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Abstract

Plants have evolved a complex series of integrated defence mechanisms against pathogens. Following recognition of a pathogen avirulence (avr) gene product by the corresponding plant resistance (R) gene product, a complex signalling network is initiated. Local inducible defences are activated and a long-distance signal is released, leading to the establishment of systemic acquired resistance (SAR) to a wide range of pathogens. SAR is marked by the accumulation of pathogenesis-related (PR) proteins. Salicylic acid (SA) is a key signalling molecule in SAR, inducing PR gene expression both locally and systemically. However, it is thought that signal transduction leading to acquired disease resistance differs for biotrophic and necrotrophic pathogens. Generally, SA is required for resistance to biotrophic pathogens only. SA-independent resistance to necrotrophic pathogens is associated with jasmonic acid (JA) and ethylene signalling and is marked by the production of antimicrobial proteins.

In order to study further the molecular basis of SAR, we have developed a method of identifying novel SAR mutants by luciferase imaging. Transgenic Arabidopsis thaliana plants expressing a PR-1a: luciferase reporter gene were generated and homozygous seed was chemically mutagenised. Mutants with perturbations in PR-1 gene expression were identified and could be divided into various classes. A novel mutant expressing PR-1 constitutively was selected for further study. cir1 (constitutively induced resistance 1) expressed both SA-dependent and SA-independent defence genes constitutively, accumulated SA to high levels and produced an increased amount of ethylene. In addition, cir1 exhibited resistance to the virulent bacterial pathogen Pseudomonas syringae pv tomato DC3000 and the virulent oomycete pathogen Peronospora parasitica Noco2. Genetic analyses indicated that cir1 is recessive and defines a mutation in a single gene. cir1 mapped to the lower arm of chromosome 4. Double mutants were produced between cir1 and SA-, JA- and ethylene-insensitive mutants. Analysis of these plants showed that SA, JA and ethylene were required for constitutive defence gene expression and disease resistance in cir1. Thus, the results obtained indicate that CIR1 acts as a negative regulator in the disease resistance signal transduction network, most likely functioning upstream of the branchpoint between the SA-dependent and SA-independent pathways.
Abbreviations

bp basepairs
ceb constitutive expression of bioluminescence
cfu colony forming units
cirl constitutively induced resistance 1
Col-0 Arabidopsis ecotype Columbia
heb high expression of bioluminescence
ISR induced systemic resistance
JA jasmonic acid
kb kilobase
KB King’s broth media
Ler Arabidopsis ecotype Landsberg erecta
luc luciferase
mRNA messenger ribonucleic acid
Me-JA methyl jasmonate
MS Murashige and Skoog media
ng nanogram
neb no expression of bioluminescence
nt nucleotide
Psm Pseudomonas syringae pv maculicola
Psi Pseudomonas syringae pv tomato
PCR polymerase chain reaction
RE restriction enzyme
RLU relative light units
rpm revolutions per minute
SA salicylic acid
SAR systemic acquired resistance
UK4 Arabidopsis ecotype Umkirch 4
U unit
Ws Arabidopsis ecotype Wassilewskija
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Chapter One
General Introduction

1.1. Introduction

It has been estimated that 12% of potential global crop production is lost annually to pre-harvest plant disease (Agrios 1997, Shah 1997). Outbreaks of disease are a result of interactions between cultivated crops and pathogens (bacteria, fungi, insects, viruses and nematodes). Despite the development and use of an increasing number of pesticides and fungicides, crop losses due to disease still continue. Plant breeding has been used to introduce resistance genes from wild populations into commercial crop cultivars, but this resistance often is not durable as pathogens are able to evolve quickly and overcome it (Agrios 1997). Plant transformation technology has been developed over the last decade for several commercially important crops and offers exciting opportunities for engineering crop protection (Shah 1997). Advances are also being made in the understanding of plant-pathogen interactions. These include the isolation of a number of resistance (R) genes and the analysis of signalling pathways leading to the hypersensitive response (HR) and systemic acquired resistance (SAR) (Hammerschmidt 1999, Martin 1999, Malek & Lawton 1998, Bent 1996, Hammond-Kosack & Jones 1996, Jackson et al. 1996, Ryals et al. 1996). Thus, further understanding of these plant disease resistance processes may pave the way for the development of transgenic crops with increased disease resistance, or novel pesticides capable of activating plant defence responses.

1.2. Disease resistance in plants

Types of disease resistance

Plants have the potential to respond to pathogen attack by employing one of two broad strategies. Firstly, structural and pre-formed chemical barriers such as saponins prevent the pathogen from gaining sustenance from the host (Hammerschmidt 1999, Glazebrook et al.1997a, Osbourn 1996). Secondly, the plant can engage a number of defence mechanisms aimed at limiting pathogen spread
(Hammerschmidt 1999, Glazebrook et al. 1997a, Hammond-Kosack & Jones 1996). These defence mechanisms include the hypersensitive response (HR), the production of reactive oxygen species (ROS), fortification of the cell wall by lignin polymerization and expression of a number of genes including those encoding pathogenesis-related (PR) proteins, thionins, defensins, glutathione-S-transferases, phenylalanine ammonia lyase (PAL) and enzymes involved in phytoalexin biosynthesis (Glazebrook et al. 1997a, Hammond-Kosack & Jones 1996). Gene-for-gene resistance is said to occur when the plant is capable of rapidly engaging these defence responses.

The use of Arabidopsis thaliana in understanding disease resistance

Arabidopsis thaliana (L.) Heynh. (Arabidopsis), a small diploid plant in the Brassicaceae family, has been developed as a model genetic system for research in plant science (Meinke et al. 1998). The reasons for this choice are many. Arabidopsis is easy to grow both in tissue culture and in the greenhouse, and many plants can be grown in a relatively small area. The entire life cycle, including seed germination, formation of a rosette plant, bolting of the main stem, flowering, silique formation and maturation of the first seeds can be completed in approximately two months (Meinke et al. 1998). Many methods for Arabidopsis research have been developed, including chemical and insertional mutagenesis, efficient transformation methods and an extensive collection of mutants with a diverse range of phenotypes (Clough & Bent 1998, Koncz et al. 1992, http://www.arabidopsis.org/). The 120-megabase Arabidopsis genome is organised into five chromosomes and contains an estimated 27 000 genes (Meinke et al. 1998). Three related constantly evolving maps of each chromosome (classical genetic, recombinant inbred and physical maps; Lister & Dean 1993, Rhee et al. 1999) containing a number of different markers are deposited at The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). A consortium of laboratories is in the process of sequencing the entire genome with the published sequence expected in December 2000, and the complete annotated sequence of chromosome 2 and 4 has already been deposited into Genbank (Lin et al. 1999, Mayer et al. 1999).
In addition to the genetic advantages of using Arabidopsis as a model system, it is a very good system for studying host defence responses to pathogen attack. A large number of virulent and avirulent bacterial, fungal and viral pathogens have been identified and used to identify many disease resistance mutants (Glazebrook et al. 1997a, Kunkel 1996). On-going analysis of these mutants is proving to be a very important tool for the dissection of signal transduction networks leading to induction of defence responses (Glazebrook et al. 1997a, Kunkel 1996).

1.3. Gene-for-Gene resistance

If during interaction with a plant, a pathogen is able to penetrate the plant and cause disease, the pathogen is said to be virulent, the plant susceptible and the interaction compatible. On the other hand, the plant may be able to activate defence responses more quickly, thus preventing the development of disease. In this case, the pathogen is said to be avirulent, the plant resistant and the interaction incompatible. In the gene-for-gene model for plant disease resistance, first proposed by Flor, an incompatible interaction has been hypothesised to result from the interaction of the product of a plant resistance (R) gene with the product of the corresponding avirulence (avr) gene (Flor 1971, Keen 1992). R-avr interactions have been observed between plants and many different pathogens including bacteria, fungi, viruses and nematodes, and in general, a specific R gene product will interact with only the corresponding avr product (Crute & Pink 1996, Keen 1992). A simple explanation of this model is that avr genes encode ligands that bind to receptors encoded by the plant R genes (Glazebrook et al. 1997a). Binding of the ligand to the receptor then stimulates a complex signal transduction cascade that may involve protein phosphorylation, ion fluxes, ROS and activation of transcription factors, culminating in the expression of defence response genes and resistance (Yang et al. 1997, Hammond-Kosack & Jones 1996). In the case of resistance to virulent pathogens, no HR is visualised and defence responses are activated more slowly (Glazebrook et al. 1997a).
Avr genes

To date, over 40 *avr* genes have been isolated from bacterial and fungal pathogens (Gabriel 1999). In addition to *avr* genes, avirulent bacterial pathogens possess hypersensitive response and pathogenicity (*hrp*) genes, which have been shown to be necessary for an avirulent bacteria to elicit an HR in a resistant plant (Collmer 1998, Lindgren 1997). The *hrp* genes encode a type III protein-secretion system that appears to be capable of delivering the *avr* protein across the bacterial and plant cell walls and into the plant cell, where it can interact with the corresponding R protein (Collmer 1998, Lindgren 1997). On the other hand, cloned fungal *avr* genes have been predicted or shown to encode extracellular proteins (Lauge & DeWit 1998, Knogge 1996). Elicitors such as oligogalactoronates are also released from plant cells following attack by a number of different fungi, resulting in plant defence responses (Knogge 1996). Although the biological function of *avr* is unknown, it is possible that *avr* genes encode pathogenicity factors (Gabriel 1999). Recently, *virPphA*, the first virulence (*vir*) gene described from the soybean bacterial pathogen *Pseudomonas syringae* pv *phaseolicola* (Pph), was isolated (Jackson et al. 1999). Pph strains cured of a 154-kb plasmid lost virulence towards previously susceptible cultivars of soybean. Restoration of virulence was achieved by complementation with a cosmid clone containing a 30-kb region of the plasmid, which had previously been shown to contain three *avr* genes (Jackson et al. 1999). Sequencing of this clone revealed three putative *vir* genes that were predicted to encode hydrophilic proteins. One gene, designated *virPphA*, achieved partial restoration of virulence when cloned on its own, and also acted as an *avr* gene in some soybean cultivars, rapidly inducing the HR (Jackson et al. 1999). This example illustrates that a bacterial virulence factor acts as an *avr* protein, presumably when it interacts with the corresponding plant *R* gene product. In addition, it seems likely that the presence of an *avr* gene would give the pathogen an advantage during a compatible interaction. In fungal pathogens, it has been proposed that *avr* proteins are required for fungal fitness in the field (Knogge 1996).
R genes

A number of R genes have been isolated from tomato, Arabidopsis, tobacco, flax, maize, barley, rice and potato (reviewed in Buell 1998, Baker et al. 1997, Bent 1996, Hammond-Kosack & Jones 1996). The majority of R genes contain a leucine-rich repeat domain (LRR), which implies a role in protein-protein interactions (Bent 1996). Additional sequence features present in some R genes include a signal sequence, nucleotide binding site (NBS), leucine zipper (LZ), transmembrane domain, glycosylation sites, a kinase domain and Toll-IL-IR homology region (TIR) (Buell 1998, Baker et al. 1997, Bent 1996). TIRs have homology to the Drosophila developmental gene Toll and the mammalian immune response gene encoding the interleukin-1 receptor (IL-IR), both of which play a role in defence (Baker et al. 1997). R genes cloned to date can be divided into five classes, which can be defined as follows: a detoxification enzyme which is activated in a gene-for-gene manner, an intracellular protein kinase, an intracellular LRR-NBS class (which can be sub-divided into LZ/NBS/LRR and TIR/NBS/LRR proteins), an extracellular LRR protein with a single membrane spanning region and short cytoplasmic carboxyl terminus, and an extracellular LRR protein with a cytoplasmic kinase domain (reviewed in Hammond-Kosack & Jones 1997). These five classes of R genes, with examples of each, are outlined in Table 1.1. The barley Mlo resistance gene to powdery mildew falls outside of this classification, as it encodes a novel protein with six membrane-spanning helices, and differs from other R genes in that it is recessive and confers resistance in a non-race specific manner (Buschages et al. 1997).
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To date, over 49 pathogen specificity loci in Arabidopsis for various pathogens have been identified and mapped (Buell 1998). These include genes conferring disease resistance to the bacterial pathogens *Pseudomonas syringae* and *Xanthomonas campestris*, the oomycete pathogens *Peronospora parasitica* and *Albugo candida*, the powdery mildew fungal pathogens *Erisiphe cichorearum*, *Erisiphe cruciferarum* and *Erisiphe orontii*, the clubroot pathogen *Plasmodiophora brassicae*, and the viral pathogens tobacco ringspot virus, turnip crinkle virus, cauliflower mosaic virus and beet curly top virus (reviewed in Buell 1998, Glazebrook et al. 1997a). Some of these Arabidopsis *R* genes have been cloned using map based strategies and these are included in Table 1.1. Of interest to the present study is the interaction between the products of the *RPM1* and *avrB* genes. It was previously established that *avrB* corresponded to the *R* gene *RPS3*, but later it was shown that *RPM1* and *RPS3* were the same gene by complementation (Bisgrove et al. 1994). Thus the *RPM1* gene represents a deviation from the gene-for-gene theory as the RPM1 protein can bind one of two *avr* proteins: the products of the *avrB* and *avrRPM1* genes (Glazebrook et al. 1997a). The RPM1 protein has been localised by epitope tagging, and is a peripheral membrane protein most likely residing on the cytoplasmic face of the plasmamembrane (Boyes et al. 1998).

### 1.4. Acquired resistance

**Systemic Acquired Resistance**

Systemic acquired resistance (SAR) is an inducible defence response that leads to broad spectrum, long-lasting systemic resistance following inoculation with an ‘immunising’ pathogen (Delaney 1997, Sticher et al. 1997, Ryals et al. 1996, Ryals et al. 1994, Ross 1961). Systemic resistance is initiated in addition to locally induced defence responses. SAR is initiated when a resistant plant is inoculated with an avirulent pathogen, leading to the formation of the HR and localised necrosis (Fig.1.1). SAR can be expressed in both monocots and dicots against a broad range of pathogens, which may differ from the SAR-inducing organism (Sticher et al. 1997). SAR has been studied extensively in tobacco, cucumber and more recently Arabidopsis, and it has been established that the time needed for the establishment of
SAR depends on both the plant and the type of pathogen. In addition, concentration of the pathogen used in inoculation influences the level of protection (Sticher et al. 1997). It has been proposed that SAR is induced by translocation of an unknown signal from the site of primary inoculation to the rest of the plant (Fig. 1.1). This signal then primes the plant for subsequent attack by the secondary, virulent pathogen, most probably by triggering a complex array of defence responses (Fig. 1.1), which include the expression of pathogenesis-related proteins and antimicrobial peptides (Ryals et al. 1994, Penninckx et al. 1996).

![Schematic diagram of SAR induced by an avirulent pathogen.](image)

**Fig. 1.1. Schematic diagram of SAR induced by an avirulent pathogen.**
Adapted from Ryals et al. (1994).

*Signals and genes associated with acquired disease resistance*

Classical SAR has been associated with salicylic acid (SA), but recently three other signalling molecules (jasmonic acid, ethylene and H₂O₂) have been intensively studied and all four molecules are thought to play very important roles in the induction of disease resistance (Dong 1998). Integration of signalling pathways leading to expression of defence genes and disease resistance are shown in Fig. 1.2.
In Arabidopsis, SA-dependent SAR is associated with production of an HR by an avirulent pathogen, expression of *PR-1*, *PR-2* and *PR-5* genes (encoding pathogenesis-related proteins) and resistance to biotrophic pathogens such as *Pseudomonas syringae* (Delaney et al. 1994), *Peroonospora parasitica* (Lawton et al. 1995), *Erisiphe orontii* (Reuber et al. 1998) and turnip crinkle virus (TCV) (Kachroo et al. 2000, Uknes et al. 1993) (Fig. 1.2). Resistance to necrotrophic fungal pathogens such as *Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium oxysporum* and *Pythium* sp (Penninckx et al. 1996, Thomma et al. 1998, Epple et al. 1995, Staswick et al. 1998) is associated with JA and ethylene signalling and expression of genes encoding antimicrobial peptides (Fig. 1.2). In addition, it has been found that production of an HR by *B. cinerea* facilitates its infection of Arabidopsis (Govrin & Levine 2000). Thus, it is likely that the HR is only important for resistance to biotrophic pathogens (Fig. 1.2). The roles of H$_2$O$_2$, SA, JA and ethylene in inducing expression of *PR* and antimicrobial genes will be discussed separately below.

**The role of reactive oxygen species**

One of the earliest responses of plant cells to pathogens is the production of reactive oxygen species (ROS). Known as the oxidative burst, O$_2^-$ accumulates initially, which is rapidly dismutated to H$_2$O$_2$ either non-enzymatically or by the action of superoxide dismutase (Grant & Loake 2000, Lamb & Dixon 1997, Low & Merida 1996, Mehdy et al. 1996). An initial, weak burst of ROS is evoked within one hour in response to inoculation with either virulent or avirulent pathogens (Mehdy et al. 1996). However, with avirulent pathogens, the oxidative burst is biphasic, with a second massive burst of ROS produced after approximately three hours (Mehdy et al. 1996). The second burst correlates with the formation of the HR and the establishment of disease resistance (Fig. 1.2).
Fig. 1.2. Model showing integration of signal transduction pathways leading to expression of defence-related genes and disease resistance in Arabidopsis.

Several sources are known to exist for the generation of ROS (reviewed in Grant & Loake 2000, Bolwell 1999). These include a plasmamembrane located NADPH oxidase, a cell wall peroxidase and amine, diamine and polyamine oxidase-type enzymes. Production of ROS is thought to involve a signal transduction cascade, including phosphorylation/dephosphorylation, G-proteins and Ca\(^{2+}\) influx (Grant & Loake 2000, Bolwell 1999).
ROS are thought to function in a number of different processes. One of these is a direct microbial effect, but there is little evidence suggesting that accumulated ROS are responsible for killing the invading pathogen (Bolwell 1999). However, it has been established that ROS are responsible for the oxidative cross-linking of cell wall hydroxyproline-rich proteins, thereby reinforcing the cell wall against pathogen secreted wall-degrading enzymes (Mehdy et al. 1996). It has also been proposed that ROS accumulation in response to avirulent pathogen inoculation initiates programmed cell death (PCD) in plant cells (Greenberg 1997) leading to the formation of the HR (Levine et al. 1994) (Fig. 1.2). However, evidence is accumulating that PCD can be uncoupled from ROS production, and in a number of systems ROS is generated without cell death (Richberg et al. 1998). Nitric oxide (NO) has been shown to potentiate ROS-mediated induction of cell death in soybean cells, suggesting that NO also plays an important role in PCD and HR formation (Delledonne et al. 1998). A ROS-mediated systemic signalling network may also mediate the establishment of SAR (Alvarez et al. 1998). Inoculation of Arabidopsis leaves with *P. syringae pv tomato* (*Pst*) expressing *avrRpt2* induced secondary oxidative bursts in distant tissues, leading to low-frequency systemic micro-HRs. Both the primary HR and the secondary micro-HRs were shown to be necessary for the development of SAR (Alvarez et al. 1998).

**The role of salicylic acid**
The plant metabolite salicylic acid (SA) plays an important role in SAR. SA increases in plants after pathogen infection in both local and systemic tissue, and studies have shown SA is required for the expression of a set of PR genes (Mauch-Mani & Metraux 1998, Delaney 1997, Sticher et al. 1997, Ryals et al. 1994) (Fig. 1.2). Moreover, application of SA or its analogues 2,6-dichloroisonicotinic acid (INA) (Kessmann et al. 1994) and benzothiadiazole (BTH) (Friedrich et al. 1996, Gorlach et al. 1996, Lawton et al. 1996) induces broad-spectrum disease resistance in plants.

SA is a product of phenylpropanoid metabolism, synthesised from phenylalanine which is initially converted to *trans*-cinnamic acid by phenylalanine ammonia lyase.
PAL (Lee et al. 1995, Klessig & Malamy 1994). PAL is a key enzyme in the phenylpropanoid pathway that also yields phytoalexins, lignins and flavanoids (Klessig & Malamy 1994). Cinnamic acid is decarboxylated to benzoic acid which is converted to SA by 2-hydroxylation (Lee et al. 1995). A SA-binding protein has been purified and its cDNA cloned (Chen et al. 1993). This indicated that the protein is a catalase and that SA specifically inhibited this activity in vitro, leading to an increase in H₂O₂ (Chen et al. 1993). Thus, SA may act in SAR by elevating the levels of H₂O₂, which may induce the expression of PR genes (Chen et al. 1993). However, this hypothesis is unlikely as studies with transgenic tobacco expressing the H₂O₂-removing enzyme catalase in the antisense orientation (and thus unable to limit H₂O₂ accumulation) showed that SA was essential for accumulation of PR1 protein (Chamnongpol et al. 1998). Furthermore, this hypothesis is unlikely, as the oxidative burst and elevation of H₂O₂ levels occurs very early after pathogen inoculation and SA accumulates at a later time point (Lee et al. 1995).

Conclusive proof that SA is required for SAR comes from experiments with transgenic tobacco and Arabidopsis plants expressing the bacterial salicylate hydroxylase gene, nahG (Delaney et al. 1994). Salicylate hydroxylase converts SA to catechol, which is inactive in SAR signalling. Transgenic nahG plants accumulate a reduced amount of SA, do not express PR-1 or display SAR in response to SAR-inducing organisms (Fig.1.2). In addition, nahG plants are also more susceptible to avirulent and virulent Pseudomonas syringae DC3000 pv maculicola, Peronospora parasitica (Delaney et al. 1994, Lawton et al. 1995), Erisiphe orontii (Reuber et al. 1998) and turnip crinkle virus (Kachroo et al. 2000) (Fig.1.2). Further proof for the role of SA in SAR comes from tobacco plants overproducing SA (Verberne et al. 2000). Tobacco plants were transformed with two bacterial genes coding for enzymes that convert chorismate into SA by a two-step process in chloroplasts. Transgenic plants showed a 500- to 1000-fold increase in SA accumulation, constitutive expression of acidic PR genes and enhanced resistance to a viral and a fungal pathogen (Verberne et al. 2000).
Evidence both for and against SA as the translocated signal in SAR have been reported (Sticher et al. 1997, Ryals et al. 1996, Klessig et al. 1994). In vivo SA-labelling studies have provided evidence that SA produced in tobacco mosaic virus (TMV)-infected tobacco leaves is transported to the upper, non-infected leaves of the plant, accounting for 70% of the accumulated SA (Shulaev et al. 1995). However, in cucumber infected with \( P. \text{syringae} \), removal of the primarily infected leaf six hours after inoculation, which is before SA accumulates, still resulted in the expression of SAR (Rasmussen et al. 1991). In addition, TMV inoculation of \( \text{nahG} \) rootstocks resulted in very little SA accumulation in infected tissue, but SAR was established in the grafted wild type scion, indicating that translocation of the systemic signal was unaffected (Vermooij et al. 1994). Once SA accumulates, it is rapidly converted to \( \beta \)-3-0-D-glucosylsalicylic acid (SAG). SAG does not appear to be active in disease resistance and thus may either represent a detoxifying step in which SA levels are maintained below toxic concentrations, or an inert SA storage compound (Sticher et al. 1997, Ryals et al. 1996). Methyl salicylate, another metabolite of SA, is produced in large quantities in infected tissue (Seskar et al. 1998, Shulaev et al. 1997), and could function as an airborne signal in establishing SAR both in the same plant and in neighbouring plants (Shulaev et al. 1997).

In addition to the direct role played by SA in mediating disease resistance, evidence is also emerging for an indirect role, where SA potentiates the induction of local defence responses (Shirasu et al. 1997, Mur et al. 1996). When transgenic tobacco plants expressing the GUS reporter gene under the control of the asparagus PR10 promoter (\( \text{AoPR-1:GUS} \)) or \( \text{PAL3} \) promoter (\( \text{PAL3:GUS} \)) were hydroponically fed with 1 to 2 mM SA for 1 to 7 days, transgenic plants exhibited enhanced expression of both defence gene promoter fusions after wounding or infection with TMV or \( P.\text{syringae pv syringae} \) (Mur et al. 1996). In addition, it was found that inclusion of SA at physiological concentrations (50\( \mu \)M) in soybean cell suspensions potentiates an early step in the activation of local defence responses to an avirulent strain of \( P.\text{syringae pv glycinea} \) (Shirasu et al. 1997). An increase in \( \text{H}_2\text{O}_2 \) accumulation, \( \text{GST1} \) and \( \text{PAL1} \) expression, and hypersensitive cell death was visualised. This led to the conclusion that SA stimulates an agonist-dependent gain control operating at an
early step in the signal transduction pathway for induction of the hypersensitive
response (Shirasu et al. 1997).

The role of jasmonic acid
Jasmonic acid (JA) and its volatile counterpart methyl jasmonate (Me-JA) (referred
to collectively as jasmonates) are distributed throughout higher plants and effect
These include petiole abscission, tendril coiling, fruit ripening, pollen germination,
root growth and plant resistance to insects and pathogens (Creelman and Mullet
1997). JA is a 12-carbon fatty acid derivative, which is synthesised via the
octadecanoid pathway from the 18-carbon substrate linoleic acid (Leon & Sanchez-
Serrano 1999). The linoleic precursor is catalysed to linolenic acid by ω-3 fatty acid
desaturases in the chloroplasts (Leon & Sanchez-Serrano 1999). Linolenic acid is
converted in turn to 12-oxo-phytodienoic acid in a multi-step enzymatic process
involving lipoxygenase, allene oxide synthase and allene oxide cyclase activity. JA
synthesis proceeds in the cytoplasm with the action of 12-oxo-phytodienoic acid
reductase and is followed by three rounds of β-oxidation which takes place in the
peroxisomes (Leon & Sanchez-Serrano 1999). The majority of the genes
corresponding to these enzymes are transcriptionally activated and some of them are
also activated by JA, allowing for feed-back regulation of the biosynthetic pathway
(Leon & Sanchez-Serrano 1999).

JA appears to play an important role as a ‘master switch’ in signal transduction
pathways in response to insects and pathogens (Wasternack & Parthier 1997).
Proteins encoded by JA-induced genes include antimicrobial peptides, phytoalexin
biosynthetic enzymes, storage proteins and stress protectants (Wasternack & Parthier
1997). The JA-dependent wound-induced formation of proteinase inhibitors has
been well characterised (Wasternack & Parthier 1997, Schaller & Ryan 1995).
Proteinase inhibitors in tomato leaves (PIN1 and PIN2) accumulate systemically
after herbivore attack and protect the plant against the digestive enzymes produced
by the insect (Schaller & Ryan 1995). The systemic proteinase inhibitor-
inducing factor has been proposed to be an 18 amino acid peptide termed systemin (Ryan
Systemin is processed from a larger precursor protein termed prosystemin upon wounding, which is followed by the accumulation of linolenic acid and the subsequent production of jasmonic acid (Schaller & Ryan 1995). Both abscisic acid (Pena-Cortes et al. 1995) and ethylene (O’Donnell et al. 1996) are also required in this process. It has also been found that jasmonates are essential for insect defence in Arabidopsis (McConn et al. 1997). The Arabidopsis \textit{fad3-2 fad7-2 fad8} triple mutant, which can not produce the \(\omega-3\) fatty acid desaturases required for conversion of linoleic acid to linolenic acid and are thus deficient in JA (McConn & Browse 1996), were infected with larvae of \textit{Bradysia impatiens} (common fungal gnat). The mutant plants were extremely susceptible to attack by this insect species. However, prior application of Me-JA protected the plants, indicating that jasmonates play a vital role in mediating resistance against \textit{Bradysia impatiens} (McConn et al. 1997).

Two jasmonic acid responsive Arabidopsis mutants have been identified. The first of these, \textit{jarl} (jasmonic acid resistant 1) (Fig.1.2) showed decreased sensitivity to Me-JA inhibition of root elongation on agar medium containing 0.1\(\mu\)M Me-JA in comparison to wild-type seedlings (Staswick et al. 1992). Genetic data indicate that \textit{jarl} defines a single recessive gene (Staswick et al. 1992). The second mutant, \textit{coil} (coronatine-insensitive 1) (Fig.1.2), was selected on agar medium containing coronatine, a phytotoxin secreted by certain strains of \textit{Pseudomonas syringae} and an analogue of JA (Feys et al. 1994). The \textit{coil} mutant is male sterile and also defines a single recessive gene (Feys et al. 1994). The \textit{COIl} gene has been cloned, and found to encode a protein containing leucine-rich repeats and a degenerate F-box motif (Xie et al. 1998). These features are characteristic of proteins that ubiquitinate proteins targeted for removal. Thus \textit{COIl} may function by degrading a repressor protein in the JA signal transduction pathway (Xie et al. 1998).

It has become apparent that jasmonates play an important role in the regulation of pathogen defences. It has been shown that jasmonates are part of the signal transduction pathway leading to the expression of the antimicrobial peptides \textit{PDF1.2} and \textit{Thi2.1} in Arabidopsis in response to the necrotrophic fungal pathogens \textit{Alternaria brassicicola}, \textit{Botrytis cinerea} and \textit{Fusarium oxysporum} (Fig.1.2) (see
below). Jasmonate signalling is also important in resistance to the soil fungus *Pythium* sp (Fig. 1.2) (Staswick et al. 1998, Vijayan et al. 1998). Both the *jar1* (Staswick et al. 1998) and *fad3-2 fad7-2 fad8* (Vijayan et al. 1998) mutants were extremely susceptible to root rot caused by *Pythium* sp, whereas wild type plants were not. JA has also been found to accumulate in tobacco after inoculation with *Pseudomonas syringae* pv. *phaseolicola* (Kenton et al. 1999). JA accumulates 3 to 9 hours after bacterial infection and is restricted to the developing HR lesion (Kenton et al. 1999). This contrasts with the systemic accumulation of JA observed in the Arabidopsis-*A. brassicicola* interaction (Penninckx et al. 1996).

**The role of ethylene**

The simple gas ethylene (C\(_2\)H\(_4\)) is a phytohormone affecting all stages of plant growth and development, including germination, senescence, abscission, flowering, fruit ripening in climacteric fruits and responses to various stresses (reviewed in Chang & Shockey 1999, Johnson & Ecker 1998, Solano & Ecker 1998, Kieber 1997). Ethylene is formed from methionine via S-adenosyl methionine and 1-aminocyclopropane-1-carboxylic acid (ACC) (Kende 1993). S-adenosyl methionine is converted to ACC by the enzyme ACC synthase, and ACC is converted to ethylene by ACC oxidase, which is also called the ethylene-forming enzyme (Kende 1993). Both enzymes are encoded by multigene families whose members are differentially regulated by external stimuli such as flooding, pathogen infection, wounding or internal stimuli such as germination, fruit ripening or senescence (Johnson & Ecker 1998). Some of these processes can also act through other hormones such as auxin and cytokinin (Johnson & Ecker 1998).

In addition to understanding the production of ethylene, considerable progress has also been made in the genetic and molecular dissection of the ethylene-response pathway. A number of loci involved in ethylene signalling have been identified on the basis of the isolation of ethylene-response mutants in Arabidopsis. These mutant screens were based on the ‘triple response’, a series of dramatic morphological changes undergone by seedlings when grown in the dark in the presence of ethylene. In Arabidopsis, the triple response includes the inhibition of hypocotyl and root
elongation, radial swelling of hypocotyl and root cells and exaggeration of the apical hook (Ecker 1995). Mutants that display a 'constitutive' triple response result either from ethylene overproduction (eto1, eto2 and eto3) or constitutive activation of the ethylene response pathway (ctr1) (Guzman & Ecker 1990, Roman et al. 1995). Mutants unable to perceive or respond to ethylene include etr1, etr2, ein2, ein3, ein4, ein5, ein6, ein7 and eir1 (Bleeker et al. 1988, Roman et al. 1995). Genetic analysis of these mutants has indicated that they act in a linear pathway (Roman et al. 1995).

In the current view, ethylene is perceived at the plasma membrane by a family of ethylene receptors that include ETR1, ETR2, EIN4, ERS1 and ERS2 (Stepanova & Ecker 2000, Chang & Shockey 1999, Solano & Ecker 1998, Kieber 1997). From the membrane, the signal is transduced to the nucleus through a series of proteins that include CTR1, EIN2, EIN5, EIN6 and EIN7 (Chang & Shockey 1999, Solano & Ecker 1998, Kieber 1997). The current model for ethylene signalling in Arabidopsis proposes that ethylene binding deactivates the receptors such that, in the absence of a positive regulatory signal from receptors, the CTR1 protein becomes inactive (Stepanova & Ecker 2000). The ethylene signal is further transduced through the positive regulator EIN2 (Stepanova & Ecker 2000). In the nucleus, the EIN3-family of transcription factors initiate the expression of the ethylene responsive genes (Stepanova & Ecker 2000, Chang & Shockey 1999, Solano & Ecker 1998).

Cloning of the ETR1 gene uncovered its similarity to two-component histidine-kinase regulators that are sensors and transducers of environmental stimuli in bacteria (Chang et al. 1993). Two component receptors consist of a sensor protein with a histidine autokinase domain and a response regulator. Activation of the histidine-kinase promotes autophosphorylation of the histidine and a subsequent transfer of the phosphoryl group to an aspartate residue in the receiver domain of the response regulator protein (Chang et al. 1993). CTR1, which is a negative regulator of the ethylene response, acts downstream of the ethylene receptors and has similarity to the Raf family of mitogen-activated protein kinase kinase kinases (MAPKKKs) (Kieber et al. 1993). This suggests that the ethylene signal is propagated through a MAP kinase cascade. Cloning of EIN2 indicates that it codes for a structurally novel protein with an amino-terminal integral membrane domain
that has similarity to the Nramp family of metal-ion transporters (Alonso et al. 1999). The \textit{EIN3} gene codes for a nuclear-localised DNA binding protein (Chao et al. 1997).

Ethylene production often correlates with plant-pathogen interactions but its function in disease resistance and/or susceptibility is unclear and appears to be dependent on the pathogen species involved. The \textit{etr1} and \textit{ein2} mutants have been used in studies aimed at determining the role of ethylene accumulation in disease resistance. Bent and co-workers infected the Arabidopsis mutants \textit{etr1} and \textit{ein2} with avirulent \textit{Pst} strains (Bent et al. 1992). Both mutants were resistant to the bacteria, suggesting that ethylene is not required for resistance against avirulent bacterial pathogens. In a further experiment where the mutants were infected with virulent \textit{Pst}, \textit{P.syringae pv maculicola} or \textit{Xanthomonas campestris pv campestris}, \textit{ein2} developed minimal disease symptoms whereas wildtype plants and \textit{etr1} showed development of disease symptoms. However, virulent \textit{Pst} grew to wildtype levels in \textit{ein2}, indicating that the transduction of the ethylene signal as defined by this part of the pathway may be involved in pathogen-induced damage but not disease resistance. Further studies with the \textit{etr1} and \textit{ein2} mutants showed normal \textit{PR} gene induction and \textit{P.parasitica} resistance in response to SA, indicating that SA-induced SAR is independent of ethylene in Arabidopsis (Lawton et al. 1994). SAR induced in Arabidopsis by \textit{Pst (avrRpt2)} against \textit{P.parasitica} was not abolished in \textit{etr1} or \textit{ein2} plants, further indicating that ethylene is not required for SA dependent SAR (Lawton et al. 1995) (Fig.1.2). Ethylene also appears to play a role in the development of disease symptoms in tomato. The tomato mutant \textit{Never ripe}, which is impaired in ethylene perception, and a transgenic tomato line expressing the ACC-deaminase gene, which directs degradation of ACC and thereby inhibits ethylene production, exhibited a reduction in disease symptoms in comparison to wild type after inoculation with virulent \textit{Pst}, \textit{X.campestris pv vesicatoria} or \textit{Fusarium oxysporum f sp lycopersici} (Lund et al. 1998).

Further evidence that ethylene has different effects depending on the plant-pathogen interaction come from a number of different sources. Tobacco transformed with the
mutant etr1 gene from Arabidopsis remained resistant to TMV, but lost resistance to the normally non-pathogenic soil-borne fungus *Pythium sylvaticum* (Knoester et al. 1998). Arabidopsis ein2 mutant plants inoculated with *Botrytis cinerea* were more susceptible than wild-type plants (Fig.1.2), whereas no increased fungal growth was observed in ein2 plants after inoculation with avirulent fungal pathogens *P. parasitica* or *Alternaria brassicicola* (Thomma et al. 1999a). In addition, ein2.1 and etr1 plants showed reduction of PDF1.2 expression and enhanced susceptibility to the virulent bacterium *Erwinia carotovora* subsp. *carotovora* (Norman-Setterblad et al. 2000). However, these ethylene-insensitive mutants exhibited a normal SAR response to *P. parasitica* infection upon pre-treatment with harpin, the product of the *hrpN* gene of *Erwinia amylovora* (Dong et al. 2000). Studies with soybean mutants were also not clear-cut. Ethylene-insensitive soybean mutants were either more resistant or more susceptible to a range of bacterial and fungal pathogens (Hoffman et al. 1999).

**Pathogenesis-related proteins**

Associated with both local resistance to an avirulent pathogen and SAR are the expression of a number of genes which encode pathogenesis-related (PR) proteins (Ward et al. 1991). In tobacco, the set of PR proteins consists of at least nine families, comprising acidic forms of PR1 (PR1a, PR1b, PR1c), β-1,3-glucanase (PR2a, PR2b, PR2c), class II chitinases (PR3a and PR3b, also called PR-Q), hevein-like protein (PR4a and PR4b), thaumatin-like protein (PR5a and PR5b), acidic and basic isoforms of class III chitinase, an extracellular β-1,3-glucanase and the basic isoform of PR1 (Ryals et al. 1996, Ward et al. 1991). In general, accumulation of acidic PR proteins is dependent on SA (Ward et al. 1991). PR1, PR2, PR3, PR4 and PR5 show antimicrobial activity in vitro, with chitinases and β-1,3-glucanases showing antifungal activity (Sticher et al. 1997). The role of PR proteins in defence have been investigated using transgenic plants expressing the corresponding gene under the control of the cauliflower mosaic virus 35S promoter, and in some cases the transgenic plants were more resistant to pathogens (Sticher et al. 1997). In particular, over-expression of *PR-1* in tobacco increased resistance to *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* but not to *Ceronomospora nicotianae* or *P. syringae* pv. *tabaci* (Alexander et al. 1993).
In Arabidopsis PR proteins dependent on the accumulation of SA comprise PR-1, PR-2 and PR-5, with PR-1 being the predominant protein (Uknes et al. 1992) (Fig. 1.2). Acidic and basic chitinases (PR3) were isolated from Arabidopsis (Samac et al. 1990). Expression of the acidic chitinase gene was not observed in untreated plants or in plants treated with ethylene or SA, whereas the basic chitinase gene was expressed constitutively in roots and systemically upon application of ethylene (Samac et al. 1990). A hevein-like gene, with 70% homology to tobacco PR4, was isolated and was found to be inducible by SA, ethylene and infection by turnip crinkle virus (Potter et al. 1993). In addition, expression of both the basic PR3 and PR4 in Arabidopsis was induced by application of methyl jasmonate or inoculation with A. brassicicola (Thomma et al. 1998).

The role of antimicrobial peptides
The production of antimicrobial peptides is thought to be an ancient and widespread defence strategy. Thionins and plant defensins are examples of antimicrobial peptides that have been isolated from plants. Thionin genes, originally identified in barley, have also been identified in Arabidopsis and are toxic to several phytopathogenic bacteria and fungi (Eppe et al. 1995). Plant defensins also have antifungal activity (Broekaert et al. 1995). Recently, a re-classification of PR-proteins has included plant defensins and thionins, and has listed them as PR-12 and PR-13 respectively (Van Loon & Van Strien 1999).

A search of Arabidopsis expressed sequenced tags (ESTs) identified two putative defensins, called plant defensin 1.1 (PDF1.1) and PDF1.2 (Penninckx et al. 1996). PDF1.1 was expressed in silique and seeds, while PDF1.2 was expressed in response to infection by the compatible fungus Alternaria brassicicola in both locally infected and systemic leaves (Penninckx et al. 1996). Expression of PDF1.2 correlated with an increase in expression of jasmonic acid in both types of leaves (Penninckx et al. 1996). PDF 1.2 was not expressed in response to SA or INA treatment, but was expressed in response to Me-JA or ethylene (Fig.1.2).
Two cDNAs encoding thionin preproteins have been isolated from Arabidopsis, and have been designated Thi2.1 and Thi2.2 respectively (Epple et al. 1995). Thi2.2 is expressed at low levels in seedlings and rosette leaves, whereas Thi2.1 is expressed in leaves, and very highly in flowers and siliques. Application of methyl jasmonate, silver nitrate and infection with Fusarium oxysporum f. sp. matthiola e induced Thi2.1 to high levels (Fig.1.2), but had no effect on Thi2.2 expression (Epple et al. 1995). Application of SA to Arabidopsis plants did not induce Thi2.1 expression, indicating that this gene is also induced via a signal transduction pathway different to that for PR proteins (Epple et al. 1995). Over-expression of the Thi2.1 gene in the susceptible Arabidopsis ecotype Columbia (Col-0) resulted in enhanced resistance to F. oxysporum f. sp. matthiola e (Epple et al. 1997a). In addition, it was found that Thi2.1 expression correlates to F. oxysporum resistance: the resistant ecotypes Mt-0 and UK-4 had 5 to 10 times higher accumulation of Thi2.1 in comparison to the susceptible ecotypes Col-0, Landsberg erecta (Ler) and Wassilewskija (Ws) (Epple et al. 1998).

Rhizobacteria-mediated Induced Systemic Resistance

Some non-pathogenic rhizobacteria can induce systemic resistance in plants that is phenotypically similar to pathogen-induced SAR. Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria and viruses in Arabidopsis, bean, carnation, cucumber, radish, tobacco and tomato under conditions in which the inducing bacteria and the challenging pathogen remain spatially separated (Pieterse & Van Loon 1999, Van Loon et al. 1998). Bacterial strains differ in their mode of action and the extent of their ability in inducing ISR, and plants differ in their ability to express ISR. Bacterial determinates of ISR include lipopolysaccharides, sideophores and SA (reviewed in van Loon 1998). Most of the ISR-inducing rhizobacteria belong to the fluorescent Pseudomonas sp. A model system has been developed in which Pseudomonas fluorescens WCS417r is used to induce ISR in Arabidopsis against Pst and F. oxysporum fsp raphani (Pieterse et al. 1996). ISR was found to be effective in nahG plants and did not induce expression of the PR-1, PR-2 and PR-5 genes, indicating that SA is not needed for ISR. Therefore, ISR may be induced via a different pathway to that of
SAR (Fig. 1.3, see below) (Van Wees et al. 1997, Pieterse et al. 1996). Studies of ISR against Pst using jar1, the ethylene insensitive mutants etr1, ein2 through ein7 and eir1, and the SAR regulatory mutant npr1 (see below), indicated that components of the jasmonate and ethylene response network are engaged successively in inducing resistance (Fig. 1.3, see below) (Knoester et al. 1999, Pieterse et al. 1998). Furthermore, ethylene responsiveness is required at the site of application of the inducing rhizobacteria and the complete known signal transduction pathway of ethylene is required for ISR (Knoester et al. 1999). ISR against Pst was abolished in the npr1 mutant, indicating that NPR1 regulates both SAR and ISR (Pieterse et al. 1998) (Fig. 1.3, see below). It has also been shown that the simultaneous activation of ISR and SAR results in an additive effect on the level of resistance against Pst (Van Wees et al. 2000). No ISR marker proteins or substantial changes in gene expression have been identified to date, but ISR was found to stimulate the expression of the jasmonate-inducible vegetative storage gene Atvsp upon challenge with Pst (Van Wees et al. 1999). A single dominant gene, ISR1, has been identified which is required for both ISR and basal resistance against Pst, suggesting that a high level of basal resistance is required for induction of ISR against Pst in Arabidopsis (Ton et al. 1999).

1.5. SAR signal transduction network

The production of mutants with abnormalities in disease resistance makes it possible to define the signal transduction network underlying SAR. Evidence is emerging for both a SA-dependent and a SA-independent, jasmonate- and ethylene-dependent signal transduction pathway leading to the establishment of disease resistance to different pathogens (Fig. 1.2) (Thomma et al. 1998, Bowling et al. 1997, Ryals et al. 1996, Penninckx et al. 1996). Fig. 1.3 outlines the current model of the defence response signalling network and the positioning of genes identified in mutant screens.
Mutants in the transduction of R-gene-mediated signals

The ndrl (nonrace-specific disease resistance) mutant, produced in the Arabidopsis ecotype Col-0, is susceptible to PstDC3000 carrying any one of four *avr* genes: *avrRpm1*, *avrRpt2*, *avrB* and *avrPph3*, as well as the Emyo2 and Emwa1 isolates of *P. parasitica* (Century et al. 1995). Col-0 plants are resistant to the Emyo2 and Emwa1 isolates, and require the presence of the *RPP4* (resistance to *P. parasitica 4*) locus for resistance (Holub et al. 1994). This indicates that the *NDR1* gene product is a part of a common signal transduction pathway that mediates resistance to both bacterial and oomycete pathogens, and is required for the function of the *RPM1*, *RPS2*, *RPS5* and *RPP4* genes. The *NDR1* gene product has been placed between the HR and SA in the signal transduction pathway (Fig.1.3). The *NDR1* gene has been isolated and cloned, and the predicted amino acid sequence suggests that NDR1 may be associated with the plasma membrane (Century et al. 1997).

Fig.1.3. Current model of the SAR signal transduction network in Arabidopsis.

Adapted from Glazebrook (1999) and Feys & Parker (2000). The position of cloned genes and the site of action of their predicted proteins are shown in capitals. Mutants, representing currently uncloned genes, are shown in lower case. For simplicity, not all mutants mentioned in the text are represented in this model.
The *eds1* (*enhanced disease susceptibility 1*) mutant, produced in the Arabidopsis ecotype Ws, supported heavy sporulation of the *P. parasitica* isolates Emoy2, Cala2, Wela3 and Noco2 normally showing an incompatible interaction with Ws (Parker et al. 1996). In Ws, the *RPP1, RPP10, RPP12* and *RPP14* loci are required for resistance to the Emoy2, Cala2, Wela3 and Noco2 isolates respectively (Holub et al. 1994). Further alleles of *eds1* were isolated in the Arabidopsis ecotype Ler (Aarts et al. 1998). Studies with the *eds1.2* and *eds1.3* mutants indicated that *RPP5*-mediated resistance to *P. parasitica* Noco2 in Landberg erecta was lost, as was *RPS4*-mediated resistance to *P. syringae pv tomato* (*avrRps4*) (Aarts et al. 1998). Aarts and co-workers also showed that *RPP4*-mediated resistance to *P. parasitica* Emwal in a Col-0 *eds1* mutant was lost (Aarts et al. 1998). This indicates that the recessive *EDSJ* locus is required for the function of the *RPP1, RPP4, RPP5, RPP10, RPP12, RPP14* and *RPS4* genes. The Ws- *eds1* mutant did not show compromised RPM1-mediated resistance to the corresponding bacterial avirulence gene *avrB* (Parker et al. 1996), but demonstrated resistance to both compatible and incompatible *P. parasitica* strains after treatment with INA (Parker et al. 1996) and showed *PR-1* expression after treatment with SA but not *Pst* (*avrRps4*) (Falk et al. 1999). This indicates that *EDS1* is a necessary component of resistance mediated by a number of *R* genes, and that *EDS1* functions upstream of SA accumulation in the SA-dependent SAR signal transduction pathway (Fig. 1.3). The *EDS1* gene has been cloned and found to encode a novel protein (Falk et al. 1999). However, it has similarity in its amino-terminal portion to the catalytic site of eukaryotic lipases belonging to the L-family (Falk et al. 1999), suggesting that *EDS1* functions by hydrolyzing a lipid molecule. It is thus possible that *EDS1* may be involved in processing JA-related fatty acid intermediates, or it may define an additional lipid-based signal transduction pathway (Falk et al. 1999).

It has been shown that the *EDS1* and *NDRI* gene products are required for the function of different resistance genes (Aarts et al. 1998). *EDS1* is required for *RPP1, RPP5, RPP10, RPP12, RPP14* (which confer resistance to *P. parasitica*), and *RPS4* which confers resistance to *Pst*(*avrRps4*) whereas *NDRI* is not required for the
function of these R genes (Aarts et al. 1998). Conversely, three NDR1-dependent R loci, RPS2, RPM1 and RPS5, operate independently of EDS1 (Aarts et al. 1998). Presented results for RPP4 are unclear and do not correlate (Aarts et al. 1998, Century et al. 1995). Interestingly, the RPP1, RPP5 and RPS4 EDS1-dependent R genes encode proteins of the TIR-NBS-LRR class, whereas NDR1-requiring R genes RPS2, RPM1 and RPS5 belongs to the LZ-NBS-LRR class (Table 1.1, Aarts et al. 1998). This indicates that at least two R-gene mediated pathways are present in Arabidopsis and implies that the requirement for either EDS1 or NDR1 is governed by R protein structure (Aarts et al. 1998) (Fig. 1.3). Further studies of the RPP7 and RPP8 loci (which also confer resistance to P. parasitica) have shown that neither EDS1 nor NDR1 are required for resistance mediated by these loci (McDowell et al. 2000). However, RPP7 and RPP8-mediated resistance is weakly suppressed in the eds1 ndr1 double mutant, suggesting that they operate additively through EDS1, NDR1 and other as yet unidentified signalling components (McDowell et al. 2000).

A further three putative signal transduction genes involved in R gene-specific disease resistance in Arabidopsis have been reported (Warren et al. 1999). Mutant plants were screened for loss of RPS5-specified resistance and five pbs (avrPphB susceptible) mutants, compromising three complementation groups, were uncovered. Mutations in PBS1 blocked RPS5 resistance only, and had no effect on resistance specified by other R genes. This indicates that PBS1 may be closely associated with the RPS5 / AvrPphB interaction (Warren et al. 1999). The pbs2 mutant appears to suppress the same set of R genes as ndr1 (RPS2, RPS5 and RPM1) (Warren et al. 1999) (Fig. 1.3). The ndr1 and pbs2 mutations are not allelic and it is likely that the products of these two genes are closely associated in the same signal transduction pathway (Fig. 1.3). The pbs3 mutation partially suppressed four P. syringae R genes (RPS2, RPS5, RPM1 and RPS4), the RPP genes and allowed higher growth of virulent Pst (Warren et al. 1999). This indicates that the PBS3 gene product functions in a pathway involved in restricting both avirulent and virulent pathogens, and may operate downstream of both EDS1 and NDR1 (Warren et al. 1999) (Fig. 1.3).
Isolation and characterisation of the dnd1 (defence, no death 1) mutant has provided evidence that the HR can be separated from gene-for-gene resistance in Arabidopsis (Yu et al. 1998). Mutant dnd1 plants infected with avirulent Pst carrying either the avrRpt2, avrRpm1 or avrB genes did not form a HR, but resistance to the avirulent bacteria was seen. Additionally, SA accumulated to higher levels and PR-1 and PR-2 were expressed constitutively (Yu et al. 1998). Mutant dnd1 plants also exhibited enhanced resistance to virulent Pst, Xanthomonas campestris, P.parasitica, Erisiphe orontii and tobacco ringspot virus. It is likely that the DND1 product functions upstream of both SA accumulation and the HR in the defence response signalling network (Fig.1.3). Recently DND1 has been cloned and it’s predicted protein structure encodes a cyclic nucleotide-gated ion channel (Clough et al. 2000). Further mutants exhibiting an altered HR have been isolated including ihrl (intermediate hr1) (Yu et al. 2000). The ihrl mutant also displays elevated resistance to a wide range of pathogens, accumulates SA to higher levels and expresses PR-1 and PR-2 to higher levels (Yu et al. 2000).

**Mutants expressing constitutive SAR**

Different and independent screens have been used to identify mutants expressing SAR constitutively (Lawton et al. 1993, Bowling et al. 1994, Silva et al. 1999). In the first of these, RNA was isolated from EMS-mutagenised M2 seedlings using northern blots and hybridized to SAR gene probes (Lawton et al. 1993). This led to the discovery of constitutively immune mutants (cim), which show constitutive expression of PR-1, PR-2 and PR-5, and resistance to pathogens. The second method entailed screening mutant transgenic plants containing the BGL2(PR-2) promoter fused to the marker gene β-glucoronidase (GUS) for constitutive GUS expression (Bowling et al. 1994). To date, the isolation and characterisation of three non-allelic cpr (constitutive expresser of PR genes) mutants have been reported: cpr1 (Bowling et al. 1994), cpr5 (Bowling et al. 1997) and cpr6-1 (Clarke et al. 1998).

In addition to constitutive GUS expression, cpr1 shows elevated expression in northern blot analysis of the PR genes, is resistant to P. parasitica Noco2 and PsmES4326 and is associated with high endogenous levels of SA (Bowling et al. 1994).
1994). Progeny of a cpr1nahG double mutant do not produce SA and do not show the cpr1 phenotype, indicating that the CPR1 gene product acts upstream of SA in the SAR signal transduction pathway (Bowling et al. 1994) (Fig.1.3). The cpr5 mutant displays spontaneous HR lesion formation and reduced trichome development in addition to the same characteristics as cpr1, which indicates that the CPR5 gene product acts upstream of SA in the SAR pathway (Fig.1.3, Bowling et al. 1997). However, the cpr5npr1 double mutants (npr1 is incapable of establishing SA-dependent SAR, see below) continue to display resistance to P. parasitica Noco2 (Bowling et al. 1997). In addition, cpr5 plants display elevated expression of PDF1.2 (Bowling et al. 1997). This led to the conclusion that the cpr5 mutation regulates constitutive expression of both a SA/NPR1 dependent pathway and a SA/NPR1 independent pathway, and that the CPR5 gene is operating upstream of the HR and the branchpoint between the two pathways (Bowling et al. 1997) (Fig.1.3). In a screen for suppressor mutations that restored resistance to a susceptible line carrying a mutation in the RPS2 resistance gene, the cpr5-2 mutant was isolated (Boch et al. 1998). Resistance gene-mediated defences, including the HR, restriction of Pst growth and induction of PR-1 gene expression were observed in cpr5-2 plants. Additionally, RPS2-avrRpt2 mediated induction of PR-1 expression was enhanced (Boch et al. 1998). Unlike cpr1 and cpr5 which are recessive mutants (Bowling et al. 1994, Bowling et al. 1997), cpr6-1 is a dominant mutant (Clarke et al. 1998). The mutant cpr6-1 displays constitutive expression of the PR and PDF1.2 genes and resistance to PsmES4326 and P. parasitica Noco2 (Clarke et al. 1998). Although bacterial resistance is not observed, PR gene expression is detected in the cpr6-1npr1 double mutant, which is suppressed when SA is removed by crossing with a nahG plant (Clarke et al. 1998). This indicates that PR gene expression has been uncoupled from the NPR1 gene product and bacterial resistance in the cpr6-1 mutant. Hence, CPR6 may regulate multiple signal transduction pathways (Fig.1.3) (Clarke et al. 1998).

The cep T-DNA mutant (constitutive expression of the PR1 gene) was isolated in the Ws ecotype background (Silva et al. 1999). The cep mutant displays constitutive expression of the PR genes, elevated levels of SA accumulation, spontaneous
development of HR-like lesions and enhanced resistance to virulent \textit{Psm} and \textit{P.parasitica} Emwa (Silva et al. 1999). Although the \textit{cep} phenotype segregated as a single recessive trait in the Ws genetic background, analysis of segregating progeny from crosses to other ecotypes revealed that the \textit{cep} phenotype was due to mutations in two genes, designated \textit{cpr20} and \textit{cpr21} (Silva et al. 1999).

\textit{Lesion mimic mutants}

Many mutants have been uncovered that spontaneously form HR lesions and some of these also expresses the \textit{PR} genes, accumulate SA and express pathogen resistance (reviewed in Delaney 1997, Glazebrook et al.1997a). Simultaneous HR formation and SAR expression may come about because the plant is capable of falsely perceiving the pathogen. Included in this class are the \textit{lsd} mutants (lesion stimulating disease response): \textit{lsd1}, \textit{lsd2}, \textit{lsd3}, \textit{lsd4}, \textit{lsd5} (Dietrich et al.1994), \textit{lsd6} and \textit{lsd7} (Weymann et al.1995) and \textit{acd} (accelerated cell death) mutants (Greenberg et al. 1994, Rate et al 1999, Greenberg et al. 2000). The \textit{acd2} recessive mutant forms lesions on older leaves only, so plants can be studied both before and after lesion development (Greenberg et al. 1994). These studies have shown that lesion formation is necessary for SA accumulation, \textit{PR} gene expression and bacterial resistance (Greenberg et al. 1994). The \textit{ACD2} gene product has been placed upstream of the HR (Fig.1.3). The \textit{acd6} mutant has been characterised more recently (Rate et al. 1999). This dominant gain-of-function mutant is dwarfed in size, displays constitutive \textit{PR-1} gene expression and shows increased resistance to both virulent and avirulent \textit{Pst} (Rate et al. 1999). These phenotypes are suppressed in \textit{acd6 nahG} plants, but are hyperactivated in \textit{acd6 nahG} plants treated with BTH, suggesting that SA may be acting with a second defence signal (Rate et al. 1999). The \textit{acd6} phenotypes are also suppressed in \textit{acd6 npri} plants, indicating that \textit{acd6} acts through \textit{npri} (Rate et al. 1999). The ACD6 product is thus thought to be necessary for the activation of the SA/NPR1-dependent part of the signalling network, and is also required for the activation of the unknown second signal (Fig.1.3). Characterisation of the recessive \textit{acd5} mutant further indicated that spontaneous lesion formation, SA accumulation and \textit{PR-1} expression are correlated (Greenberg et al. 2000). However, \textit{acd5} plants were not more resistant to \textit{P.syringae}
than wild-type plants, indicating that in acd5, cell death and defence-related processes have been uncoupled from disease resistance (Greenberg et al. 2000).

The lsd1 mutant differs from the others in that once HR lesions have formed, they spread and destroy the leaf by 'runaway' cell death, whereas in the other mutants the spread of the lesion is limited (Dietrich et al. 1994). It was found that superoxide accumulation is necessary for lesion spread in lsd1 mutants (Jabs et al. 1996). Thus, the runaway cell death phenotype seen in lsd1 plants appears to reflect an abnormal response to superoxide accumulation (Jabs et al. 1996). The LSD1 gene has been cloned and the predicted LSD1 protein encodes a zinc finger protein, which suggests a role in transcriptional activation (Dietrich et al. 1997). Consequently, it was proposed that LSD1 functions in the regulation of transcription by either repressing a death pathway or activating a death inhibiting pathway (Dietrich et al. 1997) (Fig. 1.3). More recently, it was shown that LSD1 regulates SA induction of a CuZn superoxide dismutase (SOD) (Kliebenstein et al. 1999). Thus, the spreading lesion phenotype in the lsd1 mutant may be due to the lack of upregulation of a CuZn SOD responsible for detoxifying accumulating superoxide before the superoxide triggers cell death (Kliebenstein et al. 1999).

The lsd2, lsd4, lsd6 and lsd7 mutants were crossed with nahG plants in order to observe what happens in these mutants when SA accumulation is removed (Weymann et al. 1995, Hunt et al. 1997). Although PR gene expression and pathogen resistance were suppressed in the progeny of these crosses, the mutants differed in the formation of the HR lesion phenotype: lsd2nahG and lsd4nahG continued to form lesions (Hunt et al. 1997) whereas lsd6nahG and lsd7nahG did not (Weymann et al. 1995). In addition, application of SA or INA to the lsd6nahG plants initiated HR lesion formation (Weymann et al. 1995). This result could be explained by a feedback loop in SAR signalling such that HR lesions cause SA accumulation but other factors downstream of SA may also potentiate lesion formation (Weymann et al. 1995).
The lsd5 mutant was used to isolated new mutations that suppress its cell death phenotype. Nine cell death suppressers were identified and were designated phx for the mythological bird Phoenix that arose from its ashes (Morel & Dangi 1999). Four strong suppressers of cell death and constitutive PR-1 were isolated (phx2, phx3, phx6 and phx11.1). All four phx mutants showed enhanced susceptibility to avirulent P. parasitica, but only phx2 and phx3 were more susceptible to avirulent Pst and virulent P. parasitica (Morel & Dangi 1999). It is thus likely that the PHX2 and PHX3 products define common regulators of cell death and disease resistance.

**Mutants incapable of expressing SA-dependent SAR**

Three independent screens were used to identify mutants incapable of expressing SA-dependent SAR (Cao et al. 1994, Delaney et al. 1995, Shah et al. 1997). In the first of these, the npr1 (non-expresser of PR genes) was isolated (Cao et al. 1994). Mutant transgenic plants containing the BGL-2-GUS cassette were sprayed with SA or INA and screened for GUS activity (Cao et al. 1994). npr1 mutants failed to express GUS and PR-1 (Cao et al.1994). In the second screen, the nim1 (non-inducible immunity) mutant was isolated, which failed to develop resistance to P. parasitica in response to INA pre-treatment (Delaney et al. 1995). The sail (salicylic acid-insensitive) mutant was isolated in the third screen (Shah et al. 1997). This screen utilised the SA-inducible expression of the Arabidopsis tms2 gene under the control of the PR-la promoter, which confers sensitivity to α-naphthalene acetamide (α-NAM) and results in inhibition of root growth (Shah et al. 1997). The sail mutant is insensitive to α-NAM and does not express the PR-1, PR-2 and PR-5 genes in response to SA. The npr1, nim1 and sail mutants were shown to be alleles of the same recessive gene in complementation studies (Delaney et al. 1997, Shah et al. 1997). Plants with npr1/nim1/sail mutations still accumulate SA in response to pathogen infection (Cao et al. 1994, Delaney et al. 1995). This indicates that the NPR-1/NIM-1/SAIL gene (designated NPR1) acts in the SAR signal transduction pathway downstream of SA (Fig.1.3).

NPR-1/NIM-1 was cloned by two separate map-based cloning projects (Cao et al. 1997, Ryals et al. 1997). From DNA sequence analysis, it appears that NPR-1/NIM-
I encodes a novel protein containing ankyrin repeats, which implies a role in protein-protein interactions (Cao et al. 1997). Ryals and co-workers (Ryals et al. 1997) also detected homology to the mammalian transcription factor inhibitor IκB, which has been implicated in the immune response. The NPR1 protein has been expressed in Arabidopsis under the control of the CaMV35S promoter (Cao et al. 1998). The resulting transgenic plants expressed SAR genes more strongly upon induction and showed dramatic resistance to *P.syringae* and *P.parasitica* (Cao et al. 1998).

Two mutant screens have been conducted in order to uncover suppressers of the *nprl* mutation. The dominant *ssil* (suppressor of *SA* insensitivity 1) mutant was isolated in the first of these screens as a suppressor of *nprl-5* (previously *sail*) (Shah et al. 1999). In *ssil* plants, which are small and spontaneously develop HR-like lesions, *PR-1, PR-2, PR-5* and *PDF1.2* are expressed constitutively, SA accumulates to elevated levels and plants remain resistant to *Pst* infection (Shah et al. 1999). These phenotypes remain in *ssil nprl* plants, but are abolished in *ssil nprl nahG* plants, indicating that they are dependent on SA (Shah et al. 1999). In the second screen, the recessive *snil* (suppressor of *nprl* inducible 1) mutant was isolated as a suppressor of *nprl-1* (Li et al. 1999). The *snil* mutant, which is smaller than wild type plants, expressed *PR-1, PR-2* and *PR-5* to wild type levels, accumulated SA and displayed resistance to *Psm* and *P.parasitica* only after application of SA (Li et al. 1999). The expression of *PDF1.2* was not recorded. The *SNI1* gene was cloned by map-based cloning and was found to contain a novel leucine-rich nuclear protein (Li et al. 1999).

Using NPR1 as a bait in a yeast two-hybrid screen, a subclass of transcription factors in the basic leucine zipper protein (bZIP) family were isolated (Zhou et al. 2000, Zhang et al. 1999). Small differences were found in the extent of interaction between different members of the bZIP family and NPR1 (Zhou et al. 2000, Zhang et al. 1999). It was shown that these bZIP transcription factors interacted specifically with NPR1 in yeast and *in vitro*, and that point mutations that abolished NPR1 function in Arabidopsis also abolished interactions between NPR1 and the transcription factors in the yeast two-hybrid assay (Zhang et al. 1999). It was also
shown in a gel mobility shift assay that the purified bZIP transcription factor protein, AHBP-1b, bound specifically to a SA-responsive promoter element in the Arabidopsis PR-1 gene. This indicates that NPR1 may regulate PR-1 expression by interaction with bZIP transcription factors (Zhou et al. 2000, Zhang et al. 1999). A model has been proposed whereby SNI1 represses the expression of PR genes in the absence of SA (Li et al. 1999). When SA is introduced, it activates NPR1, which represses the SNI1 repressor, allowing transcription of PR genes. SA also activates a second factor, possibly the bZIP transcription factors, that can also allow transcription of PR genes (Li et al. 1999).

SA-independent SAR

To date, no Arabidopsis mutants have been specifically isolated as being important in jasmonate- and /or ethylene-dependent SAR signalling. However, by studying the JA and ethylene-dependent expression of PDF1.2 and Thi2.1 in combination with other SAR signalling mutants, it has become apparent that SA-independent signal transduction plays a role in establishing resistance against necrotrophic fungal pathogens (Thomma et al. 1998, Penninckx et al. 1996, Epple et al. 1995).

PDF 1.2 expression was not affected by the nahG transgene, or by the npr1 or cpr1 mutations (Penninckx et al. 1996). However, the ein2 or coil mutations greatly reduced PDF 1.2 expression both locally and systemically (Penninckx et al. 1996), indicating that both jasmonates and ethylene, and not salicylic acid, are important in the signal transduction pathway leading to PDF1.2 expression. More recently, in further studies where the coil and ein2 mutants were treated with either ethylene or Me-JA, it was established that both the ethylene and jasmonate signalling pathways need to be triggered concomitantly, not sequentially, in order to activate PDF1.2 expression upon A. brassicicola infection (Penninckx et al. 1998). PDF1.2 was also expressed to a high level in Arabidopsis seedlings infected with the compatible necrotrophic fungus, Fusarium oxysporum f. sp. matthiolae (Epple et al. 1997b). In this study, PDF1.2 expression was induced in seedlings treated with Me-JA and silver nitrate, but not by SA or the ethylene-producing compound, ethephon. Furthermore, it was found that PDF1.2 was expressed in naïve mature rosette leaves,
but not in naïve seedlings (Epple et al. 1997b). It is possible that PDF1.2 is expressed at a very low level in rosette leaves, and so was only detected by Epple and co-workers (1997b) but not by Penninckx and co-workers (1996). However, it is quite clear in both studies that PDF1.2 expression is induced to higher levels by a range of compatible necrotrophic fungi.

PDF1.2 expression was further investigated by activity studies of the PDF1.2 promoter (Manners et al. 1998, Mitter et al. 1998). The PDF1.2 promoter was linked to the β-glucoronidase (GUS) reporter gene as a translational fusion and transformed into Arabidopsis (Manners et al. 1998) or tobacco (Mitter et al. 1998). Challenge of the transgenic Arabidopsis plants with *A. brassicicola* or the necrotrophic fungal pathogen *Botrytis cinerea* resulted in both local and systemic GUS expression (Manners et al. 1998). Wounding had no effect on GUS expression, while treatment with either jasmonic acid or the reactive oxygen producing compound paraquat strongly induced GUS activity (Manners et al. 1998). SA or ethylene application did not result in GUS expression (Manners et al. 1998). In contrast to the transgenic Arabidopsis, the PDF1.2:GUS transgenic tobacco expressed GUS in response to jasmonic acid and ethylene (Mitter et al. 1998), indicating that the ability of ethylene to induce PDF1.2 expression is genotype-dependent. GUS expression was also strongly induced by inoculation of the transgenic tobacco plants with *Phytophthora parasitica, Cercospora nicotianae* and TMV (Mitter et al. 1998).

In order to study the expression of the Thi2.1 gene, the cognate promoter was fused to the GUS gene and transformed into Arabidopsis (Vignutelli et al. 1998). Systemic and local GUS expression could be induced by *F. oxysporum* f.sp. *matthiolae* or by wounding in plants and in young seedlings by Me-JA application (Vignutelli et al. 1998), silver nitrate and sorbitol (Bohiman et al. 1998). Treatment of transgenic plants with inhibitors of JA biosynthesis led to reduced Thi2.1 expression, indicating that JA is an important step leading to Thi2.1 expression. The coil and *fad3-2 fad7-2 fad8* mutants were crossed separately to the Thi2.1:GUS transgenic line and GUS activity was analysed in the progeny plants (Bohiman et al. 1998). After methyl jasmonate application, no GUS activity was seen in the *coil*
background, whereas GUS activity was seen in the *fad3*-*2 fad7*-*2 fad8* mutant, confirming that the JA-dependent octadecanoid pathway regulates *Thi2.1* gene expression (Bohlman et al. 1998).

Further evidence for separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis come from studies with *A. brassicicola*, *B. cinerea* and *P. parasitica* (Thomma et al. 1998). The coil mutant showed enhanced susceptibility to virulent *A. brassicicola* and *B. cinerea* but not to *P. parasitica*, whereas nahG and npri plants showed enhanced susceptibility to *P. parasitica* but not to the other two fungal pathogens (Thomma et al. 1998).

**Cross-talk between SA-dependent and JA-dependent signalling**

Evidence that SA-dependent and JA-dependent signalling may influence each other by ‘cross-talk’ is accumulating (Bostock 1999, Malek & Dietrich 1999). Early evidence for negative cross-talk between SAR and the wound response in Arabidopsis came from pharmacological experiments where it was shown that SA inhibited wound-induced gene expression (Doares et al. 1995, Doherty et al. 1988). Furthermore, it was shown that SA prevented wound-induced gene expression in tomato by blocking synthesis of JA (Pena-Cortes et al. 1993). SA and JA also appear to have an antagonistic effect on *PR-1* gene expression in wounded tobacco leaves (Niki et al. 1998). It was found that JA induced the expression of basic *PR-1* and suppressed the expression of acidic *PR-1*, while SA was found to do the opposite (Niki et al. 1998).

Recent experiments have suggested an inverse relationship between SAR and resistance to insect herbivory (Felton et al. 1999). Transgenic plants silenced for PAL expression showed reduced SAR to TMV but enhanced grazing-induced resistance to larvae of *Heliothis virescens*. In contrast, transgenic plants overexpressing PAL exhibited enhanced SAR to TMV but larval resistance was reduced (Felton et al. 1999).
In a related experiment, separate tomato plants were infected with *Helicoverpa zea* larvae, the bacterial pathogen *Pst* or the fungal pathogen *Phytophthora infestans*, or plants were sprayed with BTH (Stout et al. 1999). The effects of these treatments on expression of defence genes and resistance to *Pst* or *H.zea* was determined (Stout et al. 1999). Inoculation with *Pst* increased resistance in the same plant to *H.zea* and *Pst*. Similarly, feeding by *H. zea* caused systemic resistance to both *H. zea* and *Pst*. In contrast, inoculation with *P. infestans* had no effect on resistance to *H. zea* and *Pst*. BTH treatment increased resistance to *Pst* but enhanced feeding of *H.zea*. *H. zea* feeding led to the systemic expression of *pin* mRNA, whereas *P. infestans* inoculation caused the accumulation of *PR* transcripts. *Pst* inoculation led to enhanced expression of both *pin* and *PR* genes (Stout et al. 1999). These results provide evidence for reciprocal induced resistance in tomato against multiple pathogens, and suggests that induced resistance to some pathogens may compromise resistance to others (Stout et al. 1999).

Analysis of Arabidopsis SAR mutants also provides evidence for interactions between SA-dependent and JA-dependent resistance. For example, the *cpr5* mutant displays constitutive expression of SA-dependent *PR-1* and the JA-dependent *PDF1.2*, which may indicate that the expression of these two genes is co-regulated (Bowling et al. 1997). This is consistent with the co-induction of both pathways in Arabidopsis by *Pst* inoculation (Maleck & Dietrich 1999). When the *cpr6* mutant was crossed with *nahG*, expression of the *PR-1* gene was significantly reduced, but *PDF1.2* expression was enhanced to levels higher than in *cpr6* alone (Clarke et al. 1998). This indicates that the elevated SA levels in *cpr6* may be suppressing *PDF1.2* expression. However, studies with the *ssil* mutant suggest that SSII may function as a switch modulating cross-talk between the SA-dependent and JA-dependent signal transduction pathways (Shah et al. 1999). Evidence for this comes from suppression of *PDF1.2* expression in the *ssil npr1 nahG* triple mutant, which could be restored by spraying the plants with BTH (Shah et al. 1999). However, it is also possible that the *ssil* mutant simply defines a common step in the signal transduction pathway, upstream of the branch point between SA-dependent and JA-dependent signal transduction. Although the *ssil* mutation is dominant, triploid
plants containing one ssii allele were phenotypically wild-type, indicating that the dominant nature of the ssii mutation was due to haploinsufficiency i.e. that one wild-type copy is insufficient to rescue the defect caused by the ssii mutation in diploid plants (Greenberg 2000). This implicates SSII as a negative regulator of SA-dependent SAR (Greenberg 2000). In conclusion, some of the current evidence for cross-talk between SA-dependent signalling and JA-dependent signalling is somewhat contradictory, but further work in this area may help elucidate the complexity of the acquired resistance signal transduction network.

Other mutants with altered disease resistance

It is very likely that other signal transduction pathways, in addition to salicylate, ethylene and jasmonate dependent responses, are important in establishing SAR. Other mutant screens have been deployed in order to uncover further signal transduction pathways.

Phytoalexin-deficient mutants

In order to determine the role of camalexin, the predominant phytoalexin produced in Arabidopsis, pad (phytoalexin deficient) mutants were isolated (Glazebrook et al. 1997b). Five complementation groups were identified. Mutations in PAD1, PAD2 and PAD4 caused enhanced susceptibility to Psm, while mutations in PAD3 and PAD4 did not (Glazebrook et al. 1997b). It was also shown that PAD1, PAD2, PAD3 and PAD4 are required for resistance to P.parasitica (Glazebrook et al. 1997b). In pad4 plants inoculated with virulent Psm, SA levels, synthesis of camalexin, and PR-1 transcript accumulation are all reduced (Zhou et al. 1998). Hence, PAD4 may play an important role in acquired resistance. No such defects were seen after inoculation with Psm (avrRpt2) (Zhou et al. 1998). Treatment of pad4 plants with SA partially reversed the camalexin deficiency and induced PR-1 expression, suggesting that PAD4 operates upstream of SA accumulation in response to infection by virulent bacteria (Fig.1.3). PAD4 may therefore participate in a positive regulatory loop that increases SA levels (Zhou et al. 1998). PAD4 has been cloned recently and the predicted protein sequence displays similarity to triacyl glycerol lipases (Jirage et al. 1999).
The pad3 mutant has also shown enhanced susceptibility to *A. brassicicola* (Thomma et al. 1999b), but not *B. cinerea* or *Erisphe orontii* (Thomma et al. 1999b, Reuber et al. 1998). Thus, PAD3 appears to be a key determinant in resistance to *A. brassicicola* (Thomma et al. 1999b). PAD3 has also recently been isolated by map-based cloning and the predicted protein appears to be a cytochrome P450 monooxygenase, similar to an enzyme from maize that catalyses synthesis of an indole-derived metabolite (Zhou et al. 1999). PAD3 expression is tightly correlated with camalexin synthesis, indicating that PAD3 may encode an enzyme required for camalexin synthesis (Zhou et al. 1999). The *PR-1* and *PDF1.2* genes were not reduced in *A. brassicicola* infected pad3 plants, indicating that camalexin production is controlled by a pathway that does not display cross-talk with SA-dependent and JA-dependent SAR (Thomma et al. 1999b).

**Enhanced disease susceptibility mutants**

In order to identify plant defence responses that limit pathogen attack, more enhanced disease susceptibility (eds) mutants that exhibit enhanced disease susceptibility to virulent *Psm* were identified (Glazebrook et al. 1996). At least 8 previously unidentified genes have been uncovered (Volko et al. 1998, Glazebrook et al. 1997a). Apart from *eds4* and *eds5*, none of the *eds* mutants showed a significant alteration in the HR or SAR responses (Rogers & Ausabel 1997, Glazebrook et al. 1996), indicating that they define a new set of defence-related functions aimed at limiting the growth of virulent pathogens. *PR-1* expression and SA accumulation was reduced in *eds5* plants following *Psm* infection but camalexin production was unaffected (Rogers & Ausabel 1997). Thus, *EDS5* probably operates upstream of SA accumulation in a way that does not interfere with camalexin accumulation (Fig. 1.3). Recent characterisation of the *eds4* mutant indicates that *EDS4* plays a role in SA-dependent SAR, as *PR-1* expression and SA accumulation following *Psm* inoculation was reduced and SAR was impaired (Gupta et al. 2000). Thus it has been proposed that *EDS4* acts in elevating SA following *Psm* inoculation, possibly by participation in an SA amplification loop (Fig. 1.3) (Gupta et al. 2000). The *eds4* and *pad4* mutants also caused heightened expression of *PDF1.2* in response to rose
bengal and Me-JA, supporting the idea that SA accumulation interferes with JA-dependent signalling (Gupta et al. 2000).

Two sid (salicylic acid induction deficient) mutants were isolated in a screen designed to quantify SA accumulation in each individual mutant (Nawrath et al. 1999). Both sid mutants did not accumulate SA in response to *Pst* (avrRpt2), were susceptible to both virulent and avirulent *Pst* and *P.parasitica* and showed reduced expression of *PR-1* but not *PR-2* and *PR-5* in response to *Pst* (avrRpt2) (Nawrath et al. 1999). In addition, both mutants were found to have a blockage in SA biosynthesis (Nawrath et al. 1999), indicating that they operated upstream of SA in the SAR signal transduction network (Fig. 1.3). The *sid1* mutant was shown to be allelic to *eds5*, but *sid2* defines a previously unidentified gene involved in SA biosynthesis and SAR signal transduction (Nawrath et al. 1999) (Fig. 1.3).

**Enhanced disease resistance mutants.**

The *edr1* (enhanced disease resistance) mutant displays enhanced resistance to *Pst* and *Erysiphe cichoracearum*, but does not constitutively express the *PR-1, PR-2* and *PR-5* SAR marker genes (Frye & Innes 1998). Thus *edr1* describes a novel mutant class. *E. cichoracearum* conidia germinate and formed extensive hyphae on *edr1* plants, but subsequent conidiophore production and sporulation is drastically reduced in comparison to wild-type plants, indicating that *EDR1* can be considered to be a ‘late-acting’ gene in resistance to powdery mildew (Frye & Innes 1998). More recently, four additional *E. cichoracearum* resistant mutants were isolated (Vogel & Sommerville 2000). The *pmr* (powdery mildew resistant) mutants did not display constitutive expression of *PR-1* or *PDF1.2* and, unlike *edr1*, were not more resistant to *Pst* (Vogel & Sommerville 2000). This indicates that the *pmr* mutants define a different disease resistance class in comparison to *edr1*. 
1.6. Aims of the project

The overall aim of the project is to further investigate the mechanism of the signal transduction network leading to the establishment of acquired resistance in Arabidopsis. The pathway beginning with the interaction between the *avrB* and *RPM1* gene products, and culminating in the local expression of *PR-1* was relevant to this project. Transgenic Arabidopsis plants expressing the luciferase reporter gene under the control of the *PR-1a* promoter (designated *PR-1a:luc*) were generated as a tool to study *PR-1a* gene expression. Real-time *PR-1a:luc* expression following inoculation with an avirulent bacterial pathogen could be monitored by detection of luc activity in an ultra low-light imaging camera. A genetic approach was taken in order to further study this pathway and mutants were generated by chemical mutagenesis. Potential mutants were identified by visualising abnormal luc activity. A group of potential mutants were chosen for characterisation and one, *cir1* (constitutively induced resistance 1) was confirmed as a true disease resistance mutant.

The following chapter (Chapter 2) details the materials and methods used in this study.

Chapter 3 outlines the production of the *PR-1a:luc* transgenic lines and describes the pattern of luc activity produced following inoculation with the avirulent bacterial pathogen, *Pst* DC3000 (*avrB*). Mutants were generated in homozygous *PR-1a:luc* plants, and M2 mutant plants were screened for abnormal luc activity following inoculation with *Pst* DC3000 (*avrB*). Several classes of potential mutants were uncovered.

Chapter 4 discusses the characterisation of two groups of these potential mutants, *neb* (no expression of bioluminescence) with a block upstream of SA in the acquired resistance signal transduction pathway, and *ceb* mutants, which showed constitutive expression of *PR-1a:luc*. Luminometer assays were conducted in order to quantify *PR-1a:luc* expression and Northern blot analysis was used to study expression of
various defence-related genes. Disease resistance assays to virulent pathogens (PstDC3000, *P. parasitica* Noco2 and *F. oxysporum* f.sp. *matthioli*) were performed. One of the *ceb* mutants, re-named *cir1*, was selected as *bona fide* SAR mutant.

Chapter 5 shows further characterisation of *cir1*. Genetic analysis was conducted by crossing *cir1* with various other Arabidopsis lines, in order to determine the segregation and allelism of *CIR1*. The relationship of *cir1* to SA, JA and ethylene was also investigated by crossing *cir1* to SA-, JA- and ethylene-insensitive mutants.

The position of the *CIR1* gene in the Arabidopsis genome was determined by mapping with SSLP and CAPS markers.

Finally, Chapter 6 discusses the results with reference to current ideas about the SAR signalling network in Arabidopsis, and the potential for its application in the development of better crops.
Chapter Two
Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, UK).

2.1. Growth of Arabidopsis thaliana

Arabidopsis thaliana (Arabidopsis) seeds of ecotype Columbia (Col-0) and Landsberg erecta (Ler) were used. All Arabidopsis transgenic lines and mutant strains used were in a Col-0 background and are outlined in Table 2.1. Most seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were placed on potting medium consisting of peat moss, vermiculite and sand (4:1:1), and allowed to vernalise for 48 hours at 4°C after which they were transferred to 20°C. Plants were placed 4 to a pot, were watered by sub-irrigation and were fertilised once a week with Phosphogen®. In order to promote the growth of healthy leaves, plants were placed under short day length conditions (10 hours light, 14 hours dark) at 20°C, otherwise plants were placed in the transgenic greenhouse under longer day length conditions (16 hours light, 8 hours dark).

2.2. Growth of Pseudomonas syringae pv tomato DC3000 (avrB) and inoculation of plants

Pseudomonas syringae pv tomato DC3000 (avrB) (PstDC3000 (avrB)) was grown on Kings broth (King et al. 1954) supplemented with 50 mg.l⁻¹ rifampicin and 50 mg.l⁻¹ kanamycin. Liquid cultures were grown on a shaker at 30°C, and cells were harvested at OD₆₀₀ equal to 0.2 (the equivalent of 10⁶ colony forming units per cm⁻² (cfu.cm⁻²). Cells were pelleted by centrifugation and re-suspended for plant inoculation in 10 mM MgCl₂. For inoculations, 6 μl of the PstDC3000 (avrB) solution were forced under the abaxial epidermis using a 1 ml syringe. Successful inoculations were visualised by the appearance of a watery area under the epidermis.
Table 2.1. Arabidopsis transgenic lines and mutant strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Phenotype</th>
<th>Reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>wild-type</td>
<td>Thomson &amp; Loake</td>
<td>Thomson &amp; Loake</td>
</tr>
<tr>
<td>Ler</td>
<td>wild-type</td>
<td>Lawton et al. 1995</td>
<td>Novartis, USA</td>
</tr>
<tr>
<td>PR-1a:luc</td>
<td>PR-1a:luc transgenic</td>
<td>Cao et al. 1995</td>
<td>Dong, Duke University</td>
</tr>
<tr>
<td>nahG</td>
<td>Salicylate hydroxylase</td>
<td>Guzman &amp; Ecker 1990</td>
<td>NASC</td>
</tr>
<tr>
<td>npr1-1</td>
<td>SAR insensitive</td>
<td>Bleeker et al. 1988</td>
<td>NASC</td>
</tr>
<tr>
<td>ein2-1</td>
<td>Ethylene insensitive</td>
<td>Staswick et al. 1992</td>
<td>Staswick, University of Nebraska</td>
</tr>
<tr>
<td>etr1-1</td>
<td>Ethylene insensitive</td>
<td>Feys et al. 1994</td>
<td>Turner, University of East Anglia</td>
</tr>
<tr>
<td>jar1-1</td>
<td>Jasmonate insensitive</td>
<td>Bowling et al. 1994</td>
<td>Dong, Duke University</td>
</tr>
<tr>
<td>coil-1</td>
<td>Jasmonate insensitive</td>
<td>Bowling et al. 1997</td>
<td>Dong, Duke University</td>
</tr>
<tr>
<td>cpr1-1</td>
<td>Constitutive SAR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpr5-1</td>
<td>Constitutive SAR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3. Treatment of plants with salicylic acid or methyl jasmonate

A 10 mM stock solution of salicylic acid (SA) was made up in 10 mM sodium phosphate buffer, pH 7.2 (Sambrook et al. 1989). A 1:10 dilution, containing 0.001% (v/v) Silwet (Ambersil Ltd, UK) and 0.001% (v/v) triton-X, was used to paint the leaves. These leaves were imaged 24 hours later for PR-1a:luc expression using the ultra-low light imaging camera or harvested for RNA extraction.

A 20 mM methyl jasmonate (Me-JA, Aldrich) stock solution was prepared in 0.1% (v/v) ethanol. A 1:200 dilution, containing 0.001% (v/v) Silwet (Union Carbide) and 0.001% (v/v) triton-X, was used to paint the leaves which were harvested after 24 hours for RNA extraction.
2.4. In vitro selection of Arabidopsis seedlings

Arabidopsis seedlings were tested *in vitro* by addition of various compounds to the tissue culture medium. PR-1a:luc transgenic seeds were tested for kanamycin resistance, nahG transgenic seeds were tested for the appearance of brown roots on MS medium supplemented with 0.5mM SA (Bowling et al. 1994), jar1 seedlings were tested for insensitivity to 10μM Me-JA (Staswick et al. 1992) and ein2.1 and etr1 mutants were selected for insensitivity to 10μM 1-aminocyclopropane-1-carboxylic acid (ACC) in the dark (Oh et al. 1997). In all cases, approximately 1000 transgenic Arabidopsis seed (20 mg) were sterilised for 20 minutes in 10 % (v/v) bleach containing 10 μl of Triton-X. Seeds were washed four times with sterile distilled water. Seeds were placed at 4°C for 2 days so vernalisation could take place. Seeds were re-suspended in 0.1% (w/v) agarose (1 ml used per 1000 seed) and plated out on MS selection plates (1 X MS salts (M5519), 0.3% (w/v) sucrose, 8g.l⁻¹ agar, 200 mg.l⁻¹ cefotaxime and 20 mg.l⁻¹ Rovral, Rhone-Poulenc Ltd). For transgenic Col-0, 50 mg.l⁻¹ kanamycin was used. After drying, plates were placed in the growth room (20°C, continuous light) for 7-10 days. Seedlings were scored for the appropriate phenotype under test and transferred to soil.

2.5. Viewing PR-1a:luc expression using the ultra-low light imaging camera

Luciferin (Biosynth AG) is the substrate for the firefly luciferase gene. Leaves, still attached to the plant, were painted *in situ* with 1 mM luciferin in a 100 mM Na-citrate buffer containing 0.001% (v/v) Silwet and 0.001% triton-X (v/v) (Ow et al. 1996). Following this, plants were placed in the dark for 30 minutes in order to allow the luciferin to dry and to minimise background bioluminescence.

In order to make imaging more easy during the mutant screen, leaves from four plants were excised and imaged simultaneously by placing the leaves on the same plate. In other cases the entire plant was imaged. Imaging was performed using a Berthold Luminograph (EG&G Wallac, Milton Keynes, UK). The Luminograph consists of an ultra-low light imaging camera attached to a dark box and a computer.
Leaves or plants were placed in the dark box for imaging. Bioluminescence images emitted from the leaves were collected for a 10 second accumulation period and integrated over a 5 second period using the Luminograph software. The image intensifier was set to 100% and gamma, flat-field and defect corrections were performed on the accumulated images in all cases. Images, saved as TIF files, were exported from the Luminograph software and processed using the Confocal Assistant (Biorad) and Adobe Photoshop software.

2.6. Luminometer assay

Luciferase activity was measured in the Berthold MicroLumat LB96P microwell plate luminometer (EG&G Wallac, Milton Keynes, UK). Crude protein extractions were made by grinding a leaf in one ml of lysis buffer (0.1M sodium phosphate pH 7.2, 5 mM DTT) (Doelling & Pikaard et al. 1993). The liquid was removed and centrifuged (12000 rpm, 5 minutes). The supernatant was used directly to measure total protein content, and was diluted 2-fold with lysis buffer to assay for luciferase activity. Total protein content was measured using the Bradford micro-assay (Sigma) using bovine serum albumin (BSA) as the protein standard. Luciferase assays were performed by mixing 100 μl of assay buffer (60 mM Tris-HCl pH 8.0, 20 mM MgCl2, 20 mM DTT, 2 mM EDTA, 2 mM ATP) (Doelling & Pikaard et al. 1993) with 100 μl of the protein extract. In another tube, 2 X assay buffer (without ATP) was mixed with an equal volume of 2 mM luciferin. Background luminescence for each sample was determined for 20 seconds prior to luciferin injection. Luciferase activity was measured in the 20 seconds following injection of the luciferin sample (100μl) into the protein sample. After background readings were subtracted, the luciferase activity for each sample was calculated as RLU per microgram of extractable protein.

2.7. RNA Gel Blot analysis

Total RNA was extracted from Arabidopsis leaves harvested from 5-week old plants using the guanadinium thiocyanate (GTC) phenol chloroform extraction method.
Leaf tissue (approximately 0.1g) was ground in liquid nitrogen using a pestle and mortar, poured into a 1.5 ml eppendorf and 0.45 ml GTC solution (4M guanadinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 0.1 M β mercaptoethanol) was added. Following mixing by vortexing for 30s, 0.05 ml 2 M sodium acetate pH4.0, 0.45 ml phenol and 0.1 ml chloroform:iso-amylalcohol (49:1) were added. After further mixing, the tubes were placed on ice for 15 min. The samples were centrifuged at 10 000 rpm for 20 min, the supernatant was removed carefully from each tube and transferred to a new tube. An equal volume of isopropanol was added to each tube, mixed and left at -20°C for at least 2 hours or overnight in order to precipitate the RNA. After incubation, the RNA was recovered by centrifugation (10000 rpm, 20 min). The pellet was re-dissolved in 0.15 ml GTC solution, and re-precipitated by the addition of 0.15ml isopropanol and storage at -20°C for 1 hour. Following centrifugation (10 000 rpm, 20 min), the RNA pellet was washed twice in 70% ethanol, dried and dissolved in 100 μl DEPC-treated water. The absorbance of each sample was measured at 260 nm, and used to calculate the concentration of RNA. Samples (10 μg) were separated on formaldehyde-agarose gels (Sambrook et al. 1989), transferred to a Hybond™-N hybridization membrane according to the instructions of the supplier (Amersham Lifesciences) and hybridized with the relevant probes (described below). Dextran sulphate (10% w/v) was included in the pre-hybridization / hybridization solution in order to allow for efficient binding of the probe (Sambrook et al. 1989). Blots were washed twice for 30 min each at 65°C in 4 X SSC, 1% (w/v) SDS, which was followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate time period. Blots were stripped by incubation in boiling 0.1% (w/v) SDS and washing in 0.5 X SSC for 30 min at room temperature, before hybridization with a subsequent probe (Sambrook et al. 1989).

Probes were prepared by amplification of appropriate sequences using PCR and directly purified using a kit (Promega). The expected PCR fragment size was verified by gel electrophoresis. Alternatively, probes were generated from plasmids by digestion with relevant restriction enzymes, identified by gel electrophoresis, and purified from the gel by freeze-thaw extraction. Sequences for the PCR primers,
templates used and the reference for each probe are shown in Table 2.2. Probes were labelled with $\alpha$-32P-dCTP by random priming using the Prime-a-Gene® labelling system (Promega). In order to identify lane-to-lane variations in the amount of RNA added and thus facilitate comparisons between lanes, each blot was probed with the ribosomal 18S (r18) probe.

Table 2.2. Reagents used in the generation of probes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Template</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>RE</th>
<th>Probe (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-1</td>
<td>TA-PR1 a</td>
<td>CTCGACCTCACACCTCTGTA a</td>
<td>TATgTACgTgTgTATgCATgATCa</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>PR-2</td>
<td>pBluescript-P2 a</td>
<td>T7</td>
<td>T3</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td>PR-5</td>
<td>pBluescript-PR5 a</td>
<td>T7</td>
<td>T3</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>GST1</td>
<td>GST1 b</td>
<td>GCCTGTAGACACATCACAC</td>
<td>CAAgACTGATCATGATTAC</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>PDF1.2</td>
<td>genomic DNA</td>
<td>TCAgACCTCAgATTTgGTCC</td>
<td>ATTACACgATTTgGACC</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>Thi2.1</td>
<td>pUC19-Thi2.1 d</td>
<td>-</td>
<td>EcoRI</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>ACS2</td>
<td>genomic DNA</td>
<td>CGAgAAgAAGACATTCACTTA</td>
<td>CgATCCTCAgTAGATgTCT</td>
<td>-</td>
<td>0.45</td>
</tr>
<tr>
<td>LOX2</td>
<td>pZL1-LOX2 f</td>
<td>-</td>
<td>Sall-NotI</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>r18</td>
<td>pBluescript-18S f</td>
<td>T7</td>
<td>T3</td>
<td>-</td>
<td>1.5</td>
</tr>
</tbody>
</table>

a Uknes et al. 1992. PR-1 was initially isolated by PCR from an Arabidopsis cDNA library with the forward and reverse primers, and cloned into the TA vector (Thomson & Loake, unpublished).

b Sharma et al. 1996, Yang et al. 1998

c Penninckx et al. 1996

d Epple et al. 1995

e Liang et al. 1992 (GenBank accession no.: M95594)
f Obtained from AIMS stock centre, stock no. 106C8T7 (GenBank accession no.: T22547)
g Pruitt & Meyerowitz 1986

2.8. Virulent disease resistance assays

2.8.1. Resistance to Pseudomonas syringae pv tomato DC3000

P.syringae pv tomato DC3000 (PstDC3000) (Whalen et al. 1991) was grown in King’s broth liquid media (King et al. 1954) supplemented with 50 mg.l$^{-1}$ rifampicin. Four week old soil-grown plants were infected with a Pst DC3000 suspension (OD$_{600}$ = 0.0002) in 10 mM MgCl$_2$ by completely infiltrating the abaxial side of the leaf with a 1 ml syringe (Cao et al. 1994). 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 uniform size (0.5 cm\(^2\)) were made from the leaf samples using a cork borer. Three leaf discs from each plant were ground in 990 \(\mu\)l 10 mM MgCl\(_2\) in a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension, and 100 \(\mu\)l of each dilution was used to inoculate King's B medium plates containing 50 mg.l\(^{-1}\) rifampicin. The plates were incubated at 30°C for 2 days, and the number of bacterial colonies for each sample was recorded. Three days prior to inoculation with \(Pst\)DC3000, some Col-0 plants were induced to activate SAR by spraying with 1 mM SA. Bacterial counts were statistically analysed using the Mann-Whitney test (Mini-tab version 12).

2.8.2. Resistance to \(P.parasitica\) Noco2

The \textit{Peronospora parasitica} Noco2 oomycete pathogen (Parker et al. 1993) was maintained in Col-0 seedlings grown under humid conditions and conidiospores were transferred to new seedlings weekly by dusting infected seedlings onto healthy seedlings. For the \textit{P.parasitica} disease resistance assays, conidiospores were harvested by vortexing infected seedlings in water. The spore concentration was determined using a haemocytometer, and adjusted to 1X10\(^5\) spores per ml. Four-week old plants grown under short day conditions (10 hours light, 14 hours dark) were sprayed with the conidiospore solution and maintained under humid conditions. Results were taken after 10 days, and infected plants were scored for the extent of downy mildew growth (visualised as conidiophore growth) based on Bowling 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 conidiophores, 3=25 to 50% of three or four leaves covered with conidiophore growth, 4=25 to 50% of all leaves covered with conidiophore growth, 5=all leaves covered with conidiophore growth. Plants in different replicates were assigned a disease index as follows: D.I.=\(\Sigma\) i X j/n, where i=infection class, j=the number of plants scored for that infection class and n=the total number of plants in the replicate (based on Epple et al. 1997a).
2.8.3. Resistance to *Fusarium oxysporum* f.sp. *matthiolae*

*Fusarium oxysporum* f.sp. *matthiolae* (Mauch-Mani & Slusarenko 1994) was maintained on potato dextrose agar plates at room temperature. Cultures were sub-cultured onto fresh medium every two to three weeks. For inoculation of plants, spores were harvested by gently rubbing the mycelial growth on each plate and re-suspension in 10 ml sterile water. Spores were separated from mycelia by centrifugation (4000 rpm, 15 minutes) and the resulting pellet, containing the spores, was re-suspended in 5 ml sterile water. Spore concentration was determined using a haemocytometer, and adjusted to 1X10⁵ spores per ml (Epple et al. 1997a). Four-week old plants grown under short day conditions (10 hours light, 14 hours dark) were sprayed with the spore solution and maintained under humid conditions. Three replicates, containing four plants each, were inoculated. Results were taken after 8 days and infected plants were scored for the number of necrotic leaves. Symptom development was expressed as the percentage of necrotic leaves per plant. Scores were statistically analysed using the Mann-Whitney test (Mini-tab version 12).

2.9. Determination of SA levels

SA measurements were performed with leaves harvested from 5-week old plants according to Bowling and co-workers (1994). Frozen leaf tissue samples (1g) were extracted with methanol, dried down and re-suspended in 0.01M H₂SO₄, and free SA levels were analysed using an HPLC method.

2.10. Determination of ethylene evolution

Ethylene measurements were performed using 5-week old plants according to Iannetta and co-workers (1999). Six plants per line were enclosed individually in glass jars and evolved ethylene was collected over a 24 hour period. One ml of head space was removed for ethylene determination by gas chromatography. After incubation, the fresh weight of each plant was determined.
2.11. Trypan blue staining

Leaf samples were boiled for 2 minutes in a lactic acid-phenol-trypan blue solution (2.5mg.ml\(^{-1}\) trypan blue, 25% (w/v) lactic acid, 23% water saturated phenol, 25% glycerol and water) (Bowling et al. 1997). After cooling for one hour, the lactic-acid-phenol-trypan blue solution was replaced with a chloral hydrate solution (25g in 10 ml water) for de-staining. After 24 hours, the chloral hydrate solution was removed and the samples were equilibrated in 70% (v/v) glycerol and mounted onto microscope slides. Stained leaves were viewed for micro-lesions, or small areas of dead tissue, using the Leica Wild M3C microscope.

2.12. Genetic analysis

Crosses were performed by dissecting and emasculating unopened buds two days prior to anthesis, and using the pistils as recipients for pollen from open donor flowers (Koorneef & Stam 1992). In order to determine the dominant/recessive nature of the mutation in the ceb candidate mutant lines, pollen from each ceb plant was used to pollinate separate wild-type PR-1a:luc transgenic plants. The number of segregation groups amongst the ceb candidate mutants was determined by crossing the ceb plants against each other (Koorneef & Stam 1992). F1 and F2 five-week old plants were tested for constitutive PR-1a:luc expression using the ultra low-light imaging camera.

Pollen from homozygous cir1 plants was used to pollinate nahG, npr1, jar1, ein2.1 and etr1 plants. As jar1, ein2.1 and etr1 do not contain the kanamycin resistance gene, F1 progeny were tested for kanamycin resistance in order to indicate that the cross had been successful. F1 seeds were germinated on MS medium supplemented with kanamycin as described above. F2 five-week old plants from all crosses were tested for constitutive PR-1a:luc expression using the ultra low-light imaging camera. All F2 plants producing constitutive luc activity were allowed to set seed and were tested further. F3 cir1:nahG seeds were tested for brown roots in germinating seedlings placed on MS medium supplemented with 0.5 mM SA as...
described above. F2 cir1:npr1 plants were tested for the presence of the npr1 mutation using a CAPS PCR marker. The npr1 mutation abolishes a NLaIII restriction site in the PCR fragment generated by this marker, thus providing a method for distinguishing npr1 from wild-type (Clarke et al. 1998, Cao et al. 1997). F2 cir1:jarl plants were identified by their small, dark green leaves typical of jar1 (Staswick et al. 1992) and constitutive PR-1a:luc expression. F3 cir1:jarl plants were tested for insensitivity to Me-JA as described above. F2 progeny from the ein2.1 X cir1 and etrl X cir1 crosses, expressing PR-1a:luc constitutively, were identified. These plants were allowed to set seed and the F3 progeny from these plants were tested for ACC insensitivity as described above. F3 cir1:ein2.1 and cir1:etrl were thus identified.

2.13. Mapping using SSLP and CAPS markers

In order to generate an F2 mapping population, cir1 was crossed to Ler. F2 plants expressing constitutive luc activity were selected for mapping. Mapping was performed using the co-dominant cleaved amplified polymorphic sequences (CAPS) protocol described by Konieczny & Ausabel (1993) and the single sequence-length polymorphisms (SSLPs) protocol described by Bell & Ecker (1994). Both protocols are based on PCR of polymorphic genomic DNA sequences between Col-0 and Ler. The PCR primers used for both types of markers and the restriction enzymes used for the CAPS protocol are outlined at The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). The markers used in the present project are listed in Table 2.3.

Genomic DNA was extracted from leaf tissue using a small-scale method (Edwards et al. 1991). Approximately 1 cm² of leaf tissue was ground in 0.5ml DNA extraction buffer with carborandum. Samples were incubated at 60°C for 10 min followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation (10 000rpm, 10 min), the DNA was precipitated from the resulting supernatant by the addition of 2X volume ethanol and 1/10 volume 3M sodium acetate, pH 5.2. After a further centrifugation step (10 000rpm, 10min), the
DNA pellet was washed in 70% (v/v) ethanol twice and finally re-suspended in 50 µl TE (Sambrook et al. 1989). RNase (10 µg) was added and samples were incubated for one hour at room temperature in order to eliminate contaminating RNA species.

Primers for the different markers are listed in Table 2.2 and all primers were manufactured by Amersham Pharmacia. PCR reactions were performed using 10X buffer, magnesium chloride and Taq polymerase (Promega) and deoxynucleotides from Boehringer (Roche Molecular Biochemicals). The PCR reactions were carried out in 10 µl volume containing 1µl of the DNA miniprep (approximately 10 ng DNA), 5 pmol of each primer, 200 µM each of four deoxynucleotides, 2mM magnesium chloride and 1 unit of Taq polymerase. PCR reactions were carried out in either a Hybaid Omn-E thermal cycler or a Peltier 200 thermal cycler. For the CAPS markers, conditions were as follows: 30s at 94°C, primer annealing for 30s at 56°C, primer elongation for 3 min at 72°C. This cycle was repeated 50 times. For the SSLPs markers, conditions were as follows: 30s at 94°C, primer annealing for 30s at 56°C, primer elongation for 1 min. This cycle was repeated 30 times. Products from the CAPS PCR reactions were digested with the relevant restriction enzymes listed in Table 2.2. All digests were performed at 37°C with restriction enzymes purchased from Promega except for BsaB1 which was purchased from New England Biolabs. Digests with BsaB1 were performed at 60°C. A restriction enzyme mix (10 µl) containing 2 µl of restriction enzyme buffer and 1.5 units of restriction enzyme were added to each 10 µl CAPS PCR tube and samples were incubated for 2 hours at the optimal temperature for the particular enzyme. For both the SSLP PCR products and the CAPS digestion products, 5 µl of loading dye (Sambrook et al. 1989) was added to each tube prior to electrophoresis. Agarose gel concentrations optimised for each marker are listed in Table 2.2. Col-0 and Ler samples were included on each gel as controls and run in adjacent bands in order to visualise polymorphisms. The 1kb DNA ladder (Gibco-BRL) was also included on each gel in order to determine the size of different PCR or restriction digestion products.
Table 2.3. List of CAPS and SSLP markers used in mapping experiments.

Restriction enzymes required for analysis of CAPS markers, gel conditions for analysis of PCR products, observed PCR products polymorphic between Col-0 and Ler and primer sequences for the markers are shown.

<table>
<thead>
<tr>
<th>Chr</th>
<th>cM *</th>
<th>Name</th>
<th>Type</th>
<th>Enzyme</th>
<th>Gel</th>
<th>Observed band size (kb)</th>
<th>Ler</th>
<th>Forward primer seq</th>
<th>Reverse primer seq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.3</td>
<td>nga63</td>
<td>SSLP</td>
<td>MC</td>
<td>2% 2h,82V</td>
<td>0.111</td>
<td>0.089</td>
<td>ACCCAAgTgATCgCCACC</td>
<td>AACCAGgcCACAgAggCg</td>
</tr>
<tr>
<td>1</td>
<td>46</td>
<td>AthS0392</td>
<td>SSLP</td>
<td>MP*</td>
<td>3% MP 4.5h,45V</td>
<td>0.142</td>
<td>0.156</td>
<td>TTTgATCgCAgCgATCTg</td>
<td>gTTCgATCgCACgCgTAAGC</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>nga128</td>
<td>SSLP</td>
<td>RSA1</td>
<td>2% 2h,82V</td>
<td>0.18</td>
<td>0.18</td>
<td>gTCgATCgCACgCgACgC</td>
<td>ATCTgAAgACCCCTTTgAggAg</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>THY1</td>
<td>CAPS</td>
<td>RSA1</td>
<td>2% 2h,82V</td>
<td>0.81</td>
<td>0.6/0.1</td>
<td>ggCgACCTgAgACCTgTAACg</td>
<td>AACCgCCACTTTCTTCTTACg</td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>nga168</td>
<td>SSLP</td>
<td>RSA1</td>
<td>2% 2h,82V</td>
<td>0.151</td>
<td>0.158</td>
<td>TACgCACgACgCgACgC</td>
<td>TgTCgATCgCACgCgCCG</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>ArLIM15</td>
<td>CAPS</td>
<td>RSA1</td>
<td>1% 2h,82V</td>
<td>0.5</td>
<td>0.5</td>
<td>gCCACgTTTTCTCgCACATCAAATC</td>
<td>TgTCgATCgCACgCgTT</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>nga707</td>
<td>SSLP</td>
<td>RSA1</td>
<td>3% MP 4.5h,45V</td>
<td>0.132</td>
<td>0.128</td>
<td>TGAATgCgAACgCCATTTgACgAgAg</td>
<td>TgTCgATCgCACgCgCg</td>
</tr>
<tr>
<td>4</td>
<td>17.7</td>
<td>GA1</td>
<td>CAPS</td>
<td>RSA1</td>
<td>1% 2h,82V</td>
<td>1.2</td>
<td>1.2</td>
<td>AACgCCTgAgACgCgACgCgAC</td>
<td>TgTCgATCgCACgCgTT</td>
</tr>
<tr>
<td>4</td>
<td>26.3</td>
<td>nga8</td>
<td>SSLP</td>
<td>RSA1</td>
<td>1% 2h,82V</td>
<td>0.154</td>
<td>0.198</td>
<td>gAggCACgACgCgACgCgACgCgAC</td>
<td>TgTCgATCgCACgCgTT</td>
</tr>
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<td>nga1111</td>
<td>SSLP</td>
<td>RSA1</td>
<td>4% MP 6.5h,45V</td>
<td>0.148</td>
<td>0.154</td>
<td>ATGATAgACgCgACgCgACgCgAC</td>
<td>TgTCgATCgCACgCgTT</td>
</tr>
<tr>
<td>4</td>
<td>57.6</td>
<td>g4539</td>
<td>CAPS</td>
<td>HindIII</td>
<td>1% 2h,82V</td>
<td>0.6</td>
<td>0.6</td>
<td>gAggAACgACgCgACgCgACgCgAC</td>
<td>TgTCgATCgCACgCgTT</td>
</tr>
<tr>
<td>4</td>
<td>63.1</td>
<td>AG</td>
<td>CAPS</td>
<td>RSA1</td>
<td>2% 2h,82V</td>
<td>1.366</td>
<td>1.366</td>
<td>CAAACgCCTTTACTGACgACgACgAC</td>
<td>CAAACgCCTTTACTGACgACgAC</td>
</tr>
<tr>
<td>4</td>
<td>75.7</td>
<td>RPS2</td>
<td>CAPS</td>
<td>BSaB1</td>
<td>2% 2h,82V</td>
<td>0.878</td>
<td>0.18/0.005</td>
<td>CTCgAgATCgCACgCgACgCgAC</td>
<td>TgTCgATCgCACgCgTT</td>
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<tr>
<td>4</td>
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<td>SSLP</td>
<td>RSA1</td>
<td>2% 2h,82V</td>
<td>0.15</td>
<td>0.14</td>
<td>gCgACgACgCgACgACgACgACgAC</td>
<td>CgACgACgACgACgACgACgACgAC</td>
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<td>26</td>
<td>nga161</td>
<td>SSLP</td>
<td>RSA1</td>
<td>2% 2h,82V</td>
<td>0.15</td>
<td>0.12</td>
<td>gTTTgATCgACgCgACgCgACgAC</td>
<td>CgACgACgACgACgACgACgAC</td>
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<td>62</td>
<td>AthS0262</td>
<td>SSLP</td>
<td>RSA1</td>
<td>3% MP 4.5h,45V</td>
<td>0.145</td>
<td>0.159</td>
<td>CTGCCACCAAATgAgACgACgACgACgACgAC</td>
<td>TgATgTgATgATgATgATg</td>
</tr>
<tr>
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<td>AthS0191</td>
<td>SSLP</td>
<td>RSA1</td>
<td>3% MP 4.5h,45V</td>
<td>0.148</td>
<td>0.156</td>
<td>TgATgTgATgATgATgATgATgATgATg</td>
<td>CTGCCACCAAATgACgACgACgAC</td>
</tr>
</tbody>
</table>

* cM, centimorgans. Position of the marker on the genetic map (Lister and Dean 1993).

* MP, MetaPhor agarose (FMC Bioproducts)
Chapter Three
Screening for Arabidopsis SAR mutants

3.1. Introduction

A genetic approach was undertaken in this project in order to study SAR. This entailed isolating Arabidopsis thaliana (Arabidopsis) mutants. In order to allow for easy isolation of candidate mutants, mutations were generated in a transgenic Arabidopsis line expressing the luciferase (luc) reporter gene under the control of the PR-1a reporter gene. Transgenic plants thus produced luciferase (luc) activity during the establishment of SAR and it was possible to identify candidate mutants with changes in PR-1a:luc expression in comparison to transgenic plants.

3.2. Production of PR-1a:luc transgenic Arabidopsis plants

Arabidopsis ecotype Columbia (Col-0) plants were transformed with a construct containing the -903bp PR-1a promoter from tobacco (Payne et al. 1988) fused to the firefly luciferase (luc) marker gene (Promega) and the ocs terminator from Agrobacterium tumefaciens (Fig.3.1) (Thomson & Loake, unpublished results). This construct also contained the kanamycin resistant gene, so transgenic seedlings could be selected for kanamycin resistance. Sixteen individual kanamycin resistant PR-1a:luc transgenic seedlings were identified (Thomson & Loake, unpublished results). All 16 lines were tested for luc activity following inoculation with Pseudomonas syringae pv. tomato DC3000 expressing the avrB avirulence gene (PstDC3000 (avrB)) (Innes et al. 1993). The avrB avirulence gene was isolated from Pseudomonas syringae pv. glycinea Race 4, a pathogen of soybean, and transferred to the virulent Arabidopsis bacterial pathogen PstDC3000 (Innes et al. 1993). Inoculation of Arabidopsis ecotype Col-0 plants with the PstDC3000 (avrB) strain resulted in visualisation of a HR 24 hours after inoculation (Innes et al. 1993). The luc gene encodes an enzyme that catalyzes the ATP-dependent oxidation of luciferin, producing light at 465 nm (Ow et al. 1986). Inoculated leaves were painted with luciferin and luc activity in real time was imaged by means of an ultra low-light
imaging camera. *PR-la:luc* transgenic plants expressed light around the HR lesion following inoculation with *Pst*DC3000 (*avrB*) (Thomson & Loake, unpublished results). A highly inducible line with low background luc activity was chosen for further study (Thomson & Loake, unpublished results). Plants from this line were allowed to self-fertilise and T2 seed was tested *in vitro* for kanamycin resistance. Over 1000 F2 seedlings were scored and it was found that the segregation ratio of kanamycin resistant seedlings to kanamycin susceptible seedlings was 3:1. This indicated that the *PR-la:luc* transformation cassette (Fig.3.1) had integrated into a single position in the Arabidopsis genome (Thomson & Loake, unpublished results). T2 plants were allowed to self-fertilise and T3 seeds from individual T2 plants were collected. T3 seedlings were scored for kanamycin resistance and T2 plants showing 100% kanamycin resistance in their T3 progeny were identified as being homozygous for the *PR-la:luc* transformation cassette. Homozygous *PR-la:luc* seeds were used for all further studies.

**Fig.3.1.** The transformation cassette used to produce *PR-la:luc* transgenic plants.

LB-left border, LUC-luciferase gene, NPTII- neomycin phosphotransferase (encoding kanamycin resistance), NOS- nopaline synthase, OCS- octapine synthase, Pro- promoter, RB- right border, Ter- terminator.

### 3.3. Expression of *PR-la:luc* in transgenic plants.

In order to use the *PR-la:luc* transgenic plants to generate mutations and screen for candidate mutants expressing abnormal luc activity during SAR, it was first necessary to describe the pattern of luc activity produced by the wild type *PR-la:luc* plants. Two methods (outlined in Chapter 2) were used to investigate the induction
of luc activity in PR-1a:luc plants: imaging with the ultra low-light camera and a luminometer assay.

SAR is initiated by inoculation of a resistant plant with an avirulent pathogen and is associated with SA accumulation and SA-dependent expression of PR-1 (Delaney 1997, Ryals et al. 1996, Ryals et al. 1994). Thus, the extent of luc activity, as an indication of PR-1 expression, was investigated in PR-1a:luc transgenic plants in response to inoculation with avirulent bacterial pathogens or treatment with SA. The luminometer assay was used in order to quantify luc activity. PR-1a:luc plants were inoculated with PstDC3000 (avrB), PstDC3000 (avrRpt2), MgCl₂ (the control) or painted with 1mM SA. Infected leaves were harvested 3 days (72 hours) after inoculation and SA-treated leaves were harvested 24 hours after application. Extracts were made and the luc activity of the extract was determined. Luciferin was injected individually into each well of the luminograph plate and the light produced in the 20 seconds following injection was recorded. When luciferase comes into contact with luciferin, a flash of light is produced (Ow et al. 1986). It was decided to record the amount of light produced for 20 seconds as both the flash of light and the stable linear production of luciferase activity would be recorded. Luc activity was calculated as the relative light units (RLU) per microgram of extractable protein produced during the 20 seconds following luciferin injection. Results obtained are outlined in Fig.3.2.
Fig. 3.2. Luc activity in PR-1a:luc plants.
Plants were inoculated with 10mM MgCl₂, PstDC3000 (avrB), Pst DC3000 (avrRpt2) or 1mM SA. Naive plants were included as a control. Leaves were harvested 72 hours after the treatment, or after 24 hours in the case of SA application. The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.

PR-1a:luc was expressed in transgenic Arabidopsis plants in response to inoculation with two avirulent bacterial pathogens or application of SA (Fig. 3.2). The naïve control PR-1a:luc plants did not show an appreciable increase in luc activity (2 RLU/µg protein) and PR-1a:luc plants inoculated with MgCl₂ showed a very small increase in luc activity (10 RLU/µg protein) (Fig. 3.2). Both PstDC3000 (avrB) and PstDC3000 (avrRpt2) induced approximately a 60-fold increase in luc activity in PR-1a:luc plants in comparison to naive PR-1a:luc plants, whereas SA application induced a 350-fold increase in luc activity (Fig. 3.2). Thus, luc activity in PR-1a:luc plants is produced by conditions known to induce PR-1 during the establishment of SAR. Luc activity can thus be taken as an accurate report of PR-1 expression during the establishment of acquired resistance in local tissue.

The local spatial-temporal expression of PR-1 in leaves inoculated with PstDC3000 (avrB) was investigated in PR-1a:luc plants. Fig. 3.3 is a time course showing the induction of PR-1a:luc expression in the 72 hours following inoculation with
**PstDC3000 (avrB).** Luc activity began 12 hours after pathogen inoculation and appeared as a streak of light in the midrib of the leaf (Fig.3.3). By 24 hours post-inoculation, luc activity had spread to a ‘C-shape’ surrounding the HR, which is visible at this time point. The intensity of light produced in this area increased over time to 72 hours post-inoculation (Fig.3.3).

![Image of camera images showing the induction of luc activity in PR-1a:luc leaves over time, following inoculation with Pst DC3000 (avrB).](image)

Leaves were painted with 1mM luciferin and images were collected for 10s in the ultra low-light imaging camera. Inoculated leaves were imaged at the time points shown (in hours). Size bar represents 5 mm.

In order to quantify **PR-1a:luc** expression after inoculation with **PstDC3000 (avrB)**, a luminometer assay was performed. **PR-1a:luc** plants were inoculated with **PstDC3000 (avrB)** and infected leaves were harvested every 12 hours for four days after inoculation. Protein extractions were performed and luc activity determined. Luc activity, calculated as RLU per microgram of extractable protein produced during the 20 seconds following luciferin injection, was plotted against time (Fig.3.4). **PR-1a:luc** expression peaked at 36 hours after inoculation with **PstDC3000 (avrB)**, which corresponded to the ‘C-shape’ of luc activity produced around the visible HR at this time (Fig.3.3). Luc activity was approximately 100-fold more than naïve **PR-1a:luc** plants at this time point (Fig.3.4). At 72 hours (3
days), luc activity was still induced to approximately 50-fold more than in naïve $PR$-$1a: luc$ plants. By 96 hours post-inoculation, luc activity had decreased (Fig.3.4). A similar pattern of luc activity was visualised in Fig.3.3 and Fig.3.4, indicating that consumption of luciferase during the time course in Fig.3.3 did not limit luc activity. Northern blot analysis confirmed that luc activity produced in $PR$-$1a: luc$ plants corresponded to the expression of $PR-1$ in Col-0 following inoculation with $Pst$ DC3000 ($avrB$) (Chini, unpublished results not shown).

![Graph showing luc activity over time](image)

**Fig.3.4.** The extent of luc activity produced by $PR$-$1a: luc$ plants over time, following inoculation with $Pst$DC3000 ($avrB$).

The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.

Attempts where made to visualise the expression of $PR$-$1a: luc$ in systemic leaves following inoculation with $Pst$DC3000 ($avrB$). Although it did appear that luc activity decreased in locally infected leaves after three days and light was observed in adjacent leaves, no clear pattern of $PR-1a: luc$ expression was established (results not shown).
3.4. Mutagenesis of PR-1a:luc transgenic plants and isolation of candidate SAR mutants.

Production of mutants
Homozygous T2 seed (approximately 10 000) were subjected to ethylmethane sulfanate (EMS) mutagenesis (Redei & Koncz 1992) (Thomson & Loake, unpublished results). EMS-mutagenised seed were grown in 12 trays (8 pots per tray). Plants were allowed to self-fertilise (thus allowing all plants carrying recessive mutant genes to become homozygous) and the M2 seed was collected. Seed from each pot was pooled.

Primary screen
M2 seed was used for the mutant screen. In order to allow for ease of screening, the seed was divided into three groups:
- Group A: Seed from tray 1,2,3 (plants screened November-December 1997)
- Group B: Seed from tray 4,5,6 (plants screened January 1998)
- Group C: Seed from tray 7,8,9,10,11,12 (plants screened February-March 1998).
Approximately 60 seeds from each pot were planted out. This amounted to 480 seeds per tray and a total of 5760 seeds.

The primary screen consisted of inoculating 4-5 week old plants, which had not yet started to bolt, with PstDC3000 (avrB). After three days, plants were imaged using the ultra low-light imaging camera for abnormal luc activity. Two categories of candidate mutants were isolated: plants that did not produce luc activity and plants that produced increased luc activity in comparison to PR-1a:luc plants (Fig.3.3). For ease of reference, the first group of candidate mutants was called neb (no expression of bioluminescence) and the second group was called heb (high expression of bioluminescence).
The ‘mutant status’ of the candidate mutant plants was verified by a second inoculation one to five days later. Candidate mutants were allowed to self-fertilise and the M3 seed was collected for further screening and confirmation of the mutant status in the next generation.

Secondary screen

The secondary screen was conducted on M3 plants from putative mutant lines. A total of 104 heb and 256 neb candidate mutants were re-tested in the secondary screen. The four steps comprising the secondary screen were:

1) A leaf from 3 different plants from each line was inoculated with PstDC3000 (avrB) and imaged after three days. If all three leaves correlated to the result obtained in the primary screen, the line was tested further. If not, it was discarded.

2) The neb plants were tested for SA-induction of PR-1α:luc expression. SA (1 μM) was applied to the neb lines in order to determine their position relative to SA in the SAR pathway. Treatment of Arabidopsis with SA has previously been shown to induce PR-1 expression after 24 hours (Uknes et al. 1992). A leaf from three different plants from each line was tested and imaged after 24 hours. Luc activity was determined and compared to SA-induction of luc activity in PR-1α:luc plants. Plants with luc activity corresponding to the wild type indicated that the probable mutation may be upstream of SA in these plants, whereas plants that did not show luc activity after application of SA indicated that the block was likely downstream of SA.

3) One possibility that the neb plants did not show induction of luc activity following inoculation with PstDC3000 (avrB) was that the PR-1α:luc transformation cassette (Fig.3.1) had been eliminated from the plants. In order to check that the neb plants still contained the transformation cassette, seeds from these plants were plated out on MS medium containing kanamycin. After 10 days, seedlings were scored for kanamycin resistance or susceptibility. Only plants showing 100% kanamycin resistance were selected as neb candidate mutants. Plants that did not show kanamycin resistance were discarded.

4) The heb plants were tested for constitutive luc activity. Three naïve heb plants from each line were painted with luciferin and imaged in the ultra low-light imaging camera. Plants that showed constitutive luc activity (i.e. induction of luc activity in
the absence of SAR induction) were placed in a new category, termed *ceb* (constitutive expression of bioluminescence).

Table 3.1. Numbers of candidate mutant plants isolated in the mutant screen.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ceb</em></td>
<td>5</td>
</tr>
<tr>
<td><em>heb</em></td>
<td>3</td>
</tr>
<tr>
<td><em>neb</em> - block upstream of SA</td>
<td>10</td>
</tr>
<tr>
<td><em>neb</em> - block downstream of SA</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
</tr>
</tbody>
</table>

A total of 37 putative mutants were isolated by the end of the secondary screen (Table 3.1). A representative *ceb*, *heb* and *neb* candidate mutant is shown in Fig.3.5. Leaves were imaged for luc activity in the ultra low-light camera. The pattern of luc activity three days after inoculation with *PstDC3000 (avrB)* is shown for the *neb* candidate (Fig.3.5b) and the *heb* candidate (Fig.3.5c) in comparison to *PR-la:luc*, which displayed a ‘C-shape’ of light surrounding the HR (Fig.3.5a). No light was seen for the *neb* candidate whereas the band of light in the *heb* candidate was larger than in the *PR-la:luc* plant (Fig.3.5). A naïve leaf from a *ceb* candidate plant showing constitutive luc activity is shown (Fig.3.5d).
A transformation cassette containing the −903 bp PR-1a promoter from tobacco (Payne et al. 1988) was fused to the luc reporter gene and the cassette was used to transform Arabidopsis. Luc activity was induced in a homozygous transgenic line by SA and inoculation with avirulent bacterial pathogens (Fig.3.2). As SAR induction by SA and inoculation with an avirulent pathogen has been well described (Ryals et al. 1996), luc activity in PR-1a:luc plants can thus be taken as a report of the establishment of SAR. Expression of the PR-1a promoter has previously been studied in transgenic tobacco. The −903bp PR-1a promoter was fused to the β-glucoronidase (GUS) reporter protein encoded by the uidA gene and used to transform tobacco (Van de Rhee et al. 1990, Uknes et al. 1993). In both cases inoculation of the transgenic plants with TMV or application of SA induced expression of PR-1a:uidA (Van de Rhee et al. 1990, Uknes et al. 1993). Thus, expression of the luc reporter gene in the PR-1a:luc transgenic Arabidopsis line corresponded to GUS expression in PR-1a:uidA transgenic tobacco lines, viz. that expression of the reporter gene was induced by both SA and inoculation with an avirulent pathogen.
Following inoculation of PR-la:luc plants with PstDC3000 (avrB), luc activity was produced around the HR (Fig.3.3). This result corresponded to TMV induction of GUS activity in PR-la:uidA transgenic tobacco, where GUS activity was greatest in the area adjacent to the TMV lesions (Uknes et al. 1993). GUS activity was also observed surrounding the HR produced when PR-la:uidA transgenic tobacco leaves were inoculated with a 50mM glycoprotein extract from Phytophthora megasperma (Costet et al. 1999). Thus, expression of a reporter gene under the control of the PR-la promoter in plants is associated with cell death following the interaction of R-avr gene products, an increase in available SA and the establishment of acquired resistance. Inoculation of PR-la:luc Arabidopsis plants with MgCl₂ did not induce luc activity (Fig.3.2), nor did rubbing of PR-la:uidA tobacco plants with carborundum induce GUS activity (Uknes et al. 1993).

Luc activity following inoculation of PR-la:luc plants with PstDC3000 (avrB) was detected after 12 hours (Fig.3.3 & Fig.3.4). Luc activity increased sharply after 24 hours, peaking at 36 hours post-inoculation, and decreased after 96 hours (Fig.3.4). The rapid induction of luc activity 12 to 24 hours post-inoculation is consistent with PR-I expression in Arabidopsis following inoculation with the avirulent bacterial pathogen P. syringae pv maculicola ES4326 (avrRpt2) (Reuber & Ausabel 1996). Expression of the PR-I gene in Arabidopsis Col-0 also increased 12 to 24 hours post-inoculation with PstDC3000 (avrB), peaked at 36 hours and decreased after 96 hours (Chini, unpublished results), further indicating that luc activity accurately reported PR-I expression during the establishment of SAR.

Recently, a -4258 bp Arabidopsis PR-I promoter was isolated and the cis-acting regulatory elements involved in INA induction were characterised by deletion analysis, linker-scanning mutagenesis and in vivo footprinting (Lebel et al. 1998). It was found that a -621bp promoter was essential for INA-inducible PR-I expression. Linker-scanning analysis showed that this region of the promoter contained a CGTCA motif, which has high homology to the recognition site of transcription factors of the basic leucine zipper class (Lebel et al. 1998). As INA is an analogue of
SA, it is likely that elements in the PR-1 promoter that are necessary for INA-inducibility, are also required for SA-induction of PR-1 expression. Deletion analysis of the PR-1a promoter indicated that a -661 bp minimal region (Uknes et al. 1993) or a -643 bp minimal region (Van de Rhee et al. 1990) was essential for SA-induction of the GUS reporter gene. In addition, functional analysis of the PR-1a promoter revealed a region of 139 bp (from -691 to -553) required for GUS expression in response to SA (Strompen et al. 1998). This region contained two TGACG motifs (the complementary sequence of CGTCA) which were found to be the recognition site of TGA1a, a tobacco transcription factor of the basic leucine zipper class (Strompen et al. 1998). This indicates that a similar core region of both the PR-1 and PR-1a promoters are required for SA-inducibility. Furthermore, both genes appear to be under the control of a similar cis-acting regulatory element.

Homozygous PR-1a:luc seed was mutagenised and M2 candidate mutant plants showing perturbations in the pattern of luc activity following inoculation with PstDC3000 (avrB) were identified. A total of 37 candidate mutants were isolated following secondary screening of M3 plants (Table 3.1). The candidate mutants could be divided into various classes (Fig.3.5). Characterisation of a group of these mutants will be presented in Chapter 4.
Chapter Four
Characterisation of neb and ceb candidate mutants

4.1. Introduction

Two groups of candidate mutants identified in this study and thought to represent new SAR mutant classes were chosen for further characterisation. The neb candidate mutants with a putative block upstream of SA in the SAR signal transduction pathway and the ceb candidates were studied further. A number of mutants have previously been uncovered that show constitutive expression of PR-1. However, for many of these mutants such as lsd1 (Dietrich et al. 1994), acd2 (Greenberg et al. 1994), cpr5 (Bowling et al. 1997) and cep (Silva et al. 1999), constitutive PR-1 gene expression is associated with the spontaneous formation of HR-like lesions. The ceb candidate mutants uncovered in this project did not display spontaneous formation of visible lesions, or accelerated cell death following inoculation with PstDC3000 (avrB), and thus represent a mutant class where cell death and constitutive PR-1 expression have been uncoupled. Furthermore, a number of different alleles of NPR1/NIM1, which acts downstream of SA in SAR, have already been uncovered in mutant screens (Cao et al. 1994, Delaney et al. 1995, Glazebrook et al. 1995, Shah et al. 1997). In order to avoid isolating further alleles of NPR1, it was decided to characterise the group of neb candidate mutants that appeared to be upstream of SA in the SAR signal transduction network.

The ceb and neb candidate mutants were studied using three approaches in order to determine if they were true SAR mutants. Firstly, the extent of PR-1a:luc expression in the mutants was quantified using the luminometer method. Secondly, the expression of SA-dependent defence genes (PR-1, PR-2 and PR-5) (Uknes et al. 1992), jasmonate-dependent genes (Thi2.1, LOX2) (Bohiman et al. 1998, Bell & Mullet 1993), a gene dependent on concomitant signalling through jasmonates and ethylene (PDF1.2) (Penninckx et al. 1998) and ACS2, a gene encoding a biosynthetic intermediate in the production of ethylene (Liang et al. 1992), were determined using Northern blot analysis. Thirdly, the resistance of the candidate mutants to a range of
Plant pathogens normally virulent on the Col-0 ecotype was determined. Disease resistance assays with the bacterial pathogen *Pseudomonas syringae pv. tomato* (Whalen et al. 1991), the biotrophic oomycete pathogen *Peronospora parasitica* Noco2 (Parker et al. 1993) and the necrotrophic fungal pathogen *Fusarium oxysporum* f.sp. *matthiolae* (Mauch-Mani & Slusarenko 1994) were performed.

4.2. Luminometer assays

*neb* candidate mutants

Four *neb* candidate mutants were selected for initial further study. They were *neb26*, *neb27*, *neb28* and *neb29*. Fig. 4.1 represents the results from luminometer assays of these four mutants and the *PR-la:luc* transgenic line under various conditions: 36 hours after inoculation with *PstDC3000 (avrB)* (a), 72 hours after *PstDC3000 (avrB)* inoculation (b) and 24 hours after 1mM SA application (c). The 36 hours post-inoculation time point was chosen as *PR-la:luc* expression was previously found to peak at this time following inoculation with *PstDC3000 (avrB)* (Fig. 3.3). The 72 hours post-inoculation time point was chosen in order to determine if the *neb* candidate mutants showed delayed increase in luc activity following inoculation with *PstDC3000 (avrB)*.

The four *neb* candidate mutants all showed a dramatic reduction in luc activity at both 36 and 72 hours post-inoculation with *PstDC3000 (avrB)* in comparison to *PR-la:luc* plants (Fig. 4.1a&amp;b). The four *neb* candidates expressed *PR-la:luc* 24 hours after application of 1mM SA (Fig. 4.1c). However, the amount of luc activity in the *neb* candidates was approximately two to seven times less than the extent of luc activity produced in the *PR-la:luc* wild-type plants treated with SA (Fig. 4.1c). In comparison, GUS activity in SA treated *npr1* mutant plants (which contain the *PR-2* promoter fused to the *uidA* gene) was approximately 15 times less than SA treated wildtype transgenic plants (Cao et al. 1994).

The luminometer results presented in Fig. 4.1 suggest that these *neb* candidate mutants are blocked in *PstDC3000 (avrB)* induction of luc activity, but not in SA.
induction of PR-1α: luc expression, and thus may define mutations in the SAR signal transduction pathway upstream of SA. Furthermore, the luminometer results presented in Fig.4.1 confirm the results obtained using the ultra low-light imaging camera to image luc activity in neb plants, which was carried out during the secondary phase of the mutant screen.

**Fig.4.1.** Luminometer assay of neb candidate mutants, assayed either 36 hours after inoculation with PstDC3000 (avrB) (a) or after 72 hours (b), and 24 hours after application of 1mM SA (c).

Each graph includes untreated control PR-1α: luc plants. The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level. Luminometer assays were repeated twice and results obtained followed a similar trend.
ceb candidate mutants

All five ceb candidate mutants were selected for further study. They were ceb1, ceb3, ceb4, ceb5 and ceb6. All five candidate mutant lines showed no morphological changes, except for ceb5 which displayed earlier senescence in comparison to wild-type Col-0 plants (results not shown). None of the plants showed macroscopic HR lesion production. Fig.4.2 represents the results from luminometer assays of naïve five-week old plants of all five candidates and the PR-la:luc transgenic line. PR-la:luc plants sprayed with 1mM SA 24 hours prior to the luminometer assay were also included (Fig.4.2). As expected, PR-la:luc plants treated with SA showed an increase in luc activity in comparison to naïve PR-la:luc plants (Fig.4.2).

Fig.4.2. Luminometer assay of ceb candidate mutants

Naïve five-week old plants were assayed, and PR-la:luc leaves treated with 1mM SA 24 hours prior to harvesting were included as a control. The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level. The luminometer assay was repeated twice and results obtained followed a similar trend.

All five ceb candidate mutants showed constitutive expression of PR-la:luc in comparison to PR-la:luc plants, ranging from five-fold greater expression in the case
of ceb6 to more than 100-fold in the case of ceb1 and ceb5 (Fig.4.2). Only ceb1 and ceb5 expressed PR-1α:luc to levels higher than the SA-treated PR-1α:luc leaves (Fig.4.2). The cpr1 and cpr5 mutants, which express constitutive SAR and also contain the PR-2 promoter fused to the uidA gene, showed a two-fold increase in GUS activity in comparison to naïve transgenic plants (Bowling et al. 1994, Bowling et al. 1997). Although it is difficult to make a direct comparison between the ceb and cpr mutants as different SAR promoter-marker transcriptional fusions were used, ceb1 and ceb5 do show a greater increase in marker protein expression in comparison to published reports of other SAR mutants.

4.3. Northern blot analysis

neb candidate mutants
Expression of the endogenous Arabidopsis PR-1 gene (Uknes et al. 1992) was determined in the four neb candidate mutants. This analysis was performed in order to confirm that the loss of luc activity following Pst DC3000 (avrB) inoculation accurately depicted PR-1 gene expression. Col-0 wildtype plants, nahG, npri and the neb candidates were inoculated with PstDC3000 (avrB). Inoculated leaves were harvested after 48 hours and total RNA was extracted. RNA samples were separated by electrophoresis, transferred to a membrane by Northern blotting and the membrane was probed with PR-1 and the ribosomal 18S gene. The latter constitutive probe was included in order to ensure that uniform loading and transfer of RNA had taken place. The result is shown in Fig.4.3. As was expected, the uninfected Col-0 sample did not show PR-1 activity, whereas Col-0 plants inoculated with PstDC3000 (avrB) did (Fig.4.3). nahG plants, which accumulate reduced levels of SA following PstDC3000 (avrB) infection (Delaney et al. 1994), did not show PR-1 expression (Fig.4.3). The npri mutant, which is blocked in SA induction of PR-1 expression (Cao et al. 1994), showed a small amount of PR-1 expression (Fig.4.3). The jar1 plants inoculated with PstDC3000 (avrB) expressed PR-1 to levels comparable to wild-type Col-0. The jar1 mutants are insensitive to jasmonate signalling (Staswick et al. 1992). Application of Me-JA did not induce PR-1 expression (Fig.4.3). These results confirm previous observations that Me-JA plays no direct role in the signal
transduction pathway leading to PR-I expression (Penninckx et al. 1996). From the luminometer data, it was expected that the neb lines would show reduced PR-I expression following Pst DC3000 (avrB) inoculation. However, all four neb lines showed increased PR-I expression following PstDC3000 (avrB) in comparison to nahG or npr1 plants and naive Col-0 plants (Fig.4.3). All four neb lines also showed increased PR-I expression following SA application (results not shown). From these results it appears that the neb lines do not contain a loss-of- function mutation in the signal transduction pathway leading to the expression of the Arabidopsis PR-I gene.

Fig.4.3. Northern blot analysis of PR-I mRNA expression in neb plants.
Col-0, neb26, neb27, neb28, neb29, npr1, nahG and jar1 plants were inoculated with PstDC3000 (avrB). Wild-type plants (Col-0) were included as a negative control and Col-0 plants were treated with 100μM Me-JA. RNA samples were consecutively probed with the PR-I and 18S probes. The table below the blot represents the fold induction of the gene expression for each sample relative to that of Col-0. The expression has been quantified using the ImageQuant software, adjusted to the expression of the loading control (r18) and normalised to the expression of Col-0, which was set to 1 unit. Northern blot analysis was repeated twice with similar results. avrB, plants inoculated with PstDC3000 (avrB).

celb candidate mutants
Leaves from naïve five-week old Col-0 plants and all five ceb plants were harvested for Northern blot analysis. Col-0, nahG, npr1 and jar1 plants were inoculated with PstDC3000 (avrB) and leaves were harvested after 48 hours. Col-0 plants were
treated with 1mM SA or 100µM Me-JA and leaves were harvested after 24 hours. Col-0 plants were also treated with 2mM H₂O₂ and leaves were harvested after 4.5 hours. Total RNA was extracted from all the samples and Northern blot analysis was performed. Expression of the SA-dependent genes PR-1, PR-2 and PR-5 (Uknes et al. 1992) and the SA-independent genes PDF1.2 (Penninckx et al. 1996) and Thi2.1 (Epel et al. 1995) were determined (Fig.4.4). Northern blot analysis was repeated at least twice on separate occasions and similar results were obtained. Expression of PDF1.2 is dependent on jasmonate and ethylene signalling (Penninckx et al. 1998) and Thi2.1 expression is dependent on jasmonate signalling (Bohlmann et al. 1998). AtLOX2 and ACS2 were also selected for Northern blot analysis (Fig.4.4). AtLOX2 encodes a lipoxigenase enzyme which catalyses the hydroperoxidation of unsaturated fatty acids and is thought to be important in the biosynthesis of jasmonic acid (Bell & Mullet 1993). AtLOX2 expression is rapidly induced in leaves following Me-JA application (Bell & Mullet 1993). Thus, a high level of expression of AtLOX2 can be taken as an indication of the presence of accumulated jasmonates. ACS2 encodes an isoform of ACC synthase and is the major form of the enzyme found in Arabidopsis vegetative tissues (Liang et al. 1992). ACC synthase is the rate-limiting enzyme responsible for conversion of methonine to ACC during the biosynthesis of ethylene (Kende 1993). Thus, increased transcription of ACS2 may indicate an increase in ethylene production in Arabidopsis plants. The blots were also probed with the glutathione S-transferase 1(GST1) probe. GSTs are enzymes that catalyze the conjugation of glutathione to a variety of toxic substrates and in plants, a subclass of GSTs has been implicated in numerous stress responses, including oxidative stress and pathogen attack (Marrs et al. 1996). GST1 expression is upregulated following inoculation with PstDC3000 (avrB) and application of hydrogen peroxide (Grant & Loake 2000, in press).

All five ceb candidate mutants displayed constitutive expression of PR-1, PR-2, PR-5, PDF1.2 and GST1 but not Thi2.1 (Fig.4.4a,b,c). All five candidates did not show substantially enhanced expression of ACS2 and only ceb5 showed enhanced expression of AtLOX2 (Fig.4.4c,d). As in Fig.4.3, control Col-0 leaves did not express PR-1 whereas Col-0 and jar1 leaves inoculated with PstDC3000 (avrB) did
Fig. 4.4. Northern blot analysis of defence mRNA expression in *ceb* plants: PR-1, PDF1.2 (a), PR-2, PR-5, GST1 (b), Thi2.1 and ACS2 (c) and AtLOX2 (d).

Naïve Col-0, *ceb*1, *ceb*3, *ceb*4, *ceb*5 and *ceb*6 leaves were harvested. As controls, Col-0, nahG, *npr1* and *jar1* plants were inoculated with *PstDC3000* (*avrB*) and leaves were harvested 48-hours post-inoculation, or Col-0 was treated with 1mM SA, 100μM Me-JA or 2mM H2O2. RNA samples were consecutively probed with the PR-1, PDF1.2 and r18 probes (a), the PR-2, PR-5, GST1 and r18 probes (b), the Thi2.1, ACS2 and r18 probes (c) and the AtLOX2 and r18 probes (d). The table below the blot represents the fold induction of the gene expression for each sample relative to that of Col-0. The expression has been quantified using the ImageQuant software, adjusted to the expression of the loading control (r18) and normalised to the expression of Col-0, which was set to 1 unit. Northern blot analysis was repeated twice in all cases with similar results. *avrB*, plants inoculated with *PstDC3000* (*avrB*).
Expression of PR-1 in the nahG + avrB sample was negligible but was increased for the npr1 + avrB sample (Fig.4.4a) in comparison to the first blot (Fig.4.3). The Col-0 control did not show expression of PDF1.2, PR-2, PR-5, GSTI and Thi2.1 as expected, and showed basal expression of ACS2 and AtLOX2 (Fig.4.4a,b,c,d). The Col-0+SA sample showed induced expression of PR-1 and weak expression of PR-2, PR-5 and GSTI (Fig.4.4a&b), whereas the Col-0+avrB sample showed induced expression of PR-1, PR-2, PR-5, GSTI, PDF1.2 and AtLOX2 but not Thi2.1 (Fig.4.4a,b,c). From previous reports, it was expected that SA application would not induce PDF1.2 expression (Penninckx et al. 1996). The Col-0+Me-JA sample showed weak induction of PR-1, PDF1.2 and GSTI expression (Fig.4.4a&b). Hydrogen peroxide application induced expression of GSTI only (Fig4.4b). The induction of GSTI expression by SA and Me-JA application most likely indicates a role for GSTI in the detoxification of these compounds. GSTI expression was higher 24 hours after inoculation of Col-0 with PstDC3000 (avrB) than 48 hours post-inoculation, confirming previous observations that GSTI is rapidly expressed following inoculation with an avirulent pathogen (Grant & Loake 2000, in press). NahG plants inoculated with PstDC3000 (avrB) showed slight induction of PDF1.2 (Fig.4.4a). It has previously been found that nahG plants inoculated with Alternaria brassicicola expressed higher levels of PDF1.2 than control nahG plants and this is thought to demonstrate a degree of cross-talk between the SA-dependent and SA-independent signal transduction pathways (Penninckx et al. 1996). In comparison to Col-0+avrB, the npr1+avrB sample showed increased expression of PDF1.2 but decreased expression of PR-1 (Fig.4.4a). The jar1+avrB sample showed a high level of PR-1 expression but no increase in PDF1.2 expression in comparison to Col-0+avrB (Fig.4.4a). This indicates that cross-talk between SA-dependent gene induction and Me-JA- dependent gene induction may be occurring in these two mutant lines. In Fig.4.4c a positive control for Thi2.1 expression was included. Plants of Arabidopsis ecotype UK4 were inoculated with Fusarium oxysporum f.sp. matthioliæ and leaves for RNA extraction were harvested after five days. Previously it has been shown that Thi2.1 is expressed following F. oxysporum f.sp. matthioliæ infection in UK4 leaves but not in Col-0 (Epple et al.
Here the UK4 + \textit{F.oxysporum} f.sp. matthiolae sample was the only sample to express \textit{Thi2.1} (Fig.4.4c).

### 4.4. Disease resistance assays

Both the \textit{neb} and \textit{ceb} candidate mutant lines were tested for resistance to the bacterial pathogen \textit{Pseudomonas syringae pv tomato} DC3000 (\textit{PstDC3000}) and the virulent oomycete pathogen \textit{Peronospora parasitica} Noco2. \textit{PstDC3000} is the causal organism of bacterial speck of tomato and bacterial growth is usually limited to the locally infected leaf (Agrios 1997). \textit{PstDC3000} is virulent on the Arabidopsis ecotype Col-0 (Whalen et al. 1991) and induced resistance to this bacterial pathogen is associated with SA accumulation and \textit{PR-1} expression (Cameron et al. 1999). \textit{Peronospora parasitica}, a member of the oomycete family \textit{Peronosporaceae}, is an obligate biotroph and causes downy mildew in \textit{Cruciferae} (Agrios 1997). Many \textit{P.parasitica} isolates have been uncovered with varying degrees of resistance to Arabidopsis ecotypes (Holub et al. 1994). \textit{P.parasitica} Noco2 is virulent on the ecotype Col-0 (Parker et al. 1993) and thus was chosen for the disease resistance analysis. Induced resistance to \textit{P.parasitica} Noco2 in Col-0 plants is also associated with SA accumulation and \textit{PR-1} expression (Bowling et al. 1994, Bowling et al. 1997, Clarke et al. 1998). It has also been suggested that \textit{PDF1.2} expression may play a role in \textit{P.parasitica} induced resistance in Col-0 (Bowling et al. 1997). As the \textit{neb} candidate mutants showed loss of \textit{PR-1a:luc} expression, it was expected that they would show enhanced susceptibility to these two pathogens. On the other hand, the \textit{ceb} candidate mutants showed constitutive expression of \textit{PR-1} and \textit{PDF1.2} and thus were expected to show enhanced resistance. The \textit{ceb} candidates only were tested for resistance to the necrotrophic fungal pathogen \textit{Fusarium oxysporum} f.sp.\textit{matthiolae} (Mauch-Mani & Slusurenko 1994).

\textit{Pseudomonas syringae pv tomato} DC3000.

Five-week old Col-0, \textit{nahG}, \textit{npr1}, \textit{ceb} and \textit{neb} plants were inoculated with \textit{Pst DC3000}. Development of disease symptoms (chlorosis, wilting of inoculated leaves) was monitored daily and development of symptoms in wildtype Col-0 was found to
be optimal at four days post-inoculation (Fig.4.5a). At this time period nahG plants had undergone extreme chlorosis and wilting in comparison to Col-0. The neb and ceb candidate mutants showed varying degrees of chlorosis (results not shown), and so it was decided to quantify bacterial growth in these mutant lines in order to compare them to Col-0. Three plants per line were inoculated with PstDC3000 and leaves were harvested after three days. This time point was chosen as PstDC3000 growth in Arabidopsis peaks three days after inoculation (Whalen et al. 1991, Delaney et al. 1994). Only neb26, neb27 and neb29 were tested from the neb lines, as insufficient seed was collected for neb28. Leaf disks were produced from each inoculated leaf, ground in 10mM MgCl₂, dilutions were made and plated out on KB media. After three days, the number of bacterial colonies were recorded as an indication of the bacterial titre in the different plants. Fig.4.5b&c outlines the results obtained for both the ceb and neb candidate mutants respectively.

In Fig.4.5b, the Col-0 samples showed a bacterial titre of $10^7$ cfu/leaf disk which corresponds to bacterial titres in Col-0 at this time point in previous studies (Bowling et al. 1997, Clarke et al. 1998). Bacterial titres in nahG and npr1 plants were greater than Col-0, which also corresponds to previous reports (Bowling et al. 1997). In both the case of nahG and npr1, a statistically significant difference in bacterial titre in comparison to Col-0 was found using the Mann-Whitney test at the 95% confidence level (Mini-tab Version 12). The neb26, neb27 and neb28 candidate mutant lines showed bacterial titres more similar to Col-0 than npr1 or nahG and thus do not appear to show an ‘enhanced disease susceptibility’ phenotype to PstDC3000 (Fig.4.5b).

The bacterial titre in the Col-0 samples in the ceb experiment ($10^5$ cfu/leaf disk, Fig.4.5c) was less than the bacterial titre in the neb experiment ($10^7$ cfu/leaf disk, Fig.4.5a). It has previously been shown that bacterial titres in Col-0 plants can vary significantly in different experiments (Glazebrook et al. 1996). However, the same trend of PstDC3000 growth can be seen in both Fig.4.5b and Fig.4.5c. In both
Fig. 4.5. Resistance of *neb* and *ceb* candidate mutants to *PstDC3000*.

a: The development of *PstDC3000* symptoms on Col-0 4 days after inoculation.
b: Graph indicating bacterial titre three days after *PstDC3000* inoculation. Col-0, *nahG*, *npr1* and three *neb* candidate mutants were tested.
c: Graph indicating bacterial titre three days after *PstDC3000* inoculation. Col-0, *nahG* and all five *ceb* candidate mutants were tested.

In all cases, values presented are the averages obtained from three plants and error bars represent the standard error between values at the 95% confidence level. Experiments were repeated twice and results obtained followed a similar trend.
experiments, the bacterial titre in *nahG* plants was greater than in Col-0 plants. The *ceb3, ceb4* and *ceb5* lines showed bacterial titres equivalent to those of Col-0 (Fig.4.5c) and *ceb1* and *ceb6* showed bacterial titres significantly less than Col-0 (Fig.4.5c). The reduction in bacterial titre in *ceb1* and *ceb6* corresponds to results for *cpr5* and *cpr6*, where bacterial titres were also reduced in the mutant lines by 1000 cfu per leaf disk in comparison to Col-0 (Bowling et al. 1997, Clarke et al. 1998).

*Peronospora parasitica Noco2*

Four-week old Col-0, *nahG, nprl, ceb* and *neb* plants were spray-inoculated with a *P. parasitica Noco2* conidiospore suspension. The plants were maintained in a humid environment for 10 days before they were visually accessed for the extent of *P. parasitica Noco2* growth. Plants were grown in three separate trays, with four plants in each tray. After 10 days, Col-0 plants typically showed downy mildew symptoms (Fig.4.5a, visualisation of sporulating conidiophores). The extent of macroscopic conidiophore development over the surface area of the leaves was scored under good light (scoring criteria outlined in Chapter 2). A disease index was applied to the scores and the results obtained are outlined in Fig.4.6b&c.

In both Fig4.6b and 4.6c, *nahG* and *nprl* plants showed a greater degree of conidiophore development in comparison to Col-0. This confirms results previously obtained for *nprl* and Col-0 (Bowling et al. 1997). None of the *neb* lines showed an increase in conidiophore development in comparison to Col-0 (Fig.4.6b). The *ceb1, ceb3, ceb4* and *ceb6* lines showed a reduction in conidiophore development in comparison to Col-0, whereas *ceb5* showed the equivalent degree of conidiophore development (Fig.4.6c). Both the *cpr5* (Bowling et al. 1997) and *cpr6* (Clarke et al. 1998) mutants showed a reduction in *P. parasitica Noco2* conidiophore development in comparison to Col-0, which is comparable to the results obtained here for *ceb1, ceb3, ceb4* and *ceb6*. 
Fig. 4.6. Resistance of *neb* and *ceb* candidate mutants to *P. parasitica* Noco2.

a: Downy mildew symptoms on Col-0 10 days after *P. parasitica* Noco2 inoculation.
b: Disease rating of *P. parasitica* Noco2 infection 10 days after inoculation. Col-0, nahG, npr1 and three *neb* candidate mutants were tested.
c: Disease rating of *P. parasitica* Noco2 infection 10 days after inoculation. Col-0, nahG and all five *ceb* candidate mutants were tested.

Scoring criteria are outlined in Chapter 2. Four plants were analysed per replicate and three disease indices representing three replicates were obtained per line. Error bars represent the standard error between replicates at the 95% confidence level. Experiments were repeated twice and results obtained followed a similar trend.
Fusarium oxysporum f.sp. matthioliæ

The soil-borne fungus *F. oxysporum* is the causal organism of vascular wilt in a wide range of plants, including many economically important crops (Agrios 1997). The fungus normally invades the roots (or leaves if spray-inoculated) and colonises the vascular tissue, causing typical symptoms: the leaves turn yellow, wilt and finally die (Fig.4.7a). The interaction between Arabidopsis and *F. oxysporum* f.sp. *matthioliæ* has been studied in some detail, and resistant and susceptible ecotypes have been identified (Mauch-Mani&Slusarenko 1994, Epple et al 1998). When leaves were spray-inoculated with a *F. oxysporum* f.sp. *matthioliæ* spore suspension and disease symptoms (wilted, necrotic leaves) were monitored eight days later, Arabidopsis ecotype Umkirch (UK4) was found to be resistant to infection with very few leaves showing disease symptoms, whereas ecotype Col-0 was susceptible (Epple et al. 1998).

In order to investigate the extent of *F. oxysporum* f.sp. *matthioliæ* resistance in the five *ceb* candidate mutants, *ceb* plants were inoculated with *F. oxysporum* f.sp. *matthioliæ* together with Col-0 and UK4 plants. Four-week old plants were sprayed-inoculated with a *F. oxysporum* f.sp. *matthioliæ* spore suspension and incubated under humid conditions for eight days, after which time the disease symptoms were recorded. The number of necrotic leaves per plant were noted as an indication of disease severity. Plants were grown in three separate trays, with four plants grown in each tray. Results are presented in Fig.4.7b. As expected, UK4 was resistant to *F. oxysporum* f.sp. *matthioliæ* inoculation, whereas Col-0 was susceptible. An average of 1.4% of leaves per UK4 plant wilted and died, whereas 19.9% of Col-0 leaves per plant died (Fig.4.7b). This amounted to a significant difference using the Mann-Whitney test at the 95% confidence level. The *ceb* lines showed a range of responses in comparison to Col-0. The *ceb3, ceb4* and *ceb6* lines had fewer necrotic leaves per plant in comparison to Col-0 which were not significantly different, whereas *ceb1* displayed a similar degree of symptom development in comparison to Col-0 (Fig.4.7b). The *ceb5* plants showed significantly enhanced susceptibility to *F. oxysporum* f.sp. *matthioliæ* in comparison to Col-0 (Fig.4.7b).
Fig. 4.7. Resistance of ceb candidate mutants to *F. oxysporum* f.sp. *matthiolae*.

a: Col-0 plant showing typical *F. oxysporum* f.sp. *matthiolae* symptoms eight days after spray-inoculation with a *F. oxysporum* f.sp. *matthiolae* spore suspension.

b: Graph indicating extent of symptom development (percentage of leaves per plant showing *F. oxysporum* f.sp. *matthiolae* symptoms). Col-0, UK4 and the five ceb candidate mutants were tested. Values presented are the averages of disease symptoms obtained for 12 plants. Error bars represent the standard error between replicates at the 95% confidence level. The experiment was repeated twice and results obtained followed a similar trend.

4.5. Segregation analysis

The ceb candidate mutants were back-crossed to the *PR-la:Luc* transgenic line in order to determine the dominant/recessive nature of the putative mutations (Koornneef & Stam 1992). Pollen was taken from the ceb donor plants and applied to pistils of
dissected PR-1a:luc flowers, which acted as the pollen recipient. F1 seeds were collected. Five-week old F1 plants were analysed for constitutive luc activity using the ultra low-light imaging camera. The results obtained are outlined in Table 4.1. For the ceb1, ceb3, ceb4 and ceb6 crosses, analysis of the F1 plants showed loss of constitutive PR-1a:luc expression in all cases, indicating that they define recessive mutations (Table 4.1). On the other hand, all 12 F1 progeny from the ceb5 X PR-1a:luc cross showed constitutive luc activity, indicating that ceb5 defines a putative dominant mutation (Table 4.1).

Table 4.1. Segregation analysis of the ceb candidate mutants.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Donor</th>
<th>Recipient</th>
<th>No. tested</th>
<th>Luc activity</th>
<th>No luc activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>ceb1</td>
<td>PR-1a:luc</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>F1</td>
<td>ceb3</td>
<td>PR-1a:luc</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>F1</td>
<td>ceb4</td>
<td>PR-1a:luc</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>F1</td>
<td>ceb5</td>
<td>PR-1a:luc</td>
<td>12</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td>ceb6</td>
<td>PR-1a:luc</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

4.6. Discussion

The neb and ceb candidate mutants were tested further in order to determine rigorously if they were true SAR mutants. Expression of defence-related genes and resistance to a range of virulent pathogens is usually used to determine the status of a potential mutant (Cao et al. 1994, Bowling et al. 1994, Clarke et al. 1998) and these criteria were applied to neb and ceb candidates.

After inoculation with PstDC3000 (avrB), the four neb candidate mutants were shown to express a reduced amount of PR-1a:luc in comparison to wildtype PR-1a:luc transgenic plants (Fig.4.1a&b). SA application induced luc activity in the neb plants (Fig.4.1c), indicating that they may define a mutant class with a block upstream of SA in the signal transduction pathway leading to SAR. However, the neb lines showed expression of PR-1 following PstDC3000 (avrB) in Northern blot
analysis (Fig.4.3). It is unlikely that this discrepancy in luminometer and Northern blot results can be explained by loss of the PR-1a:luc transformation cassette in the neb lines. The neb candidates were tested during the secondary phase of the mutant screen for sensitivity to kanamycin during seedling germination and all the lines were resistant. It is also unlikely that the PR-1a-luc sequence was mutagenised in these lines, or that silencing of the PR-1a promoter by methylation (Finnegan & McElroy 1994) has occurred as SA induced PR-1a:luc expression in all cases. It is possible that the neb candidates represent loss of upstream signalling components required for transcription of the tobacco PR-1a gene which are not required for the transcription of PR-1 in Arabidopsis. Consistent with this possibility, the neb plants tested here did not show a significant difference in resistance to PstDC3000 or P.parasitica Noco2 in comparison to Col-0, and thus can not be considered to be SAR mutants. For this reason it was decided to do no further testing of this neb candidate mutant class and to concentrate on characterising the ceb candidate mutants.

All five ceb candidate mutants expressed the SA-dependent defence genes PR-1, PR-2 and PR-5 constitutively in naïve plants (Fig.4.4a&b), indicating that they represent mutations in the SA-dependent signal transduction pathway leading to expression of these genes (Ryals et al. 1996). These mutations may be either up- or downstream of SA. Of the five ceb lines, ceb3 and ceb5 expressed PR-1 to levels higher than Col-0 plants treated with SA, indicating that the pathway in these lines is very strongly induced. The PR-1 Northern blot result for ceb5 corresponded to the luminometer data (Fig.4.2) as luc activity in ceb5 increased in comparison to PR-1a:luc plants treated with SA (Fig.4.2). The ceb1 line shows the greatest increase in luc activity in comparison to SA-treated PR-1a:luc plants (Fig.4.2), but only showed a moderate increase in PR-1 expression (Fig.4.4a). In this respect, ceb1 is analogous to cpr5, which showed a two-fold increase in GUS activity in comparison wild-type plants treated with INA, whereas constitutive PR-1 expression was significantly less than in wild-type plants treated with INA (Bowling et al. 1997).
Expression of the PDF1.2 gene is dependent on both jasmonate and ethylene signalling (Pennincix et al. 1998). All five ceb candidate mutants expressed this gene constitutively (Fig.4.4a), indicating that they represent mutations in the jasmonate and ethylene signal transduction pathway leading to PDF1.2 expression. However, all five ceb plants did not express Thi2.1 and only ceb5 showed a moderate increase in AtLOX2 expression (Fig.4.4c&d). Expression of the Thi2.1 gene is dependent on jasmonate signalling (Bohlmann et al. 1998). Although not shown in Fig.4.4d, expression of AtLOX2 was previously found to be responsive to Me-JA application (Bell & Mullet 1993), indicating that only ceb5 may contain higher levels of methyl jasmonate or other jasmonate signalling components. Expression of ACS2 is not upregulated in any of the ceb plants (Fig.4.4c), suggesting that ethylene levels may not be altered in ceb plants in comparison to wild-type. ACS2 is the major isoform of ACC synthase in Arabidopsis and wound-induced expression of ACS2 is controlled at the level of transcription (Liang et al. 1992). However, recently it has been reported that ethylene over-production may be controlled by post-transcriptional regulation of ACS isoforms under different conditions (Woeste et al. 1999a, Woeste et al. 1999b). This suggests that a lack of up-regulation in ACS2 expression does not necessarily indicate that ethylene production is not increased in the ceb plants. Thus, although expression of genes involved in the jasmonate and ethylene response is not clear, it is interesting that all five ceb lines showed constitutive expression of both PR-1 and PDF1.2 and thus may define mutations common to both SA, jasmonate and ethylene signal transduction.

None of the five ceb candidate mutants developed macroscopic lesions spontaneously, but all expressed GST1 constitutively at low levels (Fig.4.4b). Expression of GST1 can be used to report the accumulation of ROS (Grant & Loake 2000, in press). Thus, it is likely that the ceb candidates accumulate low levels of ROS, or that GSTs are playing an alternative role in removing potentially toxic signalling components accumulated due to constitutive activation of SAR.

In the resistance assay to the virulent bacterial pathogen PstDC3000, only ceb1 and ceb6 showed a significant reduction in bacterial growth in comparison to Col-0
In the resistance assay to the virulent oomycete pathogen *P. parasitica* Noco2, *ceb1, ceb3, ceb4* and *ceb6* showed a significant reduction in conidiophore development in comparison to Col-0 (Fig.4.6c). None of the *ceb* plants showed increased resistance to the necrotrophic fungal pathogen *F. oxysporum* f.sp.*matthiolae*, and *ceb5* showed significantly enhanced susceptibility to the fungus in comparison to Col-0 (Fig.4.8). This result corresponded to the lack of constitutive *Thi2.1* expression in all the *ceb* lines (Fig.4.4c), as resistance to *F. oxysporum* f.sp.*matthiolae* in Arabidopsis is associated with increased expression of *Thi2.1* (Epple et al. 1998).

It is interesting to note that *ceb5*, which shows the strongest expression of defence genes in Northern blot analysis (Fig.4.4a,b,c,d), also displayed earlier senescence in comparison to wild-type Col-0 plants (results not shown). Recently it has been shown that *PR-1* is expressed in senescing Arabidopsis plants and that *PR-1* expression under these conditions is dependent on SA signal transduction (Morris et al. 2000). Thus, it is possible that *ceb5* defines a gene important in SA-dependent senescence signal transduction. *ceb5* plants displayed no significant increase in disease resistance to *PstDC3000* (Fig.4.5c) or *P. parasitica* Noco2 (Fig.4.6c). It is likely that other defence genes, which have yet to be discovered and are required for resistance to these two pathogens, have not been induced in *ceb5*. Furthermore, *ceb5* was more susceptible to *F. oxysporum* f.sp. *matthiolae*, indicating that induction of *PR-1, PR-2, PR-5, PDF1.2* and *GST1* expression and possibly other unidentified genes may result in ‘cross-talk’ and suppression of elements involved in resistance to *F. oxysporum* f.sp. *matthiolae*. Alternatively, the early senescence phenotype of *ceb5*, resulting in enhanced necrosis in this line, may promote colonisation of the plant by *F. oxysporum* f.sp. *matthiolae*, a necrotrophic fungal pathogen. Recently it has been shown that *Botrytis cinerea*, also a necrotrophic fungal pathogen, triggered cell death and the production of an HR in Arabidopsis, thereby facilitating its colonisation of plants (Govrin & Levine 2000). It is possible that *F. oxysporum* f.sp. *matthiolae* uses a similar mechanism in pathogenesis, which is enhanced by increased cell death in *ceb5*. Thus, although *ceb5* has an interesting phenotype and bears further investigation, the aim of the current study was to characterise SAR
mutants showing enhanced disease resistance and no further characterisation of ceb5 was performed.

Taking the expression of defence-related genes and disease resistance assays into account, it was decided to select ceb1 for further characterisation. Luc activity in ceb1 was high, the defence-related genes PR-1, PR-2, PR-5, PDF1.2 and GST1 were expressed constitutively and ceb1 plants were significantly more resistant than wild-type Col-0 plants to both a virulent bacterial and oomycete pathogen. For these reasons ceb1 can be considered to be a true SAR mutant and it was decided to rename this candidate constitutively induced resistance 1 (cirl). Further detailed characterisation of cirl will be presented in Chapter 5.
5.1. Introduction

The cir1 mutant was selected from the group of ceb putative mutants for further characterisation. The SA-dependent defence related genes PR-1, PR-2 and PR-5 and the jasmonate- and ethylene-dependent gene PDF1.2 are expressed constitutively in cir1. In addition, cir1 is resistant to both a virulent bacterial pathogen, PstDC3000, and a virulent oomycete pathogen P.parasitica Noco2. Preliminary genetic analysis indicated that cir1 is a recessive mutation.

In this chapter, a more comprehensive analysis was made of the phenotype of cir1. Further segregation analyses were conducted to confirm the recessive nature of cir1. Complementation analyses of cir1 to other mutants displaying constitutive SAR were also conducted. The map position of cir1 was determined and the relationship of cir1 to SA and ethylene was investigated. The position of cir1 in the SAR signal transduction network was further investigated by creating double mutants between cir1 and npr1, ein2.1 and jar1. These double mutants were analysed for constitutive luc activity, constitutive expression of PR-1 and PDF1.2, and enhanced resistance to PstDC3000 and P.parasitica Noco2.

5.2. Visual phenotype of cir1

Soil-grown PR-1a:luc Col-0 and cir1 plants were routinely grown under short day conditions (growth conditions described in Chapter 2) and analysed at 5 weeks after planting, at which time leaves were large and fully expanded. Plants were analysed by imaging luc activity in the ultra low-light imaging camera. Maximal constitutive luc activity was obtained in five-week old naïve cir1 plants whereas no light was produced in PR-1a:luc transgenic Col-0 plants of the same age (Fig.5.1a). In comparison naïve cir1 seedlings grown in vitro did not show constitutive PR-1a:luc expression (data not shown).
Upon initial visual observation, \textit{crl} mutant plants did not appear to show any phenotypic differences to \textit{PR-la:luc} Col-0 plants. In order to record the growth features of \textit{crl} in comparison to Col-0, ten plants of both \textit{crl} and \textit{PR-la:luc} Col-0 were grown under the same conditions. The diameter of the rosette from each five-week plant was measured (Table 5.1) and representative plants were photographed (Fig.5.1b). At this point, the plants were transferred to long-day conditions and the number of days to bolting was recorded (Table 5.1). A plant was considered bolted when the stem was approximately 2cm in length. The bolt height of each plant was recorded at 8 weeks (Table 5.1).

The \textit{crl} mutant plants were slightly smaller than \textit{PR-la:luc} Col-0 in terms of both rosette size and bolt height, and bolting in \textit{crl} was initiated slightly later than in Col-0 (Table 5.1). Leaves of \textit{crl} plants had a slightly longer, thinner shape in comparison to \textit{PR-la:luc} Col-0 (Fig.5.1b).

Table 5.1. Growth features of \textit{crl} in comparison to \textit{PR-la:luc} Col-0 plants.

<table>
<thead>
<tr>
<th></th>
<th>\textit{PR-la:luc} Col-0</th>
<th>\textit{crl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosette diameter (Day 35)</td>
<td>6.71 (0.07)</td>
<td>4.91 (0.05)</td>
</tr>
<tr>
<td>Days to bolting</td>
<td>45</td>
<td>47-49</td>
</tr>
<tr>
<td>Bolt height (Day 56)</td>
<td>32.94 (1.61)</td>
<td>26.69 (2.29)</td>
</tr>
</tbody>
</table>

Measurements are shown in centimetres (cm), with the standard error at the 95% confidence level shown in parentheses. Ten plants of both \textit{crl} and Col-0 were analysed.
Fig. 5.1. The phenotype of cir1 in comparison to PR-1a:luc Col-0 plants.
a: Five-week old representative PR-1a:luc and cir1 plants were painted with 1mM luciferin. Plants were imaged for constitutive luc activity in the ultra low-light imaging camera. PR-1a:luc is shown on the left, cir1 on the right.
b: Representative five-week old PR-1a:luc Col-0 and cir1 plants. PR-1a:luc is shown on the left, cir1 on the right.

As shown in Fig.5.1b, no spontaneous HR lesion formation was visualised in cir1 leaves. However, it has been reported that micro-lesion formation, as a result of accumulating ROS, underlies SAR (Alvarez et al. 1998) and thus micro-lesion formation in cir1 may be occurring. In order to test this, leaves from four-week old naïve cir1 plants were stained with trypan blue (Cao et al. 1998). Trypan blue stains dead cells thus allowing for easy identification of potential micro-lesions. Of the ten cir1 leaves stained, no areas of dead cells could be visualised as was the case with Col-0 leaves. Trypan blue staining revealed areas of dead cells in leaves from the
The *cpr5* mutant shows spontaneous formation of lesions and a similar pattern of constitutive defence gene expression and disease resistance as *cirl* (Bowling et al. 1997). A representative trypan blue stained *cirl* leaf is shown in Fig. 5.2 and a *cpr5* leaf is shown as the positive control.

![Image](image-url)

**Fig.5.2. Comparison of micro-lesion formation between *cirl* and *cpr5*.** Leaves were stained with trypan blue, de-stained and areas of cell death were visualised using a dissecting microscope. Dead cells were visualised as areas with a dark blue colour. *cirl* is shown on the left, *cpr5* is shown on the right. Size bar represents 5mm.

These results show that the only easily identifiable phenotype of *cirl* was constitutive expression of *PR-1a:luc* in five-week old naïve plants. This phenotype was thus chosen as the method of identifying *cirl* in the progeny of crosses described in the following section.

**5.3. Genetic analysis of *cirl***

**Segregation analysis**

As recorded in Chapter 4, the *ceb* mutant lines were back-crossed to the *PR-1a:luc* Col-0 transgenic line. Loss of *PR-1a:luc* expression was detected in the F1 progeny of the *cirl X PR-1a:luc* cross, indicating that *cirl* is a recessive mutation (Koornneef & Stam 1992). In order to confirm this result, reciprocal crosses were made between *cirl* and *PR-1a:luc* Col-0 and between *cirl* and Col-0. Five-week old plants from both the F1 and the F2 progeny were analysed for constitutive luc activity using the ultra low-light imaging camera. The results obtained are outlined in Table 5.2.
Analysis of the F1 plants showed loss of constitutive luc activity in all cases, confirming that \textit{cirl} is a recessive mutation. To determine if \textit{cirl} defines a single gene, the segregation of \textit{PR-la:luc} expression was investigated in F2 populations of both types of crosses. In the case of the F2 population of the \textit{cirl} X \textit{PR-la:luc Col-0} cross, a ratio of one plant with luc activity to three plants without luc activity was obtained, indicating that the \textit{cirl} trait is controlled by a single gene and that the mutated \textit{CIRL} gene is not linked to the \textit{PR-la:luc} transgene. This result was confirmed by analysis of the F2 progeny of the \textit{cirl} X \textit{Col-0} cross. However, in this case, the \textit{PR-la:luc} transgene segregates as a monogenic dominant trait and constitutive luc activity was only visualised in three-quarters of the F2 plants homozygous for \textit{cirl} (Table 5.2).

\textit{Complementation analysis}

As documented in Chapter 4, a total of four recessive mutations displaying constitutive SAR were isolated in this study. In order to determine if \textit{cirl} is allelic to any of the other three mutants, crosses were made between \textit{cirl} and \textit{ceb3}, \textit{ceb4} or \textit{ceb6}. In addition, \textit{cirl} was crossed to both the \textit{cprl} (Bowling et al. 1994) and \textit{cpr5} (Bowling et al. 1997) mutants, which are also recessive and display constitutive SAR. \textit{cprl} has been placed downstream of the HR, but upstream of SA accumulation in the SAR signal transduction pathway (Bowling et al. 1994, Fig.1.3), whereas \textit{cpr5} has been placed upstream of the HR (Bowling et al. 1997, Fig.1.3). F1 plants from each cross were imaged using the ultra low-light imaging camera in order to identify plants producing constitutive luc activity. The rationale was that the presence of a single copy of the \textit{PR-la:luc} transgene would be sufficient to identify constitutive \textit{PR-la:luc} expression, if any of the recessive mutations were allelic to \textit{cirl} and thus unable to complement \textit{cirl} in F1 plants. The results obtained are outlined in Table 5.3. Constitutive luc activity was lost in the majority of the F1 progeny of all the crosses, except for the progeny of the \textit{cirl} X \textit{ceb3} cross, all of which showed constitutive luc activity. This indicated that \textit{cirl} and \textit{ceb3} were allelic. Although the reciprocal cross was not performed, it is unlikely that the constitutive \textit{PR-la:luc} expression in F1 plants is simply due to self-pollination of
Table 5.2 Segregation analysis of *cir*1*.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Donor</th>
<th>Recipient</th>
<th>Hypothesis</th>
<th>No. tested</th>
<th>Luc activity</th>
<th>No luc activity</th>
<th>$\chi^2$</th>
<th>$P=5%$</th>
<th>Accept?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Observed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td><em>PR-lac:</em></td>
<td><em>cir</em>1</td>
<td></td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>PR-lac:</em></td>
<td><em>cir</em>1</td>
<td></td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td><em>cir</em>1</td>
<td><em>PR-lac:</em></td>
<td>1:3$^a$</td>
<td>60</td>
<td>15</td>
<td>15</td>
<td>45</td>
<td>45</td>
<td>&lt;3.841</td>
</tr>
<tr>
<td>F1</td>
<td><em>Col-0:</em></td>
<td><em>cir</em>1</td>
<td></td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td><em>cir</em>1</td>
<td><em>Col-0:</em></td>
<td></td>
<td>16$^*$$^b$</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td><em>cir</em>1</td>
<td><em>Col-0:</em></td>
<td>3:13$^b$</td>
<td>72</td>
<td>10</td>
<td>13.5</td>
<td>62</td>
<td>58.5</td>
<td>1.116</td>
</tr>
</tbody>
</table>

The $\chi^2$ value was calculated and compared to the critical value at the 5% probability level (Ennos 2000).

$^a$ The hypothesis was that *cir*1 segregates as a monogenic recessive trait with respect to constitutive luc activity.

$^b$ The hypothesis was that *cir*1 segregates as a monogenic recessive trait. However, the *PR-lac:* transgene will segregate as a monogenic dominant trait and constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir*1 mutation.

$^*$ These F1 plants were identified as kanamycin resistant *in vitro*, thus indicating that the transformation cassette containing the *PR-lac:* gene and the kanamycin resistance gene had been donated by the *cir*1 pollen donor.
ceb3, as the 20 F1 plants analysed originated from a pool of seed from three separate siliques. The F2 progeny of cir1 X ceb3 cross was analysed for constitutive luc activity and 53 plants out of 54 showed constitutive luminescence (Table 5.3), further indicating that cir1 and ceb3 were allelic. It is likely that the single plant not showing constitutive PR-1a:luc expression was due to contamination during seed collection. Twelve F1 plants from the cir1 X cpr1 cross were examined for constitutive luc activity. One plant was found to express PR-1a:luc constitutively (Table 5.3) and the reason for this unexpected segregation is unknown. However, it appears that cir1 is not allelic to either cpr1 or cpr5.

**Table 5.3. Complementation analyses of cir1.**

<table>
<thead>
<tr>
<th>Generation</th>
<th>Donor</th>
<th>Recipient</th>
<th>No. tested</th>
<th>Luc activity</th>
<th>No luc activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>cir1</td>
<td>ceb3</td>
<td>20</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>F2</td>
<td>cir1</td>
<td>ceb3</td>
<td>54</td>
<td>53</td>
<td>1</td>
</tr>
<tr>
<td>F1</td>
<td>cir1</td>
<td>ceb4</td>
<td>24</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
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<td>cir1</td>
<td>ceb6</td>
<td>20</td>
<td>0</td>
<td>20</td>
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<tr>
<td>F1</td>
<td>cir1</td>
<td>cpr1</td>
<td>12</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>F1</td>
<td>cir1</td>
<td>cpr5</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

**Mapping cir1**

In order to determine the map position of the cir1 mutation, cir1 Col-0 plants were crossed to Landsberg erecta (Ler) plants. The F1 progeny did not exhibit constitutive PR-1a:luc expression as expected for a recessive trait (data not shown). These plants were allowed to self-pollinate and the F2 progeny of F1 plants were analysed for constitutive PR-1a:luc expression using an ultra low-light imaging camera. Initially an F2 population of 62 plants with constitutive luc activity were identified. The chromosomal location of the cir1 mutation was then mapped with the 62 plants and a variety of PCR-based markers. Linkage analysis was performed by amplification of the polymorphic DNA regions corresponding to the marker, followed by the determination of the PCR product sizes. CAPS (cleaved co-dominant amplified polymorphic sequences; Konieczny & Ausabel 1993) and SSLP (single sequence length polymorphism; Bell & Ecker 1994) markers approximately
20 to 30 centimorgans (cM) apart on the recombinant inbred genetic map (Lister & Dean 1993) were used. Markers used in this study were chosen from each of the five chromosomes and are outlined in Table 5.4. Markers were selected from databases maintained at TAIR (http://www.arabidopsis.org) and were chosen for easy identification of the polymorphic Col-0 and Ler PCR products by agarose gel electrophoresis. Because the markers are co-dominant, the genotype for all 124 chromosomes could be monitored and scored at every locus tested. Generally, a recombination frequency of less than 30% in a small population is accepted as evidence for linkage between the mutation and the marker under test (Ponce et al. 1999). However, linkage could not be identified between any of the markers tested in this population of 62 plants (results not shown). It is likely that this was due to mis-scoring of the cirl mutant phenotype (constitutive luc activity as scored in the ultra low-light imaging camera) and PR-la:luc plants were scored as cirl. Mis-scoring of the mutant phenotype is thought to be a common problem in mapping experiments (Lukowitz et al. 2000).

In order to solve this problem, a different F2 population from the cirl X Ler cross was screened and a further 24 F2 plants producing constitutive luc activity were identified. Greater care was taken to ensure that the plants were correctly scored. All plants were grown under uniform growth conditions and plants were scored for constitutive luc activity when they were exactly five weeks old. Positive controls (cirl plants) were grown in each tray and the number of leaves showing constitutive PR-la:luc expression per plant were determined. Generally, five-week old cirl plants have approximately one half of all leaves expressing PR-la:luc constitutively (Fig.5.1a). F2 plants were only scored for the mutant phenotype if they corresponded exactly to cirl in the tray undergoing analysis, namely that one half of all leaves produced constitutive luc activity. Using the 48 chromosomes contributed by these 24 plants and the PCR-based markers outlined in Table 5.4, linkage was established to markers on the lower arm of chromosome 4 (Table 5.4). In order to get a clearer idea of the map position of cirl on chromosome 4, a further 24 F2 plants expressing constitutive luc activity were identified and analysed for linkage to markers on chromosome 4 (Table 5.4). Data from these 96 chromosomes indicated
Table 5.4. Mapping *cir1* using CAPS and SSLP markers.

<table>
<thead>
<tr>
<th>Plant Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chromosome 1</th>
<th>Chromosome 2</th>
<th>Chromosome 3</th>
<th>Chromosome 4</th>
<th>Chromosome 5</th>
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<tbody>
<tr>
<td></td>
<td>nga63 AthS0392 nga128 THY1 nga168</td>
<td>AthS0392 nga128 THY1 nga168</td>
<td>AthS0392 nga128 THY1 nga168</td>
<td>AthS0392 nga128 THY1 nga168</td>
<td>AthS0392 nga128 THY1 nga168</td>
</tr>
<tr>
<td>1</td>
<td>9.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46</td>
<td>100</td>
<td>30</td>
<td>73</td>
</tr>
<tr>
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<td>C</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
</tr>
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\(\%R^{c}\) = Percentage recombination, calculated as follows: 2 X L scores + H scores / total number of chromosomes scored.

\(C = \) homozygous Col-0, H = heterozygous, L = homozygous Ler, U = Undetermined.

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\(a\) All 48 F2 plants analysed segregated as a recessive trait and exhibited the \textit{cir}I phenotype of constitutive luc activity. Plants were analysed in two separate groups: 1 to 24 and 25 to 48.

\(b\) Genetic position on the recombinant inbred (RI) map (Lister & Dean 1993). Position is indicated in centimorgans (cM).

\(c\) Genetic position on the recombinant inbred (RI) map (Lister & Dean 1993). Position is indicated in centimorgans (cM).
that *cirl* maps to a 28 cM region, approximately 9.4 cM south from nga1111 and approximately 12.5 cM north from g4539 (Table 5.4).

### 5.4. Relationship of *cirl* to SA

Treatment of a wide variety of plants with SA is known to induce *PR-1* gene expression (Ryals et al. 1996) and accumulation of SA often precedes or parallels *PR-1* gene induction during SAR in Arabidopsis (Cameron et al. 1999). Having shown that *cirl* displayed constitutive expression of *PR-1, PR-2* and *PR-5*, it was necessary to determine the position of *cirl* relative to SA in the SAR signal transduction pathway. Both a biochemical and a genetical approach were taken in order to investigate the relationship of *cirl* to SA.

**Determination of the endogenous level of SA in *cirl***

The amount of endogenous SA in five-week old naïve Col-0 and *cirl* plants was determined by HPLC analysis (Thomson, unpublished results). Col-0 plants inoculated with *Pst* DC3000 (*avrB*) two days before analysis were included as a control. As shown in Fig.5.3, levels of endogenous SA in *cirl* plants was approximately ten times higher than Col-0. Interestingly, endogenous SA levels in *cirl* were approximately the same as the level of SA accumulated in Col-0 plants inoculated with *Pst* DC3000 (*avrB*).

**The *cirl* phenotype in *nahG* plants.**

To define the role of SA in *cirl* plants, a cross was made between *cirl* and a transgenic plant containing the *nahG* gene from *Pseudomonas putida* (Delaney et al. 1994). *nahG* encodes a salicylate hydroxylase which converts SA to catechol. Plants expressing the *nahG* transgene are thus unable to accumulate SA, undergo SAR or express *PR-1* in response to avirulent pathogens (Lawton et al. 1995). As *cirl* segregates as a recessive trait, F1 plants from the *cirl X nahG* cross did not express constitutive *PR-1a:luc* (Table 5.5). F1 plants were allowed to self-polinate and F2 plants were analysed for constitutive *PR-1a:luc* expression. As *cirl* segregates as a monogenic recessive trait and the *PR-1a:luc* transgene segregates as a
Fig. 5.3. **Comparison of free SA levels in Col-0, Col-0 inoculated with *Pst DC3000 (avrB)* and *cir1***.

Col-0 plants were inoculated with *PstDC3000 (avrB)* two days prior to analysis. Leaves from five-week old plants were collected for HPLC analysis of free SA content. The values presented are the average of three replicates ± SE (micrograms SA per gram fresh weight leaf tissue).

A monogenic dominant trait, constitutive luc activity will normally only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case the hypothesis was that *nahG*, which segregates as a monogenic dominant trait, will degrade SA in three quarters of the F2 plants, leading to loss of constitutive luc activity in three quarters of the plants. Nineteen F2 plants were analysed, two of which produced constitutive luc activity (Table 5.5). This was consistent with the ratio of 3 plants expressing *PR-la:luc* to 61 plants not showing luc activity (Table 5.5). F2 plants were allowed to self-pollinate and F3 seedlings were tested for sensitivity to SA *in vitro*. When *nahG* plants are grown on 0.5mM SA *in vitro*, brown deposits are formed in the roots (Bowling et al. 1994). These deposits are phenolic by-products from the breakdown of catechol (Bowling et al. 1994). Of the 19 F3 families grown on SA, 12 showed brown deposits in the roots. The two F3 families which produced constitutive luc activity did not show brown deposits in the roots, indicating that the F2 parents did not contain the *nahG* transgene. These results indicate that loss of SA accumulation in *cir1* abolishes constitutive luc expression in *cir1*. As the visual phenotype of *cir1* (constitutive luc activity) is dependent on the accumulation of SA, it was not possible to isolate a *cir1:nahG* double mutant using the ultra low-light imaging camera.
Isolation of a cirl:npri double mutant.

The mutant npri is insensitive to SA accumulation and abolishes PR gene expression during SAR (Cao et al. 1994). In order to determine if cirl-induced constitutive luc activity is dependent on a functional NPR1 protein, a cross was made between cirl and npri plants. As both mutants segregate as recessive traits, F1 plants did not produce constitutive luc activity (Table 5.5). F1 plants were allowed to self-pollinate and F2 plants were analysed for constitutive luc activity. As cirl segregates as a monogenic recessive trait and the PR-1a:luc transgene segregates as a monogenic dominant trait, constitutive luc activity will normally only be visualised in three-quarters of the F2 plants containing the cirl mutation. In the case of the F2 population from the cirl X npri cross, two hypotheses were possible. Firstly, that npri would abolish constitutive PR-1 activity, as was the case in the cpr5:npri double mutant (Bowling et al. 1997). Secondly, that npri would not suppress constitutive PR-1 activity, as was the case in the cpr6:npri double mutant (Clarke et al. 1998). Out of a population of 54 F2 plants from the cirl X npri cross, 11 produced constitutive luc activity (Table 5.5). This data fitted both hypotheses (Table 5.5), thus making it difficult to determine the influence of npri on cirl-induced constitutive luc activity. The presence of the npri mutation in the F2 population was thus determined. The wild-type NPR1 gene contains a NalIII restriction digestion site which is abolished in the npri-i allele (Cao et al. 1997). This was the allele of the npri mutant used in the present study. Thus, restriction digestion analysis of the npri-i region amplified by PCR is a tool that can be used to determine the presence of a homozygous npri-i mutation (Clarke et al. 1998). DNA was extracted from the 11 F2 plants with constitutive luc activity and an additional 16 F2 plants selected at random from the F2 population of 54 plants. Col-0 wild-type and npri plants were also included as controls. PCR with npri-i specific primers was performed on all 29 samples and the PCR products were digested with the NalIII restriction enzyme. A 100 bp product is produced in Col-0 samples which is missing in homozygous npri-i plants (results not shown). Thus, the 27 F2 plants could be scored for the presence of the npri-i mutation. Out of the 11 F2 plants which showed constitutive luc activity, one contained the homozygous npri-i mutation. This F2 plant was thus a cirl:npri double mutant. Out of the additional
Table 5.5. Genetic analysis of cirl X nahG and cirl X npr1

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The $\chi^2$ value was calculated and compared to the critical value at the 5% probability level (Ennos 2000).

<sup>a</sup> cirl segregates as a monogenic recessive trait. In addition, the PR-1a:Luc transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the cirl mutation. In this case the hypothesis was that nahG, which segregates as a monogenic dominant trait, will degrade SA in three-quarters of the F2 plants, leading to loss of constitutive luc activity in three-quarters of the plants.

<sup>b</sup> cirl segregates as a monogenic recessive trait. In addition, the PR-1a:Luc transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the cirl mutation. The hypothesis was that npr1, which is insensitive to SA and segregates as a monogenic recessive trait, will block constitutive luc activity in one quarter of the F2 plants.

<sup>c</sup> cirl segregates as a monogenic recessive trait. In addition, the PR-1a:Luc transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the cirl mutation. In this case, the hypothesis was that npr1 did not block constitutive luc activity in the F2 plants.

*F3 seedlings from both plants were tested for sensitivity to SA in vitro. Brown roots (indicative of the presence of nahG; Bowling et al. 1994) were not detected. Both F3 families were kanamycin resistant.

**All 11 F2 plants were tested for the presence of an Nia III restriction site in the wild type NPR1 gene (Cao et al. 1997). The restriction site was lost in one of the 11 plants, indicating the presence of the npr1-1 mutation (Clarke et al. 1998).
16 F2 plants scored, three contained the homozygous *npr1-1* mutation (results not shown). As the *cirl:npr1* double mutant expressed constitutive luc activity, it could be concluded that second hypothesis was correct, namely that *npr1* does not suppress constitutive *PR-1* activity in *cirl* plants.

*The influence of SA application on constitutive PR-1 and PDF1.2 expression in cirl.*

*Cirl* expresses *PR-1a:luc*, *PR-1* and *PDF1.2* constitutively. In order to investigate further the relationship between *cirl* and SA, *cirl* and *PR-1a:luc* Col-0 plants were inoculated with *Pst* DC3000 (*avrB*) or 1mM SA. Leaf samples were assayed for luc activity using the luminometer assay (Fig.5.4b) or subjected to Northern blot analysis using the *PR-1* and *PDF1.2* probes (Fig.5.4a). In the case of the *PR-1a:luc* Col-0 plants, addition of SA or inoculation with *PstDC3000* (*avrB*) induced both luc activity (Fig.5.4b) and *PR-1* expression (Fig.5.4a), which corresponded to previous results (Fig.3.2, Fig.4.1, Fig.4.2, Fig.4.3, Fig.4.4). Inoculation of Col-0 plants with *PstDC3000* (*avrB*) induced *PDF1.2* expression, whereas SA application had no effect on *PDF1.2* expression (Fig.5.4a). This result corresponds to previous reports (Penninclx et al. 1996, Malek & Dietrich 1999). In the case of *cirl*, addition of SA or inoculation with *PstDC3000* (*avrB*) induced both luc activity (Fig.5.4b) and *PR-1* expression (Fig.5.4a) to levels approximately 10-fold higher than those found in naïve *cirl* plants. This indicates that *cirl* is somehow primed or ‘potentiated’ for SA-induction of *PR-1* expression. However, inoculation of *cirl* with *PstDC3000* (*avrB*) partially suppressed *PDF1.2* expression and SA application completely suppressed *PDF1.2* expression in comparison to naïve *cirl* plants (Fig.5.4a). Thus, applied SA in *cirl* plants appears to play a positive role in elevating *PR-1* expression, but plays a negative role by suppressing *PDF1.2* expression.
Fig. 5.4. PR-I expression is elevated and PDF1.2 expression is suppressed by SA application in cirl.

a: Northern blot analysis of PR-I and PDF1.2 mRNA expression in Col-0 and cirl. Naive plants, plants treated with 1mM SA and harvested after 24 hours, or plants inoculated with PstDC3000 (avrB) and harvested after 48 hours were analysed. RNA samples were sequentially probed with the PR-I, PDF1.2 and r18 probes. The table below the blot represents the fold induction of the gene expression for each sample relative to Col-0. The expression was quantified using the ImageQuant software, adjusted to the expression of the loading control (r18) and normalised to the expression of Col-0, which was set to 1 unit. avrB, plants inoculated with PstDC3000 (avrB).

b: Luminometer assay of cirl and PR-la:luc plants. Naive plants, plants treated with 1mM SA and harvested after 24 hours, or plants inoculated with PstDC3000 (avrB) and harvested after 36 hours were analysed for luc activity. Presented values are averages of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.
Ethylene, in combination with jasmonates, has been shown to play an important role in signal transduction leading to PDF1.2 expression (Penninckx et al. 1998). As naïve cirl plants express strong constitutive PDF1.2 expression, it was decided to investigate the level of ethylene evolution in cirl plants.

Ethylene readings using a gas chromatograph were conducted by Dr Pietro Ianetta, at the Scottish Crop Research Institute, Invergowrie, Dundee and the results obtained are outlined in Fig.5.5. As ethylene is released upon virulent pathogen infection (reviewed in Johnston & Ecker 1998), Col-0 plants inoculated with virulent PstDC3000 were included as a positive control. These plants were assayed three days after inoculation. Ethylene evolution in wild type Col-0 plants was low (Fig.5.5) and corresponded to levels previously obtained for light-grown Arabidopsis plants (Guzman & Ecker 1990). However, ethylene evolution in cirl plants was significantly more than in Col-0 plants (Fig.5.5). A one-way ANOVA test was performed which showed a statistically significant difference in ethylene evolution between Col-0 and cirl. Ethylene evolution by naïve cirl plants was equivalent to ethylene evolution by Col-0 plants inoculated with virulent PstDC3000 (Fig.5.5).

When Arabidopsis seedlings are grown in the dark in the presence of ethylene, they undergo the ‘triple response’, which consists of an inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and an exaggerated curvature of the apical hook (Ecker 1995). A number of Arabidopsis mutants have been isolated which display a constitutive triple response and greater ethylene evolution (Guzman & Ecker 1990, Kieber et al. 1993, Roman et al. 1995). In order to test cirl for a constitutive triple response in vitro, cirl seeds were plated out on to medium containing 10μM 1-amino-cyclopropane-1-carboxylic acid (ACC). ACC is the immediate precursor to ethylene in the biosynthetic pathway (Kende 1993) and its inclusion in tissue culture medium is a convenient alternative method for testing the ethylene triple response. After 5 days in the dark on MS medium supplemented with 10μM ACC, Arabidopsis seedlings have germinated and root inhibition in Col-0...
wild-type is clearly seen (Alonso et al. 1999). ACC inhibited rooting in \textit{cirl} seedlings and \textit{cirl} seedlings did not show inhibition of rooting in the absence of ACC (results not shown). Thus, \textit{cirl} responded as Col-0 wild-type to ACC and did not show constitutive expression of the triple response.

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**Fig. 5.5. Comparison of ethylene evolution in Col-0, Col-0 inoculated with \textit{PstDC3000} and \textit{cirl}.**

Five-week old plants were enclosed in a container and ethylene produced over a 24 hour period was collected. Ethylene concentration was determined by GC analysis. The values presented are the average of six replicates ± SE (nanograms ethylene produced per gram of fresh weight plant material).

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5.6. Epistasis between \textit{cirl}, \textit{ein2.1} and \textit{jarl}

*Isolation of \textit{cirl}:\textit{ein2.1} and \textit{cirl}:\textit{jarl} double mutants*

In order to determine the role of ethylene and jasmonates in constitutive \textit{PDF1.2} expression and disease resistance in \textit{cirl} plants, \textit{cirl} was crossed with the ethylene insensitive mutant \textit{ein2.1} (ethylene insensitive 2.1) (Guzman & Ecker 1990) and the Me-JA-insensitive mutant \textit{jarl} (Staswick et al. 1992). As all three mutations (\textit{cirl}, \textit{ein2.1} and \textit{jarl}) were recessive, double mutants from the \textit{cirlXein2.1} and \textit{cirlXjarl} crosses were identified in the F2 populations from each cross (Table 5.6).

As \textit{cirl} segregates as a monogenic recessive trait and the \textit{PR-1a:luc} transgene segregates as a monogenic dominant trait, constitutive luc activity will normally only
be visualised in three-quarters of the F2 plants containing the *cirl* mutation. In the case of both the segregating F2 populations from the *cirl X ein2.1* cross and the *cirl X jar1* cross, the hypothesis was that the second mutation would have no influence on *cirl*-induced constitutive luc activity. Segregation data from both F2 populations confirmed these hypotheses (Table 5.6). F2 plants which produced luc constitutively were allowed to self pollinate and F3 plants were analysed for either the *ein2.1* or *jar1* phenotype. *ein2.1* seedlings are insensitive to 10 µM ACC and produce roots *in vitro* (Alonso et al. 1999). One F3 family from the *cirl X ein2.1* cross was identified which was insensitive to ACC in all seedlings, thus indicating that it represented a *cirl:ein2.1* double mutant. This line was used in further experiments. *jar1* plants are small and dark green in comparison to Col-0 and *jar1* seedlings produce longer roots when exposed to 10 µM Me-JA *in vitro* than Col-0 seedlings (Staswick et al. 1992). One F2 plant from the *cirl X jar1* cross produced both luc constitutively and had a small dark green phenotype indicative of *jar1* (Table 5.6). F3 seedlings from the self-pollination of this F2 plant produced longer roots when exposed to 10 µM Me-JA *in vitro* than Col-0 or *cirl*, indicating that this line represented a *cirl:jar1* double mutant.

A cross was also made between the dominant ethylene-insensitive mutant *etrl* (Bleecker et al. 1988) and *cirl*. F1 plants from this cross were identified (Table 5.6) but due to time constraints, F2 plants were not analysed. Two attempts were also made to cross *cirl* with the Me-JA-insensitive mutant *coil* (Feys et al. 1994). However, *coil* plants became infected with powdery mildew in the greenhouse at both attempts and died before siliques had matured.
Table 5.6. Genetic analysis of *cir1* X *ein2.1* and *cir1* X *jar1*.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Donor</th>
<th>Recipient</th>
<th>Hypothesis</th>
<th>No. tested</th>
<th>Luc activity</th>
<th>No luc activity</th>
<th>$\chi^2$</th>
<th>$P=5%$</th>
<th>Accept?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td><em>cir1</em></td>
<td><em>ein2.1</em></td>
<td>3:13a</td>
<td>6*</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td><em>cir1</em></td>
<td><em>ein2.1</em></td>
<td>3:13b</td>
<td>22</td>
<td>5**</td>
<td>4</td>
<td>17</td>
<td>18</td>
<td>0.306</td>
</tr>
<tr>
<td>F1</td>
<td><em>cir1</em></td>
<td><em>etrl</em></td>
<td>3*</td>
<td>3*</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td><em>cir1</em></td>
<td><em>jar1</em></td>
<td>5*</td>
<td>5*</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td><em>cir1</em></td>
<td><em>jar1</em></td>
<td>3:13b</td>
<td>68</td>
<td>17***</td>
<td>13</td>
<td>51</td>
<td>55</td>
<td>1.806</td>
</tr>
</tbody>
</table>

The $\chi^2$ value was calculated and compared to the critical value at the 5% probability level (Ennos 2000).

a *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case the hypothesis was that *ein2.1* will have no effect on luc activity.

b *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case, the hypothesis was that *jar1* did not block constitutive luc activity in the F2 plants.

* These F1 plants were identified as kanamycin resistant *in vitro*, thus indicating that the transformation cassette containing the *PR-1a:luc* gene and the kanamycin resistance gene had been donated by the *cir1* pollen donor.

**F3 seedlings from these plants were tested for sensitivity to ACC *in vitro*. When grown in the dark, Col-0 seedlings do not root on medium containing ACC, whereas *ein2.1* seedlings are insensitive to ACC. One line was insensitive to ACC, thereby defining a *cir1:ein2.1* double mutant.

***One of these F2 plants also showed the *jar1* phenotype (small, dark green leaves in comparison to Col-0). F3 seedlings derived from self-pollination of this F2 plant were insensitive to Me-JA inhibition of rooting *in vitro*, thus defining a *cir1:jar1* double mutant.
Expression of PR-1 and PDF1.2 in cir1:ein2.1 and cir1:jar1.

Five-week old cir1:ein2.1 and cir1:jar1 plants were assayed for constitutive luc activity, and PR-1 and PDF1.2 expression (Fig. 5.6). Constitutive luc activity was determined using the ultra-low light imaging camera (Fig. 5.6b&d) and the luminometer assay (Fig. 5.6c&e). Both methods showed that luc activity was reduced in cir1:ein2.1 and cir1:jar1 in comparison to cir1 plants, both in the number of leaves per plant expressing PR-1a:luc (Fig. 5.6b&d) and in average luc activity (Fig. 5.6c&e). Three leaves chosen at random from a cirl, cirl:ein2.1 and cirl:jar1 plant and imaged in the ultra-low light camera are shown in Fig. 5.6. All three cirl leaves expressed PR-1a:luc constitutively in both cases (Fig. 5.6b&d), whereas only one cirl:ein2.1 leaf out of three showed luc activity (Fig. 5.6b) and two cirl:jar1 leaves showed luc activity (Fig. 5.6d). A luminometer assay of naïve cirl and cirl:ein2.1 F3 plants showed an average five-fold reduction in luc activity in cirl:ein2.1 in comparison to cirl (Fig. 5.6c). PR-1a:luc expression was reduced approximately ten-fold in cirl:jar1 F3 plants in comparison to cirl (Fig. 5.6e). Luc activity was virtually undetectable in naïve PR-1a:luc transgenic plants (Fig. 5.6c&e). Both of these results indicate that PR-1a:luc is expressed constitutively in cirl:ein2.1 and cirl:jar1 plants, but to levels much lower than in cirl. Thus, EIN2 and JAR1 appear to be partial requirements for constitutive luc activity in cirl plants.

Leaves from five-week old naïve Col-0, ein2.1, cirl and F3 cirl:ein2.1 were harvested for Northern blot analysis with the PR-1 and PDF1.2 probes. Plants from all four lines were also treated with 1mM SA or inoculated with PstDC3000 (avrB) and harvested for RNA extraction. Results from the Northern blot analysis are shown in Fig. 5.6a. Col-0 and ein2.1 samples did not express PR-1 but treatment with 1mM SA or inoculation with PstDC3000 (avrB) induced PR-1 expression. The naïve cirl sample expressed PR-1 constitutively and treatment with 1mM SA or inoculation with PstDC3000 (avrB) induced PR-1 expression to a higher level. Constitutive PR-1 expression was lost in the cirl:ein2.1 double mutant (Fig. 5.6a), indicating that EIN2 is an absolute requirement for constitutive PR-1 expression in cirl. Treatment of cirl:ein2.1 plants with 1mM SA or inoculation with PstDC3000 (avrB) induced
Fig. 5.6. Expression of PR-1 and PDF1.2, and production of luc activity in cir1:ein2.1 and cir1:jarl plants.

a: Northern blot analysis of PR-1 and PDF1.2 mRNA expression in Col-0, cir1, ein2.1, jar1 and the cir1:ein2.1 and cir1:jar1 double mutants. Naïve plants, plants treated with 1mM SA and harvested after 24 hours, or plants inoculated with PstDC3000 (avrB) and harvested after 48 hours were analysed. RNA samples were sequentially probed with the PR-1, PDF1.2 and r18 probes. The table below the blot represents the fold induction of the gene expression for each sample relative to Col-0. The expression was quantified using the ImageQuant software, adjusted to the expression of the loading control (r18) and normalised to the expression of Col-0, which was set to 1 unit.

b&d: Three representative leaves from five-week old cir1 (b&d), cir1:ein2.1 (b) and cir1:jar1 plants (d) were painted with 1mM luciferin and imaged for constitutive PR-1a:luc production in the ultra low-light imaging camera.

c&e: Luminometer assay of cir1 (c&e), PR-1a:luc (c&e), cir1:ein2.1 (c) and cir1:jar1 (e) plants. Naïve plants were analysed for luc activity. Presented values are the averages of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.
PR-1 expression (Fig. 5.6a). PDF1.2 was only expressed in the Col-0+avrB and naïve cir1 samples (Fig. 5.6a). Previously results indicated that PDF1.2 was also weakly expressed in a cir1+avrB sample (Fig. 5.4a) but this was not seen in this case. PDF1.2 was not expressed in the ein2.1+avrB sample or in the naïve cir1:ein2.1 sample (Fig. 5.6a), indicating that EIN2 is an absolute requirement for PDF1.2 expression in cir1. Furthermore, EIN2 also appears to be an absolute requirement for induction of PDF1.2 expression in response to an avirulent bacterial pathogen.

In a preliminary experiment, leaves from a jar1 plant and the F2 cir1:jar1 plant were harvested for Northern blot analysis (Fig. 5.6a). The jar1 sample did not express either PR-1 or PDF1.2 (Fig. 5.5a). Previous results (Fig. 4.4a) indicated that PR-1 and PDF1.2 expression is induced in jar1 by inoculation with PstDC3000 (avrB). Surprisingly, PR-1 expression was lost in the cir1:jar1 sample (Fig. 5.6a). Weak PDF1.2 expression was obtained in the cir1:jar1 sample (Fig. 5.6a), confirming that jasmonate signal transduction plays a partial role in PDF1.2 expression in cir1.

Disease resistance of cir1:ein2.1 and cir1:jar1.
As both the cir1:ein2.1 and cir1:jar1 double mutants showed a huge reduction or complete loss of PR-1 and PDF1.2 expression, it was decided to test them for disease resistance. cir1 plants showed enhanced resistance to both a virulent bacterial pathogen (PstDC3000) and a virulent oomycete pathogen (P. parasitica Noco2). The aim of the disease resistance assays of the two double mutants was to determine the role of ethylene and jasmonate signal transduction in cir1-induced disease resistance.

The results of the disease resistance assays of cir1:ein2.1 and cir1:jar1 to PstDC3000 and P. parasitica Noco2 are outlined in Fig. 5.7. The positive control consisted of Col-0 plants pre-treated with 1mM SA twice, five and three days prior to pathogen inoculation. Either npri or nahG plants were included as a negative control (results not shown). For the PstDC3000 resistance assay, five-week old plants were inoculated with bacteria. After three days, inoculated leaves were harvested, ground and diluted in 10mM MgCl2 buffer. Dilutions were plated out onto KB medium and the number of colony forming units (cfu) per leaf disk were
PstDC3000 bacteria grew in Col-0 and ein2.1 samples to similar levels while bacterial growth was reduced in cir1 (Fig.5.7a). However, bacterial growth in cir1:ein2.1 resembled that in ein2.1. A statistically significant difference in bacterial titre was found between cir1 and both ein2.1 and cir1:ein2.1 using the Mann-Whitney test at the 95% confidence level (Minitab Version 12). These results indicate that cir1-induced resistance to PstDC3000 is dependent on EIN2 and ethylene signal transduction. In the PstDC3000 disease resistance assay for cir1:jar1, bacteria grew in Col-0, jar1 and cir1:jar1 plants to similar levels whereas bacterial growth was reduced in cir1 (Fig.5.7c). A statistically significant difference in bacterial titre was found between cir1 and both jar1 and cir1:jar1 using the Mann-Whitney test at the 95% confidence level (Minitab Version 12). This also indicates that cir1-induced resistance to PstDC3000 is dependent on JAR1 and jasmonate signal transduction. Bacterial growth in cir1 plants in both experiments was less or equivalent to bacterial growth in Col-0 plants treated with SA (Fig.5.7a&c), indicating that cir1-induced resistance to PstDC3000 is equivalent to SA-induced resistance.

For the P.parasitica Noco2 resistance assay, four-week old plants were sprayed with a conidiospore solution. After incubation in a humid environment for 10 days, plants were scored for conidiophore production and a disease index was calculated (Fig.5.7b&d). The Col-0 and ein2.1 samples showed equivalent disease development, while cir1 and cir1:ein2.1 showed reduced disease development (Fig.5.7b). Thus, cir1-induced resistance to P.parasitica Noco2 appears to be independent of EIN2 and ethylene signal transduction. In Fig.5.7d, Col-0 and jar1 plants also showed equivalent disease development, whereas cir1 and cir1:jar1 showed a reduced disease index. Thus, cir1-induced resistance to P.parasitica Noco2 appears to be independent of JAR1 and jasmonate signal transduction. Furthermore, disease development on cir1 plants was equivalent to disease development on Col-0 plants treated with SA (Fig.5.7b&d), indicating that cir1-induced resistance to P.parasitica Noco2 is equivalent to SA-induced resistance.
Fig. 5.7. Disease resistance of *cirl:ein2.1* and *cirl:jar1*.

**a & c:** Resistance to *PstDC3000*. Col-0, ein2.1, cirl, cirl:ein2.1 and Col-0 plants treated with SA (Col-0+SA) were tested.

**b & d:** Disease rating of *P. parasitica* Noco2 infection 10 days after inoculation. Col-0, ein2.1, cirl, cirl:ein2.1 and Col-0 plants treated with SA (Col-0+SA) were tested.

Three plants per line were analysed in the *PstDC3000* assay and 12 plants per line were analysed in the *P. parasitica* Noco2 disease resistance assay. Presented values represent average values. Error bars represent the standard error between replicates at the 95% confidence level. Pathogen inoculation techniques and scoring methods are outlined in Chapter 2.

### 5.7. Discussion

The *cirl* mutant expressed PR-1, PR-2, PR-5, PDF1.2, and GST1 constitutively and showed enhanced resistance to both a virulent bacterial pathogen and a virulent oomycete pathogen. Further, more detailed characterisation of *cirl* in terms of phenotype, genetic analysis, relationship to the signalling molecules SA and ethylene
and epistatic interactions with SA-, Me-JA- and ethylene-insensitive mutants was presented in this chapter.

cirl plants do not show any dramatic changes in morphology in comparison to wild-type Col-0 (Fig. 5.1b) and do not display macro- or micro-lesion development (Fig. 5.2), but are slightly smaller in comparison to Col-0 (Table 5.1). However, cirl plants accumulated SA to levels approximately ten times higher than wild-type Col-0 (Fig. 5.3). The cpr6 mutant, which does not show spontaneous formation of macro- or micro-lesions (Clarke et al. 1998), and the cep mutant, which shows spontaneous lesions formation (Silva et al. 1999), are comparable to cirl in terms of SA accumulation. Levels of free SA in the cpr6 and cep mutants were seven times higher than in naïve Col-0 plants (Clarke et al. 1998, Silva et al. 1999). However, cpr1 and cpr5 accumulate SA to much higher levels, approximately 30 times more than naïve Col-0 plants (Bowling et al. 1994, Bowling et al. 1997). SA accumulation in Arabidopsis is often associated with formation of lesions, either to high levels in local tissue following inoculation with an avirulent pathogen (Lawton et al. 1995, Summermatter et al. 1995), to lower levels in systemic tissue during SAR (Lawton et al. 1995, Summermatter et al. 1995) or through constitutive expression in mutants such as cpr5 (Bowling et al. 1997), cep (Silva et al. 1999) and acd2 (Greenberg et al. 1994). Arabidopsis mutants accumulating SA to high levels may also display an abnormal morphology. For example, the cpr1 mutant shows a stunted morphology in comparison to Col-0 wild-type plants (Bowling et al. 1994) and cpr6 mutants show loss of apical dominance in both cpr6/CPR6 and cpr6/cpr6 plants (Clarke et al. 1998). Thus, cirl appears to describe a novel class of constitutive SAR mutants, where SA accumulates to higher levels without dramatically influencing plant morphology. It is possible that SA accumulation in cirl is below the threshold level needed to bring about a dramatic change in morphology such as stunting, or that accumulated SA in cirl has a cellular localisation that does not greatly interfere with plant development. Furthermore, it is possible that a HR-independent signalling pathway leads to accumulation of SA in cirl, in a manner similar to that in the dnd1 mutant (Yu et al. 1998). Identification and characterisation of a cirl:nahG double mutant will help to determine the role of accumulated SA in cirl. Unfortunately, a
ci1:nahG F2 double mutant could not be isolated using the ultra low-light imaging camera as constitutive luc activity was dependent on SA accumulation. Cloning and sequencing of the CIR1 gene should facilitate the production of a CIR1 PCR marker. Identification of homozygous ci1 plants with this marker in combination with the brown root phenotype of nahG plants on SA (Bowling et al.1994) could thus provide a method for identifying a ci1:nahG F2 double mutant.

Light-grown ci1 plants also evolved ethylene to levels approximately twice higher than wild-type Col-0 (Fig.5.5). Ethylene evolution in ci1 is comparable to the eto1 (ethylene overproducer 1), eto3 and ein2.1 mutants (Guzman & Ecker 1990, Kieber et al. 1993), where ethylene is also produced in light-grown plants to levels approximately twice those of wild-type. Ethylene evolution was also determined in acd5 mutant plants, which show spontaneous cell death, SA accumulation and expression of defence-related genes in older plants (Greenberg et al. 2000). Ethylene evolution in acd5 plants showing lesions was approximately 5-fold more than wild-type, whereas younger acd5 plants without lesions evolved approximately twice as much ethylene as wild-type plants (Greenberg et al. 2000).

Ethylene signal transduction through EIN2 is required for luc activity and PR-1 expression in ci1 (Fig.5.6). It is interesting to note that PR-1a:luc expression in ci1:ein2.1 is reduced in comparison to ci1 whereas PR-1 expression is abolished in ci1:ein2.1. This may reflect different ethylene signal transduction requirements for transcription of the Arabidopsis PR-1 gene in comparison to transcription of the luc reporter gene as controlled by the tobacco PR-1a promoter. Both the Arabidopsis PR-1 (Lebel et al. 1998) and tobacco PR-1a (Payne et al. 1988) promoters contain the ethylene response cis element ERELEE4 isolated in tomato (Montgomery et al. 1993), indicating that ethylene plays a role in transcription of these promoters. A 903 bp promoter fragment was used to generate the PR-1a:luc transgenic plants. It is possible that ethylene signal transduction is also required for binding of transcription repressors to cis elements upstream of the 903 fragment, which are not present in the PR-1a:luc transgenic line. If this is occurring, it would help to explain different
levels of PR-I expression obtained for cirI:ein2.1 in the luminometer and Northern blot analyses (Fig.5.6).

Results from a preliminary experiment with the cirI:jar1 F2 plant suggested that jasmonate signal transduction through JAR1 is required for PR-I expression (Fig.5.6a). This result was suprising as induction of PR-I expression by an avirulent pathogen was previously shown to be independent of jasmonate signal transduction (Pieterse et al. 1998, Thomma et al. 1998). Furthermore, PR-I expression was induced in jar1 plants by inoculation with PstDC3000 (avrB) (Fig.4.4a). Interestingly, it has been reported that expression of the acidic PR-Ib gene in tobacco is induced by either a combination of Me-JA and ethylene, or by a combination of Me-JA and SA (Xu et al. 1994). Thus, Me-JA could play a role in PR-I expression under certain conditions.

It is interesting to note that constitutive luc activity is maintained in the cirI:npr1 double mutant (Table 5.5), indicating that PR-Ia:luc expression is independent of NPR1 in cirI. Further experiments with the cirI:npr1 double mutant should help to elucidate the function of NPR1 in PR-I expression and disease resistance in cirI.

Ethylene signal transduction through EIN2 and jasmonate signal transduction through JAR1 are required for cirI-induced constitutive PDF1.2 expression (Fig.5.6a). Previously it was shown that concominant ethylene and jasmonate signal transduction were required for PDF1.2 expression in Arabidopsis (Penninckx et al. 1998). More recently, it was shown that Me-JA-treated transgenic Arabidopsis plants expressing the carboxy-end of the EIN2 protein expressed PDF1.2 to high levels (Alonso et al. 1999), indicating that EIN2 is the link between ethylene and jasmonate signal transduction leading to PDF1.2 expression. Results obtained in Fig.5.6a, where PDF1.2 expression is abolished in cirI:ein2.1, confirm this observation. Expression of PDF1.2 in the cirI:jar1 double mutant was partly

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*The Northern blot of cirI:ein2 and cirI:jar1 with the PR-I probe was repeated after submission of the thesis. PR-I expression was seen in both double mutant lines, but at a reduced level in comparison to cirI. Thus, signal transduction through EIN2 and JAR1 does not appear to be an absolute requirement for CIR1-induced PR-I expression, but does play a partial role.*
suppressed (Fig. 5.6a), indicating that jasmonate-signalling through JAR1 is only partially required for PDF1.2 expression in cir1.

Ethylene signal transduction through EIN2 and jasmonate signalling through JAR1 was required for cir1-induced enhanced resistance to PstDC3000 but was not required for resistance to P. parasitica Noco2 (Fig. 5.7). Previously it was shown that growth of avirulent and virulent PstDC3000 in ein2 was equivalent to Col-0, implying that ethylene signal transduction did not play a role in basal PstDC3000 resistance (Bent et al. 1992). PstDC3000 grew to similar levels in Col-0 and ein2.1 (Fig. 5.7a), confirming that ethylene signal transduction through EIN2 is not required for basal resistance to PstDC3000. Bacterial growth in cir1 plants was reduced but not in cir1:ein2.1 plants (Fig. 5.7a), indicating that ethylene signal transduction is required for CIR1-induced resistance to PstDC3000. SAR to virulent PstDC3000, as induced by inoculation with PstDC3000 (avrRpt2), was also abolished in ein2.1 in addition to other ethylene-insensitive mutants (Knoester et al. 1999). On the other hand, reduced P. parasitica Noco2 disease symptoms were observed for both cir1 and cir1:ein2.1 (Fig. 5.7b). Previous reports have indicated that ethylene signal transduction through EIN2 is not required for basal resistance (Thomma et al. 1998) nor SAR to P. parasitica induced by SA or PstDC3000 (avrRpt2) (Lawton et al. 1994, Lawton et al. 1995). The results in Fig. 5.7 confirm that ethylene signal transduction through EIN2 is not required for induced resistance to P. parasitica Noco2.

PstDC3000 grew to similar levels in Col-0 and jar1 (Fig. 5.7c), indicating that jasmonate signal transduction is not required for basal resistance to PstDC3000. Bacterial growth was reduced in cir1 plants but not in cir1:jar1 plants (Fig. 5.7c), indicating that JA signal transduction through JAR1 plays a role in cir1-induced SAR against PstDC3000. Previously it was shown that SAR to virulent PstDC3000, as induced by inoculation with PstDC3000 (avrRpt2), was not abolished in jar1 and thus that SAR was independent of jasmonate signal transduction (Pieterse et al. 1998). Thus, results presented in Fig. 5.7c for the cir1:jar1 mutant further suggest that cir1 may define a novel jasmonate-dependent branch in the SAR signal
transduction network. Alternatively, accumulation of signalling molecules upstream of JAR1 in the SAR signal transduction network may negatively regulate cirl-induced PstDC3000 resistance through a cross-talk mechanism.

Reduced P. parasitica Noco2 disease symptoms were observed for both cirl and cirl:jari in comparison to Col-0 and jar1 (Fig.5.7d). It has previously been reported that jasmonate signal transduction through COII is not required for resistance to P. parasitica (Thomma et al. 1998). Thus, the results in Fig.5.7d confirm that jasmonate signal transduction is not required for induced resistance to P. parasitica Noco2.

When cirl plants were treated with SA or inoculated with PstDC3000 (avrB), PR-1a:luc expression in a luminometer assay and PR-1 expression in Northern blot analysis were substantially increased in comparison to naïve cirl plants (Fig.5.4). This indicates that flux through the SA-dependent pathway is not completely ‘on’ in cirl and PR-1 expression can be boosted by the addition of SA. This response is comparable to cpr1 and cpr6. When cpr1 was treated with SA or INA, PR-2:GUS activity and PR-1 expression was increased relative to naïve cpr1 plants (Bowling et al. 1994). PR-1 expression was slightly increased in the cpr6 + INA sample relative to naïve cpr6 plants (Clarke et al. 1998). However, addition of SA completely inhibited constitutive expression of PDF1.2 in cirl (Fig.5.4), which is also analogous to PDF1.2 expression in cpr6 plants treated with INA (Clarke et al. 1998). PDF1.2 expression was also substantially reduced in cirl plants inoculated with PstDC3000 (avrB) whereas PDF1.2 expression in Col-0 was induced by avirulent Pst (Fig.5.4, Malek & Dietrich 1999). This indicates that the signal transduction pathway leading to PDF1.2 expression in cirl is completely ‘on’ and that extra flux through this pathway or flux through a cross-talk pathway results in reduction in PDF1.2 expression. Previously, it was shown that SA prevented wound-induced gene expression in tomato by blocking synthesis of JA (Pena-Cortes et al. 1993). As SA is known to accumulate following inoculation with both avirulent and virulent Pst (Cameron et al. 1999), it is possible that accumulation of SA over a threshold level in cirl may be blocking synthesis of JA, thereby blocking PDF1.2 expression. Accordingly, SA
accumulation in Col-0 inoculated with PstDC3000 may fall below the threshold level, thereby not influencing the expression of PDF1.2 by ‘cross-talk’.

Based on genetic analysis, the cir1 trait is inherited as a recessive mutation at a single locus (Table 5.2). Furthermore, it was found that the ceb3 mutation was allelic to cir1 in complementation analyses (Table 5.3). The cir1 mutation was mapped to the lower arm of chromosome 4, between the markers nga1111 and g4539 (Table 5.4). Unfortunately, no further PCR-based mapping markers were available between nga1111 and g4539 at the time of this study. However, a number of restriction fragment length polymorphisms (RFLPs), single nucleotide polymorphisms (SNPs) and visible markers are present in this 28cM region of chromosome 4 (http://www.arabidopsis.org). Utilisation of these markers and larger F2 mapping population from the cir1X Ler cross should elucidate the fine map position of cir1 (Lukowitz et al. 2000). Map-based cloning of cir1 and sequencing of the isolated CIR1 gene from wild-type Col-0, cir1 and ceb3 plants will show the position of the mutations in these alleles providing possible clues to cir1 gene function.

In addition to cir1, four other recessive mutants (cpr1, lsd1, acd2 and cpr20), which cause constitutive PR gene expression, elevated SA levels and enhanced disease resistance, have been mapped to chromosome 4 (Bowling et al. 1997, Dietrich et al. 1997, Greenberg et al. 1994, Silva et al. 1999). Of these, cpr1 mapped to approximately the same region as cir1 (Bowling et al. 1997, Table 5.4). The other three mutants mapped to positions on chromosome 4 distal to cir1 so it was decided not to perform complementation analysis (Dietrich et al. 1997, Greenberg et al. 1994, Silva et al. 1999). To determine whether the mutation carried by cpr1 was allelic to cir1, a cross was made between these two mutants and the resulting F1 plants were analysed for constitutive PR-la:luc expression (Table 5.3). An unexpected segregation was obtained, as one of the 12 F1 plants analysed showed constitutive luc activity (Table 5.3). However, it is unlikely that cir1 and cpr1 are allelic. cpr1 plants are stunted (Bowling et al. 1994) whereas cir1 plants are only slightly smaller than Col-0. None of the F1 plants from the cir1 X cpr1 cross were stunted. In
addition, cpr1 plants do not express PDF1.2 constitutively (Penninckx et al. 1996), whereas cir1 plants do. Complementation analysis indicated that cir1 and cpr5, a recessive mutant which also expresses constitutive SAR (Bowling et al. 1997), are not allelic (Table 5.3). This is consistent with mapping studies that have placed cpr5 on chromosome 5 (Bowling et al. 1997).

Two dominant mutations (acd6 and ssil) also showed constitutive PR gene expression, elevated SA levels and enhanced disease resistance, and mapped to chromosome 4 (Rate et al. 1999, Shah et al. 1999). It was decided not to perform complementation analysis between these mutants and cir1 as complementation between a dominant and a recessive mutation is difficult. It has been shown that acd6 and ssil are not allelic (Greenberg 2000). However, there is a small chance that cir1 is allelic to either acd6 or ssil as they map to approximately the same 28cM region (Table 5.4, Rate et al. 1999, Shah et al. 1999). If this is the case, cir1 and ssil or acd6 would identify different types of mutations in the same gene. The fine mapping of ACD6, SSII and CIR1 should determine whether this is the case or not.

Interestingly, cir1 maps to a position close to Major Recognition Complex H (MRC-H), one of the many clusters of disease resistance genes that have been documented in Arabidopsis (Holub & Benyon 1997). Among others, MRC-H contains the RPP5 resistance gene, which confers resistance to P. parasitica Noco2 in the Ler ecotype only (Parker et al. 1993). Four ESTs showing similarity to resistance genes (R-ESTs) have been mapped within or close to MRC-H (Botella et al. 1997). Furthermore, the sequencing of chromosome 4 and analysis of the predicted proteins has indicated a function for more than 170 genes in disease resistance and almost 200 in signal transduction (Mayer et al. 1999). Thus, the proximity of cir1 to both MRC-H and six enhanced disease resistance mutants on a chromosome rich in potential disease resistance genes is intriguing.
SAR is triggered upon infection by a necrotising avirulent pathogen and is implicated in the subsequent development of resistance to a broad range of virulent plant pathogens (reviewed in Ryals et al. 1996). Elucidation of the mechanisms underlying SAR could contribute to the exploitation of SAR in controlling plant diseases. In the work described in this thesis, a transgenic Arabidopsis line was developed expressing the luciferase reporter gene under the control of the \textit{PR-1a} promoter. This line was used to study SAR and a number of candidate SAR mutants were isolated using this line. Identification and characterisation of one mutant, \textit{cir1}, which displayed constitutive activation of SAR, has provided new insights into the signal transduction network underlying SAR.

6.1. Luciferase activity accurately reports \textit{PR-1} expression in \textit{PR-1a:luc} plants

A homozygous \textit{PR-1a:luc} transgenic Arabidopsis line was generated and in Chapter 3, expression of \textit{PR-1a:luc} was investigated. Luc activity was visualised by means of an ultra-low light imaging camera and a luminometer assay. Inoculation of \textit{PR-1a:luc} plants with well-characterised inducers of SAR (avirulent bacterial pathogens) or application of SA, induced luc activity (Fig. 3.2, Fig. 3.3, Fig. 3.4, Fig. 5.4b). In Northern blot analysis, \textit{PR-1} expression was also induced by application of SA or inoculation with the avirulent bacterial pathogen \textit{PstDC3000 (avrB)} (Fig. 4.3, Fig. 4.4a, Fig. 5.4a, Fig. 5.6a). Furthermore, luc activity and \textit{PR-1} expression was induced congruently in \textit{PR-1a:luc} plants following inoculation with \textit{PstDC3000 (avrB)} (Fig. 3.3, Fig. 3.4). Thus, luc activity accurately reports \textit{PR-1} expression in \textit{PR-1a:luc} plants.

In Chapter 3, the mutagenesis of \textit{PR-1a:luc} seed and the mutant screen of M2 and M3 progeny was described. A number of candidate mutants, falling into different classes of abnormal luc activity, were uncovered. A sub-group of candidate mutants were carefully evaluated for abnormal luc activity during SAR by investigating expression of defence-related genes (Fig. 4.3, Fig. 4.4) and disease resistance (Fig. 4.5,
Fig.4.6, Fig.4.7). Candidate mutants were only considered as bona fide SAR mutants if luc activity, defence-gene expression and disease resistance results correlated.

6.2. cirl: A novel SAR mutant

The cirl mutant was identified in Chapter 4. This mutant displayed constitutive expression of PR-1a:luc and defence-related genes (Fig.4.2, Fig.4.4). cirl also displayed constitutive activation of resistance to PstDC3000, a virulent bacterial pathogen (Fig.4.5c), and P.parasitica Noco2, a virulent oomycete pathogen (Fig.4.6c). This analysis indicated that cirl is a bona fide SAR mutant. cirl defines a monogenic recessive mutation (Table 5.2) and maps to the lower arm on chromosome 4 (Table 5.4). Complementation analysis indicated that cirl is allelic to ceb3, an additional SAR mutant isolated in this study. Results obtained in Northern blot analyses and disease resistance assays for both cirl and ceb3 are summarised in Table 6.1. Overall, cirl and ceb3 display a similar phenotype, but with subtle differences. Both cirl and ceb3 do not display spontaneous formation of HR-like lesions (Table 6.1). ceb3 displayed stronger expression of the SA-dependent genes in comparison to cirl but cirl expressed PDF1.2 more strongly than ceb3 (Fig.4.4, Table 6.1). cirl was more resistant to PstDC3000 than ceb3 (Fig.4.5, Table 6.1) but ceb3 was more resistant to P.parasitica Noco2 and F.oxysporum f.sp. matthiolae than cirl (Fig.4.6, Fig.4.7, Table 6.1). Although SA accumulation and ethylene evolution levels in ceb3 were not determined, it is likely that production of both signalling molecules will be increased in comparison to wild-type Col-0, as for cirl (Table 6.1). Map-based cloning of CIR1 and sequencing of the isolated CIR1 gene from cirl, ceb3 and wild-type Col-0 will provide comparison between different alleles and it will be interesting to determine the location of the mutations that convey these small differences in phenotype.
Table 6.1. Summary of results for \textit{cir1} and \textit{ceb3}

<table>
<thead>
<tr>
<th>SAR feature</th>
<th>\textit{cir1}</th>
<th>\textit{ceb3}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signalling molecule(^a)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>no macro- or micro-lesions</td>
<td>no macro- or micro-lesions</td>
</tr>
<tr>
<td>SA</td>
<td>++</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>ethylene</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>Me-JA</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Gene expression(^a)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{PR-1}</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>\textit{PR-2}</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>\textit{PR-5}</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>GST1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{Thl2.1}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{Lox2}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{PDF1.2}</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>\textit{ACS2}</td>
<td>- (basal expression)</td>
<td>- (basal expression)</td>
</tr>
<tr>
<td><strong>Disease susceptibility(^c)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{PstDC3000}</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>\textit{P. parasitica Noco2}</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>\textit{F. oxysporum f.sp. matthiolae}</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

\(^a\)Denotes production of signalling molecules or expression of defence-related genes.
- , no induction; + to +++ denotes extent of expression, with ++ the equivalent as \textit{Col-0} inoculated with \textit{PstDC3000 (avrB)}.
\(^b\)ND, not determined
\(^c\)Denotes approximate disease susceptibility in comparison to \textit{Col-0}. Susceptibility in \textit{Col-0} was set to +++ for all three disease resistance assays.
6.3. A model for CIR1-induced SAR signal transduction

Characterisation of cirl plants by analysing the accumulation of molecules involved in SAR signal transduction, the expression of defence-related genes, resistance to pathogens and epistatic interactions with other SAR signal transduction mutants has enabled the development of a model outlining the role of CIR1 in SAR. This proposed model, which will evolve as more information about the function of the CIR1 gene product becomes available, is outlined in Fig. 6.1.

cirl defines a recessive mutation in a single gene (Table 5.2). In a single step in a signal transduction pathway, production of a constitutive phenotype in the absence of any stimulus indicates the loss of a negative regulator (Bowler & Chua 1994). Thus, CIR1 probably encodes a negative regulator of SAR (Fig. 6.1). Signal components of SAR include ion fluxes, signal transduction via protein phosphorylation by activation of mitogen-activated protein kinase (MAPK) pathways and activation of transcription factors (Meskine & Hirt 2000, Martin 1999, Yang et al. 1997). It is likely that CIR1 encodes a component contributing to one of these processes. Map-based cloning of CIR1 and analysis of the predicted protein structure should indicate the likely function of the CIR1 protein.

cirl plants did not show spontaneous formation of HR-like macro-lesions (Fig. 5.1b) or micro-lesions (Fig. 5.2). Previous characterisation of spontaneous cell death in mutants displaying constitutive SAR has placed mutations either up-stream or down-stream of the HR in the SAR signal transduction network. Mutants displaying lesions such as acd2, cpr5 and cep (Greenberg et al. 1994, Bowling et al. 1997, Silva et al. 1999), were placed upstream of the HR (Fig. 6.1). Mutants such as cpr1 (Bowling et al. 1994) and cpr6 (Clarke et al. 1998) which do not show spontaneous lesion formation were placed downstream of the HR (Fig. 6.1). However, recent isolation of the dndl mutant, which displays constitutive expression of SAR but no HR development in response to inoculation with an avirulent bacterial pathogen (Yu et al. 1998), has brought the central role of the HR in the development of SAR into question. The DND1 gene encodes a cyclic nucleotide-gated ion channel that
probably allows passage of Ca$^{2+}$, K$^+$ and other cations (Clough et al. 2000). Transmembrane ion fluxes are one of the earliest detectable signalling events in plant defence responses (Hammond-Kosack & Jones 1996). Thus, $DND1$ is likely to act very early in the induction of SAR. However, as $cir1$ plants do not show spontaneous lesion development and a normal HR developed in $cir1$ plants inoculated with $Pst/DC3000$ ($avrB$) (results not shown), it is likely that $CIR1$ acts downstream of the HR (Fig.6.1). Furthermore, both SA (Fig.5.3) and ethylene (Fig.5.5) were found to accumulate to higher levels in $cir1$ plants in comparison to

![Diagram](image)

**Fig.6.1. Proposed model of $CIR1$-induced SAR signal transduction.**

wild-type plants, implying that \textit{CIR1} acts upstream of the production of these signal transduction molecules in the SAR signal transduction network (Fig.6.1).

In addition to increased SA accumulation, \textit{ciri} plants showed constitutive expression of the SA-dependent genes \textit{PR-1}, \textit{PR-2} and \textit{PR-5} (Fig.4.4a\&b), further indicating that \textit{CIR1} plays an important role in SA-dependent SAR signal transduction. \textit{ciri} plants also displayed constitutive expression of \textit{PR-1a:lux} (Fig.4.2, Fig.5.1a) and luc activity reported the constitutive expression of \textit{PR-1}. Analysis of the F2 progeny from a \textit{ciri} \textit{X nahG} cross (Table 5.5) indicated that constitutive luc activity (and thus \textit{PR-1} expression) was dependent on SA accumulation (Fig.6.1). As the visual phenotype of \textit{ciri} was dependent on the accumulation of SA, it was not possible to isolate a \textit{ciri:nahG} double mutant for further analysis. However, resistance to virulent \textit{PstDC3000} and \textit{P.parasitica Noco2} is associated with SA accumulation (Lawton et al. 1995), and thus it is likely that a \textit{ciri:nahG} double mutant will be susceptible to these two pathogens. On the other hand, analysis of the F2 progeny from a \textit{ciri} \textit{X npri} cross (Table 5.5) indicated that \textit{PR-1} expression was independent of NPR1 (Fig.6.1). Expression of \textit{PR-1} in the dominant mutants \textit{cpr6} and \textit{siisi} was also SA-dependent but NPR1-independent (Clarke et al. 1998, Shah et al. 1999). Two signalling pathways, one dependent on SA and NPR1, and the other dependent on SA only, have thus been proposed to control the expression of \textit{PR-1} during SAR (Shah et al. 1999, Clarke et al. 1998). Characterisation of the \textit{siisi} mutant also suggested the presence of a second SA-dependent, NPR1-independent pathway (Li et al. 1999). A model was proposed whereby SNI1 represses the expression of \textit{PR-1} in the absence of SA (Li et al. 1999, Delaney 2000). When SA is introduced, it activates NPR1, which represses the SNI1 repressor, allowing transcription of \textit{PR-1}. Another SA-dependent, but NPR1 and SNI1-independent factor was identified that could also allow \textit{PR-1} transcription (Li et al. 1999). Possibly this factor, \textit{CPR6}, \textit{SSI1} and \textit{CIR1} are all involved in a second SA-dependent, NPR1-independent pathway leading to \textit{PR-1} expression (Fig.6.1).

\textit{PR-1} expression in response to inoculation with an avirulent bacterial pathogen is neither dependent on \textit{EIN2} (Fig.5.6a, Lawton et al. 1995) nor \textit{JAR1} (Fig.4.3,
Fig. 4.4a, Pieterse et al. 1998). However, PR-1 expression in cir1 was found to be dependent on EIN2 and JAR1 (Fig. 5.6a). This implies that ethylene and jasmonates, in addition to SA, are required for signal transduction leading to PR-1 expression in cir1 (Fig. 6.1). It is thus likely that the CIR1 protein acts as a negative regulator in a SA-, ethylene- and jasmonate-dependent pathway leading to PR-1 expression, and further implies that CIR1 must be operating at an early point in the SAR signal transduction network (Fig. 6.1). The tunable dial model for the regulation of defence gene expression by SA, ethylene and jasmonates proposes that a plant is able to fine-tune its response by employing a single signal molecule or a combination of the three molecules, depending on the nature of the attacking pathogen (Reymond & Farmer 1998). CIR1 appears to fit this model. Wild-type CIR1 is a negative regulator of SAR, presumably suppressing the expression of SAR genes in healthy tissue. Following pathogen attack, the plant 'tunes' the establishment of SAR by suppressing CIR1 activity, leading to the employment of all three signalling molecules in inducing PR-1 expression. However, the pathogen that would suppress wild-type CIR1 activity has yet to be identified, as it appears that CIR1-induced PR-1 expression differs to PR-1 expression induced by inoculation with PstDC3000 (avrB).

Expression of PDF1.2 in cir1 was dependent on EIN2 and JAR1 (Fig. 5.6a), placing PDF1.2 expression downstream of ethylene signalling through EIN2 and jasmonate signalling through JAR1 (Fig. 6.1). It has previously been shown that A. brassicicola-induced PDF1.2 expression occurs independently of SA (Penninckx et al. 1996) and that concomitant jasmonate and ethylene signal transduction through EIN2 is required for PDF1.2 expression (Penninckx et al. 1998, Alonso et al. 1999). Ethylene evolution in cir1 plants was increased in comparison to wild-type Col-0 (Fig. 5.5), further indicating that PDF1.2 expression in cir1 is dependent on ethylene signal transduction through EIN2 (Fig. 6.1). Although jasmonate levels in cir1 were not investigated in this study, Northern blot analysis indicates that it is unlikely that jasmonate production is increased in cir1. Treatment of cir1 plants with SA suppressed PDF1.2 expression (Fig. 5.4a), indicating that increased SA accumulation in cir1 plants may be suppressing jasmonate levels by a cross-talk mechanism.
Expression of the Me-JA responsive genes Thi2.1 (Bohlmann et al. 1998) and AtLOX2 (Bell & Mullet 1993) were also not detected in cir1 (Fig.4.4c&d). It is possible that increased Me-JA levels in planta are required for Thi2.1 and AtLOX2 expression, but that endogenous jasmonate levels are sufficient, when ethylene signal transduction through EIN2 is enhanced, for expression of PDF1.2.

Enhanced resistance to the virulent bacterial pathogen PstDC3000 in cir1 is dependent on EIN2 and JAR1 (Fig.5.7a&c), placing PstDC3000 resistance downstream of EIN2 and JAR1 in the model (Fig.6.1). Resistance to PstDC3000 has previously been shown to be dependent on SAR signal transduction through SA and NPR1 (Cao et al. 1994, Bowling et al. 1994, Bowling et al. 1997, Clarke et al. 1998). Although PstDC3000 resistance in cir1 appears to be associated with PR-1 and PDF1.2 expression, characterisation of the cpr6 mutant indicated that PstDC3000 resistance could be uncoupled from PR-1 expression, implying that the expression of additional NPR1-dependent genes are required for PstDC3000 resistance (Clarke et al. 1998). It is likely that CIR1 also induces additional ethylene- and jasmonate-responsive genes that are required for PstDC3000 resistance.

Enhanced resistance to the virulent oomycete pathogen P.parasitica Noco2 in cir1 is independent of EIN2 and JAR1 (Fig.5.7b&d). Previously both NPR1-dependent and NPR1-independent P.parasitica resistance signalling has been reported (Cao et al. 1994, Bowling et al. 1997), although P.parasitica resistance is dependent on SA signalling (Lawton et al. 1994). It is thus likely that CIR1-induced resistance to P.parasitica Noco2 is dependent on SA signalling, either by an NPR1-dependent or an NPR1-independent signalling pathway (Fig.6.1). It has previously been proposed that NPR1-independent resistance to P.parasitica Noco2 is dependent on expression of PDF1.2 (Bowling et al. 1997). Studies with the cir1:ein2.1 and cir1:jar1 double mutants in this thesis have shown that PDF1.2 expression is suppressed without loss of P.parasitica Noco2 resistance, indicating that P.parasitica resistance is independent of PDF1.2 expression (Fig.6.1).
6.4. Future analysis of the CIR1-induced SAR model

The proposal of a model outlining the role of CIR1 in the SAR signal transduction network raises a number of additional questions. These are listed below. Due to time limitations, they could not be addressed in this thesis. Further characterisation of cir1, with the aim of answering these questions, should prove to be interesting and will help the development of the model presented in Fig.6.1.

- Expression of PR-1 in cir1 was dependent on SA but independent of NPR1. Is CIR1-induced resistance to PstDC3000 and P.parasitica Noco2 dependent on NPR1? Disease resistance assays of the cir1:npr1 double mutant should answer this question. Further Northern blot analysis should also uncover the role of SA and NPR1 in CIR1-induced constitutive PDF1.2 expression.

- Ethylene evolution in cir1 was increased in comparison to wild-type Col-0 plants and CIR1-induced ethylene signalling through EIN2 appears to play an important role in expression of PR-land PDF1.2, and resistance to PstDC3000. Is increased ethylene evolution in cir1 due to increased biosynthesis of ethylene, or is it due to a feedback loop in ethylene signal transduction? Does CIR1 operate upstream or downstream of ethylene biosynthesis? Determination of PR-1 and PDF1.2 expression, and PstDC3000 resistance in cir1 plants treated with inhibitors of ethylene biosynthesis should help to answer these questions. Furthermore, characterisation of a cir1:etr1 double mutant (which would contain a mutant ethylene receptor) would prove to be interesting.

- CIR1-induced PstDC3000 resistance and expression of PR-1 and PDF1.2 appears to be dependent on jasmonate-dependent signalling through JAR1. What role are jasmonates playing in cir1? Is the production of jasmonates increased in cir1, or are endogenous levels sufficient for CIR1-induced signal transduction? Does CIR1 operate upstream or downstream of jasmonate production? Determination of the endogenous jasmonate levels (both JA and Me-JA) in cir1 and wild-type Col-0 by GC-MS (Penninckx et al. 1996) should help to answer some of these questions. In addition, crossing cir1 with the other Me-JA insensitive mutant coil (Feys et al. 1994) and the JA-deficient triple mutant fad3-
2fad 7-2fad8 (McConn & Browse 1996), should indicate if CIR1-induced signalling is dependent on jasmonate signal transduction through JAR1 or on JAR1 only.

- cir1 plants did not show spontaneous formation of lesions and showed enhanced resistance to PstDC3000 and P.parastica Noco2 but not to F. oxysporum f.sp. matthiolae. Recently it has been shown that the dndl mutant, which shows enhanced disease resistance without cell death, was resistant to Botrytis cinerea (Govrin & Levine 2000). Is cir1 more resistant (or more susceptible) to other agriculturally important pathogens? Further disease resistance assays with additional pathogens such as B. cinerea should indicate if this is the case or not and further expand the role of CIR1 in SAR.

- cir1 plants expressed PR-1, PR-2, PR-5, GST1 and PDF1.2 genes constitutively. Do cir1 plants express additional SA-, ethylene- and jasmonate-responsive genes constitutively? In addition, CIR1-induced resistance to P.parastica Noco2 appears to be independent of PR-1 and PDF1.2 expression. Which CIR1-induced genes are responsible for P.parastica Noco2 resistance? Analysis of gene expression in cir1 plants using techniques such as cDNA amplified fragment length polymorphism (cDNA-AFLP) (Durrant et al. 2000) or cDNA microarray analysis (Reymond et al. 2000, Schenk et al. 2000) should help to unravel CIR1-induced gene expression and the position of CIR1 in the SAR signal transduction network.

6.5. SAR as a mechanism to control plant disease

Further elucidation of the mechanisms underlying SAR could contribute to the exploitation of SAR in controlling plant diseases. One example of the successful application of SAR in controlling disease in agriculture is the development of the chemical plant activator Bion. The active compound of Bion is benzothiadiazole (BTH), which is a functional analogue of SA. In most plants studied, BTH activates the SAR pathway and is effective against a wide range of plant pathogens (Friedrich et al. 1996, Gorlach et al. 1996, Lawton et al. 1996). It has also recently been shown that Me-JA application reduced disease development of several necrotrophic plant
pathogens on Arabidopsis (Thomma et al. 2000). Over-expression of SAR regulatory proteins in transgenic plants is an alternative strategy to utilising SAR in plant disease control. It has been shown that transgenic Arabidopsis plants expressing the NPR1 protein are dramatically more resistant to the virulent pathogens \textit{P.syringae} and \textit{P.parasitica} (Caò et al. 1998). Recently, the observation that simultaneous activation of SAR and rhizobacteria-mediated ISR results in a significantly enhanced level of resistance to \textit{P.syringae} (Van Wees et al. 2000) offers great potential for integrating both types of induced resistance in plant disease control.

It has been shown in Arabidopsis that the SA-dependent SAR pathway is more effective against certain pathogens (i.e. the biotrophic oomycete \textit{P.parasitica}) whereas the jasmonate-dependent pathway is directed to other pathogens (i.e. the necrotrophic fungal pathogens \textit{A.brassiciola} and \textit{B. cinerea}) (Thomma et al. 1998). However, the recent characterisation of the \textit{dnd1} mutant suggests SAR is more complex than signal transduction through two separate pathways. The \textit{dnd1} mutant displayed the hallmarks of SA-dependent SAR (accumulation of SA, constitutive \textit{PR-1} expression) (Yu et al. 1998) but was also resistant to \textit{B.cinerea} (Govrin & Levine 2000). In addition, recent cDNA microarray analysis of over 2000 defence-related genes unexpectedly revealed that the expression of a large number of genes was co-ordinated by both SA and Me-JA (Schenk et al. 2000). A better understanding of plant resistance strategies is thus required. Furthermore, although Arabidopsis is a useful tool for dissecting SAR, it is likely that crop plants will show different SAR responses, both in comparison to Arabidopsis and to different pathogens. Map-based cloning of \textit{CIR1}, the isolation of \textit{CIR1}-homologues in crop plants and the analysis of pathogen resistance in \textit{CIR1}-transgenic plants will thus be a useful goal.
Chapter Seven

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