Genomic analysis and characterisation of *adr1*, an activated *Arabidopsis* mutant overexpressing a *CC-NBS-LRR* gene that confers disease resistance and drought tolerance

Andrea Chini

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Institute of Cellular & Molecular Biology

The University of Edinburgh

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Abbreviations

4x*35S*	tetramer of *CaMV 35S* enhancer element
ABA	abscisic acid
ABRE	ABA-responsive element
At	*Arabidopsis thaliana*
avr	avirulence gene
BLAST	Basic Local Alignment Search Tool
BTH	benzothiadiazole
bZIP	Basic Leucine Zipper
CaMV	Cauliflower Mosaic Virus
CBL	calcineurin B-like
CC	Coiled-Coil
CDPK	Calcium-Dependent Protein Kinases
CNL	CC-NBS-LRR
DEX	dexamethasone
DNA	deoxyribonucleic acid
DREB	Dehydradation-Responsive Element
ET	Ethylene
GST	Glutathione S-Transferase
HR	Hypersensitive Response
HSP	Heat Shock Protein
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
KB	King's Broth media
LUC	luciferase
LRR	Leucine-Rich Repeat
MAPK	Mitogen Activated Protein Kinase
MEME	Multiple Expectation Maximisation for Motif Elicitation
Me-JA	Methyl Jasmonate
MS	Murashige and Skoog media
NBS	Nucleotide-Binding Site
PCR	Polymerase Chain Reaction
PDF	plant defensin
PR	Pathogen Related protein
Pst Dc3000	*Pseudomonas syringae* pv *tomato* DC3000
R	Resistance gene
RD	Responsive to Dehydration
RNA	ribonucleic acid
ROI	Reactive Oxygen Intermediate
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SSC	Solution of sodium citrate
TAIL-PCR	Thermal Asymmetric Interlaced PCR
TAIR	The *Arabidopsis* Information Resource
TIR	Toll/Interleukin-1 Receptor
TNL	TIR-NBS-LRR

Abstract

A transgenic *Arabidopsis* line containing a chimeric *PR-1*::*luciferase* (*LUC*) reporter gene was subjected to mutagenesis with activation tags. Screening of lines via high throughput LUC imaging identified a number of dominant *Arabidopsis* mutants that exhibited enhanced *PR-1* gene expression. Here we report the characterisation of one of these mutants, designated activated disease resistance (*adr*) 1. This line showed constitutive expression of a number of key defence marker genes and accumulated salicylic acid, but not ethylene or jasmonic acid. Furthermore, *adr1* plants exhibited resistance against the biotrophic pathogens *Peronospora parasitica* and *Erysiphe cichoracearum* but not the necrotrophic fungus *Botrytis cineria*. Analysis of a series of *adr1* double mutants suggested that *adr1*-mediated resistance against *P. parasitica* was SA-dependent, while resistance against *E. cichoracearum* was both SA-dependent and partially NPR1-dependent. The controlled, transient expression of *ADR1* conveyed striking disease resistance in the absence of yield penalty, highlighting the potential utility of this gene in crop protection.

The *ADR1* gene encoded a protein possessing a number of key features including homology to subdomains of protein kinases, a nucleotide binding domain and leucine rich repeats. Sequence analysis revealed that *ADR1* is a member of a small atypical *Arabidopsis* sub-class containing four *CC-NBS-LRR* genes. In addition, homologous genes were uncovered in many phylogenetically distant and agronomically important plant species; their sequence analysis revealed a number of consensus motifs unique and distinctive for the ADR1 family.

Furthermore, we show that either constitutive or conditional enhanced expression of *ADR1* conferred significant drought tolerance. This was not a general feature of defence-related mutants because *cir1*, *cir2* and *cpr1*, which constitutively express SAR, failed to exhibit this phenotype. Cross-tolerance was not a characteristic of *adr1* plants, rather they showed increased sensitivity to thermal and salinity stress. Hence, *adr1* activated signalling may antagonise some stress responses. Northern analysis of abiotic marker genes revealed that *DREB2A* but not *DREB1A*, *RD29A* or *RD22* were expressed in *adr1* plant lines. Moreover, *DREB2A* expression was SA-dependent but NPR1-independent. Microarray analyses, of plants containing a conditional *ADR1* allele, demonstrated that a significant number of the up-regulated genes had been previously implicated in responses to dehydration. Therefore, biotic and abiotic signalling pathways may share multiple nodes and their outputs may have significant functional overlap.

Additionally, a large activation tagged population was screened in order to isolate novel mutants altered in disease susceptibility. Here we report the characterisation of one mutant, designated activated disease susceptibility (*ads*) 1. This line was confirmed to concurrently exhibit increased susceptibility to hemi-biotrophic, necrotrophic and non-host pathogens.

"The desire to know is natural to good men ... one day the world will look upon research on plants as it now looks on human beings"

Leonardo da Vinci, 1452 – 1519

1) Introduction

1.1 General Context

Plants cannot move to escape environmental challenges; some of these biotic stresses are markedly critical. In fact, a myriad of potential pathogens, fungi, bacteria, nematodes and insects obtain products metabolised by plants, and viruses use replication systems at the host's expense. Therefore, plants have evolved sophisticated mechanisms to detect such attacks and to translate that perception into an adaptive response.

The complex and refined systems evolved by plants enable them to successfully withstand infection by the vast majority of potential pathogens. What makes this achievement truly remarkable is the sheer diversity of infection mechanisms that these pathogens use. While most immunologists may view the mammalian immune system as a pinnacle of evolution, natural selection has honed the defence system of plants over 1.6 billion years without recourse to antibodies, T cells and similar mechanisms, producing strategies no less refined or effective. Nevertheless, recent findings have highlighted remarkable similarities in the innate defence systems of plants, animals and insects (van der Biezen and Jones, 1998; Nurenberger and Brunner, 2002). It is surprising that recognition of pathogen associated molecular patterns, presence of proteins sharing similar domains in recognition complexes, MAPK-mediated activation of immune response genes and subsequent production of antimicrobial products similarly occur in both plant and animal kingdoms (Staskawicz et al., 2001; Nurenberger and Brunner, 2002).

Though plants can recognize and mount successful defence against a vast majority of pathogens, it has been estimated that at least 12% of potential global crop production is lost annually to pre-harvest plant disease (Baker et al., 1997; Shah, 1997; Trewavas, 2001). Despite the development and use of an increasing number of pesticides and fungicides, crop losses due to disease still continue. Plant breeding has been used to introduce genes from wild populations into commercial crop cultivars, but this resistance is often not durable as pathogens are able to evolve quickly and overcome it (Pink and Puddephat, 1999; Jones, 2001; McDowell and Woffenden, 2003). Nowadays agriculture, which is heavily dependent on the chemical control of a multitude of pathogens, demands unsustainable environmental and economic costs (Trewavas, 2001). In developing countries, where chemicals are often an unaffordable luxury, crop losses are often measured in term of human starvation and death. This is likely to deteriorate in the near future: global population is predicted to reach its peak by 2025 when there will be an estimated 2.3 billion extra people on the earth, with 95% of this growth occurring in less developed countries (Trewavas, 2001).

Therefore alternative strategies for sustainable agriculture, respecting the natural environmental balance and taking into account the global population increase, must be achieved. Advances in our understanding of plant defence systems and recent progresses in biotechnology offer new tools to control plant disease through the development of more efficient and environmentally friendly pesticides, resistant germplasm and the genetic engineering of plants with enhanced resistance against diseases (Stuiver and Custers, 2001; Hammond-Kosack and Parker, 2003).

Although several attempts have been made to engineer durable disease resistance, unfortunately, many of these efforts have failed due to the complexity of disease resistance signalling and the sheer diversity of pathogen infection mechanisms (Stuiver and Custers, 2001). Alternatively, transgenic plants that exhibited durable disease resistance could not be commercially exploited because of the detrimental effects on plant growth, development and crop yield (Purrington, 2000; Hammond-Kosack and Parker, 2003). Moreover, the pervasive negative reporting of so-called genetically

modified (GM) crops in the media has greatly damaged the general public opinion on potential benefits offered by progress in biotechnology. Nonetheless, "second generation" GM crops may be more acceptable to a currently mistrustful public.

The overall aim of the work described in this thesis was to gain further understanding into the genetic basis of plant defence. More indirectly, this research might contribute to the development of crops with enhanced disease resistance.

1.2 Gene-for-gene resistance and hypersensitive response

Plants are constantly subject to attack by a plethora of pathogens. If during an interaction with a plant, the pathogen is able to penetrate the plant and cause disease, the pathogen is said to be virulent, the plant susceptible and the interaction compatible. On the other hand, plant may be able to activate defence responses promptly, thus preventing the development of the disease. In this case the pathogen is said to be avirulent, the plant resistant and the interaction incompatible (Staskawicz et al., 1995; Dangl et al., 1996).

1.2.1 Gene-for-gene hypothesis

In the gene-for-gene interaction model, first proposed by Flor thirty years ago, an incompatible interaction was hypothesised to occur after the recognition of an avirulance (*avr*) gene product by the corresponding plant resistance (*R*) gene product (Flor, 1971). *R-avr* interactions have been observed between plants and many different pathogens, including bacteria, fungi, viruses and nematodes (Dangl et al., 1996; Heath, 2000; Hammond-Kosack and Parker, 2003). The mechanism for incompatible interaction requires that the *R* product specifically recognises an *avr* gene product and triggers a signal transduction cascade, which culminates in the activation of defence mechanisms and the arrest of pathogen infection (Flor, 1971; Staskawicz et al., 1995; Baker et al., 1997; Yang et al., 1997).

1.2.1 Plant R genes

R-avr interactions were hypothesised to be extremely specific, thus these molecules have been intensively investigated and during the last decade several *R* genes from model plants and crop species have been identified and cloned (Ellis et al., 2000; Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003). The *R* genes isolated to date can modulate resistance against bacteria, fungi and viruses as well as nematodes and insects (Dangl and Jones, 2001). Despite the wide range of pathogen species and the presumed difference in their pathogenicity effector molecules, *R* genes encoded only five classes of proteins (Bent, 1996; Ellis et al., 2000; Dangl and Jones, 2001). Most of these genes have been initially identified in model plants, such as *Arabidopsis*, tobacco and rice, but the presence of homologues in many divergent plant species has been now confirmed (Meyers et al., 1998; Cannon et al., 2002; Hammond-Kosack and Parker, 2003).

The largest *R* gene group, designated as class 1, encoded for nucleotide-binding site leucine-rich repeat (NBS-LRR) class of proteins (Jones, 2001; Meyers et al., 2003). The NBS domain was generally located in the central portion of the protein and was required for ATP and GTP binding (Saraste et al., 1990). Several conserved motifs have been identified in the NBS domain and they are described in Chapter 8. The LRR domain defined a conserved structural region with a variable number of carboxy-terminal LRRs, which function in direct protein-protein interaction, peptide-ligand binding and protein-carbohydrate interaction (Jones and Jones, 1996; Bent et al., 1996; Jia et al., 2000; Thomas et al., 1996). This class of R proteins was further subdivided into two subclasses based on the structure of the N-terminal region. Proteins containing a domain showing high homology with the Toll/Interleukin-1 Receptor (TIR) were classified as TIR-NBS-LRR (TNL) (Meyers et al., 1999; Pan et al., 2000a). Both *Drosophila* Toll proteins and mammalian immune response Interleukin receptors play a role in defence (Baker et al., 1997). Most of the NBS-LRR proteins lacking the TIR domain showed a coiled-coil (CC) motif in the N-terminal region; thus they were designated as CC-NBS-LRR (CNL) (Pan et al., 2000a; Meyers et al., 1999).

The R proteins which did not show NBS-LRR structure were subdivided into four further classes (Dangl and Jones, 2001). The second group consisted of several *Cf* genes that encoded for proteins with three characteristic domains: an extracelluar LRR region, a transmembrane domain and a short cytoplasmatic peptide (Jones and Jones, 1996; Dixon, 2000). *R* genes included in the third class encoded cytoplasmatic protein kinases; for example, tomato *Pto* encoded a Ser/Thr kinase whose activation conferred disease resistance (Martin et al., 1993). The fourth class of *R* genes encoded proteins showing three specific structural domains: an extracelluar LRR domain, a transmembrane motif and a cytoplasmatic protein kinase domain (Dangl and Jones, 2001). Thus this structure closely resembles the union of R proteins from the second and third class. The rice *Xa21* was the first gene of this class to be cloned (Song et al., 1995). The fifth and last class of *R* gene was the most recently uncovered and consisted of only two paralog genes, *RPW8.1* and *RPW8.2* (Xiao et al., 2001). These two genes encodes for relatively short proteins consisting of a transmembrane domain and a putative cytoplasmatic CC domain (Xiao et al., 2001).

1.2.3 The hypersensitive response

The *R* mediated recognition of avirulent pathogens initiates a signal transduction cascade that culminates in the activation of the plant defence system (Staskawicz et al., 1995; Yang et al., 1997). This response is often accompanied by a rapid cell death in and around the initial infection side, a reaction known in plants as local hypersensitive response (HR) (Dangl et al., 1996). This defence mechanism is associated with restricted pathogen growth and represents a form of programmed cell death (Heath, 2000; Loake, 2001). In cells surrounding the HR area, a number of additional local responses, such as high accumulation of phenolic compounds and cell-wall reinforcements, also occur in order to confine the pathogen and protect the plant (Dangl et al., 1996). Production of pathogenesis-related (PR) proteins, with antimicrobial functions, is also triggered (Ward et al., 1991; Hammond-Kosack and Jones, 1996).

1.3 Systemic Acquired Resistance and SA-dependent disease resistance pathway

1.3.1 Systemic Acquired Resistance

Following an incompatible interaction is the establishment of immunity to secondary infections in systemic tissues, a mechanism designated as systemic acquired resistance (SAR) (Uknes et al., 1992). This systemic response conveys long-lasting protection against a broad spectrum of normally virulent pathogens (Ryals et al., 1996). Associated with the onset of SAR are the systemic production of PR proteins and the establishment of disease resistance (Ward et al., 1991; Ryals et al., 1996). Though SAR has been extensively investigated, the translocation factor by which SAR is induced from the infection site to the rest of the plant still remains unknown.

1.3.2 The function of salicylic acid

Salicylic acid (SA) undoubtedly plays an essential role in the onset of SAR (Malamy et al., 1990; Metraux et al., 1990); however, a number of independent studies have clarified that SA is not the messenger factor that moves throughout the phloem (Vernooij et al., 1994; Ryals et al., 1995). The precise function of SA during the establishment of SAR in distal tissue is still unclear. Currently, the accumulating data supports the theory that SA functions at multiple nodes in the defence signalling network, most likely by acting as a signal amplifier (Shirasu et al., 1997). Consistent with this hypothesis, SA increases in plants after infection in both local and systemic tissues, and it is also required for the expression of PR genes (Malamy et al., 1990; Metraux et al., 1990; Ryals et al., 1996). In addition, application of SA or its analogues, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), induces broad spectrum disease resistance in plants (Uknes et al., 1992; Gorlach et al., 1996).

1.3.3 Constitutive disease resistance mutants

The isolation of loss-of-function mutants, mainly in *Arabidopsis*, exhibiting abnormalities in disease resistance has improved the understanding of the SAR transduction network and the role of SA (Glazebrook, 2001; Lorrain et al., 2003;

Hammond and Parker, 2003). A large number of mutants that accumulate high levels of SA and exhibit constitutive disease resistance have been isolated and characterise to date (Lorrain et al., 2003); thus, only the most relevant disease resistant mutants and those specifically employed in this research will be described here.

Among the initial mutants isolated, *cpr1* (constitutive of pathogen related proteins) plants exhibited dwarf phenotype, constitutive expression of several SA induced defence genes and enhanced disease resistance (Bowling et al., 1994). The *cpr1 nahG* double mutant did not accumulate SA nor showed *cpr1* phenotype, indicating that CPR1 acted upstream of SA in the SAR signalling pathway (Bowling et al., 1994). The *CPR1* gene has not been identified yet. The *cpr6* mutant line also exhibited a similar SA-dependent phenotype: constitutive expression of SA-induced defence genes and enhanced disease resistance (Clake et al., 1998). In addition to enhanced disease resistance, *cpr5*, *cpr20*, *cpr21* and *cpr22* mutant plants also showed spontaneous necrotic lesions (Bowling et al., 1997; Silva et al., 1999; Yoshioka et al., 2001).

The *lsd1* (lesion simulating disease response) mutant leaves also exhibited spontaneous necrotic lesions, which resembled those resulting from disease, but occurred in the absence of pathogen infection (Dietrich et al., 1994). The *LSD1* gene encodes for a zinc-finger-protein suggesting that LSD1 could regulate transcription in response to signals generated by pathogen-infected cells undergoing HR (Dietrich et al., 1997). Similarly to *cpr* mutants, *lsd1* plants constitutively expressed SA-responsive defence genes and showed enhanced disease resistance; however *lsd1* mutants did not reveal morphological abnormalities (Dietrich et al., 1994; 1997). The majority of mutants exhibiting constitutively active SA defence pathway and enhanced disease resistance also developed spontaneous necrotic lesions (Lorrain et al., 2003). Constitutive cell death was therefore suggested to induce SA production and, subsequentially, defence gene expression (Greenberg et al., 1994; Lorrain et al., 2003)

Constitutive disease resistance could also occur in the absence of morphological phenotype and extensive HR cell death in two mutants, *dnd1* and *cir1* (Yu et al., 1998; Murray et al., 2002). The *DND1* gene encoded for a cyclic nucleotide-gated ion channel,

indicating the involvement of ion fluxes in defence activation or in pathogen-induced cell death (Clough et al., 2000). Furthermore, a number of mutant lines, *edr1*, *pmr1*, *pmr2*, *pmr3* and *pmr4*, have been isolated that exhibited increased disease resistance in the absence of constitutive expression of defence genes and elevated SA levels (Frye and Innes, 1998; Vogel and Somerville, 2000). Therefore, disease resistance observed in these mutant lines is not due to the constitutive activation of defence responses but to the activation of an enhanced defence mechanism in response to pathogen attack (Frye and Innes, 1998; Vogel and Somerville, 2000). In summary, the isolation of several enhanced disease resistance mutant lines has allowed the dissection and a better understanding of plant defence pathways (Yun and Loake, 2002; Hammond and Parker, 2003).

1.3.4 SA deficient mutants and transgenic lines

The conclusive proof for the SA requirement to establish SAR came from the investigation of mutants incapable of responding to SA. The *Arabidopsis* and tobacco transgenic *nahG* plants, expressing a bacterial salicylate hydroxylase which converted SA into catechol, accumulated greatly reduced amount of SA, did not express PR genes or display SAR in response to SAR-inducing infections (Gaffney et al., 1993; Delaney et al., 1994; Friedrich et al., 1995). Furthermore, *nahG* plants were strikingly more susceptible to various pathogens, confirming the requirement for SA accumulation to mount plant defence responses (Delaney et al., 1994; Friedrich et al., 1995). Wild-type plants treated with catechol, the degradation product to which SA was converted in *nahG* transgenic lines, showed the loss of non-host resistance against *Pseudomonas syringae* pv *phaseolicola* (VanWees and Glazebrook, 2003). However, race specific and R mediated defence responses did not appear to be affected by catechol accumulation (VanWees and Glazebrook, 2003).

Several independent projects aimed to isolate loss-of-function mutants deficient in or insensitive to SA (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997; Nawrath and Metraux, 1999). The allelic mutants nonexpresser of *PR* genes (*npr1*), noninducible immunity (*nim1*) and salicylic acid insensitive (*sai1*) accumulated SA normally after

pathogen recognition, but were non-responsive to SA (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). In addition, *npr1* plants exhibited reduced disease resistance and loss of *PR1* expression following SA treatment (Cao et al., 1994). The *NPR1* gene encoded an ankyrin-repeat protein whose interaction with the transcription factor TGA2 was essential to activate *PR1* expression and disease resistance (Fan and Dong, 2002). NPR1 was normally present as an oligomeric cytosolic aggregate and, upon SAR induction, the change in cellular reduction potential induced NPR1 reduction into a monomeric form that could be transferred to the nucleus (Mou et al., 2003).

Two SA induction deficient (*sid1* and *sid2*) *Arabidopsis* mutants also showed decreased expression of defence genes and increased susceptibility to several pathogens (Nawrath and Metraux, 1999). *EDS5/SID1* encoded a MATE (multidrug and toxin extrusion) transporter protein that was induced by pathogen recognition and SA treatment, suggesting that EDS5 acts in a positive feedback regulation loop (Nawrath et al., 2002). *SID2/EDS16/ICS1* encoded an isochorismate synthase that was induced upon pathogen recognition in order to synthesise SA, which in turn triggered local defence and SAR (Wildermuth et al., 2001).

1.4 JA- and ET-dependent disease resistance pathways and Induced Systemic Resistance

Two additional signalling molecules, jasmonic acid (JA) and ethylene (ET) play important roles in the induction of disease resistance against several pathogens (Loake and Nurmberg, 2003; Solano and Ecker, 1998). Mutants exhibiting abnormal response to JA and ET were precious genetic tools to dissect disease resistance pathways (Stepanova and Ecker, 2000; Berger, 2002; Devoto and Turner, 2003).

1.4.1 Jasmonic acid-dependent disease resistance pathway

JA and its volatile counterpart methyl jasmonate (Me-JA) are signal molecules conserved throughout higher plants and affect many different processes (Creelman and

Mullet, 1997). JA and Me-JA are involved in the response to wounding, resistance to insects and a plethora of pathogens and are also required for plant development (Creelman and Mullet, 1997; Leon et al., 2001). Proteins encoded by JA-responsive genes include antimicrobial peptides, phytoalexin biosynthetic enzymes, storage proteins, stress protectants and wound-induced proteinase inhibitors (Loake and Nurmberg, 2003).

Both JA accumulation and plant defensin (*PDF1.2*) expression occurred in *Arabidopsis* plants 3 days after infection with *Alternaria brassicicola*, a necrotrophic fungal pathogen (Penninckx et al., 1996; Thomma et al., 1999a). Pre-treatment with JA, but not with SA or its chemical analogue INA, triggered *PDF1.2* accumulation and disease resistance against *A. brassicicola* in wild-type plants, but not in mutants insensitive to JA or ET (Penninckx et al., 1996). The *Arabidopsis fad3-2 fad7-2 fad8* triple mutant, deficient in JA production, was extremely susceptible to the necrotrophic fungus *Pythium mastophorum* (Vijayan et al., 1998). However, prior application of Me-JA restored *fad3-2 fad7-2 fad8* resistance to *P. mastophorum*, consistent with the hypothesis that jasmonates were crucial in mediating disease resistance (Vijayan et al., 1998). In addition, the *esa1* (enhanced susceptibility to *A. brassicicola*) mutant was more susceptible than wild-type plants to several necrotrophic fungi, but not to biotrophic pathogens (Tierens et al., 2002). The *esa1* enhanced susceptibility correlated with the delayed accumulation of JA and *PDF1.2* expression (Tierens et al., 2002). In contrast, *cev1* (constitutive expression of *VSP1*) mutant, which constitutively accumulated high levels of JA and *PDF1.2* transcripts, exhibited enhanced disease resistance to three *Erysiphe* isolates (Ellis and Turner, 2001). Altogether, these data confirmed the presence of a JA-dependent SA-independent disease resistance pathway.

Interestingly, separate JA- and SA- dependent defence response pathways in plants conveyed resistance against different pathogens and many studies have suggested an antagonistic correlation between these two separate signalling pathways (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Li et al., 2004). For example, *PAL* (phenylalanine ammonia-lyase) overexpression in transgenic plants enhanced resistance against larvae of *Heliothis virescens* but reduced SAR (Felton et al., 1999). In contrast,

the silencing of *PAL* induced resistance to necrotrophic pathogens but decreased SAR (Felton et al., 1999).

Microarray results also showed that SA and JA induced the expression of different sets of genes (Glazebrook et al., 2003); in addition, SA treatment inhibited the expression of many JA-responsive genes, whereas JA treatment inhibited the expression of several SA-responsive genes (Schenk et al., 2000). In this context, WRKY70 has been recently identified as a concurrent activator of SA-induced genes and repressor of JA-responsive genes, suggesting that WRKY70 is a regulator node of SA and JA antagonistic pathways (Li et al., 2004). However, a number of genes are induced by either JA or SA (Schenk et al., 2000; Glazebrook et al., 2003). It is also worth noting that some genes are synergistically induced in response to JA and SA (Schenk et al., 2000; Glazebrook et al., 2003). Therefore, the control of transcription cued by these two phytohormones appears predominantly, but not exclusively, antagonistic (Schenk et al., 2000; Glazebrook et al., 2003; Li et al., 2004).

1.4.2 Ethylene-dependent disease resistance pathway

ET is a simple gas (C_2H_4) that affects many stages of plant growth and development including: germination, senescence, abscission, flowering and response to various stresses (Solano and Ecker, 1998). ET production often occurs during plant-pathogen interactions but its functions in disease resistance or susceptibility are currently uncertain and may depend on the pathogen species involved (Thomma et al., 1999b; Berrocal-Lobo et al., 2002).

Elucidation of ET perception and signal transduction relied on the isolation and characterisation of mutants that exhibited defective ET responses (Stepanova and Ecker, 2000; Guo and Ecker, 2004). ET perception is mediated by several receptors consisting of integral membrane proteins; among them ETR1, ETR2 (ethylene receptor), EIN4 (ethylene insensitive), ERS1 and ERS2 (ethylene response sensor) (Hua and Meyerowitz, 1998). Signal transduction is accomplished via a protein kinase cascade regulated by the MAP kinase CTR1 (constitutive triple response) (Keiber et al., 1993). Finally, signal

transduction triggers the expression of transcription factors such as ERF1 (ethylene response factor) and other EREBPs (ethylene response element binding protein) which subsequently promote transcription of defence-related genes (Solano et al., 1998; Lorenzo et al., 2003).

In order to study the onset of disease resistance in plants deficient in the ET signalling pathway, the ethylene insensitive (*ein2*) *Arabidopsis* mutant was analysed (Alonso et al., 1999). Infection with *A. brassicicola* induced accumulation of *PDF1.2* and other defence genes in wild-type plants, but the induction of these genes was abolished in *ein2* mutants (Penninckx et al., 1996). Furthermore, *ein2* plants were markedly more susceptible to *Botrytis cinerea* than wild-type plants; however, challenge of *ein2* mutants with *P. parasitica* or *A. brassicicola* failed to promote fungal infection (Thomma et al., 1999b). The JA insensitive *coi1* mutant also exhibited enhanced disease susceptibility to *B. cinerea*, suggesting that both JA and ET pathways are required to convey resistance to this necrotrophic pathogen (Penninckx et al., 1998). In addition, transcription of *PDF1.2* was concurrently activated by JA and ET signalling upon pathogen infection (Penninckx et al., 1998).

The current knowledge of the ET defence response pathway suggests that this phytohormone plays an important role in the establishment of disease resistance to some but not all types of pathogens (Guo and Ecker, 2004). Furthermore, several independent studies have confirmed that ET can trigger disease resistance synergistically or antagonistically to SA and/or JA depending on specific responses to different pathogens (Penninckx et al., 1998; O'Donnell et al., 1996; Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). In this context, both ET and JA were required to trigger induced systemic resistance (ISR) (Pieterse et all., 1998).

1.4.2 Induced Systemic Resistance

In addition to the SA-dependent SAR, a novel systemic defence mechanism has been recently reported. Treatment of *Arabidopsis* plants with the nonpathogenic root-colonizing bacteria *Pseudomonas fluorescens* conferred systemic resistance against

virulent *P. syringae*; this novel defence mechanism was designated as ISR (Pieterse et al., 1998). Importantly, ISR required intact JA, ET and NPR1 signalling pathways, whereas it was independent of SA (Pieterse et al., 1998). SAR could also convey resistance against *P. syringae* and the simultaneous induction of SAR and ISR exhibited an additive effect on *P. syringae* resistance (Uknes et al., 1992; VanWees et al., 2000). These results suggested that SAR and ISR pathways were established through parallel signalling cascades that did not exhibit significant cross-talk.

1.5 *R* gene mediated disease resistance

To better understand R-mediated defence signalling, many projects aimed to identify mutants disrupted in defence signalling. Several "enhanced disease susceptibility" mutants were isolated and a number of genes that are essential for full expression of *R* gene-mediated resistance were identified (Glazebrook et al., 1996; Parker et al., 1996; Century et al., 1997; Muskett et al., 2002; Tornero et al., 2002; Tor et al., 2002).

1.5.1 The role of EDS1 and NDR1

The characterization of *eds1* and *ndr1* mutants suggested that at least two distinct R-mediated resistance pathways existed in plants (Parker et al., 1996; Glazebrook et al., 1996; Century et al., 1997). EDS1 encoded for a protein homologous to eukaryotic lipases, although the putative lipase activity has not been confirmed either *in vitro* or *in vivo* (Falk et al., 1999). EDS1 was required to establish the resistance signalling cascade induced by *TIR-NBS-LRR* genes (Feys et al., 2001). In contrast, *NDR1* encoded a putative membrane-associated protein of unknown function which was essential to establish resistance triggered by most, but not all, *CC-NBS-LRR* genes (Aarts at el., 1998).

Many *TIR-NBS-LRR* genes, such as *RPP1*, *RPP4*, *RPP5*, *RPP10*, *RPP14* and *RPS4*, were proven to convey resistance in an EDS1-dependent but NDR1-independent manner (Dodds and Schwechheimer, 2002). In addition, the *RPW8* genes that have been recently

identified also convey resistance independently to NDR1 but in an EDS1 dependent manner (Xiao et al., 2001). The two tandem *RPW8* genes encoded for short R proteins that exhibited high similarity to CC domain of CC-NBS-LRR proteins, lacked both the NBS and LRR domains and were therefore classified in a separate R gene group (Dangl and Jones, 2001; Xiao et al., 2001). Furthermore, none of the characterised *CC-NBS-LRR* genes required a functional EDS1 pathway to establish resistance (Dodds and Schwechheimer, 2002).

Several *CC-NBS-LRR* genes required a functional NDR1 protein to properly establish disease resistance: *RPM1*, *PRS2* and *RPS5* represent some examples (Dodds and Schwechheimer, 2002). Thus, EDS1 and NDR1 appeared to specify two distinct resistance signalling pathways that were activated by classes of structurally different R proteins (Aarts et al., 1998). However, at least two genes, *RPP8* and *RPP13*, encoding for proteins with CC-NBS-LRR structures triggered resistance independently of NDR1 (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). Furthermore, *RPP8* and *RPP13* are also EDS1 independent, hence suggesting the existence of at least one additional pathway which establishes *R* gene mediated resistance independently of EDS1 and NDR1. The analysis of *RPP7*-mediated disease resistance showed that the function of NDR1 and EDS1 are partially redundant, but confirmed the existence of at least one additional pathway (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001).

1.5.2 The role of RAR1 and SGT1B

Additional downstream components of the *R* mediated resistance pathway have been recently identified. In barley, the *RAR1* gene was initially isolated as necessary for the specific *Mla12* mediated resistance; however, several additional *Mla* and powdery mildew *R* genes also triggered resistance dependent on RAR1 in barley (Freialdenhoven et al., 1994; Shirasu et al., 1999). The *Arabidopsis* homolog of *RAR1* was necessary for the establishment of resistance conveyed by several *R* genes that belonged to both *CNL* and *TNL* subgroups (Muskett et al., 2002; Tor et al., 2002; Tornero et al., 2002). Therefore the RAR1 regulation of R-mediated resistance was not based on the structure specificity of the cognate R protein.

RAR1 also differed from EDS1 and NDR1 in the fact that it possessed homologues not only among several plant species, but also amongst other eukaryotic organisms (Shirasu et al., 1999). Plant *RAR1* genes encoded a protein with two conserved zinc-finger motifs, CHORD-I and CHORD-II (Shirasu et al., 1999; Muskett et al., 2002). However RAR1 lacked a C-terminal domain, designated as CS, which was present in metazoan *RAR1* homologues (Muskett et al., 2002; Tornero et al., 2002). Nevertheless, the conserved CS motif was present in a different eukaryotic protein, SGT1, suggesting an evolutionary domain division event in eukaryotic organisms (Shirasu et al., 1999; Austin et al., 2002). Indeed, co-immunoprecipitation experiments in *Arabidopsis* and barley confirmed that RAR1 and SGT1 proteins interacted both *in vitro* and *in vivo* (Azevedo et al., 2002).

Similarly to RAR1, SGT1 was necessary to establish resistance mediated by some *CC-NBS-LRR* and *TIR-NBS-LRR* genes, suggesting that SGT1 did not regulate *R*-mediated resistance based on structure specificity of R proteins (Tor et al., 2002). It is noteworthy that a number of *R* genes, such as *RPP4*, *RPP5* and *RPP21*, exhibited a requirement for both RAR1 and SGT1; therefore, RAR1 and SGT1 pathways did not appear mutually antagonistic (Dodds and Schwechheimer, 2002). As previously described, *RPP8* and *RRP13* conveyed resistance independently of EDS1 and NDR1 (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). Furthermore, *RPP8* required neither RAR1 nor SGT1. The requirement of RAR1 and SGT1 for *RPP13*-mediated resistance is still unknown (Dodds and Schwechheimer, 2002); however, these data confirmed the existence of at least one unidentified *R*-mediated resistance signalling pathway.

SGT1 was originally isolated and characterized in yeast, where it interacts with SKP1, which was identified as a component of the SKP1/Cullin/F-box protein (SCF) ubiquitin ligase complex (Kitagawa et al., 1999). The GST1/SKP1 interaction was well conserved in plants suggesting a role for ubiquitination in the plant resistance response (Azevedo et al., 2002). In this context, evidence that RPM1 protein was rapidly degraded after pathogen infection in *Arabidopsis* cells underlined the potential importance of protein degradation in plant resistance (Boyes et al., 1998). In addition, AvrRpt2 recognition caused RIN4 (RPM1 interacting protein) degradation during the activation of RPS2

mediated resistance pathway (Mackey et al., 2002; Axtell and Staskawicz, 2003). Thus, it is not surprising that proteolysis complexes are, directly or indirectly, involved in plant defence response. A recent RNA silencing study in tomato plants confirmed that SGT1 was required to convey R mediated disease resistance (Peart et al., 2002). Surprisingly, SGT1 was also required to establish non-host disease resistance: hence it was suggested that R mediated and non-host resistance might involve similar proteolytic defence mechanisms (Peart et al., 2002).

1.5.3 The role of HSP90

Recently, a yeast two-hybrid screen identified a cytosolic heat shock protein 90 (HSP90) as an interacting protein of RAR1 (Takahashi et al., 2003). Co-immunoprecipitation experiments confirmed that HSP90 was also associated with SGT1 *in vivo* (Takahashi et al., 2003). The establishment of both *RPM1*- and *RPS2*-mediated resistances in *Arabidopsis* required functional HPS90 protein and were suggested to occur via direct interaction of HSP90 with RAR1 and SGT1 (Takahashi et al., 2003). In addition, a virus-induced gene silencing investigation in tobacco plants revealed that HSP90 silencing resulted in the loss of disease resistance responses mediated by several *R* genes including *Rx*, *N* and *Pto* (Lu et al., 2003). Altogether, these results suggested that HSP90 might act in a multi-protein complex as a chaperonin, reflecting the typical role of HSP protein.

1.6 Abiotic stress tolerance pathways in plants

1.6.1 General context

As previously described, plants cannot move to escape environmental challenges; therefore plants are constantly exposed to a variety of both biotic and abiotic stresses. Among the latter, drought, salinity and extreme temperatures represent the most devastating challenges since they can reduce crop yield by more than 50% (Wang et al., 2003). An example of the devastating effect of abiotic stresses was the heat-wave that in summer of 2003 severely disrupted agriculture, with equally devastating effects in developing and developed countries (Debono et al., 2004). Therefore, understanding the plant perception of abiotic stresses and the signalling pathways that trigger an adequate adaptive response is crucial to develop new methodologies to improve crop tolerance to abiotic stresses.

1.6.2 Perception of abiotic stresses

Generally, the first step of all abiotic adaptive responses is to perceive the stress, which, in turn, induces the generation of a second messenger (Xiong et al., 2002). These molecules, often by modulating intracellular calcium levels, can regulate the transcription of several specific classes of genes (Knight and Knight, 2001). Subsequently, the products of these genes can lead to the induction of "early" physiological adaptive responses; alternatively, they can trigger the expression of transcription factors and/or phytohormone such as abscisic acid (ABA) (Zhu, 2001). In turn, these regulator molecules may establish a second set of "late" adaptive responses that complements the early-induced tolerance mechanisms.

Tolerance responses have usually been dissected as linear physiological pathways in isolation from other stresses to simplify laboratory data evaluation. Increasing molecular and genetic data are revealing an unexpected extent of cross-talk and overlap between these individual pathways that are nowadays regarded as components of a more sophisticated network of signals (Chinnusamy et al., 2004).

It is well established that several abiotic stress tolerance pathways share common elements; this is consistent with the fact that plants cannot perceive the difference between some abiotic stresses. For example, drought and freezing conditions are recognized as an equivalent dehydration stress; hence, plants are required to activate an analogous dehydration protection response to induce freezing and drought tolerance (Wang et al., 2003). However, drought and freezing stresses also induce distinct responses that are specific for each distinct abiotic condition. In general, all abiotic stresses trigger antioxidant production in response to oxidative damages. Plants therefore need to activate a similar antioxidant protection system to overcome the oxidative damage caused by many different stresses (Xiong et al., 2002).

Despite the extensive similarities between many abiotic stress pathways, plants need to perceive the different features of each singular stress to induce the most appropriate and specific adaptive response. As previously described, damage caused by oxidative stress is common to several abiotic stresses, whereas others (such as disruption of ion distribution in salinity stress) are more specific (Xiong et al., 2002; Zhu, 2001). Therefore, plants need to activate a combination of general and specific pathways in order to trigger the most appropriate adaptive response. The complexity of this multiple response is further increased by the fact that a limited number of signalling components can function as a common "node" (Chinnusamy et al., 2004). Indeed, elements such as MAP kinases can be activated by more than one signalling pathway, cooperate with different "co-factors" and induce separate physiological responses (Ludwig et al., 2004).

1.6.3 The role of calcium in stress signalling cascades

Considering the complexity and extent of the stress signal network, a number of different receptor classes are assumed (Xiong et al., 2002). However, plant receptors able to perceive any initial abiotic stress have not been confirmed yet. Cold, drought and salinity stresses quickly induce an increase in intracellular calcium concentration, suggesting that a yet unidentified membrane system functions as a stress receptor and subsequentially induces Ca^{2+} accumulation (Knight and Knight, 2000; Pei et al., 2000).

A transient increase in calcium concentration subsequently activates downstream signalling pathways via Ca^{2+}-binding proteins (Knight and Knight, 2000). Specifically, CDPKs (Ca^{2+}-dependent protein kinases) are thought to play a major role in abiotic stress tolerance pathways (Ludwig et al., 2004). This class of protein kinase contains a calmodulin-like domain with 4 conserved EF motifs that can directly bind Ca^{2+} (Cheng et al., 2002). For example, the *Arabidopsis* genome encodes at least 34 CDPKs (TAIR, 2000), several of which were confirmed to be activated by different abiotic stresses (Xiong et al., 2002). These data suggested that a transient increase of calcium could activate a kinase cascade resulting in enhanced stress tolerance. Consistent with this hypothesis, rice over-expressing CDPK7 exhibited enhanced cold, drought and salt tolerance (Saijo et al., 2000).

An additional class of proteins induced by abiotic stresses and regulated by direct Ca^{2+}-binding are CBLs (calcineurin B-like) (Luan et al., 2002). Transgenic *Arabidopsis* plants overexpressing *CBL1* were more salt and drought tolerant, whereas they showed enhanced freezing susceptibility (Cheong et al., 2003; Albrecht et al., 2003). In contrast, *cbl1* loss-of-function mutants showed enhanced freezing resistance but less drought and salt tolerance (Cheong et al., 2003). Hence, the Ca^{2+}-regulated CBL1 was hypothesised to function as a positive regulator of drought and salt responses and a negative regulator of the cold tolerance pathway (Cheong et al., 2003). These data were consistent with the hypothesis that the same signal molecule (Ca^{2+} acting as second messenger) could specifically up- or down-regulate individual adaptive responses against different abiotic stresses (Knight and Knight, 2000).

1.6.4 The role of abscisic acid in stress response signalling

It has been long known that plants undergoing abiotic stresses may produce increased levels of ABA; indeed, ABA was the first phytohormone identified to play a major role in abiotic stress signalling pathways (Rikin et al., 1975; Chen et al., 1983). ABA treatment induced the expression of several dehydration- and cold-responsive genes and may enhance plant tolerance against abiotic stresses (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). The ABA-insensitive *abi1* (abscisic acid

insensitive) mutant exhibited drought, salt and osmotic hypersensitivity (Werner and Finkelstein, 1995). Nevertheless, the response to several abiotic stress genes was not regulated by ABA and the existence of both ABA-dependent and ABA-independent signal transduction pathways was therefore suggested (Thomashow, 1999; Zhu, 2001; Xiong et al., 2002).

1.6.5 ABA dependent stress response pathways

Several ABA-responsive genes have been isolated and the promoter analysis of these genes identified an element that could induce transcription upon ABA binding; this regulatory element was designated ABRE (ABA-responsive element) (Thomashow, 1999; Shinozaki Yamaguchi-Shinozaki, 2000). The *RD* (dehydration to responsive) genes were amongst the best characterised genes that contained ABRE in their promoters (Taji et al., 1999). For example, the *RD29B* promoter contains two ABRE elements that were required to activate ABA-induced gene expression. In addition, the drought induced expression of *RD29B* was blocked in *abi* mutants, confirming the dependence of *RD29B* expression on ABA (Yamaguchi-Shinozaki and Shinozaki, 1994).

Several basic leucine zipper (bZIP) transcription factors that bound ABRE (ABA-responsive element binding protein) elements have been recently isolated (Hobo et al., 1999; Finkelstein et al., 2000; Uno et al., 2000). Two of them, named *AREB1* and *AREB2*, were highly expressed upon drought, salinity and ABA treatments and, in turn, ABRE1 and ABRE2 activated the expression of several ABRE-containing genes (Uno et al., 2000). Transgenic *Arabidopsis* lines overexpressing *ABF4/AREB2* have been recently characterised and *35S::ABF4* plants exhibited enhanced ABA sensitivity and constitutive expression of several stress-responsive genes that contained ABRE elements (Kang et al., 2002). These transgenic plants also showed a reduced transpiration rate and enhanced drought tolerance; in contrast, they were hypersensitive to moderate salt and sugar concentrations but not to osmotic stress (Kang et al., 2002). These results argued

that the binding of bZIPs to ABRE elements can co-ordinately orchestrate the activation of several ABA-dependent stress responses.

Molecular studies have uncovered the existence of at least an additional ABA-dependent abiotic stress signalling pathway, which specifically required the biosynthesis of a novel protein to activate expression of ABA-responsive genes (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002). Drought-responsive *RD22* transcription was mediated by ABA but blocked by cycloheximide, an inhibitor of protein biosynthesis (Yamaguchi-Shinozaki and Shinozaki, 1993).

1.6.6 ABA-independent stress response pathway

Independent analyses of the drought-responsive *RD29A* and the cold-inducible *COR15* promoter uncovered the presence of a motif termed dehydradation-responsive element/C-repeat (DRE/CRT) (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994). Activation of *RD29A* and *COR15* was ABA-independent, hence defining a new stress signalling pathway (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994). Several transcription factors specifically recognized DRE/CRT elements and induced gene expression (Stockinger et al., 1997; Liu et al., 1998). These transcription factors were grouped in two families; the CBF/DREB1 family, composed by CBF1/DREB1B, CBF2/DRE1C and CBF3/DREB1A and the DREB2 family (Gilmour et al., 1998; Liu et al., 1998; Shinwari et al., 1998)

CBF/DREB1 proteins possessed a DNA-binding AP2 domain and induced stress-responsive gene expression by specific binding of the DRE/CRT element (Stockinger et al., 1997; Liu et al., 1998; Shinwari et al., 1998). All *CBF/DREB1* genes were specifically induced by low temperature conditions but not by drought stress. In addition, *Arabidopsis* plants overexpressing *CBF1/DREB1B* or *CBF3/DREB1A* exhibited constitutive induction of cold-responsive genes and enhanced freezing tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). These results therefore confirmed the presence of an ABA-independent low temperature responsive signalling pathway mediated by CBF/DREB1 transcription factors.

DREB2A and DREB2B, two additional proteins that specifically recognized DRE/CRT elements, were also identified (Liu et al., 1998). In contrast to *DREB1* gene regulation, *DREB2* expression was induced by ABA treatment, dehydration and salt stress but not by cold condition (Liu et al., 1998; Nakashima et al., 2000). Transgenic *Arabidopsis* plants overexpressing *DREB2A* weakly induced stress-response genes but failed to show enhanced drought or freezing tolerance, indicating that DREB2 activation was established in a post-transcriptional manner (Liu et al., 1998). In conclusion, two distinct protein families, *CBF/DREB1* and *DREB2*, act as transcription factors in two independent signalling pathways (cold and dehydration stress pathway respectively) (Liu et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Recently, rice homologues of both *DREB1* and *DREB2* genes have been isolated and the *DREB1A* rice gene was fully functional in *Arabidopsis* plants, suggesting that the CBF/ DREB1 stress pathway was well-conserved amongst plant species (Dubouzet et al., 2003).

2) Experimental Procedures

2.1 Growth of *Arabidopsis thaliana* and plant treatments

Arabidopsis thaliana seeds of Columbia (Col-0) and Landsberg erecta (L*er*) accessions were used. Most seeds of *Arabidopsis* transgenic plants and mutant lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (Table 2.1).

Table 2.1 *Arabidopsis* accessions, mutant and transgenic lines.

Line	Accession	Phenotype	Reference	Source
Col-0	Col-0	wild-type		NASC
L*er*	L*er*	wild-type		NASC
abi1-1	L*er*	ABA insensitive	Koornneef et al., 1984	NASC
cir1	Col-0	Constitutive SAR	Murray et al., 2002	Loake, University of Edinburgh
coi1-1	Col-0	Jasmonate insensitive	Feys et al., 1994	Turner, University of East Anglia
cpr1-1	Col-0	Constitutive SAR	Bowling et al., 1994	Dong, Duke University
eds1-2	L*er*	Impaired in R-mediated signalling	Feys et al., 2001	Parker, Max Planck Institute
ein2-1	Col-0	Ethylene insensitive	Guzman and Ecker, 1990	NASC
hsp90-1	Col-0	Impaired in R-mediated signalling	Takahashi et al., 2003	Shirasu, John Innes Centre
nahG	Col-0	Salicylate hydroxylase transgene	Lawton et al., 1995	Syngenta, USA
npr1-1	Col-0	SAR insensitive	Cao et al., 1994	Dong, Duke University
ndr1-2	Col-0	Impaired in R-mediated signalling	Aarts et al., 1998	Staskawicz, Berkeley University
rar1-10	L*er*	Impaired in R-mediated signalling	Musket et al., 2002	Parker, Max Planck Institute
sgt1-3	Col-0	Impaired in R-mediated signalling	Tor et al., 2002	Holub, Warwick HRI

Seeds were placed on soil and allowed to vernalise for 48 hours at 4°C after which they were transferred into growth rooms. Plants were grown under 10-hours of light at 22°C and 14-hours of dark at 18°C. For aseptic growth, seeds were sterilised with commercial bleach for 20 minutes,

washed 4 times in distilled water and maintained 4 days in the dark at 4°C to improve germination uniformity. Plants were subsequently transferred to MS plates containing MS basal salts supplemented with 1% (w/v) sucrose and 1% (w/v) agar. All chemicals employed were purchased from Sigma-Aldrich UK or unless stated otherwise. Petri dishes were transferred to a growth chamber with 16-hours of light at 22°C and 8-hours of dark at 18°C.

A 20mM methyl jasmonate (Me-JA) stock solution was prepared in 100% (v/v) ethanol. A 1:100 dilution, containing 0.01% (v/v) Silwet (Union Carbide, UK), was used to paint plant leaves which were harvested after 48 hours for RNA extraction. To activate SAR, wild-type plants were sprayed with a 300 μM BTH solution containing 0.01% Silwet (Syngenta, CA, USA) (Gorlach et al., 1996). Leaves were collected 5 days after treatment.

TA::ADR1 and TA:: transgenic lines were treated with dexamethasone (DEX) as described by Grant et al. (2003). Briefly, a 1mM DEX stock solution was prepared in ethanol and a 1:1000 dilution, containing 0.01% (v/v) Silwet, was used to homogenously spray plant leaves.

Seeds of activation tagged mutant lines were sown in flats and selected by spraying twice a 150mg/l BASTA (Agrevo, Germany) solution; one week after germination and four days later. Resistant plants were visually identified one week after treatment.

2.1.1Generation of adr1 *double mutants*

Genetic crosses were undertaken using pollen from homozygous *adr1* plants to fertilise unopened flowers of *abi1* (Koornneef et al. 1984), *ndr1* (Century et al. 1997) and *eds1* (Parker et al. 1996) plants. Successful F1 crosses were confirmed by spraying plants with 150 μg/ml BASTA and subsequently scoring for BASTA resistance 4 days later. The presence of *abi1* was selected in the F2 generation on MS media containing 3 μM ABA (Koornneef et al. 1984). The *ndr1* and *eds1* mutations were identified by allele specific PCR (Century et al. 1997; Falk et al. 1999).

2.2 Pathogen growth and disease resistance assays

P.syringae pv *tomato* DC3000 (*Pst*DC3000) (Whalen et al. 1991) was grown in King's broth (KB) liquid media supplemented with 50 mg/l rifampicin. Four week old soil-grown plants were infected with a *Pst* DC3000 suspension (OD_{600} = 0.0003) in 10 mM $MgCl_2$ by infiltrating the abaxial side of the leaf with a 1 ml syringe (Cao et al. 1994). Three leaves per plant and three to five plants per line were infiltrated. After three days, leaves were harvested for analysis of bacterial growth. Leaf discs of uniform size ($0.5cm^2$) were made from the leaf samples using a

cork borer. Three leaf discs from each plant were ground in 990 µl 10 mM $MgCl_2$ in a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension, and 100 µl of each dilution was used to inoculate KB medium plates containing 50 mg/l rifampicin. The plates were incubated at 30°C for 2 days, and the number of bacterial colonies for each sample was recorded. Bacterial counts were statistically analysed using the Student T test (Mini-tab version 13).

Pseudomonas syringae pv *tomato* DC3000 (*avrB*) (*Pst*DC3000 (*avrB*)) was grown on Kings Broth supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin. Liquid cultures were grown on a shaker at 30°C, and cells were pelleted by centrifugation and re-suspended for plant inoculation in 10 mM $MgCl_2$ at OD_{600} equal to 0.2 (the equivalent of 10^6 colony forming units per cm^2). For inoculations, 10 µl of the *Pst*DC3000 (*avrB*) solution were forced under the abaxial epidermis using a 1 ml syringe. Successful inoculations were visualised by the appearance of a watery area under the epidermis.

Erisyphe cichoracearum UED1 (Grant et al., 2003) infection assay was performed by transferring 3-4 week old plants to the transgenic greenhouse, an environment conducive to promiscuous growth of the pathogen, and by evenly tapping spores from highly infected wild-type plants. Results were obtained after 6 days, and infected plants were scored for visual pathogen sporulation (Figure 3.4). Scoring was as follows: 0 = no pathogen sporulation, 1 = 1-2 leaves with < 25% of the surface showing pathogen sporulation, 2 = 3-4 leaves with 25-50% of the surface showing pathogen sporulation, 3 = = 3-4 leaves with > 50% of the surface showing pathogen sporulation, 4 = most of the leaves showing > 50% of the surface showing pathogen sporulation, 5 = all leaves showing > 50% of the surface showing pathogen sporulation. Plants in different replicates were assigned a disease index as follows: $D.I.=\sum i.j/n$, where i = Infection class, j = the number of plants scored for that infection class and n = the total number of plants in the replicate (based on Epple et al. 1997a). 8 to 16 plants were infected per each line; experiments were repeated three times with similar results.

For testing resistance against *Botrytis cineria* (Grant et al., 2003), six week-old *Arabidopsis* plants were inoculated with a virulent isolate (PJH2). Conidial spores (1×10^5 per ml) suspended in potato dextrose broth were sprayed onto plants until droplets ran off. Inoculated plants were incubated at 100% relative humidity for 3 days before being examined for typical necrotic lesions caused by *B. cineria* infection (Figure 3.6). Scoring was as follows: 0 = no necrotic lesions, 1 = 1-2 leaves with necrotic lesions, 2 = 3-4 leaves with necrotic lesions, 3 = 5-6 leaves with necrotic

lesions, 4 = greater than 6 leaves showing necrotic lesions, 5 = all leaves showing necrotic lesions. Plants in different replicates were assigned a disease index as follows: D.I.=∑i.j/n, where i = Infection class, j = the number of plants scored for that infection class and n = the total number of plants in the replicate (based on Epple et al. 1997a). Twenty plants were infected for each line. Experiments were repeated twice with similar results.

Peronospora parasitica Noco2 was maintained as reported in Bowling et al. (1994). Typically, 3-week-old soil-grown plants were infected by spraying a solution of $1x10^6$ conidiospores per ml. Plants were maintained in humid conditions for 10 days and results were taken at this point. Infected plants were scored by counting the number of conidiophores per leaf. Three to eight leaves per plant were scored and three to six plants per line were examined. Experiments were repeated at least three times with similar results.

Cauliflower mosaic virus BJ1 (CaMV) infection was carried out pipetting 2 µl of a 50 ng/µl CaMV solution on one of the first two true leaves (each plant received 100ng of virus). The abrasive carborundum powder (Sigma-Aldrich) was also added to the working solution. Plants were maintained at 19°C and low-light conditions for 30 days after treatment and subsequently scored for visual symptoms. Scoring was as follows: 0 = no infection, 1 = half of the leaves with symptoms, 2 = all leaves showing symptoms. Plants in different replicates were assigned a disease index as follows: D.I.=∑i.j/n, where i = Infection class, j = the number of plants scored for that infection class and n = the total number of plants in the replicate (based ob Epple et al. 1997a). 8 to 12 plants were infected for each line. Experiments were repeated twice with similar results.

Pseudomonas fluorescens pv *phaseolicola* NPS3121 (Lu et al., 2001) was grown on KB liquid media supplemented with 50 mg/l rifampicin. Four-week-old soil-grown plants were infected with a *P. fluorescens* suspension (OD_{600} = 0.0003) in 10 mM $MgCl_2$ by syringae infiltration, as described above for *Pst* DC3000 infection. Measurements of bacterial growth and statistical analysis were carried out as described for *Pst* DC3000 infection.

2.3 DNA and RNA blot analyses

2.3.1 Southern blot analysis

DNA (10µg) isolated from *ads1* mutant line was digested overnight using the restriction enzymes *ApaI*, *BamHI*, *EcoRI*, *KpnI*, *SacI*, *SpeI*, *TaqI* and *XhoI*, (Promega, UK). Digested samples were run through a 0.8% agarose gel and transferred onto a nylon membrane (Amersham, UK) according to

the supplier instructions. The membrane was pre-hybridised for 1 hour at 42°C and hybridised sequentially with two probes (described below). Probes were labelled with α-^{32}P-dCTP by random priming using the Prime-a-Gene® labelling system (Amersham, UK). Hybridization was run over-night at 65°C according to the instructions of the supplier (Promega, UK). Blots were washed twice for 30 min each at 65°C in 4 X SSC (solution of sodium citrate), 1% (w/v) SDS, which was followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate time period. Blots were stripped by incubation in boiling 0.1% (w/v) SDS and washing in 0.5 X SSC for 30 min at room temperature, before hybridization with a subsequent probe (Sambrook et al. 1989).

Two DNA fragment were employed as probes: a 339 bp fragment corresponding to the CaMV*35S* enhancer region and a 610 bp fragment corresponding to the *BAR* gene. Probes were obtained by PCR using primers 5'-GATCCCCAACATGGTGGAGCACG-3' and 5'-TAGATATCACATCAA TCCACTTGC-3' (CaMV*35S* enhancer); 5'-GAAGTCCAGCTGCCAGAAAC-3' and 5'-CACCAAATCGACTCTAGCCA-3'(*BAR* gene).

2.3.2 Northern blot analysis

Total RNA was extracted from *Arabidopsis* leaves harvested from 5-week old plants using the guanadinium thiocyanate (GTC) phenol chloroform extraction method as described in Grant et al. (2003). In summary, leaf tissue (approximately 0.3g) was ground in liquid nitrogen using a pestle and mortar, poured into a 1.5 ml eppendorf and 0.45 ml GTC solution (4M guanadinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 0.1 M ß mercaptoethanol) was added. Following mixing by vortexing, 0.05 ml 2 M sodium acetate pH4.0, 0.45 ml phenol and 0.1 ml chloroform:iso-amylalcohol (49:1) were added. The samples were centrifuged, the supernatant removed carefully and transferred to a new tube. An equal volume of isopropanol was added to each tube, mixed and left at -20°C for at least 2 hours. RNA was recovered by centrifugation and pellet was re-dissolved in 0.15 ml GTC solution, and re-precipitated by the addition of 0.15ml isopropanol and storage at -20°C for 1 hour. Following centrifugation, the RNA pellet was washed twice in 70% ethanol, dried and dissolved in 100 µl DEPC-treated water. Alternatively, RNA extraction was carried out using an RNA kit (Qiagen, CA, USA) according to the instructions of the supplier. The absorbance of each sample was measured at 260 nm, and used to calculate the concentration of RNA. Samples (10 µg) were separated on formaldehyde-agarose gels (Sambrook et al. 1989), transferred to a Hybond™-N hybidization membrane according to the instructions of the supplier (Amersham, UK) and hybridized with the relevant probes (described below). Dextran

sulphate (10% w/v) was included in the pre-hybridization / hybridization solution in order to enhance efficient binding of the probe (Sambrook et al. 1989). Blots were washed twice for 30 min each at 65°C in 4 X SSC, 1% (w/v) SDS, which was followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate period. Blots were stripped by incubation in boiling 0.1% (w/v) SDS and washing in 0.5 X SSC for 30 min at room temperature, before hybridization with a subsequent probe (Sambrook et al. 1989).

Probes were prepared by amplification of appropriate sequences using PCR and directly purified using a kit (Promega, UK) (Table2.2). Alternatively, probes were generated from plasmids by digestion with relevant restriction enzymes and purified from the gel by freeze-thaw extraction (Table2.2). Sequences for the PCR primers and templates used for each probe are reported. Probes were labelled with α-^{32}P-dCTP by random priming using the Prime-a-Gene® labelling system (Promega, UK).

Table 2.2 DNA probes employed for northern blot analysis.

Gene	Template	Forward primer	Reverse primer	RE
ADR1	pSK-ADR1	T7	T3	-
PR-1	TA-PR1	CTgCAgACTCATACACTCTgg	TATgTACgTgTgTATgCATgATC	-
GST1	pSK-GST1	GgTTCTTTAAgTgAATCTCAAA	CAAgACTCATTATCgAAgATTAC	-
PDF1.2	genomic DNA	TCATggCTAAgTTTgCTTCC	AATACACACgATTTAgCACC	-
DREB1A	pUC19-BREB1A	T7	T3	-
DREB2A	pUC19-BREB2A	T7	T3	-
RD22	pUC19-RD22	-	-	*EcoRI*
RD29A	pUC19-RD29A	-	-	*EcoRI* *HindIII*
R18	pSK-18S	T7	T3	-

2.4 Biochemical Analysis

For measurements of ethylene emission, 4-week-old plants were removed from soil, their roots were washed and each plant was subsequently placed in an air-tight 50 ml syringe. After 5 hours a 5 ml gas sample removed from the headspace was subjected to gas chromatography using a HP5980 series II gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a Poropack

N column and a flame ionisation detector. The ethylene emission measurement was performed on 15 to 20 independent plants per line and determined by comparison with known standards.

Free endogenous JA was quantified by gas chromatography/mass spectrometry. Quantification procedure was carried out by Dr Mike Beale, coordinator of the GARnet project "Metabolite profiling of *Arabidopsis*". The stable, labelled isotope, $2H_2$-JA (90.4% total JA), was employed as an internal standard. The samples were extracted three times with 70% methanol. The combined extract was then passed through a C_{18} solid phase extraction cartridge (pre-conditioned, in turn methanol and 70% methanol) and washed with 70% methanol. The combined eluate and washings were vacuum concentrated, diluted with 2.5 ml of water and acidified with 0.15 ml concentrated hydrochloric acid. The aqueous phase was extracted with chloroform and concentrated to 0. 2ml before transfer to a 0.2 ml autosampler vial. Samples were evaporated to dryness and derivatized with hexane and N-methyltrimethylsilyl-trifluoroacetamided and analysed on a Micromass GC-TOF mass spectrometer in accurate mass acquisition mode. Endogenous JA was quantified on the basis of the ration of the JA: $^2H_2$-JA integrated peak areas for the molecular ions (m/z 282.1651 and 284.1777 respectively). For each sample, three sample preparation replicates were each analysed in triplicate.

Free and total endogenous SA levels were determined by Dr Mike Beale, coordinator of the GARnet project "Metabolite profiling of *Arabidopsis*", essentially as described by Bowling et al. (1994). Briefly, frozen leaf tissue samples (1g) were extracted with methanol, dried down and re-suspended in 0.01M H_2SO_4, and free SA levels were analysed using an HPLC methodology.

2.5 Histochemical analyses

Trypan blue staining was employed to examine dead plant cells. Leaves were stained by boiling for 5 minutes in alcoholic lactophenol trypan blue (20 ml of ethanol, 10 ml of phenol, 10 ml of water, 10 ml of lactic acid and 10 mg of trypan blue). Stained leaves were cleared in chloral hydrate (2.5 g in 1 l of water) overnight at constant shaking and then mounted under coverslips in 60% glycerol (Bowling et al., 1997).

Hydrogen peroxide (H_2O_2) production was detected by histochemical staining using 3,3-diaminobenzidine (DAB) as described by Thordal-Christensen et al. (1997). Leaves were stained by 5 minutes treatment with a solution of 1 mg per ml DAB over-night in constant shaking. Leaves were then cleared by boiling in acetic/glycerol/ethanol (1/1/3 v/v/v) solution and then mounted under coverslips in 60% glycerol.

2.6 Real time *in planta* imaging of LUC activity

Leaves *PR1::LUC* transgenic plants were painted with a solution containing 1mM Luciferin (Promega, UK) and 0.01% triton X-100 and 0.03% Silwet in a 1mM sodium citrate buffer (pH 5.8). Plants were placed in the dark for 30 minutes in order to allow the luciferin to dry and to minimise background bioluminescence. All *in planta* LUC imaging was performed using an ultra low light imaging camera system (EG & G Berthold Luminograph 980). Images were collected over a 10 second time period. Microscopy imaging was carried out using Nikon Optiphot-2 microscope.

2.7 Abiotic stress treatments

2.7.1 Drought stress assay

For drought treatment, 4-week-old soil-grown plants were completely withheld from water for 15 days and re-watered at day 16 (Kang et al., 2002). To minimize experimental variation transgenic and control plants were grown in the same tray (72 plants per tray). Experiments were repeated at least three times, with similar results, using approximately 40 plants per line.

2.7.2 Extreme temperature assays

To assess heat tolerance, 4-week-old plants were transferred into a heating chamber (Forma Scientific, Ohio, USA); the temperature was increased sequentially from 22°C to 42°C, via a 5°C increase in temperature per hour (modified from Larkindale and Knight 2002). Plants were maintained in this condition for the times stated in the text.

To assess freezing tolerance, 4-week-old plants were transferred into a freezing chamber and temperature was decreased sequentially from 12°C to - 5°C (Kasuga et al., 1999). Plants were maintained in this condition for 5 hours.

2.7.3 Salt- and metal-stress assay

To examine potential salt or metal tolerance, 4-week-old soil grown plants were irrigated with an increasing concentration of either salt or metal every 4 days. The concentrations of sequentially applied sodium chloride and potassium chloride were 50 mM, 100mM and 150 mM; for sorbitol, concentrations of 100 mM, 200 mM and 300 mM were applied. For $CuSO_4$ these concentrations were 8 mM, 14 mM and 20 mM; and, for $CdCl_2$, concentrations of 12 mM, 18 mM and 25 mM were sequentially applied (modified from Song et al. 2003).

2.7.4 Measurement of transpiration rates

The aerial structure of 4-week-old plants were detached from their roots and maintained at room temperature. The weight of each aerial structure was subsequently determined every 20 minutes over a period of 5 hours (Kang et al., 2002). Each measurement was performed using 4 plants per line. Experiments were repeated at least twice with similar results.

2.8 Homology searches and sequence analyses

The bioinformatic tools from web-sites presented here (Table 2.3) were used for sequence search and analysis. Instructions were followed as detailed at site. Homology searches in *Arabidopsis* were carried out using BLAST and WU-BLAST2 at the TAIR web-site, whereas plant homology searches were conducted using the MIPS and TIGR databases. Protein sequence alignment and production of phylogenetic trees were performed using ClustalW and Phylodendron ClustalW respectively. The *Arabidopsis* Resistance Genes site was employed to evaluate sequence alignment.

Table 2.3 Web-sites used for sequence search and analysis.

Name	Function	Web-address
BLAST	Homology search in *Arabidopsis*	http://www.arabidopsis.org/Blast/
WU-BLAST2	Homology search in *Arabidopsis*	http://www.arabidopsis.org/wublast/index2.jsp
ClustalW	Alignment of multiple peptide sequences	http://www.ebi.ac.uk/clustalw/index.html
At R genes	*Arabidopsis* Resistance Genes Database	http://niblrrs.ucdavis.edu/At_RGenes/
Multiple Expectation Maximisation for Motif Elicitation (MEME)	Reveal motifs (highly conserved regions) in groups of related protein sequences	http://meme.sdsc.edu/meme/website/intro.html
Munich Information Centre for Protein Sequences (MIPS)	Plant Sequence Database and BLAST search	http://mips.gsf.de/projects/plants
The Institute for Genomics Research (TIGR)	Plant Sequence Database and BLAST search	http://www.tigr.org/tdb/euk/
Phylodendron ClustalW	Representation of the neighbour-joining trees	http://www.es.embnet.org/Services/

3) Isolation and characterization of activation tagging mutants perturbed in disease resistance

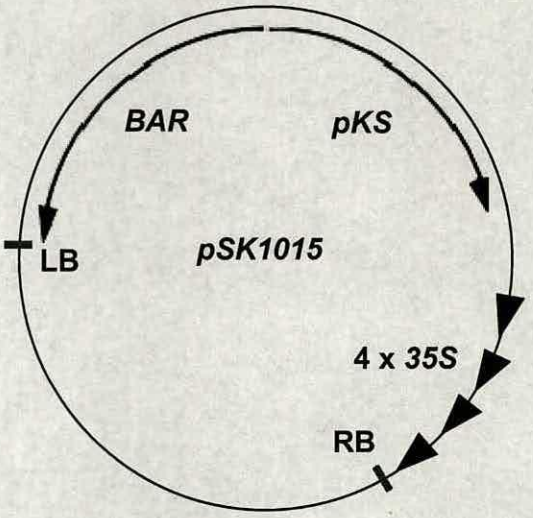
3.1 Genomic screen project

Analysis of the *Arabidopsis* genome has revealed the presence of many duplicated genes which are similar in their coding and non-coding sequences. Functional redundancy will therefore be a significant hurdle to the assignment of gene function in *Arabidopsis*, as has been the case for other higher eukaryotes (Miklos and Rubin, 1996; Ross-Macdonald et al., 1999). There is a second group of genes that are also difficult to uncover by classical screens. These genes encode proteins that function at multiple stages during the life cycle of the organism, one of which is essential for early embryo or gametophyte development. Loss-of-function mutations in these genes result in lethality. Obviously, this precludes the recovery of this mutant class from classical knock-out screens. Activation tagging is one of the approaches that have emerged to circumvent these problems (Kardailsky et al., 1999; Zhao et al., 2001; Borevits et al., 2000; Jeong et al., 2002; Grant et al., 2003: Xia et al., 2004).

3.1.1 Activation tagged methodology

Approximately 20 millions seeds were generated by floral dip transformation of Col-0 *Arabidopsis* plants with the activation tagging vector *pSK1015* (Tani et al., 2004). This was accomplished with the technical support funded by Akadix Inc (CA, USA). The *pSK1015* construct contains four repeats of the *35S* enhancer element, a pBluescriptKS (Stratagene) plasmid backbone and the *BAR* gene which conveys resistance to the commercial herbicide BASTA (Figure 3.1). A project to isolate activation tagged mutants perturbed in disease resistance/susceptibility was carried out. T_1 transformed seeds were germinated on soil flat and plants were selected by BASTA treatment seven days later. Resistant plants were transferred to pots and grown for approximately 3 weeks.

(a)



(b)

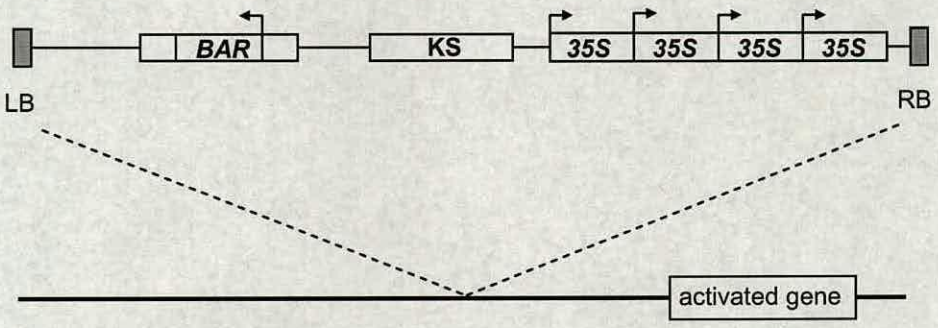


Figure 3.1The activation tagging vector pSKI015.

(a) T-DNA insertion cassette enclosed by left border (LB) and right border (RB) containing BASTA resistance gene (*BAR*), origin of replication of *E. coli* (as part of pBluescriptKS (KS) plasmid) and tetramer of the *35S* enhancer. This representation was adapted from Weigel lab web site: www.salk.edu/LABS/pbio-w/researchfs.html.

(b) Integration of the 4x*35S* enhancer region into the plant chromosome may enhance the expression of endogenous genes adjacent to the T-DNA insertion activation cassette.

Subsequently, two strategies were employed: activation tagged mutants were tested for either enhanced disease resistance or susceptibility after leaf infiltration with *Pseudomonas syringae* pv *tomato* DC3000 suspension (*Pst* DC3000 suspension of OD = 0.002 for resistance screen and OD = 0.0003 to test susceptibility). Mutants exhibiting either enhanced resistance or susceptibility were identified 3 to 4 days post infection. Seeds were collected from each individual candidate. Plants showing abnormal phenotypes were also identified and seeds collected. Particular attention was paid to plants of reduced stature or showing lesion mimic phenotypes since these developmental abnormalities are common among well-characterised disease resistance mutants (Frye and Innes, 1998; Maleck et al., 2002; Pilloff et al., 2002). Seeds of the remaining plants were collected in pools (70 plants per pool) in order to create a valuable community resource. Overall, more than half of the T_1 seed, corresponding to about 12 million seeds and 40,000 individual T_1 tagged plants (transformation rate of approximately 0.3%), were screened and ninety-one T_1 candidates were isolated. Approximately 15% of T_1 candidates were sterile, preventing further analysis. Surprisingly, only two candidates were confirmed in the following generation (T_2) and the characterisation of one of these lines is reported here.

3.1.2 Search for loss-of-function mutations

To also uncover recessive, loss-of-function mutations, T_2 plants were screened. Approximately one thousand seeds for pool were germinated; since each pool was generated by 70 T_1 plants, we expected to test approximately 16 T_2 plants, four of which were homozygous for the T-DNA insertion, per each T_1 line. 4-week-old plants were searched for lesion-mimic, abnormal or reduced phenotypes. This approach enabled the search for recessive, homozygous mutant lines that could not have been isolated in the T_1 screen.

This project was carried in collaboration with Pedro Nuremberg; 37 putative mutants were isolated. To date two recessive disease resistance mutants and two recessive enhanced disease susceptibility mutants have been confirmed following a back-cross with wild-type plants. The description of the first enhanced disease susceptibility mutant,

isolated in the T_1 screen for dominant mutations, is reported below, whereas the characterization of one tagged disease resistance mutant is described in chapter 4. In addition, another student has now followed-up the characterization of the recessive mutants identified in the T_2 screen, but these analyses will not be described here.

3.2 Isolation and characterisation of the *ads1* mutant

One T_1 mutant exhibited reduced stature and pale green serrated leaves (Figure 3.2). Furthermore, this line showed striking loss of apical dominance and reduced fertility (Figure 3.2). Approximately 300 T_2 plants were treated with BASTA 3 weeks after germination. In total, approximately 75% (226/297) of these T_2 progeny retained the mutant phenotype and were BASTA resistant, whereas all plants exhibiting a wild-type phenotype, about 25% (71/297), were BASTA susceptible. The chi-square value of T_2 plants (χ^2 = 0.194; P = 0.01 with one degree of freedom) showed a BASTA resistance:susceptible ratio that did not deviate significantly from the expected 3:1 ratio with a confidence of 99%. These results suggested that a single dominant mutation was responsible for this phenotype.

3.2.1 ads1 *exhibits enhanced disease susceptibility*

T_2 progeny was also tested for their response to *Pst* DC3000 infiltration (OD = 0.0003). All T_2 plants expressing the mutant phenotype exhibited enhanced disease susceptibility, similar to that of the enhanced disease susceptibility (*eds)* 1 mutant (Feys et al., 1994), while wild-type plants failed to show disease symptoms at this inoculation of *Pst* DC3000. Consequently, this analysis was repeated and the number of *Pst* DC3000 in infected leaves was scored three days later. As shown in Figure 3.3, this mutant is significantly more susceptible to *Pst* DC3000 than wild-type plants. This mutant line was therefore designated activated disease susceptibility (*ads*) 1.

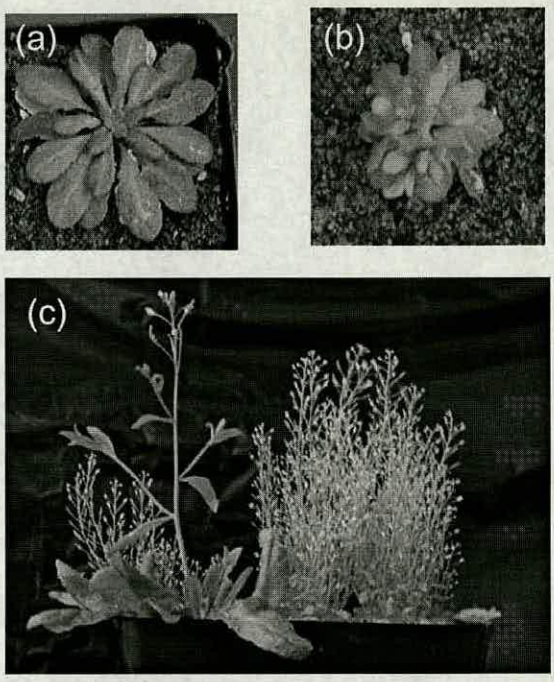


Figure 3.2 The *ads1* mutant phenotype.

25-day-old wild-type (a) and *ads1* (b) plant grown in short day condition; and 6-week-old flowering wild-type and *ads1* plants (c).

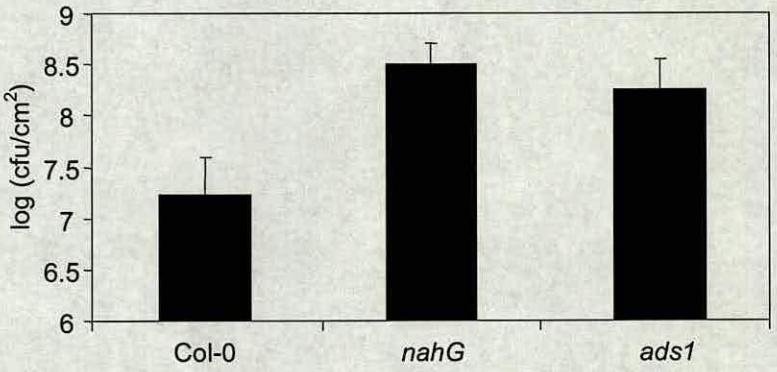


Figure 3.3 *ads1* plants exhibit enhanced susceptibility to *Pst* Dc3000.

Plants were challenged with virulent *Pst* DC3000 bacterial suspension of OD = 0.0003 by pressure infiltration. Col-0 wild-type plants and *nahG* transgenic lines were included as controls. Pathogen growth was analysed 3 days after challenge. The experiment was repeated 3 times; error bars represent standard deviations.

To investigate whether *ads1* susceptibility was specific to *Pst* DC3000, *ads1* plants were infected with different pathogens. First, *ads1* and wild-type control plants were challenged with *Erysiphe cichoracearum* UED1 (Grant et al., 2003), and infection rate was calculated 6 days later. As presented in Figure 3.4, *ads1* exhibited an infection rate similar to that of wild-type control plants. The *ads1* mutants were also infected with *Peronospora parasitica* NOCO2 (Bowling et al., 1994). Again, *ads1* did not show enhanced susceptibility compared to wild-type plants. Thus, *Pst* DC3000 was the only pathogen tested that showed enhanced growth in *ads1* plants.

To investigate the response of the *ads1* line to a necrotrophic pathogen, *ads1* and control plants were challenged with *Botrytis cinerea* PJH2 in collaboration with Pedro Nuremberg (Grant et al., 2003). Interestingly, *ads1* plants exhibited striking enhanced disease susceptibility (Figure 3.5). Wild-type Col-0 plants showed moderate *B. cinerea* lesions 5 days after infection, while *ads1* plants had collapsed and were fully covered by *B. cinerea* hyphae (Figure 3.6). Additionally, *ads1* plants showed infection symptoms 2 days post *B. cinerea* challenge, whereas wild-type plants remained healthy. These results suggested that enhanced disease susceptibility in *ads1* plants is not limited to *P. syringae*, a hemi-biotrophic pathogen, but it is also extended to the necrotrophic pathogen *B. cinerea*.

The *ads1* line was also tested for enhanced disease susceptibility against the non-host pathogen *Pseudomonas fluorescens* pv *phaseolicola* NPS3121 (Lu et al., 2001). Surprisingly, *ads1* plants supported greater *P. fluorescens* growth than wild-type plants, therefore *ads1* is also impaired in non-host disease resistance (Figure.3. 7).

3.2.2 Procedures to identify the ADS1 *gene*

From this preliminary characterisation of the *ads1* mutant, the novelty of this defence deficient mutant was evident. Thus, we initiated experiments to identify the activation tagged ADS1 gene. A Southern blot analysis was performed in order to confirm the presence of only one T-DNA insertion. Genomic DNA of *ads1* was isolated, purified and digested with 8 restriction enzymes, whose recognition site was not present in the

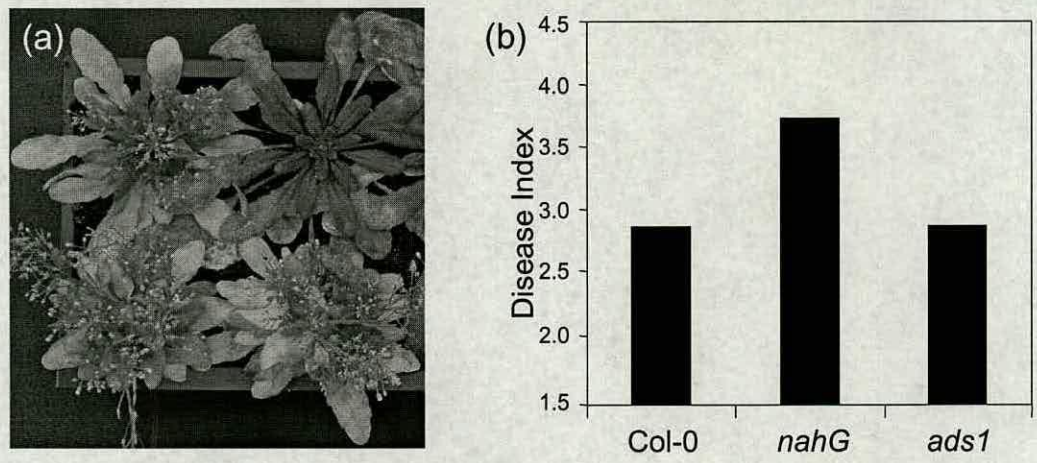


Figure 3.4 *ads1* mutants do not show enhanced susceptibility to *E. cichoracearum*.

Plants were challenged by gently dusting *E. cichoracearum* spores from the leaves of infected wild-type plants. Col-0 wild-type plants and *nahG* transgenic lines were included as controls. Pathogen growth was detected 7 days after infection in both wild-type and *ads1* plants (a).

(b) Plants were scored employing a disease index (as described in "Experimental Procedures") for visual fungal growth 7 days after dusting *E. cichoracearum* spores.

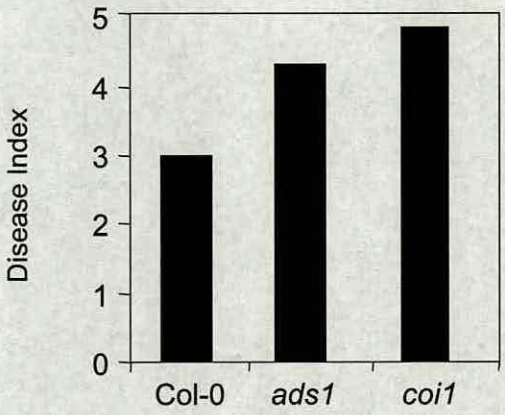


Figure 3.5 *ads1* plants exhibit enhanced susceptibility to *B. cinerea*

Plants were sprayed with a *B. cinerea* fungal solution of 10^5 spores per ml. Wild-type plants and the JA insensitive *coi1* mutants were included as controls. Plants were scored employing a disease index for visual disease symptoms 4 days post infection.

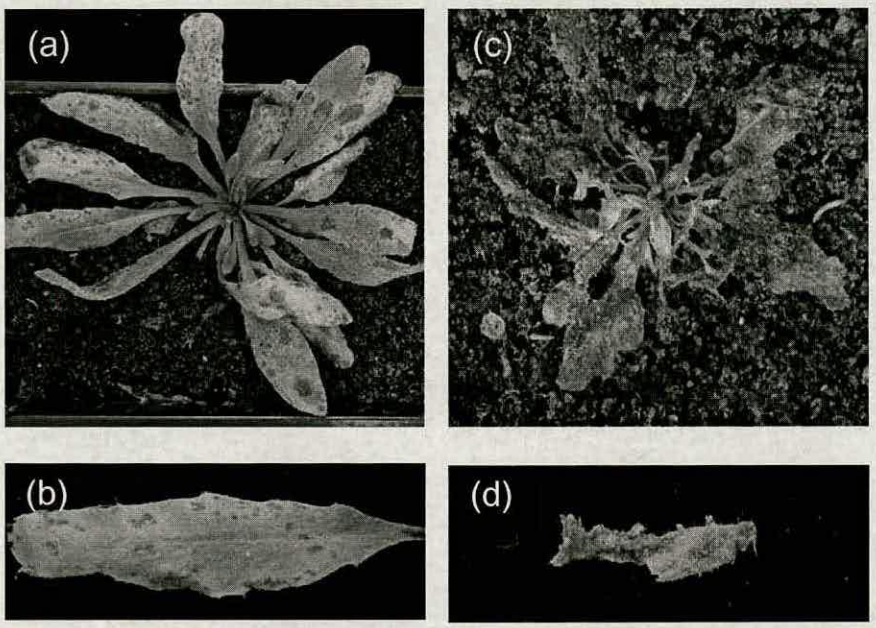


Figure 3.6 *ads1* plants exhibit enhanced susceptibility to *B. cinerea*.

Plants were infected by spraying a *B. cinerea* fungal solution of 10^5 spores per ml.

Pathogen symptoms on Col-0 control plants (a and b) and *ads1* mutants (c and d) are shown 4 days after challenge.

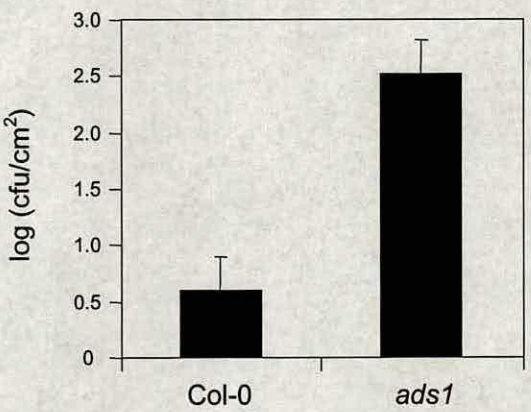


Figure 3.7 *ads1* exhibits enhanced susceptibility to the non-host pathogen *P. fluorescens*.

Plants were challenged with a non-host *P. fluorescens* by pressure infiltration of bacterial suspension of OD = 0.0003. Bacterial growth was analysed 3 days after challenge. Error bars represent standard deviations.

DNA sequence used as the probe: a 370 bp PCR product, corresponding to the *35S* enhancer element. Results showed only one specific band for every restriction enzyme employed (Figure 3.7), thus confirming the presence of only one T-DNA insertion. In addition, a specific 700 bp PCR product corresponding to the *BAR* gene, which conveys BASTA resistance and is located adjacent to the left border of the T-DNA insertion, was employed as a probe in a Southern blot analysis. Results confirmed the presence of a single and intact T-DNA insertion.

Subsequently, a TAIL-PCR procedure was employed to identify the *ADS1* gene; 6 different degenerate primers were employed in combination with specific primers from either the left border or the right border of the inserted T-DNA (Liu et al., 1995). This approach was unsuccessful. Consequently, inverse PCR was employed (Yuanxin et al., 2003). This approach also failed to amplify any specific DNA sequence. A plasmid rescue methodology was therefore employed to uncover the activation tagged *ADS1* gene (Ichikawa et al., 2003). The plasmid rescue experiments were repeated several times using the restriction enzymes *EcoRI*, *HindIII*, *KpnI*, *SpeI* and *TaqI*. Again, these experiments were unsuccessful. Another student has now followed-up this project by screening a genomic library and is currently testing candidate genes for *ADS1*.

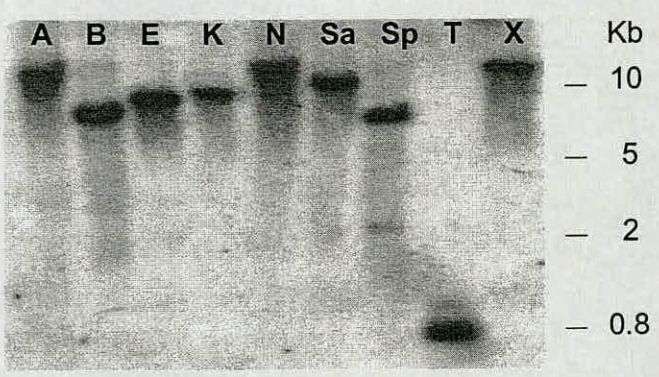


Figure 3.8 Southern blot analysis of *ads1* genomic DNA

Genomic DNA isolated from *ads1* plants (10 ng per line) was digested with *Apa*I (line A), *Bam*HI (B), *Eco*RI (E), *Kpn*I (K), *Sac*I (sa), *Spe*I (Sp), *Taq*I (T) and *Xho*I (X), run on a gel and transferred onto a membrane. This filter was subsequentially hybridised with a probe corresponding to the CaMV*35S* enhancer element present in the T-DNA insertion. The size of each band can be enferred by the ladder scale represented on the right.

3.3 Discussion

Screens of activation tagged mutant population have been a successful tool to investigate plant metabolism (Weigel et al., 2000; Tani et al., 2004). Recently, two tagged mutants perturbed in defence response have been isolated (Grant et al., 2003; Xia et al., 2004). Thus, the large-scale screen we are carrying out will enable the isolation of novel mutants and the corresponding genes. Results presented here showed that one enhanced disease resistant mutation and one enhanced susceptible mutation, among the 91 T_1 candidates, were confirmed in the following T_2 generation. Approximately 15% of T_1 candidates were sterile, preventing further analysis; these results were consistent with the conclusions of similar studies reported in the literature (Jeong et al., 2002; Maldonado et al., 2002; Nakazawa et al., 2003). However, the unexpected low number (2%) of mutant lines confirmed in T_2 was inconsistent with results from similar activation tag projects (Jeong et al., 2002; Maldonado et al., 2002; Nakazawa et al., 2003). The growing and/or environmental conditions, specific for the screen described here, probably contributed to the isolation of a greater number of false T_1 candidate lines compare to that of similar studies. In addition, it is likely that several T_2 lines lost the T-DNA insertion because approximately 20% of T_2 lines failed to retain BASTA resistance.

The *ads1* mutant showed increased susceptibility to *Pst* DC3000, which is an hemi-biotrophic pathogen, resistance against which requires the activation of plant SA-dependent defence pathway. However, *ads1* plants exhibited disease symptoms similar to that of wild-type plants following challenge with two biotrophic pathogens, *P. parasitica* and *E. cichoracearum*, resistance against which requires the establishment of plant SA-dependent defence pathway. In addition, *ads1* plants were strikingly more susceptible to *B. cinerea*, resistance against which requires the activation of JA-dependent defence response. Thus, *ads1* plants seem to exhibit enhanced susceptibility against some, but not all pathogens. Several loss-of-function mutants exhibit enhanced susceptibility to pathogens, resistance against which requires the activation of a plant SA-dependent defence pathway (Cao et al., 1994; Glazebrook et al., 1996; Aarts et al.,

1998); however, none of these mutants simultaneously show enhanced susceptibility to pathogens resistance against which requires the activation of a plant JA-dependent defence response. To our knowledge, *ads1* is the first mutant that exhibits coexisting enhanced susceptibility to distinct pathogens whose recognition requires both SA- and JA-dependent signalling pathways.

Furthermore, *ads1* permitted a massive growth of *P. fluorescens*, which is a non-host pathogen of *Arabidopsis*. Therefore, *ads1* is not only impaired in host, but also in non-host bacterial resistance. To date, only one mutant, *nho1*, has been reported to be compromised in non-host resistance (Lu et al., 2001). It has been recently established that NHO1 encodes a glycerol kinase (Kang et al., 2003). In addition, *nho1* plants showed increased susceptibility to *B. cinerea*, but exhibited a response to the hemi-biotrophic pathogen *Pst* DC3000 and the biotrophic pathogen *P. parasitica* equivalent to that of wild-type plants (Lu et al., 2001). Therefore *ads1*, to our knowledge, is the first mutant which simultaneously exhibits increased susceptibility to hemi-biotrophic, necrotrophic and non-host pathogens.

It is difficult to hypothesize how *ads1* might affect disease resistance because the *ADS1* gene has not been identified yet. The strong loss of apical dominance might suggest a role for the phytohormone auxin (Leyser, 2003). Interestingly, a growing number of reports are supporting the hypothesis that this hormone might also be involved in defence response (Cheong et al., 2002; Tiryaki and Staswick, 2002). An *Arabidopsis* mutant insensitive to JA has been shown to be allelic to the auxin-signalling deficient mutant *axr1* (Lincoln et al., 1990; Tiryaki and Staswick, 2002). This mutant was originally isolated as an auxin insensitive line, exhibited reduced leaf size, loss of apical dominance and partial sterility, due to limited pollen production (Estelle and Somerville, 1987; Lincoln et al., 1990). Several morphological phenotypes of *axr1* parallel those of *ads1*. Recently, *axr1* was shown to be more susceptible than wild-type plants to the necrotrophic pathogen *Pythium irregulare*, whose recognition requires JA (Tiryaki and Staswick, 2002). Furthermore, treatment of wild-type plants with the auxin IAA induced the expression of several JA responsive genes, such as *VSP*, *LOX* and *AOS* (Tiryaki and

Staswick, 2002). These results strongly suggest a possible overlap of JA and auxin signalling pathways. Thus a defect in the balance of auxin to cytokinin may be responsible for both the loss of apical dominance in *ads1* plants and their enhanced susceptibility to specific host and non-host pathogens. Quantification analysis of phyto-hormone levels in *ads1* plants will be required to confirm this hypothesis.

ADS1 might therefore function as a negative regulator of plant defence response. In unchallenged plants, ADS1 may be constitutively active, contributing to preserve the finely-regulated mechanism that restricts the establishment of disease resistance. Following pathogen recognition, wild-type plants may repress ADS1 activity in order to trigger disease resistance. In *ads1* mutants, the constitutive over-expression of the negative regulator ADS1 may delay the mechanism that establishes disease resistance and consequently contribute to enhanced susceptibility to several pathogens. The fact that *ads1* plants exhibited increased susceptibility to various host and non-host pathogens is consistent with this hypothesis. Nevertheless, *ads1* retained a response against *E. cichoracearum* and *P. parasitica* similar to that of wild-type plants, suggesting that ADS1 is specifically required in the establishment of defence response against some, but not all, pathogens. In order to confirm the function of ADS1 as a negative regulator of plant defence response, plants carrying a loss-of-function mutation in *ADS1* gene will be generated. Analysis of this line will elucidate whether the loss of ADS1 function will cause the activation of defence response. The expression analysis of *ADS1* in wild-type plants during compatible and incompatible interactions will be also examined. These investigations may validate the putative function of ADS1 as negative regulator of plant defence.

Alternatively, ADS1 may represent a key host compatibility factor (Ham et al., 1999; Jin et al., 2003; Gao et al, 2004). Compatibility factors are essential host proteins exploited by pathogens in order to successfully invade and infect the host system (Gao et al, 2004). The over-expression of *ADS1* gene, encoding a host compatibility factor of unknown function, may therefore be responsible for the susceptible phenotype observed in *ads1* plants. This hypothesis would be consistent with the enhanced disease susceptibility of

ads1 plants against a number of host and non-host pathogens. Host factors often interact physically with virulence factors expressed by the invading pathogen (Ham et al., 1999; Gao et al, 2004). Co-immunoprecipitation analysis of extracts from infected wild-type plants, employing a specific ADS1 antibody, might elucidate whether ADS1 physically interacts with a virulence factor. Furthermore, the structure of the ADS1 protein, inferred by genomic sequence, will also help to establish whether the activity of this protein may facilitate pathogen invasion and infection of the host plant system.

4) *ADR1* overexpression conveys broad-spectrum disease resistance

4.1 Identification and characterisation of the *adr1* mutant

A novel *Arabidopsis* transgenic line containing a chimeric *PR1::LUC* construct was generated in the Loake laboratory in order to detect the establishment of SAR in living plants by ultra low lighting imaging. This transgenic line was confirmed to mark SAR development with high fidelity and was proved to be a useful tool to identify mutants with perturbed expression of defence-related genes (Murray et al., 2002).

4.1.1 Isolation of disease resistance mutants

The screen of a large activation tagged population generated in the *PR1::LUC* transgenic background was undertaken in order to discover potentially redundant and/or essential genes which might encode key signalling components responsible for the establishment of disease resistance. It is noteworthy that all similar studies reported at that time exploited loss-of-function methodologies (such as EMS or fast neutron bombardment); hence, activation tagging screening was a novel approach in this field (Lorrain et al., 2003). A large number of *Arabidopsis* lines were screened for constitutive LUC activity via ultra low light imaging and a few lines that exhibited heritable, constitutive LUC activity were identified. One of these mutants, subsequently designated as activated disease resistance (*adr1*), was further analysed (Grant et al., 2003).

This mutant exhibited a strong phenotype consisting of reduced stature and curled leaves compared to Col-0 wild-type plants. In the T_2 generation the *adr1* phenotype segregated in a semi-dominant manner, with homozygote *adr1/adr1* plants exhibiting a more severe phenotype and greater LUC activity than hemizygote *adr1/ADR1* plants (Grant et al., 2003). Moreover, the onset of the *adr1* phenotype in both homozygous and hemizygous plants was generally observed 2 weeks after germination, prior to which time *adr1* plants were undistinguishable from wild-type (Grant et al., 2003).

4.1.2 *Characterisation of the* adr1 *mutant*

To confirm endogenous *PR1* expression, *adr1* mutants were investigated by northern blot (Grant et al., 2003). *adr1* plants constitutively expressed *PR1*, *PDF1.2* and *GST1*, which were genes respectively induced by SA, JA/ET and ROI; these three genes play important roles in the establishment of resistance against a broad spectrum of pathogens. The *adr1/adr1* mutant exhibited greater defence transcript accumulations than *adr1/ADR1* plants. Moreover, *adr1* mutants exhibited significant resistance against *Peronospora parasitica*, *Erysiphe cichoracearum* and *Pseudomonas syringae* (Grant et al., 2003). These data confirmed that *adr1* plants not only constitutively expressed defence-related genes, but also displayed enhanced resistance against a number of pathogens.

To investigate the requirement of ADR1 function for SA, JA and ET, *adr1* was crossed with transgenic *nahG* plants, compromised in SA accumulation (Delaney et al. 1994), with *npr1*, a mutant insensitive to SA (Cao et al. 1994) and with the JA and ET insensitive mutants, *coi1* and *ein2* respectively (Feys et al. 1994; Guzman and Ecker, 1990). Interestingly, the *adr1* phenotype in *adr1/ADR1 nahG* double mutants was completely suppressed; however only partial suppression was observed in *adr1/adr1 nahG* double mutants (Grant et al., 2003). Thus the accumulation of SA is required for developing the *adr1* phenotype. In *adr1/coi1*, *adr1/ein2* and *adr1/npr1* double mutant plants physical stature and leaf-curling were very similar to *adr1* mutants (Grant et al., 2003).

The expression of a number of defence-related genes in *adr1* double mutant plants was investigated via northern blot analysis (Grant et al., 2003). *GST1* transcript accumulation was completely abolished in *adr1/ADR1 nahG* and partially decreased in *adr1/adr1 nahG* double mutants. However, *GST1* expression appeared slightly higher in *adr1/ADR1 coi1* plants, but was unaffected in *adr1/ADR1 ein2* and *adr1/ADR1 npr1* double mutants. *adr1*-mediated *GST1* expression was therefore partially dependent on SA. The expression of *PR1* was unmodified in *adr1/ADR1 coi1* and *adr1/ADR1 ein2*, but it was completely abolished in both *adr1/ADR1 nahG* and *adr1/adr1 nahG*.

Moreover *adr1/ADR1 npr1* plants did not accumulate *PR1* transcripts; thus *adr1*-mediated *PR1* expression was dependent on SA and NPR1. In addition, *PDF1.2* expression was abolished in *adr1/ADR1 coi1* plants. Thus, *PDF1.2* accumulation mediated in *adr1* mutants is dependent on JA but independent on SA and ET (Grant et al., 2003).

To investigate further the role of *ADR1* in disease resistance signalling, the expression of the *ADR1* gene was analysed (Grant et al., 2003). *ADR1* exhibited a vanishingly low basal level of accumulation in Col-0 wild-type plants; however, significant upregulation of *ADR1* was observed following infection with *P. syringae* carrying the *avrB* gene and application of SA or the SA analogue BTH. A lower induction level was detected in response to wounding injury (Grant et al., 2003).

4.2 *adr1* shows broad-spectrum disease resistance to biotrophic pathogens

The establishment of disease resistance against pathogens is thought to be orchestrated by fine regulation of local and systemic concentration of specific defence signalling molecules such as SA, its conjugate SA β-glucoside (SAG), JA and ET (Yun and Loake, 2002; Devoto and Turner, 2003; Guo and Ecker, 2004). As mentioned in the introduction, *adr1* plants accumulate high transcript levels of defence genes including *PR1*, *PDF1.2* and *GST1*, whose expression has been established to be dependent on SA, ET/JA and ROI accumulation respectively (Ward et al., 1991; Penninckx et al., 1996; Grant et al., 2000). We therefore investigated whether any key defence signalling molecules accumulated in *adr1* plants. The severely reduced stature of *adr1/adr1* plants precluded the isolation of sufficient biomass for their biochemical analysis. Hence, the concentration of SA, SAG and JA were determined only in *adr1/ADR1* plants (in collaboration with Dr Mike Beale, coordinator of the GARnet project "Metabolite profiling of *Arabidopsis*"). The measurement of ET and H_2O_2 accumulation were undertaken in both *adr1/ADR1* and *adr1/adr1* mutants (at Edinburgh University).

SA has been shown to be essential for the development of SAR and, in several cases, for the successful establishment of resistance against biotrophic and hemi-biotrophic pathogens (Ryals at al., 1996; Shirasu et al., 1997). Interestingly *adr1* plants constitutively accumulated high levels of both SA and SAG, seven and eleven times greater than those detected in wild-type plants respectively (Figure 4.1). Many disease resistant, lesion mimic mutants have been reported to accumulate similar constitutive SA levels (Brodersen et al., 2002; Maleck et al., 2002; Shirano et al., 2002; Torrain et al., 2003). In contrast to these mutants, *adr1* plants do not show any macroscopic cell death under normal growth condition, thus trypan blue staining analysis was performed to search for the presence of microscopic cell death. Both *adr1/ADR1* and, to greater extend, *adr1/adr1* plants exhibited regions of cell death (Figure 4.2).

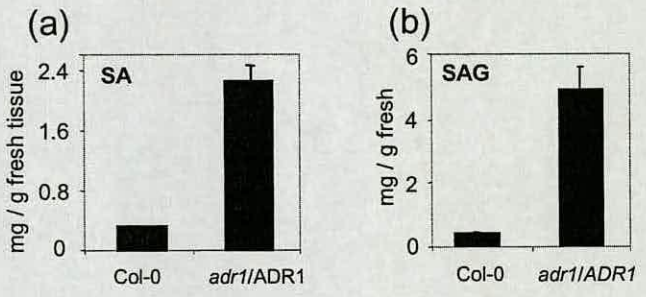


Figure 4.1 Both SA and SAG concentrations are increased in *adr1* mutants.

(a-b) SA and SAG concentrations in *adr1/ADR1* mutants and wild-type control plants are reported in (a) and (b) respectively. Error bars represent standard errors.

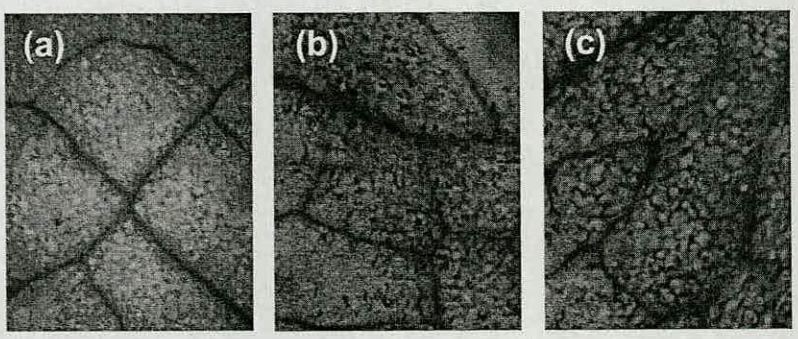


Figure 4.2 Accumulation of microscopic cell death in *adr1* mutants.

(a-c) Accumulation of microscopic cell death uncovered by trypan blue staining in representative leaves of wild-type Col-0 (a), *adr1/ADR1* (b) and *adr1/adr1* plants (c). Magnification in each figure was 10X.

Resistance to necrotrophic pathogens is dependent on JA (Devoto and Turner, 2003). In contrast to SA, however, the concentration of JA was slightly decreased in *adr1/ADR1* mutants compare to wild-type Col-0 plants (Figure 4.3a). The ET-mediated defence pathway has been reported to play a critical role in both disease symptom development and resistance against specific pathogens (Stearns and Glick, 2003; Guo and Ecker, 2004). The amount of ET released from *adr1/ADR1* and *adr1/adr1* plants was not significantly different from that released from wild-type plants (Figure. 4.3b). Thus, the *adr1* mutation does not result in a detectable increase in ET biosynthesis. Accumulation of H_2O_2 and other ROI occur following plant recognition of putative pathogens (Grant et al., 2000). 3,3-diaminobenzidine (DAB) staining analysis revealed that *adr1* plants possessed elevated H_2O_2 levels (Figure. 4.4).

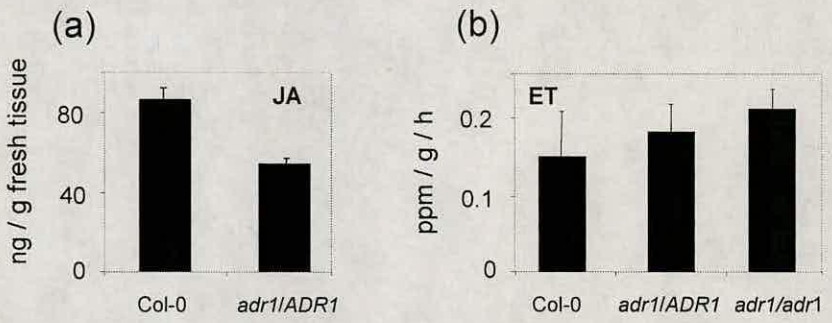


Figure 4.3 Measurement of JA and ET levels in *adr1* plants.

(a) Level of JA in *adr1/ADR1* mutants and wild-type Col-0 control plants.

(b) Amount of ET released from *adr1/ADR1*, *adr1/adr1* and wild-type Col-0 control plants.

Error bars in (a) and (b) represent standard deviations.

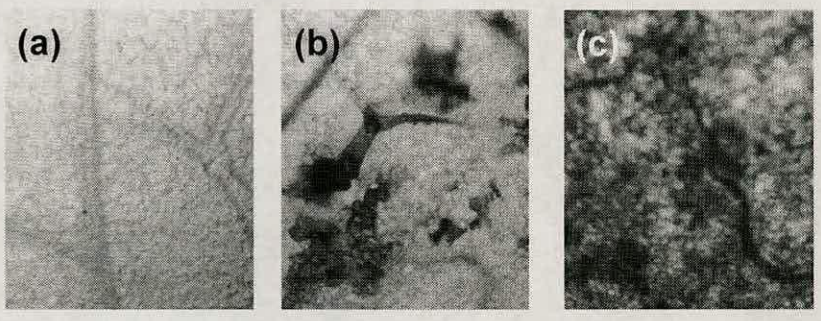


Figure 4.4 Constitutive accumulation of H_2O_2 in *adr1* mutants.

(a-c) Accumulation of H_2O_2 reported by 3,3-diaminobenzidine staining in representative leaves of wild-type Col-0 (a), *adr1/ADR1* (b) and *adr1/adr1* plants (c). Magnification in each figure was 10X.

SA and ROI accumulation strengthens previous results reporting enhanced resistance in *adr1* plants against *Peronospora parasitica* NOCO2, a biotrophic oomycete, and *Pseudomonas syringae* pv *tomato* DC3000, a hemibiotrophic bacterial pathogen (Grant et al., 2003). To extend this analysis, *adr1* response to additional pathogens, including the biotrophic fungus *Erysiphe cichoracearum* UED1, cauliflower mosaic virus (CaMV) BJ1 and *Botrytis cinerea* PJH2, a necrotrophic fungus was also investigated (Grant et al., 2003).

Wild-type plants infected with *E. cichoracearum* showed initial fungal growth four days after treatment. Seven days after pathogen challenge, control wild-type plants were considerably infected (Figure 4.5a) while *adr1* did not show any fungal growth (Figure 4.5b). At this time plants were scored employing a disease index for symptom development (Figure 4.5c). At 10 days post infection, wild-type plants were heavily infected whereas *adr1* mutants showed only modest fungal growth.

Response to CaMV was also investigated in *adr1* plants. Wild-type seedlings were infected and peculiar symptoms were evident around 20 days after virus challenge. Disease resistance was ultimately scored 30 days after treatment with CaMV. At this time, wild-type plants showed strong symptoms (Figure 4.6a), caused by the virus infection, compared to control unchallenged plants (Figure. 4.6b); in contrast, challenged *adr1* plants did not show any evident symptoms (Figure 4.6c).

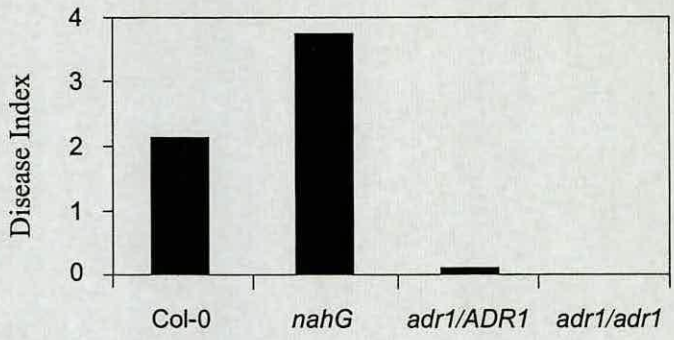


Figure 4.5 *adr1* mutants exhibit *E. cichoracearum* resistance.

Plants were gently dusted with *E. cichoracearum* spores from the leaves of infected wild-type plants. Pathogen growth was detected 6 days after infection in wild-type but not in *adr1/ADR1* or *adr1/adr1* plants. Plants were scored employing a disease index, as described in "Experimental Procedures", for visual fungal growth 7 days after dusting *E. cichoracearum* spores.

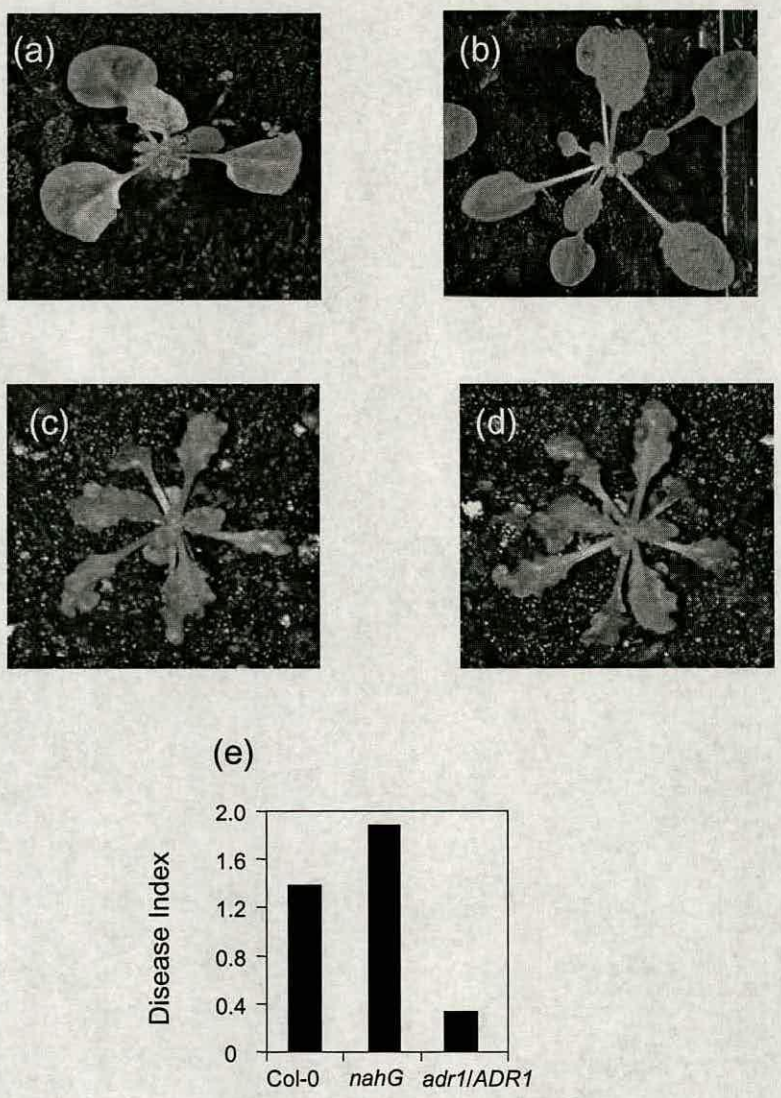


Figure 4.6 *adr1* plants show CaMV resistance.

(a-b) One of the first two leaves of 10 day old seedlings were scrubbed with a CaMV suspension of 50 ng per ml and visual symptom were assessed after 30 days. Wild-type challenged and unchallenged plants are represented in (a) and (b) respectively.

(c-d) Challenged and unchallenged *adr1/ADR1* mutants are shown in (c) and (d).

(e) Plants were scored using a disease index for symptoms, as described in "Experimental Procedures", development 30 days after CaMV challenge.

In order to investigate the response of *adr1* plant against a wide range of pathogens, *adr1* mutant resistance to the necrotrophic pathogen *B. cinerea* was also tested. Infection assays were performed with the help of Pedro Nurmberg. Plant were scored for distinct *B. cinerea* disease symptoms 4 days after infection and *adr1* lines showed disease susceptibility similar to that of wild-type plants (Figure 4.7).

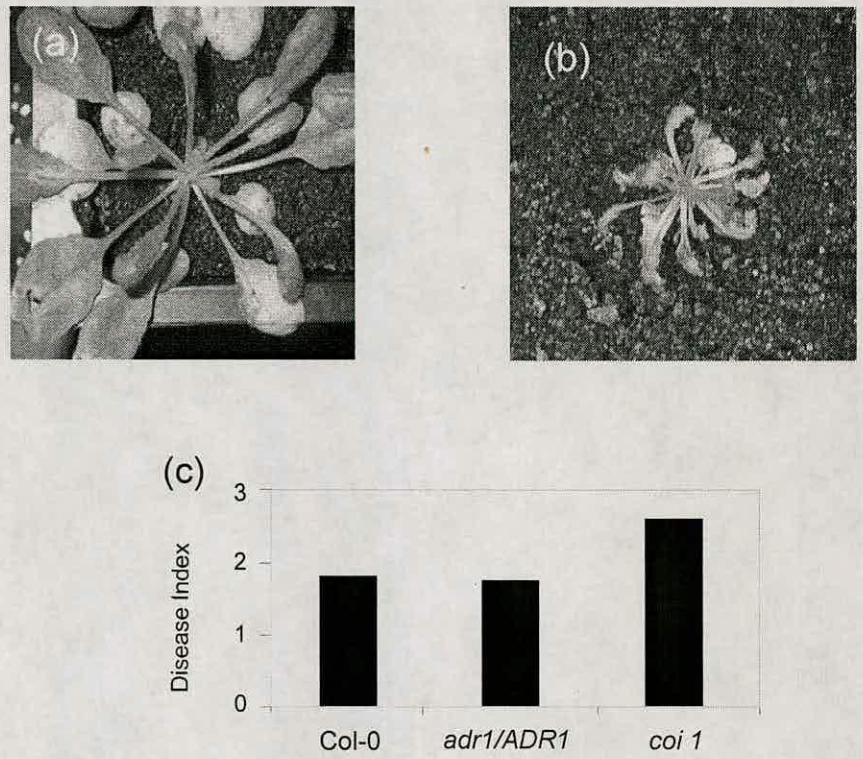


Figure 4.7 *adr1* plants do not exhibit enhanced resistance to *B. cinerea*.

(a-b) Plants were sprayed with a *B. cinerea* fungal solution of 10^5 spores per ml. Wild-type plants (a) and *adr1/ADR1* mutants (b) developed symptoms 4 days after infection.

(c) Plants were scored employing a disease index for visual disease symptoms as described in "Experimental Procedures", 4 days post infection. JA insensitive *coi1* mutants were included as negative control.

4.3 CaMV*35S::ADR1* transgenic lines exhibit enhanced disease resistance

To confirm that *ADR1* over-expression was responsible for enhanced disease resistance, a transgenic line carrying *ADR1* under the control of the constitutive cauliflower mosaic virus promoter CaMV*35S* was generated by Dr John Grant. Wild-type *Arabidopsis* plants were transformed with this construct (by Dr John Grant) and approximately 100 T_2 transgenic lines were screened for the characteristic *adr1* phenotype. All transgenic CaMV*35S::ADR1* lines were categorized into three major morphological classes. CaMV*35S::ADR1* lines belonging to the first class had the same phenotype as wild-type plants and line 23 was a representative example (Figure 4.8a). Plants from the second class showed an *adr1/ADR1* phenotype and were represented by line 36 (Figure 4.8b). Finally, transgenic plants that exhibited severely reduced stature, similar to *adr1/adr1* mutants, were grouped into a third morphological class, represented by line 10 (Figure 4.8c).

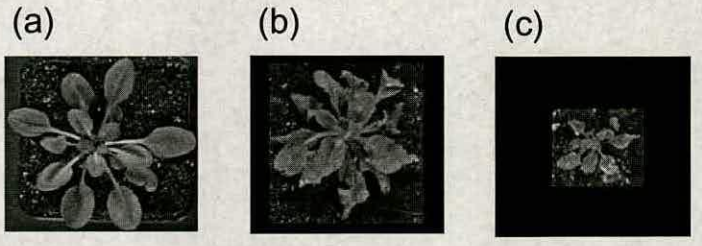


Figure 4.8 Phenotypic appearances of transgenic lines expressing *ADR1* under the control of the constitutive promoter CaMV*35S*.

(a-c) CaMV*35S::ADR1* line 23 represents the class of plants showing wild-type phenotype (a). The group of CaMV*35S::ADR1* lines that exhibit *adr1/ADR1* phenotype are denoted by line 36 (b), whereas line 10 (c) represents the class of plants showing severely reduced stature, similar to *adr1/adr1* mutants.

To confirm that defence gene expression was activated in selected CaMV*35S::ADR1* lines, gene expression analysis was performed. The magnitude of the *adr1* morphological phenotype was directly proportional to the level of *ADR1* and *PR1*

transcript accumulations (Figure 4.9). In contrast, plants displaying a wild-type phenotype, such as line 23, were unable to accumulate either *ADR1* or *PR1* transcripts. Surprisingly, three different *ADR1* transcripts, of 2.8, 1.6 and 1.4 kb respectively, were identified by northern blot analysis, suggesting differential splicing or RNA editing (Figure 4.9). In this context, the differential splicing of *ADR1* exons would be consistent with the observed transcript sizes (Grant et al., 2003); however, confirmation would require reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

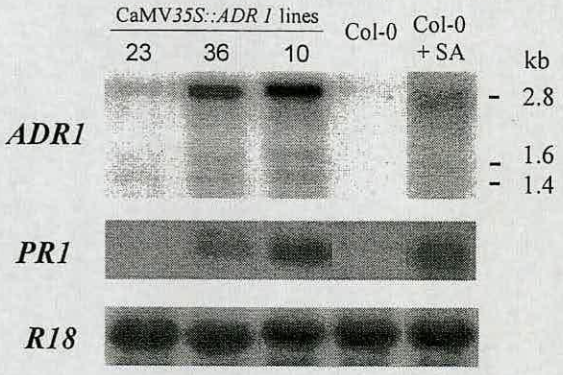


Figure 4.9 Gene expression analyses of *CaMV35S::ADR1* transgenic lines.

The expression of *ADR1* and *PR1* transcripts was enhanced in *CaMV35S::ADR1* line 36 and 10. SA treated and untreated Col-0 wild-type plants were included as controls. *R18* was used to control equal RNA loading and transfer. Arrows on the right indicate transcript sizes as inferred from RNA ladder.

In order to investigate whether *ADR1* and *PR1* expression correlated with disease resistance, a pathogenicity assay was performed by challenging selected CaMV*35S::ADR1* plant lines with *P. parasitica*. Results showed that plants with high (line 10) and intermediate (line 36) levels of *ADR1* expression displayed high and moderate disease resistance respectively (Figure 4.10). Furthermore, plants that did not accumulate *ADR1* (line 23) were as susceptible to *P. parasitica* as wild-type plants (Figure 4.10).

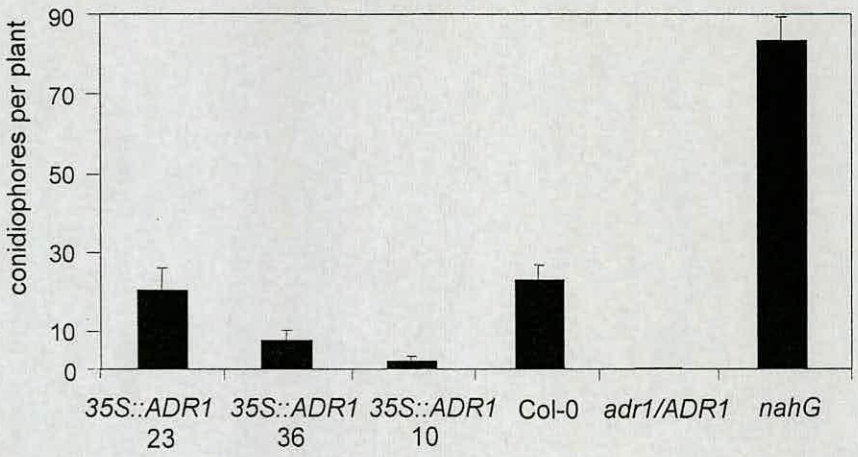


Figure 4.10 Resistance of selected *CaMV35S::ADR1* lines against *P. parasitica.*

Plants were sprayed with a *P. parasitica* NOCO2 suspension of $1x10^6$ conidiospores per ml and the numbers of conidiophores per plant were counted 10 days after treatment. Col-0 wild-type, *adr1/ADR1* and *nahG* plants were included as controls.

The presence of microscopic cell death areas and accumulation of H_2O_2, characteristic of the *adr1* mutant phenotype, were investigated in selected CaMV*35S::ADR1* lines. Results showed that line 36 and line 10 plants, which accumulated intermediate or high levels of *ADR1* transcripts, exhibited accumulation of H_2O_2 and microscopic cell death (data not shown). Consequentially, these results confirmed that *ADR1* accumulation proportionally induced expression of defence-related genes and enhanced disease resistance, which tightly co-segregated with specific abnormal development in CaMV*35S::ADR1* transgenic lines.

4.4 *ADR1* gene expression

To investigate further the potential role of *ADR1* in disease resistance, gene expression in response to defence-related stimuli was determined. Previous analysis by Dr John Grant suggested that *ADR1* was promptly expressed upon pathogen recognition, SA treatment and wounding stress. To further analyse *ADR1* expression, kinetic analysis after avirulent (*Pst* DC3000(*avrB*)) and virulent (*Pst* DC3000) infections were

performed. *ADR1* expression was detected at 1.5 hours post *Pst* DC3000(*avrB*) recognition (Figure 4.11). Similar results were obtained after plant infection with *Pst* DC3000 carrying the avirulent *Rpt2* gene. Interestingly, upon pathogen recognition *ADR1* was also expressed in unchallenged systemic tissues within 24 hours, and expression could be detected for as long as 5 days after infection. Hence, *ADR1* may function in defence signalling during both local *R* gene-mediated resistance and the establishment of systemic immunity. *ADR1* transcripts were also slightly induced 10 hours after *Pst* DC3000 infection and 3 days post *E. cichoracearum* infection. These results are consistent with the well established knowledge that both compatible and incompatible plant-pathogen interactions regulate similar set of genes, but, crucially, with different kinetics (Wan et al., 2002). BTH, an analogue of SA, substantially included *ADR1* transcription. In addition, *ADR1* expression after wounding stress was also confirmed. To investigate *ADR1* regulation by additional key defence signalling molecules, *ADR1* gene expression was analysed in wild-type plants treated with Me-JA and the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC). These experiments suggested that *ADR1* expression was not cued by JA or ET (Figure 4.11).

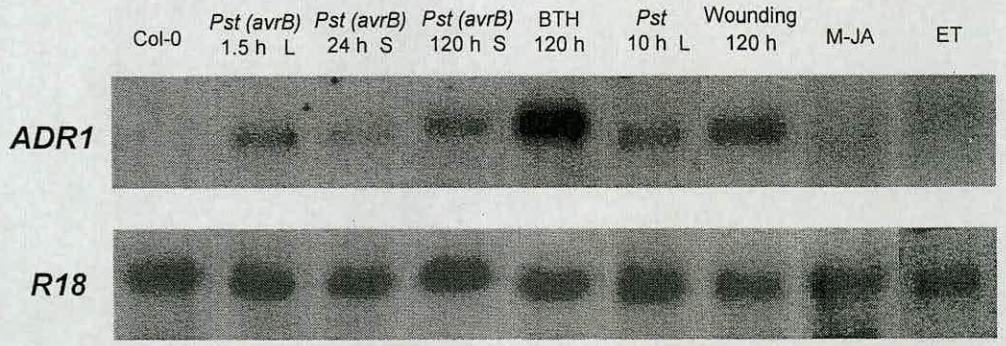


Figure 4.11 Analysis of *ADR1* gene expression in response to defence-related stimuli.

ADR1 northern blot analysis at given time in hours (h) in response to attempted infection by *Pst* (*avrB*) and *Pst* DC3000. L denotes locally challenged leaves, whereas S indicates systemically unchallenged leaves. Wild-type plants were also exogenously treated with 0.1 mM BTH, 5 μM 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor (ET) or 50 μM M-JA. *R18* was used to confirm equal RNA loading and transfer.

4.5 Characterisation of *adr1* double mutants

To investigate the individual contribution of known defence signalling pathways to the establishment of the *adr1* phenotype, Dr John Grant crossed *adr1* plants with selected *Arabidopsis* mutants compromised in defence signalling. These included transgenic *nahG* plants, depleted in SA (Delaney et al. 1994), *coi1* plants insensitive to JA (Feys et al. 1994), *ein2* plants insensitive to ethylene (Guzman and Ecker, 1990) and the SA insensitive mutant, *npr1* (Cao et al. 1994). The expression analysis of selected defence-related genes in *adr1* double mutants was already established and reported in the introduction (4.1.2). To assess the contribution of distinct defence signalling pathways to the establishment of *adr1*-mediated resistance against *P. parasita* NOCO2 and *E. cichoracearum* UED1, the panel of *adr1* double mutants was challenged with these pathogens. In addition, the level of SA, SAG, JA and ET were also determined.

Both SA and SAG concentration are constitutively increased in *adr1/ADR1* mutants compared to those detected in wild-type plants. Biochemical analysis was performed to examine whether *adr1* double mutants maintained high levels of these metabolites (in collaboration with Dr Mike Beale, coordinator of the GARnet project "Metabolite profiling of *Arabidopsis*"). As shown in Figure 4.12, both *adr1/ADR1 coi1* and *adr1/ADR1 ein2* plants retained high level of SAG. In addition, while *adr1/ADR1 coi1* plants also maintained an increased level of free SA, *adr1/ADR1 ein2* plants showed a 3-fold increase compared to *adr1/ADR1* plants. In contrast, *adr1/ADR1 npr1* double mutants exhibited a 3- and 5-fold accumulation of SA and SAG respectively, compared to *adr1/ADR1* levels. Furthermore, *adr1/adr1 nahG* double mutant does not accumulate high levels of SA nor SAG; this is due to the *NahG* transgene, which encodes an enzyme that converts SA into catechol. JA levels were also examined and only *adr1/ADR1 npr1* plants exhibited an increase in this metabolite to a level 3-fold greater than wild-type plants (Figure 4.13). Finally, ET production was measured and, as expected, *adr1/ADR1 ein2* and *adr1/adr1 ein2* plants revealed a 2-fold higher ET level. All other *adr1* double mutants did not exhibit increased ET production (Figure 4.14).

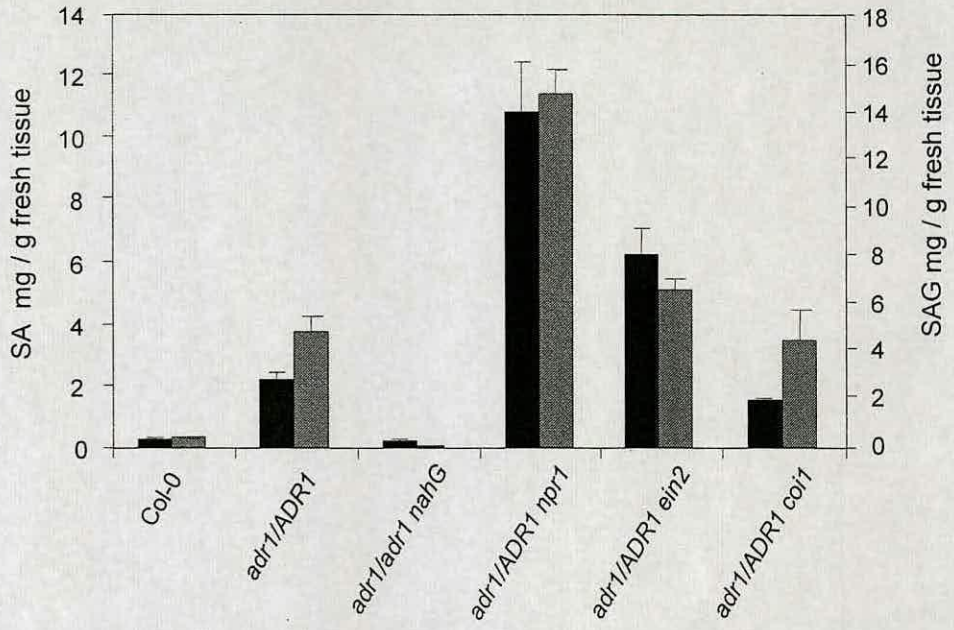


Figure 4.12 Measurement of SA and SAG levels in *adr1* double mutants.

Concentrations of SA and SAG in *adr1* mutants and wild-type plants are represented in the left-side black and right-side grey columns respectively. The scale on the left side denotes the extent of SA concentration, whereas the amount of SAG is specified by the scale on the right side of the histogram. Error bars represent standard errors.

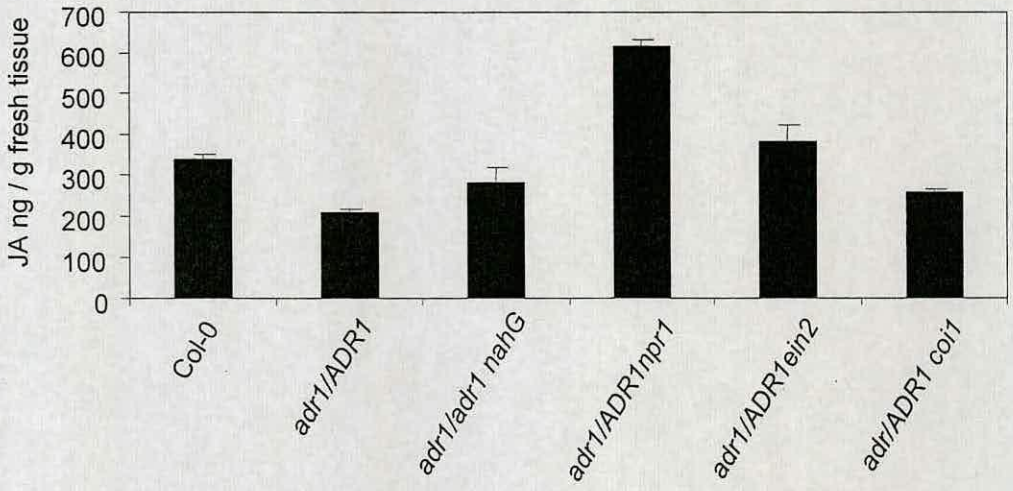


Figure 4.13 Measurement of JA levels in *adr1* double mutants.

The concentration of JA in wild-type control plants and *adr1* double mutants. Error bars represent standard errors.

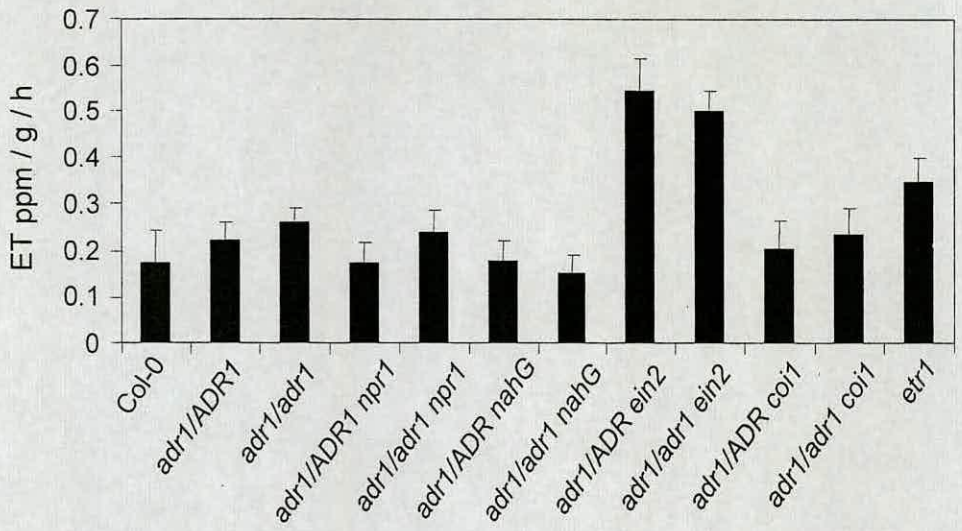


Figure 4.14 **Production of ET in *adr1* double mutants**.

Wild-type control plants, *adr1/ADR1* and *adr1/adr1* double mutants were analysed for ET production. ET insensitive *etr1* mutant plants were included as control. Error bars represent standard deviations.

Disease resistance in *adr1* double mutants was then investigated. Double mutants were challenged with *P. parasitica* NOCO2 and 10-day old *adr1/ADR1 coi1* and *adr1/ADR1 npr1* double mutants exhibited similar susceptibility as *coi1* and *npr1* plants respectively (Figure 4.15). However, *adr1/ADR1 ein2* plants showed reduced fungal growth. Interestingly, 10-day old *adr1/adr1 nahG* plants were more resistant than the immune-deficient *nahG* transgenic line. As described in the introduction, 2-week-old *adr1* seedling do not accumulate high level of *ADR1* transcripts and defence related genes; thus, 25-day-old adult plants were also challenged with *P. parasitica* (Figure 4.16). Only *adr1/adr1 nahG* showed significant fungal infection, but to a lower extent than *nahG* transgenic plants. These results therefore suggested that *ADR1* orchestrates resistance against *P. parasitica* in adult plants in a partially SA-dependent manner, but independently of JA or COI1, ET or EIN2 and NPR1. The apparent age-dependent disease resistance was most likely due to the fact that *ADR1* transcripts do not accumulate in *Arabidopsis* plants until 15 days after germination (Grant et al., 2003). This conclusion is consistent with the fact that the onset of reduced stature and curled leaves was generally observed 2 weeks after germination, prior to which time *adr1* plants were indistinguishable from wild-type.

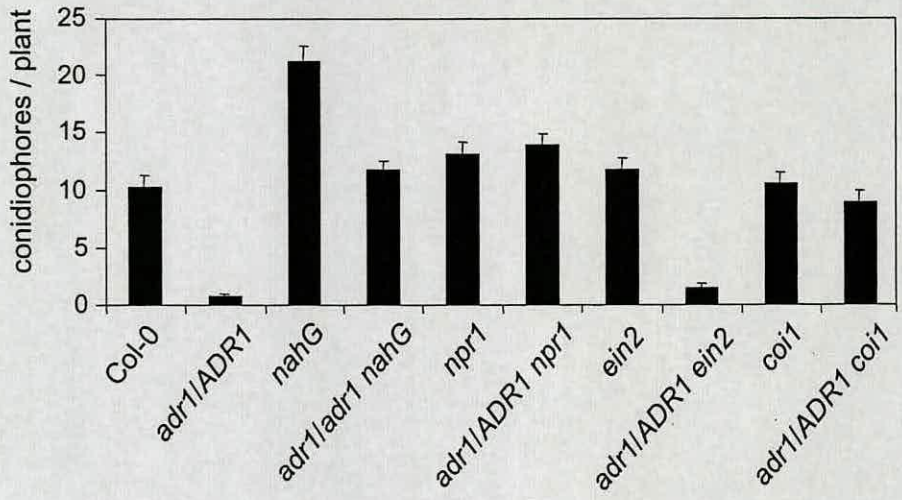


Figure 4.15 Response of 10-day old *adr1* double mutants to *P. parasitica*.

10-day-old *adr1* double mutants were challenged with a *P. parasitica* NOCO2 conidiospores suspension of $1x10^6$ spores per ml and the number of conidiophores per seedling was counted 10 days post treatment. Col-0 wild type, *adr1/ADR1*, *nahG*, *npr1*, *ein2* and *coi1* plants were included as controls. Error bars represent standard errors.

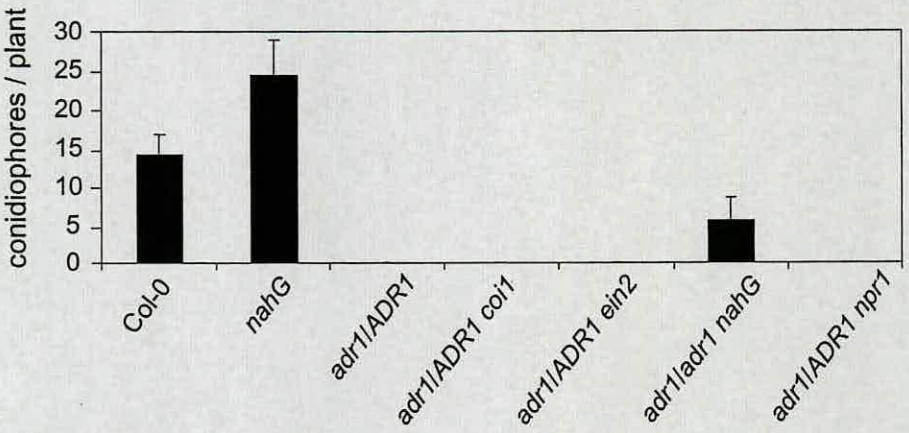


Figure 4.16 Response of 25-day old *adr1* double mutants to *P. parasitica*.

25-day-old *adr1* double mutants were challenged with a *P. parasitica* NOCO2 conidiospores suspension of $1x10^6$ spores per ml and the number of conidiophores per seedling was counted 10 days post treatment. Col-0 wild type, *nahG* and *adr1/ADR1* plants were included as controls. Error bars represent standard errors.

In addition, susceptibility to *E. cichoracearum* UED1 was also tested in *adr1* double mutants. In contrast to *P. parasitica* disease resistance results, only *adr1/ADR1 ein2* and *adr1/ADR1 coi1* double mutants retained full resistance to *E. cichoracearum* (Figure 4.17). On the other hand, *adr1/ADR1 npr1* plants showed an infection rate similar to that of wild-type plants, while *adr1/adr1 nahG* double mutants were nearly as susceptible as *nahG* plants. Consequently, these results suggested that *adr1*-mediated resistance against *E. cichoracearum* is dependent on SA, but only partially dependent on NPR1.

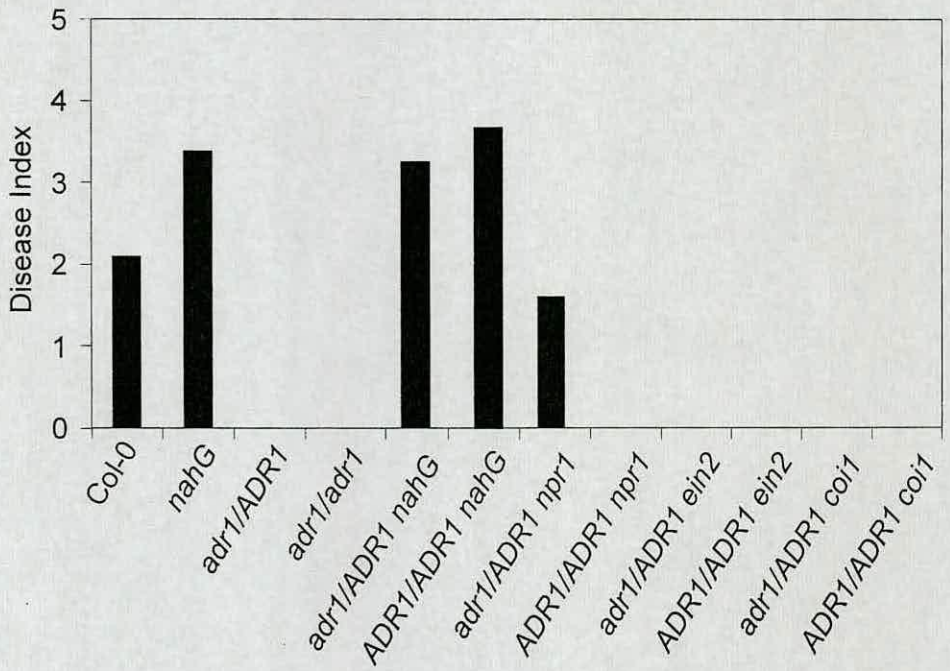


Figure 4.17 Response of *adr1* double mutants to *E. cichoracearum*.

Plants were challenged by gently dusting *E. cichoracearum* UED1 spores from the leaves of infected wild-type plants onto *adr1* double mutants. Col-0, *nahG* and *adr1/ADR1* plants were included as controls. Plants were scored employing a disease index for visual fungal growth 7 days after treatment.

Trypan blue and DAB staining analysis were performed employing *adr1* double mutants to assess the contribution of distinct defence signalling pathways to the establishment of *adr1*-mediated microscopic cell death and H_2O_2 accumulation. Figure 4.18 shows that *adr1*-mediated cell death was maintained in all double mutants apart from *adr1/adr1 nahG*. Similarly, high levels of H_2O_2 occurred in all double mutants apart from *adr1/adr1 nahG* (Figure 4.19). These results suggested that a functional SA pathway is necessary for both *adr1*-mediated cell death and ROI accumulation.

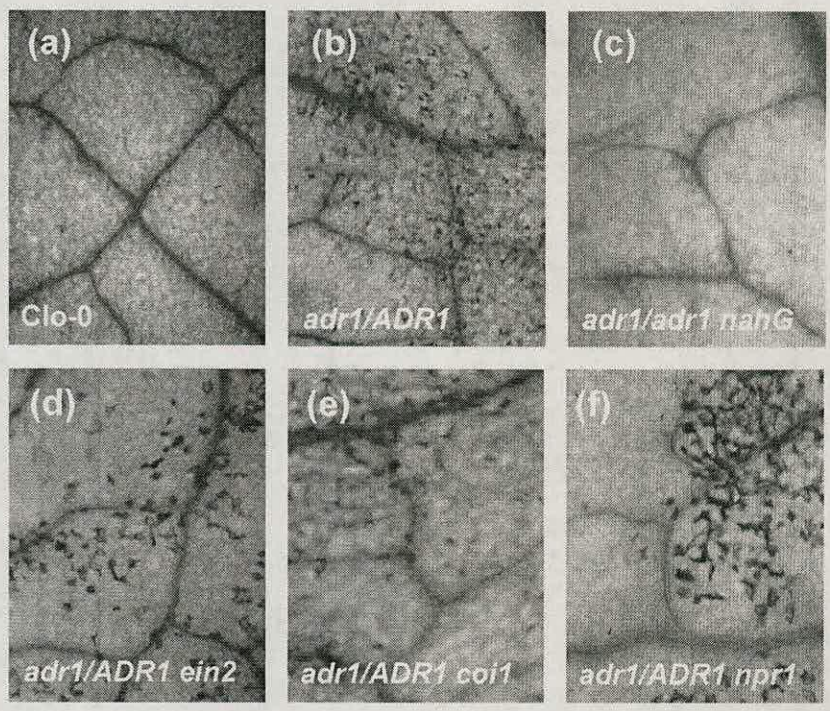


Figure 4.18 Accumulation of microscopic cell death in *adr1* doubles mutants.

(a-c) Accumulation of cell death uncovered by trypan blue staining in a representative wild-type Col-0 leaf (a), *adr1/ADR1* leaf (b) *adr1/adr1 nahG* (c), *adr1/ADR1 ein2* (d), *adr1/ADR1 coi1* (e) and *adr1/ADR1 npr1* plants (f). Magnification in each figure was 10X.

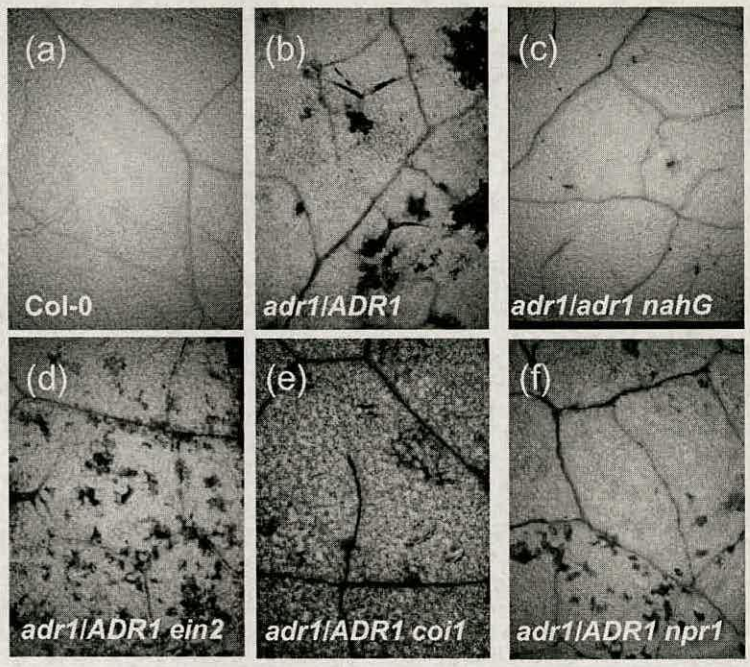


Figure 4.19 Accumulation of H_2O_2 in *adr1* double mutants.

(a-f) Accumulation of H_2O_2 reported by 3,3-diaminobenzidine staining in representative leaves of wild-type (a), *adr1/ADR1* (b), *adr1/adr1 nahG* (c), *adr1/ADR1 ein2* (d), *adr1/ADR1 coi1* (e) and *adr1/ADR1 npr1* plants (f). Magnification in each image was 10X.

4.6 Controlled *ADR1* expression establishes disease resistance without yield penalty

Constitutive induction of *ADR1* led to enhanced disease resistance but simultaneously affected plant development. Thus, we investigated whether the controlled expression of *ADR1* could orchestrate the establishment of resistance without affecting normal plant development and yield. This analysis was undertaken by employing a glucocorticoid-regulated gene transcription system (Ayoama and Chua, 1997). Dr John Grant generated a *TA::ADR1* construct which consists of the *ADR1* gene under the control of a DEX-inducible promoter (Figure 4.20). This construct was subsequentially transformed into wild type *Arabidopsis* plants carrying the *PR1::LUC* reporter construct.

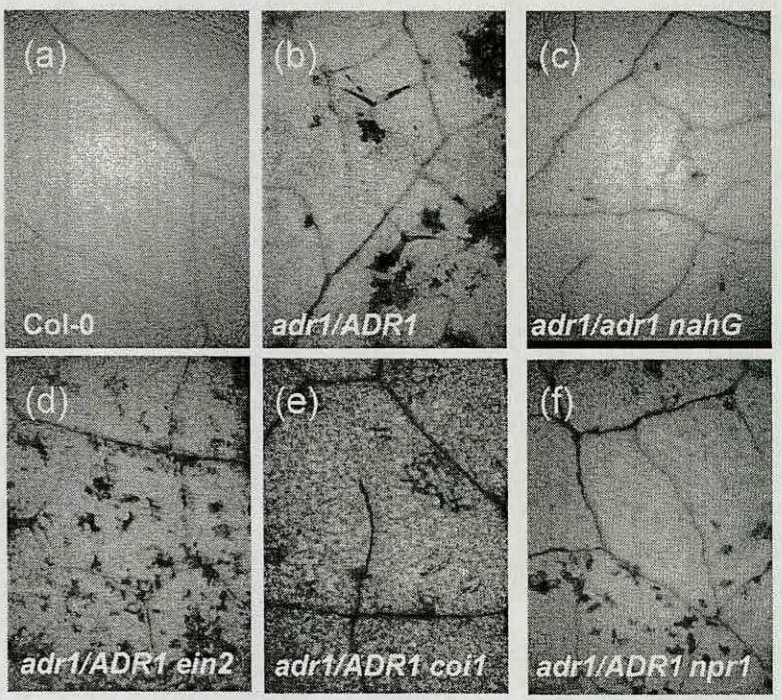


Figure 4.19 Accumulation of H_2O_2 in *adr1* double mutants.

(a-f) Accumulation of H_2O_2 reported by 3,3-diaminobenzidine staining in representative leaves of wild-type (a), *adr1/ADR1* (b), *adr1/adr1 nahG* (c), *adr1/ADR1 ein2* (d), *adr1/ADR1 coi1* (e) and *adr1/ADR1 npr1* plants (f). Magnification in each image was 10X.

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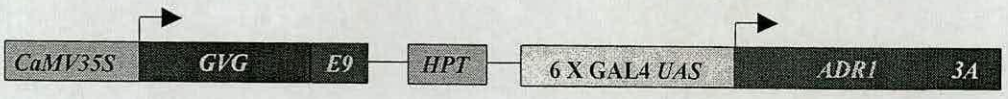


Figure 4.20 The glucocorticoid-inducible system employed for the controlled expression of *ADR1* in plants.

The schematic reproduction of the chimeric glucocorticoid-inducible system employed for the controlled, transient expression of *ADR1* in transgenic plants (*TA::ADR1* binary cassette). The same cassette deployed of *ADR1* sequence was used as control (*TA::* empty vector). *ADR1* expression is regulated by the glucocorticoid activated promoter (*6xGAL4UAS*) and *3A* denotes the pea *rbcsS*-A polyadenylation sequence. The glucocorticoid responsive transcription factor (*GVG*) is driven by *CaMV35S*, and *E9* corresponds to the pea *RBCS-E9* polyadenylation sequence. The hygromycin phosphotransferase (*HPT*) gene confers hygromycin resistance in plants (promoter and terminator not shown).

Germination analysis of T_2 and T_3 lines, grown on MS plate containing hygromycin, identified more than 20 individual homozygous *TA::ADR1* lines. Preliminary characterisation suggested that most of these *ADR1* inducible lines exhibited equivalent features, thus only line 6.17 was used for further characterisation and it will be subsequentially referred to as *TA::ADR1*. Similarly, only line 1.4, among the empty vector control lines, was used for further characterisation and it will be subsequentially referred to as *TA::*. Previous studies have reported that a high level of DEX treatment may trigger defence-related gene expression in plants (Kang et al., 1999). Transgenic lines were therefore sprayed with different DEX concentrations in order to achieve the most effective expression of *ADR1* in absence of any potential phyto-toxic consequences associated with DEX. *TA::ADR1* transgenic plants, but not *TA::* control lines, exhibited moderate HR-like lesions 48 hours after treated with 1μM DEX. None of the studies on DEX-induced gene expression system reported in the literature described any phyto-toxic effects of such a low concentration DEX treatment.

The luciferase (LUC) activity of DEX-treated plants was evaluated via an ultra low light imaging camera. DEX-treated *TA::ADR1* plants exhibited high bioluminescence at 48

hours after treatment compared to *TA::* control plants (Figure 4.21). To confirm that the observed LUC activity corresponded to *PR1* accumulation, RNA analysis was performed. Expression of *PR1* transcript was verified to occur within two days (Figure 4.23), consistent with the appearance of visual macroscopic chlorosis. Furthermore, control *TA::* plants did not show *PR1* accumulation, confirming that the optimal 1μM DEX treatment was non-phyto-toxic and did not induce defence gene expression. In addition, the kinetics of *ADR1* expression was investigated. Northern analysis revealed that *ADR1* transcripts were accumulated within 5 hours post DEX treatment (Figure 4.22), earlier than the 50-hour post DEX treatment accumulation of *PR1* transcripts.

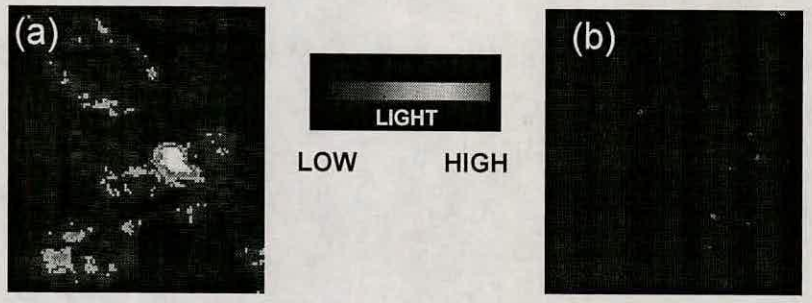


Figure 4.21 DEX treatment induced luciferase activity in *TA::ADR1* transgenic lines.

(a-b) Ultra low light imaging was employed to assess LUC activity in *TA::ADR1* (a) and *TA::* (b) plants 50 hours after treatment with 1 μM DEX solution. Colour scale represents light intensity.

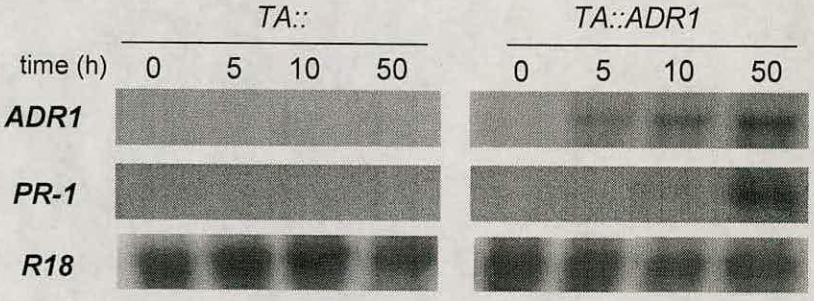


Figure 4.22 Kinetic analysis of the accumulation of *ADR1* and *PR1* transcripts.

TA::ADR1 and *TA::* plants were investigated by northern blot analysis for *ADR1* and *PR1* expression at given time in hours (h) after treatment with 1 μM DEX. *R18* was used to control equal RNA loading and transfer.

According to the gene expression kinetic results, inducible *ADR1* plants were challenged with *P. parasitica* 50 hours after 1 or 0.4 μM DEX treatment. As presented in Figure 4.23, inducible *TA::ADR1* plants showed complete disease resistance, since no fungal growth could be detected. Moreover, DEX-treated *TA::* control plants exhibited an infection rate analogous to that of wild-type plants. Transient expression of *ADR1* can therefore orchestrate disease resistance.

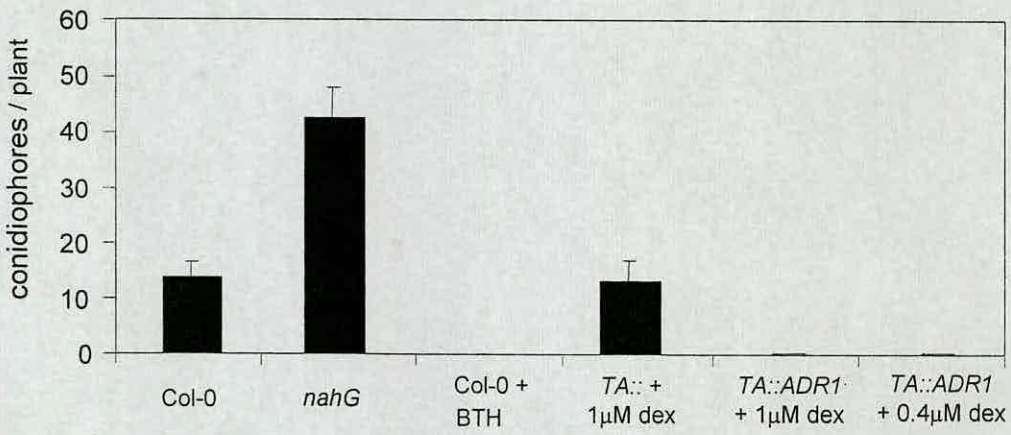


Figure 4.23 The *ADR1* inducible line showed resistance against *P. parasitica*.

TA::ADR1 transgenic plants and *TA::* control lines were treated with a *P. parasitica* NOCO2 conidiospores suspension ($1x10^6$ spores per ml) 24 hours after DEX treatment. The number of conidiophores per plant was counted 10 days after challenge. Col-0 wild-type, BTH treated Clo-0 and *nahG* plants were included as controls. Error bars represent standard errors.

Finally, we investigated whether the *adr1*-mediated disease resistance alters plant production. Seeds, from *adr1* mutants, constitutive *ADR1* overexpressing plants, *ADR1* inducible lines and wild-type control plants, were collected and weighted (Figure 4.24). As expected, *adr1* and CaMV*35S::ADR1* plants that showed enhanced resistance exhibited a significant decrease in seed production. However, the seed yield of *TA::ADR1* plants following 1 or 0.4 μM DEX treatment, which conferred disease resistance, was not significantly different to that of wild-type plants. In addition, the seed yield of DEX treated *TA::*, untreated *TA::ADR1* and *TA::* lines were also

statistically equivalent to that of wild-type plants. Thus, the transient expression of *ADR1* can convey disease resistance in the absence of significant yield penalty under the environmental conditions tested.

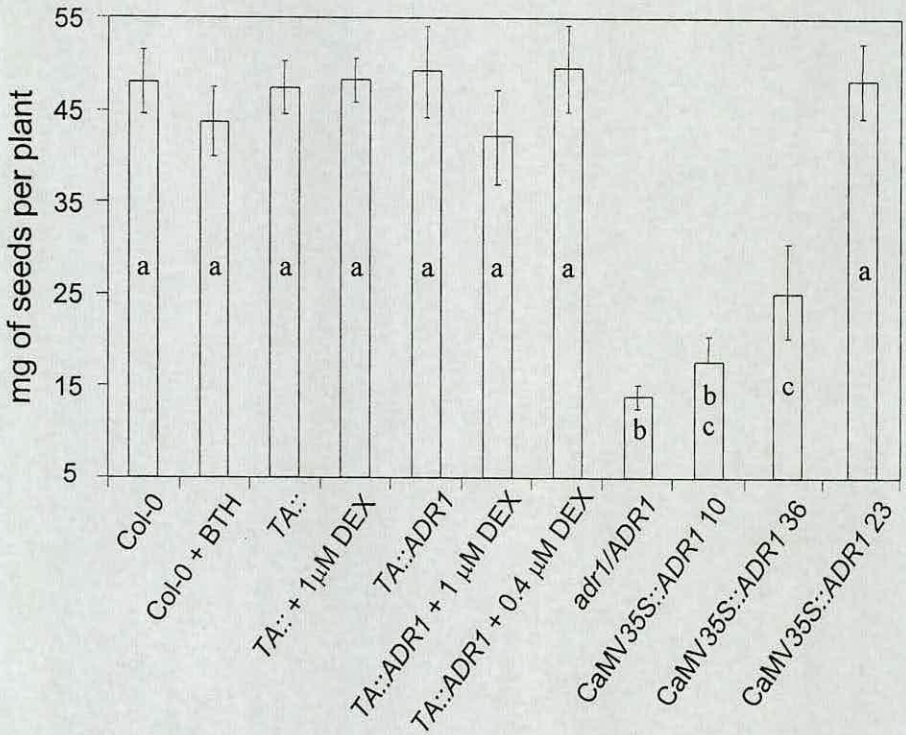


Figure 4.24 Transient expression of *ADR1* is not associated with yield penalty.
Yield, reported as grams of seeds per plant, were determined employing 10 to 25 plants per line. Mutants, transgenic lines and control plants were grown in similar conditions. Histogram bars labelled with different letters indicate data that are significantly different (P > 0.95). Error bars represent standard deviations.

4.7 Conclusions

Previous analysis established that *adr1* plants accumulated high level of *PR1*, *PDF1.2* and *GST1* transcripts. The expression of these defence-related genes has been previously reported to be induced by SA, JA/ET and ROI respectively. We therefore determined the concentration of SA, JA and SA in *adr1* plants. The defence metabolite profiling of *adr1* mutant presented here suggest that constitutive *PR1* expression may be due to the presence of microscopic cell death region and high SA level in the mutant. Similarly, constitutive accumulation of H_2O_2 may play a major role in the *GST1* constitutive

expression. Interestingly, these results indicate that neither elevated JA nor high ET production are necessary to trigger constitutive *PDF1.2* expression in *adr1* mutants.

adr1-mediated resistance to *P. parasitica* was reported previously (Grant et al., 2003). However, results presented here suggest that *ADR1* expression not only conveys resistance against this oomycete, but also against the cauliflower mosaic virus and *E. cichoracearum*, a biotrophic pathogen. In addition, *adr1* lines constitutively expressed *PDF1.2*, a key defence gene in resistance against necrotrophic fungal pathogens; however, *adr1* plants did not exhibit resistance to the necrotrophic *B. cinerea*. Antagonistic action of SA- and JA-mediated defence pathways have been previously reported (Felton et al., 1999; Li et al., 2004) and the fact that *adr1* plants exhibited slightly decreased JA levels may have resulted in increased susceptibility to *B. cinerea*. In spite of this, *adr1* did not show enhanced disease susceptibility to *B. cinerea*.

Analysis of the CaMV*35S::ADR1* transgenic lines verified that the magnitude of reduced stature, defence gene expression and disease resistance were proportional to the level of *ADR1* expression. Our results showed that all these features co-segregated. In addition, the rapid accumulation of *ADR1* transcripts following *Pst*(*avrB*) or *Pst*(*avrRpt2*) recognition significantly preceded the expression of SA-dependent marker *PR1* (Malamy et al., 1990; Metraux et al., 1990). Thus, the early accumulation of *ADR1* transcripts occurs before a significant increase in SA concentration. Exogenous SA or BTH treatment, however, highly induced both local and systemic transcription of *ADR1*. Therefore, SA-dependent defence signalling may function as a positive feedback loop, maintaining or increasing *ADR1* gene expression during the establishment of disease resistance both locally and systemically.

Previous gene expression analysis in *adr1* double mutants established that *adr1*-mediated *PR1* expression was SA- and NPR1-dependent (Grant et al., 2003). Complementary, disease resistance results presented here strongly suggested that *adr1*-mediated *E. cichoracearum* resistance was SA- and partially NPR1-dependent, but ET- and JA-independent. In contrast, *adr1*-mediated resistance against *P. parasitica* appeared to be dependent on SA, but NPR1-, ET- and JA-independent. Consistent with

these observations, cell death and ROI accumulation occurred only in *adr1* double mutants exhibiting enhanced disease resistance. Furthermore, phytohormone quantification analysis in *adr1* double mutants revealed that the disease resistance trait was generally associated with SA accumulation, which was necessary for *ADR1* transcript accumulation. It is noteworthy that *adr1/ADR1 npr1* plants showed high SA and SAG levels but failed to retain full disease resistance against *E. cichoracearum*, which may be due to the lack of accumulation of *PR1* and other SA-dependent genes in *adr1/ADR1 npr1* double mutants.

As reported in the introduction, the detrimental effect of constitutive disease resistance on plant development and yield detained the exploitation of transgenic disease resistance crops (Hammond-Kosack and Parker, 2003). Reduced yield is therefore one of the main restraints for the commercial exploitation of our knowledge in plant disease resistance. To potentially overcome this limitation, *ADR1* inducible lines were generated and analysed. Results presented here suggested that transient *ADR1* expression can trigger defence gene expression and convey disease resistance in the absence of reduced seed production.

5) The establishment of *adr1*-mediated disease resistance is NDR1-independent and EDS1-dependent

5.1 Analysis of *adr1 ndr1* and *adr1 eds1* double mutants

As described in the introduction, EDS1 is required to establish disease resistance mediated by NBS-LRR proteins possessing a TIR domain located at the C-terminus portion of the protein (Feys et al., 2001). In contrast, NBS-LRR proteins lacking the TIR domain usually exhibit a C-terminal CC domain and their ability to trigger disease resistance is generally dependent on NDR1 (Aarts at el., 1998). To test whether the establishment of disease resistance mediated by the CC-NBS-LRR ADR1 (Grant et al., 2003; Meyer et al., 2003) protein conformed with this general rule, *adr1 ndr1* and *adr1 eds1* double mutants were generated.

5.1.1 Generation of adr1 ndr1 *and* adr1 eds1 *double mutant plants*

Pollen from *adr1* plants was used to pollinate recessive, loss-of-function *ndr1* mutant (Aarts at el., 1998). Successful crossing was confirmed by BASTA selection on F_1 plants, since the activation tag construct inserted in *adr1* contains the *BAR* gene, which conveys resistance to BASTA. Seeds were then collected from individual F_1 plants and F_2 lines were investigated for morphological phenotype and BASTA resistance. As presented in Table 5.1 and 5.2, segregation of the *adr1* morphological phenotype was independent of *ndr1*, since all plants with a wild-type phenotype (~ 26%) died after BASTA treatment, whereas all plants showing an *adr1* phenotype (~ 74%) were BASTA resistant. Allele specific *ndr1* PCR analysis of ten BASTA resistance lines identified 4 independent *adr1/ADR1 ndr1/ndr1* lines and one *adr1/adr1 ndr1/ndr1* double mutant. PCR was repeated on F_3 plants, confirming these results.

A similar approach was employed to generate *adr1 eds1* double mutants; *eds1* is also a recessive, loss-of-function mutation (Feys et al., 2001). Only a minority (30%) of the F_2 plants exhibiting a wild-type phenotype were BASTA susceptible (Table 5.1 and 5.2),

suggesting that the *eds1* mutation suppressed the *adr1* phenotype. To investigate this possibility, allele specific *eds1* PCR-based marker analysis was carried out. Sixteen BASTA resistant plants expressing an *adr1* phenotype were tested. Statistically, if *adr1*-mediated reduced stature was EDS1-independent, 25% of these plants (approximately 4) should be homozygous for the loss-of-function *eds1* mutation. However, none of the BASTA resistance plants expressing an *adr1* phenotype were homozygous for *eds1*. F_2 plants homozygous for *eds1* were only identified in BASTA resistant plants exhibiting wild-type morphology. These results strongly suggest that the *adr1* morphological phenotype is EDS1-dependent.

Line	Total number of plants	BASTA susceptible	BASTA resistant
adr1 ndr1	230	60 (26.1%)	170 (73.9%)
adr1 eds1	341	76 (22.3%)	265 (77.7%)
adr1 Ler	460	122 (27.0%)	336 (73.0%)

Table 5.1 Segregation of BASTA resistance in F_2 *adr1* double mutants.

The total number of *adr1* double mutants F_2 plants investigated for BASTA resistance are reported. Values indicated in the BASTA susceptible and BASTA resistance columns represent the number and percentage of plant that did not survive BASTA treatment and BASTA resistant plants respectively.

Line	Total BASTA resistant plant	Wild-type phenotype	Weak *adr1* phenotype	Intermediate *adr1* phenotype	Severe *adr1* phenotype
adr1 ndr1	170	0 (0%)	0 (0%)	129 (75.9%)	41 (24.1%)
adr1 eds1	265	152 (57.4%)	25 (9.4%)	85 (32.1%)	3 (1.1%)
adr1 Ler	336	79 (23.5%)	139 (41.4%)	106 (31.5%)	12 (3.6%)

Table 5.2 Morphological phenotype of F_2 BASTA resistant *adr1* double mutant plants.

F_2 plants were classified in one of the four morphological groups. Both number and percentage of plants belonging to each class are shown.

Several *Arabidopsis* mutants have been reported, however, to show different levels of penetrance in different *Arabidopsis* accessions (Dietrich, personal communication; Aboul-Soud and Loake, unpublished data). The *eds1* mutant was isolated in Landsberg erecta (L*er*) accession, whereas *adr1* was selected in a Colombia (Col-0) accession; whether the *adr1* mutation was fully penetrant in the L*er* accession was unknown. To address this question, *adr1* mutants were employed to pollinate L*er* plants, F_1 lines were selected by BASTA treatment and seeds collected from individual lines. Analysis of F_2 plants showed the expected 3:1 BASTA resistance segregation (Table 5.1); however, only 75% of the BASTA resistant plants exhibited an *adr1* phenotype (Table 5.2). Therefore, the penetrance of *adr1* in L*er* accession was approximately 75%. In this context, the *EDS1*-dependent *adr1* phenotype observed in *adr1 eds1* double mutants could represent a consequence of the fact that *adr1* was not fully penentrant in L*er* accession. The analysis of 30 F_3 lines showed that each homozygous *adr1/adr1* L*er* line exhibited, to different extent, an *adr1* phenotype. Allele specific PCR analysis failed to identify any *adr1 eds1* line simultaneously showing an *adr1* phenotype and homozygous for the *eds1* mutation. Moreover, none of the analysed *adr1/adr1 eds1/eds1* lines showed an *adr1* phenotype, whereas all *adr1/adr1* L*er* plants exhibited an *adr1* phenotype. These results confirm that the *adr1* phenotype is *EDS1*-dependent.

5.1.2 Characterization of adr1 ndr1 *and* adr1 eds1 *double mutants*

To investigate whether the expression of *ADR1* transcripts paralleled the requirement for *EDS1* observed for *adr1*-mediated reduced stature, *ADR1* analysis was assessed. The accumulation of *ADR1* transcripts was high in both *adr1/ADR1 ndr1* and *adr1/adr1 ndr1* plants, whereas *ADR1* accumulation was considerably reduced in *adr1/adr1 eds1* mutants and nearly undetectable in *adr1/ADR1 eds1* plants (Figure 5.1). Consistent with *ADR1* expression, *adr1 ndr1* plants showed enhanced expression of both *PR1* and *GST1*, while *adr1 eds1* mutants failed to accumulate transcripts of these genes. Furthermore, *adr1/adr1 ndr1* showed greater defence gene expression compared to that of *adr1/ADR1 ndr1* plants, consistent with the level of *PR1* and *GST1* gene expression previously observed in hemizygous and homozygous *adr1* plants (Grant et al., 2003).

To address whether increased expression of defence genes conferred disease resistance in *adr1 ndr1* double mutants, plants were tested for disease resistance against *P. parasita*, since *adr1* conveys full resistance to this oomycete. Results showed that *adr1 ndr1* mutants were more resistant to *P. parasitica* than wild-type plants, whereas *adr1 eds1* double mutants were as susceptible as *eds1* plants (Figure 5.2). These data suggest that *adr1*-mediated resistance against *P. parasita* is *NDR1*-independent but *EDS1*-dependent. Similar results were observed following challenge of *adr1 ndr1* and *adr1 eds1* double mutants with *E. cichoracearum* and CaMV (Figure 5.3 and 5.4 respectively). In summary, the *adr1*-mediated morphological phenotype, defence gene expression and disease resistance appeared *NDR1*-independent but *EDS1*-dependent.

5.2 Generation of *adr1 rar1* and *adr1 sgt1b* double mutants

Recently, *RAR1* and *SGT1B*, two genes required in signalling NBS-LRR-mediated disease resistance, have been isolated and characterised (Muskett et al., 2002; Tor et al., 2002; Tornero et al., 2002). A number of CC-NBS-LRR (CNL) proteins require RAR1 to induce disease resistance (Tornero et al., 2002); *adr1 rar1* double mutants have therefore been generated to investigate whether *adr1*-mediated resistance was dependent on RAR1.

5.2.1 Generation of adr1 rar1 double mutant

A number of *rar1* alleles have been isolated from EMS screens (Muskett et al., 2002; Tornero et al., 2002). The *rar1-10* allele is a null mutation caused by a 5 base pair deletion, whereas only a one base pair change was responsible for all the remaining *rar1* alleles (Muskett et al., 2002; Tornero et al., 2002). F_2 progeny from an *adr1 rar1* cross would therefore be easily evaluated employing a *rar1-10* allele specific PCR analysis. Pollen from *adr1* mutants was used to pollinate *rar1-10* plants, F_1 lines were treated with BASTA and seeds from individual plants collected. Allele specific *rar1* PCR

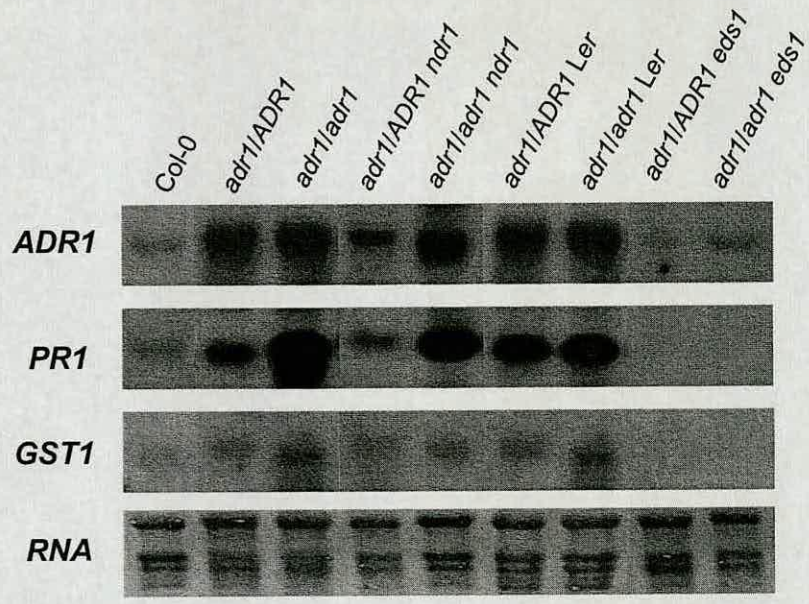


Figure 5.1 Gene expression analysis in *adr1 ndr1* and *adr1 eds1* plants.
Northern blot analysis of *adr1* double mutants was performed to investigate *ADR1, PR1* and *GST1* expression. Col-0, *adr1/ADR1* and *adr1/adr1* plants were included as control. *adr1/ADR1* L*er* represents *adr1* heterozygous mutants in L*er* accession. The RNA in each line was stained with methylene blue to confirm equal loading and transfer.

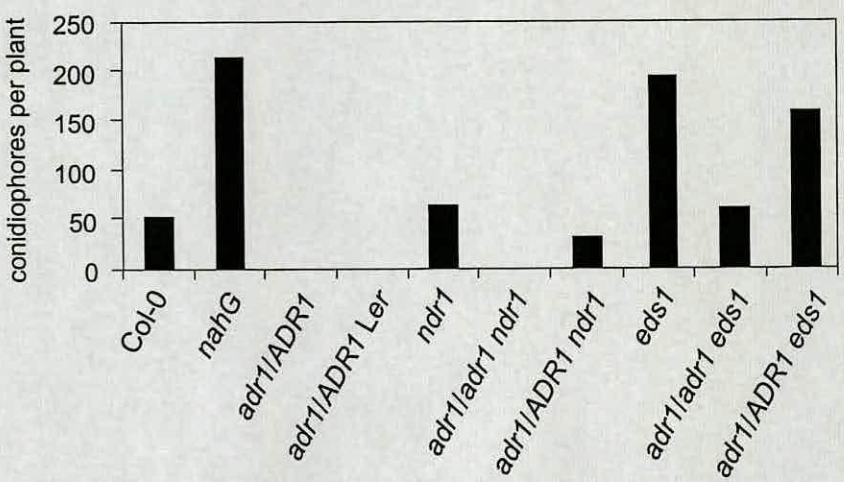


Figure 5.2 The *adr1*-mediated resistance against *P. parasitica* is NDR1-independent and EDS1-dependent.

Plants were treated with a *P. parasitica* suspension of $1x10^6$ conidiospores per ml and the number of conidiophores per plant was counted 10 days after challenge. Col-0 wild-type, *adr1/ADR1*, *nahG*, *ndr1* and *eds1* plants were included as controls.

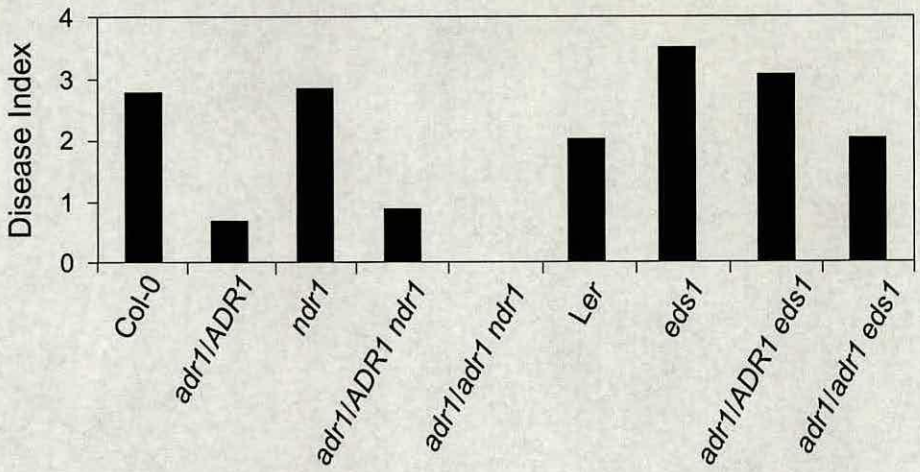


Figure 5.3 The *adr1*-mediated resistance against *E. cichoracearum* is NDR1-independent but EDS1-dependent.

Plants were challenged by gently dusting *E. cichoracearum* spores from the leaves of infected wild-type plants onto *adr1* double mutants. Col-0, L*er*, *adr1*/ADR1, *ndr1* and *eds1* plants were included as controls. Plants were scored employing a disease index for visual fungal growth 7 days after *E. cichoracearum* treatment.

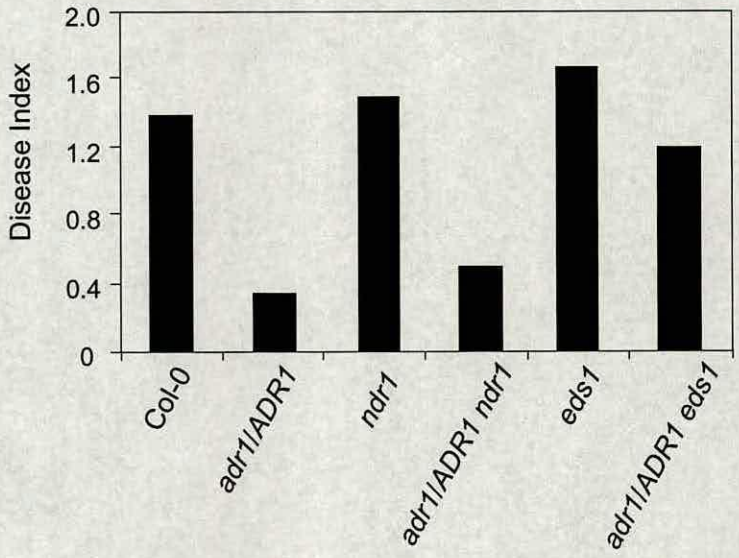


Figure 5.4 The *adr1*-mediated resistance against CaMV is NDR1-independent but EDS1-dependent.

One of the first two leaves of 10-day-old seedling was scrubbed with a CaMV suspension of 50 ng per ml. Plants were scored using a disease index for visual symptoms development 30 days after CaMV challenge. Col-0, *adr1*/*ADR1*, *ndr1* and *eds1* plants were included as controls.

analysis of T2 plants and investigation of *adr1 rar1* double mutants will be undertaken by another student.

5.2.2 Generation of an adr1 sgt1b *double mutant*

SGT1B is also required by several NBS-LRR proteins to establish disease resistance; both CNL and TNL (TIR-NBS-LRR) genes exhibited dependence on SGT1B (Tor et al., 2002). In order to address whether *adr1*-mediated defence pathway was *SGT1B*-dependent, an *adr1 sgt1b* double mutant was generated. Three *sgt1b* alleles have been isolated and all of them consisted of a single base pair mutation (Tor et al., 2002). The *sgt1b-3* mutation was, however, the only allele that completely abolished *SGT1B* expression (Tor et al., 2002). Hence, pollen from *adr1* plants was employed to fertilise *sgt1b-3* mutants, F_1 plants were selected by BASTA treatment and seeds from individual lines collected. Allele specific *sgt1b* PCR analysis of F_2 plants and investigation of *adr1 sgt1b* double mutants will be undertaken by another student.

5.3 Discussion

Several studies have established that *NBS-LRR* gene products signal through defence pathways that require either EDS1 or NDR1 (Muskett et al., 2002; Tor et al., 2002; Tornero et al., 2002). Generally, TNL-mediated disease resistance requires EDS1, while NDR1 is usually necessary to establish CNL-mediated disease resistance (Muskett et al., 2002; Tor et al., 2002; Tornero et al., 2002). As described previously, exceptions to this general rule have been reported (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001); however, none of the CNL proteins characterised to date has been shown to be EDS1-dependent. Surprisingly, the phenotypic appearance of *adr1 ndr1* and *adr1 eds1* plants suggested that the characteristic *adr1* phenotype was retained in *adr1 ndr1* but not in *adr1 eds1* plants. Accordingly, gene expression analysis showed substantial *ADR1*, *PR1* and *GST1* transcripts accumulated in *adr1 ndr1* plants, but not in *adr1 eds1* plants. Consistently, *adr1 ndr1* plants, but not *adr1 eds1* mutants, exhibited *adr1*-mediated

resistance against *P. parasitica*, *E. cichoracearum* and CaMV. In summary, *adr1 eds1* lines showed wild-type morphology, a disease response similar to that of wild-type plants and very low expression of *ADR1* and defence genes. In contrast, *adr1 ndr1* double mutants exhibit the characteristic *adr1* phenotype, increased expression of defence genes and enhanced disease resistance. Thus, ADR1 may be the first example of *CNL* gene product to signal through EDS1 but independently on NDR1.

EDS1, however, contributes to the establishment of basal disease resistance, disease resistance mediated by *R* gene products and is also required for the amplification loop that potentates SA-dependent defence responses (Parker et al., 1996; Feys et al., 2001). The overexpression of *ADR1* can induce the transcription of the defence gene *PDF1.2*, whose expression is not regulated by SA nor EDS1 (Grant et al., 2003; Falk et al., 1999). *PDF1.2* expression will therefore be studied in *adr1 eds1* mutant plants. In case constitutive *PDF1.2* expression is observed, this analysis may suggest that *adr1*-induced expression of defence genes does not require *EDS1* and therefore that *ADR1* can signal independently from *EDS1*.

In addition, to validate that ADR1 signals through EDS1 to establish disease resistance, the *TA::ADR1* line will be crossed with an *eds1* knock-out line isolated in Col-0 accession. The knock-out lines disrupted in the *EDS1* gene in a Col-0 accession have been obtained from the SALK knock-out resource. The isolation and analysis of *eds1 TA::ADR1* plants and *eds1 TA::* control lines will be carried out by another student. The investigation of immediate gene expression in *eds1 TA::ADR1* plants and *eds1 TA::* lines following DEX treatment may uncouple the function of EDS1 in early R protein signalling responses with the role of EDS1 within a amplification loop required for the establishment of basal resistance.

6) Drought tolerance established by enhanced expression of *ADR1* requires salicylic acid, EDS1 and ABI1.

6.1 Enhanced expression of *ADR1* conveys tolerance to drought stress.

The refined regulation of transcription is thought to coordinate the acclimation of plants to challenging environmental conditions (Seki et al. 2002a; Schenk et al. 2000; Tao et al. 2003). Stress research has traditionally focussed on plant responses to a single environmental challenge. In the field, however, plants are concurrently exposed to various environmental insults, hence requiring the synchronized engagement of multiple signalling pathways, connected within a complex network (Xiong et al., 2002). When considered in isolation, plant response to dehydration stress is a relatively well-characterised phenomenon (Hasegawa et al. 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al. 2002). The existence of at least two abscisic acid (ABA)-dependent and two ABA-independent signalling pathways in response to drought is well-established (Giraudat et al. 1994; Shinozaki and Yammaguchi-Shinozaki, 2000; Xiong et al. 2002). These pathways have been extensively described in Chapter 1.

6.1.1 adr1 *plants exhibited drought tolerance*

During the growth and maintenance of *adr1* plants, this mutant line appeared more drought tolerant than wild-type plants. Analyses to further investigate this observation were therefore carried out. Wild-type Col-0 plants were severely withered following water withdrawal for 11-days, while both hemizygous *adr1/ADR1* and homozygous *adr1/adr1* plants remained healthy. After 15-days without water, all wild-type plants had died, however, both *adr1/ADR1* and *adr1/adr1* plants were all still viable, although exhibiting signs of wilting. On day-16, wild-type and *adr1* plants were re-watered and scored for survival the following day (Figure 6.1a). Approximately 100% of *adr1* plants survived, whereas only 6% wild-type Col-0 plants were still viable (Figure 6.1a).

6.1.2 Enhanced expression of ADR1 *confers drought tolerance*

In order to confirm the drought tolerant phenotype of *adr1* plants, we examined selected lines containing a CaMV*35S*::*ADR1* transgene exhibiting significant differences in the magnitude of *ADR1* transcript accumulation. The *35S*::*ADR1* line 10 showed high levels of *ADR1* expression; line 36 an intermediate level; and no *ADR1* transcripts were detected in line 23 plants following gene expression analysis (Chapter 4, Figure 4.9). Both *35S*::*ADR1* line 10 and 36 plants exhibited significantly increased drought tolerance compared to wild-type Col-0 plants, whereas the line 10 plants exhibited greater drought tolerance than line 36 plants (Figure 6.1a). In contrast, the line 23 plants, which failed to accumulate *ADR1* transcripts, did not show significant difference to wild-type plants with respect to drought tolerance (Figure 6.1a).

6.1.3 Conditional expression of ADR1 *is sufficient to trigger drought tolerance*

Several *Arabidopsis* mutants have been uncovered that, like *adr1*, exhibit broad-spectrum disease resistance. Response of *cpr1* (Bowling et al. 1994), *cir1* (Murray et al. 2002) and *cir2* (Tani, 2004) mutant lines to dehydration stress was therefore compared to that of *adr1* plants. The survival rate for *adr1* plants was 99%, whereas only 4%, 3% and 8% of *cpr1*, *cir1* and *cir2* plants respectively, remained viable following 15 days without water (Figure 6.1b). Furthermore, only 9% of wild-type Col-0 plants sprayed with the SAR inducing chemical benzothiadiazole (BTH) survived (Gorlach et al., 1996) (Figure 6.1b). This suggested that drought tolerance was not a general feature of *Arabidopsis* plants exhibiting broad-spectrum disease resistance.

As *adr1* mutant plants were smaller than wild-type plants, we investigated if the drought tolerant phenotype expressed by this mutant line was a consequence of its reduced stature. To address this issue, we employed the *TA::ADR1* transgenic line, described in Chapter 4, expressing *ADR1* under the control of a glucocorticoid-mediated transcription induction system (Aoyama and Chua, 1997). Four week-old *TA*::*ADR1* plants, which were physically indistinguishable from wild-type plants, were sprayed with DEX and scored for survival following water withdrawal for 15 days (Figure 6.1c). Only 5% of

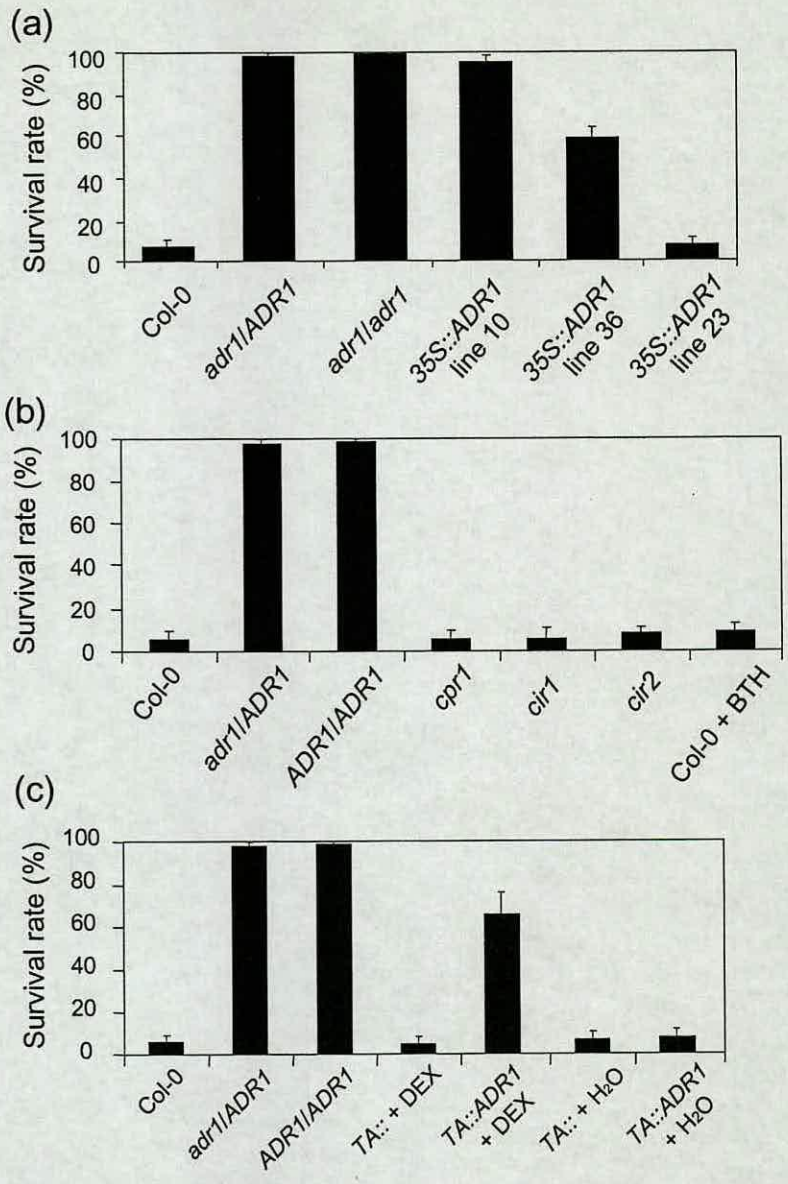


Figure 6.1 Plant survival rate following 15-days of water withdraw.

Plant survival rate was calculated following 15 days of water withdraw. Col-0 wild-type plants, hemizygous and homozygous *adr1* mutants were always included as controls. The experiment was repeated three times with similar results. Error bars represent standard errors.

(a) Plant survival rate of 35S::*ADR1* lines following 15 days of water withdrawal.

(b) Exposure of BTH-treated wild-type plants, *cpr1*, *cir1* and *cir2* mutant lines to 15 days of water withdrawal, with the survival rate shown per each given plant line.

(c) Plant survival rate of DEX-treated *TA::ADR1* and *TA::* plants. Water-treated *TA::ADR1* and *TA::* controls were also included.

Col-0 wild-type plants remained viable in contrast to more than 95% of both *adr1/adr1* and *adr1/ADR1* plants (Figure 6.1c). The DEX-treated *TA::ADR1* line showed a survival rate of 65%, while only 4% of plants containing an empty vector cassette, *TA::*, survived (Figure 6.1c). As control, *TA::ADR1* and *TA::* plants were sprayed with water and failed to show drought tolerance (Figure 6.1c). The conditional expression of *ADR1* can therefore convey significant drought tolerance in the absence of any reduction in plant stature.

6.1.4 Transpiration rate in adr1 *plants*

Stomata closure in response to drought stress is an important survival strategy because it limits water loss through reduced transpiration (Leung and Giraudat, 1998). A decreased transpiration rate might therefore contribute to the establishment of drought tolerance in *adr1* plants. To investigate this possibility, the fresh weight loss of detached wild-type and *adr1* plants was evaluated and their transpiration rate determined (Kang et al., 2002). At five hours post detachment, *adr1* plants exhibited a 38% decrease in fresh weight, not significantly different from the 36% decrease of Col-0 plants (Figure 6.2). In contrast, the rate of weight loss in the ABA-insensitive *abi1* plants was 69% at five hours post detachment (Figure 6.2). Therefore, decreased rate of water loss does not appear to contribute to the establishment of drought tolerance in *adr1* plants.

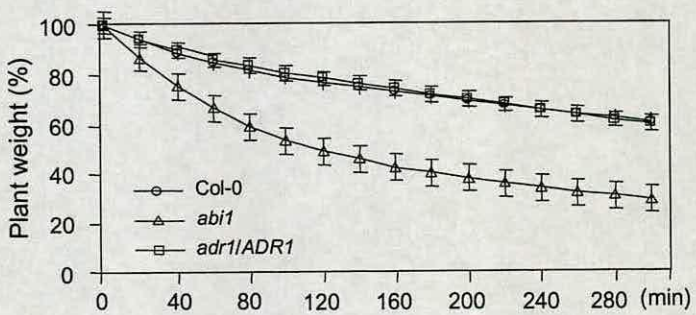


Figure 6.2 Transpiration rate of *adr1* mutant line
Transpiration rate of 4-week-old soil grown Col-0, *abi1* and *adr1* plants is presented. The transpiration rate was calculated as the relative loss of weight throughout a period of 5 hours. Data points represent averages of duplicate experiments, with standard errors shown as bars.

6.2 Abiotic cross-tolerance in *adr1* plants

It has been previously reported that some transgenic plant lines show tolerance to multiple environmental stresses (Roxas et al. 1997; Kasuga et al., 1999; Bowler and Fluhr, 2000; Chinnusamy et al., 2004), a phenomenon termed cross-tolerance. To examine if *adr1* plants exhibited cross-tolerance, the response of this mutant line to additional abiotic insults was investigated.

6.2.1 Response of adr1 *to heat stress*

Heat tolerance was examined by determining the survival rate at given times following exposure to a temperature of 42℃ (Larkindale and Knight, 2002). The survival rate of both hemizygous and homozygous *adr1* plants was indistinguishable from that of wild-type Col-0 plants following either 9 hours or 11.5 hours exposure to a temperature of 42℃ (Figure 6.3a). After 16 hours at 42 ℃, however, the survival rate of both hemizygous and homozygous *adr1* plants was significantly less than that of Col-0 plants. Hence, *adr1* lines may show a small increased sensitivity to heat stress. To test this hypothesis, the heat response in *TA::ADR1* and *TA::* plants was also examined (Figure 6.3a). The conditional expression of *ADR1* in the *TA::ADR1* line was found to convey significant heat sensitivity in the absence of any reduction in plant stature.

6.2.2 Response of adr1 *to heavy metal and freezing stresses*

We also examined the response of *adr1* plants to heavy metals. After exposure to an increasing gradient of $CuSO_4$ concentrations (Song et al., 2003), the survival rate of hemizygous and homozygous *adr1* plants was indistinguishable from that of Col-0 plants (Figure 6.3b). A similar result was obtained following exposure to an increasing gradient of $CdCl_2$ concentrations (Song et al., 2003) (Figure 6.3c). Therefore, neither *adr1/ADR1* nor *adr1/adr1* plants exhibited either increased tolerance or sensitivity to heavy metal stress. In a similar fashion, *adr1* plants also failed to exhibit significant tolerance to freezing (Kasuga et al., 1999) (Figure 6.3d).

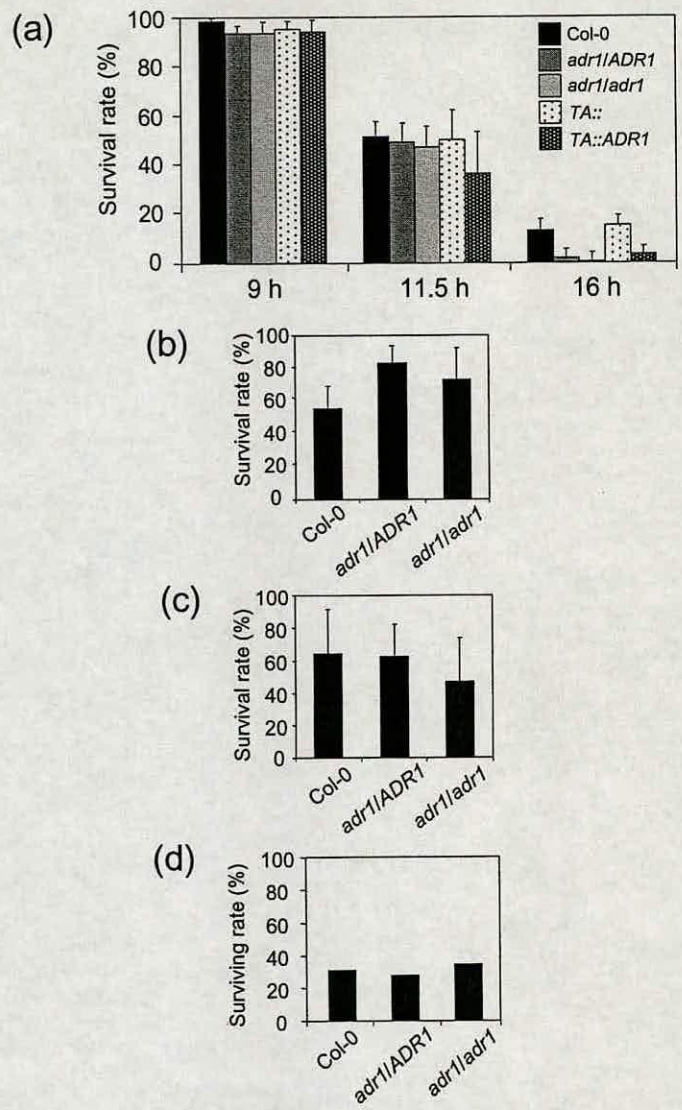


Figure 6.3 *adr1* plants do not show cross-tolerance

(a) Exposure of wild-type Col-0 and *adr1* plants to thermal stress. Following a gradient of increasing temperate, plants were maintained at 42°C for 9, 11.5 or 16 hours. The survival rate for Col-0 (■), *adr1* hemizygous (▩), *adr1* homozygous (▨), DEX-treated *TA::* (⊡) and DEX-treated *TA::ADR1* (▩) plants for a given time of exposure are shown.

(b) The survival rate of Col-0, hemizygous and homozygous *adr1* plants following treatment with an increasing concentration gradient of $CuSO_4$.

(c) Exposure of Col-0, hemizygous and homozygous *adr1* plants to an increasing concentration gradient of $CdCl_2$, with the survival rate shown for each given plant line.

(d) The survival rate of Col-0, hemizygous and homozygous *adr1* plants following 5 hours at freezing temperature, as described in experimental procedures

Experiments were repeated three times, showing similar results. Data points represent averages, with standard errors shown as bars.

6.2.3 Response of adr1 *plants to salinity*

To examine the response of *adr1* plants to NaCl stress, this line was germinated on a range of NaCl concentrations from 0 to 200 mM (Figure 6.4). Germination of wild-type Col-0 and *adr1* plants was not affected by NaCl concentrations of 90 mM or less. However, the germination rate of both Col-0 and *adr1* plants were significantly reduced at concentrations of 100 mM NaCl or greater; germination was completely inhibited at 140 mM NaCl (Figure 6.4). These results showed that the germination of *adr1* plants was undistinguishable to that of wild-type plants and *adr1* mutants did not exhibit either NaCl resistance or hypersensitivity.

The expression of *ADR1* gene was relatively weak in *adr1* seedlings (Grant et al. 2003), therefore the response of 4-week old *adr1* plants to a range of NaCl concentrations was also examined. Adult *adr1* plants were strikingly hypersensitive to NaCl, with homozygous *adr1* plants more sensitive than hemizygous plants (Figure 6.5). For example, at 150 mM NaCl, all *adr1/adr1* plants died, while 12% of *adr1/ADR1* plants survived. In contrast, the survival rate of Col-0 plants was 92%. Similar results were obtained following conditional expression of *ADR1* in the *TA::ADR1* line. In this case the rate of survival was 47% for DEXtreated *TA::ADR1* plants compared to 88% for DEXtreated *TA::* control plants (Figure 6.5).

The response of adult *adr1* plants to KCl was also investigated in an equivalent set of experiments. Analogous to the response to NaCl, *adr1* plants also showed hypersensitivity to KCl (Figure 6.5). Again, homozygous *adr1* plants were more sensitive than hemizygous *adr1* plants. Furthermore, the *TA::ADR1* but not the *TA::* line was also hypersensitive to KCl (Figure 6.5). To investigate if the observed hypersensitivity to either NaCl or KCl was ionic or osmotic in nature, we examined the response of *adr1* plants to a concentration of sorbitol that provided a similar osmotic pressure (Figure 6.5). The response of both *adr1/ADR1* and *adr1/adr1* plants to sorbitol were indistinguishable from Col-0 plants. The response of *adr1* plants to both NaCl and KCl therefore appeared to be ionic in nature.

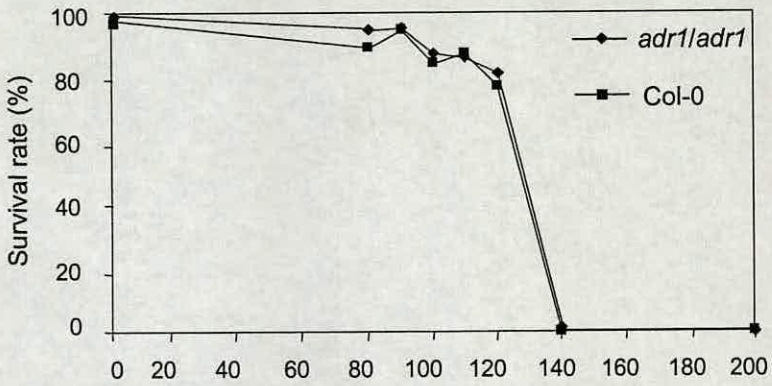


Figure 6.4 Impact of NaCl on the germination of Col-0 and homozygous *adr1* plants.
Col-0 and *adr1* seed were sown in MS plates containing the indicated concentration of NaCl. The germination rate is shown for each plant line. The experiments were repeated twice with similar results.

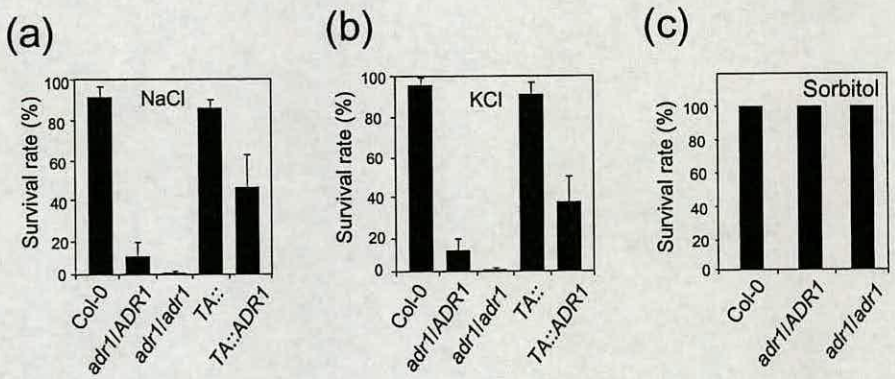


Figure 6.5 Response of *adr1* plants to salt and osmotic stresses.
Effect of NaCl treatment, as described in experimental procedures, on 4-week-old soil grown Col-0 plants, heterozygous and homozygous *adr1* lines.
(a) The survival rate in response to NaCl treatment for Clo-0 plants, *adr1,* DEX-treated *TA::ADR1* and DEX-treated *TA::* lines.
(b) Impact of KCl treatment, as described in experimental procedures, on Col-0 plants, *adr1*, DEX-treated *TA::ADR1* and *TA::* lines. The survival rate for each plant line is shown.
(c) The survival rate of 4-week-old, soil grown Col-0 plants and *adr1* lines to sorbitol treatment, as described in experimental procedures.
Each experiment was repeated three times with similar results. Data points represent averages, with standard errors shown as bars.

6.3 Drought responsive gene expression in *adr1* plants

Drought stress promotes a significant reprogramming of plant transcription (Seki et al. 2002a; Seki et al. 2002b), we therefore examined if *adr1* plants constitutively expressed key abiotic response genes. The expression of the cold-inducible *DREB1A* gene was not detected in Col-0 wild-type plants, hemizygous or homozygous *adr1* lines (Liu et al., 1998) (Figure 6.6). In contrast, the expression of the drought-responsive *DREB2A* gene was detected in *adr1/adr1* plants but not in *adr1/ADR1* plants (Liu et al., 1998) (Figure 6.6). *adr1* plants were also examined for the expression of the ABA-dependent and ABA-responsive genes *RD22* and *RD29A* respectively (Yamaguchi-Shinozaki and Shinozaki, 1994; Abe et al. 1997). In contrast to *DREB2A*, *RD22* or *RD29A* transcripts were not up-regulated in either hemizygous or homozygous *adr1* plants (Figure 6.6). Equivalent to *DREB2A* regulation, the expression of both *RD29A* and *RD22* increased in drought stressed Col-0 plants. In contrast, the accumulation of the cold-responsive *DREB1A* gene was not detected in drought stressed wild-type plants (Figure 6.6).

*6.3.1 Drought responsive gene expression in CaMV*35S::ADR1 *and* TA::ADR1 *transgenic lines*

To confirm these results, we examined the expression of these marker genes in the CaMV*35S*::*ADR1* lines, employed to transgenically reconstruct the *adr1* phenotype. The extent of *DREB2A* transcript accumulation correlated with the strength of *ADR1* gene expression. As presented in Figure 6.7, *35S*::*ADR1* line 10, which strongly expressed *ADR1*, showed high accumulation of *DREB2A* transcripts. In contrast, *DREB2A* expression was reduced in line 36 and undetectable in line 23, in which the accumulation of *ADR1* transcripts was reduced and absent respectively (Figure 6.7).

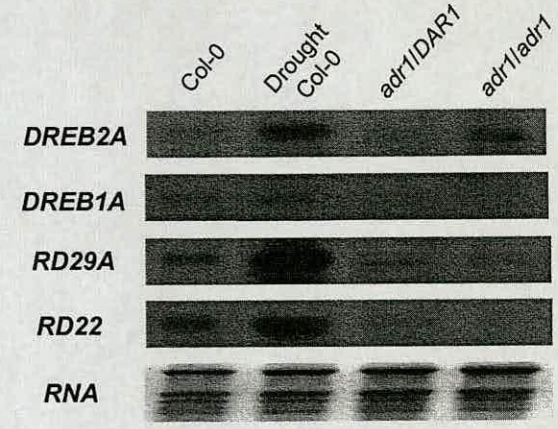


Figure 6.6 Analysis of abiotic gene expression in *adr1* lines.
Expression analysis of *DREB1A*, *DREB2A*, *RD29A* and *RD22* gene in Col-0 wild-type, drought-stressed Col-0, *adr1* hemizygous and homozygous lines. The RNA in each lane of the blots was stained with methylene blue to confirm equal loading and transfer.

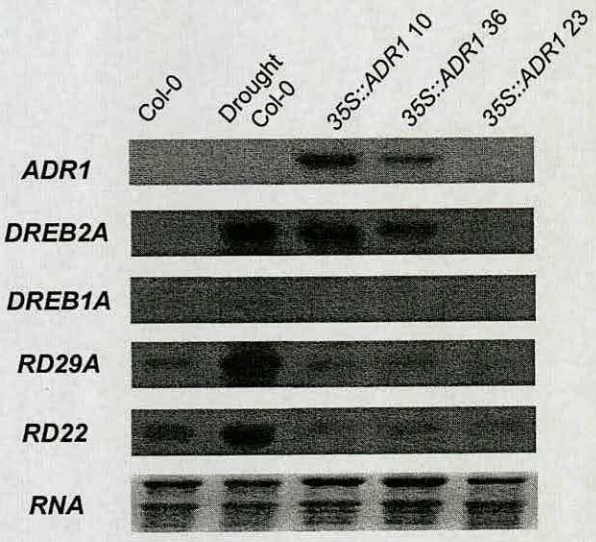


Figure 6.7 Analysis of abiotic gene expression in *35S::ADR1* lines.
Accumulation of *ADR1, DREB2A*, *BREB2A*, *RD29A* and *RD22* transcripts in wild-type Col-0, drought-stressed Col-0 and selected CaMV*35S::ADR1* transgenic lines. The RNA in each lane of the blots was stained with methylene blue to confirm equal loading and transfer.

The possibility that chronic effects caused by the *adr1* mutation might result in indirect activation of *DREB2A* gene expression was also tested employing the *TA::ADR1* transgenic line. The exogenous application of DEX to this line resulted in *DREB2A* transcript accumulation within 50 hours; whereas no *DREB2A* transcripts were detected in DEX treated *TA*:: control plants (Figure 6.8). The expression of *DREB1A*, *RD29* or *RD22* genes were not detected in the CaMV*35S*::*ADR1* lines or the DEX treated *TA*::*ADR1* plants, confirming the results obtained from the gene expression analysis of *adr1* plants.

The constitutive expression of a number of key defence-related genes and the subsequent establishment of disease resistance in *adr1* plants is dependent upon elevated SA levels (Grant et al. 2003). We therefore examined whether the expression of the *DREB2A* gene was also SA-dependent. The application of benzothiadiazole (BTH), a functional analogue of SA (Gorlach et al. 1996), to wild-type Col-0 plants triggered significant accumulation of *DREB2A* and weak accumulation of *RD29A* transcripts. In contrast, the transcription of neither *DREB1A* nor *RD22* was detected (Figure 6.9).

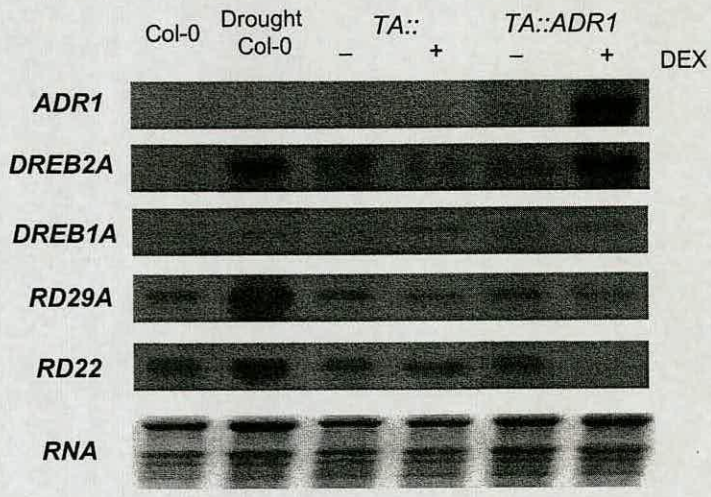


Figure 6.8 Analysis of abiotic gene expression in *TA::* and *TA::ADR1* lines.
Gel blot analysis of *ADR1, DREB2A, DREB2A, RD29A* and *RD22* gene expression in Col-0, drought-stressed Col-0 plants and in transgenic lines containing the *TA::* or *TA::ADR1* transgenes. DEX + represent plants 50 hours post 1μM DEX treatment, while DEX – represents untreated plants. The RNA in each lane of the blots was stained with methylene blue to confirm equal loading and transfer.

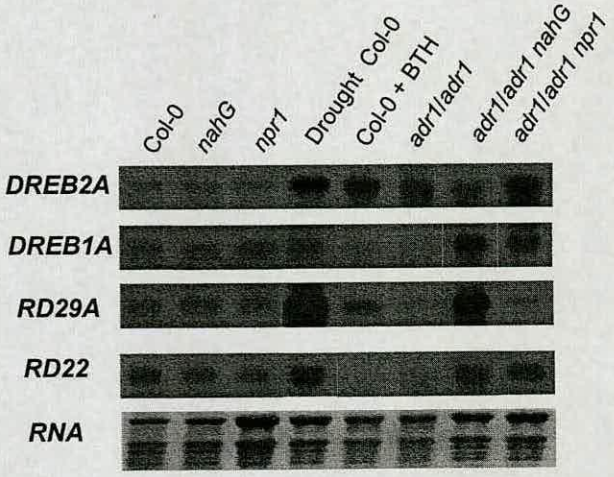


Figure 6.9 Analysis of abiotic gene expression post BTH treatment.
Accumulation of *DREB1A*, *DREB2A*, *RD29A* and *RD22* transcripts in wild-type Col-0, *nahG* and *npr1* mutants, drought-stressed Col-0, wild-type plants treated with 300 μM BTH, *adr1* lines, *adr1 nahG* and *adr1 npr1* double mutant plants. The RNA in each lane of the blots was stained with methylene blue to confirm equal loading and transfer.

6.4 Response of *adr1* double mutants to drought stress

To further analyse *adr1*-activated signalling, the response of a panel of *adr1* double mutants to drought stress was investigated. These mutant lines were either hemizygous or homozygous for *adr1* and also contained one of the following mutations: *ein2* (Guzman and Ecker, 1990), which conveys ethylene (ET) insensitivity; *ndr1* (Century et al. 1997), required for CC-NBS-LRR signalling; *eds1* (Parker et al., 1996), required for TIR-NBS-LRR signalling; *coi1* (Feys et al. 1994), which conveys jasmonate (JA) insensitivity; and, *abi1* (Koornneef et al. 1984), which conveys ABA insensitivity. Additional *adr1* lines contained either the *nahG* transgene, which depletes SA (Delaney et al. 1994) or the *npr1* mutation, which conveys insensitivity to SA (Cao et al. 1994). These mutant lines, apart from *adr1 abi1*, have been described in Chapter 4 and 5.

The *abi1* mutation confers ABA insensitivity (Koornneef et al. 1984); *adr1 abi1* double mutants were therefore germinated and selected on ABA plates. The reduced stature of *adr1/adr1 abi1* and *adr1/ADR1 abi1* plants paralleled that of hemizygous and homozygous *adr1* mutant lines. Furthermore, *ADR1* gene expression in *adr1 abi1* plants is equivalent to that observed in hemizygous and homozygous *adr1* plants (Figure 6.11).

6.4.1 Drought tolerance in adr1 *double mutant plants*

This panel of double mutants were scored for their survival rate following 15 days of water withdrawal (Figure 6.12). Homozygous *adr1* plants containing either a *nahG* transgene, *eds1* or *abi1* mutations exhibited a survival rate of 88%, 72% and 70% respectively. This compared to a survival rate of approximately 100% for homozygous *adr1* plants. The effects of the *nahG* transgene, *eds1* or *abi1* mutations were more pronounced in hemizygous *adr1* plants, where the survival rate was decreased to 31%, 23% and 5% respectively (Figure 6.12). In contrast, the *npr1*, *ndr1*, *ein2* or *coi1* mutations failed to show any significant impact on the survival rate of either hemizygous or homozygous *adr1* plants. Therefore, SA, EDS1 and ABI1 appear to be required for the establishment of *adr1*-mediated drought tolerance, whereas NPR1, NDR1, EIN2 and COI1 are dispensable.

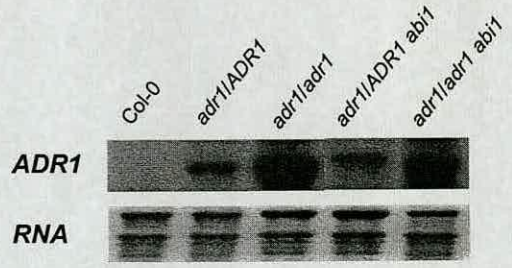


Figure 6.11 Expression of *ADR1* gene in *adr1 abi1* mutant lines.
Accumulation of *ADR1* transcripts was examined in wild-type Col-0, *adr1/ADR1*, *adr1/adr1* and hemizygous and homozygous *adr1 abi1* mutant lines. The RNA in each lane of the blots was stained with methylene blue to confirm equal loading and transfer.

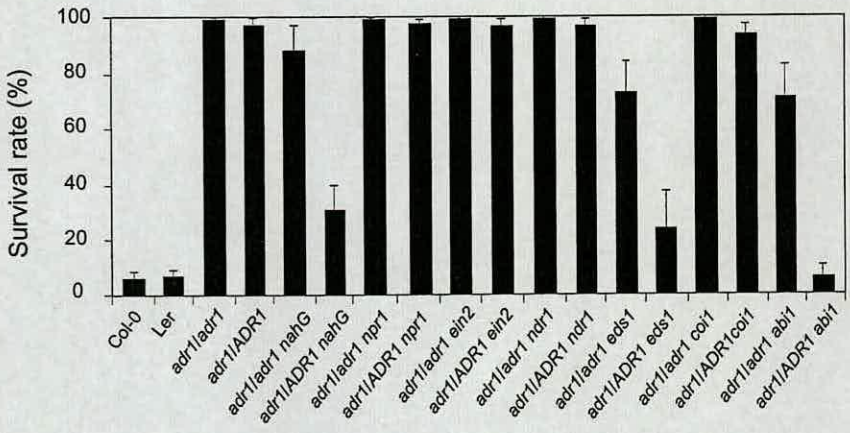


Figure 6.12 Drought tolerance of *adr1* double mutant lines.
The survival rate of *adr1* homozygous and hemizygous double mutant lines following 15 days of water withdrawal. Each data point represents the mean of duplicate experiments, with standard errors shown as bars.

6.4.2 Drought responsive gene expression in adr1 nahG *and* adr1 npr1 *double mutants*

To investigate further the putative role of SA in *adr1*-mediated drought tolerance, expression of drought-responsive genes was also examined in homozygous *adr1* plants containing a *nahG* transgene and the *npr1* mutation (Figure 6.9). *DREB2A* gene expression was abolished in *adr1/adr1 nahG* plants (Delaney et al. 1994), which depletes SA. However, in an *adr1/adr1 npr1* double mutant, *DREB2A* expression was unaffected. Plants possessing the *npr1* mutation are insensitive to SA (Cao et al. 1994). Interestingly, in *adr1/adr1 nahG* plants significant *DREB1A* and *RD29A* but not *RD22* transcripts were detected, whereas the induction of *RD29A* but not *DREB1A* was abolished in *adr1/adr1 npr1* plants (Figure 6.9).

6.5 Discussion

An activation tagged allele of *adr1* exhibited both broad-spectrum disease resistance (Grant et al. 2003) and significant drought tolerance. To our knowledge this is the first example of an *Arabidopsis* mutant to simultaneously express both of these key agricultural input traits. Importantly, this does not appear to be a general feature of *Arabidopsis* mutants that show broad-spectrum disease resistance, because *cir1* (Murray et al. 2002), *cir2* (Tani, 2004) or *cpr1* (Bowling et al. 1994) plants, which constitutively express SAR, do not exhibit increased drought tolerance. Therefore, this phenomenon may be specific for *ADR1* or a limited number of *R* gene signalling pathways. Recent reports have suggested possible connections between the establishment of disease resistance and drought tolerance. However, this topic will be extensively discussed in Chapter 9, whereas only the *adr1*-mediated drought tolerance will be discussed here.

Interestingly, *adr1* alleles do not appear to convey cross-tolerance against other abiotic stresses. Adult *adr1* plants exhibited enhanced tolerance to dehydration, while showing increased sensitivity to thermal stress and hypersensitivity to NaCl. Therefore, *adr1* activated signalling may antagonise signal transmission through other abiotic pathways. Expression analysis of stress marker genes in *adr1* lines provided insights into this signal cross-talk. In *adr1*, CaMV*35S*::*ADR1* and DEXtreated *TA::ADR1* plants, significant *DREB2A* transcript accumulation was detected. In contrast, the expression of *DREB1A*, *RD29* or *RD22* genes was not observed in any of these lines. The ABA-independent, dehydration responsive signalling pathway, marked by *DREB2A* expression, therefore appeared to be engaged in these plants. However, there was no evidence for the activation of the signalling pathways that orchestrate the ABA-independent expression of *DREB1A* and the ABA-dependent induction of *RD22* and *RD29A*. The *RD29A* promoter contains a DRE motif, which is a specific binding site for DREB2A. However, the accumulation of *DREB2* transcripts in *adr1* lines did not result in activation of *RD29A* expression. This might reflect the requirement of DREB2A for a dehydration induced, post-translational modification (Liu et al. 1998). The absence of

RD29A expression in *adr1* lines suggested this modification step may not occur in *adr1* plants.

Broad-spectrum disease resistance is established in *adr1* plants via a SA-dependent mechanism (Grant et al. 2003). Gene expression analysis revealed that *DREB2A* transcripts were induced in Col-0 plants following exogenous application of the SA analogue BTH. In contrast, no transcripts for either *DREB1A* or *RD22* were detected in BTH-treated plants. *RD29A* transcripts accumulated only weakly, supporting the results from a recent study (Borsani et al. 2001). In *adr1 nahG* double mutants, where endogenous SA was depleted, *DREB2A* transcript accumulation was abolished. This observation suggests that *DREB2A* expression in *adr1* plants may be mediated by SA. While a functional NPR1 protein is required for the expression of many SA regulated defence genes (Cao et al. 1994), *DREB2A* transcript accumulation was not reduced in an *adr1 npr1* double mutant line. *adr1*-mediated *DREB2A* gene expression could therefore be regulated by a SA-dependent but NPR1-independent signalling pathway. SA may function to amplify ROI accumulation during the expression of disease resistance (Shirasu et al. 1997; Grant and Loake, 2000). Also, ROIs have been reported to cue *DREB2A* expression (Desikan et al. 2001). As ROI concentrations are high in *adr1* plants (Grant et al. 2003), *DREB2A* expression might result from SA amplified ROI synthesis, which would suggest that *DREB2A* expression is under redox control. It is noteworthy that both ROIs and NO have also been implicated as second messengers in the regulation of stomata aperture (Pei et al. 2000; Desikan et al. 2002). The exogenous application of BTH alone, however, was insufficient to trigger significant drought tolerance. Therefore, additional SA-independent mechanisms may also be active in *adr1* plant lines.

The requirement for SA in the establishment of drought tolerance in *adr1* plants was also supported from the analysis of a series of double mutants and transgenic lines. The *adr1 nahG* mutant line exhibited significantly decreased *adr1*-mediated drought tolerance. Furthermore, drought tolerance was also substantially attenuated in *adr1/ADR1 eds1* double mutant line. EDS1 is required for signalling by the TIR-NBS-

LRR class of R proteins (Aarts et al. 1998), the establishment of non-host resistance in *Arabidopsis* against wheat powdery mildew (Yin et al. 2003) and the development of basal disease resistance (Parker et al. 1996). EDS1 is a putative lipase which is thought to function within a SA-dependent amplification loop (Feys et al. 2001; Loake, 2001). As extensively discussed in Chapter 9, EDS1 may be required in the signal amplification loop that triggers the accumulation of elevated levels of SA, subsequently activates *adr1*-mediated signal pathway and confers drought tolerance in *adr1* plants. In contrast, NDR1, which is required for signalling by the CC-NBS-LRR class of R proteins (Aarts et al. 1998), does not appear to be required for drought tolerance established by *adr1*, because the both *adr1/ADR1 ndr1* and *adr1/adr1 ndr1* mutant lines were not compromised in drought tolerance. It is noteworthy that the requirement for EDS1, but not for NDR1, observed in the establishment of the *adr1*-mediated drought tolerance parallels their requirement in the activation of *adr1*-mediated disease resistance described previously. Therefore, the engagement of the pathway mediated by ADR1, which leads to the establishment of both disease resistance and drought tolerance, appears to signal through EDS1 but not NDR1.

NPR1, which is also required for SA signal transmission (Cao et al. 1994), appears to be dispensable for the establishment of *adr1*-mediated drought tolerance, because *adr1 npr1* lines were not significantly decreased in drought tolerance. Furthermore, hemizygous and homozygous *adr1* plants containing either *coi1* or *ein2*, were also not significantly different from *adr1* plants with respect to drought tolerance. JA and ET are therefore also unlikely to play a role in *adr1*-mediated tolerance to dehydration stress. In contrast, drought tolerance was strongly reduced in *adr1 nahG* lines. However, *adr1* lines which contained the *nahG* transgene were still significantly more drought tolerant than Col-0 plants, suggesting additional, SA-independent mechanisms also contributed to dehydration tolerance. In this context, double mutant analysis also suggested a requirement for ABA to establish *adr1*-mediated drought tolerance. In homozygous and hemizygous *adr1* plants the *abi1* mutation reduced the survival rate following 15-days of water withdrawal to 70% and 5% respectively. Therefore, additional ABA-

independent mechanisms can convey increased drought tolerance in plants homozygous for *adr1*.

Interestingly, northern analysis also suggested that SA might function as a negative regulator of some abiotic responsive signalling pathways in *adr1* mutant line. For example, in the *adr1/adr1 nahG* double mutant, *RD29A* and *DREB1A* but not *RD22* transcripts were detected, suggesting SA may suppress *RD29A* and *DREB1A* gene expression in *adr1* plants. As *DREB1A* but not *RD29A* transcripts accumulated in *adr1 npr1* plants, NPR1 function may be required for the SA-dependent suppression of *DREB1A* but not *RD29A*. SA may therefore function as both a positive and negative regulator of distinct abiotic response pathways. The SA-dependent suppression of some abiotic stress signalling pathways may explain why adult *adr1* plants exhibited increased sensitivity to NaCl. This hypothesis is consistent with the observation that *nahG* plants showed increased tolerance to salt and other osmotic stresses (Borsani et al. 2001).

The establishment of both drought tolerance and salt hyper-sensitivity following engagement of the *adr1*-signal pathway is a good example of the complex signalling network and overlap among signalling pathways activated in response to abiotic stresses (Knight and Knight, 2001; Xiong et al., 2002). Uncovering the identities and connection diversities of the common nodes of this signalling network may be important for rational crop design.

7) Gene expression profiling in *TA::ADR1* transgenic lines

7.1 Microarray analysis of DEX treated *TA::ADR1* plants

Microarray analysis represents a reliable technology that enables the simultaneous transcription analysis of several thousand genes. This technique has been successfully employed to study plant response to both biotic and abiotic challenges (Wan et al., 2002; Seki et al., 2004) and it also representes a precious tool to characterize disease resistant mutants and transgenic lines (Brodersen et al., 2002; Lorenzo et al., 2003). To search for the expression of additional drought responsive and defence related genes in *adr1* plants, a microarray experiment was undertaken. This experiment was carried out in collaboration with Dr Seki, coordinator of the Microarray Unit at the Riken Center (Japan). This research group developed an *Arabidopsis* microarray tool containing over 7,000 independent full-length cDNA groups that enabled the identification of many genes responsive to different environmental challenges (Seki et al., 2002a; Seki et al., 2002b; Oono et al., 2003; Seki et al., 2004).

7.1.1 Gene expression profiling of TA::ADR1 *versus* TA:: *plants*

The severely reduced stature of *adr1* plants, similar to that of most disease resistance mutants, may alter the expression of several genes induced indirectly due to the chronic *ADR1* over-expression. Consistent with this assumption, several well-characterized genes involved in development were identified in microarray studies of disease resistant mutants (Schenk et al., 2000; Brodersen et al., 2002). In order to aid the identification of genes that are directly regulated by the expression of *ADR1*, plants containing the conditional *ADR1* allele were employed to perform the transcriptome analysis. The genomic-scale mRNA levels of *TA::* and *TA::ADR1* lines, 50 hours after DEX treatment, were compared to determine cDNA groups that exhibited significant difference in gene expression.

Microarray results obtained at the Riken Center were subsequently analysed at the University of Edinburgh. Expression data were standardized employing a λ DNA

control fragment and approximately 5% (n = 336) of the cDNA groups, corresponding to 317 genes, exhibited significantly increased transcript accumulation in DEX-treated *TA::ADR1* plants with an expression ratio at least 2-fold greater than in *TA::* plants. The extensive list of up-regulated genes is reported in Appendix 1, whereas the genes exhibiting an expression ratio at least 3-fold greater in *TA::ADR1* versus *TA::* plants are presented in Table 7.1.

TA::ADR1/TA:: expression ratio	AGI code	RAFL code	Gene name	Coded protein
13.9	At2g14610	RAFL06-68-J19	*PR1*	pathogenesis-related protein
8.9	At4g12470	RAFL11-11-M23		lipid transfer protein (LTP)
7.0	At2g29350	RAFL06-14-B04	*SAG13*	alcohol dehydrogenase
5.5	At2g43570	RAFL04-12-G16		glycosyl hydrolase (chitinase)
4.8	At4g02380	RAFL06-13-N20	*SAG21*	late embryo abundant protein
4.6	At1g75040	RAFL04-13-G17	*PR5*	thaumatin-like protein
4.3	At1g75750	RAFL11-10-B10	*GASA1*	GA-responsive GAST1 protein
4.2	At2g43510	RAFL05-18-K08		trypsin inhibitor-related
4.2	At1g02920	RAFL07-14-E05	*GST11*	glutathione S-transferase
4.0	At3g30775	RAFL05-17-E01	*EDR5*	proline dehydrogenase
3.9	At1g73260	RAFL11-04-I22		trypsin inhibitor propeptide
3.9	At4g30270	RAFL04-09-O24	*MERI5B*	endo xyloglucan transferase
3.8	At1g02930	RAFL05-16-O07	*EDR11/GST1*	glutathione S-transferase
3.7	At3g22660	RAFL08-09-M05		lipid transfer protein (LTP)
3.6	At1g28580	RAFL05-21-O19		putative lipase
3.3	At5g39670	RAFL09-10-B14		calcium-binding EF-hand protein
3.1	At1g54100	RAFL08-15-L09	*ALDH*	aldehyde dehydrogenase
3.1	At4g39670	RAFL05-01-D05		expressed protein
3.0	At3g49120	RAFL09-07-G15		peroxidase
3.0	At5g10760	RAFL06-12-P24		nucleoid DNA-binding protein

Table 7.1 Genes showing increased mRNA levels in DEX treated *TA::ADR1* plants. The genes exhibiting an expression ratio at least 3-fold greater in *TA::ADR1* plants versus *TA::* control lines are listed. Induction fold, codes, name and predicted encoded protein of each gene are reported.

Furthermore, approximately 0.7% (n =45) of the cDNA groups, corresponding to 39 genes, exhibited significant expression reduction in DEXtreated *TA::ADR1* plants with an expression ratio at least 2-fold lower than *TA::* plants. The list of the down-regulated genes is also reported in Appendix 1.

7.1.2 Validation of microarray results

As previously reported in chapter 4, both *PR1* and *GST1* transcripts were significantly accumulated in DEXtreated *TA::ADR1* plants, whereas they were undetectable in DEX treated *TA::* control lines. Consistent with these results, the gene exhibiting the most elevated transcript increase, 14-fold, in DEXtreated *TA::ADR1* versus *TA::* plants was *PR1*. In addition, the transcription level of *GST1* was estimated to be 4-fold greater in DEXtreated *TA::ADR1* lines compared to that in the DEXtreated *TA::* control plants. The expression of selected genes were confirmed by northen blot analysis (data not shown).

7.2 Analysis of genes induced by transient *ADR1* expression

Among the 317 *Arabidopsis* genes exhibiting significantly increased expression in DEX treated *TA::ADR1* plants, only 77 genes are characterized and described in the literature; whereas the large majority of these genes have not been characterised. The results of several microarrays, exploited to study plant response to biotic and abiotic stresses, were searched for the transcription levels of the genes significantly accumulated in DEX treated *TA::ADR1* plants. Specifically, the microarray data employed originated from analyses of plant response to drought stress (Seki et al., 2002a), salt stress (Seki et al., 2002a), osmotic stress (Oono et al., 2003), re-hydration stress (Oono et al., 2003), ABA treatment (Seki et al., 2002b) and cold stress (Chen et al., 2002; Fowler and Thomashow, 2002; Seki et al., 2002a). Several of these studies were conducted at the Riken Center; this fact facilitated the comparison of the transcription levels because the set of full-length cDNAs analysed and technical procedures were identical. In addition, we also employed microarray data from studies investigating plant response to biotic stresses such as treatment with SA (Schenk et al., 2000; Chen et al., 2002), BTH (Schenk et al.,

2000), JA (Schenk et al., 2000), ET (Schenk et al., 2000), plant response to pathogen infection (Schenk et al., 2000), analysis of disease resistance mutants such as *acd11* (Brodersen et al., 2002), *cim5*, *cim6* and *cim11* (Schenk et al., 2000) and the enhanced resistant line *35S::ERF1* (Lorenzo et al., 2003). Results of this investigation are reported below.

7.2.1 Description of genes showing increased mRNA levels in DEX treated TA::ADR1 *versus* TA:: *plants*

Among the genes exhibiting an expression ratio of at least 3-fold greater in DEX treated *TA::ADR1* versus *TA::* plants, twelve transcripts (60%), represented defence-related genes (Ishes et al., 1992; Murray et al., 2002) (Figure 7.1). Interestingly, ten transcripts (50%) corresponded to genes responsive to drought (Kiyosue et al., 1993; Kiyosue et al., 1996; Kirch et L., 2001), whereas thirteen (65%) genes were responsive to abiotic stresses other than drought (such as high salinity, ABA treatment, temperature shock or osmotic stress) (Pei et L., 2000; Wagner et al., 2002). It is noteworthy that the expression of 7 genes (35%) were induced by both biotic and abiotic stresses. These results were proportional to those obtained from the analysis of the 317 genes exhibiting an expression ratio at least 2-fold greater in DEX treated *TA::ADR1* versus *TA::* plants (Figure 7.2). The number of genes involved in defence, drought and abiotic stress responses was 68 (21.5%), 49 (15.5%) and 57 (18%) respectively, whereas the expression of 30 genes (9.5%) was regulated by both biotic and abiotic stresses. To illustrate further genes up-regulated by the transient expression of *ADR1*, a short description of each group presented in Figure 7.2 is reported below.

The first and largest group represents 203 genes that have not been previously identified as responsive to biotic challenges or abiotic stresses; 83 genes encoded proteins of unknown function. Genes encoding for putative proteins that might mediate *ADR1*-induced disease resistance and/or drought tolerance included: *At1g10570* (up-regulated 2.3 fold greater in DEX treated *TA::ADR1* versus *TA::* plants) which encodes for a protein phosphateses 2, two protein kinase genes, *APK2A* and *At2g39660* (both up-regulated 2 fold) (Ito et al., 1997) and *ZIK4* kinase (up-regulated 2 fold), encoding a

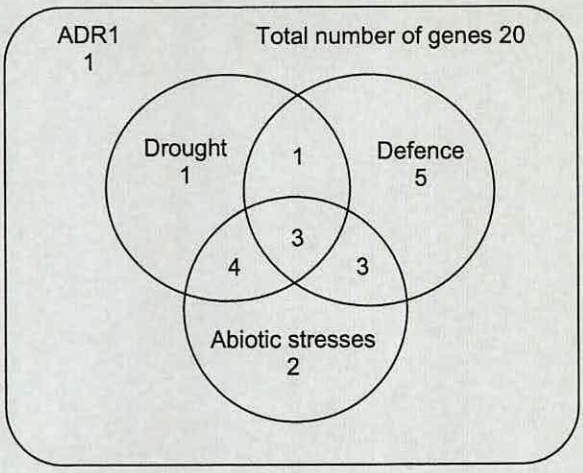


Figure 7.1 Classification of the 20 genes showing an expression ratio at least 3-fold greater in DEX treated *TA::ADR1* versus *TA::* plants

Genes responsive to drought, defence and abiotic stress were divided into 7 groups: drought-responsive gene (1 gene); defence-related genes (5 genes); abiotic stresses-inducible genes (2 genes); drought- and defence-inducible gene (1 gene); drought- and abiotic stresses-responsive genes (5 genes); defence-related and abiotic stresses-inducible genes (3 genes); genes responsive to drought, biotic and abiotic stresses (3 genes).

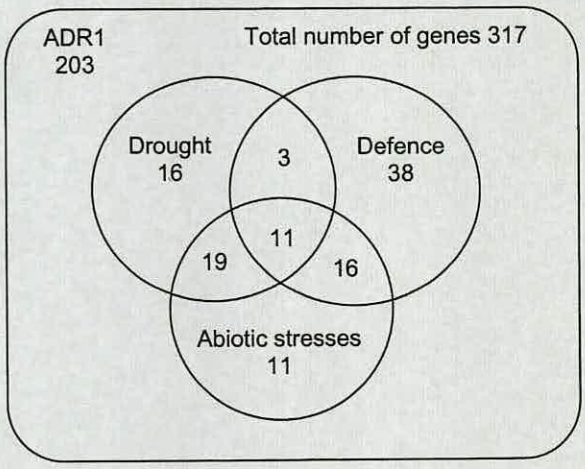


Figure 7.2 Classification of the 317 genes showing an expression ratio at least 2-fold greater in DEX treated *TA::ADR1* versus *TA::* plants

Genes responsive to drought, defence and abiotic stress were divided into 7 groups: drought-responsive genes (16 genes); defence-related genes (38 genes); abiotic stresses-inducible genes (11 genes); drought- and defence-inducible genes (3 genes); drought- and abiotic stresses-responsive genes (19 genes); defence-related and abiotic stresses-inducible genes (19 genes); genes responsive to drought, biotic and abiotic stresses (11 genes).

mitogen activated protein kinase (Nakamichi et al., 2002). These proteins may play a role in the putative kinase signalling cascades activated by *ADR1* expression. In addition, several proteins might play a role in calcium-dependent signal transduction activated by *ADR1*; *GBF2* (up-regulated 2 fold), which encodes a bZIP transcription factor regulated by Ca^{2+} (Jakoby et al., 2002), *At1g73800*, *At4g3100* and *At5g57580* (up-regulated 2.1, 2.1 and 2 fold respectively) three genes encoding for calmodulin-binding proteins, *CAX1* (up-regulated 2 fold) a calcium antiporter gene (Hirschi et al., 1996), *CAM9* (2.1 fold up-regulated) encoding for a calmodulin protein (Zielinski, 2002) and At5g39670 (up-regulated 3.3 fold), that encodes for a calcium-binding EF-hand protein. Furthermore, the *At1g28580* gene, which encodes for a putative lipase, was up-regulated 3.6 fold greater in DEXtreated *TA::ADR1* versus *TA::* plants. This protein exhibits the same structure as EDS1 and PAD4, suggesting that At1g28580 might also have a similar function in disease resistance signalling (Falk et al., 1999; Jirage et al., 1999).

The second group consists of 38 genes that were previously identified to play a role in disease resistance. Transcripts that exhibited greater accumulation in DEXtreated *TA::ADR1* versus *TA::* plants included several well-characterised defence-related genes: *PR1* (14 fold up-regulated), *PR2* (2.7 fold up-regulated), *PR5* (4.6 fold up-regulated), *PDF1.1* (2.3 fold up-regulated) and *PDF1.2* (2.7 fold up-regulated) (Knes et al., 1992; Epple et al., 1997b). In addition, the following genes were also accumulated in DEX treated *TA::ADR1* plants: *ICS1* (2.2 fold up-regulated) that encodes for an isochorismate synthase which is required for defence-mediated SA synthesis (Wildermuth et al., 2001), *RAP2.2* (2.2 fold up-regulated) which encodes for an AP2 domain-containing protein highly induced by SA, JA, ET and during the establishment of systemic resistance (Okamuro et al., 1997), and *PAD4* (2 fold up-regulated) which encodes for a putative lipase required in basal defence (Jirage et al., 1999).

Sixteen genes composed the group of transcripts that are specifically responsive to drought stress. Among these *At5g04760* (2 fold up-regulated) encodes a myb transcription factor (Okamuro et al., 1997), *At3g49110* (2.7 fold up-regulated) encodes a peroxidase and three genes, *At3g49110*, *At4g00740* and *At5g06050* (2.1, 2 and 2.1 fold

up-regulated respectively), share high sequence homology with *ERD* (early responsive to drought) 3 (Taji et al., 1999).

The fourth class grouped eleven genes that are responsive to abiotic stresses other than drought. This class included: *At4g12470* (9 fold up-regulated) which encodes for a protease inhibitor/seed storage/lipid transfer protein, *ERD5* (4 fold up-regulated) which is highly responsive to osmotic stress (Kiyosue et al., 1996) and *BXL1* (2.3 fold up-regulated) encoding for a xylosidase specifically expressed in tissues undergoing secondary wall thickening (Goujon et al., 2003). It is noteworthy that cell wall thickening is also an important mechanism of plant defence against invading pathogens (Schmelzer, 2002; Goujon et al., 2003; Vorwerk et al., 2004).

Three transcripts, that exhibited greater accumulation in DEXtreated *TA::ADR1* versus *TA::* plants, encode for proteins that are involved in both drought stress and defence responses. These transcripts are *At2g43570* (5.5 fold up-regulated) encoding a chitinase, *POX* (3.3 fold up regulated) which encodes a proline oxidase (Verbruggen et al., 1996) and *TRX5* (2.5 fold up-regulated) which encodes a cytosolic thioredoxin that reduces disulfide bridges of target proteins (Rivera-Madrid et al., 1995). This class of genes could be particularly important since the over-expression of *ADR1* confers both disease resistance and drought tolerance.

Sixteen transcripts were responsive to both biotic challenges and abiotic stresses other than drought. The most significant genes of this group are listed below: *GST11* (4.2 fold up-regulated) encodes a glutathione transferase (Wagner et al., 2002), *COR8.6* (2 fold up-regulated) encodes a glycine-rich protein highly induced by cold and several biotic stresses (Quigley et al., 1991), *PIP3* (2 fold up-regulated) encodes a salt-stress-inducible aquaporin (Pih et al., 1999) and *PRXR2* (2 fold up-regulated) encodes a peroxidase.

In addition, nineteen transcripts, that exhibited greater accumulation in DEXtreated *TA::ADR1* versus *TA::* plants, have been previously identified as responsive to multiple abiotic stresses, including drought stress. Among these transcripts we identified *ABF4/AREB2* (2 fold up-regulated), which encodes an ABA-responsive ABRE-binding bZIP transcription factor (Uno et al., 2000), *NCED3* (2 fold up-regulated) which encodes

a key enzyme in the ABA biosynthesis, the 9-cis-epoxycarotenoid dioxygenase 3 (Iuchi et al., 2001; Tan et al., 2003), *ALDH* (3.1 fold up-regulated) which encodes for an aldehyde dehydrogenase isoform (Kirch et al., 2001) and *RD20* (2.3 fold up-regulated) a calcium-binding EF-hand gene induced by abscisic acid during dehydration (Takahashi et al., 2000).

Finally, eleven were characterised as responsive to multiple abiotic stresses, drought and biotic challenges. Among them, *SAG21* (3.3 fold up-regulated) encodes a late embryogenesis abundant protein (Weaver et al., 1998), *At5g20230* (2.6 fold up-regulated) encodes a blue copper binding protein and *GPX* (2.1 fold up-regulated) encodes a glutathione peroxidase (Rodriguez Milla et al., 2003).

7.3 Investigation of genes down-regulated by transient expression of ADR1

Microarray analysis identified 38 genes exhibiting an expression ratio at least 2-fold lower in DEXtreated *TA::ADR1* versus *TA::* plants (see Appendix 1). The transient expression of *ADR1* repressed the transcription of several genes involved in the photosynthetic process. In particular, seven genes encoding for chlorophyll a/b-binding proteins, *CAB1*, *CAB2*, *LHCB2.1*, *LHCB2.2*, *LHCB3*, *At2g34420* and *At4g10340* (2.8, 2, 2.2, 2.7, 2, 2, and 2.1 fold down-regulated respectively) (Brusslan and Tobin, 1992; Anderson and Kay, 1995; Yu et al., 2001; Jackowski et al., 2001) and *At1g31330* and *At3g16140* (3.2, and 2 fold down-regulated respectively), that encode for two photosystem subunit precursors, were down-regulated in DEXtreated *TA::ADR1* plants. In this context, the establishment of systemic resistance in wild-type plants and the constitutive activation of disease resistance in mutant lines caused a decrease in the activity of the photosynthetic machinery (Maleck et al., 2000; Brodersen et al., 2002; Cartieaux et al., 2003). In particular, the fact that transcription of *LHC* genes were repressed by both SA and BTH treatments is consistent with the observation that *adr1* mutants accumulate elevated levels of SA (Maleck et al., 2000; Grant et al., 2003).

In addition, the expression of three genes involved in cold acclimatisation were also repressed by the transient expression of *ADR1*. The transcription rates of the

RD29A/COR78, COR15A and *COR413* genes were down-regulated 2.6, 2.3 and 2 fold respectively in DEX treated *TA::ADR1* plants compared to *TA::* lines (Yamaguchi-Shinozaki and Shinozaki, 1993; Steponkus et al., 1998; Breton et al., 2003).

It is noteworthy that three heat shock protein genes were also repressed by the transient expression of *ADR1*. The *HSP90.2*, *HSP90.3* and *HSP90.4* genes (all 2.3 fold down-regulated) encode for heat shock proteins that were only modestly induced by heat but were significantly up-regulated by NaCl, drought and ABA (Takahashi et al., 2003). In addition, HSP90.2 physically associates with RPM1 and modulates disease resistance signalling mediated by RPM1 (Hubert et al, 2003). The *HSP90.2*, *HSP90.3* and *HSP90.4* genes encode for proteins that are 97% identical and are located in physical proximity on *Arabidopsis* chromosome 5. These proteins share more than 75% sequence identity with HSP90.1, a chaperone acting in the NBS-LRR-mediated signalling pathway by physically interacting with both RAR1 and SGT1B (Takahashi et al., 2003).

Consistent with the antagonistic regulation that orchestrates the establishment of the SA and JA defence pathways, the expression of a group of JA-responsive genes was repressed by the transient expression of *ADR1* (Felton et al, 1999: Li et al., 2004). *VSP1*, *VSP2* and *At2g4000* were down-regulated 2.1, 4.1 and 2.1 fold respectively. Both *VSP1* and *VSP2* genes encode for proteins with anti-fungal and anti-nematode activities (Ellis and Turner, 2001). Furthermore, *TGG1* and *TGG2* (2 and 2.4 fold down-regulated) are also JA-responsive genes and they encode for the only two *Arabidopsis* myrosinases that catalyse the hydrolysis of glucosinolates into compounds toxic to numerous microbes and herbivores (Husebye et al., 2002).

7.4 Discussion

The gene expression profiling reported here showed that transient expression of *ADR1* can orchestrate the transcription rate of many genes responsive to both biotic and abiotic stresses. This observation is consistent with the fact that *adr1* mutant plants are simultaneously disease resistant, drought tolerant and salt hyper-sensitive. To elucidate the *adr1*-mediated mechanism conferring drought tolerance, we focused on genes encoding proteins involved in signalling in response to dehydration stress. *NCED3*, which was highly induced by drought stress, encodes a dioxygenase involved in ABA biosynthesis (Iuchi et al., 2001). The transgenic lines overexpressing *NCED3* accumulated high levels of ABA and showed increased drought tolerance (Iuchi et al., 2001). The high expression of the *NCED3* transcripts observed in DEXtreated *TA::ADR1* plants might confer drought tolerance by increasing ABA biosynthesis. In addition, the ABRE binding factor ABF4, which regulates ABA-mediated stress responses, was also up-regulated by transient expression of *ADR1*. Transgenic lines over-expressing *ABRE* exhibited a significant increase in drought tolerance (Kang et al., 2002). Moreover, *35S::ABRE* transgenic plants exhibited greater salt hyper-sensitivity compared to that of wild-type plants; however, they retained a response to osmotic stress caused by mannitol treatment, similar to that observed for wild-type plants (Kang et al., 2002). An equivalent hyper-sensitivity to ionic stress and wild-type response to osmotic stress was observed in *adr1* mutant plants (Chini et al., 2004). These data therefore suggested that the ABF4-mediated stress signalling might contribute to *adr1*-mediated drought tolerance and salt hyper-sensitivity. However, measurements of ABF4 activity and ABA levels in *adr1* mutants and DEXtreated *TA::ADR1* plants will be required to verify this hypothesis.

Five genes encoding for calcium-binding proteins were significantly up-regulated in DEXtreated *TA::ADR1* versus *TA::* plants, a number of these proteins might represent elements integral to the signalling pathway orchestrated by *ADR1*. Alternatively, the high transcription rate of genes encoding calcium-binding proteins in DEXtreated *TA::ADR1* plants might be the consequence of high levels of ROS induced by *ADR1*

expression. This hypothesis would be consistent with the fact that ROS can activate Ca^{2+}-mediated signalling (Dolmetsch et al., 2001).

The *VSP1* and *VSP2* genes are highly expressed in wild-type *Arabidopsis* plants following JA treatment and also in *cev1* mutants, which show increased JA accumulation and enhanced resistance to several *Erysiphe* isolates (Ellis and Turner, 2001). The transcription of both *VSP1* and *VSP2* genes was significantly repressed in DEXtreated *TA::ADR1* versus *TA::* plants, consistent with the antagonistic regulation that orchestrates the establishment of SA- and JA-dependent defence pathways (Felton et al, 1999: Li et al., 2004). The *VSP2* gene encodes for an effector of salt tolerance whose transcription is up-regulated during salt stress adaptation in wild-type plants and repressed in the salt hyper-sensitive mutants *sos* (salt overly sensitive) (Ishitani et al., 2000). The negative regulation of *VSP2* expression in DEXtreated *TA::ADR1* plants might therefore contribute to the salt-hypersensitive phenotype of *adr1* mutants. Altogether, these results suggest that expression of *VSP* genes might be regulated by both biotic and abiotic defence pathways.

The over-expression of *ADR1* triggers accumulation of SA in *adr1* plants. It was therefore surprisingly that the expression of three JA-responsive genes, *PDF1.2*, *TRX5* and *GASA1*, were up-regulated in DEXtreated *TA::ADR1* versus *TA::* plants. Moreover, the *adr1*-mediated accumulation of *PDF1.2* transcripts was COI1-dependent because it was abolished in *adr1 coi1* double mutants (Grant et al., 2003). The expression of JA-responsive genes can be regulated by the cyclopentenone 12-oxo-phytodienoate (OPDA) in a COI1-dependent manner (Stintzi et al., 2001). The expression of *PDF1.2*, *TRX5* and *GASA1* genes may therefore be engaged via the selective accumulation of OPDA or a related molecule in *adr1* mutants. In this context, the expression of these genes would not be antagonised by the activation of the SA-dependent defence pathway in *adr1* plants. To address this possibility, further biochemical analysis of *adr1* mutants will be required. Alternatively, the transcription of a number of defence genes such as *PDF1.2*, *TRX5* and *GASA1* might be orchestrated by multiple, overlapping signals, thus the expression of these genes may be incompatible with the over-simplified antagonistic model of SA- and JA-dependent defence pathways.

Finally, it is noteworthy that three heat shock protein genes, *HSP90.2*, *HSP90.3* and *HSP90.4* showed significantly reduced expression in DEX-treated *TA::ADR1* lines versus *TA::* plants. The heat shock protein HSP90.1 shares high sequence homology to HSP.2, HSP.3 and HSP.4 and these four HSPs form a defined family within the *Arabidopsis* genome (Takahashi et al., 2003). The HSP90.1 protein was identified as an element required in R-mediated defence signalling (Takahashi et al., 2003). This protein physically interacts with both RAR1 and SGT1B and acts as a chaperone to establish R-mediated resistance (Takahashi et al., 2003). Unfortunately, the analysis of the cDNA representing the *HSP90.1* gene failed in our microarray analysis, hence it remains unknown whether DEX-treated *TA::ADR1* plants repressed the transcription of *HSP90.1*. Recent results suggest the requirement of *HSP90.2* in RPM1-mediated defence signalling (Hubert et al, 2003). Moreover, the *hsp90.1* knock-out line retained only partial avrRpt2 recognition (Takahashi et al., 2003). The *HSP90.3* gene has been previously identified as *ERD8*, whose transcription was up-regulated by dehydration stress in an ABA-dependent manner (Kiyosue et al., 1994). Finally, the expression of both *HSP90.2* and *HSP90.3* genes is repressed in wild-type plants following SA treatment and the establishment of disease resistance (Schenk et al., 2000). There is therefore mounting independent evidence that the four HSP90 isoforms might act as a dual regulatory system in both biotic and abiotic defence signalling (Kiyosue et al., 1994; Schenk et al., 2000; Hubert et al, 2003; Takahashi et al., 2003).

The observation that transient *ADR1* expression repressed the transcription of three *HSP90* genes is not inconsistent with the ADR1 role in the establishment of disease resitance. The degradation of RPM1 has been previously shown to occur during the onset of HR mediated by a number of *avr/R* product interactions (Boyes et al., 1998). This study suggests the presence of a negative feedback loop controlling the extent of HR and the overall resistance response. The repression of *HSP90* genes during the establishment of *adr1*-mediated resistance is consistent with the hypothesis of a negative regulatory mechanism controlling cell death and amplitude of defence responses following pathogen recognition. This hypothesis is also consistant with the observation that HSP90 genes are down-regulated in response to SA treatment (Schenk et al., 2000).

8) Structural and phylogenetic analysis ofADR1 protein

8.1 Identification of the *ADR1* family in the *Arabidopsis thaliana* genome

The most prevalent class of plant resistance genes encodes for proteins that contain a nucleotide-binding site (NBS) domain located in the central portion of the protein and a C-terminal leucine-rich repeat domain (LRR) (Dangl and Jones, 2001). *NBS-LRR* genes are abundant in all plant species and they represent approximately 0.5% of the whole *Arabidopsis* genome (Cannon et al., 2002; Pan et al., 2000b; Meyers et al., 2003). NBS-LRR proteins were divided into two groups based on the presence of an N-terminal region that showed high homology to the Toll/Interleukin-1 Receptor (TIR) domain (Pan et al., 2000a). Most of the non-TIR NBS-LRR proteins exhibited a coiled-coil (CC) domain in the N-terminal region (Meyers et al., 1999). Recently, all *NBS-LRR* genes from the complete *Arabidopsis* genome were identified and predicted to encode for 83 TIR-NBS-LRR (TNL) and 51 CC-NBS-LRR (CNL) proteins (Meyers et al., 2003).

The complete set of *NBS-LRR* genes were employed for phylogenetic analysis and proteins encoded by these 149 *NBS-LRR* genes were also searched for conserved motifs (Meyers et al., 2003). The overall results revealed a greater degree of diversity among the CNL proteins compared to that uncovered among TNL proteins (Meyers et al., 2003). This observation reflected a more ancient origin of the CNL group compared to that of the TNL group. Furthermore, four distinct, CNL sub-groups (CNL clade A, B, C and D) were identified in *Arabidopsis* and their presences were confirmed in several plant species (clade N1, N2, N3 and N4) (Cannon et al., 2002; Zhu et al., 2002; Meyers et al., 2003; Ashfield et al., 2004). The *ADR1* gene belonged to the smallest and least characterised of these clades, designated as CNL-A in *Arabidopsis* and non-TIR N4 in the entire plant kingdom (Cannon et al., 2002; Baumgarten et al., 2003; Meyers et al., 2003).

8.2 Identification of *ADR1* homologs in *Arabidopsis thaliana*

In June 2000 Dr Grant identified two putative *ADR1 Arabidopsis* homologues genes, *At4g33300*, *ADR1-LIKE1* (*ADR1-L1*), and *At5g04720*, *ADR1-LIKE2* (*ADR1-L2*), that showed 69 and 67 % sequence identity to *ADR1* respectively (Grant et al., 2003). "The *Arabidopsis* Initiative Resource" (TAIR) released the first complete sequence of the *Arabidopsis* genome in December 2000 (TAIR, 200); in addition, many annotations are being regularly updated. Therefore, the TAIR database was searched for *ADR1* homologues employing the "Blast" programme.

Blast results confirmed the high homology of *ADR1-L1* and *ADR1-L2* to *ADR1* and identified only one additional *Arabidopsis* gene that exhibited significant identity to *ADR1*. The *At5g47280* gene showed 67% sequence identity to *ADR1* and was therefore named *ADR1-LIKE3* (*ADR1-L3*). *ADR1-L3* was predicted to encode a 623 amino acid protein which, in contrast to the 787 amino acid ADR1 protein, lacked the 180 amino acidic portion of the N-terminal region of the protein corresponding to the CC domain. Comparative analyses of ADR1-L1, ADR1-L2 and ADR1-L3 proteins revealed 68, 65 and 66% sequence identity (81, 80 and 71% homology) to ADR1 respectively. Consistent with these observations, ADR1 and the three ADR1-LIKE proteins will be subsequentially referred to as ADR1 proteins or ADR1 family.

The comprehensive study on *Arabidopsis NBS-LRR* genes identified two additional genes, *At5g66900* and *At5g66910*, as members of the CNL-A group, which correspond to the *ADR1* clade (Meyers et al., 2003). These two paralog genes were located in physical proximity and shared 91% sequence identity. However, AT5g66900 and AT5g66910 proteins respectively showed 31 and 30% sequence identity to ADR1 compared to 68, 65 and 66% identity of ADR1-L1, ADR1-L2 and ADR1-L3 respectively. Significant sequence homology among ADR1 proteins, AT5g66900 and AT5g66910 was identified only in the P-loop and kinase 2 domains, which were motifs commonly conserved among all NBS-LRR proteins (Meyers et al., 1999; Pan et al., 2000a). On the other hand, *ADR1* genes, *AT5g66900* and *AT5g66910* presented the same number and location of the four introns (Meyers et al., 2003). In addition, the six *CNL-A*

genes encoded for the only 6 NBS-LRR proteins, out of 162 investigated, that contained a glutamine (Q) instead of a methionine (M) as third residue in the MHDV domain (Meyers et al., 2003). This methionine was conserved among all remaining 156 *Arabidopsis* NBS-LRR proteins and was integral to the core sequence liable for the MHDV domain name itself (Meyers et al., 2003).

Overall, these results suggested that AT5g66900 and AT5g66910 were more similar to the ADR1 proteins than any other NBS-LRR protein. For further investigations, these two tandem genes should, however, be considered as a different sub-group, as initially proposed by Mondragon-Palomino and co-workers (Mondragon-Palomino et al., 2002). This hypothesis was consistent with the phylogenetic results in which the CNL-A group was markedly divided into two sub-groups; the first represented by the four *ADR1* genes and the second sub-group consisted of the paralog *AT5g66900* and *AT5g66910* genes (Mondragon-Palomino et al., 2002; Baumgarten et al., 2003; Meyers et al., 2003).

8.3 Analysis of the CC, NBS and LRR domains of the ADR1 proteins

Comparative analysis of the NBS domain of numerous plant NBS-LRR proteins identified eight conserved motifs (P-loop, RNBS-A, -B, -C, -D, kinase 2, GLPL and MHDV) and computational analysis additionally predicted 30 putative motifs specific for individual NBS-LRR clades (Meyers et al., 1999; Pan et al., 2000b; Meyers et al., 2003). The presence of six conserved motifs (P-loop, kinase 2, RNBS-A, GLPL, RNBS-D and MHDV) in the NBS domain of ADR1 was previously reported (Grant et al., 2003). The amino acid sequences of the four ADR1 proteins were further evaluated employing the programme MEME (Multiple Expectation Maximisation for Motif Elicitation) (Bailey and Elkan, 1995) and results are described below in order in which domains are located in the proteins, starting at the N terminus (see supplementary CD data for complete MEME output).

8.3.1 Analysis of the CC, NBS domains

MEME analysis did not identify any significant conserved motif within the N-terminal CC domain (from ADR1 M1 to L187). In contrast, MEME analysis of the NBS domain (from ADR1 F188 to N472) identified seven motifs, six of which corresponded to the well established, conserved motifs previously reported for ADR1 (P-loop, kinase 2, RNBS-A, GLPL, RNBS-D and MHDV) (Grant et al., 2003). Comparative analysis of these six domains of the ADR1 proteins and those of the complete set of the *Arabidopsis* NBS-LRR proteins, revealed specific differences in the sequence of the RBNS-D and MHDV motifs unique for the ADR1 family (Figure 8.1 and Table 8.1). Furthermore, the presence of one additional ADR1 specific motif was uncovered. This putative motif (from ADR1 L221 to L234) was designated as TVS, according to the core sequence, and is reported in Table 8.1 (marked with "blue box" in Figure 8.1).

To assess the reliability of this putative motif, the conservation rate of the TVS motif among the ADR1 proteins was compared to that of the previously established, conserved NBS motifs. Among the four ADR1 proteins, the P-loop, kinase2, RNBS-A, GLPL, RNBS-D and MHDV motifs exhibited 74, 89, 50, 67, 52 and 71% sequence identity respectively (79, 89, 77, 93, 86 and 100% homology). The putative TVS motif showed 71% sequence identity (93% homology) among the four ADR1 proteins, greater than any other of the conserved NBS motifs apart from the P-loop and kinase 2 motifs. These results therefore confirmed that the novel TVS motif was significantly conserved within the ADR1 family. Furthermore, the TVS motif spanned a similar, but more defined, amino acid sequence, designated as putative NBS-22 motif (Meyers et al., 2003).

8.3.2 Analysis of the NBS-LRR linker domain

The end of the NBS domain is conventionally assumed to match with the end of the MHDV motif (Meyers et al., 2003; Ashfield et al., 2004). The LRR domain, however, started approximately 40 to 65 amino acids C-terminal to the MHDV motif and this inter-domain region was designated as the NL linker (NBS-LRR linker) (Meyers et al., 2003). The motif for this linker was conserved within the different CNL classes but

varied among classes (Meyers et al., 2003). Within the ADR1 family, the NL linker was 78 amino acid long (from ADR1 R473 to S551) and MEME analysis identified two putative motifs, designated LMP and PKAE according to the core sequence of each motif (Figure 8.1 and Table 8.1). The MEME statistical analysis confirmed that the ADR1 NL linker was the most conserved among the NL linkers of the four clades representing all *Arabidopsis* CNL proteins (Meyers et al., 2003). In addition, the LMP and PKAE putative motifs showed 80 and 71% sequence identity respectively (90 and 95% homology) among the ADR1 family proteins. Hence, these two motifs exhibited greater conservation than previously established NBS motifs, apart from the P-loop and kinase 2 motifs.

8.3.3 Analysis of the LRR domain

The LRR domain in R proteins may mediate direct or indirect interactions with pathogen molecules (Jia et al., 2000; Dangl and Jones, 2001). Structurally, individual LRRs formed repeats of β-strand-loop and α-helix-loop units, with non-leucine residues exposed and responsible for protein recognition (Thomas et al., 1996; Michelmore and Meyers, 1998; Kobe and Kajava, 2001). Comparative analysis of *NBS-LRR* genes from tomato, lettuce, rice and *Arabidopsis* revealed that the non-leucine residues of the LRR are hypervariable and subject to positive selection (Parniske et al., 1997; Meyers et al., 1998; Wang et al., 1998; Noel et al., 1999; Ellis et al., 2000).

Genome wide analysis of *Arabidopsis NBS-LRR* genes established that the NBS domain was significantly more conserved compared to the variable LRR domain (Meyers et al., 1999; Pan et al., 2000a; Meyers et al., 2003). The comparative sequence analysis of the LRR domains (from ADR1 R552 to L734) of the four ADR1 proteins showed a striking sequence identity of 57%. In contrast the NBS domain (from ADR1 F188 to N472), which was theoretically the most conserved domain within all NBS-LRR proteins, exhibited 50% sequence identity within the four ADR1 proteins. The hypervariable LRR domain therefore showed greater conservation in the ADR1 family compared to that of

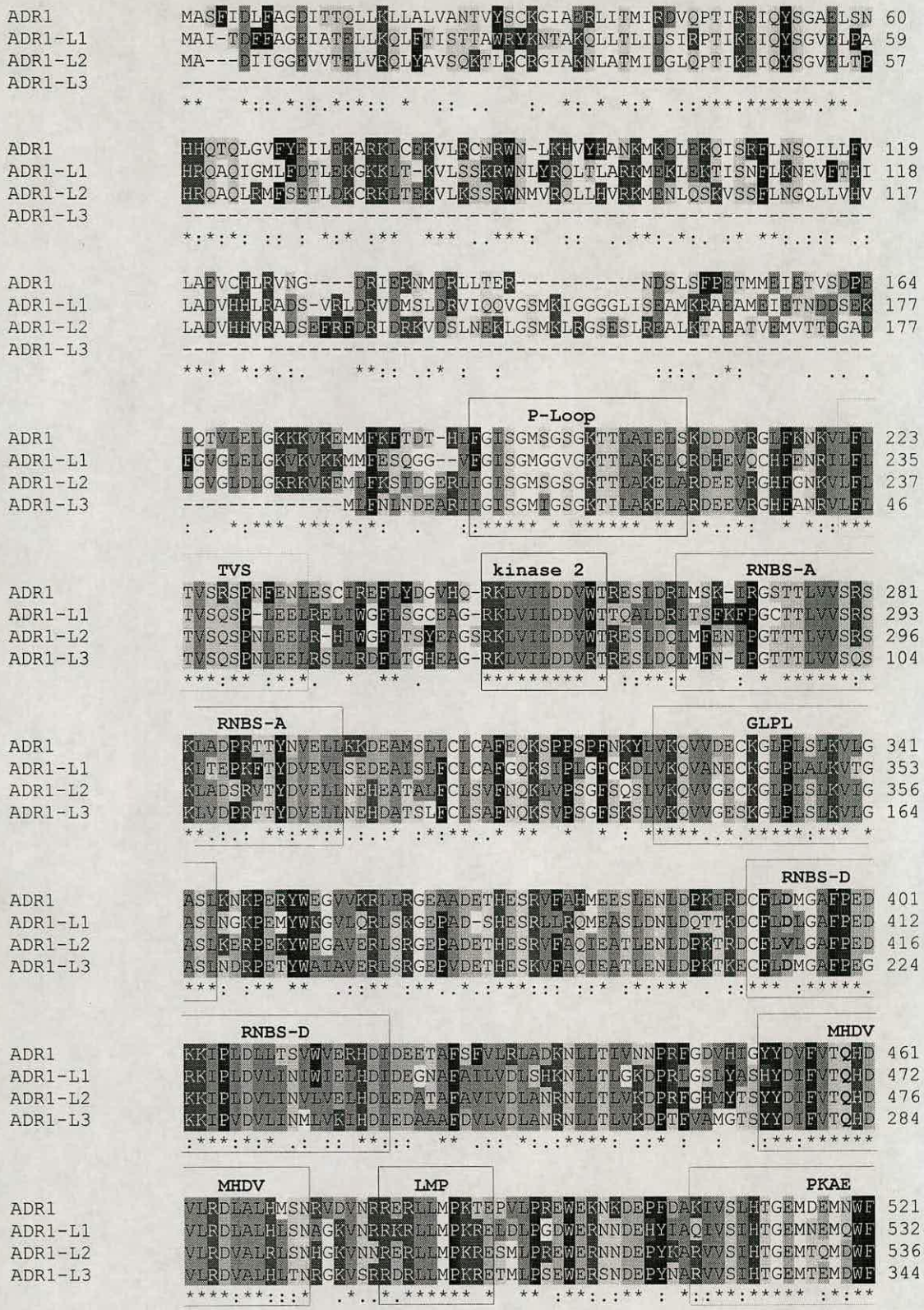
the NBS domain and, to my knowledge, this is the first report describing this unique property.

Comprehensive analysis of the complete set of *Arabidopsis* NBS-LRR proteins identified At1g69550, At5g44510 and At2g14080 as the subclass that exhibited the greatest sequence identity (45%) and homology (70%) in the LRR domain, apart from proteins encoded by tandem paralog genes which normally showed greater identity (Meyers et al., 2003). Results presented here clearly identified the ADR1 family as the subclass of NBS-LRR genes that exhibited the greatest sequence identity (57%) and homology (83%) in the LRR domain. These conclusions were consistent with the fact that the ADR1 family was the only CNL clade that did not exhibit significant positive selection in the LRR domain (Mondragon-Palomino et al., 2003).

Motif Name	Protein Group	Consensus Sequence
TVS	ADR1 family	LFLTVSQSPNLEEL
	CC-NBS-LRR	not present
RNBS-D	ADR1 family	CFLDLGAFPEDKKIPLDVLINVWVEIHDI
		||| |||| | | | |
	CC-NBS-LRR	CFLYCALFPEDYEIxKEKLIDYWIAEGFI
MHDV	ADR1 family	YYDVFVTQHDVLRDLALHLSN
		| ||| | ||
	CC-NBS-LRR	-----VKMHDVVREMALWIA-
LMP	ADR1 family	VNRRERLLMPKRE
	CC-NBS-LRR	not present
PKAE	ADR1 family	IVSIHTGEMDEMDWFDMDLPKAEVLILNFSSDNYVLPPFI
	CC-NBS-LRR	not present

Table 8.1 Consensus sequence of the conserved motifs in the NBS domain.

The consensus sequence of selected motifs, specific for the ADR1 family, are presented in the upper line, while the typical sequences for CC-NBS-LRR proteins (Meyer et al. 2003) are displayed in the lower line. Underlined residues indicate the core sequences that established the motif name.


ADR1
ADR1-L1
ADR1-L2
ADR1-L3
P-Loop
TVS
kinase 2
RNBS-A
RNBS-A
GLPL
RNBS-D
RNBS-D
MHDV
MHDV
LMP
PKAE

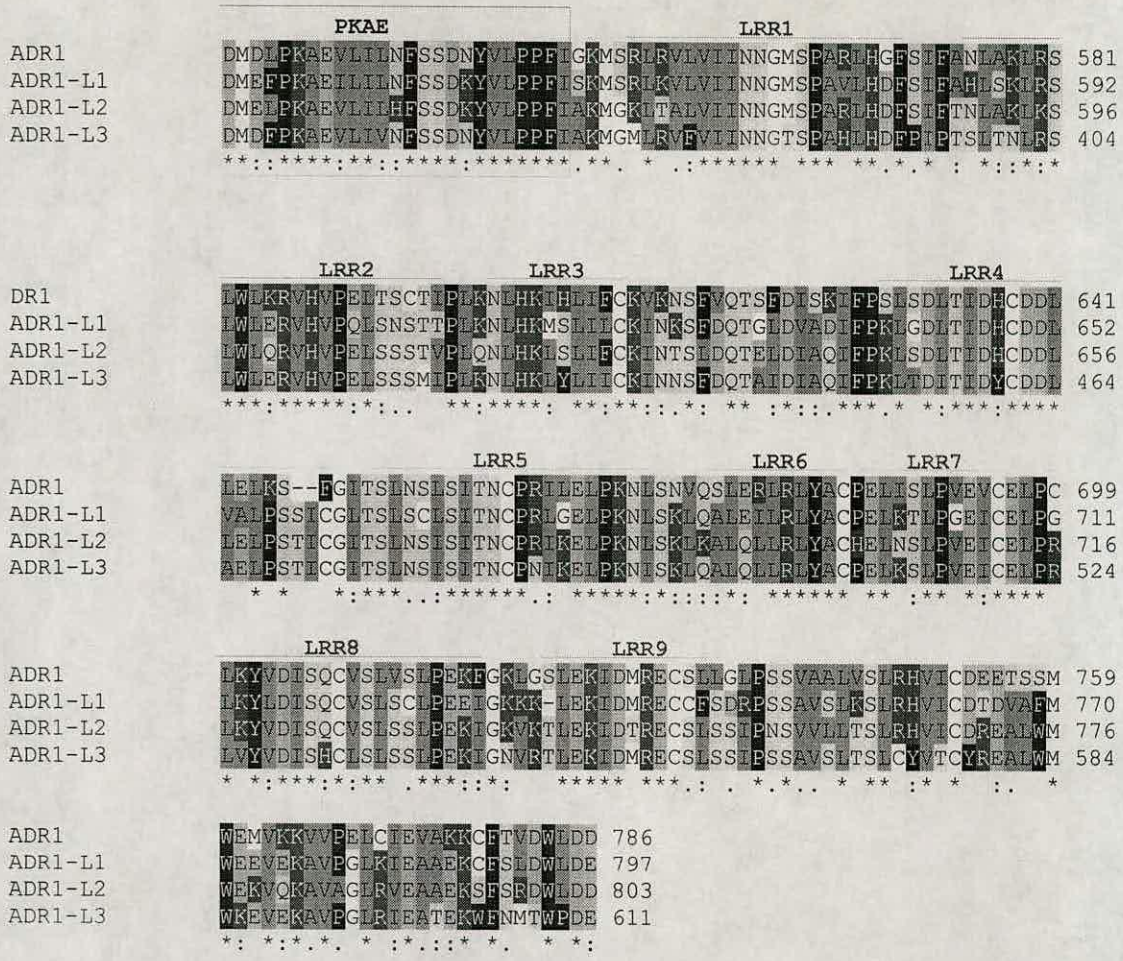


Figure 8.1 Sequence alignment of ADR1, ADR1-L1, ADR1-L2 and ADR1-L3 proteins.

Alignment of the complete sequence of ADR1 proteins performed employing the ClustalW programme (Thomson et al., 1994). The "*" symbol marked residues identical among all sequences; the ":" symbol identified residues modified by conserved substitutions; the "." symbol residues labelled residues changed by semi-conserved substitutions. The six previously established, conserved motifs of the NBS domain (P-loop, kinase2, RNBS-A, GLPL, RNBS-D and MHDV) were marked with "black boxes". "Blue boxes" identified novel conserved motifs (TVS, LMP and PKAE). Individual LRR were marked by red lines. Hydrophobic residues (A, I, L and V) are represented in green, basic residues (K, R, H) in red, ring-containing residues (P, F, Y, W) in blue, acid residues (D, E) in grey, alcoholic and amino-containing residues (S, T, Q, N) in yellow, small and S-containing residues (M, G, C) in white.

8.4 ADR1 homologs among species of the plant kingdom

In order to identify putative *ADR1* homologs in different plant species database search analyses were carried out. Both the full ADR1 protein sequence and the specific sequences of the conserved RNBS-D, MHDV and PKEA motifs were employed for Blast searches. Two public databases were exploited as sequence resources: The Institute for Genomic Research (TIGR) and the Munich Information Center for Protein Sequences (MIPS) databases. At the time the search was conducted, in September 2003, 37 different plant genome databases were available. In order to minimize errors due to insufficient sequence comparison, only clones exhibiting a minimal 200 amino acid length were selected for further analyses.

8.4.1 Identification of ADR1 homologs in different plant species

First, database searches employing the sequences of RPM1, RPS2, RPS5, RPP8 (representatives of the CC-NBS-LRR subfamily), RPS4, RPP1 and RPP13 (members of the TIR-NBS-LRR subfamily) were run. All these NBS-LRR proteins exhibited the greatest sequence identity of approximately 20-25% (30-40% homology) to clones of different plant species. Therefore an empirical two-fold threshold of 50% identity and/or 70% similarity was selected to identify *ADR1* homologs. Fungal, bacterial, yeast and mammalian database were employed as negative controls and they failed to identify any clone exhibiting high sequence identity to ADR1. One individual sequence from 12 different plant species showed great sequence identity to ADR1, whereas results from the remaining 25 plant databases failed to identify sequences sharing significant identity to ADR1. Details of the identified clones are reported in Table 8.2.

To assess whether these clones could represent *ADR1* homologs, the four ADR1 proteins were aligned with the amino acid sequences of the 12 putative ADR1 homologs (Appendix II). The difference in length of these 12 plant clones precluded a comprehensive analysis of the full-length protein sequence (Figure 8.2). Therefore, only specific motifs present in the majority of these sequences were analysed. The RNBS-D, MHDV and PKAE domains met these criteria, and multiple alignments are shown in

Plant Species	Plant Family	Common Name	Accession Number	Length	Database Source
Oryza sativa	*Poaceae*	Rice	TC149181	656	MIPS TIGR
Medicago truncatula	*Fabaceae*	Barrel medic	TC87505	647	TIGR
Triticum aestivum	*Poaceae*	Wheat	TC107913	574	TIGR
Sorghum bicolor	*Poaceae*	Sorghum	TC57671	530	TIGR
Glycine max	*Fabaceae*	Soybean	TC195419	433	TIGR
Zea mays	*Poaceae*	Maize	BG836496	359	MIPS
Solanum tuberosum	*Solanaceae*	Potato	TC62931	332	TIGR
Lycopersicon esculentum	*Solanaceae*	Tomato	AW039749	293	MIPS
Pinus taeda	*Pinaceae*	Loblolly Pine	AW043275	291	MIPS
Gossypium hirsutum	*Malvoideae*	Cotton	CD486153	258	TIGR
Vitis vinifera	*Vitaceae*	Wine grape	CA32EN0005	244	TIGR
Lotus japonicus	*Fabaceae*	Lotus	AV417020	234	MIPS

Table 8.2 List of plant sequences homologous to *Arabidopsis* ADR1.

The accession number and taxonomic details are reported for each plant sequence showing high homology to ADR1. The length of the amino acid sequence and the database from which these sequences were identified are also reported.

Figure 8.3, 8.4 and 8.5 respectively. The degree of conservation of these motifs among all plant sequences, with the exception of the *Pinus taeda* AW043275, was surprisingly high. The RNBS-D motif was greatly conserved among several phylogenetically distant species (Figure 8.3). The MHDV motif was also significantly conserved; it is remarkable that all sequences exhibited the specific glutamine (Q) residue in the third position of this motif, which was a unique characteristic of the ADR1 family (Figure 8.4). Equally, the novel PKAE motif, which corresponded to the ADR1 clade-specific NL linker domain, exhibited a significantly high conservation level (Figure 8.5).

Altogether these results strongly suggested that the plant sequences described here corresponded to *ADR1* homologs, or homologs of an *ADR1-LIKE* gene. Consistent with this hypothesis, a comprehensive phylogenetical analysis of the plant *NBS-LRR* genes confirmed the existence of the non-TIR N4 clade, which correspond to the *ADR1* clade, among many plant species (Cannon et al., 2002). Within the non-TIR N4 clade the only sequence not isolated from *Arabidopsis* was the *M. truncatula* EST AW685945, which corresponded to a shorter clone of the *M. truncatula* TC87505 sequence described here (Cannon et al., 2002).

8.5 Phylogenetic analysis of ADR1 homologs

A phylogenetic analysis was employed to assess whether the sequences identified in different plant species were homologous to *ADR1* or to a distinct *ADR1-LIKE* gene. Initially, the sequence identities between each *Arabidopsis* ADR1 protein and each plant sequence clone were calculated. *At5g66900* and *At5g66910* genes, which were classified as CNL-A members, were included as control. Only one sequence, the *Pinus taeda* AW043275, exhibited similar identity rate to the four ADR1 proteins, At5g66900 and At5g66910, arguing that the pine sequence might represent a homolog of *At5g66900* or *At5g66910* (Table 8.3). The remaining 11 plant clones showed substantially greater identity to ADR1 proteins than At5g66900 or At5g66910 (Table 8.3). Each individual plant clone exhibited a sequence identity to ADR1 of 47 to 59%, and they also showed

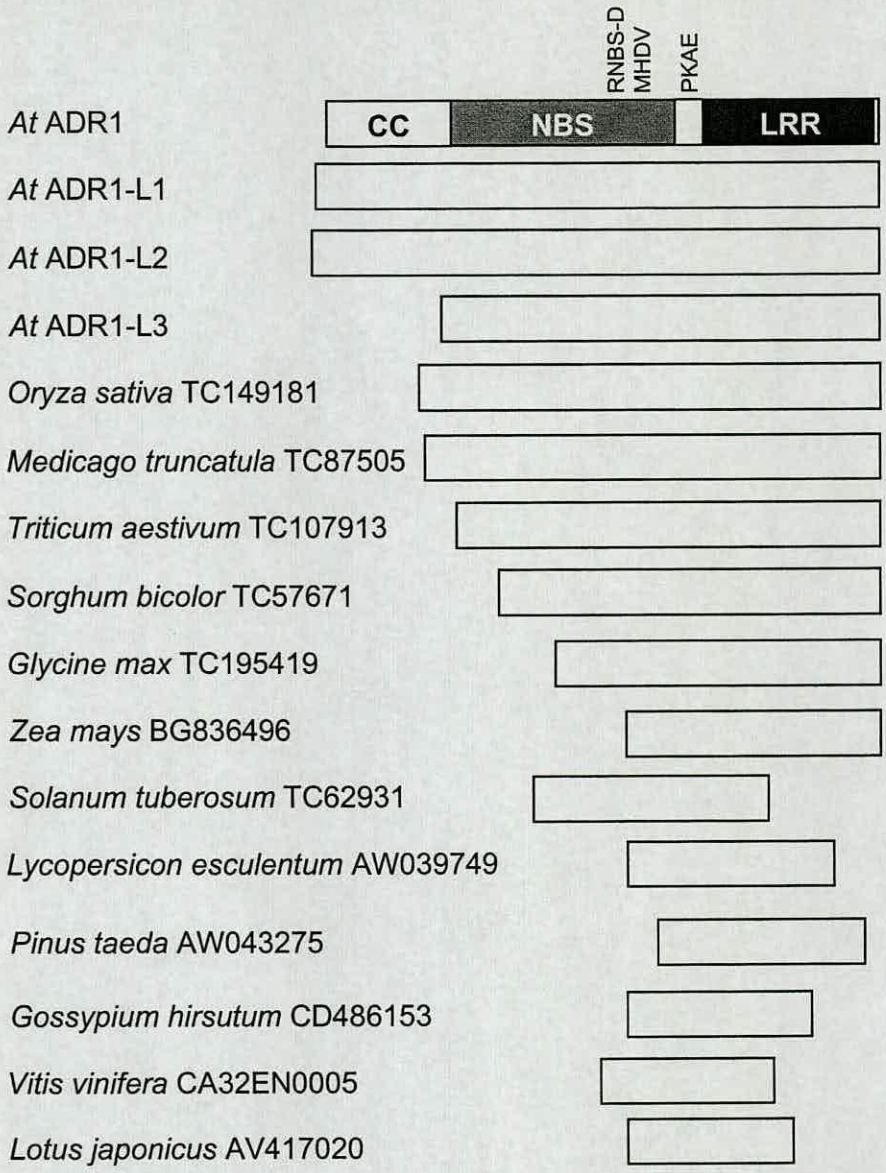


Figure 8.2 Representation of plant sequences homologous to *Arabidopsis* ADR1.

The amino acidic sequences are schematically represented by rectangles, whose dimension is proportional to the length of the sequence (1cm = 100 residues). The position of the CC, NBS and LRR domains and the location of the RNBS-D, MHDV and PKAE motifs within the ADR1 protein are also indicated.

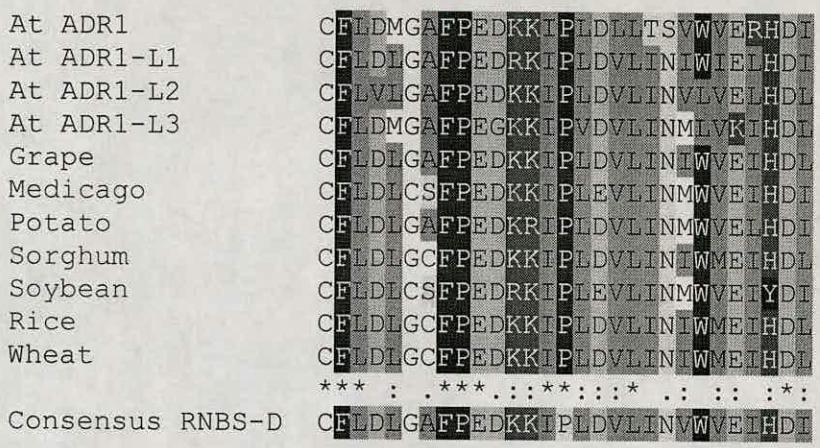


Figure 8.3 Multiple alignment of the RNBS-D motif

The amino acid sequences corresponding to the RNBS-D motif of all available plant clones were aligned employing the ClustalW programme. The consensus sequence was also extrapolated. The symbology is identical to that described in Figure 8.1. The accession number (fully reported in Table 8.2) was omitted.

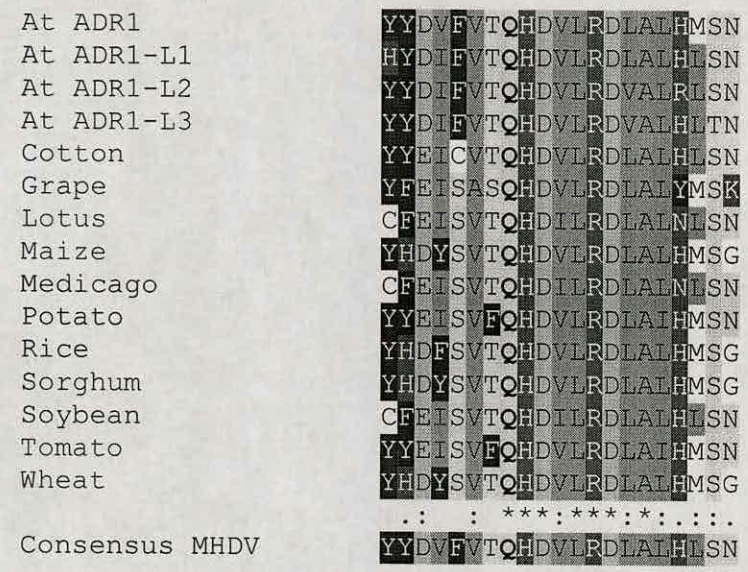


Figure 8.4 Multiple alignment of the MHDV motif

The MHDV motif sequences of all plant clones were aligned employing the ClustalW programme and the consensus sequence was also extrapolated. The symbology is identical to that described in Figure 8.1.

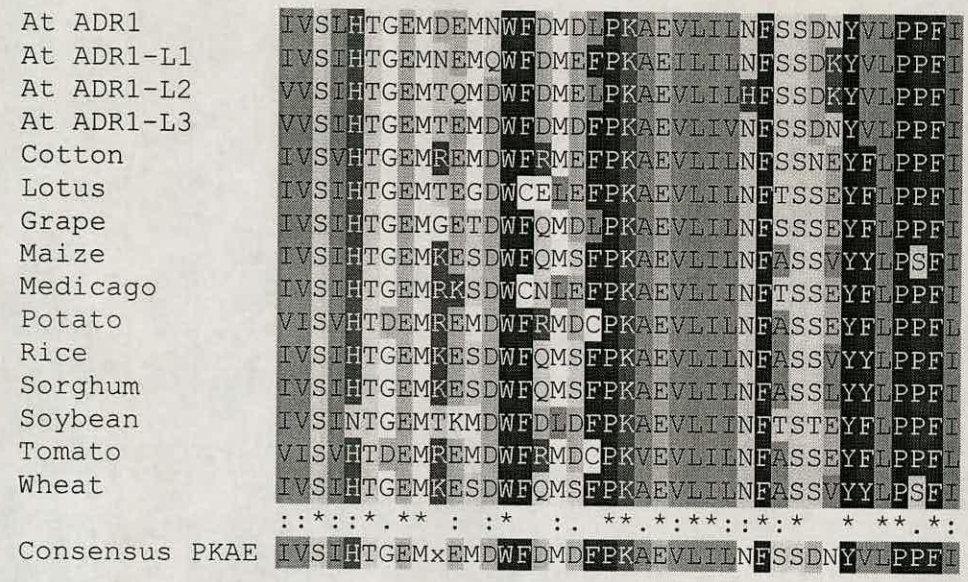


Figure 8.5 Multiple alignment of the PKAE motif.

The amino acid sequence alignment of the PKAE motif of all plant clones was performed employing the ClustalW programme. The consensus sequence was also extrapolated. The symbology is identical to that described in Figure 8.1.

50 to 63%, 49 to 59% and 46 to 56% sequence identity to ADR1-L1, ADR1-L2 and ADR1-L3 respectively (Table 8.3). In addition ADR1-L1 showed greater sequence identity to each individual plant clone compared to that of ADR1, ADR1-L2 or ADR1-L3. These results therefore suggested that the isolated plant sequences might be orthologs of *ADR1-L1*.

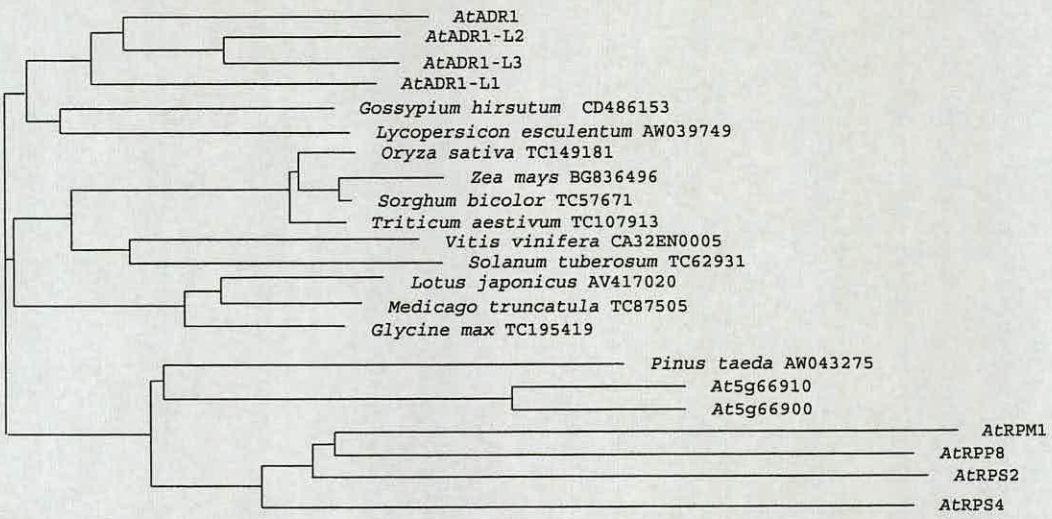
In addition, the most conserved portion among *Arabidopsis* ADR1 (the NBS domain, the NL linker and the LRR domain) and the corresponding region of the 12 plant sequences were employed in a phylogenetic analysis. At5g66900 and At5g66910 proteins, which showed the greatest sequence identity to ADR1 among all *Arabidopsis* NBS-LRR proteins, were included as controls. RMP1, RPP8 and RPS2 were included as representative protein of the CNL-B, CNL-C and CNL-D clade respectively, whereas RPS4 was employed as an out-grouped control from the TNL family. The phylogenetic results showed that all identified plant sequences, apart from the *Pinus taeda*

control sequences grouped in distant branches of the phylogenetic tree (Figure 8.6). In addition, *ADR1-L1* was the *Arabidopsis* gene that exhibited greater phylogenetic proximity to all sequences from different plant species. These data therefore confirmed that all the plant sequences reported here corresponded to *ADR1* homologs, possibly to *ADR1-L1* orthologs.

	ADR1	ADR1-L1	ADR1-L2	ADR1-L3	At5g669000	At5g669100
Glycine max	49.4%	54.5%	50.8%	49.4%	36.7%	35.6%
Gossypium hirsutum	58.9%	62.4%	59.3%	55.8%	37.2%	36.8%
Lotus japonicus	49.1%	55.6%	55.6%	53.9%	32.9%	34.2%
Lycopersicon esculentum	52.9%	56.0%	53.6%	51.5%	37.5%	37.2%
Medicago truncatula	46.8%	50.1%	49.2%	46.2%	33.2%	31.8%
Oryza sativa	50.0%	53.3%	49.7%	47.6%	34.55	33.3%
Pinus taeda	41.2%	46.4%	42.3%	43.6%	38.8%	37.8%
Solanum tuberosum	53.9%	56.9%	55.4%	52.1%	36.5%	33.4%
Sorghum bicolor	53.2%	57.6%	53.4%	51.3%	36.0%	34.3%
Triticum aestivum	52.3%	54.5%	50.2%	48.8%	35.2%	34.1%
Vitis vinifera	52.9%	53.7%	54.1%	53.7%	34.0%	33.6%
Zea mays	52.1%	52.7%	50.4%	47.6%	38.7%	37.1%

Table 8.3 Sequence identity rate between *Arabidopsis* ADR1 proteins and putative plant homologs.

(a)



(b)

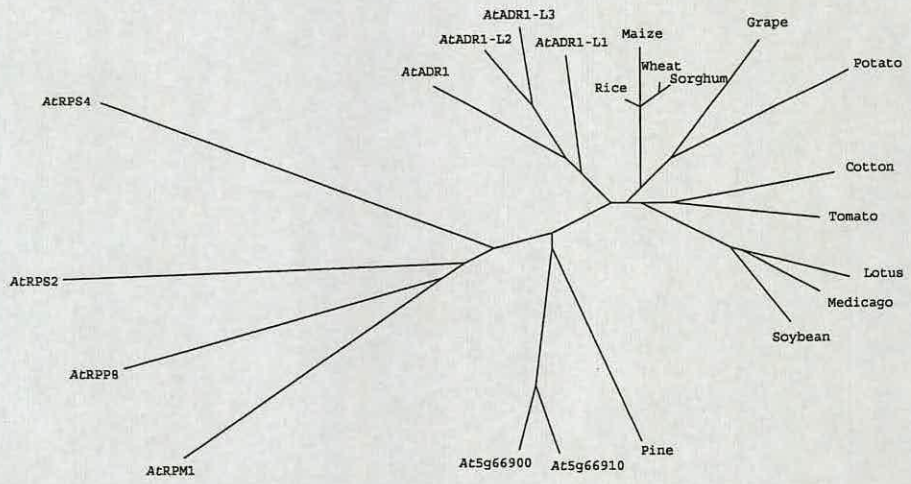


Figure 8.6 Phylogenetic tree of selected NBS-LRR plant sequences

(a) Phenogram representation of the neighbour-joining tree was constructed according to the method of Saitou and Nei (1987). The aligned amino acidic sequences of the NBS-NR-LRR portion of each protein were created by ClustalW and they were subsequentially analysed by Phylodendron ClustalW to generate the phylogenetic tree. Branch lengths are proportional to the genetic distance. The accession number (reported in Table 8.2) was omitted.

(b) Diagram representation of the neighbour-joining tree generated employing the same parameter as described in (a). Branch lengths are proportional to the genetic distance.

8.5 Discussion

The availability of the complete *Arabidopsis* genome sequence and the ever-increasing sequence data from many other plant species have opened terrific opportunities for comprehensive phylogenetic analysis (TAIR, 2000; Whitelaw et al., 2003; Kikuchi et al., 2003). The number of complete sequences, EST and partial genomic sequences of *NBS-LRR* genes identified in the last decade was particularly prosperous (Bai et al., 2002; Bendahmane et al., 2002; Cannon et al., 2002; Meyers et al., 2003; Mondragon-Palomino et al., 2003). Several conserved motifs in the NBS domain of the proteins encoded by *NBS-LRR* genes have been identified and large scale functional analyses have been employed to characterise the function of these motifs (Meyers et al., 1999; Pan et al., 2000; Tornero et al., 2002; Hammond-Kosack and Parker, 2003).

To date, the most extensive investigation of an *NBS-LRR* gene was *RPM1*; 95 independent *rpm1* alleles were isolated and the number of loss-of-function mutations in the NBS domains was significantly greater than in other, less conserved domains of the protein (Grant et al., 1995; Tornero et al., 2002). However, this nearly saturating analysis of the *RPM1* gene failed to isolate any loss-of-function mutation in the MHDV motif located in the NBS domain (Tornero et al., 2002). A complementary large scale analysis on the *Rx* potato gene resulted in the isolation of 7 independent gain-of-function alleles (Bendahmane et al., 2002). Three of these mutations occurred in the NBS domain and they were all localized in the RNBS-D and MHDV motifs, suggesting that the alteration of these putative negative regulator sequences might lead to gain-of-function phenotypes (Bendahmane et al., 2002). The modification of one residue adjacent to the methionine (M) in the MHDV core motif was responsible for one gain-of-function mutation (Bendahmane et al., 2002). Hence, the MHDV motif might function as a regulatory component. The different, perhaps unique MHDV motif sequence reported here for ADR1 proteins might reflect different protein regulation. To confirm this hypothesis, individual amino acid substitution analysis and subsequent studies of ADR1 function will be required.

The presence of the non-TIR NBS clade N4 was confirmed in many plant species (Cannon et al., 2002; Zhu et al., 2002). The N4 clade, which also contained sequences from *M. truncatula*, corresponded to the *ADR1* group (Cannon et al., 2002). The failure to identify homologs of the N4 clade from several *Poaceae* families (database search took place in January 2001) suggested that the N4 clade was lost in the monocot lineage (Cannon et al., 2002). Here the identification of genes exhibiting significant homology to *ADR1*, hence members of the N4 clade, was reported in four *Poaceae* species (*Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum* and *Zea mays*). The previous conclusion that the N4 clade was absent in the monocot lineage therefore resulted from the limitation of plant sequences. Our results confirmed the presence of the N4 clade in several monocot species.

The identification of only one *ADR1* homolog in several plant species argues that *ADR1* may be an ancient gene, conserved among plant species. The unique observation that *ADR1* could orchestrate broad disease resistance and simultaneously conferred drought tolerance is consistent this hypothesis. Only the *Arabidopsis* and rice genomes have been extensively sequenced to date, whereas several plant databases contain a limited amount of sequences (TAIR 2000; Kikuchi et al., 2003). The failure to identify additional *ADR1* homologs might therefore result from a limitation of sequences available to date. The stringent threshold employed to search for *ADR1* homologs might also have prevented the identification of additional *ADR1* sequences.

The four *Arabidopsis ADR1* genes exhibited great sequence homology and they formed a distinct phylogenetic clade (Mondragon-Palomino et al., 2002; Grant et al., 2003). These observations suggested that the presence of four *ADR1* genes resulted from sequence duplication. The recent duplications of *Arabidopsis NBS-LRR* genes normally occurred in physical chromosomal proximity. In contrast, gene duplications in distant chromosomal positions were largely the consequence of segmental chromosome duplication and rearrangement, rather than the independent duplication of individual genes (Baumgarten et al., 2003; Meyers et al., 2003). The four *ADR1* genes were located

in distant positions on three separate chromosomes; therefore *ADR1* gene duplication was not the consequence of recent, local chromosome duplication or rearrangement.

Comprehensive *Arabidopsis* analysis also revealed that a minority (18 genes) of duplicated *NBS-LRR* homologs failed to show physical proximity, as described here for *ADR1* genes (Baumgarten et al., 2003; Meyers et al., 2003). However, many of these genes were located in segmentally duplicated regions of the genome (Baumgarten et al., 2003; Meyers et al., 2003). The failure of the *ADR1* genes to show physical proximity was therefore most likely due to segmental chromosome duplication and not a consequence of duplication of individual genes, which was an exceptionally rare cause of *NBS-LRR* gene duplication in *Arabidopsis* (Baumgarten et al., 2003; Meyers et al., 2003).

In conclusion, results reported here suggested that *ADR1* is an ancient gene conserved among several plant species and that the presence of four homologous *ADR1* genes in *Arabidopsis* was most likely the consequence of segmental duplication.

9) Discussion

9.1 Gene-for-gene hypothesis: putative role of ADR1 and NBS-LRRs

In this concluding chapter, I aim to integrate our current understanding of *ADR1* function into disease resistance and abiotic stress signal transduction. A comprehensive model describing the potential role of ADR1 is also presented. Finally, recent reports hinting at possible crosstalk between biotic and abiotic stress signalling pathways are also examined. In contrast, the *ads1* mutant data are not taken into account in this section, having been comprehensively discussed in Chapter 3.

9.1.1 Overexpression of ADR1 *conveys broad-spectrum disease resistance*

ADR1 belongs to the *NBS-LRR* gene family that consists of 149 *Arabidopsis* genes (Meyers et al., 2003) and represents one of the largest and yet elusive classes of plant genes. The conventional hypothesis regards *NBS-LRRs* as resistance genes encoding for proteins involved in the specific recognition of avirulence gene products secreted during pathogen attack (Flor, 1971; Jones and Dangl, 2001).

Data presented here show that *ADR1* overexpression confers disease resistance against several pathogens. In addition to *adr1*, a number of transgenic plants expressing *NBS-LRR* genes under the control of the CaMV*35S* promoter have been described (Mindrinos et al., 1994; Ellis et al., 1999; Stokes et al., 2000). However, a broad-spectrum resistance similar to that exhibited by *adr1* plants has only been reported for transgenic plants ectopically expressing *At4g16890* (Stokes et al., 2002). The overexpression of this *Arabidopsis NBS-LRR* gene, which maps to an *R* gene cluster on chromosome 4, conveyed broad-spectrum disease resistance in the heritable but metastable *bal* epigenetic variant (Stokes et al., 2002). In addition, the CaMV*35S*-driven overexpression of *Pto*, an *R* gene encoding a serine/threonine kinase, has also been reported to confer broad-spectrum disease resistance (Tang et al., 1999).

9.1.2 Role of NBS-LRRs in plants

NBS-LRRs may also hold additional and more complex functions to that of recognition of specific avirulence factors. For example, a point mutation in the *RPS5* gene partially compromised gene-for-gene resistance to several bacterial and fungal isolates, whereas the conventional hypothesis would predict only the loss of resistance to bacteria carrying the avirulence gene *AvrPphB*, which is recognised by *RPS5* (Warren et al., 1998). It is therefore possible that RPS5 retains an additional function that enables the establishment of disease resistance in response to the recognition of several bacterial and fungal isolates. In this context, the "guard hypothesis" proposes a role for NBS-LRR proteins as general component of defence responses (van der Biezen and Jones, 1998; Dangl and Jones, 2001). In this model, NBS-LRRs perceive (or "guard") a complex of proteins (or "gardee" molecules) that can interact with avirulence factors. Plant recognition of avirulence molecules modifies the gardee complex and triggers defence responses that activate the NBS-LRR guard protein (Dangl and Jones, 2001). This hypothesis is also consistent with the requirement for the NBS-LRR protein Prf (guard) to trigger defence responses following the interaction between AvrPto and Pto (gardee molecules) (Martin et al., 1993; Salmeron et al., 1996; van der Biezen and Jones, 1998).

The hypothesis of a more complex and diverse role for NBS-LRR proteins is firmly consistent with the data presented within this work. *ADR1* is rapidly induced prior to SA accumulation following pathogen attack and also by wounding and SA treatment. In contrast, ET and JA do not appear to trigger *ADR1* expression. Moreover, the transient expression of *ADR1* results in enhanced disease resistance, perhaps not unsurprisingly in itself, but it also confers increased drought tolerance and salt sensitivity. These observations have not been reported for any other *NBS-LRR* genes investigated to date. It is therefore possible that these features may be unique for the *ADR1* gene.

These observations are reinforced by fact that ADR1 and the three ADR1-LIKE proteins exhibit distinct domains observed only among NBS-LRR proteins belonging to the small ADR1 class. In addition, data presented within this work suggest that *ADR1* is an ancient gene conserved among several phylogenetically distant plant species. It is

therefore tempting to speculate that the well-conserved and unique domains of ADR1 and ADR1-LIKE proteins may retain putative functions required for the establishment of broad-spectrum disease resistance and increased drought tolerance. This hypothesis is consistent with the systemic induction of *ADR1* after pathogen recognition and the fact that SA, which is a general defence phytohormone, can mediate *ADR1* expression (Grant et al., 2003). Alternatively, these unique ADR1 domains may be required for the recognition of evolutionary conserved ligands, such as PAMPs (pathogen-associated molecular patterns), preserved amongst several different pathogens (Nurnberger and Brunner, 2002). In this scenario, ADR1 may retain a role similar to that of animal Toll-like receptors in pathogen recognition (Baker et al., 1997; Janssens and Beyaert, 2003). Furthermore, ADR1 may also possess other unknown functions in addition to the role played in disease resistance.

Several questions still remain unanswered regarding the role of ADR1. Firstly, it will be important to uncover whether the expression profile and the overexpression phenotype of *ADR1* are unique for this transcript, conserved among *ADR1-LIKE* genes or common to a large number of *NBS-LRR* genes. In addition, analysis of transgenic plants blocked in *ADR1* expression might also help to understand ADR1 function.

9.2 Models of the putative ADR1 function

9.2.1 Putative regulation of ADR1 *during the establishment of defence resistance*

From the results presented within this work, it is possible to speculate on the position of ADR1 in signal transduction pathways which lead to disease resistance and drought tolerance (Figure 9.1). The expression of *ADR1* is likely induced immediately after the recognition of avirulence gene products, since *ADR1* expression was observed within 1.5 hours following *Pst* DC3000*(avrB)* challenge. There is an absence of experimental evidence to suggest that avirulence factors are directly perceived by ADR1. However, it cannot be ruled out that an avirulence factor may be recognised directly by ADR1 or via

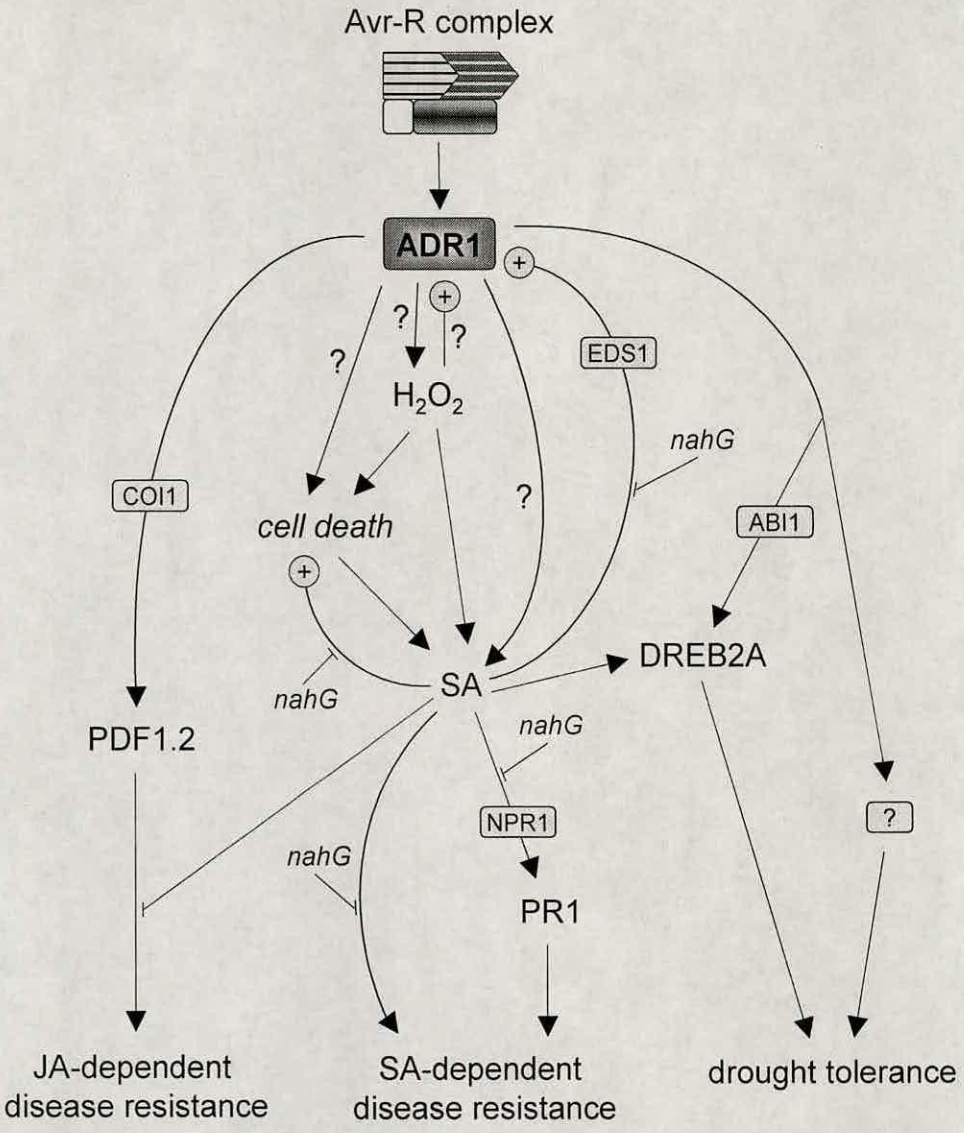


Figure 9.1 Proposed model of *adr1*-mediated signalling pathway.

This schematic model integrates ADR1 into different signal transduction pathways which engaged disease resistance downstream of the Avr-R interaction. A simplified representation of the drought tolerance transduction pathway is also included. —⊕ defines positive feedback loops, whereas —⊣ indicates negative regulations. Box defines proteins that are required for the establishment of pathways in which they are presented.

a multiprotein recognition complex. The rapid accumulation of *ADR1* might also be triggered or amplified by an "early" defence signalling molecule, such as H_2O_2, which is induced within one hour following *Pst* DC3000*(avrB)* challenge (Grant et al., 2000). In contrast, the accumulation of SA that follows the recognition of avirulence factors is a "late" defence response (Delaney et al., 1995; Shah and Klessing, 1996). Hence, the SA-induced transcription of *ADR1* most likely acts through a SA-mediated positive feedback loop (Grant et al., 2003). The initial *ADR1* expression and defence response may be subsequently amplified by this SA-mediated feedback loop (Shirasu et al., 1997). Recognition of avirulence factors also triggers programmed cell death, which in turn induces SA production and may therefore indirectly stimulate *ADR1* expression reinforcing the SA-mediated amplification loop.

This hypothesis is strengthened by the analysis of *adr1 eds1* and *adr1 nahG* double mutants. Both SA accumulation and EDS1 are required for the *adr1*-mediated activation of downstream pathways, most likely because SA and EDS1 are required in order to establish a SA-mediated feedback loop (Grant et al., 2003; Chini et al., 2004). Indeed, EDS1 has been previously found to function within a SA-mediated amplification loop (Feys et al., 2001; Loake, 2001). In contrast NDR1, which is usually required for signalling by CC-NBS-LRR proteins (Aarts et al., 1999), does not appear to be required to establish *adr1*-mediated disease resistance, since *adr1 ndr1* plants are not compromised in enhanced defence resistance. Alternatively, *adr1*-mediated disease resistance may be established through both NDR1-independent and NDR1-dependent pathways.

In summary, the current model for ADR1 signalling suggests an early induction of *ADR1* expression following plant recognition of avirulence factors; however it remains unknown whether ADR1 directly perceives avirulence factors. Plant recognition of avirulence factors also triggers production of signalling molecules, such as ROIs, and induces early defence responses, which in turn promote the accumulation of SA. Increased SA levels, not only activates downstream defence pathways, but also reinforces the expression of *ADR1* via a SA-mediated positive feedback loop. Although

experimental evidence is absent, it is assumed that a negative regulator mechanism limits the amplification of defence responses that otherwise would result in plant death.

9.2.2 Putative molecular function of ADR1 in the establishment of disease resistance

ADR1 encodes a protein with an NBS-LRR structure; it is therefore reasonable to assume that ADR1 acts at a similar level of the defence pathway to the Avr-R interaction. In this context, the obvious speculation is that signal transduction pathways, which are normally triggered following avirulence recognition, are constitutively activated in *adr1* mutants. However, the precise molecular mechanism by which ADR1 acts remains unidentified. Two hypotheses ascribing putative mechanisms by which ADR1 may lead to the constitutive activation of downstream signalling pathways are contemplated below.

Sequence analysis of ADR1 suggests that the LRR domain may function in ligand binding or protein-protein interaction (Jones and Jones, 1996; Thomas et al., 1996), whereas the CC region may play a role in dimerization or protein-protein interactions (Jones and Jones, 1996; Lupas, 1996). In addition, the CDPK-like domain may activate the downstream pathways through a phosphorylation cascade. However, the CPDK-like domain of ADR1 lacks the highly conserved serine/threonine kinase subdomain VII that includes the kinase active site (Hanks et al., 1988). It is therefore unlikely that ADR1 retains kinase activity; hence, additional proteins, such as protein kinases, may be required to activate downstream pathways.

Recently, NBS proteins have been revealed to possess ATPase activity and this novel class of kinases only share homology with the ATP-binding site of conventional protein kinases (Ryazanov et al., 1997). It is therefore possible that ADR1 and additional proteins possessing a NBS domain, which contains an ATP-binding site, might also be included in this novel ATPase class. At present, studies of the putative ATPase activity of NBS-LRRs are however absent and further analyses will therefore be required to verify this hypothesis. It is more likely that an ADR1-interacting protein may possess kinase activity, which may be required to initiate defence responses.

9.2.3 ADR1 *may signal through a putative multiprotein complex*

Nonetheless, ADR1 might act within and function via a multiprotein complex in order to activate downstream response pathways. In this context, both the CC and LRR domains of ADR1 may play a role in protein-protein interactions (Jones and Jones, 1996; Kopp and Medzhitov, 1999). Recently, RIN4 has been shown to bind the R proteins Rpm1 and Rpt2 in addition to a still unidentified protein (Ellis and Dodds, 2003). Furthermore, RIN4 also appears to interact directly with the avirulence protein AvrB, AvrRPM1 and AvrRpt2; this interaction causes RIN4 modification or degradation which in turn activates downstream defence pathways (Mackey et al., 2002; Mackey et al., 2003; Axtell et al., 2003). These two models are not mutually exclusive.

In a similar fashion ADR1 might also function in a multiprotein complex that finely regulates the activation of downstream disease resistance pathways. The overexpression of *ADR1* may result in the modification and/or constitutive activation of this putative multiprotein complex that would switch on downstream defence pathways. The SA-mediated amplification loop, which appears necessary to amplify *ADR1* expression, might be also required for the direct or indirect post-transcriptional modification, regulation and/or activation of this putative multiprotein complex. Indeed, both *adr1 nahG* and *adr1 eds1* plants, which are compromised in the SA-mediated amplification loop, accumulate moderate levels of *ADR1* transcripts but fail to activate downstream resistance pathways. This hypothesis is therefore consistent with the observation that the SA-mediated amplification loop is required to activate *adr1*-mediated disease resistance.

9.2.4 Putative regulator mechanism of adr1*-mediated drought tolerance*

Broad-spectrum disease resistance is established in *adr1* plants via a SA-dependent mechanism (Grant et al., 2003). In addition, gene expression analysis revealed that *DREB2A* transcription was induced in wild-type plants following exogenous application of the SA analogue, BTH (Chini et al., 2004). A putative role for SA in the establishment of drought tolerance in *adr1* plants was therefore examined through the analysis of a series of *adr1* double mutants and transgenic lines.

adr1 lines containing either the *nahG* transgene or the *eds1* mutation, but not *npr1* mutation, exhibited substantially attenuated *adr1*-mediated drought tolerance. The *adr1*-mediated drought tolerance signalling therefore appears to be dependent on SA and EDS1 but independent of NPR1 (Figure 9.1). NPR1 is required to induce defence gene expression and systemic resistance via a signalling pathway downstream from SA (Cao et al., 1994), whereas EDS1 is required for signalling by TIR-NBS-LRR proteins to establish disease resistance (Parkers et al. 1996). However, EDS1 also functions within a SA-dependent amplification loop (Feys et al. 2001; Loake, 2001). The SA-mediated signal amplification loop necessary to establish *adr1*-mediated disease resistance may therefore be also required for the development of drought tolerance in *adr1* plants. In order to address whether SA is a key signal in *adr1*-mediated drought tolerance or a regulator of *ADR1* gene expression further analyses, such as characterization of *TA::ADR1 nahG* or CaMV*35S::ADR1 nahG* lines, will be required.

In addition, drought tolerance in *adr1* lines is significantly reduced, but not completely abolished, by *abi1*. These results suggest that both ABI1-dependent and ABI1-independent mechanisms can induce drought tolerance in *adr1* plants. In addition, a still unidentified SA- and ABI1-independent mechanism might also contribute to the establishment of *adr1*-mediated dehydration tolerance (Figure 9.1). However, analysis of *adr1 nahG abi1* triple mutant line will be required to confirm this hypothesis.

9.3 Biotic and abiotic resistance pathway crosstalk

The main focus of this final section is the analysis of cross-talk between signalling pathways that regulate defence responses against biotic and abiotic stresses. Indeed, data presented within this thesis encourages the reassessment of the traditional perception of biotic and abiotic stress signalling pathways regarded as distinct disciplines. In addition, ever-increasing genetic and genomic evidence that hints at possible connections between the establishment of disease resistance and drought tolerance are also discussed (Chinnusamy et al., 2004; Seki et al., 2004). In agreement with these observations,

inoculation of *Arabidopsis* plants with growth-promoting rhizobacteria has been shown to enhance protection against both *Erwinia carotovora* and dehydration stress (Timmusk and Wagner, 1999).

9.3.1 Biotic and abiotic stress pathways can regulate similar subsets of genes

Several pathogen-responsive genes were induced by abiotic stress and vice-versa. For example, chestnut cysteine protease *CSC*, rice *PIB* genes, fig tree *TRI* trypsin inhibitor, *Brassica napus PGIP* genes and pepper *SAR82A* were all regulated by both pathogen attack and abiotic stresses (Pernas et al., 2000; Wang et al., 2001; Kim et al., 2003a; Li et al., 2003; Lee and Hwang, 2003). Specifically, chestnut *CSC* expression was triggered in response to fungal infection, wounding, cold, JA and salt treatments (Pernas et al., 2000). *TRI* transcription in fig tree was upregulated by wounding and SA treatment but downregulated by dehydration stress (Kim et al., 2003a). In contrast, *B. napus PGIP* genes were induced in response to flea beetle infection, mechanical wounding and low temperature treatment (Li et al., 2003). The *NBS-LRR PIB* genes were highly transcribed in response to salt stress, SA, JA, ET and ABA treatments, whereas low temperature downregulated the expression of *PIB* genes (Wang et al., 2001).

Overall, these data suggested that defence gene expression was up-regulated by environmental conditions that would favour pathogen infection. However, gene regulation reflects the nature of the pathogen infection, which is favoured in different environmental conditions. This hypothesis is consistent with the different regulation of cold-responsive genes upon attack from different pathogens (Wang et al., 2001; Li et al., 2003). Low temperature may increase the infection success of one group of pathogens, whereas it might be detrimental to the infection process of a different group of pathogens.

Alternatively, defence genes might protect plants from damage, such as oxidative stress, caused by both biotic infection and abiotic disturbances. Consistent with this hypothesis, most of the 7 *Arabidopsis* glutathione peroxidase genes exhibited increased expression in high salt and osmotic conditions and they were also induced by ABA, SA or JA

treatments (Rodriguez-Milla et al., 2003). Similarly, the fig tree peroxidase gene was up-regulated upon wounding, dehydration, JA and ABA treatments (Kim et al., 2003a). Peroxidase activity might therefore protect plants from increased oxidant concentration triggered by both biotic and abiotic stresses. Consistent with this hypothesis, the overexpression of the reductase *YK1* gene in transgenic rice lines enhanced the plant antioxidants defence system, which in turn conferred both resistance against rice blast and tolerance to salinity and UV treatments (Uchimiya et al., 2002).

9.3.2 Biotic and abiotic defence pathways can be co-ordinately modulated by the same regulator elements

It is therefore currently acknowledged that biotic and abiotic stress signalling pathways can regulate the activation of a common subset of defence genes (Chinnusamy et al., 2004; Seki et al., 2004). Furthermore, these two signalling pathways might be modulated by the same regulatory mechanisms. For example, the tobacco MEK2 kinase is activated in response to pathogen recognition and abiotic stress sensing (Kim et al., 2003b). In turn, MEK2 induced ET biosynthesis and the expression of several defence genes. It is noteworthy that different subsets of genes were activated by either biotic or abiotic stresses via the same MEK2 signalling pathway (Kim et al., 2003b). Similarly, the constitutive overexpression of the tobacco transcription factor *TSI1* leads to increased pathogen-related gene transcription, disease resistance and improved tolerance to salt stress (Park et al., 2001). These results strengthened the hypothesis that overlapping signalling cascades could be required for responses to biotic and abiotic stresses (Parker et al., 2001; Kim et al., 2003b; Chinnusamy et al., 2004).

However, it has been shown that the same kinase cascade could positively regulate disease resistance and negatively regulate abiotic stress tolerance pathways. The silencing of the *MAPK5* gene in rice plants resulted in kinase activity suppression, constitutive accumulation of defence genes transcripts, broad-spectrum disease resistance and enhanced hypersensitivity to dehydration, salt and cold stress (Xiong and Yang, 2003). In contrast, rice plants overexpressing *MAPK5* exhibited increased kinase activity and enhanced tolerance to dehydration, cold and salt stresses (Xiong and Yang,

2003). Therefore, the MAP kinase MAPK5 acts as a signalling regulator that can positively modulate drought, salt and cold tolerance and negatively regulate disease resistance. These results are also consistent with the hypothesis that the same signalling cascade can be responsible for the fine regulation, either activation or suppression, of separate biotic and abiotic defence pathways.

A potential role for ABA in plant disease resistance has not been explored in detail. However, a recent study reported that *Arabidopsis* plants pre-treated with ABA or exposed to drought conditions showed increased susceptibility to *Pst* DC3000(*avrB*) (Mohr and Cahill, 2003). In addition, *aba1* plants exhibited enhanced resistance to the virulent pathogen *P. parasitica* (Mohr and Cahill, 2003). Therefore, an increased concentration of ABA could alter the establishment of plant defence in response to pathogen attack.

9.3.3 The use of microarray technology to investigate cross-talk between biotic and abiotic stress pathways

Microarray technology has been employed to analyse the expression patterns of several thousand genes following specific biotic and abiotic stress conditions and an ever-increasing amount of data has been accumulated (Bray, 2002; Wan et al., 2002; Seki et al., 2004). Several independent studies investigated the complex gene expression reprogramming that *Arabidopsis* plants orchestrate in response to dehydration, salt, cold and ABA treatment (Chen et al., 2002; Fowler and Thomashow, 2002; Seki et al., 2002a; Seki et al., 2002b). These studies revealed that many genes encoding proteins with anti-pathogenic function were significantly upregulated in response to abiotic stresses; for example, *PR1*-like, *RPS4*-like, nematode resistance and *PGIP* genes (Chen et al., 2002; Fowler and Thomashow, 2002; Seki et al., 2002a). The transcription of several genes encoding detoxifying enzymes, such as glutathione S-transferases and peroxidases, was also triggered following abiotic stress challenge.

In a similar fashion, several transcriptomic studies analysed gene expression following pathogen attack or treatments with defence hormones such as SA, JA and ET (Maleck et

al., 2000; Schenk et al., 2000; Reymond et al., 2000; Cheong et al., 2002; Narusaka et al., 2003). Gene expression of a number of constitutive disease resistance mutants was also investigated (Maleck et al., 2000; Brodersen et al., 2002; Lorenzo et al., 2003). Response to biotic challenges resulted in the transcriptional modulation of many genes encoding proteins responsive to different abiotic stresses. For example, several *RD*, *EDR*, catalase and ascorbate-peroxidase genes were upregulated. On the other hand, a number of abiotic stress responsive genes were downregulated following pathogen challenge (Maleck et al., 2000; Schenk et al., 2000; Seki et al., 2033a).

These results were therefore consistent with the hypothesis that separate abiotic and biotic signalling pathways could co-ordinately modulate the expression of overlapping subsets of stress responsive genes. In particular, a group of genes was tightly co-regulated by biotic and abiotic stresses; these genes encode proteins required for the detoxification mechanisms that prevent cellular damage caused by oxidative stress, a common consequence of both biotic and abiotic challenges. The previously described microarray results of plants transiently expressing *ADR1* are also firmly consistent with these reports.

9.3.4 Conclusion

In conclusion, the establishment of both disease resistance and drought tolerance following engagement of the *adr1*-mediated signalling pathway suggests, at least in some cases, there may be significant overlap between biotic and abiotic stress defence pathways. However, the study of biotic and abiotic pathway cross-talk prompts numerous questions. The coordinate studies of stress responses employing recently available microarrays containing the complete set of *Arabidopsis* genes and the simultaneous quantification of several phyto-hormones and signalling molecules will provide a better understanding of the complex cross-talk established among distinct signalling pathways (Schmelz et al., 2003; Trethewey, 2004; Hilson, et al., 2003; Craigon et al, 2004). Uncovering the identity and regulation of common nodes modulating biotic and abiotic stress signalling may be crucial for rational crop design.

10) References

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