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Interactions of AtRGL1, a Negative Regulator of Gibberellic Acid Signalling

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Abstract

Arabidopsis thaliana AtRGL1 (repressor of *ga1-3* like-1) is a negative regulator of the signal transduction pathway of the plant hormone gibberellin. AtRGL1 belongs to the DELLA subfamily within the GRAS family of plant regulatory proteins. There are four other DELLA proteins, including AtRGA (repressor of *ga1-3*) and AtRGL2, encoded by the *A. thaliana* genome. Previous studies provided evidence that the DELLA proteins are nuclear localised and are functionally divided into N- and C-terminal domains. The N-terminal domain perceives the gibberellin signal, while the C-terminal domain functions as a negative regulator of transcription and also as a possible dimerisation domain. Previous studies have also shown that *AtRGA*, *AtRGL1*, and *AtRGL2* function together in the regulation of the development of the inflorescence and that *AtRGL1* is primarily expressed in this tissue.

To investigate how DELLA proteins function in gibberellin signalling, I sought plant proteins that interact with AtRGL1. Two proteins, p24 (24 kDa) and p64 (64 kDa), were isolated from wild-type plant nuclear extracts by affinity to the N-terminal 121 amino acid residues of AtRGL1. The identity of these two proteins remains to be established. To investigate the interactions of the C-terminal domain of AtRGL1 an anti-AtRGL1 polyclonal antiserum was developed for co-immunoprecipitation experiments. However, AtRGL1 was not detectable in plant nuclear extracts from the inflorescence of wild-type plants, precluding this approach.

The possibility of DELLA protein dimerisation was also investigated using AtRGA, AtRGL1, and AtRGL2 in yeast 2-hybrid experiments. Yeast 2-hybrid protein interaction results suggest that AtRGA, AtRGL1, and AtRGL2 do not form homo- or hetero-dimers. Complexities encountered with this approach could make these results invalid, so these interactions require further investigation.

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Abbreviations

3-AT	3-amino-1,2,4-trazole
ABA	Abscisic acid
AD	GAL4 activation domain
AMP	Ampicillin
$AmSO_4$	Ammonium sulfate
AP	Alkaline phosphatase
AtASK1	*A. thaliana* Arabidopsis Skp like-1
AtGAI	*A. thaliana* Gibberellic acid insensitive
AtGAMYB33	*A. thaliana* Gibberellic acid induced MYB-33
AtGAMYB65	*A. thaliana* Gibberellic acid induced MYB-65
AtGCR1	*A. thaliana* G-protein coupled receptor-1
AtGPA	*A. thaliana* G-protein α subunit
AtHDA19	*A. thaliana* Histone deacetylase-19
AtLFY	*A. thaliana* Leafy
AtLRP	*A. thaliana* Lateral root primordium
AtPKL	*A. thaliana* Pickle
AtrgaΔ17	*A. thaliana* Repressor of *ga1-3*, deletion of 17 amino acid DELLA motif
AtRGL1	*A. thaliana* Repressor of *ga1-3*-1
Atrgl1Δ17	*A. thaliana* Repressor of *ga1-3*-1, deletion of 17 amino acid DELLA motif
AtRGL2	*A. thaliana* Repressor of *ga1-3*-2
AtRGL3	*A. thaliana* Repressor of *ga1-3*-3
AtSHI	*A. thaliana* Short internodes
AtSLY1	*A. thaliana* Sleepy-1
AtSLY2	*A. thaliana* Sleepy-2
AtSNY	*A. thaliana* Sneezy (AtSLY2)
AtSPY	*A. thaliana* Spindly
BD	GAL4 DNA binding domain
BnSCL1	*B. napus* Scarecrow like-1
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
c-Myc	Mammalian c-Myc oncogene epitope

Co-IP	Co-immunoprecipitation
Col-0	Columbia-0
DAPI	4',6-Diamidino-2-phenylindole
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GA	Gibberellic acid
GAL4	GAL4 transcription factor
GARE	Gibberellic acid responsive element
GFP	Green Fluorescent Protein
HA	Influenza hemagglutin epitope
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGP	Heterotrimeric G-protein
HRP	Horseradish peroxidase
HvCDPK1	*H. vulgare* Calcium dependent protein kinase-1
HvGAMYB	*H. vulgare* Gibberellic acid induced MYB
HvHSIMYB	*H. vulgare* Spindly interacting MYB
HvHSINAC	*H. vulgare* Spindly interacting NAC
HvSAD	*H. vulgare* Sad
HvSLN1	*H. vulgare* Slender-1
HvSPY	*H. vulgare* Spindly
IgG	Immunoglobulin G
Imidazole	1,3-Diaza-2,4-cyclopentadiene
IPTG	Isopropylthio–β-D-galactoside
KAN	Kanamycin
Ler-0	Landsberg *erecta*-0
MALDI TOF	Matrix assisted laser desorption ionisation time of flight
MBP	Maltose-binding protein
miR159	Micro ribonucleic acid 159
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid

MWCO	Molecular weight cut-off
NHS	*N*-hydroxysuccinimide
NLS	Nuclear localisation signal
O-GlcNAc	*O*-linked *N*-acetyl glucosamine
ONPG	*O*-Nitrophenol-galactoside
OsD1	*O. sativa* Dwarf-1
OsDOF3	*O. sativa* DNA binding with one finger-3
OsGID2	*O. sativa* Gibberellic acid insensitive dwarf-2
OsSLR1	*O. sativa* Slender rice-1
p24	24 kDa AtRGL1-interacting protein
p53	Murine p53
p64	64 kDa AtRGL1-interacting protein
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Poly-ethylene glycol
PMSF	Phenylmethylsulphonylfluoride
SCF	Skp-Cullin-F-box
SD	Synthetic dropout
SDS	Sodium dodecyl sulfate
StPHOR1	*S. tuberosum* Photoperiod-responsive-1
T antigen	SV40 large T antigen
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween-20
Tris	Tris (hydroxymethyl) aminomethane
TrxA	Thyrodoxin domain A
UV	Ultra violet

Note on Nomenclature

Gene names and symbols are written in *italics*, and proteins are in standard text. Wild-type genes and products are in uppercase, mutants are in lower case. For plant species, the two letters preceding a gene/protein refer to the species. At, *Arabidopsis thaliana*. St, *Solanum tuberosum*. Hv, *Hordeum vulgare*. Os, *Orysa sativa*. Bn, *Brassica napus*.

Chapter 1. Introduction

1.1 Introduction to plant hormones

Plant hormones are small organic molecules that regulate the growth and development of plants. There are several types of plant hormones, and these mediate plant growth and environmental responses differently. One type of plant hormones are the auxins, which are produced in young leaves, seeds, and the root cap. Auxins regulate the growth and differentiation of many plant tissues; promoting growth and development of flowers, fruit, stems and leaves, and inducing root growth when at low levels.

Abscisic acid (ABA) is a plant hormone that is responsible for dormancy of seeds and floral buds. ABA functions during seed development to promote protein storage and seed dormancy. Stored proteins required for germination cannot be released until ABA is overcome by drying, moist chilling, and light.

Ethylene is plant hormone that is unusual in that it is a gas. Ethylene production occurs during fruit ripening, and is also induced by auxin and by environmental stresses. Ethylene promotes fruit ripening, and also the growth of roots and the formation of root hairs.

The Gibberellins are a large family of tetracyclic diterpenoids, named gibberellic acids (GAs). There are several forms of gibberellic acids, including inactive precursors, active GAs, and catabolically inactivated products. Active GAs perform a role during the germination of seeds, opposing the dormancy due to ABA; they also promote cell elongation important for root growth, stem growth, and floral tissue development. The focus of this thesis is the interactions of the *Arabidopsis thaliana* protein AtRGL1 (Repressor of *ga1-3* like-1). This protein belongs to the DELLA protein family that is central to the mechanism by which GA regulates plant development.

1.2 Gibberellins act through a signal transduction pathway.

Although there is significant knowledge of the biosynthetic pathway for GA production and inactivation (reviewed by Hedden and Kamiya, 1997), the means by which GA induces cell elongation and differentiation is poorly characterised. The current experimental evidence has established that the response of plant cells to GA involves a signalling pathway that alters gene regulation, ultimately resulting in changes in cell shape and fate.

Mutations of several genes encoding putative GA signalling components have been identified in many plants, including the model dicot *A. thaliana*, and the monocots, rice (*Orysa sativa*) and barley (*Hordeum vulgare*). Several commercial crop varieties termed the 'green revolution' lines, due to increased crop yields, carry mutations in GA signalling components including the DELLA proteins. These mutations cause reduction in GA responses resulting in dwarfed phenotypes (Figure 1-1) that cannot be corrected by exogenous GA treatment (insensitive to GA), suggesting that the wild-type gene product is a component of GA signalling. Other mutants have constitutive GA responses, but not elevated GA levels; these plants have a slender phenotype (Figure 1-1). This also implies that the wild-type gene product is involved in GA signalling.

A proposed model for GA signalling, as shown in figure 1-2, involves active GA perception at the plasma membrane by a receptor protein. The perception of GA results in secondary cytoplasmic signalling. Secondary signals enter the nucleus where they result in degradation of the DELLA proteins. DELLA proteins normally repress GA-induced responses by transcriptional regulation until they are degraded on GA signalling. Therefore, DELLA proteins are central to the regulation of plant development by GA, and this is why intense research effort has been concentrated on them in recent years.

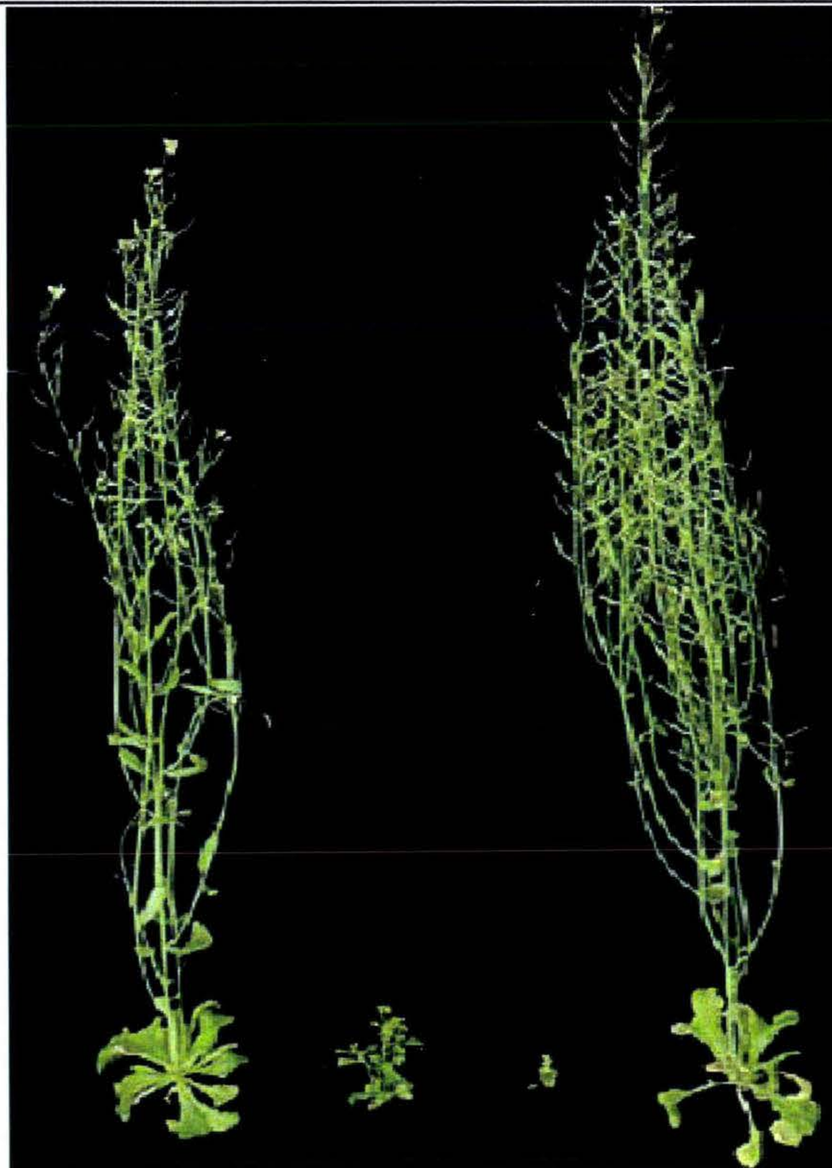


Figure 1-1 Phenotypes of *A. thaliana* GA signalling mutants. The dwarf plant has a gain-of-function mutation in a GA signalling pathway component, the DELLA gene *AtGAI* (*Atgai-1*) (GA-insensitive). The severe dwarf is a transgenic line over-expressing a gain-of-function mutant form of a second DELLA gene *AtRGL1* (*Atrgl1Δ17*) (deletion of 17 amino acid DELLA motif). The slender plant is the same transgenic line as the severe dwarf, but has undergone gene silencing of the overexpressed mutant *Atrgl1Δ17* and endogenous wild-type *AtRGL1* genes. (Wen and Chang, 2002).

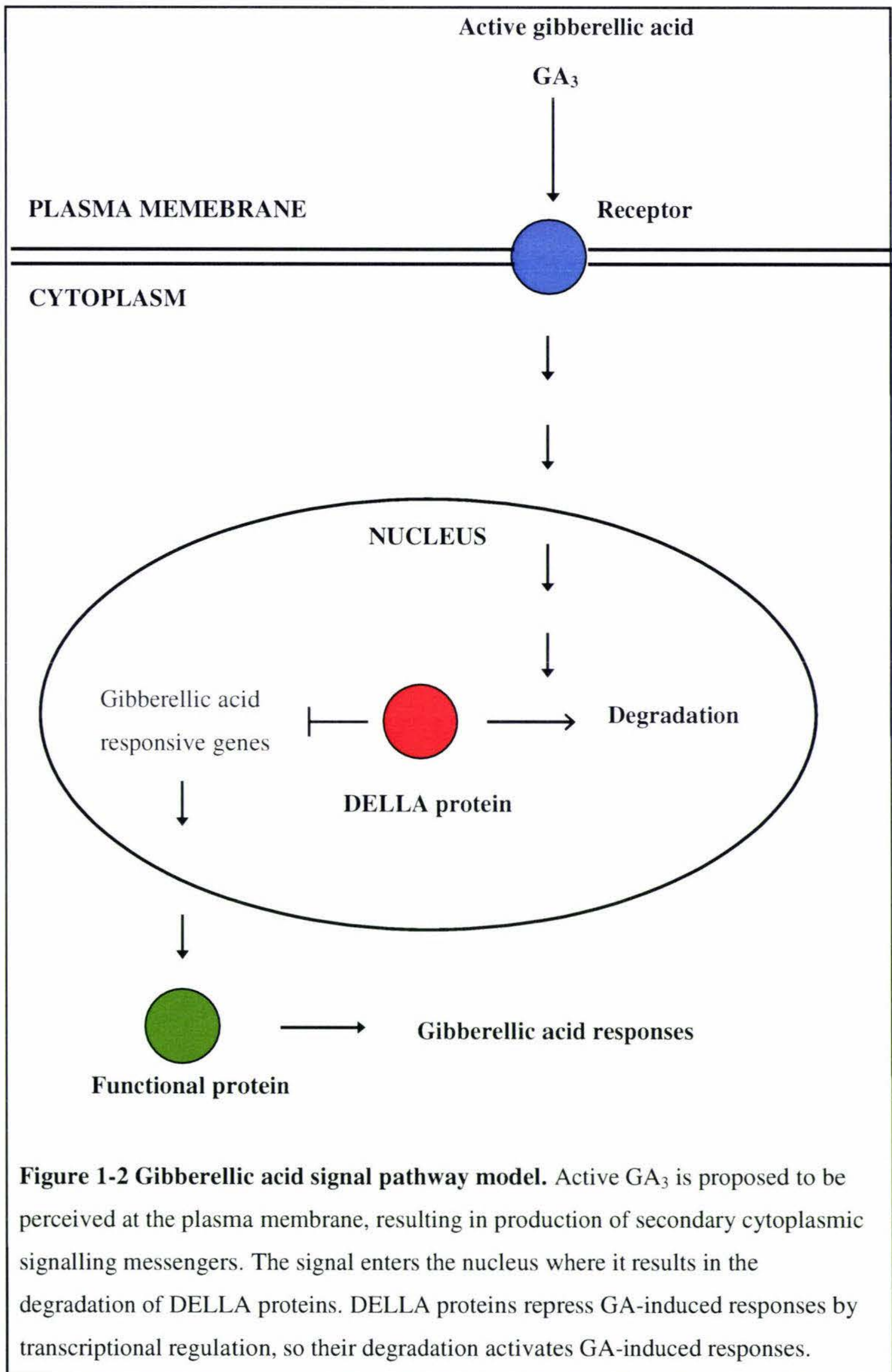


Figure 1-2 Gibberellic acid signal pathway model. Active GA_3 is proposed to be perceived at the plasma membrane, resulting in production of secondary cytoplasmic signalling messengers. The signal enters the nucleus where it results in the degradation of DELLA proteins. DELLA proteins repress GA-induced responses by transcriptional regulation, so their degradation activates GA-induced responses.

1.2.1 Production of gibberellic acids

It has been demonstrated using gas chromatography, a technique that can detect and measure different classes of GA, that GA production is limited to developing tissues (Ogawa *et. al.,* 2003). However, this method cannot be used to detect the exact cell types that produce GA, due to the inability to test specific cell types and layers within organs. A more accurate mapping of GA synthesis has been performed by Kaneko *et. al.* (2003). This group used promoter:GUS reporter lines to examine spatial expression of the enzyme GA3-oxidase, required for the final step of GA_3 (active GA) synthesis. Histochemical analysis of GUS expression and *in situ* hybridization of mRNA transcripts allowed precise localisation of the expression of the enzyme. Interestingly, the GA3-oxidase transcript co-localised with the expression of GA-inducible genes. This suggests that active GA is produced in, or close to the tissues where it functions to produce growth responses.

1.2.2 Cell perception of gibberellic acids

Despite years of research, a GA receptor has not yet been unequivocally identified. GA-binding proteins have been located both at the plasma membrane, and in the cytoplasm, but there is no further evidence that any of them function as receptors in GA signalling. Aleurone tissue in seeds contains stored nutrients that are released during GA-dependent germination. A study performed with GA-coated sepharose beads showed that these beads were able to stimulate aleurone cells, indicating a cell surface receptor (Lovegrove *et. al.*, 1998). Another study also concluded that the GA perception site is extracellular, as microinjection of GA_3 into aleurone cells could not activate α amylase expression while extracellular application could (Gilroy *et. al.*, 1994).

Rather than a cell surface receptor perceiving active GA, it may be possible that GA perceived at the cell surface is covalently modified and transported into the cell. A covalent modification on entering the cell may also be required for activation of GA to induce GA-dependent responses. Studies by Schneider *et. al.* (2000) show that GAs are modified to form glucosides and glucosyl ethers in the plant. They also showed

that an inactive precursor, GA_{20}, as an *O*-glucoside, can be taken up and metabolised to GA_1 and GA_{29} that still have the *O*-glucoside attached (Schneider *et. al.*, 2000). This has been proposed as a mechanism for storage of GAs, but it also could be a mechanism for cell uptake and activation. More research is required before either hypothesis can be supported.

Genetic studies suggest that a heterotrimeric G protein (HGP) may have a role in GA signalling (Ueguchi-Tanaka et. al., 2000). Analyses of completely sequenced genomes of *O. sativa* and *A. thaliana* have identified only one putative HGP complex made of an α, a β, and two γ subunits (Kato *et. al.*, 2004). This is in contrast to mammals whose genomes encode for many different heterotrimeric G-protein complexes (reviewed by Offermanns, 2003). Genetic studies indicate that the HGP functions in almost every plant hormone signalling pathway (Ullah *et. al.*, 2001). *OsD1* (dwarf1) is a rice gene encoding the α subunit of the HGP (Ueguchi-Tanaka *et. al.*, 2000). The *OsD1* mutant, *Osd1*, has a GA-insensitive dwarf phenotype (Ueguchi-Tanaka *et. al.*, 2000). This shows that the HGP is involved in the response to GA, possibly GA signalling, as the phenotype suggests it is essential for GA-induction of responses. HGP may not be required in all GA-induced responses, as Nanjo *et. al.* (2004) have shown that some GA-induced genes are still GA upregulated in the *Osd1* mutant, while others are not. How the HGP may function, and its role in GA signalling is yet to be shown.

A putative plasma membrane receptor from *A. thaliana*, AtGCR1 (G-protein coupled receptor-1), has been implicated in GA signalling, but has not been shown to bind GA (Colucci *et. al.*, 2002; Chen *et. al.*, 2004). AtGCR1 is a seven transmembrane receptor homologue localised to the plasma membrane of epidermal cells and vascular tissue (Chen *et. al.*, 2004). AtGCR1 has been shown to interact with AtGPA1 (G-protein α subunit), the α subunit of the HGP in *A. thaliana*. However, epistatic analyses suggest that AtGCR1 can also function in GA signalling independently of the HGP (Pandey and Assmann *et. al.*, 2004; Chen *et. al.*, 2004).

This evidence suggests that GA may be perceived at the plasma membrane by a receptor:G-protein complex, but no physical GA binding has been shown to AtGCR1

or the HGP. All the published results together suggest that there may be multiple mechanisms for perception of GA, rather than one simple GA receptor. Hormone receptors for auxins and mammalian steroid hormones are nuclear proteins, so it may be possible that a GA receptor could be located in the nucleus in a similar fashion (Dharmasiri *et. al.,* 2005; Kepinski and Leyser, 2005; Shank and Paschal, 2005).

1.2.3 Cytoplasmic secondary messengers

Several secondary effectors, including cGMP (cyclic guanosine monophosphate), Ca^{2+} and Calmodulin, have been implicated in GA signalling in the cytoplasm. The levels of cGMP were shown to rise upon GA treatment of aleurone. This cGMP increase was demonstrated to be required, but not sufficient for GA-dependent induction of the target genes (Penson *et. al.*, 1996). Gomez-Cadenas *et. al.* (2001) showed that application of the Guanylyl cyclase inhibitor LY83583 that lowers cGMP levels, prevented induction of GA-inducible genes. This evidence suggests that the GA signal in aleurone involves cGMP.

The rising of the cytoplasmic Ca^{2+} concentration and activation of Calmodulin have been implicated in GA signalling. Calmodulin mRNA and protein concentration were shown to be rapidly increased upon GA signalling, but by definition this is an indirect induction as it requires *de novo* protein synthesis (Schuurink *et. al.*, 1996). It is likely that Calmodulin is required for later GA-dependent responses. The cytoplasmic concentration of Ca^{2+} in aleurone cells greatly increases on GA treatment and this is required for activation of GA-induced genes, suggesting its rising cytoplasmic concentration is important to the GA signalling process (Gilroy, 1996). One cytoplasmic kinase HvCDPK1 (Ca^{2+} dependent protein kinase-1) has been shown to mediate GA regulation of vacuolar functions, via Ca^{2+}; however, its effect is down stream of transcriptional regulation, so it is part of the GA response, not GA signalling (McCubbin *et. al.*, 2004).

Most of this evidence points to Ca^{2+} and Calmodulin having roles in GA responses and not GA signalling. It is possible that Ca^{2+} is involved in both GA signalling, and in assisting GA-induced responses.

It is possible that other secondary messengers including glucose are involved in GA signalling. However, there is no experimental evidence directly implicating them as important components of the GA signal pathway as yet. α amylases are a GA-induced gene family that encode similar enzymes required for seed germination, and Glucose has been shown to inhibit their GA-induction (Loreti *et. al.*, 2000). The GA-induced gene *HvGAMYB* (GA-induced MYB) is not downregulated by glucose (Loreti *et. al.*, 2000). *HvGAMYB* is upstream of α amylase in GA signalling; suggesting that glucose induced repression of α amylase is independent of the GA signalling pathway (discussed section 1.2.5).

There is currently no evidence for how the GA signal enters the nucleus. Signal transport may involve cGMP and Ca^{2+} concentrations, but it is probable that a protein signal cascade is involved in conjunction. Experiments performed by Gubler *et. al.* (2002) suggest that a signal transduction pathway including protein kinases and phosphatases is involved in GA signalling. This supports the hypothesis that signalling involves more than rising Ca^{2+} and cGMP concentrations, but to date only one cytoplasmic protein has been implicated in GA signalling, the protein StPHOR1.

The StPHOR1 (photoperiod-responsive-1) protein of potato (*Solanum tuberosum*) was identified to be translocated from cytoplasm to nucleus upon GA signalling (Amador *et. al.*, 2001). Downregulation of StPHOR1 expression by antisense mRNA produces GA resistant dwarf plants (Amador *et. al.*, 2001). StPHOR1 has homology to an ARM repeat protein, Armadillo, from Drosophila. Armadillo is a regulator of gene expression involved in segment polarity and tissue differentiation in Drosophila, so if there is functional conservation between invertebrates and plants, StPHOR1 may regulate gene expression on GA signal perception (Amador *et. al.*, 2001). *StPHOR1* was shown to be expressed in most tissues, and is also upregulated during Short Days. StPHOR1 has been suggested to be a positive regulator of GA signalling and may be a U-box protein, a class of proteins that little is known about (Monte *et. al.*, 2003). U-box proteins may act as E4 ubiquitin ligases that bind ubiquitinated proteins and aid their poly-ubiquitination. Alternatively, they may act as an E3 ubiquitin ligase involved in targeted protein degradation. GFP-StPHOR1 (Green fluorescent protein fusion) acquires nuclear localisation upon GA perception, making it a possible

candidate for a GA signal carrier from cytoplasm to nucleus. There are three *A. thaliana StPHOR1* homologues, *AtHIM1, 2, 3* (also known as *AtPUB28, 29, 27*) (Monte *et. al.*, 2003). Therefore, the function of StPHOR1 may be conserved between plant species.

In summary, many cytoplasmic signalling factors have been implicated in GA signalling, but how the signal enters the nucleus has not yet been shown. It is possible that a protein such as StPHOR1 enters the nucleus after being altered in the cytoplasm on GA signalling. Alternatively GA may enter the nucleus directly or bound to a carrier protein. Other small molecules, such as Ca^{2+} or cGMP may also be the messengers that carry the GA perception information into the nucleus.

1.2.4 Nuclear molecular switch

A family of proteins named 'DELLA' after a conserved amino acid sequence is central to nuclear GA signalling. DELLA proteins act as negative regulators and block the GA-induced gene expression until GA signalling occurs. This family of proteins and their role in GA signalling is discussed in detail in latter sections of this chapter.

The first DELLA protein that was discovered in *A. thaliana* was AtGAI (GA-insensitive). The mutant *Atgai-1* is a gain-of-function mutant that results in dwarf plants that are insensitive to exogenous GA_3 treatment (Koornneef *et. al.*, 1985). The *A. thaliana* genome encodes five DELLA proteins AtGAI, AtRGA (repressor of ga1-3), AtRGL1, AtRGL2, and AtRGL3 (Dill and Sun, 2001; Lee *et. al.*, 2002; Wen and Chang, 2002). The gain-of-function mutant of *AtGAI*, *Atgai-1*, lacks the 17 amino acid conserved 'DELLA' motif that defines the DELLA protein family (Peng *et. al.*, 1997). Mutations of the DELLA motif of *AtRGA* and *AtRGL1* produce similar phenotypes to that of *Atgai-1* (Silverstone *et. al.*, 1997; Wen and Chang, 2002). The DELLA family is a subfamily of a large protein family named the GRAS proteins (after GAI, RGA, and Scarecrow), that are putative transcriptional regulators unique to plants (Pysh *et. al.*, 1999). GFP fusions of DELLA proteins have revealed that DELLA proteins are localised exclusively to the nucleus (Dill *et. al.*, 2001; Fleck *et. al.*, 2002; Wen and Chang, 2002).
Another nuclear factor implicated in GA signalling is a putative F-box protein. F-box

proteins are a component of a SCF (Skp-Cullin-F-box) E3 ubiquitin ligase complex that targets specific proteins for degradation by the proteasome (reviewed by Kipreos and Pagano, 2000). *Osgid2-1* (GA-insensitive dwarf-2) is a severely dwarfed rice mutant that is insensitive to GA treatments, and the normal gene product is a putative F-box protein (Sasaki *et. al.*, 2003). The *A. thaliana* homologue of *OsGID2* is *AtSLY1* (Sleepy-1). Loss-of-function *Atsly1* mutants are GA insensitive dwarf plants, suggesting that the normal gene product is required for GA signalling (McGinnis *et. al.*, 2003). *AtSLY1* also has a functionally redundant homologue *AtSLY2* (also known as Sneezy, *AtSNY*), though this gene is normally expressed at very low levels and so does not compensate for null alleles of *AtSLY1* unless overexpressed (Strader et. al., 2004). The function of AtSLY1 and OsGID2 is discussed in detail latter in section 1.4, pertaining to degradation of DELLA proteins.

Other nuclear proteins have been implicated in GA signalling, and these may have roles in alterations to the functions of the DELLA proteins. Tall slender plants result from loss-of-function mutations of *AtSPY* (Spindly) (Swain *et. al.*, 2001). In *Atspy* mutants most of the normally GA-dependent developmental processes become constitutive and GA-independent (Swain *et. al.*, 2002). Overexpression of *AtSPY* interferes with seed germination, suggesting that *AtSPY* may block the GA signal (Swain *et. al.*, 2002). *AtSPY* is normally expressed in all tissues of the plant, with protein present predominantly in the nucleus but also in the cytoplasm (Swain *et. al.*, 2002). AtSPY is a putative *O*-GlcNAc (*O*-linked *N*-acetyl glucosamine) transferase predicted to add GlcNAc moieties to serine and theronine residues.

Based on epistatic analysis and the hypothesised *O*-GlcNAc activity of *AtSPY*/AtSPY, it has been proposed that AtSPY acts to repress the GA-induced responses by protection of possible phosphorylation sites on the DELLA proteins. However, there is no direct evidence to show that DELLA proteins are covalently modified with *O*-GlcNAc moieties. HvSPY has been shown to bind to two transcription factors in barley, HvHSINAC (SPY interacting NAC class transcription factor), and HvHSIMYB (SPY interacting MYB class transcription factor) (Robertson, 2004). *HvHSINAC* is down-regulated on GA signalling, while HvHSIMYB binds to the promoter of an α amylase gene (a GA-inducible gene) as a complex with HvSPY and

represses gene expression (Robertson, 2004). This suggests that HvSPY may act at several points of the GA signalling pathway.

AtSHI (Short internodes) is a gene of *A. thaliana* that is implicated in GA signaling. A 35S:GUS fusion of functional *AtSHI* demonstrated a GA-insensitive dwarf phenotype, suggesting that the AtSHI protein acts to repress GA signalling (Fridborg *et. al.*, 1999; Fridborg *et. al.*, 2001). *AtSHI* is normally expressed in most tissues at a low level, but is upregulated in root and shoot primordia (Fridborg *et. al.*, 2001). Null mutants have a normal phenotype, possibly due to genetic/functional redundancy. Fridborg *et. al.* (2001) describe *AtSHI* belonging to a family of at least 9 genes, including *AtLRP* (Lateral root primordium). The proteins encoded by genes in this family contain a putative Zn^{2+} binding RING finger motif similar to a mammalian protein, COP1, but with several significant differences in the domain (Fridborg *et. al.*, 2001). COP1 functions as a component of the SCF E3 ubiquitin ligase in mammals that targets proteins for degradation (Seo *et. al.*, 2003). This suggests that AtSHI may be part of an SCF E3 complex, possibly including AtSLY1, involved in GA signalling. However, overexpression results indicate that AtSHI may interfere with the function of an SCF E3 complex, though this could be an artifact of overexpression. AtSHI and its homologues carry a C-terminal domain named IGGH that is unique to plants, and so are named IGGH proteins (Fridborg *et. al.*, 2001).

Induction of GA responses involves gene regulation, but none of the above nuclear proteins currently implicated in GA signalling are suggested to have DNA binding activity. Therefore, it is unclear as to how they function to regulate gene expression of target genes.

A putative chromatin-remodeling factor, encoded by *AtPKL* (Pickle), has been implicated in GA signalling (Ogas *et. al.*, 1999). However, due to the possible involvement of *AtPKL* in multiple pathways it is difficult to tell how it may function in GA signalling. AtPKL may be recruited to target genes to induce repression, but this still leaves the DNA binding protein that is required for targeting unknown.

1.2.5 Gene regulation and cell responses

The proposed GA signal transduction pathway is suggested to result in plant growth responses by altering gene expression of target genes. This likely involves the expression of key transcriptional regulators that each alters the expression of several down stream genes that encode plant structural and enzymatic proteins. It is believed that DELLA proteins repress the transcription of the primary key transcriptional regulators, preventing expression of the downstream targets.

In the aleurone tissue of seeds the effect of GA is to release nutrients for the seed to germinate. The enzymes required for this effect are α-amylases, which are not expressed until after initiation of GA signalling (Kaneko *et. al.*, 2002). However, in other tissues expression of different genes is required for different GA responses. In the inflorescence the floral identity gene *AtLFY* (Leafy) is required to be expressed for floral development (reviewed in Yanofsky, 1995). This gene is normally activated under long day light conditions, but is also activated by GA under short day conditions (Wilson *et. al.*, 1992; Blázquez *et. al.*, 1998). Interestingly most α-amylases and *AtLFY* contain GA responsive elements in their promoter regions, and a MYB class transcriptional activator binds to these, as discussed below.

HvGAMYB from *H. vulgare* is a transcription factor of the MYB family that is upregulated by active GA (Gubler *et. al.*, 2002). It was identified as having GA-induced expression in barley aleurone cells by measuring mRNA levels from plants treated with GA, or the GA biosynthesis inhibitor Paclobutrazol (Gubler *et. al.*, 2002). HvGAMYB has been shown to bind to the promoter of α-amylase genes and activate their expression. The DNA binding site of HvGAMYB is a consensus sequence GATTGACTTGACC determined *in vivo* and *in vitro*, termed the GA response element (GARE), (Gubler *et. al.*, 1995). AtGAMYB33 was shown to bind to a GARE present in the promoter of *AtLFY* (Blázquez and Weigel, 2000; Gocal *et. al.*, 2001)

HvGAMYB, and also AtGAMYB33 and AtGAMYB65 (two of several *A. thaliana* homologues), have been shown to be required for GA-dependent floral development (Murray *et. al.*, 2003; Millar *et. al.*, 2005). Millar *et. al.* (2005) looked at the localised

expression of *AtGAMYB33* and *AtGAMYB65* by histochemical analysis of promoter:GUS fusions. These authors also localised the expression of the intact (wild-type) proteins in the inflorescence, to specific cell types, the same that were found to be sites of active GA production (Millar *et. al.*, 2005). There were complexities with GUS reporter constructs having slightly different localisation if fused to the normal gene coding sequence. This suggests that in addition to the promoter, some internal elements of the MYB genes are involved with their regulation.

Achard *et. al.* (2004) investigated the role of miRNA in GA signalling. They showed that a miRNA, named miR159, is conserved among plants and targets a conserved region of the GAMYBs mRNA. They also showed that GA upregulates miR159 by opposing DELLA function; this seems to be the opposite of what would be expected, but it is possible that this is a negative feed-back loop contributing to gauging GA-induced gene expression (Achard *et. al.*, 2004). The miR159 is also possibly upregulated by GAMYB itself, as the promoter of the precursor gene contains putative GAMYB binding elements (Achard *et. al.*, 2004). Another possibility is that the miR159 may function to regulate tissue specificity of expression of GAMYB rather than level, as proposed by Millar *et. al.*, (2005), who analyzed the distribution of the GAMYB mRNA in tissue sections, as opposed to whole tissue analysis carried out previously (Achard *et. al.*, 2004).

Additional transcription factors have been implicated in the regulation of GA-induced genes. HvSAD and its homologue OsDOF3 (DNA binding with one finger-3) have been shown to be bound in complex with GAMYB to the α amylase promoter as co-activators (Isabel-LaMoneda et. al., 2003; Washio, 2003; Diaz et. al., 2005). Additional activators and repressors of the expression of α amylase genes have been identified. One of them is HvHRT, a putative zinc finger (DNA binding domain) protein that may repress α amylase gene expression (Raventos et. al., 1998). Another transcription factor, OsWRKY71, was identified to repress α amylase gene expression, and is degraded on GA signalling (Zhang *et. al.,* 2004). Some of these factors may regulate GA-induced gene expression independently, but in addition to, the function of the DELLA proteins in GA signalling, such as OsWRKY71 (Zhang *et. al.,* 2004).

1.2.6 Regulation of gibberellin responses by other plant hormones

There is evidence that other plant hormones affect plant responses to GA. In *H. vulgare* aleurone cells it has been shown, by monitoring α amylase mRNA levels, that ABA opposes GA to counteract germination (Gubler *et. al.*, 2002). Overexpression of *HvPKABA1*, which encodes an ABA-induced protein kinase, blocks GA-induction of *HvGAMYB* gene expression (Gomez-Cadenas et al 2001). This experimental evidence suggests the possible mechanism by which ABA opposes GA signalling.

Auxins regulate GA responses, possibly by promoting GA signalling. In *A. thaliana,* low auxin levels from the shoot apex are required for growth of roots. GA is also required for proper root growth, but the full effect of GA on roots requires auxin (Fu and Harberd, 2003). GA responses in roots are regulated by the DELLA genes *AtGAI* and *AtRGA*, as root growth is GA independent in null mutants of these genes (Fu and Harberd, 2003). Experiments using GFP-AtRGA fusions showed that the gain in root growth-promoting effect of GA by auxin acts in part by auxin enhancing GA-induced responses via de-stabilisation of AtRGA (Fu and Harberd, 2003), i.e. auxin increases GA-induced degradation of DELLA proteins.

In *A. thaliana,* the hormone ethylene may interfere with the GA signaling pathway for apical hook formation and root growth. Ethylene competes against GA signalling, inhibiting the elongation involved in apical hook formation on one side of the stem (Vriezen *et. al.*, 2004). Ethylene was shown to interfere with GA signalling by stablising DELLA proteins, again using GFP-DELLA fusion proteins (Achard *et. al.*, 2003; Vriezen *et. al.*, 2004).

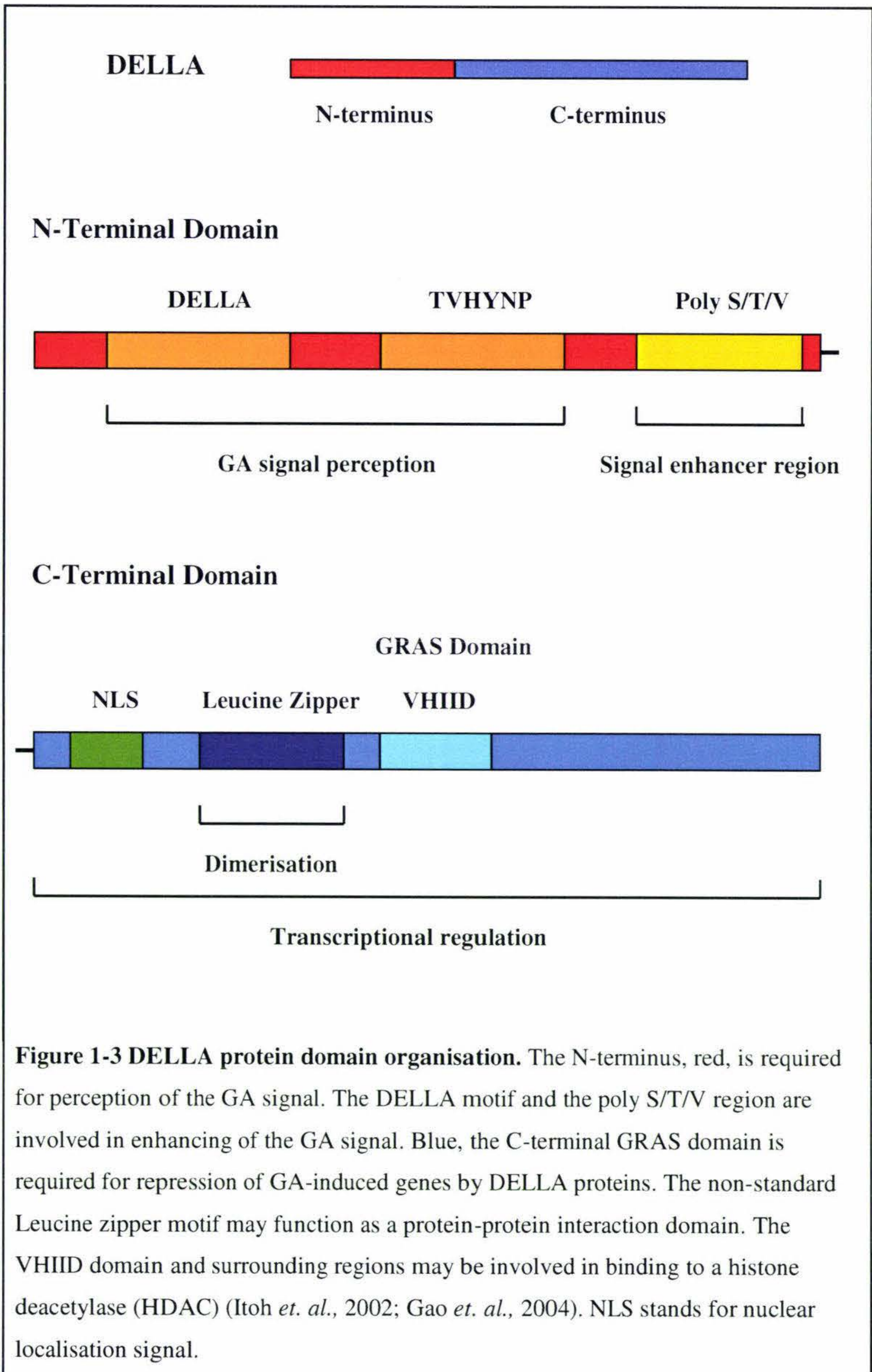


Figure 1-3 DELLA protein domain organisation. The N-terminus, red, is required for perception of the GA signal. The DELLA motif and the poly S/T/V region are involved in enhancing of the GA signal. Blue, the C-terminal GRAS domain is required for repression of GA-induced genes by DELLA proteins. The non-standard Leucine zipper motif may function as a protein-protein interaction domain. The VHIID domain and surrounding regions may be involved in binding to a histone deacetylase (HDAC) (Itoh *et. al.*, 2002; Gao *et. al.*, 2004). NLS stands for nuclear localisation signal.

1.3 Organisation of DELLA proteins

The DELLA subfamily has a unique N-terminal region, including two conserved motifs, DELLA, and TVHYNP (Figure 1-3). Based on genetic and structure-function analyses, distinct functions were assigned to the N- and C-terminal domains of DELLA proteins. DELLA proteins appear to repress GA-dependent genes through its C-terminal domain, whereas the N-terminal located DELLA motif perceives the GA signal that inactivates the repressor function.

Monocots and some dicots have only one DELLA gene. Rice has *OsSLR1* (Slender rice-1), and barley *HvSLN1* (Slender barley-1) (Itoh *et. al.*, 2002; Gubler *et. al.*, 2002). Deletion analysis of *OsSLR1* by Itoh *et. al.* (2002) showed that the DELLA and TVHYNP motifs and a non conserved spacer region between them are all required for GA signal perception as mutations resulted in GA-insensitive plants. Deletion of a region rich in serine and theronine residues (poly S/T/V) results in dwarf plants, but these plants are sensitive to GA. Therefore, the poly S/T/V region likely perceives regulatory signals that aid GA signalling, so this region contributes to the ability of DELLA proteins to respond to the perceived GA signal.

Mutations of the C-terminal domain of OsSLR1 resulted in recessive slender phenotypes, corresponding to constitutive GA responses (Itoh *et. al.*, 2002). Therefore, the C-terminal portion is a repressor of GA responses, presumably acting at the level of gene expression. A non-standard Leucine zipper sequence in the C-terminal domain of OsSLR1 may act as a dimerisation region, as has been suggested by the results of yeast 2-hybrid assays (Itoh *et. al.*, 2002). Results with GFP fusions also suggest that this region is involved in GA signalling as well as DELLA repressor function as OsSLR1 protein lacking the Leucine zipper is not degraded on GA treatment, while the plants had slender phenotype (Itoh *et. al.*, 2002).

DELLA proteins contain no typical DNA binding motifs, and have not been shown to interact with DNA. Hence, DELLA proteins may repress transcription indirectly by recruiting or modifying other DNA binding proteins, or they may contain a yet uncharacterised DNA binding domain.

OsSLR1 contains two putative nuclear localisation signals (NLS). Deletion of either putative NLS in GFP-SLR1 fusions revealed that neither affected nuclear import. A double NLS deletion was not performed (Itoh *et. al.*, 2002), so both NLS may be functional, or neither required for nuclear import. The DELLA protein AtRGL1 does not contain a putative NLS, yet GFP fusions are exclusively nuclear localised (Wen and Chang, 2002). This suggests that AtRGL1 has an atypical NLS, or that DELLA proteins may have an additional method to enter the nucleus.

Another protein of the GRAS family, BnSCL1 (from *Brassica napus*), which is involved in response to the hormone auxin, has been shown to bind to histone deacetylase (HDAC) AtHDA19 (Gao *et. al.*, 2004). Histone deacetylases act in complexes to modify histones, resulting in gene repression (reviewed by Li *et. al.*, 2002). Interestingly, the AtHDA19-binding region was narrowed down to being the Leucine zipper and the VHIID motif, which are conserved in GRAS family proteins, including the DELLA proteins (Gao *et. al.*, 2004). As described previously, genetic analysis implicates this region in the repressor function in DELLA proteins (Itoh *et. al.*, 2002). Therefore, GRAS (and DELLA) proteins may function as part of a complex containing a histone deacetylase to repress gene expression of target genes.

1.4 Degradation of DELLA proteins

DELLA proteins act as negative regulators of GA signalling, and must be inactivated for GA responses to occur. To establish if DELLA proteins are degraded on GA signalling, several experiments with both GFP fusions and native proteins have been performed. Experiments with GFP fusions have shown that GFP-AtRGA, AtGAI-GFP, and OsSLR1-GFP all rapidly disappeared from plant nuclei on treatment of plants with exogenous GA_3 (Dill *et. al.*, 2001; Itoh *et. al.*, 2002; Fu *et. al.*, 2004). However, the GFP-AtRGL1 protein was shown to not be degraded on GA treatment, and the AtGAI-GFP fusion was very resistant to GA-induced degradation (Fleck and Harberd, 2002; Wen and Chang, 2002; Fu *et. al.*, 2004). This discrepancy points to the drawbacks of GFP fusions, as the fusion proteins do not always behave as the native protein. Phenotypical analyses of the lines were performed, but *A. thaliana* has five DELLA proteins with partially overlapping functions. Due to this, it may be possible that phenotypes were masked or misinterpreted. For those GFP fusions that

were degraded on GA_3 treatment, degradation was reported to begin within 30min of treatment, and to be complete within 4 hours (Dill et. al., 2001).

An alternative method to GFP fusions was to monitor the endogenous DELLA proteins. Using western blotting, it has been shown that resident AtRGA, AtGAI, and AtRGL2 proteins of *A. thaliana* are degraded upon GA signalling (Dill *et. al.*, 2004; Tyler *et. al.*, 2004). These DELLA proteins are in fact are only detectable in null mutants of GA biosynthesis (*Atga1-3*) or the F-box protein-encoding loss-of-function mutant whose product is implicated in DELLA protein degradation, *Atsly1-10* (Dill *et. al.*, 2004; Tyler *et. al.*, 2004). In rice, the single DELLA protein OsSLR1 is detected at a low level in wild-type plants, and is also rapidly degraded on treatment with GA_3 (Sasaki *et. al.*, 2003). In agreement with these findings, the gain-of-function mutant proteins of AtGAI (Atgai-1) and AtRGA (AtrgaΔ17, deletion of the 17 amino acid DELLA motif) which cannot perceive the GA signal are not degraded on GA signalling (Dill *et. al.*, 2004; Fu *et. al.*, 2004). It was also observed that the *Atgai-1* encoded mutant protein had two forms with different molecular weights depending on the phosphorylation state (Fu *et. al.*, 2004).

Genetic analysis of mutants in GA signalling implicated the involvement of an F-box protein, AtSLY1, and the rice homologue OsGID2 (Steber *et. al.*, 1998; McGinnis *et. al.*, 2003; Sasaki *et. al.*, 2003). F-box proteins are a family of proteins with a conserved F-box domain and are involved in the recruiting of proteins to an SCF E3 complex (Figure 1-4) (reviewed by Hershko and Ciechanover, 1998; Kipreos and Pagano, 2000). E3 SCF complexes are one type of E3 ubiquitin ligases that function to covalently modify recruited proteins, adding a poly-ubiquitin chain. Proteins with poly-ubiquitin chains are tagged for degradation by a large protein complex, the proteasome. In this way F-box proteins recognise specific proteins and target them for degradation.

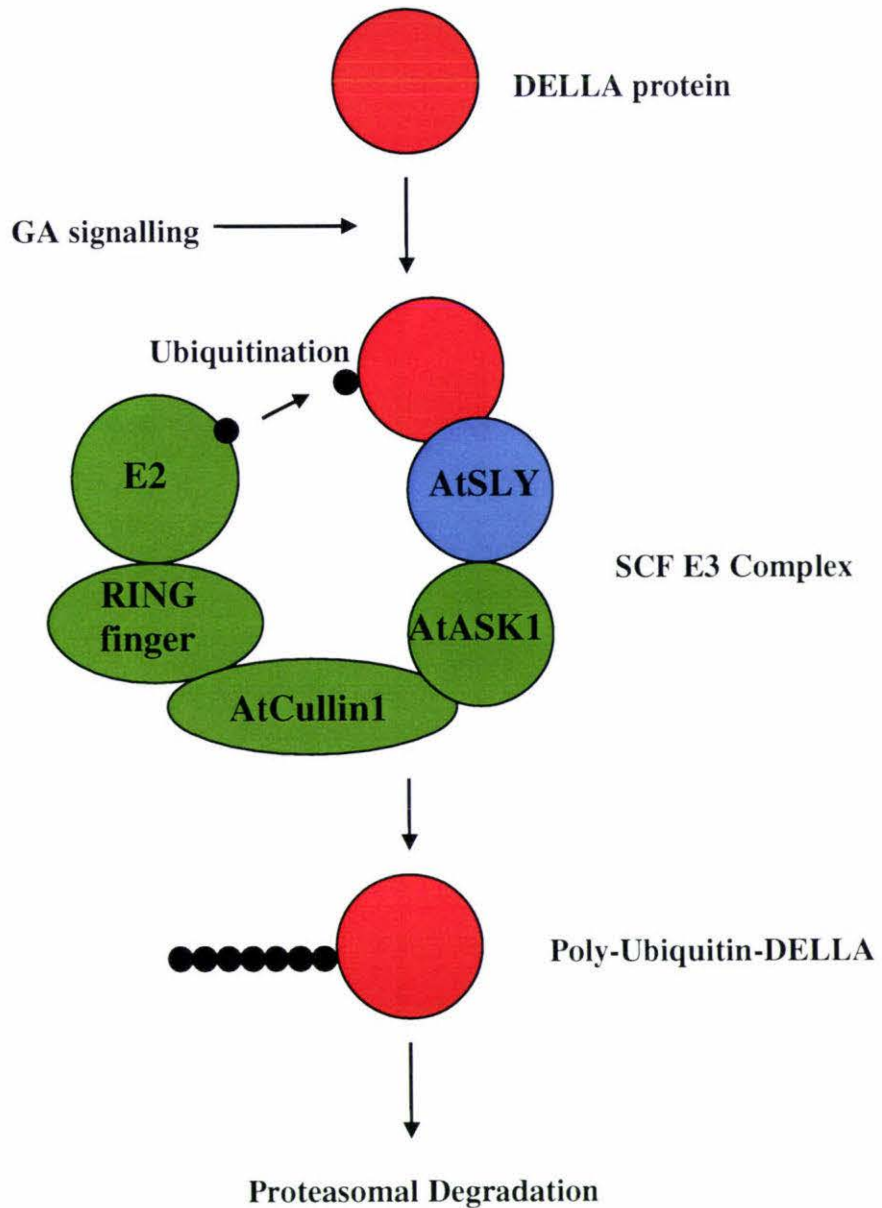


Figure 1-4 Structure and function of an SCF E3 ubiquitin ligase complex. The F-box protein (AtSLY1, blue) recruits a target protein (DELLA, red) to the complex. The target protein is then poly-ubiquitinated by the E2 ubiquitin ligase. Poly-ubiquitinated proteins are degraded by the proteasome (Gomi *et. al.,* 2004).

Analysis of DELLA proteins in mutant backgrounds was used to investigate the functional relationships between the two classes of proteins, DELLA and F-box. In the *Atsly1-10*/*Osgid2-1* loss-of-function genetic backgrounds, both endogenous and GFP fusions of AtRGA, AtGAI, and OsSLR1 proteins are present at a higher level and the amount does not decrease on GA treatment (Dill et. al, 2004; Fu et. al., 2004; Gomi et. al., 2004). This evidence suggests that DELLA proteins are no longer degraded on GA signalling in the absence of the F-box proteins (Dill et. al, 2004; Fu et. al., 2004; Gomi et. al., 2004). It has also been shown that DELLA protein OsSLR1 accumulates in a phosphorylated form in the F-box loss-of-function mutant *Osgid2-1* (Sasaki *et. al.,* 2003). Recent results by the same group indicate that the phosphorylation is not a result of GA signalling, as both unphosphorylated and phosphorylated forms accumulated in GA deficient callus tissue (Itoh *et. al.,* 2005).

These studies strongly suggest that the F-box proteins, AtSLY1 and OsGID2, target DELLA proteins for degradation on GA signalling via the proteasome. To strengthen this argument, a point mutant of AtSLY1 was found that acts as the genetic suppresser of GA biosynthetic mutant *Atga1-3* (Swain *et. al.,* 2004). This mutant, *Atsly1-d* is a gain-of-function mutant, and is also able to restore the degradation of DELLA gain-of-function of GA-insensitive mutant proteins Atgai-1 and AtrgaΔ17. (Dill *et. al.,* 2004; Fu *et. al.,* 2004). Consistent with the degradation of GA-insensitive proteins in *Atsly1-d* plants, *Atsly1-d* dependent degradation of Atgai-1 and AtrgaΔ17 also occurs in the GA-biosynthetic mutant background, *Atga1-3* (Dill *et. al.,* 2004; Fu *et. al.,* 2004). Therefore, the degradation of DELLA proteins by the gain-of-function Atsly1-d F-box protein mutant is GA-independent.

Yeast 2-hybrid and *in vitro* pull-down experiments have shown that the F-box proteins AtSLY1, Atsly1-d, and OsGID2 can bind to DELLA proteins from the same species (Gomi *et. al.,* 2004; Dill *et. al.,* 2004; Fu *et. al.,* 2004; Tyler *et. al.,* 2004). In pull-down experiments wild-type AtSLY1 and OsGID2 could only bind with high affinity to phosphorylated AtGAI and OsSLR1 protein, respectively (Gomi *et. al.,* 2004; Fu *et. al.,* 2004). In contrast, the gain-of-function mutant protein, Atsly1-d, is able to bind with high affinity to unphosphorylated and phosphorylated DELLA proteins in pull-down experiments (Fu *et. al.,* 2004). The interaction of AtSLY1 or

Atsly1-d with the DELLA proteins AtRGL1, 2, and 3 have not yet been investigated using pull-down assays or genetics. However, yeast 2-hybrid experiments show that Atsly1-d, but not AtSLY1, is able to interact with all the AtRGLs (Tyler *et. al.*, 2004). Therefore, it is likely that AtSLY1 interacts with all five *A. thaliana* DELLA proteins in a similar fashion.

A novel gain-of-function GA insensitive *DELLA* mutant was isolated from *Brassica rapa* (Muangprom *et. al.*, 2005). Unexpectedly, it is a point mutation slightly C-terminal of the VHIID motif. Furthermore, when the analogous mutation was introduced into *A. thaliana* in *AtGAI*, it was also a gain-of-function mutation. This mutant is different to deletion of the DELLA motif, in that its phenotypic effect is not overcome by the *AtSLY1* (F-box protein) gain-of-function mutant, *Atsly1-d*, which suppresses DELLA motif gain-of-function mutations (Muangprom *et. al.*, 2005). This implicates the DELLA protein mutated site in AtSLY1 binding. An alternative interpretation is that the Glutamine to Arginine mutation results in a drastic change to the AtGAI protein structure that alters the AtSLY1 binding surface. Yeast 2-hybrid and pull-down experiments using AtSLY1 and Atsly1-d suggested the binding site to be somewhere within the C-terminal third of the AtGAI protein, which does not include the VHIID motif. This evidence argues in favor of changes to the binding site rather than its deletion. However, proteins have complex, not linear, structure so both the VHIID and very C-terminal regions of AtGAI could be involved in AtSLY1 binding.

Recent work by Itoh *et. al.* (2005) suggests that phosphorylation targets on DELLA proteins are primarily serine residues in the poly S/T/V region. However, phosphorylation of these residues is not a result of GA signalling (Itoh *et. al.*, 2005). Other possible phosphorylation targets may be aspartic acid (D), theronine (T), histidine (H), or tyrosine (Y), all of which are present in the DELLA and TVHYNP motifs. Phosphorylated DELLA proteins have a higher affinity for the F-box proteins (Fu et. al., 2004) so this is in agreement with genetic evidence that the poly S/T/V region is involved in increasing the effect of the GA signal (Itoh et. al., 2002). The phosphorylation of this motif may be the mechanism by which other hormones such as auxin and ethylene affect DELLA protein stability. The GA-induced degradation of OsSLR1 was shown to be due to an increased affinity for OsGID2. This leaves open

the question of how GA signalling increases the affinity of OsGID2 for OsSLR1. The GA-induced degradation of OsSLR1 does not involve phosphorylation as it is not prevented by phosphorylation inhibitors (Itoh *et. al.*, 2005).

In further exploration of interactions of DELLA proteins with the ubiquitination /protein degradation pathways, AtSLY1 was shown to bind AtASK1 (Arabidopsis Skp like-1) (Fu *et. al.*, 2004). Skp1 is a component of the SCF E3 enzyme complex (reviewed by Kipreos and Pagano, 2000). This newly established interaction supports a model in which the F-box proteins recruit DELLA proteins for proteasomal degradation. Interestingly another F-box protein, AtTIR1, is involved in auxin signalling. AtTIR1 also binds to AtASK1 and targets AUX/IAA transcriptional regulators for proteasomal degradation (Dharmasiri *et. al.*, 2005; Kepinski and Leyser, 2005). Recently it was found that the AtTIR1 F-box protein was binding directly to auxin resulting in increased affinity of the complex for AUX/IAA proteins (Dharmasiri *et. al.*, 2005; Kepinski and Leyser, 2005). By analogy, it would be worth exploring whether GA is capable of enhancement of DELLA-AtSLY1/OsGID2 interaction by direct binding. However, published results suggest that the increased affinity is due to an alteration to the DELLA proteins and not the F-box, so GA may bind directly to the DELLA proteins.

1.5 Developmental functions of DELLA proteins

The *A. thaliana* genome encodes five DELLA proteins, while monocots and many dicots only encode one. Why some dicots have multiple DELLA proteins is a question that needs to be answered. Several studies have attempted to allocate specific functions to each DELLA protein in *A. thaliana* by detection of *AtDELLA* mRNA in specific tissues. However, these experiments only showed the presence of DELLA-encoding mRNAs, not the functional proteins themselves. Tyler *et. al.* (2004) performed quantitative analysis of all five *AtDELLA* mRNAs in several different tissues. The results of this investigation showed *AtRGA* to be expressed at a constitutive level in all tissues. The second DELLA gene, *AtGAI*, was expressed at the highest level in germinating seeds, and at a moderate level in all other tissues. The third DELLA gene, *AtRGL1*, was expressed at a moderate level in germinating seedlings, and a high level in flowers and siliques. The fourth DELLA gene, *AtRGL2*,

was expressed at a constitutive level in germinating seeds, and a high level in flowers and siliques. Finally, the fifth DELLA gene, *AtRGL3* was expressed at a high level in germinating seeds, but not in any other tissues. These quantitative results are in agreement with previously published *in situ* and semi-quantitative DELLA mRNA analyses (Wen and Chang, 2002; Lee *et. al.,* 2002).

Combinations of mutations have been used to analyse the role that *AtDELLA* genes have in plant development. Null mutations to individual *AtDELLA* genes can rescue aspects of the *Atga1-3* (GA biosynthesis null mutant) dwarf phenotype, but only if the mutated *AtDELLA* gene is involved in that aspect of the phenotype. This is due to the fact that the DELLA protein in question would normally repress the wild-type phenotype until GA signalling occurred. Null mutations of single *AtDELLA* genes show only small changes in phenotype to that of either wild-type or *Atga1-3* parent lines. However, the double-null mutant of *AtGAI* and *AtRGA* rescues most of the wild-type stem elongation phenotype to a dwarf *Atga1-3* parent line (Cheng *et. al.*, 2004). Furthermore, additional null mutations of *AtRGL1* and *AtRGL2* were required for complete rescue of the wild-type stem phenotype (Cheng *et. al.*, 2004).

Similarly, in the development of floral tissues more than one *AtDELLA* gene null mutation is required to restore fertility to near-wild-type in an *Atga1-3* parent line. However, the requirement for particular DELLA mutation combinations is different to that for the stem elongation. Loss of *AtRGL2*, *AtRGA*, and to a small extent, *AtRGL1*, is required for full recovery of wild-type phenotype (Cheng *et. al.*, 2004).

The study of auxin induction of root development described earlier showed that root elongation is mediated through *AtGAI* and *AtRGA*, but also at least one of the other *AtRGL* genes, or other factors (Fu and Harberd, 2003). Seed germination has been shown to be largely repressed by *AtRGL2,* with null mutations of *AtRGL2* being able to germinate in the absence of GA_3 (Lee *et. al.*, 2002).

Ait-ali *et. al.* (2003) examined the effect of the gain-of-function *Atgai-1* mutation in different tissues and developmental stages using a transgene under the control of the alcohol-inducible expression system. This method allowed monitoring of the effect on GA responses in particular tissues at particular stages of development and monitor

plant phenotypes. Using this method the authors showed that *Atgai-1* overexpression inhibits growth in young plants.

Altogether, the current experimental evidence suggests that the *AtDELLA* genes are partially functionally redundant, but do have different tissue expression patterns. The sites of gene expression are similar to the sites of effects on plant development, and all *AtDELLA* genes expressed in a particular tissue need to carry null mutation to rescue a GA-deficient background.

Recently it was shown that *Atgai-1* mRNA can be transported between tissues via the phloem; the gain-of-function *Atgai-1* was shown to affect plants by grafting of transgenic tissues to wild-type plants (Haywood *et. al.,* 2005). These finding suggest that the tissues of *AtDELLA*-mRNA expression may not necessarily be the target of DELLA protein expression and action.

Many mutations to genes involved in GA signalling in *A. thaliana* (*Atga1-3*, *Atgai-1*, *Atrga*Δ*17*) have been produced in the Landsberg *erecta*-0 (L*er*-0) background. L*er*-0 plants are naturally partial dwarf due to a mutation in a gene encoding a putative transmembrane protein of unknown function, *AtERECTA* (Fridborg *et. al.,* 2001). L*er*-0 is used as dwarfing mutant phenotypes are more severe in this background, making them easier to detect. However, this may cause misinterpretations as the *Ater-0* mutation is involved in dwarfing of plants, and may interfere with the GA-signalling pathway, so observations in this background could be inaccurate.

1.6 Speculative model of the Gibberellic acid signalling pathway

I present here a speculative model of the gibberellic acid signalling pathway based on the current evidence (Figure 1-5). It is thought that, in the absence of GA, the DELLA family proteins repress the expression of key GA-induced genes, including GAMYBs, through a yet unknown mechanism.

When GA is present it is possibly perceived at two sites, firstly at the plasma membrane by an unknown receptor:G protein complex, and secondly within the cell. How or whether GA enters the cell is yet unknown. If GA does cross the plasma membrane; the process may involve a transporter and/or enzymatic modification. The perception at the plasma membrane is suggested to result in elevated Ca^{2+} and cGMP cytoplasmic concentrations and activation of a putative protein kinase/phosphatase signalling pathway. Somehow these changes result in the transition of the signal into the nucleus, possibly by the putative protein AtHIM (StPHOR1 homologue) or GA itself. Nuclear events result in changes to the DELLA proteins that increase their affinity for the F-Box protein AtSLY1. AtSLY1 is part of a SCF E3 ubiquitin ligase complex that results in tagging of DELLA proteins for degradation by the proteasome.

The degradation of DELLA proteins relieves the repression exerted on the expression of GAMYBs and other key GA-responsive genes. GAMYBs act to alter gene expression, resulting in the observed responses to GA. A second signal, possibly from cross-signalling of other plant hormones activates a kinase which phosphorylates the DELLA proteins, resulting in an additional increase in affinity of AtSLY1 for DELLA proteins on GA signalling. It is possible that the phosphorylation signal is reversible, with some signals reducing phosphorylation or inducing de-phosphorylation.

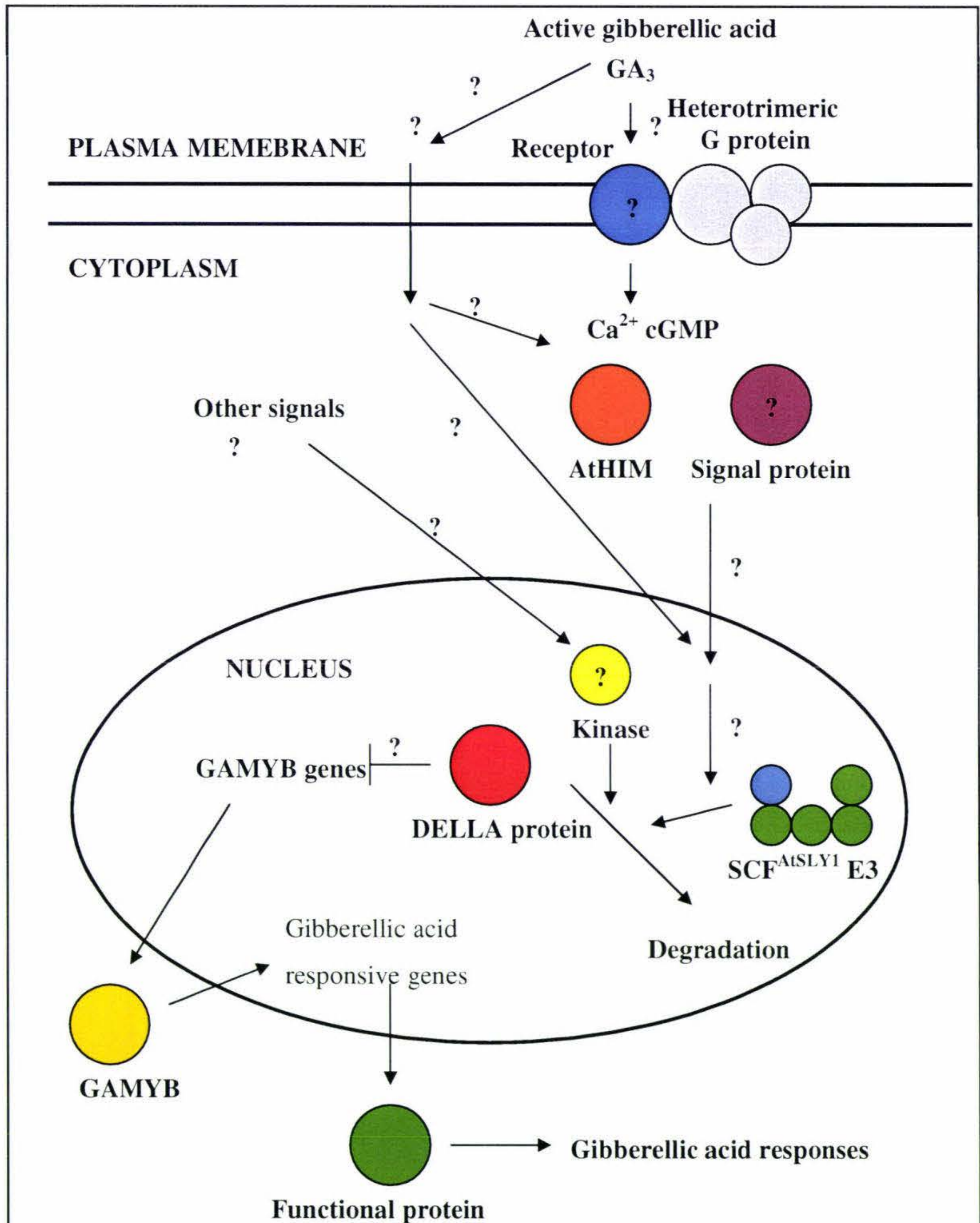


Figure 1-5 Detailed speculative gibberellic acid signal pathway model. Active GA_3 may be perceived at the plasma membrane, resulting in production of secondary cytoplasmic signalling messengers. The signal enters the nucleus where it results in the degradation of DELLA proteins. DELLA proteins repress GA-induced responses by transcriptional regulation, so degradation activates GA-induced responses

1.7 How do DELLA proteins function?

The current knowledge of DELLA protein function is very limited. This section discusses several important aspects of GA signalling that warrant exploration.

Inactivation of DELLA proteins upon GA signalling has been shown to be required for normal developmental processes. Their roles as repressors are required for proper timing of plant development. Without DELLA proteins, the plants lack the regulation of development by GA. GA-dependent development is required for the regulation of growth, such as when it is beneficial to the plant to halt growth during stress conditions. The mechanism responsible for DELLA protein degradation has been shown to be through an F-box/ubiquitin/proteasome pathway, but how DELLA proteins are targeted to this pathway by GA perception by the cell is yet unknown. Therefore, in the interest of understanding the GA signalling pathway it is important to discover the components required for DELLA protein targeting for degradation.

Another important question is how do DELLA proteins act as negative regulators of GA signalling? They are putative transcriptional regulators, but do they actually bind DNA? This is yet to be determined, and may be valuable in understanding DELLA protein function. In search of answering these questions, it would be necessary to identify:

a) Other regulatory or structural components of chromatin that take part in the DELLA protein-mediated gene regulation;
b) Target genes that are directly regulated by DELLA proteins

Another unresolved question is why some dicot plants have multiple DELLA proteins? Multiple DELLA proteins may be the result of chromosome duplications and so may be completely functionally redundant. However, the DELLA genes of *A. thaliana* have been shown to have different tissue expression and the encoded proteins have divergent N-terminal domains. Therefore, the role of specialisation of DELLA family members is an interesting question. OsSLR1 has been shown to homo-dimerise in yeast 2-hybrid experiments through a conserved non-standard Leucine zipper (Itoh *et. al.*, 2002). Based on this finding, it is possible that in *A. thaliana* different DELLA proteins act as homo- and hetero-dimers to regulate different targets. This

combinatory strategy is very common among in the regulatory protein families such as the bZIP class transcription factors, and the proteins with the closest relation to DELLA/GRAS proteins, the mammalian nuclear receptors (Siberil *et. al.*, 2001; Nettes and Greene, 2005).

1.7.1 Hypotheses.

Two testable hypotheses can be postulated to begin deciphering the functions of the DELLA proteins that are discussed above:

1. DELLA proteins perceive the GA signal and regulate target gene expression through direct interaction with other nuclear proteins.

2. DELLA proteins function as homo- and hetero-dimers in *A. thaliana*.

To test these hypotheses, two aims for this thesis have been defined:

1.7.2 Aim 1: Identification of proteins that interact with AtRGL1.

My research focuses on AtRGL1, a DELLA protein of *A. thaliana* that is involved in floral development. To investigate the mechanisms by which AtRGL1 protein activity is regulated, AtRGL1-interacting proteins were isolated by affinity purification. A soluble recombinant N-terminal domain of AtRGL1 was used as a bait to successfully isolate two novel proteins, and a possible third protein. Immunoprecipitation experiments were also planned for the isolation of GA signalling components associated with native AtRGL1; however, the resident AtRGL1 was not detectable in wild-type nuclear plant extracts using the antisera that were raised against this protein, precluding this approach.

1.7.3 Aim 2: Investigation of DELLA - DELLA interactions.

A yeast 2-hybrid system was utilised to establish whether AtRGL1-interacts with either AtRGL2 or AtRGA.

Chapter 2. Materials and Methods

2.1 Materials and reagents

All analytical grade chemicals were sourced from Sigma, BDH, or Merck. Bacteriological grade media was obtained from BD. Antibiotics and isopropylthio-β-D-galactoside (IPTG) were sourced from Applichem. Reagents for preparation of poly-acrylamide gels were obtained from Biorad. Enzymes and reagents for molecular biology were sourced from Roche, New England Biolabs, or Invitrogen. Antibody-enzyme conjugates were supplied by either Sigma or Amersham Biosciences. Anti-DELLA antibody 5E1 and anti rabbit-IgG-horseradish peroxidase conjugate were kindly supplied by Dr William Jones at HortResearch, Palmerston North.

2.2 General methods

2.2.1 Molecular biology

Agarose gel electrophoresis was used as described by Sambrook *et. al.* (1987) to separate DNA fragments by size for identification, quantification, and purification. 0.7 % w/v agarose gels were routinely used. DNA samples were measured for concentration and purity using a UV spectrophotometer (Nanodrop, Nanodrop).

2.2.2 Protein electrophoresis

Protein electrophoresis procedures were used as described by Sambrook *et. al.* (1987). Proteins were separated by 10 % SDS-PAGE (Sodium dodecyl sulfate-Poly-acrylamide gel electrophoresis), using either Glycine (Laemmli, 1970), or 8 % Tricine (Schägger and von Jagow, 1987) gel systems. 8 % Tricine SDS-PAGE gels were modified from the work by Schägger and von Jagow (1987) by reducing the acrylamide ratio (bis-acrylamide ratio from 6 to 3 %) and absolute acrylamide concentration. Native protein samples were separated by Native-PAGE. Gels were either stained with coomassie brilliant blue R250, or with SYPRO ruby gel stain.

Alternatively protein from unstained gels was transferred to nitrocellulose filters for western blotting.

2.2.3 Western blotting

Western blotting was performed as described by Sambrook *et. al,* (1987). The Tris (tris (hydroxymethyl) aminomethane) Buffered Saline (TBS) was modified to TBST (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % v/v Tween-20). The addition of 5 % w/v non-fat milk powder was used as a blocking agent. Dilutions of primary and secondary antibodies are detailed in Table 5. Alkaline phosphatase (AP) detection was performed as described by Sambrook *et. al.* (1987). Horseradish peroxidase (HRP) detection was performed using the ECL Plus detection system (Amersham Biosciences).

2.3 Bacterial strains and culture conditions

All plasmids used in this thesis are listed in Table 1. Bacterial strains are listed in Table 2. All primers used were obtained from either Invitrogen or Clontech and are listed in Table 3. *Escherichia coli* strains were cultured in either 2xYT (Gibco) or Rich Media (2 gL^{-1} glucose, 10 gL^{-1} tryptone, 5 gL^{-1} NaCl, 5 gL^{-1} yeast extract). Antibiotics used were ampicillin (AMP), 100 μgmL^{-1}, or kanamycin (KAN), 50 μgmL^{-1}. Liquid cultures were aerated by shaking (200 rpm). Solid media plates were made by the addition of 10 gL^{-1} bacteriological grade agarose (Oxoid). *E. coli* liquid cultures and solid media were incubated overnight at 37°C except where noted otherwise. *E. coli* strains were stored long term by addition of dimethyl sulphoxide (DMSO) to 7 % v/v and immediate freezing at -80°C.

2.4 Plant material and growth conditions

Wild-type *Arabidopsis thaliana* Colombia seeds were suspended in half strength Murashige and Skoog media supplemented with Gamborg's vitamins and chilled at 4°C for 4 days. Seeds were then sown on moist potting mix and placed in a mist chamber for 7 days at 25°C under 16 hours light/ 8 hours darkness. After this time

pots were removed from the mist chamber and kept under the same growth conditions.

2.5 Yeast strains and culture conditions

All *Saccharomyces cerevisiae* strains used in this thesis are listed in table 4. *S. cerevisiae* were cultured in either YPD (10 gL^{-1} yeast extract, 20 gL^{-1} peptone, 20 gL^{-1} glucose) or synthetic dropout (SD) media (26.7 gL^{-1}minimal nitrogen base (Clontech), 20 gL^{-1} glucose, 0.6 gL^{-1} appropriate amino mixture (Clontech)). Solid media plates were made by the addition of 20 gL^{-1} bacteriological grade agarose (Oxoid). Liquid cultures were aerated by shaking (300 rpm) and incubated overnight at 30°C unless noted otherwise. *S. cerevisiae* grown on solid media were incubated at 30°C for 3-4 days. Yeast strains were stored by addition of sterile glycerol to 25 % v/v to overnight cultures and freezing at -80°C

2.6 Bacterial recombinant protein expression

Chemically competent *E. coli* were transformed using procedures described by Sambrook *et. al.*, (1987). *E. coli* strains used for protein expression were: TUNER (Novagen) for vectors with a *tac* promoter, and TUNER (DE3) (Novagen) for vectors with a T7 promoter. Both these strains allow induction of the recombinant protein expression with IPTG.

Individual transformants were cultured in antibiotic containing 2xYT media (Gibco). To express recombinant proteins, overnight cultures were diluted 1:100 into 1 L Rich media and grown to an OD_{600} (Optical density, 600 nm wavelength) of 0.3. Expression was induced by the addition of IPTG to a concentration of 0.3 mM and incubation at 37°C for a further 2 hours. Bacteria were harvested by centrifugation (4000 g/ 20 min/ 4°C). Cells were resuspended in 25 mL ice cold Column Buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA (Ethylenediaminetetraacetic acid)) including protease inhibitors (1 mM DTT (1,4-Dithiothreitol), 0.2 mM PMSF (Phenylmethylsulphonylfluoride), general use protease inhibitor cocktail, Sigma (note: use rate 10 µL 10x solution per mL)) then frozen at –80°C. Cells were thawed

on ice and sonicated for a total time of 8 min using 8 sec bursts, 20 % power, and fine probe (Virsonic digital 475, Virtis). Crude bacterial lysate was either; aliquoted and frozen –80°C, or centrifuged (10000 g/ 20 min/ 4°C). Supernatant was diluted with a further 25 mL Column Buffer including protease inhibitors and this crude lysate was immediately used for protein purification.

2.6.1 Purification of MBP-LacZ

The construct use for MBP-LacZ (maltose-binding protein, β-galactosidase) expression was the pMALc2x plasmid (New England Biolabs). The pMALc2x plasmid expresses the MBP-LacZ fusion under the control of the *tac* promoter.

The crude lysate from 500 mL of induced culture was loaded through a 0.2 μm filter onto an amylose column (2.5 mL bed volume, New England Biolabs) connected to a low-pressure chromatography system (Econo System, Biorad). Amylose/Maltose-binding proteins were eluted specifically using column buffer containing 10 mM Maltose. Fractions were analysed by SDS-PAGE with coomassie blue staining. Fractions containing the expected 50.8 kDa protein were pooled (7.5 mL) and concentrated to 2.5 mL by centrifugal concentrator (4000 g/ 20 min/ 4°C; 5 kDa MWCO Vivaspin 20, Vivascience). Finally, the sample buffer was exchanged with PBS pH 7.4 supplemented with protease inhibitors using a PD10 column (Amersham Biosciences). The purified MBP-LacZ protein was aliquoted, glycerol was added to 10 % v/v, and aliquots frozen at –80°C. All stages of purification were performed on ice or at 4°C with the exception of Diethylaminoethyl (DEAE) columns.

2.6.2 Purification of MBP-AtRGL1$^{(1-121)}$

A construct for MBP-AtRGL1$^{(1-121)}$ expression was developed by J. Rakonjac (HortResearch, 2002). This construct is the pMALc2x plasmid (New England Biolabs) carrying the N-terminal domain of AtRGL1 (amino acids 1-121). The pMALc2x plasmid expresses the MBP-AtRGL1$^{(1-121)}$ fusion under the control of the *tac* promoter.

Crude lysate from 1 L of induced culture was filtered through a 0.2 μm pore filter. Filtered lysate (50 mL) was loaded onto a DEAE column (Pharmacia) connected to an AKTA low pressure chromatography system (Pharmacia), and eluted with an NaCl gradient between column buffer (50 mM NaCl) and high salt column buffer (1 M NaCl). Collected fractions were analysed by SDS-PAGE visualised by coomassie blue staining. Those fractions containing MBP-AtRGL1$^{(1-121)}$ (the major band) were pooled (100 mL), then loaded onto an amylose column (New England Biolabs, prepacked 100 mL bed volume) for affinity purification via the MBP tag. The MBP-AtRGL1$^{(1-121)}$ fusions was eluted with column buffer containing 10 mM Maltose. Fractions containing protein (as determined by UV absorbance) were pooled, concentrated by centrifugal concentrator to 2.5 mL (4000 g/ 60 min/ 4°C; 10 kDa MWCO Vivaspin 20, Vivascience), and then buffer exchanged back to the column buffer using a PD10 column (Amersham). The concentrated sample (3.5 mL) was again loaded on to a DEAE column, and eluted as before. Fractions were analysed by SDS-PAGE and coomassie staining and those containing pure MBP-AtRGL1$^{(1-121)}$ protein were pooled and concentrated by centrifugal concentrator (4000 g/ several hours/ 4°C; 10 kDa MWCO Vivaspin 20, Vivascience). Concentrated purified MBP-AtRGL1$^{(1-121)}$ was buffer exchanged to PBS pH 7.5 using a PD10 column (Amersham Biosciences) and glycerol added to 10 % v/v. Purity of the protein was examined by SDS-PAGE and the concentration was determined using DC protein assay (Biorad). The protein aliquots were stored at -80°C.

2.6.3 Purification of AtRGL1$^{(1-121)}$-TrxA

A construct for AtRGL1$^{(1-121)}$-TrxA (thyrodoxin domain A) expression (pPMB0350 (pDJS012)) was developed by N. Frearson (HortResearch). This construct is the pETM-20 plasmid carrying the N-terminal domain of AtRGL1 (amino acids 1-121). pDJS012 expresses the AtRGL1$^{(1-121)}$-TrxA Histidine tag fusion under the control of the T7 promoter.

Crude lysate from 1 L of induced culture was filtered through a 0.2 μm pore filter, then loaded onto a DEAE A50 column (Diethylaminoethyl-Sephadex, Sigma) connected to a low pressure chromatography system (Biorad, Econo system). Proteins

were eluted with a NaCl concentration gradient between column buffer (50 mM NaCl) and high salt column buffer (1 M NaCl). Eluted fractions were analysed by SDS-PAGE with coomassie blue staining and those containing AtRGL1$^{(1-121)}$-TrxA (the major band) were pooled (total 150 mL) and concentrated by centrifugal concentrator (4000 g/ several hours/ 4°C; 5 kDa MWCO Vivaspin 20, Vivascience) to 2.5 mL. Concentrated AtRGL1$^{(1-121)}$-TrxA was exchanged into nickel column buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl) using a PD10 column. The resulting sample (3.5 mL) was then loaded onto a nickel column (3 mL bed volume, chelating sepharose fast flow, Amersham biosciences). After washing with nickel column buffer, bound proteins were eluted using nickel elution buffer (20 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 500 mM Imidazole (1,3-Diaza-2,4-cyclopentadiene)). The eluted sample (2.5 mL) was buffer exchanged into PBS pH 7.4 supplemented with protease inhibitors using a PD10 column. Glycerol was added to 10% v/v and aliquots were stored at -80°C. Purity of the protein was examined by SDS-PAGE using coomassie blue staining and the protein concentration was determined using DC protein assay (Biorad).

2.7 Extraction of plant nuclear proteins

Plant material from various above ground tissues of wild-type *A. thaliana* was collected and frozen in liquid nitrogen. Nuclei were extracted by differential centrifugation as described by Busk and Pages (1997), but with several modifications. Nuclei isolation buffer (Buffer A, Busk and Pages, 1997) was used with the addition of 1.0 % w/v Poly Ethylene Glycol (PEG) 4000 to remove phenolic compounds that interfere with recovery of proteins and protein assays. General use protease inhibitor cocktail (Sigma) was included in all buffers. Filtering of plant tissue suspension was not performed in a microcentrifuge, as sample volumes were too large. Instead the suspension was filtered through 4 layers of 23-25 μm pore filter cloth and squeezed to recover most of the liquid. The filtrate was then centrifuged (4000 g/ 20 min/ 4°C) and the pellet resuspended in nuclei isolation buffer. The final nuclei wash step was omitted as this procedure resulted in loss of a significant proportion of isolated nuclei. Nuclei were observed by light microscopy using methylene green stain and florescent microscopy, using 0.2 μgmL^{-1} DAPI stain (4',6-Diamidino-2-phenylindole). To aid

lysis, nuclei were resuspended in a hypotonic buffer (20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)/ KOH pH 7.8, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, general use protease inhibitors (Sigma)) instead of nuclei storage buffer before freezing at –80°C. To avoid reduction of antibody disulfide bonds, preparations used for immunoprecipitations were performed with the omission of DTT from any buffers following the sucrose gradient, and the nuclei were finally resuspended in PBS pH 7.4 before freezing at –80°C.

Lysis of isolated nuclei was performed using freeze thawing and salt extraction. Nuclei frozen in hypotonic buffer were thawed on ice and centrifuged (12000 g/ 10 min/ 4°C). The supernatant was moved to a new tube and the pellet resuspended in salt buffer (hypotonic buffer, 100 mM NaCl), incubated on ice for 30 min and pipetted vigorously, then centrifuged (12000 g/ 10min/ 4°C). The resulting supernatant was combined with that of the hypotonic buffer lysis. The pellet was resuspended in high salt buffer (hypotonic buffer, 1 M NaCl). The nuclei were again incubated on ice for 30 min and then centrifuged, and the supernatant added to the combined first two. This is the soluble nuclear lysate. The soluble nuclear lysate was used immediately and has a buffer composition of 20 mM Hepes/ KOH pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, general use protease inhibitors (Sigma). Nuclei suspended and frozen in PBS pH 7.4 for immunoprecipitations were lysed by thawing on ice and debris was removed by centrifuging (12000 g/ 10 min/ 4°C).

The protein concentration of these plant nuclear extract samples cannot be determined by the DC protein assay (Biorad), due to the formation of an unknown yellow precipitate when the reaction reagents were added. This precipitate interfered with absorbance measurements. Protein concentration was instead estimated by SDS PAGE using coomassie blue staining, or Tricine SDS-PAGE using SYPROruby (Biorad).

2.8 Affinity Purification

Affinity purification was used to purify plant proteins that interact with the N-terminal domain of AtRGL1. Affinity columns were prepared using purified BSA, MBP-LacZ, or MBP-AtRGL1$^{(1-121)}$ cross-linked to amino link plus resin (Amino link plus kit, Pierce) through lysine residues. Plant nuclear extract, prepared as described above, was incubated with protein coated resin for 2 hours at 4°C on a nutator. Unbound extract was removed by either gravity flow (column) or centrifugation (1000 g/ 5 sec/ 4°C; Micro spin columns, Pierce), and washed using 20 mM Hepes/ KOH pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, complete protease inhibitor cocktail. Proteins were eluted using a low pH buffer (IgG pure elution buffer, Pierce) and immediately pH neutralised with 1 M Tris-HCl pH 8.0 (to a final concentration of 50 mM), then separated by Tricine PAGE and visualised by SYPROruby staining.

2.9 Mass Spectroscopy

Sample preparation by in-gel tryptic digestion and mass spectrometry analysis was performed as described by Jimenez *et. al.* (1998a, 1998b). Protein bands were excised from Tricine-PAGE gels stained with either coomassie blue or SYPROruby gel stain. Tryptic digests were analysed by MALDI TOF (matrix assisted laser desorption ionisation, time of flight) mass spectrometry using a Micromass M@LDI-Reflectron. Mass fingerprints were used to search the Swiss Prot peptide mass fingerprints database to identify proteins. Alternatively undigested gel fragments were sent to the Mass Spectroscopy Facility (BMSF) at the University of New South Wales for analysis by liquid chromatography/ mass spectroscopy.

2.10 Anti-AtRGL1 polyclonal antibodies

Two Rabbits were immunised with 1 mg of purified MBP-AtRGL1$^{(1-121)}$ by Richard Broadhurst at AgResearch, Ruakura. Serum was collected after 3 months. The serum obtained was tested for reaction against MBP-LacZ, MBP-AtRGL1$^{(1-121)}$, AtRGL1 (*E. coli* expressed full length insoluble), and AtGAI (*E. coli* expressed full length

insoluble), and plant nuclear extract by western blot analysis of SDS-PAGE separated proteins.

Serum IgG was purified by $AmSO_4$ purification. This involves 'salting out' of the IgG by addition of one volume ice cold saturated Ammonium Sulphate slowly with stirring on ice. The sample was then centrifuged (10000 g/ 10 min/ 4°C). The pellet was re-dissolved in two volumes 100 mM Tris-HCl pH 8.0, then dialysed against 3 changes of 40 volumes of PBS pH 7.4 overnight at 4°C using 12-14 kDa cut off dialysis tubing (Servapore, Serva).

Purified IgG was affinity purified against AtRGL1$^{(1-121)}$-TrxA, using pierce amino link plus resin. Seven mg AtRGL1$^{(1-121)}$-TrxA was cross linked through lysine residues to 1 mL bed volume NHS resin (*N*-hydroxysuccinimide, amino link plus, Pierce) overnight at 4°C using 50 mM Sodium Borohydride in PBS pH 7.4 on a nutator. The antibody (Purified IgG fraction) was loaded, unbound flow-through reloaded three times, and the column washed with 3 volumes PBS pH 7.4 at 4°C. Purified antibodies were eluted off the column using a low pH buffer (IgG pure elution buffer, Pierce). The eluted fraction was immediately buffer neutralised by addition of 1 M Tris pH 8.0 to a concentration of 50 mM, concentrated by centrifugal concentrator (4000 g/ 20 min/ 4°C; 5 kDa MWCO Vivaspin 20, Vivascience) and buffer exchanged into PBS pH 7.4 using a PD10 column (Amersham Biosciences). The purified anti-AtRGL1$^{(-121)}$ was tested for reaction against MBP-LacZ, AtRGL1$^{(1-121)}$, and plant extracts by western blot. The purified anti-AtRGL1$^{(1-121)}$ was also tested as an immunoprecipitating antibody on plant extracts and AtRGL1$^{(1-121)}$-TrxA, using the Pierce co-found kit.

2.11 Cloning of full length DELLA genes

The full length coding sequences of *AtRGA, AtRGL1*, and *AtRGL2* were amplified for insertion into yeast 2-hybrid vectors. *AtRGA* and *AtRGL2* were amplified from wild-type Columbia (Col-0) variety, while *AtRGL1* was amplified from Landsberg *erecta* (L*er*-0) variety. The reason for this is that *AtRGL1* L*er*-0 and Col-0 translated sequence were predicted to be the same, so an already existing vector containing an

AtRGL1 L*er*-0 clone was used as a template for ease. There are two Col-0 protein sequences for AtRGL1 listed by NCBI, one encoded by a cDNA sequence matches that encoded by the L*er*-0 cDNA, but the genomic Col-0 sequence contains one amino acid substitution (Threonine22-Methionine). Therefore, it is not known which is the correct sequence.

Full-length *AtRGL1* (wild-type L*er*-0) coding sequence was PCR amplified from pDJS005. The primers DS005 and DS006 were used to generate a 5' XmaI + 1 nt and a 3' SacI + 1 nt for insertion in frame into the multiple cloning site of pACT2. The primers DS005 and DS007 were used to generate a 5' XmaI + 1 nt and a 3' SalI + 1 nt for in frame insertion into the multiple cloning site of pGBKT7. The plasmid pDJS005 was digested with BamHI and BglI to release a 2.5 kb fragment containing the full length AtRGL1 coding sequence. This 2.5 kb fragment was separated by agarose gel electrophoresis, visualised by ethidium bromide staining, then DNA products were excised and purified using the Wizard SV gel purification kit (Promega). Seventy five ng of purified 2.5 kb fragment was used as a template for a 50 μL PCR reaction using PWO polymerase (Roche) as detailed in the manufacturers instructions, with 400 nM primers. The PCR conditions used were a 94°C hot start for 4min, followed by 35 cycles at 94°C (30 sec), 60°C (30 sec), 72°C (105 sec), with a final 5 min at 72°C using an FTS thermal sequencer (Corbett Research).

Full-length *AtRGL2* (wild-type Col-0) coding sequence was PCR amplified from genomic DNA. The primers DS003 and DS004 were used to generate a 5' NcoI + 2 nt and a 3' XmaI + 2 nt for in frame insertion into the multiple cloning sites of both pACT2 and pGBKT7. Two hundred ng genomic DNA (supplied by Chris Kirk, HortResearch) was used as a template for a 50 μL PCR reaction using platinum pfx polymerase (Invitrogen) with a final buffer concentration of 2 times and 400 nM primers. PCR conditions used were 94°C for 4 min, followed by 35 cycles at 94°C (15 sec), 55°C (30 sec), 68°C (120 sec), with a final 5 min at 68°C using a Mastercycler (Eppendorf).

Full-length *AtRGA* (wild-type Col-0) coding sequence was PCR amplified from genomic DNA. The primers DS010 and DS011 were used to generate a 5' NcoI + 2 nt

and a 3' XmaI + 2 nt for insertion in frame into the multiple cloning sites of both pACT2 and pGBKT7. Two hundred ng genomic DNA was used as a template for a 50 μL PCR reaction using platinum pfx polymerase (Invitrogen) with a final buffer concentration of 1.5 times, a 1x concentration of PCR enhancer solution, and 400 nM primers. PCR conditions used were 94°C for 2 min, followed by 35 cycles at 94°C (15 sec), 55°C (30 sec), 68°C (120 sec), with a final 5 min at 68°C using a Mastercycler (Eppendorf).

PCR products were separated by agarose gel electrophoresis, and then viewed by ethidium bromide staining. DNA bands were excised and purified using the Wizard SV gel purification kit.

The *AtRGL1* product produced from the PCR using primers DS005 and DS007 was blunt-end ligated into pTOPO using the TOPO II blunt-ended ligation kit (Invitrogen). Pure plasmid from transformants was prepared using a high pure plasmid isolation kit (Roche). Presence of the correct 1.5 kb insert was determined by agarose gel electrophoresis of plasmid DNA. Plasmid was digested with XmaI and SalI to release the *AtRGL1* fragment.

Restriction digests were performed at 37°C for 2 hours to overnight in the appropriate New England Biolabs buffer with addition of BSA (Bovine serum albumin) for efficient enzyme action. Reaction volumes were 10 μL, 1-4 μg DNA for preparative digests of PCR products and 10-20 μL, 10-20 μg DNA for plasmids using 5 units of each enzyme per 10 μL reaction (enzymes were obtained from either New England Biolabs or Roche). All products were separated by agarose gel electrophoresis, and then the DNA was excised and purified using the Wizard SV gel purification kit (Promega).

The *AtRGL2* and *AtRGA* PCR products were both digested with NcoI and XmaI, whereas the *AtRGL1* fragment produced by PCR using DS005 and DS006 was digested with XmaI and SacI. The yeast 2-hybrid vectors were each digested with two different sets of restriction endonucleases: pACT2 with XmaI and SacI, or NcoI and XmaI; and pGBKT7 with XmaI and SalI, or NcoI and XmaI.

Digested full length DELLA coding sequences were ligated into the respective digested vectors, at a ratio of: 3 moles fragment / 1 mole vector. Ligations were performed at room temperature for 1 hour using 1 unit T4 DNA ligase (Invitrogen) in 1x ligation buffer with a total of 200-1000 ng DNA. Ligations were transformed into chemically competent *E. coli* TG1 and selected for on media containing antibiotic. Plasmid DNA was purified from transformation candidates using a high pure plasmid isolation kit (Roche). Correct insert size of 1.5 kb for *AtRGL1*, 1.6 kb for *AtRGL2*, and 1.7 kb for *AtRGA* were determined by agarose gel electrophoresis of purified plasmid. Purified plasmid was sequenced in forward and reverse directions using the sequencing primers, DS008 forward and DS009 reverse for pACT2, and T7 forward (Clontech) and 3' DNA BD sequencing primer (Clontech) reverse for pGBKT7. Plasmids encoding correctly *in silico* translated protein sequence were selected.

2.12 Yeast 2-hybrid experiments

Competent *S. cerevisiae* CG1945 cells were produced by the LiCl method described in the Clontech yeast-2-hybrid matchmaker two-hybrid system 3 kit. 100 ng of each purified plasmid was transformed into LiCl competent *S. cerevisiae* CG1945 by heat shock and plated on synthetic dropout medium (20 gL-1 glucose, 20 gL-1 agarose, 26.7 gL-1 minimal SD base (Clontech), appropriate quantities of amino acid mixtures lacking specific amino acids) The plates were incubated for 3 days at 30°C, selecting for growth in the absence of Leucine (pACT2, pCL1, pGADT7-T antigen), or Tryptophan (pGBKT7). Transformation frequencies were determined to detect toxicity. Urea/SDS protein extracts (as described in the Clontech yeast-2-hybrid matchmaker manual) of transformants containing pACT2 with DELLA gene inserts were analysed by western blotting using the anti-DELLA motif monoclonal antibody 5E1. Transformants expressing DELLA proteins from pACT2 vectors, and also yeast carrying the empty pACT2 vector were transformed with pGBKT7 and derived clones and selected on synthetic dropout medium lacking Leucine and Tryptophan. Double transformants were analysed by western blotting using anti-HA (Santa Cruz Biotechnology) and anti-c-Myc antibodies (Sigma).

2.12.1 *HIS3* reporter gene assay

Several independent yeast transformants from each transformation were cultured and plated on synthetic dropout medium lacking Leucine, Tryptophan, and Histidine, with addition of various concentrations of the Histidine synthesis inhibitor 3-AT (3-amino-1,2,4-trazole) (0, 1, 2, 5, 10, 30, 60 mM). *HIS3* reporter gene activation was scored based on the presence of colonies growing in the presence of differing levels of 3-AT. Yeast double transformation mixtures were also parallel plated on synthetic dropout medium lacking Leucine, Tryptophan, and Histidine with 5mM 3-AT to confirm observations.

2.12.2 *LACZ* reporter gene assay

Three transformants of each vector combination were measured for *LACZ* reporter gene (encoding β-galactosidase) activity as described by the yeast 2-hybrid match maker 3 kit (Clontech). *S. cerevisiae* were cultured in SD media, lacking the appropriate amino acids to retain plasmids, then diluted and incubated for a 3 hour 30°C culturing in rich media to allow β-galactosidase expression without the limitation of amino acid quantities. Cells are then collected and lysed. The β-galactosidase enzyme activity was determined by an *in vitro O*-Nitrophenol-galactoside (ONPG) assay in triplicate.

Table 1

Plasmids		
Name	Description	Reference
pCL1	wild-type full-length *GAL4* gene in a YCp50 derivative, *LEU2,* amp^r	Fields and Song, 1989
pACT2	$GAL4^{(768\text{-}881)}$AD, *LEU2*, amp^r, HA epitope tag	Li *et. al.*, 1994
pGBKT7	$GAL4^{(1\text{-}147)}$DNA-BD, *TRP1*, kan^r, c-Myc epitope tag	Clontech; Louret *et. al.*, 1997
pGBKT7 p53	murine $p53^{(72\text{-}390)}$ in pGBKT7, *TRP1*, kan^r	Clontech
pGADT7-T	SV40 large $T\text{-}antigen^{(84\text{-}708)}$ in pGADT7, *LEU2*, amp^r	Clontech
pMALc2x	$MALE_{(\Delta\ \text{signal sequence})}$, amp^r	New England Biolabs
pDJS001	*A. thaliana* $AtRGL1^{(1\text{-}121)}$ in pMALc2x, amp^r	Unpublished, HortResearch pPMB7235
pDJS002	*A. thaliana* $AtRGL1^{(1\text{-}512)}$ pET21a, amp^r	Unpublished, HortResearch pPMB0316
pDJS003	*A. thaliana* $AtGAI^{(1\text{-}533)}$ in pET36b, kan^r	Unpublished, HortResearch pPMB0229
pDJS005	*A. thaliana* $AtRGL1^{(1\text{-}512)}$ in pGEX-6P-2, amp^r	Unpublished, HortResearch pPMB0241
pDJS006	*A. thaliana* $AtRGL1^{(1\text{-}512)}$ in pACT2, *LEU2*, amp_r, HA epitope tag	This study
pDJS007	*A. thaliana* $AtRGL1^{(1\text{-}512)}$ in pGBKT7, *TRP1*, kan^r, c-Myc epitope tag	This study
pDJS008	*A. thaliana* $AtRGL2^{(1\text{-}547)}$ in pACT2, *LEU2*, amp^r, HA epitope tag	This study
pDJS009	*A. thaliana* $AtRGL2^{(1\text{-}547)}$ in pGBKT7, *TRP1*, kan^r, c-Myc epitope tag	This study
pDJS010	*A. thaliana* $AtRGA^{(1\text{-}587)}$ in pACT2, *LEU2*, amp^r, HA epitope tag	This study
pDJS011	*A. thaliana* $AtRGA^{(1\text{-}587)}$ in pGBKT7, *TRP1*, kan^r, c-Myc epitope tag	This study
pDJS012	*A. thaliana* $AtRGL1^{(1\text{-}121)}$ in pETM-20, kan^r	Unpublished, HortResearch pPMB0350

Table 2

	Bacterial strains
Strain	Genotype
TUNER	*F- ompT hsdS(rBmB-) gal dcm lacY1*
TUNER (DE3)	*F- ompT hsdS(rBmB-) gal dcm lacY1* (DE3)
TG1	K12, D(*lac-pro*), *supE44*, *thi*, *hsdR 5*, F' *traD36*, *proA+B+*, *lacIq*, *lac*ZDM14
K1915	TUNER (pDJS001)
K1916	TUNER (DE3) (pDJS002)
K1917	TUNER (DE3) (pDJS003)
K1918	TUNER (pMALc2x)
K1963	TG1 (pDJS006)
K1964	TG1 (pDJS007)
K1965	TG1 (pDJS008)
K1966	TG1 (pDJS009)
K1967	TG1 (pDJS010)
K1968	TG1 (pDJS011)
K1973	TUNER (DE3) (pDJS012)

Table 3

Primers

Name	Sequence	Restriction site	Target
T7	TAATACGACTCACTATAGGGC	Sequencing	pGBKT7
3' DNA BD	TAAGAGTCACTTTAAAATTTGTAT	Sequencing	pGBKT7
DS003	CATGCCATGGCAATGAAGAGAGGATACGGAGAAAC	NcoI	At*RGL2*
DS004	TCCCCGGGTTTCAGGCGAGTTTCCACGCCG	XmaI	At*RGL2*
DS005	TCCCCGGGTATGAAGAGAGAGCACAACCACC	XmaI	At*RGL1*
DS006	CGAGCTCGTTATTCCACACGATTGATTCGCC	SacI	At*RGL1*
DS007	ACGCGTCGACGTTATTCCACACGATTGATTCGCC	SalI	At*RGL1*
DS008	CTATCTATTCGATGATGAAGATAC	Sequencing	pACT2
DS009	AGTTGAAGTGAACTTGCGGGGTT	Sequencing	pACT2
DS010	CATGCCATGGCAATGAAGAGAGATCATCACCAA	NcoI	At*RGA*
DS011	TCCCCCGGGTTTCAGTGCGCCGCCGTCGAGAGT	XmaI	At*RGA*

Table 4

Yeast strains		
Strain	Genotype	Reference
CG1945	MATa, *ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, cyhr2,* LYS2:GAL1$_{uas}$-GAL1$_{tata}$-HIS3, URA3:GAL4 $_{17mers\ (x3)}$ - CYC1$_{tata}$-LacZ	Feilotter *et. al.,* 1994
SC001	CG1945 (pCL1)	This study
SC002	CG1945 (pACT2)	This study
SC003	CG1945 (pGBKT7)	This study
SC004	CG1945 (pGBKT7-p53) (pGADT7-T)	This study
SC005	CG1945 (pDJS006)	This study
SC006	CG1945 (pDJS007)	This study
SC007	CG1945 (pDJS008)	This study
SC008	CG1945 (pDJS009)	This study
SC009	CG1945 (pDJS010)	This study
SC010	CG1945 (pDJS011)	This study
SC011	CG1945 (pACT2) (pGBKT7)	This study
SC012	CG1945 (pACT2) (pDJS007)	This study
SC013	CG1945 (pACT2) (pDJS009)	This study
SC014	CG1945 (pACT2) (pDJS011)	This study
SC015	CG1945 (pDJS006) (pGBKT7)	This study
SC016	CG1945 (pDJS006) (pDJS007)	This study
SC017	CG1945 (pDJS006) (pDJS009)	This study
SC018	CG1945 (pDJS006) (pDJS011)	This study
SC019	CG1945 (pDJS008) (pGBKT7)	This study
SC020	CG1945 (pDJS008) (pDJS007)	This study
SC021	CG1945 (pDJS008) (pDJS009)	This study
SC022	CG1945 (pDJS008) (pDJS011)	This study
SC023	CG1945 (pDJS010) (pGBKT7)	This study
SC024	CG1945 (pDJS010) (pDJS007)	This study
SC025	CG1945 (pDJS010) (pDJS009)	This study
SC026	CG1945 (pDJS010) (pDJS011)	This study

Table 5

Antibodies			
Name	Description	Target	Dilution
'5E1'	Anti-DELLA motif mouse monoclonal (Hort Research)	DELLA motif	1 μgmL^{-1}
Anti-MBP-AtRGL1 serum	Anti-MBP-AtRGL1$^{(1-121)}$ rabbit serum	MBP, DELLA	1:1000
Anti-MBP-AtRGL1 IgG	Anti-MBP-AtRGL1$^{(1-121)}$ rabbit serum, IgG fraction	MBP, DELLA	1:500 (equivalent to 1:1000 serum)
Anti-AtRGL1	Anti-AtRGL1 affinity purified rabbit polyclonal antibodies	DELLA	1:5000 (equivalent to 1:1000 serum)
Anti-c-Myc	Anti-c-Myc affinity purified rabbit polyclonal antibodies (Sigma)	c-Myc	1 μgmL^{-1}
Anti-HA	Anti-HA affinity purified rabbit polyclonal antibodies (Santa Cruz Biotechnology)	HA	1 μgmL^{-1}
Anti-Histone H3	Anti-Histone H3 trimethyl-Lysine 9 antibodies (Abcam)	Histone H3	0.5 μgmL^{-1}
Anti-mouse-HRP	Anti-mouse IgG horseradish peroxidase conjugate	Mouse IgG	1:5000
Anti-mouse-AP	Anti-mouse IgG alkaline phosphatase conjugate	Mouse IgG	1:5000
Anti-rabbit-HRP	Anti-rabbit IgG horseradish peroxidase conjugate	Rabbit IgG	1:5000
Anti-rabbit-AP	Anti-rabbit IgG alkaline phosphatase conjugate	Rabbit IgG	1:5000

Chapter 3. Results

3.1 Affinity purification of AtRGL1-interacting proteins

The focus of this thesis is to further the understanding of GA signalling by investigating the interactions of DELLA proteins. The first aim was to identify proteins that interact with the *A. thaliana* DELLA protein AtRGL1. *AtRGL1* mRNA is primarily expressed in the inflorescence, and *AtRGL1* is involved in the regulation of GA-dependent inflorescence development (Wen and Chang, 2002; Tyler *et. al.*, 2004). AtRGL1 was selected over other DELLA proteins due to the relative lack of research on this protein in comparison to AtRGA and AtGAI. The model dicot, *A. thaliana*, was used as its genome has been completely sequenced, allowing purified proteins to be identified using MALDI-TOF (matrix assisted laser desorption ionisation-time of flight) mass spectrometry of tryptic digests (peptide mass fingerprinting).

When the work on this thesis was initiated no DELLA interacting proteins had been identified. However, since this work began, it has been reported that the F-box proteins AtSLY1 (*A. thaliana*) and OsGID2 (*O. sativa*) interact with the DELLA proteins AtRGA/AtGAI, and OsSLR1 respectively (Dill *et. al.*, 2004; Fu *et. al.*, 2004; Gomi *et. al.*, 2004). This interaction is required for degradation of DELLA proteins, likely via the poly-ubiquitination-proteasome pathway. The affinity of the F-box AtSLY1 for the DELLA proteins AtGAI and AtRGA is increased on GA signalling, but the mechanism for this increase has not yet been revealed.

Aside from the mechanism of DELLA protein degradation, two other groups of interactions remain to be investigated. First are the interactions required for perception of a GA signal and possibly other hormonal or environmental signals by DELLA proteins. Genetic studies suggest these interactions involve the N-terminal domain of the DELLA proteins (Itoh *et. al.*, 2002). Second are the interactions required for regulation of target gene expression by DELLA proteins. To identify these groups of interacting proteins, co-affinity/affinity purification using AtRGL1 as a bait and co-immunoprecipitation (Co-IP) using anti-AtRGL1 antibodies were used.

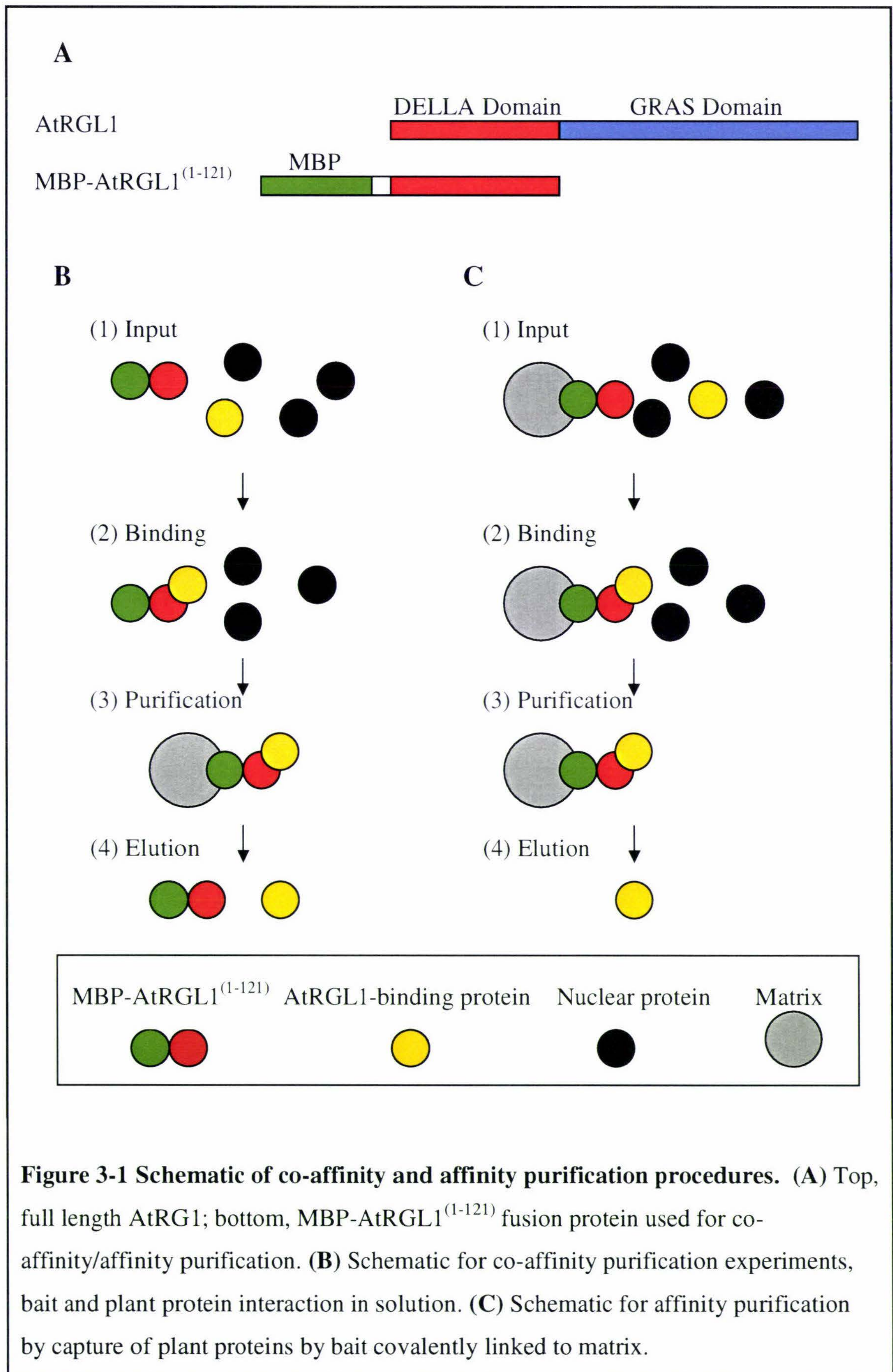


Figure 3-1 Schematic of co-affinity and affinity purification procedures. **(A)** Top, full length AtRG1; bottom, MBP-AtRGL1$^{(1-121)}$ fusion protein used for co-affinity/affinity purification. **(B)** Schematic for co-affinity purification experiments, bait and plant protein interaction in solution. **(C)** Schematic for affinity purification by capture of plant proteins by bait covalently linked to matrix.

Co-affinity purification ('pull-down') procedures were used to isolate new proteins that interact directly with AtRGL1. The 'bait' protein AtRGL1 was expressed as a recombinant fusion in *E. coli* and purified. Full-length AtRGL1 and other DELLA proteins were difficult to express in several systems tried (HortResearch plant immunology group, unpublished observations). However, the N-terminal domain of AtRGL1 and other DELLA proteins were expressed at high levels and were soluble, suggesting that these truncated proteins are correctly folded and suitable for use as bait. The motifs present in the expressed N-terminal domain of AtRGL1 are expected to be required for perception of GA (DELLA and TVHYNP) and regulatory signals (S/T/V) (Itoh *et. al.,* 2002; Wen and Chang, 2002). For this reason, proteins with roles in transmitting the GA or regulator signals to the DELLA proteins are anticipated to be isolated by affinity purification with this bait. The bait compromises the first 121 amino acid residues of AtRGL1 fused to the *E. coli* MBP (Maltose-binding protein) purification and solubility tag (Figure 3-1).

AtRGL1 protein is localised to the nucleus (Wen and Chang *et. al.,* 2002). Therefore, plant nuclear extract was prepared as the source of AtRGL1-interacting proteins. Co-affinity purification involves purification of a 'bait' protein from solution with the proteins of interest bound to it. Two methods for co-affinity purification were attempted but were unsuccessful in the isolation of AtRGL1-interacting proteins. This was due to a problem with interference of the bait protein in elutions. Therefore, I developed and performed direct affinity purification experiments to isolate AtRGL1-interacting proteins. In affinity purification experiments the bait was covalently cross-linked to a matrix allowing increased efficiency of AtRGL1-interacting protein recovery and separation of the bait protein from interacting proteins. The schematics for these two procedures are shown in figure 3-1.

3.1.1 Expression and purification of the AtRGL1 'bait' protein

As stated above, the N-terminal domain of AtRGL1 (residues 1-121) was expressed in *E. coli* in a soluble form. The recombinant protein MBP-AtRGL1$^{(1-121)}$ was successfully expressed and purified to greater than 95 % purity using a combination of ion exchange chromatography and amylose affinity purification (not shown). 15 mg of purified MBP-AtRGL1$^{(1-121)}$ fusion was obtained from 1 L of induced *E. coli*

culture. The purified fusion protein matched the predicted molecular weight of 53.9 kDa and is soluble (Figure 3-2). Even though the major portion of the bait protein appeared to be stable, upon overnight incubation under affinity purification experimental conditions (4°C), degradation products of 37 kDa, 48 kDa, and 51 kDa were detectable (Figure 3-2.). Co-affinity and affinity purification experiments to isolate AtRGL1-interacting proteins were typically performed using 2-4 hour incubations. Therefore, it is noteworthy to be aware of the potential presence of bait degradation products in elutions when interpreting results.

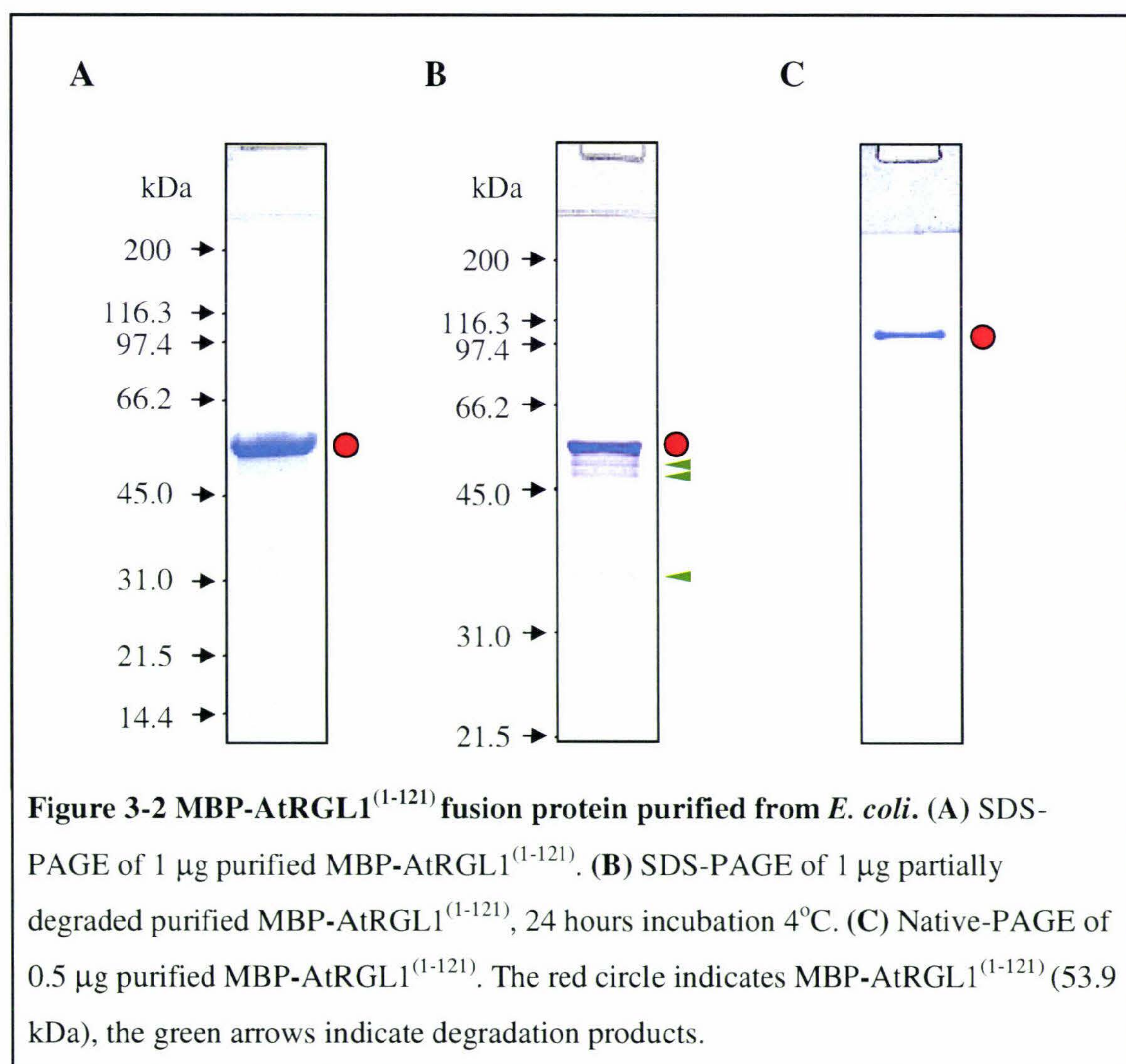


Figure 3-2 MBP-AtRGL1$^{(1-121)}$ fusion protein purified from *E. coli*. (**A**) SDS-PAGE of 1 μg purified MBP-AtRGL1$^{(1-121)}$. (**B**) SDS-PAGE of 1 μg partially degraded purified MBP-AtRGL1$^{(1-121)}$, 24 hours incubation 4°C. (**C**) Native-PAGE of 0.5 μg purified MBP-AtRGL1$^{(1-121)}$. The red circle indicates MBP-AtRGL1$^{(1-121)}$ (53.9 kDa), the green arrows indicate degradation products.

3.1.2 Preparation of plant nuclear extract

To purify AtRGL1-interacting proteins by affinity purification they must be in a soluble and native form. Because AtRGL1 is a nuclear localised protein (Wen and Chang, 2002) it is expected that interacting proteins are also nuclear. Plant nuclear extract was used for the affinity purification experiments, as it contains highly concentrated native nuclear proteins, and so will likely contain highly concentrated AtRGL1-interacting proteins.

To obtain plant nuclear proteins, intact plant nuclei were prepared by isolation using differential centrifugation (see methods section 2.7). Nuclei were then lysed by freeze-thawing in hypotonic buffer and the remaining proteins were extracted using step-wise increases in salt concentration (Figure 3-3, B). The presence of nuclear proteins was confirmed by the detection of histone H3 by western blotting using an appropriate commercial antibody (Figure 3-3, A).

Nuclear proteins were extracted from two types of tissue; whole above-ground tissue of mature 4-6 week old 'bolting' plants (referred to as 'whole plant'), and inflorescence tissues (which include the inflorescence meristem, floral meristems, and immature flowers). Mature plants have undergone GA signalling and so are expected to contain putative GA signalling proteins that interact with the N-terminal domain of DELLA proteins. The inflorescence is the primary site of *AtRGL1* mRNA expression making it the most likely site of AtRGL1 binding proteins. Inflorescences also proved to be the best tissue for nuclear protein preparation due to the high nuclei to cell volume ratio, yielding approximately ten fold as much protein per gram of plant tissue as did whole plant tissue.

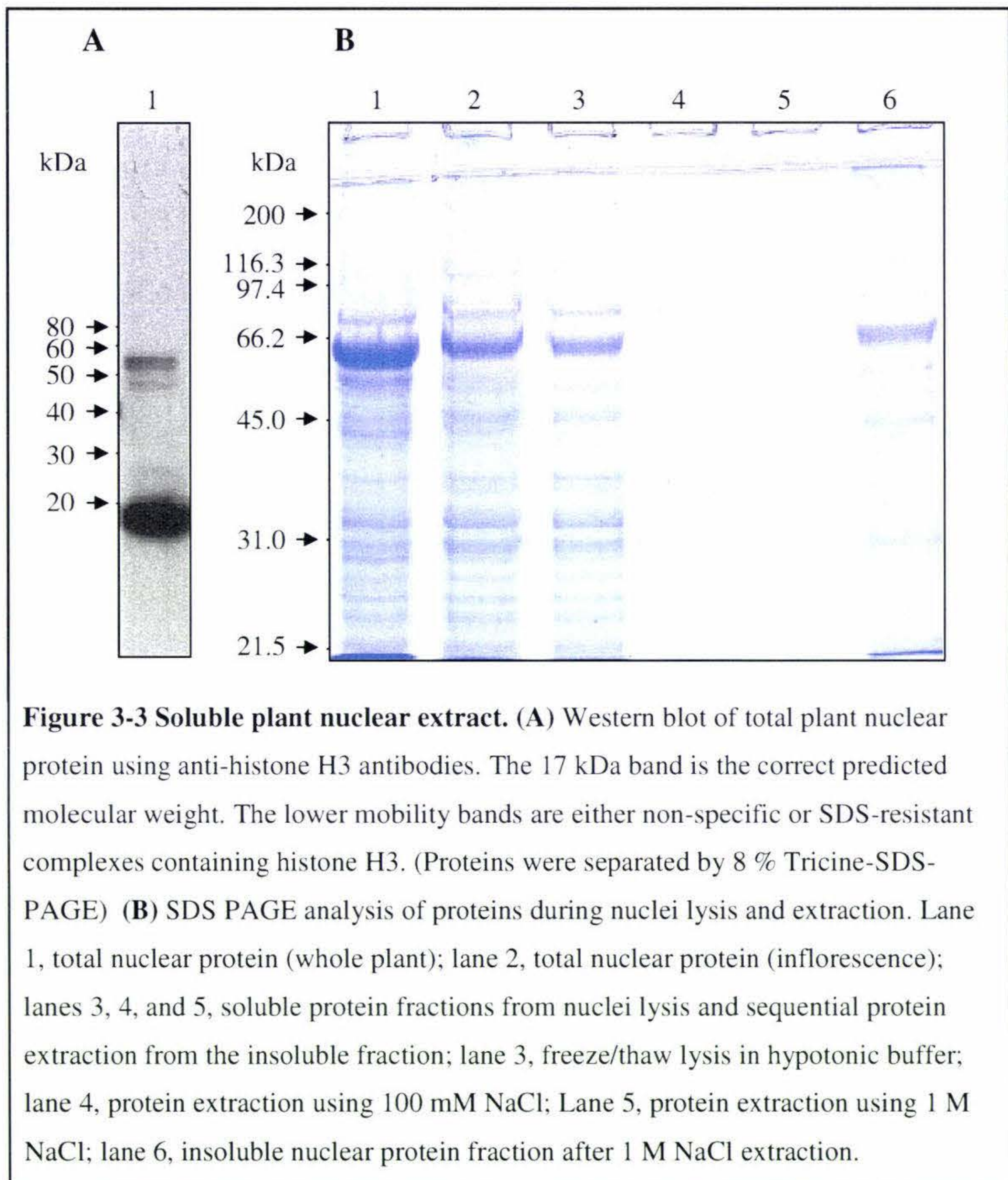


Figure 3-3 Soluble plant nuclear extract. (A) Western blot of total plant nuclear protein using anti-histone H3 antibodies. The 17 kDa band is the correct predicted molecular weight. The lower mobility bands are either non-specific or SDS-resistant complexes containing histone H3. (Proteins were separated by 8 % Tricine-SDS-PAGE) **(B)** SDS PAGE analysis of proteins during nuclei lysis and extraction. Lane 1, total nuclear protein (whole plant); lane 2, total nuclear protein (inflorescence); lanes 3, 4, and 5, soluble protein fractions from nuclei lysis and sequential protein extraction from the insoluble fraction; lane 3, freeze/thaw lysis in hypotonic buffer; lane 4, protein extraction using 100 mM NaCl; Lane 5, protein extraction using 1 M NaCl; lane 6, insoluble nuclear protein fraction after 1 M NaCl extraction.

3.1.3 Affinity purification of AtRGL1-interacting proteins

Two methods for co-affinity purification of AtRGL1-interacting proteins were attempted. Initially the MBP-AtRGL1$^{(1-121)}$ bait protein was covalently modified by biotinylation of Lysine residues, allowing capturing of the bait and associated plant nuclear proteins by binding of biotin groups to Streptavidin-coated magnetic beads. The advantage of magnetic beads over other matrices is that they are rapidly and easily separated from solution using magnetic particle separators (magnets). However, the disadvantage of magnetic beads is their low binding capacity (μg range; amount dependent on quantity of beads and the specific protein used) for the bait protein, as opposed to milligrams of bait protein required for efficient co-affinity purification experiments. No AtRGL1-interacting proteins were isolated using this method (data not shown).

To address the problem of the low binding capacity of the matrix, a second co-affinity purification protocol was developed using amylose resin instead of magnetic beads. One mL of amylose resin has a 3 mg binding capacity for MBP fusion proteins, at least two orders of magnitude higher than that of magnetic beads. Another advantage of amylose resin is that all the bait molecules will bind in an orientation that favourably exposes the N-terminal domain of AtRGL1, allowing maximal chance for nuclear proteins to interact. The disadvantage of this approach is that the elution of AtRGL1-interacting proteins will also elute the bait protein from the matrix. The bait is present in large amounts (high μg-mg), so when the elution is analysed by SDS-PAGE the AtRGL1-interacting proteins present in low amounts (ng) will be masked by the bait and its slight impurities. Furthermore, plant nuclear extract contains amylose-binding proteins that also co-purify with the bait and AtRGL1-interacting proteins, interfering with the identification of AtRGL1-interacting proteins (data not shown).

Due the problems encountered with co-affinity purification, affinity purification was performed instead, using MBP-AtRGL1$^{(1-121)}$ bait protein covalently linked to high-capacity NHS resin (10 mg protein per mL resin, see methods section 2.8). Covalent

cross-linking of the bait protein prevents its release during elution of AtRGL1-interacting proteins (Figure 3-1).

Affinity purification of AtRGL1-interacting proteins from nuclear extract of the whole plant successfully eluted one specific MBP-AtRGL1$^{(1-121)}$ binding protein with an approximate molecular weight of 24 kDa (Figure 3-4 A, C). The control columns were coated with either bovine serum albumin (BSA; Figure 3-4 B) or MBP-LacZ (Figure 3-4 D; MBP-β-galactosidase, expressed and purified as described in methods section 2.6.1) covalently cross-linked to the resin to identify proteins non-specifically interacting with the resin or the MBP domain. No 24 kDa proteins were eluted in either of these control experiments. This rules out the possibility that the 24 kDa protein interacts with the MBP domain of the bait protein or the resin. Therefore, this protein is likely interacting specifically with the N-terminal domain of AtRGL1. The 24 kDa protein was reproducibly obtained in two other similar experiments, confirming its interaction with the N-terminal 121 amino acids of AtRGL1.

The reason for not showing the MBP-LacZ bait in figure 3-4 D is that this semi-purified protein did not show a distinct band when stained with SYPROruby due to strong staining of the entire lane. It is clearly discernable when stained with coomassie blue (see Figure 3-5, E).

In these experiments, a set of bands 55-65 kDa in size could be detected in all lanes, including the loading buffer only lane (Figure 3-4, E). This indicates a systematic contamination. These 55-65 kDa proteins, suspected to be human keratin, are of a low abundance and were only detected due to the low nanogram sensitivity of the SYPROruby stain used to visualise the proteins.

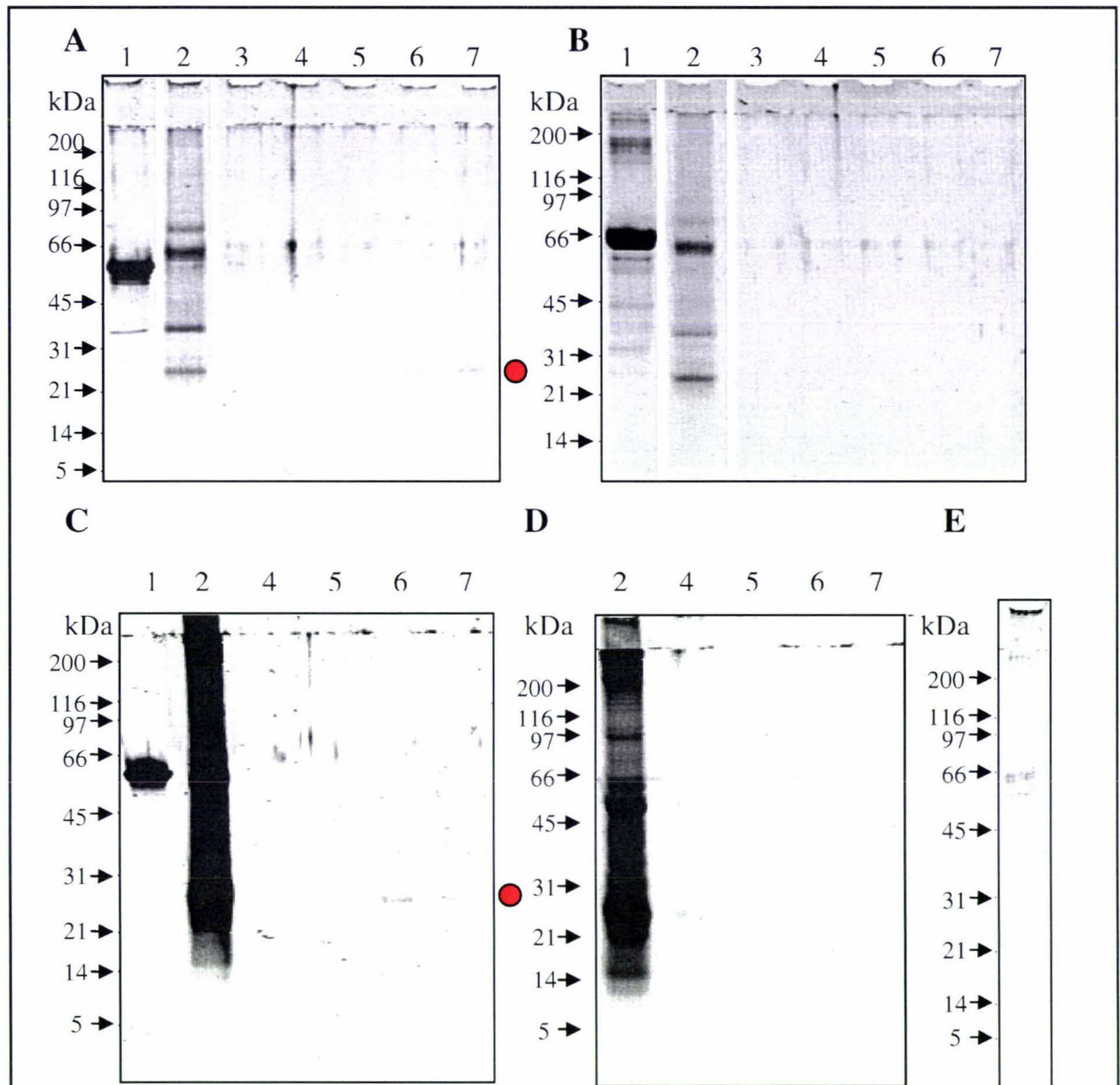


Figure 3-4 Affinity purification of MBP-AtRGL1$^{(1\text{-}121)}$-interacting proteins from whole plant nuclear extract. A. B. C. D. Lane 1, 2 μg of bait prior to cross-linking to the matrix; lane 2, input plant nuclear protein; lane 3, final wash; lane 4, 5, 6, and 7, sequential low pH elutions. **(A)** Small scale affinity purification using 400 μg MBP-AtRGL1$^{(1-121)}$ as bait and 700 μg plant nuclear extract (input 0.3 %, wash 0.3 %, elutions 10 % of the total sample was loaded). **(B)** Control affinity purification for **A,** BSA was used as bait. **(C)** Large scale affinity purification using 2 mg MBP-AtRGL1$^{(1-121)}$ as bait and 12 mg plant nuclear extract (input 0.15 %, elutions 10 % of the total sample loaded). **(D)** Control affinity purification for **C**, MBP-LacZ used as bait (not shown). **(E)** 5 μL contaminated SDS-PAGE loading buffer. Red circles (A., C.) indicate 24 kDa eluted protein. Gels stained with SYPROruby gel stain.

The affinity purification of AtRGL1$^{(1-121)}$-interacting proteins from nuclear extract prepared from inflorescence tissue was performed using large and small scale experiments. In both experiments parallel control purifications were performed using resin coated with MBP-LacZ to allow elimination of MBP and resin binding proteins. The combination of these two affinity purifications isolated three putative AtRGL1$^{(1-121)}$-interacting proteins with the approximate molecular weights of 24, 48, and 64 kDa (Figure 3-5). The large scale affinity purification, using approximately 7 mg of plant nuclear extract, resulted in elution of 48 and 64 kDa proteins (Figure 3-5, A). The small scale experiment, using approximately 0.7 mg of plant nuclear extract, eluted a 24, and a 64 kDa protein (Figure 3-5, C). The 32/34 kDa bands present in the first elution of the small scale experiment (Figure 3-5, C, lane 4 asterix) do not appear to be protein bands and have not been seen in any other experiments. Therefore, the presence of 32/34 kDa interacting proteins will be discounted unless they are detected in future affinity purification experiments.

It is unclear why the different preparations yielded different sized proteins. The absence of the 24 kDa protein in the elutions of the large scale experiment clearly cannot be accounted for by low protein amounts. Therefore, there must have been a difference in experimental conditions between the large and small scale experiments that prevent its isolation in the large scale experiment. One noted difference between these experiments, other than scale, was the season when tissue was collected. Inflorescence tissue was collected during late winter for the large scale experiment, whereas the collection was carried out during autumn for the small scale experiment. Although plants were grown under semi-controlled conditions, the seasonal conditions still affect plant growth, particularly light. GA signalling is involved in inflorescence development primarily during short days, i.e. winter conditions, so GA signalling may have been different in the plants collected during different seasons. The contamination of putative Human keratin was removed for the smaller scale affinity purification from inflorescence tissue, confirming that the 24 kDa and 64 kDa proteins were from the plant nuclear extract, and not the contaminating source.

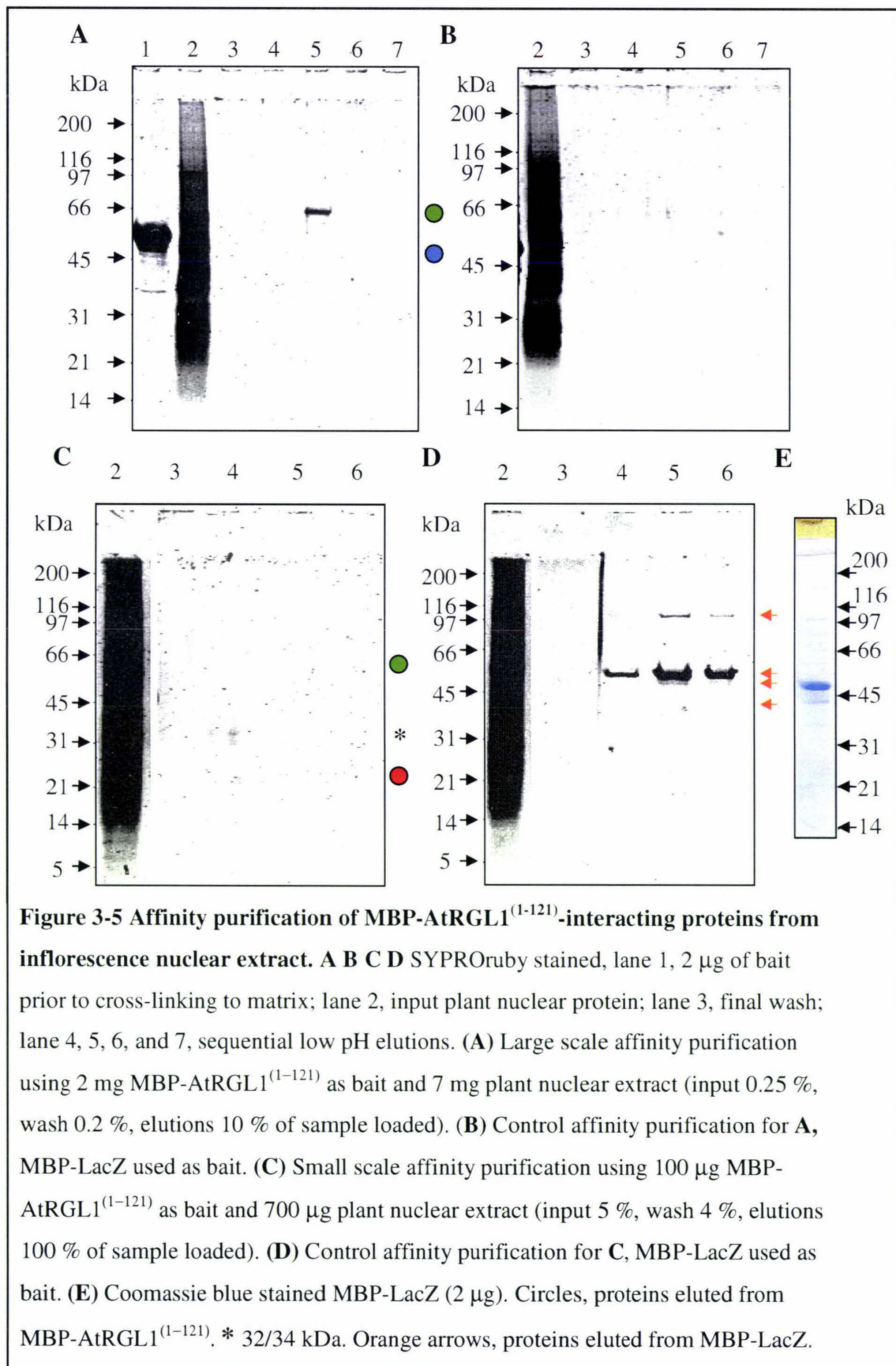


Figure 3-5 Affinity purification of MBP-AtRGL1$^{(1\text{-}121)}$-interacting proteins from inflorescence nuclear extract. A B C D SYPROruby stained, lane 1, 2 μg of bait prior to cross-linking to matrix; lane 2, input plant nuclear protein; lane 3, final wash; lane 4, 5, 6, and 7, sequential low pH elutions. **(A)** Large scale affinity purification using 2 mg MBP-AtRGL1$^{(1-121)}$ as bait and 7 mg plant nuclear extract (input 0.25 %, wash 0.2 %, elutions 10 % of sample loaded). **(B)** Control affinity purification for **A**, MBP-LacZ used as bait. **(C)** Small scale affinity purification using 100 μg MBP-AtRGL1$^{(1-121)}$ as bait and 700 μg plant nuclear extract (input 5 %, wash 4 %, elutions 100 % of sample loaded). **(D)** Control affinity purification for **C**, MBP-LacZ used as bait. **(E)** Coomassie blue stained MBP-LacZ (2 μg). Circles, proteins eluted from MBP-AtRGL1$^{(1-121)}$. * 32/34 kDa. Orange arrows, proteins eluted from MBP-LacZ.

A 48 kDa protein was only eluted once, in the largest scale experiment when using 2mg MBP-AtRGL1$^{(1-121)}$ as bait and 7 mg of inflorescence nuclear extract. It is plausible that this 48 kDa protein is of lower abundance than the other eluted proteins (24 and 64 kDa) in plant extracts, or that it has a lower affinity for AtRGL1$^{(1-121)}$. The eluted 48 kDa protein has a similar molecular weight to one of the MBP-AtRGL1$^{(1-121)}$ degradation products, so it may be a bait degradation product rather than a AtRGL1-interacting protein.

In the control experiments, in which MBP-LacZ fusion protein was used as bait, four proteins, 43, 50, 55 and 100 kDa in size were detected in the elutions of the small scale experiment (Figure 3-5, D). Why these proteins were not eluted in other control experiments is unclear. It is possible that the 50 kDa protein is the 48 kDa protein present in the elutions from experiments using MBP-AtRGL1$^{(1-121)}$as bait, though it is more likely the MBP-LacZ bait protein released from the NHS resin due to incomplete cross-linking. However, without any evidence that the similar sized proteins eluted from the MBP-LacZ affinity control and the MBP-AtRGL1$^{(1-121)}$ affinity experiments are the same protein a 48 kDa AtRGL1-interacting protein cannot be discounted. The identity of these proteins can only be resolved by identification using MALDI-TOF mass spectroscopy or N-terminal peptide sequencing.

The 64 kDa protein (hence referred to as p64) was consistently affinity purified from plant extract obtained from the inflorescence, but not from the whole plant nuclear extract. This suggests that the p64 protein may only be present in the inflorescence. To resolve this issue, p64 will need to be identified and a probe designed to analyse its expression.

The AtRGL1-interacting proteins were purified in very low amounts. The 24 kDa protein (hence referred to as p24) was eluted in quantities of approximately one to several nanograms, based on the protein being close to the detection limit of the SYPROruby stain used, which is one nanogram. Approximately 10 ng was purified from 1 mg of nuclear extract, so this protein has an abundance of approximately 0.001 % or 1/100000 in the nucleus. (This is a very crude estimation; the amount of p24 in tissue could be higher as only a fraction may be affinity purified). Similarly for p64,

up to 50ng was purified from 1 mg of nuclear extract; hence this protein could have an abundance of approximately 0.005 % or 1/20000 in the nucleus.

To identify these purified proteins, MALDI TOF mass spectrometry analysis was performed. This procedure is used to analyse protein bands of interest after separation by SDS PAGE. Gel slices containing the protein band are excised and treated with trypsin which digests the protein into peptides. The masses of the peptides are then determined by MADLI TOF mass spectrometry and compared to a database of predicted tryptic digest peptide masses (tryptic digest mass fingerprint database) generated from *A. thaliana* translated genomic sequence. Unfortunately attempts to identify AtRGL1-interacting proteins have failed due to low amounts of protein (low nanograms). The weak signal was further masked due to accidental contamination after separation that occurred at the protein analysis facility (University of New South Wales, Australia).

The p24 protein does not match the predicted weight of any protein product of any gene currently implicated by genetic analyses to be involved in GA signalling (AtSPY, AtSLY1, AtSHI, AtGPA1), and so may be a novel component. The p64 protein is close to the predicted molecular weight of the DELLA protein RGA; however, AtRGA is not present in the tissues used (See below, section 3.2, it would be expected to be detected in plant extracts by 5E1), so it is possible that p64 is also a yet unknown component of GA signalling. Therefore, these two proteins, p24 and p64, may be novel GA signalling components and their identification may reveal the mechanisms by which GA signalling is perceived by the N-terminal domain of AtRGL1 and perhaps other DELLA proteins.

3.2 Antibody characterisation

The affinity purification experiments performed as part of this thesis were limited to investigating the interactions of the N-terminal 121 amino acid residues of AtRGL1. However, as discussed earlier, the C-terminal domain has been implicated in the roles of DELLA proteins in gene repression. Co-immunoprecipitation of endogenous plant AtRGL1 should allow co-purification of interacting proteins binding to either the N-terminal or C-terminal domain (Figure 3-6).

Immunoprecipitation can also be used to study the post-translational modifications of AtRGL1 in addition to protein interactions. As endogenous AtRGL1 would be immunoprecipitated from plant tissue, the AtRGL1 protein will carry any covalent modifications that occur *in vivo*. Post-translational modifications such as phosphorylation, glycosylation, ubiquitination, and enzymatic cleavage can be identified using western blotting and mass spectroscopy analysis of purified protein. To perform both these experiments, co-immunoprecipitation and analysis of modifications, an immunoprecipitating antibody is required, and AtRGL1 must be present in plant extracts at a detectable level.

3.2.1 Characterisation of an anti-DELLA motif monoclonal antibody 5E1

A monoclonal mouse antibody (5E1) was developed at HortResearch against a 12 amino acid peptide from the DELLA motif. This antibody was selected for its high affinity for *E. coli*-expressed N-terminal domains of DELLA proteins and it detects the N-terminal domains of AtGAI, AtRGA, AtRGL1, and AtRGL2 with a high affinity, but not AtRGL3 (HortResearch plant immunology group, unpublished results). In the course of research for this thesis, the monoclonal antibody 5E1 was characterised for recognition of proteins present in the plant nuclear extract. Very faint bands that did not correlate with the expected molecular weights of DELLA proteins were obtained repeatedly (Figure 3-7, A, and data not shown). Further characterisation will need to be performed with mutant *A. thaliana* plants with deletions in individual DELLA protein encoding genes to verify the identity of proteins, and GA biosynthesis mutants to have accumulated DELLA protein levels.

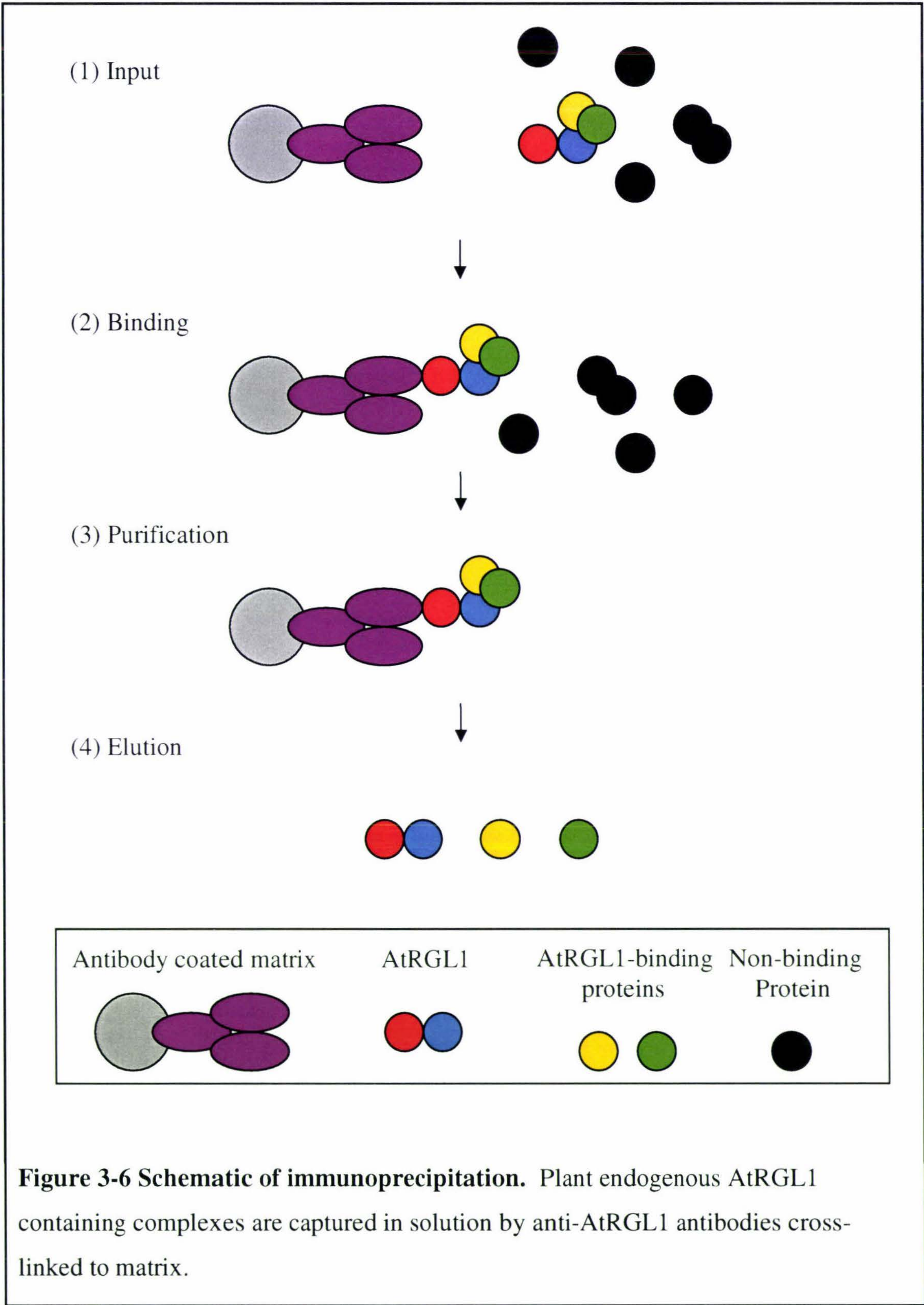


Figure 3-6 Schematic of immunoprecipitation. Plant endogenous AtRGL1 containing complexes are captured in solution by anti-AtRGL1 antibodies cross-linked to matrix.

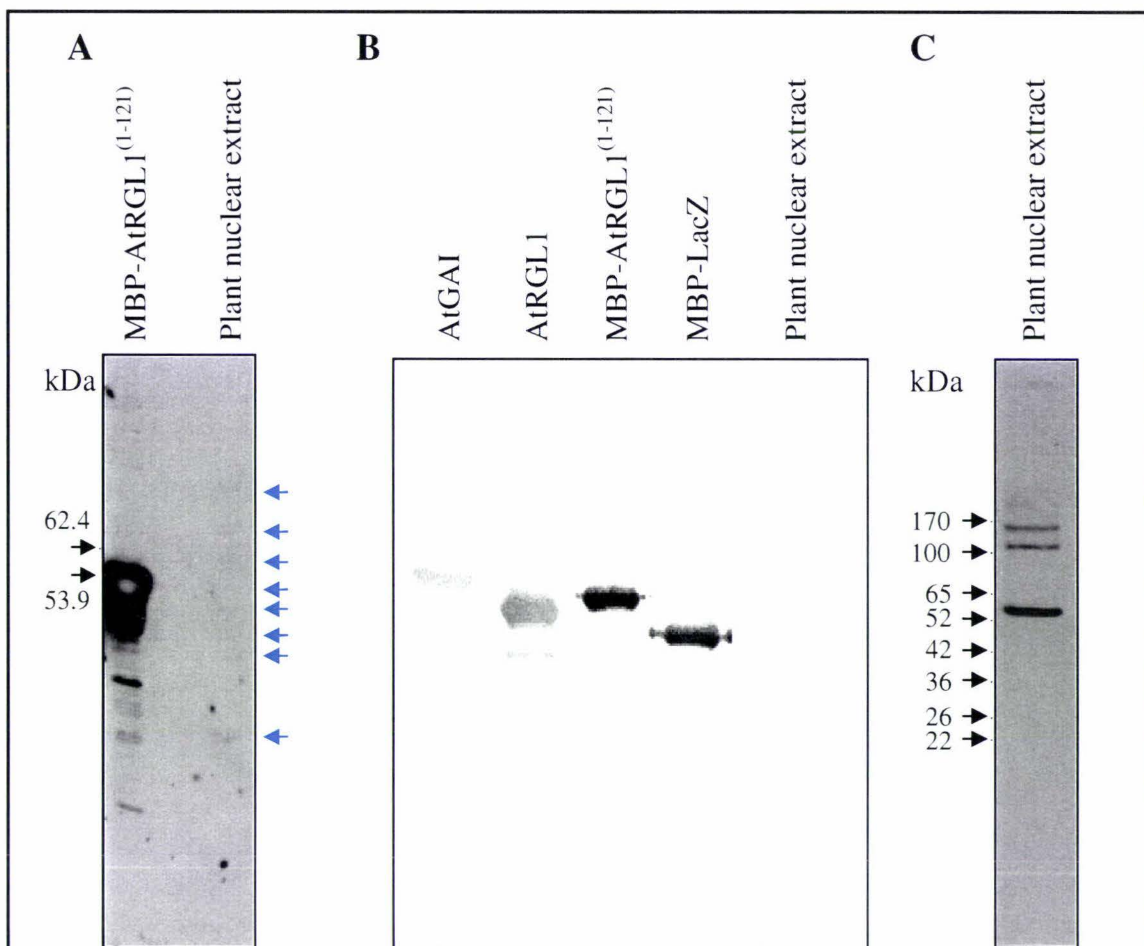


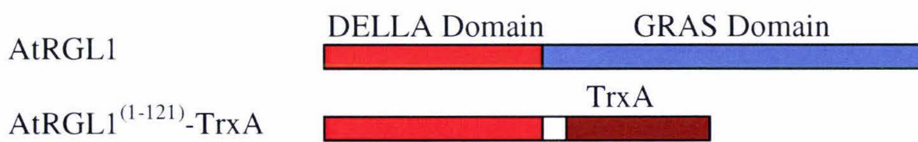
Figure 3-7 Western blot analysis of plant nuclear extract using anti-DELLA antibodies. **(A)** Western blot using anti-DELLA motif monoclonal antibody 5E1 (1 μgmL^{-1}) of 2 μg purified MBP-AtRGL1$^{(1-121)}$, 2 μg soluble plant nuclear protein. Blue arrows indicate bands detected in plant nuclear extract. **(B)** Western blot using anti-MBP-AtRGL1$^{(1-121)}$ polyclonal antiserum (1/1000 dilution) of crude *E. coli* lysate expressing AtGAI or AtRGL1; 100 ng of purified MBP-AtRGL1$^{(1-121)}$ and 100 ng of purified MBP-LacZ, and 2 μg soluble plant nuclear protein. **(C)** Western blot of 1 μg soluble plant nuclear protein using anti-MBP-AtRGL1$^{(1-121)}$ antiserum IgG fraction (1/500 dilution - equivalent to 1/1000 serum).

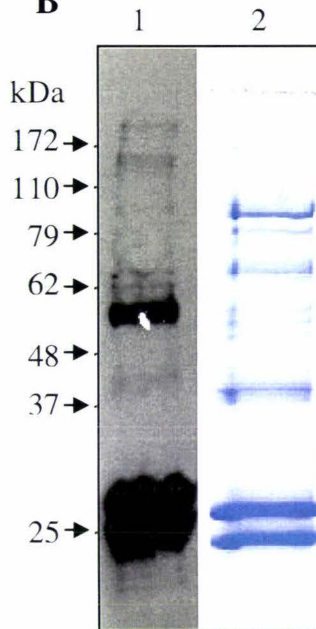
3.2.2 Production of an anti-AtRGL1 polyclonal antibody

The monoclonal antibody 5E1 recognises only a single epitope, and if this is blocked by covalent modification then the protein will not be recognised by the antibody in western blots or immunoprecipitation experiments. In contrast polyclonal antibodies are usually composed of multiple clones of antibodies that bind to multiple different epitopes. Therefore, if an epitope is blocked by covalent modification, interacting protein or protein conformation, then the remaining epitopes are available for binding of cognate antibodies to immunoprecipitate the target protein or detect it by western blotting. For this reason a polyclonal antiserum was developed by immunisation of rabbits with the purified recombinant fusion protein MBP-AtRGL1$^{(1-121)}$.

This polyclonal anti-MBP-AtRGL1$^{(1-121)}$ serum recognises MBP, and the DELLA proteins AtRGL1 and AtGAI expressed in *E. coli* (Figure 3-7, B). Other DELLA proteins may also be detected but they were not investigated. MBP is strongly immunogenic, and so it is expected that a strong immune response would be produced against MBP in addition to that against AtRGL1$^{(1-121)}$ by the rabbit . The detection of non-AtRGL1 DELLA proteins is expected as the N-terminal domains of all *A. thaliana* DELLA proteins contain conserved motifs.

Anti-MBP-AtRGL1$^{(1-121)}$ serum detected proteins in plant nuclear extract (Figure 3-7, B, C). IgG was fractionated from other serum proteins as described in methods section 2.10. This purified IgG fraction contains the polyclonal antibodies, and detects three proteins in plant nuclear extract, 56 kDa, 110 kDa, and 150 kDa. The sizes of these bands correlate to the predicted size of AtRGL1 (56.8 kDa), and two higher molecular weight bands that could be explained by the existence of an SDS-resistant dimer and a protein complex containing AtRGL1. Unfortunately, the IgG fraction has a strong response to MBP, so the anti-AtRGL1$^{(1-121)}$ polyclonal antibodies were affinity purified from the IgG fraction to remove antibodies against MBP. Affinity purification was performed using purified AtRGL1$^{(1-121)}$-TrxA fusion protein (Figure 3-8) cross-linked to NHS resin. AtRGL1$^{(1-121)}$-TrxA fusion was expressed in *E. coli* and purified from extract using a combination of ion-exchange chromatography and affinity chromatography. The affinity purified anti-AtRGL1$^{(1-121)}$ polyclonal antibodies comprised a fraction of approximately 0.7 % of the total serum IgG.





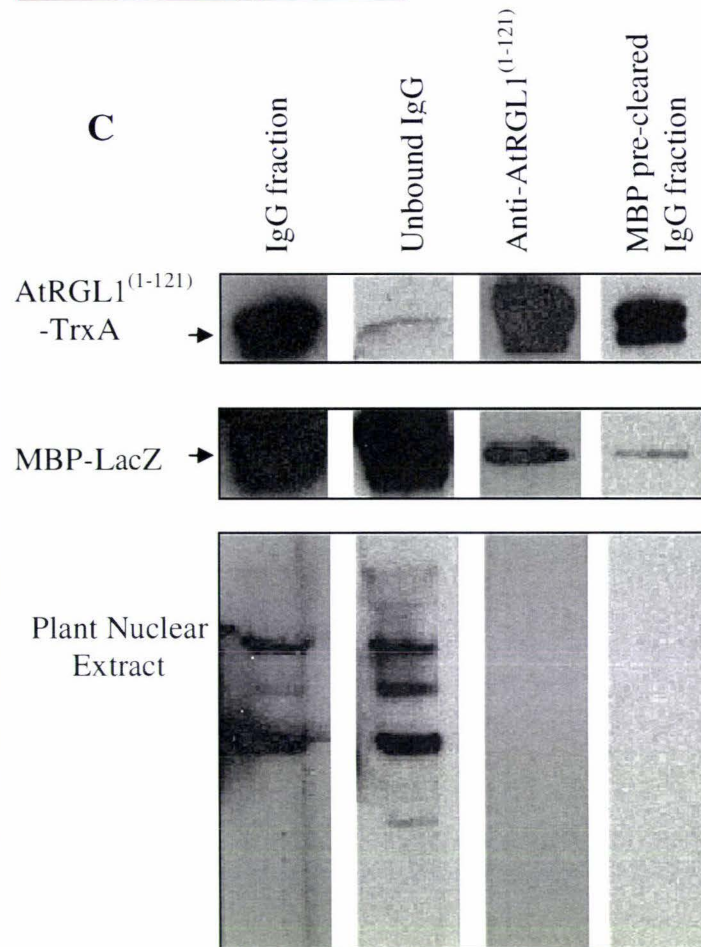


Figure 3-8 Fractionation and determination of the specificity of anti-MBP-AtRGL1$^{(1-121)}$ antiserum. (A) Top, schematic of full length AtRGL1. Bottom, schematic of AtRGL1$^{(1-121)}$-TrxA fusion **(B)** 1, western blot of purified AtRGL1$^{(1-121)}$-TrxA (1 μg) using anti-MBP-AtRGL1$^{(1-121)}$ serum IgG fraction. 2, coomassie blue stained gel showing purified AtRGL1$^{(1-121)}$-TrxA (1 μg). Expected size of AtRGL1$^{(1-121)}$-TrxA is 25 kDa. **(C)** Top to bottom: western blot of AtRGL1$^{(1-121)}$-TrxA (1 μg), MBP-LacZ (1 μg), and plant nuclear extract (1 μg). Left to right: antibody fractions from affinity purification of anti-MBP-AtRGL1$^{(1-121)}$ serum using AtRGL1$^{(1-121)}$-TrxA cross-linked to resin: IgG fraction (total IgG from serum), Unbound IgG (affinity purification flow-through fraction), Anti-AtRGL1$^{(1-121)}$ (AtRGL1$^{(1-121)}$-TrxA column eluate fraction), MBP pre-cleared IgG fraction (total IgG fraction pre-incubated with MBP-LacZ). Antibodies diluted equivalent to a 1/1000 dilution of serum.

Characterisation of the antibody fractions from purification reveals that affinity-purified anti-AtRGL1$^{(1-121)}$ polyclonal antibodies do not detect proteins in plant nuclear extract (Figure 3-8). To ascertain if the proteins detected in plant nuclear extract by the total IgG fraction are in fact recognised due to MBP-like epitopes, antibodies that recognise MBP epitopes were blocked prior to western blotting by incubation with MBP-LacZ fusion protein in solution. This sequestering of the anti-MBP antibodies resulted in loss of response of the total IgG fraction to plant nuclear proteins, but not to AtRGL1$^{(1-121)}$-TrxA (Figure 3-8, C). Therefore, the plant nuclear proteins detected did indeed have MBP-like epitopes, and so no DELLA proteins were detected in plant nuclear extract.

Similar results were obtained when using the anti-DELLA motif monoclonal antibody 5E1. These results indicate that neither the polyclonal nor the monoclonal antibodies can be used for the immunoprecipitation of AtRGL1 or other DELLA proteins from wild-type plant nuclear extract. The absence of DELLA proteins in wild-type plant tissues and methods for overexpression and hindering GA-induced degradation are discussed in the discussion sections 4.1 and 4.4.

3.2.3 Characterisation of the capacity of anti-AtRGL1$^{(1-121)}$ polyclonal antibodies to immunoprecipitate the antigen from solution.

Despite the absence of detectable levels of DELLA proteins in plant nuclear extracts it is worth determining if the anti-AtRGL1$^{(1-121)}$ polyclonal antibodies are able to immunoprecipitate the antigen from solution for use in future work. The reason for this is that plant extracts may be produced with detectable levels of DELLA proteins, as discussed in sections 4.1 and 4.4.

Not all antisera are suitable for immunoprecipitating the antigen from solution, due to the conformation of the antigen being different in solution (folded), relative to the state during Western blotting (denatured). Western blotting involves detection of antigen that has been denatured, and so the antigen may be unfolded, or only partially refolded. For this reason an antibody may detect a protein by western blot, but not in solution, but also vice versa. To establish whether anti-AtRGL1$^{(1-121)}$ polyclonal antibodies are suitable for immunoprecipitation, AtRGL1$^{(1-121)}$-TrxA was

immunoprecipitated from spiked plant nuclear extract (Figure 3-9). This procedure was successful, with approximately 10-30 % of the total AtRGL1$^{(1-121)}$-TrxA recovered from the plant extract when using 2 μg of fusion protein and 25 μg of purified anti-AtRGL1$^{(1-121)}$ polyclonal antibody cross-linked to NHS resin.

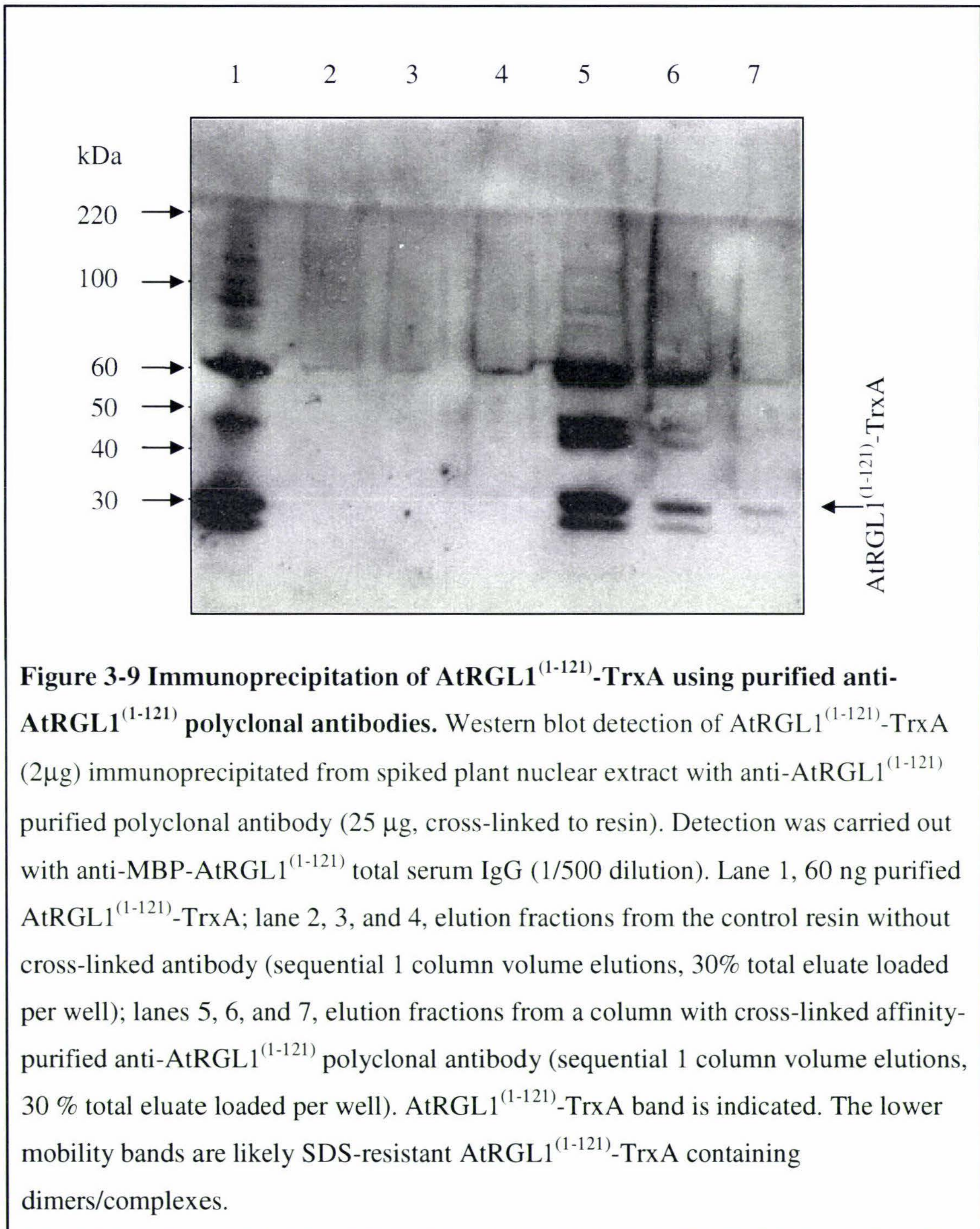


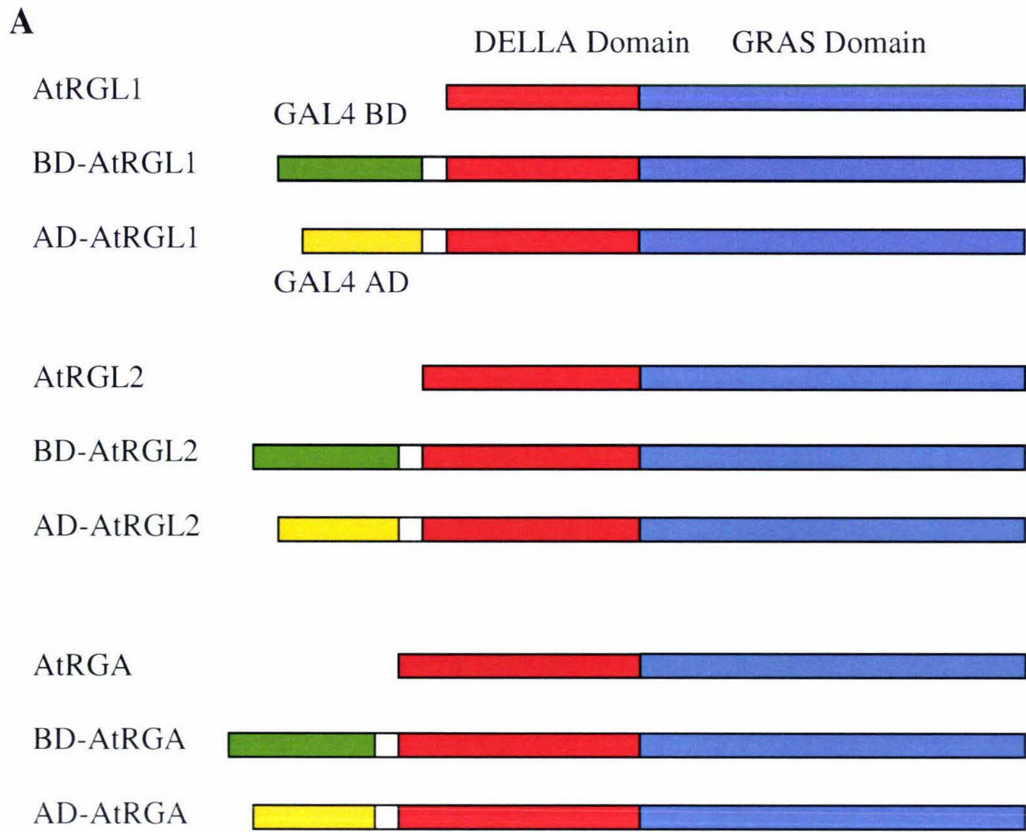
Figure 3-9 Immunoprecipitation of AtRGL1$^{(1-121)}$-TrxA using purified anti-AtRGL1$^{(1-121)}$ polyclonal antibodies. Western blot detection of AtRGL1$^{(1-121)}$-TrxA (2μg) immunoprecipitated from spiked plant nuclear extract with anti-AtRGL1$^{(1-121)}$ purified polyclonal antibody (25 μg, cross-linked to resin). Detection was carried out with anti-MBP-AtRGL1$^{(1-121)}$ total serum IgG (1/500 dilution). Lane 1, 60 ng purified AtRGL1$^{(1-121)}$-TrxA; lane 2, 3, and 4, elution fractions from the control resin without cross-linked antibody (sequential 1 column volume elutions, 30% total eluate loaded per well); lanes 5, 6, and 7, elution fractions from a column with cross-linked affinity-purified anti-AtRGL1$^{(1-121)}$ polyclonal antibody (sequential 1 column volume elutions, 30 % total eluate loaded per well). AtRGL1$^{(1-121)}$-TrxA band is indicated. The lower mobility bands are likely SDS-resistant AtRGL1$^{(1-121)}$-TrxA containing dimers/complexes.

The immunoprecipitation protocol utilised cross-linking of antibodies directly to the NHS resin via a covalent bond through Lysine residues, hence the orientation of the antibodies was random. This would have inevitably blocked the antigen binding site of a portion of the antibody molecules. To improve the efficiency for future immunoprecipitation experiments, the antibody will be cross-linked in the correct orientation via indirect cross-linking to the resin through a Protein A bridge. Protein A binds exclusively to the Fc portion of the antibody that is not involved with the antigen binding sites. Therefore, the antigen-binding sites of all antibodies bound to the column will be free to interact with the antigen.

3.3 Analysis of DELLA-DELLA protein interactions

At the start of the research for this thesis (2003) the only interaction of a DELLA protein that had been shown was the homo-dimerisation of OsSLR1 in a yeast 2-hybrid experiment (Itoh *et. al.,* 2002). Therefore, it was of interest to investigate whether other DELLA proteins form dimers as this could be important to DELLA protein function. The genomes of some dicot plants, such as *A. thaliana*, encode multiple DELLA proteins. Therefore, multiple DELLA proteins may form hetero-dimers with functionally important consequences. Analysis of plant phenotypes of multiple DELLA gene knockouts in a GA-deficient background revealed that *AtRGA*, *AtRGL1*, and *AtRGL2* all have overlapping roles in inflorescence development (Cheng *et. al.,* 2004, Tyler *et. al.,* 2004). For this reason, during 2004, I selected these three DELLA proteins for investigation of DELLA-DELLA protein interaction as the second aim of this thesis.

To determine whether the three DELLA proteins, AtRGA, AtRGL1, and AtRGL2 interact, a yeast 2-hybrid system was used. The yeast 2-hybrid system exploits the recruitment of two domains of a transcription factor together by the interaction of the two proteins being investigated (Figure 3-10). Proteins are expressed as recombinant fusions to the transcription factor GAL4, as either DNA binding domain (BD) or GAL4 activation domain (AD) fusions. If the proteins interact then the BD fusion is able to recruit the AD to the GAL4 induced promoter of a reporter gene, activating transcription.



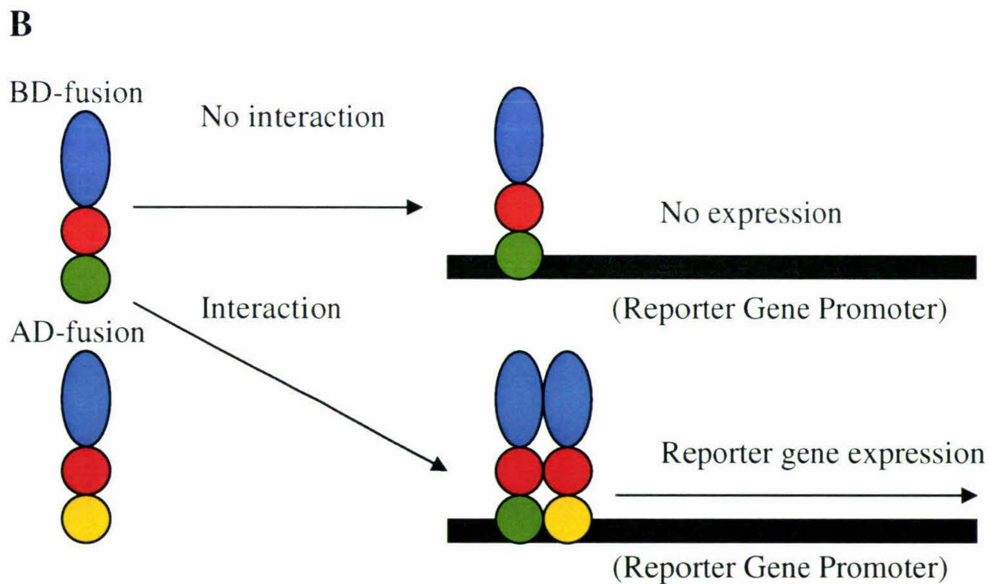


Figure 3-10 Schematic of yeast 2-hybrid analysis of DELLA-DELLA protein interactions. (**A**) Schematics of full length AtDELLA proteins, GAL4 DNA binding domain fusions (BD), and GAL4 Activation domain fusions (AD). (**B**) Schematic of yeast 2-hybrid protein interaction reporter gene system.

3.3.1 Expression of DELLA proteins in *Saccharomyces cerevisiae*

The three *A. thaliana* DELLA genes of interest, *AtRGA*, *AtRGL1*, and *AtRGL2*, were cloned into the yeast 2-hybrid system vectors and transformed into a yeast reporter strain, CG1945. To confirm the presence of vectors and expression of the DELLA proteins, yeast cell extract from the cultures containing the relevant plasmids were analysed by western blotting. AD domain fusions were detected using the anti-DELLA motif antibody 5E1. The predicted protein sizes were: AD-AtRGA 81.2 kDa, AD-AtRGL1 74.3 kDa, and AD-AtRGL2 77.9 kDa. However, both AtRGA and AtRGL2 had lower mobility than expected (Figure 3-11). AD domain fusions contain an HA epitope tag, and western blotting with an anti-HA polyclonal confirmed the presence of the AD fusion DELLA proteins (Figure 3-11, 3-12).

The BD-DELLA fusions were more difficult to extract from yeast, requiring heating during SDS/UREA extraction. Heat extraction is normally only required for membrane protein extraction from yeast. This suggests that they are present in either an SDS-resistant complex or inclusion bodies. The BD fusions of AtRGA and AtRGL2 were found to have an intrinsic ability to activate transcription in the absence of an interacting AD fusion protein (see below). Therefore, at least a portion of the expressed BD-AtRGA and BD-AtRGL2 fusion proteins are folded correctly, so an SDS resistant complex is a more likely explanation for difficulties in BD fusion extraction. BD fusions contain a c-Myc epitope tag and western blotting using a anti-c-Myc antibody confirmed the presence of proteins at approximately the expected molecular weights (BD-AtRGA, 84.4 kDa; BD-AtRGL1, 77.7 kDa; BD-AtRGL2, 80.9 kDa), though again both AtRGA and AtRGL2 had lower mobility than expected (Figure 3-12). For unknown reasons the heat extraction caused an extremely high background signal when detection was attempted with the anti-DELLA motif monoclonal 5E1 (data not shown). In summary, GAL4 AD and BD fusions of DELLA proteins are expressed in yeast cells.

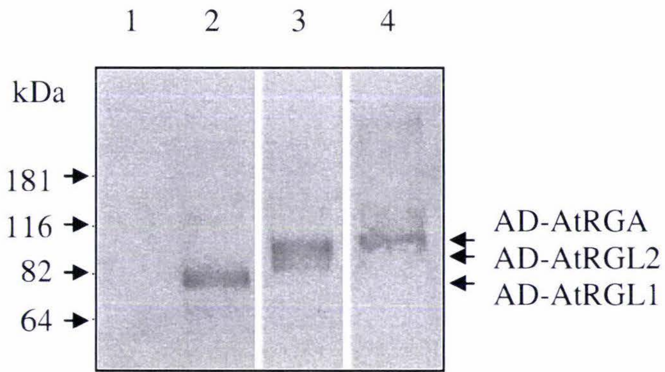


Figure 3-11 Western blot detection of AD-DELLA protein fusions expressed in yeast. Western blot of yeast crude protein extracts from CG1945 transformants using anti-DELLA motif monoclonal antibody 5E1. Lane 1, pACT2 (empty); lane 2, pDJS006 (AD-AtRGL1); lane 3, pDJS008 (AD-AtRGL2); lane 4, pDJS010 (AD-AtRGA.

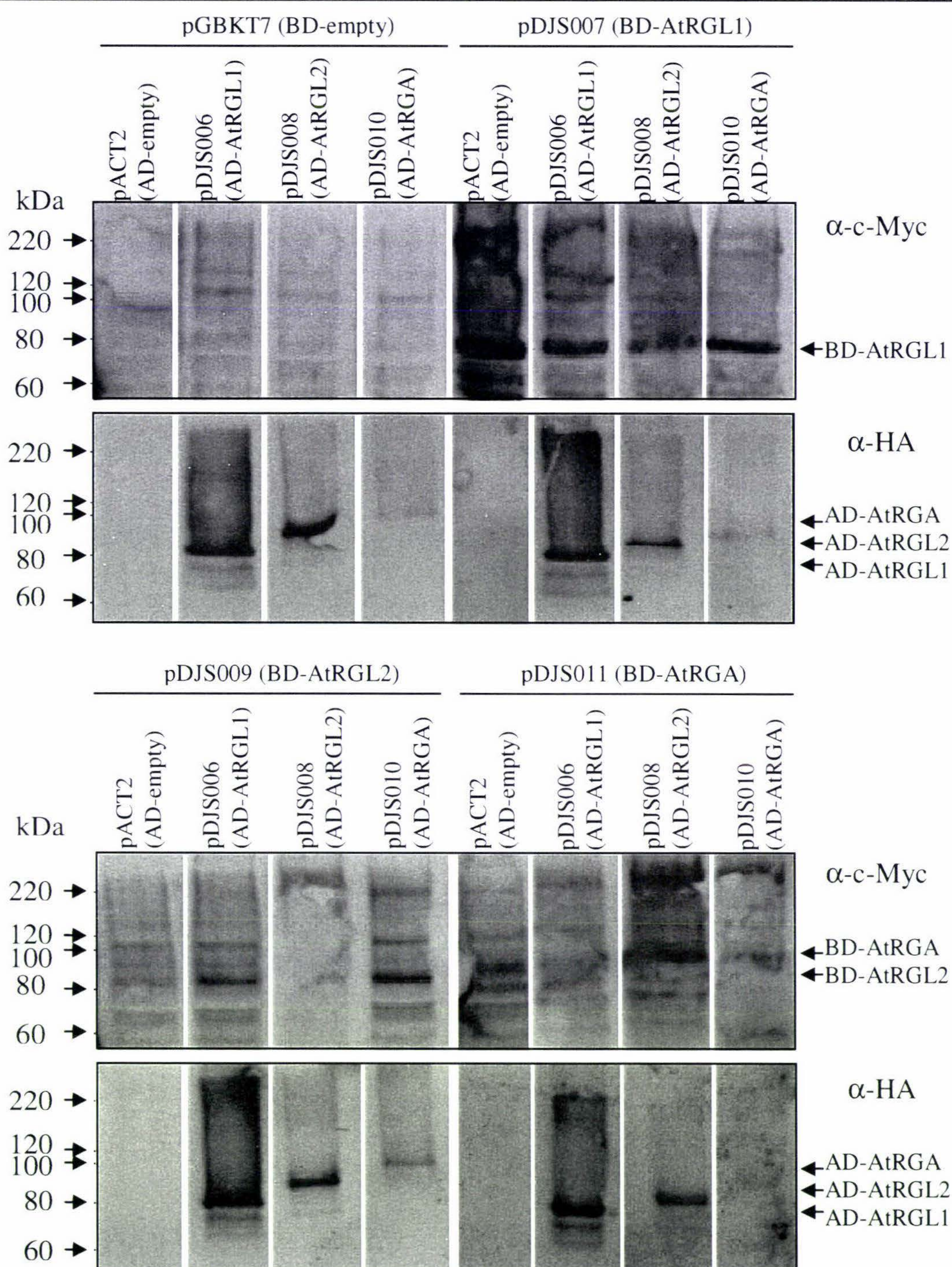


Figure 3-12 Western blot detection of AD- and BD-DELLA protein fusions expressed in yeast. Western blot of yeast crude protein extracts from CG1945 double transformants using anti-c-Myc (1 μgmL^{-1}) and anti-HA (1 μgmL^{-1}).

3.3.2 Yeast 2-Hybrid analysis of DELLA-DELLA protein interactions

All combinations of BD-DELLA and AD-DELLA fusions were analysed for activation strength on two reporter genes, *HIS3* and *LACZ*. The *HIS3* reporter gene is able to detect weak protein-protein interactions. This reporter gene consists of a GAL4 promoter controlling a gene encoding for one of the enzymes of the histidine biosynthesis pathway, Imidazoleglycerol-phosphate dehydratase. Yeast transformants are grown on minimal media lacking histidine in the presence of various concentrations of 3-AT, a chemical that inhibits the expression of the reporter gene by an unknown mechanism. Transformants with higher activation are able to grow on media containing higher amounts of the inhibitor. Table 6 shows that both BD-AtRGA and BD-AtRGL2 had strong autoactivation beyond the activation range measurable by the *HIS3* reporter gene. In contrast, BD-AtRGL1demonstrated weak autoactivation of the *HIS3* reporter gene, within the range of what would be considered a weak interaction. The combinations of AD-DELLA fusions with BD-AtRGL1 showed that there was no significant increase in activation (Table 6). There was one exception; a reduction in 3-AT resistance was observed when AD-AtRGA or AtRGL2 were co-expressed with BD-AtRGL1. The reason for this could be due to the growth inhibition resulting from the DELLA protein expression as discussed below.

The *HIS3* reporter gene has a narrow range and is only semi-quantitative. A second reporter gene, *LACZ*, was utilised to quantitatively determine the reporter gene activation and to establish whether AD-DELLA fusions increase the reporter gene activation above the level of autoactivation. The level of activation of the *LACZ* reporter gene can be measured accurately by an enzymatic assay for β-galactosidase, the protein encoded by *LACZ*. β-galactosidase enzyme activity measurement showed the autoactivation of BD-AtRGA to be equivalent to the activation resulting from the positive interaction control, the BD-p53 and AD-large T antigen fusions (Figure 3-13). The BD-AtRGL2 had an even stronger autoactivation, approximately three times that of the positive interaction control. The BD-AtRGL1 fusion demonstrated a weak autoactivation, much lower than that of the other two BD-DELLA fusions (approximately 1/8th the positive interaction control), in agreement with the *HIS3* reporter gene results. The co-expression of AD-DELLA fusions with the BD-DELLA

fusions did not result in any statistically significant differences to reporter gene activation. A replica experiment using a separate set of yeast transformants was also analysed, with similar results.

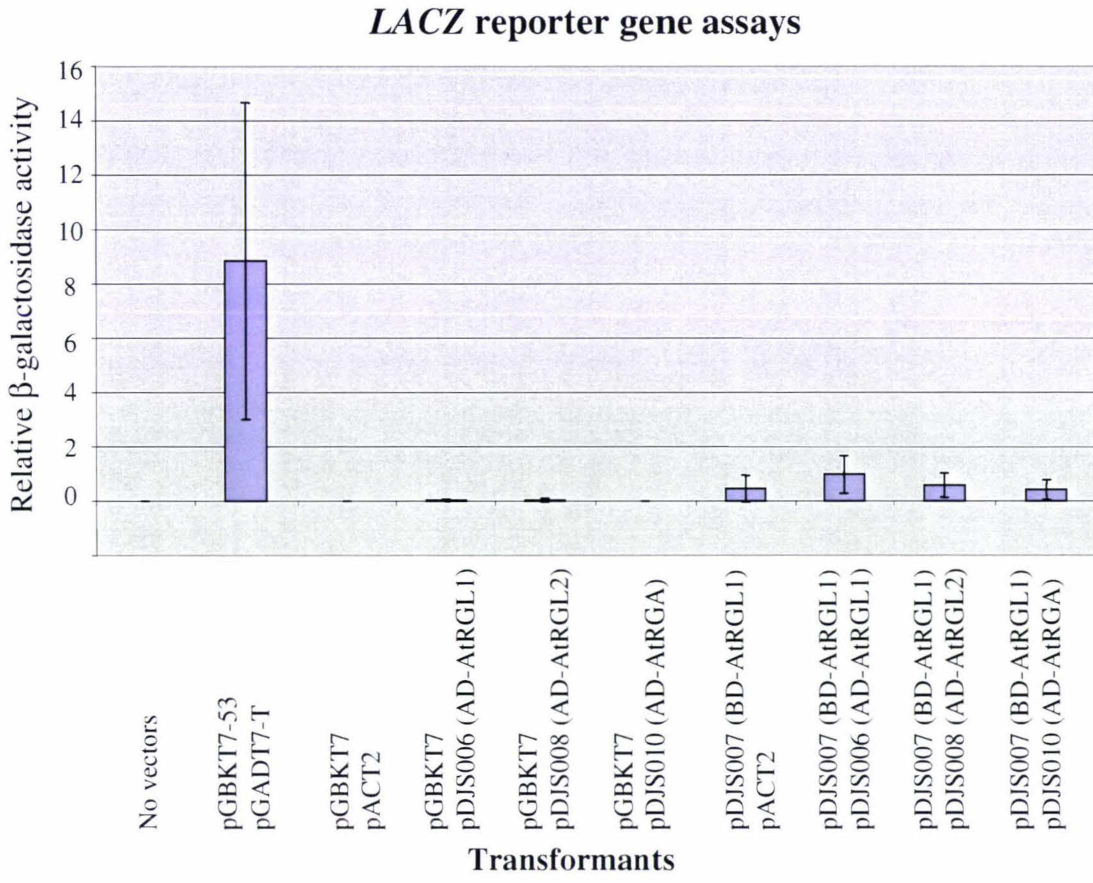
Surprisingly, some decrease in reporter gene activation was noted, although this data is not statistically significant due to the large variation in individual values of the enzyme assays. There was a slight increase in LACZ reporter gene activation when BD-AtRGL1 was co-expressed with AD-AtRGL1, in comparison to BD-AtRGL1 alone. However, as this is within one standard deviation the difference is not statistically significant. The effect of expressed DELLA protein fusions on yeast growth is noteworthy. DELLA proteins, especially AtRGA, restricted the maximal growth of yeast in/on media that provides restricted nitrogen sources (yeast transformants are grown on minimal media for the retention of plasmids). Abnormal cell division was also observed, with cells clumping even when grown under vigorous shaking (data not shown). This altered growth was not associated with either the AD or BD fusions, but more specifically with the DELLA protein portions of the fusions. AtRGA had the most drastic phenotype, AtRGL2 less so, while AtRGL1 did not have a noticeable effect on yeast growth.

The simplest explanation of the results obtained is that DELLA proteins do not form homo-dimers or hetero-dimers. However, there are several other interpretations possible from these results, so the experiment is inconclusive. Possible explanations and approaches to establishing whether DELLA protein dimers form are discussed in Chapter 4, sections 4.2 and 4.5.

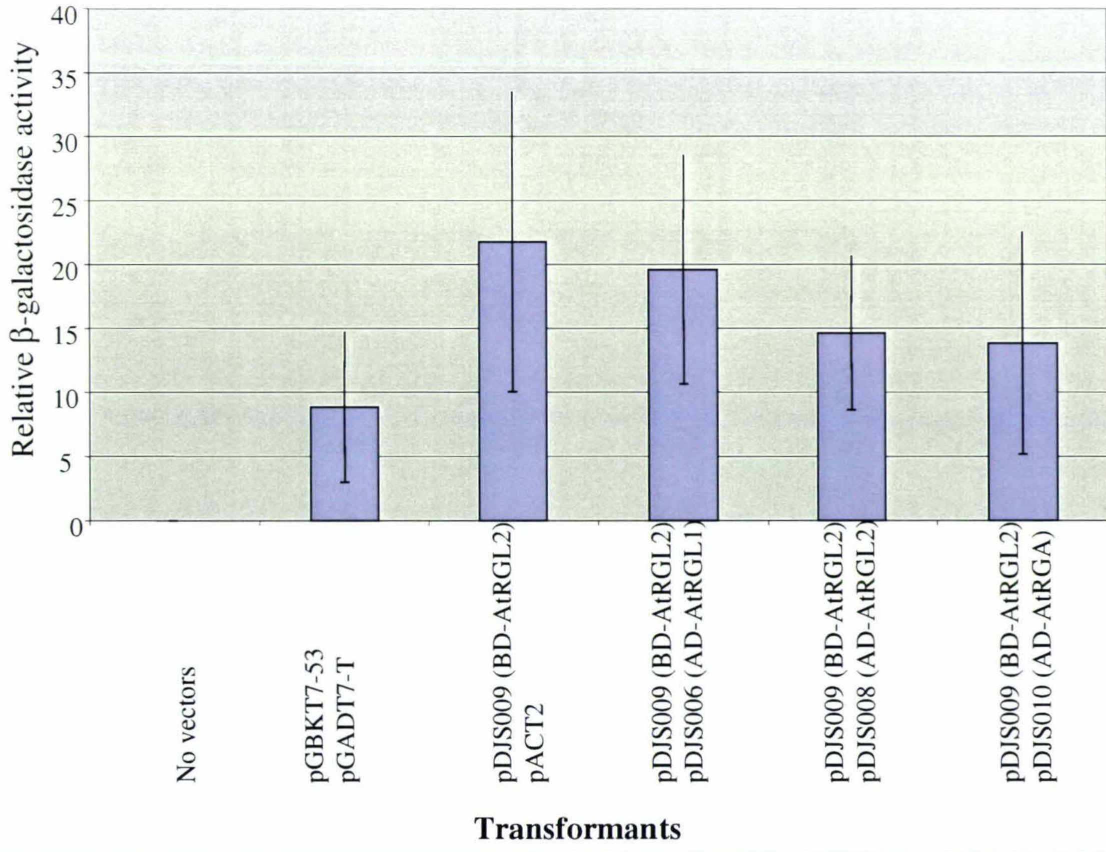
Table 6

HIS3 reporter gene assay of DELLA-DELLA protein interactions

Vectors	Expressed proteins	3-AT resistance (mM)
no vectors		0
pGBKT7-p53 and pGADT7-T	BD-p53 and AD-T-antigen	60
pCL1	full length GAL4	60
pGBKT7 and pACT2	BD and AD	0
pGBKT7 and pDJS006	BD and AD-AtRGL1	0
pGBKT7 and pDJS008	BD and AD-AtRGL2	0
pGBKT7 and pDJS010	BD and AD-AtRGA	0
pDJS007 and pACT2	BD-AtRGL1 and AD	10
pDJS007 and pDJS006	BD-AtRGL1 and AD-AtRGL1	10
pDJS007 and pDJS008	BD-AtRGL1 and AD-AtRGL2	2
pDJS007 and pDJS010	BD-AtRGL1 and AD-AtRGA	2
pDJS009 and pACT2	BD-AtRGL2 and AD	60
pDJS009 and pDJS006	BD-AtRGL2 and AD-AtRGL1	60
pDJS009 and pDJS008	BD-AtRGL2 and AD-AtRGL2	60
pDJS009 and pDJS010	BD-AtRGL2 and AD-AtRGA	60
pDJS011 and pACT2	BD-AtRGA and AD	60
pDJS011 and pDJS006	BD-AtRGA and AD-AtRGL1	60
pDJS011 and pDJS008	BD-AtRGA and AD-AtRGL2	60
pDJS011 and pDJS010	BD-AtRGA and AD-AtRGA	60



LACZ reporter gene assays
Relative β-galactosidase activity
16
14
12
10
8
6
4
2
0
No vectors
pGBKT7-53
pGADT7-T
pGBKT7
pACT2
pGBKT7
pDJS006 (AD-AtRGL1)
pGBKT7
pDJS008 (AD-AtRGL2)
pGBKT7
pDJS010 (AD-AtRGA)
pDJS007 (BD-AtRGL1)
pACT2
pDJS007 (BD-AtRGL1)
pDJS006 (AD-AtRGL1)
pDJS007 (BD-AtRGL1)
pDJS008 (AD-AtRGL2)
pDJS007 (BD-AtRGL1)
pDJS010 (AD-AtRGA)
Transformants



LACZ reporter gene assays
Relative β-galactosidase activity
40
35
30
25
20
15
10
5
0
No vectors
pGBKT7-53
pGADT7-T
pDJS009 (BD-AtRGL2)
pACT2
pDJS009 (BD-AtRGL2)
pDJS006 (AD-AtRGL1)
pDJS009 (BD-AtRGL2)
pDJS008 (AD-AtRGL2)
pDJS009 (BD-AtRGL2)
pDJS010 (AD-AtRGA)
Transformants

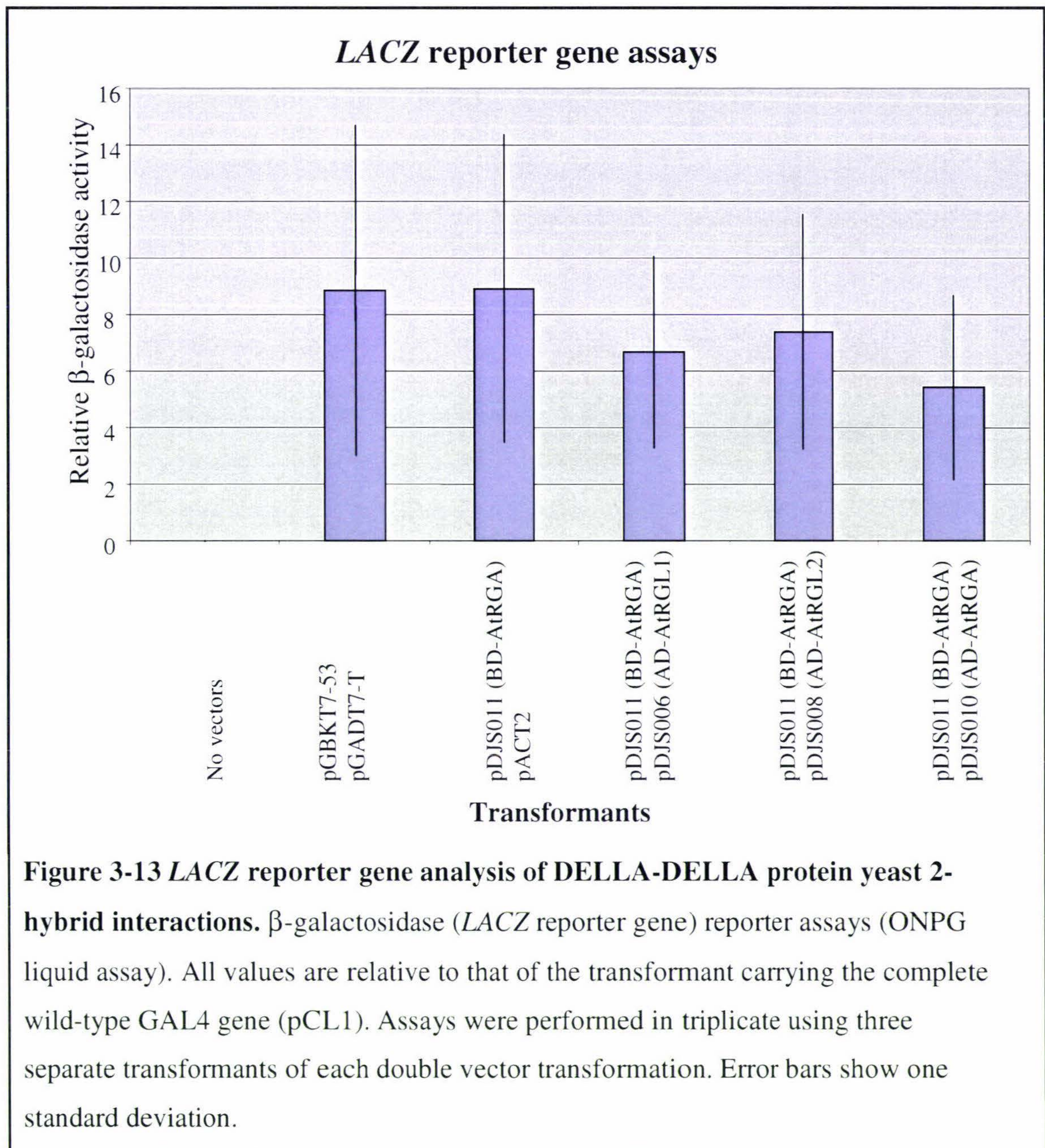


Figure 3-13 *LACZ* reporter gene analysis of DELLA-DELLA protein yeast 2-hybrid interactions. β-galactosidase (*LACZ* reporter gene) reporter assays (ONPG liquid assay). All values are relative to that of the transformant carrying the complete wild-type GAL4 gene (pCL1). Assays were performed in triplicate using three separate transformants of each double vector transformation. Error bars show one standard deviation.

Chapter 4. Discussion

4.1 Isolation of AtRGL1-interacting proteins

Genetic studies have implicated the DELLA protein family to be central to GA signalling (Koornneef *et. al.*, 1985; Silverstone *et. al.*, 1998; Dill and Sun, 2001; Itoh *et. al.*, 2002; Lee *et. al.*, 2002; Wen and Chang, 2002). However, very little is known on how DELLA proteins function to repress transcription, or how they perceive the GA signal. These functions are presumably dependent on other proteins interacting with the DELLA proteins. At the beginning of this research, the only direct interaction shown for DELLA proteins was OsSLR1 homo-dimer formation in a yeast 2-hybrid experiment (Itoh *et. al.*, 2002).

To understand the mechanism by which DELLA proteins function, this thesis sought to isolate proteins that interact with the DELLA protein AtRGL1. AtRGL1 is a nuclear protein (Wen and Chang, 2002), so plant nuclear extract was used for experimental procedures to isolate AtRGL1-interacting proteins. Two approaches were used to attempt to purify AtRGL1-interacting proteins. Co-affinity/affinity purification was used to purify proteins by their affinity for the N-terminal domain of AtRGL1, and co-immunoprecipitation was used to purify endogenous AtRGL1 containing complexes.

Two proteins, p24 and p64, were isolated from plant nuclear extracts through interaction with the recombinant fusion protein MBP-AtRGL1$^{(1-121)}$ by affinity purification. These plant proteins were found to be binding specifically to the N-terminal domain of AtRGL1 and not the MBP fusion tag. The DELLA motif, a non conserved spacer, and the TVHYNP motif that are present in the first 121 amino acids of AtRGL1 are required for GA signal perception. This suggests that the two new proteins isolated may be GA signalling components that mediate or regulate GA signal perception by AtRGL1. A third protein, 48 kDa in size, was also isolated by interaction with MBP-AtRGL1$^{(1-121)}$. However, a similar sized protein was purified by control purifications and furthermore, there is a 48 kDa degradation product of MBP-AtRGL1$^{(1-121)}$. Further work to identify this protein will be necessary to establish

whether the 48 kDa protein is an AtRGL1-interacting protein. The U-Box proteins AtHIM1, AtHIM2 and AtHIM3, are approximately 47 kDa in size and are implicated in GA signalling, based on homology to StPHOR1 (Monte *et. al.,* 2003). For this reason, it is of interest to identify this 48 kDa protein.

The AtRGL1$^{(1\text{-}121)}$-interacting proteins p24 and p64 proteins do not match the predicted molecular weights of any *A. thaliana* proteins implicated in GA signalling (AtSPY, AtSLY1, AtSHI, AtGPA1, and AtGCR1), so they may be novel factors that interact with the N-terminal domain of DELLA proteins. Attempts were made to identify the two affinity purified proteins by mass spectroscopy of tryptic digests, but the small quantities isolated prevented identification. Further attempts to purify larger amounts of these two protein and the possible 48 kDa protein will need to be performed to allow identification.

The p24 protein was purified from plant nuclear extract prepared from both total above ground tissue of mature plants (bolting) and inflorescences. Bolting plants were selected because GA signalling should be active as these plants are undergoing GA-dependent stem elongation and inflorescence development. Tissues that are not undergoing GA signalling, such as seedlings, were not used in this thesis for affinity purification procedures. These tissues were not used as they are less likely to contain GA-dependent signalling proteins that can bind to the N-terminal domain of AtRGL1. To confirm a role of p24 in GA signalling, further experiments to characterise this protein will need to be performed once it is identified.

The p64 protein was only purified from plant nuclear extract prepared from inflorescences and not total above ground tissue. There are several possible explanations for this result: a) p64 may have localised expression to the inflorescence; b) p64 may require an inflorescence specific modification of either itself or AtRGL1 for their interaction to occur. The inflorescence is the primary site of *AtRGL1* expression and function (Tyler *et. al.,* 2004). Thus, if p64 specifically interacts with AtRGL1, it is likely that p64 would also be primarily expressed in this tissue. The role of p64 in GA signalling will need to be established once it is identified. As p64 may have limited tissue expression or tissues of favourable affinity for AtRGL1, it is possible that this protein could function in regulation of GA signalling, rather than

GA signalling itself. As discussed in the introduction section 1.2.6, the degradation of DELLA proteins is enhanced by auxin and reduced by ethylene. The N-terminal domain of DELLA proteins is required for GA-induced degradation, so the N-terminal binding protein p64 may mediate one of these signals from other hormone pathways.

It is important, once these AtRGL1-interacting proteins are identified, that interactions and interpretations are subjected to further experimental investigation. Currently there is no structural information on DELLA proteins, but it is reasonable to expect that they could have a complex 3-dimensional structure that does not have separate N-terminal and C-terminal domains. This could render the interactions of the bait biologically irrelevant. This possibility is ruled out by the finding that overexpression of the OsSLR1 N-terminus alone in wild-type rice is able to reduce GA signalling, likely by sequestering the signal. This supports the idea that the N-terminal domain functions separately (Itoh *et. al.,* 2002).

Co-immunoprecipitation experiments were planned as a second approach to investigate the interactions of AtRGL1, particularly the C-terminal domain. An antibody suitable for these experiments was developed, but the undetectable level of AtRGL1 protein in wild-type plant extracts precluded this continuation of this experiment. This finding that no DELLA proteins are detectable is in agreement with recently published results that show that DELLA proteins are not detectable in most wild-type plant tissues (Tyler et. al., 2004, Dill et. al., 2004).

During this research, one DELLA interacting protein has been reported. The F-box proteins AtSLY1/OsGID2 from *A. thaliana* and *O. sativa* have been shown to interact with AtRGA, AtGAI, and OsSLR1 respectively (Gomi *et. al,* 2004; Fu *et. al.,* 2004; Dill *et. al.,* 2004). The interactions of DELLA proteins involved in gene regulation, GA signal perception, and other regulatory signals remain to be discovered.

4.2 Investigation of DELLA-DELLA interactions

Work published on the DELLA protein OsSLR1 showed that OsSLR1 homo-dimerises in a yeast 2-hybrid experiment (Itoh *et. al.*, 2002). Yeast 2-hybrid experiments were performed as part of this thesis to investigate whether dimerisation also occurs for other DELLA proteins. Some dicots, such as *A. thaliana*, have multiple genes encoding DELLA proteins. The role of multiple DELLA genes is unknown, but recent published results suggest that they have some functional redundancy (Tyler *et. al.*, 2004). The DELLA genes of *A. thaliana* do have differing but overlapping expression patterns (Tyler *et. al.*, 2004). Given that DELLA proteins do form homo-dimers it may be possible that they also form hetero-dimers that are functionally important.

Work by Tyler *et. al.* (2004) demonstrated that deletion of *AtRGA, AtRGL1,* and *AtRGL2* rescued a wild-type phenotype to the inflorescence of the GA biosynthetic deficient dwarf mutant *ga1-3*. This implies that these DELLA proteins function together in inflorescence development and possibly interact with each other. Yeast 2-hybrid experiments were used to test this hypothesis by investigating if these three DELLA proteins of *A. thaliana*, AtRGA, AtRGL1, and AtRGL2, are involved in homo- or hetero-dimerisation.

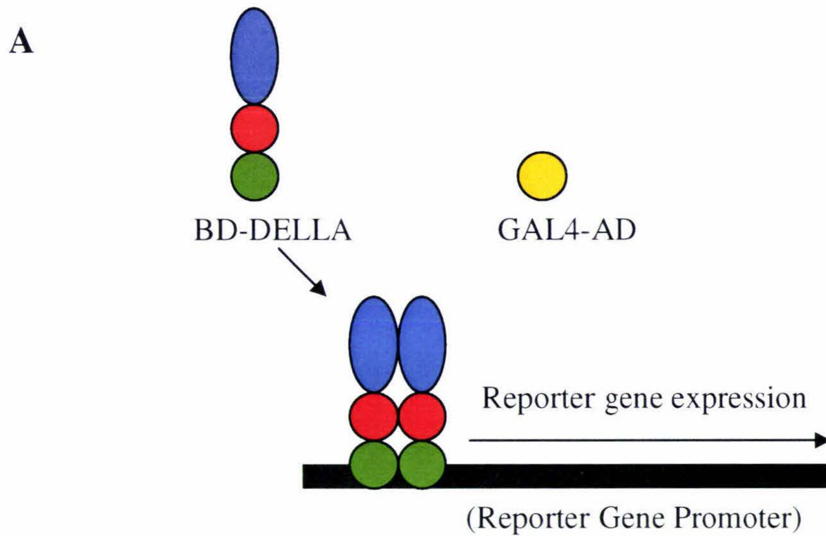
All three DELLA proteins were successfully expressed as both GAL4 DNA binding (BD) domain and activation domain (AD) fusions. The published results for OsSLR1 dimerisation showed that the BD-OsSLR1 fusion exhibited autoactivation, i.e. no interacting protein was required for reporter gene activation (Itoh *et. al.*, 2002). AtGAI was also shown to autoactivate in yeast 2-hybrid experiments by Fu *et. al.* (2004). This thesis shows that autoactivation also occurs with AtRGA and AtRGL2. Autoactivation by DELLA proteins suggests that conserved motifs allow interaction with unknown yeast transcription factors or general transcriptional machinery that may be conserved between yeast and plants. This conclusion strengthens the prediction of DELLA proteins acting as transcriptional regulators. As AtRGA and AtRGL2 are functional they must be soluble and able to enter the nucleus. The reasons for the lack of autoactivation by AtRGL1 are unknown. This could be due to

mis-folding when expressed in yeast, or AtRGL1 lacks the conserved motif required for autoactivation by the other DELLA proteins.

Reporter gene assays performed as part of this thesis showed no significant increase above autoactivation levels when both BD-DELLA and AD-DELLA fusions were expressed. These results indicate that no homo-dimerisation or hetero-dimerisation occurs between the DELLA proteins AtRGL1, AtRGL2, and AtRGA. However, the reporter gene assays may have been invalid due to complications as discussed below.

Expression of DELLA proteins affected the growth of yeast, particularly interfering with cell division. Truong *et. al.,* 1997, isolated AtRGA and AtGAI in a screen for components of plant nitrogen metabolism. The experiment involved rescue of yeast nitrogen metabolic mutants (*gln3, gdh1*), though this rescue was not due to complementation of the mutated factors. It should be noted that yeast 2-hybrid experiments involve growth of yeast on minimal media so the source of nitrogen is limited to Ammonium Nitrate and specific amino acids so interference with nitrogen metabolism could explain effects on cell growth. Reporter gene assays could be susceptible to the growth and nutritional status of the yeast.

Reporter gene assays may not be suitable for investigating the interaction of proteins that form homo-dimers, especially proteins with autoactivation in the yeast 2-hybrid system. The reporter gene assay results obtained in this thesis could be explained by the formation of three dimer species in each assay: BD-BD, BD-AD, and AD-AD (Figure 4-1). Reporter gene assays are used to detect the presence of the BD-AD complex, which would only comprise a portion of the dimers in this experiment. Additionally the BD-BD dimer would be expected to have greater affinity for the reporter gene promoter, so this complex would be the dominant species bound to reporter genes. A small amount of BD-AD dimers binding may be detectable in the reporter gene assays; however, the autoactivation of BD-DELLA fusions could mask this.



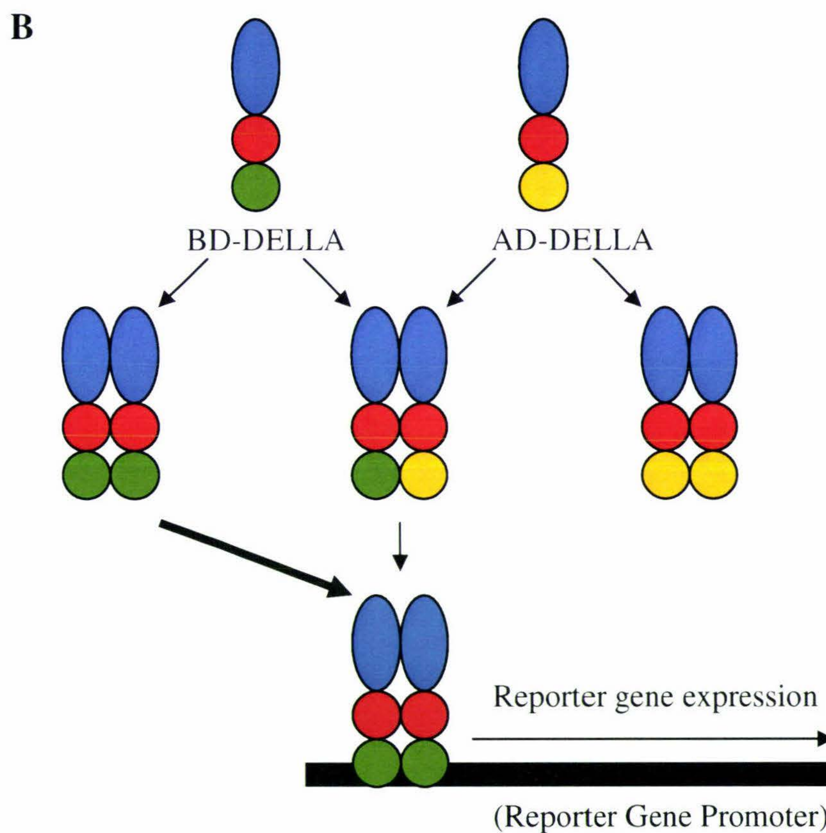


Figure 4-1 Schematic of possible DELLA-DELLA dimer interactions with reporter gene promoters. A. Expression of BD-DELLA fusion forms dimer that interacts with DNA (GAL4 binding sites) of the reporter gene promoter, resulting in autoactivation of the reporter gene. **B.** Expression of BD-DELLA and AD-DELLA fusions results in formation of three dimer species. The higher DNA affinity of the BD-DELLA-BD-DELLA dimer prevents the lower affinity BD-DELLA-AD-DELLA from binding to the promoter.

Another explanation for the lack of interactions could be that the AD-DELLA fusions did not have access to the BD-DELLA fusions. The fusions may have been insoluble, modified, or being unable to enter the nucleus. However, this is unlikely, because recent publications have shown successfully the interaction of BD-AtSLY1 with AD-AtRGA and AD-AtGAI using yeast 2-hybrid (Dill *et. al.,* 2004). This suggests that AD-DELLA fusions are soluble and able to enter the nucleus. In conclusion, the yeast 2-hybrid results from this thesis require further investigation.

4.3 Future identification of AtRGL1-interacting proteins

Two proteins were isolated through interaction with AtRGL1 using affinity purification. To confirm these interactions and to discover their roles in GA signalling they must first be identified before further experiments can be performed. Identification will involve purification of larger quantities of these new proteins followed by mass spectroscopy analysis. There have been several reports in literature of identifying proteins by mass spectroscopy from low nanogram (sub-picomole) quantities. Therefore, the prospect for identification of AtRGL1-interacting proteins is promising (Shevchenko *et. al.,* 1996; Sumner *et. al.,* 2002).

Once AtRGL1-interacting proteins have been identified, further investigation into their function in the GA signalling pathway will be performed. The tissue expression of the genes encoding these new proteins can be determined by both quantitative RT-PCR and *in situ* hybridisation. To confirm a role in GA signalling, null mutant and 35S overexpression lines will be produced. The expected phenotype of null mutants of positive GA signalling factors would be a GA insensitive dwarf. Overexpression lines would be expected to have a slender phenotype with enhanced GA responses. There may be complexities with this approach and obtained phenotypes if the *A. thaliana* genome encodes for functionally redundant homologues, or if overexpression results in saturation that blocks the GA signal.

To confirm the interaction of newly identified proteins with AtRGL1, and to test interactions with other DELLA proteins, biochemical techniques will be used. The AtRGL1-interacting proteins will be expressed as recombinant proteins in *E. coli* to

allow for *in vitro* experiments. Pull-down experiments can be performed using *E. coli* extracts from bacteria expressing AtRGL1-interacting protein using purified MBP-AtRGL1$^{(1-121)}$ as bait similar to the experiments performed in this thesis. Similarly pull-down experiments can be reversed, using p24 or p64 as bait to pull-down AtRGL1 and also test for interactions with other DELLA proteins. It is possible that some of these interactions are not limited to AtRGL1 as they may involve the conserved motifs of DELLA proteins. However, it could be expected that there are interactions unique to each DELLA protein that involve non-conserved motifs. *In vitro* mutagenesis of conserved and unique DELLA protein motifs will be performed to identify those involved in protein-protein interactions, as deletion will prevent pull-down interactions.

Complications may be encountered with *in vitro* pull-down procedures if the interactions require other plant proteins, GA signalling dependent modifications, or secondary signalling molecules. Nuclear extract specific plant proteins (i.e. AtSPY) or secondary signalling molecules (i.e. cGMP, Ca^{2+}) may be added to the system. If pull-down conditions cannot be produced using *E. coli* expressed proteins, then further pull-down experiments from plant tissue will need to be performed.

4.4 Plans for immunoprecipitation of AtRGL1

Antibodies suitable for immunoprecipitation of AtRGL1 were developed as part of this thesis. However, the undetectable quantities of AtRGL1 protein in wild-type plant tissue prevented the use of these antibodies for co-immunoprecipitation experiments. Recent publications show that AtRGA, AtGAI, and AtRGL2 are detectable in tissues of the GA biosynthetic mutant *Atga1-3* (Tyler *et. al.*, 2004). AtRGA and AtGAI have also been shown to accumulate in tissues of *Atsly1* null mutants (Dill *et. al.*, 2004). AtRGA was also shown to be detectable in 8-day-old *A. thaliana* seedlings (Dill *et. al.*, 2004). The rice DELLA protein OsSLR1 was detectable in callus tissue, presumably because this tissue lacks GA biosynthesis (Itoh *et. al.*, 2005). Therefore, these tissues may be used for immunoprecipitation of AtRGL1. *Atga1-3,* 8-day-old wild-type seedlings, and wild-type callus tissue will be analysed for presence of AtRGL1 at a detectable limit. Unfortunately *AtRGL1* is primarily expressed in the

inflorescence so AtRGL1 may only be detectable in this tissue in the At*ga1-3* mutant line. The use of *Atsly1* plants would be desirable, but if the authors do not wish to supply these plants then null mutants will need to be produced, if this is the case a different approach will be taken, as described below.

Transgenic overexpression of *AtRGL1* provides an additional approach to increase levels of detectable AtRGL1 protein. To avoid difficulties with dwarfism and infertility that result from *AtRGL1* overexpression (Wen and Chang, 2002), AtRGL1 will be expressed as a glucocorticoid receptor (GR) fusion. GR fusions are retained in the cytoplasm until treatment with dexamethasone induces nuclear import, Yu *et. al.* (2004) successfully used this technique for overexpression of AtRGA. Overexpression of GR-AtRGA did not affect plant growth until dexamethasone treatment induced nuclear localisation.

Further AtRGL1-interacting proteins may be co-immunoprecipitated with AtRGL1 from plant tissue. Proteins purified by immunoprecipitation can be identified by mass spectroscopy as for affinity purified proteins isolated in this thesis. The anti-AtRGL1 polyclonal antibody developed likely sees multiple epitopes on the N-terminus of AtRGL1. Therefore, binding of proteins to this region of AtRGL1 should not prevent immunoprecipitation of these complexes. Proteins that interact with the C-terminal residues of AtRGL1 should not hinder immunoprecipitation of AtRGL1 containing complexes as this region was not present in the immunogen used to raise the antibodies. It is likely that anti-AtRGL1 polyclonal antibodies will immunoprecipitate other *A. thaliana* DELLA proteins as there are conserved motifs in the N-terminal regions of these proteins. Co-immunoprecipitated proteins could include any proteins interacting with both the N and C-terminal of AtRGL1, so new proteins involved in GA signalling and DELLA dependent gene repression may be isolated.

Immunoprecipitation of native AtRGL1 from the plant will allow mapping of covalent modifications that may regulate DELLA protein function. Recent literature reports phosphorylation of DELLA proteins, which has been mapped to serine residues of the poly S/T/V region of OsSLR1 in rice (Itoh *et. al.,* 2005). It is expected that other covalent modifications of DELLA proteins occur such as Ubiquitination, glycosylation, and phosphorylation of other amino acid residues. Ubiquitination is a

modification that should result from the recruitment of DELLA proteins to the SCF^{SLY1} E3 Ubiquitin ligase as discussed in section 1.4. Glycosylation is a covalent linkage of carbohydrate groups to amino acid residues. The involvement of glycosylation in GA signalling is predicted due to a constitutive GA response phenotype of mutants of the *O*-GlcNAc transferase AtSPY (Swain *et. al.,* 2002). How AtSPY functions in GA signalling has not yet been shown, though it has been proposed that DELLA proteins may be modified by this enzyme. Aspartic acid (D) is a conserved amino acid in the DELLA motif, a motif required for GA signal perception. Although it has not yet been proposed, it may be possible that the aspartic acid residue present in the DELLA motif may be a target for phosphorylation.

If DELLA proteins regulate transcription as part of a complex associated with chromatin it may be possible to identify the genetic elements directly regulated by AtRGL1. DNA present in complexes can be purified from the plant by chromatin immunoprecipitation (CHIP) and the DNA then identified. Unfortunately the targets of DELLA proteins are unknown, though likely candidates are the GAMYB genes which are expressed on GA signalling (Gubler *et. al.*, 2002; Murray *et. al.,* 2003; Millar *et. al.*, 2005). CHIP only enriches DNA elements that co-purify with the target protein, so detecting and identifying an unknown enriched DNA fragment will be very difficult. For this reason, CHIP will only be performed if a transcription factor with DNA binding is purified in complex with AtRGL1 by co-immunoprecipitation. The identity of co-purified transcription factors will give some clue as to the DNA sequence recognised by the complex, and so the possible promoter regions that could be probed for in CHIP experiments.

To discover genes that may be regulated by a putative AtRGL1-transcription factor complex, *in vitro* DNA binding studies using partially random sequences (based on the transcription factors putative DNA binding recognition) may show the DNA sequence bound by the complex. This DNA sequence can then be used to search the genomic sequence of *A. thaliana* to determine possible target promoter regions of GA regulated genes. The promoter sequences of these genes can be probed for by PCR and quantitative analysis of DNA enriched by anti-AtRGL1 polyclonal in CHIP experiments.

4.5 Further investigation of yeast 2-hybrid experiments

Yeast 2-hybrid experiments performed as part of this thesis suggested no dimerisation of the DELLA proteins AtRGL1, AtRGL2, and AtRGA. However, due to complexities discussed earlier these results require further investigation. Reporter gene assays may not be able to detect the BD-DELLA-AD-DELLA dimer so another approach is required. To specifically detect this complex co-immunoprecipitation can be used. BD fusions have a c-Myc epitope tag and AD fusions have an HA epitope tag. Immunoprecipitation using anti-HA antibodies followed by western blot detection with anti-c-Myc antibodies should detect the presence of a BD-DELLA-AD-DELLA dimer. Although truncations could be used to remove the autoactivation from DELLA proteins in yeast 2-hybrid experiments, this could also remove their putative ability to dimerise, or result in biologically irrelevant results.

The BD-AtRGL1 fusion exhibits a weak autoactivation, as shown in this thesis. The autoactivation is low enough that yeast expressing this protein cannot growth on low levels of 3-AT, an inhibitor of the *HIS3* reporter gene, when grown on media lacking histidine. This will allow a classical yeast 2-hybrid screen of AtRGL1 against an *A. thaliana* cDNA library to identify AtRGL1-interacting proteins.

Chapter 5. Conclusion

This thesis focused on the interactions of AtRGL1, a protein of the DELLA family that acts as a represser of GA signalling. My first aim was to identify proteins that interact with AtRGL1. Using affinity purification, I successfully isolated two *A. thaliana* nuclear proteins, p24 and p64, that bind to the N-terminal domain of AtRGL1 (residues 1-121). The N-terminal domain of DELLA proteins is implicated in perceiving the GA signal, suggesting that the isolated proteins may be components of GA signalling. This evidence of AtRGL1-interacting proteins may aid the discovery of the molecular interactions of AtRGL1, and ultimately the mechanism by which DELLA proteins sense the GA signal that targets them for degradation. The GA-induced degradation of DELLA proteins is essential for relief of repression of GA-induced genes, making these interactions important to understanding of GA signalling. The focus of further research will be aimed at the identification these AtRGL1-interacting proteins and characterisation of their role in the GA signalling pathway.

Co-immunoprecipitation of AtRGL1 containing complexes from plant tissue was planned as a second approach to identify further AtRGL1-interacting proteins. An anti-AtRGL1 polyclonal antibody was developed and determined suitable for immunoprecipitation experiments. However, endogenous AtRGL1 in plant extracts was below the detection level, precluding the use of immunoprecipitation experiments from wild-type *A. thaliana* tissue. Further research will involve production of plant extracts that contain detectable levels of AtRGL1; either by use of mutant backgrounds, GA deficient tissues, or transgenic inducible overexpression lines. These tissues can then be used for co-immunoprecipitation of AtRGL1 and proteins interacting with either the N- or C-terminal domains. The C-terminal domain of DELLA proteins is implicated in repression of GA-induced genes, so identification of proteins interacting with this domain may reveal how DELLA proteins repress gene expression.

My second aim was to investigate the interactions of AtRGL1 with other DELLA proteins to determine if dimers and hetero-dimers form. Full length AtRGL1, AtRGL2, and AtRGA were successfully expressed as GAL4 domain fusions in S.

cerevisiae. Reporter gene assays did not show any interactions. There were complications associated with the use of reporter genes in this study that may make these results invalid. Further experiments will need to be performed to establish if DELLA proteins form dimers, this will include the use of co-immunoprecipitation techniques. Additionally it may be possible to screen for AtRGL1-interacting proteins from an *A. thaliana* library in a classical yeast 2-hybrid experiment.

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