl destination vector and made to a final volume of 8 µl with TE buffer. 2 µl of LR clonase (Invitrogen) was added and reactions incubated at RT for 1h. Reactions were terminated using 1 µl of Proteinase K (Invitrogen) and 37 °C incubation for 10m. Reactions were then transformed into chemically competent TOP10 *E.coli (*section 2.4.2).

*For further information on the LR reaction, see *figure 3.3*

2.4.4 Transformation of chemically competent Agrobacterium tumefaciens GV3101

Agrobacterium GV3101 was transformed to contain plasmid DNA (in expression vectors) for transient expression (*section 2.5.3*) or floral dip transformation (*section 2.6.4*). Approximately 1 µg plasmid DNA was added to *Agrobacterium* cells. Cells were then incubated in ice, liquid nitrogen and at 37 °C, each for 5min. 1 ml of sterile LB media was added and cells incubated at 28 °C for 2h with shaking (220rpm). Approximately 200 µl of culture was spread onto LB agar plates supplemented with 50 µg ml^{-1} rifampicin, 25 µg ml^{-1} gentamicin (GV3101 selection) and appropriate selection antibiotics for expression vector (*table 2.2*). Plates were incubated at 28 °C for 48h.

2.4.5 Small-scale preparation of plasmid DNA (Miniprep)

Bacterial colonies were selected from LB agar plates using a sterile inoculation loop and cultured in 10 ml LB media containing appropriate antibiotics. *E.coli* cultures were incubated overnight at 37 °C with shaking (200 rpm). DNA was extracted using the QIAprep® Spin Miniprep Kit (Qiagen), eluted in 35 µl nuclease-free water and stored at -20 °C.

2.4.6 Midi scale preparation of plasmid DNA (Midiprep)

Bacterial colonies were selected from LB agar plates using a sterile inoculation loop and cultured in 50 ml LB media containing the appropriate antibiotics. *E.coli* cultures were incubated overnight at 37 °C with shaking (200 rpm). DNA was extracted using the QIAprep® Spin Midiprep Kit (Qiagen), eluted in 35 µl nuclease-free water and stored at -20 °C.

2.4.7 Preparation of bacterial colonies for PCR screening and extraction of plasmid DNA

Bacterial colonies were removed from LB agar plates using a sterile inoculation loop and diluted in 20 µl nuclease-free water. For screening, 1 µl of this sample was used as PCR template (*section 2.2.2*). The same loop was used to inoculate sterile LB media containing appropriate antibiotics for subsequent plasmid preparation after overnight incubation (*section 2.4.5*).

2.5 Protein-Protein Interaction Assays

2.5.1 Transformation of yeast strain (Saccharomyces cerevisiae) AH109 for use in the yeast-two hybrid (Y-2H) system

The Y-2H system is used to investigate the interaction between two proteins using the gateway compatible destination vectors pDEST22 and pDEST32. Genes of interest were initially transferred to pDEST22 and pDEST32 via LR reaction (*section 2.4.3*) for transformation into chemically competent *E.coli* (section 2.4.2) for subsequent plasmid preparation (*section 2.4.6*). Approximately 2 µg of plasmid was added to a sterile 1.5 ml eppendorf (Starlab) and 270 µl of transformation solution [240 µl 50% PEG 3350, 30 µl 10x TE pH 7.6, 30 µl 1M lithium acetate pH 7.6] added. Using a sterile inoculation loop, a 2 mm ball of yeast cells was removed from YPD agar plate and placed into 30 µl of water. The yeast solution was then added to the transformation solution. Cells were briefly vortexed, then heat shocked at 42 °C for 15min. Cells were centrifuged at 1,000x g for 5min, supernatant removed and cells resuspended in 500 µl YPD (Clonetech) then

left at RT for 5h. Cells were re-centrifuged at 1,000x g for 5min, supernatant removed and cells resuspended in 0.8% NaCl for overnight incubation at RT in the dark.

Following overnight incubation, cells were centrifuged at 1,000x g for 5min. 400 µl of supernatant discarded and the pellet re-suspended in remaining 0.8% NaCl solution. Cells were spread onto SD (Clonetech) selection plates supplemented with double dropout (DO) (-leucine, -tryptophan, Clonetech). Plates were incubated for 3-5 days at 30 °C until large colonies developed.

2.5.2 Y-2H interaction assay

For the interaction assay, a single yeast colony was suspended in 500 µl 0.8 % NaCl, then 5 µl spotted onto both double (SD –leucine, -tryptophan, Clonetech), and triple (SD –leucine, -tryptophan, -histidine, Clonetech) selection plates*. Triple selection plates were supplemented with 40 mg/ml X-α-Gal and 100 mM 3-AT. Plates were incubated for 3-5 days at 30 °C until large colonies developed.

*Explanation of DO selection, see *section 3.1*

2.5.3 Infiltration of Agrobacterium for infiltration into Nicotiana benthamiana (N.benthamiana)

Agrobacterium transformed with chosen vectors were cultured overnight in LB media at 28 °C with shaking (220rpm). Entire culture was spun down at 5,000x g, supernatant removed, and pellet initially re-suspended in 5 ml 10 mM $MgCl_2$. The culture was then diluted to an OD_{600} of 0.6 with 10 mM $MgCl_2$. Cultures to be co-infiltrated were mixed

and 1 µl of 200 mM acetosyringone per 1 ml of culture added, enhancing transformation efficiency. Reactions were incubated at RT for 3h.

Agrobacterium cultures were infiltrated into the underside of *N. benthamiana* leaves and left for 2-4 days before harvesting for protein extraction.

2.5.4 Protein extraction from N. benthamiana

Approximately 1 g of frozen leaf tissue was ground, then 3 ml of protein extraction buffer [500 µl 1M Tris-Cl, 1500 µl 5M NaCl, 20 µl 0.5M EDTA, 1000 µl 50% Glycerol, 100 µl 1M DTT, 100 µl 50% Triton X-100, 1x proteinase-inhibitor tablet (Roche)] and a level spatula of PVPP (approx 3 g) (Sigma) added. Homogenate was transferred to a sterile 1.5 ml eppendorf and centrifuged at 14,000 x g for 15min at 4 °C. Supernatant was then transferred to a new sterile eppendorf.

2.5.5 Co-Immunoprecipitation (Co-IP) assay

Approximately 400 µl of total cell lysate was incubated at 4 °C with 35 µl of MACS MicroBeads (Miltenyi Biotec) with desired protein tag (either α-HA, α-GFP) for 15min, inverting sample every 2min. Samples were transferred to µMACS column and then placed in the magnetic field of a µMACS Separator (Miltenyi Biotec). Samples passed through columns via gravity flow, then columns washed three times with 200 µl of protein extraction buffer. Target proteins were eluted with 80 µl elution buffer (MiltenyiBiotec), pre-heated to 98 °C (to denature protein) for SDS separation and subsequent detection via western blotting.

2.5.6 SDS-PAGE

Protein samples were loaded onto a 10 % separating sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) electrophoresis gel [3.5 ml H_20, 3.4 ml 30 % acrylamide solution (Sigma), 2.6 ml, 1.5M Tris pH 8.8, 100 µl 10% SDS, 100 µl 10% ammonium persulphate (APS, Fisher), 8 µl TEMED (Fisher)]. Input proteins were loaded with 4x SDS loading buffer. Samples were electrophoresed for approximately 3h at 80 v in 1x SDS loading buffer for full protein separation. Overnight transfer onto polyvinylidene fluoride (PVDF, Fisher) membrane was carried out in 1x transfer buffer, at 30 v with 4 °C incubation.

2.5.6 Western blotting

Membranes were blocked in Tris-buffered saline (TBS) containing 0.1 % Tween 20 (Fisher) and 5 % non-fat milk powder (TBST) for 1h, then incubated with primary antibodies [1:5,000 (α-HA) – 1:10,000 (α-GFP) dilution in TBST] for 3h at RT. Primary antibodies were removed following three x 5min washes with TBST, then membranes incubated with secondary antibodies (anti-rat IgG hrp, anti-rabbit IgG hrp, 1:10,000 dilution) for 1h at RT. Secondary antibodies were removed following three x 5min washes with TBST. The membranes were then incubated with ECL solution (ThermoScientific) for 2min for detection via chemiluminescence (blots will fluoresce when exposed to markers on secondary antibody). Membranes were then exposed in a dark room.

2.6 *Arabidopsis* growth and manipulation

2.6.1 Vapour-phase sterilisation of Arabidopsis seeds

100 ml of 13 % Sodium Hypochlorite (Fisher) and 3 ml 36 % HCl were mixed in a conical flask, seeds were added, then immediately sealed in an airtight container for overnight seed sterilisation.

2.6.2 Growth conditions

Seeds were placed on either soil or ½ x Murashige & Skoog (MS, Melford) media (pre-sterilised) and stratified for 2-3 days at 4 °C to synchronise germination. Plants were grown under long day conditions (LD 12 hours light, 12 hours dark) at a temperature of 22-24 °C in either a dedicated growth room or Sanyo growth cabinet with a photosynthesis photon flux density (PPFD) of 120nm.

2.6.3 Thermo tolerance assays

Seeds were geminated on ½ MS media (*section 2.6.2*). Following germination, plants were left to grow for 10-12 days in LD conditions. Plants were heat shocked at 45 °C for up to one hour, with time points every 5min, and then immediately returned to growth cabinet. Tolerances to heat treatment were recorded for the next 4-6 days.

2.6.4 Floral dip transformation

10ml of LB media was inoculated with *Agrobacterium* transformed with chosen constructs (*section 2.4.4*) with appropriate selection antibiotics and were incubated

overnight at 28 °C with shaking (220 rpm). 5 ml of this culture was used to inoculate 500 ml of LB media (with appropriate selection) and again incubated overnight at 28 °C with shaking (220 rpm). Entire culture was centrifuged at 14,000 x g for 10min, supernatant removed and pellet re-suspended in 1 L 5 % sucrose. 200 µl of Silwett-L77 was then added. After removing any open flowers or siliques, the aerial plant structures were dipped into the *Agrobacterium* culture for approximately 30s. Plants were left on their side for one day, then returned upright and watered for 3 weeks before being left to dry out for seed collection.

CHAPTER III:

Identification of DELLA WRKY TF interactions; Protein domain interaction analysis

3.1 Introduction

This chapter describes the background and initial identification of DELLA and ATWRKY TF proteins interacting *in vitro,* via the yeast-2 hybrid (Y-2H) system, the use of which was widespread throughout this project. Initially, protein-protein interactions (PPIs) were studied using this technique, and subsequently verified via co-immunoprecipitation (co-IP) assays. Following on from the identification of putative targets, the protein domains where PPIs were specifically occurring were investigated, again via Y-2H analysis.

The Y-2H system has been extensively utilised since its initial development (*Fields & Song, 1989*) as a major tool for the detection of PPIs, using *Saccharomyces cerevisiae* (yeast) as a host organism. The use of this system has resulted in the publication in a vast array of documented interacting partners (*Brückner et al. 2009*), with the relatively simple concepts behind the molecular basis of it design revolutionising protein-interaction analysis. The key element behind this system is the use of the GAL4 yeast transcription factor, which was originally shown to bind to the upstream reporter sequence (UAS) and hence activate gene transcription in the presence of galactose. When the N- and C-terminals of GAL4 are separated into two fragments it was identified that although the N-terminal is able to bind DNA, transcription is not initiated, with the C-terminal fragment mediating this latter function. Such observations resulted in the characterisation of two distinct GAL4 domains; the N-terminal DNA binding domain (BD) and the C-terminal transcriptional activation domain (AD). Additionally, non-covalent interactions between the fragments reconstitutes a functional GAL4 (*Keegan et al. 1986*), hence the properties of this transcription factor have been exploited to analyse PPIs. The fusion of two potential interacting proteins to the BD and AD of GAL4, forming bait and prey plasmids respectively, in turn reforms the active

transcription factor and drives reporter gene transcription if said proteins are interacting (*Fields & Song, 1989*). See *figure 3.1*.

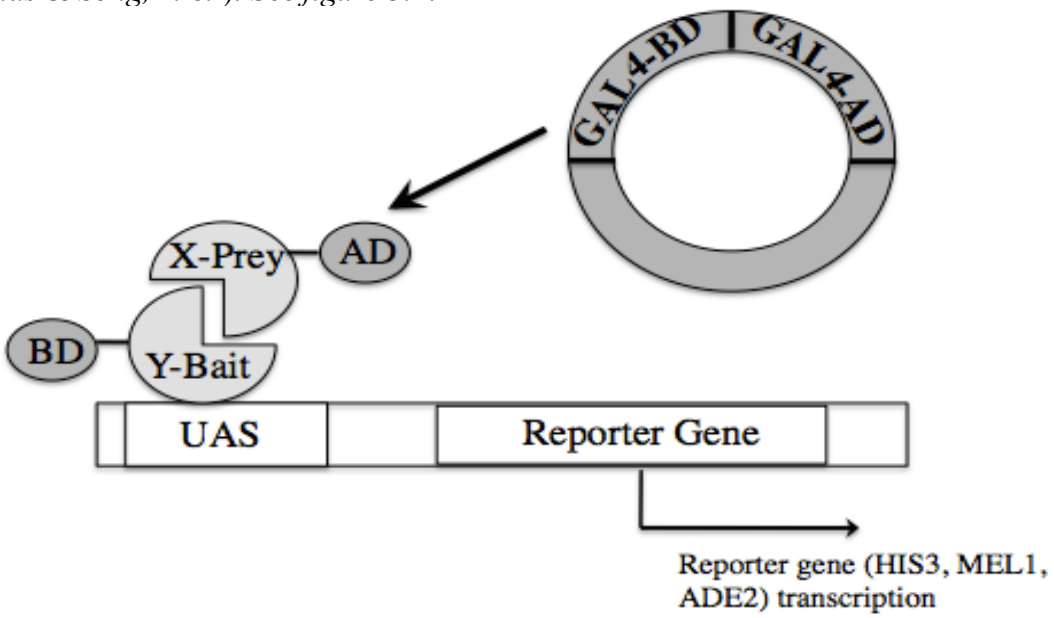


Figure 3.1: Representation of interaction between bait and prey plasmids. *Protein-protein interaction reforms the GAL4 transcription factor, bringing together the BD and AD, hence initiating the transcription of the yeast reporter genes.*

The Y-2H vectors used in this study, pDEST22 and pDEST32, were supplied by Invitrogen and hence were Gateway® compatible, enabling quick and effective cloning of genes of interest into said vectors. The formation of both bait and prey plasmids, fused in frame with the GAL4 BD and AD, were then used in the system. As shown in *figure 3.2,* both pDEST22 and pDEST32 have different means for selection. Unlike bacterial systems, which feature antibiotic resistance, nutritional markers are used for selection in yeast. TRP1 and LEU2, involved in tryptophan and leucine biosynthesis, are incorporated into pDEST22 and pDEST32 vectors respectively, with the presence of these genes then used to select for transformed yeast. When grown on double drop-out (DO) selection plates, supplemented with a nutrient mix lacking both leucine and tryptophan, only yeast cells co-transformed with both pDEST22 and pDEST32 are able to grow, as they are capable of synthesising these essential amino acids.

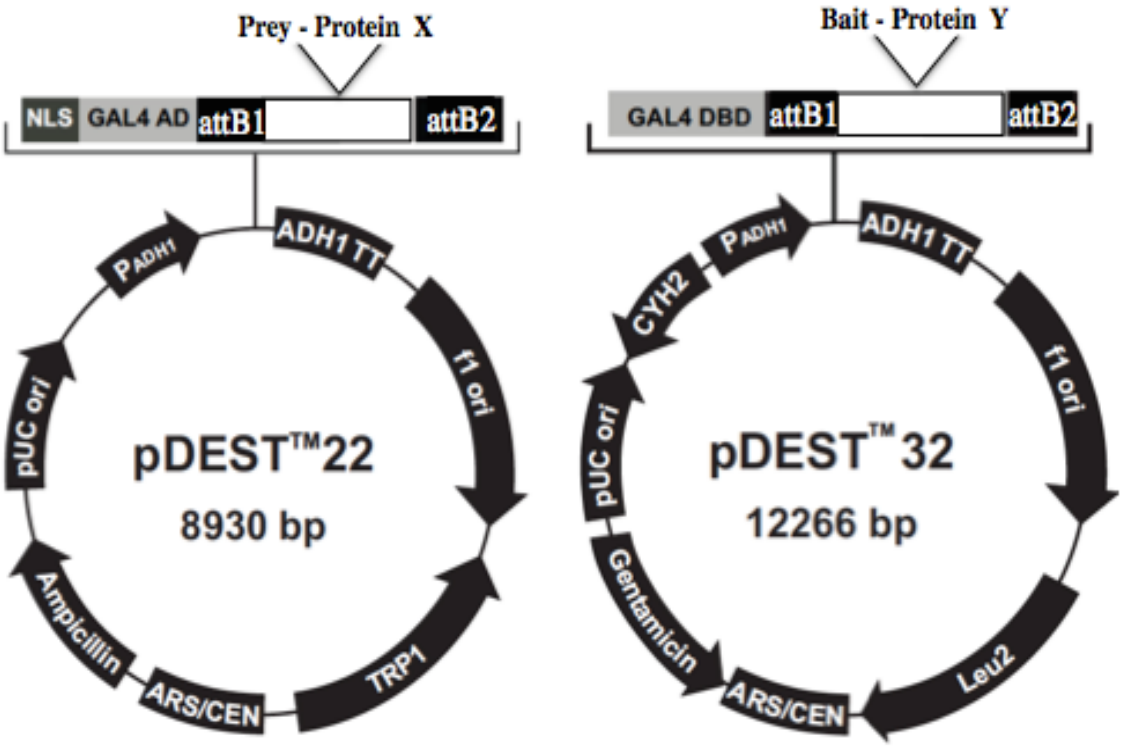


Figure 3.2: Vector maps for bait and prey pDEST22 and pDEST32 vectors used in Y-2H interaction assays. *Prey and bait proteins were sub-cloned into pDEST22 and pDEST32 in frame with the N-termini GAL4AD and GAL4BD respectively. Adapted from Invitrogen.com*

Regardless of growth in the presence of double DO selection, which is a prerequisite for subsequent interaction assays, identification of interacting proteins is dependent, in part, upon the yeast strain used. Throughout this particular study, AH109 (Clonetec), a genetically modified yeast strain, was utilised, which features three reporter genes, HIS3, MEL1 and ADE2. It is the transcription of these reporter genes, activated via GAL4, and the subsequent specific downstream responses, which enables interaction to be identified. One particular method for identification is growth on triple DO selection, which lacks histidine in addition to leucine and tryptophan. The growth of yeast on such plates indicates interaction as the transcription of the HIS3 reporter gene, required for histidine biosynthesis, in theory only occurs if the two domains of GAL4 are reconstituted to form the active transcription factor. The strength of such

interactions can be assessed via the addition of 3-Amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 reporter gene product, imidazole glycerol phosphate dehydratase (*Brückner et al. 2009*). In varying the supplemented concentrations of 3-AT, the degree to which protein interactions are occurring can be analysed. Additionally, the use of 3-AT limits the possibility of BD self-activation of the HIS3 reporter, and hence increases the probability that any interaction occurring is in fact a true result. Another means for assessing interaction is the addition of X-α-Gal into the selection media, which is used to visualise the products of MEL1 transcription. MEL1 encodes α-galactosidase, an enzyme involved in saccharide hydrolysis. The presence of this enzyme, in response to GAL4 transcriptional activation of MEL1, results in the hydrolysis of X-α-Gal, which subsequently results in the yeast colonies developing blue colouration (*Aho et al. 1997*).

This chapter describes the interactions between various AtWRKY TF and the *AtRGA* DELLA protein, as identified using the Y-2H system. Of those AtWRKYs baited against *AtRGA*, *AtWRKY26* showed strong interaction and hence proved a good candidate for further study. The interaction between *AtWRKY26* and both *AtRGA* and *AtGAI* was subsequently proven *in vivo* via co-IP assays. Following on from this, the identification of interaction between the different DELLA protein domains and *AtWRKY26* was then assessed, again using the Y-2H system to identify interaction.

3.2 Results

3.2.1 Cloning of target genes and conversion to Gateway® compatible destination vectors

Originally, preliminary screening identified two AtWRKY TF, *AtWRKY47* and *AtWRKY72*, as potential DELLA targets (*Nelis et al. unpublished data*). Due to these findings, said proteins were chosen as prey for *AtRGA*, one of the major DELLAs, in the Y-2H system. These three genes, *AtWRKY47, AtWRKY72* and *AtRGA*, were amplified using cDNA, which lacks introns, synthesised from RNA extracted from the Col-0 *Arabidopsis* ecotype. Cloning primers used for gene amplification contained a four base pair sequence, CACC, on the 5' end of the forward primer for subsequent directional cloning into the pENTR/d-TOPO® vector, part of the Gateway® cloning system (Invitrogen).

The Gateway® cloning system utilises the properties of bacteriophage lambda for site-specific recombination (*Landy, 1989*), enabling genes of interest to be effectively transferred between various expression vectors. After an initial entry clone is formed in frame with the lambda attachment sites (att sites), subsequent recombination to form expression constructs is performing via the LR reaction. This reaction, catalysed by LR Clonase™ II, results in the DNA fragment of the entry clone, flanked by attL sites, being transferred to a destination vector flanked by attB sites (*Karmi et al. 2007*) The basis of this reaction is shown in *figure 3.3*.

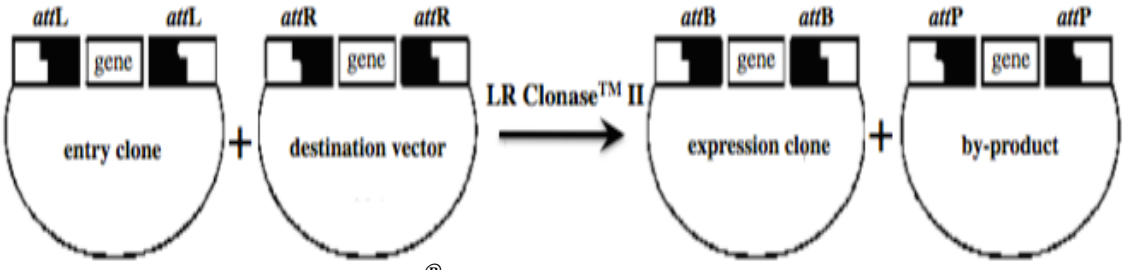


***Figure 3.3: overview of Gateway® LR reaction**. Following the LR reaction entry clones are converted to expression clones. This reaction is catalysed by LR Clonase™ II, allowing site-specific recombination of the att sites. Adapted from Invitrogen.com.*

Those AtWRKY TF (*AtWRKY47, AtWRKY72*) chosen as potential DELLA targets were transferred to pDEST22 and pDEST32, which, as previously mentioned, are vectors used for protein expression and subsequent detection of PPI using the Y-2H system. Additionally, other ATWRKY TF; *AtWRKY8, AtWRKY26, AtWRKY28, AtWRKY33, AtWRKY48*, previously cloned by another lab member, were also baited against *AtRGA*, thus increasing the likelihood of identifying interacting proteins.

3.2.2 in vitro Y-2H screening for protein-protein interactions

One issue associated with the Y-2H system is the potential for auto-activation, whereby a protein can activate reporter gene transcription, regardless of whether PPIs are occurring. The frequency of this problem has been approximated at 5% of proteins having latent activating ability. However, when generating fragments of functional proteins, such as random fragments used in Y-2H libraries, this figure is believed to be much higher (*Criekinge & Beyaert, 1999*). Additionally, it could be argued that transcription factors, which as described in their name regulate gene transcription, are a class of proteins that likely exhibit high levels of auto-activation. With this is mind, it is imperative to check for auto-activation to validate any results, which can be achieved by baiting plasmids against empty vectors. As *AtRGA* was the commonality between

assays, it made sense to test for its ability to auto-activate in both of the yeast expression vectors, pDEST22 and pDEST32. As shown in *figure 3.4, AtRGA* auto-activates in pDEST32, the vector containing the GAL4 BD. Yeast growth in histidine lacking selection, and the blue colouration of the colonies are both indicators of auto-activation, showing that HIS3 and MEL1 transcription has been initiated. Additionally, supplementation with 100 mM 3-AT, which is usually sufficient to control auto-activation, does not suppress this interaction and hence the use of *AtRGA* in pDEST32 is unsuitable as a bait clone. However, as also shown in *figure 3.4*, *AtRGA* in pDEST22 does not exhibit any auto-activation and hence is a suitable prey clone for subsequent work.

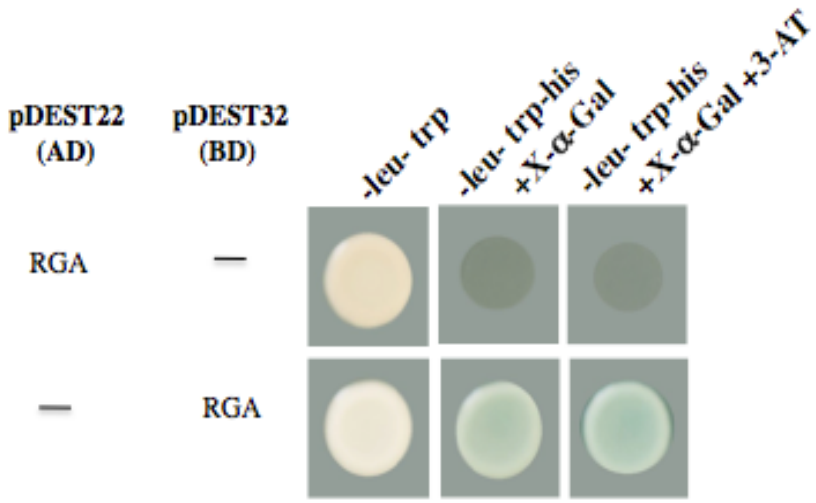


Figure 3.4: Y-2H assay testing for AtRGA auto-activation in both bait and prey (pDEST22 & pDEST32) when baited against empty opposing vector. *AtRGA auto-activates in pDEST32, but not in pDEST22.*

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT

With the identification that *AtRGA*-pDEST32 auto-activates, and hence is unsuitable as a bait clone, it was necessary for the subsequent interaction assays that the AtWRKY TF be cloned into pDEST32. Regardless, testing for AtWRKY TF auto-activation in pDEST32 was required and hence these plasmids were baited against empty pDEST22 vectors. Unfortunately, *AtWRKY8, AtWRKY28, AtWRKY33* and *AtWRKY48* in pDEST32 all auto-activated, as shown in *figure 3.5*, and it was therefore impossible to identify whether interaction with *AtRGA* was occurring using the Y-2H system.

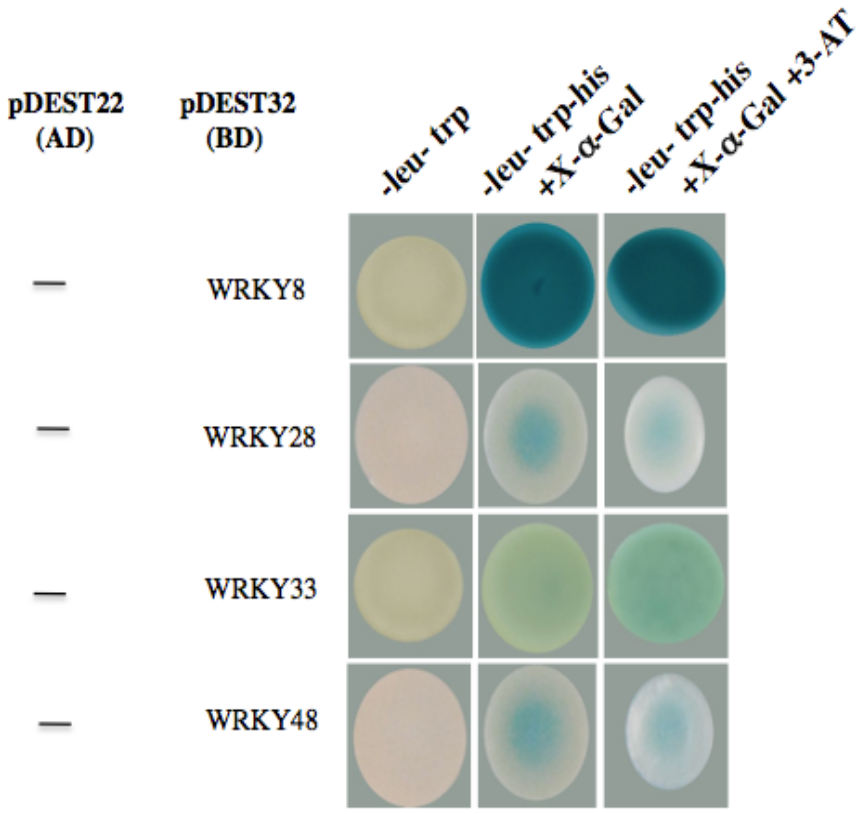


Figure 3.5: Y-2H assay showing auto-activation exhibited in AtWRKY8, AtWRKY28, AtWRKY33 and AtWRKY48 clones in pDEST32, when baited against empty prey vectors (pDEST22).

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT

One point of note is the varying degree to which the blue colouration is exhibited in the triple DO selection supplemented with X-α-gal, as seen in *figure 3.5*. It is suggested that variations in colour could indicate the relative strength of a particular interaction, however this is difficult to quantify and hence to form such conclusions is unreliable. For example the duration of 28 °C incubation for colony growth is one such variable (as growth rates were not static), which could explain in the differences exhibited. Unless the duration of incubation is specified, variations may result in increased hydrolysis of X-α-Gal via α-galactosidase, thus enhancing any blue colouration seen. For the assays shown in this study, incubation time was flexible and hence could account for the variations. As the blue colonies seen here is a result of auto-activation, and not PPIs, this point is not necessarily valid, nevertheless it is of note for future assays which do indicate true interaction.

Luckily, not all AtWRKY TF showed auto-activation, such as *AtWRKY47* and *AtWRKY72;* the interaction between these proteins and *AtRGA* is shown in *figure 3.6*. However, the strength of interaction occurring here is not strong as supplementation with 100 mM 3-AT is sufficient to inhibit the HIS3 product and hence cease growth on triple DO plates.

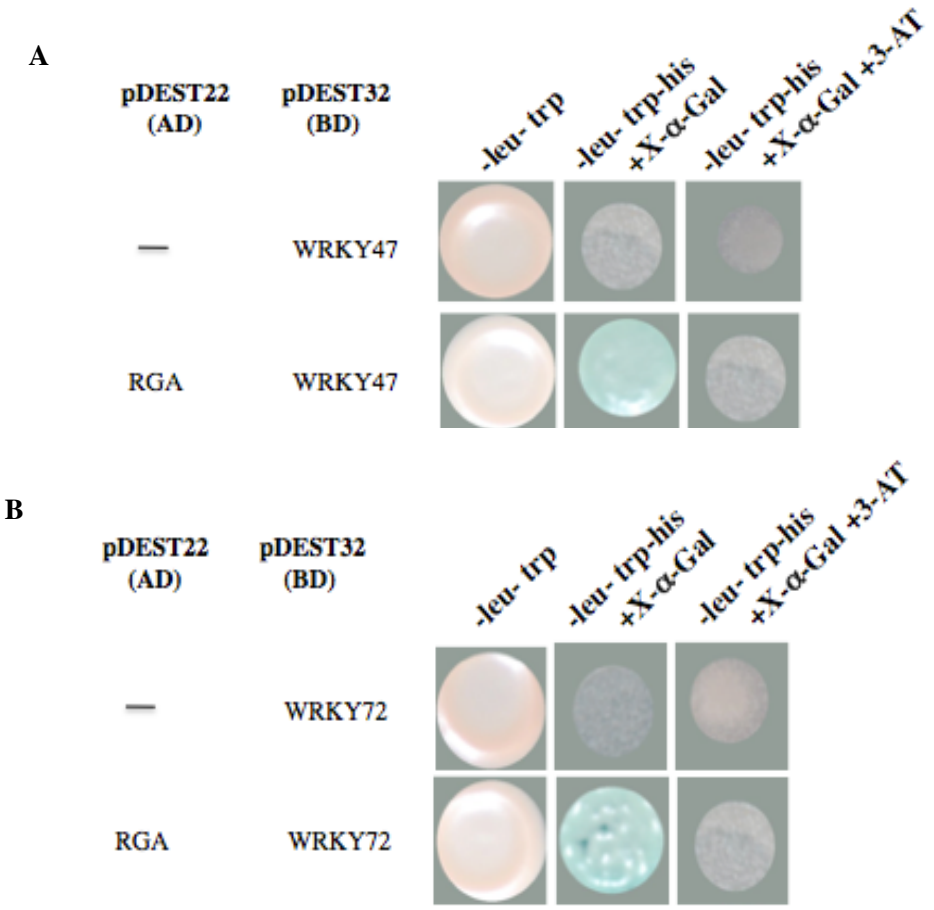


Figure 3.6: Y-2H assays for AtWRKY47 & AtWRKY72 baited against AtRGA. *Both* ***a.*** *AtWRKY47 and* ***b.*** *AtWRKY72, exhibit weak interaction with AtRGA. Colony growth on triple DO selection and blue colouration in the presence of X-α-gal indicates PPI. However, supplementation with 3-AT inhibits interaction.*

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT

The final AtWRKY TF studied was *AtWRKY26*, which unlike both *AtWRKY47* and *AtWRKY72,* showed strong interaction with *AtRGA*. Initially it did appear that *AtWRKY26*-pDEST32 auto-activated, however, supplementation with 100 mM 3-AT was sufficient to inhibit this interaction. With this in mind, subsequent baiting against *AtRGA* could be performed so long as any potential interactions included 3-AT

selection. As shown in *figure 3.7*, *AtRGA*-pDEST22 interaction with *AtWRKY26*-pDEST32 is not inhibited when 3-AT is supplied and hence this likely shows true interaction between these proteins.

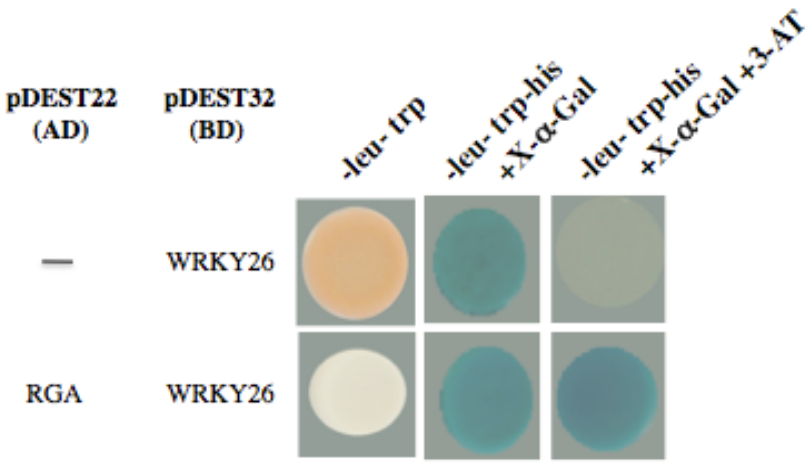


Figure 3.7: Y-2H assay for AtWRKY26 baited against AtRGA. *WRKY26 exhibits strong interaction with AtRGA, which is not inhibited when triple DO is supplemented with 3-AT.*

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT

3.2.3 AtWRKY26 physically interacts with AtRGA in vivo

To further determine the interaction between *AtWRKY26* with *AtRGA*, a co-IP assay was performed to isolate these interacting proteins *in planta*. Co-IPs are a highly useful method for protein detection and purification. The principle of a Co-IP is reasonably simple; an antibody against a specific protein forms an immune complex with its target, the protein is then precipitated out of solution using microbeads. Any

other macromolecules, such as interacting protein targets of said protein, will similarly precipitate. Eluted protein samples can then be separated via SDS-PAGE and visualised by western blotting.

AtRGA and *AtWRKY26* were transferred to pEG201 and pEG104 vectors respectively. These pEARLEYGATE vectors are Gateway® compatible and can be used for various techniques, such as immunoprecipitation or protein localisation, due to the different tags each possesses (*Earley et al 2006*), of which pEG201 and pEG104 utilise HA and YFP respectively. 35S-HA-RGA and 35S-YFP-WRKY26 were co-expressed in *N. benthamiana* and the recombinant protein extracted was then immunoprecipitated with α-GFP* MACS MicroBeads. Tissue co-infiltrated with 35S:HA-*RGA* and free GFP was included as a negative control and similarly immunoprecipitated with α-GFP. Subsequently, samples were analysed via SDS-PAGE followed by western blotting with α-HA and α-GFP antibodies. Individual proteins can be detected via the differences in size of each protein (kDa), of which separation is obtained via the SDS-PAGE process.

Figure 3.8.a and *3.8.b* show both input (not treated with GFP MicroBeads) and IP (treated with GFP MicroBeads) samples, which have then been probed with α-GFP or α-HA antibodies respectively. Immunoprecipitated YFP-*WRKY26* can be seen in the far right-hand lane of *figure 3.8.a,* with the label RGA:WRKY26. The corresponding lane in *figure 3.8.b* shows the detection of HA-RGA with α-HA. As GFP beads were used for immunoprecipitation, it is only possible to detect HA-tagged proteins, blotted with α-HA antibodies, if it has been co-immunoprecipitated with a GFP-tagged protein, in this instance YFP-WRKY26. Additionally, the control, RGA:GFP, does not show the same corresponding HA-RGA band when co-expressed with free GFP (specific bands of interest have been highlighted and descirbed). Bearing these facts in mind, it can be concluded that *AtWRKY26* and *AtRGA* physically interact when over-expressed in *N.*

benthamiana leaves. One additional Co-IP and western blot repeat was performed, again showing the same interaction between *AtWRKY26* and *AtRGA*.

(**NB. YFP-tagged proteins can be immunoprecipitated using GFP MicroBeads and blotted with α-GFP antibodies*).

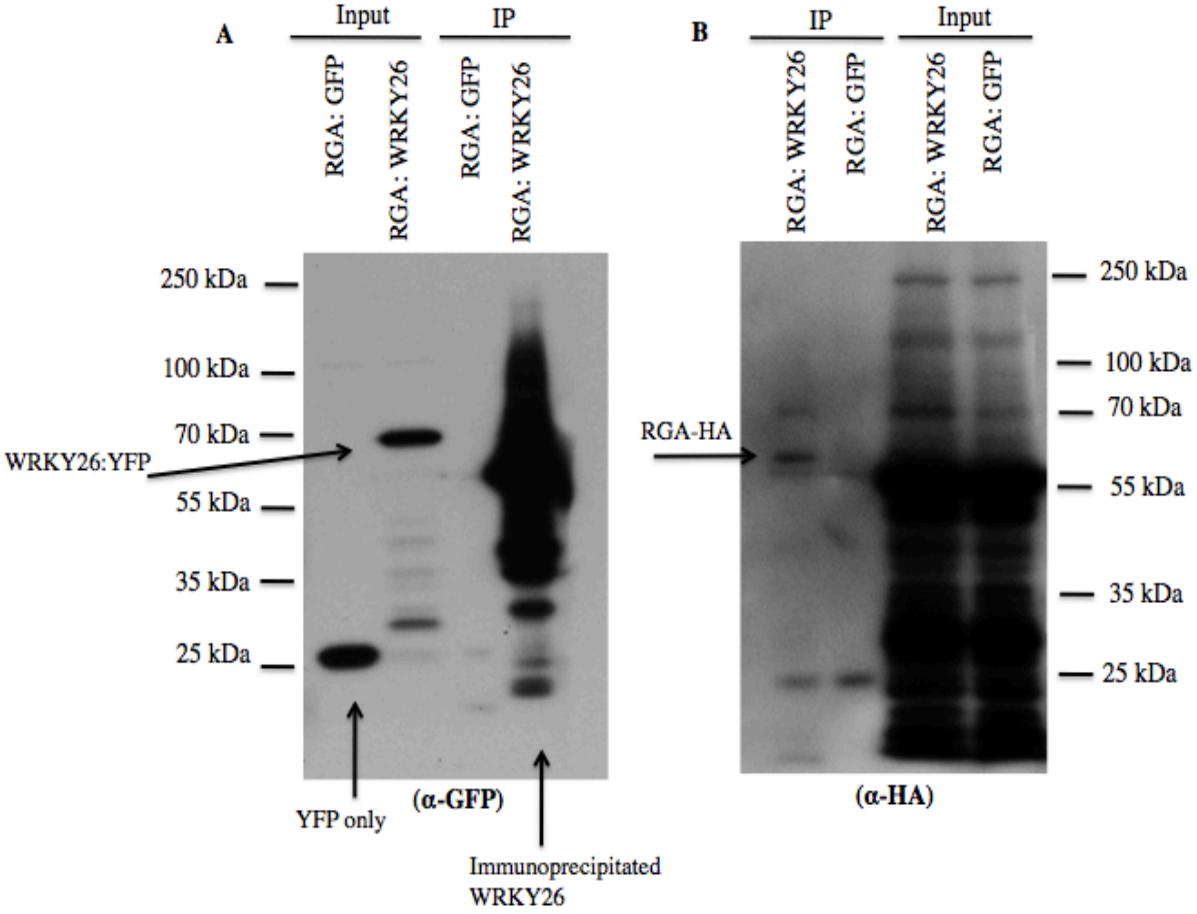


Figure 3.8: co-IP of AtRGA and AtWRKY26 in planta. *HA-RGA was transiently co-expressed with a GFP control (RGA:GFP) or with YFP:WRKY26 (RGA:WRKY26) in N. benth. The recombinant protein extract was immunoprecipitated with α-GFP MACS MicroBeads. The input and IP protein samples were analysed via western blotting using antibodies for* ***a.*** *GFP and* ***b.*** *HA. Arrows indicate the detection of individual bands.*

One issue with this blots is the lack of free RGA:GFP visual in the IP samples of *figure 3.8.a*. Had time permitted, optimisation of these reactions would have been carried out, allowing clarification for the reasons behind this issue.

3.2.4 AtWRKY26 physically interacts with AtGAI in vivo

After discovering that *AtWRKY26* and *AtRGA* were interacting *in plant*, the next aim was to identify whether any additional DELLA proteins also showed such relationships. The *AtGAI* DELLA was chosen as a potential additional target, as in sharing 82 % sequence homology with *AtRGA*, was likely candidate for also showing such interaction. A co-IP assay was again preformed following the same conditions and controls as the previous experimental design, however in this instance substituting *AtRGA* for *AtGAI*. As shown in *figure 3.9.b,* when HA-GAI is co-expressed with YFP-WRKY26, GAI is immunoprecipitated. Again, HA-GAI is not immunoprecipitated when co-expressed with free GFP. Hence, it can also be concluded that *AtGAI* and *AtWRKY26* physically interact when over-expressed in *N. benthamiana.* One additional Co-IP and western blot repeat was performed, again showing the same interaction between *AtWRKY26* and *AtGAI*.

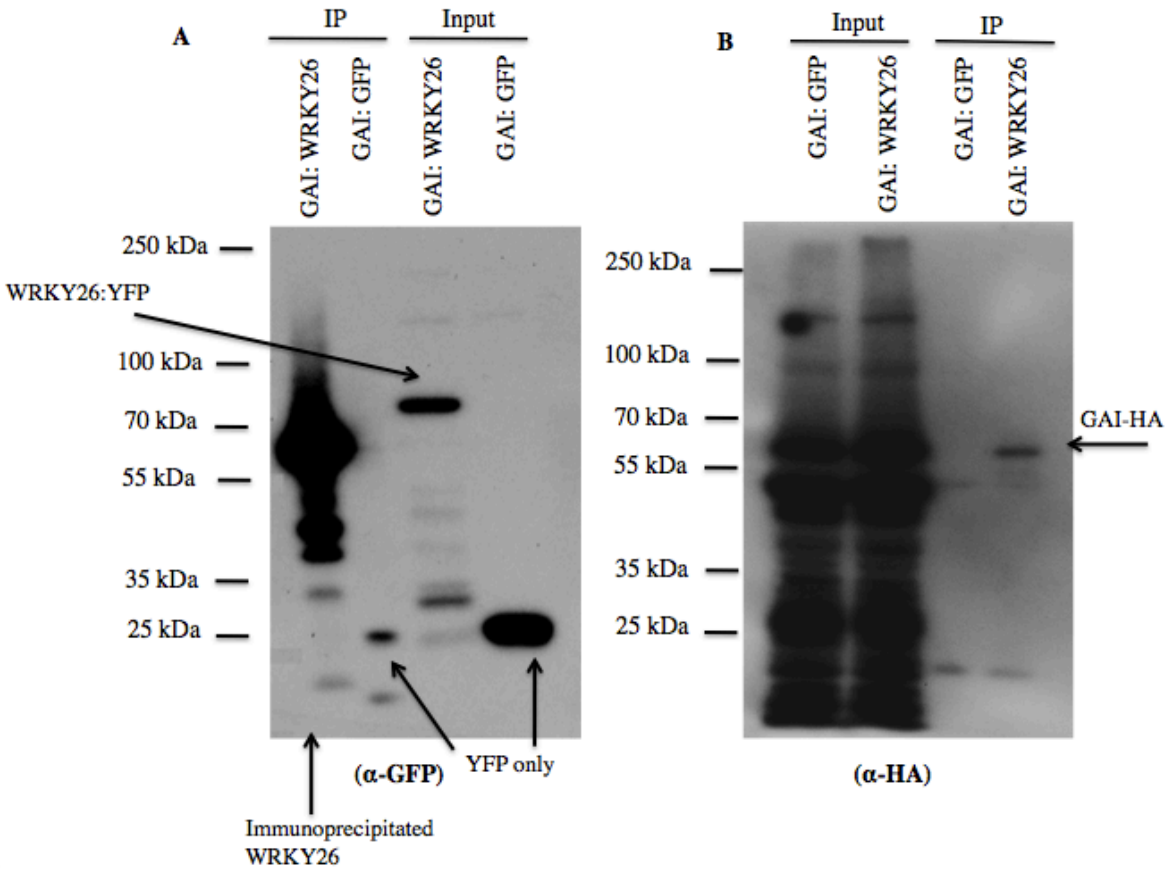


Figure 3.9: co-IP of AtGAI and AtWRKY26 in planta. *HA-GAI was transiently co-expressed with a GFP control (GAI:GFP), or with YFP:WRKY26 (GAI:WRKY26) in N. benth. The recombinant protein extract was immunoprecipitated with α-GFP MACS MicroBeads. The input and IP protein samples were analysed via western blotting using antibodies for* ***a.*** *GFP and* ***b.*** *HA. Arrows indicate the detection of individual bands.*

The band smearing seen in both *figure 3.8* and *figure 3.9* is likely due to protein degradation or non-specific antibody binding. Optimising elution and loading less protein when running the SDS-PAGE gel should reduce the smearing seen, as should reducing the exposure during blot visualisation.

3.2.5 Domain mutation strategy

After identifying that *AtWRKY26* and DELLA proteins show strong interaction, in contrast to *AtWRKY47* and *AtWRKY72* where only weak interaction was highlighted, the specific protein domains where such interaction occurs was then investigated. Both *AtWRKY26* and DELLAs have two functional protein domains, a schematic of which can be seen in *figure 3.10*. The two domains found in DELLAs, the example depicted here being *AtRGA*, are the DELLA and GRAS domains. Both domains have distinct functions, from regulating GA-dependent protein degradation via the DELLA domain, to protein dimerisation and regulation via the GRAS domain (*Pysc et al.1999)*. The function of the two AtWRKY DNA binding domains of *AtWRKY26* are less well characterised; while it has been demonstrated that the C-terminal domain is the major regulator of DNA-binding, the function of the N-terminal domain is less well characterised (*Eulgem et al. 2000*). Certainly there has been no research carried out on the difference these two domains exhibit in the AtWRKY of this particular study.

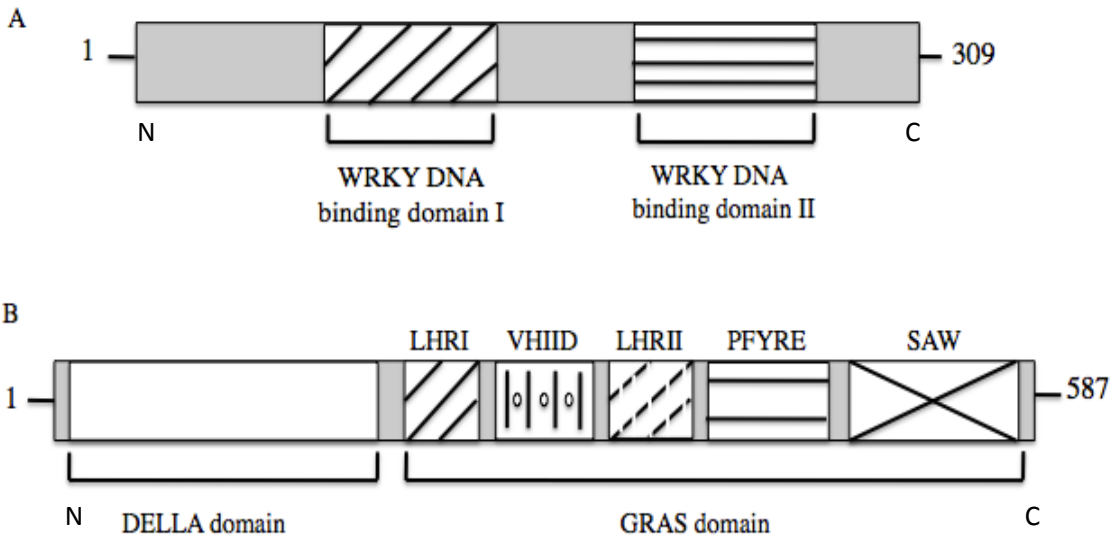


Figure 3.10: Schematic of a. AtWRKY26 & b. AtRGA. *AtWRKY26 has two DNA-binding domains. The two domains found in all DELLAs are the DELLA and GRAS domains. The DELLA domain is required for GA-dependent DELLA degradation, whereas the GRAS domain has various functions. The GRAS domain can be further subdivided into five distinct sequence motifs.*

In order to determine domain interaction, the full-length proteins were sub-divided, resulting in the expression of single domains when subsequently baited in the Y-2H system. For *AtRGA*, three separate mutations to the full-length protein were cloned, a depiction of which is shown in *figure 3.11*. Both *II* and *III* contain the coding sequence for the DELLA and GRAS domains alone, whereas *I* also includes the initial nucleotides before the DELLA domain. All clones were amplified using primers which included both the start and stop codons on the 5' and 3' primers respectively, to enable accurate protein synthesis and subsequent expression in yeast. The gel image shown in *figure 3.12* shows the three plasmid clones, the sizes of which can be compared to the full-length *AtRGA* protein.

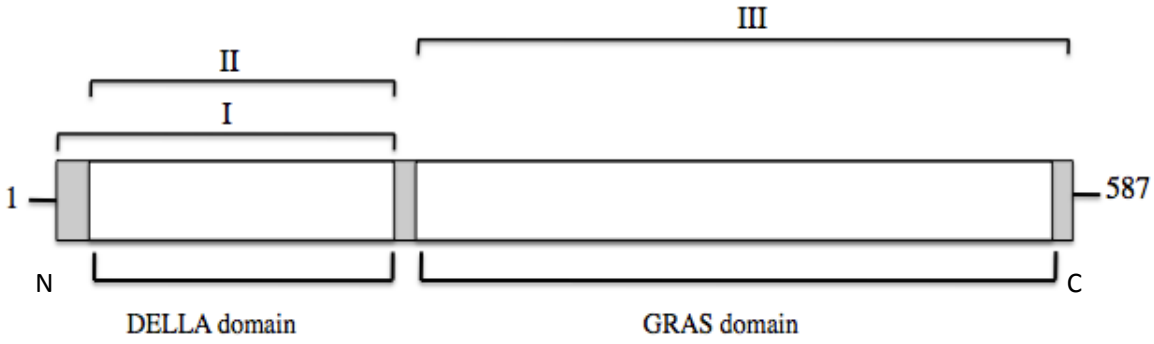


Figure 3.11: Schematic of AtRGA domain mutations. *AtRGA was divided into three separate truncated proteins; I includes the N-termini of the protein terminating at the end of the DELLA domain, whereas II includes only the DELLA domain. III includes only the GRAS domain.*

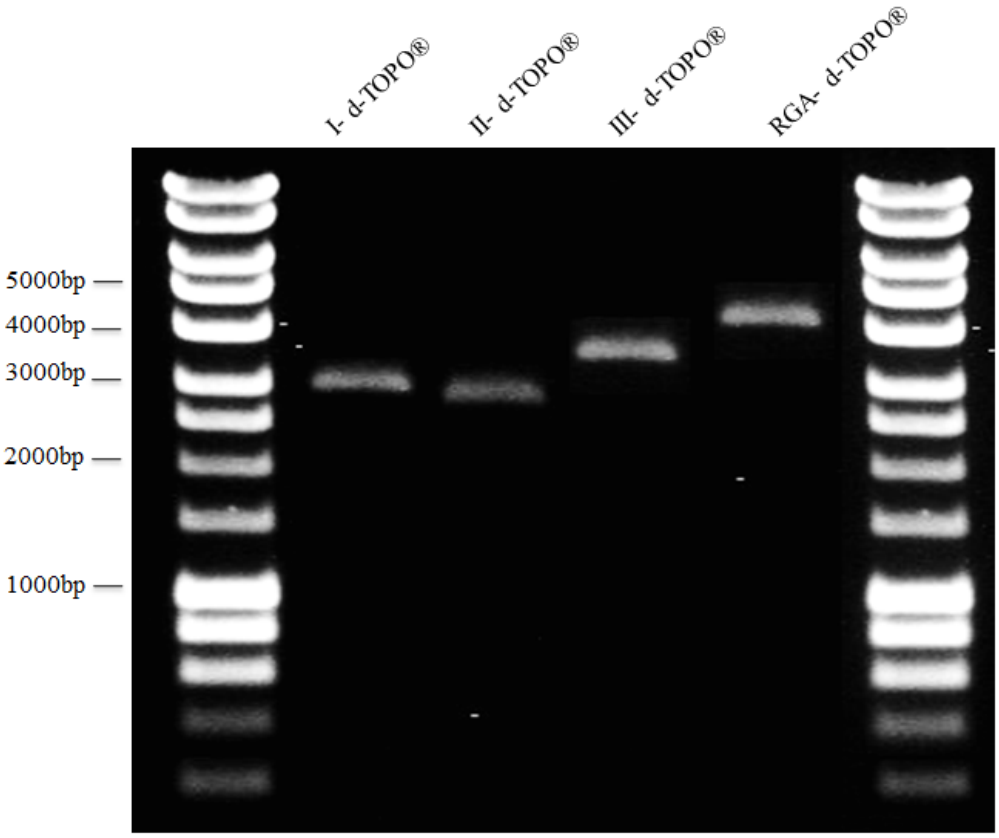


Figure 3.12: Gel electrophoresis image showing the AtRGA mutated domains. *Plasmids were linearised using PvuI restriction endonuclease (NEB)*

The same strategy was used for the division of the two *ATWRKY26* domains, as shown in *figure 3.13.* Initially only the DNA binding domain II was cloned for Y-2H analysis, as it has been characterised that this domain predominantly regulates DNA binding.

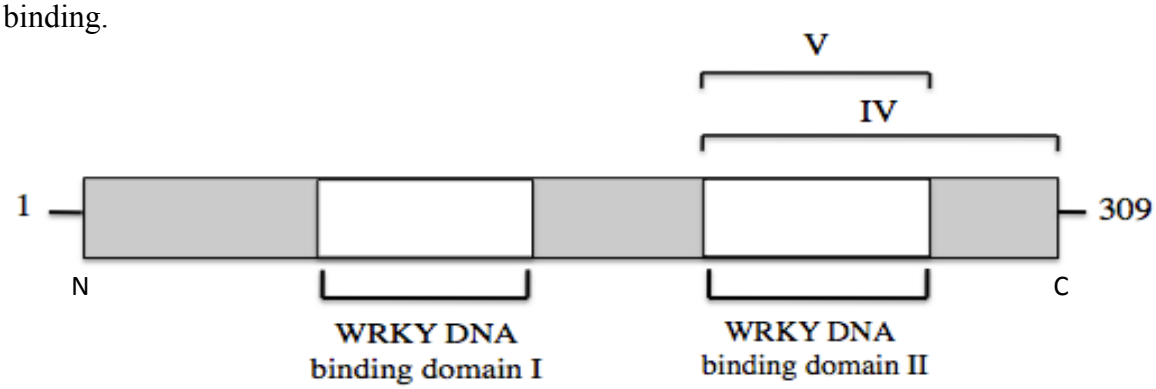


Figure 3.13: Schematic of WRKY domain mutations. ***At****WRKY26 was divided into two truncated proteins; IV contains the start of WRKY binding domain II until the C-termini, whereas V only includes the domain*

2.2.6 AtRGA DELLA and GRAS domains interact with AtWRKY26 in vitro

The three *AtRGA* domain mutations, *I-III*, were originally baited against empty pDEST32 to check for auto-activation, which, as shown in *figure 3.14,* does not occur. *AtRGA*-pDEST22 and *AtWRKY26*-pDEST32, included in the first two rows of this figure, had previously shown interaction, and hence this combination was used for this assay. Regardless, for any true interactions, the growth of colonies in media supplemented with 3-AT, the concentration used throughout this study being 100mM, was required as *AtWRKY26*-pDEST32 weakly auto-activates. As shown, the colony growth of clone *I* is inhibited in the presence of 3-AT, hence it cannot be concluded if whether these proteins are interacting. However both *II* and *III*, which encompass only the DELLA and GRAS domains, have grown in the presence of 3-AT. This likely indicates interaction between both these domains and *AtWRKY26*.

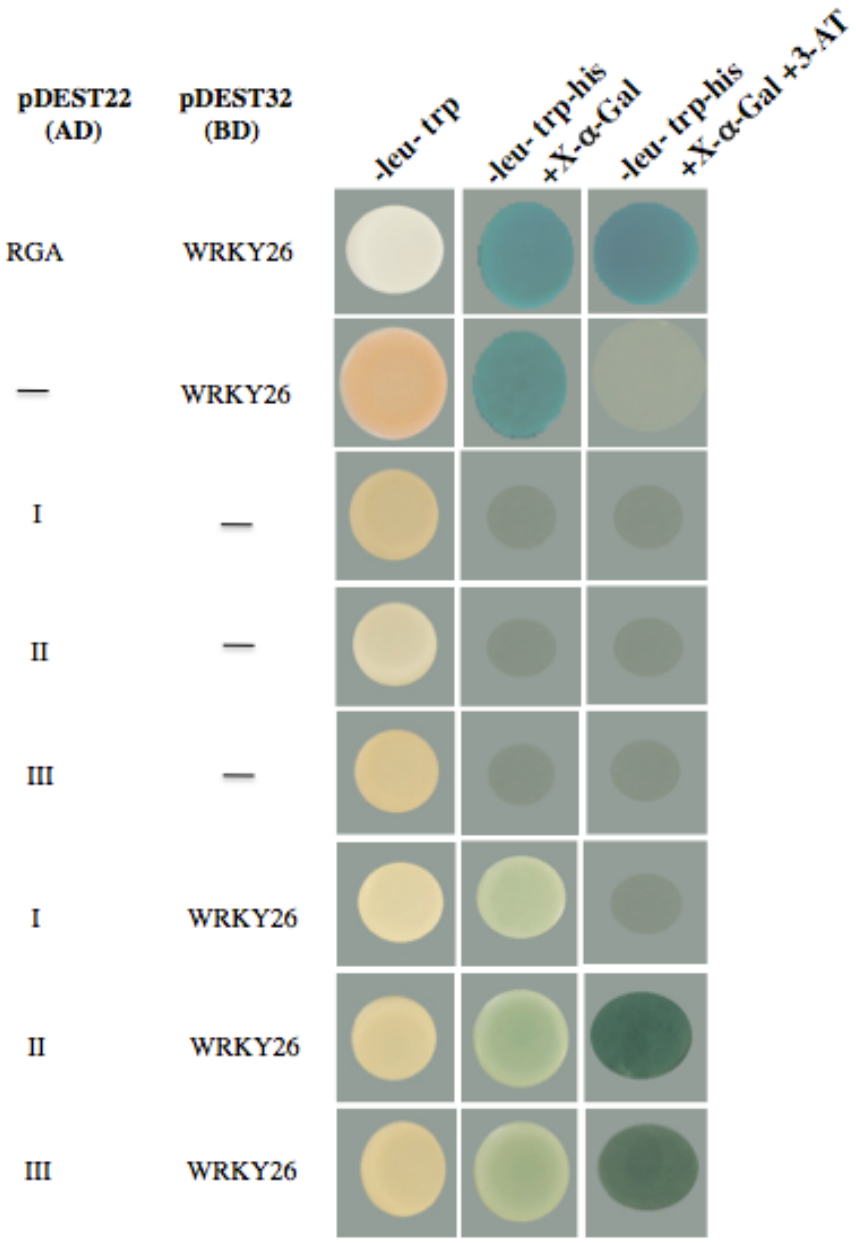


Figure 3.14: Y-2H assay for AtRGA domain mutations baited against AtWRKY26. *Both DELLA and GRAS domains indicate interaction with WRKY26, as interaction is not inhibited when triple DO is supplemented with 3-AT. Clone I, which includes the initial nucleotides of the protein coding sequence, is inhibited by 3-AT. Both clones II and III, which encode the DELLA and GRAS domains alone, are not inhibited by 3-AT.*

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT

3.2.7 AtWRKY33 physically interacts with AtRGA in vivo

The interaction between *AtWRKY33* and *AtRGA*, which could not be shown via Y-2H due to *AtWRKY33* auto-activation (see *figure 3.5*), was demonstrated using a Co-Immunoprecipitation (Co-IP) as the beginnings of some preliminary work on this protein. 35S:HA-YFP-AT*ATRGA* was co-expressed with 35S:YFP-WRKY33, then immunoprecipitated with α-HA MACS MicroBeads. Tissues co-infiltrated with 35S:HA-YFP-*RGA* and free GFP was included as a negative control and similarly immunoprecipitated with α-HA. Unlike the previous co-IP assays, in which both AtWRKY and DELLA proteins included single tags, in this instance *AtRGA* was expressed in pEG101, which utilises both HA and YFP tags. Because of this, both *AtRGA* and *AtWRKY33* proteins are visualised on the same blot after probing with α-GFP antibodies, as shown in *figure 3.15.b. A*dditionally, as also shown in *figure 3.15.b,* protein has not been detected in the input lanes. This could be due to insufficient protein concentrations for detection by α-GFP. Concentrating said protein samples may result in detection; hence further work on this interaction is needed. Regardless, the immunoprecipitation of *AtWRKY33* with *AtRGA* tentatively highlights the physical interaction between these proteins when over-expressed in *N. benthamiana.*

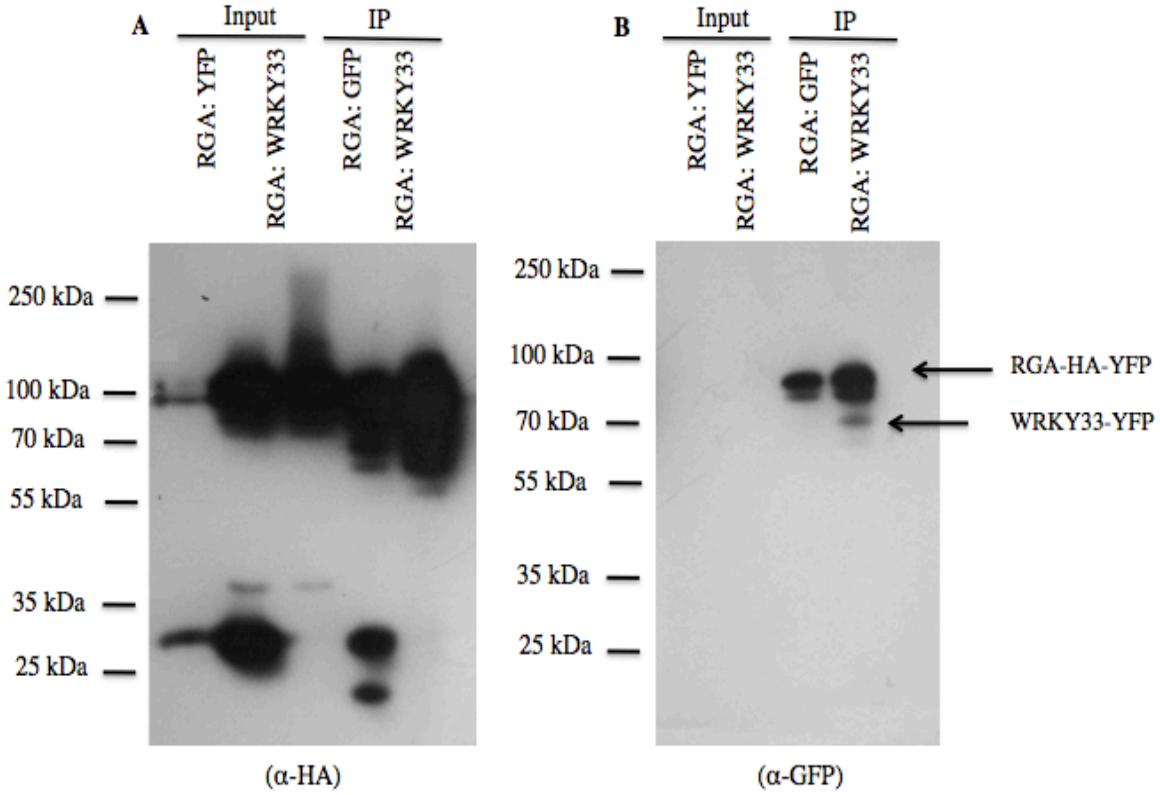


Figure 3.15: co-IP of AtRGA and AtWRKY33 in planta. *HA-YFP-RGA was transiently co-expressed with a GFP control (RGA:GFP) or with YFP:WRKY33 (RGA:WRKY33) in N. benth. The recombinant protein extract was immunoprecipitated with α-HA MACS MicroBeads. The input and IP protein samples were analysed via western blotting using antibodies for* ***a.*** *HA and* ***b.*** *GFP.*

3.3 Discussion

3.3.1 Y-2H screening is used to assess protein-protein interactions in vitro

The initial aim of work carried out in this chapter was to identify whether any AtWRKY TF were interacting with the *AtRGA* DELLA protein, using the Y-2H system to assess interaction. As *AtRGA* auto-activated in pDEST32 (*figure 3.4*), all subsequent interaction assays required the *AtRGA*-pDEST22: AtWRKY-pDEST32 combination of plasmids, with all AtWRKY TF initially tested for auto-activation in pDEST32. The high level of auto-activation exhibited by the transcription factors was an initial set back, with four out of the seven AtWRKY TF auto-activating in pDEST32 (*figure 3.5*). However, neither *AtWRKY47* nor *AtWRKY72* auto-activated, and *AtWRKY26* auto-activation was inhibited via the supplementation with 100 mM 3-AT. Each of these three AtWRKY TF were shown to be interacting with *AtRGA in vitro (figure 3.6 & figure 3.7).*

Regardless that interaction between both *AtWRKY47* and *AtWRKY72* with *AtRGA* (*figure 3.6*) was shown, the inhibition of growth after supplementation with 3-AT indicated weak interaction between said proteins. However, the relatively high concentration of 100 mM 3-AT in the selection media is likely the reason behind this, and hence it would be interesting to see if altering the concentration of 3-AT would cease to inhibit interaction. Repeating interaction assays with the inclusion of several concentrations of 3-AT between 0 mM, where interaction occurs, to 100 mM, where interaction is inhibited, would help to elucidate the relative strength of PPI between both *AtWRKY47* and *AtWRKY72* with *AtRGA*. Additionally, further proof of these interactions could be proven via the use of co-IP assays.

The interaction between *AtWRKY26* and *AtRGA* (*figure 3.7*) was shown to be stronger than that of both *AtWRKY47* and *AtWRKY72*. The weak auto-activation of *AtWRKY26*-pDEST32 was inhibited by 100 mM 3-AT, conversely colony growth was not inhibited in the interaction assays between *AtWRKY26* and *AtRGA* when 3-AT was supplemented, thus indicating true interaction. Additionally, although data is not shown, this particular *AtWRKY26* and *AtRGA* assay was repeated numerous times, each showing the same interaction results. Again, the relative strength of interaction could be assessed via altering 3-AT concentrations, in this case potentially above that of 100 mM, to identify at what concentration interaction is repressed.

To further elucidate protein interactions, the domains where interaction between *AtRGA* and *AtWRKY26* occurs was identified. Initially the two *AtRGA* domains, the DELLA and GRAS domains, were cloned and the three truncated proteins (*figure 3.11*), *I-III*, were expressed in pDEST22 for subsequent baiting against *AtWRKY26*-pDEST32. As previously demonstrated, *AtRGA* and *AtWRKY26* interact (*figure 3.7*), however the weak auto-activation of *AtWRKY26*-pDEST32 required the addition of 3-AT for the actual interaction assays. This same plasmid combination was utilised in the Y-2H assay. The DELLA domain, involved in GA-dependent DELLA degradation (*Dill et al. 2001*), has in previous studies, been generally shown not to be involved in modulating PPIs. One such study similarly mutated the two *AtRGA* domains and identified the GRAS domain being the element responsible for interaction with *BZR1,* a transcription factor regulating brassinosteroid-responsive gene expression (*Bai et al. 2012*). Additional Y-2H studies have shown that the GRAS domain interacts directly with *SLY1,* a component of the SCF complex that regulates DELLA protein degradation (*Dill et al. 2004*)**.** Apart from interaction with the GA receptor, GID1, which is mediated via the DELLA domain (*Willige et al. 2007*), there seems to be little literature documenting

additional DELLA domain interacting partners. With these points in mind, the initial hypothesis was that the GRAS domain region would show interaction with *AtWRKY26* however, from the Y-2H data this may not be the case. Interestingly, data indicated interaction between *AtWRKY26* and both the DELLA (clone *II*) and GRAS (clone *III*) domains, as shown via colony growth in the triple DO selection supplemented with 100mM 3-AT (*figure 3.14*). However, this same interaction was not see in the opposing vector combination (*AtWRKY26*-pDEST22: *AtRGA* mutant-pDEST32), where colony growth was inhibited in the triple DO media, regardless of 3-AT presence (*appendix II; figure 1*). The reason for this is unclear; it could be that this lack of supposed interaction is a false negative, a relatively common issue associated with Y-2H. Also the potential for incorrect protein folding could also similarly result in no identifiable interaction. With this in mind, further analysis is required. One way interaction with both GRAS and DELLA domains could be proven is by utilising different protein-protein interaction techniques, such as complementation assays, or again via co-IPs. The use of the latter however may prove difficult, as protein expression would need to be relatively high for detection. Alternatively, a different Y-2H strategy could be carried out, whereby rather than expressing protein fragments, PCR-site directed mutagenesis could be utilised to mutate each domain sequentially. Supposing such mutational techniques were sufficient to knockout function, hence limiting binding to one domain only, the potential problem of incorrect protein folding could be overcome, and thus the integral 3D protein structure maintained. If time had allowed this would have been the next step in the analyses of the DELLA and GRAS domain functions. Additionally, it would have been interesting to analyse the function of the various GRAS domain sequence motifs, assessing which of the five motifs were involved in the regulation of binding with *AtWRKY26*.

The AtWRKY26 DNA domain responsible for interaction with *AtRGA* was not identified using Y-2H. Two clones were created, IV and V (*figure 3.13*), which encompassed only the N-terminal DNA binding domain II. Both *IV*-pDEST32 and *V*-pDEST32 failed to co-transform with empty-pDEST22 and hence auto-activation of these plasmids could not be assessed. Regardless, this proved not to be an issue, as when subsequently baiting *IV*-pDEST32 against *AtRGA*-pDEST22, which unlike *V*-pDEST32 did co-transform, interaction was not identified (*appendix II, figure 2*). Similarly, baiting both of these clones against *AtRGA*-pDEST32 was not possible as previous study (*figure 3.4*) showed auto-activation of this plasmid, regardless of 3-AT presence. The fact that so many of these clones failed to co-transform was cause for concern, and could indicate an issue with the mutational strategy, or indeed with the transformation strategy, although this had previously caused no concerns. However, the reason for no identifiable interaction could very well be that the DNA binding domain II is not responsible for interaction with *AtRGA*. Although the N-terminal domain is generally considered to control the majority of protein binding (*Rushton et al. 2000*), certain studies have implicated the role of the C-terminal domain. One such example has demonstrated that the C-terminal of *AtWRKY33* mediates the binding of various proteins involved in the responses to pathogen attack (*Chi et al. 2013*), hence the idea that the C-terminal domain is always redundant has proven to be false. Again, with hindsight it would have been a more stringent method had site-directed mutagenesis been utilised to knockout the DNA-binding domains sequentially, thus maintaining 3D protein structure.

Ultimately after baiting single domains against opposing whole length proteins it would be prudent then to bait single domains against each other, hence unequivocally proving interaction between said domains.

3.3.2 Co-IP assays can identify the interaction of AtWRKY26 and AtWRKY33 with AtDELLA proteins

The use of a co-IP assay to demonstrate interaction between *AtWRKY26* and *AtRGA* (*figure 3.8*) follows on the Y-2H data, giving confidence that interaction between these proteins occurs not only *in vivo*, but *in planta* also. This is especially important when reminded of the weak auto-activation of *AtWRKY26*-pDEST32, which although is inhibited by 3-AT, could be seen as a potential flaw in this result. Similarly, it was interesting to see that *AtGAI* also interacts with *AtWRKY26* (*figure 3.9*) *in vivo*. When comparing α-GFP immunoprecipitated *RGA*-HA and GAI-HA, detected after blotting with α-HA (*figure 3.8.b & figure 3.9.b*), it appears that the expression levels in the *AtRGA* blot are lower that of *AtGAI*. The strength of the *AtRGA* signal is much lower than that of *AtGAI*, although each had an equivalent exposure time of approximately five minutes. However protein concentrations in both input and IP samples were not quantified before loading onto SDS-PAGE gels for subsequent blotting. It would have been useful to quantify said samples, and hence standardise protein concentrations between both the *AtRGA* and *AtGAI* blots. Additionally, if time had allowed, further repeats of these Co-IPs and subsequent blots would have been beneficial to optimise conditions for protein expression would also have been beneficial.

As the interaction between *AtWRKY26* with both *AtRGA* and *AtGAI* was shown, it would be interesting to see if any of the remaining three DELLA proteins; *AtRGL1*, *AtRGL2* and *AtRGL3*, also interact with *AtWRKY26*. As mentioned previously, although there is crossover between expression profiles throughout the different stages of development, some DELLAs are only expressed in certain tissues or stages of development. One example of this is RGL2, which is involved in the regulation of seed

germination (*Tyler et al. 2004*), a process which neither *AtRGA* nor *AtGAI* seemingly regulate. If AtWRKY26 was shown to interact with additional DELLAs, the function of this protein may be further elucidated. The potential functions of the *AtWRKY26* protein shall be discussed in further detail in the following chapter.

The additional co-IP shown, indicating interaction between *AtWRKY33* and *AtRGA* (*figure 3.15*) was the result of preliminary work on this AtWRKY TF, which ultimately was continued by another lab member. This blot does appear to show interaction between these proteins, however the weakness of the *AtWRKY33* signal is slight cause for concern, and hence a repeat of this assay is required. Also, one argument against interaction identified is that the *AtWRKY33* band is potentially degradated *AtRGA* protein. However with the size differences obtained it could tentatively be suggested this is not the case. Similarly, the overexposure of the blot is not ideal. Finally, as *AtWRKY33* was tagged with YFP (pEG104), and *AtRGA* with both YFP and HA (pEG101), the resulting detection of both proteins on the same α-GFP blot is not ideal. Nevertheless, the immunoprecipitation of *AtWRKY33* with α-HA and the subsequent detection in the α-GFP blot (*figure 3.15.b*) appears to show interaction. Before any further work on these interacting partners is carried out, the movement of *AtRGA* into a vector with a single tag, for example pEG201, would be a positive move forward.

CHAPTER IV:

Elucidating the function of the AtWRKY26 protein

4.1 Introduction

WRKY transcription factors, as previously mentioned, have a wide array of functions, from modulating responses to biotic and abiotic stresses, to regulating various developmental processes. Initially this chapter discusses background material, documenting the rather limited information regarding the potential functions of the *WRKY26* protein. One publication (*Li et al. 2011*) details the regulation of heat tolerances as a function of *WRKY26,* and, with this in mind, data presented here attempted to repeat and consequently continue with this work. In turn, the relationship between DELLAs and *WRKY26*, as described and identified in chapter III, shall be discussed, with the potential for DELLA modulation of the WRKY response analysed. Additionally, bioinformatic data shall also be presented.

Although certain WRKY TF have received quite extensive research, there is little evidence relating to the function of At*WRKY26*. However, one recent publication discusses the role of *AtWRKY26*, in conjunction with *AtWRKY25* and *AtWRKY33*, relating its role in response to high temperatures stress. Initially it was shown that the relative expression of *AtWRKY26* increases upon 48 °C treatment, with *Atwrky26* knockouts showing decreased tolerances to high temperature. This latter phenotype was demonstrated by comparing survival rates of mutant and wild type plants, see *figure 4.1.a* and *figure 4.1.b*. However, a *Atwkry26* genotype alone seems insufficient to alter the expression of various heat-inducible marker genes, such as Hsp70, *Hsp101* and *Zat10*. A combination of double and triple knockouts with both *Atwrky25* and *Atwrky33* demonstrated a reduction in marker gene expression, with the *wrky25wrky26wrky33* triple mutant exhibiting the greatest thermosensitivity (*Li et al. 2011*). This highlights the somewhat functional redundancy of these genes in the regulation of thermotolerance.

Additionally, constitutive expression of *AtWRKY26*, under control of the 35S promoter, results in a less vigorous phenotype than wild type, with plants exhibiting smaller size and lighter fresh weight. A similar phenotype is also shown with 35S:WRKY25, but not in 35S:WRKY33, lines, see *figure 4.2 (Li et al. 2011)*. The size reduction exhibited in plants over-expressing At*WRKY26* is an interesting point, posing the question of the role of the DELLA proteins, modulators of growth, in this repression. The possible effects of altering *AtWRKY26* expression *in planta* shall be addressed later in this chapter.

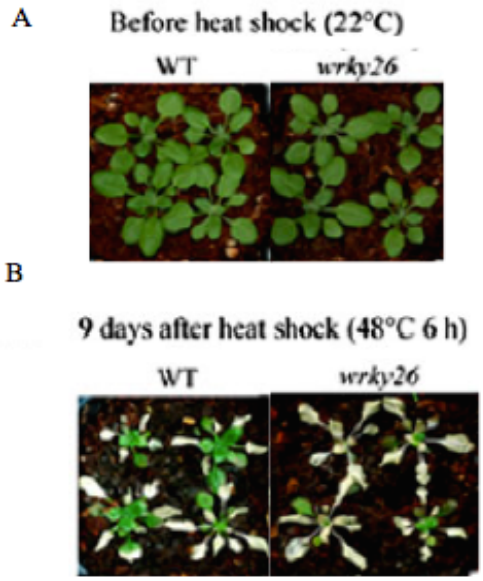


Figure 4.1: Survival of wild type (WT) and WRKY26 mutants (wrky26). *WT and wrky26 null mutants were* ***a.*** *germinated and grown for 25 days at 22 ℃ followed by heat shock treatment of 48 ℃ for 6 hours.* ***b.*** *The survival rates of WT and mutants were recorded 9 days post heat treatment. wrky26 plants exhibited decreased survival rates compared to WT (Li et al. 2011).*

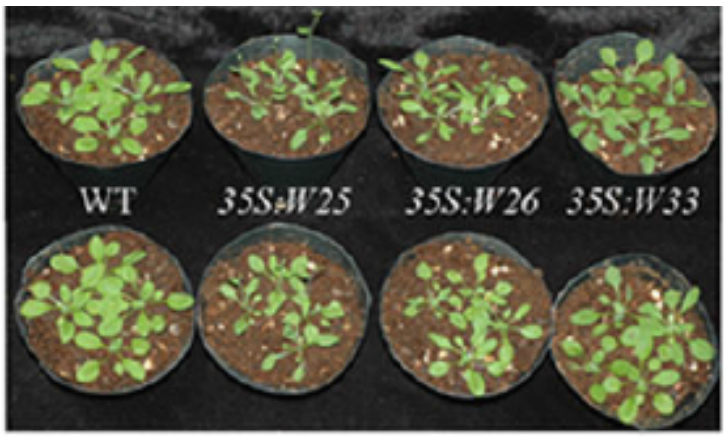


Figure 4.2: Phenotype of WRKY25, WRKY26 and WRKY33 overexpressors. *35S:WRKY25 and 35S:WRKY26 plants have a weaker phenotype compared to WT, whereas 35S:WRKY33 exhibits no altered phenotype (Li et al. 2011).*

The link between genotype and phenotype is often quite difficult to prove, however, certain techniques can be utilised to causally demonstrate gene function *in planta*. Insertional mutagenesis is one such technique, used to disrupt gene function, and hence analyse whether plants lacking specific genes result in a particular phenotype shown. A major technique for this kind of mutagenesis is the use of T-DNA insertions, which have been widely used to produce many thousands of independent transgenic lines, spanning the *Arabidopsis* genome. The insertion of foreign T-DNA, generally between 5-25kb, is generally sufficient to cause a dramatic disruption of gene function, hence producing a null mutant for said gene (*Krysan et al. 1999*). An additional benefit of using T-DNA insertion is the ability to screen for homozygous and heterozygous individuals, using combinations of primers specific to both the gene in question, and the T-DNA sequence. An example of a T-DNA insertion into *WRKY26* is shown in *figure 4.3.*

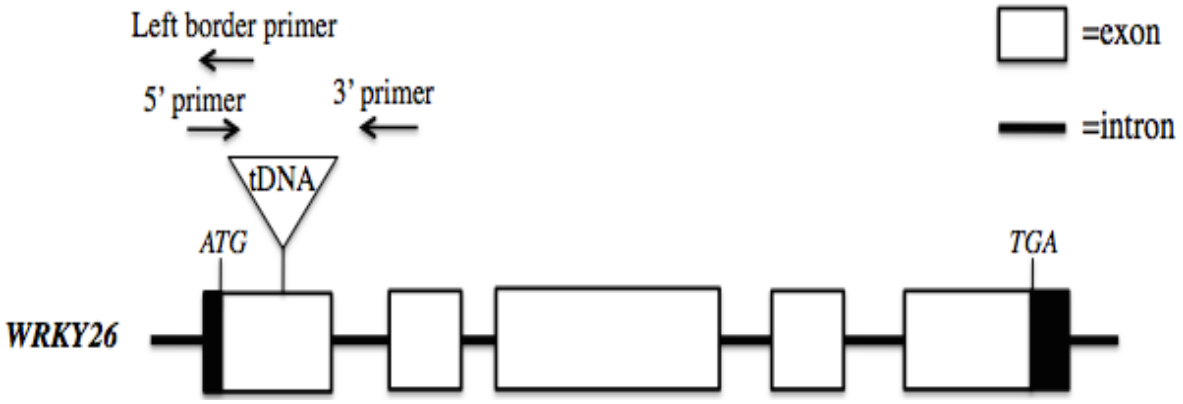


Figure 4.3: Schematic of WRKY26 loss of function mutant. *t-DNA SALK_063386 insertion into WRKY26 exon results in a null mutant. Insertion is identified via PCR using a combination of 5', 3' and left border primer (T-DNA specific).*

This chapter describes potential functions of the *AtWRKY26* protein. Previous publications, stating roles in thermotolerance, has been described, hence the use of *Atwrky26* gene knockouts in various thermotolerance assays was assessed. Additionally, preliminary qPCR data is presented.

4.2 Results

4.2.1 Identification of WRKY26 gene knockouts

To obtain a *AtWRKY26* gene knockout, three independent T-DNA SALK insertion lines (SALK_063386, SALK_063378, SALK_137675) were obtained from NASC. Each line was chosen as the T-DNA sequences were exon insertions, hence were unlikely to be spliced out and thus provide a knockout phenotype. Only one of these lines had previously been described, namely SALK_137675, used in the *Li et al.* publication. gDNA from the putative T-DNA insertions was extracted from the three SALK lines for subsequent genotyping, with samples initially tested using actin primers (act_F & act_R) as shown in *figure 4.4.a*. Gene presence was identified using At*WRKY26* gene specific 5' and 3' primers (W26_geF & W26_geR, see *table 2.1*), as shown in *figure 4.4.b*, of which samples 1-5 showed no presence of the At*WRKY26* gene; due to the large T-DNA insertion, the gene sequence is far too large to amplify with standard PCR, hence can be used as a test with these gene specific primers. Further clarification of gene presence was analysed using a three-primer combination, using a T-DNA left border primer (SALK_geW26, amplifies out from the primer in the 5' direction) along with both the 5' and 3' WRKY26 gene specific primers, as shown in *figure 4.4.c*. The benefit of this three-primer combination is that it enables the detection of homozygotes and heterozygotes for the insertion, in addition to WT individuals, on a single gel. As shown in *figure 4.4.c,* individuals 1-5 have only one band, corresponding to the left border and forward primer target amplification. The two bands found in 6-8 identify these plants as heterozygotes; hence have the insertion on only one chromosome. Individuals 1-5 are wild type and hence lack the T-DNA insert all together.

Of the three T-DNA lines genotyped here, corresponding to eighty-three individual plants, only the SALK_063386 insertion produced *AtWRKY26* knockouts; individuals 1-12 in *figure 4.4* depict some of these. Seeds collected from these plants were subsequently used for various thermotolerance assays.

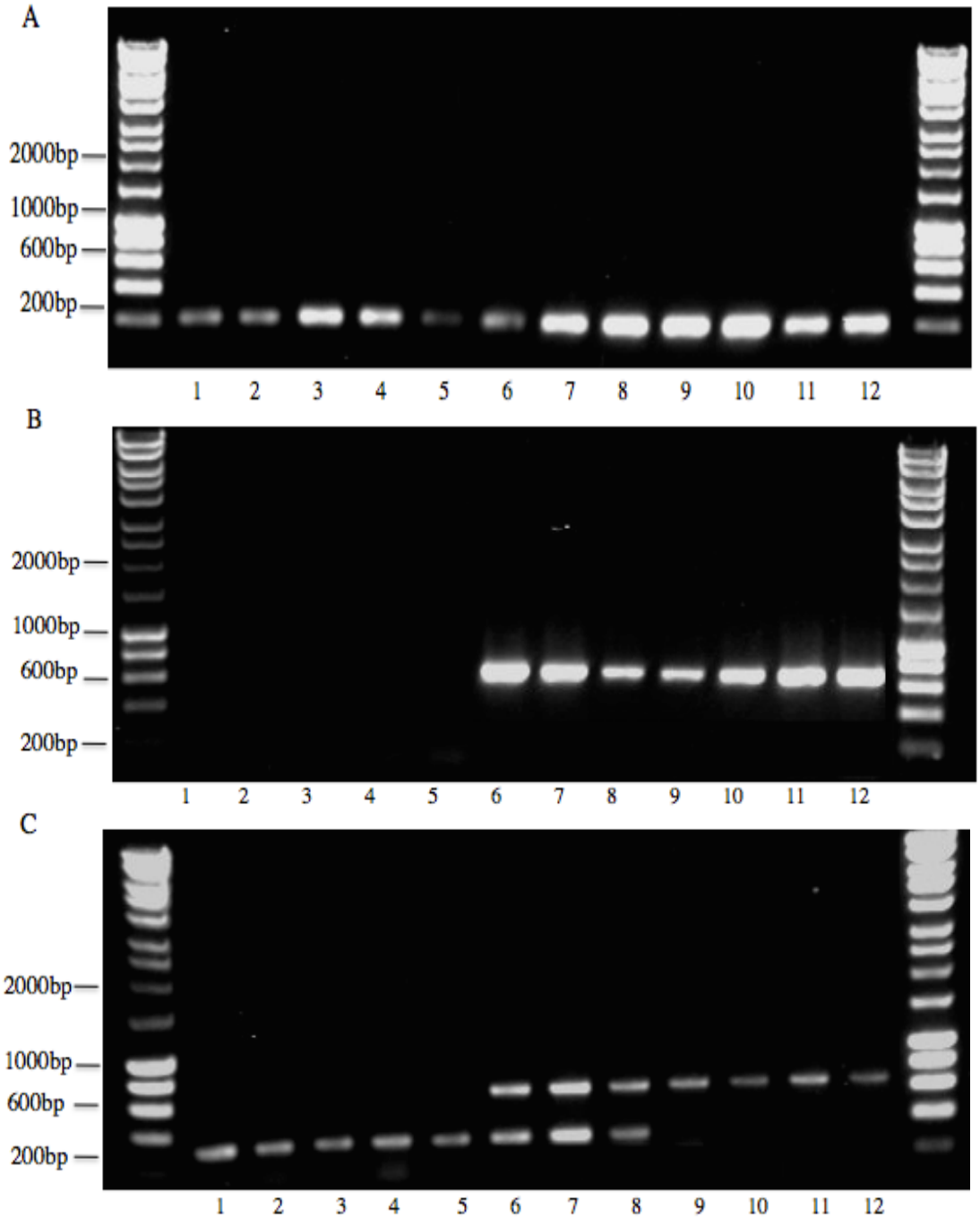


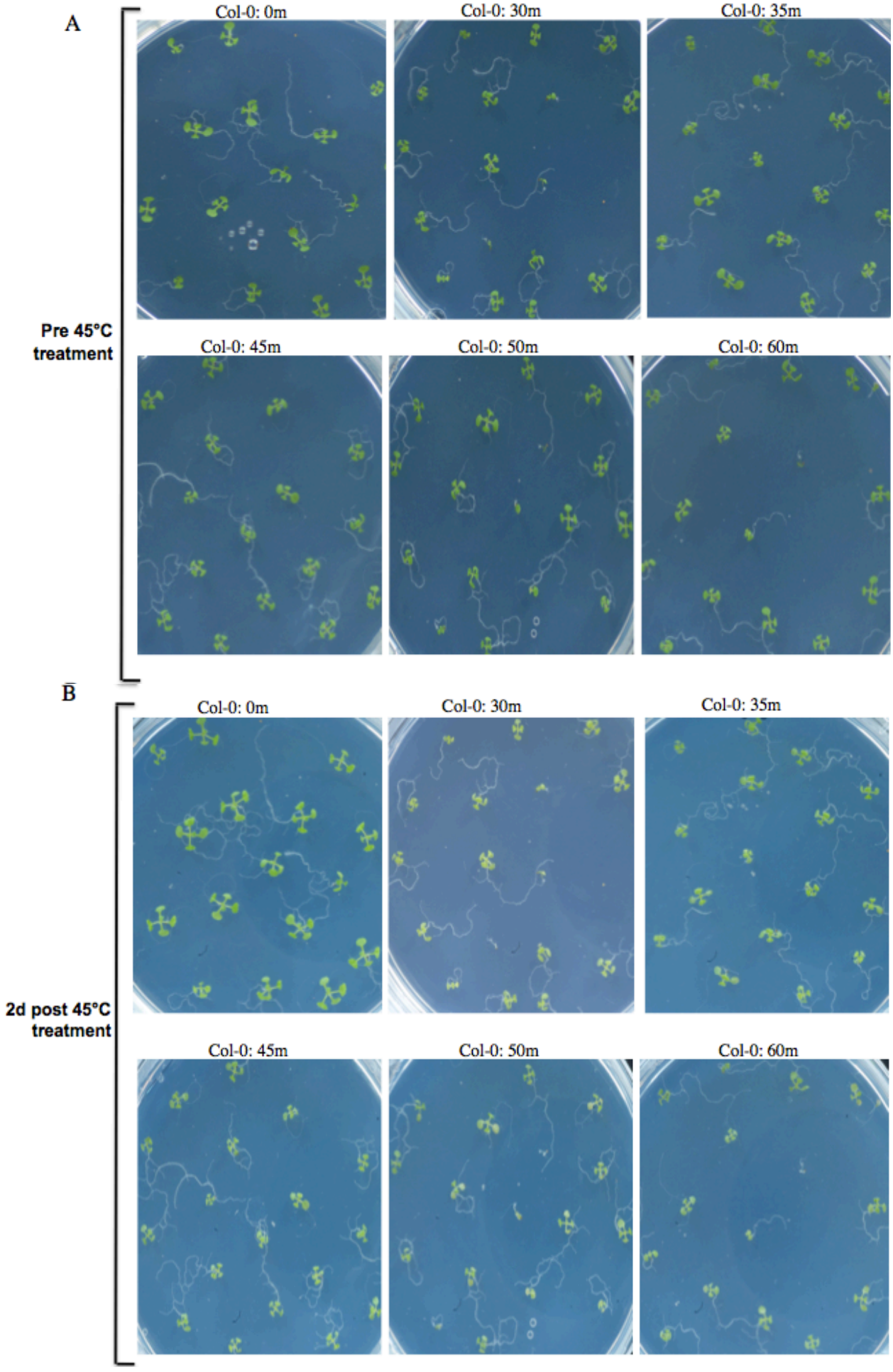
Figure 4.4: Identification of SALK_063386 WRKY26 mutants using PCR. *a. actin control to test cDNA fidelity b. WRKY26 gene presence in cDNA sample tested using 5' & 3' WRKY26 specific primers c. presence of t-DNA insertion tested using t-DNA specific left border primer and 5' & 3' specific WRKY26 primers.*

cDNA samples 1-5 depict homozygous individuals for the insertion; 6-8 heterozygous individuals & 9-12 lack the insertion and hence are wild type for the WRKY26 gene.

4.2.2 Assessment of Col-0 seedlings sensitivity to heat shock treatment of 45 ℃

Heat treatment of 45 °C has been previously described as a lethal shock if heat acclimation has not been pre-acquired (*Silva-Correia et al. 2014*), hence was chosen as a suitable temperature to test survival rates amongst seedlings at varying time points. Col-0 seeds were germinated on ½ MS plates and grown in long day conditions for twelve days, before being heat-treated for up to 1h. Survival rates were then assessed over the following six days. Time points of 30min, 35min, 45min, 50min and 60min are shown in *figure 4.5*.

The proportion of seedlings remaining green and hence photosynthetically viable is a simple measure of seedling survival, when compared to control plates, which are shown in *figure 4.5.a*. Two days post 45 °C heat treatment there is little obvious seedling death, as shown in *figure 4.5.b*. However, by day six seedling death is initiated; in seedlings heat shocked for 30min, the apex of some of the leaves had turned white, indicating a loss of photosynthetic ability. By 45min the loss is more noticeable, and by 50min all seedlings became completely unviable, *figure 4.5.c*. Repeats of this assay were carried out, with similar results obtained.


A
Col-0: 0m
Col-0: 30m
Col-0: 35m
Pre 45°C treatment
Col-0: 45m
Col-0: 50m
Col-0: 60m
B
Col-0: 0m
Col-0: 30m
Col-0: 35m
2d post 45°C treatment
Col-0: 45m
Col-0: 50m
Col-0: 60m

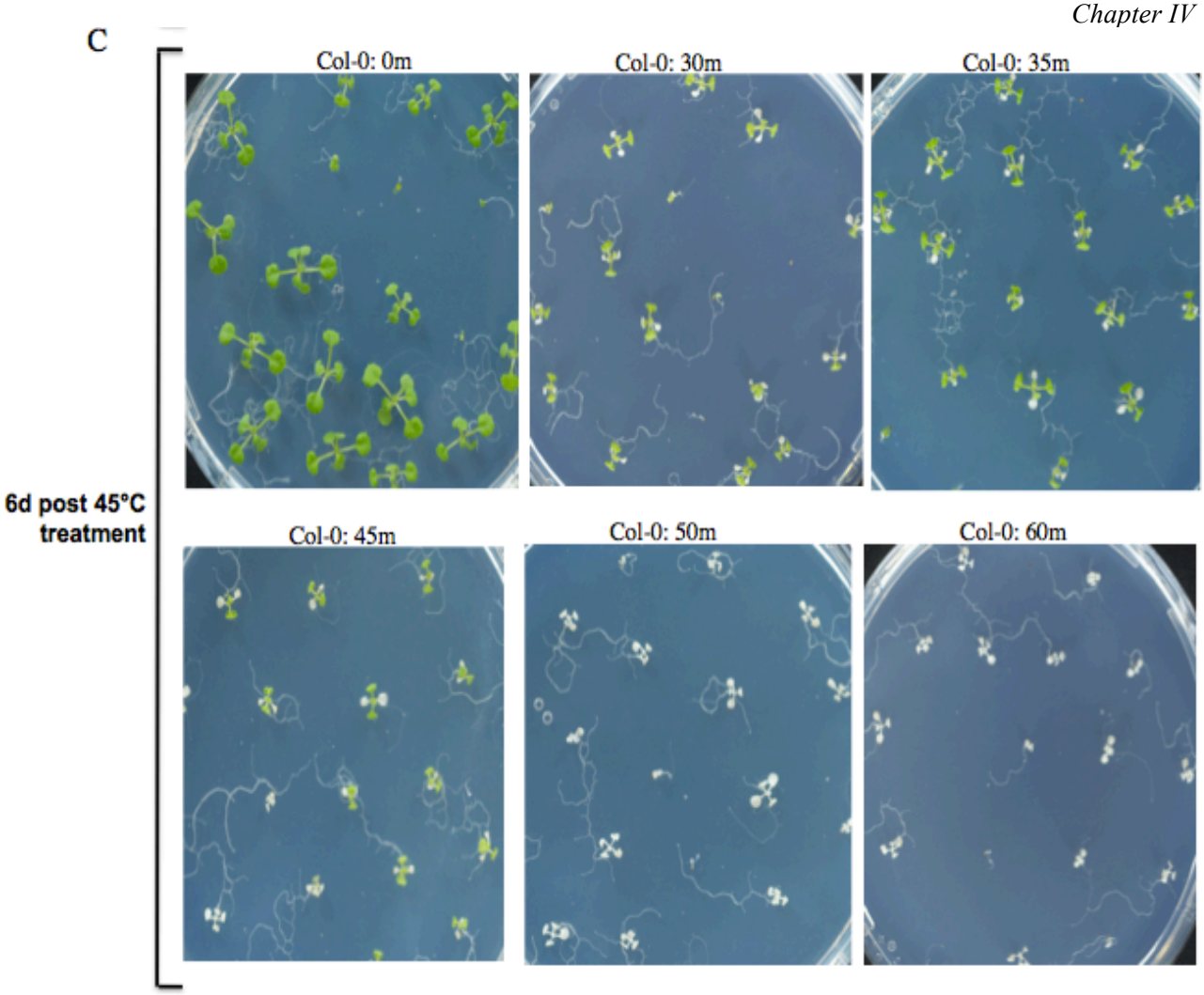


Figure 4.5: Thermotolerance of Col-0 seedlings. *Seedlings were germinated on ½ MS plates for 12 days before being heat shocked at 45 ℃ up to 1hour.* ***a****. pre-heat treatment control* ***b****. 2 days post heat-treatment &* ***c.*** *6 days post heat-treatment*

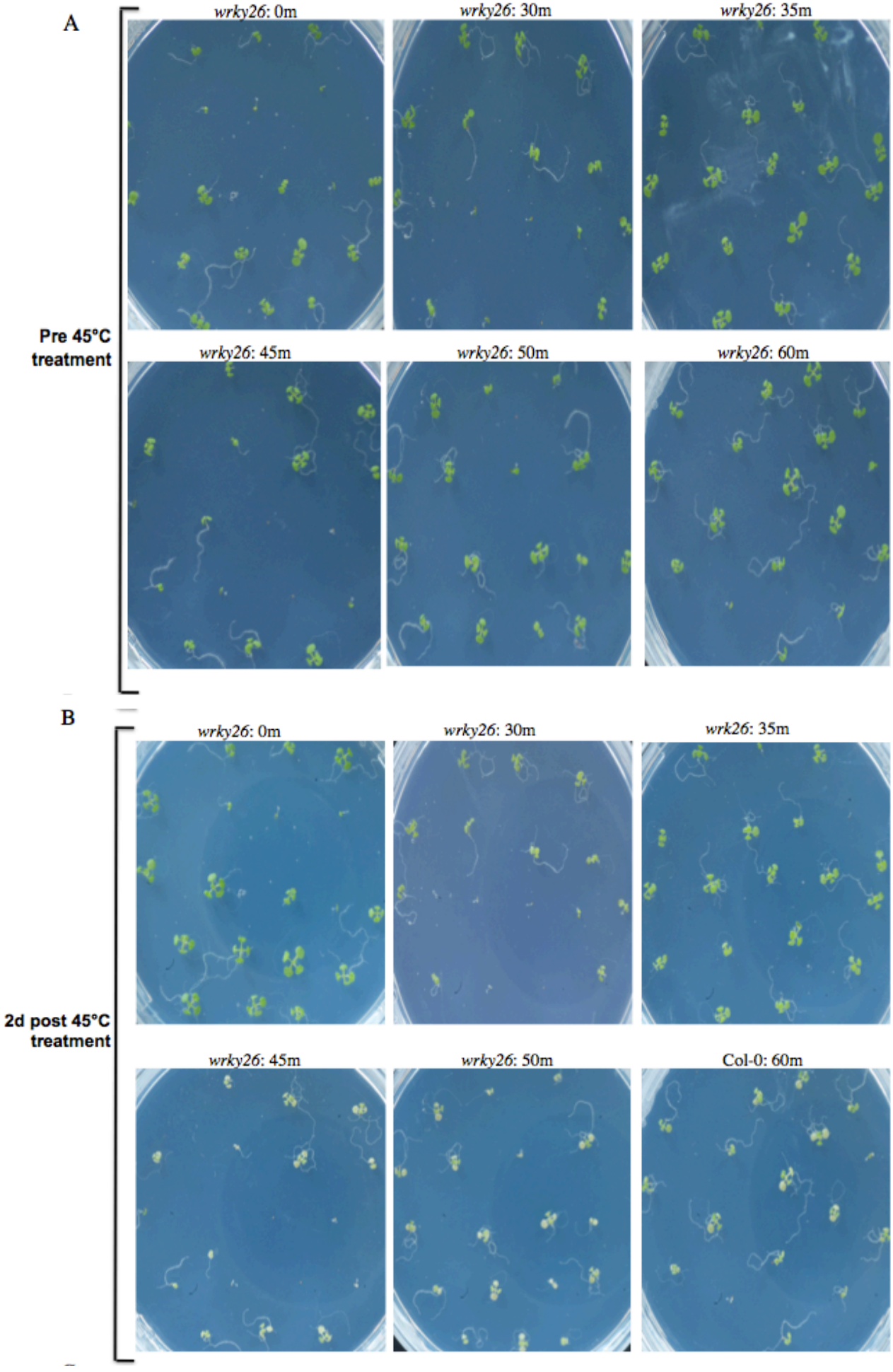
There is little obvious seedling death noticeable 2 days post treatment (figure 4.5.b). By day 6, seedling death is noticeable by 30min, with full loss of photosynthetic ability seen by 50min (figure 4.5.c).

4.2.3 SALK_063386 Atwrky26 mutants have a decreased tolerance to 45 ℃ heat shock treatment in comparison to wild type Col-0

As previously described (*Li et al. 2011*) the *WRKY26* T-DNA insertion SALK_137675 produced a null knockout that showed a decreased tolerance to high temperature stresses. This same phenomenon was demonstrated using the SALK_063386 line identified here as a *AtWRKY26* mutant. *Atwrky26* seeds were germinated on ½ MS plates and grown in long day conditions for twelve days, before being heat-treated for up to 1h. Survival rates were then assessed over the following six days and again time points of 30min, 35min, 45min, 50min and 60min are shown (*figure 4.6).*

Germination of *Atwrky26* mutants was unimpaired and seedling size was comparable to wild type. As can be seen in *figure 4.6.b*, the loss of green pigmentation is noticeable two days post treatment, especially 45min and after, this is in contrast with Col-0 seedlings where there was little evidence of impaired photosynthetic activity after two days. Six days post 45 °C treatment seedlings appear unviable starting from 30min, in a similar fashion to Col-0. By 45min all seedlings have lost photosynthetic ability, *figure 4.6.c*. As *wrky26* mutants show unviability earlier, in terms of both recovery time and the time point at which seedling death occurs, it can be concluded that these mutants show a decreased tolerance to heat shock treatment compared to wild type Col-0. Again, repeats were carried out showing similar patterns of thermotolerance.

One point of note, *AtWRKY26* gene knock outs were not verified using qPCR; hence for absolute surety that knock outs were indeed identified, additional study is required using qPCR to assess that no *AtWRKY26* gene transcription was present.


A
wrky26: 0m
wrky26: 30m
wrky26: 35m
Pre 45°C treatment
wrky26: 45m
wrky26: 50m
wrky26: 60m
B
wrky26: 0m
wrky26: 30m
wrk26: 35m
2d post 45°C treatment
wrky26: 45m
wrky26: 50m
Col-0: 60m

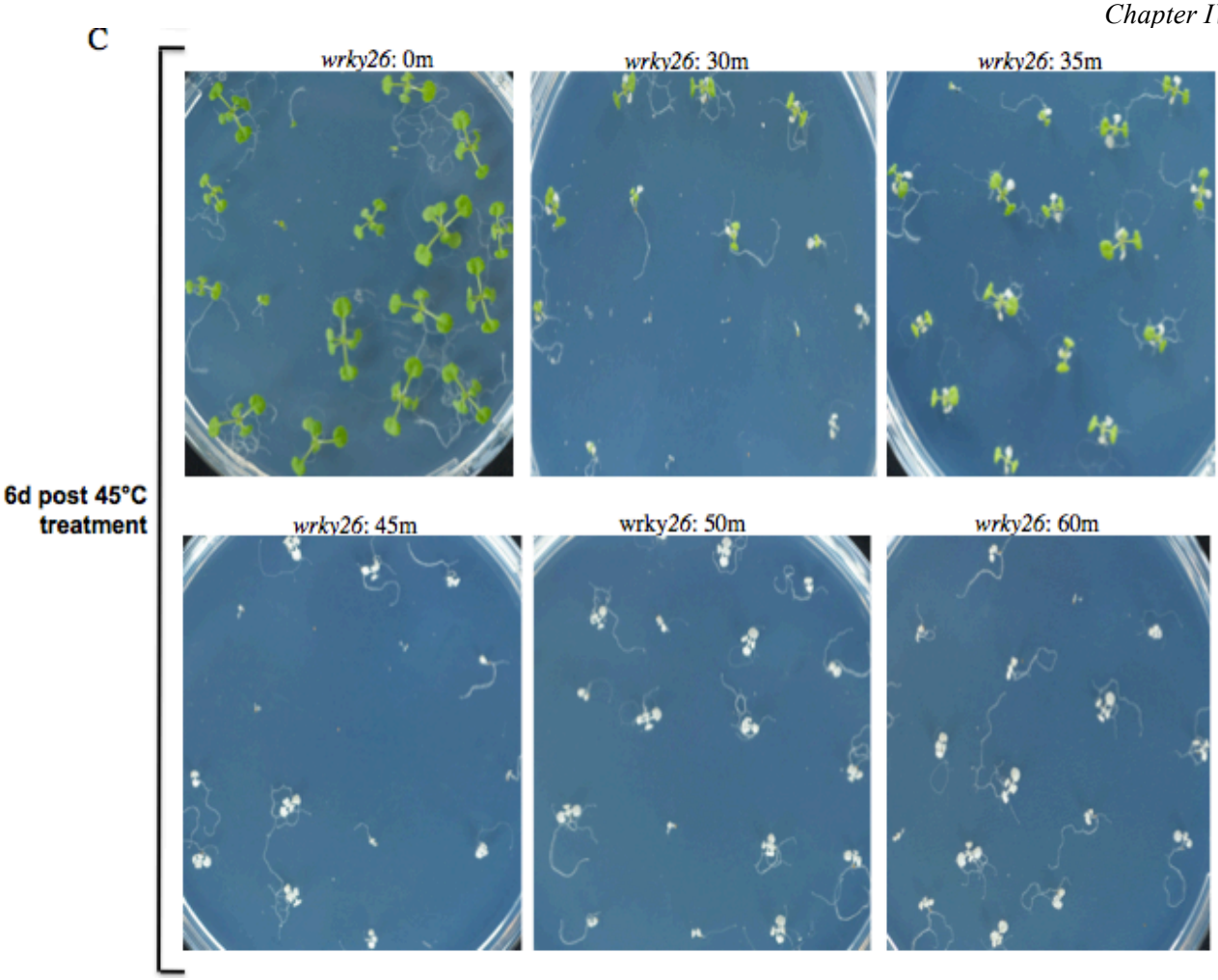


Figure 4.6: Thermotolerance of wrky26 seedlings. *Seedlings were germinated on ½ MS plates for 12 days before being heat shocked at 45 ℃ up to 1hour.* ***a****. pre-heat treatment control* ***b****. 2 days post heat-treatment &* ***c.*** *6 days post heat-treatment.*

Seedling death is noticeable 2d post treatment, with a loss of photosynthetic ability seen (figure 4.6.b). By day 6, seedling death is noticeable by 30min, with full loss of photosynthetic ability seen by 45min (figure 4.6.c).

4.2.4 qPCR analysis of heat shocked Col-0 and rga gai

The link between GA signalling and abiotic stress tolerance has been described, as mentioned within the initial chapter, with GA degradation resulting in DELLA protein accumulation (*Achard et al. 2008*) and hence growth attenuation. Additionally, the identified interaction between *AtWRKY26* and DELLAs poses a potential further link between stress and developmental responses. The idea that modulating either of these responses, with the aim to improve a plants response to abiotic stress, potentially via altering growth patterns, is an interesting area of research. With these points in mind, work carried out in this section was looking to see if there was a quantitative difference between *AtWRKY26* gene expression in Col-0 and the DELLA mutants, *rga gai,* when heat stress was applied.

Seedlings were heat shocked at 45 °C for 1 hour. Samples were immediately frozen for RNA extraction and subsequent cDNA synthesis. qPCR using standardised 200 μg/μl cDNA and data normalisation using Actin as an endogenous control was carried out. This latter step is imperative for gene quantification, and enables sample to sample errors between qPCR runs to be accounted for. For this, the C_t (threshold cycle; relative concentration of target gene in the qPCR reaction) value for the target gene sequence is divided by the C_t value derived from the control sequence, hence allowing direct comparison between values. qPCR analysis for the pre-treatment controls and post 45 °C heat shocked samples are shown in figures *figure 4.7* and *figure 4.8* respectively.

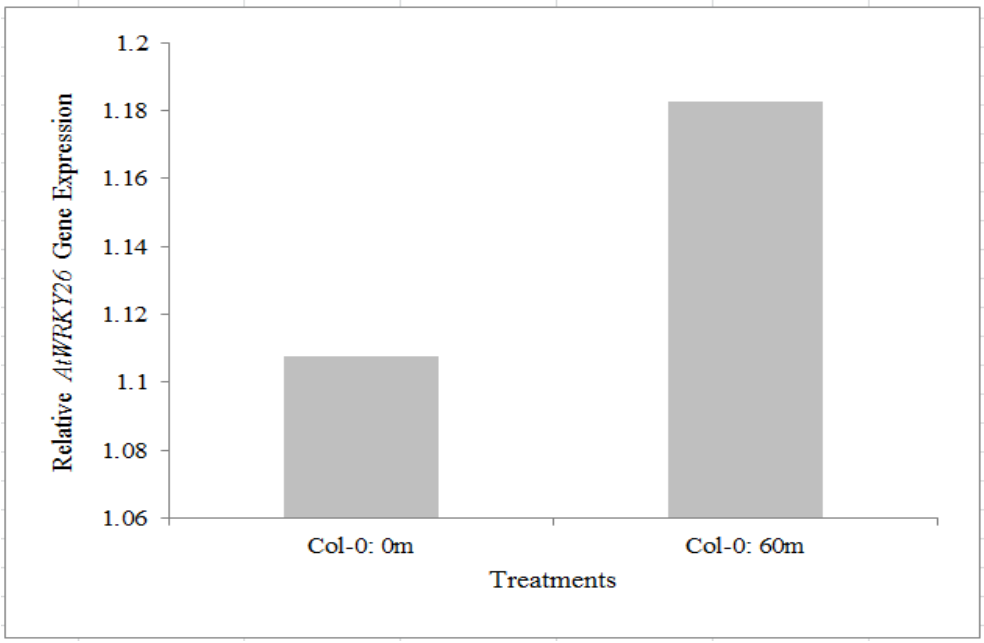


Figure 4.7: qPCR analysis of heat responsive AtWRKY26 expression in Col-0. *Total RNA was isolated from 1h 45 ℃ heat-treated leaves. Actin was used as an internal control for normalisation of gene expression.*

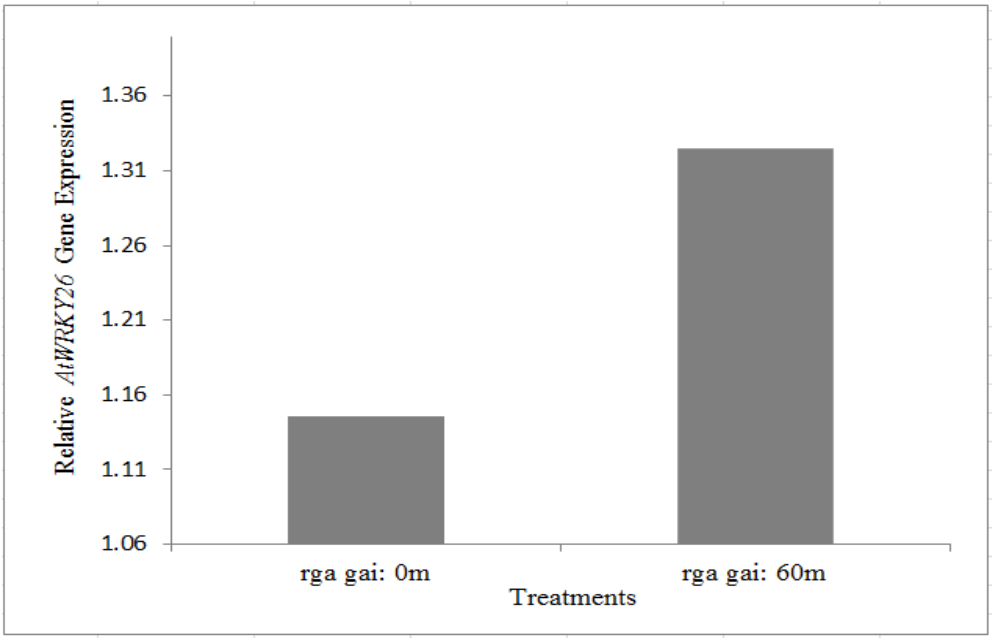


Figure 4.8: qPCR analysis of heat responsive AtWRKY26 expression in rga gai lines. *Total RNA was isolated from 1h 45 ℃ heat-treated leaves. Actin was used as an internal control for normalisation of gene expression.*

Firstly, the relative expression of *AtWRKY26* in the pre-treated Col-0 (*figure 4.7*), and *rga gai* controls (*figure 4.8*), exhibited very slight differences in expression profiles; 1.13 and 1.15 respectively. Secondly, the expression of *AtWRKY26* seems to increase upon 45°C treatment in both Col-0 and *rga gai*, with expression in the DELLA mutants greater than that of the wild type seedlings (1.18 compared with 1.32). However, there is no statistical significance in this data; usually a 2-fold difference in gene expression should be given as a minimum, which was not attained here. As previous studies have shown that an increase in *AtWRKY26* gene expression upon heat treatment is induced (*Li et al. 2011*), it was initially surprising that this was not found in this study also. I suggest that altering the thermotolerance assay would account for the lack of significance, and repeating this experiment using grown plants, rather than seedlings, would give a different result. However, due to time constraints, it was not possible to repeat this qPCR, or to utilise a different means of sample collection. Additionally, had there been statistical significance between, repeats of all qPCR would have been required for averages and error bars to be included for any true validation of results.

4.3 Discussion

4.3.1 AtWRKY26 knockouts can be used to assess gene function under stress conditions

After the identification of the interaction between *AtWRKY26* and DELLAs, work conducted in this chapter aimed to further elucidate the function of *AtWRKY26 in planta*, and to assess the potential role of the DELLAs in relation to this. Previous studies have shown that *AtWRKY26* positively regulates thermotolerance in *Arabidopsis* (*Li et al. 2011*). There has, however, been minimal additional evidence of the function of this protein, neither has the link between DELLAs and WRKY TF been explored previously.

The initial identification of *AtWRKY26* knockouts proved to be a difficult process. Although three independent SALK lines were obtained from NASC, of the eighty-three plants genotyped, only in one line, SALK_063386, were knock outs identified. These plants were identified using combinations of gene specific and SALK specific left border primers, of which homozygous, heterozygous and wild type individuals can be distinguished with such a combination (*figure 4.4.b & 4.4.c*). The ability to test potential gene function using knock outs such as these is a useful tool for elucidating function, enabling phenotype to be attributed to a specific genotype.

A preliminary thermotolerance assay for Col-0 enabled the subsequent tolerance of *wrky26* seedlings to be compared with wild type. As shown, the ability of seedlings to withstand such treatment, as prior pre-acclimated was not carried out, was dependent on the duration of 45 °C heat shock. Additionally, the days of recovery also highlighted differences, as little evidence of seedling death is evident two days post treatments, regardless of heat shock duration. Six days post treatments photosynthetic ability is

remarkably reduced in seedlings subjected to just 30min heat shock, with the leaf apex of the majority of seedlings turning white, indicating unviability. By 50min all seedlings have become unviable (*figure 4.5.c*).

The initial Col-0 thermotolerance assays allowed for direct comparison with the *Atwrky26* SALK mutants. Firstly it appears that *Atwrky26* succumb to heat shock at a faster rate than that of wild type (*figure 4.6.b*) with seedlings two days post heat shock exhibiting symptoms not shown in Col-0 (*figure 4.5.b*). Additionally, the time point after which all photosynthetic ability is initially lost is earlier than wild type (45min and 50min respectively) as shown in *figure 4.6.c*. This data indicates that SALK_063386 *Atwkry26* mutants, like the mutant described in *Li et al*., showed decreased tolerances to heat shock. However, due to difference in heat shock method (seedlings compared to established plants) it is difficult to make a quantitative direct comparison.

A point of note is the age of plants used in these assays. Seedlings were stratified and then germinated, with the approximate age of the seedlings, up to the point of heat shock, between 12-14 days. An interesting experiment would be to see if varying plant age would resulted in different tolerances to heat stress, both in seedlings on ½ MS plates, or fully grown plant moved on to soil. This could potentially show variation in how quickly the plant loses viability, or in how long high temperatures can be withstood.

Another potential variable exhibited with *Atwrky26* is the temperature ranges these mutants could withstand. Heat shock of 45 °C has been descried as a lethal shock (*Silva-Correia et al. 2014*); however percentage seedling survival in Col-0 lines decreases after 42 °C, indicating that wild type plants have a basal tolerance to heat shock up to this point. It would be interesting to see if *Atwkry26* mutants follow a

similar trend, and only decrease their viability after 42 °C, or indeed they succumb to temperatures lower than this. In other words, an assessment of the basal heat tolerance of these mutants would show if there was greater variability shown in this genotype. Additionally, a plant ability to withstand high, and indeed low, temperatures can be greatly improved if prior pre-acclimation is acquired; hence the ability of these mutants to withstand shock after pre-acclimation would also be an interesting point.

A final point in relation to thermotolerance assays is the survival patterns exhibited in both DELLA and GA mutant plants. Initially, both *rga gai* and *ga1-3,* double DELLA knock-outs and GA biosynthetic mutants respectively, were going to be assessed for thermotolerance in the same manner as Col-0 and the *AtWRKY26* mutants. Preliminary assays (*data not shown*) suggested that little difference between *rga gai* and Col-0; however insufficient time ultimately made this impossible to test further. Additionally, the actual germination of *ga1-3* mutants proved a difficult task. *ga1-3* require GA supplementation for germination, however the seeds obtained failed to germinate, regardless of GA concentration supplied. To fully assess the link between DELLA, GA and *AtWRKY26* it would be useful to perform a full thermotolerance assay with these mutants, in addition to the other lines screened.

4.3.2 qPCR analysis can be utilised to assess expression profiles of various lines after heat shock treatment

The final aim of work conducted in this thesis aimed to show whether the expression of *AtWRKY26* varied between Col-0 and *rga gai,* with the use of qPCR data to analyse this. The expression of *AtWRKY26* appeared to increase upon heat treatment, with the relative expression of *AtWRKY26* in *rga gai* mutants post heat shock apparently

greater than that of wild type plants. However, the statistical significance threshold was not reached, and hence it is not possible to conclude any difference is relative gene expression upon heat shock treatment. This experiment requires attention, with a different technique utilised, such as the use of fully-grown plants rather than seedlings. This would aim to clarify whether a tentative working hypothesis on the roles of DELLAs in the regulation of *AtWRKY26* is correct, namely that heat-activated *AtWRKY26* is being sequestered by the DELLAs. Clarification on the expression of the *rga gai* DELLA mutants, in comparison to Col-0, would initially give weight to this hypothesis, however further experiments are imperative.

In order to further test this hypothesis on the roles of DELLA-*AtWRKY26* interaction, I would propose a few simple additional experiments. Firstly, qPCR analysis of *AtWRKY26* in various additional DELLA lines post heat shock treatment could be performed. Although physical interaction between *AtWRKY26* and the three additional DELLA proteins; *AtRGL1*, *AtRGL2* and *AtRGL3*, has not been identified, it is likely that at least one of these DELLAs, if not all, interacts with *AtWRKY26*. With this in mind, it would be interesting to see the differences in gene expression in various combinations of these mutant lines. Secondly, the differences in relative gene expression in GA mutants, where DELLAs are maintained at low concentrations, would be useful to compare, especially if these expression profiles showed the opposing results to the DELLA mutants, i.e. down-regulation of *AtWRKY26* after heat shock. Finally, various Col-0 lines could be supplemented with various concentrations of GA pre-heat shock, with the aim to assess whether wild type lines can have altered expression profiles of *AtWRKY26*, modulated via GA-dependent degradation of DELLA proteins.

CHAPTER V:

FINAL CONCLUSIONS AND FUTURE WORK

5.1 Final conclusions

As described in chapter I, the role DELLA proteins play in the negative regulation of GA-dependent development has received a great deal of research focus. A functional DELLA domain, specifically the DELLA motif at the N-termini of the domain, is required for recognition via the GA receptor, GID1. Various signalling pathways ultimately result in the degradation of DELLAs via their poly-ubiquitination and subsequent degradation via the 26S proteasome (*Hochstrasser, 2000*). DELLA degradation results in the inhibitory effects of these proteins to be lifted and hence GA-dependent developmental responses to be initiated. Regulation is intrinsically linked with a plants ability to cope with stress, both biotic and abiotic. This field has becoming increasingly more relevant, with research focused on how plants modulate growth patterns to survive a perceived stress. GA biosynthetic mutants, such as *gai1-3,* have been utilised to demonstrate that plants with increased DELLA levels are better able to cope with osmotic stress compared to wild type plants (*Achard et al. 2006*). Additionally, the cross talk between various hormonal pathways has also been described in relation to abiotic stress tolerances. XERICO, a downstream target of the DELLAs, regulates both drought tolerances and ABA biosynthesis, hence forming a link between the antagonist hormonal pair, GA and ABA, and stress tolerances, in *Arabidopsis* (*Ko et al. 2006*).

WRKY transcription factors, as also described in chapter I, have been identified as major regulators of various plant processes, both developmental and stress induced, with the presence of WRKY TF in *Arabidopsis* particularly well characterised (*Ulker & Somssich, 2004).* WRKY TF induced regulation of both biotic and abiotic stresses is of particular interest, with roles in regulating biotic stress particularly well characterised (*Eulgem & Somssich, 2007*).

The aim of the work conducted for this thesis initially attempted to demonstrate that DELLA proteins and WKRY TF showed protein-protein interaction *in planta.* Preliminary screenings had indicated this likely to be the case (*Nelis et al unpublished data*), however the likely purpose of such interactions has yet to be identified. Additionally, certain studies have shown interaction between both WRKY TF and the rice DELLA protein, SLR1 (*Zentella et al. 2007*). Also, WRKY TF have been shown to regulate GA signalling (*Zhang et al. 2004*).

Initial screening conducted in this study used the Y-2H system to identify the interaction of *AtWRKY47*, *AtWRK72* and *AtWRKY26* with *AtRGA*. Weak interaction was exhibited in both *AtWRKY47* and *AtWRKY72 (figure 3.6)*. The interaction of *AtWRKY26* and *AtRGA*, as demonstrated in the Y-2H (*figure 3.7*), was relatively strong and uninhibited when 100 mM 3-AT, a competitive inhibitor of the *HIS3* reporter gene product, was included in the selection media. This interaction was also demonstrated *in plant*a via the use of Co-IP assays and subsequent western blotting (*figure 3.8)*. Interestingly, interaction with an additional DELLA, *AtGAI*, was also shown *in planta* (*figure 3.9*), posing the question if the three other Arabidopsis DELLAs showed such interaction with *AtWRKY26*. Furthermore, due to plasmid auto-activation, the interaction between *AtWRKY33* and *AtRGA* could not be shown *in vitro*; regardless an additional Co-IP assay does seem to show that this interaction occurs (*figure 3.14*). *AtWRKY33* is a relatively well studied transcription factor, with evidence to show that is involved in the regulation of both biotic stress responses, specifically against pathogen infection (*Mao et al. 2011*), and in the regulation of thermotolerance (*Li et al. 2011*). Further work on the function of this protein, specifically in relation to the role of the DELLAs, would be an interesting area of research.

Both DELLAs and the WRKY TF in question, *AtWRKY26*, have two protein domains. Ultimately, it proved interesting to assess which of these specific domains were interacting. Domain deletion techniques were utilised to bait single domains against each other, again using the Y-2H system to identify interaction. Data from the Y-2H seemed to indicate that both the *AtRGA* domains, the DELLA and GRAS domains, were interacting with the full length *AtWRKY26* protein (*figure 3.13*). Initially, it was hypothesised that the GRAS domain, involved in various processes from protein regulation to dimerization (*Bolle, 2004*), would be the site where interaction with the DNA binding domains of *AtWRKY26* would be localised. This was mainly hypothesised due to the limited evidence showing that the DELLA domain is involved in any additional processes, other than the regulation of GA signalling. Data presented here indicates the potential for DELLA interaction with both of these domains; additional work conducted *in planta*, such as the use of Co-IPs, would unequivocally prove this to be the case.

After demonstrating the interaction between *AtWRKY26* and *AtRGA*, both *in vitro* and *in planta*, the additional aim of work conducted for this thesis was to further elucidate the function of *AtWRKY26*, and to assess the potential regulation between *AtWRKY26* and DELLAs. One publication had demonstrated that *AtWRKY26* gene knockouts have a reduced thermotolerance (*Li et al. 2011*). Using an different SALK T-DNA insertion line to the one described in this publication, a decreased tolerance to 45 °C heat shock was also demonstrated in *Atwrky26* mutants (*figure 4.5* and *figure 4.6*)

The potential role of the DELLA proteins in the regulation of the *AtWRKY26* was also assessed using preliminary qPCR data, analysing the differences in relative gene expression in DELLA mutants and wild type. However, the design of these

experiments requires alteration before any conclusive evidence of altered gene expression can be obtained.

5.2 Future Work

Some proposed future experiments have been previously outlined within the discussion sections of both chapters III and IV. These mainly relate to further clarification of the data obtained, such as utilising a different mutational strategy for the domain analysis or repeating the various qPCR analyses.

One additional experiment not previously mentioned is to assess plant phenotype when *AtWRKY26* is overexpressed in wild type and various mutant lines. This experiment was initiated, however due to the time scales that such experiments require, it was not possible to obtain any workable data. The introduction of 35S:WRKY26 into various lines (Col-0, *SALK_wrky26, rga gai, gai1-5, 2ots)* was carried out via floral dip transformation. The overall aim was to see the effect of overexpression, and hence subsequently assess how DELLA levels are modulated in these plants compared to the norm. This, in conjunction with the other additional experiments previously proposed, would enable further clarification of the roles of the DELLA – *AtWRKY26* interaction. Furthermore, whether said interaction can be modulated to alter, and ultimately enhance, the stress responses exhibited in *Arabidopsis thaliana,* could be defined.

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Appendices I

List of buffers and reagents (Alphabetical order)

200mM Acetosyringone	Dissolve 0.03924g acetosyringone (Sigma) in 1ml DMSO (Sigma) Store aliquots in freezer
1M 3-AT	Dissolve 840mg 3-AT ( )in 10ml deionised H_2O Filter sterilize and store at -20°C
DEPC H_2O	Add 0.1% DEPC (Sigma) to 1L dH_2O Incubate overnight at RT and autoclave
10x DNA Loading Dye	Dissolved 0.025g of xylene cyanol and 0.025g of Bromophenol Blue in 6.25ml dH_2O Add 1.25ml 10% SDS and 12.5ml glycerol Store aliquots in the fridge
10mM dNTPs	Mix 10µl dATP, dCTP, dGTP, dTTP with 60µl DEPC H_2O. Store at -20°C
0.5M EDTA	18.6 g $EDTA.Na_2.2H_2O$ Add 80ml dH_2O Adjust to pH8 with 1M NaOH Adjust to a final volume of 100ml with dH_2O and autoclave
1/2 x Murashige & Skoog (MS) medium	Dissolve 2.15g MS powder (Melford) and 12g agar in 1L of dH_2O and autoclave

LB Agar	Dissolve 5g LB powder and 3g of agar (Melford) in 250ml dH_2O and autoclave
LB Media	Dissolve 5g LB powder (Fisher) in 250ml dH_2O and autoclave
Oligo dT	Mix 10µl Oligo dT with 90µl DEPC H_2O Store at -20°C
SD Base Agar	Dissolve 26.7g SD base (Clonetec) and 12g agar in 1L of dH_2O For double drop out add 0.64g –leu -trp (Clonetec) For triple drop out add 0.62g –leu –trp –his (Clonetec) Autoclave
4x SDS Loading Buffer	2ml 1M Tris-Cl pH 6.8 4ml glycerol 0.8g SDS 1ml 0.5M EDTA 0.4ml β-mercaptoethanol A touch of bromophenol blue Adjust to a final volume of 10ml with dH_2O and autoclave Store aliquot at -20°C.
10x SDS-PAGE Running Buffer	30.4g Tris 144g Glycine 20g SDS Adjust to 1L with dH_2O
1x SDS-PAGE	100ml 10x SDS-PAGE

Running Buffer	Adjust to 1L with dH_2O
SOC Media	2g trypton 0.5g yeast extract 0.2ml 5M NaCl 0.25ml 1M KCl 1ml of 1M $MgCl_2$ 1ml of 1M $MgSO_4$ 2ml of 1M glucose Adjust to 100ml with dH_2O and autoclave
1x TE Buffer	1ml 1M Tris pH8.0 400µl 0.25 M EDTA Adjust to 100ml with dH_2O and autoclave
10x Transfer Buffer	30.5g Tris base 144g Glycine Adjust to 1L with dH_2O
1x Transfer Buffer	100ml 10x Transfer 200ml Methanol Adjust to 1L with dH_2O
10x Tris-Buffered Saline (TBS)	Dissolve 60g Tris and 90g NaCl in 800ml dH_2O Adjust to pH7.4 with 5M HCl Adjust to a final volume of 1L with dH_2O
1x Tris-Buffered Saline with Tween20 (TBST)	100ml 10x TBS Adjust to 1L with dH_2O

	Add 1ml Tween20 (Fisher)
YPD Agar	Dissolve 50g YPD (Clonetec) and 12g agar in 1L dH_2O and autoclave
YPD Media	Dissolve 50g YPD in 1L dH_2O and autoclave
40mg/ml X-α-gal	Dissolve 120mg X-α-gal (Glycosynth) in 3ml DMF (Fisher)

Appendix II

Additional figures

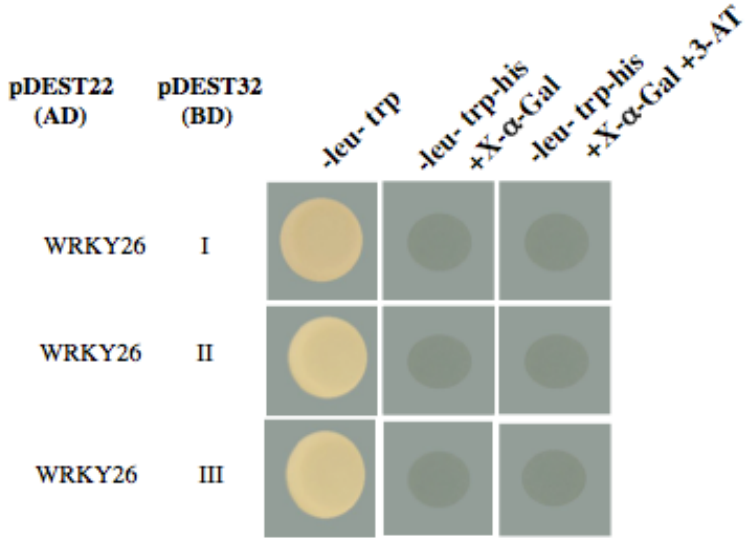

Figure 1: Y-2H for WRKY26-pDEST22 baited against mutant RGA-pDEST32. *Colony growth in triple DO selection was inhibited in this vector combination, regardless of 3-AT presence.*

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT

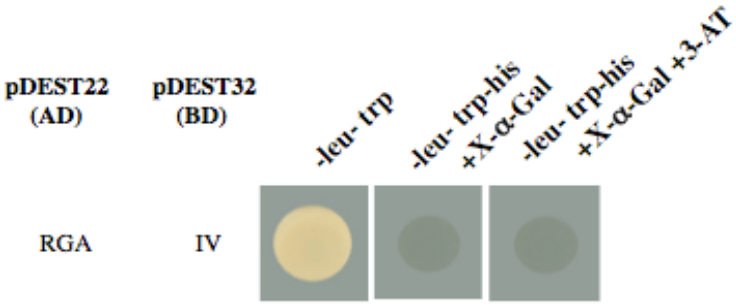

Figure 2: Y-2H for RGA-pDEST22 baited against mutant WRKY26-pDEST32. *Colony growth in triple DO selection was inhibited in this vector combination, regardless of 3-AT presence.*

– indicates empty vector. Triple DO –leu –trp –his plates supplemented with 40 mg/ml X-α-gal and 100 mM 3-AT