A GENETIC DISSECTION OF SIGNAL TRANSDUCTION PATHWAYS UNDERLYING THE OXIDATIVE BURST, COGNATE REDOX SIGNALLING, AND ESTABLISHMENT OF SYSTEMIC ACQUIRED RESISTANCE

JOHN J. GRANT

DOCTOR OF PHILOSOPHY

INSTITUTE OF CELL AND MOLECULAR BIOLOGY
UNIVERSITY OF EDINBURGH
SEPTEMBER 2000

CONTENTS

DECLARATION

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university.

John J. Grant

ACKNOWLEDGEMENTS

I would especially like to thank my supervisor Dr Gary Loake for his advice, support and unfailing optimism over the course of my PhD. I am also grateful to the individual members of the Loake group for their invaluable advice and also for making the lab the entertaining and cosmopolitan place that it is. In addition, many people in the department have helped me over the course of my studies, too numerous to mention here. Finally, I would like to thank my family and friends for their moral support and for keeping me relatively sane throughout this period.

PUBLICATIONS ARISING FROM THIS WORK

Grant, JJ & Loake, GJ (2000) - *Role of ROIs and Cognate Redox Signaling in Disease Resistance*
Plant Physiology Vol. 124, No. 1, pp. 21-30.

Grant, JJ & Loake, GJ - *The Oxidative Burst and Cognate Redox Signalling Reported by Luciferase Imaging: Identification of a Signal Network that Functions Independently of Ethylene, SA and JA but is Dependent on MAPKK Activity*
Plant Journal Vol. 24, No. 5, pp. 569-82.

Grant, JJ, Basu, D, Loake, GJ - *Transient expression of ADR1 engages a systemic signalling network that establishes broad spectrum resistance*
(manuscript in preparation)

Grant, JJ, Basu, D, Loake, GJ - *Resistance to both biotic and abiotic stress stimuli in plants via the ectopic expression of ADR1*
(manuscript in preparation)

RELATED PAPERS

Grant, RL & Grant, JJ (2000) - *Biotechnology Review*
Pulp & Paper International Vol. 42, No. 8, pp. 29-30.

Grant, RL & Grant, JJ (1999) - *Biotechnology Review*
Pulp & Paper International, Vol. 41, No. 8, pp. 44-45.

PATENTS

Inventors:	Grant, JJ, & Loake, GJ
	ADR1 protein and nucleic acid sequences: compositions and methods for plant pathogen and drought resistance.
Status:	UK patent pending (filed by Cruikshank & Fairweather patent attorneys)

ABBREVIATIONS

4x*35S*	tetramer of *CaMV 35S* enhancer region
a.a.	amino acid
ABA	abscisic acid
A.t.	*Arabidopsis thaliana*
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pair
CaMV	Cauliflower Mosaic Virus
cDNA	complimentary DNA
Col-0	*Arabidopsis* ecotype Columbia
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EST	expressed sequence tag
kb	kilobase
kD	kilodalton
KB	King's broth media
Ler	*Arabidopsis* ecotype Landsberg
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog (media/plates)
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
Poly(A)	polyadenylate
RNA	ribonucleic acid
RTPCR	reverse transcription polymerase chain reaction
RE	restriction enzyme
T-DNA	transfer DNA
UTR	untranslated region
Ws-0	*Arabidopsis* ecotype Wassilewskija

ABSTRACT

A genetic dissection of signal transduction pathways underlying the oxidative burst, cognate redox signalling, and establishment of systemic acquired resistance

Recognition of avirulent microbial pathogens activates an oxidative burst, leading to the accumulation of reactive oxygen intermediates (ROIs), which are thought to integrate a diverse set of defence mechanisms resulting in the establishment of plant disease resistance. Two contrasting experimental strategies were devised to dissect genetic mechanisms governing these signal transduction pathways.

Firstly, a novel transgenic *Arabidopsis* line containing a *GST1*::*LUC* transgene was developed and employed to report the temporal and spatial dynamics of ROI accumulation and cognate redox signalling in response to attempted infection by avirulent strains of *Pseudomonas syringae* pv. *tomato* (*Pst*). Strong engagement of the oxidative burst was dependent on the presence of functional *Pst hrpS* and *hrpA* gene products. Experiments employing specific pharmacological agents suggested at least two distinct sources, including a NADPH oxidase and a peroxidase-type enzyme, contributed to the generation of redox cues. The analysis of *GST1*::*LUC* gene expression in specific mutant backgrounds suggested engagement of the oxidative burst and cognate redox signalling functioned independently of ethylene, salicylic acid and methyl jasmonate in local *RPM1* mediated resistance. In contrast, studies using a panel of specific protein kinase and phosphatase inhibitors revealed mitogen activated protein kinase kinase (MAPKK) activity was required for the activation of the ROI-regulated genes *GST1* and *PAL1* in response to redox cues. Thus the engagement of a redox signalling network dependent on MAPKK activity may contribute to the establishment of plant disease resistance and the development of cellular protectant mechanisms.

Secondly, Activation Tagging was employed in conjunction with the reporter gene line *PR1a*::*LUC*, to uncover a mutant with constitutive defence gene expression. This mutant, subsequently named activated disease resistance 1-dominant (*adr1-D*), was shown to have enhanced resistance to fungal and bacterial pathogens. *adr1-D* mutants were also shown to have enhanced drought tolerance, and as such are believed to be the first plants engineered with elevated resistance to both disease and drought stress. Accumulation of different defence related transcripts in *adr1-D* lines introgressed into different mutant backgrounds suggested that the gross phenotype is largely but not exclusively mediated by SA accumulation. The corresponding *ADR1* gene was cloned and its overexpression was shown to recapitulate the *adr1-D* phenotype. Furthermore, *ADR1* was shown to be induced following pathogen attack and wounding. Potential biotechnological applications of the *ADR1* gene, shown to have close homologues in agronomically important crops, are also discussed.

SYNTAX USED IN THESIS
(in accordance with the journal *GENETICS*)

Type	**Format**	**Example**
Wildtype gene	Italics, uppercase	*NDR1*
Mutant gene/plant	Italics, lowercase	*eds1*
Protein	Non-italics, 1st letter uppercase	Gst1
Activation tagged mutant	Lowercase italics,-D (for dominant)	*adr1-D*

However, there are a number of exceptions to the above conventions which were adopted if widespread throughout the literature:

Resistance genes
Some as normal genes (e.g. *RPM1*, *RPP5*, *RPS2*); others with first letter in uppercase and following letters in lower case (e.g. *Cf-9*, *Xa21*, *Pto*, *Prf*)

Avirulence genes
1st letter is uppercase (e.g. *AvrB*, *AvrRpt2*)

***Hrp* genes**
Wildtype with 1st letter in uppercase (e.g. *HrpA*) ; for mutant genes *hrp* is lowercase (*hrpA*)

Other exceptions (gene/protein)
PR1, *PR5* etc./PR1, PR5 etc
rbohA/RbohA
$gp91^{phox}$/$gp91^{phox}$, $p22^{phox}$/$p22^{phox}$etc
OsRac1/OsRac1

This last grouping is not exhaustive and there are other genes, usually those with more than four letters, that do not follow the orthodox convention in the text.

1) INTRODUCTION

"Whoever could make two ears of corn, or two blades of grass grow upon a spot of ground where only one grew before; would deserve better of Mankind, and do more essential service for his country, than the whole race of politicians put together."

Gulliver's Travels (Swift, 1727)

i) Context

Despite the use of sophisticated and intensive crop protection measures, around 13% of crops are lost globally every year to insect predation and microbial diseases. Agriculture is still heavily reliant on chemical control of such pathogens, with current annual global expenditure estimated at around $8.7 billion (Shah et al. 1995). In developing countries, where chemicals are often an unaffordable luxury, crop losses are often measured in terms of famine and death. This is likely to be exacerbated in coming years: by 2025 there will be an extra 2.3 billion people on the earth with 95% of this growth in the less developed countries. The environment is also inextricably bound into the equation in both the developed and the developing world as chemical pesticides may be detrimental to natural habitats. More indirectly, greater efficiency of land use would necessitate a reduction in the conversion of marginal areas such as rainforests to agricultural use.

Recent advances in biotechnology offer new potential to control plant disease through the development of more efficient and environmentally friendly pesticides, the identification of resistant germplasm, and the genetic engineering of plants with enhanced resistance to disease. There have been a number of early successes of genetically engineering resistance into crops. The two strategies to date that have been commercially exploited are crop plants expressing the Bt toxin gene derived from the bacterium *Bacillus thuringiensis* for insect resistance (de Maagd *et al.* 1999) and coat-protein mediated resistance for protection against viruses (Fitchen & Beachy 1993).

Whilst both examples illustrate the potential of genetic engineering in this context, they have two major draw-backs. Firstly, the transgenic crops are only resistant to a limited range of pests and are thus susceptible to attack by other pathogens. Secondly, as the engineered resistance is dependent on the action of a single protein, pathogens are liable to rapidly evolve resistance, a problem which is already coming to light in *Bt* crops (Frutos *et al.* 1999). Therefore such strategies may only provide a short-term solution. Moreover, a common theme of negative reporting of so-called genetically modified (GM) crops in the media concerns the use of genes of non-plant origin in crop plants. Conceivably, 'second generation' GM crops engineered with genes of plant origin may be more palatable to a currently mistrustful public.

The overall remit of the work subsequently outlined in this thesis was to gain further understanding into the genetic processes underlying plant defence against disease. More indirectly, such research might contribute to the development of crops with enhanced endogenous protection against pathogens.

ii) The gene-for-gene concept

Plants are constantly subject to attack by a plethora of microbial organisms, including fungi, bacteria, and viruses, and have evolved an array of sophisticated defence mechanisms to protect themselves against disease. Physical barriers such as the waxy cuticle may prevent pathogen ingress in the first instance (Jackson & Taylor, 1996), and preformed anti-microbial chemicals such as saponins may also inhibit the pathogen's attempts at colonisation (Osbourn, 1996). Assuming the pathogen is able to overcome these defences and the plant in turn can support its particular niche requirements, two principle outcomes are possible: successful colonisation resulting in disease, or the plant may be resistant to infection. If the interaction is 'incompatible' the pathogen is said to be avirulent; conversely only so-called 'virulent' pathogens may cause disease in a compatible interaction.

Such plant-pathogen interactions are exquisitely specific and are thought to be the result of a single recognition event. In an incompatible interaction, an avirulence (*Avr*) gene product encoded by the pathogen is thought to interact directly or

indirectly with a resistance (*R*) gene product encoded by the plant. This event triggers a signal transduction pathway that unleashes a battery of defences both locally during the hypersensitive response (HR) and systemically in a process known as systemic acquired resistance (SAR). If the plant is lacking in the relevant *R*-gene, such defences are induced significantly later following the onset of disease symptoms and are much reduced in magnitude (Ryals, 1996).

iii) Resistance genes

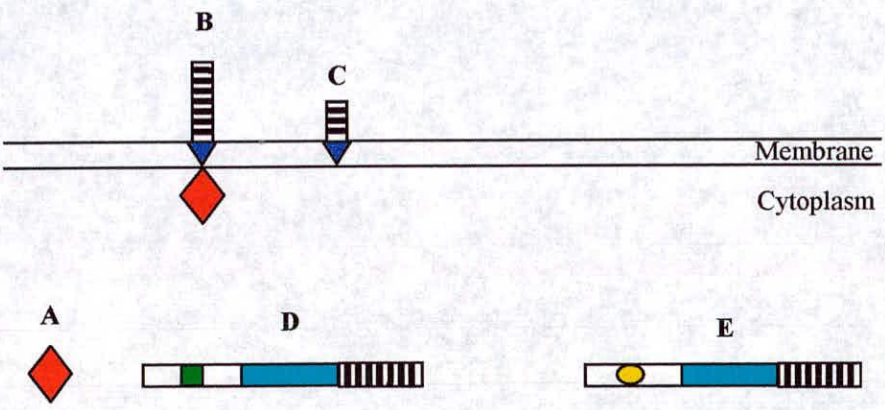
Although over 20 *R*-genes from seven plant species have been cloned to date (Martin 1999), surprisingly little is known about their precise function. The first *R*-gene to be cloned, *Hm1*, was shown to confer resistance to Race 1 strains of the fungal pathogen *Cochliobolus carbonum* in maize and was subsequently shown to encode a NADPH-dependent reductase that inactivates a potent toxin produced by the fungus (Johal and Briggs, 1992). However, as *Hm1* is functionally distinct from all other *R*-genes which are all thought to be involved in signal transduction and does not function in a classical *Avr*-dependent manner, it will not be classified as an *R*-gene for the remainder of this study.

The different classes of *R*-genes identified to date that function in an *Avr*-gene dependent manner are summarised in Figure 1. Probably the best characterised is *Pto* of tomato, the first 'classical' *R*-gene to be cloned, which recognises the *AvrPto* gene product of *Pseudomonas syringae* (Martin et al., 1993). *Pto* encodes a cytoplasmic serine-threonine kinase suggesting it plays a role in a phosphorylation cascade, and has been shown to exhibit kinase activity *in vitro* (Loh & Martin 1995). Direct physical interaction between the Pto and AvrPto proteins has been demonstrated by means of a yeast two-hyrid assay (Scofield *et al.* 1996; Tang *et al.* 1996).

All other *R*-genes share one common feature, a region composed of leucine rich repeats (LRR), and can be sub-grouped according to the additional structural domains they possess. LRRs consist of leucines and other hydrophobic residues at regularly spaced intervals (described in further detail in Chapter 7), which are

thought to specify protein-protein interactions (Kobe and Deisenhofer, 1994). Analysis of the crystalline structure of another LRR-containing protein, porcine ribonuclease inhibitor, has suggested that it is the interstitial residues between the conserved leucines that determine specificity of ligand binding (Kobe and Deisenhofer, 1993), a model which might be equally applicable to plant LRRs (Bent, 1996).

Aside from *Pto*, only one other *R*-gene has been shown to have a kinase domain, *Xa21* of rice, which encodes a membrane-bound receptor kinase-like protein (Song *et al.* 1995). It has an external LRR domain which is postulated to interact with an Avr protein, which may in turn activate the cytoplasmic kinase domain triggering a signal



Serine-threonine kinase	**A** - Serine-threonine kinase (e.g. *Pto* (Martin *et al.*, 1993))
Transmembrane domain	**B** - Receptor-like kinase (e.g. *Xa21* (Song *et al.*, 1995))
Leucine Rich Repeat region	**C** - Extracellular LRR (e.g. *Cf-9* (Jones *et al.*, 1994))
Nucleotide Binding Site region	**D** - TIR-NBS-LRR (e.g. *N* (Whitam *et al.*, 1994))
Coiled-coil/Leucine Zipper region	**E** - CC-NBS-LRR (e.g. *RPM1* (Grant *et al.*, 1995))
Toll/Interleukin-1 receptor homology	

Figure 1: The different classes of *R*-genes and their structural domains

Schematic shows the five characterised classes of *R*-genes and their corresponding structural domains (Hammond-Kosack & Jones, 1997). Drawings not to scale and N- and C-terminal regions are not shown.

transduction cascade culminating in various defence responses. The *Cf R*-genes of tomato (e.g. *Cf-9*, *Cf-2*) that convey resistance to the fungus *Cladosporium fulvum* also encode extracellular LRRs but lack any obvious domains that could effect downstream signal transduction (Jones *et al.* 1994; Dixon *et al.* 1996).

A possible model for signal transduction incorporating both types of membrane-bound protein has been devised by drawing analogies to the CLAVATA (CLV) system in *Arabidopsis* which is involved in development of the shoot meristem (Jeong *et al.*, 1999). In the CLV system, CLV1 encoding a LRR receptor kinase similar to *Xa21* binds CLV2, a membrane spanning anchor that is structurally analogous to the *Cf* genes. A small extracellular protein, CLV3, may act as a ligand to activate the CLV complex, a role that might conceivably be performed by an avirulence protein in *R*-gene interactions (Ellis *et al.,* 2000). This model is supported by the recent observation that one member of the *Xa21*-complex that conveys partial resistance to bacterial blight, *Xa21D*, encodes a truncated homologue of *Xa21* that is structurally analogous to Cf proteins (Wang *et al.*, 1998).

The most prolific class of *R*-genes contain a nucleotide binding site (NBS) located at the N terminus of the protein in addition to the LRR at the C terminus. The nucleotide binding domain primarily consists of the P-loop that is required for phosphate binding of ATP and GTP and is common to a vast array of proteins of diverse organisms (Saraste *et al.* 1990; Walker *et al.* 1982). A number of conserved motifs have been identified within the NBS domain (Traut *et al.* 1994) that are described more fully in Chapter 7. Intriguingly, these motifs are also found in the pro-apoptotic genes *APAF-1* and *CED-4* of humans and *C. elegans* respectively, suggesting that regulation of these cell-death pathways might share conserved elements (van der Biezen & Jones, 1998).

R-genes containing NBS-LRRs can be further sub-divided according to whether they possess an amino terminus coiled-coil (CC) domain (e.g. *RPM1* (Grant *et al.* 1995)) or region with homology to Toll/Interleukin-1 receptors (TIR) (e.g. *N* of tobacco (Whitam *et al.* 1994)). Toll and interleukin receptor proteins are known to be

involved in non-specific cell-immunity in animals and it has been postulated that plant TIRs may be involved in signal transduction (Young, 2000). However, recent data suggests that this region may play a role in pathogen recognition, as two alleles of the flax resistance gene *L* have different specificities for distinct flax rust isolates and yet only differ in the their respective TIR regions (Ellis *et al.* 1999).

Coiled-coil (CC) structures are known to promote dimerization and facilitate protein-protein interactions (Alber, 1992), however little is known about their role in *R*-gene signal transduction. Leucine zippers (LZ) comprise a sub-set of CC structures characterised by a predominance of leucine residues at position d of the heptad repeat. Although all CC-NBS-LRR *R*-genes to date have been classified as LZ-NBS-LRRs, it has recently been noted that of the identified *R*-genes, only *RPS2* possesses a *bona fide* leucine zipper and the remainder have CC domains. Interestingly, whilst TIR-NBS-LRR genes comprise 75% of NBS-LRRs, they have so far only been found in dicots despite exhaustive searching in cereals. In contrast, NBS-LRRs with a CC domain are widespread throughout the angiosperms (Pan *et al.*, 2000).

Intriguingly, these two classes of NBS-LRR *R*-genes appear to have different specificities in terms of their immediate downstream effectors. Pathogenicity analysis revealed that the mutant *eds1* is compromised in its ability to mount a resistance response that is mediated by TIR-NBS-LRRs, while resistance conferred by CC-NBS-LRRs is abrogated in the *ndr1* mutant background. This correlation is not perfect, as resistance to at least one CC-NBS-LRR is not compromised by either *eds1* or *ndr1*, and resistance conveyed by the *EDS1*-dependent genes, *RPP4* and *RPP5*, was slightly impaired by *ndr1*, implicating a degree of cross-talk between the two pathways. However, this finding does indicate that signalling following *R*-gene recognition is mediated by at least two distinct signalling pathways and may lead to further understanding of how this class of *R*-gene functions (Aarts *et al.*, 1998).

Whilst the specific roles of *R*-genes in the disease resistance signal transduction pathway remain elusive, some insights have been gleaned from studies in tomato. *Pto* and a related serine/threonine protein kinase Fen (conferring sensitivity to the

herbicide Fenthion) have been shown to both be dependent on an additional gene, *Prf* that encodes a CC-NBS-LRR (Salmeron *et al.* 1994). This implicates both LRR-containing proteins and protein kinases as components of the same signalling pathway and this relationship is further underlined by the fact that the Xa21 protein contains both kinase and LRR domains. It is conceivable that other NBS-LRR genes may also have respective protein kinase partners. Furthermore, recent studies in protoplasts have also demonstrated that Rps2 co-immunoprecipitates with its respective avirulence gene product AvrRpt2, in addition to an unknown 75kD plant protein (Leister and Katagiri, 2000). This suggests that the formation of a complex involving at least three proteins may be necessary for the elicitation of a resistant response.

iv) Avirulence genes

Although numerous *Avr* genes have been cloned in the last decade, comparatively little is known about the biochemical function of their corresponding proteins. Some *Avr* genes act as pathogenicity factors (e.g. *AvrRpm1*) (Lorang *et al.*, 1994) which may account for their evolutionary conservation, though this is not always the case (Kjemtrup *et al.*, 2000). Some *Avr* genes can be lost with no obvious penalty, for example the *Avr9* gene of *Cladosporium fulvum* appears to be dispensable and is lacking in some races virulent on *Cf-9* tomato (De Wit *et al.* 1995). Others such as *AvrBs2* play a disease causing role and contribute to the virulence of the pathogen in a compatible plant-pathogen interaction (Kearney & Staskawicz 1990). However, a specific role in virulence has not yet been assigned to many avirulence proteins (Alfano & Collmer, 1996). This is an important consideration from an agricultural perspective as the effectiveness of single *R*-genes in preventing disease is often compromised by shifts in pathogen populations towards individuals lacking avirulence, and hence may not be a durable strategy for engineering crop resistance.

Delivery of bacterial avirulence proteins to the interior of plant cells is mediated by a type III secretory system analogous to that employed by Gram-negative bacterial pathogens of animals (Fenselau *et al.*, 1992). Genes encoding components of this secretory system in bacterial phytopathogens are referred to as hypersensitive

response and pathogenicity (*Hrp*) genes, many of which have homologues in animal bacterial pathogens (Galan & Collmer, 1999). The function of a number of *Hrp* genes has been identified, for example *HrpA* encodes the major structural protein of the pilus, which acts as an appendage through which the bacteria may secrete virulence factors (Roine *et al.* 1997). Moreover, the regulation of this gene is known to be mediated in part by the *HrpS* gene (Wei *et al.* 2000).

v) The hypersensitive response & systemic acquired resistance

Following recognition of an avirulent pathogen, a signal transduction cascade is rapidly induced that triggers the hypersensitive response (HR) of the plant. This is characterised by localised cell death and necrosis of plant tissue, arresting further pathogen spread. Pathogens may be killed directly from exposure to toxic compounds and in the case of biotrophs this may be exacerbated by nutrient deprivation (Hammond-Kosack & Jones 1996).

Whilst there is a wealth of data to implicate the hypersensitive cell death as a vital component in the containment of biotrophic pathogens, recent evidence suggests that cell death may in fact enhance the ability of necrotrophic pathogens to colonise plant tissue. Growth of the necrotrophic fungal pathogen *Botrytis cinerea* was shown to be increased when co-inoculated with an avirulent strain of the biotrophic bacteria *Pseudomonas syringae*. This effect was not observed when the fungus was co-inoculated with *hrp-* strains that are unable to elicit an HR response (Govrin & Levine, 2000). Moreover, fungal growth was also suppressed in the HR-deficient mutant *dnd1* (Yu *et al.*, 1998) suggesting that cell death may be beneficial to necrotrophs who use dead plant tissue as a food source (Govrin & Levine, 2000).

Following HR formation is the establishment of immunity to secondary infections in systemic tissues, termed systemic acquired resistance (SAR), which conveys protection against a broad spectrum of normally virulent microbial pathogens (Ryals, 1996). Associated with the onset of SAR is the production of so-called pathogenesis-related (PR) proteins. Direct anti-microbial function both *in vivo* and *in vitro* has been demonstrated for a number of PR proteins such as PR1a of tobacco

(Alexander *et al.* 1993; Niderman *et al.*, 1995). Expression of some *PR* genes is tightly correlated with the onset of SAR making them useful marker genes (Ward *et al.* 1991; Uknes *et al.* 1992).

vi) The role of salicylic acid in SAR

Although there is significant evidence to implicate salicylic acid (SA) as a key signalling molecule in the establishment of SAR, the exact nature of its role remains to be rigorously established. There is a considerable amount of data to suggest a strong correlation between the local and systemic concentration of SA and the establishment of SAR (e.g. Malamy *et al.* 1990; Rasmussen *et al.* 1991; Yalpani *et al.* 1993). In addition, there is much evidence to suggest that exogenous SA can induce SAR gene expression (Uknes *et al.* 1992) and SAR (Vernooij *et al.* 1995). This relationship is reinforced by observations in transgenic plants that express the enzyme salicylate hydroxylase (*nahG*) which catalyses the breakdown of SA to the inactive metabolite catechol. Such plants not only are unable to accumulate free SA, but they are incapable of mounting a SAR response when infected with an avirulent pathogen (Gaffney *et al.* 1993; Friedrich *et al.* 1995), suggesting that SA accumulation is required for SAR induction. Intriguingly, depletion of SA also causes a breakdown of gene-for-gene resistance. Inoculation of *nahG Arabidopsis* with an incompatible isolate of *Peronospora parasitica*, leads to the development of severe disease symptoms, whereas wildtype plants are unaffected (Delaney *et al.* 1994).

Whilst it is clear that SA is required both locally in gene-for-gene resistance and systemically in SAR, other studies appear to refute the idea that SA is the translocated signal that induces SAR in leaves distal to the site of infection. Firstly, primary leaves of cucumber infected with *P. syringae* can be removed at 6hr after inoculation, before SA accumulates in the phloem, yet systemic increases in SA and SAR gene expression remain unaffected (Rasmussen *et al.* 1991). This finding is reinforced by grafting experiments between *nahG* and wildtype tobacco plants. When a *nahG* scion was grafted on to wildtype rootstocks, no SAR nor *PR* gene expression was detected in the leaves of the scion, following immunisation of leaves

of the rootstock with TMV. Conversely, plants with a *nahG* genotype rootstock still show SAR and *PR* gene induction in the wildtype scion following immunisation of leaves below the graft junction (Vernooij *et al.* 1994).

The isolation of mutants with aberrant disease resistance has further consolidated the role of SA in this signalling pathway. A number of mutants such as *cpr1*, *cpr5* and *cpr6*, all exhibit enhanced SA accumulation, constitutive expression of *PR* genes and resistance to bacterial and fungal pathogens (Bowling *et al.* 1994; Bowling *et al.* 1997; Clarke *et al.* 1998). Conversely, the mutants *pad4* and *npr1* (also called *nim1*) which have defects in SA accumulation and SA-dependent defence responses respectively, display enhanced susceptibility to bacterial and fungal pathogens (Glazebrook *et al.* 1997; Cao *et al.* 1994).

A significant body of evidence suggests that SA may also have an agonistic role in activating downstream defence responses. Hypersensitive cell death, and accumulation of H_2O_2 and transcripts of the defence-related genes phenylanaline ammonia-lyase (*PAL*) and glutathione *S*-transferase (*GST*) were all shown to be potentiated by addition of 50μM SA to soybean cell suspension cultures following infection by *P. syringae* pv *glycinae*. Production of peroxide was also synergistically activated by cantharadin, a phosphatase 2A inhibitor, in a manner that was cycloheximide-insensitive, suggesting that engagement of the oxidative burst in this context may require protein phosphorylation without *de novo* protein synthesis (Shirasu *et al.*, 1997).

These studies are apparently in conflict with previous assumptions that SA accumulation occurs long after activation of the oxidative burst (Dempsey *et al.*, 1999). This paradox might be reconciled by observations of tobacco plants infected with avirulent *P. syringae* pv. *phaseolicola*. In this system, a small transient rise in SA was observed that overlapped with the second phase of the oxidative burst and occurred prior to the sustained increase in SA previously documented (Draper, 1997). It has been postulated that this early induction of SA may potentiate the oxidative burst itself, which in turn may drive further SA production in a self-

amplification loop ultimately resulting in cell death, a process termed the oxidative cell death (OCD) cycle (Draper, 1997; Van Camp *et al.,* 1998).

While SA clearly plays an integral role in dicot disease signalling, its role in monocots is more ambiguous. Application of SA was shown to induce *PR* gene expression in rice (Matsuta *et al.*, 1991), maize (Morris *et al.*, 1998) and to a limited extent in barley (Vallélian-Bindschedler *et al.*, 1998). Furthermore, the functional analogues of SA 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), are known to convey resistance to certain pathogens in wheat (Gorlach *et al.*, 1996), barley (Wasternack *et al.*, 1994; Kogel *et al.*, 1994), and maize (Morris *et al.*, 1998). However, SA levels remain unchanged in rice following infection with either a fungal or bacterial pathogen (Silverman *et al.*, 1995). As rice is known to produce constitutively high levels of SA, it is hypothesised that defence responses may be activated by changes in sensitivity to SA, rather than a rise in SA levels *per se* (Chen *et al.,* 1997).

Recent evidence has also implicated lipid signalling in the SAR signal transduction pathway. Two *Arabidopsis* genes, designated *PAD4* and *EDS1*, which are required for resistance against virulent and avirulent pathogens respectively, have been shown to encode products with homology to phospholipases (Jirage *et al.* 1999; Falk *et al.* 1999). Furthermore, gene products of *PAD4* and *EDS1* have been shown to interact in yeast two-hybrid analysis (J. Parker - pers. comm.). Moreover, a lipid-transfer protein has been shown to be required for the establishment of SAR following the HR (Cameron *et al.* - unpublished). Current research in mutant analysis and use of microarrays is likely to shed more light on this section of the signalling pathway.

vii) The role of redox signalling in disease resistance

The production of Reactive Oxygen Intermediates (ROI) and subsequent cognate redox signalling underpins both the formation of the HR and establishment of SAR (Grant & Loake, 2000). This is initiated by the engagement of the so-called oxidative burst, a rapid biphasic production of ROIs, primarily superoxide (O_2^-) and hydrogen peroxide (H_2O_2), at the site of attempted infection (Apostol *et al.*, 1989;

Levine *et al.*, 1994). Multiple cellular functions have been ascribed to ROIs including the oxidative cross-linking of cell wall structural proteins (Bradley *et al.*, 1992) and direct antimicrobial activity (Peng and Kuc, 1992). Moreover, the accumulation of ROIs may also initiate the development of hypersensitive cell death (Jabs *et al.*, 1996; Levine *et al.*, 1994) and engage the deployment of cellular protectant functions in distal cells to limit cell death expansion (Tenhaken *et al.*, 1995; Kliebenstein *et al.*, 1999).

Reactive nitrogen intermediates (RNIs) have recently been proposed to function in concert with ROIs to both potentiate cell death and induce the expression of specific defence genes (Bolwell, 1999). Nitric oxide (NO) has been shown to accumulate during incompatible but not compatible plant:pathogen interactions and pharmacological inhibitors of NOS have been shown to ameliorate the establishment of plant disease resistance (Delledonne *et al.*, 1998). NO was also observed to potentiate ROI-mediated induction of cell death in soybean cells (Delledonne *et al.*, 1998). Thus ROIs may function in combination with NO to drive the development of host cell death during the formation of the HR, possibly after reacting together to form highly toxic peroxynitrite ($ONOO^-$). However, the identity of the agent(s) that directly execute plant cells still remains to be rigorously established.

Additional support for a role of NO in disease resistance is derived from the observation that the transient accumulation of NO in tobacco plants via the injection of mammalian NOS elaborated resistance against a previously virulent strain of tobacco mosaic virus (Durner *et al.*, 1998). To date however, a plant gene encoding NOS has not been identified, suggesting NO generation might occur by an alternative mechanism, such as respiration, de-nitrification or nitrogen fixation, via the production of NO_2.

viii) Production of ROI

While some of the cellular consequences of ROI accumulation have been uncovered, the identity of the molecular machinery underlying the oxidative burst remains to be rigorously established. Recent evidence has implicated a number of possible

mechanisms including: a plasma membrane located NADPH-dependant oxidase (Keller *et al.*, 1998; Groom *et al.*, 1996); a cell wall peroxidase (Bolwell and Wojtaszek 1997; McLusky *et al.*, 1999); an extracellular germin-like oxalate oxidase (Zhang *et al.*, 1995); and apoplastic amine, diamine and polyamine oxidase-type enzymes (Allan and Fluhr, 1997; Tipping *et al.*, 1995).

Of these proposed mechanisms the NADPH-dependant oxidase system, similar to that present in mammalian neutrophils, has received the most attention. Homologues of the mammalian $gp91^{phox}$ subunit of the NADPH oxidase complex have recently been identified in both rice and *Arabidopsis* (Groom *et al.*, 1996; Keller *et al.*, 1998). In other plant species there is accumulating evidence for the involvement of apoplastic peroxidases in the oxidative burst. These enzymes have been shown to produce H_2O_2 at an alkaline pH, as found in the apoplast during an incompatible interaction (Bolwell and Wojtaszek 1997), and may be directly secreted to the sites of attempted microbial infection (McLusky *et al.*, 1999).

ix) Regulation of ROI

Due to the highly cytotoxic and reactive nature of ROIs, their accumulation must be under tight control (Figure 2). Studies employing pharmacological agents have shown the plant oxidative burst to be regulated, at least in part, by a phosphorylation/dephosphorylation poise (Levine *et al.*, 1994). Phospholipases are thought to be intimately involved in the activation of the mammalian NADPH oxidase complex, which prompted studies to investigate if these enzymes have a similar function in plants. No role for phospholipase D has been identified, which is particularly important in mammals. However, inhibitors of phospholipase A have been shown to blunt the oxidative burst in tobacco suspension cells in response to specific recognition of the Cf-9 elicitor of *Cladosporium fulvum* (Piedras *et al.*, 1998). Moreover, a role for phospholipase C mediated production of inositol 1,4, 5-triphosphate in engagement of the plant oxidative burst has also been proposed (Legendre *et al.*, 1993).

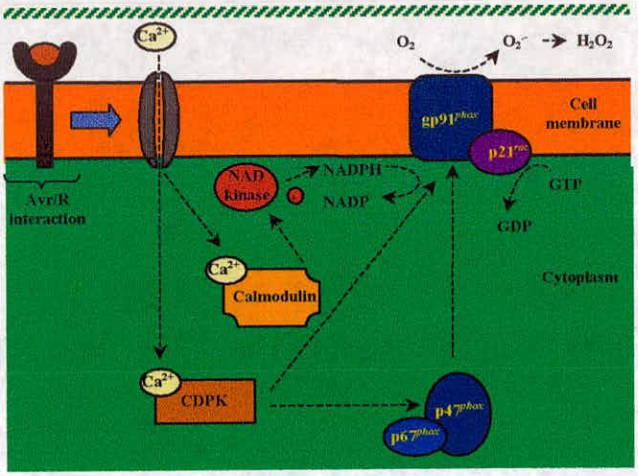


Figure 2. Schematic model for engagement of the NADPH oxidase-dependent oxidative burst in plants.

Pathogen recognition results in an influx of Ca^{2+} which may activate both the production of NADPH via NAD kinase and the translocation of $p67^{phox}$ and $p47^{phox}$ from the cytosol to the plasma membrane. Moreover, Ca^{2+} may also activate $gp91^{phox}$ directly, by binding to the two EF-hand motifs present in this protein, or indirectly via phosphorylation, following the Ca^{2+}-mediated activation of a specific CDPK. The small GTP-binding protein $p21^{rac}$ may also make an important contribution to the activation of the NADPH oxidase complex.

A striking feature of the plant homologue of $gp91^{phox}$, designated *rbohA*, is the presence of an extended amino terminus which is followed by two Ca^{2+}-binding EF hand motifs (Keller *et al.*, 1998), where E and F refer to two of the seven α-helices found in extracellular calcium binding modules (Kretsinger, 1996). This suggests Ca^{2+} may play an important role in the regulation of NADPH oxidase activity (Keller *et al.*, 1998). Structural predictions of RbohA suggest that the two EF hand motifs are located within the cytosol. The rapid accumulation of cytosolic Ca^{2+} in responses to elicitors and avirulent pathogens has been well documented (Zimmermann *et al.* 1997; Xu and Heath 1998). Thus Ca^{2+} release following pathogen recognition may drive a conformation change in RbohA leading to transient O_2^- production.

Recent evidence has also been presented suggesting that Ca^{2+} may modulate the NADPH oxidase complex via a more indirect route. NAD kinase catalyses the final step in the production of NADPH and the activity of this enzyme is dependent on the Ca^{2+}-binding protein calmodulin. Transgenic plants containing a constitutively active synthetic calmodulin have been generated and shown to possess increased

basal and induced levels of NADPH in response to elicitor treatment, resulting in elevated levels of ROI production (Harding *et al.* 1997). Hence, Ca^{2+} may also regulate the NADPH oxidase complex indirectly by elevating the concentration of available NADPH via modulation of NAD kinase activity.

Biochemical studies of the human NADPH oxidase complex have identified two further cognate proteins: the small GTP binding protein $p21^{rac}$ and the GDP-dissociation inhibitor factor rhoGDI. Thus GTP binding and its subsequent hydrolysis to GDP may play an important role in modulating O_2^- production. Recently, a number of rice genes have been identified as homologues of human $p21^{rac}$ and dominant gain-of-function and dominant negative forms of one such gene, designated *OsRac1*, have been expressed in rice cell cultures and transgenic plants. Intriguingly, while expression of constitutively active *OsRac1* resulted in the production of ROI, expression of the dominant negative form ameliorated ROI generation (Kawasaki *et al.*, 1999). Hence, OsRac1 may function to modulate the oxidative burst in rice by regulating the activity of an NADPH oxidase complex.

The active movement of the $p47^{phox}$ and $p67^{phox}$ components of the NADPH oxidase complex from the cytosol to the plasma membrane is thought to be a key point of regulation in neutrophils. Antibodies raised against these proteins, which cross-react with proteins of a similar mass in plants, have been employed to show translocation of these proteins may also occur to the plasma membrane of tomato, in response to race-specific elicitors of the leaf mould pathogen *Cladosporium fulvum* (Xing *et al.*, 1997). In animals, phosphorylation of these proteins has been proposed to initiate their translocation to the plasma membrane. In neutrophils protein kinase C is thought to phosphorylate these proteins, while in tomato this process may be mediated via a Ca^{2+}-dependent protein kinase (CDPK) (Xing *et al.*, 1997).

Unfortunately, plant gene homologues encoding $p47^{phox}$ and $p67^{phox}$ have yet to be uncovered. In this context, an alternative mechanism for the activation of the plant NADPH oxidase independent of these proteins has been proposed. The *R*-gene dependent activation of a CDPK which functions upstream of the oxidative burst has

recently been reported (Romeis *et al.* 2000). While the molecular target of this CDPK remains enigmatic, it may phosphorylate the plant NADPH oxidase, as this protein possesses CDPK phosphorylation signature sites. Hence, this kinase may directly contribute to the activation of NADPH oxidase activity (Figure 2).

Mechanisms regulating the production of ROIs via the modulation of cell wall associated peroxidase activity are also now beginning to emerge. Peroxidase isoforms have been isolated that actively produce H_2O_2 *in vitro* at an alkaline pH (Bolwell and Wojtaszek, 1997). This is an important criteria, because following pathogen recognition there is a rapid alkanisation of the apoplast. Hence, an increase in apoplastic pH, mediated via plasma membrane ion channels, could engage the activity of the relevant peroxidase isoforms, driving the production of H_2O_2. The delivery of substrate(s) to the apoplast may provide another powerful mechanism for the regulation of peroxidase dependent ROI production, although the existence of such a regulatory mechanism remains to be established.

A recent study has highlighted the possible co-localisation of peroxidase activity and H_2O_2 accumulation at *Botrytis allii* infection sites in onion epidermal cells (McLusky *et al.*, 1999). The directed secretion of peroxidases to sites of attempted pathogen infection could thus provide an elegant mechanism for regulating peroxidase-dependent ROI generation. Finally, like the NADPH oxidase complex, the activation of apoplastic peroxidase activity may also be coupled to Ca^{2+} fluxes, as Ca^{2+} is required for optimal enzyme activity.

Genetic evidence for the involvement of ROIs in the HR has been provided by studies employing the recessive *lsd1* lesion mimic mutant of *Arabidopsis*. The accumulation of O_2^- preceded the onset of cell death and the local accumulation of O_2^- but not H_2O_2 in *lsd1* was sufficient to initiate the development of runaway cell death in this mutant (Jabs *et al.*, 1996). Thus runaway cell death in *lsd1* plants probably reflects abnormal accumulation of O_2^- and an inability to respond to signals derived from it. *LSD1* has been shown to encode a zinc finger transcription factor

and may function by monitoring a superoxide-dependent signal and negatively regulating a cell death pathway.

Recently, some of the targets of *LSD1* gene function have begun to emerge: *LSD1* has been shown to be required for the induction of CuZn superoxide dismutases in response to SA accumulation (Kliebenstein, *et al.*, 1999). Thus the runaway cell death phenotype in *lsd1* probably reflects the accumulation of O_2^- to a critical threshold concentration, due to a reduction in CuZnSOD activity, which engages the mechanism(s) underlying runaway cell death in this mutant. However it is currently unclear how this mechanism operates because O_2^- production is presumed to be extracellular and the target CuZnSOD intracellular. Furthermore, O_2^- will not typically cross biological membranes due to its inherent charge.

x) Role of ROI in systemic acquired resistance

In addition to their proposed role in local, *R*-gene mediated resistance, the oxidative burst and cognate redox signalling may also play a pivotal function in the establishment of systemic acquired resistance (SAR). This was highlighted by an elegant series of experiments in transgenic tobacco plants which contained an antisense catalase gene (Chamnongpol *et al.*, 1998). Exposure of these plants to high light levels for two days resulted in visible necrosis and induced PR-proteins in light shielded local and systemic leaf tissues. In contrast, exposure to high light levels for 4 hours induced PR-proteins in local light shielded but not systemic tissue, in the absence of necrosis. Thus the ROI-mediated activation of SAR genes could be uncoupled from cell death in local tissues. However, local ROI-mediated cell death was necessary for the accumulation of PR proteins in systemic tissues.

Studies employing *Arabidopsis* have placed similar observations in a more biological context (Alvarez *et al.*, 1998). Engagement of a local oxidative burst in response to an avirulent isolate of *P.syringae pv. tomato* (*Pst*) induced "micro-bursts" in systemic leaf tissue. These "micro-bursts" drove the formation of "micro-HRs" which preceded the establishment of SAR. Co-infiltration of the NADPH oxidase inhibitor diphenylene iodinium (DPI) with avirulent *Pst* ameliorated engagement of a

local oxidative burst and blocked the formation of systemic "micro-bursts" and the development of SAR. In the corresponding gain-of-function experiment local infiltration of an H_2O_2 generating system induced systemic "micro-bursts" and subsequently SAR. Hence, a ROI-mediated systemic signalling network may also mediate the establishment of plant immunity. ROI accumulation may therefore integrate a plethora of local and systemic defence responses (Figure 3).

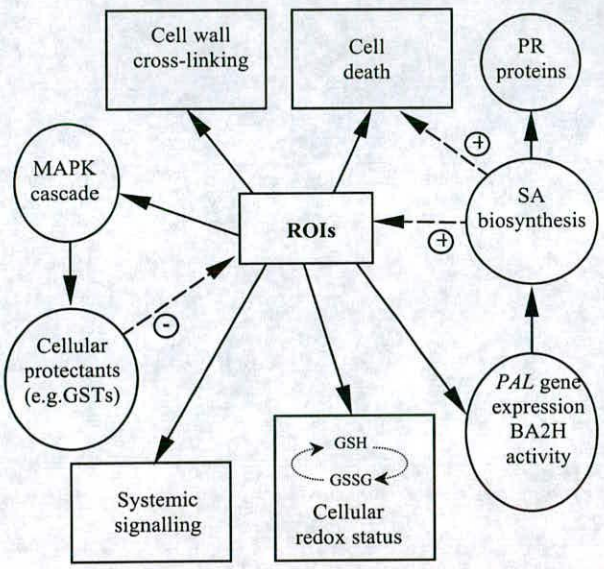


Figure 3.: Functional integration of defence responses by reactive oxygen intermediates during the establishment of plant disease resistance.

SA, salicylic acid; BA2H, benzoic acid 2-hydroxylase; *PAL*, phenylalanine ammonia lyase; MAPK, mitogen activated protein kinase; GSH/GSSG, reduced and oxidised forms of glutathione respectively.

xi) SA-independent disease resistant pathway

Plant defence responses are not universally mediated by salicylic acid and alternative pathways are known to exist that function independently of SA. A subset of pathogens induce defence gene expression that is mediated by components of the jasmonic acid (JA) and ethylene signalling pathways (Creelman & Mullet, 1997). JA has previously been characterised as an important component of the wound-response and insect-herbivory pathways (Doares *et al.*, 1995). JA and its methyl ester, methyl-jasmonate (MeJA), have been shown to confer local and systemic protection against the fungal pathogen *Phytophthora infestans* in potato and tomato (Cohen *et al.*, 1993). Moreover, exogenous application of JA causes induction of the anti-

microbial peptides thionin Thi2.1 (Epple *et al.* 1995) and defensin Pdf1.2 (Penninckx *et al.* 1996) which are not inducible by SA. Furthermore, inoculation of *Arabidopsis* with the avirulent fungal pathogen, *Alternaria brassicola*, induces *PDF1.2* independently of SA. Lastly, studies using the ethylene insensitive mutant *ein2*, and *coi1*, a mutant insensitive to coronatine (an analogue of JA), suggested that JA and ethylene are required concomitantly for *PDF*1.2 expression of the defensin gene (Penninckx *et al.* 1996; Penninckx *et al.* 1998).

Recent data suggest that ethylene might play a more influential role in conveying resistance to necrotrophic pathogens. *ein2* mutants were found to be markedly more susceptible as compared with wildtype to the fungal necrotroph, *Botrytis cinerea.* In contrast, resistance to avirulent strains of a biotrophic oomycete, *P.parasitica*, and an avirulent necrotrophic fungus, *Alternaria brassicicola*, remained unaffected (Thomma *et al.* 1999).

There is considerable evidence for a degree of cross-talk between the SA and JA signalling pathways (Dong, 1998). For example, the mutants *eds4* and *pad4* which have reduced ability to accumulate SA have been shown to display enhanced responses to the inducers of JA expression, Me-JA and rose bengal (Gupta *et al.* 2000). This relationship was placed in a more biological context by a series of experiments in tobacco whereby phenylalanine ammonia-lyase (Pal) expression, a precursor in SA biosynthesis, was manipulated to affect corresponding levels of SAR. Plants with reduced SAR displayed greater resistance to grazing-induced resistance to larvae of *Heliothis virescens*, whilst in plants with an elevated SAR response the reverse was true (Felton *et al.* 1999). However this apparent antagonism between SA- and JA-mediated defence pathways is not incontrovertible. A recent study showed that simultaneous activation of SAR and JA-dependent induced systemic resistant (ISR) via treatment with the *Pst*(*AvrRpt2*) and the non-pathogenic *Pseudomonas rhizobacteria* WCS417r respectively, resulted in additive resistance against virulent *Pst* (van Wees *et al.,* 2000). Further work is now required to characterise cross-talk between SA-and JA-dependent disease resistance pathways,

not least because of the obvious implications it has regarding the potential to transfer of this technology to engineer pathogen and pest resistant crops.

xi) Summary

Perhaps one of the most neglected areas of plant disease resistance has been research into the genetic mechanisms underlying the production of ROI and cognate redox signalling. Despite their obvious importance in mediating the defence response (as well as in many other signalling pathways), the mechanism by which ROI are generated is still disputed. Moreover, data has been collated from a variety of different pathosystems and as such is difficult to consolidate and draw meaningful universal conclusions. In addition, almost nothing is known about the genetics of ROI perception and the defence pathways engaged immediately after ROI accumulation.

To address these questions, we constructed a novel transgenic *Arabidopsis* line that faithfully reports the oxidative burst and cognate redox signalling. This technology was employed in conjunction with different pharmacological inhibitors, in a variety of mutant backgrounds, and with different pathosystems and inducing chemicals to gain further insights into the genetic determinants of this pathway. This transgenic line has also been deployed in a mutant screen to uncover genes involved in ROI production and perception (not described herein).

Activation Tagging in a designer background was also employed to identify mutants that display elevated resistance to microbial pathogens. One such mutant was isolated, subsequently named *adr1-D* (*activated disease resistance* 1-dominant), and was shown to be resistant to bacterial and fungal pathogens. *adr1-D* also displayed strongly enhanced drought tolerance and as such is believed to be the first mutant isolated that is resistant to both disease and drought stress. Furthermore, *ADR1* was shown to be induced following pathogen attack, wounding, and treatment with SA or BTH. *ADR1* encodes a novel signal transduction gene that is thought to act as a

global regulator effecting plant responses to biotic and abiotic stress. As such, the potential for the commercial exploitation of this gene is also considered.

2) MATERIALS & METHODS

i) Reagents

Unless otherwise stated, all reagents used were supplied by Sigma (Poole, Dorset, UK).

ii) Nucleic acid analysis

Total RNA was extracted from 4-6 week old plants according to standard procedures (Reuber and Ausubel, 1996). For Northern analysis, 12 μg total RNA samples were separated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Amersham) exactly according to manufacturers instructions (Hybond booklet). ^{32}P-labelled DNA probes were prepared using a Prime-a-Gene® labelling kit (Promega). Hybridisation conditions and stringency washes (always at 65°C) were as described by Ausubel *et al,*. 1996. Blot hybridizations were quantified with a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA) in conjunction with ImageQuant 3.3 software and normalised with reference to *R18* hybridisation.

Sequencing reactions were prepared and run on a HYBAID Omnigene Thermocycler using the PERKIN-ELMER ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, according to the manufacturers instructions. Sequence data was transferred to the UNIX-based GCG package for further analysis.

iii) Cloning of the *GST1* promoter

A cosmid library of *Arabidopsis* (ecotype Ws) genomic DNA (Arabidopsis Stock Center, Ohio) was screened at 65°C with a ^{32}P-labelled gene-specific probe of the *GST1* gene. The fragment was obtained by PCR amplification of the 3' untranslated region of a template *GST1* cDNA clone using primers previously designed by Sharma *et al.* (1996). DNA from a positive clone was isolated and subjected to restriction analysis followed by hybridisation with the *GST1* gene-specific probe. An 8 kb *KpnI* fragment containing *GST1* was sub-cloned from a positive cosmid into

pBluescript SK$^-$ (Stratagene), further digested with *EcoRI* and resulting fragments sub-cloned into pBluescript SK$^-$ and sequenced (see also Figure 5A-D, chapter 3).

iv) Generation of *GST1*::*LUC* transgenic plants

PCR was used to amplify a 909 bp promoter fragment (*GST1*) with *Sac*I and *Nco*I sites at respective 5' and 3' ends using the following primers:

5'primer-5'-TATAGAGCTCGGAAACAGCTATGACCATG-3'

3'primer-5'- TTGATTCCTGCCATGGGTTAATACTGTGT-3'

This fragment was subsequently sub-cloned into pART7 (Gleave 1992) containing a *LUC* reporter gene and *OCS* termination sequence and transgenic plants generated by 'floral dip' (Clough and Bent 1998).

v) Cross-pollination and selection of mutant backgrounds

Genetic crosses were undertaken by dissecting and emasculating unopened flower buds and then using the remaining pistils as recipients for pollen from 4 opened flowers. Transgenics with a selectable marker (i.e. *GST1*::*LUC* and *adr1-D*) were always used as the pollen donor and mutant lines as recipient, enabling kanamycin and Basta selection as appropriate, on MS plates or soil respectively. Successful crosses were allowed to self and homozygous transgenic plants were screened from their progeny.

Progeny containing *nahG* were identified by virtue of their brown deposits in root tissue when grown on MS media containing 1mM salicylic acid (Bowling *et al.*, 1994). The ethylene insensitive mutants *etr1* and *ein2-1* were selected at 2-3 weeks on MS plates containing 50μM ACC by virtue of having significantly longer roots than wildtype plants. Mutants successfully introgressed into the *coi1* background were selected by normal growth on MS plates containing 200μM Me-JA in comparison to stunted wildtype plants. The *npr1-1* mutant background was selected by *Nla*III digestion of PCR product of genomic DNA amplified by CAPS markers (Li *et al.*, 1999). Appropriate controls were always used to check selection method.

vi) Treatment of Plants

Bacteria were maintained as previously described (Dangl *et al.* 1992). Mutant *hrp Pseudomonas* strains were transformed with the pVB01 plasmid by electroporation as described by Keen *et al.* (1990). Bacteria were inoculated into individual leaves by pressure infiltration using a 1 ml syringe; 10 µl of bacteria were infiltrated into the abaxial leaf surface.

Aspergillus niger glucose oxidase 2.5U/ml was added to 2.5mM D-glucose in 20mM Na phosphate buffer pH (6.5), immediately prior to infiltration. Catalase (bovine liver) (300U/ml), superoxide dismutase, (25U/ml) DPI (3µM), sodium azide (1µM), K252a (Calbiochem) (1µM), staurosporine (Calbiochem) (1µM), and PD98059 (Calbiochem) 100µM, sodium nitroprusside (0.5mM) in 10mM Tris-HCl pH7.5, putrescine (1mM), L-arginine (1mM), L-NNA (100µM) and cantharidin (Calbiochem) (5µM) were co-inoculated with 10µl of bacterial suspension or H_2O_2-generating system as appropriate. Controls were also carried out using buffers alone. Bacterial growth curves were used to ensure inhibitors did not adversely affect bacteria. BTH (Novartis) was painted on leaves as a 300µM solution containing 0.01% Silwet (Union Carbide). DAB staining was as described by Thordal-Christensen *et al.* (1997) and leaves photographed in 80% glycerol. Imaging of autofluorescence was performed using a Polyvar confocal microscope (Reichert-Jung) with a UV light source at x100 magnification.

vii) Real time *in planta* imaging of LUC activity

Leaves of *GST1*::*LUC* or *PR1a*::*LUC* transgenic plants were painted with a solution containing 1mM Luciferin (Promega) and 0.01% triton X-100 and 0.03% Silwet (Union Carbide) in a 1mM sodium citrate buffer (pH 5.8). All *in planta* LUC imaging was performed using an ultra low light imaging camera system (EG & G Berthold Luminograph 980). Images were routinely collected over a 1s (*GST1*::*LUC*) or 10s (*PR1a*::*LUC*) time period. Microscopy imaging was carried out using Nikon Optiphot-2 microscope.

viii) Activation tagging mutagenesis and screen

Agrobacterium GV3101 transformed with the pSKI015 vector (gift from I. Kardailsky) was grown overnight at 30°C in LB media containing 50 $mg.l^{-1}$ kanamycin (to select for the helper plasmid) and 50 mg/l ampicillin (to select for the pSKI015 binary vector) and used to transform *PR1a*::*LUC* by floral dip method (Clough & Bent, 1998). Seed harvested from transformed plants was sown in flats and selected by 100mg/l Basta (Agrevo) spraying two weeks after germination, then three times at four day intervals. Resistant plants were visually inspected for any abnormalities, sub-planted and F_2 seed collected in individual lines. Mutant screen was carried out by sowing pools containing around 10 seed from 25 different F_2 lines in 8cm high pots. Two weeks post-germination seedlings were painted with luciferin and imaged for constitutive luciferase activity.

ix) Fungal pathogenicity assays

Peronospora parasitica NOCO2 (gift from Jane Parker) was maintained on Col-0 seedlings grown in majenta jars. For the *P.parasitica* disease resistance assays, conidiospores were harvested by vortexing infected seedlings in water. Spore concentration was determined using a haemocytometer, and resuspended in sterile distilled water to $1x10^5$ spores per ml. Four-week old plants grown under short day conditions were sprayed with the conidiospore solution and placed in trays covered with Saran wrap to maintain a humid environment. Fungal growth on plant leaves (visualised as condiophore growth) was scored 10 days post-infection using qualitative method adapted from Cao et al. (1997). Scoring was as follows: 0 - no infection, 1 - less than 25% of one leaf with conidiophore growth, 2 - 25 to 50% of one or two leaves covered with condiophores, 3 - 25 to 50 % of three or four leaves covered with conidiophore growth, 4 - 25 to 50% of all leaves covered with condiophore growth, 5 - all leaves covered with conidiophore growth. Plants in different replicates were assigned a disease index as follows: $D.I.=\sum iXj/n$, 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.* 1997).

Powdery mildew (*Erisyphe cichoracearum*) infection assay was performed by simply transferring 4 week old plants to the transgenic greenhouse, an environment conducive to promiscuous growth of the pathogen. Plants were scored ten days later by number of leaves infected.

x) Bacterial pathogenicity assay

P. syringae pv. *tomato* DC3000 (*Pst*) was grown in King's broth (KB) liquid media (King, 1954) supplemented with 50 $mg.l^{-1}$ rifampicin. Four week old plants were infected with a *Pst* suspension (OD_{600} = 0.0002) in 10 mM $MgCl_2$ by pressure infiltration of the abaxial side of the leaf with a 1ml syringe. Three leaves per plant, and three plants per line were infiltrated. After three days, plants were inspected for development of symptoms. Leaves were also harvested at this time point for analysis of bacterial growth. Leaf discs of the same size ($0.5cm^2$) were made from these samples using a cork borer. Three leaf discs from each plant were ground in 990µl 10 mM $MgCl_2$ in a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension, and 100µl of each dilution was plated onto KB 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.

xi) Cloning of the *ADR1* genomic clone

Performed exactly as described in chapter 6, paragraphs 1-4 and Figure 25A-E.

xii) Homology searches and sequence analysis

The following web-sites (Table 1) were used and instructions followed as detailed at site. Coiled-coil structure prediction was performed with COILS (Lupas, 1997) and scans were made with variable window sizes of 14-28 residues using the MTIDK matrix. GCG10 and Genejockey were also used for sequence analysis and alignment purposes.

Name	*Function*	*Web address*
BLAST	Homology searches in plants	www.arabidopsis.org/blast/
BLAST2	Homology in all organisms	www.blast.genome.ad.jp/
Genscan	Identification of ORFs	genes.mit.edu/GENSCAN.html
Proscan	Protein motifs	pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html
PLACE	Motifs in promoter	www.dna.affrc.go.jp/htdocs/PLACE
ISREC COILS Server	Prediction of coiled-coil regions (Lupas's method)	www.ch.embnet.org/software/COILS_form.html
PSORT prediction	N-glycosylation sites and sub-cellular localisation	psort.nibb.ac.jp/form.html
ClustalW	Alignment of multiple peptide sequences	www2.ebi.ac.uk/clustalw/
Boxshade 3.21	Shading of conserved a.a.'s in different sequences	www.ch.embnet.org/software/BOX_form.html

Table1: Web-sites used for sequence analysis

xiii) Generation of *ADR1* transgenic lines

PCR amplification was performed in capillary tubes using a Rapidcycler (Idaho Technology) exactly according to manufacturers instructions. The 5.5 kb *Spe*I/*Pst*I fragment containing the *ADR1* genomic clone was used as a template (100ng/ul) and the concentration of $MgCl_2$ in the buffer was 3mM. Cycles were as follows:

a) 94°C - 15"; b) 94°C - 15"; c) 65°C - 30";d) 72°C - 1'30" (Repeat b-d for 30 cycles); e) 72°C - 1'45"

The expression cassettes were created as outlined below, transformed into *Agrobacterium* by freeze-thaw method which was then used to transform *PR1a*::*LUC* lines by 'floral dip' method.

i) <u>*CaMV 35S::ADR1*</u> - PCR was used to amplify the *ADR1* genomic fragment with *Kpn*I and *BamH*I immediately adjacent to the start (ATG) and stop codon respectively using the following primers (bold indicates start/stop codons, italics shows RE recognition site):

*Kpn*I primer -5'- GCTTA*GGTACC*AAGATCGGTCTCG**AT** -3'

*BamH*I primer -5'- GCGAA*GGATCC*AGAAGC**CTA**ATCGTC -3'

This fragment was subsequently sub-cloned into *Kpn*I/*BamH*I sites of the MCS of pART7 (Gleave 1992) downstream of the *CaMV 35S* promoter. The *CaMV 35S::ADR1::OCS* cassette was sub-cloned by *Not*I digestion to pGreen and this binary vector was then used to transform *Agrobacterium* containing the *trans* functioning plamid pJIC Sa_Rep (Hellens *et al.*, 2000). Transgenic plants generated were selected by spraying with 100mg/l Basta (Agrevo)..

ii) <u>pTA7001::*ADR1*</u> - PCR was used to amplify the *ADR1* genomic fragment with *Sal*I and *Spe*I immediately adjacent to the start (ATG) and stop codon respectively using the following primers:

*Sal*I primer -5'- GCTTA*GTCGAC*AAGATCGGTCTCG**AT** -3'

*Spe*I primer -5'- GCGAA*ACTAGT*AGAAGC**CTA**ATCGTCA -3'

This fragment was subsequently sub-cloned into *Xho*I/*Spe*I sites of pTA7001 (gift from N-H Chua; McNellis *et al.*, 1998) and this cassette pTA7001::*ADR1* used to transform *Agrobacterium*. Transgenic plants were selected on MS plates containing 20μg/ml hygromycinB (Calbiochem). Transgenics were selected at two weeks then transferred to 50μg/ml kanamycin MS plates for seven days to ensure that *PR1a::LUC* cassette was intact. Seedlings were then transferred to MS plates containing 30μM Dexamethasome (DEX). Luciferase imaging was performed ten days later in comparison to the following negative controls: empty vector pTA7001 (*PR1a::LUC*) on DEX plates; pTA7001::*ADR1* (*PR1a::LUC*) on non-DEX plates.

xiv) Drought stress assay

Around 20 seedlings (14 days old) of both *adr1-D* and Col-0 plants were transplanted into two separate halves of the same tray under short day conditions. Watering was stopped 11 days later and differences recorded. Dead plants were

confirmed by failure to resuscitate on resumption of watering. This experiment was also carried out under long day conditions. In a different experiment, 6-8 seedlings of Col-0, *adr1-D*, *adr1-DxnahG*, *adr1-Dxetr1* and *adr1-Dxnpr1* were subplanted to separate segments of the same tray and assay performed as described above. Assays were also performed in 8cm high pots (4 plants/pot) using Col-0, *adr1-D*, and *coi1*, under long and short day conditions.

3) GENERATION OF A NOVEL ROI-REPORTER SYSTEM

i) Introduction

To facilitate genetic dissection of the dynamic events surrounding HR formation, we engineered a novel transgenic *Arabidopsis* line that would report accumulation of a gene tightly correlated with redox signalling during the oxidative burst. The principle advantages of such an approach are: spatial and temporal accumulation of marker transcripts can be imaged in real-time in a non-destructive manner; experiments can be performed *in planta* and thus results are more likely to reflect mechanisms that are actually occurring in the plant; it is less labour intensive than comparable techniques; high-throughput saturating mutant screens can be carried out with transgenic lines. To engineer a reporter cassette for subsequent plant transformation, the promoter region of the marker gene was fused to the firefly luciferase gene (Millar *et al.* 1995a). Such transgenic plants may also be utilised for high-throughput mutant screens (e.g. Millar *et al.,* 1995b; Ishitani *et al.* 1997).

ii) Identification of a ROI/Avr-inducible reporter gene

Marker genes are a useful means of monitoring dynamic events in a signal transduction pathway. The *PR* genes have been used extensively to monitor SAR responses (Ryals, 1996), whilst genes encoding glutathione S-transferases (*GSTs*) have been employed as molecular markers for the oxidative burst (e.g. Jabs *et al.* 1996; Alvarez *et al.* 1998). Gsts are enzymes ubiquitous to nearly all aerobic organisms whose main function is to detoxify toxic compounds by catalysing the nucleophilic attack of the sulphur atom of reduced glutathione (GSH) to the electrophilic centre of the substrate. Gsts may often also function as peroxidases by means of the GSH-dependent reduction of H_2O_2 (Marrs, 1996).

A gene encoding a glutathione S-transferase, designated *GST1* was previously isolated in a differential screen to uncover genes rapidly induced following avirulent pathogen attack (Greenberg *et al.,* 1994; Yu & Ausubel - unpublished). It had also previously been shown to be induced by O_3 in a SA-dependent manner probably via the accumulation of ROI (Sharma *et al.* 1996). As *GST1* was rapidly induced during

the establishment of the HR and was implicated in oxidative stress responses, it was deemed to be a suitable marker gene.

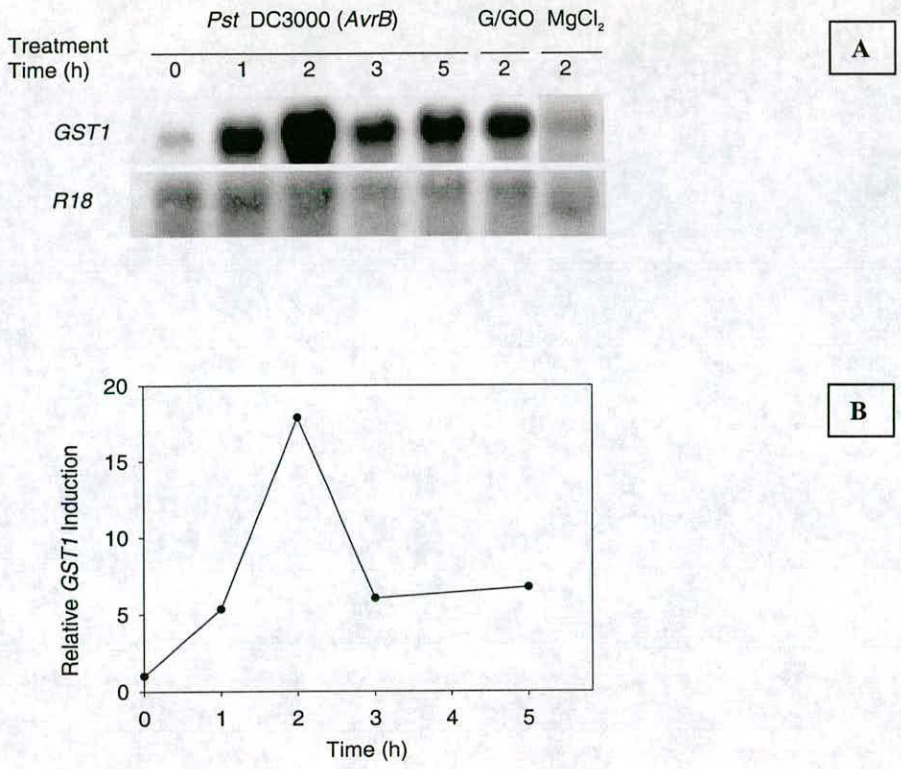


Figure 4: Rapid induction of *GST1* transcripts following oxidative burst or inoculation with H_2O_2-generating system.
(**A**) Northern blot hybridisation of *GST1* transcripts following inoculation with *Pst* DC3000(*AvrB*) at different time intervals post-inoculation and with Glucose/Glucose Oxidase (G/GO), and mock inoculation with 10mM $MgCl_2$.
(**B**) Time course for accumulation of *GST1* transcripts (quantified using phosphorImage and normalised to *R18*) in leaves challenged with *Pst* DC3000(*AvrB*).

To further characterise the expression pattern of *GST1*, Northern blot analysis was performed using a *GST1* gene-specific probe corresponding to a fragment of the 3' untranslated region (UTR) of the gene (Sharma *et al.* 1996). Levels of *GST1* transcript accumulation were quantified by using phosphorImager analysis and normalised to ribosomal *R18*. *GST1* mRNA was shown to accumulate within one hour of inoculation with *Pseudomonas* syringae pv. *tomato* (*Pst*) strain DC3000 expressing the *AvrB* avirulence gene (Tamaki *et al.* 1991) and showed maximum 18-

fold accumulation at 2-3 hours (Figure 4A&B). Mock inoculation with $MgCl_2$ alone only caused a five-fold induction of *GST1*.

Leaf infiltration with the H_2O_2-generating system glucose/glucose oxidase (G/GO) strongly induced the accumulation of *GST1* transcripts after 2 hours (Figure 4a). DAB staining (performed by G.Loake) was used to confirm the temporal accumulation of endogenous H_2O_2 in response to *Pst* DC3000(*AvrB*) was congruent with *GST1* expression (Figure 9B&C).

iii) Cloning of the *GST1* promoter region

Having confirmed that *GST1* expression was tightly correlated with the oxidative burst and accumulation of H_2O_2, the corresponding upstream regulatory promoter region was isolated (Figure 5). The *GST1* gene-specific probe was employed to identify the corresponding genomic clone from a genomic cosmid library (Schulz *et al.*, 1994). Chapter 42 of the library gave a positive band following Southern hybridisation and colony blotting was performed to obtain a single cosmid clone containing the *GST1* genomic region (Figure 5A).

DNA from the positive colony was subject to restriction digest by 10 different enzymes (*Cla*I, *EcoR*I, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sma*I, *Spe*I, *Xba*I, and *Xho*I) which were probed with the *GST1* gene-specific probe by Southern hybridisation. A positive *Kpn*I fragment of approximately 8 kb was selected for further analysis and cloned to pBluescriptSK$^-$. This construct was further digested with *EcoR*I and products of 5 kb, 2 kb, 1 kb, and 0.35 kb sub-cloned to pBluescriptSK (Figure 5B). Sequencing was carried out on the *Kpn*I/*EcoR*I fragments using T3 and T7 primers enabling the fragments to be ordered relative to one another as detailed in the schematic (Figure 5C).

*Sac*I and *Nco*I sites were engineered at the 5' and 3' ends of the *GST1* promoter by PCR mutagenesis to enable sub-cloning into the reporter cassette. The *GST1* promoter was transcriptionally fused to the firefly luciferase (*LUC*) reporter gene

(Figure 5D) which had previously been cloned into the pART7 plasmid upstream of an *OCS* terminator region (Gleave *et al.* 1992; Thomson & Loake - unpublished).

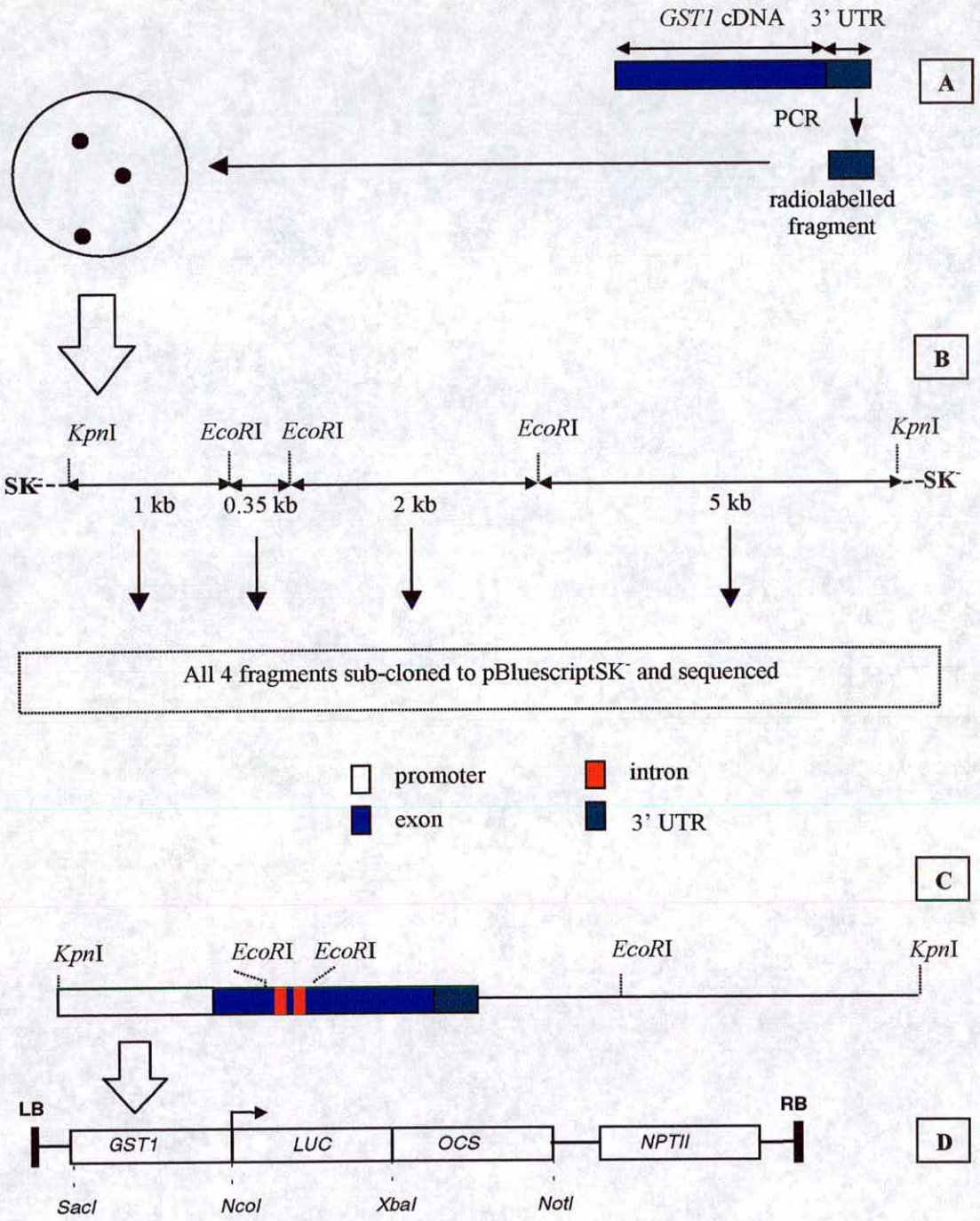


Figure 5: Cloning of the *GST1* promoter and construction of the reporter-gene cassette
A) The 3'UTR of *GST1* was used to probe a cosmid library and a single positive colony isolated.
B) An 8 kb *Kpn*I fragment from the single colony containing *GST1* is cloned into pBluescript, then cut with *EcoR*I and fragments sub-cloned to pBluescriptSK-.
C) Coding regions of *GST1* genomic clone identified following assembly of sequenced contig.
D) The *GST1* promoter in the pART27 binary transformation cassette including: left border (LB), right border (RB), luciferase (*LUC*), and octopine synthase (OCS) terminator. Neomycin phosphotransferase (*NPTII*) gene confers kanamycin resistance in plants.

Deletions were made in the resulting *GST1*::*LUC* cassette to enable sequencing of the entire promoter region and functional analysis of putative regulatory motifs that were identified. Digestion by *BstX*I and *EcoR*I followed by incubation with Exonuclease III at staggered timepoints was performed in order to generate fragments differing in size by ~200 bp, which were subsequently transformed into *E. coli* DH5α. Sequencing of the deletions was carried out enabling a contiguous overlapping sequence of the *GST1* genomic clone to be assembled (Figure 6).

A 909 bp promoter sequence upstream of the translation start site was uncovered, the translated region was 1092 bp in length and contained two introns of 92 bp and 110 bp (Fig. 5C). The *GST1* gene is composed of two introns and three exons: in accordance with evolutionary classification by Droog, this gene encodes a type I plant Gst (Droog *et al.*, 1993). This class of Gst is thought to function as a key cellular protectant and other members of this class are inducible by pathogen attack, wounding and lipid peroxidation (Marrs, 1996). During the course of this work a *GST* from a different *Arabidopsis* accession (Y11727) with 98% sequence homology to *GST1* was reported, (Yang *et al.* 1998), suggesting these sequences encode the same protein.

iv) Identification of putative regulatory motifs in the *GST1* promoter

A number of putative regulatory motifs were found in the promoter region of *GST1* which were in concordance with the diverse stimuli known to induce other Gsts (Marrs, 1996). A G-box motif was located at -411 (all positions of motifs are given in relation to translation start site). This element is present in many diverse plant genes that respond to an array of stimuli and has been shown to bind basic leucine zipper (bZIP) proteins (Droge-Laser *et al.* 1997). It is thought to be important for transcriptional activation *per se*, rather than conveying responsiveness to specific stimuli (Menkens *et al.* 1995). Subsequent analysis of transgenic plants transformed with deletions of the *GST1* promoter fused to luciferase showed that a 200 bp region containing the G-box was required for background and inducible *GST1*::*LUC* activity (data not shown).

```
-909  GGTACCAAAAGAAGAAGAATTAATCTTATAATCTTGTGATGTACTTTCGG
-859  TGATTTCTTTAAGTTTGATGTTATGAATAAAAGGCAAAGCTTTTGCAAAA
-809  ATCACTCTTTTTTTTTGCCATAATGATTTCAAAATTCCAAAGAATAATATA
                              ERE
-759  TACTTCAATATACATGTCACAAGATTAAATGTCAAAGTTGTTTATAATGA
-709  GCATTTTTGTGGATGTGGAAAATGTGAAAATTACTCTGTTCCTTTGAATG
-659  TTTCTATACGAAATTATAGTTAGGATTTAGTAATGTATTTCTCCATAATT
-609  ATGCTAAATTTAGTTAGTTACTTCAATATGATTAAATACTTTATTGACCC
-559  CAAATTTGTAATTGTACCAGATTGTCAAAAGTGTGAAACCAAATTTTCTT
-509  TTCTTTTGATATTGTTTTGTTTCTTATTATCTCTCGTTCTATCGAATGAT
-459  CTAACTCGAGCATCCAACGATCTAACTCGAGCATCAACGATCCACGTGGA
                                                G-BOX
-409  CCCAACAACGTCGGTCAGAGTTTGACTAGTAGATGAAGGACTATTCTTGT
-359  GGTCGTTGTCACGCGGTGGCTGATATTTTCTCTTATTTTTATTTTATTTT
-309  AACTATTTTTTACGTTATATTTAAGTCTTGAACCAATAGAAACGACGAAT
-259  CATACATTACTCAGCTTGACTTTGAAATAATCCTATAACAAAAGAGCATT
                              ERE
-209  CCAAGAATTTTATCCAAAAAACAAAATAAAAAAGAGTATTCAAGCTTGGT
-159  GGCGCCGTTTGTTTTTGGTTTATTCACTAAAGTTACTCTGTTTTAGTTGT
-109  ATAAATACACACTCCCATTTGTGTATTTCTTTTCATCAATCACAAAGATC
 -59  TCTCTACTTCAATAAATCTCCACCTTACTTTAAGAACAAGAAAAACACAG
                                        TCA
  -9  TATTAACAATGGCAGGAATCAAAGTTTTCGGTCACCCAGCTTCCACAGCC
            M  A  G  I  K  V  F  G  H  P  A  S  T  A
 +59  ACTAGAAGAGTTCTCATCGCTCTTCACGAGAAGAATGTCGACTTTGAATT
      T  R  R  V  L  I  A  L  H  E  K  N  V  D  F  E  F
+109  CGTTCATGTCGAGCTCAAAGATGGTGAACACAAGAAAGAGCCTTTCATCC
       V  H  V  E  L  K  D  G  E  H  K  K  E  P  F  I  L
+159  TTCGCAACgtgagtacataaaacatctgtcaagacaaaatattatatttc
         R  N
+209  atctagatactgaatcttggtcttaacaatcttgaatattgtttttgcag
+259  CCCTTTGGTAAAGTTCCAGCCTTTGAAGATGGAGACTTCAAGATTTTCGg
      P  F  G  K  V  P  A  F  E  D  G  D  F  K  I  F  E
+309  taaatacaaatatatatcattatagtcatgtttacaaatttttggtttta
+359  tgatcattgcaataatagaaagcagaaacactcaaaaatgtttttttttt
+409  ggtgggcagAATCAAGAGCAATTACTCAATACATAGCTCATGAATTCTCA
                  S  R  A  I  T  Q  Y  I  A  H  E  F  S
+459  GACAAAGGAAACAACCTTCTCTCAACTGGCAAGGACATGGCGATCATAGC
      D  K  G  N  N  L  L  S  T  G  K  D  M  A  I  I  A
+509  CATGGGCATTGAAATTGAGTCGCATGAGTTTGACCCAGTTGGTTCAAAGC
       M  G  I  E  I  E  S  H  E  F  D  P  V  G  S  K  L
+559  TTGTTTGGGAGCAAGTCTTAAAGCCTTTGTATGGTATGACCACAGACAAA
         V  W  E  Q  V  L  K  P  L  Y  G  M  T  T  D  K
+609  ACCGTTGTTGAAGAAGAAGAGGCTAAGCTAGCCAAAGTCCTCGATGTTTA
      T  V  V  E  E  E  E  E  A  K  L  A  K  V  L  D  V  Y
+659  CGAACACAGGCTTGGTGAGTCCAAGTATTTGGCTTCTGACCACTTCACTT
       E  H  R  L  G  E  S  K  Y  L  A  S  D  H  F  T  L
+709  TGGTCGATCTTCACACTATCCCTGTGATTCAATACTTACTTGGAACTCCA
         V  D  L  H  T  I  P  V  I  Q  Y  L  L  G  T  P
+759  ACTAAGAAACTCTTCGACGAGCGTCCACATGTGAGTGCTTGGGTTGCTGA
      T  K  K  L  F  D  E  R  P  H  V  S  A  W  V  A  D
+809  CATCACTTCAAGGCCTTCTGCTCAGAAGGTTCTTTAAGTGAATCTCAAAC
       I  T  S  R  P  S  A  Q  K  V  L  *
+859  TATTTATCGATCAGAAGATGTAATAAAAGTGGCGATGACCTAATTGCCCA
+909  ATTCTCATAAACTCTGTTTTTCTTTTTGTGTTTACTCTGTTTTTCTGCTG
+959  CCGAGTGTTTGATTTGAATGTAATCTTCGATAATGAGTCTTGATATAATA
+1009 AAATATTTCTTATCTTAAAGTTTAACTACCAATTAATGTTGATCTCATTC
+1059 TTCAAAGTTGACTATCATTCTCCTAAGTCTTGAACGTTGACTTCCAAATT
```

Figure 6: The *GST1* genomic clone

Numbers refer to nucleotide position in relation to translation start site. Introns are indicated in lower case. The TATA box and two polyadenlyation sites are shaded and motifs in the promoter are in bold. An asterisk indicates the stop codon.

A putative TCA element was found at the 3' end of the *GST1* promoter (-28). This motif is highly conserved in over 30 different stress-inducible genes, including tobacco *PR1a*. This sequence has been shown to bind tobacco nuclear protein (TCA-1), and this binding activity was increased in the presence of SA (Goldsbrough *et al.* 1993). In addition, two ethylene responsive elements were located at -787 and -240. This motif has previously been shown to be essential for ethylene responsiveness when incorporated into a heterologous promoter (Ohmetakagi & Shinshi, 1995). The presence of the TCA and ERE motifs is in accordance with previous observations that *GST1* is induced following treatment with ethylene and SA (Conklin & Last, 1995).

iv) *GST1*::*LUC* plants faithfully report redox signalling following pathogen attack

The reporter gene cassette, *GST1*::*LUC*::*OCS*, was cloned into the binary plant vector pART27 (Gleave *et al.* 1992). This was then transformed into *Agrobacterium tumifaciens* which was used to transform *Arabidopsis* accession Col-0, and over 100 transgenics were generated. Analysis of 20 of these lines revealed that whilst there was some variation in the background level of *GST1*::*LUC* (probably due to different points of integration into the plant genome), relative induction in response to different stimuli remained constant (data not shown). One of these lines was selected for further analysis.

Expression of the *GST1*::*LUC* cassette was quantified using an ultra low light imaging camera system, following leaf inoculation with *Pst* DC3000 expressing either the *AvrB* or *AvrRpt2* (Innes *et al.* 1993) avirulence genes (Figure 7). The temporal profile of Luc activity established following *Pst* DC3000(*AvrB*) inoculation was congruent with the expression of the endogenous *GST1* gene: Luc activity was first detected approximately 45 minutes post-inoculation, with a maximal induction of approximately 20 fold at 2-3 hours, followed by a steady decay of Luc activity. Similar results were obtained following inoculation of *Pst* DC3000(*AvrRpt2*), although the maximum Luc activity measured was approximately 25% less than

following *Pst* DC3000(*AvrB*) inoculation. Infiltration with $MgCl_2$ or virulent *Pst* DC3000 resulted in an approximately 5 fold increase in Luc activity.

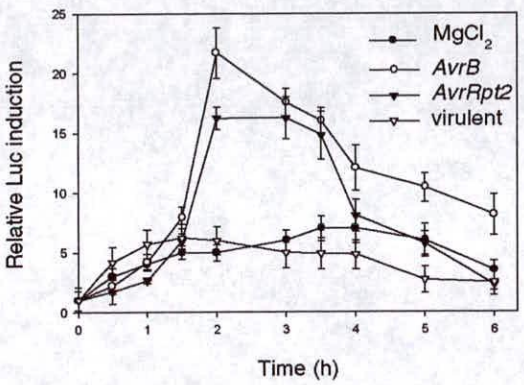


Figure 7: The *GST1::LUC* reporter cassette faithfully reports accumulation of H_2O_2 *in planta*. Time course of *GST1::LUC* induction post inoculation with $MgCl_2$, *Pst* DC3000 harbouring the *AvrB* or *AvrRpt2* avirulence genes and virulent *Pst* DC3000.

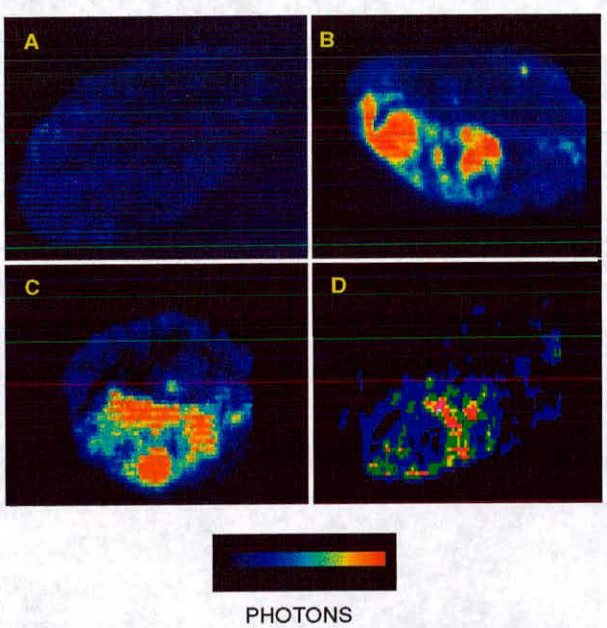


Figure 8: *GST1::LUC* induction is dependent on accumulation of H_2O_2 but not O_2^-. **(A)(B)(C)(D)** Bioluminescent images of *GST1::LUC* induction collected following inoculation with *Pst* DC3000(*AvrB*) at 0 hours (**A**), 2 hours (**B**), and at 2 hours with superoxide dismutase (**C**) or catalase (**D**).

In order to confirm ROI accumulation is responsible for activation of the *GST1::LUC* transgene, two enzymatic scavengers, catalase (CAT) and superoxide dismutase (SOD) were independently co-inoculated with *Pst* DC3000(*AvrB*) (Figure 8). While Luc activity was substantially reduced following co-inoculation of CAT (Figure 8D), in contrast SOD did not significantly diminish Luc activity (Figure 8C). This observation suggested that accumulation of H_2O_2 rather than O_2^- was the major cue responsible for *GST1::LUC* induction.

v) A functional bacterial secretion system is necessary to engage oxidative burst and downstream signalling

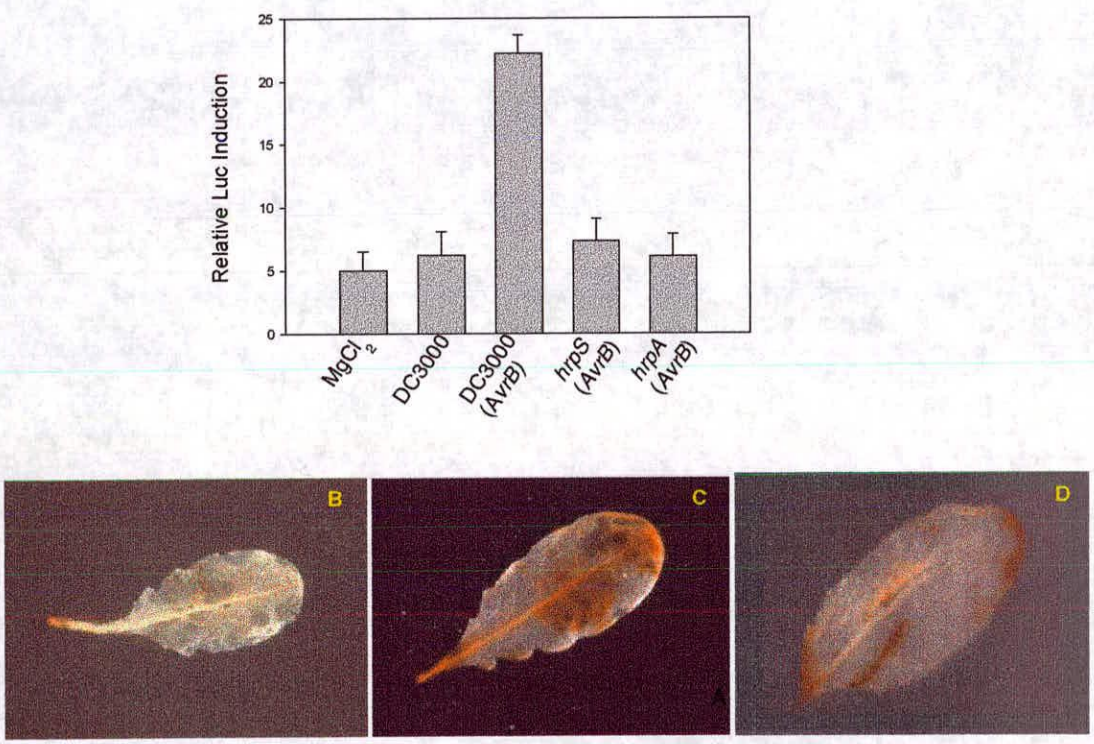


Figure 9.*GST1::LUC* induction is dependent on functional *HrpS* and *HrpA* gene products.
(A) *GST1::LUC* induction measured 2 hours after mock inoculation with $MgCl_2$, *Pst* DC3000, and *Pst* DC3000(*AvrB*) with null mutation in *HrpS* or *HrpA* gene respectively.
DAB staining carried out in leaves challenged with *Pst* DC3000(*AvrB*) at 0 hours (**B**), 2 hours (**C**), and with *hrpS* mutant (transformed with pAvrB plasmid) at 2 hours (**D**).

The hypersensitive response and pathogenicity (*Hrp*) genes of Gram-negative plant pathogenic bacteria are thought to encode a type III protein-secretion system that may deliver avirulence proteins inside host plant cells (Fenselau *et al.*, 1992). This prompted us to examine if engagement of the oxidative burst and cognate redox signalling was *Hrp* gene dependent. Strains of *Pst* DC3000 possessing a null mutation within either the *HrpA* or *HrpS* gene (Roine *et al.*, 1997) were transformed with plasmid pVB01 containing the *AvrB* gene (Innes *et al.*, 1993) and assessed for their ability to engage the oxidative burst. Inoculation of *Pst* DC3000(*AvrB*) *hrpA* or *hrpS* mutants failed to induce Luc activity above the levels recorded for *Pst* DC3000 and $MgCl_2$ control inoculations (Figure 9A).

Moreover, no significant accumulation of H_2O_2 was detected by DAB staining (performed by G.Loake) following inoculation with *Pst* DC3000(*AvrB*) *hrpS* (Figure 9D) nor *hrpA* strains (data not shown). Therefore, functional *HrpA* and *HrpS* gene products are required for successful engagement of the oxidative burst during *AvrB/RPM1* mediated disease resistance.

vi) *GST1::LUC* expression shows distinct changes in spatial expression during development of the HR

Using time lapse image capture for 24 hrs, we determined the temporal and spatial profile of Luc activity established during the dynamic events associated with the hypersensitive response (HR). Key images are shown in Figure 10. Interestingly, the presence of Luc activity within directly challenged cells at 8 hours post *Pst* DC3000(*AvrB*) inoculation, suggested *GST1* transcript accumulation preceded their subsequent programmed execution during the HR (Figure 10B&C). This observation differs from that predicted by previous models in which *GST* gene expression was proposed to occur only in the distal unchallenged cells (Tenhaken *et al.* 1995).

The onset of hypersensitive cell death usually occurred between 7-12 hours post *Pst* DC3000(*AvrB*) inoculation and resulted in a rapid reduction of Luc activity in directly challenged cells during the phase of lesion spread. At 16 hours post inoculation, viable unchallenged cells delimiting the developed HR lesion continued

to express Luc activity, probably reflecting the impact of other defence signals temporally resolved from ROIs (Figure 10C). Observation of this cellular margin,

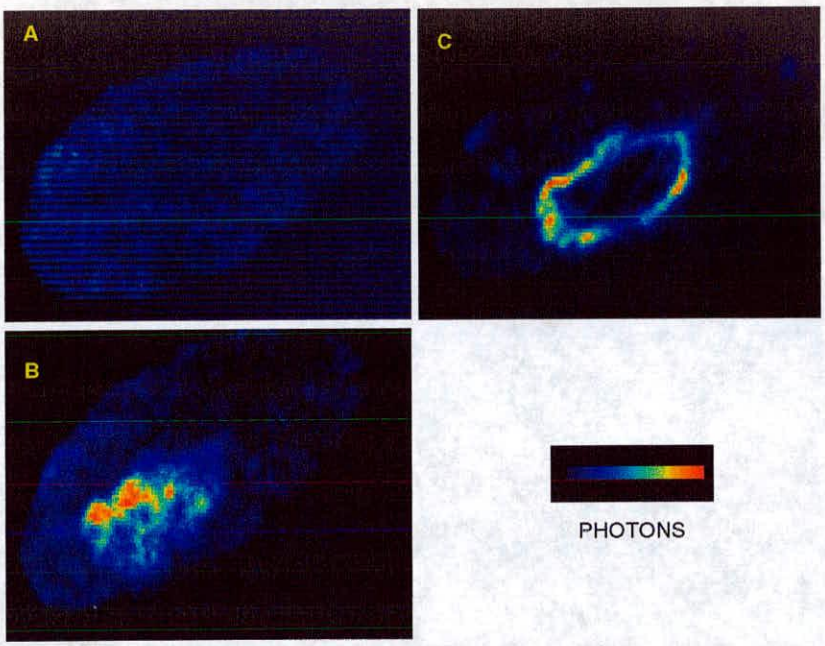


Figure 10: Spatial expression of *GST1::LUC* changes with onset of the hypersensitive response. (**A**)(**B**)(**C**) Bioluminescent images of *GST1::LUC* induction collected following inoculation with *Pst* DC3000(*AvrB*) at 0 hours (**A**), 8 hours (**B**) and 16 hours (**C**).

using a light microscope attachment, revealed the width of Luc activity was approximately 20 cells (data not shown).

Having previously demonstrated that the *GST1::LUC* transgenic line faithfully reports accumulation of H_2O_2 and immediate signalling events, it could be employed in conjunction with mutant bacteria, plants and loss and gain of function inhibitor studies. Inoculation of *Pst* DC3000(*AvrB*) containing mutations in either *HrpA* or *HrpS* failed to result in significant ROI accumulation or strong induction of Luc activity. The *HrpS* and *HrpA* gene products are thought to be required for the development and structural integrity respectively of the Hrp pilus, a filamentous surface appendage which may function as a conduit for the delivery of *Pst avr* gene products to the inside of plant cells (Roine *et al.*, 1997). Hence, Hrp pilus formation and possibly the successful delivery of Avr proteins may constitute a prerequisite for successful engagement of phase II of the oxidative burst.

vii) Discussion

To aid the functional dissection of the oxidative burst and cognate redox signalling, we developed a novel *GST1::LUC* transgenic line to report, in real time, these dynamic cellular processes during the establishment of plant disease resistance. Leaf infiltration of $MgCl_2$ or virulent *Pst* DC3000 resulted in a small but reproducible increase in Luc activity. This response probably reflected engagement of the so-called phase I oxidative burst, which is thought to be associated with wounding or inoculation with virulent microbial pathogens. In contrast, the large increase in Luc activity induced following *Pst* DC3000(*AvrB*/*AvrRpt2*) inoculation probably resulted from engagement of the phase II burst, which is thought to correlate with the establishment of disease resistance (Levine *et al.*, 1994).

Co-inoculation of the H_2O_2 scavenging enzyme CAT with *Pst* DC3000(*AvrB*) significantly blunted the induction of Luc activity, confirming ROIs functioned as the signal mediating *GST1::LUC* gene expression. Due to size exclusion, infiltrated CAT was not expected to enter plant cells. The source of the ROI signal is therefore presumably the apoplast. Co-inoculation of the O_2^- scavenger SOD with *Pst* DC3000(*AvrB*) in contrast, did not significantly decrease Luc activity. Hence, H_2O_2 rather than O_2^- is likely to be the ROI that cues the activation of *GST1::LUC* expression.

In bacteria and yeast compelling evidence suggests ROI can engage distinct redox signalling pathways. For example, H_2O_2 signalling is mediated through the transcription factor OxyR in *Escherichia coli* (Storz *et al.*, 1990), while the *SoxR* and *SoxS* gene products are required for the perception of O_2^- (Demple and Amabile-Cuevas, 1991). In a similar fashion, the redox signalling pathway mediating the activation of *GST1*, a prominent marker for the establishment of local and systemic disease resistance, may be engaged specifically by apoplastic H_2O_2.

Having established that the *GST1::LUC* transgenic line faithfully reports accumulation of H_2O_2 and immediate signalling events, it was employed in

conjunction with mutant bacteria impaired in the delivery of avirulence proteins. Inoculation of *Pst* DC3000(*AvrB*) containing mutations in either *HrpA* or *HrpS* failed to result in significant ROI accumulation or strong induction of Luc activity. The *HrpS* and *HrpA* gene products are thought to be required for the development and structural integrity respectively of the Hrp pilus, a filamentous surface appendage which may function as a conduit for the delivery of *Pst Avr* gene products to the inside of plant cells (Roine *et al.*, 1997). Hence, Hrp pilus formation and possibly the successful delivery of Avr proteins may constitute a prerequisite for successful engagement of phase II of the oxidative burst.

4) *GST1*::*LUC* TRANSGENICS AS A TOOL TO DISSECT REDOX SIGNALLING

i) At least two distinct sources of redox cues activate *GST1* gene expression

A number of possible sources may contribute to ROI accumulation during the pathogen activated oxidative burst including a NADPH-dependent oxidase (Keller *et al.*, 1998; Groom *et al.*, 1996), cell-wall bound peroxidases (Bolwell and Wojitaszek 1997) and apoplastic amine oxidase-type enzymes (Allan and Fluhr 1997). The contribution of these enzymes to ROI accumulation in *Arabidopsis* has not previously been directly compared. Co-inoculation of diphenylene iodonium (DPI), a specific inhibitor of the NADPH-dependent oxidase complex with *Pst* DC3000(*AvrB*) reduced Luc activity by 36% compared to the value obtained with inoculation of *Pst* DC3000(*AvrB*) alone (Figure 11). In a similar experiment, co-inoculation with sodium azide (NaN_3), a specific peroxidase inhibitor, reduced Luc activity by 28% (Figure 11).

Conversely, no reduction in Luc activity was observed when these phamacological agents were individually co-inoculated with H_2O_2, suggesting these agents inhibited H_2O_2 production not perception (data not shown). Neither DPI or NaN_3 blocked the induction of Luc activity in an *Arabidopsis* line containing a *PR1a*::*LUC* marker gene, suggesting these agents did not inhibit either *Pst* DC3000 or Luc activity *in planta* (data not shown). Amines can induce ROI production by acting as substrates for amine oxidases (Allan and Fluhr, 1997). We therefore tested the ability of putrescine and arginine to activate *GST1*::*LUC* gene expression. Infiltration of these amines failed to induce Luc activity above the levels for control inoculations (Figure 11).

The emerging evidence suggests NO may function as a key signal in disease resistance (Delledone *et al.*, 1998; Durner *et al.*, 1998). This prompted us to examine if NO could impact *GST1*::*LUC* gene expression. Infiltration of the NO donor sodium nitroprusside (SNP) failed to induce significant Luc activity and co-inoculation of the NO scavenger N^{ω}-nitro-L-arginine (L-NNA) with

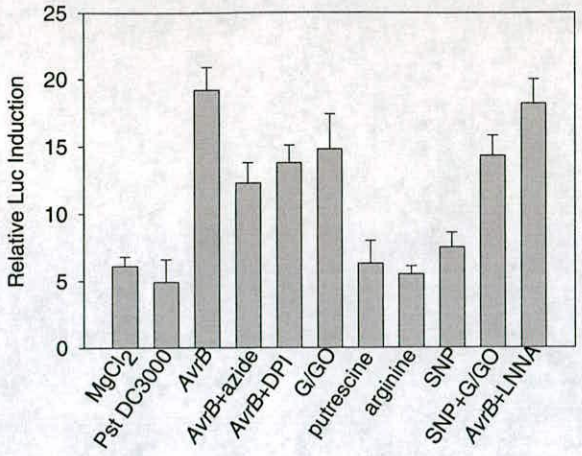


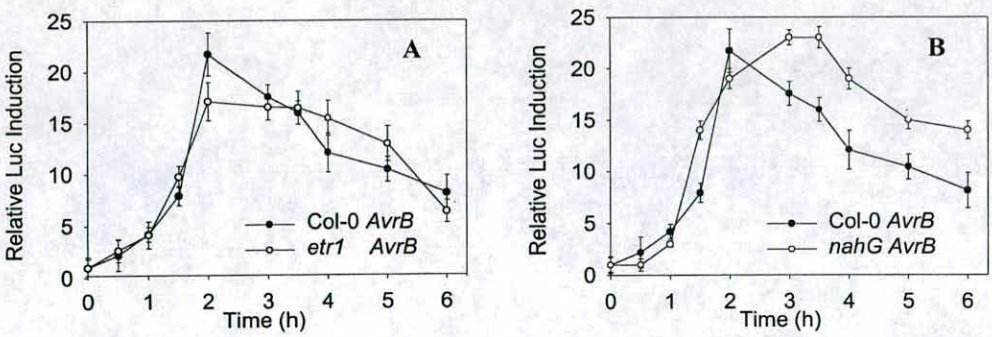
Figure 11: *GST1* expression is dependent on two distinct sources of ROIs but is not potentiated by nitric oxide.

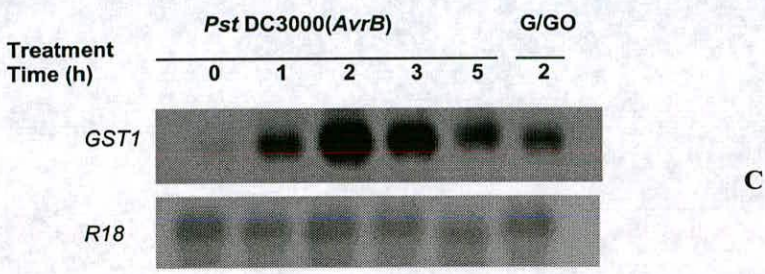
Relative *GST1*::*LUC* induction 2 hours post-inoculation with $MgCl_2$, *Pst* DC3000, *Pst* DC3000(*AvrB*), *Pst* DC3000(*AvrB*)+DPI, *Pst* DC3000(*AvrB*)+Sodium Azide, G/GO, Putrescine, L-Arginine, Sodium nitorprusside (SNP), SNP+G/GO, and *Pst* DC3000(*AvrB*)+L-NNA.

Pst DC3000(*AvrB*) did not significantly blunt the activation of Luc activity (Figure 11). Moreover, co-inoculation with G/GO and SNP did not significantly potentiate the induction of Luc activity compared to G/GO alone. In total, these observations suggested that NO did not directly or indirectly impact the redox signalling network that mediates *GST1* gene expression. Our results therefore suggested that at least two distinct enzymatic sources including an NADPH oxidase and a peroxidase-type enzyme generated the redox cues that engaged *GST1* gene expression.

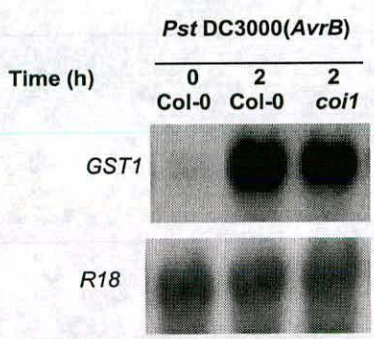
ii) Engagement of the oxidative burst and cognate redox signalling is independent of ethylene, SA or Me-JA

Ethylene, Me-JA and SA are thought to play key roles in plant defence signalling (Xu *et al.*, 1994; Gaffney *et al.*, 1993). We therefore investigated if any of these signal molecules mediated either the activation of the oxidative burst or the subsequent transduction of redox signals. *Arabidopsis* Col-0 plants containing the *GST1*::*LUC* transgene were crossed with plants containing the ethylene insensitive allele *etr1* (Bleecker *et al.*, 1988) and with plants containing the *nahG* transgene,





C



D

Figure 12: ROI mediated induction of *GST1* is not dependent on ethylene, SA or Me-JA.

(A) Time course of *GST1::LUC* induction in *etr1* mutant background *cf.* Col-0 wild-type.

(B) Time course of *GST1::LUC* induction in *nahG* mutant background *cf.* Col-0.

(C) Northern blot hybridisation of *GST1* transcripts following inoculation with *Pst* DC3000(*AvrB*) at different time intervals, and G/GO in *nahG* background.

(D) Northern blot hybridisation following inoculation with *Pst* DC3000(*AvrB*) at 0 hours in Col-0, and at 2 hours in Col-0 and *coi1* mutant.

which converts SA to catechol (an inactive metabolite with respect to defence signalling) (Gaffney *et al.*, 1993). These plants were infiltrated with *Pst*(*AvrB*) and Luc activity recorded over time using an ultra low light imaging camera system. No consistent differences were observed in the profile of *GST1*::*LUC* gene expression in either the *etr1* or *nahG* genetic backgrounds compared to the wildtype Col-0 accession (Figure 12A and 12B). These observations suggested that neither ethylene nor SA impacted either the engagement of the oxidative burst or the subsequent transduction of redox signals.

We also undertook Northern analysis of *GST1* gene expression in *nahG* plants as an alternative experimental strategy to confirm our previous data. The accumulation of *GST1* transcripts in *nahG* plants closely paralleled that observed for wildtype Col-0 plants (Figure 12C), with maximum *GST1* transcript accumulation at 2-3 hours post *Pst*(*AvrB*) inoculation. Furthermore, infiltration of G/GO into *nahG* plants induced maximum *GST1* transcript accumulation at approximately 2 hours, similar to that obtained for wildtype Col-0 plants (Figure 12C). These experiments therefore confirmed our previous observations, which in total showed that local SA accretion was not necessary for the engagement of *GST1*::*LUC* gene expression in response to redox cues. Northern analysis of *GST1* gene expression in the *Arabidopsis* mutant *coi1* (Feys *et al.*, 1994), which is insensitive to Me-JA, was also indistinguishable from that observed with wildtype Col-0 plants (Figure 12D). Hence, the lipid signalling molecule Me-JA also does not appear to play a role in either the activation of the oxidative burst or the subsequent transduction of redox cues.

iii) ROI-induced gene expression is dependent on MAPKK activity

A key feature of redox signalling in animal cells is the pivotal role played by protein kinase cascades (Bauskin *et al.*, 1991). We therefore tested a variety of specific protein kinase inhibitors for their ability to blunt H_2O_2 induced *GST1* gene expression. Col-0 *GST1*::*LUC* plants were inoculated with either G/GO or H_2O_2 in the presence or absence of a given kinase inhibitor and the relative induction of *GST1*::*LUC* gene expression was subsequently determined by measuring the level of Luc activity 2 hours post inoculation.

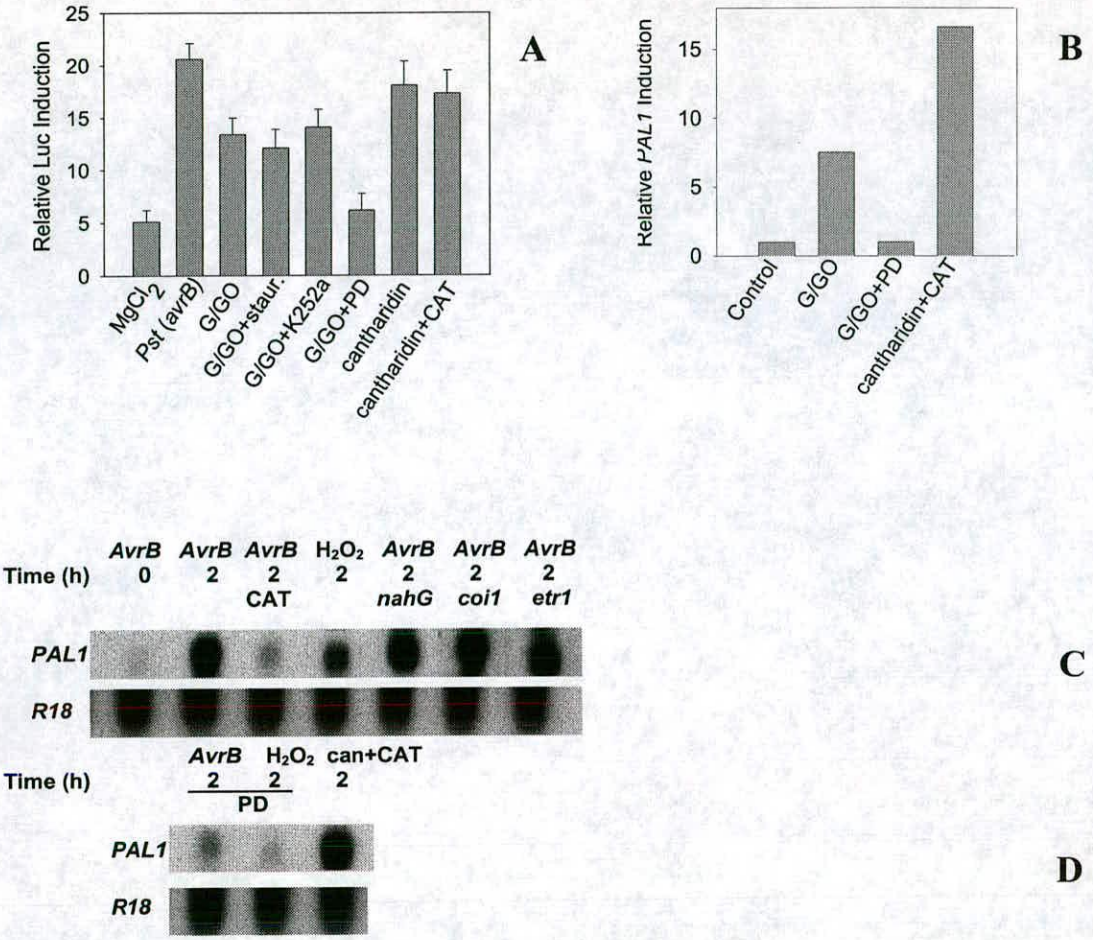


Figure 13: ROI-induced *GST1* and *PAL1* gene expression is dependent on MAPKK activity.

(**A**) *GST1::LUC* induction 2 hours post-inoculation with $MgCl_2$, *Pst* DC3000(*AvrB*), G/GO, G/GO+staursoporine, G/GO+K252a, G/GO+PD98059, cantharidin, and cantharidin+catalase.

(**B**) Northern hybridisation of *PAL1* transcripts following inoculation with *Pst* DC3000(*AvrB*) at 0 hours, *Pst* DC3000(*AvrB*), *Pst* DC3000(*AvrB*)+catalase, H_2O_2, and *Pst* DC3000(*AvrB*) in *nahG*, *coi1* and *etr1* respectively (all at 2 hours).

(**C**) Northern hybridisation of *PAL1* transcripts 2 hours post-inoculation with *Pst* DC3000(*AvrB*)+PD98059, G/GO+PD98059, and cantharidin+catalase.

(**D**) Quantification of *PAL1* transcripts (normalised to *R18*) 0 hours post-inoculation with G/GO (control), G/GO (2 hours), G/GO+PD98059 (2 hours), and cantharidin+catalase (2 hours).

Neither staurosporine nor K252a, two broad spectrum kinase inhibitors, which are widely used in studies of plant biology, significantly inhibited the induction of Luc activity. These two pharmacological agents are well characterised inhibitors of protein kinase C (PKC), protein kinase A (PKA), calmodulin dependent kinase (CaMK) and protein kinase G (PKG). In contrast, co-inoculation of G/GO with PD98059, a specific inhibitor of mitogen activated protein kinase kinases (MAPKKs) (Cohen, 1997), strongly blunted the induction of Luc activity by 46% (Figure 13A). Therefore, a MAP kinase cascade may be an integral component of the redox signalling network that engages *GST1* gene expression in response to ROI accumulation.

To explore this possibility further, we undertook a complementary gain-of-function experiment, using the specific phosphatase 2A inhibitor cantharidin, to examine if this pharmacological agent could induce *GST1*::*LUC* gene expression in the absence of redox cues. To discriminate between activation of the oxidative burst (which is also regulated by a phosphorylation cascade (Levine *et al.*, 1994)) and engagement of redox signalling, cantharidin was co-inoculated with the H_2O_2 enzyme scavenger catalase (CAT), in order to blunt any H_2O_2 accumulation resulting from possible engagement of the oxidative burst machinery. Inoculation of 300 units/ml of CAT had previously been demonstrated to strongly diminish the magnitude of *GST1*::*LUC* gene induction in response to H_2O_2 generated in response to *Pst* DC3000(*AvrB*) inoculation (Figure 8D). The results clearly demonstrated that cantharidin strongly induced Luc activity even in the presence of CAT (Figure 13A). The expression of the *GST1* gene may therefore be regulated by a poise between phosphorylation/dephosphorylation. Similar results were obtained using Northern blot analysis (data not shown).

In addition, we also examined the response of a phenylalanine ammonia-lyase (*PAL1*) gene, which has been previously demonstrated to be strongly induced in response to ozone in a SA-independent manner (Sharma *et al.*, 1996). Inoculation with *Pst* DC3000(*AvrB*) resulted in strong accumulation of *PAL1* transcripts within two hours (Figure 13C). Revealingly, co-inoculation of *Pst* DC3000(*AvrB*) with

CAT significantly blunted *PAL1* gene expression, while inoculation of G/GO strongly induced the expression of this gene. Thus, ROIs may constitute a key signal for the activation of *PAL1* in response to *Pst* DC3000(*AvrB*) inoculation.

In a similar fashion to *GST1*, engagement of *PAL1* gene expression in response to *Pst* DC3000(*AvrB*) inoculation was not abrogated in either a *nahG*, *coi1* or *etr1* genetic background (Figure 13C). We therefore investigated if the engagement of *PAL1* gene expression by ROIs also depended on MAPKK activity. Co-inoculation of PD98059 with either *Pst* DC3000(*AvrB*) or G/GO significantly reduced the induction of *PAL1* compared to that observed with either alone (Figure 13B&D).

In the corresponding gain-of-function experiment, inoculation of cantharidin in the presence of CAT strongly induced *PAL1* gene expression in the absence of H_2O_2. Quantification of these results using phosphorImage analysis, revealed that PD98059 decreased G/GO induction of *PAL1* by 99.5%. In contrast, cantharidin in the presence of CAT activated *PAL1* gene expression by 17-fold (Figure 13B). The sum of the data therefore suggests that MAPKK activity is an integral component of the signal network that couples the induction of both *PAL1* and *GST1* gene expression to redox cues.

To confirm the data derived from the deployment of pharmacological agents, we also determined MAPK activity directly by means of in-gel kinase assay (experiments performed by B-Y Wook - data not shown). Two kinases that bound the myelin basic protein (MBP) substrate of approximately 48- and 46-kD were rapidly induced by leaf infiltration of G/GO from a low basal level of activity. Induction of both bands was unchanged in the *etr1* and *coi1* mutant backgrounds. In the *nahG* background however, while the basal activity of the 46-kD MBP kinase was similar to that determined for Col-0 plants, the relative induction of this kinase was consistently reduced. Furthermore, PD98059 was shown to significantly blunt G/GO-mediated induction of these two MBP kinases. Subsequent results obtained by means of Western analysis using a radiolabelled antibody (Phospho-p44/42 MAP kinase (Thr202/Tyr204) Antibody - New England Biolabs) raised against

phosphorylated MAPK were entirely consistent with the data derived from the in-gel kinase assays. Thus, PD98059 may blunt activation of *GST1* and *PAL1* expression by inhibiting MAPKK activity and consequently suppressing the activation of the two MAPKs.

iv) Discussion

While it is becoming increasingly apparent that ROI integrate a diverse set of complementary defence mechanisms, the identity of the molecular machinery underlying the oxidative burst still remains to be rigorously established. In bean and cotton the pathogen activated oxidative burst is cyanide sensitive and apoplastic peroxidases are thought to be a direct source of ROIs in these cases (Bolwell and Wojtaszek 1997; Martinez *et al.*, 1998). However, a DPI inhibited NADPH oxidase activity has been proposed to mediate the oxidative burst in soybean and tobacco cell suspension cultures (Levine *et al.*, 1994; Piedras *et al.*, 1998).

As many of the reported studies to date have been undertaken in different experimental systems and have focused on only one possible enzymatic mechanism, we employed a variety of pharmacological agents to investigate the enzymatic source(s) of the redox cues responsible for engaging *GST1* gene expression. Co-inoculation of either the NADPH oxidase inhibitor DPI or the peroxidase inhibitor NaN_3 in combination with *Pst* DC3000(*AvrB*/*AvrRpt2*) significantly decreased the induction of *GST1* gene expression. In contrast, no role could be found for the generation of ROIs via amine oxidase-type enzymes. Subject to the usual caveats associated with the deployment of these pharmacological agents (Barcelo, 1998), the data suggested that both an NADPH oxidase and a peroxidase-type enzyme contributed to the generation of redox signals that cued the engagement of *GST1* gene expression in response to attempted *Pst* DC300(*AvrB*) infection. Hence, mechanistically, the oxidative burst in *Arabidopsis* may resemble that of lettuce, which may also generate ROIs via both an NADPH oxidase system and apoplastic peroxidases (Bestwick *et al.*, 1998). It will be interesting to assess if the contribution of these enzymes to ROI production varies in response to different pathogens.

Recently, NO has been proposed to potentiate the induction of hypersensitive cell death in soybean cells by ROIs and function independently of such intermediates to induce gene expression during the establishment of disease resistance (Delledone *et al.*, 1998; Durner *et al.*, 1998). The deployment of both gain- and loss-of-function experiments however did not identify a direct function for NO in the engagement of *GST1* gene expression. Moreover, NO was found not to potentiate the activation of *GST1* via ROIs. No direct or indirect role for NO in the redox regulation of *GST1* was therefore established. Thus, the key redox responsive switch(es) integral to the signalling network regulating *GST1* gene expression are more likely to constitute critical regulatory thiols, the preferred targets of ROIs, rather than iron targets, the prototypic preference of NO (Stamler 1994).

Ethylene, SA and Me-JA are thought to constitute key defence signalling molecules that function to coordinate a diverse array of defence mechanisms which underlie the establishment of disease resistance (Xu *et al.*, 1994; Gaffney *et al.*, 1993; Feys *et al.*, 1994). We therefore examined the potential role of these molecules on both the generation and perception of ROIs by monitoring *GST1* gene expression in *coi1* and *etr1* plants, which are insensitive to ethylene and Me-JA respectively. Neither mutant background significantly impacted either the magnitude of induction or the temporal expression profile established by the *GST1* gene in response to inoculation with *Pst* DC3000(*AvrB/AvrRpt2*). Moreover, similar observations were derived from Northern analysis of the redox responsive *PAL1* gene. Thus, engagement of the oxidative burst and cognate redox signalling may occur independently of Me-JA and ethylene. These conclusions however, do not preclude a potential role for these key defence signalling molecules in the engagement of the oxidative burst and cognate redox signalling in tissues exhibiting either local or systemic acquired resistance, where their respective concentrations may exceed a critical threshold value.

Similar experiments were undertaken in *nahG* plants, which show reduced accumulation of SA in response to attempted pathogen invasion (Gaffney *et al.*, 1993). Neither expression of a *GST1*::*LUC* reporter gene nor the accumulation of endogenous *gst1* transcripts were affected in a *nahG* genetic background. Hence, a

suppression in the local accretion of SA was found not to impact either ROI production or the subsequent transmission of redox signals. These observations contrasted with previous studies that have suggested SA accumulation may potentiate the oxidative burst (reviewed in Van Camp *et al.*, 1998). In cucumber hypocotyls however, this phenomenon required an 18 hour conditioning process that depended on *de novo* protein synthesis (Fauth *et al.*, 1996). This mechanism would therefore probably not be operational in our experimental system. In contrast, co-application of SA in conjunction with an avirulent pathogen has been reported to potentiate the oxidative burst without a prior conditioning step in soybean suspension cultures (Shirasu *et al.*, 1997). However, the required SA concentration has only been measured routinely in tissues surrounding local HR lesions (Malamy *et al.*, 1990). Thus, this mechanism also may not be operational in naive tissue but deployed following the development of acquired resistance in local and possibly systemic tissues. Our observations of redox responsive gene expression in naive tissue of *nahG* transgenic plants would be consistent with this hypothesis.

A pivotal role for MAPKs in disease resistance has recently begun to emerge (Ligterink *et al.*, 1997; Romeis *et al.*, 1999), although these kinases are thought to function independently of the oxidative burst. Interestingly, it has recently been reported that the overexpression of constitutively active deletion mutants of a mitogen activated protein kinase, kinase, kinase (MAPKKK), activated 2 of the 6 co-overexpressed MAPKs tested in *Arabidopsis* protoplasts exposed to acute H_2O_2 stress (Kovtun *et al.*, 2000). Moreover, exposure of tobacco cell suspension cultures to necrosis inducing concentrations of ozone has also recently been proposed to stimulate MAPK activity (Samuel *et al.*, 2000).

In the context of plant disease resistance, our observations have extended these studies by demonstrating that sustained production of sub-lethal concentrations of ROIs, mimicking the kinetics of the oxidative burst, resulted in the rapid activation of 48- and 46-kD MBP kinase activities in *Arabidopsis* leaf tissue. The substrate specificity and molecular mass of these kinases predicted a MAPK identity, later confirmed by Western blot analysis using an antibody raised against a

phosphorylated MAPK. Moreover, PD98059, an inhibitor of MAPKKs, completely suppressed the activation of both kinases, suggesting MAPKK activity is a prerequisite for their activation, further underscoring their proposed MAPK identity. The molecular mass and activation kinetics of the 48- and 46-kD MBP kinases suggest they belong to the stress activated class of MAPKs in *Arabidopsis* (Mizoguchi *et al.*, 1997), which contain orthologues of the SA inducible (SIPK) and wound inducible (WIPK) MAPKs from tobacco, which may be important mediators of SA and Me-JA-dependent signalling respectively (Zhang and Klessig, 1997; Seo *et al.*, 1995).

The induction of the redox responsive genes *GST1* and *PAL1* were found to occur independently of SA, Me-JA and ethylene. We therefore investigated the activation of the 48- and 46-kD kinases in *nahG*, *coi1* and *etr1* genetic backgrounds in response to sustained ROI accumulation. Activation of the 48-kD kinase in these genetic backgrounds paralleled that which occurred in wildtype plants. Hence, this kinase is activated independently of the action of SA, Me-JA and ethylene. In contrast, while the activation of the 46-kD kinase was similar in *coi1* and *etr1* mutants compared to that of wildtype plants, in a *nahG* genetic background the activation of this kinase was consistently reduced in comparison with wildtype plants. Hence, SA may be either necessary or sufficient for maximum activation of this kinase. The 48- and 46-kD kinase activities can therefore be resolved in the absence of SA. As the induction of *GST1* and *PAL1* genes are not affected within a *nahG* genetic background, the 48-kD rather than the 46-kD kinase probably undertakes a predominant functional role in the transmission of redox cues. A MAPK module may therefore be a key feature of the redox signalling pathway engaged following activation of the oxidative burst during the establishment of disease resistance.

In animal cells there is compelling evidence for both direct and indirect mechanisms for the regulation of gene expression in response to changes in cellular redox status. In response to severe hyperoxic states, key cysteine residues of redox modulated transcription factors may become oxidised, effecting changes in the expression profile of their target genes (Abate *et al.*, 1990). In contrast, signal transmission in

response to lower levels of ROIs may require the action of specific protein kinases. Recently, ROIs have been shown to engage the stress-activated class of MAPKs (SAPKs), including the c-Jun N-terminal kinase (JNK) group, that contribute to a MAPK cascade activated in response to specific environmental stresses (Klotz *et al.*, 1999). Hence our data highlighting a pivotal role for a MAPK module in ROI-mediated signalling during the establishment of disease resistance suggests significant parallels may exist in the transduction of stress induced redox signals in plants and animals.

Local ROI accumulation has recently been shown to lead to the establishment of acquired resistance in both local and systemic tissues (Chamnongpol *et al.,* 1998; Alvarez et al., 1998). This mechanism is thought to operate via the SA-dependent induction of acidic PR proteins (Chamnongpol *et al.*, 1998) and the production of SA precursors is known to be a major function of PAL during the development of disease resistance (Mauch-Mani and Slusarenko 1996). We have demonstrated that expression of the *Arabidopsis PAL1* gene is mediated via redox cues following engagement of the oxidative burst. Moreover, this redox signalling network functions independently of SA, Me-JA and ethylene but is dependent on a 48-kD MAPK activity. Thus, local ROI production during the oxidative burst and reiterated systemic microbursts may activate the expression of *PAL1*, thereby driving the biosynthesis of SA, leading to the accumulation of PR proteins and the subsequent establishment of acquired resistance in both local and systemic tissues. The oxidative burst may therefore engage a bifurcating redox signalling pathway that orchestrates the production of both key defence signals and pivotal antioxidant defences, leading to the development of both disease resistance and the limitation of HR lesion formation respectively.

The *GST1*::*LUC* transgenic line described here has been deployed to undertake a saturating genetic screen to uncover mutations that impact redox signal transmission through this network (screen performed by B-W Yun). The characterisation of the corresponding mutants should provide significant insights into the mechanisms underlying redox signalling during the establishment of plant disease resistance.

5) ISOLATION AND CHARACTERISATION OF A NOVEL DISEASE RESISTANT MUTANT BY ACTIVATION TAGGING

i) Introduction

Mutant screens have been used extensively as a means of dissecting genetic pathways in a wide array of different organisms. In plants this has often taken the form of EMS mutagenesis, neutron particle bombardment and T-DNA tagging. EMS and irradiation are efficient methods of generating large numbers of mutations that effectively saturate the genome. However, a significant drawback of these methods is the subsequent lengthy process of gene-cloning associated with chromosome walking. Cloning genes from T-DNA tagged plants is comparatively more straightforward, although chromosomal rearrangements may make plasmid rescue difficult (Feldmann, 1991).

One major shortcoming of all three methods of mutant isolation is that they are largely confined to the isolation of loss-of-function mutations. As such, genes whose function is essential during multiple stages of the plant life-cycle will not be uncovered if their disruption results in a lethal phenotype. In addition, sequencing of the *Arabidopsis* genome has revealed a high degree of apparent gene duplication (Bevan *et al.* 1998), so loss of function screens rarely identify genes that act redundantly.

Activation tagging has recently been developed as a means of isolating gain-of-function mutations and corresponding genes (Kakimoto 1996; Kardailsky *et al.* 1999; Weigel *et al.* 2000). In addition to gain-of-function mutants, insertional loss-of-function knockouts can also be uncovered in the T_2 generation. The binary vector pSKI015 (Figure 14A) (Walden *et al.*, 1994; Weigel *et al.* 2000) has been designed specifically for this purpose: between the left and right border is a tetramer of the *CaMV 35S* enhancer (Fang *et al.* 1989) that is randomly integrated in the plant genome. Endogenous expression of genes immediately adjacent to the site of insertion should thus be significantly enhanced (Figure 14B). Also integrated into the transformation cassette is the *BAR* gene, facilitating high-throughput screening of

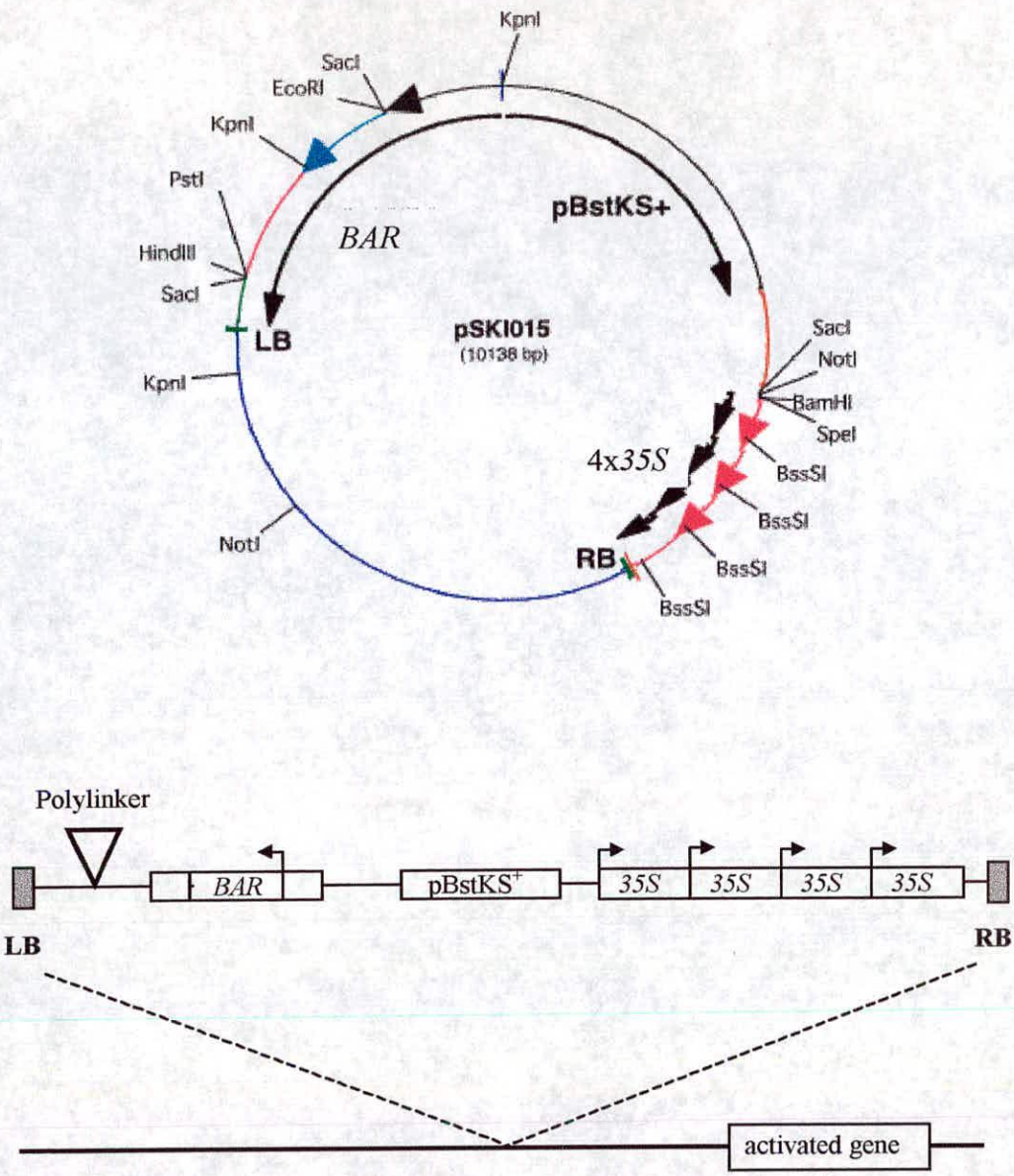


Figure 14: The activation tagging vector pSKI015

(**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 pBstKS$^+$ plasmid) and tetramer of the *35S* enhancer (from Weigel lab web site: www.salk.edu/LABS/pbio-w/researchfs.html).

(**B**) Endogenous expression of genes adjacent to the 4x*35S* enhancer region may be enhanced following integration of the activation cassette into the plant chromosome.

primary transformants by Basta herbicide selection. In addition, the *oriV* origin of replication of *E. coli* enables regions of the plant genome flanking the T-DNA point of insertion to be cloned by plasmid rescue.

ii) Isolation of a mutant displaying spontaneous lesion formation

Approximately 3,000 novel transgenic *Arabidopsis* lines were generated containing the binary cassette of pSKI015 randomly integrated into their genome. A transgenic *PR1a::LUC* line was used for transformation, comprising the tobacco *PR1a* promoter (Uknes *et al.*, 1993) fused to the firefly luciferase gene (Thomson & Loake, unpublished). Such plants had previously been shown to faithfully report accumulation of *Arabidopsis PR1* transcripts following pathogen attack and had been successfully deployed in an EMS mutant screen to isolate SAR signalling mutants (Murray *et al.* - unpublished). These lines were screened in the first (T_1) generation for visual peculiarities and constitutive luciferase activity.

One mutant (subsequently named *adr1-D* for activated disease resistance *1-d*ominant), characterised by its dwarf phenotype, curled leaves, constitutive luciferase activity (Figure 15) and the development of the spontaneous formation of small necrotic lesions under high light conditions was isolated for further analysis. A number of lesion mimic (LM) mutants exhibiting a similar morphological phenotype have previously been isolated, most notably the accelerated cell death (*acd*) mutants (Greenberg *et al.* 1994) and lesions simulating disease resistance response (*lsd*) mutants (Dietrich *et al.* 1994). Such mutants often display elevated levels of SA and enhanced disease resistance (Greenberg 1994 *et al.*; Dietrich *et al.* 1994). Furthermore, no gain-of-function mutant of this type, as distinct from the loss of a negative repressor such as *lsd1* (Jabs *et al.*, 1996), has previously been reported making it an intriguing candidate for further analysis.

Onset of the *adr1-D* phenotype was observed to occur 2-3 weeks after germination prior to which time mutants are indistinguishable from wildtype. From this point, mutants became progressively stunted, and the development of leaf curling was

apparent, with adult plants significantly dwarfed compared to wildtype (Figure 16). Interestingly, the phenotype appeared to be semi-dominant: in homozygotes (i.e. two

Figure 16: The *adr1-D* mutant in comparison to wildtype Col-0

copies of 4x*35S*) leaf curling and dwarfing were greatly exacerbated as compared with the hemizygote and lesions formed even in the absence of high light conditions (Figure 18). Semi-dominance has also been observed in a number of other activation tagged mutants including another disease resistant mutant, *cdr1* (Weigel *et al.*, 2000). Unlike *lsd1*, the phenotype was not strictly dictated by day-length (Jabs *et al.* 1996).

The *adr1-D* mutant was allowed to self-pollinate and the F_2 generation analysed. Basta resistance in the progeny always segregated with lesion formation and constitutive luciferase expression suggesting that the gene conveying this phenotype was tagged. Furthermore, progeny segregated 3:1 ($Basta^R$:$Basta^S$) in 120 lines tested, suggesting T-DNA insertion had occurred at one locus only. *adr1-D* was back-crossed to wildtype Col-0 and progeny allowed to self-fertilise. The resulting F_2 generation of the successful cross were selected for Basta resistance and scored for loss of *PR1a*::*LUC* cassette by lack of constitutive luciferase activity, and these plants were crossed to transgenic *GST1*::*LUC* plants. Basta resistant progeny of successful crosses showed constitutive Luc activity (Figure 17) that also segregated with lesion formation and Basta resistance.

The mutant was crossed to a number of different mutant backgrounds, and progeny of successful crosses allowed to self-pollinate before selecting for homozygotes with

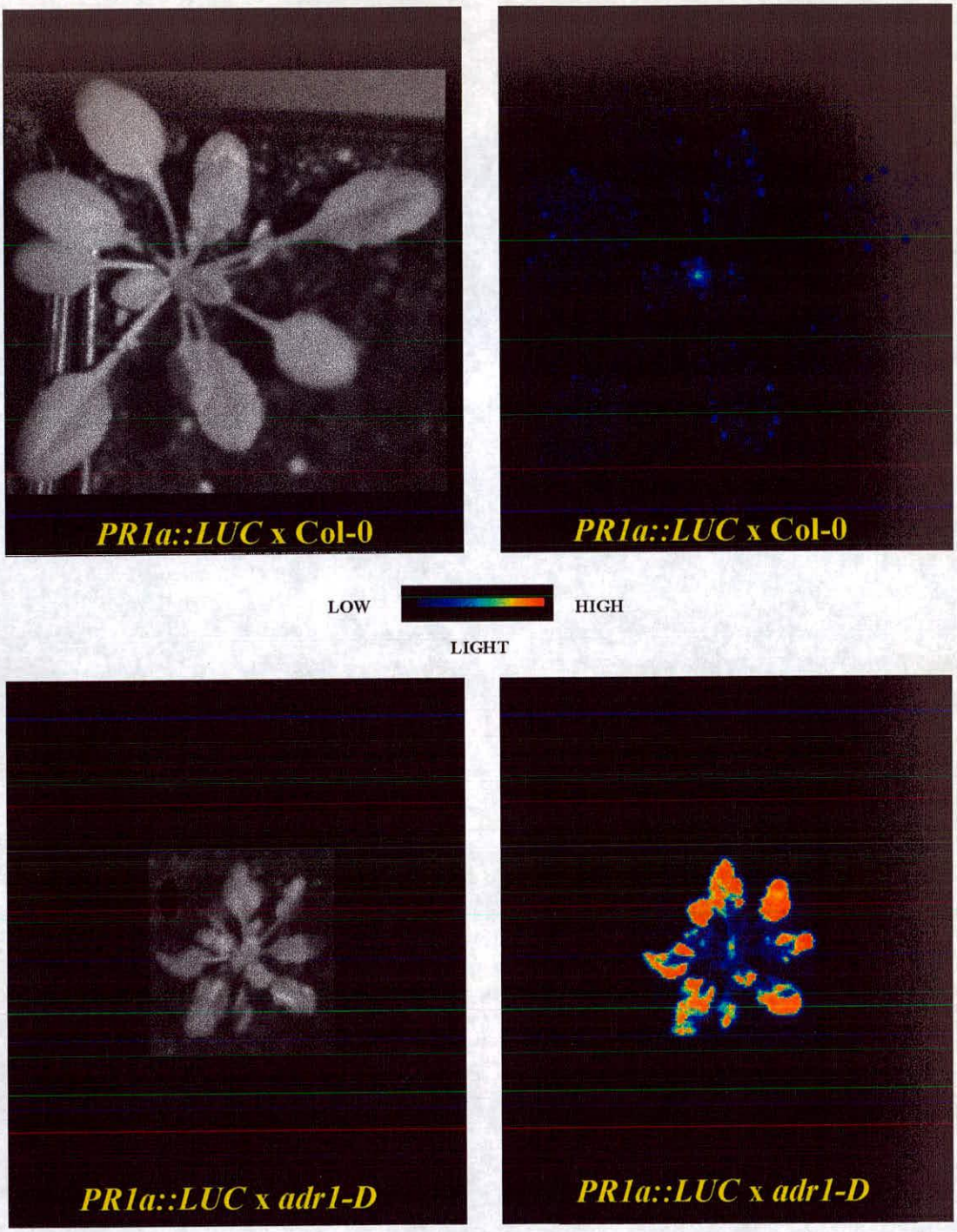


Figure 15: The *adr1-D* mutant constitutively expresses a *PR1a*::*LUC* reporter gene as shown by luciferase imaging

Images routinely collected over 10 seconds exposure. Light intensity corresponds to accumulation of *PR1* gene expression (Thompson and Loake - unpublished).

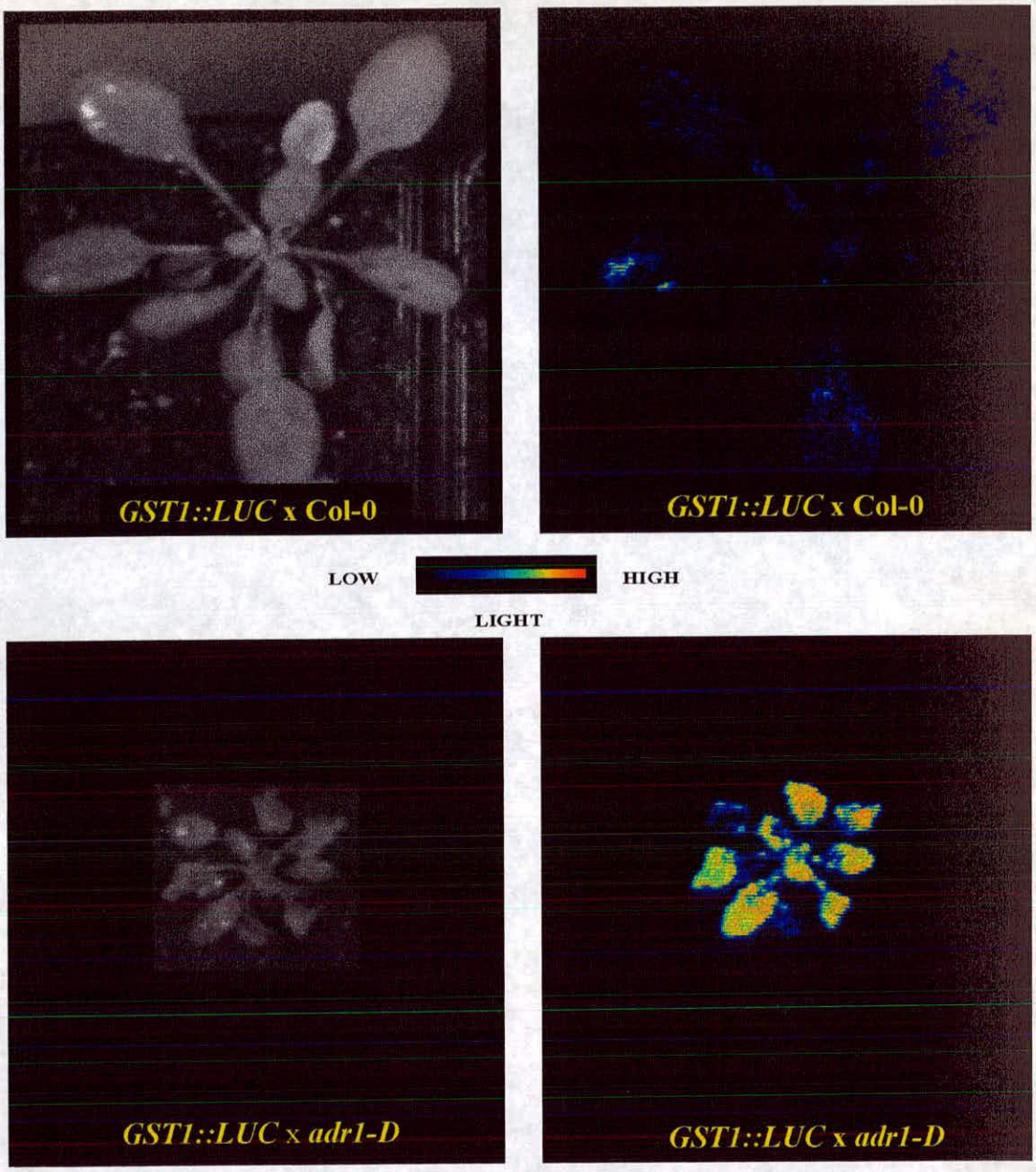


Figure 17: The *adr1-D* mutant constitutively expresses a *GST1*::*LUC* reporter gene as shown by luciferase imaging
Bioluminescent images collected over 1 second. Light intensity corresponds to *GST1* expression (Chapter 3).

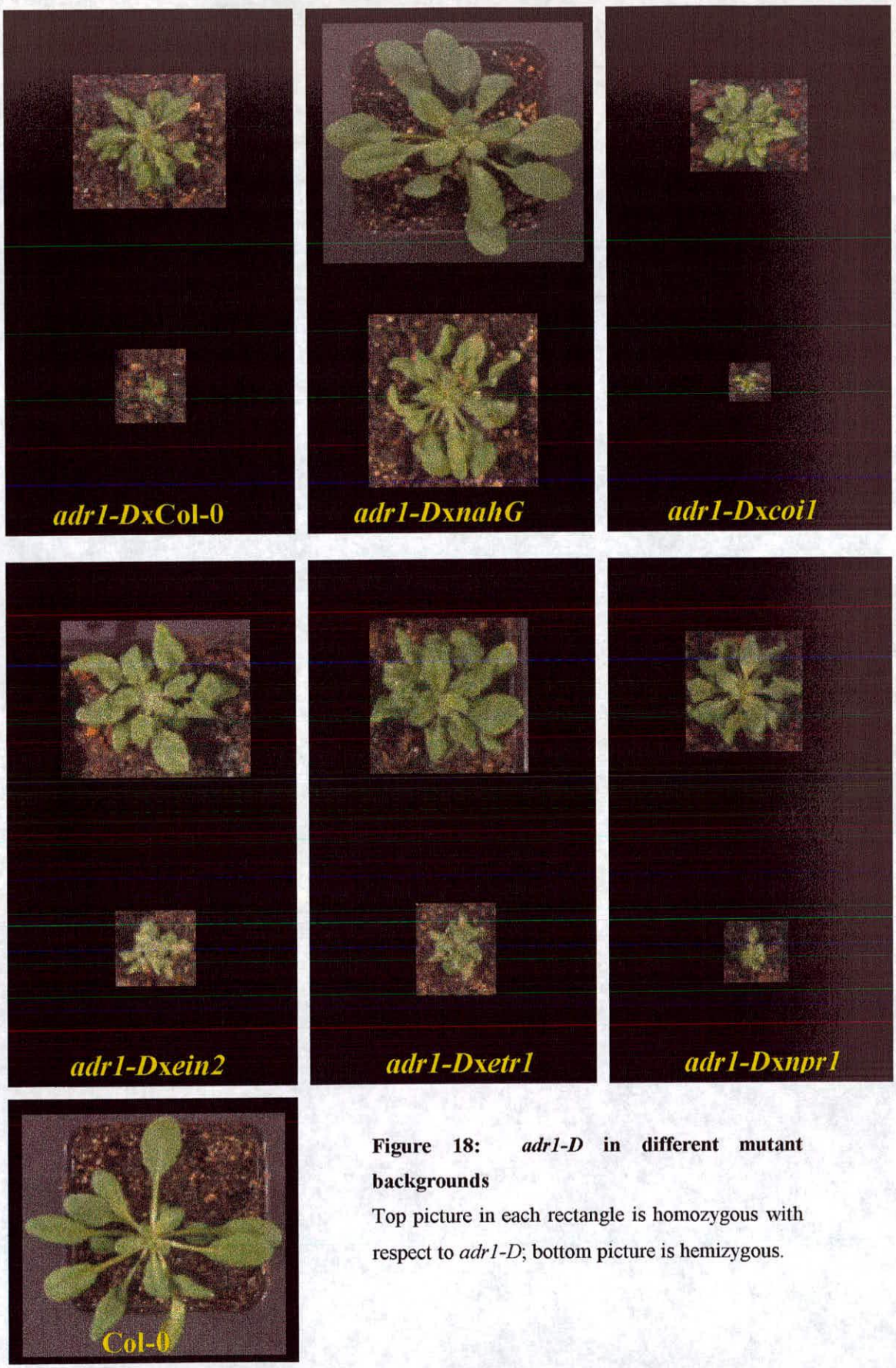


Figure 18: *adr1-D* in different mutant backgrounds

Top picture in each rectangle is homozygous with respect to *adr1-D*; bottom picture is hemizygous.

respect to mutant background. The mutants (*nahG*, *etr1*, *ein2-1*, *coi1*, *npr1*) were chosen for crossing purposes as they have all been implicated in disease resistance pathways (chapters 1 and 4). The double mutants were characterised at 4 weeks in terms of severity of the *adr1-D* visible phenotype and accumulation of defence-related transcripts. In the case of *adr1-DxnahG* mutants, H_2O_2 accumulation and lesion proliferation were also assessed, and bacterial and fungal pathogenicity assays performed.

iii) Morphological phenotype is mediated by SA, ethylene, and *NPR1*, but not by JA signalling

The gross morphology of double mutants relative to wildtype is summarised in the panel of photos in Figure 18. Some lesion mimic mutants such as *lsd6* and *lsd7* have previously been shown to lose the visible phenotype in the *nahG* background, suggesting that a SA-regulated positive feedback mechanism mediates lesion formation (Weymann *et al.*, 1995). This phenomenon was also observed in hemizygous *adr1-DxnahG* plants which were indistinguishable from Col-0x*nahG*. However, homozygous *adr1-DxnahG* displayed a significant degree of leaf-curling, slight reduction in size, and lesion development was visible under conditions of high light, suggesting that SA is not completely broken down by the salicylate hydroxylase.

Application of INA, an analogue of SA that is not a substrate for salicylate hydroxylase, has previously been shown to reiterate lesion development in *nahG*x*lsd6* and *nahG*x*lsd7* (Weymann *et al.* 1995). Due to the unavailability of this compound, its functional analogue benzothiadiazole (BTH) (active ingredient of crop protectant, Bion™) (Schweizer *et al.* 1999; Lawton *et al.* 1996) was used in a similar recapitulation experiment (Figure 19). Leaves of the *adr1-DxnahG* double mutant developed lesions approximately five days after BTH application whilst no significant difference was observed in *nahG*+BTH nor *adr1-DxnahG*+silwet control plants (data not shown). Intriguingly, emergent and systemic leaves also showed lesion formation in *adr1-DxnahG* plants despite not having been directly treated.

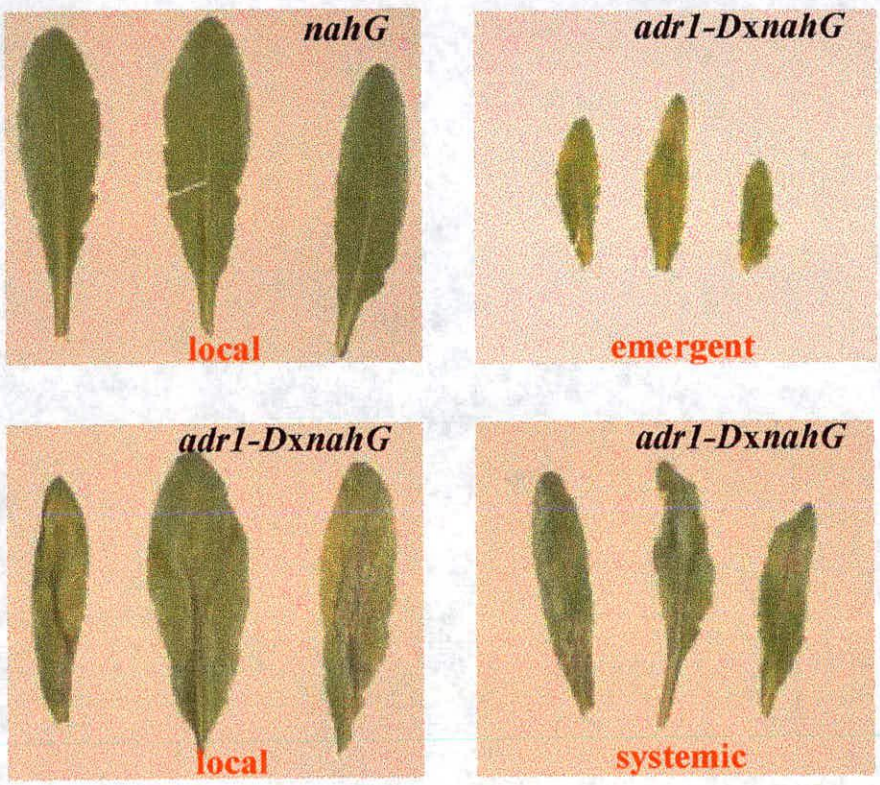


Figure 19: Local application of BTH causes systemic induction of cell death

Leaves of *adr1-DxnahG* plants were shown to undergo cell death when painted with 300μM BTH within 4-5 days of application. Emergent and systemic leaves also showed a similar cell death phenotype. *nahG* controls were not affected. Photos taken 7 days after BTH application.

The formation of micro-lesions has previously been observed in naive leaves distal to those undergoing the hypersensitive response (Alvarez *et al.*, 1998), thus this observation in BTH-treated *adr1-DxnahG* double mutants may in effect be an amplified reiteration of lesions mediated by a similar enigmatic diffusible signal.

Lesion development, dwarfing and leaf-curling were partially attenuated in *ein2*, *etr1*, and *npr1* mutant backgrounds, though not to the same extent shown by *nahG*. *adr1-Dxnpr1* double mutants also exhibited a pronounced yellowing chlorosis which increased in severity towards the centre of the rosette. A similar effect has been reported in the lesion mimic mutants *cpr5* and *acd6* crossed with *npr1* (Bowling *et al.*, 1997; Rate *et al.*, 1999), suggesting that *npr1* mutants are hypersensitive to SA phytotoxicity. *coi1* did not appear to significantly block the LM phenotype though natural variation within the *adr1-Dxcoi1* population appeared greater than observed for other double mutants.

H_2O_2 accumulation was assayed in *adr1-D* in Col-0 and *nahG* backgrounds (Figure 20). DAB staining was greatly enhanced in the *adr1-D* mutant but confined to the periphery of the leaf being largely absent in the region surrounding the main vein in the hemizygote. Staining was intensified in the homozygote but more homogenous in distribution. No staining was evident in the *nahG* background for the hemizygote, though the homozygote exhibited a light brown staining suggesting that excess H_2O_2 accumulation was not completely abrogated.

UV confocal microscopy revealed the presence of discreet patches of autofluorescent tissue in the *adr1-D* mutant but absent in the wildtype (Figure 21). This is indicative of the biosynthesis or deposition of secondary metabolites such as phytoalexins and is associated with cells undergoing the hypersensitive response (Dietrich *et al.*, 1994). Regions of autofluorescence were around 10 cells in diameter, whereas in the homozygote the frequency and size of lesions was approximately double. No such micro-lesions were observed in the *adr1-DxnahG* double mutant implicating SA in mediating hypersensitive cell death.

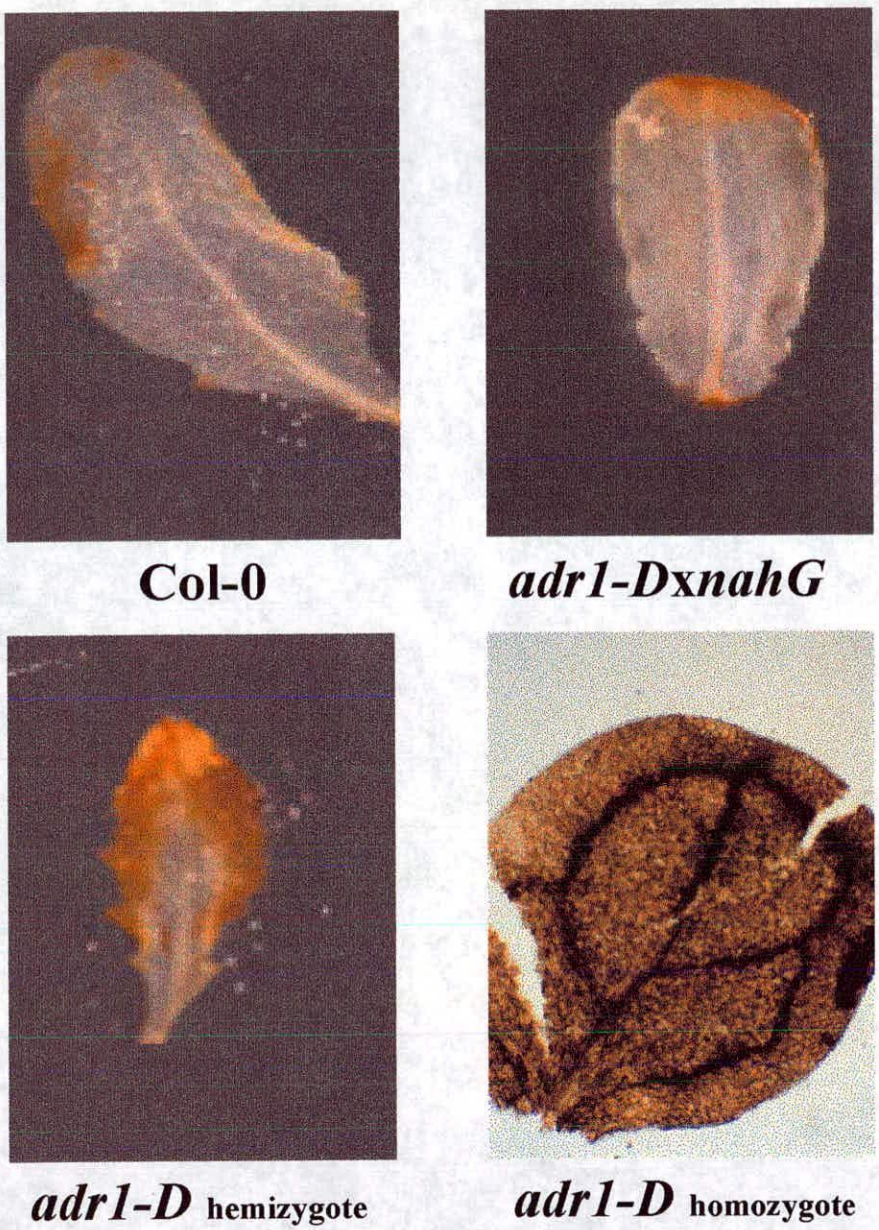


Figure 20: H_2O_2 accumulation and lesion development is SA-dependent

DAB staining was performed to assess H_2O_2 accumulation. *adr1-DxnahG* is hemizygous with respect to the *ADR1* gene. Photo of *adr1-D* homozygote taken at x4 magnification.

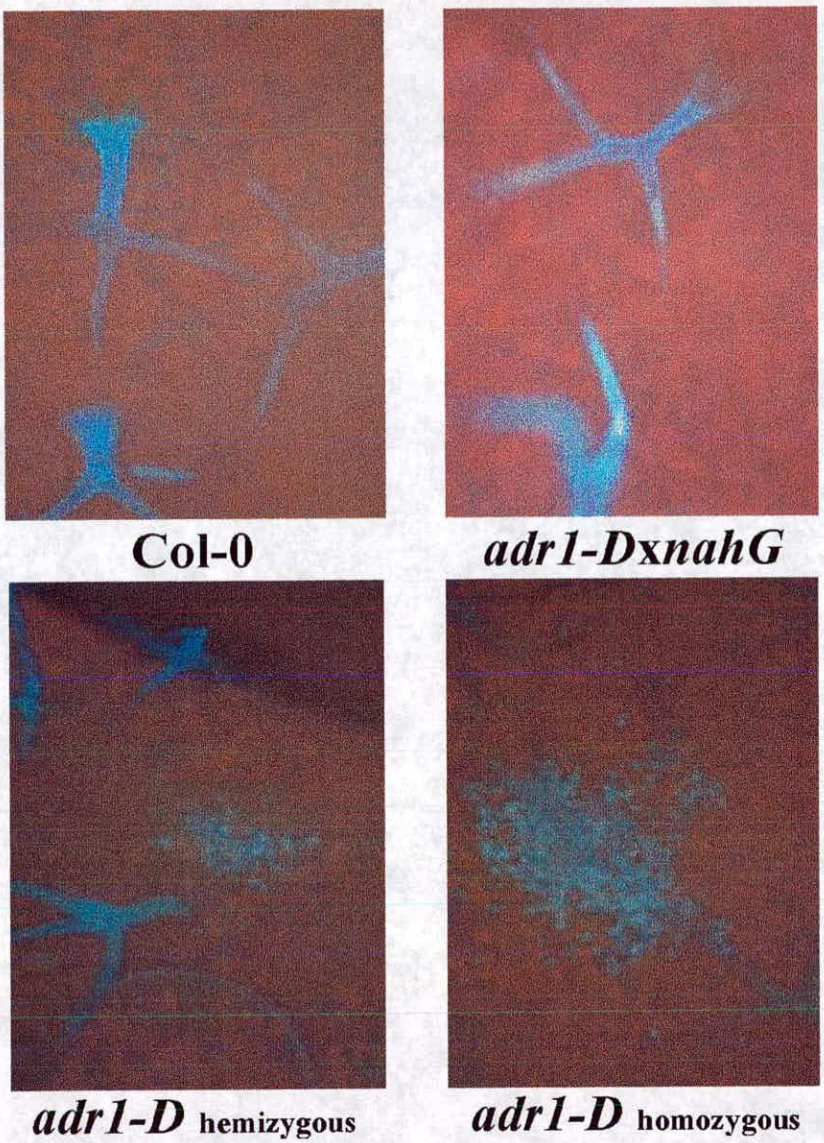


Figure 21: Lesion formation is SA-dependent

Cell death in *adr1-D* mutant is visualised as clusters of autofluorescent cells using a confocal microscope as described in Chapter 2. *adr1-DxnahG* is hemizygous with respect to *ADR1* gene.

iv) Defence genes are strongly are upregulated in the *adr1-D* mutant

The proliferation of mRNA transcripts of five different genes was measured by Northern analysis in *adr1-D* double mutants. *GST1*, *PR1*, and *PDF1.2* were chosen as markers of H_2O_2 accumulation, SAR and JA-dependent signalling respectively (chapter 1). Probing with *ADR1* was performed to assess if co-dominance was mediated at the transcriptional level and whether *ADR1* might be activated by a feedback mechanism. This was carried out after cloning of the *ADR1* gene described in the following chapter. The results of the Northern analysis are summarised in Figure 22.

High levels of *GST1* transcript were shown to accumulate in the *adr1-D* mutant with significantly greater amounts present in the homozygote than the hemizygote. *adr1-D* seedlings also over-express *GST1* but not to the same extent as adult plants. Expression in *adr1-D* mutants was significantly reduced but not completely blocked in the *nahG* background. *GST1* expression appeared to be slightly higher in *adr1-Dxcoi1*, but was unaffected in *ein2*, *etr1*, and *npr1* backgrounds. This apparent insensitivity and only partial reduction of *GST1* in homozygous *adr1-DxnahG* is in contrast to *PR1* and may reflect different induction specificities of the respective genes. For example, *GST*s may be induced by ROI, lipid peroxides, ethylene, and jasmonic acid (Marrs, 1996) which may not necessarily be reduced in the *nahG* background.

The expression pattern of *PR1* was broadly similar to that of *GST1*, however relative induction of *PR1* was significantly higher in *adr1-D* mutants than *GST1*. This was not the case in *adr1-D* seedlings in which *GST1* but not *PR1* induction was evident. *nahG* completely abolished *PR1* gene expression in both the *adr1-D* homozygote and hemizygote thus underlining its dependency on SA accumulation. Expression also appeared slightly reduced in *coi1*, *ein2*, and *etr1*, but significantly lower in the *npr1* background.

Transcript levels of *PDF1.2* contrasted markedly with *GST1* and *PR1* expression patterns, reflecting the different specificities of this SA-independent gene. All

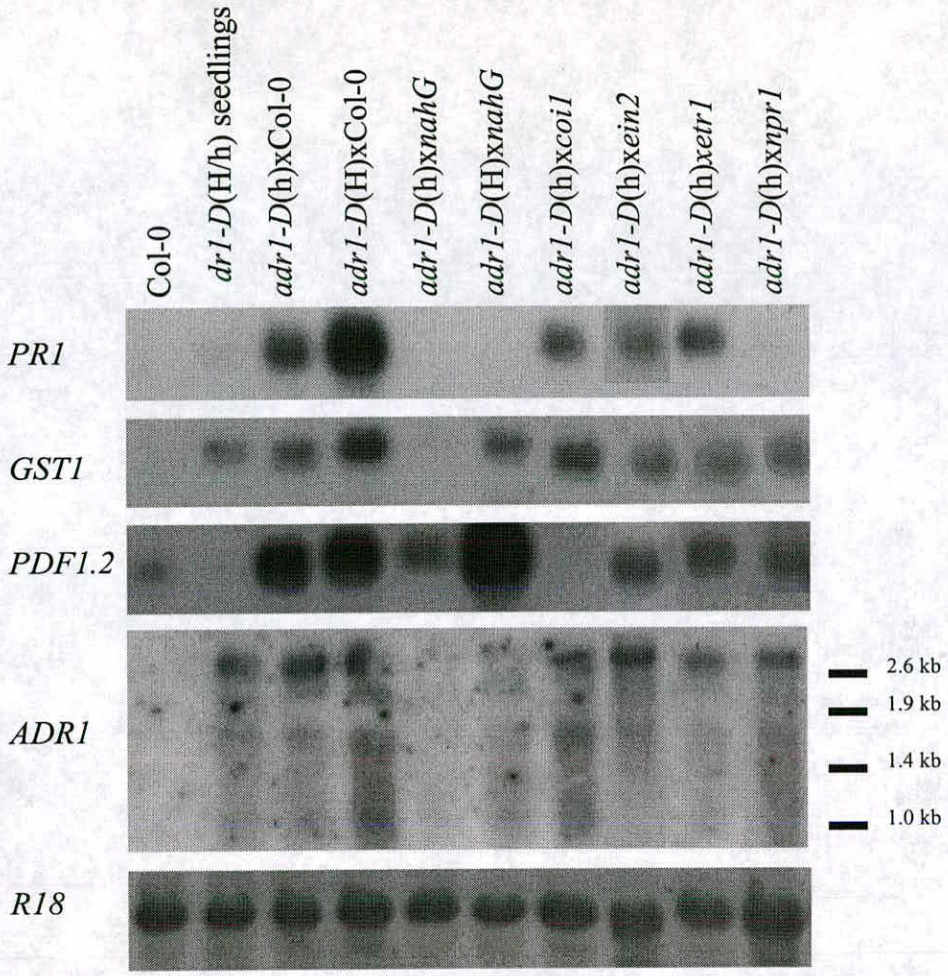


Figure 22: Expression of defence-related transcripts in different mutant backgrounds
Northern analysis shows mRNA levels of *PR1*, *GST1*, *PDF1.2*, *ADR1*, and *R18*, in mutant backgrounds as indicated.

(H) - homozygous

(h) - hemizygous

Approximate size on right as indicated by RNA ladder (Promega #G3191).

mutants showed high expression of *PDF1.2* except for *adr1-Dxcoi1* in which expression was entirely abolished, as would be expected for this JA-dependent gene. The *ein2*, *etr1*, and *npr1* mutant backgrounds appeared to partially attenuate expression of *PDF1.2*. The results for *adr1-DxnahG* are more ambiguous as expression appears to be reduced in the hemizygote but increased in the homozygote relative to *adr1-D*xCol-0. Surprisingly, expression in *adr1-D* seedlings was non-existent despite being evident in naïve adult Col-0 plants suggesting that *PDF1.2* expression may be developmentally regulated.

As expected, *ADR1* gene expression was upregulated in all *adr1-D* mutant backgrounds but could not be detected in Col-0. Although relative induction of *ADR1* is high, the net gene expression of *ADR1* was much less than was evident for all the defence genes tested. This may testify to the potency of *ADR1* as a signalling gene if such a dramatic phenotype can be attained by relatively low gene expression. Intriguingly, *ADR1* appears to be multiply spliced as at least 4 putative transcripts were observed. However, it was not possible to reconcile the transcript sizes that were obtained with possible splicing mechanisms.

ADR1 gene expression varied in different genetic backgrounds and broadly correlated with phenotype severity. *ADR1* expression was stronger in homozygotes than hemizygotes indicating that gene load dictates severity of phenotype to some degree. However, *ADR1* expression was only marginally lower in the phenotypically normal seedlings, suggesting that initiation of the LM phenotype at the seedling stage may depend on attaining a sufficient threshold of pro-death effectors such as SA and ROI. Like *GST1* and *PR1* expression, *ADR1* was significantly abrogated by *nahG* suggesting that the *ADR1* gene is SA-inducible, a fact which was later confirmed (chapter 6), thus creating a positive feedback loop absent in a *nahG* background. However, unlike *PR1* SA-dependent activation of *ADR1* is independent of *NPR1*. Overall, the *ADR1* expression pattern was almost identical to that of *GST1* so it is tempting to speculate that both are regulated by similar cues, and it is interesting to note that the promoters of both genes contain TCA elements, at -19 in *GST1* and at -93 and -33 in *ADR1* with respect to translational start site.

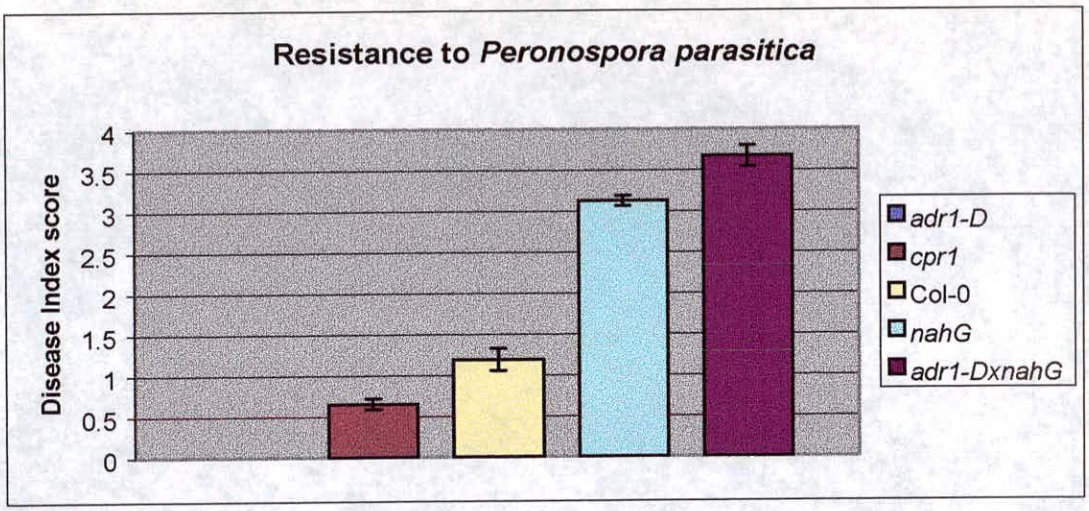
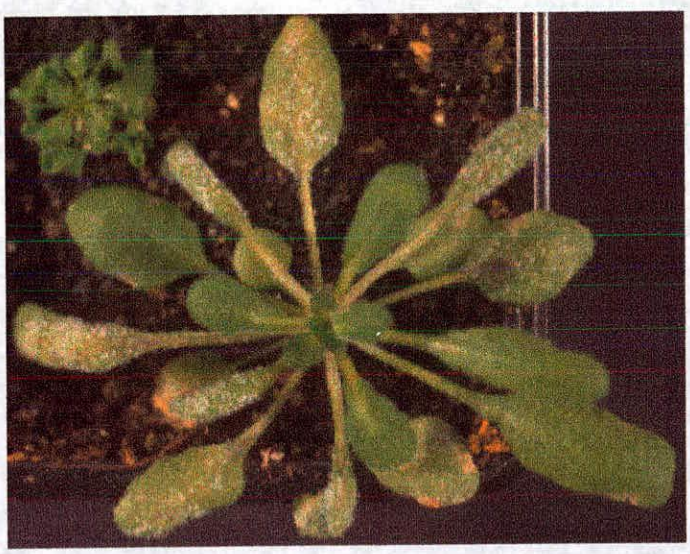


Figure 23: *adr1-D* conveys resistance to *Pernospora parasitica* (downy mildew)

(A) Disease index scored for multiple replicates according to method devised by Cao *et al.* 1997. No bar is visible for *adr1-D* as disease index score was 0 - no disease symptoms visible on any leaves.



(B) 10 days following spraying with fungal spore suspension, no infection is evident on *adr1-D* mutant (top left), whereas *adr1-DxnahG* is heavily infected and dies soon after.

v) *adr1-D* mutants display enhanced resistance to fungal pathogens

The overexpression of defence transcripts and hyperaccumulation of SA prompted us to examine whether *adr1-D* mutants exhibit enhanced resistance to virulent pathogens. This was assessed by scoring disease symptoms in *adr1-D* in comparison to Col-0, *nahG*, hemizygous *adr1-DxnahG*, and the disease resistant mutant *cpr1* (Bowling *et al.*, 1997) following infection with the biotrophic oomycete *Peronospora parasitica*. The scoring method took into account number of leaves infected and proportion of leaf covered.

The results of the *P. parasitica* resistance assay are summarised in Figure 23A&B. There was no sign of fungal infection on any of the *adr1-D* plants. However, this resistance was dramatically abrogated in the *nahG* background which was the most susceptible of the lines. *nahG* was also significantly more susceptible than Col-0 with around three times the disease index score of the wildtype. Conversely, *cpr1* was marginally more resistant than Col-0 with approximately half the disease index score of wildtype. *adr1-D* also exhibited enhanced resistance to an isolate of *Erysiphe cichoracearum* as compared with Col-0 that was also abolished in the *nahG* background (data not shown).

vi) *adr1-D* mutants display enhanced resistance to a bacterial pathogen

adr1-D plants were also deployed in a bacterial disease resistance assay using virulent *Pst DC3000* pv. *tomato*, the causal agent of bacterial speck disease in tomato. Adult *adr1-D*, *adr1-DxnahG*, and Col-0 plants were hand infiltrated with a bacterial suspension of 10^{-3} cfu/ml and visually scored for disease symptoms. Leaf disks from individual lines were also homogenised and plated at different dilutions to assess bacterial growth. As shown in Figure 24 bacterial growth in *adr1-D* was almost an order of magnitude less than Col-0, whereas the *adr1-DxnahG* was significantly more susceptible than wildtype. Leaves of Col-0 plants exhibited yellowing chlorosis symptomatic of this disease. Some leaves of *adr1-D* plants displayed visible signs of infection though disease symptoms were less severe and fewer leaves were diseased as compared with wildtype. Resistance did not seem as robust as conveyed against the fungal pathogens which may reflect problems

associated with infiltrating the small leaves of the mutant, or variations between conditions of growth chambers.

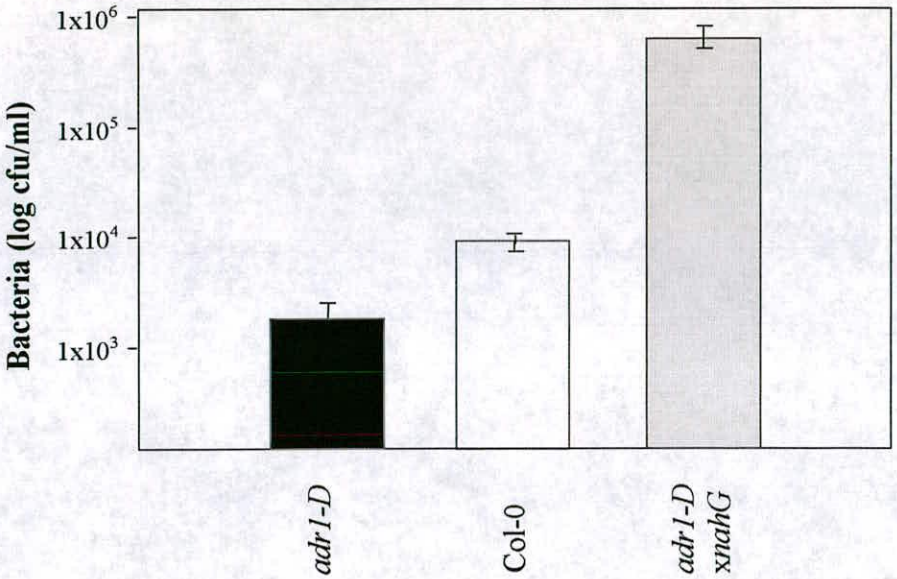


Figure 24: *adr1-D* displays enhanced resistance to *Pseudomonas syringae* pv. *tomato*
Virulent *P. syringae* were inoculated into leaves with ~10^3 cfu/ml and colonies counted three days later following plating of homogenised leaf disks on KB agar at 30°C.

vii) Discussion

The aforementioned data clearly indicate that SA accumulation underpins almost all aspects of the *adr1-D* phenotype. This is evident in terms of H_2O_2 accumulation, lesion formation, *GST1* and *PR1* transcript levels, and disease resistance to two fungal pathogens, and virulent *Pst* DC3000, which were all significantly enhanced in the *adr1-D* mutant and markedly reduced in *adr1-DxnahG* mutants relative to wildtype. Furthermore, all these characteristics with the exception of disease resistance which was not tested, were further enhanced in the *adr1-D* homozygote which probably reflects greater SA accumulation than in the hemizygote. Only *PDF1.2* expression appeared unaffected by SA, which is perhaps not surprising given that its regulation is largely JA-dependent (Chapter 1).

Ethylene signalling also appears to regulate the *adr1-D* phenotype though to a much lesser extent than SA. The visible phenotype was partially attenuated in both the

ethylene insensitive mutants *ein2* and *etr1*. Furthermore, accumulation of *PR1*, *PDF1.2* transcripts but not of *GST1* also appeared slightly decreased. Induction of these transcripts appears to correlate with severity of the visual phenotype although it remains to be established whether ethylene insensitivity affects gene induction directly, or indirectly by virtue of attenuating the LM phenotype. Thus, there appears to be some evidence for a degree of cross-talk between ethylene and SA signalling pathways. However, to determine the epistatic position of *ein2/etr1* relative to SA accumulation would require analysis of the triple mutant *adr1-D* (homozygote)x*ein2/etr1*x*nahG* or SA quantification in *adr1-Dxein2/etr1* in comparison to *adr1-D*.

JA signalling did not appear to have a big impact on the gross phenotype of *adr1-D*, though there appeared to be a greater variation of morphology within a *adr1-Dxcoi1* population, so this was difficult to ascertain conclusively. *PR1* expression was slightly reduced suggesting possible cross-talk between SA and JA pathways, whilst expression of the JA-dependent gene *PDF1.2* was entirely abolished in the *coi1* background.

Prior results suggest that *ADR1* functions upstream of *NPR1* in the signalling pathway and this is reinforced by data from the *adr1-Dxnpr1* cross. Although *adr1-Dxnpr1* mutants were characterised by a yellowing chlorosis, they appeared to have fewer lesions. Moreover, and leaf curling and dwarfing were not as exacerbated as in *adr1-D*. Furthermore, *PR1* transcript levels were markedly reduced whilst *GST1*, and *PDF1.2* were slightly lower in *adr1-Dxnpr1* mutants. These observations may be attributed to the fact that in some respects *npr1* is insensitive to SA, in terms of defence responses (Bowling *et al.* 1997) and yet apparently hypersensitive to the phytotoxic effects of high SA levels. One possible explanation for this apparent paradox is that *NPR1* might be involved in the perception of SA thus activating a negative feedback mechanism to prevent accumulation of high levels of SA.

To conclude, the regulation of a number of different aspects of the *adr1-D* phenotype is largely dictated by SA, although ethylene, JA and *NPR1* signalling all appear to play minor roles. Furthermore, *ADR1* is likely to be upstream of the H_2O_2 accumulation, HR formation, and SA production in the signalling pathway. In addition, the overexpression of *PDF1.2* in *adr1-D* mutants suggests that *ADR1* also functions upstream of the branch point between SA and JA signalling pathways. The interaction of these different signalling pathways and will be discussed further in the final chapter.

6) STRUCTURE AND FUNCTION OF THE *ADR1* GENE

i) Cloning of the *ADR1* gene

Plasmid rescue was carried out using *EcoR*I-digested *adr1-D* genomic DNA to obtain sequence downstream from the point of T-DNA insertion. 21 positive colonies were obtained and digestion with *EcoRI/BamHI* revealed that all contained a 650 bp fragment downstream from the 4x*35S* enhancers (Figure 25A) confirming that only one T-DNA insertion event had taken place. Two of these colonies also released 3 kb and 4 kb fragments respectively following *EcoR*I digestion, possibly due to incomplete digestion of the genomic DNA during plasmid rescue. Digests with *EcoR*V and *Xba*I also confirmed the tetramer of *35S* enhancers was intact (data not shown). A 2.2 kb *EcoRI/BamHI* fragment containing the 4x*35S* enhancers and rescued DNA was cloned into pBluescript SK$^-$ for sequencing (Figure 25B).

Homology searches using this sequence revealed that this area had not already been sequenced by the *Arabidopsis* Genome Initiative (AGI). Sequence immediately adjacent to the 4x*35S* enhancers was exactly homologous to EST AI995729 which encodes a gene with homology to serine carboxypeptidases. However, the T-DNA appeared to have inserted into the coding region of this gene thus it was unlikely that this disrupted gene would give rise to a functional gene product and therefore probably did not account for the dominant mutant phenotype.

Sequence further downstream from the 4x*35S* enhancers was obtained by probing a Ws-0 cosmid genomic library (as described in chapter 3). A 650 bp *Xba*I fragment (Figure 25C) was used to probe the library and chapter 46 was shown to give a positive band. A single colony from this chapter was isolated by probing with the *Xba*I fragment. DNA from this positive colony was digested with *Spe*I in tandem with 13 other restriction enzymes (*BamH*I, *Cla*I, *Hind*III, *EcoR*I, *Kpn*I, *Not*I, *Pst*I, *Sac*I, *Sac*II, *Sma*I, *Spe*I, *Xba*I, and *Xho*I), the digests run on a gel and Southern hybridisation performed. This blot was probed with a 400 bp *SpeI/Xba*I fragment (Figure 25D) to isolate fragments downstream of the region already rescued. Autoradiography revealed positive bands ranging in size from 1 kb to 6 kb and one

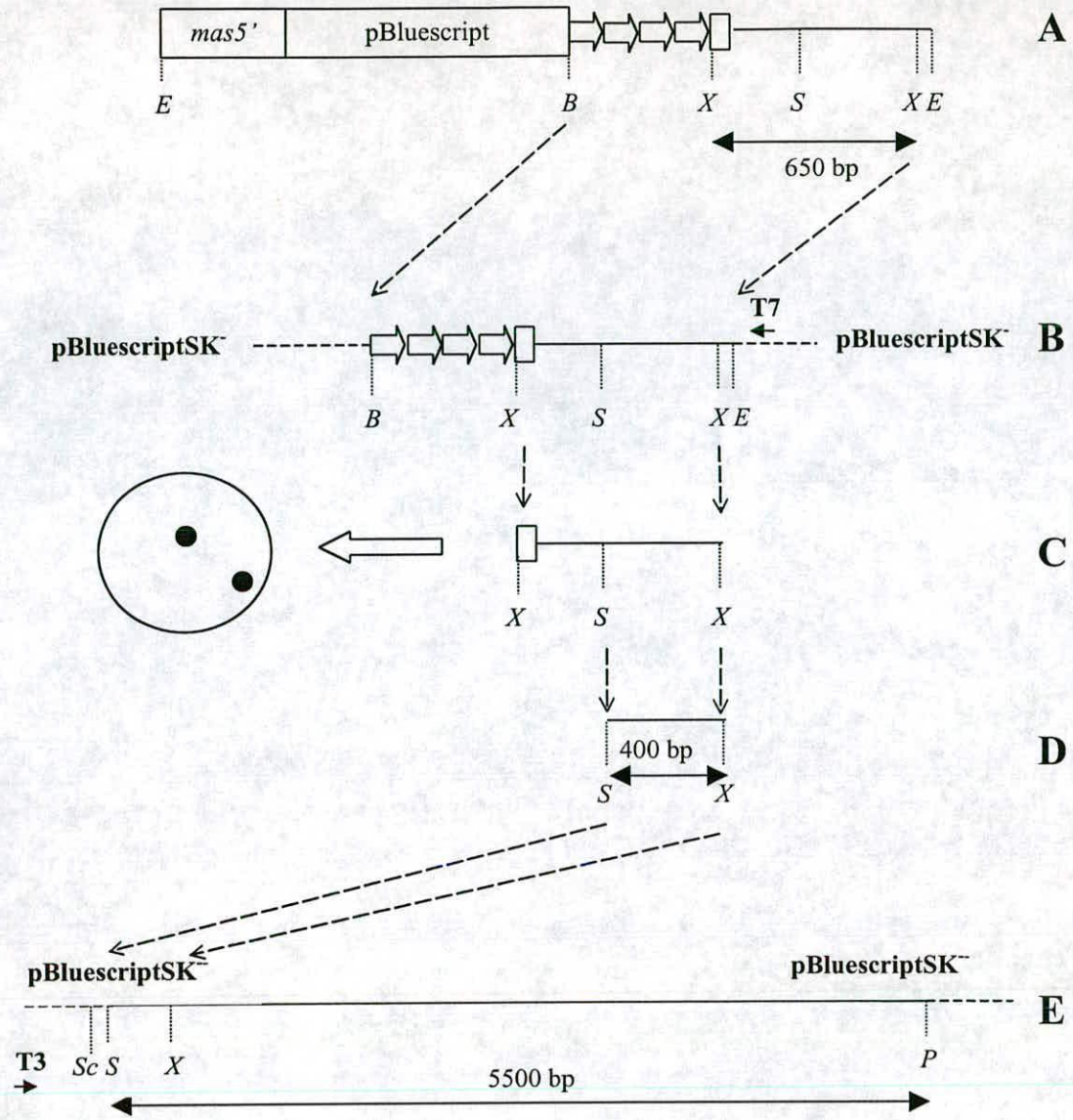


Figure 25: Cloning of *ADR1* by plasmid rescue and genomic library screening

A) The rescued plasmid containing mas5' promoter, pBluescript, 4x*35S* and ~650 bp of *ADR1* genomic DNA.

B) A 2.3 kb *EcoRI/BamHI* fragment containing enhancers and rescued DNA is cloned to pBluescript. Sequencing is performed with this construct using the T7 primer.

C) A 650 bp *Xba*I fragment of rescued DNA is used to probe a cosmid genomic library and a single colony is isolated.

D) A 400 bp *Spe*I/*Xba*I fragment was used to probe DNA from the positive cosmid previously digested with *Spe*I and in conjunction with a number of different enzymes.

E) A 5.5 kb *Spe*I/*Pst*I positive fragment is cloned to pBluescript. A nested set of deletions is generated which are sequenced using the T3 primer.

Abbreviations: *B - BamHI; E - EcoRI; Sc - SacI; S - SpeI; P - PstI; X - XbaI;*

*Spe*I/*Pst*I band of 5.5 kb was cloned to pBluecript SK⁻ for further analysis (Figure 25E).

The 5.5 kb fragment was sequenced to determine whether it contained any genes that might account for the *adr1-D* phenotype. The 5.5 kb fragment cloned into pBluescript SK⁻ was digested with *Sac*I and *Spe*I then incubated with Exonuclease III to generate a nested set of deletions. Timepoints for incubation with the exonuclease were chosen so as to generate fragments differing in size by 300-400 bp, which were subsequently transformed into *E. coli* DH5α. Sequencing was performed using the T3 primer and a contiguous overlapping sequence assembled (Appendix 1).

The sequenced contig was analysed for the presence of open reading frames (ORFs) by Genscan on the web-server. The 5.5 kb fragment was predicted to contain a gene designated *C* of 2787 bp approximately 700 bp downstream from the 4x*35S* enhancers (Figure 26), which was later shown to encode the *ADR1* gene (Appendix I). A 500 bp fragment of a gene was identified 1411 bp downstream from the stop codon of *C*, referred to as gene *D*. Homology searches indicated that *C* had homology to *R*-genes whilst *D* was similar to genes encoding transposon-like proteins. Gene *C* was thus a suitable candidate for further analysis as it was immediately adjacent to the *35S* enhancers and was possibly implicated in disease resistance signalling by virtue of its sequence. Moreover, gene *C* had very high homology (98-100%) at the nucleotide level to 3 ESTs (F19983; N96117; Z25604) in the database suggesting it encoded an expressed gene, while no ESTs were found for gene *D*.

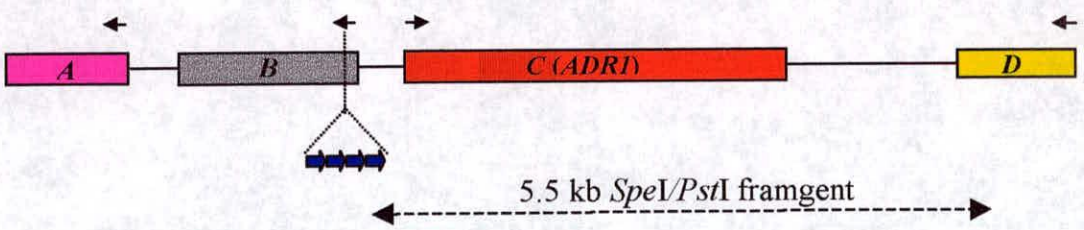


Figure 26: The relative position of *ADR1* in the genome

Schematic shows genes adjacent to *ADR1*, the point of T-DNA integration (depicted by 4 block arrows), and the 5.5 kb fragment cloned from the genomic library. Small arrows (←) indicate direction of gene transcription.

During the course of this work AGI released the sequence of BAC F10C21 which was shown to contain the isolated 5.5 kb sequence. This BAC is located on the top arm of chromosome I near genetic marker mi423a. Although the *ADR1* and F10C21 sequences are from different ecotypes (Ws-0 and Col-0 respectively) they differ by only 1 nucleotide (G_{1413} to T_{1413} which changes a methionine to an isoleucine) in the entire 2787 bp gene sequence. Sequence from this BAC was used to determine what genes were located upstream of *ADR1*. Gene *B* was predicted to encode a serine carboxypeptidase of 225 amino acid residues and further upstream was located a gene designated '*A*' (Figure 26) with no homology to any known genes.

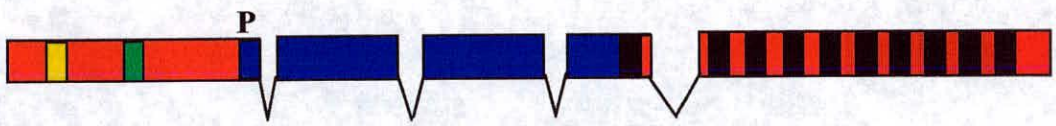


Figure 27: Structure of the *ADR1* genomic clone
Schematic shows five exons (rectangle boxes) and four introns (diagonal lines). Motifs as indicated below:

CDPK homology
nucleotide binding site region
P P-loop
coiled-coil region
leucine rich repeat

BlastX homology searches using the entire *ADR1* translated nucleotide sequence revealed two distant homologues of *ADR1* in *Arabidopsis*, AL162972 on chromosome 5 (62%) and AL161583 on chromosome 4 (56%) subsequently referred to as *ADR1-LIKE1* (*ADR1-L1*) and *ADR1-LIKE2* (*ADR1-L2*) respectively. Both genes encode disease-resistance like proteins and a number of ESTs exist for each suggesting they both encode functional genes (Table 2). The higher similarity between translated *ADR1* and the ESTs of its two homologues is probably a more accurate reflection of the degree of homology as they are not distorted by the introns which show no conservation.

Gene C, later named Activated Disease Resistance 1 (*ADR1*), is predicted by Genscan to be 2787 bp in length from start to stop codon and contains five exons and

four introns (Figure 27; Appendix I). Sequence obtained from cDNA clone (RTPCR and sequencing performed by D. Basu) revealed that the full length transcript of *ADR1* is 2361 bp, which is consistent with a transcript size of ~ 2.4 kb which was subsequently obtained by RTPCR using primers immediately adjacent to putative start and stop codons (data not shown). As the full length transcript obtained by Northern analysis was shown to be approximately 2.8 kb this suggests that there is a relatively large combined 5' and 3' untranslated region of 400 bp.

ii) *ADR1* encodes a novel gene product with CDPK, CC, NBS, and LRR domains

The Adr1 protein is made up of four principle regions: the N terminus (residues 1-187) containing a coiled-coil (CC) region (Lupas, 1996); a nucleotide binding site (188-463) (NBS) (van der Biezen & Jones 1998); a region containing leucine rich repeats (464-736) (LRRs) (Jones & Jones 1997) (Figure 29), followed by a small region at the C-terminus (737-787) containing no obvious domains.

The N terminus had no strong resemblance to other genes in the protein database except for a small region (residues 28-52) which shared significant identity (44%) to a domain highly conserved amongst plant calcium-dependent protein kinases (CDPKs) (Figure 28). This homology was significantly lower in Adr1-L1 and Adr1-L2 suggesting conservation is confined to Adr1. The peptide sequences shown were selected for box-shade comparison as they represent closest homology to Adr1 in this region: overall (Adr1-L1); to known proteins (AtCDPK3, Urao *et al.* 1994); to monocot proteins (maize CDPK); to non-plant proteins (human leucine zipper-serine/threonine protein kinase (Ruiz-Perez *et al.* 2000)).

A recent wealth of evidence has implicated an important role for calcium signalling in the establishment of disease resistance (reviewed in Grant & Loake, 2000). However, the region of similarity with CDPKs is relatively small (25 aa) and is confined to sub-domains II and III of the kinase (Hanks & Quinn, 1991). This region does not correspond with the catalytic site of kinases (sub-domain VII) and may be involved in substrate binding or necessary for proper conformation of the kinase

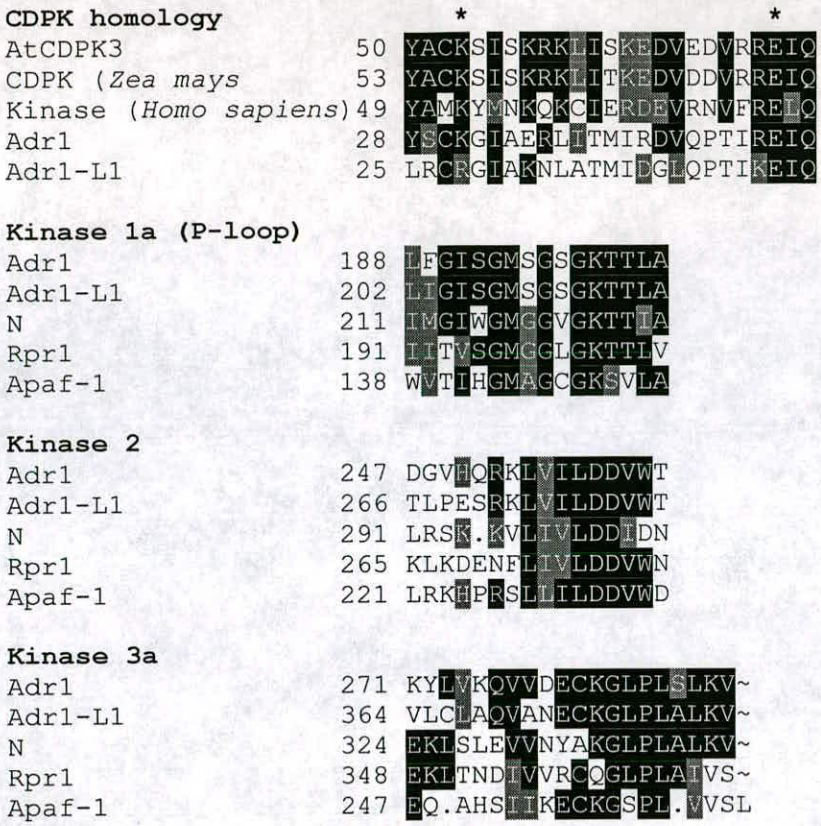


Figure 28: Conserved motifs in Adr1

Box shade was performed to highlight homologies between peptide residues of Adr1 and other genes. Grey boxes indicate similar residues, black boxes indicate identical residues. GenBank accession numbers as follows: AtCDPK3 (BAA05918), CDPK (*Zea mays*) (T03271), Adr1-L1 (AL162972), N (A54810), Rpr1 (BAA75812), Apaf-1 (AF013263).
Number refers to amino acid residue position in translated sequence. An asterisk (*) indicates the K and E residues that are highly conserved in serine threonine kinases (Hanks and Quinn, 1991).
N.B. translated sequence of *ADR1-L1* is based on computer prediction.

```
VLRDLALHMSNRV            463
RLRVLVIINNGMSPARLHGF     552
KLRSLWLKRVHVPELTSCTI     579
NLHKIHLIFC               602
SLSDLTIDHCDDLLELKSIF     629
SLNSLSITNCPRILELPKNL     652
SLERLRLYAC               677
ELISLPVEVC               687
CLKYVDISQCVSLVSLPEKF     701
SLEKIDMRECSLL            725

xLxxLxLxxCxxLxxLxxxx     consensus
```

Fig 29: The ten imperfect LRRs of the Adr1 peptide sequence

L stands for leucine though can include isoleucine and other aliphatic residues (F,V,M) and x stands for any other residue (Jones & Jones 1997). Number refers to amino acid residue position in peptide sequence.

(A.Harmon - pers. comm.). The region of homology does, however, have a conserved lysine (K) and glutamic acid (E) residue in sub-domains II and III respectively that are present in the same position (in sub-domains II and III respectively) in virtually all known serine-threonine kinases (Hanks & Quinn, 1991). It is also interesting to note that in most serine-threonine kinases sub-domain I is a nucleotide binding site (P-loop) and in Adr1 a P-loop is also closely associated with this region, located downstream from sub-domain III.

NBS-LRRs form a prolific class of genes in plants that includes many of the known *R*-genes. It is estimated that there are as many as 200-300 different NBS-LRRs in *Arabidopsis* comprising ~1% of the genome and tentative estimates in rice place this number as high as 1500 (Young, 2000). The presence of a coiled-coil (CC) structure at the amino terminus (96-112) classifies Adr1 as a Group I (or non-TIR) NBS-LRR, which is widespread throughout the angiosperms. Leucine does not predominate at position d of the heptad repeat so Adr1 does not belong to the sub-class of CCs known as leucine zippers (LZ) (Pan *et al.* 2000).

The domains that constitute the NBS were previously shown to be conserved between *R*-genes and pro-apoptotic factors, Apaf-1 & CED-4 (van der Biezen & Jones, 1998). Conserved residues with other selected proteins are highlighted in Figure 28. These peptide sequences were selected for comparison as they represent the closest homology to Adr1: overall, Adr1-L1; of known proteins, N (Whitam *et al.,* 1994); of monocots Rpr1 (Sakamoto *et al.* 1999); and of non-plant proteins, Apaf-1 (Zou *et al.,* 1997). These motifs are distributed between predicted exons 1-4 (Appendix I). A kinase 1a (P-loop) (consensus GXXXXGKT[T/S]) is present which is common to an array of ATP/GTP binding genes (Saraste *et al.*, 1990). In addition there is a kinase 2 domain and a HD motif located further downstream. The kinase 3a domain, and motifs 2, 4 and 5 are also present though these domains are less well conserved.

In total, nine imperfect LRRs of 20 aa were found, all but one located in the fifth exon. The broad consensus (x**L**xx**L**x**L**xx**C**xx**L**xx**L**xxxx) is suggestive of LRRs

belonging to cytoplasmic rather than extracytoplasmic proteins (Jones & Jones 1997). The Adr1 protein is not predicted to be highly glycosylated having only three putative N-linked glycosylation sites (consensus NXS/T), as compared with 22 in the plasma-membrane localized Cf-9 protein (Piedras *et al.,* 2000). Moreover, no cleavable signal sequence is apparent in the Adr1 peptide sequence. Taken together these results suggest that the Adr1 protein is localised within the cytoplasm.

iii) The *ADR1* gene is highly conserved in different agronomically important plant species

TBlastN was performed to compare the Adr1 protein sequence against all higher plant DNA sequences translated in all three reading frames. In total eight different species besides *Arabidopsis* were shown to have transcribed sequences in the form of expressed sequence tags (ESTs) in the database with significant identity (>40% at the predicted amino acid level) to Adr1. The distribution of hits reflects the fact that sequencing projects have concentrated on agronomically important crops or model species (e.g. *L. japonicus* and *M. trunculata*). Most of the ESTs with predicted identity to Adr1 were short sequences at the 3' end suggesting that the ESTs are not full length or that only one sequencing run was performed. The only ESTs from *Arabidopsis* with high identity to *ADR1* were *ADR1-L2* (71%) and *ADR1-L1* (68%).

From an evolutionary perspective the high degree of apparent conservation in the monocot species (Sorghum and maize) and a gymnosperm (loblolly pine), is particularly intriguing, given that evolutionary branchpoints from dicots and angiosperms are thought to have occurred approximately 150 million years ago (Cronquist, 1971). Furthermore, the fact that one of the highest predicted identities to Adr1 was found in a monocot suggests that genes with an even greater degree of homology may exist in the much less divergent dicot species. Interestingly, when a similar TBlastN search was performed for a cross-section of known *R*-genes (e.g. *RPS2*, *Prf*, *Cf-9* and *N*), the only ESTs with high identity (>40%) in other species were confined to the same family i.e. *Brassicaceae* and *Solanaceae*.

When searches against genes of known function (performed by BlastP search) were performed, the Adr1 was shown to share most identity with NBS-LRR *R*-genes. This reflects the fact that with one exception, *RPR1*, the only functionally

Genbank Accesssion	Plant species	Predicted identity (%)	Conserved area in Adr1
AV536466	*A. thaliana* (Adr1-L2)	71	434-575
AV546640	*A. thaliana* (Adr1-L1)	68	662-787
AI163353	*Populus tremula x P. tremuloides*	66	460-533
AW676969	*Sorghum bicolor*	62	386-525
AW039749	*Lycopersicon esculentum*	56	420-580
AW598621	*Glycine max*	55	659-736
AW410194	*Lotus japonicus*	52	419-789
AI855190	*Zea mays*	50	563-718
AW684410	*Medicago trunculata*	48	446-647
AI813065	*Pinus taeda*	40	494-611

Table 2: ESTs from other plant species with homology to *ADR1*

TblastN was performed against all *Viridiplantae* sequences in GenBank (www.arabidopsis.org/blast/). Identity refers to percentage of exactly conserved residues based on predicted amino acid sequence of the ESTs. All hits are listed as obtained with default settings of BLAST. Multiple hits were evident in some species but only the highest predicted identities are shown. 'Conserved area in Adr1' refers to the residue positions in Adr1 with identity to the translated EST.

characterised NBS-LRRs are *R*-genes. However, the *R*-gene with highest identity to Adr1 (N of tobacco) only shares 24% identity, largely confined to specific domains of the NBS and conserved leucines in the LRR.

iv) Motifs in the *ADR1* promoter

The 5' regulatory region of *ADR1* was analysed for regulatory motifs that might account for the pattern of inducible expression previously observed. An ASF-1 motif

(TGACG) was found at -592 relative to translation start site. This motif is thought to be involved in the transcriptional activation of several genes by auxin and/or salicylic acid (Terzaghi and Cashmore, 1995). Additional support for SA-mediated transcription of *ADR1* comes from the presence of two TCA-elements (-32 & -93) containing 8/10 nucleotides conforming to the consensus TCATCTTCTT as previously found in the *GST1* promoter (Chapter 3). Lastly, a motif (CACATG) found at -177 was shown to be necessary for drought/ABA-induction of the dehydration-responsive gene *RD22* in *Arabidopsis*, and binds a drought-regulated Myc transcription factor (Abe *et al.*, 1997). Although these are only computer predictions, it is interesting to note that *ADR1* expression was shown to be regulated by SA and its overexpression conveys substantial drought tolerance.

v) The *ADR1* gene is inducible by BTH, SA, and pathogen attack

A putative role of *ADR1* in disease resistance signalling was consolidated following Northern blot analysis following different treatments (Figure 30).

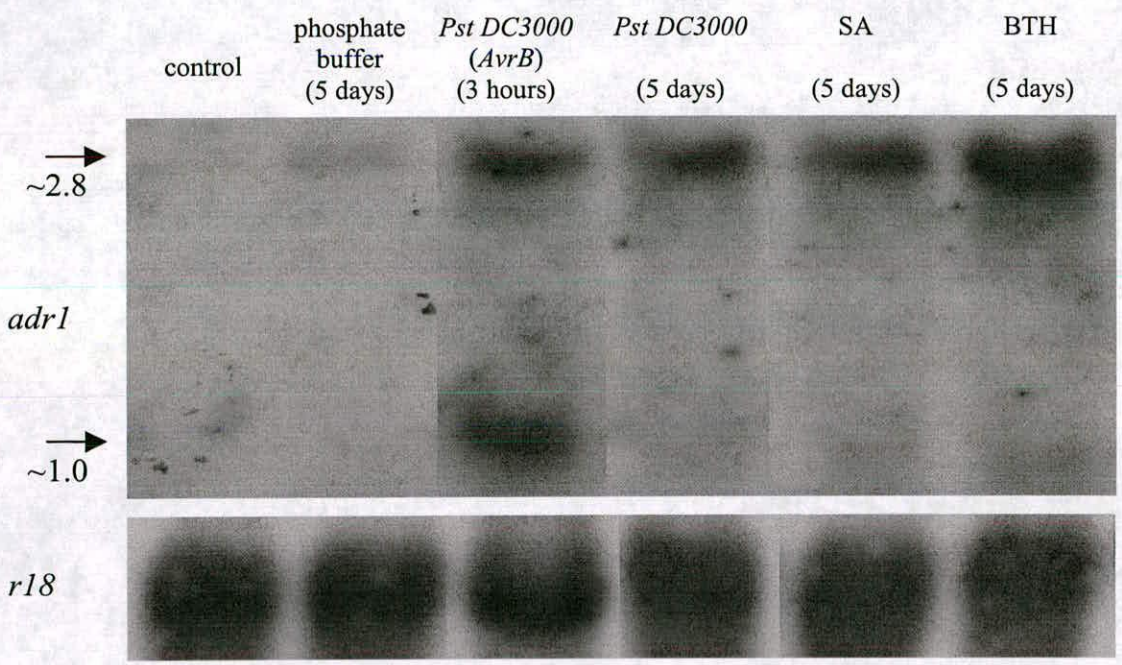


Figure 30: Induction of *ADR1* in response to different treatments

Treatments (from left): control - naive Col-0 plants; 1mM phosphate buffer pH5.8; *Pst DC3000(AvrB)*; *Pst DC3000*; salicylic acid 1mM (infiltrated in 1mM phosphate buffer pH5.8); BTH 300μM painted on leaves (in 0.01% silwet). Arrows indicate approximate sizes as inferred from RNA ladder (not shown).

ADR1 was shown to have a vanishingly low basal level of transcription in naive Col-0 plants, however, significant up-regulation of *ADR1* was observed following treatment with avirulent bacteria (*Pst* DC3000(*AvrB*)) 3 hours post-inoculation, and by virulent *Pst* DC3000, SA, and BTH, all 5 days after treatment. A small induction was observed following inoculation with 1mM phosphate buffer (used as a negative control for SA treatment), suggesting that *ADR1* induction might also be mediated by a wound response pathway. Only two of the four previously observed transcripts were evident which is probably because endogenous expression of *ADR1* is relatively weak. Intriguingly, relative induction of the 1 kb transcript was significantly stronger following treatment with *Pst DC3000*(*AvrB*) at three hours as compared with other treatments. Similar results were also obtained with *Pst DC3000*(*AvrRpt2*) at three hours (not shown). This suggests that *ADR1* may be differentially spliced in response to different stimuli such as SA or pathogen recognition, although further confirmation is required by means of Northern analysis using polyA$^+$ RNA and sequencing of respective transcripts.

vi) Discussion

As a putative role for *ADR1* will be discussed in the concluding chapter, here we consider sequence and functional similarities with *R*-genes and other uncharacterised NBS-LRRs. There is evidence both to suggest that *ADR1* might encode a *R*-gene that recognises a hitherto unknown pathogen-derived elicitor, but perhaps stronger evidence to refute it. All *R*-genes classified as such have been cloned by a functional approach, i.e. they were shown to be essential to confer resistance against specific pathovar(s) of pathogen (e.g. *N* specifies resistance to tobacco mosaic virus). Endogenous expression of *ADR1* has not been shown to be specific for a particular pathovar although theoretically it may confer resistance to an as yet unknown or extinct pathovar.

Sequence homology searches identify similarities to a large number of NBS-LRR *R*-genes. However, overall identity is weak, and is largely confined to specific domains of the NBS and the leucine residues of the LRR. Genes containing LRRs are the largest known class to have been found in *Arabidopsis* (Bevan *et al.* 1998) and the

NBS is common to a large number of genes from a diverse array of organisms. The *Arabidopsis* genome is thought to contain around 200-300 NBS-LRR genes (Pan *et al.* 2000). Whilst a significant proportion of these are likely to encode *R*-genes and also serve as a large gene pool to keep pace with continually evolving avirulence genes, it is conceivable that many also have additional roles. However as no other purpose outside of *Avr* gene recognition has been definitively ascribed to NBS-LRRs, it is difficult to assign other possible roles to this class.

Furthermore, most but not all *R*-genes, such as the *RPP1* complex of *Arabidopsis* ecotype Ws-0, have been shown to occur in clusters (Botella *et al.*, 1998) as this favours evolution by intergenic crossover enabling the plant to keep pace with rapidly evolving avirulence genes (Richter and Ronald, 2000). Thus, many *R*-genes have virtually identical genes in the immediate vicinity, e.g. *Cf-2* of tomato (Dixon *et al.*, 1996). *ADR1* has no close homologues, and the sequence of adjacent genes suggests they have completely unrelated functions.

Another apparent difference with classical NBS-LRR *R*-genes is the region of homology shared with the kinase domain of CDPKs. Whilst the functional significance of this domain has yet to be demonstrated, comparison with the overexpression phenotype of other *R*-genes lends additional support to the hypothesis that *ADR1* may possess domains involved in signal transduction that are absent in NBS-LRR *R*-genes. Overexpression of four other NBS-LRRs has previously been reported, both from the TIR class (*N* and *L6*) and CC class (*Prf* and *RPS2*). Of 82 *CaMV 35S::RPS2* transgenics generated, only 5 were able to complement a non-functional mutant and no constitutive resistance or lesion formation was observed. I fact many of the transgenics were actually *RPS2*-suppressed (Mindrinos *et al*, 1994; Katagiri - pers. comm.). Tomato plants with 1 or 2 additional copies of *Prf* displayed elevated disease resistance and SA accumulation. No LM phenotype was observed, however, it is not known whether this can be attributed to a relatively lower upregulation of *Prf* (Oldroyd & Staskawicz, 1998). Transgenic *CaMV 35S::L6* flax plants show no change in resistance phenotype as compared with the endogenous *L6* gene and do not exhibit lesion formation (Ellis *et al.,* 1999; Ellis - pers. comm.).

Moreover, *CaMV 35S::N* plants failed to confer complete TMV resistance and no lesion development is reported (Dinesh-Kumar and Baker, 2000).

These results are in contrast with tomato plants overexpressing the serine-threonine kinase, *Pto*, which display constitutive resistant responses and spontaneous cell death (Tang *et al.*, 1999). Thus, it is possible that an additional signalling event such as protein phosphorylation is necessary to effect a pro-death pathway which may be mediated by the CDPK-like domain in *ADR1*. An alternative hypothesis is that *ADR1* might encode a gene responsible for coordinating resistance responses for multiple resistance genes, much as *Pto* and *Fen* resistance are both dependent on *Prf* in tomato (Salmeron *et al.,* 1996). In support of this theory is the fact that of five reports of NBS-LRR overexpression, only *Prf* and *ADR1* confer broad spectrum resistance. Furthermore, it is interesting to note that both genes, unlike most NBS-LRR *R*-genes, possess relatively long N-termini that do not strongly resemble other sequences found in protein databases. Analysis of anti-sense *ADR1* lines is now required to assess whether resistance responses are compromised when *ADR1*-signalling is down-regulated.

From an evolutionary standpoint, *ADR1* appears distinct not only from *R*-genes but also from other members of the NBS-LRR gene family. A phylogenetic analysis of NBS sequences from NBS-LRRs classifies *ADR1-L1* and *ADR1-L2* (*ADR1* had not been sequenced at time of publication) as belonging to an evolutionarily distinct clade (Meyers *et al.,* 1999). Furthermore, despite extensive analysis of over 20 NBS-LRRs and membrane-bound LRRs (not shown), only *ADR1* shares high identity at the predicted amino acid level to monocot genes.

Lastly, from a functional point of view, the fact that *ADR1* is inducible following various stimuli further sets it apart from *R*-genes. Whilst pathogen inducibility has also been observed for two other *R*-genes conferring nematode resistance in sugar beet, $HS^{pro\text{-}1}$ (Cai *et al.*, 1997) and against bacterial blight in rice, *Xa1* (Yoshimura *et al.*, 1998), lack of data for other *R*-genes suggests that this is the exception rather than the rule. Furthermore, non-*R*-gene NBS-LRRs have been shown to be inducible by pathogen attack and SA in rice and rye (Sakamoto *et al.,* 1999). This is one

example of recent data that has challenged former preconceptions regarding *R*-genes, reinforcing the notion that NBS-LRRs may have diverse roles not merely confined to pathogen recognition in the case of *R*-genes.

To conclude, it is ultimately impossible to determine if *ADR1* encodes a *R*-gene without being able to show that this gene is directly responsible for recognition of a specific avirulence gene product. Antisense *ADR1* plants (engineered by D.Basu) are currently being assayed and a knock-out line is being screened for, which may help clarify the role of *ADR1* in disease resistance. However, the implication of *ADR1* in the mediation of multiple stress responses heralds a new paradigm of NBS-LRR analysis. It is anticipated that with the advent of sophisticated functional genomics techniques of analysis, further light will be shed on the enigmatic NBS-LRR gene family both in and outwith pathogen resistance.

7) THE ROLE OF *ADR1* IN ABIOTIC STRESS SIGNALLING

i) The *adr1-D* mutant has enhanced resistance to drought stress

Parallels have recently been drawn between the pathways governing responses to abiotic stress and disease. A number of signals are common to both pathways, such as an increase in cytostolic calcium, production of ROI, activation of MAPK cascades, and upregulation of antioxidant genes (reviewed in Bowler & Fluhr, 2000). This fact coupled with the identification of a putative binding site for a drought-responsive Myc transcription factor in the *ADR1* promoter prompted us to investigate whether *adr1-D* was altered in its ability to withstand drought conditions.

A drought stress assay was carried out using 23 Col-0 and 19 *adr1-D* plants grown in two halves of the same tray under short day conditions (Figure 31). Watering was stopped 25 days after germination and the tray was transferred to long day conditions. Severe wilting was first evident 11 days later in Col-0 but *adr1-D* plants appeared normal. At +15 days all wildtype plants were dead and severe wilting was evident in *adr1-D* plants. Watering was resumed at this point to confirm plant death. In a different experiment in which plants were grown in 8 cm high pots (4 plants/pot in 6 pots) under long day conditions, *adr1-D* plants were shown to survive for up to a week longer than Col-0. Drought resistance has yet to be confirmed in *CaMV 35S::ADR1* transgenics (Chapter 8) so it is not yet known whether drought resistance is some aberrant phenotype resulting from the activation tagging process.

Similar results were obtained under short day conditions. However, drought tolerance in *adr1-D* relative to Col-0 appeared enhanced under long days. This may be due to a greater propensity for the mutant to initiate bolting under long day conditions as compared with Col-0 which could in turn limit water loss and/or reduce water requirement, though this hypothesis has yet to be proven.

ii) SA, *NPR1*, JA, and ethylene are not key determinants of *ADR1*-mediated drought tolerance

The *adr1-D* lines that had been introgressed into different mutant backgrounds

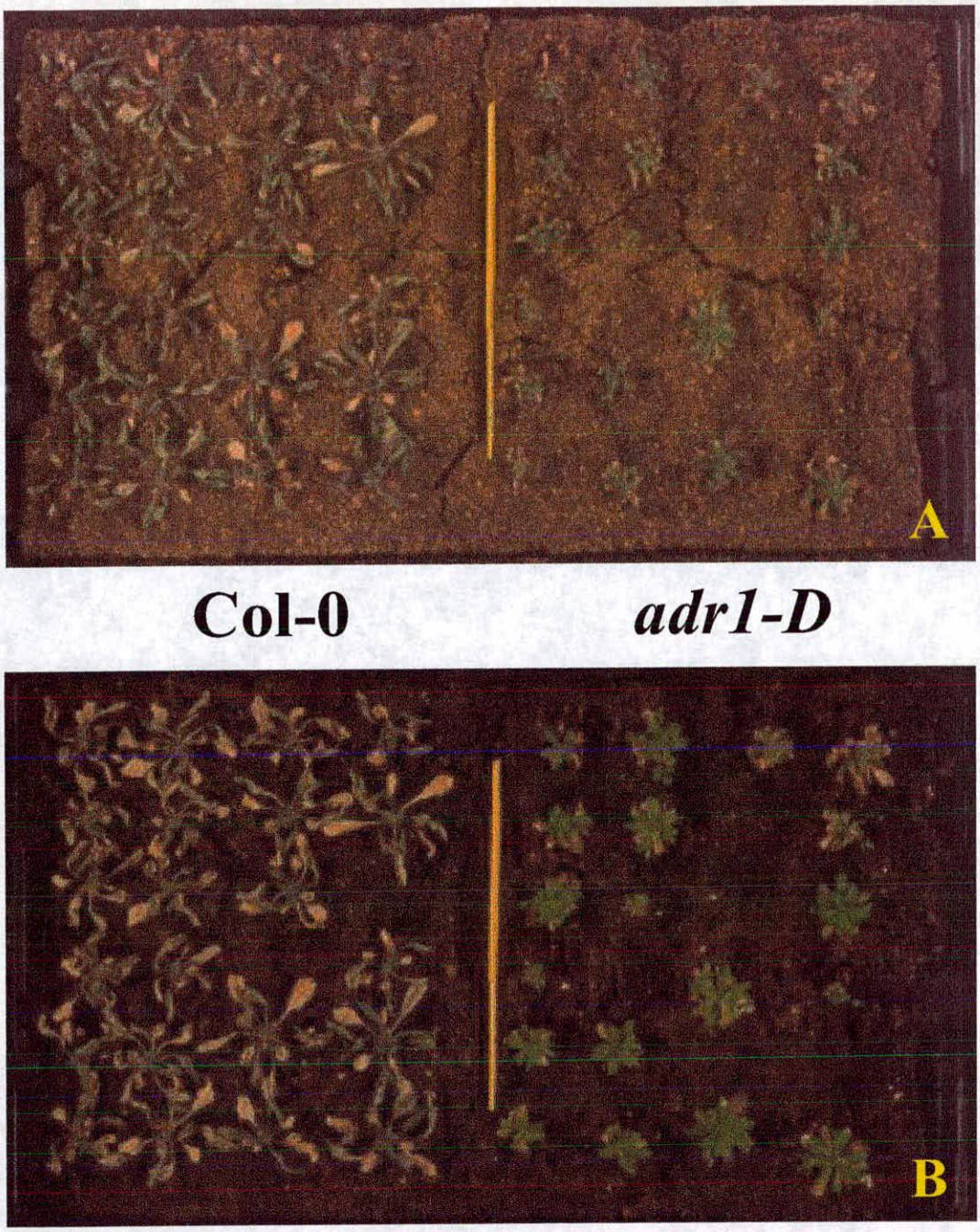


Figure 31: The *adr1-D* mutant has enhanced drought tolerance
(A) 23/23 Col-0 and 0/19 *adr1-D* plants are dead 16 days post-watering.

(B) Re-watered plants at day 17 confirm death is confined to Col-0.

(described in Chapter 5) were deployed in drought assays to assess what signal transduction pathways might govern *ADR1*-mediated drought resistance. Between 6-8 lines of Col-0, *adr1-D*, *adr1-DxnahG*, *adr1-Dxetr1*, and *adr1-Dxnpr1*, were sub-planted at 14 days post-germination into different segments of the same tray and watering was stopped 11 days later (results not shown). 16 days after watering was stopped, all Col-0 plants but none of the other lines were dead, although some exhibited severe wilting. After 17 days between 1-3 plants of all the mutant lines except *adr1-Dxnpr1* were dead. Dead plants were confirmed by resumption of watering. In a separate experiment, *adr1-Dxcoi1* mutants were shown not to be significantly compromised in drought resistance. Two novel disease resistant mutants which accumulate high levels of SA (Murray *et al.*, - unpublished) were also tested for drought tolerance, but did not appear to have enhanced protection as compared with wild-type (results not shown).

From this data we can conclude that neither SA, JA, ethylene, nor *NPR1* significantly abrogate *ADR1*-mediated drought resistance. Furthermore, the fact that *adr1-DxnahG* plants show drought resistance suggests that tolerance is not a function of size. However, due to the small numbers of plants used in the assay, we cannot conclusively state whether differences between the *adr1-D* double mutant backgrounds are actually significant. Therefore, the study should be repeated using larger numbers of plants in each class. As two of the four identified pathways governing drought resistance are mediated by abscisic acid (ABA) (Shinozaki & Yamaguchi-Shinozaki, 2000), *adr1-D* has also been crossed into the ABA-insensitive mutant *abi1* (Koornneef *et al.*, 1984) to determine whether *ADR1*-mediated drought resistance is compromised in this background. Moreover, the induction of stress-associated genes will also be examined.

iii) Discussion

In this chapter we have shown that the *adr1-D* mutant displays significantly enhanced resistance to drought. *adr1-D* mutants appeared to survive drought conditions 1-2 days longer than wildtype plants under a short-day light regime and

this difference was accentuated under long-day conditions. This is believed to be the first report of a mutant that is resistant to both disease and drought.

The mechanism by which *ADR1* is able to confer drought tolerance when overexpressed remains open to conjecture, and it is conceivable that this protection is specifically engaged by the *ADR1* gene. This is particularly pertinent in the light of the CDPK-like domain located in the *ADR1* gene and a number of reports in the literature that correlate induction of *CDPK*s with drought and cold stress (e.g. Pestenacz and Erdei, 1996; Li and Komatsu, 2000). Indeed expression of a rice *CDPK* gene was recently shown to confer salt, drought and cold tolerance (Saijo *et al.*, 2000).

It remains to be determined if drought resistance is common among disease resistant plants or whether this phenotype is specific to the overexpression of *ADR1*. Conversely, it is possible that in some instances elevated abiotic stress resistance may correlate with disease resistance. Such cross-tolerance assays will help localise genes epistatically in stress signalling pathways, assist in defining a branch-point for abiotic and disease signalling, and further advance our understanding of cross-talk in stress resistance. These results may have a significant impact on current thinking regarding drought and disease resistance pathways. In the past, abiotic stress and pathogen resistance signalling have been considered separate disciplines and very little research has been carried out encompassing both fields. These findings may help redress this somewhat limited perspective and encourage an approach that incorporates both areas.

A number of other models, not mutually exclusive to one another, may also account for *ADR1*-mediated drought resistance. Recently published data of *Avr9*/*Cf-9* rapidly elicited (*ACRE*) genes by means of cDNA amplified fragment length polymorphism (AFLP) analysis in tobacco cell cultures may provide a clue as to a possible mechanism of *ADR1*-mediated drought resistance. One such *ACRE* gene, subsequently shown to be rapidly and transiently induced in *Avr9*-treated *Cf-9* tobacco cell suspension cultures in a DPI-insensitive manner, was shown to have

99% homology to the *DREB1A* gene (Durrant *et al.*, 2000). This stress responsive transcription factor has previously been shown to mediate cold and drought responses. Furthermore, overexpression of *DREB1A* by the *CaMV 35S* promoter was shown to convey enhanced freezing and drought tolerance (Liu *et al.*, 1998).

Further support for a rapidly induced stress response following the Avr-R interaction is implicated by the recent report of an *Arabidopsis relA/spoT* homologue, *At-RSH1* that was shown to specifically interact with the NBS of the *R*-gene, *RPP5,* in a yeast two-hybrid assay. *relA/spoT* genes determine the levels of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), which are effector nucleotides of the prokaryote stringent response, which are known to be activated under conditions of environmental stress in bacteria. Furthermore, At-RSH1 was able to complement an *E.coli relA* mutant to enable growth on minimal SMG medium that is inhibited in the mutant. Perhaps somewhat surprisingly given the degree of conservation between motifs in NBS domains, no interaction was observed when the NBS from other *R*-genes was used as bait (van der Biezen *et al.,* 2000).

However, the only direct evidence for induction of a drought-inducible gene in the *adr1-D* mutant is the overexpression of *GST1,* which was originally isolated as the early response to drought gene *ERD11* (Kiyosue *et al.*, 1993). This is reinforced by data showing that the overexpression of a cDNA with Gst and glutathione peroxidase activity was able to enhance seedling growth compared with wildtype plants under conditions of salt and chilling stress (Roxas *et al.*, 1997). However, Northern analysis showed that *GST1* induction in *adr1-DxnahG* was substantially reduced, yet this double mutant did not seem significantly different from *adr1-D* in terms of drought tolerance. Furthermore, whilst the induction of antioxidants such as Gsts may enhance stress tolerance, this may well be negated by additional oxidative stress in *adr1-D* mutants. DAB staining had previously shown that H_2O_2 accumulated to a much higher extent than in wildtype plants despite high induction of *GST1,* suggesting that the redox 'balance' at least in terms of peroxide was tipped in favour of ROIs. Thus, overall the evidence does not appear to be strongly in favour of an antioxidant-mediated mechanism of drought tolerance in *adr1-D.* However, this

does not rule out possible stress protection afforded by other antioxidants such as superoxide dismutases (SODs), which have previously been shown to convey protection to abiotic stresses when overexpressed (reviewed in Allen *et al.*, 1997).

Further reports also hint at similarities between gene induction following biotic and abiotic stress. Two such examples include the observation that enhanced protection against the bacterial pathogen E*rwinia carotovora* and dehydration stress was conferred by the inoculation of *Arabidopsis* plants with plant-growth-promoting rhizobacteria (PGPR) (Timmusk and Wagner, 2000). Furthermore, different abiotic stresses caused the induction of cystatin in *Castanea sativa*, a protein previously characterised as an anti-fungal agent (Pernas *et al.,* 2000). This is in addition to a plethora of biochemical processes previously mentioned that are common to both pathogen and abiotic induced stress responses of plants.

Mechanisms that convey resistance to both pathogens and drought might also be mediated at the physical level. For example, it seems logical to suggest that a plant cell with high turgor pressure would be more resistant to penetration by a fungal appressorium than a more flaccid drought-stressed cell, though this remains to be proven. In addition, some pathogen defence responses could be an indirect consequence of drought stress mechanisms. For example, in most pathosystems such as *Xanthomonas campestris* pv. *campestris/Arabidopsis* and *Peronospora parasitica/Brassica oleracea*, bacterial and fungal infection occurs via the stomata (Hugouvieux *et al.,* 1998; Achar, 1998), which might be inhibited if stomatal pores were closed as a result of drought stress.

From an evolutionary perspective the degree of overlap between abiotic and biotic stress responses is perhaps not that surprising. If a plant is being subject to abiotic insults it is may be more vulnerable to pathogen attack and thus it makes sense to activate defences that counteract both threats. This notion is reinforced by the fact that some fungal diseases of plants are often triggered by drought stress, such as the fungal stalk rots. Indeed varieties of sorghum with enhanced drought tolerance are also more resistant to fungal stalk rot (Diourte *et al.,* 1995).

To conclude, we have shown here that *ADR1*-mediated drought protection is apparently independent of SA, JA, ethylene and *NPR1*. However, the analysis of abiotic stress associated gene expression in *adr1-D* introgressed into mutants with aberrant stress responses (e.g. *abi1* (Koornneff *et al.*, 1984)) is now required to determine more precisely the role of *ADR1* in drought signalling. Work is also currently in progress to determine whether *adr1-D* is also more tolerant of heat, freezing and high salt stress which should also facilitate the genetic dissection of abiotic stress tolerance in *adr1-D*. As drought is second only to disease in terms of natural causes of crop losses worldwide, this finding also has obvious important implications from a biotechnological perspective which are discussed further in the final chapter.

8) MANIPULATION OF *ADR1* EXPRESSION

i) Introduction

In Chapter 6 the *ADR1* candidate gene was shown to be strongly upregulated in the *adr1-D* mutant. However, to prove that this gene was directly responsible for the *adr1-D* phenotype, it was necessary to show that its overexpression could recapitulate the *adr1-D* phenotype. This would require the generation of a large number of transgenic plants overexpressing the candidate *ADR1* gene which would be likely to exhibit a spectrum of phenotypes with respect to the disease architecture as previously observed for background levels of luciferase activity in *GST1::LUC* lines (data not shown). Therefore, *ADR1* overexpressing lines might also help answer pertinent questions about *ADR1*-mediated disease resistance. For example, whether it is possible to uncouple lesion development from disease resistance and to what extent does severity of lesions correlate both with resistance and *PR1::LUC* expression. In addition, the disease resistant phenotype has potential for use in a biotechnological context, as a means of conveying broad spectrum resistance to crop plants. Thus, it is important to investigate whether the yield penalty associated with the dwarf phenotype can be attenuated without substantially compromising disease resistance.

As a complementary approach, an inducible system was also employed to transiently express *ADR1*. Controlled expression of *ADR1* might thus confer disease resistance without the yield penalty associated with constitutive overexpression. This would demonstrate the 'proof of principle' for the use of a transient induction system that could ultimately be engineered into crop plants enabling a farmer to activate resistance at will using an analogous but non-toxic system.

To achieve these aims, the *ADR1* genomic clone was introduced into *Arabidopsis* under the two different expression regimes. Firstly, the entire *CaMV 35S* promoter (Odell *et al.*, 1985) was used to convey constitutive ectopic expression of *ADR1*. Secondly, the glucocorticoid-inducible *GVG* gene was employed to drive downstream transcription of the *ADR1* gene following treatment with the synthetic

steroid dexamethasone (DEX) (Aoyama & Chua, 1997). The *GVG* system had previously been used in a similar context whereby dexamethasone-mediated *AvrRpt2* expression was shown to drive downstream defence responses in transgenic plants (McNellis *et al.*, 1998).

ii) Constitutive expression of the *ADR1* gene recapitulates the lesion mimic phenotype

The overexpression cassette was constructed by engineering *Kpn*I and *BamH*I sites by PCR immediately adjacent to the respective start and stop codons of the *ADR1* genomic clone. This fragment was then cloned into the multiple cloning site (MCS) of pART7 (Gleave, 1992) to generate the overexpression cassette *CaMV 35S*::*ADR1*::*OCS* (Figure 32). This construct was cloned into the *Not*I site of pGREEN (Basta^R) which was transformed into an *Agrobacterium* strain containing the trans-acting plasmid, pJIC Sa_Rep (Hellens *et al.*, 2000). The resulting construct was then transformed into genetic background *PR1a*::*LUC* by *Agrobacterium* transformation to facilitate subsequent screening of transgenics by means of luciferase imaging.

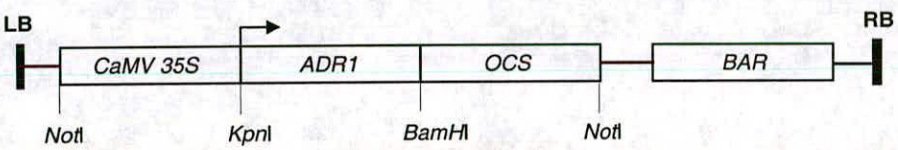


Figure 32: The *ADR1* overexpression cassette
The *ADR1* genomic clone was ligated into the *Kpn*I/*BamH*I site of the MCS of pART7 to generate the overexpression cassette *CaMV 35S*::*ADR1*::*OCS*. This was then cloned into the *Not*I site of the binary vector pGREEN, also incorporating the *bar* gene (promoter and terminator of *bar* omitted) for Basta resistance between the left and right border.

In total 110 *CaMV 35S*::*ADR1* transgenics were generated which were characterised with respect to morphology, luciferase activity, and disease resistance. This was carried out in the F_1 generation due to time limitations but will be repeated in the progeny. A range of visible phenotypes was observed which were grouped into four

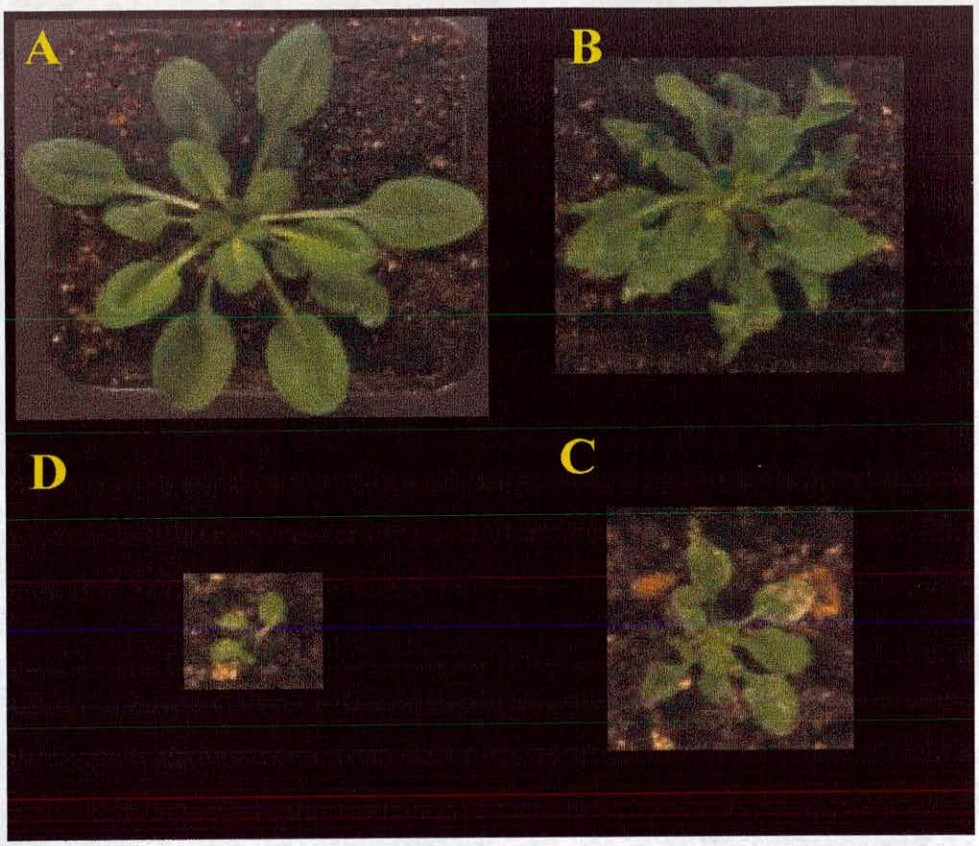


Figure 33: Constitutive expression of the *ADR1* candidate gene recapitulates the *adr1*-D phenotype
Plants transformed with the construct *CaMV 35S::ADR1* show a spectrum of phenotypes which were grouped into four classes A, B, C, D according to severity.

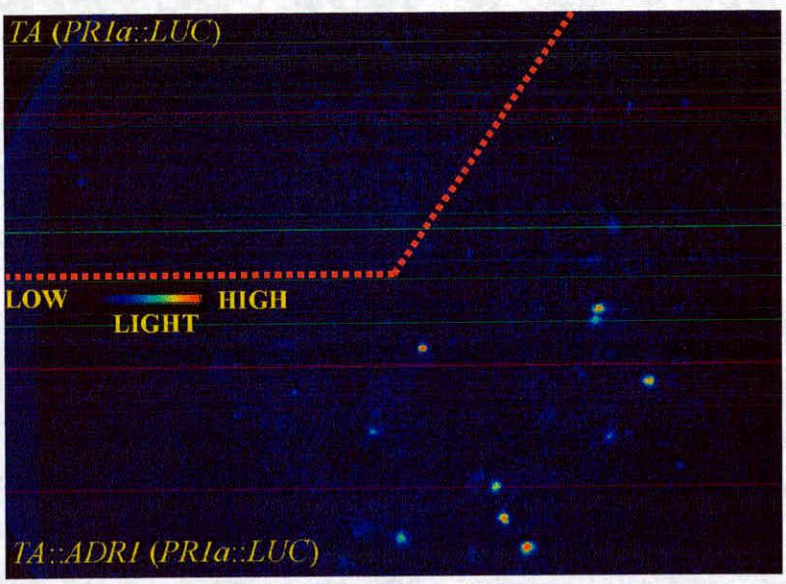


Figure 35: Transient expression of *ADR1* induces *PR1* gene expression
Luciferase activity is evident in 0/4 seedlings transformed with empty vector *TA* (top left) and 5/10 seedlings transformed with *TA::ADR1* (bottom right) after they have been transferred to a MS plate containing 30μM DEX.

distinct classes as depicted in Figure 33: A) normal; B) slight dwarfing, leaf curling, lesions apparent under long-day conditions; C) severe dwarfing, visible lesions (equivalent to hemizygous *adr1-D* mutant); D) very severe dwarfing, lesions, often non-viable (equivalent to homozygous *adr1-D* mutant). In total 46% of transgenic plants exhibited some form of aberrant morphology or LM phenotype (classes B-D). The spectrum of phenotypes obtained is probably a consequence of the level of endogenous transcription in the area of T-DNA insertion and number of insertional events that have taken place. In addition, different insertion events at identical positions in the plant genome may also give rise to different levels of expression.

iii) *CaMV 35S::ADR1* transgenics have elevated *PR1::LUC* expression and enhanced resistance to a fungal pathogen

Two leaves of every plant were imaged for luciferase activity with reference to *PR1a::LUC* (Col-0) control plants. In total 46% of plants belonging to phenotypic classes B-D but none from class A had significantly elevated luciferase expression. Previous observations had indicated that luciferase imaging was less sensitive than Northern analysis, thus it is likely a number of transgenic lines had elevated accumulation of *pr1* transcripts that could not be detected by ultra-low light imaging camera.

Disease resistance in transgenic lines was recorded 14 days after transferring plants to an environment conducive to powdery mildew infection. Plants were scored as follows: 0 - No infection; 1 - 1-2 leaves infected; 2 - mild infection on 3 or more leaves; 3 - heavy infection on 3 or more leaves. There was a significant correlation between *adr1-D* phenotype and resistance: 100% of plants of class A, but only 7% of B-D had infection scores of 2 or 3. Furthermore, resistance was shown to correlate strongly with severity of the phenotype, as D was the only class to exhibit zero infection. However, it should be noted that plants were scored at 8 weeks by which stage it had previously been observed that senescing leaves of *adr1-D* mutants may become infected, as was the case in this instance in classes B & D.

iv) Transient expression of *ADR1* induces *PR1*::*LUC* gene expression

A glucocorticoid-inducible transcription system has been developed enabling a much greater degree of control of transgene expression in plants in comparison with constitutive promoters such as *CaMV 35S*. This expression system consists of two components: a glucocorticoid-regulated transcription factor, GVG, containing the DNA binding domain of the yeast transcription factor GAL4, the transactivation domain of herpes viral protein VP16 and the receptor domain of the rat glucorticoid receptor. The second component of the system consists of a promoter containing six GAL4 DNA binding sites which drives downstream expression of the gene of interest. Following addition of glucocorticoid, the constitutively expressed GVG protein is activated upon binding with the steroid, which in turn binds to the GAL4 binding sites driving transcription of the downstream gene of interest (Aoyama & Chua, 1997; McNellis *et al.*, 1998).

The *ADR1* genomic clone was cloned into the binary vector pTA7001 containing the GVG expression system, and this cassette was then used to transform *PR1a*::*LUC* transgenics by *Agrobacterium*-mediated transformation. Firstly *Sal*I and *Spe*I sites were engineered by PCR at the 5' and 3' ends respectively of the *ADR1* genomic clone. This fragment was then cloned into the *Xho*I (which is compatible with a *Sal*I sticky end) and *Spe*I sites of the vector pTA7001 to generate the construct *TA*::*ADR1* (Figure 34). In addition to this construct, *PR1a*::*LUC* plants were also transformed with the empty vector pTA7001 as a negative control to generate lines subsequently referred to as *TA*.

Again due to time limitations, *TA*::*ADR1* transgenics could only be screened as primary transformants purely to ascertain that the system was working rather than to obtain any data regarding *ADR1* expression. Transformants were selected on hygromycin MS plates then transferred at two weeks to kanamycin MS plates to select for the *TA*::*ADR1* and *PR1a*::*LUC* cassettes respectively. Seedlings were then transferred to MS plates containing 30μM Dexamethasome (DEX). No discernible difference was observed when luciferase imaging was performed three days later. Imaging 14 days later revealed that 5/10 *TA*::*ADR1* plants, but 0/4 *TA* lines displayed

luciferase activity (Figure 35). No luciferase activity was evident in the negative controls *TA* on DEX plates and *TA*::*ADR1* on plates lacking DEX (data not shown).

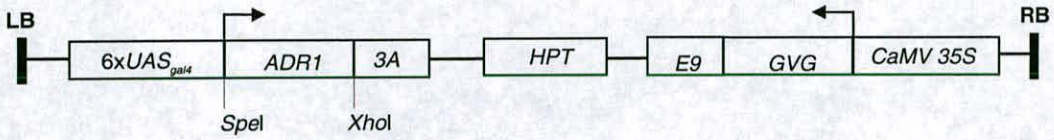


Figure 34: The glucocorticoid-inducible system for transient expression of *ADR1* in plants (adapted from McNellis *et al.*, 1998)

The *TA*::*ADR1* binary cassette depicted in the schematic was engineered by cloning *ADR1* into the *Spe*I/*Xho*I site of the vector pTA7001 between the glucocorticoid activated promoter (6xUAS_{gal4}) and pea *rbcsS*-A polyadenylation sequence (3A). The glucocorticoid responsive transcription factor (*GVG*) is driven by *CaMV 35S,* 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).

This experiment confirms that the DEX system can be used to drive defence gene expression via *ADR1* induction. Furthermore, expression is dependent on the presence of both the *ADR1* gene and application of DEX. There appeared to be a long time delay before *PR1*::*LUC* induction was evident given that maximal gene induction directly mediated by DEX (i.e. *ADR1* expression) is likely to occur 24 hours after DEX treatment (Aoyama and Chua, 1997), although imaging was not carried out between 3-14 days. However, it is likely that *PR1* transcripts accumulate much sooner than is possible to detect by the less sensitive means of luciferase imaging. Furthermore, *ADR1* expression is not being directly measured but induction of a downstream gene. In addition, it was previously observed that *PR1* gene expression was evident in *ADR1* adult plants but not seedlings thus DEX-induced defence gene induction in the *TA*::*ADR1* transgenics might also be partly developmentally regulated.

v) Discussion

The fact that overexpression of the *ADR1* candidate gene was able to recapitulate lesion development and luciferase expression confirms that this gene is responsible

for the *ADR1* phenotype. Although a large proportion of transgenics appeared phenotypically normal, this may be attributable to poor expression of *ADR1* possibly arising from integration of the transgene in a region of low transcriptional activity. An alternative explanation for the lack of any observable phenotype in *CaMV 35S::ADR1* transgenics is that *ADR1* expression maybe inhibited by sense suppression (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). It is worth noting that in another reported instance of *CaMV 35S*-mediated overexpression of a NBS-LRR, *RPS2*, only 6% of transgenics were able to complement a *RPS2*-null mutation. Furthermore, most of the wildtype plants transformed with *CaMV 35S::RPS2* were unable to mount a HR in response to *AvrRpt2* suggesting that sense suppression might be occurring (Mindrinos *et al.*, 1994). When inoculated with *Pst* DC3000(*AvrB*), *CaMV 35S::ADR1* transgenics were not found to be compromised in HR formation. Thus if sense suppression is occurring it does not appear to manifest itself by negating gene-for-gene interactions, at least in the case of *AvrB-RPM1*. Further studies need to be carried in the F_2 generation to determine whether any of the transgenic lines are compromised in their ability to mount broad spectrum resistance or drought tolerance.

Time limitations meant that only crude luciferase and disease resistance assays could be performed on the F_1 generation. However, to draw conclusions regarding the relationship between *ADR1* expression, the induction of other defence genes, and disease resistance, multiple plants from different transgenic lines would have to be assayed in the F_2 or F_3 generation. It would also be interesting to determine whether *CaMV 35S::ADR1* transgenics exhibit co-dominance like the *adr1-D* mutant. Lastly whilst at the morphological level *CaMV 35S::ADR1* transgenics appear similar to the *adr1-D* mutant it would be worthwhile to analyse differences in gene expression at the developmental level as at least in other example, enhancement of endogenous gene expression in an activation tagged mutant differed from constitutive ectopic expression (Weigel *et al.*, 2000).

We have also demonstrated in principle that *PR1* may be induced by DEX in *TA::ADR1* transgenics but not in *TA* lines. However, Northern analysis and disease

resistance assays must be performed in multiple transgenic homozygote lines in the F_3 generation before the usefulness of this system can be assessed. Furthermore, the DEX inducible system is far from optimal because of associated pleiotropic effects including growth retardation and induction of defence genes *PDF1.2, PR5*, but not *PR1*, observed in plants containing the empty vector following DEX application (Kang *et al.,* 1999). However, these shortcomings can be readily addressed by the use of appropriate controls. Alternative approaches for engineering resistance without compromising yield are discussed in the final chapter.

9) DISCUSSION

i) Introduction

In this concluding chapter, I explore a number of different themes that have arisen over the course of this work. However the *GST1*::*LUC* data is largely ignored in this section having been covered comprehensively earlier on. Firstly, I attempt to integrate current understanding of *ADR1* signalling into disease resistance and abiotic stress signal transduction pathways, and ascribe a putative function for the endogenous *ADR1* gene. Secondly, a model is formulated to account for overexpression of the *ADR1* gene giving rise to the *adr1-D* mutant phenotype. Finally, the potential for biotechnological exploitation of *ADR1*-derived technology is discussed.

ii) *ADR1* encodes a putative early regulator integrating multiple stress pathways

Having analysed *ADR1* from sequence, evolutionary, and functional perspectives we are now in a position to ascribe a putative function to this gene. The NBS-LRR gene family to which *ADR1* belongs is estimated to comprise 200-300 genes in *Arabidopsis,* (Young, 2000), making it one of the most prolific and yet enigmatic classes of plant genes. In the past, this class of genes has generally been considered in a resistance gene context due to lack of data pertaining to other NBS-LRRs, however data presented in this work suggests this is too great an oversimplification. Indeed established preconceptions of *R*-gene functions are continually being challenged in the light of new data that increasingly hints at diverse functions outside of their hypothesised role in *Avr* gene recognition. One such example of this is the observation that a point mutation in the resistance gene *RPS5* was shown to partially compromise gene-for-gene resistance to several bacterial and fungal isolates. This is in apparent contradiction to the proposed model of race-specific recognition which would predict that only resistance to bacteria harbouring *AvrPphB*, the avirulent gene recognised by *RPS5*, would be compromised (Warren *et al.,* 1998).

This notion of a more diverse and complicated role for NBS-LRRs is strongly reinforced by data presented within this work. Here we show that *ADR1* is rapidly inducible prior to SA accumulation following pathogen attack and also by wounding, BTH, and drought. Furthermore the overexpression of the *ADR1* gene resulted in constitutive activation of defences, perhaps not unsurprising in itself, but also conveyed protection to drought.

These results demand a reappraisal of the current perception of defence and abiotic stress signalling commonly treated as distinct disciplines. Furthermore, the results underline the use of activation tagging as a powerful functional genomics tool as it is probable that *adr1-D* would not have been isolated by knock-out mutagenesis. This can be inferred from the fact that no NBS-LRR other than *R*-genes is known to have been isolated by EMS or T-DNA disease resistance mutant screens despite the fact that they comprise 1% of the genome. This could be attributable to a degree of redundancy which may arise from overlapping functions of genes within this large gene family.

The fact that *ADR1* apparently regulates disease and drought resistance and is also activated by wounding suggests that it might act as a global regulatory gene to activate different pathways following diverse forms of environmental insult. This then alerts the plant to any impending danger, be it microbial, abiotic or perhaps even wounding as a result of insect feeding, thus causing the plant to activate its defences. Thus it may be advantageous to ensure against drought stress upon pathogen attack as dehydrated plants may be more disease susceptible (Chapter 7) and vice versa. One caveat in this theory is that whilst *ADR1* has been shown to be inducible by pathogen attack, it has yet to be shown to be expressed during drought stress. Thus it is conceivable, although unlikely, that drought tolerance may be a pleiotropic effect caused by the massive upregulation of *ADR1*, and not associated with the endogenous expression of *ADR1*.

A second intriguing point for analysis is the issue of differential splicing observed in response to avirulent pathogen attack in comparison with the other treatments.

Results suggest (Chapter 6) that a truncated form of *ADR1* is induced within 3 hours of pathogen recognition in addition to the full length transcript. These observations reflect similar data regarding tobacco *N* splicing following tobacco mosaic virus (TMV) infection. The full length TIR-NBS-LRR (N_S) transcript was more prevalent for the first three hours of TMV infection, whereas a truncated form lacking the LRR (N_L) predominated 4-8 hours post-infection (Dinesh-Kumar & Baker, 2000). In addition, a transcript with 82% identity to N_S was shown to be induced during Avr9- and Cf9-mediated defence responses in tobacco cell cultures (Durrant *et al.*, 2000). Thus it is possible to speculate that truncated *ADR1* might be more responsive to signals specifically associated with the Avr-R interaction, whereas the full-length *ADR1* transcript could be induced by a plethora of signalling events associated with the establishment of SAR, including ROI/RNI generation, ion fluxes, protein kinase cascades, and SA accumulation. A timecourse of *ADR1* expression during HR establishment and analysis using a similar suite of ROI/RNI and protein kinase inhibitors as used previously, would help identify the exact signals required for the rapid induction of this gene.

A number of questions still remain unanswered regarding *ADR1* in relation to other NBS-LRRs. Firstly, is the expression pattern and overexpression phenotype of *ADR1* unique to this gene and maybe its close homologues (Table 2), or common to a large proportion of NBS-LRR genes. If the latter scenario is true then it is likely that a number of similar mutants would have been identified by the dearth of activation tagging projects in progress. This has not been the case so far suggesting that *ADR1* may possess some unique feature. Perhaps more pertinent is the question regarding function of other uncharacterised NBS-LRRs which are likely to have some role other than acting as a genetic reservoir of *R*-gene-like genes enabling the plant to keep pace with the continuing diversification of pathogen avirulence genes. This might be addressed by taking a more directed functional approach such as overexpressing/antisensing sample NBS-LRRS from the different evolutionary clades.

Sequence comparison of the *ADR1* gene would appear to distinguish it from other NBS-LRRs analysed to date. Of particular prominence is the region with homology to CDPKs. Although this region of conservation is small and confined to two sub-domains, it is more highly conserved that many of the other sub-domains that make-up the NBS (van der Biezen & Jones, 1998). Furthermore, it contains the only two residues in these sub-domains that are ubiquitous to virtually all serine threonine kinases. In addition, the sub-domains are located near to the P-loop like in all serine threonine kinases. Moreover, overexpression of four other NBS-LRRs does not confer a LM phenotype (Chapter 6). Lastly the CDPK-like region appears to be present in all four *ADR1* transcripts (data not shown). Taken together, this data presents a compelling case for the *adr1-D* phenotype being in part mediated by a unique structural domain which may be the CDPK-like region. This area could hypothetically function in tandem with the P-loop to activate downstream effectors possibly by means of a phosphorylation cascade. Kinase assays using the purified ADR1 protein are now required to ascertain if this is indeed the case.

The uniqueness of *ADR1* is reinforced by the high degree of conservation in at least two monocot species which was not found in over 20 other NBS-LRRs tested to date. Moreover, *ADR1-L1* and *ADR1-L2* also retain this conservation with a diverse number of species including monocots. Proof of the evolutionary divergence of the clade to which *ADR1* belongs has been previously documented (Meyers *et al.,* 1999). The explanation for the existence of this distinct clade containing high conservation between genes of different classes may be central to resolving the question of the role of NBS-LRR genes.

Recent data suggests that even highly conserved NBS-LRRs may have divergent functions. *At-RSH1* was recently shown to interact specifically with the NBS of the *R*-gene *RPP5* in a yeast two-hybrid assay, whilst NBS sites of other *Arabidopsis R*-genes including the closely conserved expressed homologue, *RPP5-ColF* (92% similarity), failed to elicit a positive response (Van der Biezen *et al.*, 2000). Thus it would appear that not only do LRRs seem to be highly specific in terms of their ligand specificity (Ellis *et al.*, 2000), but individual NBSs may also differ

considerably in terms of their molecular interactions. It is anticipated that further yeast two-hybrid assays using both characterised *R*-genes and other NBS-LRRs may help illuminate functions of this class of genes in coming years.

From the previously mentioned data it is possible to speculate on the position of *ADR1* in a signal transduction pathway as illustrated in the schematic in Figure 36. *ADR1* is likely to be induced immediately downstream of the Avr-R complex as it was previously shown to be expressed within 3 hours of pathogen attack.

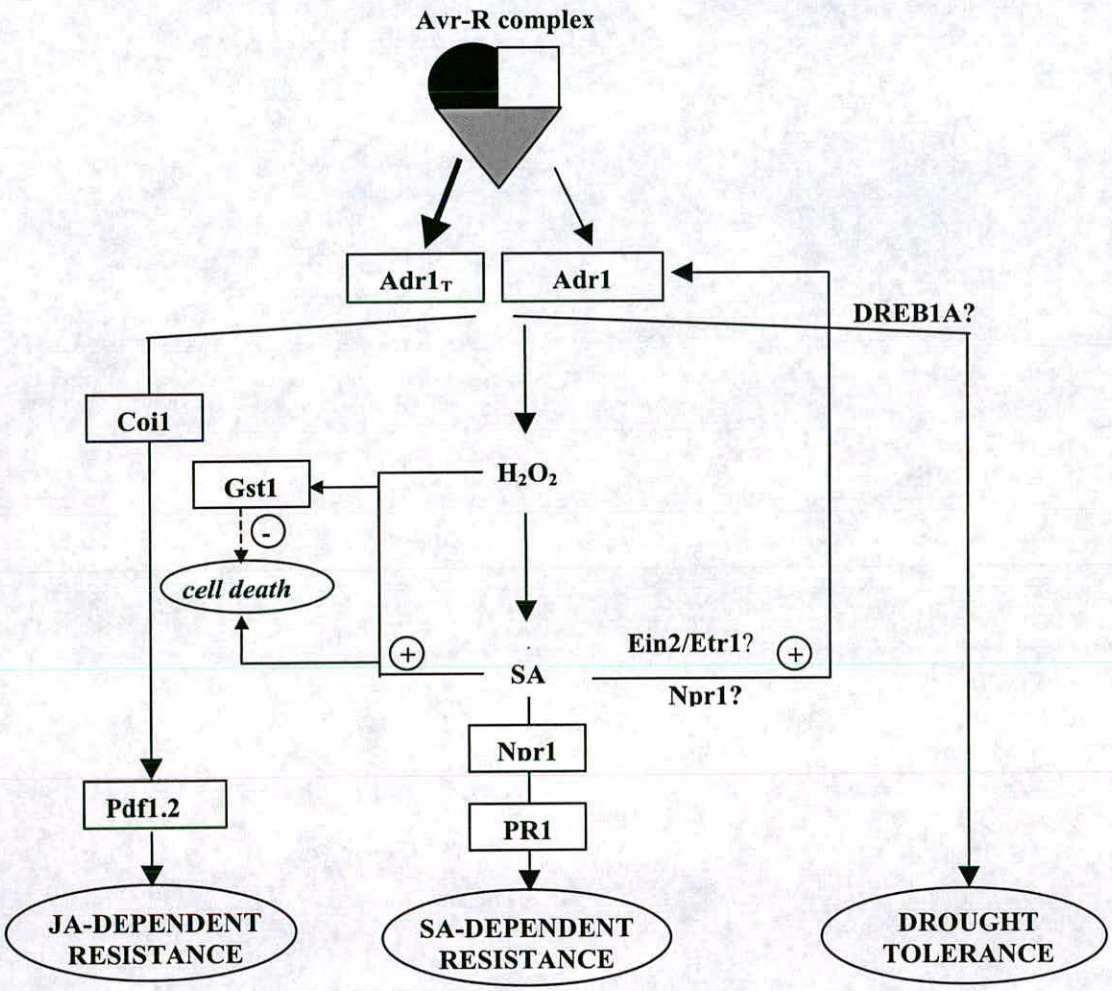


Figure 36: ADR1 mediates multiple pathways involved in disease and drought resistance
Speculative model integrates Adr1 into different signal transduction pathways downstream of the Avr-R interaction. Dashed line indicates negative feedback pathways; ⊖ ⊕ define positive and negative feedback loops respectively. Adr1$_T$ refers to the truncated form of Adr1.

However, induction of *ADR1* might also occur downstream of H_2O_2, as at three hours post-inoculation there is also substantial accumulation of ROI during the oxidative burst (Chapter 2). There is convincing evidence to support a SA-mediated positive feedback loop that induces *ADR1*, probably giving rise to hypersensitive cell death from the combined effect of ROI and SA as well as other effectors. Gst1 and other cellular protectants probably act to negatively regulate this pathway to prevent runaway cell death in distal cells. In the *adr1-D* mutant however, this balance is tipped in favour of a pro-death pathway, which may be exacerbated by additional ROI generated by photo-oxidation, giving rise to localised regions of tissue necrosis under high-light conditions.

At present it is not possible to determine the exact nature of negative regulation mediated by *ein2*, *etr1*, or *npr1* in attenuating the *adr1-D* phenotype but it is conceivable that these genes may be involved in the SA-positive feedback loop. *pdf1.2* expression and drought tolerance are both mediated independently of SA, the former via a JA-dependent signalling pathway. The mechanisms of drought tolerance in the *adr1-D* mutant remain enigmatic, though by analogy to *Avr9/Cf-9*-induced gene expression (Durrant *et al.*, 2000) it is tempting to speculate that the DREB1A transcription factor may mediate this process. One major caveat in the model as a whole is that it is based on data obtained from the *adr1-D* mutant and endogenous expression of the *ADR1* gene and it may not necessarily be possible to reconcile these two different systems.

This last point raises the pertinent question of how exactly at the molecular level does *ADR1* overexpression give rise to the observed phenotype. The obvious assumption is that signal transduction pathways normally activated following Avr recognition are constitutively switched on. Thus, assuming that *ADR1* acts at a similar section of the pathway to the Avr-R interaction, there are a number of potential mechanisms to account for overexpression leading to constitutive activation of downstream pathways. Assuming that the LRR of the endogenous ADR1 protein functions in ligand binding, the NBS and potentially the CDPK-like domain may then activate a downstream effector by means of phosporylation. This may or may

not require an additional protein, as the NBS lacks the recognised signature sequence for the catalytic site of a serine-threonine kinase.

Overexpression might thus activate downstream pathways by one of two mechanisms which are not mutually exclusive. Firstly, if the putative phosphorylation cascade is held in check by constitutive phosphatase activity, upregulation of *ADR1* would override this means of negative regulation thus switching on downstream pathways. Alternatively, the Adr1 protein may become activated following autophosphorylation, possibly via dimerisation mediated by the coiled-coil domain, and thus phosphorylated Adr1 may then be capable of activating downstream signalling components.

Whilst these hypotheses remain unsubstantiated, there is some degree of corroboration from the literature. Firstly overexpression of *Pto* confers a similar phenotype to that of *ADR1* (Tang *et al.,* 1999) and the Pto protein, albeit a *bona fide* protein kinase, has been shown to be capable of autophosphorylation (Sessa *et al.,* 2000). Indeed autophosphorylation is also known to modulate protein-protein interactions as in the case of the receptor-like kinase RLK5 of *Arabidopsis* (Stone *et al.,* 1994), and thus could conceivably affect the specificity of ligand binding. Furthermore, a novel class of kinases have been identified that only share homology with the ATP-binding site of conventional protein kinases (Ryazanov *et al.,* 1997 and references therein) so it is conceivable that Adr1 and other proteins with a NBS might also fall into this category. However, at present biochemical data for NBS-LRRs specifically pertaining to kinase activity remains conspicuously absent.

To conclude, a wealth of questions remain to be answered pertaining to the exact mechanism by which *ADR1* activates signal transduction pathways leading to disease and drought resistance. It is anticipated that biochemical studies, in depth analysis of *ADR1* endogenous expression as outlined, transgenic antisense lines, and the use of the inducible system to dissect the early events surrounding activation of *ADR1*, should yield novel insights into *ADR1* signalling. Yeast two-hybrid analysis using different portions of *ADR1* as bait might also produce interesting findings, given the

evolutionary divergence of *ADR1* from other NBS-LRRs. Lastly, a suppresser screen could be used to isolate mutants that block the *adr1-D* phenotype. This approach has previously been used to isolate suppressers of the cell death mutant *lsd5*, which were shown to convey altered patterns of disease susceptibility in response to different pathogens (Morel and Dangl, 1999).

iii) *ADR1* from a biotechnological perspective

ADR1 is believed to be the first report of a plant mutant that conveys resistance to both disease and drought stress. As these two factors comprise the two greatest environmental causes of crop losses, there is considerable potential for the commercial exploitation of this technology. Furthermore, preliminary results indicate that the mutant may also be resistant to freezing and high salinity (D.Basu - unpublished), problems often associated with land under irrigation and or prone to frost. However, we must first consider the applicability of the technology from a scientific perspective. Such a feasibility study should also take into account other factors, such as existing alternatives, perceived benefits to both farmer and consumer, and public acceptance of such genetically modified (GM) foods, however there is insufficient scope for in depth discussion here.

One of the most important questions to address regarding the feasibility of *ADR1* as a method of crop protection, is whether the technology will actually function in agronomically important crop species. This could be ascertained either using the *Arabidopsis ADR1* gene in a heterologous system, or using the genes predicted to high identity with *ADR1* at the amino acid level (Table 2) in the endogenous plant species. The apparent high conservation of *ADR1* in other plant species including monocots and even gymnosperms is encouraging when considered in comparison with data of heterologous expression of another NBS-LRR, the *Bs2* gene of pepper. In addition to pepper, this gene is also able to convey gene-specific resistance to bacterial spot in tomato and tobacco (Tai *et al.*, 1999). This is despite TblastN searches revealing that the closest known *Bs2* homlogue in tomato shares only 43% identity with the pepper gene and no close homologues are apparent in tobacco (results not shown). However, as all three plants belong to the *Solanaceae* family, it

is possible that this reiteration of function might be more dependent on conservation of downstream signalling machinery rather than the *Bs2* gene itself.

Assuming function is conserved between evolutionary diverse plant species, another important consideration is the need to manipulate expression so as to generate plants which retain the beneficial attributes without a yield penalty. Preliminary data suggests that there is a strong correlation between disease resistance and severity of LM phenotype, including dwarf stature and associated low yield. However observations in the F_1 generation of *CaMV 35S::ADR1* transgenics indicate that it may be possible to have moderately enhanced resistance with no yield penalty, though this remains to be substantiated in the F_2 generation. Indeed, it is interesting to note that only five-fold rises in *PR1* expression mediated by overexpression of the NBS-LRR *Prf* are required to confer bacterial resistance in tomato with no associated yield penalty (Oldroyd & Staskawicz, 1998). This strategem could equally be applied to *ADR1* by transforming *Arabidopsis* with the gene under the control of its native promoter and screening for disease resistance in plants of normal stature.

An inducible expression system has been employed in an attempt to address the resistance:yield issue. Preliminary results have shown that transient expression can induce *PR1* gene expression in seedlings grown on DEX plates. Furthermore DEX treatment of adult plants does not adversely affect size or yield (results not shown). Crucially however, it remains to be determined whether transient induction of *ADR1* in adult plants can convey broad spectrum disease resistance without compromising yield. Support for this assumption can be gleaned from the observation that overexpression of another activation tagged gene, *CDR1*, was shown to cause stunting and a LM phenotype when overexpressed. However, when *CDR1* was transiently expressed using the DEX system, resistance was attained in plants without compromising size (Xia *et al.*, - unpublished). If the same is true for *ADR1* then an environmentally friendly analogous system, such as the ethanol-inducible promoter (Salter *et al.*, 1998), could be a viable alternative for use in crop plants. Indeed in some instances, transient rather than constitutive expression of defence

pathways may be obligatory because pathogenesis-related proteins are known plant allergens (Hoffman-Sommergruber, 2000).

Aside from constitutive or chemically inducible transient expression there are a number of alternative options for engineering *ADR1*-mediated resistance without yield penalties. Other examples include the use of tissue specific promoters such as the leaf-specific promoter *RBCS-3C* (Dai *et al.,* 2000); a promoter of a gene that is switched on by virulent pathogens such as *GST1*, perhaps as a tetramer to enhance expression; or even circadian-regulated promoter such as *CAB2* (Millar *et al.*, 1992). Lastly, *ADR1* gene expression under the control of a tetramer of the *ADR1* promoter might significantly enhance upregulation of endogenous defence responses following attempted infection.

One last issue for consideration is the matter of pleiotropic effects mediated by *ADR1,* in that defence, abiotic stress, and conceivably other pathways are constitutively activated in the *ADR1* mutant. In some cases it may be beneficial to have both defence and drought response pathways activated, for example, if the crop is prone to drought or infection by a disease that is provoked by dehydration stress (e.g. stalk rot in sorghum). However, drought protection in crops grown in latitudes not subject to drought might amount to an unwanted drain on the plant's resources. Thus it would be interesting to determine whether it is possible to uncouple disease and drought resistance. One approach might be to overexpress different transcripts of *ADR1* to determine if different portions of the gene govern different signalling pathways. Genes downstream of the disease/drought resistance branchpoint could also be isolated by means of a suppresser screen or yeast two-hybrid experiments.

To conclude, I have shown that whilst *ADR1*-based technology shows considerable potential for use in a crop-protection context on a global scale, further experiments, as outlined above, are required for complete validation. Whilst the need for increased agricultural productivity in both developed and developing world was highlighted in the first chapter, altruistic and capitalistic motives might seem too diametrically opposed to be mutually compatible. However, the royalty-free

donation of technology to subsistence farmers in the developing world might serve as a shrewd public relations strategy for the embattled agbiotech companies. This concept has apparently already been realised by Monsanto and Novartis who have waived their intellectual property rights associated with the much publicised vitamin A-enriched 'Golden Rice' (Nomile, 2000).

APPENDIX I: SEQUENCE OF *ADR1* GENOMIC CLONE

```
-689  ataccattaa gtaatttgaa gaaatatata aaatattaat caaaacgcgt
-639  aaaaagagta aagtgatctt tgatgattag atctacaaat cgacgtcaaa
                                                 ASF-1 motif
-589  aggaaaaaaa aaaaaaaagc tacaaaataa acattgaaaa agttcgagtc
-539  cctaatccct atatagttta tacacatatc aacaccaaat aatcacaaga
-489  aaaaaaaatt ataaacaagg ttgcatatgg gatcgttttt ctttcccaat
-439  tagttaaatt agatagagat agcgaccaaa aaaaacaaga ttagaggtat
-389  taatccaaat aggatttagc taactttaaa caattacact cccaaagtga
-339  ttgaacccgt atggccgtat tagggaatat cgtaatttag agtttgtcta
-289  gataatcgaa ttcgaaaaaa aactactaaa tcgatctgtt tgataataag
-239  tgaaaaactg atacgtaacc ataagatcca tacattctct gaaatctatt
-189  acaaagctat tacatgtgtt accgcagaaa caaaacttta aaacactaaa
                MYC
-139  cgacaaaacg tatgttattg gaagttgcta agaaacttcg tcttcttcgc
                                              TCA motif
 -89  tattttctaa tctaaaaacg tatcaaccct tattaccact caaccatctc
 -39  actttctttt ttctgtctta cactccaaga tcggtctcg
       TCA motif
   1 ATGGCTTCGTTCATAGATCTTTTCGCCGGCGACATCACGACGCAACTCTTAAAGCTGCTC
   1 M  A  S  F  I  D  L  F  A  G  D  I  T  T  Q  L  L  K  L  L

  61 GCTCTAGTAGCTAACACAGTCTACAGTTGCAAAGGAATCGCCGAACGACTGATCACGATG
  21 A  L  V  A  N  T  V  Y  S  C  K  G  I  A  E  R  L  I  T  M
                                          CDPK homology
 121 ATCAGAGACGTTCAACCAACGATCAGGGAGATCCAATACAGTGGCGCGGAGCTGAGTAAC
  41 I  R  D  V  Q  P  T  I  R  E  I  Q  Y  S  G  A  E  L  S  N

 181 CATCACCAAACTCAACTGGGAGTATTCTATGAGATCTTGGAGAAAGCAAGAAAGCTATGT
  61 H  H  Q  T  Q  L  G  V  F  Y  E  I  L  E  K  A  R  K  L  C

 241 GAAAAGGTTTTAAGATGCAATAGGTGGAACCTTAAACATGTGTACCACGCGAATAAGATG
  81 E  K  V  L  R  C  N  R  W  N  L  K  H  V  Y  H  A  N  K  M

 301 AAGGATCTCGAGAAACAAATATCTCGTTTCCTCAACAGCCAGATTTTGCTCTTTGTTCTT
 101 K  D  L  E  K  Q  I  S  R  F  L  N  S  Q  I  L  L  F  V  L
       coiled-coil region
 361 GCTGAGGTATGTCATCTACGGGTCAATGGTGACAGGATTGAGAGGAATATGGATAGACTG
 121 A  E  V  C  H  L  R  V  N  G  D  R  I  E  R  N  M  D  R  L

 421 TTGACTGAGCGGAATGATTCTTTGTCGTTTCCGGAGACTATGATGGAGATTGAGACGGTA
 141 L  T  E  R  N  D  S  L  S  F  P  E  T  M  M  E  I  E  T  V

 481 AGCGATCCGGAGATTCAGACGGTATTGGAATTGGGGAAGAAAAAGGTGAAGGAGATGATG
 161 S  D  P  E  I  Q  T  V  L  E  L  G  K  K  K  V  K  E  M  M

 541 TTTAAGTTTACGGATACACATTTGTTTGGGATCTCTGGAATGAGCGGTTCAGGGAAAACC
 181 F  K  F  T  D  T  H  L  F  G  I  S  G  M  S  G  S  G  K  T
                                          Kinase 1a
 601 ACTCTTGCAATAGAGCTTTCAAAGGACGATGATGTTCGAGgtttgtgaagttttattgaa
 201 T  L  A  I  E  L  S  K  D  D  D  V  R  G

 661 ttttgttttgtgttgatggtcatgagttctaatcaagatttttgtgcagGACTCTTTAAG
 215                                                  L  F  K

 721 AATAAGGTTTTGTTTTTGACTGTGTCACGGTCTCCGAATTTTGAGAATTTGGAGTCTTGT
 218 N  K  V  L  F  L  T  V  S  R  S  P  N  F  E  N  L  E  S  C

 781 ATACGAGAATTTCTTTATGATGGAGTTCATCAACGGAAGCTAGTGATTCTTGATGATGTT
 238 I  R  E  F  L  Y  D  G  V  H  Q  R  K  L  V  I  L  D  D  V
                                          Kinase 2
 841 TGGACAAGGGAGTCCTTGGACAGGCTGATGTCTAAAATTCGTGGAAGCACTACTTTAGTA
 258 W  T  R  E  S  L  D  R  L  M  S  K  I  R  G  S  T  T  L  V
                                          Kinase 3a
 901 GTGTCACGGTCCAAGCTTGCAGATCCTAGAACCACCTACAATGTGGAATTATTAAAGAAA
 278 V  S  R  S  K  L  A  D  P  R  T  T  Y  N  V  E  L  L  K  K

 961 GATGAAGCAATGTCTCTTTTGTGTCTCTGTGCTTTCGAACAAAAGTCCCCGCCTTCTCCA
 298 D  E  A  M  S  L  L  C  L  C  A  F  E  Q  K  S  P  P  S  P
                Motif 4
1021 TTCAACAAATATTTGGTGAAGCAGgtactaatgccttttataaatatttgaatctgtaa
 318 F  N  K  Y  L  V  K  Q
                Motif 3
```

```
1081 ctaaaaacacactttgcttccaaatactaaaattgttactctgtttggtagGTTGTTGAT
 327                                                    V  V  D

1141 GAGTGTAAAGGTTTACCTTTATCTTTGAAAGTTCTTGGTGCTTCGTTAAAAAACAAACCT
 329 E  C  K  G  L  P  L  S  L  K  V  L  G  A  S  L  K  N  K  P
                HD Motif
1201 GAAAGATATTGGGAAGGCGTAGTGAAGAGGTTATTAAGAGGAGAAGCTGCTGATGAAACT
 349 E  R  Y  W  E  G  V  V  K  R  L  L  R  G  E  A  A  D  E  T

1261 CATGAGAGCAGAGTGTTTGCTCATATGGAAGAAAGTCTAGAAAACCTCGACCCGAAAATC
 369 H  E  S  R  V  F  A  H  M  E  E  S  L  E  N  L  D  P  K  I
                            Motif 2
1321 CGAGACTGTTTCTTGGATATGGGTGCTTTCCCTGAAGACAAGAAGATCCCTCTTGATCTT
 389 R  D  C  F  L  D  M  G  A  F  P  E  D  K  K  I  P  L  D  L

1381 CTCACGAGCGTGTGGGTTGAGAGGCATGATATTGACGAGGAAACTGCGTTTTCCTTTGTT
 409 L  T  S  V  W  V  E  R  H  D  I  D  E  E  T  A  F  S  F  V

1441 CTTCGTTTAGCTGACAAGAATCTCCTTACTATAGTGAACAATCCGAGgtattcgctcttt
 429 L  R  L  A  D  K  N  L  L  T  I  V  N  N  P  R

1501 tatgactttgtctcttgtaccaacacttcagtgggattaatcatatcataaatttgcagG

1561 TTTGGCGATGTGCACATTGGCTACTATGATGTATTTGTGACGCAACACGATGTTTTGAGA
 445 F  G  D  V  H  I  G  Y  Y  D  V  F  V  T  Q  H  D  V  L  R
                                              Motif 5
1621 GACCTAGCCCTTCATATGTCCAATCGTGTGGACGTAAATAGGAGAGAGCGGTTATTAATG
 465 D  L  A  L  H  M  S  N  R  V  D  V  N  R  R  E  R  L  L  M

1681 CCAAAAACAGAGCCAGTGCTTCCAAGAGAATGGGAAAAGAATAAAGATGAGCCATTTGAT
 485 P  K  T  E  P  V  L  P  R  E  W  E  K  N  K  D  E  P  F  D

1741 GCCAAGATAGTTTCCCTTCATACAGgcaagacttctttaactttaaatgaatttggttag
 505 A  K  I  V  S  L  H  T  G
1801 tgactagagtcttcaagttgatacgagccaaagatttagtctttgaactcgtaatcctag
1861 actttcttaacgtgataaataagaaagccactaaaataaaaccaaaacacctaaaactct
1921 tatcacgtatcaggtttgatagatttttttgtgtctttagGGGAAATGGATGAAATGAAT
 514                                                E  M  D  E  M  N

1981 TGGTTTGACATGGACCTCCCTAAGGCAGAAGTTTTAATACTGAACTTCTCTTCGGACAAC
 520 W  F  D  M  D  L  P  K  A  E  V  L  I  L  N  F  S  S  D  N

2041 TACGTCTTGCCACCATTTATTGGTAAGATGAGTAGACTCAGGGTGCTCGTGATTATCAAC
 540 Y  V  L  P  P  F  I  G  K  M  S  R  L  R  V  L  V  I  I  N

2101 AATGGCATGTCTCCTGCGCGTCTACATGGCTTCTCCATCTTTGCCAATTTGGCCAAACTG
 560 N  G  M  S  P  A  R  L  H  G  F  S  I  F  A  N  L  A  K  L

2161 AGGAGTCTCTGGCTCAAGAGGGTACATGTCCCTGAACTCACCAGCTGCACCATTCCACTG
 580 R  S  L  W  L  K  R  V  H  V  P  E  L  T  S  C  T  I  P  L

2221 AAAAACCTGCACAAGATACATCTAATCTTTTGTAAGGTCAAGAACAGCTTTGTTCAGACG
 600 K  N  L  H  K  I  H  L  I  F  C  K  V  K  N  S  F  V  Q  T

2281 TCATTCGACATCTCGAAAATATTCCCAAGCTTGTCTGATCTCACTATTGATCACTGTGAT
 620 S  F  D  I  S  K  I  F  P  S  L  S  D  L  T  I  D  H  C  D

2341 GATCTTTTGGAACTAAAATCCATATTTGGAATAACCTCTCTCAACTCTCTCAGCATAACC
 640 D  L  L  E  L  K  S  I  F  G  I  T  S  L  N  S  L  S  I  T

2401 AACTGTCCACGGATTCTTGAATTGCCCAAGAATTTGAGTAACGTACAGTCCCTTGAACGT
 660 N  C  P  R  I  L  E  L  P  K  N  L  S  N  V  Q  S  L  E  R

2461 CTAAGGTTATATGCCTGCCCCGAGCTGATATCCCTCCCGGTCGAAGTTTGTGAGCTGCCA
 680 L  R  L  Y  A  C  P  E  L  I  S  L  P  V  E  V  C  E  L  P

2521 TGTCTAAAGTACGTTGACATTTCACAGTGTGTCAGCCTGGTTTCTCTTCCTGAAAAGTTT
 700 C  L  K  Y  V  D  I  S  Q  C  V  S  L  V  S  L  P  E  K  F

2581 GGAAAGCTAGGGAGTCTTGAGAAAATTGACATGAGAGAATGCAGTTTATTGGGTTTACCA
 720 G  K  L  G  S  L  E  K  I  D  M  R  E  C  S  L  L  G  L  P

2641 AGTTCTGTAGCTGCACTTGTGTCTCTACGCCATGTCATTTGCGATGAGGAGACTTCGTCT
```

```
 740 S  S  V  A  A  L  V  S  L  R  H  V  I  C  D  E  E  T  S  S

2701 ATGTGGGAAATGGTCAAGAAGGTGGTTCCTGAACTTTGCATTGAAGTCGCCAAAAAATGC
 760 M  W  E  M  V  K  K  V  V  P  E  L  C  I  E  V  A  K  K  C

2761 TTCACCGTGGATTGGCTTGACGATTAGgctcttgtaatcattgatcagcagtaattagag
 780 F  T  V  D  W  L  D  D  *
2820 cgagttagagcgagttatttgaagtttggaatgtttctctctatatataagatcattttt
2980 agctatccatcttaactttaagaagagttgttttctgtttatgaagttaatgcactggtt
3040 ctaggagcggagccaattaaggaggaggggatcatttaaccccataacattataatttt
3100 ttaattataaggtaaattacctaataattattaatgttttggtataaactttctaagttg
3160 accccatgtcatgccctagttttctacttttgatcccattcatatttgtttttctctcaa
3220 cattaatttctttttccatgtttattttgtgctttcctaacaaatgattacaaatctctag
3280 tattgattacacattaggcttttcacatattgattgtttttcttctcattggatatcat
3340 gtttgtttttttgtccttcttcttcttcaaaatcgtttttacatatatttctatgtaagtcaag
3400 aattgttttataaataagcctaatgtgtaataaatagaac
```

Appendix I: Sequence of the *ADR1* genomic clone

Numbers relate to position with respect to translation start site; non-translated regions (i.e. promoter, introns and 5' and 3' UTR) in lower case; motifs in promoter and amino acid sequence indicated in bold, LRRs underlined. Putative TATA box and two polyadenylation signals are shaded. *Xho*I site in italics (307) indicating that region approx. 1kb upstream from here used as *ADR1* probe in Northern analysis.

BIBLIOGRAPHY

Ausubel FM, Brent R, Kingston RE, *et al*, editors. Current Protocols in Molecular Biology. New York: John Wiley and Sons, (1996)

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **95,**(17) 10306-11.

Abate, C., Patel, L., Rauscher, F.J. and Curran, T. (1990) Redox regulation of FOS and JUN DNA-binding activity *in vitro*. *Science* **249,** 1157-61.

Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D. and Shinozaki, K. (1997) Role of *Arabidopsis* MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* **9,**(10) 1859-68.

Achar, P.N. (1998) Effects of temperature on germination of *Peronospora parasitica* conidia and infection of *Brassica oleracea*. *Journal Of Phytopathology-Phytopathologische Zeitschrift* **146,**(2-3) 137-41.

Alber, T. (1992) Structure of the leucine zipper. *Curr Opin Genet Dev* **2,**(2) 205-10.

Alexander, D., Goodman, R.M., Gutrella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahlgoy, P., Luntz, T., Ward, E. *et al.* (1993) Increased tolerance to 2 oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein-1a. *Proc. Natl. Acad. Sci. USA* **90,**(15) 7327-31.

Alfano, J.R. and Collmer, A. (1996) Bacterial pathogens in plants: Life up against the wall. *Plant Cell* **8,**(10) 1683-98.

Allan, A.C. and Fluhr, R. (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* **9,** 1559-72.

Allen, R.D., Webb, R.P. and Schake, S.A. (1997) Use of transgenic plants to study antioxidant defenses. *Free Rad. Biol. Med.* **23,**(3) 473-9.

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92,** 773-84.

Aoyama, T. and Chua, N.H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant Journal* **11,**(3) 605-12.

Apostol, I., Heinstein, P.F. and Low, P.S. (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant-cells - role in defense and signal transduction. *Plant Physiol.* **90,** 109-16.

Barcelo, A.R. (1998) Use and misuse of peroxidase inhibitors. *Trends In Plant Sci.* **3,** 418

Bauskin, A.R., Alkalay, I. and Benneriah, Y. (1991) Redox regulation of a protein tyrosine kinase in the endoplasmic-reticulum. *Cell* **66,** 685-96.

Bestwick, C.S., Brown, I.R. and Mansfield, J.W. (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. *Plant Physiol.* **118,**(3) 1067-78.

Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W. *et al.* (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* **391,**(6666) 485-8.

Bleecker, A.B., Estelle, M.A., Somerville, C. and Kende, H. (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis-thaliana*. *Science* **241,** 1086-9.

Bolwell, G.P. (1999) Role of active oxygen species and NO in plant defence responses. *Curr. Op. Plant Biol.* **2,**(4) 287-94.

Bolwell, G.P. and Wojtaszek, P. (1997) Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. *Physiol. Mole. Plant Path.* **51,** 347-66.

Botella, M.A., Parker, J.E., Frost, L.N., Bittner-Eddy, P.D., Beynon, J.L., Daniels, M.J., Holub, E.B. and Jones, J.D.G. (1998) Three genes of the *Arabidopsis* RPP1 complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell* **10,**(11) 1847-60.

Bowler, C. and Fluhr, R. (2000) The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends In Plant Sci.* **5,**(6) 241-6.

Bowling, S.A., Clarke, J.D., Liu, Y.D., Klessig, D.F. and Dong, X.N. (1997) The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* **9,**(9) 1573-84.

Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F. and Dong, X.I. (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired-resistance. *Plant Cell* **6,**(12) 1845-57.

Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992) Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant-cell wall protein - a novel, rapid defense response. *Cell* **70,** 21-30.

Cai, D.G., Kleine, M., Kifle, S., Harloff, H.J., Sandal, N.N., Marcker, K.A., KleinLankhorst, R.M., Salentijn, E.M.J., Lange, W., Stiekema, W.J. *et al.* (1997) Positional cloning of a gene for nematode resistance in sugar beet. *Science* **275,**(5301) 832-4.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X.N. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6,**(11) 1583-92.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X.N. (1997) The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88,**(1) 57-63.

Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., VanMontagu, A., Inze, D. and VanCamp, W. (1998) Defense activation and enhanced pathogen tolerance induced by H2O2 in transgenic tobacco. *Proc. Natl. Acad. Sci. USA* **95,** 5818-23.

Chen, Z.X., Iyer, S., Caplan, A., Klessig, D.F. and Fan, B.F. (1997) Differential accumulation of salicylic acid and salicylic acid-sensitive catalase in different rice tissues. *Plant Physiol.* **114,**(1) 193-201.

Clarke, J.D., Liu, Y.D., Klessig, D.F. and Dong, X.N. (1998) Uncoupling PR gene expression from NPR1 and bacterial resistance: Characterization of the dominant *Arabidopsis cpr6-1* mutant. *Plant Cell* **10,**(4) 557-69.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16,** 735-43.

Cohen, P. (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.* **7,**(9) 353-61.

Conklin, P.L. and Last, R.L. (1995) Differential accumulation of antioxidant messenger-rnas in *Arabidopsis-thaliana* exposed to ozone. *Plant Physiol.* **109,**(1) 203-12.

Creelman, R.A. and Mullet, J.E. (1997) Biosynthesis and action of jasmonates in plants. *Ann. Rev. Plant Physiol. Plant Mole. Biol.* **48,** 355-81.

Cronquist, A. Introductory Botany. **2nd,**New York: Harper & Row, (1971)

Dai, Z.Y., Hooker, B.S., Anderson, D.B. and Thomas, S.R. (2000) Expression of *Acidothermus cellulolyticus* endoglucanase E1 in transgenic tobacco: biochemical characteristics and physiological effects. *Transgenic Research* **9,**(1) 43-54.

Dangl, J.L., Ritter, C., Gibbon, M.J., Mur, L.A.J., Wood, J.R., Goss, S., Mansfield, J., Taylor, J.D. and Vivian, A. (1992) Functional homologs of the *Arabidopsis Rpm1* disease resistance gene in bean and pea. *Plant Cell* **4,**(11) 1359-69.

de Maagd, R.A., Bosch, D. and Stiekema, W. (1999) *Bacillus thuringiensis* toxin-mediated insect resistance in plants. *Trends Plant Sci.* **4,**(1) 9-13.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E. *et al.* (1994) A central role of salicylic-acid in plant-disease resistance. *Science* **266,**(5188) 1247-50.

Delledonne, M., Xia, Y.J., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394,** 585-8.

Demple, B. and Amabile-Cuevas, C.F. (1991) Redox redux - the control of oxidative stress responses. *Cell* **67,** 837-9.

Dempsey, D.A., Shah, J. and Klessig, D.F. (1999) Salicylic acid and disease resistance in plants. *Crit. Rev. Plant Sci.* **18,**(4) 547-75.

De-Wit, P.J.G.M., Joosten, M.H.A.J., Honee, G., Vossen, P.J.M.J., Cozijnsen, T.J., Koomangersmann, M. and Vogelsang, R. (1995) Molecular aspects of avirulence genes of the tomato pathogen *Cladosporium fulvum*. *Can. Jour. Bot.-Rev.* **73,** S490-4.

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A. and Dangl, J.L. (1994) *Arabidopsis* mutants simulating disease resistance response. *Cell* **77,**(4) 565-77.

Dinesh-Kumar, S.P. and Baker, B.J. (2000) Alternatively spliced *N* resistance gene transcripts: Their possible role in tobacco mosaic virus resistance. *Proc. Natl. Acad. Sci. USA* **97,**(4) 1908-13.

Diourte, M., Starr, J.L., Jeger, M.J., Stack, J.P. and Rosenow, D.T. (1995) Charcoal rot (*Macrophomina-phaseolina*) Resistance and the effects of water-stress on disease development in sorghum. *Plant Pathology* **44,**(1) 196-202.

Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K. and Jones, J.D.G. (1996) The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine rich repeat proteins. *Cell* **84,**(3) 451-9.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. (1995) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. USA* **92,**(10) 4095-8.

Dong, X.N. (1998) SA, JA, ethylene, and disease resistance in plants. *Current Opinion In Plant Biology* **1,**(4) 316-23.

Draper, J. (1997) Salicylate, superoxide synthesis and cell suicide in plant defence. *Trends In Plant Science* **2,**(5) 162-5.

Droge-Laser, W., Kaiser, A., Lindsay, W., Halkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A. and Lamb, C. (1997) Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. *EMBO Journal* **16,**(4) 726-38.

Droog, F.N.J., Hooykaas, P.J.J., Libbenga, K.R. and Vanderzaal, E.J. (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione S-transferases and one member indeed shows *in vitro* GST activity. *Plant Molecular Biology* **21,** 965-72.

Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. USA* **95,** 10328-33.

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E. and Jones, J.D.G. (2000) cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* **12,**(6) 963-77.

Ellis, J., Dodds, P. and Pryor, T. (2000) Structure, function and evolution of plant disease resistance genes. *Current Opinion In Plant Biology* **3,**(4) 278-84.

Ellis, J.G., Lawrence, G.J., Luck, J.E. and Dodds, P.N. (1999) Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. *Plant Cell* **11,**(3) 495-506.

Epple, P., Apel, K. and Bohlmann, H. (1995) An *Arabidopsis-thaliana* thionin gene is inducible via a signal-transduction pathway different from that for pathogenesis-related proteins. *Plant Physiol.*. **109,**(3) 813-20.

Epple, P., Apel, K. and Bohlmann, H. (1997) Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell* **9,**(4) 509-20.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J. and Parker, J.E. (1999) EDS1, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. USA* **96,**(6) 3292-7.

Fauth, M., Merten, A., Hahn, M.G., Jeblick, W. and Kauss, H. (1996) Competence for elicitation of H_2O_2 in hypocotyls of cucumber is induced by breaching the cuticle and is enhanced by salicylic acid. *Plant Physiol.*. **110,**(2) 347-54.

Feldmann, K.A. (1991) T-DNA insertion mutagenesis in *arabidopsis* - mutational spectrum. *Plant Journal* **1,**(1) 71-82.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C. and Dixon, R.A. (1999) Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology* **9,**(6) 317-20.

Fenselau, S., Balbo, I. and Bonas, U. (1992) Determinants of pathogenicity in *Xanthomonas-campestris* pv *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Molecular Plant-Microbe Interactions* **5,** 390-6.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male-sterile, insensitive to Methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* **6,** 751-9.

Fitchen, J.H. and Beachy, R.N. (1993) Genetically-engineered protection against viruses in transgenic plants. *Annual Review Of Microbiology* **47,** 739-63.

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A. and Ryals, J. (1995) Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Molecular Biology* **29,**(5) 959-68.

Frutos, R., Rang, C. and Royer, F. (1999) Managing insect resistance to plants producing Bacillus thuringiensis toxins. *Critical Reviews In Biotechnology* **19,**(3) 227-76.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic-acid for the induction of systemic acquired- resistance. *Science* **261,** 754-6.

Galan, J.E. and Collmer, A. (1999) Type III secretion machines: Bacterial devices for protein delivery into host cells. *Science* **284,**(5418) 1322-8.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R. and Ausubel, F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics* **146,**(1) 381-92.

Gleave, A.P. (1992) A versatile binary vector system with a T-DNA organizational-structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Molecular Biology* **20,** 1203-7.

Goldsbrough, A.P., Albrecht, H. and Stratford, R. (1993) Salicylic acid-inducible binding of a tobacco nuclear-protein to a 10-bp sequence which is highly conserved amongst stress-inducible genes. *Plant Journal* **3,**(4) 563-71.

Gorlach, J., Volrath, S., KnaufBeiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. *et al.* (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* **8,**(4) 629-43.

Govrin, E.M. and Levine, A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. *Current Biology* **10,**(13) 751-7.

Grant, J.J. and Loake, G.J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol* **124,**(1) 21-30.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W. and Dangl, J.L. (1995) Structure of the *arabidopsis Rpm1* gene enabling dual-specificity disease resistance. *Science* **269,**(5225) 843-6.

Greenberg, J.T., Guo, A.L., Klessig, D.F. and Ausubel, F.M. (1994) Programmed cell-death in plants - a pathogen-triggered response activated coordinately with multiple defense functions. *Cell* **77,** 551-63.

Groom, Q.J., Torres, M.A., FordhamSkelton, A.P., HammondKosack, K.E., Robinson, N.J. and Jones, J.D.G. (1996) *Rboha* a rice homologue of the mammalian gp91^{Phox} respiratory burst oxidase gene. *Plant Journal* **10,** 515-22.

Gupta, V., Willits, M.G. and Glazebrook, J. (2000) *Arabidopsis thaliana EDS4* contributes to salicylic acid (SA) -dependent expression of defense responses: Evidence for inhibition of jasmonic acid signaling by SA. *Molecular Plant-Microbe Interactions* **13,**(5) 503-11.

Hammond-Kosack, K.E. and Jones, J.D.G. (1996) Resistance gene-dependent plant defense responses. *Plant Cell* **8,** 1773-91.

Hammond-Kosack, K.E. and Jones, J.D.G. (1997) Plant disease resistance genes. *Annual Review Of Plant Physiol. And Plant Molecular Biology* **48,** 575-607.

Hanks, S.K. and Quinn, A.M. (1991) Protein-kinase catalytic domain sequence database -identification of conserved features of primary structure and classification of family members. *Methods In Enzymology* **200,** 38-62.

Harding, S.A., Oh, S.H. and Roberts, D.M. (1997) Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species. *EMBO Journal* **16,**(6) 1137-44.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42,**(6) 819-32.

Hoffmann-Sommergruber, K. (2000) Plant allergens and pathogenesis-related proteins - What do they have in common? *International Archives Of Allergy And Immunology* **122,**(3) 155-66.

Hugouvieux, V., Barber, C.E. and Daniels, M.J. (1998) Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: A system for studying early infection events in bacterial pathogenesis. *Molecular Plant-Microbe Interactions* **11,**(6) 537-43.

Innes, R.W., Bisgrove, S.R., Smith, N.M., Bent, A.F., Staskawicz, B.J. and Liu, Y.C. (1993) Identification of a disease resistance locus in *Arabidopsis* that is functionally homologous to the *Rpg1* locus of soybean. *Plant Journal* **4,** 813-20.

Ishitani, M., Xiong, L.M., Stevenson, B. and Zhu, J.K. (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: Interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell* **9,**(11) 1935-49.

Jabs, T., Dietrich, R.A. and Dangl, J.L. (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* **273,** 1853-6.

Jackson, A.O. and Taylor, C.B. (1996) Plant-microbe interactions: Life and death at the interface. *Plant Cell* **8,**(10) 1651-68.

Jeong, S., Trotochaud, A.E. and Clark, S.E. (1999) The *Arabidopsis CLAVATA2* gene encodes a receptor-like protein required for the stability of the *CLAVATA1* receptor-like kinase. *Plant Cell* **11,**(10) 1925-33.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. USA* **96,**(23) 13583-8.

Johal, G.S. and Briggs, S.P. (1992) Reductase-activity encoded by the hm1 disease resistance gene in maize. *Science* **258,**(5084) 985-7.

Jones, D.A. and Jones, J.D.G. (1997) The role of leucine rich repeat proteins in plant defences. *Advances In Botanical Research Incorporating Advances In Plant Pathology, Vol 24* **24,** 89-167.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balintkurti, P.J. and Jones, J.D.G. (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* **266,**(5186) 789-93.

Kakimoto, T., (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* **274,** (5289) 982-985.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999) Activation tagging of the floral inducer FT. *Science* **286,**(5446) 1962-5.

Kawasaki, T., Henmi, K., Ono, E., Hatakeyama, S., Iwano, M., Satoh, H. and Shimamoto, K. (1999) The small GTP-binding protein Rac is a regulator of cell death in plants. *Proc. Natl. Acad. Sci. USA* **96,**(19) 10922-6.

Kearney, B. and Staskawicz, B.J. (1990) Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*. *Nature* **346,**(6282) 385-6.

Keen, N.T., Shen, H. and Cooksey, D.A. Glover DM, eds. Molecular Plant Pathology, A Practical Approach. (1990) Introduction of cloned DNA into plant pathogenic bacteria. 45-50.

Keller, T., Damude, H.G., Werner, D., Doerner, P., Dixon, R.A. and Lamb, C. (1998) A plant homolog of the neutrophil nadph oxidase $gp91^{phox}$ subunit gene encodes a plasma membrane protein with Ca^{2+}-binding motifs. *Plant Cell* **10,** 255-66.

King, E.D., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of phycocyanin and fluorescin. *J. Lab. Clin.* **44,** 301-7.

Kiyosue, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993) Characterization of 2 cDNAs (*erd11* and *erd13*) for dehydration-inducible genes that encode putative glutathione S-transferases in *Arabidopsis-thaliana* L. *Febs Letters* **335,**(2) 189-92.

Kjemtrup, S., Nimchuk, Z. and Dangl, J.L. (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Current Opinion In Microbiology* **3,**(1) 73-8.

Kliebenstein, D.J., Dietrich, R.A., Martin, A.C., Last, R.L. and Dangl, J.L. (1999) *Lsd*1 regulates salicylic acid induction of copper zinc superoxide dismutase in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions* **12,** 1022-6.

Klotz, L.O., Pellieux, C., Briviba, K., Pierlot, C., Aubry, J.M. and Sies, H. (1999) Mitogen-activated protein kinase (p38-, JNK-, ERK-) activation pattern induced by extracellular and intracellular singlet oxygen and UVA. *European Journal Of Biochemistry* **260,** 917-22.

Kobe, B. and Deisenhofer, J. (1993) Crystal-structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature* **366,**(6457) 751-6.

Kobe, B. and Deisenhofer, J. (1994) The leucine-rich repeat - a versatile binding motif. *Trends In Biochemical Sciences* **19,**(10) 415-21.

Koch, E. and Slusarenko, A. (1990) *Arabidopsis* is susceptible to infection by a downy mildew fungus. *Plant Cell* **2,**(5) 437-45.

Kogel, K.H., Beckhove, U., Dreschers, J., Munch, S. and Romme, Y. (1994) Acquired-resistance in barley - the resistance mechanism induced by 2,6-dichloroisonicotinic acid is a phenocopy of a genetically based mechanism governing race-specific powdery mildew resistance. *Plant Physiol.* **106,**(4) 1269-77.

Koornneef, M., Reuling, G. and Karssen, C.M. (1984) The isolation and characterization of abscisic-acid insensitive mutants of A*rabidopsis thaliana*. *Physiologia Plantarum* **61,**(3) 377-83.

Kovtun, Y., Chiu, W.L., Tena, G. and Sheen, J. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA* **97,**(6) 2940-5.

Kretsinger, R.H., (1996) EF-hands reach out. Nat. Struc. Biol. **3** (1) 12-15.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal* **10,**(1) 71-82.

Legendre, L., Yueh, Y.G., Crain, R., Haddock, N., Heinstein, P.F. and Low, P.S. (1993) Phospholipase-c activation during elicitation of the oxidative burst in cultured plant cells. *Journal Of Biological Chemistry* **268,**(33) 24559-63.

Leister, R.T. and Katagiri, F. (2000) A resistance gene product of the nucleotide binding site -leucine rich repeats class can form a complex with bacterial avirulence proteins *in vivo*. *Plant Journal* **22,**(4) 345-54.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79,** 583-93.

Li, W.G. and Komatsu, S. (2000) Cold stress induced calcium-dependent protein kinase(s) in rice (*Oryza sativa* L.) seedling stem tissues. *Theoretical And Applied Genetics* **101,**(3) 355-63.

Li, X., Zhang, Y.L., Clarke, J.D., Li, Y. and Dong, X.N. (1999) Identification and cloning of a negative regulator of systemic acquired resistance, *SNII1*, through a screen for suppressors of *npr1-1*. *Cell* **98,**(3) 329-39.

Ligterink, W., Kroj, T., zurNieden, U., Hirt, H. and Scheel, D. (1997) Receptor-mediated activation of a map kinase in pathogen defense of plants. *Science* **276,** 2054-7.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* **10,**(8) 1391-406.

Loh, Y.T. and Martin, G.B. (1995) The *Pto* bacterial-resistance gene and the FEN insecticide sensitivity gene encode functional protein-kinases with serine/threonine specificity. *Plant Physiol.* **108,**(4) 1735-9.

Lorang, J.M., Shen, H., Kobayashi, D., Cooksey, D. and Keen, N.T. (1994) *avrA* and *avrE* in *Pseudomonas-syringae* pv *tomato* pt23 play a role in virulence on tomato plants. *Molecular Plant-Microbe Interactions* **7,**(4) 508-15.

Lupas, A. (1996) Prediction and analysis of coiled-coil structures. *Computer Methods For Macromolecular Sequence Analysis* **266,** 513-25.

Malamy, J., CARR, J.P., Klessig, D.F. and Raskin, I. (1990) Salicylic acid - a likely endogenous signal in the resistance response of tobacco to viral-infection. *Science* **250,**(4983) 1002-4.

Marrs, K.A. (1996) The functions and regulation of glutathione S-transferases in plants. *Annual Review Of Plant Physiol. And Plant Molecular* **47,** 127-58.

Martin, G.B. (1999) Functional analysis of plant disease resistance genes and their downstream effectors. *Current Opinion In Plant Biology* **2,**(4) 273-9.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T.Y., Earle, E.D. and Tanksley, S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262,**(5138) 1432-6.

Martinez, C., Montillet, J.L., Bresson, E., Agnel, J.P., Dai, G.H., Daniel, J.F., Geiger, J.P. and Nicole, M. (1998) Apoplastic peroxidase generates superoxide anions in cells of cotton cotyledons undergoing the hypersensitive reaction to *Xanthomonas campestris* pv. *malvacearum* race 18. *Molecular Plant-Microbe Interactions* **11,**(11) 1038-47.

Matsuta, C., Van den Bulcke, M., Bauw, G., Van Montagu, M. and Caplan, A. (1991) Differential effects of elicitors on the viability of rice suspension cells. *Plant Physiol.* **97,** 619-29.

Mauch-Mani, B. and Slusarenko, A.J. (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis to Peronospora parasitica*. *Plant Cell* **8,**(2) 203-12.

McLusky, S.R., Bennett, M.H., Beale, M.H., Lewis, M.J., Gaskin, P. and Mansfield, J.W. (1999) Cell wall alterations and localized accumulation of feruloyl-3'-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarisation, peroxidase activity and suppression of flavonoid biosynthesis. *Plant Journal* **17,** 523-34.

McNellis, T.W., Mudgett, M.B., Li, K., Aoyama, T., Horvath, D., Chua, N.H. and Staskawicz, B.J. (1998) Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death. *Plant Journal* **14,**(2) 247-57.

Menkens, A.E., Schindler, U. and Cashmore, A.R. (1995) The G-box - a ubiquitous regulatory dna element in plants bound by the GBF family of bZIP proteins. *Trends In Biochemical Sciences* **20,**(12) 506-10.

Meyers, B.C., Dickerman, A.W., Michelmore, R.W., Sivaramakrishnan, S., Sobral, B.W. and Young, N.D. (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant Journal* **20,**(3) 317-32.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.H. and Kay, S.A. (1995) circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science* **267,**(5201) 1161-3.

Millar, A.J., Short, S.R., Chua, N.H. and Kay, S.A. (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell* **4,**(9) 1075-87.

Millar, A.J., Straume, M., Chory, J., Chua, N.H. and KAY, S.A. (1995) The regulation of circadian period by phototransduction pathways in *Arabidopsis*. *Science* **267,**(5201) 1163-6.

Mindrinos, M., Katagiri, F., YU, G.L. and Ausubel, F.M. (1994) The *A.thaliana* disease resistance gene *rps2* encodes a protein containing a nucleotide binding site and leucine rich repeats. *Cell* **78,**(6) 1089-99.

Mizoguchi, T., Ichimura, K. and Shinozaki, K. (1997) Environmental stress response in plants: The role of mitogen-activated protein kinases. *Trends In Biotechnology* **15,**(1) 15-9.

Morel, J.B. and Dangl, J.L. (1999) Suppressors of the *Arabidopsis lsd5* cell death mutation identify genes involved in regulating disease resistance responses. *Genetics* **151,**(1) 305-19.

Morris, S.W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C.C., Frederiksen, R.A., Bhandhufalck, A., Hulbert, S. and Uknes, S. (1998) Induced resistance responses in maize. *Molecular Plant-Microbe Interactions* **11,**(7) 643-58.

Napoli, C., Lemieux, C. and jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. *Plant Cell* **2,**(4) 279-89.

Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B. and Mosinger, E. (1995) Pathogenesis-related PR-1 proteins are antifungal - isolation and characterization of 3 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. *Plant Physiol.* **108,**(1) 17-27.

Normile, D. (2000) Agriculture biotechnology - Monsanto donates its share of golden rice. *Science* **289,**(5481) 843-+.

Odell, J.T., Nagy, F. and Chua, N.H. (1985) Identification of DNA sequences required for activity of the Cauliflower mosaic virus 35S promoter. *Nature* **313,**(6005) 810-2.

Ohmetakagi, M. and Shinshi, H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* **7,**(2) 173-82.

Oldroyd, G.E.D. and Staskawicz, B.J. (1998) Genetically engineered broad spectrum disease resistance in tomato. *Proc. Natl. Acad. Sci. USA* **95,**(17) 10300-5.

Osbourn, A.E. (1996) Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **8,**(10) 1821-31.

Pan, Q.L., Wendel, J. and Fluhr, R. (2000) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *Journal Of Molecular Evolution* **50,**(3) 203-13.

Peng, M. and Kuc, J. (1992) Peroxidase-generated hydrogen-peroxide as a source of antifungal activity invitro and on tobacco leaf-disks. *Phytopathology* **82,** 696-9.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., DeSamblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M. and Broekaert, W.F. (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* **8,**(12) 2309-23.

Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Metraux, J.P. and Broekaert, W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* **10,**(12) 2103-13.

Pernas, M., Sanchez-Mong, R. and Salcedo, G. (2000) Biotic and abiotic stress can induce cystatin expression in chestnut. *Febs Letters* **467,**(2-3) 206-10.

Pestenacz, A. and Erdei, L. (1996) Calcium-dependent protein kinase in maize and sorghum induced by polyethylene glycol. *Physiologia Plantarum* **97,**(2) 360-4.

Piedras, P., Hammond-Kosack, K.E., Harrison, K. and Jones, J.D.G. (1998) Rapid, *Cf-9*- and *Avr9*-dependent production of active oxygen species in tobacco suspension cultures. *Molecular Plant-Microbe Interactions* **11,**(12) 1155-66.

Piedras, P., Rivas, S., Droge, S., Hillmer, S. and Jones, J.D.G. (2000) Functional, c-myc-tagged *Cf-9* resistance gene products are plasma membrane localized and glycosylated. *Plant Journal* **21,**(6) 529-36.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991) Systemic induction of salicylic-acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. *Plant Physiol.* **97,**(4) 1342-7.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S. and Greenberg, J.T. (1999) The gain-of-function *Arabidopsis acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. *Plant Cell* **11,**(9) 1695-708.

Reuber, T.L. and Ausubel, F.M. (1996) Isolation of *Arabidopsis* genes that differentiate between resistance responses mediated by the *RPS2* and *RPM1* disease resistance genes. *Plant Cell* **8,**(2) 241-9.

Richter, T.E. and Ronald, P.C. (2000) The evolution of disease resistance genes. *Plant Molecular Biology* **42,**(1) 195-204.

Roine, E., Wei, W.S., Yuan, J., Nurmiaho-Lassila, E.L., Kalkkinen, N., Romantschuk, M. and He, S.Y. (1997) Hrp pilus: An hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv *tomato* DC3000. *Proc. Natl. Acad. Sci. USA* **94,**(7) 3459-64.

Romeis, T., Piedras, P. and Jones, J.D.G. (2000) Resistance gene-dependent activation of a calcium dependent protein kinase in the plant defense response. *Plant Cell* **12,**(5) 803-15.

Romeis, T., Piedras, P., Zhang, S.Q., Klessig, D.F., Hirt, H. and Jones, J.D.G. (1999) Rapid *avr9*- and *Cf-9*-dependent activation of map kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. *Plant Cell* **11,** 273-87.

Roxas, V.P., Smith, R.K., Allen, E.R. and Allen, R.D. (1997) Overexpression of glutathione S-transferase glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotechnology* **15,**(10) 988-91.

Ruiz-Perez, V.L., Ide, S.E., Strom, T.M., Lorenz, B., Wilson, D., Woods, K., King, L., Francomano, C., Freisinger, P., Spranger, S. *et al.* (2000) Mutations in a new gene in Ellis-van Creveld syndrome and Weyers acrodental dysostosis. *Nature Genetics* **24,**(3) 283-6.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell* **8,**(10) 1809-19.

Ryazanov, A.G., Ward, M.D., Mendola, C.E., Pavur, K.S., Dorovkov, M.V., Wiedmann, M., ErdjumentBromage, H., Tempst, P., Parmer, T.G., Prostko, C.R. *et al.* (1997) Identification of a new class of protein kinases represented by eukaryotic elongation factor-2 kinase. *Proc. Natl. Acad. Sci. USA* **94,**(10) 4884-9.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant Journal* **23,**(3) 319-27.

Sakamoto, K., Tada, Y., Yokozeki, Y., Akagi, H., Hayashi, N., Fujimura, T. and Ichikawa, N. (1999) Chemical induction of disease resistance in rice is correlated with the expression of a gene encoding a nucleotide binding site and leucine-rich repeats. *Plant Molecular Biology* **40,**(5) 847-55.

Salmeron, J.M., Oldroyd, G.E.D., Rommens, C.M.T., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D. and Staskawicz, B.J. (1996) Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell* **86,**(1) 123-33.

Salter, M.G., Paine, J.A., Riddell, K.V., Jepson, I., Greenland, A.J., Caddick, M.X. and Tomsett, A.B. (1998) Characterisation of the ethanol-inducible *alc* gene expression system for transgenic plants. *Plant Journal* **16,**(1) 127-32.

Samuel, M.A., Miles, G.P. and Ellis, B.E. (2000) Ozone treatment rapidly activates MAP kinase signalling in plants. *Plant Journal* **22,**(4) 367-76.

Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) The P-loop - a common motif in ATP-binding and GTP-binding proteins. *Trends In Biochemical Sciences* **15,**(11) 430-4.

Schulz, B., Bennett, M.J., Dilkes, B.P. and Feldmann, K.A. Plant Molecular Biology Manual. Belgium: Kluwer Academic Publishers, (1994) T-DNA tagging in *Arabidopsis thaliana*: cloning by gene disruption.

Schweizer, P., Schlagenhauf, E., Schaffrath, U. and Dudler, R. (1999) Different patterns of host genes are induced in rice by *Pseudomonas syringae*, a biological inducer of resistance, and the chemical inducer benzothiadiazole (BTH) . *European Journal Of Plant Pathology* **105,**(7) 659-65.

Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W. and Staskawicz, B.J. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274,**(5295) 2063-5.

Seo, S., Okamoto, N., Seto, H., Ishizuka, K., Sano, H. and Ohashi, Y. (1995) Tobacco MAP kinase - a possible mediator in wound signal-transduction pathways. *Science* **270,**(5244) 1988-92.

Sessa, G., D'Ascenzo, M. and Martin, G.B. (2000) Thr38 and Ser198 are Pto autophosphorylation sites required for the *AvrPto-Pto*-mediated hypersensitive response. *EMBO Journal* **19,**(10) 2257-69.

Shah, D.M., Rommens, C.M.T. and Beachy, R.N. (1995) Resistance to diseases and insects in transgenic plants -progress and applications to agriculture. *Trends In Biotechnology* **13,**(9) 362-8.

Sharma, Y.K., Leon, J., Raskin, I. and Davis, K.R. (1996) Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. *Proc. Natl. Acad. Sci. USA* **93,** 5099-104.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current Opinion In Plant Biology* **3,**(3) 217-23.

Shirasu, K., Nakajima, H., Rajasekhar, V.K., Dixon, R.A. and Lamb, C. (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell* **9,**(2) 261-70.

Silverman, P., Seskar, M., Kanter, D., Schweizer, P., Metraux, J.P. and Raskin, I. (1995) Salicylic acid in rice - biosynthesis, conjugation, and possible role. *Plant Physiol.* **108,**(2) 633-9.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H. *et al.* (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* **270,**(5243) 1804-6.

Stamler, J.S. (1994) Redox signaling - nitrosylation and related target interactions of nitric oxide. *Cell* **78,**(6) 931-6.

Stone, J.M., Collinge, M.A., Smith, R.D., Horn, M.A. and Walker, J.C. (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine threonine receptor kinase. *Science* **266,**(5186) 793-5.

Storz, G., Tartaglia, L.A. and Ames, B.N. (1990) Transcriptional regulator of oxidative stress-inducible genes -direct activation by oxidation. *Science* **248,** 189-94.

Swift,J. (1726) Travels into several remote nations of the world. In four parts. By Lemuel Gulliver

Tai, T.H., Dahlbeck, D., Clark, E.T., Gajiwala, P., Pasion, R., Whalen, M.C., Stall, R.E. and Staskawicz, B.J. (1999) Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. *Proc. Natl. Acad. Sci. USA* **96,**(24) 14153-8.

Tamaki, S.J., Kobayashi, D.Y. and Keen, N.T. (1991) Sequence domains required for the activity of avirulence genes avrB and avrC from *Pseudomonas syringae* pv *glycinea*. *Journal Of Bacteriology* **173,**(1) 301-7.

Tang, X.Y., Frederick, R.D., Zhou, J.M., Halterman, D.A., Jia, Y.L. and Martin, G.B. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274,**(5295) 2060-3.

Tang, X.Y., Xie, M.T., Kim, Y.J., Zhou, J.M., Klessig, D.F. and Martin, G.B. (1999) Overexpression of *Pto* activates defense responses and confers broad resistance. *Plant Cell* **11,**(1) 15-29.

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C. (1995) Function of the oxidative burst in hypersensitive disease resistance. *Proceedings Of* **92,** 4158-63.

Terzaghi, W.B. and Cashmore, A.R. (1995) Light-regulated transcription. *Annual Review Of Plant Physiol. And Plant Molecular Biology* **46,** 445-74.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.J. and Broekaert, W.F. (1999) Requirement of functional *ethylene insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiol.* **121,**(4) 1093-101.

Thordal-Christensen, H., Zhang, Z.G., Wei, Y.D. and Collinge, D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley powdery mildew interaction. *Plant Journal* **11,** 1187-94.

Timmusk, S. and Wagner, E.G.H. (1999) The plant growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: A possible connection between biotic and abiotic stress responses. *MPMI* **12,**(11) 951-9.

Tipping, A.J. and McPherson, M.J. (1995) Cloning and molecular analysis of the pea seedling copper amine oxidase. *Jour. Biol. Chem.* **270,** 16939-46.

Traut, T.W. (1994) The functions and consensus motifs of 9 types of peptide segments that form different types of nucleotide binding sites. *Eur. Jour. Biochem.***222,**(1) 9-19.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompson-Taylor, H., Potter, S., Ward, E. and Ryals, J. (1993) Regulation of pathogenesis related protein 1a gene-expression in tobacco. *Plant Cell* **5,**(2) 159-69.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. (1992) Acquired resistance in *Arabidopsis*. *Plant Cell* **4,**(6) 645-56.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. (1994) An *Arabidopsis thaliana* cDNA encoding Ca^{2+}-dependent protein-kinase. *Plant Physiol.* **105,**(4) 1461-2.

Vallelian-Bindschedler, L., Metraux, J.P. and Schweizer, P. (1998) Salicylic acid accumulation in barley is pathogen specific but not required for defense-gene activation. *Molecular Plant-Microbe Interactions* **11,**(7) 702-5.

Van Camp, W., Van Montagu, M. and Inze, D. (1998) H_2O_2 and NO: redox signals in disease resistance. *Trends In Plant Science* **3,**(9) 330-4.

Van der Biezen, E.A. and Jones, J.D.G. (1998) The NB-ARC domain: A novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. *Current Biology* **8,**(7) R226-7.

Van der Biezen, E.A., Sun, J.H., Coleman, M.J., Bibb, M.J. and Jones, J.D.G. (2000) *Arabidopsis RelA/SpoT* homologs implicate (p) ppGpp in plant signaling. *Proc. Natl. Acad. Sci. USA* **97,**(7) 3747-52.

Van Wees, S.C.M., de Swart, E.A.M., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M.J. (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA* **97,**(15) 8711-6.

Vanderkrol, A.R., Mur, L.A., Beld, M., Mol, J.N.M. and Stuitje, A.R. (1990) Flavonoid genes in petunia - addition of a limited number of gene copies may lead to a suppression of gene-expression. *Plant Cell* **2,**(4) 291-9.

Vernooij, B., Friedrich, L., GOY, P.A., Staub, T., Kessmann, H. and Ryals, J. (1995) 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic-acid. *Molecular Plant-Microbe Interactions* **8,**(2) 228-34.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditzjawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* **6,**(7) 959-65.

Walden, R., Fritze, K., Hayashi, H., Miklashevichs, E., Harling, H. and Schell, J. (1994) Activation tagging - a means of isolating genes implicated as playing a role in plant growth and development. *Plant Molecular Biology* **26,**(5) 1521-8.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha-subunits and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* **1,**(8) 945-51.

Wang, G.L., Ruan, D.L., Song, W.Y., Sideris, S., Chen, L.L., Pi, L.Y., Zhang, S.P., Zhang, Z., Fauquet, C., Gaut, B.S. *et al.* (1998) *Xa21D* encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. *Plant Cell* **10,**(5) 765-79.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahlgoy, P., Metraux, J.P. and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* **3,**(10) 1085-94.

Warren, R.F., Henk, A., Mowery, P., Holub, E. and Innes, R.W. (1998) A mutation within the leucine rich repeat domain of the *Arabidopsis* disease resistance gene *RPS5* partially suppresses multiple bacterial and downy mildew resistance genes. *Plant Cell* **10,**(9) 1439-52.

Wasternack, C., Atzorn, R., Jarosch, B. and Kogel, K.H. (1994) Induction of a thionin, the jasmonate-induced 6 kda protein of barley by 2,6-dichloroisonicotinic acid. *Journal Of Phytopathology-Phytopathologische Zeitschrift* **140,**(3) 280-4.

Wei, W.S., Plovanich-Jones, A., Deng, W.L., Jin, Q.L., Collmer, A., Huang, H.C. and He, S.Y. (2000) The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato. Proc. Natl. Acad. Sci. USA* **97,**(5) 2247-52.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M. *et al.* (2000) Activation tagging in *Arabidopsis*. *Plant Physiol.* **122,**(4) 1003-13.

Weymann, K., Hunt, M., Uknes, S., Neuenschwander, U., Lawton, K., Steiner, H.Y. and Ryals, J. (1995) Suppression and restoration of lesion formation in *Arabidopsis Isd* mutants. *Plant Cell* **7,**(12) 2013-22.

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C. and Baker, B. (1994) The product of the tobacco mosaic virus resistance gene N -similarity to Toll and the Interleukin-1 receptor. *Cell* **78,**(6) 1101-15.

Xing, T., Higgins, V.J. and Blumwald, E. (1997) Race-specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. *Plant Cell* **9,**(2) 249-59.

Xu, H.X. and Heath, M.C. (1998) Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. *Plant Cell* **10,**(4) 585-97.

Xu, Y., Chang, P.F.L., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M. and Bressan, R.A. (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* **6,** 1077-85.

Yalpani, N., Shulaev, V. and Raskin, I. (1993) Endogenous salicylic acid levels correlate with accumulation of pathogenesis-related proteins and virus resistance in tobacco. *Phytopathology* **83,**(7) 702-8.

Yang, K.Y., Kim, E.Y., Kim, C.S., Guh, J.O., Kim, K.C. and Cho, B.H. (1998) Characterization of a glutathione S-transferase gene *ATGST 1* in *Arabidopsis thaliana*. *Plant Cell Reports* **17,**(9) 700-4.

Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z.X., Kono, I., Kurata, N., Yano, M., Iwata, N. and Sasaki, T. (1998) Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. *Proc. Natl. Acad. Sci. USA* **95,**(4) 1663-8.

Young, N.D. (2000) The genetic architecture of resistance. *Current Opinion In Plant Biology* **3,**(4) 285-90.

Yu, I.C., Parker, J. and Bent, A.F. (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proc. Natl. Acad. Sci. USA* **95,**(13) 7819-24.

Zhang, S.Q. and Klessig, D.F. (1997) Salicylic acid activates a 48-kD MAP kinase in tobacco. *Plant Cell* **9,**(5) 809-24.

Zhang, Z.G., Collinge, D.B. and Thordal-Christensen, H. (1995) Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *Plant Journal* **8,**(1) 139-45.

Zimmermann, S., Nurnberger, T., Frachisse, J.M., Wirtz, W., Guern, J., Hedrich, R. and Scheel, D. (1997) Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defense. *Proc. Natl. Acad. Sci. USA* **94,**(6) 2751-5.

Zou, H., Henzel, W.J., Liu, X.S., Lutschg, A. and Wang, X.D. (1997) Apaf-1, a human protein homologous to *C.elegans* Ced-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* **90,**(3) 405-13.