Characterization of the role of *ACR4*, a receptor like kinase in *Arabidopsis thaliana*

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Declaration

I clearly declare that this is my own work and that any contribution by others is clearly acknowledged.

Contents

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Abbreviations

ABA	abscisic acid
APS	ammonium persulphate
BFA	Brefeldin A
bp	base pair
BSA	Bovine serum albumin
cDNA	complimentary DNA
EDTA	ethylenediaminetetraacetic acid
GA	gibberellic acid
GFP	GREEN FLUORESCENT PROTEIN
HR	hypersensitive response
JA	jasmonic acid
kb	kilobase
L	litre
LB	Lauria Broth
mg	milligram
mM	millimolar
MS	Murashige and Skoog
PBST	phosphate buffered saline Tween
PCD	programmed cell death
PCR	polymerase chain reaction
SA	salicylic acid
SAGs	senescence-associated genes
SDS	sodium dodecyl-sulphate
SEM	scanning electron microscopy
T-DNA	transfer DNA
TEMED	Tetramethylethylenediamine
UTR	untranslated region
v	volts
X-gal	4-bromo-4-chloro-3-inodyl-β-D-galactopyranoside
YEP	yeast extract peptone

Abstract

To allow co-ordinated development, plant cells must sense and respond to environmental conditions and internal cues. One means of perceiving signals is through cell-surface receptors, including receptor like kinases (RLKs). In order to understand RLK mediated signal transduction pathway, the RLK encoded by the *Arabidopsis CRINKLY4 (ACR4)* gene was studied. The *ACR4* gene has been implicated in epidermal specification. However *acr4* null mutants show only a subtle phenotype consisting of disruption of cellular organization of sepal margins and ovule integuments. To investigate if *ACR4* acts redundantly with the related receptor like kinases [*Arabidopsis thaliana CR4-RELATED1* (*AtCRR1*), *AtCRR2, AtCRR3* and *AtCRR4*], quadruple and quintuple mutants of *ACR4* and *ACR4* related kinases were generated. Characterization of these mutants showed that *ACR4* does not act redundantly with its related receptor like kinases with respect to shoot development.

The pathway via which ACR4 transduces developmental cues is not known. In order to elucidate this signal transduction pathway, a detailed analysis was carried out to analyze the physical interaction between ACR4 and its putative binding partner, ACR4 INTERACTING PARTNER1 (AIP1). To build a complete picture of the relationship between ACR4 and AIP1, in depth analyses of expression patterns, mutant phenotypes, genetic interactions, subcellular localizations and protein behaviour were performed. Although gene expression and protein localization studies supported possible interaction between ACR4 and AIP1, it was not possible to show this interaction by co-immunoprecipitation.

Many developmentally important RLKs have been shown to play independent roles in plant defence. In order to investigate other possible roles of *ACR4*, pathogen challenge experiments were carried out. It was found that *ACR4* operates as a negative regulator of defence responses against the necrotrophic pathogen, *B. cinerea* and as a positive regulator against *P. syringae*. It is already known that resistance to biotropic and necrotrophic pathogen involves different and to some extent, antagonistic signalling pathways. It is therefore possible that the ACR4 receptor might interact with other proteins that regulate specific defence responses. To investigate any possible epidermal defect of *acr4* leaves, chlorophyll leaching experiments were carried out and showed that *acr4* leaves have a permeable cuticle. To further understand the nature of cuticular defect of *acr4* leaves, a detailed study of the cuticular lipid composition of *acr4* leaves by ESI-MS was initiated. This may help ascertain whether epidermal defects in *acr4* leaves are responsible for resistance against *B. cinerea.*

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Chapter – 1

General introduction

1.1 Introduction

Plant cells continually respond to a stream of signals to alter their physiology, morphology and development. These stimuli include both internal and external signals for example light, temperature, mineral nutrients, turgor pressure, wounding and growth regulators. These signals vary dramatically in intensity both spatially and temporally. The ability of a cell to respond to a stimulus is modulated by developmental age, prevailing environmental condition as well as other factors for example, day length. Therefore many signals interact cooperatively and synergistically to produce the final response. For example, the decision of a seed to germinate involves integration of many physiological and environmental factors, by several different seed tissues. If signalling is defective, leading to germination at an inappropriate time, the decision is irreversible and could be fatal. Tight regulation of processes such as seed germination in plants, reflects the presence of a complex signal recognition and transduction system.

Signal transduction, the means by which cells respond to signals, involves conveying information within cells, between cells and throughout the plant. Plants, unlike animals, lack blood, lymphatic flow and nervous systems. Instead, plants possess a variety of other mechanisms allowing cell-cell communication (Fig 1.1), (Robert and Friml, 2009; Busch and Benfey, 2010). Intercellular communication can be mediated by direct transport between the cytoplasm of two neighbouring cells

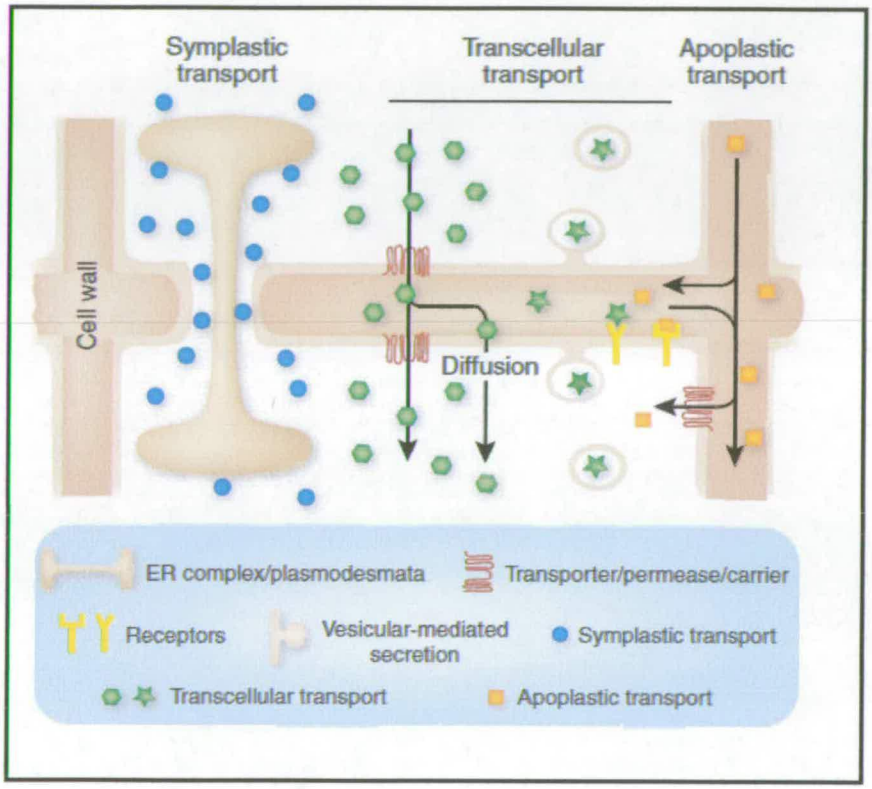

Fig 1.1: Modes of intercellular transport in plants.

Symplastic transport is mediated by plasmodesmata that connect adjacent cells. A modified endoplasmic reticulum forms part of the plasmodesmatal structure. Apoplastic transport involves passive diffusion of molecules in the extracellular space, the cell wall. Transcellular transport combines apoplastic transport with a secretion- and endocytosis-based or channel- and carrier-based transport pathway to cross plasma membranes.
Schematic taken from Robert and Friml (2009).

through plasmodesmata (symplastic transport). The movement of several types of regulatory signalling molecules including peptides, small proteins and RNAs are mediated by plasmodesmata (Maule, 2008; Lucas *et al.,* 1995). The plasmodesmata-based movement of transcription factors, such as SHORT-ROOT (SHR) (Nakajima *et al.,* 2001) and CAPRICE (Kurata *et al.*, 2005) is important for radial root and epidermis patterning respectively. Cell-cell communication can also be mediated by the signalling molecules that travel via the extracellular matrix, the apoplast. In this case, a cell secretes a signalling molecule, or a molecule leading to the production of a signal, and the target cell perceives the signal typically via a membrane localized receptor. Hormones such as brassinosteroids (Symons *et al.*, 2008) and peptides such as CLAVATA3 (Rojo *et al.,* 2002) are the examples of this type of transport. In addition, transcellular transport mechanisms also exist, which involve transport of molecules through the plasma-membrane by import-export mechanisms such as membrane diffusion, secretion and receptor or transporter mediated systems (Robert and Friml, 2009). Auxin, a phytohormone appears to be transported via a transcellular transport mechanism, although there is also evidence for apoplastic auxin signalling involving an auxin receptor (Yamagami *et al.,* 2004; Robert and Friml, 2009).

In plants, Receptor Like Kinases (RLKs) that serve as the binding sites for apoplastic signalling molecules or ligands (small peptides, some hormones), play an important role in the integration of signals. Perception of a ligand by a receptor kinase generally activates the receptor, causing activation or inactivation of downstream target proteins and ultimately resulting in either a local cytoplasmic response or a change in gene regulation – thereby transducing extracellular stimuli or signals into the cell. RLK-mediated signalling plays a fundamental role in plant development as well as in defence (De Smet *et al.,* 2009; Afzal *et al.,* 2008).

The question I would like to address in this section is how RLKs are activated and how they transduce signals inside the cells of plants such as *Arabidopsis thaliana*. First, I will give a brief general introduction regarding RLKs in *Arabidopsis*. Next, the mechanism of RLK-mediated signalling in plants will be explored.

1.2 Receptor like kinases in plants

Receptor like kinases (RLKs), also known as receptor protein kinases, exist in both plants and animals. While differences exist among the kinase structures resolved to date, all share some trademark features - an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic kinase domain. The predominant RLKs present in animals are the Receptor Tyrosine Kinases (RTKs), although Receptor Serine/threonine Kinases (RSKs) also exist in animals (Becraft, 2002). Almost all known RLKs in plants show serine/threonine kinase activity (Becraft, 2002). However, recent research revealed that several of these serine/threonine kinases can also undergo autophosphorylation on tyrosine residues. BRI1 (BRASSINOSTEROID INSENSITIVE1), which is the brassinosteroid (BR) receptor (discussed in depth below), possesses serine/threonine autophosphorylation sites and phosphorylation of these residues is known to be essential for kinase activity (Wang *et al.,* 2005a). However, recent research has also established the role of tyrosine phosphorylation of BRI1 receptor in BR signalling (Oh *et al.,* 2009). Therefore, it appears that some plant RLKs are likely to utilize both serine/threonine and tyrosine autophosphorylation sites in signalling, although the source of the tyrosine kinase activity implicated, is not yet clear.

The analysis of the whole genome sequence revealed that there are more than 400 RLK - encoding genes in *Arabidopsis* (Shiu and Bleecker, 2003). Plant RLKs have a separate evolutionary origin to that of animal RTKs and RSKs. Plant RLKs form a monophyletic clade that contains the PELLE-related cytoplasmic kinases of animals, based on the kinase domain phylogeny (Shiu and Bleecker, 2001). These independent origins are supported by the identities of downstream signalling components, which are clearly different in plants and animals. Despite these dissimilarities, RLKs in plants and RTKs in animals share many common features and behavioural characteristics suggestive of considerable convergent evolution (Cock *et al.,* 2002).

The extracellular domains of plant RLKs are extremely diverse, and it is thought that the variations in structures may enable them to respond selectively to many different signals. Based on the structures of the extracellular domains, the plant RLK superfamily has been classified into various groups (Shiu and Bleecker, 2003).

The Leucine-Rich Repeats RLKs (LRR-RLKs) contain a LRR repeat motif which is thought to be engaged in protein-protein interactions (Kobe and Deisenhofer, 1994). LRR-RLKs are the largest class of plant RLKs, with more than 200 members (Shiu and Bleecker, 2001). Only a few of them have been characterized so far. The LRR-RLKs that have been shown to play role in plant development include CLAVATA1 (CLV1) which controls cell differentiation in the shoot apical meristem (Clark, 2001), ERECTA which regulates organ size and shape and stomatal patterning (Shpak *et al.,* 2004; Shpak *et al.,* 2005), and BRI1, which is involved in brassinosteroid perception (Kim *et al.,* 2010). Other LRR-RLKs have been identified as playing roles in plant defence. For example, FLAGELLIN SENSITIVE2 (FLS2) acts as a receptor for bacterial flagellin, a pathogen-associated molecular pattern (PAMP) and induces innate immune responses (Gómez-Gómez and Boller, 2000).

The CR4 (CRINKLY4)-class of RLKs are characterised by the presence of an extracellular region containing seven repeats of ~39 amino acids ('crinkly' repeats) which are hypothesized to form a Regulator of Chromatin Condensation1 (RCC1)-like propeller structure (McCarty and Chory, 2000; Gifford *et al.,* 2005). These proteins also contain a domain showing some similarity to the extracellular cysteine-rich repeat domain of TNFR (Tumor Necrosis Factor Receptor) (Becraft *et al.,* 1996; McCarthy and Chory, 2000). In maize, *CR4* is required for normal epidermal differentiation (Becraft *et al.,* 1996). The *Arabidopsis* genome contains several genes related to *CR4* (Cao *et al.*, 2005). This family forms the subject of this thesis and is further discussed in Section 1.5.

The S-RLKs possess an extracellular S (Self-incompatibility) domain homologous to the Self-incompatibility-Locus Glycoprotein (SLG) of *Brassica* (Stein *et al.*, 1991). In *Brassica* SCR (S-locus Cysteine Rich protein), that functions as a ligand for SRK (S-locus Receptor like Kinase), are involved in self-incompatibility recognition (Nasrallah, 2000). Recently, it has been shown that the loss of self-incompatibility in the predominantly selfing plant *Arabidopsis thaliana* results from the disruption of *SCR* (Tsuchimatsu *et al.*, 2010).

The *Arabidopsis* Wall Associated Receptor Kinases (WAKs) represent the EGF (Epidermal Growth Factor) class which contains proteins with EGF-like repeats in the extracellular domain. In animals, EGFRs (Epidermal Growth Factor Receptors) bind to the EGF- related peptide growth factors and are involved in cell differentiation and proliferation (Olayioye *et al.*, 2000). In *Arabidopsis*, members of the WAK family are thought to interact with the cell wall components (Anderson *et al.*, 2001) and one member of this family, *WAK1* has been shown to bind pectin *in vitro* (Decreux and Messiaen, 2005). WAKs have also been found to be involved in cell elongation (Lally *et al.*, 2001; Kohorn *et al.*, 2006) and early stages of pathogen response (He *et al.*, 1998).

The Lectin class RLKs possess an extracellular lectin domain and are thought to bind oligosaccharides (Hervé *et al.*, 1999). Members of this family have been shown to play diverse roles in plant development and defence (Navarro-Gochicoa *et al.*, 2003; Chen *et al.*, 2006; Wàn *et al.*, 2008).

In addition to WAK and Lectin class RLKs, there are also other RLKs in *Arabidopsis* that may have a role in binding cell wall components for example the Proline-rich Extensin-like Receptor Kinases (PERK) family (Silva and Goring, 2002) and the *Catharanthus roseus* RLK1 (CrRLK1) family (Schulze-Muth *et al.*, 1996). PERKs have an extracellular proline rich domain that shares sequence similarity with plant extensins and are thought to be associated with the cell wall (Silva and Goring,

2002). The best-studied member of this family in *Arabidopsis*, PERK4 is proposed to interact with pectin and negatively influence root cell elongation (Bai *et al.*, 2009). An ortholog of *PERK* in *Brassica napus*, *BnPERK1* is implicated in early stages of pathogen response and mechanical stress response (Silva and Goring, 2002). The members of the *Arabidopsis* CrRLK1 family closely resemble the RLKs identified in *Catharanthus roseus* (Hématy *et al.*, 2008). Among them, FER (FERONIA) is found to be involved in male-female interaction during pollen tube reception (Escobar-Restrepo *et al.*, 2007) whereas THE1 (THESEUS1) is thought to act as a sensor for cell wall integrity (Hematy *et al.*, 2007). Recently, three members of CrRLK1 family in *Arabidopsis;* FER, THE1 and HERCULES Receptor Kinase 1 (HERK1) have been shown to be required for optimal cell elongation (Guo *et al.*, 2009).

Interestingly, among all the extracellular motifs found in plant RLKs, only the EGF motif is present in both plants and animals. Again, this is consistent with the independent evolutionary origins of RLKs in animals and plants.

1.3 Receptor like kinases: signal transduction mechanisms

The signalling pathway mediated by the RLK, BRI1 represents the first complete RLK-mediated signal transduction pathway identified in plants. Our understanding of the activation, signal transduction and regulation of signalling activity of plant RLKs is very limited. Some of the plant RLKs have been shown to play diverse developmental and physiological responses, suggesting the presence of signal transduction mechanisms that are both complex and precisely regulated. Here, our current understanding of the molecular mechanism involved in RLK mediated signalling is explored.

1.3.1 Ligands

A ligand is any molecule which binds to another molecule to form a larger complex. However, in the context of receptor-kinase research, the term ligand tends

to be used to describe any molecule that, by binding to the extracellular domain of a receptor, elicits a response in the cytoplasm (usually, but not always, through phosphorylation of the receptor target protein). Ligands could be almost any type of molecule present in the extracellular matrix, although peptides and carbohydrate moieties appear to be the most common ligands in many biological systems, possibly because their complexity allows specificity in signalling. The *Arabidopsis* genome contains more than 1000 genes encoding putative secreted peptides (Lease and Walker, 2006). So far, the functions of only a few of them are known. Systemin is the first identified signalling peptide in plants. In tomatoes, systemin has been found to bind the receptor SR160, a Leucine-Rich Repeat (LRR) receptor kinase and plays role in systemic wound responses (Scheer and Ryan, 1999).

The secreted peptide CLAVATA3 (CLV3) which regulates stem cell fate in the shoot apical meristem in *Arabidopsis* is processed into a 13-amino-acid peptide (mCLV3) and undergoes post-translational glycosylation which is critical for its biological activity and its high-affinity binding to the RLK, CLV1 (Fig 1.2), (Ogawa *et al.*, 2008; Ohyama *et al.*, 2009).

Another secreted peptide, SCR interacts with the receptor, SRK and plays a central role in pollen self-incompatibility (Takayama *et al.*, 2001). TPD1 (TAPETUM DETERMINANT1), a small, putatively secreted protein interacts with EMS1 (EXCESS MICROSPOROCYTES1), a leucine-rich repeat receptor-like kinase and is essential for anther cell fate determination (Yang *et al.*, 2003; Jia *et al.*, 2008).

In addition to peptides with endogenous origins, bacterial elicitors have been shown to act as ligands for plant RLKs. Flg22, a conserved 22-amino acid epitope of bacterial flagellin (Chinchilla *et al.*, 2006) and elf18/elf26, the N-terminal amino acids of bacterial elongation factor EF-Tu (Kunze *et al.*, 2004) are percieved by plant LRR-RLKs and elicit defence responses efficiently.

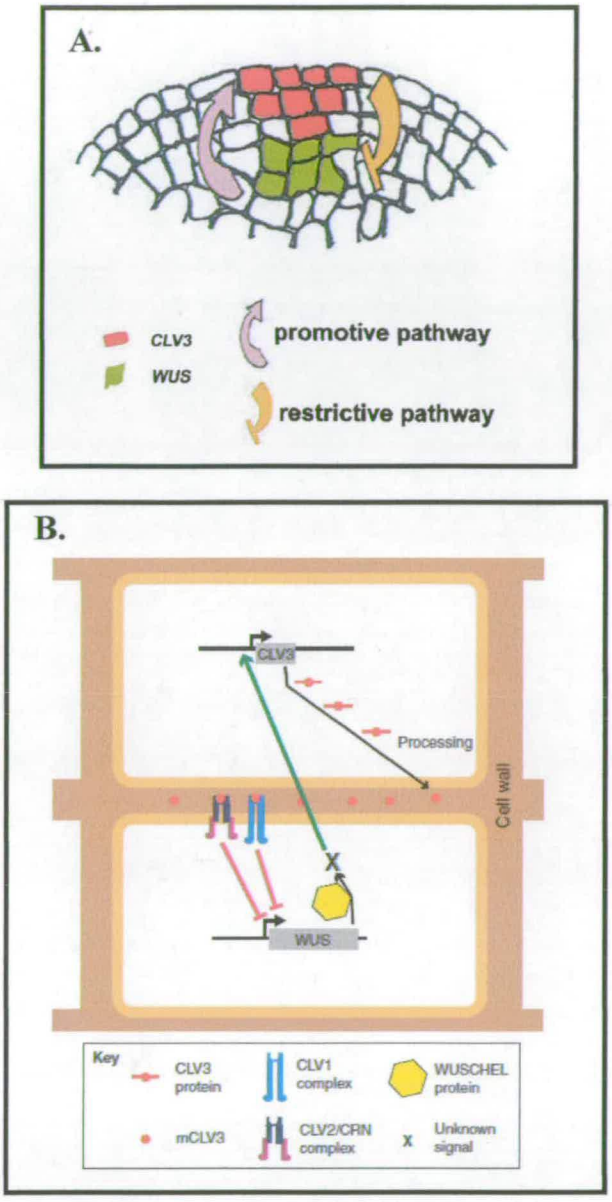

Fig 1.2: The receptor-like kinase cascade mediated by the CLV pathway, controls the size of the central stem cell pool in the shoot apical meristem.

(A) The expression of the stem cell marker, *CLV3* (red) in the shoot apical meristem requires *WUS* (*WUSCHEL*) expression (green). Schematic taken from Waites and Simon (2000). (B) The transcription factor WUS leads to the production of an unknown signal (X) that results in the transcription of *CLV3* in neighbouring cells. CLV3 protein is processed into the mature CLV3 peptide (mCLV3), which subsequently binds to CLV1 and to CLV2/CRN (CORYNE) receptor complexes. This event eventually causes the repression of *WUS*. Schematic taken from Busch and Benfey (2010).

As mentioned above, not all ligands are peptides. The steroid hormone, brassinolide is the most biologically active brassinosteroid and acts as a ligand for the LRR-RLK, BRI1 (Kinoshita *et al.,* 2005). Other examples of non peptidic ligands include bacterial Nod factors, which are lipochitin oligosaccharides and play roles in nodule development in leguminous plants. The Nod factors are proposed to be perceived by an RLK, NODULATION RECEPTOR KINASE (NORK) (Endre *et al.,* 2002).

In biological systems, activation of receptors by their ligands can initiate signalling pathways. Ligand-receptor interactions, therefore, are a focus for cell signalling studies. Identification of ligands for receptor kinases is still a challenge. Extracellular proteins including ligands often undergo structurally important post-translational modifications (Li *et al.,* 1996) and thereby these types of interactions are undetectable by yeast two hybrid screening. The affinity of interactions between ligands and cell surface receptor proteins can also be very low (Wyer *et al.,* 1999). The ligand-receptor interactions are, therefore, often overlooked by affinity purification. Some other approaches can only be used to confirm the interaction between known putative ligands and their receptors. One such approach is cell based technique where cells are being transfected separately, with the two differentially labelled proteins under study (Fehon *et al.,* 1990). Another technique which can be used to study ligand-receptor interactions in live cells is fluorescence correlation spectroscopy (Pramanik, 2004). Future understanding of extracellular ligand-receptor interactions would allow us to understand how ligands play roles in cell-cell signalling in *Arabidopsis*.

1.3.2 Receptor oligomerization

Receptor like kinases sense chemical signals through the extracellular ligand binding domain. How does the receptor-ligand interaction on the outside of the cell activate a signal transduction cascade inside the cell? Very little is known regarding the molecular mechanisms of plant receptor like kinase activation. Current models are based on the activation of animal receptor protein kinases where ligand binding

usually causes receptor kinases to form homo- or hetero-oligomers, followed by trans- or auto-phosphorylation. The mammalian Transforming Growth Factor β (TGF-β) receptor serine/threonine kinases exist as homodimers in the absence of the ligand. TGF-β binding induces the formation of hetero-tetramer, the activated receptor complex (Massague, 1998). In the case of Epidermal Growth Factor Receptor (EGFR), a receptor tyrosine kinase, ligand binding induces receptor dimerization and this interaction is mediated by specific residues in the 'dimerisation loop', leading to rapid autophosphorylation (Schlessinger, 2002; Olsen *et al.,* 2006).

Despite their independent evolutionary origin, some evidence also exists to support a role for receptor oligomerisation in signalling through plant receptor kinases. Receptor oligomerization and hyperphosphorylation have been demonstrated in the well studied plant RLK, BRI1. The binding of brassinosteroid to BRI1, causes hetero-oligomeration of BRI1 with BRI1-ASSOCIATED KINASE1 (BAK1) and hyperphosphorylation of BRI1 and BAK1 is induced (Nam and Li, 2002; Wang *et al.,* 2008; Yun *et al.,* 2009). Thus, BRI1 associates with BAK1 in a ligand dependent manner. Furthermore, BAK1 is also required for the immune responses triggered by a conserved 22-amino acid epitope (flg22) of bacterial flagellin (Chinchilla *et al.,* 2007). Perception of flg22 leads to the heteromerization of FLAGELLIN SENSITIVE2 (FLS2) with BAK1 and this heteromerization is tightly connected with the phosphorylation of the FLS2-BAK1 complex (Schulze *et al.,* 2010). Thus, the plant receptor kinase, BAK1 heterodimerizes with the brassinosteroid receptor, BRI1 and the flagellin receptor, FLS2 in a ligand dependent manner suggesting that different ligands can induce receptor oligomerization in different combinations. In the CLAVATA pathway (Fig 1.2), which regulates the stem cell population in shoot apical meristem, the RLK CLV1 is proposed to form heterodimers with the LRR-receptor like protein CLV2 (Jeong *et al.,* 1999) and biochemical evidence suggests

that the small secreted peptide CLV3 acts as a ligand (Ogawa *et al.*, 2008). Recently Muller *et al.*, (2008) proposed that the receptor kinase CORYNE (CRN) which lacks a distinct extracellular domain interacts with CLV2 via their transmembrane domains and transmits the CLV3 signal independently of CLV1.

Recent evidence suggests that the kinase activity of receptor like kinases is not always required for ligand binding. In FLS2 signalling, a kinase dead version of BAK1 can associate with FLS2, although activation of downstream signalling is blocked (Schulze *et al.*, 2010). Treatment of Arabidopsis cells using protein kinase inhibitors inhibits *de novo* phosphorylation of BAK1 and FLS2, but it does not inhibit ligand-induced heteromerization providing further evidence that the phosphorylation of BAK1 and FLS2 is not a prerequisite for stable complex formation (Schulze *et al.*, 2010).

To understand the mechanism of receptor multimerization, various novel approaches can be taken. In recent years, photon counting histogram analysis and dual-colour fluorescence cross correlation spectroscopy have been employed to determine oligomerization of any protein in living cells. Using this technique Hink *et al* (2008) have shown that 20% of the BRI1 protein exists as a homodimer in the absence of exogenous brassinosteroids, although no oligomeric structures were detected for BAK1. Another technique, which is quite extensively used in animal systems to investigate the oligomeric form of a protein, is Blue native polyacrylamide gel electrophoresis (BN-PAGE) (Schagger and von Jagow, 1991; Darie *et al.*, 2008). BN-PAGE allows separation of protein complexes in their native conformation, thereby allowing investigation of the multi-protein complex state.

1.3.3 Signalling through receptor like kinases: activation of target genes

Cell to cell communication mediated by RLKs ultimately affects dowstream targets, which can be either transcription factors involved in regulating gene expression, or, alternatively, cytoplasmic molecules involved in producing more local responses. Immediate downstream signal transduction by RLKs involves reversible phosphorylation of target proteins and in some cases involves the activation of Mitogen Activated Protein Kinase (MAPK) cascades to ultimately regulate transcription. In Arabidopsis defence responses, the expression of the transcription factors WRKY22/WRKY29, in response to the activated flagellin receptor FLS2, depends on a MAP kinase signalling cascades that involves MAP kinase kinase kinase MEKK1, MKK4 (MAP KINASE KINASE4)/MKK5 and MPK3 (MAP KINASE3)/ MPK6 (Asai *et al.*, 2002). In stomatal development, the putative receptors TMM (TOO MANY MOUTHS) and ERECTA are proposed to signal via a MAPK signalling cascade containing the MAP kinase kinase kinase YDA (YODA), to regulate the expression of transcription factors *SPCH (SPEECHLESS), MUTE* and *FAMA* (Fig 1.3), (Casson and Gray, 2008). In no case to date, however, has a direct molecular link been established between receptor kinase activation and a MAPK cascade in plants.

The RLK, BRI1- mediated signalling pathway is one of the only plant RLKs for which the direct substrates are known. Perception of brassinosteroids (BR) by the receptor BRI1 (Li *et al.,* 1997; Kinoshita *et al.,* 2005) causes activation of the BRI1/BAK1 kinase complex by transphosphorylation (Wang *et al.,* 2005a, 2008) and activated BRI1 phosphorylates BSKs (BRASSINOSTEROID SIGNALLING KINASES) (Tang *et al.,* 2008). This leads to the activation of the BSU1 (BRI1 SUPPRESSOR PROTEIN1) phosphatase which dephosphorylates and thus inactivates the kinase BIN2 (BRASSINOSTEROID INSENSITIVE2), resulting in nuclear accumulation of unphosphorylated BZR (BRASSINAZOLE RESISTANT) transcription factors to regulate brassinosteroid responsive gene expression (Fig 1.4), (Tang *et al.,* 2008).

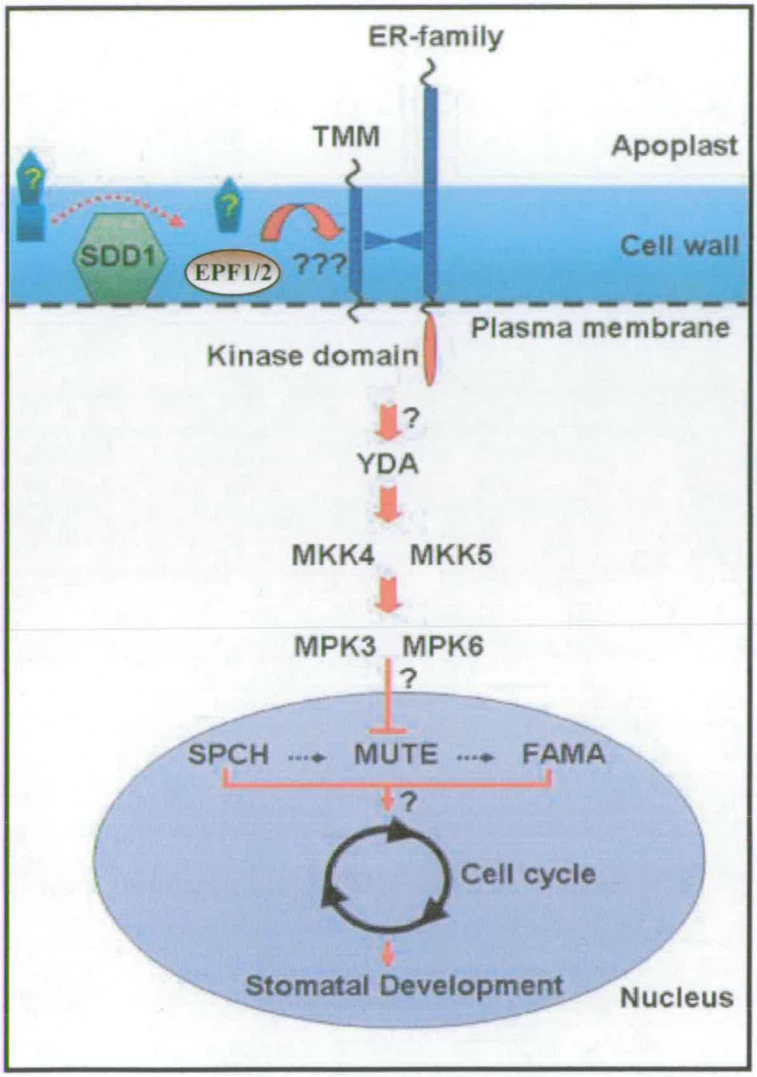

Fig 1.3: Receptor-like kinases play role in stomata development.
The RLKs TMM (TOO MANY MOUTHS) and ER (ERECTA) family members affect the initial entry division. An unknown ligand, processed by SDD1 (STOMATAL DENSITY AND DISTRIBUTION1) and the ligands EPF1 (EPIDERMAL PATTERNING FACTOR1) and EPF2 are secreted from the meristemoid or guard mother cell and this positional signal is interpreted by nearby cells. EPF1 and EPF2 are probably perceived by a TMM–ER-family complex. Activation of the TMM–ER family complex stimulates a MAPK signalling cascade starting with the MAPKKK, YDA that in turn activates MKK4/MKK5 and then MPK3/MPK6. This MAPK cascade negatively regulates stomatal development by an unknown mechanism, but may target the bHLH transcription factors; *SPCH*, *MUTE* and *FAMA*, though it is possible that the expression of the receptors may in turn be regulated by these genes. It is likely that the cell cycle is also a target of the stomatal developmental pathway. Question marks indicate unknown interactions and components. Schematic adapted from Casson and Gray (2008).

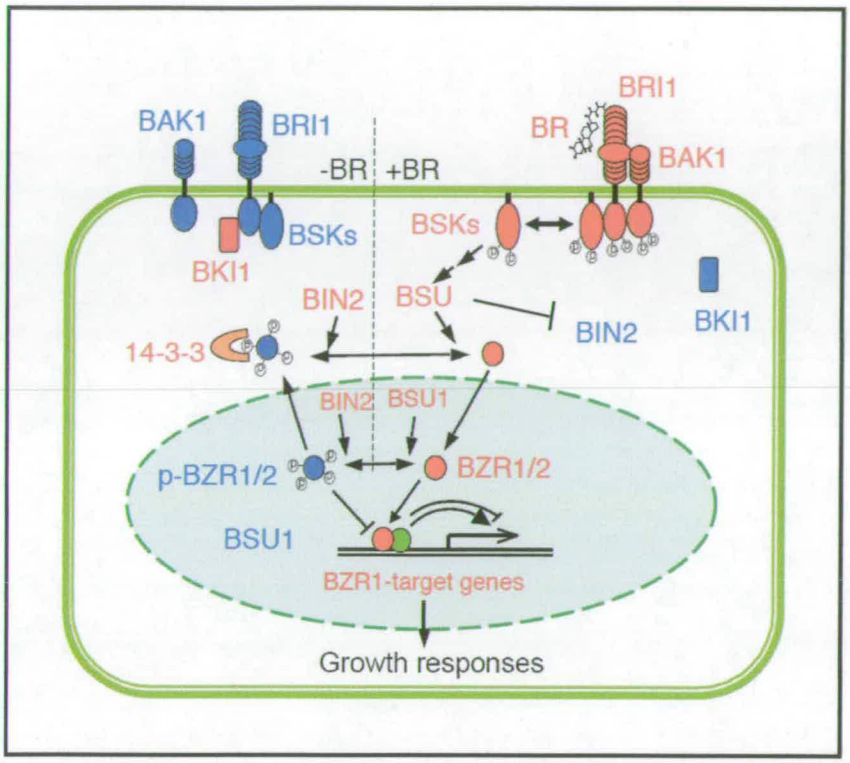

Fig 1.4: The RLK, BRI1 mediated signal transduction pathway.

In the absence of BR (−BR), BRI1 associates with BSKs in an inactive state; an inhibitor, BRI1 KINASE INHIBITOR 1 (BKI1) interacts with inactive forms of BRI1. As a consequence BSU is inactive, BIN2 phosphorylates BZR1 and BZR2 to inhibit their DNA binding activity and promote their cytoplasmic retention by the 14-3-3 proteins.
BR-binding (+BR) to BRI1 induces association with BAK1 and dissociation of BKI1. Transphosphorylation between BRI1 and BAK1 activates BRI1 which phosphorylates BSKs. Phosphorylated BSKs dissociate from BRI1, bind to BSU1 presumably to enhance BSU1 phosphatase activity. Activated BSU1 inhibits BIN2 through dephosphorylation, leading to the accumulation of BZR1 and BZR2 in the nucleus, which regulate BR-responsive gene expression. Schematic adapted from Tang *et al.* (2008).

1.3.4 Regulating RLK signalling activity

Cell-cell communication by RLKs is one of the most important mechanisms by which a cell achieves its intended developmental fate and position and is also important in defence mechanisms. How is the correct number of RLKs activated at any specific time? How is the signalling activity regulated temporally? And do subcellular spatial compartments exist to separate signalling cascades within the cytoplasm? In principle, signalling by RLKs depends on the availability of ligands, RLKs and signal transducing components or targets; within each category many distinct mechanisms are involved which could further modulate the activity of a specific cascade. These include post translational modification and selective accumulation/degradation of signalling components. Positive and negative feedback loops are also likely to act at various levels to allow precise regulation.

The distribution of ligands plays an important role in RLK activation (Freeman and Gurdon, 2002). In addition, cell-cell signalling also depends on the accumulation of RLKs. Our understanding of the distribution of known ligands for plant RLKs activation is very limited. In root epidermal patterning, for example- it is postulated that the differential localization of a putative ligand for the SCRAMBLED (SCM) RLK, in combination with the accumulation of the SCM receptors, trigger hair and non hair cell fate of the root epidermis (Fig 1.5), (Kwak and Schiefelbein, 2008). In the CLAVATA signalling pathway, *WUS* expression acts non-cell autonomously to promote the expression of *CLV3* in the outer-most cells of the shoot apical meristem. The ligand, CLV3, then activates a signal transduction pathway that regulates stem cell fate and *WUS* expression in the shoot apical meristem (Fig 1.2), (Schoof *et al.,* 2000). Another example involves, PXY (PHLOEM INTERCALATED WITH XYLEM), an RLK which is expressed in dividing procambial cells, and functions to generate the spatial information required for oriented cell divisions during vascular development (Fisher and Turner, 2007). PXY suppresses the differentiation of vascular stem cells into xylem cells on perception of the ligand CLE41 (CLV3-LIKE 41), which is secreted from the phloem cells (Fig 1.5), (Hirakawa *et al.,* 2008; Etchells and Turner, 2010). In *pxy* mutant, as a consequence of the disturbed signalling, the separation of xylem and phloem is lost (Fisher and Turner, 2007).

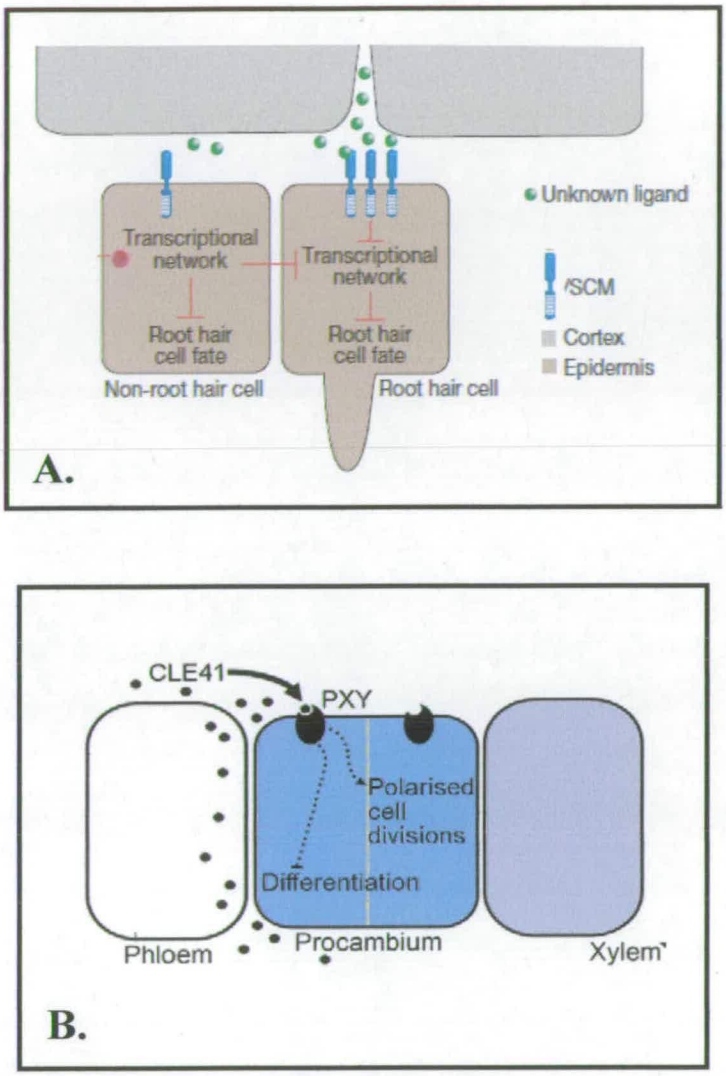

Fig 1.5: Distribution of ligands and accumulation of RLKs in regulating RLKs mediated signalling.

(A) On perception of an unknown ligand, the receptor-like kinase SCM blocks the transcriptional network that is responsible for preventing root hair cell fate. Schematic adapted from De Smet *et al.* (2009).
(B) PXY affects the plane of cell division in the procambium and inhibits subsequent differentiation into xylem on perception of the CLE41 ligand, thus providing positional information to the dividing cell. Schematic taken from Etchells and Turner (2010).

RLK-mediated signalling activity also depends on the availability of receptors in a given cell/tissue. In animal cells, activated receptors are internalised and sorted in endosomes, either for recycling back to the plasma membrane or for degradation within lysosomes (Le Roy and Wrana, 2005). Thus, attenuation of RLK mediated signalling can be achieved by endocytic removal of activated receptors. Research in the last few years has revealed that endocytosis of RLKs also occurs in plants and this may control receptor recycling and availability. Both constitutive and ligand regulated receptor endocytosis exist in plants. The internalization of the brassinosteroid receptor BRI1 is thought to occur constitutively, with exogenous application of the ligand, brassinolide causing no change in its behaviour (Geldner *et al.,* 2007). Another RLK, ACR4 (Arabidopsis CRINKLY4) is thought to be internalized in a constitutive fashion (Gifford *et al.,* 2005). However, since no ligand has been identified yet, it could be that the internalization of ACR4 is dependent on ligand binding. The internalization of both BRI1 and ACR4 is sensitive to Brefeldin A (BFA), an inhibitor of endosomal trafficking (Nebenführ *et al.,* 2002). In contrast to constitutively endocytosed receptors, the mammalian EGF receptor is rapidly endocytosed only upon binding to its ligand, EGF. Similarly, in *Arabidopsis*, the internalization of the defence related receptor, FLS2 is triggered by binding to its ligand, flg22, an active epitope of bacterial flagellin (Chinchilla *et al.*, 2006) and this endocytosis is further followed by receptor degradation (Robatzek *et al.,* 2006).

In addition to signal termination, the endocytosis of membrane bound receptors can also be important to activate signal transduction pathways. In mammals, the activated EGFR is rapidly internalized into endosomes and this endosomal localization activates downstream signalling cascades, leading to cell survival (Wang *et al.,* 2002). In *Arabidopsis*, although both FLS2 (Robatzek *et al.,* 2006) and ACR4 (Gifford *et al.,* 2005) receptors undergo internalization, there is no direct evidence demonstrating that endosomal signalling is required to activate downstream signalling cascades. The only plant RLK that has been shown to use endosomes as signalling compartments is BRI1. Geldner *et al.* (2007) reported that the endosomal localization of BRI1 is correlated with the activation of the signalling pathway (Fig 1.6).

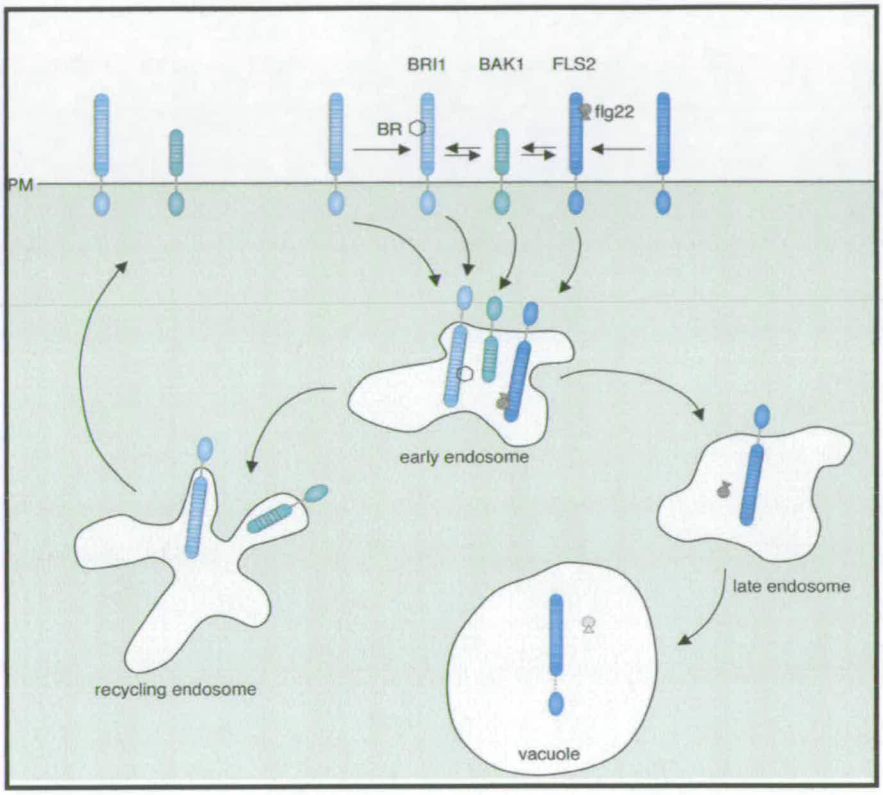

Fig 1.6: A model of the subcellular trafficking of the RLKs - BRI1, BAK1, and FLS2 in *Arabidopsis*.
The receptor like kinases BRI1 and FLS2 on perception of brassinosteroid (BR) and bacterial flagellin (flg22) respectively, form ligand-dependent complexes with the coreceptor BAK1. BRI1 and BAK1 internalizes to endosomal compartments and are recycled back to the PM. BRI1 relocalization is independent of ligand binding, but this endosomal localization activates downstream signalling cascades. Upon ligand activation, FLS2 internalizes into endosomes and is further sorted for degradation. Schematic taken from Geldner and Robatzek (2008).

Regulation of RLK signalling activity could also depend on specific signalling inhibitors. Our understanding of known inhibitors regulating plant RLK signalling activity is very limited. In *Drosophila*, signalling mediated by the protein kinase EGF receptor depends on several inhibitors. One of them KEKKON1 (KEK1) (Ghiglione *et al.,* 1999), a transmembrane protein, which is expressed in response to the EGFR signalling pathway, acts in a negative feedback loop to modulate the activity of the EGFR tyrosine kinase. KEK1 forms a heterodimer with the EGF receptor, preventing ligand binding and thereby interferes with the signal transduction pathway (Ghiglione *et al.*, 2003). In *Arabidopsis*, the well characterized BRI1-mediated signalling pathway is thought to be negatively regulated by the inhibitor, BRI1 KINASE INHIBITOR1 (BKI1), which associates with BRI1 when BR levels are low (Wang *et al.,* 2006).

Signal transduction by RLKs can also be regulated by specific phosphatases, for example Kinase-Associated Protein Phosphatase (KAPP). The CLV1 RLK has been shown to interact with KAPP both *in vitro* and *in vivo* (Stone *et al.,* 1998; Trotochaud *et al.,* 1999). In the case of FLS2 signalling, *in vitro* analysis suggests a possible interaction between KAPP and the kinase domain of FLS2 (Gomez-Gomez *et al.,* 2001). In addition, plants over-expressing KAPP mimic the kinase null mutant of FLS2 (a missense mutant that lacked autophosphorylation activity) in their responsiveness to flg22. This suggests that the kinase activity of FLS2 is required for FLS2 signalling and that KAPP possible functions as a negative regulator in this pathway. However, in no case to date, KAPP has been shown to associate with BRI1 or ACR4.

In summary, RLKs mediated signalling in plants share some basic features with its animal counterparts for example – receptor dimerization following ligand binding, autophosphorylation in receptor activation, and the existence of inhibitors and phosphatases that down-regulate receptor activity. In addition, like animals there are some RLKs in plants that are involved in more than one process. For example, TOLL-like receptors (TLRs) in mammals, are involved in both development as well as in immune response (Aderem *et al.,* 2000; Larsen *et al.,* 2007). Similarly in plants,

interactions with different partners allow RLKs to be involved in more than one signalling cascade. For instance, heterodimerization between BAK1 and BRI1 is critical for brassinosteroid signalling (Li *et al.*, 2002), whereas interaction between BAK1 and FLS2 plays role in plant defence responses (Chinchilla *et al.*, 2007). This suggested the existence of very tight regulation of RLK mediated signalling in plants. In addition, there are some developmental processes in plants where a single process is mediated by more than one RLK-mediated signalling pathways in a redundant manner. For example maintenance of stem cell pool in the shoot apical meristem is mediated by both CLV1 and CRN (Clark *et al.*, 1997; Muller *et al.*, 2008). To enhance our understanding of cell-cell signalling by RLKs, various high throughput techniques like whole genome transcriptomic, proteomic and metabolomic analysis have been employed in recent years. In addition, computational modelling approaches have been used to predict cellular signalling mechanisms. Future understanding of how signal transduction by RLKs is linked to transcriptional networks would give us a clearer picture of RLK mediated signalling in plants.

1.4 Plant epidermal specification: how is it achieved?

RLK mediated signalling has been shown to be involved in plant epidermal specification. The specification of the plant epidermal layer involves signalling during early embryogenesis that specifies the outer epidermal layer (protoderm) and inner (provascular and ground) tissues. This is thought to be followed by continual cell-cell signalling for subsequent maintenance and elaboration of epidermal organization. The signalling elements that regulate these processes are discussed in the following section.

The plant epidermal cells form an outer cell layer which protects the underlying cells from biotic and abiotic stresses while allowing the exchange of gas and water via stomata. These epidermal cells are also differentiated into a range of specialized cell types to play multiple roles. For example - the root epidermis forms

root hairs and these root hairs are specialized for absorption of water and mineral nutrients. Others develop into stomatal guard cells and trichomes. The epidermis of the aerial part of the plant is covered by a hydrophobic layer termed cuticle, which is located on the surface of the external cell wall. The cuticle is composed of cuticular wax, a complex mixture of very long chain fatty acid derivatives and cutin, a polyester that is made up of C16 and C18 ω-hydroxylated fatty acids and may carry additional hydroxy- or epoxy groups in mid-chain positions (Nawrath, 2006). The cuticle is thought to play multiple roles including prevention of water loss as well as protection against mechanical damage, pathogens and herbivorous insects. However, in recent years several cuticular defective mutants have been shown to be resistant to *Botrytis cinerea,* a necrotrophic pathogen suggesting that an interrupted cuticular membrane could allow the diffusion of effector molecules across the cuticle resulting in reduced susceptibility (Bessire *et al.,* 2007; Voisin *et al.,* 2009). This is further discussed in Chapter 6.

In *Arabidopsis*, the morphological differentiation of the outer epidermal layer and inner layers of the developing embryo becomes visible at the 16-cell Dermatogen stage and is characterized by differential gene expression. The outermost cells express a distinct set of genes including the homeobox transcription factors, *ARABIDOPSIS THALIANA MERISTEM L1* (*ATML1*) and *PROTODERMAL FACTOR2* (*PDF2*) (Fig 1.7), (Abe *et al.,* 2003). Although the knockout single mutants of *ATML1* and *PDF2* show normal shoot development, the double mutants have severe defects in shoot epidermal cell differentiation, suggesting that these genes play an important role in maintaining the outermost cell layer identity of the developing embryo by regulating the transcription of downstream target genes (Abe *et al.,* 2003). It has been found that the ATML1 and PDF2 proteins can bind an 8bp cis-regulatory element that resembles a homeodomain binding site *in vitro* (Abe *et al.,* 2001; Abe *et al.,* 2003). This homeodomain binding site is called an L1 box and is found in the promoters of *ATML1* and *PDF2* as well as those of *PROTODERMAL FACTOR1* (*PDF1*), *LIPID TRANSFER PROTEIN1* (*LTP1*) and *ETTIN* (*ETT*) all of which are expressed specifically in the embryonic protoderm (Reviewed in Ingram, 2004). L1 boxes are also found in the promoters of *FIDDLEHEAD* (*FDH*),

PRESSED FLOWER (*PRS*) and *SCARECROW* (*SCR*) which are all expressed in post-embryonic meristem and/or organ primordial L1 (Ingram, 2004). The promoters of *ATML1* and *PDF2* also contain consensus binding sites for the homeobox protein WUS (Abe *et al.*, 2003). *WUS* is expressed specifically in the apical 4 "inside" cells at the 16-cell stage embryo (Mayer *et al.*, 1998). Therefore WUS or WUS-related proteins could negatively regulate *ATML1* and *PDF2* in the "inside" cells leading to protodermal specific expression of *ATML1* and *PDF2*. Alternatively, WUS-related proteins expressed in the embryonic epidermis, such as WOX9 (WUSCHEL RELATED HOMEOBOX 9), (Haecker *et al.*, 2004) could act as transcriptional activators. However, it is not still clear how the expression of *ATML1* and *PDF2* is initiated and maintained or how the expression is repressed in the inside cells.

Specification of the epidermal cell fate during embryogenesis may involve positional signals from the tissue surrounding the embryo and maintenance of the epidermal layer after germination could involve signals from neighbouring epidermal cells and/or from underlying cell layers. It has been proposed that differentiation and maintenance of epidermal cell fate could involve cell-cell signalling by the RLK, CRINKLY4 (CR4) (Fig 1.7). In maize, *cr4* mutants show abnormal epidermal development and therefore, it is thought that *CR4* is involved in a signalling pathway that specifies and/or maintains outer cell layer behaviour during embryogenesis (Becraft *et al.*, 1996). The expression of the *Arabidopsis* ortholog of *CR4*, *Arabidopsis CRINKLY4* (*ACR4*) is restricted to protodermal cells during embryogenesis and the outer layer of the shoot apical meristem and organ primordia later in the development. Mutating this gene affects the organization of cell layers in the outer layer of the ovules and sepal margins (Gifford *et al.*, 2003; Watanabe *et al.*, 2004). Thus, *ACR4* appears to be involved in regulating the behaviour of the epidermal cell layer and possibly also in epidermal specification. Since ACR4 is likely to act as a component in an intercellular signalling cascade, it is perhaps surprising that the expression of *ACR4* is restricted to the L1. This observation might suggest the presence of positive feedback loops which are involved in L1 specification and highlights the question of how the expression of *ACR4* is

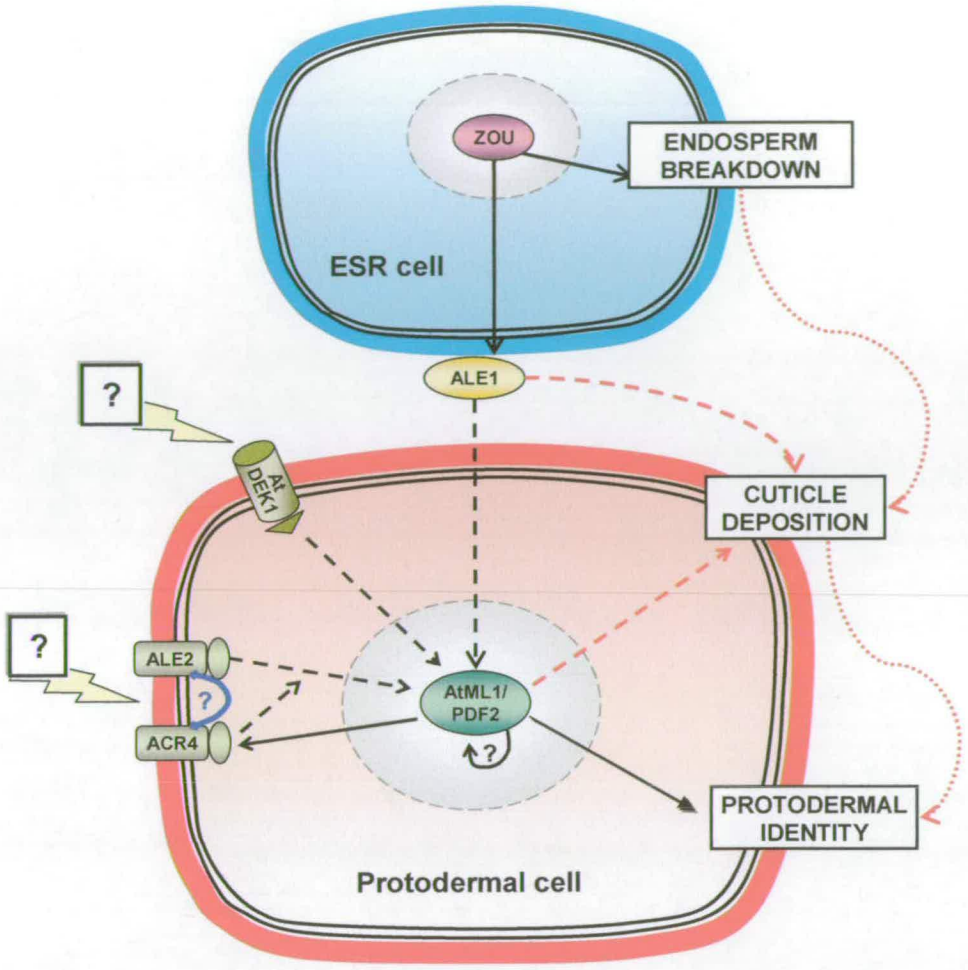

Fig 1.7: Schematic representation of epidermal specification in *Arabidopsis thaliana*.
In the protoderm, which is the outer cell layer of the embryo, several receptor-like kinases, such as ACR4, ALE2, the phytocalpain AtDEK1 are thought to perceive unknown positional signals necessary for the specification of this tissue layer. These two RLKs ACR4 and ALE2 could interact (blue arrow). The subtilisin-like serine protease, ALE1, expressed in the endosperm in response to the activity of the transcription factor ZOU, is thought to process a signal molecule perceived by the embryo, and necessary for normal cuticle deposition. Cuticle deposition itself appears to be necessary for maintaining protodermal identity, although the underlying mechanism is unclear. The transcription factors AtML1 and PDF2 are required for the acquisition of protodermal identity. ACR4, ALE2 and AtDEK1 are thought to be involved in the expression of protodermal genes such as *AtML1* and *PDF2*. AtML1 and PDF2 also promote the expression of *ACR4* suggesting the presence of a positive feedback loop. Nuclei shaded grey, cytoplasm pale red/blue, cell wall solid red/blue. Solid black arrows indicate direct/indirect transcriptional control. Intermittent black arrows show direct/indirect signalling pathways with some experimental/genetic support. Red intermittent and dotted arrows indicate functions proposed after analysis of mutant phenotypes. Schematic taken from Javelle *et al.* (2010).

maintained in the outer cell layer. Analysis of the cis regulatory elements of *ACR4* indicates that it has an L1 box (Abe *et al.,* 2001), the binding site of the homeobox transcription factor, ATML1 and PDF2. RT-PCR analysis revealed that in *atml1 pdf2* double mutant seedlings, there is no expression of *ACR4* (Abe *et al.,* 2003). One indirect possibility is that in *atml1 pdf2* double mutant there is no epidermal cell layer and so there is no *ACR4* expression. Another possibility is that ATML1 and/or PDF2 is directly required for *ACR4* expression. The current work in Ingram lab has shown that ATML1 can bind to the promoter of *ACR4*, allowing the expression of *ACR4*. In *acr4* mutants, there is slightly lower *ATML1* expression than in wild-type (Gwyneth Ingram personal communication). This suggests the existence of a feed back loop between *ACR4* and *ATML1*, which is currently the subject of further investigation in the Ingram lab.

Another gene which is thought to act in overlapping pathways with *ACR4* in specifying epidermal cell fate is *A. thaliana DEFECTIVE KERNEL 1 (AtDEK1)*, (Fig 1.7). *AtDEK1* is predicted to encode a membrane-anchored calpain-like cysteine proteinase (Lid *et al.*, 2002). Mutation in *AtDEK1* causes early embryo lethality. Using an RNAi approach to create milder phenotype, where early embryogenesis can occur normally, it has been shown that *AtDEK1* is required for maintenance of epidermal identity of the developing embryo as well as meristem development. *AtDEK1* RNAi in an *acr4* mutant background leads to an exacerbated phenotype suggesting that there could be synergistic genetic interaction between *AtDEK1* and *ACR4* (Johnson *et al.,* 2005).

Another gene which plays an important role in regulating the differentiation of protoderm is *ABNORMAL LEAF SHAPE 2 (ALE2)* (Fig 1.7), (Tanaka *et al.,* 2007). *ALE2* encodes an RLK and mutation of this gene causes various epidermal defects, including disorganization of epidermis-related tissues, defects in the leaf cuticle and the fusion of organs. Based on the genetic analysis of *acr4 ale2* double mutants, Tanaka *et al.* (2007) proposed that *ACR4* and *ALE2* might function in the same process. However, further experiments are required to understand the molecular interaction between these two RLKs.

During embryogenesis, specification of the epidermis may involve signals from the tissue surrounding the embryo. One gene that has been proposed to play a role in this respect is *ABNORMAL LEAF SHAPE 1 (ALE1)* (Fig 1.7), (Tanaka *et al.*, 2001). *ALE1* encodes a subtilisin-like serine protease. Gene expression analysis shows that *ALE1* is expressed in endosperm cells adjacent to the embryo. Loss of *ALE1* function results in impaired cuticle formation on the embryonic cotyledons and resulting in conditional seedling lethality. The ALE1 protein is predicted to be apoplastic and could be involved in the processing of a ligand which is required for the proper differentiation of the epidermis. Since *ACR4* and *ALE1* show a synergistic genetic interaction, it has been proposed that *ACR4* and *ALE1* might be involved in two major pathways that promote epidermal differentiation in *Arabidopsis* (Watanabe *et al.*, 2004).

Another gene acting in this pathway is a bHLH transcription factor *ZHOUPI (ZOU)* (Fig 1.7), (Yang *et al.*, 2008). *ZOU,* like *ALE1,* is expressed exclusively in the endosperm of developing seeds and *zou* mutant embryos have defects in cotyledon cuticle formation and in epidermal cell adhesion. They also lack the endosperm degradation usually observed in the region directly surrounding the developing embryo. The *zou* mutants lack *ALE1* expression, and are epistatic to *ale1* mutants, suggesting that one function of *ZOU* is to allow formation of a normal cuticular membrane in an *ALE1*-dependent manner. Whether this function is dependent or independnent of the role of *ZOU* in regulating endosperm cell death remains to be resolved (Yang *et al.*, 2008). The exact role of *ZOU* is currently the subject of investigation in the Ingram lab.

Thus, complex interlinked signal transduction pathways are involved in the regulation of epidermal differentiation. As mentioned earlier, the RLK, ACR4 is one of the candidate regulators of epidermal specification in *Arabidopsis*. Research in the last few years has revealed that *ACR4* plays diverse roles in plant development. This gene has been a major axis of research in the Ingram lab for several years, and is the

primary subject of this thesis. Our current understanding of the function of *ACR4* gene in plant development based on expression patterns, mutant phenotypes, genetic interactions, subcellular localizations and protein behaviour will be discussed in the next section.

1.5 ACR4: a unique plant receptor like kinase

The gene *ACR4* was first identified as an ortholog of maize *CRINKLY4 (CR4)* through sequence comparison (Tanaka *et al.,* 2002; Gifford *et al.,* 2003). Maize *CR4* encodes a receptor like kinase and *cr4* null mutants show defects in epidermal differentiation. Therefore, it is thought that *CR4* is involved in a signalling pathway that specifies or maintains outer cell layer behaviour during embryogenesis and subsequent plant development (Becraft *et al.*, 1996). Like *CR4, ACR4* also encodes a receptor like kinase. Research in the last few years has revealed that ACR4 mediated cell-cell signalling plays diverse roles in plant development, in addition to its roles in shoot epidermal formation (Gifford *et al.,* 2003; De Smet *et al.*, 2008; Stahl *et al.,* 2009a).

1.5.1 ACR4 in maintenance of the epidermal layer

The analysis of the expression pattern of *ACR4* early in the embryonic development indicates that *ACR4* starts to be transcribed at, or even before, the 8 cell/ dermatogen stage of embryogenesis, and continues to be expressed at high levels until the torpedo stage, when it becomes restricted to the shoot and root apical meristem (Gifford *et al.,* 2003). This suggests that *ACR4* could have a role in the specification of the epidermal layer. Post-embryonically in the shoot, *ACR4* is expressed in the L1 of shoot apical meristems, inflorescence meristems and floral meristems. In the mature ovule, *ACR4* is expressed in the outer integument and the inner integument and endothelium - all of which are L1-derived tissues. Therefore, it is thought that *ACR4* could be involved in a signalling pathway that specifies or

maintains outer cell layer behaviour; a supposition which is borne out by the fact that mutations in its likely maize ortholog show severe defects in epidermal development.

Surprisingly, given the strong phenotypes associated with loss of *CR4* function in maize, loss of *ACR4* function leads to quite a subtle phenotype with no changes in gross shoot morphology. However, scanning electron microscopy (SEM) revealed disruption in the cellular organisation in the ovule integuments and the sepal margins (Gifford *et al.,* 2003). A manifestation of this defect in ovule integuments is the subsequent formation of round seeds in *acr4* mutants, compared to wild type seeds which are elliptical. In *acr4* mutants, the ovule integuments do not elongate properly and thus fail to provide enough space for the embryo sac to develop and so the *acr4* mutant seeds are round. This disruption is interesting since the ovule integuments and the sepal margins are the only structures in *Arabidopsis* composed of two appressed L1 layers. These structures may therefore be more sensitive to disruption in signalling within the L1 layer than other organs, where signalling from the L2 could potentially compensate disruption of L1-layer signalling (Gifford *et al.,* 2003). Although *ACR4* expression is observed in the epidermal layer of all the meristematic tissues of the shoot, there is no strong evidence for a loss of epidermal identity in *acr4* mutants. The mild phenotype of *acr4* mutants has led to speculation that *ACR4* could act redundantly with other *ACR4* related kinases (Gifford *et al.,* 2003).

1.5.2 ACR4, initiation of lateral roots and maintenance of the stem cell niche

The initiation of lateral roots involves *de novo* formation of new meristems starting from a limited number of pericycle lateral root founder cells (Fukaki *et al.,* 2007). Transcriptome profiling of sorted pericycle cells undergoing lateral root initiation led to the identification of *ACR4* as a key factor in promoting formative cell

divisions in the pericycle (De Smet *et al.*, 2008). Mutation in *ACR4* exhibited a significant increase (19%) in the total number of lateral roots. Further analysis of *ACR4* over-expressed line as well as investigation of mutants that lack asymmetric pericycle cell divisions suggested that *ACR4* plays role both in promoting formative cell divisions in the pericycle and in suppressing proliferative cell divisions in nearby pericycle cells once organogenesis has been started. Although other members of the *ACR4* gene family (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*) were not identified in the transcript profiling, double and triple mutants of *ACR4* and *ACR4* related kinases exhibited a slight increase in the number of lateral roots compared to *acr4* single mutant suggesting that there may be some degree of redundancy among the members of the *ACR4* gene family (De Smet *et al.*, 2008).

The ACR4 receptor also functions in the root apical meristem to maintain the stem cell pool. Mutation in *ACR4* causes the formation of an additional layer of columella stem cells in the root meristem, suggesting that *ACR4* might play a role in regulating distal root stem cell fate (De Smet *et al.*, 2008). Further research by Stahl *et al.* (2009a) demonstrated that ACR4 is a target of *CLE40* signalling. *CLE40* encodes a potentially secreted protein that is related to CLV3 (Hobe *et al.*, 2003). *CLE40* was shown to be expressed in the columella cells, and the *cle40* mutant shows multiple layers of columella stem cells and an enlarged WOX5 expression pattern (Stahl *et al.*, 2009a). WOX5 is a root-specific WUS-like homeobox transcription factor, expressed in the quiescent centre (QC) of the Arabidopsis root and involved in the maintenance of the root stem cells (Sarkar *et al.*, 2007). The presence of CLE40 peptide causes increased *ACR4* expression, and a displacement of expression into the QC. This alteration in *ACR4* expression paralleled the proximal displacement of *WOX5* expression. Stahl *et al.* (2009a, 2009b) proposed that CLE40 and ACR4 form a ligand–receptor pair that regulates *WOX5* expression levels and controls the balance between proliferation and differentiation of distal root stem cells (Fig 1.8).

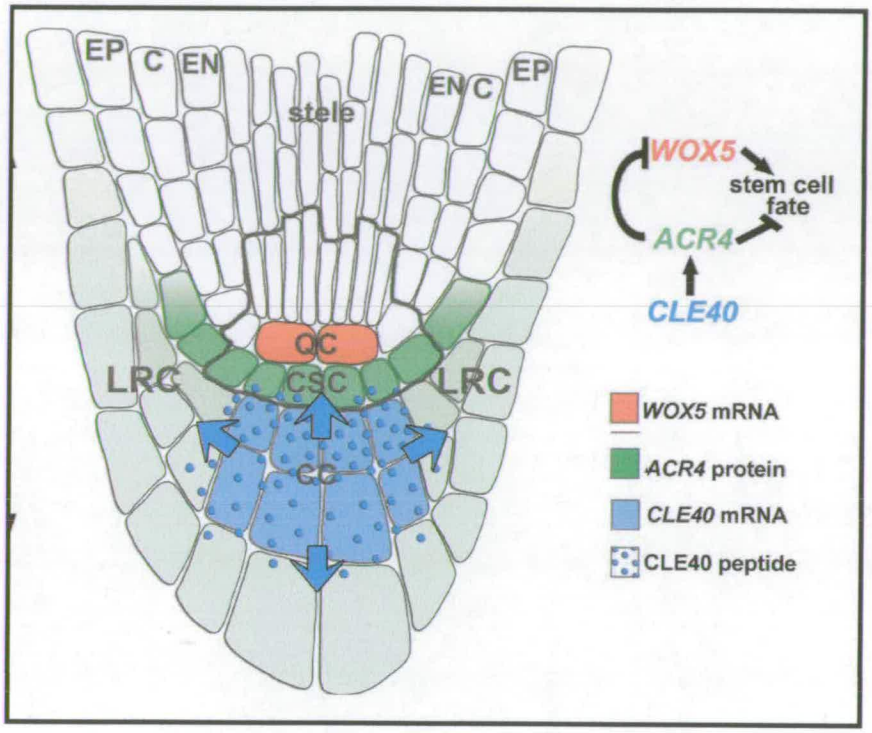

Fig 1.8: The RLK, ACR4 mediated signalling controls stem cell fate in the root meristem.

ACR4 controls *WOX5* expression in the quiescent centre and consequently, proliferation of columella stem cells (the cell layer just below the quiescent centre) through a CLE40-dependent mechanism.
Arrows indicate CLE40p movement into surrounding cells. The stem cells surrounding the QC are outlined by a grey line. QC, quiescent centre; CSC, columella stem cells; CC, columella cells; LRC, lateral root cap; EP, epidermis; C, cortex; EN, endodermis. Schematic taken from Stahl and Simon, (2009b).

1.5.3 Structure and behaviour of ACR4

ACR4 protein carries the hallmarks of a receptor kinase: an extracellular domain, a transmembrane domain, and an intracellular Ser/Thr protein kinase domain (Tanaka *et al.*, 2002). The extracellular (N-terminal) region consists of two domains– 7 repeats of 39 amino acids termed "crinkly repeats" (Cao *et al.*, 2005) and a TNFR –like region which contains three cysteine rich repeats (Fig 1.9). The 'crinkly repeats' are predicted to form a β-propeller structure that has been proposed to participate in protein–protein interactions (McCarty and Chory, 2000; Gifford *et al.*, 2005). The predicted cytoplasmic domain has a serine/threonine kinase catalytic domain and a C-terminal domain of unknown function. *In vitro* analysis has revealed that ACR4 has a functional kinase domain (Gifford *et al.*, 2003; Cao *et al.*, 2005).

A detailed analysis of the functional significance of various domains of ACR4 revealed that the extracellular crinkly repeat domain is vital for ACR4 function (Gifford *et al.*, 2005). It is thought that this crinkly repeat domain might act as a ligand binding domain. Through ACR4:GFP localization studies, it was found that ACR4 is a membrane localized receptor like kinase and is rapidly internalized and cleaved. A deletion of the 'crinkly repeats' renders ACR4 non-functional and it seems to be stabilized rather than endocytosed or cleaved. Therefore, it is speculated that ligand binding or signal perception by the 'crinkly repeat' could mediate internalisation and cleavage of the ACR4 receptor. This internalization in turn could participate either in signal termination by sequestering activated receptors into internal vesicles (Gifford *et al.*, 2005), or alternatively, in signal enhancement, in a mechanism analogous to that proposed for BRI1 signalling (Geldner *et al.*, 2007).

Fig 1.9: Diagrammatic representation of ACR4 & ACR4-related proteins (AtCRR1, AtCRR2, AtCRR3 and AtCRR4).

The predicted amino acid sequence of *ACR4* encoded protein consists of 7 repeats of 39 amino acids termed 'crinkly repeats', three repeats similar to the extracellular region of TNFR, a transmembrane domain, an active kinase domain and a C-terminal region. None of the ACR4 related kinases in Arabidopsis (AtCRR1, AtCRR2, AtCRR3 and AtCRR4) share the conserved C-terminal region. Both AtCRR1 and AtCRR2 have a high degree of sequence similarity to ACR4. However, both of them are missing critical residues in the kinase domain and are therefore predicted to be kinase inactive. Among ACR4 related kinases, both AtCRR3 and AtCRR4 conserve only the first TNFR like repeat but not the second or third.
Schematic taken from Nicholas C.R. Meuli, Masters thesis, 2006.

1.5.4 *ACR4*, a member of the *ACR4* gene family

The receptor like kinase, *ACR4* belongs to a *CRINKLY*-like gene family in *Arabidopsis* (Shiu and Bleecker, 2001). By homology comparison, it was found that there are four other genes in *Arabidopsis*, which are closely related to *ACR4* (Fig 1.9), (Cao *et al.*, 2005). These *ACR4* related receptor like kinases are - *Arabidopsis thaliana CR4-RELATED1* (*AtCRR1*), *AtCRR2, AtCRR3* and *AtCRR4.* The function of *ACR4* related receptor like kinases, analyzed here, is largely unknown, although some of them may act redundantly with *ACR4* in the formation of lateral roots (De Smet *et al.*, 2008).

Although the ACR4-like kinases form a distinct family (Fig 1.10), and share distinct structural characteristics, they are not highly conserved at the amino-acid level, suggesting that they did not arise from recent gene duplications. Among the four homologs of ACR4, AtCRR1 shares 23.6%, AtCRR2 25.2%, AtCRR3 23.7%, and AtCRR4 23.1% identity at the amino acid level with ACR4. AtCRR1 and AtCRR2 are predicted to be kinase inactive due to missing critical residues in the kinase domain known to be important for activation (Hanks *et al.*, 1988). However, AtCRR3 and AtCRR4 are kinase active (Cao *et al.*, 2005). The extracellular crinkly repeat and TNFR-like repeat of ACR4 share higher sequence similarity with AtCRR1 and AtCRR2, than with AtCRR3 and AtCRR4. A short proline rich domain, which could function in protein-protein interactions (Kay *et al.*, 2000), is present in the extracellular domain of AtCRR4. This proline rich domain is present neither in ACR4 nor in three other ACR4-related kinases. AtCRR4 is more closely related to CYTOKININ RELATED KINASE1 (CRK1) in *Nicotiana tabacum* than to any of the other ACR4 related receptor like kinases in *Arabidopsis* (Fig 1.10) (Schäfer and Schmülling, 2002). None of the ACR4-related kinases possesses a C-terminal domain equivalent to that which is present in ACR4. In summary, ACR4 related kinases conserve almost all the sequence motifs to the ACR4, with the exception of the C-terminal domain, and part of the TNFR-like domain (in case of AtCRR3 and AtCRR4).

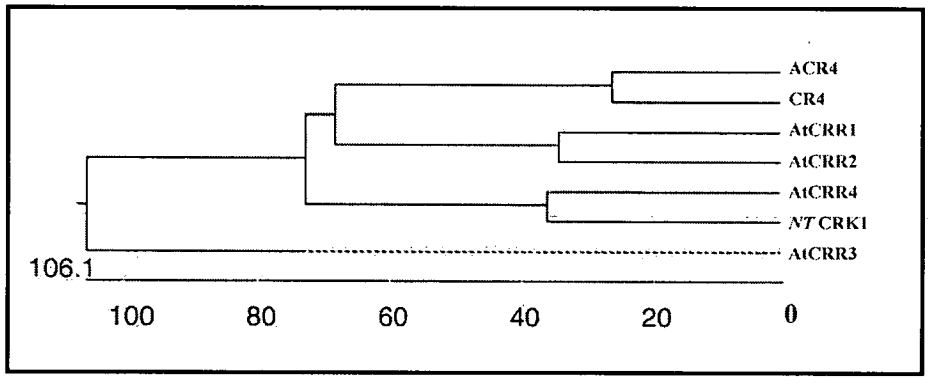

Fig 1.10: The phylogenetic analysis of the ACR4-related kinases from *Arabidopsis* (ACR4, AtCRR1, AtCRR2, AtCRR3 and AtCRR4), maize CR4, and *Nicotiana tabacum* CRK1.

The 7 proteins fall into 3 major clusters; the first contains two subclades, one consisting of the two CR4 proteins and the other contain AtCRR1 and AtCRR2. No ortholog of AtCRR1 and AtCRR2 is found in the maize and tobacco databases. The second major cluster is less related to ACR4 and contains AtCRR4 and *Nicotiana tabacum* CRK1. Thus, CRK1 appears to be a potential ortholog of AtCRR4. The rest is AtCRR3 and no ortholog is found in maize and tobacco databases.
Schematic taken from Miriam Gifford, PhD thesis, 2004.

1.6 Project aim

ACR4 has been identified as a candidate gene that plays diverse roles in plant development. The focus of my PhD project was to further investigate the role of *ACR4* and *ACR4* related genes (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*) in plant development as well as plant defence.

To investigate if *ACR4* acts redundantly with *ACR4* related receptor like kinases, the Ingram lab generated single, double and triple mutants of *ACR4* and *ACR4* related kinases. After I started, I attempted to characterise the quadruple and quintuple mutants to determine if there is extensive redundancy among the members of *ACR4* gene family in plant developmental processes, particularly with respect to shoot development. Next, physical interactions between the members of the *ACR4* family were addressed. Thirdly, to decipher the mechanism of ACR4 mediated signal transduction pathway, a detailed analysis was carried out to analyze the physical interaction between ACR4 and its putative binding partner, AIP1 (ACR4 INTERACTING PARTNER 1). Next, a possible role of a member of the *ACR4* gene family, *AtCRR4* was investigated in senescence. In recent years, many developmentally important RLKs have been shown to play independent roles in plant defence. Therefore, any possible role of *ACR4* in plant defence mechanisms was investigated. Finally, the role of *AIP1* with respect to seed germination was investigated.

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Chapter - 2

Materials and methods

2.1 Plant material, growth condition and genetic analysis

2.1.1 Plant culture and growth condition

Dry seeds were sterilized in 70% ethanol + 0.5% Triton-X-100 for 15 minutes. Seeds were then washed twice in 95% ethanol for 5 minutes, dried on sterile Whatman filter paper in a sterile hood and scattered onto Murashige and Skoog (MS) agar plates [0.5x MS salts, 0.6% sucrose, 1% plant micro agar, pH 5.7 (adjusted with KOH) and any appropriate antibiotics]. The plates were stratified for 3-days at 4°C before being transferred to growth room (22°C and constant light) for 10 days. Seedlings were then transferred to soil (3 parts soil, 1 part sand, 1 part perlite with Intercept fungicide) and grown at 22°C, 50% humidity with either 16 hrs light/ 8 hrs dark (long day) or 8hrs light/16 hrs dark (short day). Trays were covered with clear plastic covers for 3-4 days and then covers removed.

Alternatively for liquid culture, seeds were sterilized and stratified in 0.1% agar for 3 days, then 10-12 seeds were transferred to sterile conical flask containing 10 ml MS media [0.5x MS salt, 0.6% sucrose, pH 5.7 (adjusted with KOH)].

For measurement of root length and the number of lateral roots, twenty sterile *A. thaliana* seeds were sown per plate and plates were kept in a cold room for 3 days and then transferred and placed at 45° orientation under continuous light for 9 days and the number of lateral roots were counted.

2.1.2 Mutant alleles

The homozygous *acr4-2* (Gifford *et al.*, 2003), *crr1-3, crr2-2, crr3-2* and *crk1-3* (De Smet *et al.*, 2008) alleles were available in the lab. The *aip1-2* (GK-157D06) allele was obtained from the GABI-KAT T-DNA insertion collection (Rosso *et al.*, 2003) and ordered from the Nottingham *Arabidopsis* Stock Centre (NASC), UK. For PCR based genotyping, DNA was extracted from plants according to section 2.2.1 and PCR was performed according to section 2.2.2. Table 2.1 represents details of insertion lines used and Table 2.2 shows details of the primer combinations. Sequences of all primers used are listed in Table 2.3.

For examining the cuticular wax composition, homozygous mutant seeds of *lacerata* (*lcr*), and *bodyguard* (*bdg*) and heterozygous seeds of *fiddlehead* (*fdh*) were kindly provided by Alexander Yephremov, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany.

For senescence experiment, homozygous mutant seeds for *ore12-1, ahk3-1* and *AHK3* over-expressing line, AHK3-OX40 (*35s-AHK3*) were kindly provided by Prof. Hong Gil Nam, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Korea.

Table 2.1: Details of T-DNA insertion lines

Gene	Accession number	Allele	Knockout collection	Seed stock number	Ecotype	Insertion site
ACR4	At3g59420	*acr4-2* (Gifford *et al.*, 2003)	SAIL	SAIL_240_B04	Columbia-0	249bp downstream of *ACR4* ATG
AIP1	At3g20370	*aip1-1*	FLAG	FLAG_465C02	Wassilewskija (Ws)	956bp downstream of *AIP1* ATG
AIP1	At3g20370	*aip1-2*	GABI-Kat	GK-157D06	Columbia-0	1550bp downstream of *AIP1* ATG
AtCRR1	At3g09780	*crr1-3* (De Smet *et al.*, 2008)	SALK	SALK_10957	Columbia-0	1323bp downstream of *CRR1* ATG
AtCRR2	At2g39180	*crr2-2* (De Smet *et al.*, 2008)	SALK	SALK_101588	Columbia-0	329bp downstream of *CRR2* ATG
AtCRR3	At3g55950	*crr3-2* (De Smet *et al.*, 2008)	SALK	SALK_020817	Columbia-0	952bp downstream of *CRR3* ATG
AtCRR4	At5g47850	*crk1-3* (De Smet *et al.*, 2008)	SALK	SALK_068632	Columbia-0	1844bp downstream of *CRR4* ATG

Table 2.2: Details of primer combinations and product sizes used in PCR genotyping

Gene	Allele	Wild-type PCR primers	Wild-type band size (approx)	Mutant PCR primers	Mutant band size (approx)
ACR4	*acr4-2*	CR4PDEL7, CR-370	1 kb	CR4PDEL7, SAIL LB	1 kb
AIP1	*aip1-2*	MATH F, MATH R	1.5 kb	MATH R, GABI LB	0.4 kb
AtCRR1	*crr1-3*	CRR1-5, CRR1+1669	1.7 kb	CRR1+1669, SALK LB	0.3 kb
AtCRR2	*crr2-2*	CRR2-5, CRR2+594	0.6 kb	CRR2-5, SALK LB	0.3 kb
AtCRR3	*crr3-2*	CRR3-3, CRR3-5	2.4 kb	CRR3-3, SALK LB	1.6 kb
AtCRR4	*crk1-3*	CRK1-3, CRK1+1020	1.3 kb	CRK1-3, SALK LB	0.4 kb

Table 2.3 Details of primers used

Primer name	Sequence	Description
CR4PDEL7	5' GTCGACTTTGATAAGCTCCATGTCTC 3'	565bp upstream of *ACR4* ATG
CR-370	5' CTTCTAAATACTCAGCTCC 3'	*ACR4* ORF
CR-5	5' TTTGAAAAGAATGAGAATGTTCG 3'	5' *ACR4* ORF
CR-930	5' AAGTCCCTGTGAGAACTC 3'	*ACR4* ORF
CRK1-5	5' GGTACCACATGGCACTTACCATCTCAATCTC 3'	5' *CRR4* ORF + Kpn I site
CRK1-3	5' TCATCGTCGAGAAGTGTTTGATCGAGATACGGTC 3'	3' *CRR4* ORF + Sal I site and in frame stop
CRK1+1020	5' GAAAAGAAATGATGGAGAG 3'	*CRR4* ORF used for 568632 genotyping
CRR1-5	5' GGTACCACATGGAAACCCGTTGCTCTCTC 3'	5' *CRR1* ORF + Kpn I site
CRR1-3	5' TCATCCTCGAGAGATCAATGCATCTCTTGCGAC 3'	3' *CRR1* ORF + Xho I site and in frame stop
CRR1+1669	5' GGATTATGGTTGCTGCGTTT 3'	*CRR1* ORF designed for *crr1-2* genotyping
CRR2-5	5' GGTACCACATGCAACCCAATTCTCACATC 3'	5' *CRR2* ORF + Kpn I site
CRR2-3	5' TCATCGTCGAGAAGTGAGGCCAGACTTGACG 3'	3' *CRR2* ORF + Sal I site and in frame stop
CRR2+594	5' TCCCCAACAAACCAAGTCTC 3'	*CRR2* ORF designed for *crr2-2* genotyping
CRR2+2432	5' TCCGTGTTTTTATTACTTTGACAATC 3'	*CRR2* 3' UTR
CRR3-5	5' GGTACCACATGAAGAGGTTTATCAACTCAAC 3'	5' *CRR3* ORF + Kpn I site
CRR3-3	5' TCATCCTCGAGCAGAGACAATAGAACAAATGCC 3'	3' *CRR3* ORF + Xho I site and in frame stop
CRK1-1900	5'- ACAACCAAGAGCGATGTTTACAG -3'	*CRR4* ORF
MATH-738	5'ATCACTGCGAGTTTGGTGTTGATG 3'	*AIP1* ORF
pMATH-5	5' TAAGCTTTTGCATAAATGACAGAGAAGGGG 3'	*AIP1* promoter oligo
pMATH-3	5' TTCTAGAGTTGGTTTTGTGTATGAAGTTTGCAG 3'	*AIP1* promoter oligo
pMATH-int	5' ACTCAACTCTTCCAAACG 3'	*AIP1* promoter oligo
MATH F	5' TCTAGAACATGGCGAGCCACTACAGAAAC 3'	*AIP1* ORF
MATH R	5' TCATCGTCGACTCGGGAAGTACTTGGTTGAAG 3'	*AIP1* ORF
AHK3-F	5'-GATGGGTTGGAACGTGTTAGTC-3'	*AHK3* ORF oligo
AHK3-R	5'-TGTTCAACACGTGGAACTACTTC-3'	*AHK3* ORF oligo
ore12-1 F	5'-AAGGTTGGACTATTAGGAAGATG -3'	*AHK3* ORF oligo (*ore12-1* sequencing)
ore12-1 R	5'- GTAGTGAGTGTAGACCATGATAG -3'	*AHK3* ORF oligo (*ore12-1* sequencing)
FLAG LB	5' CTACAAATTGCCTTTTCTTATCGAC 3'	FLAG t-DNA left border oligo
SALK LB	5' TGGTTCACGTAGTGGGCCATCG 3'	SALK t-DNA left border oligo
SAIL LB2	5' GCTTCCTATTATATCTTCCCAAATTACCAATACA 3'	SAIL t-DNA left border oligo
GFP-R	5' TTTAGTGTTTGTATAGTTCATC 3'	*GFP* marker ORF oligo
HIS-R2	5' ATGATGATGAGCACCTCCATG 3'	*HIS* marker ORF oligo
MYC-R2	5' TAAGTTTCTGTTCTCCACCTCC 3'	*MYC* marker ORF oligo
TUBF	5' GTTCTTGATAACGAGGCCTT 3'	*TUBULIN3* ORF
TUBR	5' ACCTTCTTCCTCATCCTCG 3'	*TUBULIN3* ORF
LOX2-F	5'-GCATCCTCATTTCCGCTACACCA-3'	*LOX2* ORF
LOX2-R	5'-TCCGCACTTCACTCCACCATCCT-3'	*LOX2* ORF
B-cin-tub5	5'-TGAGATTGTCCATCTTCAAACC-3'	*B. cinerea TUBULIN* ORF
B-cin-tub3	5'-TCAAGAACTTGGTCGACAAGC-3'	*B. cinerea TUBULIN* ORF
SAG12-F	5'-CAGCTGCGGATGTTGTTG-3'	*SAG12* ORF
SAG12-R	5'-CCACTTTCTCCCCATTTTG-3'	*SAG12* ORF
SEN4-F	5'-CCTGGTAACTCTGCAGGAACAGTCAC-3'	*SEN4* ORF
SEN4-R	5'-GCATTCCTTAGGAGCTCCCTGTGG-3'	*SEN4* ORF
eIFB221	5'-GAACTCATCTTGTCCTCAAGTA-3'	*EIF4A1* ORF
eIFT22	5'-TTCGCTCTTCTCTTTGCTCTC-3'	*EIF4A1* ORF

2.1.3 Crossing lines

On secondary inflorescences, everything except 3-4 flowers was removed. These flowers were emasculated and the next day carpels hand pollinated with pollen from two different flowers of the donor plant. Once siliques had browned and the seeds had become visible, the siliques were removed and stored in a seed bag for a week before being transferred to soil.

2.1.4 Seed germination assay

For germination, *Arabidopsis* seeds were placed in 9 cm plastic Petri dishes on two layers of Whatman filter paper moistened with 3 ml water and incubated at 4°C for 4 days, followed by incubation under constant fluorescent light at 22°C. Germination was scored using a dissection microscope based on radical emergence.

For germination test in the dark, sterilized seeds were sown on 0.5x MS salts (Sigma-Aldrich)/0.8% agar (MS-agar) plates and the plates were wrapped with two layers of aluminium foil and left at 4°C for 3 days. The plates were then exposed to constant white light for 4 hours at 22°C and wrapped again with aluminium foil, left at 22°C for 3 days and germination rate was scored.

For seed germination assay in presence of gibberellic acid (GA_3)/paraquat/ ABA, seeds of similar age were stratified in 0.1% agar for 3 days at 4°C and sown on MS-agar plates containing 0, 50, 100 μM of GA_3 (Sigma) or 0, 0.5, 1 μM paraquat (Sigma) or 0, 1, 3 μM ABA (Sigma). The plates were then incubated at 22°C under constant light and germination rate was determined.

To investigate seed germination in the absence of nitrogen or phosphorus, seeds of similar age were stratified in 0.1% agar for 3 days at 4°C and sown on agar plates with and without nitrogen and incubated at 22°C under constant light and germination rate was determined. Media for N-deprivation experiments were based on synthetic medium containing 2 mM MES, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM

K_2HPO_4, 6.2 mg/L H_3BO_4, 0.025 mg/L $CoCl_2.6H_2O$ 0.025 mg/L $CuSO_4.5H_2O$, 37.2 mg/L $Na_2EDTA.2H_2O$, 27.8 mg/L $FeSO_4.7H_2O$, 370 mg/L $MnSO_4.7H_2O$, 0.25 mg/L $NaMoO_4.2H_2O$, 0.83 mg/L NaI, 8.6 mg/L $ZnSO_4.7H_2O$; pH 5.7 with KOH), and supplemented with 1mM NH_4NO_3 for control media containing nitrogen.

2.1.5 Leaf senescence assay

For natural senescence assay, plants were grown in an environmentally controlled growth room at 22°C with a 16hr light/ 8hr dark cycle (long day growth room) or 8hr light/ 16hr dark cycle (short day growth room). Leaves were carefully observed at different stages of development.

For dark-induced senescence assay, two different approaches were taken. One was detached leaf dark-induced senescence assay and the other was intact whole rosette dark-induced senescence assay. For detached leaf dark-induced senescence assay, the 3rd and 4th leaves at 12 days after leaf emergence were detached and floated on 3 mM MES (2-*N*-morpholino ethanesulfonic acid) buffer (pH 5.7) in the dark for 5 days (Kim *et al.,* 2006). For whole rosette dark-induced senescence assay, the whole rosette was excised, placed on wet filter paper in a Petri dish and then the Petri dishes were sealed using parafilm, covered with two layers of aluminium foil for 3 days (Dr. Vicky Buchanan-Wollaston, the University of Warwick, UK personal communication).

For assays of the onset of leaf senescence, total chlorophyll content was measured by immersing the leaves in 80% ethanol solution and heating at 65°C for 2 hours and absorbance of each sample was measured at 645 nm and 663 nm, as described in Section 2.4.2.

2.1.6 Seed clearing

The siliques were dissected using a dissection microscope and the developing seeds were cleared using chloral hydrate (2.5 grams of chloral hydrate in 1 ml of 30% glycerol) to determine the developmental stage of the embryo.

2.2 DNA techniques

2.2.1 Genomic DNA extraction

Three leaves were placed in a microcentrifuge tube, snap frozen in liquid nitrogen and stored at -80°C. Frozen tissue was crushed into a fine powder using mini-pestle, pre-cooled in liquid nitrogen and 500 µl of extraction buffer (50mM EDTA, 0.1 M NaCl, 0.1M Tris-HCl, 1% SDS) was added and refrozen in liquid nitrogen. Each tube was thawed on the bench and placed at 65°C for 5 minutes to complete thawing. 500µl phenol/chloroform (equilibrated phenol: chloroform: isoamylalcohol 25:24:1) was added, the tube vortexed, left to stand for 5 minutes and vortexed again. The tube was centrifuged for 5 minutes at 20,800 rcf and the upper (aqueous) phase (400 µl) removed to a fresh microcentrifuge tube and 50 µl 3M NaAc (pH 5.2) and 350 µl isopropanol was added. The tube was inverted gently and then centrifuged at 20,800 rcf for five minutes. The pellet was rinsed with 70% ethanol, then air dried before being dissolved in 50 µl R40 [TE (10 mM Tris-HCl pH 8, 1 mM EDTA) 5µg/ml ribonuclease A] and stored at -20°C.

2.2.2 PCR reaction

The following PCR constituents were made as a master-mix: 2 µl 10x PCR buffer [10 mM KCl, 20 mM Tris pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton-X-100], 1 µl 10 µM forward primer, 1µl 10µM reverse primer, 0.4µl 10mM dNTPs (10mM of each: dATP, dCTP, dGTP, dTTP), 1µl Taq polymerase enzyme (Promega, USA), 12.5 µl dH_2O; pipetted into a PCR tube, then 1µl of plasmid/genomic DNA was added. Primers were designed by eye, making sure that no secondary structures were formed, with an optimal ratio of 30-50% dGTP/dCTP and a G-clamp at the 3′ end. PCR reactions were run in a PTC-200 (MJ Research) PCR machine using PCR program: 94°C for 4 minutes followed by 35 cycles of [94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute per expected 1kb], 72°C for 5 minutes and 4°C forever.

Alternatively, for high fidelity PCR reactions, Pfu Turbo (Stratagene, USA) was used for amplification. The PCR reaction contained the following: 1 µl of plasmid/ genomic DNA, 2 µl 10x PFU Turbo PCR buffer, 1 µl 10x PFU polymerase, 1µl 10µM forward primer, 1µl 10µM reverse primer, 0.4 µl dNTP and 14µl dH_2O.

2.2.3 RNA extraction

For extraction of RNA from leaves or siliques, about 60 mg tissue was placed in a microcentrifuge tube, snap frozen in liquid nitrogen and crushed into a fine powder using mini-pestle and 600 µl TRIzol reagent (Invitrogen) was added. Then, 120 µl of chloroform was added, the tube vortexed for 15 seconds and then left at room temperature for 2-3 minutes. The tube was centrifuged at 20,800 rcf at 4°C for 15 minutes. The upper (aqueous) phase was removed to a fresh microcentrifuge tube and 200 µl of 0.8 M sodium citrate/1.2 M NaCl (half volume of the aqueous phase), 200µl isopropanol (half volume of the aqueous phase) was added and mixed by inverting the tube. The tube was left at room temperature for 10 minutes and centrifuged at 20,800 rcf at 4°C for 10 minutes. The supernatant was discarded and 1ml 75% EtOH in diethyl pyrocarbonate (DEPC)-treated water added to the pellet. The tube was vortexed briefly and centrifuged at 20,800 rcf at 4°C for 10 minutes. The ethanol was removed and the pellet briefly air dried and dissolved in 30µl DEPC-H_2O. LiCl was added to a final concentration of 2M and RNA precipitated at 4°C overnight. The tube was spun down at 20,800 rcf at 4°C for 15 minutes. The supernatant was discarded and 1ml 75% EtOH in DEPC-treated water added to the pellet. The tube was centrifuged at 20,800 rcf at 4°C for 10 minutes. The ethanol was removed and the pellet briefly air dried and dissolved in 30 µl DEPC-H_2O. RNA was quantified using the NanoDrop ND-1000 and stored at -80°C.

For extraction of RNA from siliques, RNeasy columns (Qiagen) were used according to manufacturer's instructions. The cDNA was synthesized from 0.5 g total RNA primed with oligo-dT primer using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions.

Table 2.4: Details of primers used in RT-PCR (See Table 2.3 for primer sequences)

Gene	Primer 1	Primer 2
AtCRR4	CRK1-3	CRK1+1020
SAG12	SAG12-F	SAG12-R
SEN4	SEN4-F	SEN4-R
EIF4A1	eIFB221	eIFT22
LOX2	LOX2-F	LOX2-R
B. cinerea TUBULIN	B-cin-tub3	B-cin-tub5
AIP1	MATH-738	MATH-R
TUBULIN	TUBF	TUBR

2.2.4 Digestion and ligation of DNA

Restriction digestions were carried out with the buffer, bovine serum albumin and temperature conditions suggested by the manufacturer (NEB/Promega). Double digests were carried out in the buffer recommended by the manufacturer (NEB/Promega) or if appropriate done sequentially.

Ligations were carried out with T4 DNA ligase (NEB) according to the manufacturers' instructions. Typically 10 µl reaction was set up with 10-20ng linearized plasmid DNA, 50-60 ng vector DNA, 1 µl T4 DNA ligase buffer (NEB) and 1 µl 10x T4 DNA ligase buffer (NEB) and incubated overnight at 16°C and 5 µl transformed into competent *E. coli* cells.

2.2.5 Agarose gel electrophoresis of DNA

1% agarose gel was made by dissolving agarose powder (Melford, Ipswich, UK) in TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA). DNA samples were loaded after addition of 1/10 volume of loading buffer (40% w/v sucrose, 0.25% bromophenol blue). Diagnostic gels were run at 80 V, gels for isolation of cleaved DNA fragments were run at 60 V. Gels were viewed on a UV transilluminator.

2.2.6 Transformation of plasmids into *E. coli* and *Agrobacteria*

Transformation of vector into competent DH5α *E. coli* cells was performed by heat shock. 100 µl *E. coli* competent cells were thawed on ice for 30 minutes. Competent cell were made according to Inoue (1990). 5µl of the ligation was added to the competent cells and mixed gently by flicking the tube. The tube was incubated on ice for 30 minutes and the cells were then heat shocked at 42°C for 1 minute and returned to ice. One ml of Luria Broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl; pH7) was added and the cells were incubated at 37°C for 45 minutes. Meanwhile, solid LB (LB containing 1% bactoagar) plates containing appropriate antibiotics were prepared. Ampicillin was added to a final concentration of 100 µg/ml or kanamycin to 50 µg/ml. After incubation, the cells were pelleted by centrifugation at 5,200 rcf for 3 minutes, resuspended in 100 µl LB and plated onto LB agar plates. For selection of transformed bacteria which carry a vector selection (e.g.- pGEMT-easy), blue/ white selection was performed by adding 100 µl X-gal solution (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) at a concentration of 20 mg/ml in dimethylformamaide [DMF] in 50 ml media. Colonies were picked using a sterile toothpick and inoculated into culture tubes containing 3 ml of liquid LB (containing antibiotics). Tubes were incubated overnight at 37°C on a shaking incubator.

For transformation into *Agrobacterium tumefaciens* cells, 100 µl of competent *Agrobacterium* cells were thawed on ice for 90 minutes and 1 µg plasmid DNA was added and mixed by flicking. Cells were then incubated on ice for 30 minutes, before they were snap frozen in $N_2(l)$ for one minute, then thawed at 37°C for a few minutes. One ml of YEP (1.0% bactopeptone, 1.0% yeast extract, 0.5% NaCl; pH 7.5) was added and the cells were incubated at 28°C on a shaking incubator for three hours. 10 µl of the sample was plated onto a solid YEP plate (YEP containing 1% bactoagar), containing appropriate antibiotics (gentamycin to select *Agrobacterium* cells at 80 µg/ml and kanamycin to select colonies containing the binary vector at 50 µg/ml). The remaining sample was centrifuged at 4,600 rcf for 1 minute to harvest cells, resuspended and plated onto a second YEP plate. Plates were

incubated upside down at 28°C for three days. Resultant colonies were incubated into 3 ml of liquid YEP (containing antibiotics). Tubes were incubated overnight at 28°C on a shaking incubator.

2.2.7 Preparation of plasmid DNA from bacterial cultures

To extract vector DNA from *E. coli* bacteria, 1.5 ml of fresh overnight culture was centrifuged at 4,600 rcf for three minutes and the pelleted cells were resuspended in 350 µl boiling buffer (8% w/v sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris- HCl) with 0.01% w/v lysozyme. The suspension was boiled for 1 minute, placed immediately on ice for 2 minutes and then centrifuged at 20,800 rcf for 20 minutes. The pellet was removed with an autoclaved toothpick and 40µl 3M NaAc (pH5.2) and 400 µl isopropanol were added. The tube was inverted gently, then centrifuged at 20,800 rcf for five minutes. The pellet was rinsed with 70% ethanol, then air dried before being dissolved in 50 µl R40.

For large scale purification of plasmid DNA, a single colony or alternatively 1.5 ml culture were used to inoculate 50 ml media and grown overnight at 37°C with shaking. A QIAfilter Plasmid Midi Kit (Qiagen) was used according to the manufacturer's instructions.

In order to extract DNA from *Agrobacterium* cells, 1.5 ml of fresh overnight culture was centrifuged at 20,800 rcf for three minutes to harvest the cells. Cells were resuspended in 100 µl ice-cold lysis buffer P1 (50 mM Tris-HCl, 10 mM EDTA containing 1/5 volume lysozyme). This was incubated at room temperature for 30 minutes, before 200 µl buffer P2 (0.2 M NaOH, 1% SDS) was added. This was mixed by inverting several times and the tubes kept on ice for 5 minutes. 150 µl ice cold buffer P3 (KAc, pH 4.8) was added, the tubes vortexed upside down for 10 seconds, stored on ice for 5 minutes, then centrifuged at 20,800 rcf for 5 minutes at 4°C. The supernatant was removed to a sterile tube, 315 µl isopropanol added, mixed by inverting and then centrifuged at 20,800 rcf for 10 minutes 4°C. The DNA pellet was then rinsed in 70% ethanol, air dried and resuspended in 10 µl R40.

For long term storage of bacterial lines, glycerol stock containing equal volumes of 80% glycerol and bacterial suspension was made, snap frozen in liquid N_2 and stored at -80°C.

2.2.8 Sequencing of plasmid DNA

DNA was cleaned using a QIAEX II Gel Extraction kit (Qiagen) following the manufacturer's instructions. Gel electrophoresis was performed to quantify the purified DNA. A sequencing PCR reaction was set up as follows: 50 ng DNA, 2 µl BigDye version 3.1 sequencing mix (Applied Biosystems), 1 µl primer (universal or reverse or gene specific primer), dH_2O upto 5 µl. The following PCR program was used: 96°C for 2 minutes followed by 30 cycles of [96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes]. 10 µl dH_2O was added to the reaction and sequencing was carried out by the School of Biological Sciences Sequencing Service (SBSSS) at the University of Edinburgh, UK.

2.2.9 *Agrobacterium* mediated plant transformation

Plants were transformed with the appropriate constructs by using an *Agrobacterium* mediated floral dipping technique (Clough and Bent, 1998) using *Agrobacterium* strain GV3101. Transformed *Agrobacterium* lines were grown on YEP plates for 2 days at 28°C and then inoculated stepwise into 3 ml, 50 ml and finally into 500 ml of YEP media with appropriate antibiotic for overnight. The culture was spun down at 4,200 rcf for 20 minutes at 4°C and the bacterial pellet resuspended in 500 ml 0.5X MS with 200 µl Silwet detergent (Lehle Seeds, Round Rock, USA). Plants were grown in short day condition for 5 weeks to enhance vegetative rosette leaf size and to increase the no of auxiliary buds that form during flowering. Plants were shaved to stimulate auxiliary inflorescences formation and after 2 weeks, plants were dipped in the bacterial solution. After 1 week, the same plants were retransformed with the same bacterial line.

Transformed plants carrying the appropriate vector were selected on 0.5×MS plates containing 200 mg/l Timentin (GlaxoSmithKline, Darmford, UK) and appropriate antibiotic either 15mg/ml hygromycin B (Calbiochem, Darmstadt, Germany) or 50 μg/ml kanamycin for plasmid selection, according to the vector present. Transformants were visible as plants able to develop true leaves (resistant to the antibiotic). Resultant T_1 lines harbouring a single insertion were selected on antibiotic containing plates for a ratio of 3 resistant plants: 1 susceptible plant. Resultant T_2 lines homozygous for the insertion were selected for a ratio of 100% resistant plants.

2.3 Generation of constructs

2.3.1 Expression pattern analysis

For promoter expression analysis, the AIP1 promoter was amplified with pMATH-5 and pMATH-3, cloned into pGEMT-easy (Promega) and sequenced. The AIP1 promoter was then removed using XbaI and HindIII and cloned into pMD4 (Gifford *et al.*, 2003), which contained the H2B:YFP sequence (Boisnard-Lorig *et al.*, 2001), creating construct JZ 6-1 and transformed line JZ4.

2.3.2 Complementation analysis

For complementation studies, homozygous *aip1-2* plants were transformed with the *pAIP1-AIP1:GFP* construct (gycerol stock JZ2-5, see Section 2.3.3 for details of the construction) where the transgene conferred a kanamycin antibiotic resistance.

2.3.3 Generation of epitiope tagged variants

For protein-protein interaction and protein localization studies, the following epitope tagged variants were generated: *pAIP1-AIP1:GFP*, *35s-AIP1:GFP*, *pACR4-ACR4:GFP*, *pACR4-ACR4:MYC*, *35s-AtCRR4:GFP* and *35s-AtCRR4:MYC*.

pAIP1-AIP1:GFP

The AIP1 promoter was amplified with pMATH-5 and pMATH-3 and the product was cloned into pGEMT-easy (Promega) and sequenced. After the sequence was confirmed, the fragment was digested from pGEMT-easy using HindIII and XbaI, cloned into HindIII/XbaI- cut pBIBHyg and pBIBKan (Becker, 1990), creating construct JZ8 and JZ18.The full-length *AIP1* ORF fused with GFP was cloned into pGEMT-easy (Promega) by Gwyneth C Ingram. The *AIP1:GFP* was cloned into pGEM-9Z vector (Promega) and removed as XbaI/SpeI fragment. This was cloned into XbaI-cut JZ8(Hyg) and JZ18(Kan), creating binary constructs JZ1-4 and JZ2-5 respectively and transformed plants were named as JZ1 and JZ5 respectively.

35s-AIP1:GFP

The full-length *AIP1* ORF fused with GFP was cloned into pGEMT-easy (Promega) by Gwyneth C Ingram. The AIP1:GFP was removed as XbaI/Spe1 fragment and inserted into two different XbaI/SpeI-cut binary vectors containing the 35s promoter, pBIBKan and pBIBHyg. This therefore resulted in the creation of two binary constructs: JZ4-1 (*35s-AIP1:GFP* in pBIBHyg) and JZ5-8 (*35s-AIP1:GFP* in pBIBKan) and transformed plants were called JZ3 and JZ6.

pACR4-ACR4:GFP

To generate *pACR4-ACR4:GFP* construct carrying kanamycin resistant transgene, the fragment *pACR4-ACR4:GFP* was removed from MD11a1 (Gifford *et al.,* 2003) using SalI. The plasmid MD11a1 contained *pACR4-ACR4:GFP* fragment, where the T-DNA carried a hygromycin resistance gene. The Sal1 digested *pACR4-ACR4:GFP* was cloned into a SalI cut, dCIP treated PTV50Kan and this generated JZ51 binary construct and JZ51 *Arabidopsis* transformed line.

pACR4-ACR4:MYC

The full-length ACR4 ORF fused with 2xMYC was cloned into pGEMT-easy (Promega) by Gwyneth C Ingram. The *ACR4:MYC* sequence was digested from pGEMT-easy using SacI and XbaI, cloned into MD6 (Gifford *et al.,* 2003), which

contained the ACR4 promoter sequence, generating JZ3-1 binary construct and JZ2 Arabidopsis transformed line.

35s-AtCRR4:GFP

To generate *35s-AtCRR4:GFP* construct, Gwyneth C. Ingram cloned the full-length AtCRR4 fused with GFP into pGEMT-easy (Promega). The *AtCRR4:GFP* was digested from pGEMT-easy using SalI, cloned into dCIP-treated, XhoI digested pGEM-7Z vector (Promega) and removed as XbaI/KpnI fragment. This was cloned into XbaI/KpnI-cut binary vector pBIBHyg (Becker, 1990) and pBIBKan (Becker, 1990), creating JZ13 (Hyg) and JZ14 (Kan) binary constructs and generating JZ13(Hyg) and JZ14 (Kan) Arabidopsis transformed lines respectively.

35s- AtCRR4:MYC

To create *35s-AtCRR4:MYC* construct, Gwyneth C. Ingram cloned the full-length *AtCRR4* fused with 2xMYC tag into pGEMT-easy (Promega). The *AtCRR4:MYC* was digested from pGEMT-easy using Sal1, cloned into dCIP-treated, XhoI digested pGEM-7Z vector (Promega) and removed as XbaI/KpnI fragment. This was cloned into XbaI/KpnI-cut binary vector pBIBHyg (Becker, 1990), creating binary construct JZ11-3 and transformed plant line JZ17.

Table 2.5: A list of the transgenic lines (Bold F3 lines were used for investigation).

TAGGED LINE	GLYCEROL STOCK OF *AGROBACTERIUM*	ANTIBIOTIC RESISTANCE	TRANSFORMED ALL RESISTANT LINES (F3 PLANTS)
35s-AIP1:GFP	JZ4-1	Hyg	**JZ3-3-9** JZ3-3-8
35s-AIP1:GFP	JZ5-8	Kan	JZ6-7-4 JZ6-7-5
pAIP1-AIP1:GFP	JZ1-4	Hyg	**JZ1- 2-4** JZ1-43-5
pAIP1-AIP1:GFP	JZ2-5	Kan	**JZ5-2-4** JZ5-2-5 JZ5-2-2

Tagged line	Glycerol stock of *Agrobacterium*	Antibiotic Resistance	Transformed all resistant lines (F3 plants)
pAIP1-H2B:YFP	JZ6-1	Hyg	**JZ 4-6-8** JZ4-9-5 JZ4-6-11
pACR4-ACR4:GFP	JZ51	Kan	JZ51-6-13 JZ51-6-14 **JZ51-4-7**
pACR4-ACR4:MYC	JZ3-1	Hyg	**JZ2-13-6** JZ2-21-4
35s-CRR4:GFP	JZ13-2	Hyg	JZ 13-14-7 JZ 13-11-10
35s-CRR4:GFP	JZ14-2	Kan	JZ14-17-5-1 JZ14-17-5-2
35s-CRR4:MYC	JZ11-3	Hyg	JZ17-6 (1-12) JZ17-16 (1-12)

2.3.4 Brefeldin A treatment

Two day old seedlings were incubated in a solution of 100 μM Brefeldin A (BFA) (Sigma) for 30 minutes to two hours. The working BFA stock was made by diluting 10 mM stock in water.

2.4 Assessment of cuticle permeability

2.4.1 Toluidine blue staining

Toluidine blue staining was adapted from a previously described protocol (Tanaka *et al*, 2004). Long day grown 4-week old Arabidopsis leaves were stained with 5 μl droplets of a 0.025% solution of toluidine blue in 1/4 PDB on the adaxial sites of the leaves for different time periods (2h, 12h and 24h) and then leaves were washed with water.

2.4.2 Chlorophyll leaching assays

For chlorophyll-leaching assays, four week old rosettes were excised at the hypocotyl, weighed and immersed in 80% ethanol solution. Samples were agitated gently and at 10 min, 30 min, 60 min and 90 min following immersion. Then the samples were heated at 65°C for 2 hours to extract all the chlorophyll and 1.5 ml aliquots of the ethanol solution removed. Total chlorophyll content in each sample was determined by measuring absorbance at 645 nm and 663 nm using a spectrophotometer (Beckman DU 520), (Arnon, 1949). Chlorophyll content (μgml^{-1} fresh leaf weight) was calculated using the following formula: Total chlorophyll = (20.2 × D645 + 8.02 × D663), where D = absorbance. Then percentage of total extracted chlorophyll at 10 min, 30 min, 60 min and 90 min was calculated from the total chlorophyll content at 2 hrs.

2.4.3 Measurement of water loss

This protocol was adapted from Bessire *et al.* (2007). Four week old rosettes were excised at the hypocotyl and the water loss was measured by weighing plants at different time points. Then, the sample was dried to measure the total water content. 100% equals the total water content.

2.4.4 Analysis of cuticular wax composition

The protocol for was adapted from Javelle M. (2009). Third and fourth rosettes of four week old long day grown plants were weighed and cuticular waxes were extracted by immersing tissues for 30 sec in 20 ml of chloroform (Sigma). Extracts were dried using a speed vac and dried samples were sent to the Logan lab, School of Chemistry, University of Edinburgh, UK for ESI-MS analysis.

2.5 Protein localization and functional analysis

2.5.1 Protein extraction

A pre-chilled mortar and pestle was used to grind 20 mg tissue to a fine powder in 200 µl extraction buffer (0.05 M Tris-HCl pH 7.5, 0.15 M NaCl,

containing Complete-Mini protease inhibitor cocktail, 1 tablet per 10 ml solution), (Roche). Cell debris was pelleted at 20,800 rcf for 10 mins at 4°C. The supernatant was recovered to a fresh tube, 6x loading dye (350 mM Tris-HCl pH 6.8, 10.28% w/v SDS, 36% w/v glycerol, 0.6 M dithiothreitol (DTT), 0.012% w/v bromophenol blue) added. The protein was denatured by heating at 85°C for 5 minutes, centrifuged for 5 minutes 20,800 rcf and either loaded onto the gel or stored at - 80°C.

2.5.2 Western blotting

Using the BIORAD mini-protean 3 system, protein gels were prepared and run as follows – to make the gel, 'resolving' gel [10% w/v acryl/bis-acrylamide 37.5:1 (Seven Biotech Ltd, Kiddiminister, UK), 0.375M Tris pH 8.8, 0.1% w/v SDS, 0.1% w/v APS, 0.01%v/v TEMED] was overlaid with 1/5 depth of 'stacking' gel (6% w/v bis/acrlamide, 0.125M Tris pH 6.8, 0.1% w/v SDS, 0.1% w/v APS, 0.01% v/v TEMED). This 10% acrylamide gel was used for separation of protein ranging from 40 to 150 kDa whereas for seed proteins which are only 10-20 kDa in size, 18% acrylamide gel was used to allow better resolution. Protein samples were denatured by heating at 85°C for 5 minutes, then centrifuged for 5 minutes 20,800 rcf prior to loading onto the gel. The gel was run for 2 hrs at a fixed voltage of 110 V at room temperature in running buffer (0.6% TRIS, 0.288% glycine, 1.0% w/v SDS; pH 8.3).

For Coomassie staining, gels were soaked in staining solution (45% methanol, 10% acetic acid, 0.25% Coomassie Brillent Blue R-250) for 30 minutes followed by incubation in destaining solution (30% methanol, 10% acetic acid) overnight. Stained gels were air dried between sheets of cellophane (Sigma).

For western blot analysis, separated protein was transferred to Hybond Nitrocellulose membrane in a BIORAD (USA) mini-protean 3 transfer cassette and tank; buffer containing 1.44% glycine, 0.3% TRIS, 20% v/v methanol was used to soak membrane and gel prior to transfer. Transfer was carried out at 4°C in transfer buffer (1.44% glycine, 0.3% TRIS, 0.1% SDS, 20% v/v methanol) at 65 V for one hour. Protein transfer and ladder position was monitored by rinsing the membrane briefly in Ponceau stain (0.1 w/v Ponceau, 5% v/v acetic acid), followed by rinsing in

Phosphate Buffered Saline with Tween (PBST: 1.5 mM KH_2PO_4, 150 mM NaCl, 5 mM Na_2HPO_4, 11 mM KCl, 0.02% Tween-20; pH 7.2). For MYC tag, the membrane was incubated in stripping buffer (100mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl at pH 6.7) at 55°C for 15 mins to denature the protein, as it permits detection of the protein by anti-MYC monoclonal antibody (Kaur & Bachhawat, 2009). For GFP tag, this additional step was skipped. The membrane was blocked by rinsing for 2 hours at room temperature in PBST milk (5% Sainsbury milk powder in PBST). For GFP tag, the membrane was incubated in 1/1000 dilution primary antibody (anti-GFP sheep polyclonal antibody, Roche) and for MYC tag, in 1/500 dilution primary antibody, (anti-MYC mouse polyclonal antibody, Roche) in PBST milk at 4°C overnight on a rolling incubator. Then the membrane was washed using PBST buffer for 15 minutes with three changes, followed by incubation with either 1/10,000 dilution of HRP linked anti-sheep secondary antibody (Jackson ImmunoResearch Laboratories, Inc) or 1/5,000 dilution of HRP linked anti-mouse secondary antibody (GE Healthcare, UK) in PBST milk for 1h at room temperature on a rolling incubator. The membrane was then washed using PBST buffer for 15 minutes with three changes and soaked in equal volumes of ECL detection reagents 1 and 2 (ECL Western Blotting substrate, Pierce) for two minutes, then exposed to photographic film (CL-Xposure; thermo Scientific) in the dark for a period of five minutes to 2 hours. The film was developed using a Konica developing machine (Konica Medical Film Processor, SRX-101A).

2.5.3 Silver staining

For high sensitivity staining of SDS-PAGE gels, the SilverSnap II kit (Pierce) was used according to the manufacturer's instructions.

2.5.4 Blue native PAGE

20 mg tissue was ground in 200 µl of BN-PAGE extraction buffer (50 mM Bis tris pH 7, 50 mM NaCl, 10% w/v glycerol, 0.001% Ponceau S and 1% w/v Digitonin) with protease cocktail inhibitor using chilled mortar and pestle. The

sample was then spun down at 20,000g for 20 minutes at 4°C. The sample was mixed with 10x loading dye (750 mM □-aminocaproic acid, 5% Coomassie G-250) and loaded on a pre-cast gradient gel (Native PAGE Novex 4-16% Bis-Tris gradient gels, Invitrogen). The gel was run at 150 V at 4°C with blue cathode buffer (50 mM Bis tris pH 7, 50 mM Tricine, 0.02% w/v Coomassie G-250) and anode buffer (50 mM Bis tris pH 7, 50 mM Tricine). After 45 minutes, when the gel front had reached 1/3 the way down the gel, the blue cathode buffer was replaced with the colourless cathode buffer (50 mM Bis tris pH 7, 50 mM Tricine) and voltage increased to 250 V for the remainder of the run.

For western blot analysis, separated protein was then transferred to PVDF membrane using a BIORAD mini-protean 3 transfer cassette and tank. The PVDF membrane was soaked in methanol for 30 seconds, briefly rinsed in deionized water and then placed in 1X transfer buffer (NuPAGE Transfer buffer, Invitrogen) for several minutes. Gel, blotting pads and filter papers were also soaked in 1X transfer buffer. Transfer was carried out at 4°C in transfer buffer (NuPAGE Transfer buffer, Invitrogen) at 52 V for 2 hours at 4°C. Following transfer, the membrane was soaked in 8% acetic acid for 15 minutes to fix protein, then rinsed in water and proceed to do immunodetection as mentioned in Section 2.5.2.

2.5.5 Immunoprecipitation

Fresh tissue was homogenized in 1x cold lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.1% Triton X-100, complete EDTA-free protease inhibitor tablet cocktail) using a chilled mortar and pestle. The homogenate was clarified by centrifugation at 12,000g for 15 min at 4°C. 100 µl of pre-equilibrated protein-G agarose slurry was added to 1 ml protein extract and allowed to rotate for 1h at 4°C. This pre-clearing step reduces non-specific binding of proteins to the protein-G agarose. Next spinning at 3,000g for 30 sec pelleted the protein-G agarose beads and the supernatant was transferred to a fresh tube.

The protein sample was mixed with 50 µl anti-GFP (µMACS GFP tag protein isolation kit, Miltenyi Biotec) and incubated on ice for 30 mins. Meanwhile, the µMACS columns were placed in the magnetic field of the µMACS Separator and equilibrated with 200 µl lysis buffer. The protein samples were then applied to the column. The magnetically labelled GFP-fusion proteins with their interacting partners are retained on the column. Washing the column 4 times with 200 µl lysis buffer and once with Tris-HCl pH 7.5 buffer removes any non-specific interacting proteins. 20 µl of 0.1 M Triethylamine, pH 11.8 was applied to the column and incubated for 5 mins at room temperature. The immuno-complex was finally eluted from the column with 50µl of 0.1M Triethylamine, pH 11.8 and the eluate was collected in an Eppendorf tube containing 3 µl of 1M MES, pH 3 for neutralisation. This step was repeated twice and 5 µl was analyzed by western blotting.

2.6 Pathogen challenge experiments

2.6.1 Inoculation with *B. cinerea*

The glycerol stock of *B. cinerea* (PJH2) (Nurmberg *et al.*, 2007) was kindly provided by Loake Lab, IMPS, University of Edinburgh, UK. The pathogen was grown on half strength Potato Dextrose Broth (PDB) and plates were incubated under continuous light for 10-12 days. To prepare fungal spore suspensions, colonies were washed with sterile water (1 plate with 15 ml of water) and filtered twice using Minar cloth to remove mycelium and centrifuged at 4000g for 10 minutes to pellet the spores. The pellet was resuspended in 10 ml of ½ PDB and 1/10 dilution of the spore suspension was either sprayed or 5 µl droplet was inoculated onto 6-week old short day grown leaves. Control plants were inoculated with just ½ PDB. The plants that were kept covered with clear plastic cover to ensure high humidity for 4 days.

To assess disease symptom development, plants were scored as follows, 0= no necrotic lesions, 1= plants showing small dry lesions, 2= plants showing a mix of small & medium size lesions, 3= plants showing medium size and spreading lesions,

4= plants showing predominantly spreading lesions, 5= plants showing predominantly wide necrotic lesions. Scoring was adapted from You *et al.* (2009).

Trypan blue staining was performed as previously described (based on Keogh *et al.,* 1980). Samples were submerged in lactic acid–phenol–trypan blue solution (2.5 mg/ml trypan blue, 25% lactic acid, 25% water-saturated phenol, 25% water and 25% glycerol) and boiled for 1 min. Then the stained leaves were dipped into the chloral hydrate solution (2.5 g/l) and left overnight or longer depending on the intensity of the staining. The de-stained leaves were mounted onto glass-slide by adding 60% glycerol and were covered by a coverslip over it.

Chapter -3
Investigating potential redundancy between members of *ACR4* gene family

3.1 Introduction

3.2 Results

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3.3 Discussion

Chapter – 3

Investigating potential redundancy between members of *ACR4* gene family

3.1 Introduction

As mentioned earlier, *ACR4* is expressed in the outer layer of the embryo, early in embryonic development. Later in development, *ACR4* expression is observed in the outer layer of all meristematic tissues and organ primordia of the shoot (Gifford *et al.,* 2003). Therefore, it is thought that *ACR4* could play an important role in the specification and maintenance of the epidermal layer, which is born out to some extent by shoot phenotypes. In the root, *ACR4* expression is observed in the epidermis, quiescent centre and lateral root cap (Gifford *et al.,* 2003). Research in the last few years has revealed that *ACR4* is involved in regulating asymmetric cell division during lateral root initiation and mutation of this gene results in an increase in the number of lateral roots (De Smet *et al.*, 2008). *ACR4* is also required to maintain stem cells at the root tip meristem (De Smet *et al.*, 2008; Stahl *et al.,* 2009a). Thus, *ACR4* plays two distinct roles in the root, one is the maintenance of root tip meristems and the other is generation of lateral roots.

Although *ACR4* expression is observed in the epidermal layer of all the meristematic tissues of the shoot, the loss of *ACR4* function shows quite a subtle shoot phenotype, causing disruption of cellular organization in ovule integuments and sepal margins. A manifestation of the defect in ovule integuments is round seeds, observed in *acr4* mutants whereas the wild type seeds are elliptical (Gifford *et al.*, 2003). Why does a gene which is widely expressed in all the meristematic tissues show such a subtle phenotype? One possible explanation is based on the fact that in plants, as well as in other organisms, the mutation of a single gene often results in no discernable phenotype, because its activity can be readily replaced by that of other genes that control the affected developmental process in a redundant manner (Cutler and McCourt, 2005). Therefore, the lack of phenotypic defects in *acr4* mutants in the epidermal layer of the shoot could be due to functional overlap of *ACR4* either with other close homologues or with other molecularly unrelated but functionally analogous genes. In the former case, this would indicate that closely related family members can functionally replace (at least in part) the mutated gene. The obvious test for such redundancy is to generate multiple mutant combinations for all genes suspected to be functionally related with *ACR4*. However, it is difficult to predict functionally redundant members. As mentioned earlier, four ACR4 related kinases (AtCRR1, AtCRR2, AtCRR3 and AtCRR4) share all the domains present in ACR4, with the exception of the C-terminal domain, and (in the case of AtCRR3, and AtCRR4) part of the TNFR-like domain. Therefore, these proteins represent the most obvious candidate receptors to act redundantly with ACR4.

To investigate redundancy among the members of the *ACR4* gene family, several approaches were taken in the lab. Firstly, the expression pattern of the four *ACR4* related genes was analyzed. Secondly, the T-DNA insertion mutants of these genes were characterized. In order to test functional redundancy among these genes, various combinations of multiple mutants were generated. Finally, physical interaction between the members of the *ACR4* gene family was investigated.

3.2 Results

3.2.1 Expression pattern of *ACR4* related receptor like kinases

To study the expression pattern of *ACR4* related kinases, two different approaches were taken in the lab. The first approach was *in situ* mRNA hybridization using gene specific probes and the second approach was gene expression pattern analysis using a reporter gene under the control of gene specific promoter. In addition, recently available transcriptomic and proteomic data was analyzed to determine the role of these genes in plant development.

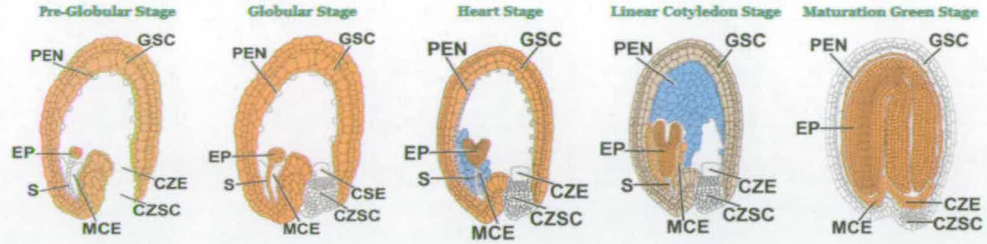
The *ACR4* gene is known to be expressed in the outer layer all the meristematic tissues including the shoot apical meristem (Gifford *et al.*, 2003). To determine the mRNA distribution of *ACR4* related kinases (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*) in the shoot apical meristem, RNA *in situ* hybridization was carried out by Nicholas C.R. Meuli. This *in situ* experiment indicated that all four *ACR4* related kinases- *AtCRR1, AtCRR2, AtCRR3* and *AtCRR4* are strongly expressed in the shoot apical meristem, suggesting that, *ACR4* related kinases could act in the same pathway as *ACR4*.

To further investigate the detailed expression pattern of these four *ACR4* related kinases and to compare the expression pattern of these genes with *ACR4,* the promoter-reporter marker lines were generated. Since our hypothesis was that ACR4 related kinases act in the similar pathway with ACR4, we were interested in ascertaining whether *ACR4* and related kinases show overlapping patterns of gene expression. The putative promoter sequence of each of the four *ACR4* related kinases (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*) were fused with the nuclear localized marker Histone2B:Yellow Fluorescent Protein (H2B:YFP), (Boisnard-Lorig *et al.*, 2001). All these promoter-reporter constructs, i.e. - *pAtCRR1-H2B:YFP, pAtCRR2-H2B:YFP, pAtCRR3- H2B:YFP* and *pAtCRR4-H2B:YFP* were made by previous lab members in the Ingram Lab. After the plants had been transformed with the constructs, T_2 generation plants carrying the transgene were selected using antibiotic

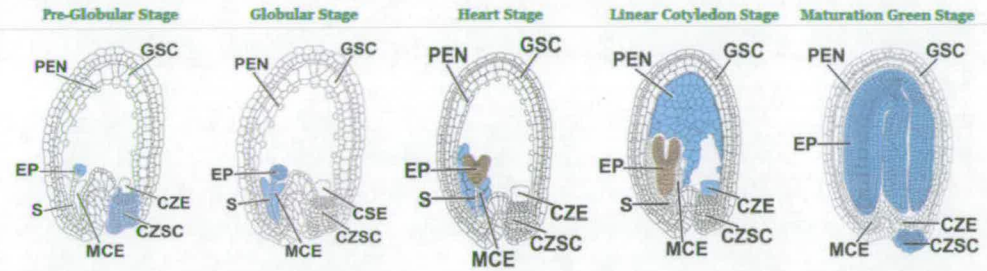
resistance. In the T_3 generation, homozygous transgenic lines were identified and promoter activity was studied in roots and inflorescences using confocal microscopy. No reproducible expression was observed for any of the four genes in any tissues, and no further experiments were carried out on any of these four promoter-reporter transgenic lines.

In recent years, extensive transcriptomic and proteomic analysis of *Arabidopsis* genes have provided an extremely powerful tool to study the expression pattern of *ACR4* and related kinases during plant development which could provide clues regarding their biological roles as well as functional redundancy. As mentioned earlier, homozygous *acr4* mutants show defects in ovule integuments and a manifestation of this defect is round seeds. To investigate whether *ACR4* related kinases act in an overlapping pathway with *ACR4* during seed development, publicly available transcriptome data was analyzed. A detailed analysis of transcripts from different seed compartments (embryo, endosperm, seed coat), regions (embryo proper, suspensor), and tissue types (epidermis, inner integument, outer integument, endothelium) at different developmental stages (globular, heart, cotyledon, and early maturation) by Goldberg and Harada laboratories (http://seedgenenetwork.net/) suggested that during seed development, the expression of *AtCRR1, AtCRR2* and *AtCRR4* are very low, however, the expression pattern of *AtCRR3* shows some similarity to *ACR4* (Fig 3.1). During early stages of seed development, both *ACR4* and *AtCRR3* are expressed in the embryo proper as well as in the seed coat and peripheral endosperm until the late heart stage, whereas in the mature green stage, they are expressed in the embryo proper and in the endosperm. Among *AtCRR1, AtCRR2* and *AtCRR4* genes, *AtCRR1* is expressed in the embryo proper at low level during the entire seed development process and there is also some expression in the endosperm; *AtCRR2* is also expressed in the embryo proper, however the expression goes down as development proceeds with very high level of expression in the preglobular stage and no expression in the mature green stage, whereas *AtCRR4* shows only a very low level of expression of in the endosperm during seed development (Fig 3.1).

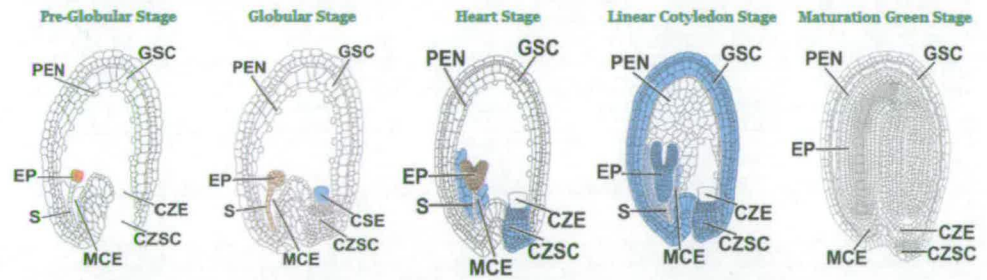
A. *ACR4*



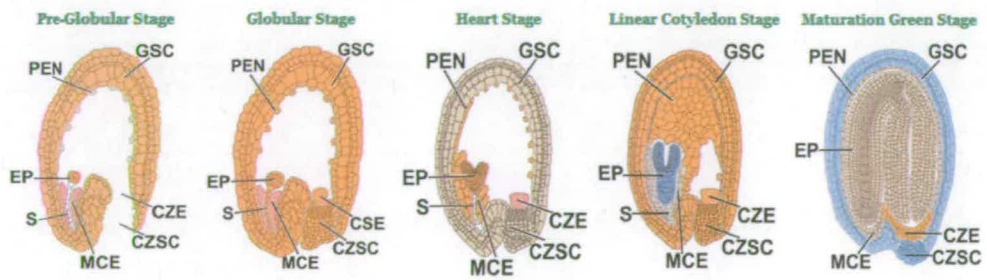
B. *AtCRR1*



C. *AtCRR2*



D. *AtCRR3*



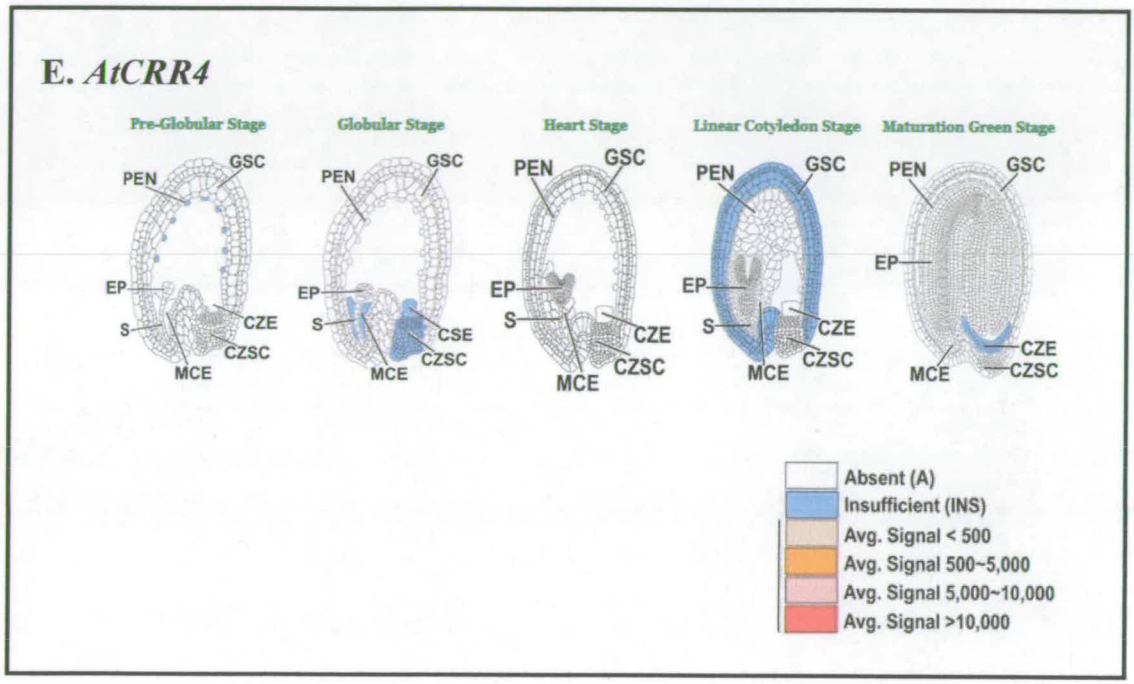


Fig 3.1: Expression profiles of *ACR4* and *ACR4* related kinases (A. *ACR4*, B. *AtCRR1*, C. *AtCRR2*, D. *AtCRR3* and E. *AtCRR4)* in different seed regions and compartments during *Arabidopsis* seed development (Goldberg and Haranda Laboratories; http://seedgenenetwork.net).

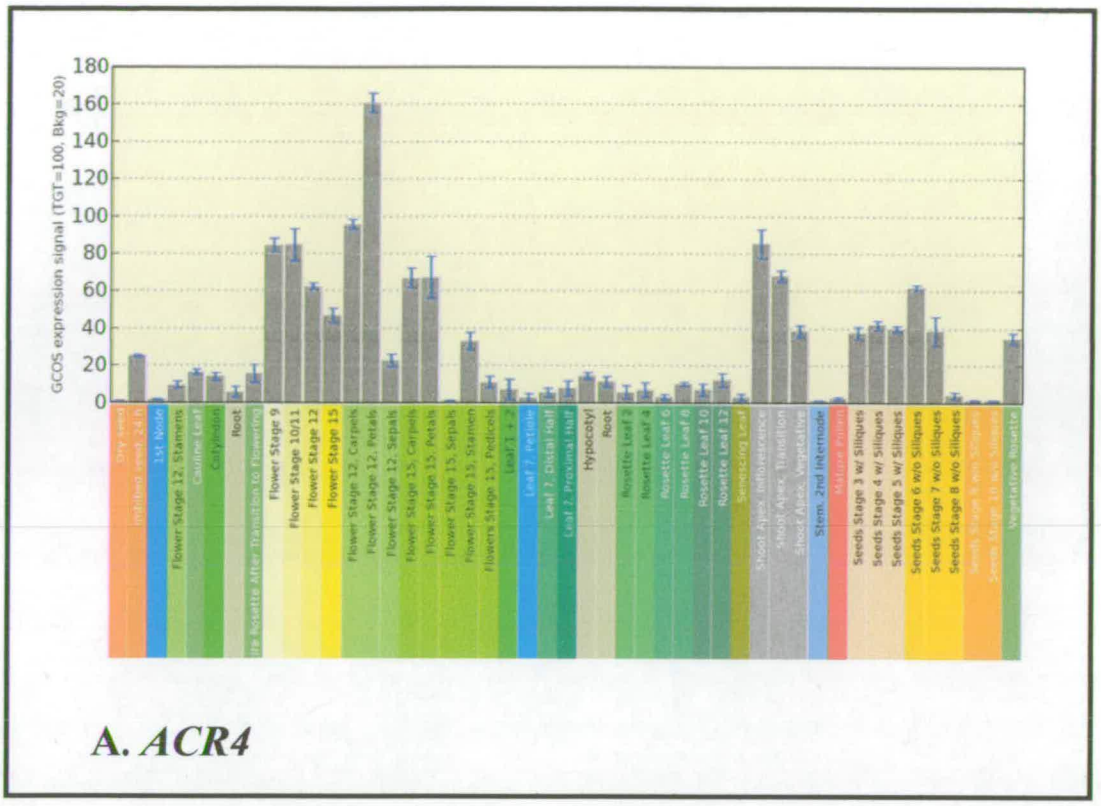
Abbreviation of Tissue/Compartment:
CZE - Chalazal Endosperm; CZSC - Chalazal Seed Coat; EP - Embryo Proper; GSC - General Seed Coat; MCE - Micropylar Endosperm; PEN - Peripheral Endosperm; S - Suspensor; WS - Whole Seed.

To investigate whether *ACR4* and related kinases are involved in regulating other aspects of plant development, recently available transcriptome and proteomic data was analyzed to investigate the expression pattern of these genes during different developmental stages because co-expressed members of the *ACR4* gene family could act in a redundant manner. As shown by Gifford *et al.* (2003), *ACR4* is strongly expressed in inflorescences and floral meristem tissues (Fig 3.2), (Winter *et al.,* 2007). Among *ACR4* related kinases, only *AtCRR3* is highly expressed in inflorescences. During seed germination, *AtCRR1* shows strong expression in imbibed seeds, however its expression goes down as germination proceeds. Among other genes, *AtCRR2* and *AtCRR3* also show some expression in cotyledons, however like *AtCRR1,* no strong expression was observed in imbibed seeds.

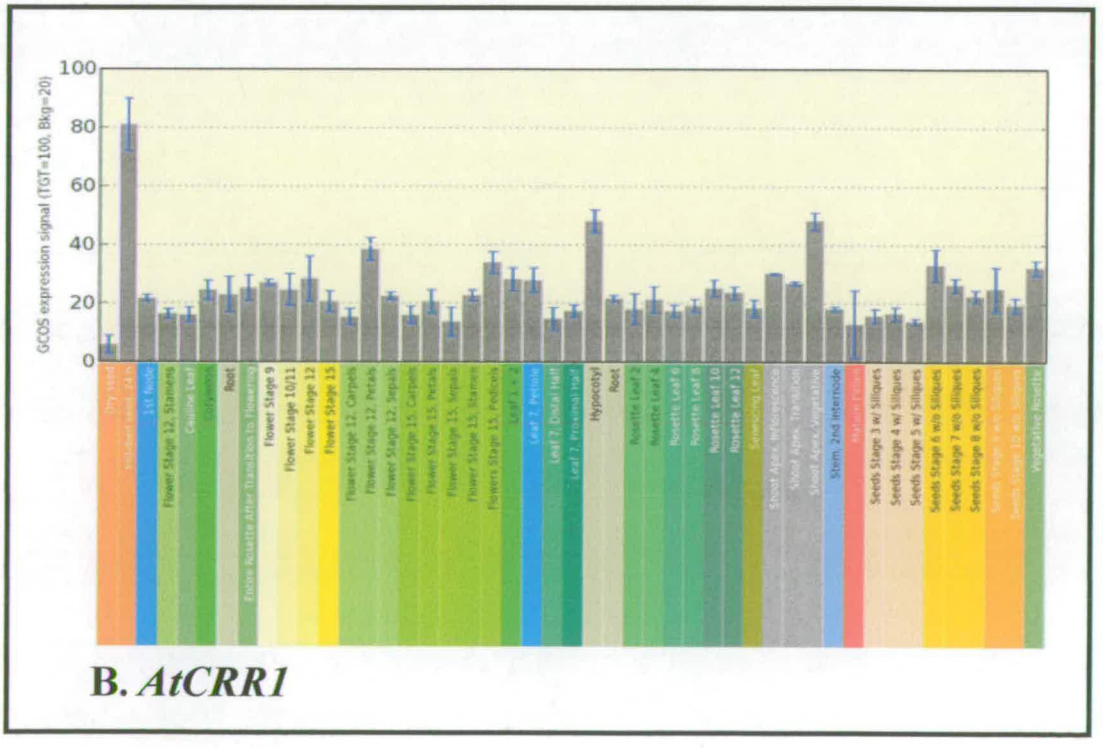
ACR4 is known to be involved in the initiation of lateral roots and the maintenance of stem cell niche at the root tip meristem (De Smet *et al.*, 2008; Stahl *et al.,* 2009a). De Smet *et al.* (2008) reported that there is some redundancy among the members of the *ACR4* gene family in the initiation of lateral roots. Surprisingly, given the root phenotypes of *acr4* mutants, *ACR4* expression levels in the root are quite low compared to other family members such as *AtCRR2* and *AtCRR3*.

In senescing leaves, both *AtCRR3* and *AtCRR4* are quite strongly expressed. However, the detailed transcriptome data provided by van der Graaff *et al.* (2006) suggest that *AtCRR3* is highly expressed in both mature and senescing leaves whereas *AtCRR4* shows massive up-regulation just in senescing leaves. Further experiments are required to understand potential redundancy between *AtCRR4* and *AtCRR3* in leaf senescence and a possible role of *AtCRR4* in senescence is discussed in Chapter-5.

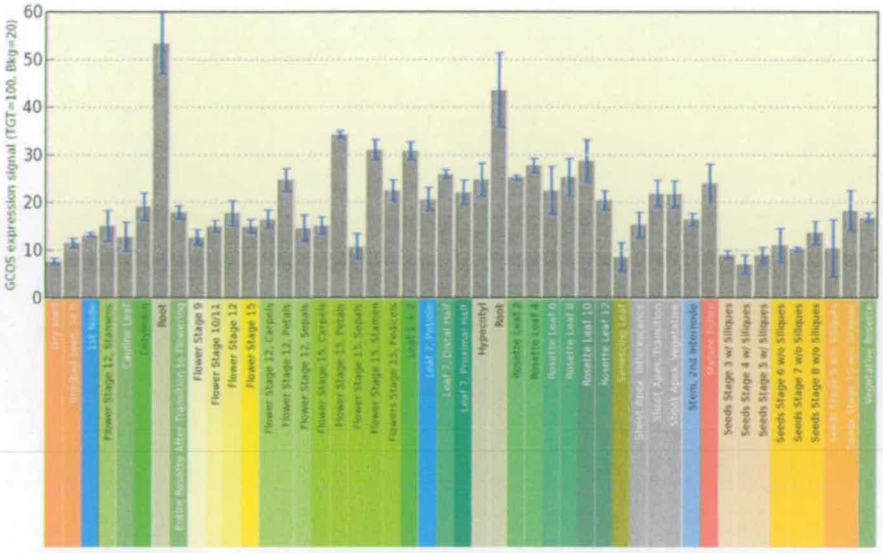
I also explored whether any proteomic data exists for ACR4 and ACR4 related kinases (AtCRR1, AtCRR2, AtCRR3 and AtCRR4) (Baerenfaller *et al.,* 2008). The proteomic analysis performed by Baerenfaller *et al.* (2008) showed the detection of ACR4-derived peptide although the levels of ACR4 protein are very low. No proteomic data is available for ACR4 related kinases (AtCRR1, AtCRR2, AtCRR3 and AtCRR4) (Fig 3.3).



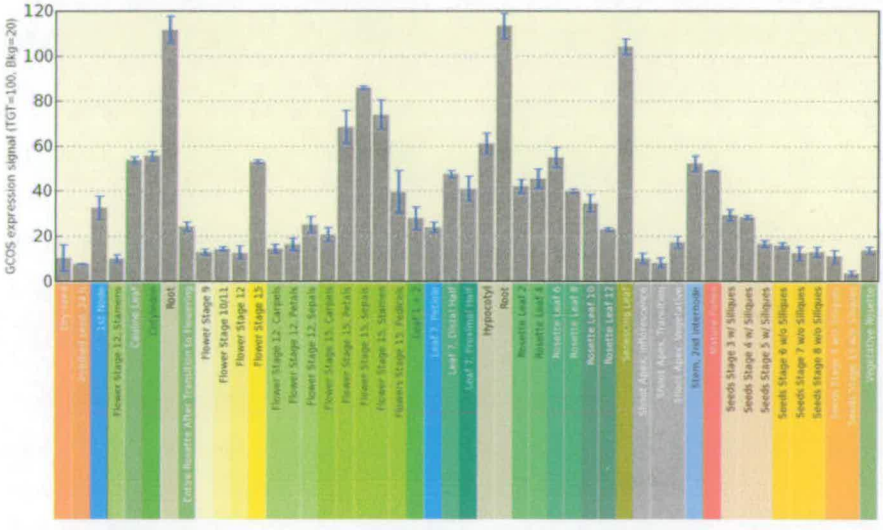
A. *ACR4*



B. *AtCRR1*



C. *AtCRR2*



D. *AtCRR3*

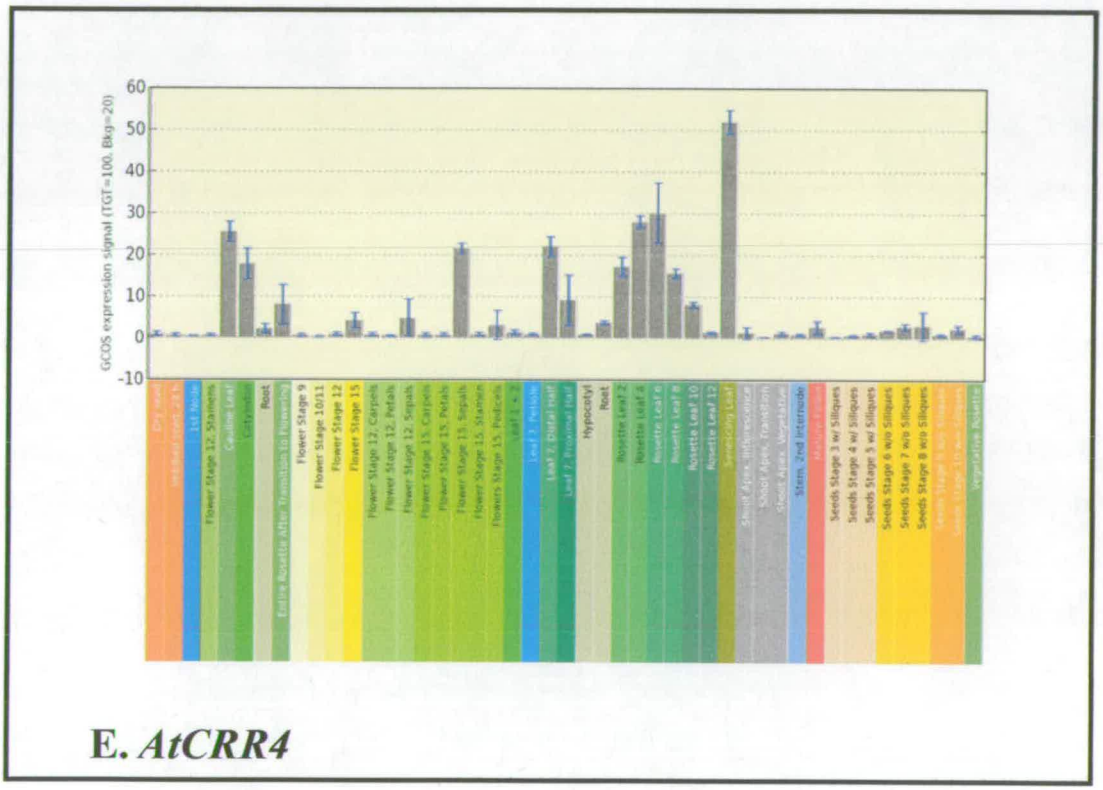


Fig 3.2: Expression profile of *ACR4* and *ACR4* related kinases (A. *ACR4*, B. *AtCRR1*, C. *AtCRR2*, D. *AtCRR3* and E. *AtCRR4*) retrieved from publicly available eFP browser (Winter *et al.*, 2007).

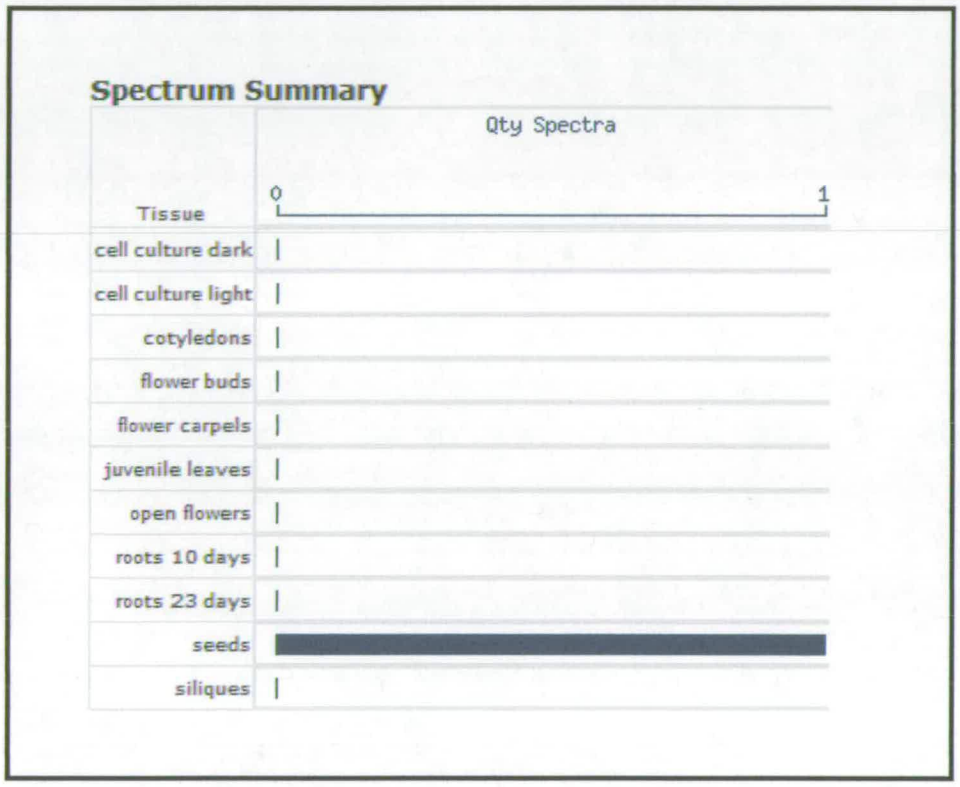


Fig 3.3: Spectrum summary from AtProteome (Baerenfaller *et al.*, 2008) showing accumulation of peptides from ACR4.

Only ACR4-derived peptides have been detected in seeds. No proteomic data is available for ACR4 related kinases (AtCRR1, AtCRR2, AtCRR3 and AtCRR4).

3.2.2 Characterizing multiple mutants of *ACR4* and its related kinases

For an initial functional analysis of *ACR4* related kinases (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*), the Ingram lab concentrated on single mutant analysis. T-DNA insertion alleles of *crr1-3, crr2-2, crr3-2* and *crk1-3* were ordered. Fig 3.4 represents the position and the orientation of the site of T-DNA insertion and Table 2.1 shows details of insertion lines used in the studies. Table 2.2 and Table 2.3 show details of the primer combinations and primer sequences respectively.To determine if loss of function in *ACR4* related genes leads to acr4 like phenotypes, siliques were dissected and the developing seeds were examined. None of the single mutants showed an acr4 like mutant phenotype. This work was carried out by Nicholas C.R. Meuli.

There are several possible explanations for the observed wild type phenotype of *atcrr1, atcrr2, atcrr3* and *atcrr4* single mutants. One possibility was that these genes play no role in aspects of plant development addressed by our phenotypic analysis. A second possibility was functional redundancy among the members of this family, as they possess similar domain organization and show overlapping expression patterns. This second possibility can be tested by studying double (or multiple) mutants of genes suspected to act redundantly. We decided to address this question systematically for the entire set of five *ACR4* and *ACR4* related genes for which we had available mutants. The Ingram Lab systematically made double and triple mutant combinations. All the double and triple mutants along with *Col-0* plants were grown in the long day (22°C with a 16hr light/ 8hr dark cycle) and the short day (22°C with a 8hr light/ 16hr dark cycle) growth room and were carefully observed during growth. It was found that double and triple mutant combinations with a mutant *acr4* gene, produce round seeds, like *acr4* single mutants. No exacerbation of *acr4* single mutant phenotype was observed. The double and triple mutants of *ACR4* related kinases resembled wild type and the seeds were also elliptical like wild type seeds. Taking these results together, it was thought that possibly members of the *ACR4* gene family do not act redundantly, at least for the shoot phenotype. This work was carried out by Nicholas C.R. Meuli and Gwyneth C Ingram.

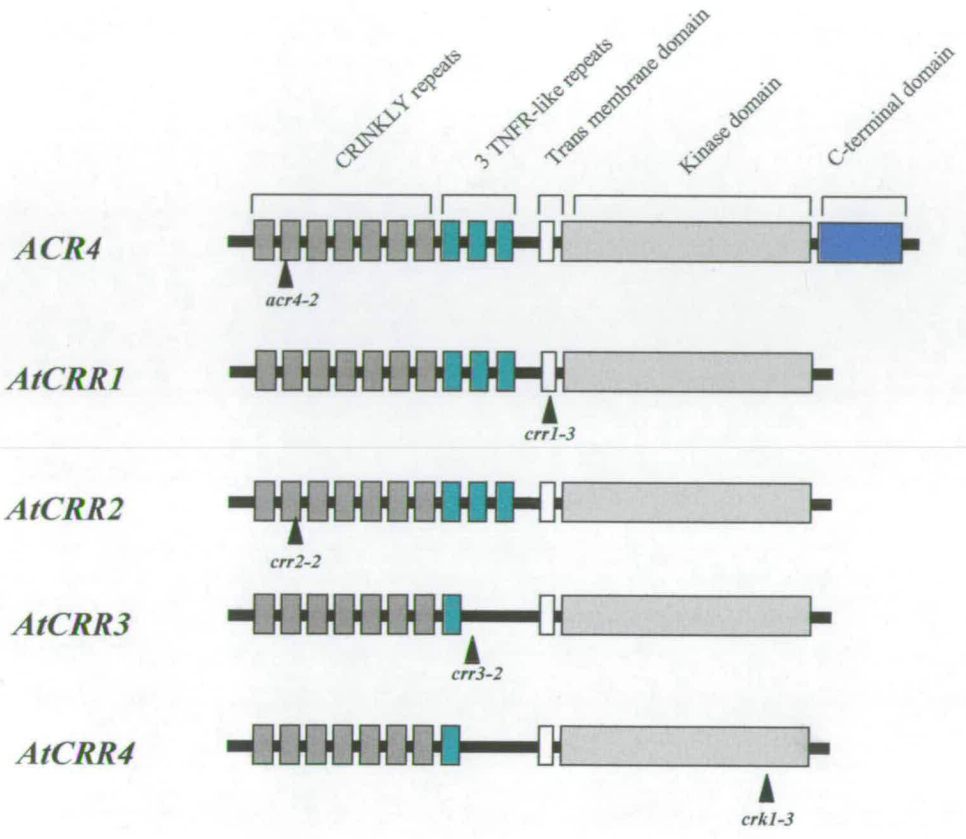


Fig 3.4: The structure of ACR4 and ACR4 related kinases and the position of T-DNA insertion.

Schematic taken from figure by Nicholas C.R. Meuli.

To test extensive redundancy among the members of the *ACR4* gene family, I decided to generate quadruple and quintuple mutant combinations. First, triple mutants were crossed to generate the five possible quadruple mutants and then quadruple mutants were crossed together to generate a quintuple mutant. The genotypes of F_1 plants from these crossings were confirmed by PCR based genotyping. The selfed seeds from the F_1 were collected, grown up and screened by PCR. Once confirmed, genotyped homozygous quadruple and quintuple mutants were planted out and the plants were examined for a phenotype. The homozygous quadruple and quintuple mutants showed no obvious change in gross morphology and appeared wild type. To examine whether any of these quadruple and quintuple mutants show an exacerbation of *acr4* single mutant phenotype, siliques were dissected and the developing seeds were examined to investigate if there is any additional defect. The developing seeds of homozygous quadruple mutant (*atcrr1 atcrr2 atcrr3 atcrr4)* were elliptical like wild type seeds, not round like *acr4* seeds, whereas quadruple mutant combinations containing *acr4 (acr4 atcrr1 atcrr2 atcrr3, acr4 atcrr2 atcrr3 atcrr4, acr4 atcrr1 atcrr2 atcrr4* and *acr4 atcrr1 atcrr3 atcrr4)* and the quintuple mutant (*acr4 atcrr1 atcrr2 atcrr3 atcrr4*) displayed the characteristic *acr4* mutant phenotypes, such as round seeds. This suggests that possibly there is no genetic interaction among the five *ACR4* and *ACR4* related genes, at least with respect to shoot development.

To uncover the hidden function of *ACR4* and its related kinases, mutant seedlings were exposed to a number of stress conditions. Publicly available microarray data (Zimmermann *et al.*, 2004) revealed that *ACR4* and *ACR4* related genes are differentially expressed under both biotic and abiotic stresses (Fig 3.5). Many biotic and abiotic stresses lead to an accumulation of Reactive Oxygen Intermediate (ROI), for example – singlet oxygen (O_2 ^{1}), hydrogen peroxide (H_2O_2) or hydroxyl radical ($HO^{\cdot}$) as signalling molecules to control various processes including hypersensitivity induced programmed cell death and senescence (Mittler, 2002; Bhattacharjee, 2005; Apel and Hirt, 2004). It was proposed that exposing mutants of *acr4* and its related kinases to different oxidative stress producing agents could reveal the role of these RLKs in regulatory pathways. An artificial method of

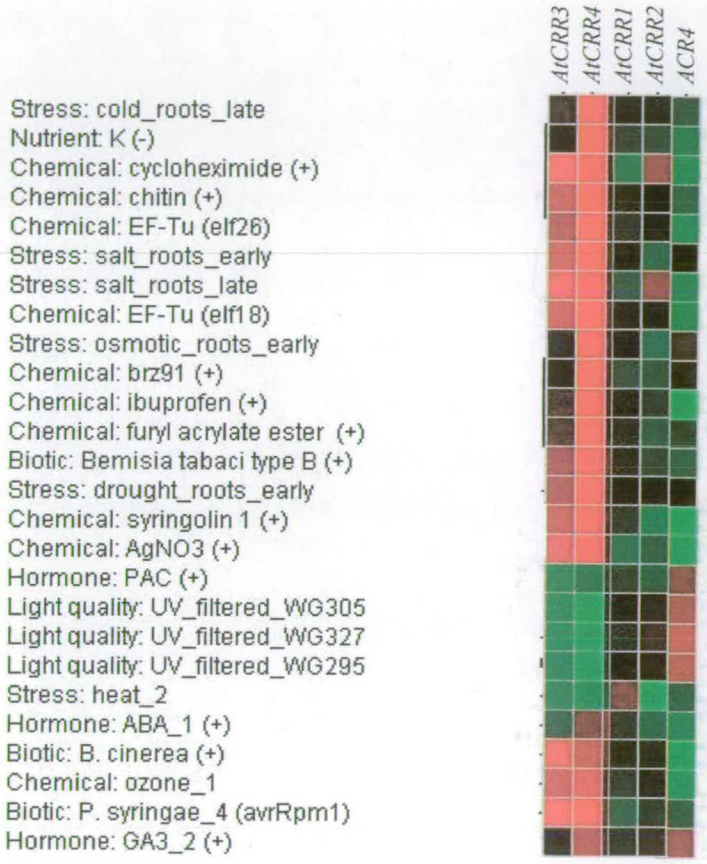


Fig 3.5: Differential expression pattern of *ACR4* and *ACR4* related genes.

Microarray data from GENEVESTIGATOR (Zimmermann *et al.*, 2004).

generating this oxidative stress in plants is to expose the plants to different oxidative stress producing agents, for example – paraquat, hydrogen peroxide etc. In my experiments H_2O_2, paraquat and Rose Bengal were used as ROI-inducing chemicals to compare the sensitivity of *Col-0* and *acr4* seedlings. Paraquat is a non-selective herbicide that predominantly induces the formation of superperoxide (O_2^-) in green plants (Babbs *et al.*, 1989). In contrast, Rose Bengal is a xanthene dye that mainly generates singlet oxygen ($^1O_2$) in photosynthetic plants (Knox and Dodge, 1984).

To assess the sensitivity of *Col-0* and *acr4* to paraquat, Rose Bengal and H_2O_2, seeds were stratified in water for four days before spreading them onto media containing different concentration of paraquat or Rose Bengal or H_2O_2. No difference was found between mutant and wild type while germinating seeds in presence of Rose Bengal or H_2O_2. Both wild type and mutants germinated properly and developed green cotyledons. However, it was found that in presence of paraquat both wild type and mutants germinated, but after 1 week, most of the cotyledons of *acr4* mutants turned yellowish while wild type didn't show such phenotype (Fig 3.6). As mentioned earlier, paraquat mainly induces the formation of superoxide free radicals whereas Rose Bengal generates singlet oxygen. My results therefore indicated that *acr4* is sensitive to superoxide but not to singlet oxygen or H_2O_2. Watanabe *et al.* (2004) has previously reported that cotyledons and young leaves of *acr4* mutants (18 days old) are permeable to the hydrophilic dye, toluidine blue. This suggested that the enhanced susceptibility of *acr4* to paraquat could be due to an epidermal defect. To further investigate whether the sensitivity of *acr4* to paraquat is due to the defect in the epidermis, the seedlings of *Col-0*, *acr4* and *ale1*, a mutant with known cuticular defects (Tanaka *et al.*, 2001) was exposed to paraquat. It was found that the cotyledons and young leaves of *ale1* turned yellow in the presence of paraquat in a very similar pattern to those of *acr4* mutants. This suggested that *acr4* mutants could have some defects in the epidermis which makes it more susceptible to paraquat. To investigate how *ACR4* related kinases behave in presence of paraquat, triple mutant seedling of *ACR4* and its related kinases were exposed to paraquat. The preliminary results suggested that triple mutants are slightly more sensitive to paraquat compared to *acr4*. It would be interesting to repeat this experiment using

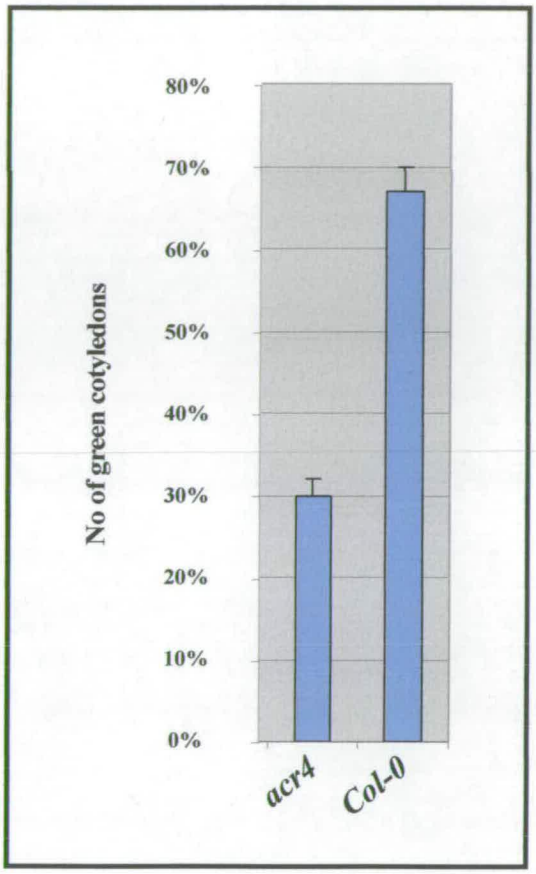


Fig 3.6: A graphical representation of the sensitivity of *Col-0* and *acr4* mutant seedlings to paraquat.

Col-0 and *acr4* seeds were stratified in water for four days before spreading them onto media containing paraquat. Although both wild type and mutants germinated properly, after 1 week most of the cotyledons of *acr4* mutants turned yellowish while wild type *Col-0* developed expanded green cotyledons. Error bars represent mean ± standard error (n = 50-70).

single, double, triple, quadruple and quintuple mutants of *ACR4* and related kinases. Analysis of the leaf epidermal defect based on chlorophyll leaching suggested that *acr4* leaves possess a permeable cuticle and this is further discussed in Chapter-6.

3.2.3 Investigating physical interaction of ACR4 with related receptor like kinases

One possible explanation for the lack of redundancy between ACR4 and related kinases, is that in fact, proteins of the ACR4-related family could interact interchangeably with ACR4 in complexes that mediate ACR4 signalling. In support of this hypothesis, the first cysteine-rich repeat of the TNFR-like domain of ACR4, which is conserved throughout the ACR4-like family (see Fig 1.9) has been shown, in TNFR, to mediate ligand independent oligomerization (Chan *et al.,* 2000). To improve our understanding of the ACR4 mediated signal transduction pathway, I therefore planned to investigated whether ACR4 could interact *in vivo* either with itself, or with any of its four related RLKs i.e. - AtCRR1, AtCRR2, AtCRR3 and AtCRR4. To do this, various constructs designed to express tagged variants of ACR4 and its related kinases (e.g. – *pACR4-ACR4:GFP*, *35s-AtCRR1:HIS etc.*) were systematically generated. I proposed to cross expressing lines in order to generate double transgenic plants carrying two different tags (e.g. – *pACR4-ACR4:GFP* and *p35S-AtCRR4:MYC*) to determine possible physical interactions by co-immunoprecipitation.

3.2.3.1 Epitope tagged variants of ACR4 related kinases are undetectable in western blots

As no functional promoters had been identified for the *ACR4* related genes, a constitutive 35s CaMV promoter was used for the expression of tagged versions of these proteins. *Arabidopsis* plants carrying *35s-AtCRR1:6xHIS, 35s-AtCRR1:2xMYC, 35s-AtCRR2:2xMYC, 35s-AtCRR2:6xHis, 35s-AtCRR3:GFP, 35s-AtCRR3:2xMYC*

and *35s-AtCRR3:6xHis* were generated by previous lab members in the Lab. After I started my project, I grew plants to isolate homozygous transgenic lines. Unfortunately, very few primary transformants had been generated for each line. Homozygous transgenic lines expressing GFP tagged proteins were assessed by fluorescence of roots using confocal microscopy, but no obvious expression was detectable. For all transgenic lines, western blots were carried out to look at protein accumulation. Total protein was extracted from seedling tissues of wild type and transgenic plants and immunodetection was carried out using either anti-GFP polyclonal or anti-MYC or anti-HIS monoclonal antibody. However, no signal was detectable in the immunoblot, despite the fact that RT-PCR studies suggested that transgenes were being expressed (G. Ingram personal communication).

Because of my particular interest in the potential role of *AtCRR4* in senescence (Chapter 5), I generated constructs to express GFP tagged AtCRR4 and MYC tagged AtCRR4. To construct the *GFP* tagged line, a CaMV 35s promoter fused with AtCRR4 was placed upstream of the *GFP* ORF and transformed into both pBIB(Hyg) and pBIB(Kan) (Becker, 1990) binary vectors. To investigate the homodimerization of AtCRR4, a 2xMYC tagged AtCRR4 line was also constructed. The aim was to generate double transgenic lines in future, expressing both AtCRR4:GFP and AtCRR4:MYC. To generate a 'MYC' tagged AtCRR4 expressing line, *AtCRR4* fused with a *2xMYC* ORF was placed under the control of a CaMV 35s promoter and *Col-0* plants were transformed with this construct. T_2 generation plants carrying the transgene were selected using antibiotic resistance. In the T_3 generation, homozygous transgenic lines were selected. RT-PCR was performed using RNA extracted from 7-week old long day grown rosette leaves of *35s-AtCRR4:MYC* line and *Col-0* (Fig 3.7). In parallel, to select strongly expressed AtCRR4:GFP line, RT-PCR was carried out using 4-week old leaf tissues (G. Ingram personal communication). To do western blot, total protein extracted from 6 week old leaves of *Col-0* and *35s-AtCRR4:GFP* line and western blot analysis was performed using anti-GFP polyclonal antibody. However, no signal was detectable. Investigation of the expression of the *AtCRR4* gene (Section 5.2) suggests that *AtCRR4* is strongly expressed in senescing leaves. The material used in western blots was mature leaves,

but not senescing leaves. Thus further experiments using protein extracts from senescing leaf tissues are required to determine the behavior of AtCRR4 protein as well as to investigate if AtCRR4 forms homodimers. Unfortunately, the high levels of proteolytic enzymes produced in senescent tissues may make this experiment technically challenging.

3.2.3.2 Investigating homo-dimerization of ACR4 receptors

In both animal and plant systems, receptor kinases have been shown to homodimerize during signal transduction (Schlessinger, 2002; Wang *et al.*, 2005b). Recently, it is found that an *Arabidopsis* Lectin Receptor-like Kinase, LecRK-b2 functions as a homodimer and the extracellular lectin domain is important for the dimerization of LecRK-b2 proteins in plants (Deng *et al.*, 2009). Stokes *et al.* (2008) reported that the transmembrane domain of ACR4 can homodimerize *in vitro*. However it is still unclear whether the ACR4 mediated signal transduction pathway *in vivo* requires homo-dimerization of ACR4. To determine the physical interaction between ACR4 receptors *in vivo*, we planned to do co-immunoprecipitation.

3.2.3.2.1 Generating double transgenic lines expressing ACR4:GFP and ACR4:MYC

To investigate the homodimerization of ACR4 receptors *in vivo* by co-immunoprecipitation, a range of epitope tagged lines were constructed. I aimed to generate a line expressing an ACR4:MYC fusion under the *ACR4* promoter, where the transgene is associated with a hygromycin resistance gene and a line expressing an ACR4:GFP fusion under the *ACR4* promoter where the transgene is associated with a kanamycin resistance gene to facilitate selection of double transgenic lines expressing both the ACR4:GFP and ACR4:MYC tag.

To generate a 'MYC' tagged ACR4-expressing line, *ACR4* fused with a *2xMYC* ORF was placed under the control of the *ACR4* promoter and *Col-0* plants

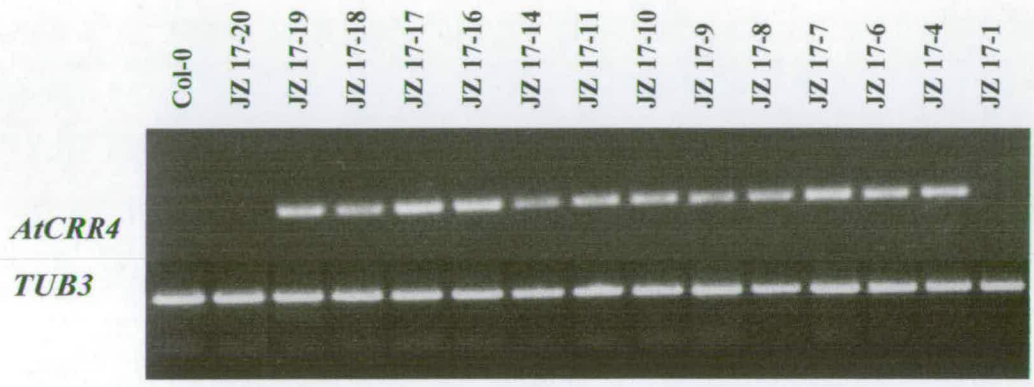


Fig 3.7: The expression of AtCRR4:MYC in different *35s-AtCRR4:MYC* transgenic lines (JZ 17).

Total RNA was extracted from ~7week old long day grown rosette leaves of *Col-* as well as independent *35s-AtCRR4:MYC* lines (JZ 17-20, JZ 17-19, JZ 17-18, JZ 17-17, JZ 17-16, JZ 17-14, JZ 17-11, JZ 17-10, JZ 17-9, JZ 17-8, JZ 17-7, JZ 17-6, JZ 17-4 and JZ 17-1) and cDNA synthesized. PCR reactions were carried out using *AtCRR4* specific 5′ primer CRK1-1900 and MYC specific 3′ primer MYC-R2.

TUBULIN3 was shown as an internal control.

were transformed with this construct. T_1 and T_2 generation plants carrying the transgene were selected using antibiotic resistance for hygromycin. In the T_3 generation, homozygous transgenic lines were assessed by immunoblotting to select the strongly expressed lines.

In order to generate double transgenic plants expressing both ACR4:GFP and ACR4:MYC, single transgenic plants carrying *pACR4-ACR4:MYC* were transformed with the *pACR4-ACR4:GFP* construct. After harvesting, the seeds were plated onto media containing both hygromycin and kanamycin and the resistant plants were transferred to soil. The expression of GFP in the primary transformed double transgenic plants was assessed by confocal microscopy and highly expressing lines were selected.

3.2.3.2.2 Co- immunoprecipitation to determine homo-dimerization of ACR4

Co-immunoprecipitation provides a reliable technique to establish the authentic protein-protein interactions *in vivo*. In order to investigate whether ACR4 exists as a homodimer within the cell, co-immunoprecipitation was performed using transgenic lines expressing both ACR4:MYC and ACR4:GFP.

To assess the accumulation of ACR4:MYC as well as ACR4:GFP in the double transgenic lines harbouring *pACR4-ACR4:MYC* and *pACR4-ACR4:GFP*, western blot analysis was performed using total protein extracted from floral meristem tissues (Fig 3.8) and a strongly expressing line was selected for co-immunoprecipitation assays. To do co-immunoprecipitation, total protein extracted from the floral meristem tissues of wild type *Col-0*, transgenic line expressing ACR4:GFP and double transgenic plants expressing both ACR4:GFP and ACR4:MYC was immunoprecipitated using anti-GFP microbeads (μMACS GFP tag protein isolation kit, Miltenyi Biotec) to capture ACR4:GFP and any differentially bound proteins. As shown in Fig 3.9, ACR4:GFP can be immunoprecipitated with the anti-GFP antibody. The immunoprecipitate was subjected to immunoblotting

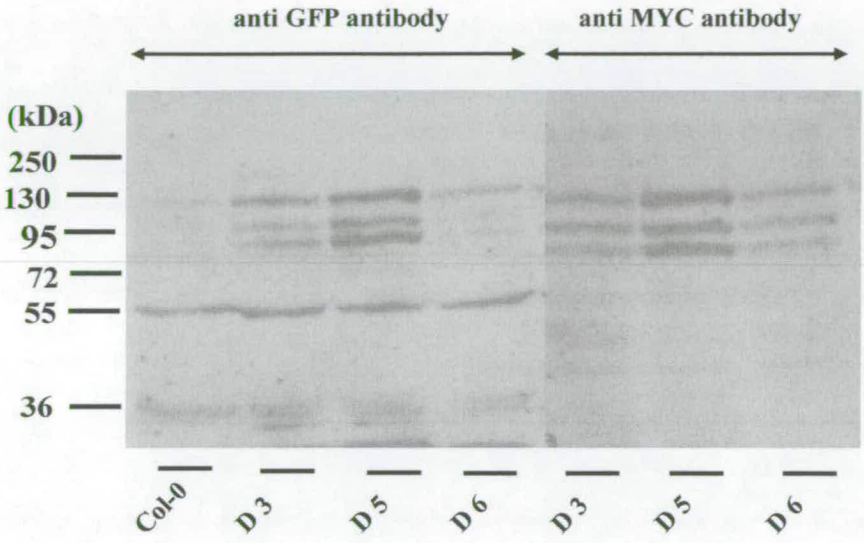


Fig 3.8: Western blot analysis of the accumulation of ACR4:GFP and ACR4:MYC in the double transgenic lines (D3, D5 & D6) carrying *pACR4-ACR4:GFP* and *pACR4-ACR4:MYC*.

Total protein from floral meristem tissues of wild type *Col-0* and double transgenic lines harbouring *pACR4-ACR4:GFP & pACR4-ACR4:MYC* (D3, D5 & D6) was subjected to SDS-PAGE, followed by western blotting using anti-GFP and anti-MYC antibody to detect ACR4:GFP and ACR4:MYC fusion protein respectively. The full length ACR4:GFP & ACR4:MYC are estimated to be about 125kDa and 98kDa respectively. Although the full length protein was detectable, several smaller bands suggested that ACR4 undergoes cleavage in the extracellular domain. Detailed about the cleavage sites are shown in Fig 4.9. As high level of accumulation was observed for D 5 line, this line was used for co-immunoprecipitation studies.

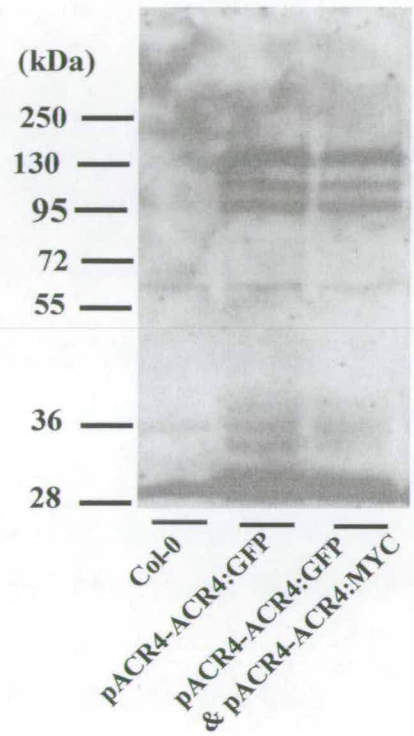


Fig 3.9: ACR4:GFP from the transgenic line *pACR4-ACR4:GFP* and the double transgenic line carrying *pACR4-ACR4:GFP* and *pACR4-ACR4:MYC* can be immunoprecipitated using anti-GFP microbeads.

Total protein extracted from inflorescence and floral meristem tissues of wild type *Col-0,* transgenic line *pACR4-ACR4:GFP* and double transgenic line harbouring *pACR4-ACR4:GFP* and *pACR4-ACR4:MYC* (D5) was immunoprecipitated using anti-GFP microbeads and a small amount of the immunoprecipitate was analyzed by SDS-PAGE, followed by western blot analysis using anti-GFP antibody.

with an anti-MYC antibody to try to detect ACR4:MYC in the immunoprecipitated samples, however no signal was detectable with the anti-MYC antibody. The inability to detect MYC-tag in the immunoprecipitate could be due to the experimental error since no signal was even detectable for the transgenic plants expressing ACR4:MYC. The immunoprecipitation was repeated, however as the plants were quite old, the signal for anti-GFP antibody was quite weak. This suggested that the immunoprecipitation experiment needs to be repeated using floral meristem tissues of young plants to investigate whether ACR4 exists as a homodimer.

3.3 Discussion

Here, potential genetic redundancy among the members of the *ACR4* gene family was investigated and we have shown that *ACR4* does not act redundantly with its related receptor like kinases (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*) with respect to shoot development. To elucidate ACR4 receptor mediated signal transduction pathways, dimerization of ACR4 receptors in ACR4 mediated signalling was investigated.

Genetic interactions among *ACR4* family members

The subtle and restricted phenotype of *acr4* mutants, compared to its more or less ubiquitous expression pattern, prompted us to investigate whether *ACR4* acts redundantly with related receptor like kinases. There is evidence for functional redundancy among the members of multigene families in both plants and animals (Guo *et al.*, 2009; Kristiansen *et al.*, 2005). Within the *ACR4* family, double, triple, quadruple and quintuple mutants have been generated. However, no enhancement of *acr4* single mutant phenotype was observed in any of these mutants. The lack of any severe phenotype in the quintuple mutant is striking. The quintuple mutant is viable and fertile and shows no further enhancement of the defects observed in *acr4* or novel phenotypes compared to either single or multiple mutants. This suggests that we did not miss any other interactions with respect to the shoot development

phenotypes analyzed here. However, *ACR4* is also involved in the formation of lateral roots. De Smet *et al.* (2008), in collaboration with the Ingram lab, reported that *acr4* exhibits a significant increase in the total number of lateral root meristems/cm and some double and triple mutant combinations of *ACR4* and its related kinases show a slight increase in the number of lateral root meristem. This suggests that there could be some redundancy among the members of *ACR4* family with respect to the root development. As the root development aspect of *ACR4* function is being analysed collaboratively in other laboratories, no further experiment using quadruple and quintuple mutants to study root development was carried out in the Ingram lab.

Functional redundancy has been demonstrated for several RLK genes in *Arabidopsis*. This redundancy is sometimes observed among related kinases. *ERECTA*, a leucine-rich repeat RLK in *Arabidopsis* acts redundantly with its homologs *ERECTA-LIKE1 (ERL1)* and *ERL2* in regulating organ growth (Shpak *et al.,* 2003; Shpak *et al.,* 2004). Loss-of-function of all these three genes leads to a dramatic reduction in plant and organ size, and defects in the differentiation of floral organs. However, the *ERECTA* family of receptor-like kinases plays overlapping but distinct roles to control stomatal patterning (Shpak *et al.,* 2005). The single mutants *erl1* and *erl2* show slight defects in stomata development, acting partially redundantly with *erecta* in this process. Thus redundancy among homologs can differ in different tissues. In some cases, receptor like kinases do not function redundantly with related genes. *SCRAMBLED (SCM)*, a receptor-like kinase which is required for root epidermal patterning, does not act redundantly with its two most closely related genes *STRUBBELIG RECEPTOR FAMILY 1* (*SRF1*) and *SRF3* (Kwak and Schiefelbein, 2007). SRF1 and SRF3 possess a similar structural organization and approximately 40% overall amino acid identity to SCM (Chevalier *et al.*, 2005). In the case of *ACR4*, our result suggested that *ACR4* do not act redundantly with its related kinase with respect to shoot development, although De Smet *et al.* (2008) reported that *ACR4* related kinases act redundantly with *ACR4* in the formation of lateral roots. This suggested that the redundant role of *ACR4* and *ACR4* related kinases could differ in different tissues. Alternatively, ACR4 could act in a complex

with related kinases, but redundantly with unrelated proteins. Enhancer screening could help identify such factors.

Physical interactions among ACR4 family members

How does ACR4 transduce signals inside the cell? Does it form a homodimeric complex or does it heterodimerize with any of its related kinases? As mentioned earlier, analysis of the structural features of ACR4 suggested that it has three repeats which are very similar to the extracellular domain of TNFR. In animal system, TNFRs are known to form trimers and transduce signal inside the cell (Banner *et al.,* 1993). This suggests that TNFR like domains of ACR4 and its related kinases could play a similar role. Our understanding of the *in vivo* protein-protein interactions among the members of ACR4 family is still unclear. It is possible that ACR4 mediated signalling involves formation of homodimers or heterodimers with its related kinases. As mentioned earlier, ACR4 is a kinase active, plasma membrane bound protein (Cao *et al.,* 2005) and therefore its kinase domain can phosphorylate other proteins. Among the four ACR4 related kinases included in our studies, AtCRR1 and AtCRR2 contain a deletion in their kinase domain and these proteins are kinase inactive while AtCRR3 and AtCRR4 are kinase active (Cao *et al.,* 2005). However, examples are available indicating that the kinase activities are not required for receptor kinases to function. One example in *Arabidopsis* is CLAVATA2 (CLV2) which lacks a cytoplasmic kinase domain. CLV2 interacts with CLV1 forming a complex that recognizes the signalling peptide CLAVATA3 (Clark, 2001; Ogawa *et al.,* 2008). It is possible that both AtCRR1 and AtCRR2 may function as receptors in the absence of kinase activity and transmit downstream signals possibly by hetero-dimerization with kinase active partners. *In vitro* studies revealed that ACR4 can phosphorylate the kinase domain of AtCRR2 (Cao *et al.,* 2005).

To investigate physical interaction among the members of *ACR4* gene family, epitope tagged variants of ACR4 and its related kinases were constructed. Unfortunately as ACR4 related kinases were not detectable in the western blot, we were unable to determine the physical association of ACR4 with related kinases. Only the transgenic lines expressing ACR4:GFP and ACR4:MYC were found to be

detectable. Therefore, double transgenic lines expressing both ACR4:GFP and ACR4:MYC were generated to investigate homo-dimerization of ACR4 by Co-immunoprecipitation. To this end, double transgenic plants expressing both ACR4:GFP and ACR4:MYC were generated. Optimization of co-immunoprecipitation experiment is required to determine if ACR4 exists as a homodimer inside the cell.

Our inability to detect fusion proteins of ACR4-related proteins expressed under the 35s promoter, despite high levels of expression of transcripts, is rather intriguing. Gifford *et al.* (2005) showed that ACR4 undergoes rapid turnover, and this is consistent with the fact that very few proteomics studies have pinpointed peptides form ACR4. The fact that none of the ACR4-related proteins has been detected by proteomics, despite the fact that some family members are expressed more strongly than *ACR4* at the transcript level, could therefore indicate that, like ACR4, these proteins are also rapidly turned over within the cell. Why a class of RLKs should show such apparent behaviour is unclear.

Chapter -4

Analyzing interactions between ACR4 and its putative binding partner, AIP1

4.1 Introduction

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4.3 Discussion

Chapter – 4

Analyzing interactions between ACR4 and its putative binding partner, AIP1

4.1 Introduction

As mentioned earlier, ACR4 (Arabidopsis CRINKLY4) is a membrane bound receptor which plays diverse roles in plant development, including maintenance of root stem cell populations (Stahl *et al.,* 2009), formation of lateral roots (De Smet *et al.*, 2008), and development of the ovule integuments (Gifford *et al.,* 2003). Part of my planned project involved investigating how ACR4 mediated signalling is transduced, and identifying potential regulatory components interacting with ACR4. In order to identify the potential downstream targets of ACR4, a yeast-two-hybrid (Y2H) screen was carried out by Samuel Dean, a project student in the Ingram lab. To carry out Y2H screening, the cytoplasmic C-terminal tail of ACR4, downstream of the kinase domain, was used as bait to screen a prey library from *Arabidopsis* seedlings. The rationale behind this strategy was that, since the C-terminal domain of ACR4 is not present in other ACR4-like proteins, it might interact with a unique subset of regulators specific to ACR4 signalling. In several repetitions, it was found that the C-terminal domain of ACR4 interacts with a protein of unknown function,

encoded by the gene *At3g20370*. This interaction was re-tested and confirmed in constructs where the entire cytoplasmic region of the ACR4 was used as a bait, although the interaction was found to be considerably weaker with this construct (Gwyneth Ingram personal communication). Attempts to test the interaction *in vitro* using protein expressed in bacteria failed due to the extreme toxicity of the ACR4 C-terminal domain in several bacterial expression systems tested (Yvonne Stewart and Gwyneth Ingram, unpublished results). Since *At3g20370* had not been named, it was decided to name it *ACR4 INTERACTING PARTNER 1* (*AIP1*).

4.1.1 AIP1 is a MATH domain containing protein of unknown function

Literature and database searches revealed that AIP1 is a member of the MATH family, consisting of two MATH domains in its C-terminus (Oelmüller *et al.*, 2005). The MATH domain is an independent folding motif and is defined by the observed homology between the COOH-terminal region of meprins and the TRAF-C domains of TRAF (Tumor necrosis factor Receptor-Associated Factor) proteins (Uren and Vaux, 1996, Sunnerhagen *et al.*, 2002). However, meprins and TRAFs are protein families that appear to be absent in *Arabidopsis* (Zapata *et al.*, 2001). Meprins are tissue-specific metalloendopeptidases, which cleave a variety of bioactive peptides including cytokines, growth factors, bioactive peptides, hormones, and extracellular matrix proteins and thus they are involved in developmental and pathological processes (Bond and Beynon, 1995). TRAFs constitute a family of adapter proteins and were first identified as signal transducers for TNFRs (Tumor Necrosis Factor Receptors). TRAF family proteins regulate several functions of the TNFR superfamily, apparently by linking the cytosolic domain of TNFR receptors to downstream signalling cascades. They participate in a broad variety of cellular processes, such as cell growth signalling and apoptosis (Arch *et al.*, 1998).

Although the specific function of the MATH domain is still unknown, Sunnerhagen *et al.* (2002) have suggested that MATH domains are involved in protein–protein interactions, both between proteins containing MATH domains and between MATH domains and other protein motifs. In plants, MATH domains are

found in combination with a variety of protein-protein interaction motifs e.g. - BTB/POZ (Broad complex, Tramtrack, Bric-a-brac/POx virus and Zinc finger) motif, indicating their potential involvement in diverse biological processes. Furukawa *et al.* (2003) and Xu *et al.* (2003) reported the interaction of CULLIN3 (CUL3) with BTB/POZ proteins containing MATH domains. CUL3, a component of the E3-ubiquitin ligase complex, is implicated in the destruction of a large number of regulatory proteins (Xu *et al.,* 2003). Thus MATH could be involved in linking specific protein substrates to ubiquitin ligase complexes.

Intensive literature searches showed that AIP1 had, in fact, previously been named as "MATH protein" by Peskan-Berghofer *et al.* (2004). Since MATH is actually the name of the domain present in AIP1, we will continue to use the name AIP1. Peskan-Berghofer *et al.* (2004) and Oelmüller *et al.* (2005) reported that AIP1 is one of the proteins whose accumulation is rapidly upregulated upon interaction of *A. thaliana* roots with *Piriformospora indica*, an endophytic fungus of the *Sebacinaceae* family. The detailed mechanism of the role of this protein in plant–fungal symbiotic association, if any, remains unclear. As interaction of the Arabidopsis root with this fungus causes rapid accumulation of AIP1, it was speculated that AIP1 protein could have a role in early recognition of the fungus (Oelmüller *et al.,* 2005). As *P. indica* is a rare fungus with great economic value and groups working with *P. indica* were not willing to collaborate, we have been unable to test whether AIP1 and ACR4 are implicated in the recognition of the *P. indica.*

4.1.2 MATH domain containing proteins are localized in the diverse cellular compartments in *Arabidopsis*

The *Arabidopsis* genome sequence revealed the presence of fifty-nine MATH domain containing proteins (Fig 4.1). Some of these proteins are predicted to contain only one MATH domain whereas others contain as many as four MATH domains (Oelmüller *et al.,* 2005). Very little is known about the function of these proteins although ubiquitin specific protease 12 was found to contain a MATH domain (Yan

et al., 2000), again suggesting a potential link to protein turnover. Members of the MATH domain containing protein families have been predicted to be localized in several different cellular compartments including the plasma membrane and/or extracellular space, endoplasmic reticulum, golgi apparatus, cytosol, nucleus and organellar membranes (Oelmüller *et al.*, 2005). Again this suggests that MATH proteins may be involved in diverse cellular processes, although it is possible that predictions suffer from a degree of inaccuracy.

4.1.3 AIP1 as a potential interacting partner of ACR4

As mentioned earlier, AIP1 was found as a potential interacting partner of ACR4 in yeast two hybrid assays. AIP1 was considered as a potentially interesting candidate because it has MATH motifs which contain TRAF homology domains (Sunnerhagen *et al.*, 2002). This is particularly intriguing since the extracellular domain of ACR4 has putative TNFR-like repeats, resembling domains in the extracellular portion of TNFRs (Tanaka *et al.*, 2002). TRAFs associate with the intracellular domain of TNFRs and it was therefore attractive to hypothesise that the intracellular domain of ACR4 could interact with the MATH domains of AIP1, even though the intracellular domains of ACR4 and TNFRs have no obvious homology. Another reason for choosing AIP1 to study further was the evidence that in *Arabidopsis*, BTB/POZ-MATH domain containing proteins can interact with the Cullin proteins, AtCUL3a and AtCUL3b and can assemble to form functional E3 ligases (Weber *et al.*, 2005). Gifford *et al.* (2005) reported that functionally active ACR4 undergoes rapid internalization and cleavage. Since AIP1 contains MATH domains, it is possible that the interaction of ACR4 with AIP1 is important for turnover of ACR4.

Since testing the interaction of ACR4 and AIP1 *in vitro* has proved challenging, three different approaches were taken to test the interaction *in planta*. The first approach was to identify if there is any genetic link between *ACR4* and *AIP1*. In order to investigate a potential genetic link, double mutants between *acr4* and *aip1* were generated and any genetic interaction was tested by comparing the phenotypes of single and double mutants.

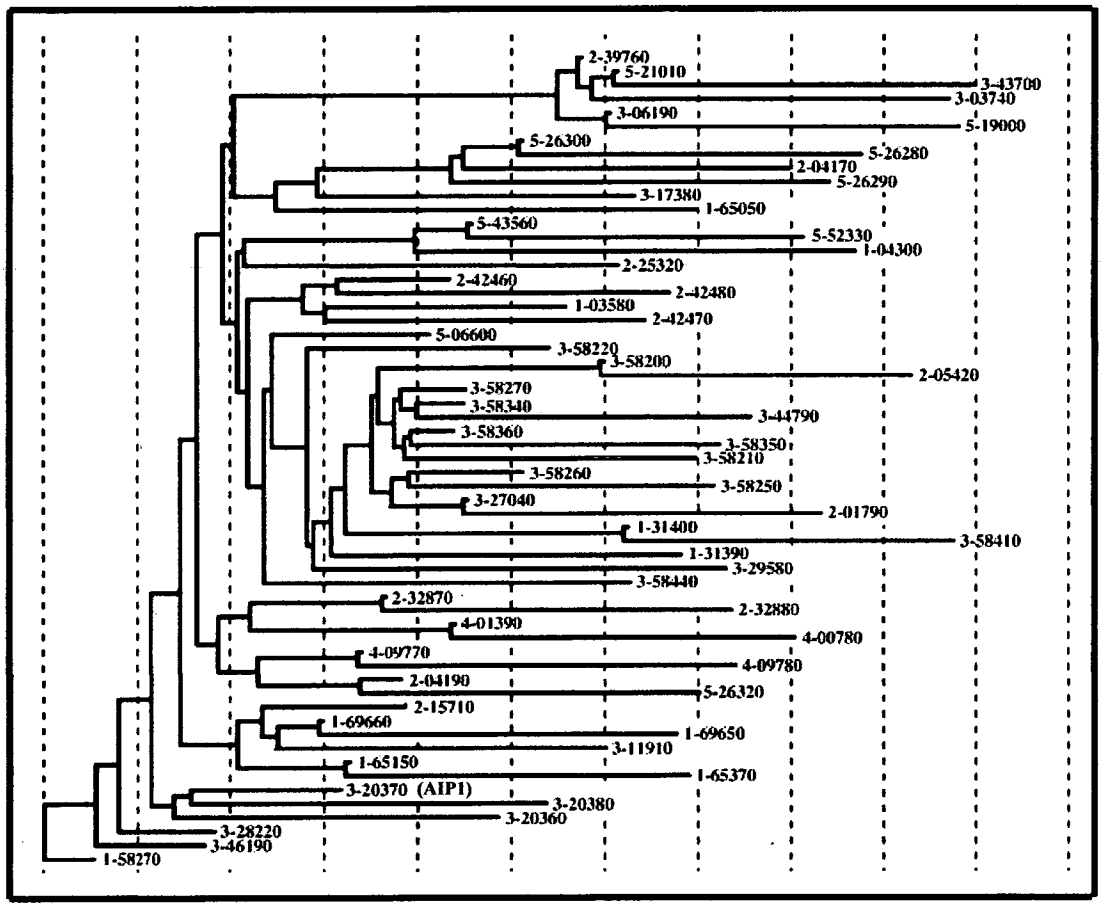

Fig 4.1: Phylogenetic tree analyses of the MATH domain containing proteins from *Arabidopsis*. AIP1 (At3g20370) shows high degree of similarity to At3g20360 and At3g20380 (Oelmüller *et al.*, 2005).

The second approach was to investigate how the ACR4 protein behaves in an *aip1* mutant background as well as how AIP1 behaves in an *acr4* background. This experiment was designed to check whether the processing of ACR4 is dependent on its interaction with AIP1 and similarly if the accumulation of AIP1 is regulated by the presence of ACR4.

Finally, to test the *in vivo* interaction between ACR4 and AIP1 in plants, I aimed to use a co-immunoprecipitation approach. In addition, blue native polyacrylamide gel electrophoresis (BN-PAGE) (Schagger and von Jagow, 1991) was carried out in order to analyze whether either protein might be present in a complex under native physiological condition. Through BN-PAGE, I aimed to test whether any protein complexes identified for either AIP1 or ACR4 changed in size in the absence of the other protein, implying the presence of the two proteins in the same complex.

4.2 Results

4.2.1 *AIP1* encodes a protein with two MATH domains

Sequence analysis has revealed that *AIP1* encodes a protein of 379 amino acids, consisting of an N-terminal signal peptide and two MATH domains in tandem in the C-terminus (Fig 4.2). To identify *genes* related to the Arabidopsis *AIP1*, a multiple sequence analysis performed by Gillespie *et al.* (2005), reported that the predicted amino acid sequence of AIP1 (At3g20370) is similar to a family of proteins of unknown function, present in both *Brassica napus* and *A. thaliana,* which can be divided into three groups based on sequence homology (Fig 4.3). Group 1 (Fig 4.3, top four sequences) includes the sequence from *B. napus* as well as three from *A. thaliana* (including AIP1), and these four genes are approximately 60% identical (Gillespie *et al.*, 2005). The next five sequences belong to group 2. These two groups have common residues throughout their sequences, not only within the conserved MATH domains. The last sequence shown in the alignment (Fig 4.3) is the sole

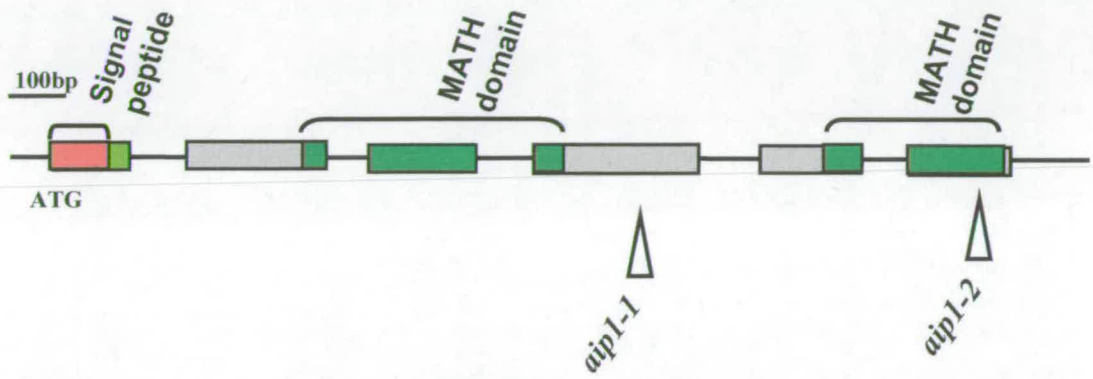

Fig 4.2: Schematic representation of *AIP1*.

Exons are represented boxes. The site of T-DNA insertion is indicated by open triangle. Other features indicated: red box, location of predicted signal peptide; green box, MATH domains.
N-terminal signal peptide and MATH domains were predicted using SignalP (Emanuelsson *et al.*, 2007) and SMART tool (Schultz *et al.*, 1998) respectively.

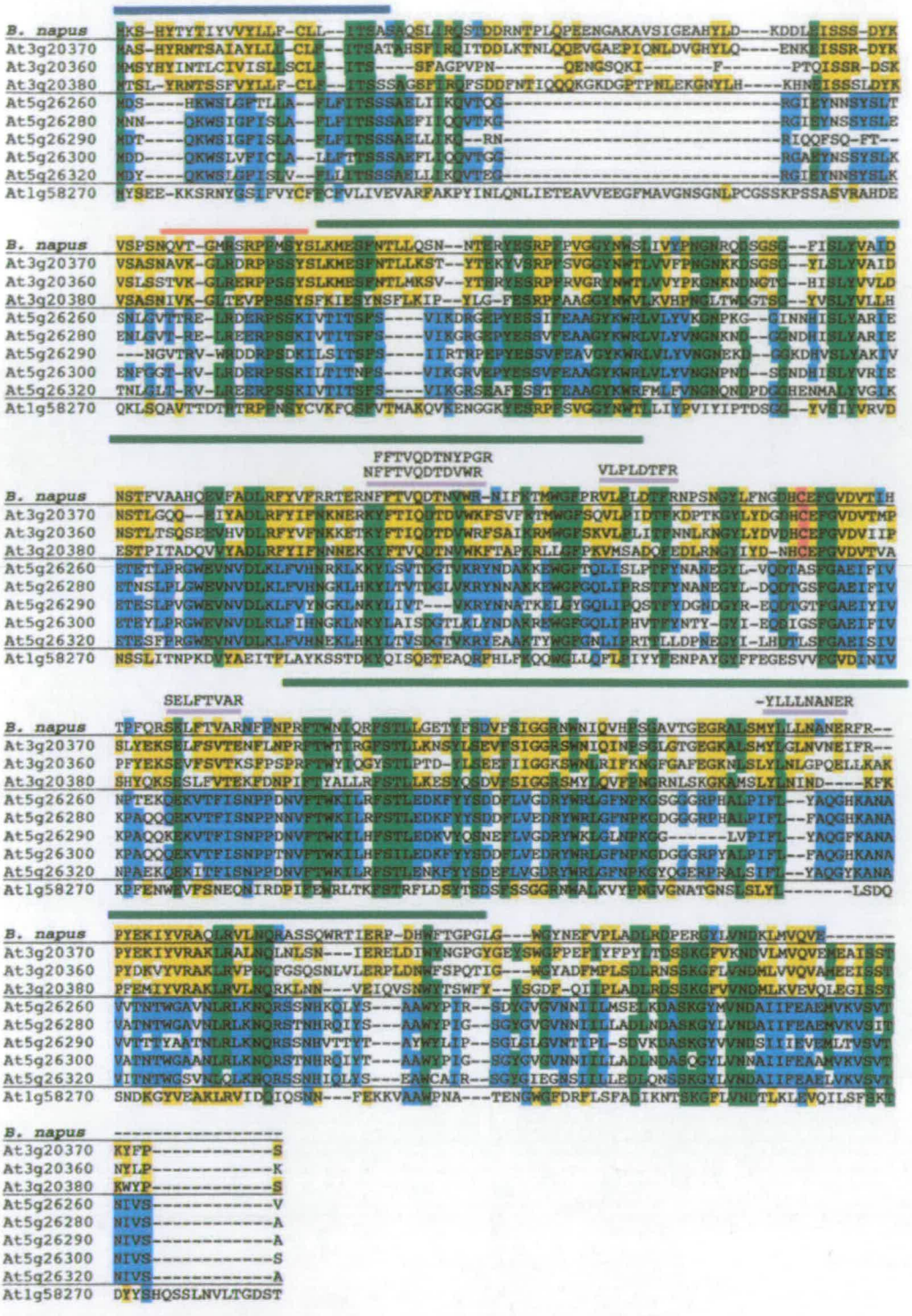

Fig 4.3: The predicted protein sequence of AIP1 contains domains which are conserved in *Brassica napus* as well as in *Arabidopsis thaliana.*

The location of predicted signal peptides is indicated by blue bar, N-terminal sequence determined by Edman degradation of *B. napus* protein in red bar and two MATH domains in green bars. All proteins predicted from *Brassica* and *Arabidopsis* genome contain two MATH domains and a predicted N-terminal signal peptide. Sequences were divided into three groups as indicated by horizontal lines between sets. The single sequence in group 3 is the least conserved; residues common to either of the other groups are color coded yellow (group 1), blue (group 2), or green (both groups) (Gillespie *et al.*, 2005).

representative of group 3, and it is also the least conserved family member. Through N-terminal sequencing, it was found that the N-terminal amino acid sequence corresponds to amino acids 78–92 in the original *B. napus* predicted protein sequence suggesting that the N-terminus could undergo proteolytic processing (Gillespie *et al.*, 2005). Since this protein shows high degree of similarity to AIP1, it is possible that the N-terminus of AIP1 also undergoes proteolytic cleavage, which is further discussed in Section 4.2.5.1.

The phylogenetic tree analysis of *Arabidopsis* sequences (Fig 4.1) as well as the multiple sequence alignment by Gillespie *et al.* (2005), (Fig 4.3) indicated that *AIP1 (At3g20370)* is very closely related to two other adjacent genes in Arabidopsis, which are *At3g20360* and *At3g20380*. AIP1 has 58% identity to At3g20360 and 56% with respect to At3g20380. Therefore, it is possible that *AIP1* can act redundantly with *At3g20360* and *At3g20380*. Since no published data is available on the expression or the role of *AIP1* or its close homologues, we looked at the publicly available transcriptome and proteome data. No transcriptome data is available regarding the expression pattern of *At3g20360* and *At3g20380*, suggesting that they are expressed at very low levels, in very few cells, or during a very restricted temporal window (Zimmermann *et al.*, 2004; Winter *et al.*, 2007). Proteome data from *Arabidopsis* indicated that both AIP1 and the protein encoded by *At3g20380* are accumulated in the root (Fig 4.4) (Baerenfaller *et al.*, 2008). It should be noted that AIP1 accumulates to levels about 10 times higher than the protein encoded by *At3g20380*. In addition, AIP1 is detected in the cotyledons whereas the protein encoded by *At3g20380* does not show such accumulation, suggesting that *AIP1* might not act completely redundantly with *At3g20380*. We also cannot, however, exclude the possibility that *AIP1* acts redundantly with *At3g20380* with respect to root development. No proteomic data is available for *At3g20360*, suggesting that *At3g20360* is expressed at very low level or in response to certain stimuli which have not yet been identified. Due to time constrains, I concentrated on characterization of the function of *AIP1*.

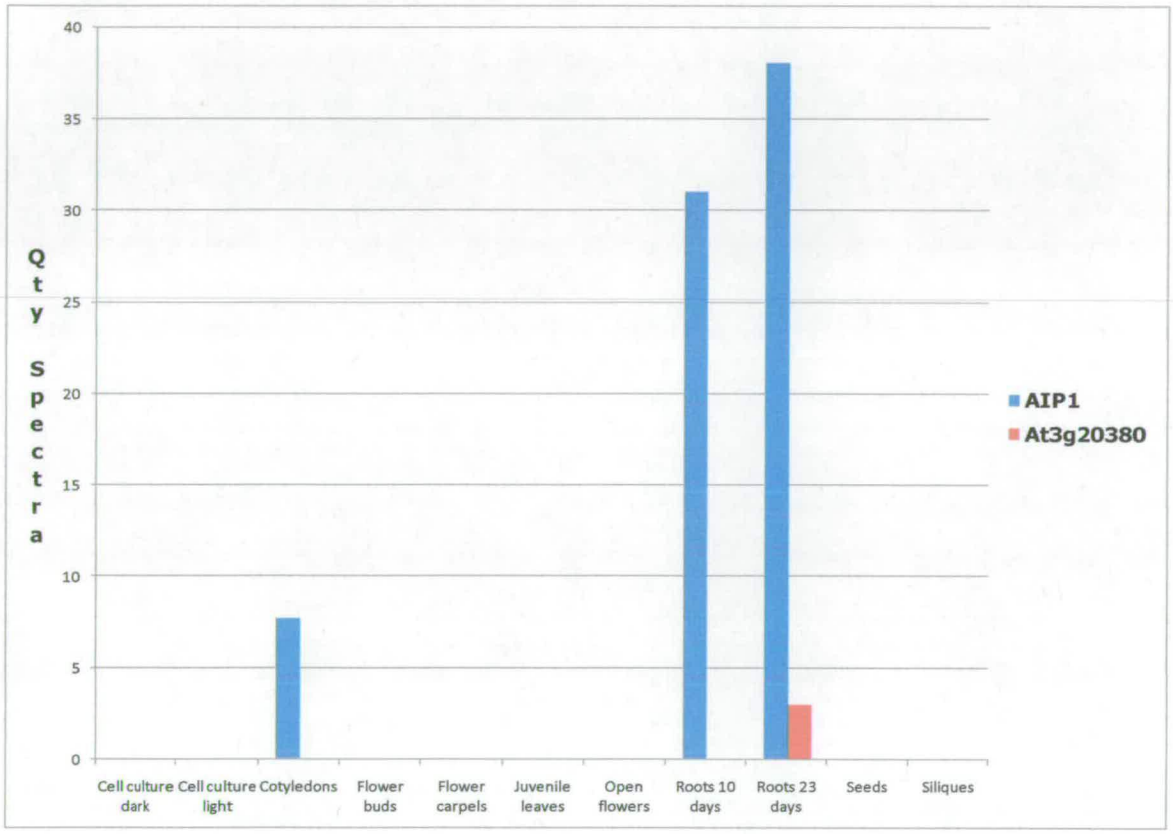

Fig 4.4: Spectrum summary from AtProteome (Baerenfaller *et al.*, 2008) showing accumulation of peptides from AIP1 and At2g20380.

AIP1 is accumulated in cotyledons as well as in roots whereas At3g20380 is found to be present at low level in roots. At3g20360 were not identified in this study.

4.2.2 Identification of an *aip1* knockout mutant

To determine the effect of the loss of *AIP1* function, two publicly available *aip1* alleles were investigated. The mutant alleles used are presented in Table 2.1. The *aip1-1* line was obtained from the FLAG T-DNA insertion collection (Samson *et al.*, 2002). The allele *aip1-1* (FLAG_465C02) was predicted to contain a T-DNA insertion in the 4th exon (Fig 4.2). Another allele, *aip1-2* (GK-157D06), was obtained from the GABI-KAT T-DNA insertion collection (Rosso *et al.*, 2003). The allele *aip1-2* was predicted to contain a T-DNA insertion in the 6th exon (Fig 4.2). Seeds from both of these lines described above were grown and DNA was extracted for PCR based genotyping to identify homozygous, heterozygous and wild type plants. Table 2.2 and Table 2.3 show details of the primer combinations and primer sequences used respectively. No T-DNA insertion was detected in plants grown from seed provided for the *aip1-1* allele (Nicholas C.R. Meuli pers. comm.). As a result, the *aip1-1* allele was abandoned and not used for further analysis. For *aip1-2*, several homozygous mutants were identified by PCR based genotyping. In order to test the expression of *AIP1* in the homozygous *aip1-2* line, RT-PCR analysis was carried out. RNA was extracted from homozygous *aip1-2* seedlings. To do RT-PCR, both tubulin primers and gene specific primers were designed to amplify intron containing regions in order to allow differentiation of products from genomic DNA and cDNA. Fig 4.5 shows the RT-PCR results. In no cases were bands detected from genomic DNA contamination. The *aip1-2* line showed no expression of *AIP1*, therefore *aip1-2* was considered as a genuine knockout line.

4.2.3 A homozygous null mutant for *AIP1* does not show acr4 like phenotype

As AIP1 was identified as a binding partner of ACR4, detailed analysis was carried out to determine if AIP1 functions in the same pathway as ACR4. As the first approach, single mutant of *aip1* was examined. To determine whether the mutant had any acr4-like seed phenotype (Gifford *et al.*, 2003) siliques were dissected and developing seeds were examined. It was found that the developing seeds of the homozygous *aip1-2* were elliptical like wild type, not round like *acr4* seeds.

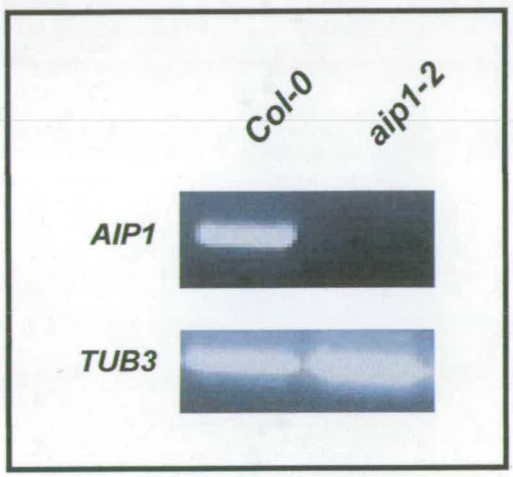

Fig 4.5 : RT-PCR analysis of the expression of *AIP1* gene in homozygous *aip1-2* line. The control bands (*TUBULIN3*) are shown in the bottom panel.

As mentioned earlier, *ACR4* is also known to be involved in the formation of lateral roots and *acr4-2* mutants exhibit an increased number of lateral roots (De Smet *et al.*, 2008). The *aip1-2* mutants were examined to test whether they show any acr4 like phenotype in the root and no enhancement in the number of lateral roots was seen in the *aip1-2* mutant (Fig 4.6).

To further investigate any possible genetic link between *ACR4* and *AIP1*, crosses of *aip1-2* mutants to the null *acr4-2* allele were made to generate double mutants. In the T_3 generation, homozygous double mutants were identified through PCR based genotyping. The double mutant plants were analyzed to determine whether any enhancement or alternation of the acr4 phenotype was observed. The double mutant plants did not show any gross morphological defect and were indistinguishable from wild type. Developing seeds of *acr4-2 aip1-2* double mutant plants were like those of *acr4-2* single mutants (data not shown). In addition, the number of lateral roots was compared with each of the single mutants. All *acr4-2 aip1-2* double mutant plants exhibited the same phenotype to those of the *acr4* single mutants (Fig 4.6).

4.2.4 Expression pattern of *AIP1*

A detailed analysis of the *AIP1* expression pattern was carried out to compare the expression pattern of *AIP1* with that of *ACR4.* Since our hypothesis is that AIP1 is a potential downstream target or regulator of ACR4, we were interested in ascertaining whether *ACR4* and *AIP1* show overlapping patterns of gene expression. In order to do this, *AIP1* marker constructs were assembled. The first was composed of the *AIP1* promoter driving the expression of a full length *AIP1* cDNA fused at its C-terminal to *GFP*. The second was composed of the *AIP1* promoter directly driving the expression of a nuclear localized marker Histone2B:Yellow Fluorescent Protein (H2B:YFP) (Boisnard-Lorig *et al.,* 2001). Lines containing a construct composed of the *ACR4* promoter driving the expression of H2B:YFP were already available in the lab (Gifford *et al.*, 2003). I also constructed new lines in which the *ACR4* promoter drives expression of an ACR4:GFP fusion protein.

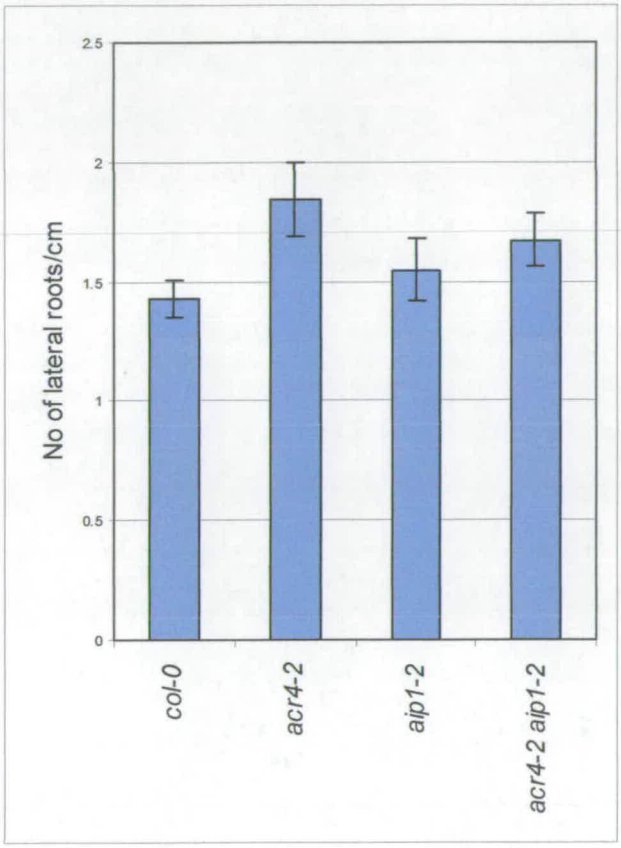

Fig 4.6: Lateral root phenotypes of *Col-0, acr4-2, aip1-2* and *acr4-2 aip1-2.*

Seedlings were grown on half strength Murashige and Skoog (MS) agar medium at 45° oriented square plates under continuous light for 9 days and the no of lateral roots per cm were counted. Error bars represent mean ± standard error (n = 15-20).

4.2.4.1 Generating reporter lines

In the lab a binary vector containing the H2B:YFP (MD4) fusion in a pBIB (hyg) vector had already been constructed (Gifford *et al.*, 2003). The full length *AIP1* promoter (1kb) was placed upstream of the H2B:YFP fusion protein to give JZ4. H2B:YFP is a stable nuclear localized marker and should allow clear visualization of the *AIP* promoter expression pattern (Boisnard-Lorig *et al.*, 2001).

To generate a construct for the expression of AIP1:GFP fusion protein, a full length cDNA was fused with the *GFP* ORF and placed downstream of the *AIP1* promoter in pBIB(hyg) to give JZ5. In a second construct the *AIP1:GFP* fusion was placed downstream of the cauliflower mosaic virus (CaMV) 35s promoter to give JZ3. This *35s-AIP1:GFP* line was made as a back-up in case the promoter sequence of *AIP1* was not able to drive expression. To compare the expression of *AIP1* with *ACR4*, *ACR4* fused with *GFP* was expressed under its own promoter and named as JZ51. Details of the generation of constructs are provided in Section 2.3.

After the plants had been transformed with the constructs, T_1 and T_2 plants carrying the transgene were selected using antibiotic resistance. In the T_3 generation, homozygous transgenic lines were assessed by relative fluorescence of roots using confocal microscopy and strongly expressing lines were selected. In parallel, western blot analysis was carried out using stably transformed lines to check the accumulation of protein in different lines. This western blot results supported the observations from confocal microscopy.

4.2.4.2 *ACR4* and *AIP1* show similar but not identical patterns of gene expression in roots

As microarray data indicates that both *ACR4* and *AIP1* are highly expressed in the root (Zimmermann *et al.*, 2004) and ACR4 was also found to interact with AIP1 in Y2H screening of an *Arabidopsis* seedling library, young root samples were used for comparison of the expression pattern of *AIP1* and *ACR4*. Plants expressing the H2B:YFP marker under the control of a gene specific promoter were used to compare the expression patterns. In the root meristem, *ACR4* is known to be

expressed in the innermost layers of the columella, the lateral root cap, the root epidermis and the quiescent centre (Gifford *et al.*, 2003). In contrast, H2B:YFP expression driven by the *AIP1* promoter indicates that the expression of *AIP1* is restricted to the outer layers of the columella as well as the lateral root cap. No expression is detectable in the quiescent centre (Fig 4.7). There is also strong expression in the epidermis. Therefore comparison of the expression pattern of *ACR4* and *AIP1* clearly indicates that although *ACR4* and *AIP1* do not have identical patterns of expression, their expression is overlapping. In particular, both *ACR4* and *AIP1* are expressed in the epidermis and lateral root cap, as well as in the middle layer of columella cells.

4.2.5 Behaviour of AIP1 and ACR4 proteins

In order to study ACR4 and AIP1 protein in more detail, two different approaches were taken. One was immunoblot analysis, and the other was localization studies to determine if both ACR4 and AIP1 were localized to the same intracellular compartment.

4.2.5.1 Immunoblot analyses of the AIP1 and ACR4 proteins

To determine the behaviour of AIP1 and ACR4 at the protein level, immunoblot analysis was carried out. To determine the behaviour of AIP1 protein, total protein was extracted from 3 day old seedlings expressing AIP1:GFP. Full length AIP1:GFP was expected to give a band of approx. 66 kDa (Fig 4.8). Predicted molecular weights were calculated using EditSeq (Lasergene). The full length 66-kDa band was detecteable only when AIP1:GFP is expressed under the 35s promoter. The 35s promoter is predicted to cause ectopic expression of the *AIP1* gene and this may explain why the full length AIP1:GFP was detectable. In both *pAIP1-AIP1:GFP* and *35s-AIP1:GFP* lines, the most intense band was observed at approx. 60 kDa. This suggests that the N-terminus of AIP1 may undergo proteolytic processing. A weak band of ~ 40kDa was also observed when AIP1 was expressed under 35s promoter possibly indicating the presence of another cleavage site between the two MATH domains.

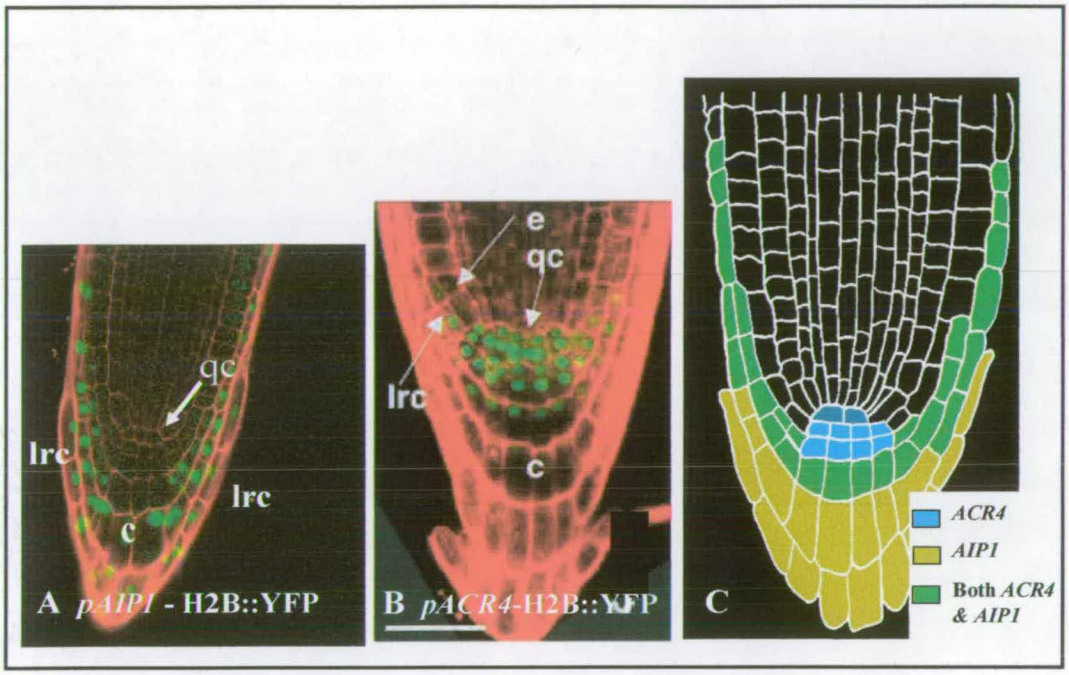

Fig 4.7: Expression of *AIP1* and *ACR4* overlaps.

Expression pattern of *AIP1* (A) and *ACR4* (B) in root meristem and epidermis. Confocal image (A) H2B::YFP expression (green) driven by the *AIP1* promoter indicates that the expression of *AIP1* in the outer layers of the columella (c) as well as in lateral root cap (lrc). No expression is detectable in the quiescent centre. There is also strong expression in the epidermis (e). The *ACR4* promoter fused to H2B::YFP (B) shows expression in the innermost layers of the columella (c), the lateral root cap (lrc), the root epidermis (not shown) and the quiescent centre (qc). Relative expression pattern of *ACR4* & *AIP1* (C).

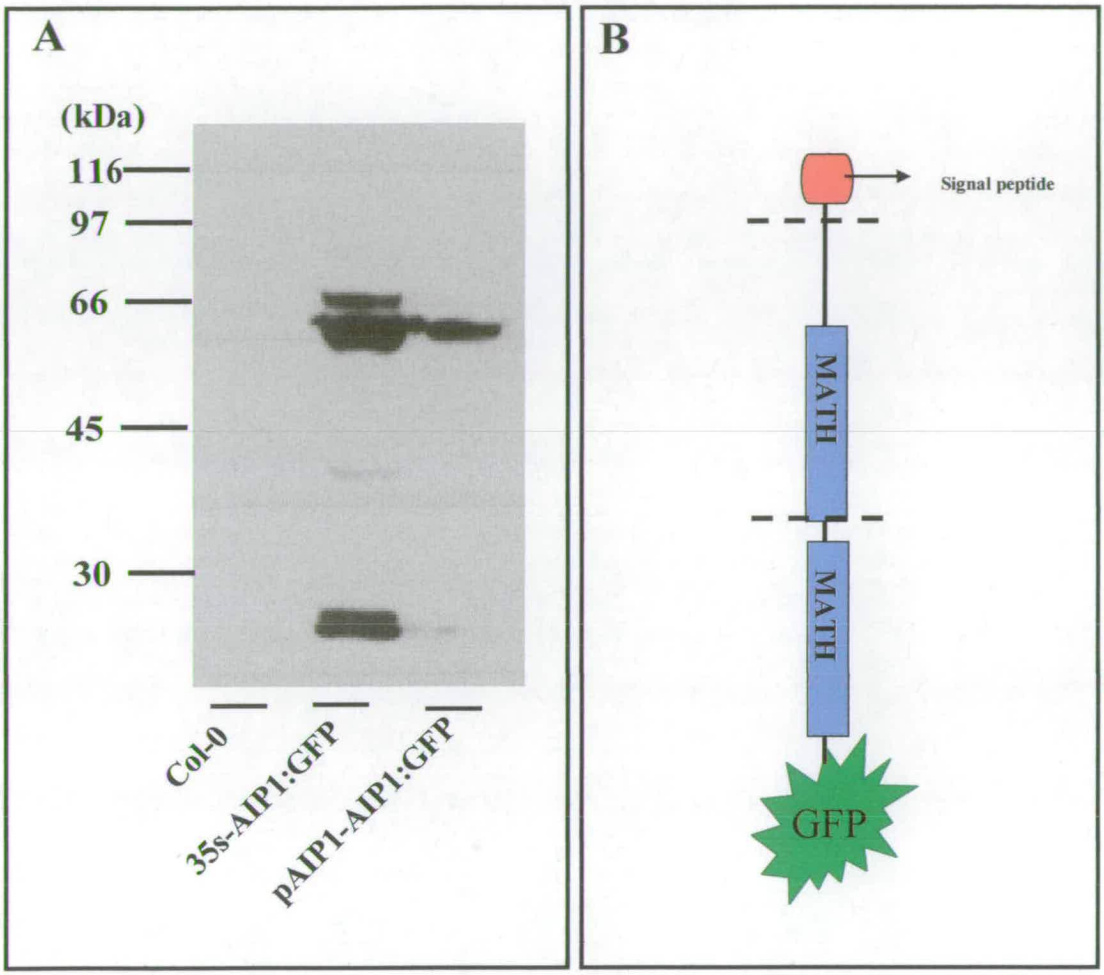

Fig 4.8: AIP1 protein undergoes cleavage.

Total protein was extracted from 3-days old equal number of seedlings of *Col-0, 35s-AIP:GFP* & *pAIP-AIP:GFP* lines and western blot was performed using anti-GFP antibody (A). The full length AIP1:GFP is predicted to be 66 kDa. The full length 66-kDa band was detecteable only when AIP1:GFP is expressed under the 35s promoter. The 35s promoter may cause ectopic expression of the *AIP1* gene. In both *pAIP1-AIP1:GFP* and *35s-AIP1:GFP* lines, the most intense band was observed at approx. 60 kDa. This suggests that the N-terminus of AIP1 undergoes proteolytic processing. A weak band of ~ 40kDa was also observed when AIP1 was expressed under 35s promoter possibly indicating the presence of another cleavage site between two MATH domains (B). A smaller band of 28 kDa was observed which correspond to free GFP.

In order to address the possibility that ACR4 could be subjected to processing within the cell, protein extracted from the inflorescences of *Col-0* and *pACR4-ACR4:GFP* was subjected to western blotting using anti-GFP antibody. The full length ACR4:GFP was expected to give a band of 125 kDa. Although the full length protein was detectable, several smaller bands of 105 kDa, 95 kDa and 80 kDa were also observed (Fig 4.9). This suggested that ACR4 undergoes cleavage in the extracellular domain. This is consistent with the observations made previously in the laboratory suggesting that the ACR4 protein is rapidly modified and turned over in wild-type plants (Gifford *et al.,* 2005). A smaller band of 28 kDa was observed corresponding to GFP, which may be cleaved off during protein export. A comparison of the stability of full-length ACR4 tagged with GFP (*pACR4-ACR4:GFP*) to the cytoplasmic domain deleted version (*pACR4-ACR4ΔC:GFP*) suggested that the ACR4 receptor where the cytoplasmic domain is deleted is far more stabilized than the full length protein (Fig 4.10).

4.2.5.2 Both ACR4 and AIP1 are localized in an intracellular compartment

Protein localization studies were carried out to see if ACR4 and AIP1 could be localized to the same intracellular compartment. For this experiment, a stably transformed transgenic line expressing an AIP1:GFP fusion under the *AIP1* promoter and a line expressing an ACR4:GFP fusion under the *ACR4* promoter were used.

The localization of AIP1:GFP and ACR4:GFP were examined in root tissues using confocal laser scanning microscopy (Fig 4.11). The confocal images were taken by Gwyneth C. Ingram. Both in the root epidermis and root meristem AIP1:GFP fusion proteins were localized in a vesicular intracellular compartment rather than in the plasma-membrane. In contrast, ACR4:GFP fusion proteins were localized both in the plasma-membrane and in a vesicular intracellular compartments of the root epidermis and root meristem. The later observation is consistent with published results (Gifford *et al.,* 2005).

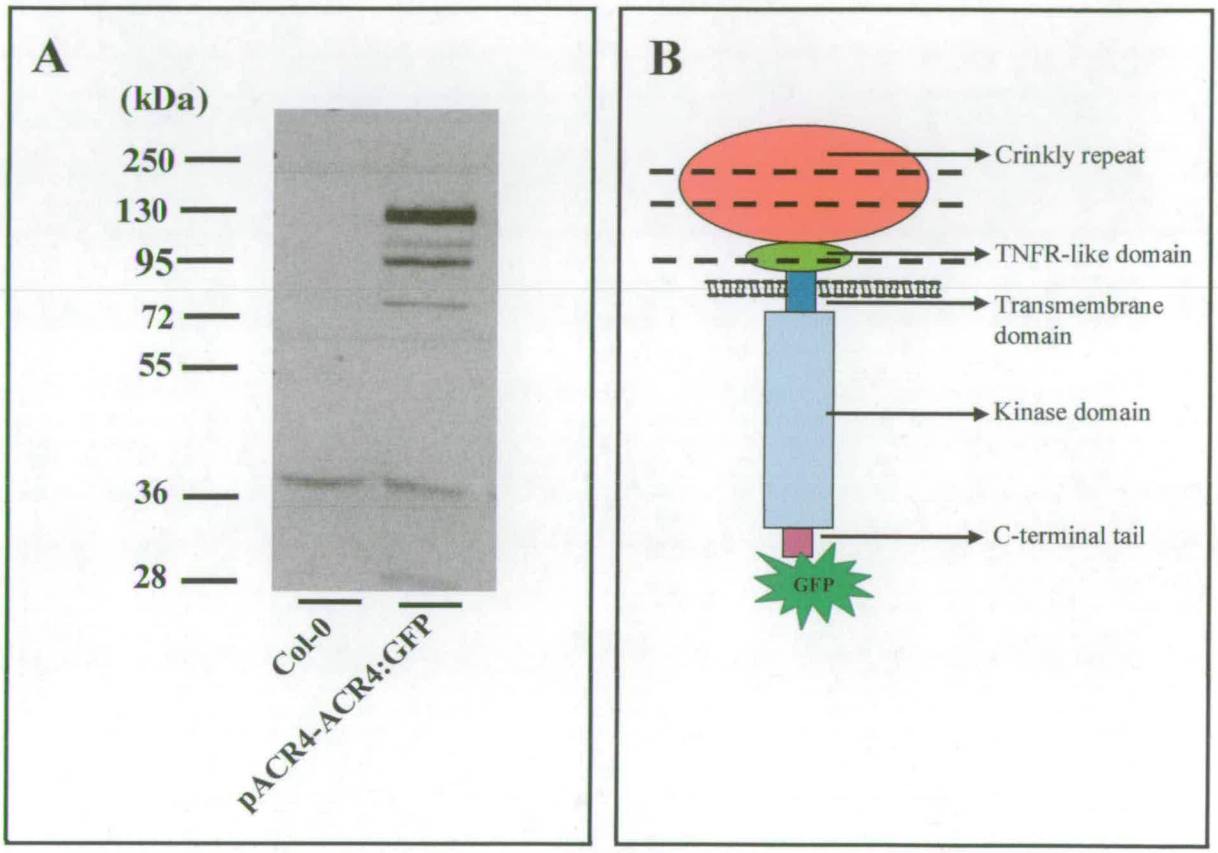

Fig 4.9: Western blot of extracted ACR4:GFP protein from inflorescences and floral meristem tissues, using anti-GFP antibody (A).
The full length ACR4:GFP is estimated to be 125 kDa. Although the full length protein was detectable, several smaller bands of 105 kDa, 95 kDa and 80 kDa were also observed suggesting that ACR4 undergoes cleavage in the extracellular domain (B). A smaller band of 28 kDa was observed which corresponds to GFP.

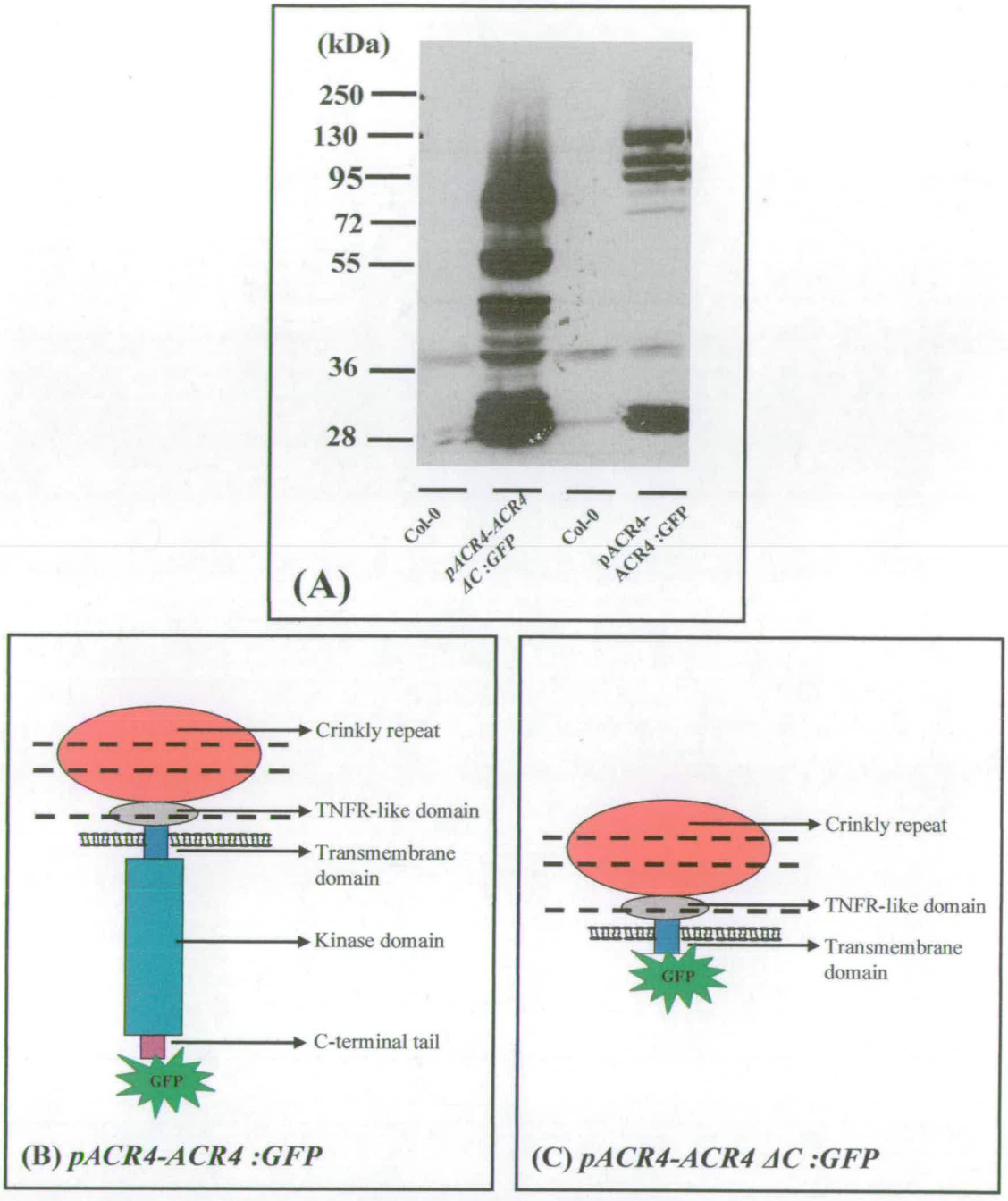

Fig 4.10: Western blot analysis of GFP tagged protein.

Total protein was extracted from the floral meristem tissues of *Col-0*, GFP tagged ACR4 line (*pACR4-ACR4:GFP)* and GFP tagged ACR4 line where the cytoplasmic domain is deleted (*pACR4-ACR4 ΔC :GFP*) and western blot was performed using anti-GFP antibody (A). For *pACR4-ACR4:GFP* line, a 125 kDa band was observed which corresponds to the full length protein and several smaller bands of 105 kDa, 95 kDa and 80 kDa suggests possible cleavages in the extracellular domain (A, B). For *pACR4-ACR4 ΔC :GFP* line, several smaller bands suggests putative cleavage in the extracellular domain (A, C). Interestingly, full length ACR4 is less stabilized compared to the ACR4 version where the cytoplasmic domain is deleted. Although there is no loading control, total protein was extracted from equal amount of tissue.

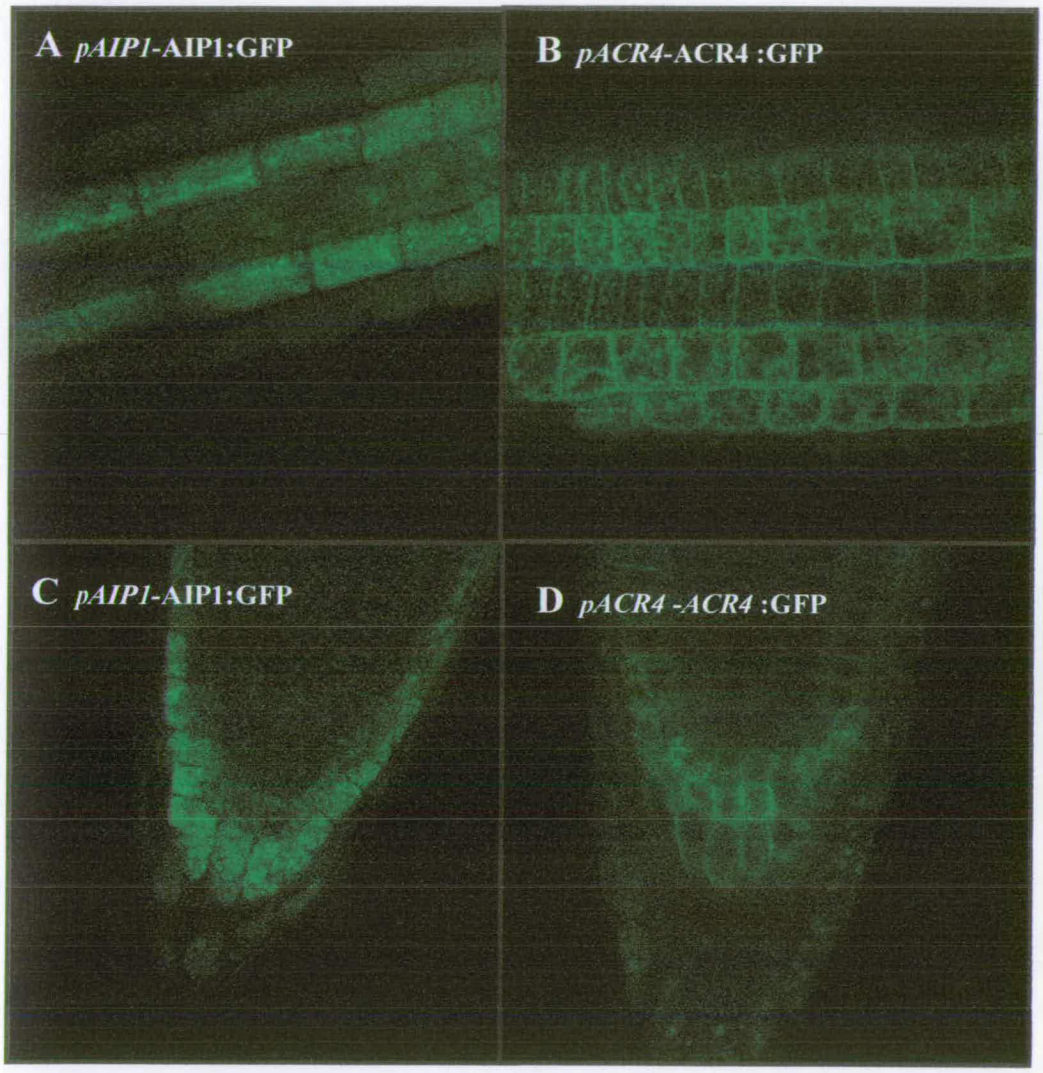

Fig 4.11: Protein localization studies.
Protein localisation in lines harbouring pAIP1-AIP1:GFP (A, C) and pACR4-ACR4:GFP (B, D) in root cells. Localization of AIP1:GFP fusion proteins (green) in the root epidermis (A) and root meristem (C) clearly indicates that the fused protein is localized in the intracellular compartment rather that in the plasma-membrane. ACR4:GFP fusion proteins (green) in the root epidermis (B) and root meristem (D) are localized both in the plasma-membrane and in an intracellular compartment. Some, but not all, of this intracellular ACR4:GFP has previously been shown to be in BFA-sensitive endocytosed vesicles (Gifford *et al.*, 2005).

Investigation of the endocytic behaviour of ACR4 using BFA indicated that ACR4 is internalized via a brefeldin A (BFA) sensitive pathway (Gifford *et al.*, 2005). BFA, an inhibitor of vesicle movement is commonly used in defining endosomal compartments (Robinson *et al.*, 2008). In animal cells, BFA causes structural changes of the endomembrane system and in particular the fusion of the Golgi apparatus with other endomembranes (Lippincott-Schwartz *et al.*, 1990). BFA appears to have different effects in plant cells. In plant cells, BFA acts to inhibit secretion and endosomal recycling of membrane bound proteins by inhibiting BFA-sensitive ARF-GEFs (Adenosine Ribosylation Factor-Guanine nucleotide Exchange Factors) (Jackson and Casanova, 2000). After BFA treatment of roots, vesicle aggregates form inside the cell, which are thought to consist of two structures. The most studied immediate effect is the formation of an endoplasmic reticulum-Golgi hybrid compartments. Subsequently the trans Golgi network derived vesicles merge with the components of the endocytic pathway to form 'BFA compartments' (Nebenführ *et al.*, 2002; Robinson *et al.*, 2008). Gifford *et al.* (2005) have shown that treatment of *Arabidopsis* roots stably expressing ACR4:GFP with BFA results in the accumulation of GFP signal into BFA compartments. In order to investigate whether AIP1 is also localized in the BFA compartments, roots of *pAIP1-AIP1:GFP* transgenic seedlings were treated with BFA for 30 minutes and no difference was observed in the localization of AIP1:GFP with and without BFA treatment. This suggested that the intracellular behaviour of AIP1:GFP is BFA insensitive.

4.2.6 Behaviour of ACR4 in an *aip1* mutant background

As mentioned earlier, functionally active ACR4 undergoes rapid internalization and cleavage (Gifford *et al.*, 2005). It was hypothesized that AIP1 protein which possesses MATH domains can interact with ACR4 and this interaction could cause endocytosis and/or degradation of ACR4. To test this hypothesis, behaviour of ACR4 protein was examined in the *aip1-2* mutant by confocal microscopy.

As mentioned earlier, ACR4 is known to be internalized via a brefeldin A (BFA) sensitive pathway (Gifford *et al.,* 2005). To test whether the absence of AIP1 disrupt the BFA-sensitive endocytic localization of ACR4, transgenic seedlings, *pACR4-ACR4:GFP* in wild type background and *pACR4-ACR4:GFP* in *aip1* background were treated with BFA for 30 minutes and no difference was observed in the localization of ACR4:GFP in wild type compared to the *aip1* mutant background. This suggested that the internalization of ACR4:GFP via a BFA sensitive pathway is independent of the interaction of ACR4 with AIP1.

4.2.7 Behaviour of AIP1 in an *acr4* mutant background

To address the possibility that ACR4 is a target of AIP1 and interaction with ACR4 is important for localization and proteolytic processing of AIP1, the behaviour of AIP1 was analyzed in an *acr4* mutant background.

To generate *acr4* mutants expressing AIP1:GFP, homozygous *acr4-2* plants were crossed to a transgenic line carrying a full length AIP1 ORF fused with GFP, under the control of the *AIP1* promoter (JZ 5-2-4). This T-DNA carries a kanamycin resistance gene. In the T_3 generation, plants were selected by spreading them onto media containing kanamycin to select lines which are homozygous for the transgene. Homozygous resistant plants were then genotyped by PCR to identify *acr4-2* homozygous line. The double homozygous lines i.e. homozygous for *acr4-2* as well as homozygous for the transgene *pAIP1-AIP1:GFP* were grown in the long day growth room. These transgenic lines did not show any gross morphological defect. Siliques were opened and the developing seeds were examined. It was found that the developing seeds of the *acr4-2* mutants expressing *AIP1:GFP* were round, just like *acr4* seeds (data not shown). The confocal microscopy studies suggested that there is no difference in the localization of AIP1:GFP in *acr4* mutant background compared to the wild type background (Fig 4.12). To investigate whether ACR4-AIP1 protein-protein interaction is important for the cleavage pattern of AIP1, immunoblot analysis was performed using *Col-0* plants expressing GFP

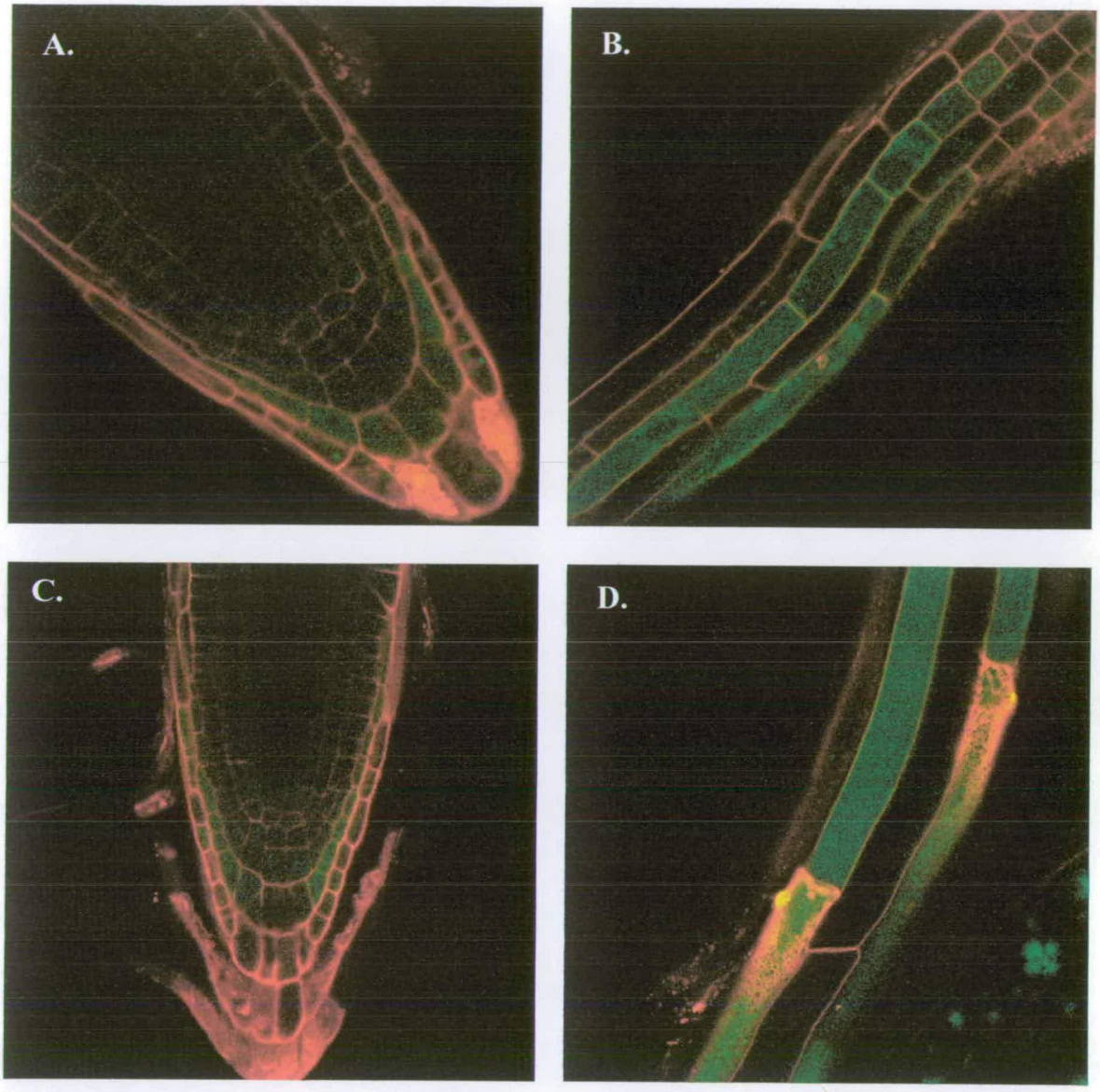

Fig 4.12: **Expression of AIP1:GFP in wild type (A, B) and *acr4* mutant (C, D).** Confocal images of the expression pattern of AIP1:GFP (green) driven by the *AIP1* promoter indicates that the expression of *AIP1* in the outer layers of the columella as well as in the lateral root cap (lrc) of root meristem (A) and in the root hair cells of root epidermis (B). The expression pattern of *AIP1* remains unchanged in the *acr4* mutant background (C, D).

tagged AIP1 as well as *acr4* mutants expressing AIP1:GFP. Total protein was extracted from 3 day old seedlings and immuno-detection was carried out using polyclonal anti-GFP antibodies. No difference was observed in the cleavage pattern of AIP1 (data not shown). This suggested that the interaction with ACR4 is not important for the cleavage of AIP1.

4.2.8 Analyzing *in vivo* interaction between ACR4 and AIP1

Our gene expression analysis and localization studies have produced no results which would preclude the possibility that AIP1 could be an interacting partner of ACR4. Both proteins are expressed in the same subset of root cells, and both are localized to an intracellular compartment. To demonstrate the interaction directly *in planta,* several complementary experiments were carried out. One approach was co-immunoprecipitation, which was chosen to examine direct interaction between ACR4 and AIP1 in *Arabidopsis*. In order to gain further insights into the interaction between ACR4 and AIP1, protein samples were run by BN-PAGE. BN-PAGE allows visualization of different multi-protein complexes by immunoblotting.

4.2.8.1 Co-immunoprecipitation

To confirm the *in vivo* protein-protein interaction between ACR4 and AIP1 by co-immunoprecipitation, epitope tagged lines were constructed to facilitate detection of potential interactions using different commercially available antibodies. A stably transformed *pACR4-ACR4:MYC* line carrying hygromycin resistance gene (JZ 2-21-4) an a *pAIP1-AIP1:GFP* line carrying kanamycin resistant gene (JZ 5-2-4) were crossed to generate double transgenic line. In T_3 generation, seeds were spread onto media containing both hygromycin and kanamycin to select homozygous double transgenic lines. These double transgenic lines expressing both AIP1:GFP and ACR4:MYC were grown in liquid culture for 10 days and the total protein extract prepared specifically from root tissues was subjected to immunoblotting with the anti-GFP antibody and anti- MYC antibody separately to test the expression of each gene in different double transgenic lines. The western blot indicated that

AIP1:GFP is detectable in the root tissues of these double transgenic lines, but no detectable expression was observed in the root tissue in any of the ACR4:MYC lines. Still co-immunoprecipitation was performed to test the possibility that the enrichment of ACR4:MYC could make the 'MYC' tag detectable.

To do co-immunoprecipitation, total protein from wild type and transgenic plants expressing both AIP1:GFP and ACR4:MYC was immunoprecipitated using anti-GFP microbeads (μMACS GFP tag protein isolation kit, Miltenyi Biotec) to capture AIP1:GFP and any differentially bound proteins. The immunoprecipitate was subjected to immunoblotting with an anti MYC antibody to try to detect ACR4:MYC in the immunoprecipitated samples. However, no signal was obtained using anti-MYC antibody. Therefore, it has not been possible to show protein-protein interaction between ACR4 and AIP1 by co-immunoprecipitation. It should be noted that in our hands, detection of ACR4:MYC, even in single transgenic lines is technically challenging and necessitates modifications to the blotting procedure (described in Section 2.5.2).

4.2.8.2 Blue-native PAGE

For in depth analysis of the interaction between ACR4 and AIP1 under physiological conditions, native proteomic analysis was performed using BN-PAGE. BN-PAGE is a powerful tool for investigating protein-protein interactions under physiological conditions (Schagger and von Jagow, 1991; Darie *et al.*, 2008). A range of experiments were carried out in the lab to clarify the protein-protein interaction between ACR4 and AIP1 by BN-PAGE.

As the first approach, total protein extracted from floral meristems expressing GFP tagged ACR4 under its own promoter and *Col-0* was used for BN-PAGE followed by western blot to determine if BN-PAGE allows successful separation of protein complexes. The western blot image (Fig 4.13) clearly indicated that ACR4 is present as a protein complex with other unknown proteins. As mentioned earlier, SDS-PAGE analysis of ACR4-GFP gives a band of 125 kDa, which corresponds to the full length protein. Several smaller bands of 105 kDa, 95 kDa and 80 kDa were

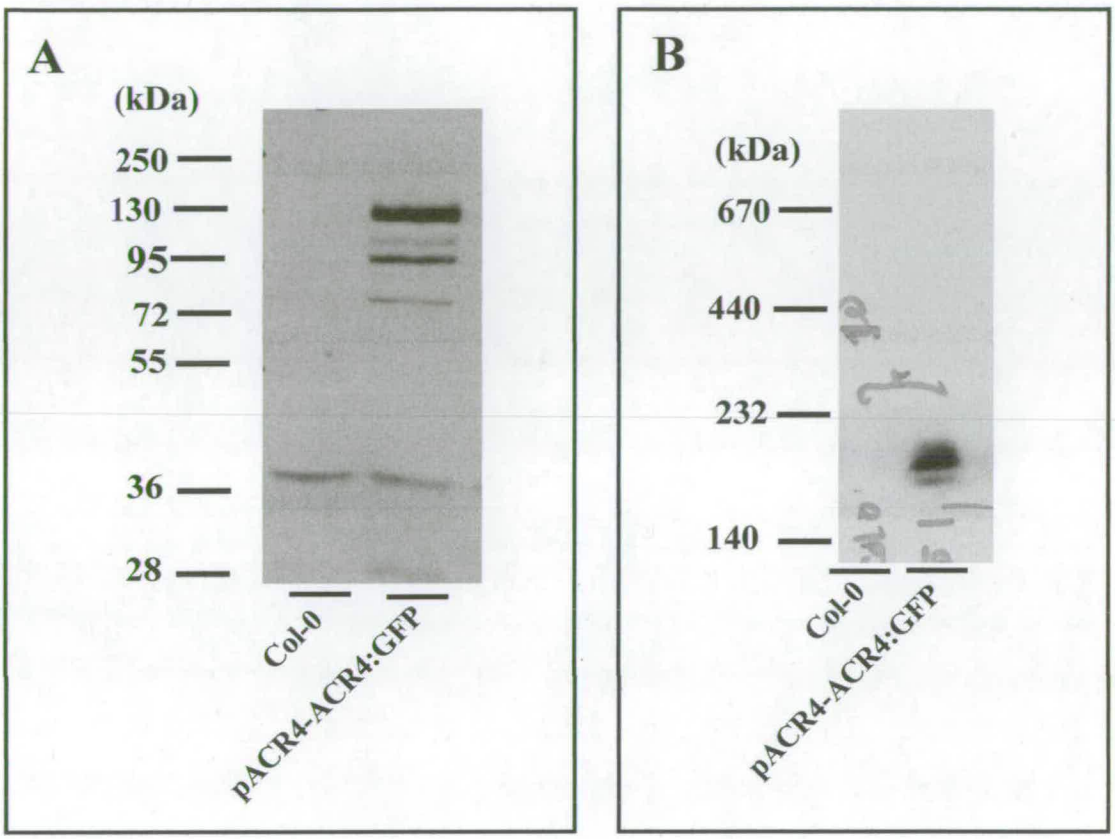

Fig 4.13: Multimerization of ACR4 protein.
Total protein from inflorescence and floral meristem tissues of *Col-0* and transgenic line *pACR4-ACR4:GFP* were subjected to SDS-PAGE (A) and Blue Native gel electrophoresis (B), followed by western blotting using anti-GFP antibody to detect ACR4:GFP fusion protein. The full length ACR4:GFP is estimated to be 125 kDa. SDS-PAGE (A) indicated that although the full length protein was detectable, several smaller bands of 105 kDa, 95 kDa and 80 kDa were also observed suggesting that ACR4 undergoes cleavage in the extracellular domain. A smaller band of 28 kDa was observed which corresponds to GFP. In the native gel (B), two bands of approximately 200 kDa were observed which may represent the complex formed with other ACR4 binding partners.

also detectable. However native-PAGE showed the presence of higher molecular weight protein complex. This suggested that ACR4 is present in a complex with other unknown proteins. In contrast, BN-PAGE followed by western blot analysis of protein samples from *pAIP-AIP1:GFP* seedlings didn't show any band in the western blot. This may be due to the fact that, although denatured AIP1:GFP is readily detectable on western blots, in its native form the GFP epitope may be masked in some way, preventing recognition by the antibody. Similar phenomena have been observed by other groups for example, Tetlow *et al.* (2004).

Although our BN-PAGE results clearly indicated that ACR4 forms a complex with other unknown proteins in inflorescences, due to financial constrain, we were unable to do MS analysis to identify the components of the multi-protein complexes of ACR4. To overcome this limitation, an indirect approach was taken in the lab. The idea was to extract protein samples from *aip1-2* seedlings expressing GFP tagged ACR4 as well as wild type seedlings expressing ACR4:GFP. A comparison of the high molecular weight protein bands obtained from wild type plants expressing ACR4:GFP protein with *aip1* mutants expressing ACR4:GFP protein could allow us to identify the protein-protein interaction between AIP1 and ACR4. If AIP1 is present as a component of ACR4 protein complex, BN-PAGE and western blot analysis would reveal a decrease in size of the high molecular weight band obtained from *aip1-2* tissues expressing GFP tagged ACR4. To this end, I generated *aip1* mutants expressing ACR4:GFP, however, I was unable to do this experiment as ACR4:GFP from seedling tissues is quite hard to detect in the western blot following BN-PAGE due to the low level of expression of *ACR4* gene in the seedlings. In contrast, *AIP1* is highly expressed in seedlings.

4.3 Discussion

The present study provided information regarding the potential interaction between ACR4 and AIP1. No downstream target of ACR4 has previously been identified in the literature. Our current investigation is hence the first approach to

characterize a potential interaction of ACR4 with a putative downstream signalling component, AIP1.

To investigate any potential genetic link between *ACR4* and *AIP1,* characterization of *aip1-2* single mutant and *acr4 aip1-2* double mutants were carried out. It was found that the developing seeds of the homozygous *aip1-2* were elliptical like wild type, not round like *acr4* seeds whereas *acr4 aip1-2* double mutant seeds were just like *acr4* single mutant. Given the fact that *AIP1* does not appear to be expressed during ovule development (when the seed defects in *acr4* mutants originate), this result is unsurprising. Unlike *acr4* mutants, the *aip1-2* mutants didn't show any enhancement in the number of lateral roots compared to wild-type. The *acr4-2 aip1-2* double mutant plants exhibited the same phenotype to that of *acr4* single mutants (Fig 4.6). These results suggested two possibilities. One possibility could be that AIP1, being a putative interacting partner of ACR4, could act downstream of ACR4, therefore no enhancement of mutant phenotype was expected in the *acr4-2 aip1-2* double mutant. The second possibility could be that there is no direct genetic link between *ACR4* and *AIP1*.

The next question was the expression pattern of *ACR4* and *AIP1*. An *in vivo* protein-protein interaction could only really be supported if both potential interactors are present in the same cells during normal plant development. Detailed analysis was carried out to compare the expression pattern of *ACR4* and *AIP1*. Promoter-reporter constructs were generated and compared. This analysis indicated that the expression pattern of *ACR4* and *AIP1* is somewhat similar, although not totally identical (Fig 4.7). Both *ACR4* and *AIP1* are expressed in the lateral roots cap, the epidermis, and in the middle cell-layer of the columella. Moreover, protein localization studies revealed that both ACR4 and AIP1 are localized in intracellular vesicular compartments (Fig 4.11). The BFA sensitive endosomal localization of ACR4:GFP is not altered in the absence of AIP1. However, it is not altogether inconceivable that ACR4 could interact with AIP1 in an intracellular compartment, since Gifford *et al.* (2005) reported that a subset of ACR4-containing vesicles, like AIP1 containing vesicles, are BFA insensitive.

Since *in vitro* methods aimed at verifying the interaction between AIP1 and ACR4 has previously failed in the laboratory, largely due to the toxicity of the ACR4 N-terminus in bacteria, I decided to try and confirm the interaction *in vivo.* In the first instance, co-immunoprecipitation was performed. The lysate prepared from seedlings expressing both ACR4:MYC and AIP1:GFP was used for immunoprecipitation with the anti-GFP polyclonal antibody and the immunoprecipitate was subjected to immunoblotting using the anti-MYC antibody to demonstrate the interactions of AIP1 with ACR4. Since no signal was obtained, it was impossible to draw any conclusion regarding the potential interaction between ACR4 and AIP1. It remains possible that ACR4 is an interacting partner of AIP1, however the ACR4:MYC was undetectable in the immunoprecipitate due to the low level of expression of *ACR4* in seedlings.

As a complementary experiment to analyze the *in vivo* interaction between ACR4 and AIP1, BN-PAGE was performed. BN-PAGE allows separation of multi-protein complexes in their native conformation, based on the external charge induced by Coomassie Blue dye and according to their mass (Schagger and von Jagow, 1991; Darie *et al.,* 2008). Relatively transient protein-protein interactions can be detected by this technique. Through BN-PAGE analysis of protein extracts from floral meristem tissues expressing ACR4:GFP under its own promoter, I have provided evidence that ACR4 is capable of forming a approxomately 200 kDa protein complex, compared to the 125kDa monomer size of ACR4:GFP. This suggests that ACR4 protein forms complex with other unknown proteins. To identify interacting partners, BN-PAGE is sometimes combined with MS analysis to identify interacting partners. However due to financial constrain, no MS analysis was performed in this project. As a parallel experiment, protein extracts from seedlings expressing AIP1:GFP under its own promoter were subjected to BN-PAGE, followed by western blot. However, no band was obtained. As mentioned earlier, BN-PAGE separates protein complexes while keeping their native conformation, it is possible that the folded 3-D structure of AIP1:GFP masks antigenic sites in GFP and so GFP was not detectable by anti-GFP antibody and no band appeared in the western blot. Similar phenomenon has previously been described by other group (Tetlow *et al.,* 2004).

In this chapter I have shown that both AIP1 and ACR4 undergo specific protein cleavage events. The results of Gifford *et al.* (2005) indicate that both endocytosis and processing of ACR4 are dependent upon protein functionality, since mutations in the extracellular domain which eliminate protein function, also eliminate protein turnover and processing. As discussed in the introduction, several lines of evidence link MATH domains to potential roles in protein turnover or, at least ubiquitination in plants. We therefore asked whether the behaviour of ACR4 is dependent on its interaction with AIP1. The confocal microscopic images indicated that the localization of ACR4:GFP is not altered in the *aip1* mutant background (data not shown). However, these results do not exclude the possibility that AIP1 mediate ACR4 signalling in a fashion similar to the mediation of TNFR signalling by TRAF proteins in mammals. In animal system, ubiquitination of TRAF is known to be associated with protein degradation (Karin and Gallagher, 2009).

Taken together, the current study provides new evidence and information regarding the multi-protein complex status of ACR4. This information is important for further efforts in defining precisely how ACR4 transmits signals inside the cell.

Chapter – 5

Characterization of the role of *AtCRR4*, a member of the *ACR4* gene family, in leaf senescence

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Chapter - 5

Characterization of the role of *AtCRR4*, a member of the *ACR4* gene family, in leaf senescence

5.1 Introduction

5.1.1 An overview of senescence

Leaf senescence is a highly-regulated sequence of biochemical and physiological events leading to death or the end of the life span (Smart, 1994). It can be defined as an organized degenerative process which allows maximum recovery of nutrients from the senescing tissues for recycling to the parts of the plant that survive (Smart, 1994). During senescence, cells undergo a major transition from carbon assimilation and other anabolic reactions to a catabolic pattern. The catabolic pattern of the senescing organs involves chlorophyll degradation and chloroplast breakdown (Hörtensteiner, 2006). The hydrolysis of proteins and nucleic acids leads to the redistribution of nitrogen and phosphorus from the degraded products to developing organs (Smart, 1994; Gan and Amasino, 1997). Photosynthesis declines and the ordered disintegration of the intracellular organelles leads to the disruption of cellular homeostasis, ultimately causing cell death.

Leaf senescence can be considered as an example of Programmed Cell Death (PCD) (Yen and Yang, 1998). The term PCD is broadly defined as a cell death process when cell suicide pathways are activated as part of normal growth and development or in response to invading pathogens (Beers, 1997; Greenberg, 1996). Almost all phases of the plant life cycle, from germination through vegetative and reproductive development are influenced by PCD including embryogenesis, floral organ abscision, root cap sloughing, senescence, and the development of gametophytes and vascular tissue (Beers, 1997; Greenberg, 1996). PCD is also observed during the hypersensitive response (HR), the cell death that is triggered in plant cells in and around the point of attempted infection by some pathogens (Greenberg, 1996; Shirasu and Schulze-Lefert, 2000). Thus, senescence and cell death associated with the HR are just two examples of PCD that occur in plants. The PCD which occurs during leaf senescence has certain features which distinguish it from HR associated cell death (Lim *et al.,* 2007; Quirino *et al.*, 1999). Senescence involves large-scale but ordered disassembly of cellular components in the senescing tissues and it allows maximum recovery of nutrients. In contrast, the PCD seen in HR, is extremely rapid (allowing no time for nutrient recycling) and is triggered in a very localised fashion around the point of attempted infection by some pathogens, causing localized cell death to block further spread of infection (Lam *et al.,* 2001). The HR has been linked to apoptotic cell death pathways in animals. This apoptosis-like pathway is generally thought to be associated with the production and perception of Salicylic Acid (SA), and caspase like proteases are presumed to have a central role in the cell death process (Love *et al.,* 2008). By contrast, plant senescence has been compared to another form of cell death in animals, autophagic cell death. This is an exaggerated form of autophagy that, in animals, involves formation of autophagosomes which sequester parts of the cytoplasm for nutrient recycling (Love *et al.,* 2008). Autophagic-like cell death in plants is thought to be controlled by the production and perception of Jasmonic Acid (JA). Thus, PCD processes in plants are likely to be relatively diverse. One obvious question is, then, how the cell death pathways associated with senescence differ from other forms of cell death at the molecular level. Genome wide expression analysis of genes associated with

senescence suggested that some defence related genes are induced during leaf senescence (Quirino *et al.*, 1999). However subgroups of genes associated with senescence are not induced during HR-associated PCD, and, likewise some genes associated with HR are not induced during senescence (Pontier *et al.*, 1999). Thus, leaf senescence and HR-associate cell death occur via distinct, but overlapping pathways.

Leaf senescence is controlled by both internal factors (including age, developmental cues, and plant growth regulators) and environmental factors (including light and temperature stress, dehydration, nutrient stress, and pathogen infection (Beers and McDowell, 2001; Smart, 1994; Pic *et al.*, 2002; Lim *et al.*, 2007). Thus, the ultimate decision to initiate senescence in a plant depends on the interplay of various signals including but certainly not limited to, endogenous growth regulators. Among the different growth regulators, cytokinin appears to play a major role in delaying senescence. The endogenous cytokinin level is dramatically lowered in most senescing tissues and the exogenous application or endogenous overexpression of cytokinin delays senescence (Gan and Amasino, 1995, 1997). In contrast to cytokinin, ethylene acts to promote senescence. The importance of ethylene signalling during senescence is illustrated by the delayed senescence phenotype of ethylene-insensitive mutants (Oh *et al.*, 1997; Grbic and Bleecker, 1995). Other growth regulators, such as JA, abscisic acid (ABA) and SA, that are closely linked to biotic and abiotic stress responses, have also been found to have roles in senescence (He *et al.*, 2002; Morris *et al.*, 2000; Buchanan-Wollaston *et al.*, 2005). A role for JA in leaf senescence was indicated by the observation that exogenous application of JA induces leaf senescence, and this induction requires an intact JA signalling pathway (He *et al.*, 2002). Similarly, ABA acts to promote senescence and induces the expression of senescence associated genes (Buchanan-Wollaston *et al.*, 2005; van der Graaff *et al.*, 2006). The SA-signalling pathway is also active during senescence (Morris *et al.*, 2000). A transcriptome analysis using senescing *Arabidopsis* leaves from wild-type plants and SA-deficient mutants revealed that the expression of many Senescence-Associated Genes (SAGs) is

dependent on the SA-signalling pathways (Buchanan-Wollaston *et al.,* 2005). The growth regulator, auxin may also play a role in modulating the senescence process. Recently, it was found that a member of the Auxin Response Factor (ARF) protein family, ARF2 positively regulates leaf senescence in *Arabidopsis* (Lim *et al.,* 2010).

Leaf senescence is regarded as a developmental strategy for plant fitness. Studying leaf senescence not only improves our understanding about this fundamental and complex developmental process, but could also lead to the identification of the strategies to manipulate senescence for increasing crop yield.

5.1.2 *AtCRR4,* a possible role in senescence

Similar to other known developmental programs in plants, the senescence program may involve signal transduction pathways where cells perceive extracellular signal(s). Such signals could be required to ensure the tissue level co-ordination of senescence, and may involve increased levels of certain proteins encoded by SAGs. Research in the last few years suggested that signal transduction by RLKs could play an important role in this process (Robatzek and Somssich, 2002; Ouelhadj *et al.,* 2007). Whole genome transcriptome analysis revealed that there are 91 RLK genes in *Arabidopsis*, which are induced during natural leaf senescence (van der Graaff *et al.,* 2006). Some of these RLKs have previously been shown to be involved in pathogen defence responses. Several members of the leucine rich repeat RLK family are also found to be upregulated during natural senescence. This group includes *SENESCENCE INDUCED RECEPTOR LIKE KINASE (SIRK)* (Robatzek and Somssich, 2002). *SIRK* is the only characterized RLK in *Arabidopsis*, which is induced during natural leaf senescence. The developmental expression of *SIRK* is strongly induced specifically during leaf senescence and the high *SIRK* expression is dependent on the transcriptional regulator, WRKY6. WRKY6 belongs to a zinc-finger-type class of protein family, known as the WRKY family. Members of this family appear to be involved in the regulation of certain plant processes, such as

pathogen defence, wound response, and senescence (Eulgem *et al.*, 2000). By analyzing a promoter-reporter construct, it was found that the expression of *SIRK* is induced by the bacterial elicitor, flg22 and WRKY6 is also thought to play a role in this response (Robatzek and Somssich, 2002). More recently, through transcriptome analysis Buchanan-Wollaston *et al.* (2005) reported that the induced expression of *SIRK* is mediated by SA-signalling pathway. However, the role of SIRK in senescence is still unclear.

Here, the potential role of a new signalling pathway mediated by the AtCRR4 receptor during leaf senescence in *Arabidopsis* is reported. Through RT-PCR analysis, it was confirmed that the expression of *AtCRR4* is induced in senescing leaves. An analysis of the cis regulatory element of *AtCRR4* using PLACE, a database of plant cis-acting regulatory DNA elements (Higo *et al.,* 1999) indicates the presence of four W–boxes, the binding site for WRKY transcription factors (Rushton *et al.,* 2010).

5.1.2.1 Microarray data suggest that *AtCRR4* is induced in senescing leaves

In recent years, microarray technology has become an important tool in describing the scope of biological phenomenon and identifying candidate genes involved in particular functions. Combined with time courses, this technology can identify genes that are associated with particular stages of development. For example, van der Graaff *et al.* (2006) performed a genome-wide expression analysis of *A. thaliana* genes to identify genes that are involved in senescence. In this microarray analysis, it was found that an *ACR4* related kinase, *AtCRR4* is highly expressed in senescing leaves. According to their data, the expression of *AtCRR4* in young leaves is low and expression increases as the leaf grows, culminating in a dramatic increase in expression in senescing leaves. This suggests that the accumulation of *AtCRR4* transcript occurs in an age dependent manner.

5.1.2.2 *AtCRR4* could be regulated by cytokinin

As mentioned earlier, cytokinin is known to play an important role in senescence. Physiological studies have shown that during leaf senescence, endogenous cytokinin levels decrease (Van Staden, 1973). Microarray analysis suggested that *AtCRR4* is upregulated as senescence progresses (van der Graaff *et al.,* 2006). Therefore it is possible that cytokinin acts to negatively regulate the expression of *AtCRR4.* This hypothesis is borne out by a study showing that *CYTOKININ RELATED KINASE1 (CRK1)* in *Nicotiana tabacum,* which is the likely ortholog of *Arabidopsis AtCRR4,* is negatively regulated by cytokinin (Schäfer and Schmülling, 2002). Further evidence for the role of cytokinin in regulating the expression of *AtCRR4* came from the published analysis of a gain of function mutant (named *ore12*) resulting in the constitutive activation of an *Arabidopsis* cytokinin receptor, AHK3 (ARABIDOPSIS HISTIDINE KINASE 3). Transcriptome data indicate that the expression of *AtCRR4* is reduced in *ore12* mutant leaves which show a delay in leaf senescence (Kim *et al.,* 2006).

5.1.2.2.1 *CRK1*, an ortholog of *AtCRR4* in tobacco is regulated by cytokinin

As mentioned earlier, *AtCRR4* encodes an RLK and consists of several prominent domains – an amino terminal signal peptide; an extracellular domain containing 7 repeats of 39 amino acids, termed "crinkly repeats"; a transmembrane domain and a cytoplasmic kinase domain with serine/threonine kinase activity (Cao *et al.,* 2005). Through sequence analysis, it was found that the protein sequence of AtCRR4 shares a high degree of sequence similarity to tobacco CRK1 and it is more closely related to CRK1 than to any other member of the ACR4 family from *Arabidopsis* (Fig 1.10), (Schäfer and Schmülling, 2002). The cytoplasmic domain shares a high degree of identity (59% identical amino acids). Both AtCRR4 and

CRK1 share all of the conserved residues in the kinase domain that are necessary for the predicted kinase activity for serine-threonine kinases (Hanks *et al.,* 1988). The extracellular domain is also conserved with 41% identity. The N-terminal signal peptide is well conserved between AtCRR4 and CRK1. A short proline rich domain between the crinkly repeats and the transmembrane domain, which could function in protein-protein interactions is present in both AtCRR4 and CRK1 (Kay *et al.,* 2000). In summary, AtCRR4 conserves all sequence motifs to the tobacco CRK1 and so is likely to be functionally analogous.

The signalling pathways, in which AtCRR4 and CRK1 are involved, are presently unknown. Schäfer and Schmülling (2002) reported that in tobacco cell culture, *CRK1* transcript level is rapidly down regulated by exogenous cytokinin. This rapid change suggested that *CRK1* might act as an early response gene to cytokinin. Inhibitor studies revealed that the addition of cyclohexamide inhibits this regulation by cytokinin suggesting that a labile repressor might be involved in controlling the expression of *CRK1*. However, the detailed mechanism of cytokinin mediated repression of *CRK1* is still unknown. As *AtCRR4* is one of the closest ortholog of *CRK1*, it is possible that cytokinin is also involved in regulating the expression of *AtCRR4* in *Arabidopsis*.

5.1.2.2.2 *AtCRR4* is down regulated in *ore12* mutants

In *Arabidopsis*, cytokinin perception and transduction mechanisms are mediated by the cytokinin receptors AHK2, AHK3, and AHK4/CRE1 (Inoue *et al.*, 2001; Yamada *et al.,* 2001). Among these three cytokinin receptors, AHK3 plays a major role in controlling cytokinin mediated leaf senescence (Kim *et al.,* 2006; Riefler *et al.*, 2006). The unique role of *AHK3* in senescence was uncovered by studies of the gain of function allele *ore12-1* (Kim *et al.,* 2006). The *ore12-1* mutant plants constitutively respond to cytokinin and show a delay in senescence. To determine whether senescence specific expression of *AtCRR4* is regulated by cytokinin, I looked at the publicly available transcriptome database from *A. thaliana*.

It was found that the expression of *AtCRR4* is down regulated to a significant level in 3-week old *ore12-1* plants compared to wild-type (Kim *et al.*, 2006).

To investigate the role of *AtCRR4* in senescence, several approaches were taken in the lab. The first approach was to confirm the high level of expression of *AtCRR4* in senescing leaves by RT-PCR. Secondly, senescence phenotypes associated with loss of *AtCRR4* gene function were investigated. Finally, the possible involvement of the *Arabidopsis* cytokinin receptor, AHK3 in regulating the expression of *AtCRR4* was studied.

5.2 Results

5.2.1 RT-PCR confirms that *AtCRR4* is highly expressed during natural leaf senescence

As mentioned earlier, genome-wide expression analysis of genes involved in senescence suggested that *AtCRR4* is one of the genes which are highly expressed in senescing leaves (van der Graaff *et al.*, 2006). To confirm this transcriptome data, RT-PCR was performed. Total RNA was extracted from rosette leaves of plants grown under long-day conditions, at 1, 4, 6 and 10 weeks after germination. At the 1-week stage, under our growth room conditions, Arabidopsis had two small true leaves. At 4-weeks after germination, the visible signs of the emergence of inflorescences were observed. At the 6 week stage, the primary inflorescence had a height of about 4cm. No visible sign of leaf senescence was observed at this stage. At 10-weeks, the rosette leaves started to senesce, showing visible signs of yellowing. At this stage RNA was extracted from leaves where 1/3 of each leaf was yellow. Fig 5.1 shows the transcript

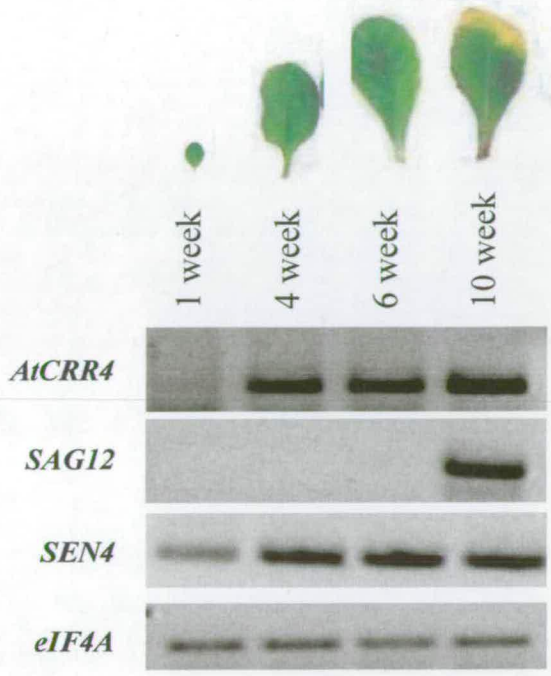

Fig 5.1: Age dependent changes of *AtCRR4* expression in leaves.

Total RNA was extracted from 1 week, 4 week, 6 week and 10 week (senescing) old long day grown *Col-0* rosette leaves. The expression of *AtCRR4* increases with leaf age. *SAG12 and SEN4* represent senescence specific marker genes. *eIF4A* was shown as an internal control.

level of *AtCRR4* gene at various stages of development. No expression of the *AtCRR4* gene was observed at 1 week stage. The expression of *AtCRR4* was first observed at 4 week after germination and remained at a similar level at the 6 week stage. However, the transcript level of *AtCRR4* was very high in 10 week old senescing leaves. The relative abundance of *AtCRR4* at different developmental stages suggested that the *AtCRR4* gene might be involved in the process of senescence.

Leaf senescence is known to be accompanied by the increased expression of Senescence Associated Genes (SAGs) (Nam, 1997). The expression pattern of *AtCRR4* in different developmental ages was compared with two SAGs, *SAG12* (*SENESCENCE-ASSOCIATED GENE 12*) and *SEN4 (SENESCENCE-ASSOCIATED GENE 4),* both of which have been shown to be up-regulated during senescence (Gan and Amasino, 1997; Park *et al.*, 1998). *SAG12* encodes a senescence-specific cysteine-protease. As shown in Fig. 5.1, the expression of *AtCRR4* was gradually up-regulated with the progression of senescence whereas the expression of *SEN4* was slightly up-regulated with the progression of senescence and *SAG12* was found to be expressed only in senescent leaves. These results supported the idea that *AtCRR4* could be involved in the progression of senescence.

5.2.2 The *atcrr4* mutant leaves may show defects in senescence

The gene expression analysis clearly indicated that *AtCRR4* is highly expressed in senescing leaves (Fig 5.1). I therefore tested whether the loss of *AtCRR4* function might cause an altered leaf senescence phenotype.

To investigate the senescence phenotype of *atcrr4* mutants, it was decided to compare the senescence of *atcrr4* mutant leaves to a number of known mutants or over expression lines with altered senescence phenotypes. Three different lines *ore12-1, ahk3-1*, and AHK3-OX40 were used as controls, in addition to wild type

Col-0. As mentioned earlier, *ore12-1* is a gain of function allele of *AHK3* showing a delay in senescence under normal growth condition (Kim *et al.,* 2006). The mutant *ahk3-1* is a loss of function allele of *AHK3* and has been reported to show an early senescence phenotype after dark induced senescence treatment, but not during natural senescence (Kim *et al.,* 2006). The AHK3-OX40 line expresses *AHK3* under the CaMV 35s promoter. AHK3-OX40 lines have been reported to recapitulate the *ore12-1* mutant phenotype during dark-induced senescence, however comparison of the senescence phenotype of *ore12-1* and AHK3-OX40 lines after dark treatment clearly indicated that *ore12-1* shows a stronger senescence phenotype than AHK3-OX40 (Kim *et al.,* 2006).

Homozygous mutant seeds for *ore12-1, ahk3-1* and *AHK3* over-expressing line, AHK3-OX40 (*35s-AHK3*) were kindly provided by Prof. Hong Gil Nam, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Korea. PCR based genotyping as well as sequencing were performed to confirm the genotypes of mutants and over expressed lines. Table 2.3 shows details of the primer sequences used.

To investigate if *atcrr4* mutant plants show any natural senescence phenotype *atcrr4, ahk3-1*, AHK3-OX40 and wild type plants were grown in an environmentally controlled growth room at 22°C with a 16hr light/ 8hr dark cycle (long day growth room). All the plants were grown in a "mixed" conformation in the same tray to minimize the effects of environmental heterogeneity. Leaves were carefully observed at different stages of development. Neither the *atcrr4* mutant nor the control line *ore12-1* showed any difference from the wild type, despite the fact that it has previously been reported that *ore12-1* shows a delay in senescence under normal growth conditions (Kim *et al.,* 2006). This experiment was repeated in another growth room, with the same outcome. Since senescence is regulated by a wide variety of environmental inputs, it seems probable that conditions in our growth rooms are unsuitable for this type of experiment.

As the next approach, I looked at senescence after a dark induced senescence treatment. In many studies, darkness is used to accelerate the senescence program, although genome wide expression analysis suggested that there are some differences in gene expression between natural and dark induced leaf senescence (Gan and Amasino, 1997; van der Graaff *et al.,* 2006). For the dark induced senescence assay, the 3rd and 4th leaves at 12 days after leaf emergence were detached and floated on 3 mM MES (2-*N*-morpholino ethanesulfonic acid) buffer (pH 5.7) in the dark for 3 days. After dark treatment *ore12-1* mutants showed a clear delay in senescence compared to the wild type, but no early senescence phenotype was observed for *ahk3-1* mutant, no delay in senescence phenotype was found in the AHK3-OX40 line and no difference from wild-type was observed for *atccr4* mutant plants. In a parallel experiment, I carried out a dark induced senescence experiment using intact rosettes. To do this experiment, the whole rosettes of 5 week old short day grown plants were excised and placed on wet filter paper in a Petri dish. The Petri dishes were sealed using parafilm and covered with two layers of aluminium foil to exclude light. Leaves were observed after 3, 5 and 7 days. Photographs were taken after 7 days dark treatment and are shown in Fig 5.2. As before, *ore12-1* lines showed a clear delay in senescence compared to the wild type. However, no difference was observed among *Col-0, atcrr4, ahk3-1* and AHK3-OX40.

As a quantitative measure of the onset of senescence, total chlorophyll content was measured from the dark treated leaves of *Col-0, atcrr4, ore12-1, ahk3-1* and AHK3OX40 and is represented in Fig 5.3. As expected from the images (Fig 5.2), the only significant difference was observed between *ore12* and *Col-0.* However, no difference was observed between *atcrr4* and *Col-0, ahk3* and *Col-0* or AHK3-OX40 and *Col-0.* This was unexpected because according to the published results of Kim *et al.* (2006), AHK3-OX40 should show a significant delay in senescence and *ahk3-1* should show an early senescence phenotype under dark treatment. As *ahk3-1* and AHK3-OX40 did not show any phenotype, it was not possible to draw any meaningful conclusions regarding the senescence phenotype of *atcrr4.*

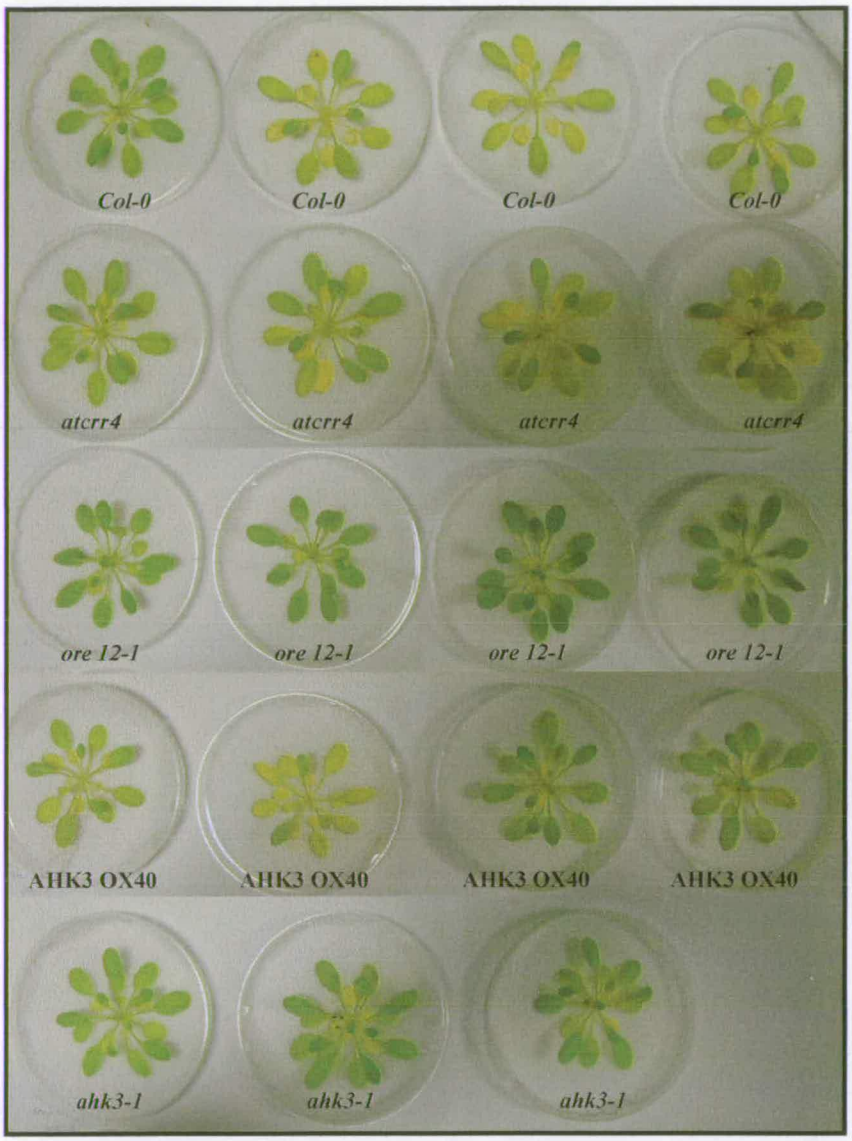

Fig 5.2: The *atcrr4* mutant does not show any delayed senescence phenotype under dark induced senescence treatment.
The whole shoot of 5 week old short day grown *Col-0, atcrr4, ore12-1,* AHK3 OX40 and *ahk3-1* plants were excised and were kept on wet filter paper in a Petri dish. The Petri dishes were sealed by parafilm and covered by two layers of aluminium foil.
Photographs were taken after 7 days of dark incubation.

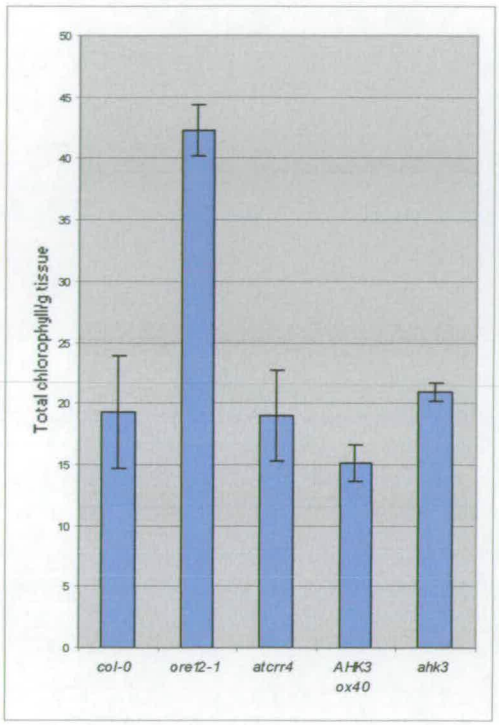

Fig 5.3: Total chlorophyll content of *Col-0*, *ore12-1*, *atcrr4*, AHK3-OX40 and *ahk3* whole shoots was measured after 7 days dark incubation.

The whole shoots of 5 week old short day grown *Col-0*, *ore12-1*, *atcrr4*, AHK3-OX40 and *ahk3* plants were excised and kept on wet filter paper in a Petri dish. The Petri dishes were sealed by parafilm, covered by two layers of aluminium foil. Total chlorophyll content was measured after 7 days dark incubation. No difference was observed between *atcrr4* and *Col-0*; *ahk3* and *Col-0*; AHK3-OX40 and *Col-0*. This is quite unexpected because according to the published paper of Kim *et al.* (2006), AHK3-OX40 should show a delayed senescence phenotype and *ahk3* should show an early senescence phenotype following dark incubation. The only mutant that behaved in the expected manner is *ore12-1*. As shown by Kim *et al.* (2006), *ore12-1* showed a delayed senescence phenotype and accordingly higher chlorophyll content than *Col-0* after 7 days dark incubation.
Error bars represent mean ± standard error (n = 6)

5.2.3 Over-expression of *AtCRR4* does not rescue the delay senescence phenotype of *ore12*

As mentioned in the introduction, the expression of *AtCRR4* has been shown to be reduced in 3-week old *ore12* leaves. The down-regulation of *AtCRR4* in *ore12-1* suggests two possibilities. One possibility is that *AtCRR4* expression is lower in *ore12-1* compared to *Col-0* simply because *ore12-1* shows a delay in senescence and *AtCRR4* is only highly expressed in senescing leaves, i.e. there is no direct link between the activation of the cytokinin receptor and the expression of *AtCRR4*. The other possibility is that *AtCRR4* expression is negatively regulated by the activated cytokinin receptor AHK3. So, the constitutively activated state of AHK3 in *ore12-1* plants constitutively down-regulates the expression of *AtCRR4*. In this case, *AtCRR4* could be a downstream target of AHK3 receptor.

To investigate if there is a direct link between the down regulation of *AtCRR4* expression and the delay senescence phenotype of *ore12,* I decided to generate *ore12-1* lines over-expressing *AtCRR4*. If *AtCRR4* were a downstream target of AHK3 receptor and if the down regulation of *AtCRR4* in *ore12* mutant were responsible for the delayed senescence phenotype of *ore12*, then over-expression of *AtCRR4* might rescue the delayed senescence phenotype of *ore12-1*.

As mentioned earlier, homozygous mutant seeds for *ore12-1* lines were kindly provided by Prof. Hong Gil Nam, Pohang University of Science and Technology, Pohang, Korea. To generate an *AtCRR4* over expressing line, an *AtCRR4:GFP* fusion was placed under the control of the CaMV 35s promoter. Homozygous transgenic lines were obtained in the T_3 generation and RT-PCR was performed using 4-week old leaves to confirm the level of expression of *AtCRR4*. To investigate whether overexpression of *AtCRR4* was able to rescue the delayed senescence phenotype of *ore12-1*, homozygous *ore12-1* plants were crossed to a *35S-AtCRR4:GFP* line where the transgene is associated with a hygromycin resistance gene. T_3 plants homozygous for the transgene were selected by plating seeds from the T_2 generation on medium containing hygromycin. These homozygous

resistant plants were then genotyped by sequencing to identify lines that were also homozygous for *ore12-1*.

Lines homozygous for *ore12-1* as well as the *35s-AtCRR4:GFP* transgene were grown in the long day growth room and compared with *Col-0* and *ore12* plants. However, as expected from previous experiments, no senescence phenotype was observed under these conditions. In my hands, the delayed senescence phenotype of *ore12* was only observed after dark induced senescence treatments. To assess the senescence phenotype of *ore12* plants over-expressing the AtCRR4:GFP fusion, the 3rd and 4th rosette leaves from 5-week old short day grown *Col-0*, *ore12* and *ore12* plants expressing AtCRR4:GFP were floated onto 3 mM MES buffer (pH 5.7) in the dark for 5 days. No difference was found between *ore12* mutants and *ore12* mutants expressing AtCRR4:GFP.

The inability of *AtCRR4* over-expressing line to rescue the *ore12* phenotype suggested that the down regulation of *AtCRR4* in *ore12* mutant is not directly associated with delayed senescence. In other words, the expression of *AtCRR4* is less in 3-week old *ore12* mutants than *Col-0* leaves possibly because *ore12* shows a delay in senescence and *AtCRR4* is only highly expressed in senescing leaves.

5.3 Discussion

Genes whose transcription increases in abundance during senescence are referred to as Senescence-Associated Genes (SAGs) (Lohman *et al.,* 1994). My RT-PCR results confirm that the transcript level of *AtCRR4* increases dramatically in abundance during senescence. Thus, *AtCRR4* can be considered as a SAG and may be implicated in *Arabidopsis* leaf senescence.

A comparison of the transcript level of the Arabidopsis *AtCRR4* gene at different stages of leaf development indicated that *AtCRR4* is first expressed in 4-

week old leaves, i.e. adult leaves (Fig 5.1). However, the transcript level goes up as the leaves start to senescence and at this stage, the expression of *AtCRR4* is very similar to that of the Arabidopsis senescence specific marker gene, *SAG12.* The gene, *SAG12* is considered to be a reliable marker for natural leaf senescence because its expression is strictly associated with senescence (Zhou *et al.*, 2009; Pontier *et al.*, 1999). The other senescence specific marker gene that I used was *SEN4.* Some authors suggested *SEN4* as a marker for dark induced senescence treatments (Park *et al.*, 1998; Lim *et al.*, 2010). In my RT-PCR, no major differences were observed in the abundance of *SEN4* transcript between samples generated from 4, 6 and 10 week old leaves. Therefore, my results suggested that *SEN4* is not a good marker for natural leaf senescence.

To investigate the role of *AtCRR4* in leaf senescence, the natural and dark induced senescence phenotypes of an *atcrr4* mutant were compared with those of *Col-0*, the known delayed and early senescence mutants *ore12-1, ahk3-1* and also with the over-expressed line AHK3-OX40 (Kim *et al.*, 2006). Unfortunately, in our growth room conditions *ore12-1, ahk3-1* and AHK3-OX40 behaved just like wild type and did not show delayed or early senescence phenotypes. In addition, although *ore12-1* showed a delayed senescence phenotype after dark treatment, no phenotype was observed in *ahk3-1* and AHK3-OX40 even under these conditions. The mutant *atcrr4* behaved just like wild type both during natural senescence and dark induced senescence. However, since the control lines *ahk3-1* and AHK3-OX40 did not show phenotypes, it is not strictly possible to draw any conclusions regarding the role of *AtCRR4* in leaf senescence. In the future, it would be useful to repeat natural senescence experiments in different growth chambers, and possibly also at different temperatures, since environmental differences may explain our inability to show the observed senescence phenotypes. It would also be interesting to investigate senescence phenotypes of other *atcrr4* alleles. As mentioned in Chapter-3, I have generated *35s-AtCRR4:GFP* and *35s-AtCRR4:MYC* lines to investigate protein-protein interactions. It might be interesting to compare the senescence phenotype of these over-expressed lines with *Col-0.*

As discussed in Chapter-1, the amino acid sequence of the AtCRR4 protein shows some similarity to the AtCRR3 protein and it is also quite closely related to the three other proteins of the ACR4 family (Fig 1.10). Therefore it is possible that AtCRR4 could act redundantly with AtCRR3 or with other ACR4 related receptor like kinases. The detail transcriptome data provided by van der Graaff *et al.* (2006) suggested that *AtCRR3* is highly expressed in both mature and senescing leaves. The analysis of the expression pattern of *AtCRR3* in leaves of different age using quantitative RT-PCR might be important to confirm this transcriptome data. It would also be interesting to investigate the senescence phenotype of the double mutant, *atcrr3/atcrr4* to test whether the senescence phenotype of *atcrr4* is masked by the expression of *AtCRR3*.

Leaf senescence is an integral part of plant development. Although my results indicate an increase in the accumulation of *AtCRR4* transcript in Arabidopsis leaf in an age dependent manner, the function of this gene in the progression of senescence is still unknown. To identify the precise roles of this novel RLK gene, the following questions need to be addressed.

How does *AtCRR4* behave under various senescence-affecting conditions?

It is already known that the senescence associated genes are differentially regulated by different senescence-inducing factors, for example - ABA, ethylene, cytokinin, methyl jasmonate, wounding, dehydration and dark treatment (Park *et al.*, 1998; Weaver *et al.*, 1998). Miller *et al.* (1999) reported that the expression of the well characterized senescence specific marker, *SAG12* is not induced by chronic ozone treatment, although it is induced during natural senescence process. Therefore, to further test the correlation between *AtCRR4* gene expression and leaf senescence, it would be interesting to test the expression of *AtCRR4* during different artificially induced senescence treatment, for example - dark treatment or hormonal treatment. This could help us to determine whether *AtCRR4* acts as a universal marker for senescence.

Does cytokinin regulate the expression of *AtCRR4*?

As mentioned earlier, a possible link between *AtCRR4* expression and cytokinin was predicted as a close ortholog of *AtCRR4* in tobacco, *CRK1* is regulated by cytokinin. Cytokinins are well-known as anti-senescence factors (Gan and Amasino, 1997). To investigate whether *AtCRR4* acts in the same pathway as the senescence associated cytokinin receptor AHK3, I tried to rescue the delayed senescence phenotype of *AHK3* gain of function mutant, *ore12* by overexpressing *AtCRR4* using the *35s* promoter. No rescue was observed; an observation which could be explained in a variety of ways. Firstly, reduced *AtCRR4* expression simply might not be the cause of the delayed senescence phenotype of *ore12-1* mutants. However, it should also be noted that although the expression of the *35s* promoter is generally considered ubiquitous, it is not clear that high levels of gene expression from this promoter are maintained immediately prior to, and during senescence. So, the inability of *35s-AtCRR4* to rescue the delayed senescence phenotype of *ore12* is not a definite proof that overexpression of AtCRR4 during senescence cannot rescue *ore12-1*. It should also be borne in mind that large numbers of genes are mis-regulated in *ore12-1* mutants, various combinations of which might explain the delayed senescence phenotype that they exhibit. Further experiments are required to understand the involvement of cytokinin in regulating the expression of *AtCRR4*.

Is *AtCRR4* involved in defence responses?

As mentioned earlier, some senescence specific genes have been identified as defence-related genes and some overlap between these two pathways has been suggested (Quirino *et al.*, 2000). An RLK in *Arabidopsis*, *SIRK* has been shown to be associated with both senescence and pathogen defence (Robatzek and Somssich, 2002; Asai *et al.*, 2002). The expression of *SIRK* is induced within 30 minutes of the application of flg22, a peptide representing the elicitor-active domain of bacterial flagellin (Asai *et al.*, 2002). *In silico* gene expression analysis also revealed that *AtCRR4* is rapidly upregulated to a significant level by flg22 (Zipfel *et al.*, 2004),

which is discussed in Section 6.1.2. Thus, *AtCRR4* could be involved in both senescence and defence associated cell death pathways.

In summary, my study revealed a possible role of an RLK, *AtCRR4* in senescence. At the present time, this is solely based on the expression of *AtCRR4* during leaf senescence, since experiments aimed at analyzing senescence phenotypes proved difficult under our growth conditions. RLKs are thought to play a key role in perception and transduction of extracellular signals. Major challenges for the future include deciphering the biological functions of the *AtCRR4* gene product. In addition to *AtCRR4*, only one other receptor-like kinase in *Arabidopsis* has been specifically shown to be linked to leaf senescence and accordingly named *SIRK* (Robatzek and Somssich, 2002). The role of the signal transduction pathways mediated by both SIRK and AtCRR4 remain unclear. Understanding the targets of AtCRR4 signalling could help us to unlock the mysteries of this RLK mediated signalling pathway during senescence.

Chapter – 6

Determining the role of the developmental regulator, *ACR4* & its related kinases in pathogen perception

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Chapter - 6

Determining the role of the developmental regulator, *ACR4* & its related kinases in pathogen perception

6.1 Introduction

Precise activation of Receptor Like Kinases (RLKs) plays a vital role in both development and the response to pathogens in all higher organisms. However, relatively little is known about how these activities are controlled. Here, the possibility of the involvement of *ACR4* in the plant immune response is investigated.

6.1.1 Dual regulators: development & defence

In both plant and animal systems, some developmentally important receptors also play roles in defence or disease resistance (Aderem *et al.*, 2000; Chinchilla *et al.*, 2007). In animal systems, the first dual-function receptor to be identified was TOLL from *Drosophila melanogaster*, which is not only involved in dorsal-ventral patterning (Hashimoto *et al.*, 1988) but also in defence against fungal and bacterial infections (Lemaitre *et al.*, 1996; Michel *et al.*, 2001). The mammalian orthologues of TOLL, which contains a leucine-rich repeat (LRR) domain (Medzhitov *et al.*,

1997), are known as TOLL-like receptors (TLRs) (Rock *et al.,* 1998). Although TLRs are mainly known as initiators of the innate immune response to pathogens (Aderem *et al.,* 2000), recent reports revealed the intriguing roles of TLRs in the development of the nervous system (Larsen *et al.,* 2007). Thus, both TOLL in *Drosophila* and TLRs in mammals, are involved in development as well as in host defence (Aderem *et al.,* 2000).

In plants, similar to their animal counterparts, several leucine-rich repeat domain containing receptors play dual roles in development and defence. Some of these LRR receptors have an intracellular kinase domain, and are known as LRR-Receptor Like Kinases (LRR-RLKs). In *Arabidopsis*, the LRR-RLKs represent the largest class of RLKs, consisting of over 200 members, and can be subdivided into 13 subfamilies (Shiu and Bleecker, 2001). With some notable exceptions, the functions of these RLKs are largely unknown. One *Arabidopsis* LRR-RLK, BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE1 (BAK1) mediates both developmental and defence related signalling (Nam and Li, 2002; Li *et al.*, 2002; Chinchilla *et al.,* 2007). Similarly, *ERECTA*, a second *Arabidopsis* LRR-RLK which regulates organ shape, inflorescence architecture and stomatal patterning (Shpak *et al.,* 2003, 2004, 2005), has also been found to be involved in resistance to bacterial wilt (Godiard *et al.,* 2003). Dual functions of LRR motif containing receptors are also present in other plant species. In tomato the systemin receptor SR160, a membrane-localized LRR-RLK, plays role in systemic wound response mediated by systemin (Scheer and Ryan, 1999), a signalling molecule involved in defence responses. SR160 is the tomato ortholog of *Arabidopsis* BRI1 (Scheer and Ryan, 2002) and, like its *Arabidopsis* orthologue, is also thought to be involved in brassinosteroid signalling (Montoya *et al.,* 2002).

Although recent studies reveal an increasing number of RLKs implicated in both disease resistance and development, our understanding of how an RLK playing a central role in development fits into defence-related signalling cascades is still unclear. RLK mediated signalling could involve potential cross talk between disease and developmental pathways or each RLK could recognize multiple ligands,

resulting in the formation of multiple different homodimer and heterodimer combinations and activation of multiple discrete signalling cascades. As mentioned earlier, BAK1 acts as a co-receptor of the brassinolide perceiving receptor, BRI1 and, in this capacity, plays roles in plant developmental regulation (Nam and Li, 2002; Li *et al.,* 2002). BAK1 also forms a complex with the flagellin receptor FLS2, to control plant immunity (Chinchilla *et al.,* 2007). Thus BAK1 acts as a common receptor for these two distinct signalling pathways in a specific ligand/partner dependent manner. Detailed analysis of other plant RLKs could improve our global understanding of how a single receptor can be involved in multiple biochemical pathways.

6.1.2 *ACR4* and its related kinases: A possible role in defence

As mentioned in Chapter-1, our current understanding of ACR4 receptor is limited to its role in plant development. Although no published data are available on the role of *ACR4* or its orthologs in plant defence, in the last few years microarray data (Zimmermann *et al.*, 2004; Winter *et al.,* 2007) have revealed that *ACR4* and related genes are differentially expressed in response to different pathogens (Fig 6.1). This suggests a possible involvement of the members of the *ACR4* gene family in plant immunity. Evidence for a potential role of *ACR4* related genes in defence response came from the study of Zipfel *et al.* (2004, 2006). Genome wide expression analysis of *Arabidopsis* genes in response to flg22, a peptide representing the elicitor-active domain of bacterial flagellin, showed that two of the *ACR4* related genes, *AtCRR3* and *AtCRR4* are upregulated to a significant level within 30 minutes of flg22 treatment (Zipfel *et al.,* 2004). Interestingly, a similar transcriptional up regulation of *AtCRR3* and *AtCRR4* was also observed following treatment with a second peptidic elicitor, elf18, which represents an active fragment of bacterial EF-TU, a pathogen associated molecular pattern (PAMP) recognized by *Arabidopsis* EF-TU RECEPTOR (EFR) (Zipfel *et al.,* 2006). This rapid up regulation of *AtCRR3* and *AtCRR4* in response to two different bacterial elicitors indicates that these genes could be involved in defence responses. A separate microarray analysis suggested that the expression of *ACR4* goes up within 4 hours of inoculation with the virulent

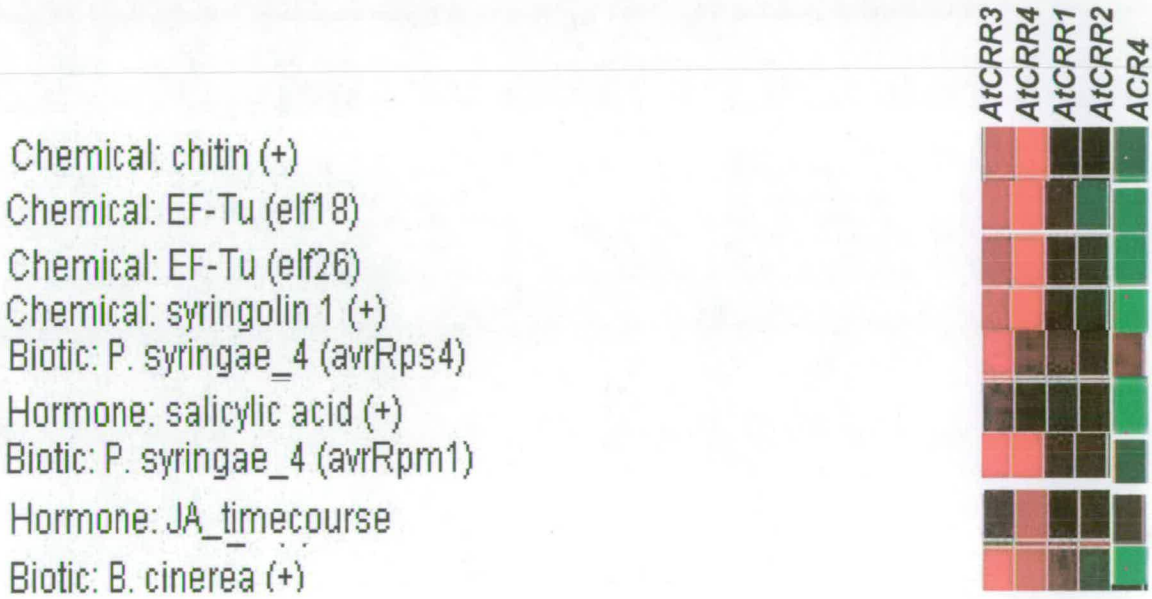


Fig 6.1: Expression pattern of *ACR4* and *ACR4* related genes in response to pathogen, pathogen associated molecular pattern & hormones involved in plant defence (Zimmermann *et al.*, 2004).

bacterium, *Pseudomonas syringae* pv *maculicola* (Winter *et al.*, 2007). *ACR4* and its related kinases are also differentially expressed in response to *B. cinerea,* for example, *ACR4* expression is down regulated while *AtCRR3* and *AtCRR4* genes are significantly upregulated (Fig 6.1) (Zimmermann *et al.*, 2004). Thus, in response to the biotropic pathogen *Pseudomonas syringae,* the expression of *ACR4* is upregulated. However, in response to necrotrophic pathogen *B. cinerea, ACR4* is down regulated. In contrast, the expression of both *AtCRR3* and *AtCRR4* increases in response to both biotropic bacterial elicitors and necrotrophic pathogens. Although far from conclusive, together these transcriptome data suggest that *ACR4* and related genes which are known to be involved in development, could also have a role in plant defence. In this capacity, members of the *ACR4* gene family could be involved in primary plant-pathogen interactions by recognizing bacterial or fungal elicitors, or could be regulated as a downstream response to primary pathogen perception.

In order to clarify the potential involvement of *ACR4* in plant immunity, several approaches were taken. Firstly, *acr4* null mutants were challenged with *Botrytis cinerea,* a necrotrophic fungal pathogen, to identify how this mutant behaves in response to the pathogen. Secondly, any possible defects in the epidermal layer of the *acr4* null mutant leaves were analyzed to determine if the defective cuticular layer plays a role in plant immunity. Finally, a direct role for *ACR4* in pathogen response was investigated.

6.2 Results

6.2.1 A mutation in *ACR4* shows reduced susceptibility to *B. cinerea*

As mentioned earlier, the expression of *ACR4* shows a decrease in response to *B. cinerea,* a necrotrophic pathogen (Fig 6.1), (Zimmermann *et al.*, 2004). Interestingly, a similar down regulation of *ACR4* was observed in an unpublished transcriptome data provided by Dr. Katherine Denby, University of Warwick, UK (personal communication). This suggested a possible role for *ACR4* in defence against *B. cinerea.* To understand if there is any potential link between the function

of *ACR4* and susceptibility to *B. cinerea*, it was decided to inoculate both wild type and *acr4* null mutants with *B. cinerea* spores.

Pathogen challenge experiments are not usually carried out in the Ingram lab. As a result, several different approaches were taken to optimize the technique of inoculating plants with the pathogen. The glycerol stock of *B. cinerea* was kindly provided by members of the laboratory of Professor Gary Loake, IMPS, University of Edinburgh, UK.

Initially 6-week old short day grown wild type and *acr4* mutant plants were sprayed with a *B. cinerea* spore suspension (as described in Nurmberg *et al.,* 2007). It was found that the size and the number of droplets of fungal suspension on different leaves were not equal, making samples difficult to compare. Therefore, it was hard to draw any conclusion from these experiments. As an alternative means of ensuring equal number and equal size of droplets, it was decided to inoculate detached leaves with 5µl droplets of the spore suspension. Ferrari *et al.* (2003) previously used this technique to inoculate *Arabidopsis* leaves with *B. cinerea.* Detached leaves from 6-week old, short day grown, wild type and *acr4* mutant plants were placed onto wet filter papers and were inoculated with two 5µl droplet of the fungal suspension. Although this approach ensured that all the leaves were equally inoculated, the limitation was that some of the leaves were in closer contact with the wet filter paper than others. Controls showed that the wet filter paper itself could cause leaf yellowing. As a result it was hard to conclude if the yellowing of leaves was due to the fungal infection or simply because the leaves were in close contact with the wet filter paper. This suggested that the drop inoculation onto detached leaves is not entirely satisfactory. To ensure the best consistency, it was therefore decided to inoculate the 6-week old attached leaves of wild type *Col-0* and *acr4* mutant with two droplets of the 5µl fungal suspension. As a second control, an *asymmetric leaves1* (*as1*) mutant line was used, which had previously been reported to show resistance to *B. cinerea* (Nurmberg *et al.,* 2007). The progression of disease was monitored and photographs were taken 5 days after inoculation, it was found that disease symptoms were less severe on *acr4* mutant leaves than on the wild type

leaves (Fig 6.2) and no difference was observed between *Col-0* and *as1* (Fig 6.3).

To assess disease symptom development, plants were scored for their disease severity three days after inoculation, using a scale of 0 to 5. Scoring was adapted from You *et al.* (2009), where 0= no necrotic lesions, 1= plants showing small dry lesions, 2= plants showing a mix of small & medium size lesions, 3= plants showing medium size and spreading lesions, 4= plants showing predominantly spreading lesions, 5= plants showing predominantly wide necrotic lesions. Scoring overall symptom development with a disease index showed that *acr4* mutants were less susceptible to *B. cinerea* than wild-type (Fig 6.4).

To facilitate visualization of cell death caused by the *B. cinerea* infection and to provide a more satisfactory means of quantifying disease progression, leaf samples were stained with trypan blue (Fig 6.5), (Koch and Slusarenko, 1990) and the ImageJ program (http://rsbweb.nih.gov/ij/) was used to measure the area of necrosis. Surprisingly, the area of necrosis in the *acr4* mutant leaves was not significantly different to that in *Col-0* plants (Fig 6.6). In addition, the area of necrosis of *acr4* is quite similar to the control line, *as1*, despite the fact that it has previously been reported that *as1* shows resistance to *B. cinerea* (Nurmberg *et al.*, 2007). However, assessment of the progression of fungal growth by scoring disease symptom clearly indicated that *acr4* mutants are less susceptible compared to wild type. It would therefore be interesting to measure the area of necrosis at different time points after fungal infection as this could provide a more meaningful result.

As another means of quantifying disease development, attempts were made to assess the extent of hyphal growth by determining the accumulation of RNA derived from a *B. cinerea TUBULIN* gene. The expression of *B. cinerea TUBULIN* directly correlates with the extent of *B. cinerea* growth (Benito *et al.*, 1998). As the *B. cinerea TUBULIN* gene shares considerable similarities with the plant *TUBULIN* gene, pairwise sequence alignment was performed at the NCBI website using BLAST (Altschul *et al.*, 1990) and primers were designed that were specific to a *B. cinerea β-TUBULIN* gene (GenBank accession number Z69263).

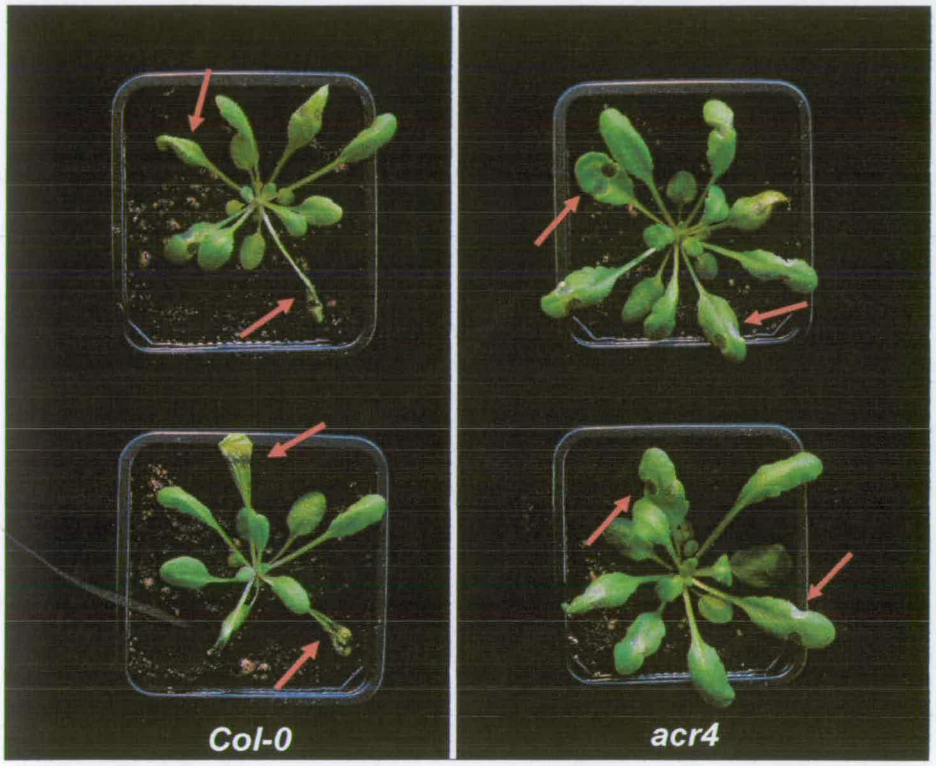


Fig 6.2: The mutant, *acr4* exhibits decreased susceptibility to *B. cinerea.*

The *Arabidopsis* lines *Col-0* and *acr4* (6-week old short day grown) were challenged with two droplets of the 5µl spore suspension of *B. cinerea.* Photograph was taken 5 days after inoculation ($n = 6$ and the experiment was repeated three times with similar results).

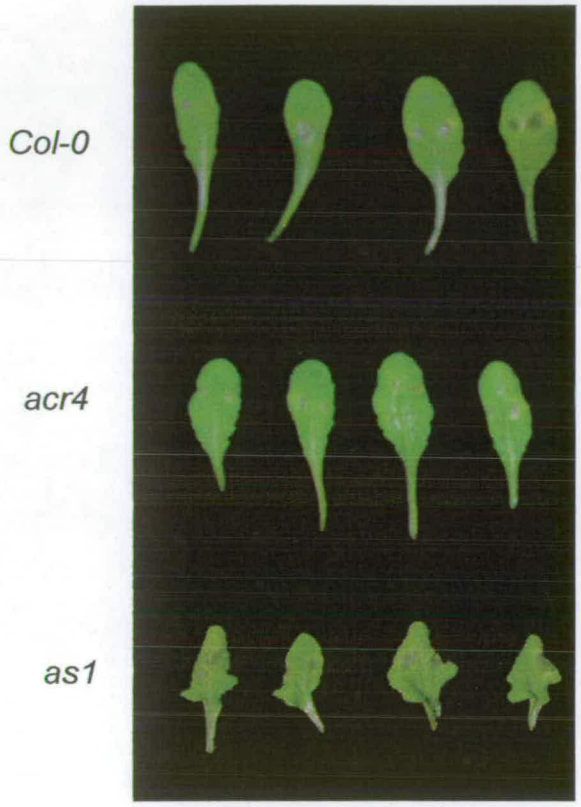


Fig 6.3: The *acr4* mutant exhibits decreased susceptibility to *B. cinerea.*

The *Arabidopsis* lines *Col-0, acr4* and *as1* (6-week old short day grown) were challenged with two droplets of the 5µl spore suspension of *B. cinerea.* Photograph was taken 3 days after inoculation. Although *acr4* shows decreased susceptibility to *B. cinerea* (the photograph is not very clear), no difference has been observed between *Col-0* and *as1.*

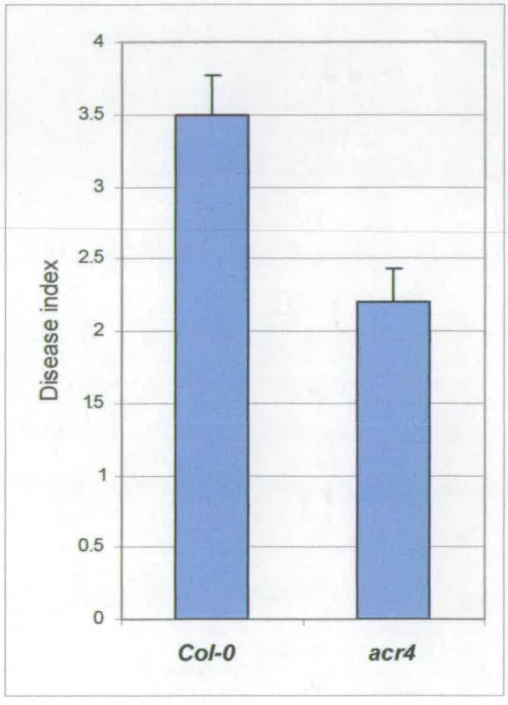


Fig 6.4: Scoring overall symptom development with a disease index indicated that *acr4* mutants show reduced susceptibility to *B. cinerea*.

Col-0 and *acr4* plants were challenged with a spore suspension of *B. cinerea* and scored for disease development at 3 days after inoculation. Scoring overall symptom development is based on 0= no necrotic lesions, 1= plants showing small dry lesions, 2= plants showing a mix of small & medium size lesions, 3= plants showing medium size and spreading lesions, 4= plants showing predominantly spreading lesions, 5= plants showing predominantly wide necrotic lesions. Error bars represent mean ± standard error (n = 15).

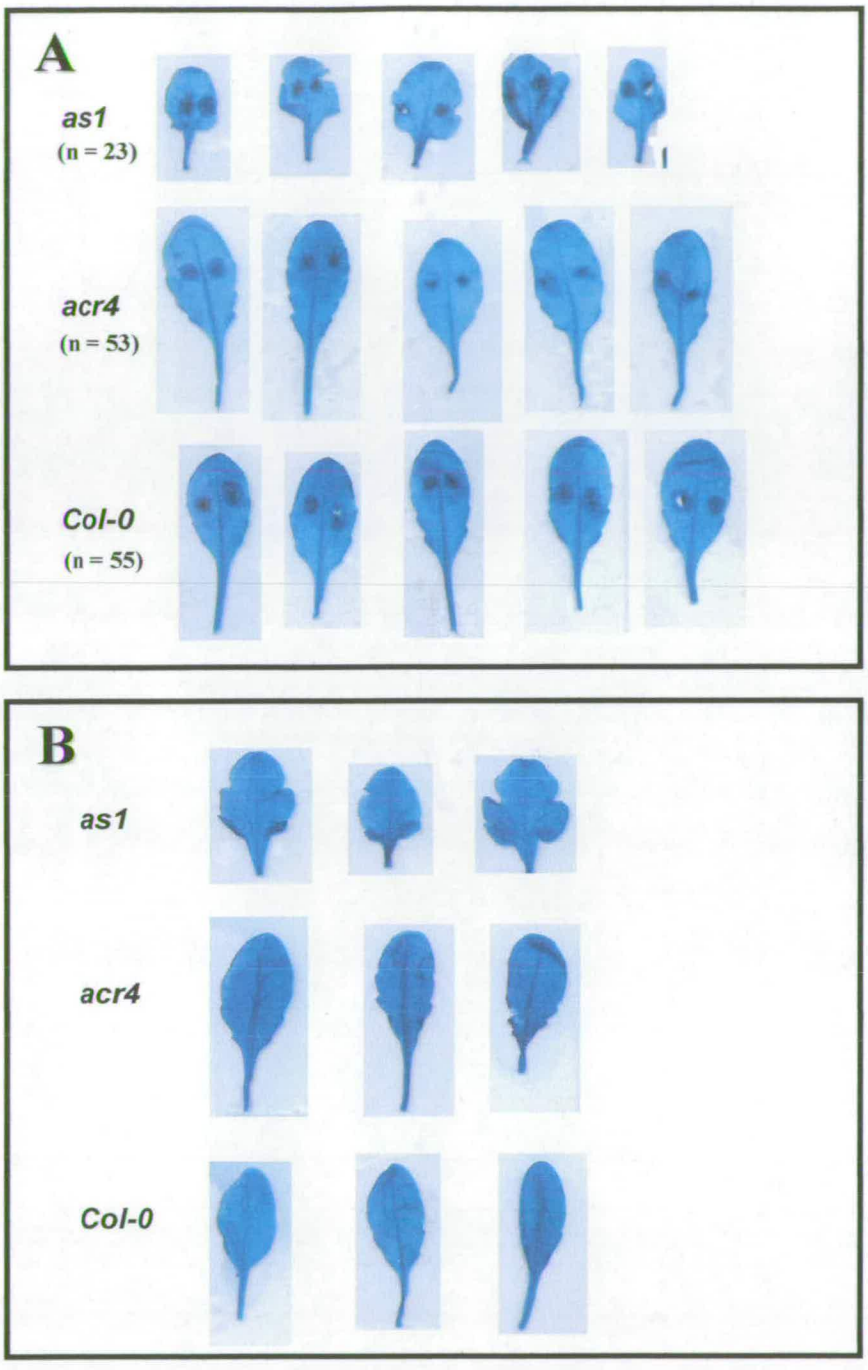


Fig 6.5: Trypan blue stained leaves, which marks dead plant cells, revealing lesion development.

6-week old short day grown *Col-0*, *acr4* and *as1* plants were challenged with either two droplets of a spore suspension of *B. cinerea* (A) or mock inoculation solution that lacked spores (B). After 4 days of inoculation, leaves were stained with trypan blue.

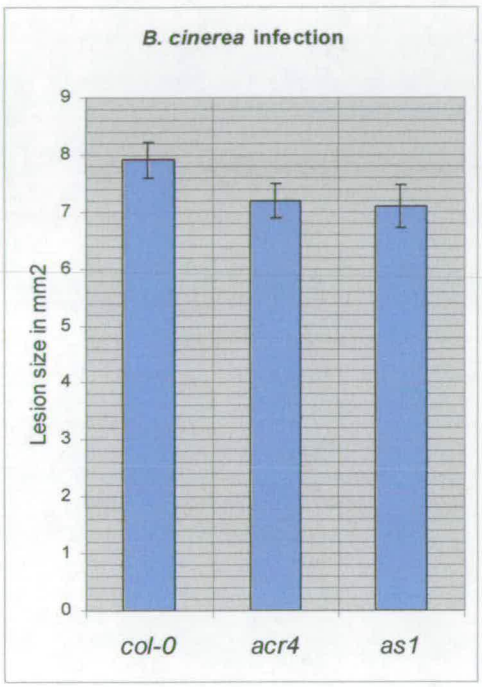


Fig 6.6: Quantification of the extent of *B. cinerea* generated lesions in the indicated plant genotypes.

The *Arabidopsis* lines *Col-0*, *acr4* and *as1* were drop-inoculated with a *B. cinerea* spore suspension and leaves were stained with trypan blue after 4 days of inoculation and the lesion size was measured using ImageJ program (n= 23-55). Error bars represent mean ± standard error.

Table 2.3 shows details of the primer sequences. Total RNA was extracted from *Col-0* and *acr4* mutant leaves without fungal infection and 4 days after fungal inoculation. Although our RT-PCR clearly indicated increased expression of the fungal *TUBULIN* gene in *Col-0* compared to *acr4* after fungal inoculation, no expression was found for the control plant tubulin gene after fungal infection. One possible reason could be the degradation of plant RNA by *B. cinerea* mediated necrosis. Publicly available transcriptome data available on the eFP browser (Winter *et al.,* 2007)) suggests that the expression of plant *TUBULIN BETA CHAIN 3* (*TUB3*, *AT5G62700*) is down-regulated 48 hours after *B. cinerea* infection. This could also explain why no expression was detectable for plant *TUB3* gene at 4 days after inoculation. It would be interesting to determine the expression of plant *TUB3* gene as well as fungal *TUBULIN* gene in the fungal inoculated leaves at earlier time points, however time constraints, and difficulties in generating satisfactory plant material meant that this was not possible.

6.2.2 Do *acr4* leaves have any defect in the leaf cuticular layer?

The leaf cuticle is generally considered to act as a physical barrier against pathogens (Martin, 1964). However, in recent years several publications have reported a potential link between the presence of a permeable cuticle layer in *Arabidopsis* and resistance to *B. cinerea* (Bessire *et al.,* 2007; Tang *et al.,* 2007; Chassot *et al.,* 2007). By analyzing different cuticular defective mutants, Voisin *et al.* (2009) suggested that mutants with increased cuticular permeability, assessed in terms of chlorophyll leaching rate and toluidine blue permeability, show enhanced resistance to *B. cinerea* compared to the wild type plants. However, why the presence of abnormally permeable cuticle provides protection against *B. cinerea* is still unclear.

As mentioned earlier, *ACR4* is expressed in epidermis-related tissues of various organs, including leaves (Tanaka *et al.*, 2002; Gifford *et al.*, 2003). The *acr4* mutant leaves look similar to wild type. No cuticular defect has yet been reported for mature *acr4* mutant leaves. However, investigation of younger leaves revealed that

young *acr4* mutant leaves and cotyledons (18 days old) are more permeable to the hydrophilic dye, toluidine blue than wild-type (Watanabe *et al.,* 2004). This suggested that the mature *acr4* mutant leaves could have defect in the cuticular layer and that this might explain the observed resistance phenotype of *acr4* to *B. cinerea.* To investigate the leaf cuticular permeability of mature leaves, several indirect approaches were taken in the lab. These included leaching of chlorophyll from leaves, staining of leaves with toluidine blue and transpirational water loss. To further understand the type of leaf cuticular defect that was being observed, the analysis of the cuticular wax composition in *acr4* mutant leaves by electron spray mass spectrometry, and comparison of the cuticular wax composition with mutants of known cuticular defects, has recently been initiated.

6.2.2.1 An *acr4* mutant shows more rapid chlorophyll leaching compared to wild type

As an indirect means of estimating leaf cuticular permeability, I performed an assay which measures chlorophyll leaching into alcohol (Bessire *et al.*, 2007). Four week old rosettes were excised at the hypocotyl and immersed in 80% ethanol. Chlorophyll leaching was measured spectrophotometrically. Fig 6.7 shows that *acr4* leaves lost chlorophyll faster than comparable *Col-0* leaves. This rapid chlorophyll leaching suggested that mature *acr4* leaves could have some defect in the leaf cuticle.

6.2.2.2 The *acr4* leaves are permeable to toluidine blue

To further assess the permeability of the cuticle, leaves were stained with toluidine blue (Tanaka *et al.*, 2004). Toluidine blue is a hydrophilic dye which can only enter leaf tissues in the presence of a discontinuous or permeable leaf cuticular layer.

The toluidine blue staining was performed as described by Bessire *et al.* (2007). Four week old long day grown *acr4* and *Col-0* plants were used for this

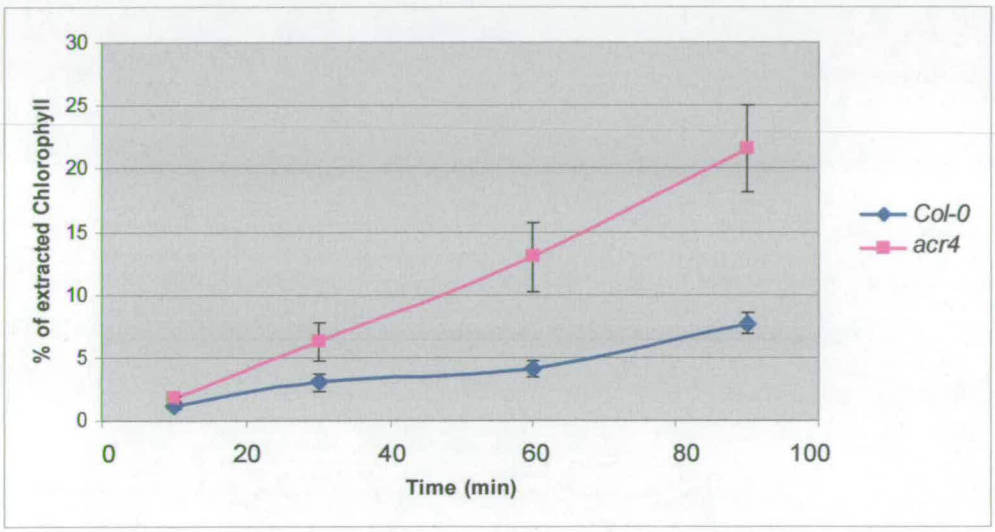


Fig 6.7: Measurement of chlorophyll leaching from *Col-0* and *acr4* rosette leaves.

Four week old long day grown rosettes were excised at the hypocotyl and immersed in 80% ethanol. Chlorophyll leaching was measured spectrophotometrically. The results are mean % values ± standard error of at least six replicates.

study. The toluidine blue staining solution was directly applied onto the adaxial side of the leaf as 5µl droplets, left overnight and then washed with water. It was found that *acr4* mutant leaves were stained with the dye, but no staining was found for *Col-0* leaves (Fig 6.8). This experiment was carried out once (n = 6-8). This study corroborated and extended the findings that *acr4* mutants could have defective cuticular layer which is permeable to toluidine blue as previously shown by Watanabe *et al.* (2004) using 18 days old plants.

6.2.2.3 The *acr4* leaves do not show enhanced cuticular water loss

The plant cuticle, covering the outer epidermal layer, plays an important role in protecting the plant from water loss (Goodwin and Jenks, 2005). Some mutants with permeable cuticles loose water more quickly than wild type plants. One such mutant is *lacs2-3* which has been shown to have an abnormally high cuticular transpiration rate (Bessire *et al.*, 2007).

To measure the water loss from *acr4* leaves, five week old, long day grown rosettes were excised at the hypocotyl and water loss was measured by weighing plants at different time points. Loss of water was calculated as a percentage of the total water content and 100% was considered to be the total water content of a plant (Fig 6.9). No significant difference was found between *Col-0* and *acr4*.

6.2.2.4 Examining the cuticular wax composition of *acr4* mutant leaves

Results from chlorophyll leaching and toluidine blue staining experiments suggested that *acr4* mutant leaves could have defects in cuticular lipid composition.

To investigate the cuticular wax composition of *acr4* mutant leaves, an approach based on Electrospray ionization mass spectrometry (ESI-MS) was planned in collaboration with Dr Logan Mackay, SIRCAMS Facility Director, School of Chemistry, University of Edinburgh, UK. The ESI-MS technique is one of the most

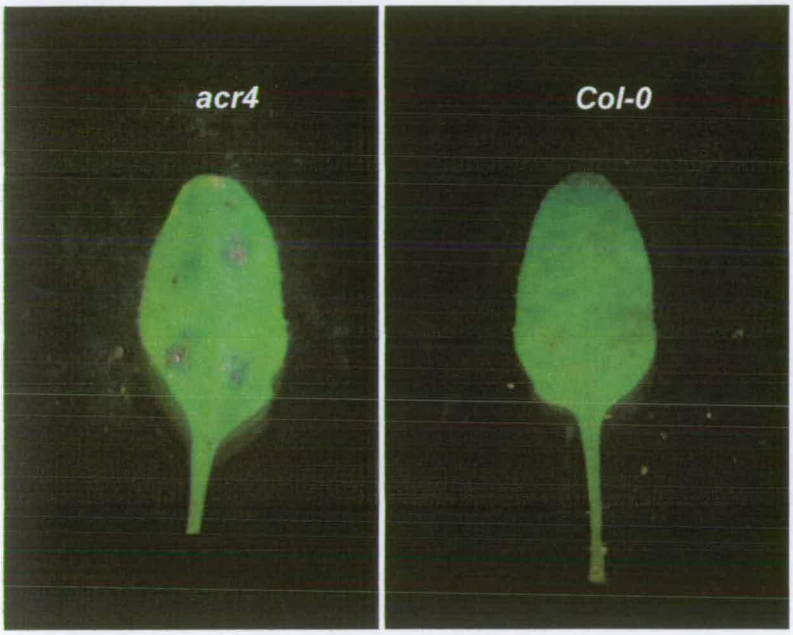


Fig 6.8: Permeability of *acr4* leaves to toluidine blue.

Droplets of a toluidine blue solution were incubated on the leaves of 4 week old long day grown plants overnight and then washed with water (n = 6-8).

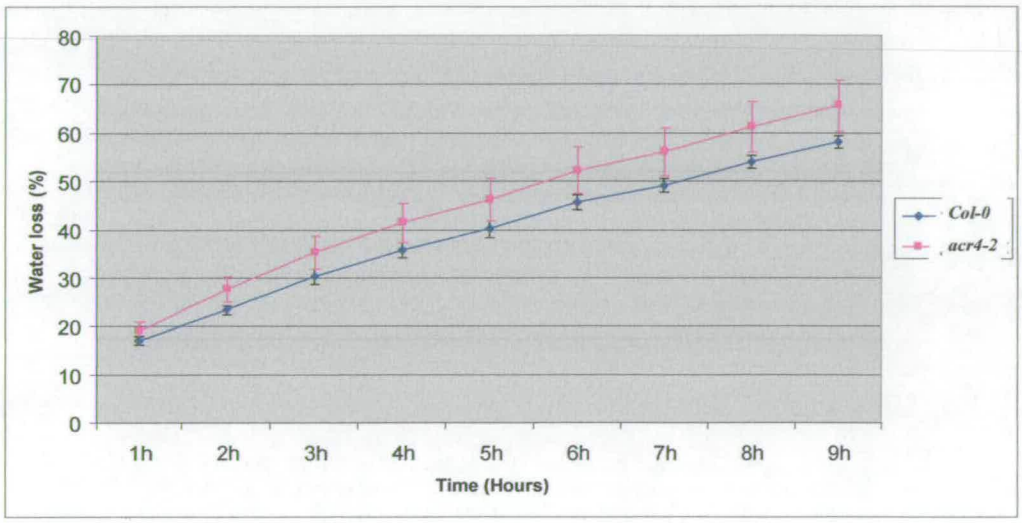


Fig 6.9: Measurement of cuticular water loss from *Col-0* and *acr4* rosette leaves.

Loss of water from 5 week-old long day grown rosettes of *Col-0* and *acr4* plants was measured at the indicated time points. The results are mean % values ± standard error of at least six replicates (the experiment was repeated twice with similar results).

sensitive, discriminating and direct methods for the qualitative and quantitative analysis of lipids from biological samples (Han and Gross 1994; Kim *et al.,* 1994). Although the technique has so far been successfully used to analyze lipid composition in yeast (Schneiter *et al.,* 1999) and human cell membranes (Han and Gross, 1994), nobody has yet used this technique to analyze the lipid composition of plant cuticles. This is surprising, since this method has the advantage that it allows the detection of very large range of molecules compared to the Gas Chromatography-MS techniques used to analyze cuticles to date, which has a maximum size detection limit of around 600 kDa. It is entirely possible that whole classes of high molecular weight molecules present in plant cuticles have not yet been characterized. We therefore aimed to use ESI-MS both to investigate leaf cuticular composition of *acr4* mutant leaves as well as a means to develop a new technique for the use of ESI-MS for both qualitative and quantitative analysis of leaf cuticular lipid composition in plants.

To extract cuticular waxes, leaves of similar age and area, grown in identical conditions, were immersed in 5ml of pure chloroform (Sigma) for 30 seconds and then the extract was dried-down using a speed vac. with a condensing trap (Javelle, 2009). Dried samples were sent to the Logan lab, School of Chemistry, University of Edinburgh for ESI-MS analysis.

To further investigate the altered properties of the cuticle of *acr4* mutants, I decided to compare the wax composition of *acr4* mutant leaf cuticle with a number of *Arabidopsis* mutants known to be defective in the biosynthesis of cuticular polyesters. Homozygous mutant seeds of *lacerata* (*lcr*) and *bodyguard* (*bdg*) and heterozygous mutant seeds of *fiddlehead* (*fdh*) were kindly provided by Alexander Yephremov, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany. All these three mutants are defective in the biosynthesis of cuticular polyesters and show an over-accumulation of cuticular wax and cutin monomers (Kurdyukov *et al.,* 2006; Voisin *et al.*, 2009). Comparing the lipid compositions of *acr4* mutant leaves to the known cuticular defective mutants could help us to understand defects in the lipid composition of *acr4* leaves and this could ultimately open the door to investigate whether certain types of cuticular defect allow rapid diffusion of antifungal

compounds or elicitors or act as a physical barrier for the penetration of fungal hyphae. Analysis of the lipid extracts from *Col-0, acr4, fdh*, *lcr*, and *bdg* has recently been started in the Logan lab.

6.2.3 How does *acr4* behave in response to biotropic pathogen, *Pseudomonas syringae*?

To test whether *ACR4* function affects responses to other pathogens, *acr4* mutant plants were challenged with the virulent biotropic bacterium, *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000. The experiment performed by another lab (Rüdiger Simon, Heinrich-Heine Universität, Germany personal communication) suggested that *acr4* mutant plants are slightly more susceptible to *P. syringae* compared to wild type. These experiments need to be repeated, but problems with strain contamination made this impossible. However, if it is confirmed that *acr4* mutant plants show an increased susceptibility to *Pseudomonas syringae,* this would indicate that *ACR4* operates as a positive regulator of defence responses against *P. syringae*. Our studies have shown that *ACR4* operates as a negative regulator of defence responses against the necrotrophic pathogen, *B. cinerea*. The antagonistic behaviour of *acr4* mutants to the necrotrophic pathogen *B. cinerea* and biotropic pathogen *P. syringae* is interesting. It is already known that resistance to biotropic and necrotrophic pathogen involves different and to some extent, antagonistic signalling pathways (Thomma *et al.,* 1998). It is possible that *ACR4* could act as an early response gene to pathogen attack and regulate particular hormonal signalling pathway.

6.2.4 Transcriptome profiling of *acr4*

Our pathogen challenge experiments indicated that *acr4* mutant plants are less susceptible to *B. cinerea* than wild type (Fig 6.2) and data from both publicly available resources, and the Denby Lab, University of Warwick, UK suggests that

ACR4 expression level starts to go down soon after inoculation with *B. cinerea.* Based on these observations, several alternative hypotheses were proposed. One possibility is that *acr4* mutants show constitutive up regulation of some pathogen response pathways, leading to decreased susceptibility to some pathogens. An alternative hypothesis is that ACR4, being a receptor, could interact with other proteins that are normally involved in repressing defence responses in the absence of attack, preventing a premature increase of immune responses and associated costs. In either case, it is logical to propose that *ACR4* expression could be down-regulated as part of the response to pathogen attack.

I was interested to know whether *acr4* mutants showed increased expression of genes known to be involved in pathogen-induced defence responses. Due to financial constraints, we were unable to carry out transcriptome profiling of the *acr4* and wild type leaves prior-to and after *B. cinerea* infection to assess changes in gene expression. As an alternative, I looked at the microarray results from a preliminary study undertaken by previous members of the Ingram Lab in 2004, where total RNA was extracted from floral meristems of wild-type and *acr4* plants. This microarray data was sub-optimal for our studies, firstly because it measured differential gene expression between wild type and *acr4* floral meristem tissues, not leaf tissues, and secondly because there were no biological replicates. However, the data appeared to be of high quality, based on internal controls and I therefore used it to ascertain whether any enrichment of genes known to be involved in defence responses was detectable. The microarray data showed that about 40 genes were significantly up-regulated and 22 genes (including *ACR4)* significantly down-regulated in the mutant compared to wild type. Among the up-regulated genes, one interesting candidate gene which was found to be upregulated in the *acr4* mutant was *LIPOXYGENASE2* (*LOX2*). *LOX2* encodes an essential enzyme in jasmonic acid biosynthesis, and its level of expression is correlated with the level of jasmonic acid production (Bell *et al.,* 1995).

To test whether the elevated expression of *LOX2* in *acr4* floral meristem was also reflected in *acr4* leaves, RT-PCR was performed using wild type and *acr4* leaf

samples prior to pathogen infection. No major difference was found in the expression of *LOX2* between wild type and *acr4* mutant (Fig 6.10) which suggested that the over-expression of *LOX2* in *acr4* floral meristem was not maintained in *acr4* leaves.

6.3 Discussion

In this chapter, I have shown that *acr4* mutant plants are less susceptible to *B. cinerea* infection than wild-type plants. This resistance phenotype is quite striking, given the conventional view that RLKs are normally involved in mediating resistance responses to pathogens. The mechanism underlying this resistance phenotype is still unclear. In recent years, research on cuticular defective mutants has revealed that several distinct *Arabidopsis* lines with defective cuticle are resistant to *B. cinerea* (Bessire *et al.*, 2007; Tang *et al.*, 2007; Chassot *et al.*, 2007; Voisin *et al.*, 2009). Chassot *et al.* (2007) reported that transgenic *Arabidopsis* plants constitutively expressing fungal cutinase targeted to their cell walls (CUTE plants) show resistance to *B. cinerea* and that this resistance is independent of the defence related signalling pathways involving jasmonic acid, ethylene or salicylic acid. Resistance to *B. cinerea* is thought to involve the jasmonic acid and ethylene signalling pathways (Thomma *et al.*, 1998; Alonso *et al.*, 2003). Kurdyukov *et al.* (2006) have shown that cuticle defective *bodyguard* (*bdg*) mutants are resistant to *B. cinerea* and the mutant exhibits enhanced accumulation of cuticular waxes compared to the wild type plants. Detailed analyses of *bdg* and CUTE plants have suggested that the modification of cuticle in these plants may allow release of antifungal compounds onto the leaf surface. A direct link between permeable cuticle and resistance to *B. cinerea* came from the study of Bessire *et al.* (2007). They have shown that permeable cuticle allows diffusion of antifungal compounds that interfere with fungal growth and hence the mutants with increased permeability are resistant to *B. cinerea.* A detailed transcriptome analysis of the cuticular defective *lcr, bdg* and *fdh* mutants by Voisin *et al.* (2009) indicated that a number of defence related genes are induced in these cuticular defective mutants which suggests a possible role of these genes in basal resistance.

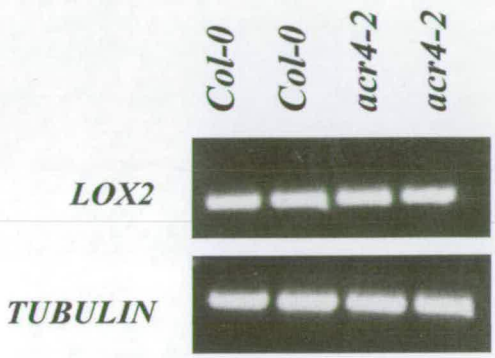


Fig 6.10: RT-PCR analysis of *LOX2* transcript accumulation in the *Col-0* and *acr4-2* leaves.

Total RNA was extracted from 4 week old long day grown leaves of *Col-0* and *acr4-2*. No difference was observed in the expression of *LOX2*.

As mentioned before, *acr4* leaves look much like wild type. However our studies have revealed that *acr4* leaves have increased cuticular permeability, as identified by the increased chlorophyll leaching and permeability to the dye, toluidine blue. Here it is to be noted that the cuticular defect of *acr4* leaves appears to allow rapid chlorophyll leaching and permeability to toluidine blue, but does not show enhanced water loss. It is not known whether other mutants also exhibit this type of discrepancy. Like *acr4* mutant leaves, *fdh, lcr* and *bdg* also show rapid chlorophyll leaching; however the cuticular water loss behaviour of *fdh*, *lcr* and *bdg* is not known (Voisin *et al.,* 2009). The findings that mature *acr4* leaves have a defective cuticular phenotype, extends the previous studies by Watanabe *et al.* (2004) showing that young *acr4* mutant leaves (18 days old) are permeable to the hydrophilic dye, toluidine blue. It also provides a potential explanation for the *Botrytis* resistance phenotype of *acr4* mutants and supports the notion that cuticle defects could, themselves, activate defence responses. To further understand the nature of cuticular defect of *acr4* leaves, a detailed study of the cuticular lipid composition of *acr4* leaves by ESI-MS has recently been initiated.

A second important question is that of how *acr4* mutants behave in response to other pathogens. Pathogen challenge experiments using *P. synringae* revealed that *acr4* is more sensitive to *P. synringae* than wild type. The difference in the behaviour of *acr4* mutants to necrotrophic pathogen, *B. cinerea* and biotropic pathogen, *P. syringae* is interesting in the light of research which has revealed that the mode of defence deployed by plants distinguishes between necrotrophs and biotrophs and is controlled by several different mediators (Thomma *et al.,* 1998). Although the production of these mediators involves a complex signalling network, research on *Arabidopsis* has led us to the notion that salicylic acid primarily activates defence responses to biotropic pathogens, such as *Hyaloperonospora parasitica* and *Pseudomonas syringae*, whereas jasmonic acid/ethylene mainly activates defence responses to necrotrophic pathogens such as *B. cinerea* and *Fusarium oxysporum* (Thomma *et al.,* 1998, 2001). The salicylate and jasmonate pathways are, to some extent, antagonistic defence responses (Spoel *et al.,* 2003). As a result of negative cross talk between salicylic acid and jasmonic acid, activation of the salicylic acid

response renders a plant more susceptible to pathogens that requires jasmonic acid dependent defences and *vice versa*. This is supported by the observed enhanced resistant phenotype of *botrytis-induced kinase1 (bik1)* mutants to a virulent bacterial pathogen *Pseudomonas syringae* pv *tomato* and their severe susceptibility to necrotrophic fungal pathogens. The pathogen responses of *bik1* are mostly dependent on salicylic acid levels (Veronese *et al.,* 2006). Another mutant, *as1,* which shows resistance to *B. cinerea,* requires intact jasmonic acid mediated defence responses. These *as1* mutant plants are more susceptible to *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (Nurmberg *et al.,* 2007). In my study, I have shown that *ACR4* operates as a negative regulator against the necrotrophic pathogen, *B. cinerea* and it could act as a positive regulator of disease resistance against *P. syringae.* However the detailed mechanism requires further investigation.

The microarray analysis produced by the Ingram Lab in 2004 indicated that in *acr4* mutant floral meristem tissues the expression of *LOX2*, a gene encoding an essential enzyme in jasmonic acid biosynthesis, is upregulated. Jasmonic acid signalling is required for resistance against *Botrytis* (Thomma *et al.,* 1998). A direct role of *LOX2* in *Botrytis* resistance was demonstrated by Yang *et al.* (2007). They reported that mutation of the *A. thaliana PHOSPHOLIPASE A1 (AtPLA1)* gene which encodes an acid hydrolase, shows decreased expression of *LOX2* and exhibits reduced accumulation of basal jasmonic acid and enhanced susceptibility to *Botrytis*. This suggested that the high level of expression of *LOX2* in *acr4* mutant could potentially have a role in resistance against *Botrytis*. To determine the expression of *LOX2* in *acr4* leaves, RT-PCR was performed using wild type and *acr4* leaf samples prior to pathogen infection and no major difference was found in the expression of *LOX2* between wild type and *acr4* mutant (Fig 6.10). However, repetition of these results using quantitative RT-PCR might give more meaningful results. It would also be interesting to cross *acr4* mutants with mutants involved in jasmonic acid perception or *lox2* mutants to test definitively whether the resistance of *acr4* plants to *B. cinerea* could be caused by over-expression of *LOX2.*

Reactive oxygen species (ROS) are often produced during early stages of plant-pathogen interactions and play roles in mediating plant defence responses (Lamb and Dixon, 1997). Some studies suggest that ROS are important for plant resistance against *B. cinerea*. Malolepsza and Urbanek (2002) reported that chemical induction of the formation of ROS using *O*-hydroxyethylorutin in tomato (*Solanum lycopersicon*) resulted in enhanced resistance against *Botrytis*. There is also conflicting evidence, however, that the generation of ROS increases the efficiency of the HR and facilitates *B. cinerea* colonization (Govrin and Levine, 2000). Whether *ACR4* function impacts on the HR is currently unknown, and no transcriptome data is available regarding the role of *ACR4* in ROS production. However, we cannot exclude the possibility that *B. cinerea* resistant phenotype of *acr4* is mediated by the generation of ROS or that the *acr4* mutation interferes with the HR response. As discussed in Section 3.2.2, *acr4* seedlings show enhanced sensitivity to paraquat and their sensitivity is similar to the cuticular defective mutant, *ale1*. It is therefore probable that the sensitivity of *acr4* seedlings to paraquat is merely due to the defective cuticle allowing increased paraquat penetration.

In summary, my studies indicated a possible role of a developmental regulator, *ACR4* in plant defence. This is based on the reduced susceptibility of the *acr4* mutant plants to *B. cinerea*. My studies also revealed that *acr4* leaves could have defects in the epidermal layer, as identified by the increased chlorophyll leaching analysis. At present, it is not known whether the defective cuticle of *acr4* leaves allows enhanced diffusion of antifungal compounds.

Finally, there remains an open question regarding the functional integration of a developmental regulator in defence. In future, understanding the targets of ACR4 could help us to uncover how ACR4 is involved in defence and whether the role of ACR4 in defence is truly independent of its developmental roles.

Chapter - 7

AIP1, a possible role in seed storage protein breakdown

7.1 Introduction

7.2 Results

7.2.1 AIP1 is transiently accumulated during seed germination

7.2.2 An *aip1* mutant shows abnormal accumulation of storage proteins in seeds

7.2.3 The *aip1* mutant seeds may have a defect in seed germination

7.2.4 Identifying potential binding partners of AIP1 during seed germination

7.3 Discussion

Chapter – 7

AIP1, a possible role in seed storage protein breakdown

7.1 Introduction

As mentioned in Chapter-4, AIP1 (ACR4 INTERACTING PARTNER 1) was originally identified as an interacting partner of ACR4 through Yeast-two-Hybrid (Y2H) screening. Recent literature searches showed that AIP1 had, in fact, previously been identified as a seed protein, localized in the internalized membranes of the protein storage vacuole (PSV) of mature *Brassica napus* and *Arabidopsis thaliana* seeds (Gillespie *et al.*, 2005). Gillespie *et al.* (2005) named the likely *Brassica* orthologues of AIP1 as 'Brassicaceae PSV-Embedded Protein (BPEP)' since they first isolated this protein from the protein storage vacuoles of *Brassica* seeds.

The protein storage vacuoles of plant seeds serve as the site of protein accumulation during seed development and this reserve is a source of nutrients for the developing seedling during seed germination (Torrent *et al.*, 1989). Structurally, these storage vacuoles are complex multivesicular organelles and in most seeds, they contain three morphologically distinct regions: matrix, crystalloid and globoid (Fig 7.1), (Pernollet, 1978; Jiang *et al.*, 2001). The matrix contains many soluble storage

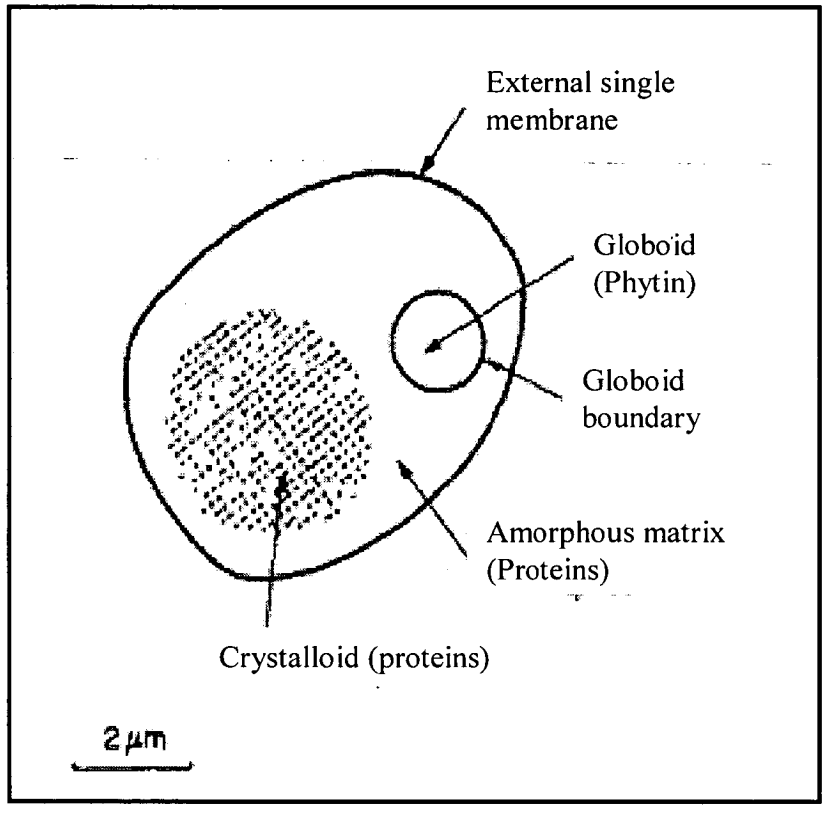


Fig 7.1: Schematic view of a typical protein storage vacuole with two types of inclusions, globoid and crystalloid (Pernollet, 1978).

proteins whereas crystalloids and globoids are two morphologically distinct types of intra-organellar inclusions. Crystalloids are composed of unknown proteins organized in a lattice structure and globoids contain phytate, a salt of phytic acid and its cations (mostly Mg^{2+}, K^{+}, and Ca^{2+}). In mature seeds, up to 90% of the total phosphorus present in the seed is stored as phytates and during seed germination, they serve as an important source of phosphorus for the germinating embryo (Otegui *et al.*, 2002). The PSVs of some plant species appear to lack distinct crystalloids (Müntz, 2007).

To identify proteins associated with the protein storage vacuole, Gillespie *et al.* (2005) attempted to isolate protein storage vacuoles from mature *Arabidopsis* seeds. However, due to the technical difficulties in isolating large quantities of protein from *Arabidopsis* seeds, they chose *Brassica napus*, a close homologue of *A. thaliana*. Through sucrose density gradient centrifugation, they showed that a 45kDa protein, which was named BPEP, was associated with the crystalloid and globoid fractions of these vesicles. A further purification of the phytate crystals from the globoid protein fraction by heating in SDS followed by solubilization of phytate in glycine solution, pH 2.5 and analysis of this fraction by SDS-PAGE indicated that the BPEP protein was, at least partially, localized to this fraction. Mass spectrometry analysis followed by database search confirmed that BPEP is an orthologue of the Arabidopsis protein encoded by the gene *At3g20370*, a gene we named as *AIP1* (Chapter-4). As mentioned in Chapter-4 (Fig 4.3), BPEP and AIP1 have an identical domain structure and are composed of an N-terminal signal peptide and two tandem MATH domains. N-terminal sequencing of the BPEP protein indicated that the protein undergoes proteolytic processing between the two MATH domains. As the cleavage site is immediately C-terminal to an asparagine residue, it was hypothesized that the protein is cleaved by an asparaginyl endopeptidase (Gillespie *et al.*, 2005).

To study the localization of BPEP and AIP1 protein in *B. napus* and *A. thaliana* seeds respectively, Gillespie *et al.* (2005) raised antibodies using a synthetic peptide specific to the amino acids 76-89 of the AIP1 protein sequence and this

antibody was named as anti-BPEP antibody. Immunoblot analysis indicated that this anti-BPEP antibody can also recognize BPEP of *B. napus*. To localize BPEP and AIP1 in *B. napus* and *A. thaliana* seed sections respectively, immunolocalization studies were performed using anti-BPEP antibody and antibodies specific to other biochemical markers for example, α- TIP (Tonoplast Intrinsic Protein), a marker for the PSV tonoplast (Jauh *et al.,* 1999); $^{\gamma}$-TIP, a marker for the globoid membranes of PSVs (Jiang *et al.,* 2001) and DIP (Dark Intrinsic Protein) for crystalloids (Jiang *et al.,* 2000). Based on the co-localization of BPEP and AIP1 with α- TIP, DIP and $^{\gamma}$-TIP markers, Gillespie *et al.* (2005) demonstrated that both BPEP and AIP1 are associated with the PSV tonoplast as well as with the crystalloid like structures and globoids of both *Brassica* and *Arabidopsis* seeds.

As Gillespie *et al.* (2005) suggested that AIP1 is associated with the PSV of mature Arabidopsis seeds, we hypothesized that AIP1 could have a role in the accumulation and/or breakdown of storage reserves. To dissect the role of *AIP1* with respect to seed development/germination, three different approached were taken. The first approach was to determine the expression pattern of *AIP1* as well as accumulation of AIP1 during seed development and germination. Secondly, accumulation of seed storage proteins in *Col-0* and an *aip1* mutant seeds was investigated. Finally, GFP tagged AIP1 was immunoprecipitated using anti-GFP microbeads, as a preliminary step to MS analysis to identify the interacting partners of AIP1 during seed germination.

7.2 Results

7.2.1 AIP1 is transiently accumulated during seed germination

Consistent with our hypothesis that AIP1 could play a role in the metabolism of seed storage proteins, *in silico* data indicated a massive up regulation in *AIP1* expression during the early stages of seed germination (Le *et al.,* 2010). In order to verify this data, the gene expression pattern of *AIP1* was examined during seed

germination using our promoter–reporter construct. As described in Chapter-4, an *AIP1* promoter-reporter construct was generated by fusing the full length *AIP1* promoter (1kb) with the nuclear localized marker, Histone2B:Yellow Fluorescent Protein (H2B:YFP), (Boisnard-Lorig *et al.,* 2001). *Col-0* plants carrying this construct (*pAIP1-H2B:YFP*) were used to investigate the expression of *AIP1* during seed germination. Using confocal laser scanning microscopy, the expression pattern of *AIP1* was assessed in dry seeds, stratified seeds (seeds left in water at 4°C for 2 days) and during the early stages of seedling germination and growth (Fig 7.2). No detectable expression of H2B:YFP was observed in dry seeds, but this tissue is very difficult to visualize. In imbibed seeds, the expression of *AIP1* was undetectable (this tissue has a high level of autofluorescence), but its level increased markedly during seed germination and reaches high levels in the cotyledons of 2 day old seedlings. Strong expression was also detectable in the cotyledons of 4 day old seedlings, however the H2B:YFP marker is not suitable as a means of monitoring a steep decline in the *AIP1* expression pattern since H2B:YFP is integrated in the nuclear chromatin and can often be detected in nuclei long after expression has been lost (G. Ingram personal communication).

To test whether the expression pattern of the *AIP1* gene in germinating seeds is reflected in AIP1 protein accumulation, and to gain further insights into the temporal pattern of AIP1 accumulation, we used lines described in Chapter 4, which express a GFP fusion to AIP1 under the control of the *AIP1* promoter. Western blot analysis was carried out on these plants. One hundred seeds of either *Col-0* or *pAIP-AIP:GFP* carrying lines were stratified in water for 4 days and then transferred to conical flasks containing 0.5xMS liquid media. The flasks were then gently agitated under constant light at 22°C. Samples were collected at different time points and western blot analysis was performed using an anti-GFP antibody (Fig 7.3). Consistent with results from promoter reporter fusion lines, the AIP1:GFP protein was not detectable in freshly imbibed seeds. At 2-days after stratification, the accumulation of AIP1:GFP was very high and it then tailed off as germination proceeded with no detectable AIP1:GFP seen at day 7 after germination. Interestingly, the full length AIP1:GFP protein is predicted to have a molecular weight of around 66 kDa. A 66- kDa band was only detectable at day 2 after

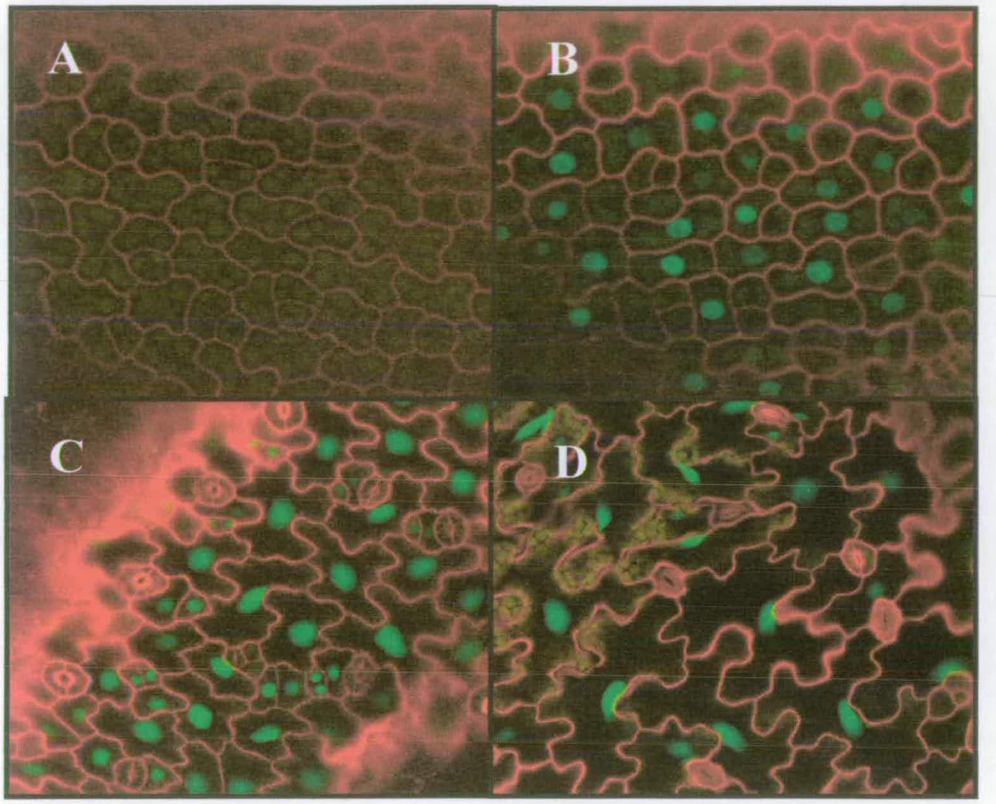


Fig 7.2: *AIP1* shows transient expression during seed germination.

Confocal images of the expression of *AIP1* using the promoter reporter construct *pAIP1-H2B:YFP* in the cotyledons of just imbibed seeds (A), 1 day old seedlings (B), 2 day old seedling (C) and 4 day old seedlings (D).

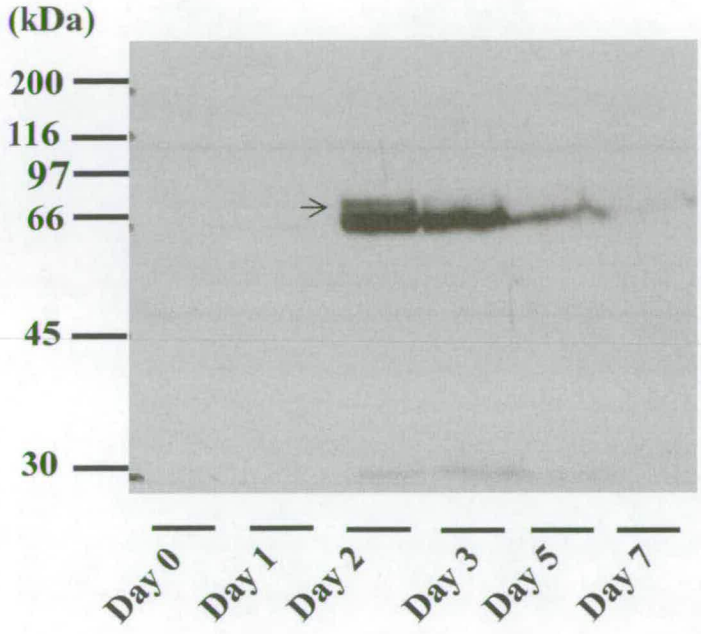


Fig 7.3: Western blot analysis of transient AIP1 protein accumulation during seed germination.

One hundred seeds of *Col-0* and *pAIP-AIP1:GFP* were stratified in water for 4 days and then transferred into conical flasks containing 0.5x MS (liq) media. Samples were collected at different time points and western blot was performed using anti-GFP antibody. AIP:GFP protein (66 kDa) is first detectable at day 2, but goes off slowly as germination proceeds.
The full length AIP1:GFP is predicted to be 66 kDa. The full length 66 kDa band (black arrow) was only detecteable at day 2, however its intensity is much less than the intensity of 60 kDa band, suggesting that the N-terminus of AIP1 undergoes proteolytic processing. A weak band of 28 kDa was also observed which correspond to GFP.

germination, however, even at this timepoint its intensity was much less than that of a slighly smaller 60-kDa species, suggesting that the N-terminus of AIP1 may undergo proteolytic processing. A very weak band of 28 kDa was also observed which corresponds to free GFP.

In summary, the accumulation of AIP1 is strictly developmentally regulated. This transient accumulation of AIP1 is further supported by the transcriptome data by Le *et al.,* (2010) which show that *AIP1* transcript levels are very low in developing seeds, but increase dramatically in 2 days old seedlings.

To ascertain whether AIP1 protein accumulates during seed development, the protein level of AIP1 was monitored by western blot analysis. Total protein extracted from siliques of *Col-0* and transgenic lines (*pAIP1-AIP1:GFP*) expressing AIP1:GFP, and western blot analysis was performed using anti-GFP antibody. This experiment was repeated several times using siliques of different ages covering the entire of seed development phase. In no case, was accumulation of AIP1:GFP observed during any stage of seed development including seed maturation.

To investigate the transcript level of *AIP1* during seed development, RT-PCR was performed. Developing seeds from young siliques were cleared using chloral hydrate to determine the embryonic developmental stage. As shown in Fig 7.4, the transcript level of *AIP1* is low in developing seeds and this may explain why AIP1:GFP was not detectable in the western blot of *pAIP1-AIP1:GFP* transgenic line. This is consistent with the detailed analyses of transcripts from every *Arabidopsis* seed compartments (embryo, endosperm, seed coat), regions (embryo proper, suspensor), and tissue types (epidermis, inner integument, outer integument, endothelium) at different developmental stages (globular, heart, cotyledon, and early maturation) released by the Goldberg and Harada Laboratories (http://seedgenenetwork.net/). These data indicate that *AIP1* is not significantly expressed during seed development or during seed maturation stages (Fig 7.5).

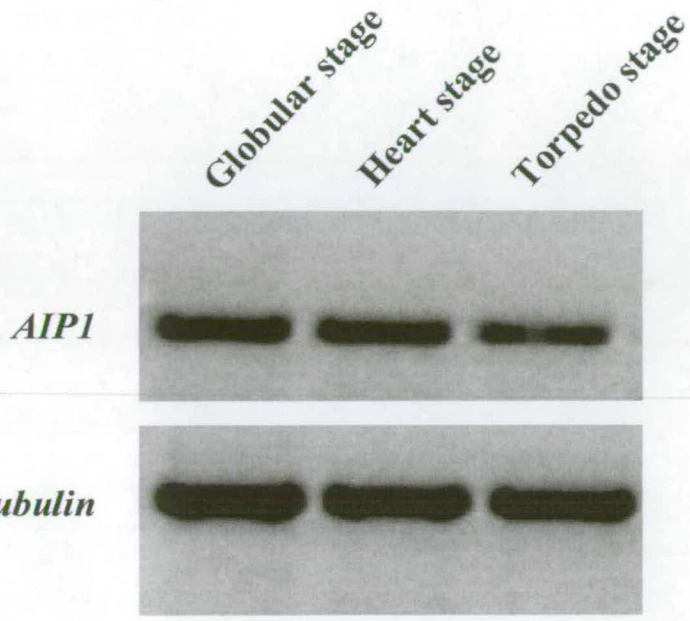


Fig 7.4: RT-PCR analysis of *AIP1* gene expression in wild-type siliques at different stages of seed development.

RNA was isolated from siliques containing embryos at the preglobular stage (silique 1-2), and siliques containing embryos at late the globular stage (silique 4-5) and siliques containing embryos at the torpedo stage (silique 7-8). The siliques were dissected and the developing seeds were cleared using cloral hydrate to determine the developmental stage of the embryo.

β-Tubulin was shown as an internal control.

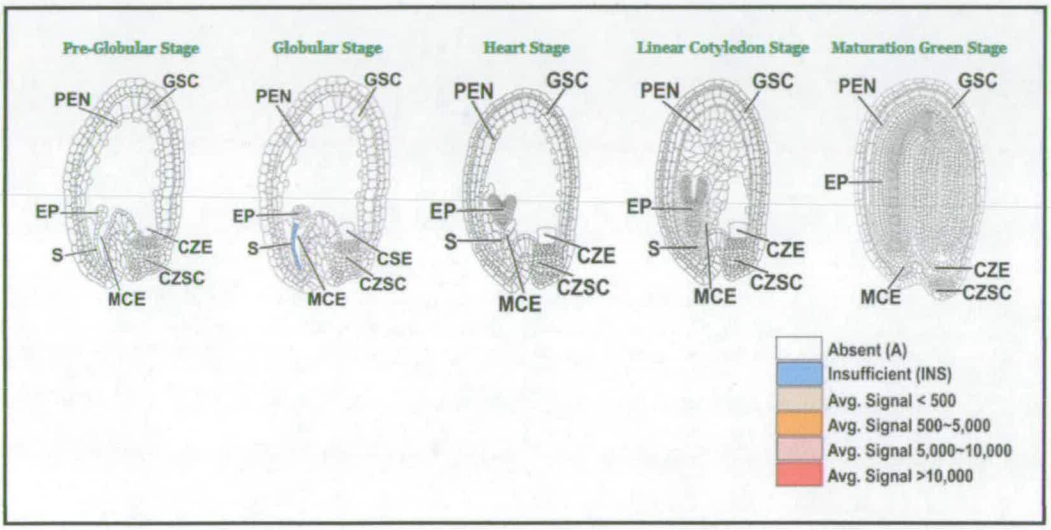


Fig 7.5: Detailed analyses of *AIP1* transcripts from different seed regions and compartments during *Arabidopsis* seed development (Goldberg and Haranda Laboratories; http://seedgenenetwork.net).

The transcriptome data suggested that there is some expression of *AIP1* in the suspensor at the globular stage of seed development. However the expression is very low.

Abbreviation of Tissue/Compartment:

CZE - Chalazal Endosperm; CZSC - Chalazal Seed Coat; EP - Embryo Proper; GSC - General Seed Coat; MCE - Micropylar Endosperm; PEN - Peripheral Endosperm; S - Suspensor; WS - Whole Seed.

7.2.2 An *aip1* mutant shows abnormal accumulation of storage protein in seeds

The seed storage proteins of *Arabidopsis* fall into two major classes; 2S albumins and 12S globulins. These proteins represent the major storage reserve in *Arabidopsis* seeds and are stored in dedicated subcellular compartments during seed filling and maturation (Hughes & Galau, 1989). During germination, these reserves are degraded to provide support for seedling growth.

To investigate the role of *AIP1* during seed germination, the protein profiles of the seed storage proteins in *aip1-2* and *Col-0* seeds and young seedlings at different time points during seed germination was monitored. The null mutant *aip1-2* allele was used in this study since RT-PCR analysis showed that this allele produces no detectable *AIP1* transcript (Section 4.2.2, Fig 4.5).

To analyze the developmental changes in seed storage protein accumulation and breakdown in wild type *Col-0* and *aip1-2* mutant seeds and seedlings, one hundred seeds of *Col-0* or *aip1-2* were stratified in water for 4 days at 4°C and then transferred into conical flasks containing 10ml liquid MS media and the flasks were kept in a growth room (22°C and constant light) with gentle agitation. Total protein was extracted from seeds and seedlings of *Col-0* and *aip1-2* at different time points during germination and was subjected to SDS-PAGE, followed by coomassie blue staining. This technique has previously been used by Shimada *et al.* (2003) to examine the seed storage protein breakdown during *Arabidopsis* seed germination. As shown in Fig 7.6, *aip1-2* mutant seeds accumulated significantly higher levels of 2S albumin compared to the levels in wild-type. In addition, marginally lower levels of 12S globulins were observed. These phenotypes were seen in several repetitions carried out using different batches of seeds harvested from plants grown in identical conditions. A comparison of the breakdown of 2S seed storage protein in *Col-0* and *aip1-2* indicated that there may also be a slight delay in 2S seed storage protein breakdown in the mutant background (comparing 0h time point with 24h time point). This suggested that *AIP1* could have role in storage protein mobilization.

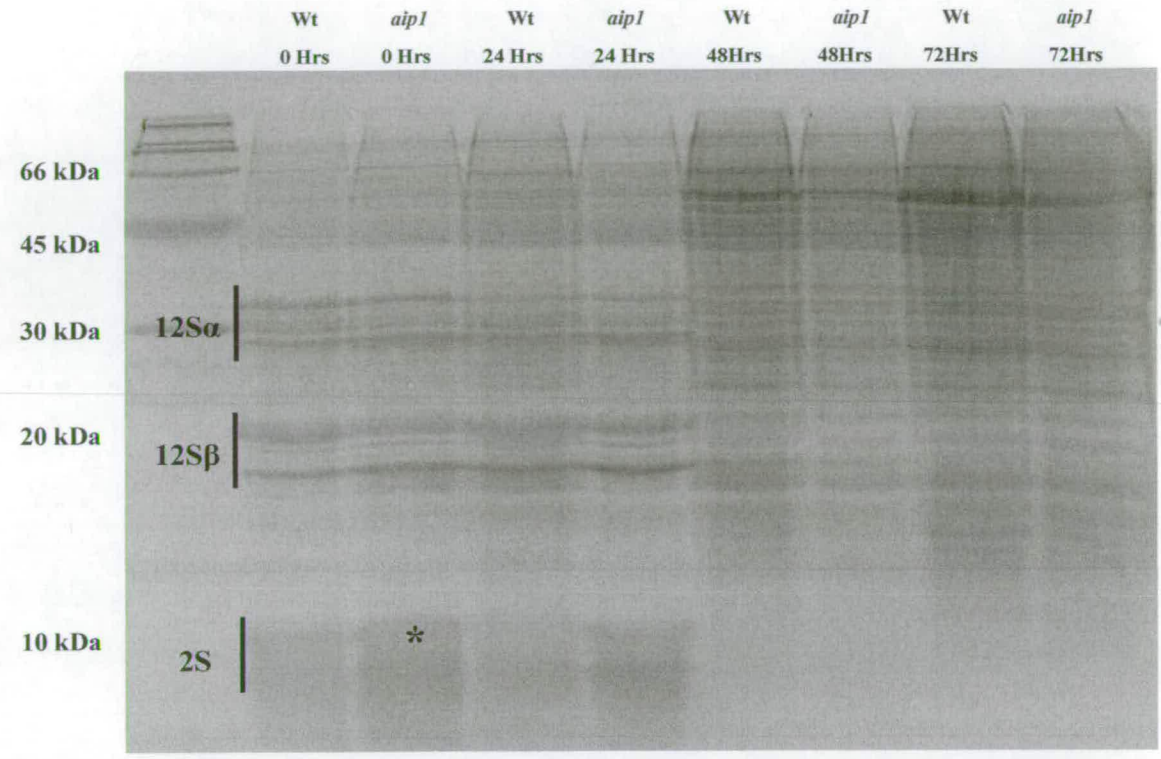


Fig 7.6: The *aip1-2* mutant seeds show abnormal accumulation and breakdown of seed storage proteins.

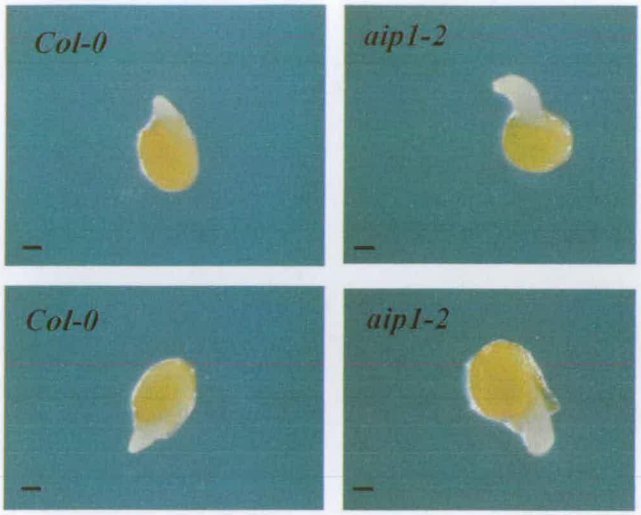
The storage protein profile in wild type and *aip1-2* mutant seeds was monitored during seed germination. Asterisk indicates that just imbibed seeds of *aip1-2* mutant have higher 2S albumin content compared to the level in wild type. There is also a slight delay in 2S seed storage protein breakdown in the mutant background (comparing 0hr timepoint with 24hr timepoint). The expression of *AIP1* during germination may therefore indicate a role in storage protein immobilization.
Repetitions of this experiment as well as complementation analysis are required to confirm this phenotype.

To investigate whether the phenotype seen in *aip1-2* mutant i.e. abnormal accumulation of 2S albumin, is due to the loss of *AIP1* expression, complementation analysis was carried out to see if an introduced copy of *AIP1* could rescue the phenotype. For complementation analysis, homozygous *aip1-2* plants were transformed with the *pAIP1-AIP1:GFP* construct (see Section 2.3 for details of transgene construction) where the transgene conferred a kanamycin antibiotic resistance. T_3 generation plants were plated on media containing kanamycin to identify plants homozygous for the *AIP1* transgene. To compare the seed storage protein profile of *Col-0*, *aip1-2* and *aip1-2* carrying *pAIP1-AIP1:GFP*, one hundred similar aged seeds from each background were stratified in water for 4 days at 4°C and total protein was extracted from these imbibed seeds and subjected to SDS-PAGE, followed by coomassie blue staining. However, the phenotype previously observed in *aip1-2* seeds, i.e. abnormal accumulation of 2S albumin was not clear. Although this experiment was performed using seeds of similar age, as all the lines i.e. *Col-0*, *aip1-2* and *aip1-2* carrying *pAIP1-AIP1:GFP* transgene were not grown in the same tray, a slight difference in seed harvest could explain this inconsistent result. Growing all the lines in the same tray, to ensure equal environmental effects, and repeating this whole experiment using after-ripened seeds (dry seeds left in the dark for at least 2 weeks; Gwyneth Ingram personal communication) could provide more meaningful results.

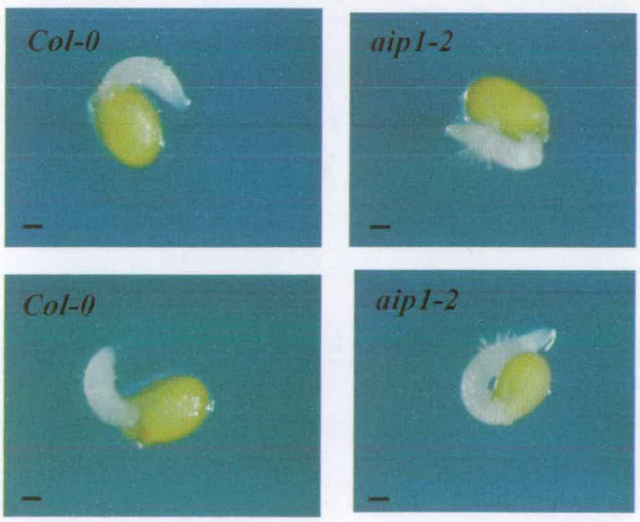
7.2.3 The *aip1* mutant seeds may have defects in seed germination

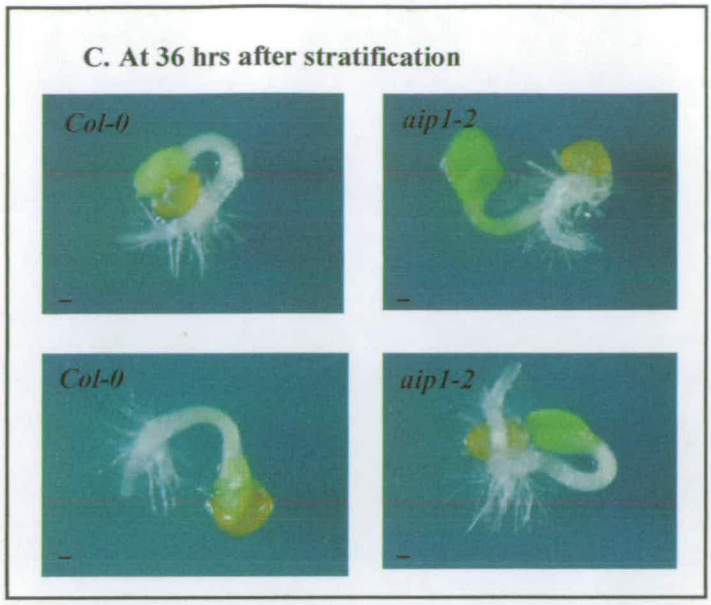
To investigate whether, *aip1-2* shows any defect in germination, wild type *Col-0* and *aip1-2* seeds of similar age were plated onto wet filter paper in a Petri dish, the plates stratified at 4°C for 4 days and then transferred to the 22°C growth room under constant light. It was found that *Col-0* seeds germinated within 24 hrs while the mutants did not germinate at all. This suggested that *aip1-2* shows a serious defect in seed germination. However, when the light in the growth cabinet was replaced with a new one, it was found that *aip1-2* mutants show an early germination phenotype (Fig 7.7, 7.8). Overall, these results indicated that *AIP1* could play role in regulating seed germination in a light dependent manner. However, further experiments using very tightly controlled lighting conditions would be required to confirm this phenotype.

A. At 15 hrs after stratification



B. At 19 hrs after stratification





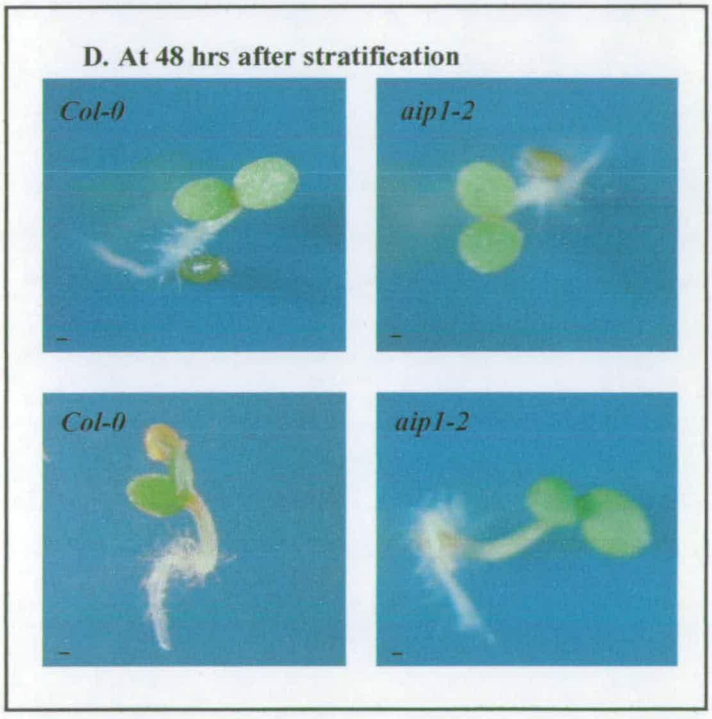


Fig 7.7: The *aip1-2* mutant seeds germinate earlier compared to wild type *Col-0* during seed germination.

The wild type *Col-0* and *aip1-2* mutant seeds were placed in 9 cm plastic Petri dishes on two layers of Whatman filter paper moistened with 3 ml water and incubated at 4°C for 4 days, followed by incubation under constant fluorescent light at 22°C. The radical emergence was examined at different time points during seed germination using a dissection microscope. The time course analysis indicated that radical emergence is slightly earlier in *aip1-2* mutant compared to wild type *Col-0*. Repetition of this experiment as well as complementation analysis is required to confirm this phenotype. Scale bars: 100 µm.

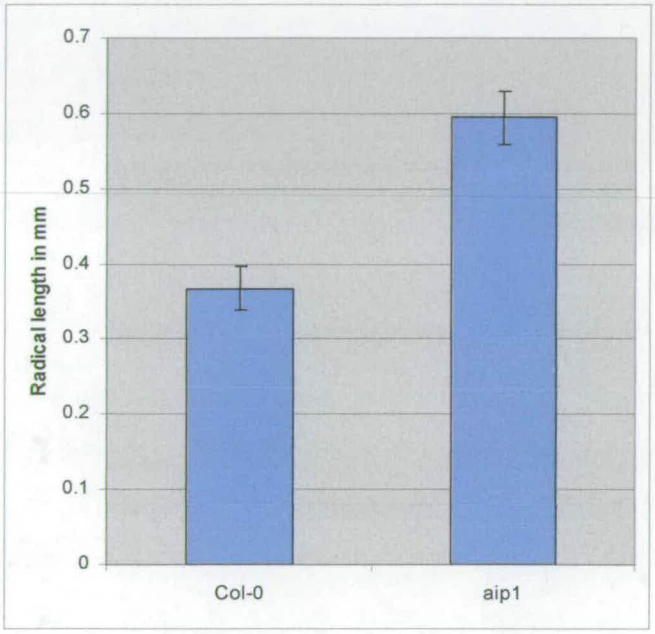


Fig 7.8: Radical emergence occurs slightly earlier in a*ip1-2* mutants compared to wild type *Col-0* and the graph represents the length of the emerged radical at 19hrs after stratification.

The wild type *Col-0* and *aip1-2* mutant seeds were placed in 9 cm plastic Petri dishes on two layers of Whatman filter papers moistened with 3 ml water and incubated at 4°C for 4 days, followed by incubation under constant fluorescent light at 22°C. The length of emerged radical was measured using ImageJ software at 19 hrs after stratification.

Error bars represent mean ± standard error (n = 30-45).

To gain further insight into the role of *AIP1* in seed germination, I looked at the publicly available transcriptome data. It was found that the expression of *AIP1* is down regulated to a significant level in thne gibberellin (GA) biosynthesis mutant, *ga1-3* (Zimmermann *et al.*, 2004). The *ga1* mutants are defective in an early step of the GA biosynthesis pathway (Sun and Kamiya, 1994) and *ga1-3* seeds fail to germinate in the absence of exogenous GA (Koornneef and van der Veen, 1980). The abnormally low *AIP1* expression in *ga1-3* imbibed seeds is further supported by the microarray data from Halliday lab, University of Edinburgh, UK (Eve-Marie Josse personal communication). However, it should be borne in mind that *ga1-3* mutants show a serious defect in germination (Koornneef and van der Veen, 1980). Since *AIP1* is only expressed during germination, any mutant showing a delay in this process would likely to show a decrease in *AIP1* expression in a time-course against wild-type. Therefore, to further investigate if GA has any abnormal effect on *aip1* seed germination, *Col-0* and *aip1-2* seeds of similar age were stratified in 0.1% agar for 3 days at 4°C and sown on MS-agar plates containing either GA or the largely antagonistic growth substance Abscissic Acid (ABA). However, no difference in response from wild-type was observed for *aip1-2* mutant seeds. It therefore seems likely that the reduction in *AIP1* expression in lines defective in GA biosynthesis is an indirect effect of delayed germination, rather than an indication that *AIP1* is implicated in GA signalling.

As mentioned earlier, Gillespie *et al.* (2005) have suggested that AIP1 is embedded in the phytate crystals within the globoid compartment of the PSV. The phytate crystals are an important source of phosphorus for the germinating embryo (Otegui *et al.*, 2002). It was therefore possible that AIP1 might play a role in the breakdown of phytate to release phosphorus upon seed germination. To investigate how *aip1* seeds behave in the absence of phosphorus, *Col-0* and *aip1-2* mutant seeds of similar age were germinated on media with and without phosphorus (see Section 2.1.4 for details about the media composition). However, no major difference in germination or post-germinative growth was observed between *Col-0* and *aip1*. This suggested that the loss of *AIP1* function does not interrupt phosphorus utilizaton from phytate crystals.

Because of the observed differences in storage protein breakdown between *Col-0* and *aip1* seeds, we also decided to test whether mutant seeds might be more sensitive to nitrogen deficiency than wild-type. Seed germination was investigated on media lacking a nitrogen source. Sterilized seeds of the similar age were germinated on plates lacking nitrogen or sucrose or both nitrogen and sucrose, and complete 0.5x MS with containing both nitrogen and sucrose as a control (see Section 2.1.4 for details about the media composition). However, no difference was seen between the behaviour of *Col-0* and *aip1-2* mutant lines on these media. Therefore, it appeared that *AIP1* does not play a limiting role in the utilization of nutrients from seeds during seed germination.

7.2.4 Identifying potential binding partners of AIP1 during seed germination

As mentioned earlier, *AIP1* encodes a protein with two MATH domains in its C-terminus. Based on structure analysis by homology modelling, Sunnerhagen *et al.* (2002) proposed that MATH domains can act as scaffolds for binding other proteins, resulting in the formation of protein complexes. It indicates that AIP1 could form protein complex with other proteins. Identification of the components of this protein complex could help us to understand how this protein functions at the cellular level.

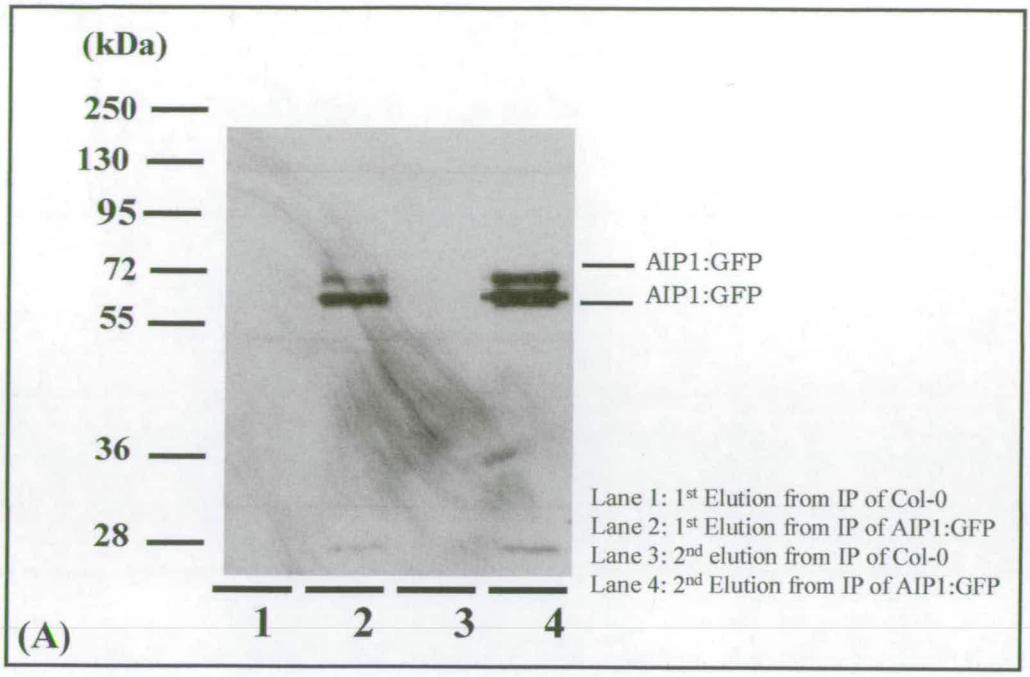
Our gene expression analysis by confocal microscopy (Fig 7.2) and protein accumulation studies by western blot (Fig 7.3) strongly suggested that AIP1 could have an important role during seed germination. In order to investigate the role of AIP1 protein in more detail during seed germination, we were interested in identifying the binding partners of AIP1. To identify the interacting components of AIP1 signalling, I planned to do immunoprecipitation, followed by Mass Spectrometry using Matrix Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF). The requirement for this analysis is the expression of a tagged protein, which can be used to capture and pull down its binding partners. As mentioned in Chapter- 4 and above, I have generated transgenic plants expressing

AIP1 fused with GFP under its own promoter and the expression of AIP1:GFP in these transgenic lines was confirmed in seedlings by western blotting.

In order to capture seed germination specific interactors of AIP1, total protein was immunoprecipitated from 3 day old *pAIP1-AIP1:GFP* line and wild type *Col-0* seedlings using anti-GFP microbeads (μMACS GFP tag protein isolation kit, Miltenyi Biotec) to capture differentially bound proteins with AIP1. The reason for using *Col-0* was to exclude the false positive interactors. A small amount of the immunoprecipitate was analyzed by SDS-PAGE, followed by western blot analysis. Fig 7.9 shows that AIP1:GFP can be immunoprecipitated and that no non-specific signal was detectable for *Col-0.* The immunoprecipitate was then subjected to silver staining which clearly indicated that additional protein can be pulled down (Fig 7.9). In order to take this project further, a research grant proposal was submitted to the Small Project Grant, University of Edinburgh Development Trust, UK. Unfortunately, as the grant application was unsuccessful, due to financial constrain it was not possible to carry out MS analysis.

7.3 Discussion

Here, my results indicated that AIP1 is transiently accumulated during seed germination. The accumulation of AIP1 is not observed in dry seeds or imbibed seeds but is highly induced at 48 hours during seed germination. Afterwards, the AIP1 protein level appears to decline and at 7 days after germination, no AIP1:GFP was detectable. In our growth cabinet (22°C and constant light), the emergence of the radical from the seed coat during seed germination is first observed at about 13 hours after stratification. At 36 hours, cotyledons start to emerge from the seed coat and by 48 hours, two separated cotyledons are observed. My results suggest that the accumulation of AIP1, which is centered at a particular developmental stage, i.e. during cotyledon emergence, could be correlated with seed storage protein break down.



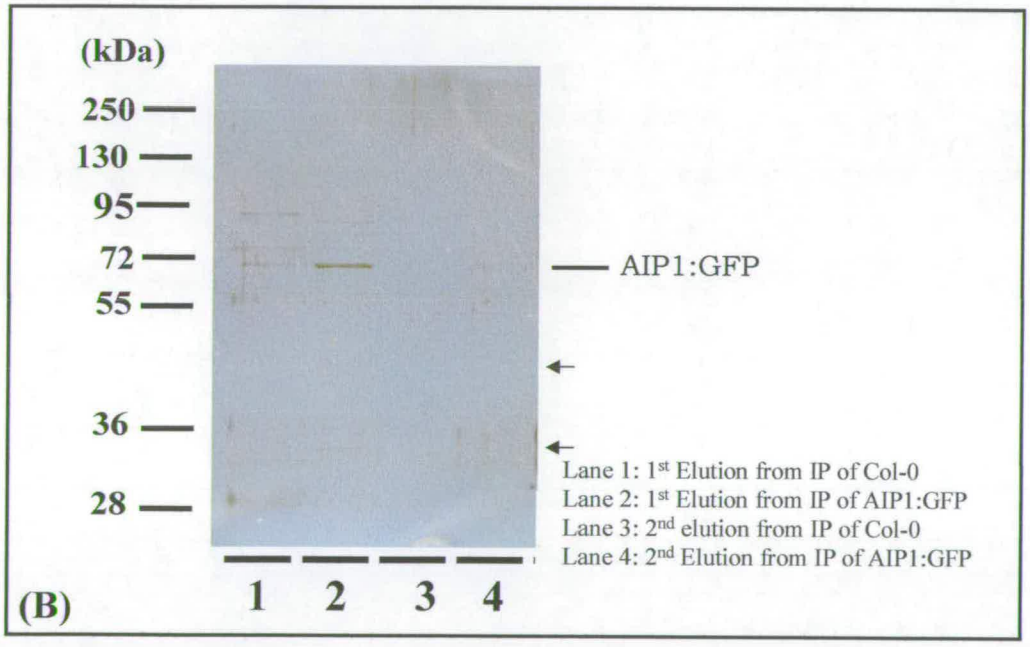


Fig 7.9: AIP1:GFP from *pAIP1-AIP1:GFP* transgenic line can be immunoprecipitated using anti-GFP microbeads.
Total protein extracted from 3 days old wild type *Col-0* and *pAIP1-AIP1:GFP* transgenic seedlings was immunoprecipitated using anti-GFP microbeads and a small amount of the immunoprecipitate was analyzed by SDS-PAGE, followed by western blot analysis using anti-GFP antibody (A). First and second elution represent eluting AIP1:GFP from the cloumn of anti-GFP microbeads. The immunoprecipitate was also subjected to silver staining (B) which clearly indicated that additional protein can be pulled down. The arrow-head indicated potential AIP1 interactors in Lane2 (the image is not very clear in the printed copy).

Investigation of the seed storage protein profile of germinating *Col-0* and *aip1* mutant seeds suggested that freshly imbibed *aip1-2* mutant seeds have higher 2S albumin content compared to the level in wild type. However no major difference was observed for 12S globulin. As germination proceeds, the breakdown of 2S seed storage protein is quite slow in the mutant background compared to the wild type *Col-0* (Fig 7.6). This experiment was carried out several times using two different batches of seeds. However when this experiment was repeated using *Col-0*, *aip1-2* and *aip1-2* harbouring *pAIP1-AIP1:GFP*, the observed abnormal accumulation of 2S albumin in *aip1-2* seeds was not clear and this inconsistent result could be due to a slight difference in seed harvest. Therefore, due to the observed variability in penetrance of this phenotype more repetitions are required.

Research in the last few decades revealed that seed germination is a complex developmental process which is controlled by endogenous hormonal cues and external environmental stimuli such as water, low temperature, and light. Among the photohormones, GA and ABA have the most pronounced effects on *Arabidopsis* seed germination (Koornneef *et al.*, 2002). ABA establishes and maintains seed dormancy, whereas GA has the opposite effect, breaking dormancy and inducing seed germination. As mentioned earlier, publicly available transcriptome data suggested that the expression of *AIP1* is down regulated in *ga1-3* imbibed seeds. However, the *AIP1* gene does not appear to be involved in GA or ABA signalling, as sensitivity to these hormones is not altered in the *aip1* mutant when germination rates were scored in the presence of these hormones. Detailed time-course analysis is required to understand whether the expression of *AIP1* is induced in wild type imbibed seeds as well as to study the expression level of *AIP1* in GA treated and GA non-treated *ga1-3* mutant. It would also be interesting to look at *aip1* mutant seed germination in presence of GA biosynthesis inhibitor, paclobutrazol (Hedden and Graebe, 1985).

Expression of the *AIP1* gene appears to be regulated by light. Transcriptome data suggested that the expression of *AIP1* is induced to a significant level when 4 day-old dark incubated plants were exposed to continuous white light or far-red light (Winter *et al.*, 2007). This likely correlates with de-etiolation, and raises the interesting possibility that *AIP1*, rather than being an agent of storage protein

breakdown, could be involved in the redeployment of nutrients during seedling greening. Since light induces the expression of many genes including the genes which are not directly light regulated, further experiments are required to ascertain the role of light in regulating the expression of *AIP1* gene. In my experiments, the *aip1-2* mutant behaved differently when the light in the growth cabinet was replaced by a new one. As shown in Fig 7.7 and Fig 7.8, *aip1* mutant shows a slightly early germination phenotype. A very recent repetition of the seed germination experiment using *Col-0*, *aip1* and the *aip1* complemented line, indicated that *aip1* showed a delay in seed germination and this phenotype is not observed in the *aip1* mutants harbouring *pAIP1-AIP1:GFP*. This suggested that the *aip1* seeds could have a defect in seed germination which possibly depends on the intensity of light. Further analysis of seed germination in a strictly controlled growth cabinet using light of different intensities and wavelengths could therefore give meaningful and reproducible results.

Functional redundancy between *AIP1* with other genes cannot be ruled out since there are many genes in *Arabidopsis* that act in a redundant manner (Cutler and McCourt, 2005). As mentioned in Chapter-4, the amino acid sequence of AIP1 shows high degree sequence similarity to the proteins encoded by *At3g20360* and *At3g20380.* However, no accumulation of either *At3g20360* or *At3g20380* derived protein has been observed in the germinating seed (Baerenfaller *et al.,* 2008). It was therefore considered unlikely that *AIP1* acts redundantly with these structurally related genes. However, functional redundancy with structurally unrelated but functionally analogous genes cannot be ruled out.

As mentioned earlier, AIP1 is one of the proteins which are rapidly accumulated in response to *Piriformospora indica,* an endophytic fungas. Literature searches revealed that one other protein that is rapidly accumulated in response to *Piriformospora indica* is the beta-glucosidase, PYK10 (Peškan-Berghöfer *et al.*, 2004). Interestingly, the expression pattern of *PYK10* in young seedlings is very similar to *AIP1*. Like *AIP1*, *PYK10* is first expressed in 2-day-old seedlings and the expression is observed throughout the whole seedlings until the seedlings are 5 day old and from day 5 on, the expression becomes restricted in the root (Nitz *et al.,*

2001). Thus, PYK10 and AIP1 likely function in the same developmental processes although their exact roles are still unclear.

As mentioned in Chapter-4, AIP1 was originally identified as a potential binding partner of ACR4. It is therefore possible that *acr4* seeds could have defects similar to those identified for *aip1*. However, the clarification of ACR4 functions in seed germination has proved challenging because *acr4* mutants seeds have morphological defects i.e. seeds are irregular in both shape and size. Any defect in seed germination could therefore be hard to interpret since the observed phenotype could be due to the morphological defect rather than the physiological defect. However, transcriptome data suggested that the expression of *ACR4* gene is relatively low during seed germination. Therefore, it seems unlikely that ACR4 acts as an interacting partner of AIP1 during seed germination.

In summary, *AIP1* is one of a group of genes which are transiently expressed during seed germination. Mutation of this gene may cause abnormal accumulation and/or breakdown of storage proteins in seeds and this could result in the defects in seed germination. Repetitions of these experiments are required to confirm the phenotype of *aip1* mutant seeds. Also, understanding the targets of AIP1 could help us to determine the role of AIP1 during seed germination.

Chapter - 8

General discussion

8.1 *ACR4* does not act redundantly with *ACR4* related kinases in epidermal specification

8.2 ACR4 appears to undergo endocytosis through interaction of the cytoplasmic domain with unknown proteins

8.3 *ACR4* could have a role in plant defence

Chapter -8

General discussion

8.1 *ACR4* does not act redundantly with *ACR4* related kinases in epidermal specification

ACR4 was originally thought to be a candidate gene in epidermal specification in *Arabidopsis* due to its high degree of sequence similarity to *CR4* gene which appeared to play a role in epidermal development in maize (Becraft *et al.*, 1996). The widespread expression of *ACR4* in the outer epidermal layer further supports the involvement of ACR4 in a signalling pathway that is involved in epidermal specification (Gifford *et al.,* 2003). Surprisingly, given the strong phenotypes associated with loss of *CR4* function in maize i.e. severe defects in epidermal differentiation (Becraft *et al.*, 1996), loss of *ACR4* function leads to a quite subtle phenotype with no changes in gross shoot morphology. It was postulated that the relatively week phenotype seen in *acr4* mutants could be due to functional overlap between ACR4 and ACR4 related kinases (Gifford *et al.,* 2003). In this thesis, I have tested this hypothesis. The phenotype seen in quadruple and quintuple mutants of *ACR4* and *ACR4* related kinase-encoding genes, i.e. no exacerbation of

the acr4 phenotype, was not consistent with the proposed functional redundancy in epidermal specification. This is further supported by the recently available transcriptome data which suggested that the expression patterns of genes encoding *ACR4* related kinases are not very similar to that of *ACR4* and therefore a functional redundancy between ACR4 and ACR4 related kinases in epidermal specification is not expected.

ACR4 is also found to be involved in the regulation of lateral root initiation and in this pathway *ACR4* shows some degree of redundancy with the members of the *ACR4* gene family (*AtCRR1, AtCRR2, AtCRR3* and *AtCRR4*), (De Smet *et al.*, 2008). This result fits with the idea that the redundancy among homologs can differ in different tissues. The *ERECTA* family of RLKs in *Arabidopsis* acts redundantly in regulating organ growth (Shpak *et al.*, 2003; Shpak *et al.*, 2004), however such extensive redundancy is not observed in stomatal patterning (Shpak *et al.*, 2005).

Although less frequently, genetic redundancy can be observed among functionally analogous but unrelated genetic sequences (Galperin *et al.*, 1998). This type of redundancy has not been reported for plant or animal RLKs studied so far. However it cannot be ruled out that *ACR4* acts redundantly with unrelated genes of similar function. One such possible gene which could act redundantly with *ACR4* is *ALE2.* However, the epidermis related defects of *acr4 ale2* double mutants do not show any enhancement compared to the either of the single mutant phenotype (Tanaka *et al.*, 2007). It was, therefore, proposed that these two RLKs ACR4 and ALE2 could act in the same pathway. Two other RLKs, which could act redundantly with ACR4 are GASSHO1 (GSO1) and GSO2, because of the similar phenotype in each single mutants (Tsuwamoto *et al.*, 2008). Further experiments are required to understand the relationship of these two RLKs with ACR4.

8.2 ACR4 appears to undergo endocytosis through interaction of the cytoplasmic domain with unknown protein

ACR4 encodes a membrane localized protein with an extracellular domain consisting of seven repeats, termed 'crinkly repeats' and a domain homologous to the extracellular domain of TNFR. The extracellular region of an RLK is generally thought to be the ligand binding domain. A detailed analysis of the cleavage pattern of ACR4 receptor suggested that ACR4 undergoes cleavage in the extracellular domain, possibly twice in the 'crinkly repeat domain' and once in the 'TNFR domain' (Fig 4.9). Gifford *et al.* (2005) have shown that a mutant where the 'crinkly repeat' domain is deleted, unable to complement the *acr4* phenotype. Therefore, cleavage in the extracellular domain is possibly a crucial requirement for ACR4 receptor function. This cleavage pattern in the extracellular domain is of interest as this is the first demonstration of potential cleavage in the extracellular domain of an RLK among plant RLKs studied to date. In animal system, NOTCH represents a membrane bound protein which has been shown to undergo ligand-induced extracellular cleavage and subsequent intracellular cleavage (Shimizu *et al.,* 2002). It is therefore possible that an extracellular protease is involved in the ACR4 mediated signal transduction pathway, although there is no evidence for this so far. An alternative hypothesis is that the ligand binding can recruit a protease causing cleavage in the extracellular domain. Stahl *et al.* (2009a) proposed CLE40 as a potential ligand of ACR4 in the maintenance of the stem cell niche in the root apical meristem. However, the biological relevance of CLE40 ligand binding and cleavage in the extracellular domain of ACR4 is yet to be uncovered.

As discussed in Chapter-1, ACR4 receptor is continually internalised from the plasma membrane and degraded or cleaved (Gifford *et al.,* 2005) and receptor endocytosis is one of the common mechanisms by which the behaviour of RLKs can be modulated. As shown in Fig 4.10, the full-length ACR4 tagged with GFP (*pACR4-ACR4:GFP*) is less stabilized than the cytoplasmic domain deleted version (*pACR4-ACR4ΔC:GFP*). This is further supported by protein accumulation studies using confocal microscopy (Gwyneth Ingram pers. comm.). Therefore, it seems that

the degradation of ACR4 is regulated by the interaction of the cytoplasmic domain with an unknown protein.

Another important angle to explain the endocytic behaviour of ACR4 receptor is that like other RLKs, the endosomal localization of ACR4 could be linked with the activation of downstream targets. In both plant and animal systems, endocytosis of membrane bound receptors has been shown to activate signal transduction pathways (Wang *et al.,* 2002; Geldner *et al.,* 2007). Therefore, it cannot be ruled out that ACR4 receptor could signal from endocytic vesicles and that is followed by receptor degradation in a manner similar to the RLK, BRI1.

Here one important question is which downstream target is actually involved in regulating ACR4 endocytosis. As mentioned in Chapter-1, protein phosphatases, e.g. KAPP, are suggested as potential downstream targets of CLV1 and FLS2 (Stone *et al.,* 1998; Gomez-Gomez *et al.,* 2001). However, interaction with KAPP has not been shown to regulate degradation, suggesting that a different mechanism might regulate ACR4 internalization and degradation. The potential downstream target of ACR4 is yet to be identified.

The fact that the functional ACR4 receptor undergoes internalization and/or degradation and that the cytoplasmic domain is important in this process, suggests that a downstream target of ACR4 is a protein that directs the receptor to degrade. In this capacity, AIP1 which is known to interact with ACR4 in Y2H screening, was considered as a potential regulatory component interacting with ACR4 since AIP1 has MATH domains and MATH domains are known to interact with proteins involved in the destruction of a large number of regulatory proteins (Xu *et al.,* 2003). Although the gene expression and protein accumulation studies suggested potential interaction between these two proteins, I was unable to show this interaction *in vivo* by co-immunoprecipitation due to the low level of expression of *ACR4* gene in the root. To do co-immunoprecipitation, I generated a double transgenic line expressing AIP1:GFP and ACR4:MYC. MYC tagged ACR4 was always quite hard to detect in the root tissue due to the low level of expression of *ACR4* and this made the overall

co-immunoprocipitation experiment technically challenging. Generation of other epitope tagged lines could be important to determine the protein protein interaction between ACR4 and AIP1.

8.3 *ACR4* could have a role in plant defence

The pathogen challenge experiments suggested that *acr4* mutant plants show reduced susceptibility to the necrotrophic pathogen, *B. cinerea* compared to wild type. In addition, some of my preliminary experiments and research from other labs revealed that *acr4* mutants exhibit enhanced susceptibility to the biotropic pathogen, *P. syringae.* The leaves of *acr4* mutants have a permeable cuticle, as identified by the chlorophyll leaching experiment.

Based on these observations, several alternative hypotheses were proposed. One possibility is that *ACR4* could be involved in repressing/enhancing particular defence related signalling pathway. This is similar to what was seen in the cuticular defective *resurrection1* (*rst1*) (Mang *et al.,* 2009). The *rst1* mutant leaves have significantly elevated amounts of cutin monomers and these mutants exhibit enhanced susceptibility to biotropic pathogens and enhanced resistance to the necrotrophic fungal pathogens due to an attenuation of salicylic acid-dependent defence responses and enhancement of JA-dependent defence. Therefore, a potential link between *ACR4* expression and suppression of particular defence related hormonal signalling pathway is worthy of further investigation. It is possible that *ACR4* expression could potentially suppress the JA dependent defence pathway in the absence of *B. cinerea* infection, preventing premature immune responses and this could explain why *acr4* mutant plants show reduced susceptibility to *B. cinerea.* This speculation is supported by the transcriptome data showing enhanced expression of *LOX2* in floral meristem tissues of *acr4.* Analysis of the expression level of *LOX2* in *acr4* leaves compared to wild type using quantitative RT-PCR could reveal whether the resistance of *acr4* plants to *B. cinerea* is caused by the over-expression of *LOX2.* Additionally, detailed transcriptomic analysis of genes which are misexpressd in

acr4 mutants as well as proteomic analysis of ACR4 targets with and without *B. cinerea* infection could shed light on the role of *ACR4* in defence responses.

Another important angle to explain the reduced susceptibility phenotype of *acr4* to *B. cinerea* is the defective cuticular composition of *acr4* leaves. This is supported by the recent observation that cuticular defective mutants with permeable cuticle are resistant to *B. cinerea* (Bessire *et al.,* 2007; Voisin *et al.,* 2009). It has been proposed that a defective epidermal layer could allow enhanced diffusion of antifungal compounds onto the leaf surfaces and so cuticular defective mutants are less susceptible to *B. cinerea* (Fig 8.1). It is therefore reasonably possible that *acr4* mutants show reduced susceptibility to *B. cinerea* due to defective cuticles. The analysis of the cuticular lipid composition of *acr4* leaves as well as known mutants with defective cuticles by ESI-MS analysis which is already started, will be continued in the lab.

A potential functional redundancy among the members of the *ACR4* gene family in plant defence cannot be ruled out. This is supported by the observation that *ACR4* related kinases act redundantly with *ACR4* in the formation of lateral roots (De Smet *et al.,* 2008), although no such redundancy was observed in epidermal specification. Transcriptome data suggested that *ACR4* and *ACR4* related kinases are differentially expressed in response to different pathogens, however very similar expression patterns were observed for *AtCRR3* and *AtCRR4*. Challenging multiple combinations of knock out lines using different pathogens might elucidate any potential redundancy among the members of *ACR4* gene family in plant defence.

To summarize, *ACR4* encodes a cell-surface receptor that functions in a signalling pathway that specifies and maintains epidermal cell layer and this function of *ACR4* does not act redundantly with *ACR4* related kinases. Although no specific ligand has been identified yet, ACR4 may undergo endocytosis upon ligand induced cleavage in the extracellular domain and interaction of the cytoplasmic domain with unknown protein. Like other dual regulators in *Arabidopsis* (Nurmberg *et al.,* 2007; Chen *et al.,* 2005; Mang *et al.,* 2009), the developmental regulator ACR4 could also be involved in defence responses, although how these two pathways are interlinked, remains unknown.

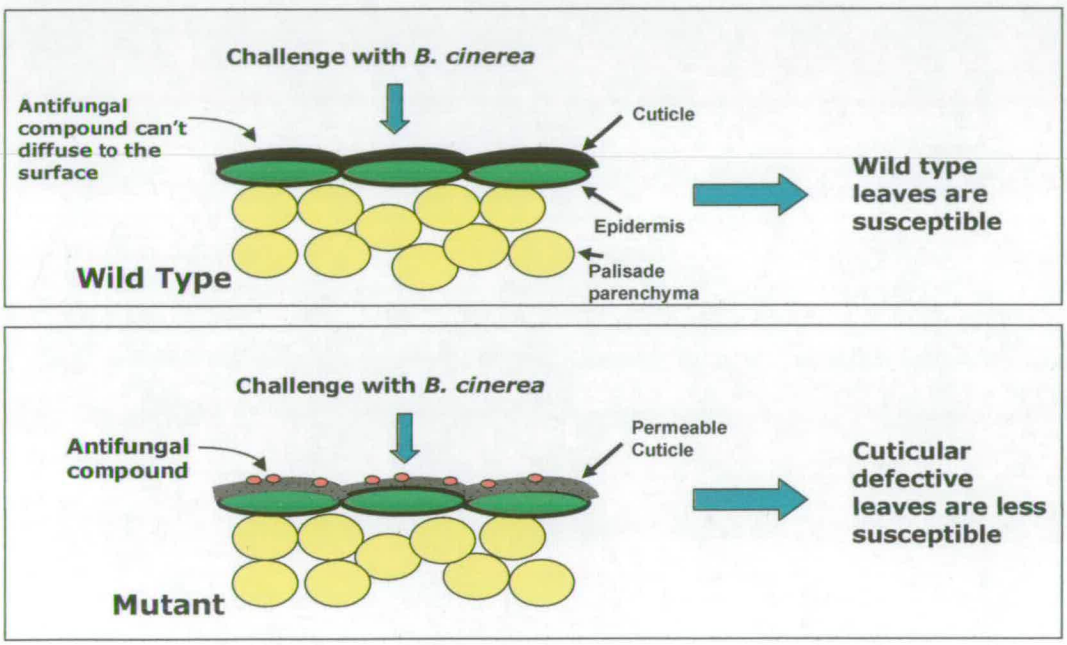


Fig 8.1: A schematic illustration of the proposed *B. cinerea* resistance phenotype of cuticular defective mutants.
Bessire *et al.* (2007) have shown that cuticular defective mutants allow enhanced diffusion of antifungal compounds onto the leaf surface and therefore leaves with permeable cuticle are less susceptible to *B. cinerea*.

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