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Title: Investigation of defence mechanisms against *Botrytis cinerea* in *Arabidopsis thaliana*

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Abstract
Disease resistance in plants has been extensively studied for the past century with many new and exciting results being discovered each year. A plant utilises both preformed and induced defence responses to resist pathogen attack but researchers have focused on dissecting the induced defence response pathway. The complex signal transduction pathway underlying the establishment of resistance to a wide range of pathogen attack is currently being dissected using Arabidopsis thaliana as a model organism. Arabidopsis mutants displaying altered disease resistance response to pathogen infections can help us to get a better understanding of the genetic and molecular basis of the disease resistance pathway. Extensive research has shown that accumulation of 3 signalling molecules are vitally important for establishing a resistance response, as aberrant signalling or accumulation of salicylic acid, ethylene or jasmonic acid leads to an altered resistance response. Researchers continue to isolate and characterise defence-related mutants to piece together the intricate puzzle of defence-signalling components.

A dominant Arabidopsis mutant, constitutive induced resistance 3 (cir3), had been isolated from an ethylmethane sulfonate (EMS) mutagenised transgenic line expressing luciferase under the control of the PR-1 promoter (PR-1::LUC). This mutant displayed constitutive PR-1::LUC gene expression. Initial studies had indicated that increased expression of defence related genes (PR-1, PR-2, and PR-5) in cir3 was uncoupled from disease resistance as these mutants were
at no advantage to wild type plants when challenged with two biotrophic pathogens. Cir3 also displayed increased expression of PDF1.2, a plant defensin gene involved in resistance to necrotrophic pathogen attack. The cir3 mutant was therefore further characterized to confirm this phenotype and to unravel CIR3 in the defence-signalling network. Increased endogenous PR-1 mRNA expression was still apparent and the pattern of expression correlated with PR-1 protein levels. Increased luciferase activity of cir3 is SA-dependent as double mutants cir3 X nahG and cir3 x apr1-1 displayed decreased luciferase activity in comparison to cir3 plants. Cir3 plants displayed higher resistance to infection by the necrotrophic pathogen, Botrytis cinerea. B. cinerea assays of cir3 x nahG and cir3 x ein2 indicate that cir3 resistance to B. cinerea is both SA and Eth dependent as these double mutants were more susceptible to B. cinerea infection than cir3. Preliminary mapping analysis reveals that this novel mutation is located on chromosome III.
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Chapter 1

Introduction

Research dedicated to unravelling plant-pathogen interactions has been underway for many years. The scientific community has been intrigued by a plant's ability to evolve various physiological and molecular mechanisms to withstand pathogen attack (Glazebrook et al., 1997a). The ultimate goal would be to utilize information gained from molecular studies to create productive crop plants with enhanced defence mechanisms. With the availability and continual discovery of powerful genomic tools and techniques, the past few years have been flooded with the identification of genes involved in defence signalling. A large proportion of these defence genes identified has emanated from research of various Arabidopsis-pathogen interactions (Glazebrook et al., 1997a).

Extensive molecular tools and resources together with the availability of the entire Arabidopsis genomic sequence (Arabidopsis Genome Initiative, 2000) has contributed to the success of Arabidopsis as an ideal model system to study. Arabidopsis is prone to infection by biotrophic pathogens such as Pseudomonas syringae (extracts nutrients from living plant cells) or necrotrophic pathogens such as Botrytis cinerea (scavenges dead cells for nutrients) (Glazebrook et al., 1997a). Arabidopsis activates a common defence response to all pathogens but specific resistance strategies are employed in response to particular pathogens (Thomma et al., 1996). Tremendous research efforts have been employed to dissect the specific resistance pathways activated in response to either biotrophic or necrotrophic pathogen attack. This chapter will review what has been
discovered thus far and how the components of each specific pathway interact to produce an effective set of responses to each pathogen.

Plants have the ability to induce multiple defence responses via complex signalling networks to prevent both local disease symptoms and subsequent secondary infections of distal tissues. Arabidopsis induced resistance against biotrophic pathogen attack has been more extensively studied and therefore will be used as a basis for this introduction and comparisons will be drawn to the less understood Arabidopsis-B. cinerea interaction.

Firstly, how does a plant recognise the pathogen at the molecular level to activate a defence response to biotrophic or necrotrophic pathogen attack? The biotrophic pathogen, *P. syringae*, injects virulence proteins into host plant cells via the type III secretion proteins (Gallan & Collmer, 1999). A compatible interaction occurs if the host plant is unable to recognize the virulence protein, resulting in manifestation of disease symptoms. If the plant harbours a corresponding resistance (R) protein that recognizes the pathogen's virulence protein, we refer to the virulence protein as an avirulence (Avr) protein. This recognition event is the initial step in the gene-for-gene resistance pathway that results in an incompatible interaction between resistant plants and avirulent pathogens (Dangl & Jones, 2001). Two models depicting how the recognition event takes place have been proposed. The "elicitor-receptor" model is based on the hypothesis that a direct interaction occurs between the pathogen-derived Avr
gene products and the receptor-like R protein. However, the number of known R-genes is much fewer than the vast number of known avirulent proteins. *Pseudomonas syringae pv. tomato* alone is hypothesised to have more than 30 Avr proteins and therefore it is hypothesized that one R protein recognizes more than one avirulence protein (Zwiesler-Vollick et al., 2002). The second model is the “Guard” model. This model suggests that multiple Avr proteins target a key plant protein involved in basal defence. When the Avr protein attacks its target, the R-protein detects the interaction and initiates a signalling cascade (Dangl & Jones, 2001). To date, there has been no evidence of direct interaction between an R and Avr protein pair, whereas the findings of the RPM1/RPM1-interacting 4 (RIN4) complex makes the guard model hypothesis more plausible (Mackey et al., 2002). Both avrRPM1 and avrB proteins are able to bind RIN4, causing RIN4 to become hyper phosphorylated. It is been shown that the R protein, RPM1 recognizes the conformational changes of RIN4 and triggers the rapid activation of complex molecular strategies to prevent both local and systemic disease caused by *P. syringae* (Mackey et al., 2002).

Infection of *Arabidopsis* by the necrotrophic pathogen *B. cinerea* is similar to a compatible *P. syringae* interaction. *B. cinerea* gains entry to the host plant without rapidly activating a resistance response via a gene-for-gene interaction and is therefore able to cause disease symptoms on the host plant. *B. cinerea* produces toxins and extra-cellular enzymes such as polygalacturonase (PG) and cutinase that degrade pectin in the plant’s cell wall (McKeen, 1974; Rijkenberg et
As an initial local defence mechanism, the plant secretes PG inhibiting proteins (PGIP's). B. cinerea has various forms of the PG enzyme and therefore the plant may or may not have PGIP's that are able to inhibit the specific PG's activity. Transgenic Arabidopsis plants over expressing bean PGIP display increased local resistance to B. cinerea infection indicating a partial role for PGIP's in the resistance response (Ferrari et al., 2002).

A compatible P. syringae interaction or B. cinerea infection results in disease symptoms as absence of the initial gene-for-gene recognition event does not allow for the rapid containment of the invading pathogen. The following two plant resistance responses are thought to assist in preventing the spread of P. syringae from the site of infection during an incompatible interaction are not observed during compatible interactions where the pathogen is able to cause spreading disease symptoms. The mechanisms employed by the host plant during an incompatible interaction are the production of reactive oxygen intermediates (ROI), such as hydrogen peroxide (H₂O₂) and superoxide molecules (O₂⁻), and initiating a form of programmed cell death called the hypersensitive response (HR) to destroy the infected cell thereby starving the invading pathogen, as it requires the host’s metabolism and cellular functions to proliferate (Greenberg, 1997). Mutation of the HYPERSENSITIVE RESPONSE-LIKE LESIONS 1 (HRL1) gene leads to increased expression of AtrbohD, a gene encoding the major subunit of the NADPH complex. Increased NADPH activity leads to accumulation of ROI, specifically O₂⁻, which correlates with enhanced
resistance to pathogen attack (Devadas et al., 2002). Mutation of the Lesion Simulating Disease (LSD), Copine (CPN) and Accelerated Cell Death (ACD) loci result in developmentally and environmentally controlled initiation of HR-like cell death in the absence of pathogen infection (Dietrich et al., 1994; Greenberg & Ausubel, 1993). The existence of various mutants that mimic HR-like cell death suggests that the HR is under genetic control. LSD1 has been shown to be a negative regulator of HR cell death as mutation of the LSD1 gene results in runaway cell death propagated by the presence of ROI (Jabs et al., 1996; Dietrich et al., 1997). Spontaneous lesion formation on Isd1 was correlated with increased resistance to virulent pathogens, suggesting that the absence of the HR during compatible interactions is responsible for allowing the pathogen to cause disease. Conditional lesion formation and increased resistance to virulent pathogen attack was also seen in cpn1-1 mutants and some of the acd mutants (Greenberg & Ausubel, 1993; Jambunathan et al., 2001).

Production of ROI and formation of the HR are advantageous for limiting the spread of biotrophic pathogens to the site of infection during an incompatible interaction but have been shown to facilitate infection by saprophytic pathogens such as B. cinerea (Govrin & Levine, 2000). B. cinerea has been shown to produce lethal levels of ROI as a mechanism to gain entry to the host plant and therefore production of ROI by the host plant would facilitate the infection process as seen by the increased susceptibility of mutants unable to control ROI accumulation (ten Have et al., 1998, Deighton et al., 2001, Epple et al., 2003). It
is hypothesised that *B. cinerea* infection could also be aided by the presence of HR micro lesions on the plants’ surface, as the patches of decaying tissue are readily available nutrient sources for the pathogen to feed on and proliferate. *Arabidopsis* plants over-expressing anti-apoptotic proteins, which delay cell death, and mutants that are unable to elicit an HR such as *defence no death 1* (*dnd1*) mutants, display increased resistance to *B. cinerea* which reaffirms the hypothesis that the HR, in contrast to biotrophic pathogens, facilitates *B. cinerea* infection (Govrin & Levine, 2000; Dickman et al., 2001).

During the employment of local defence mechanisms of ROI production and HR development, the host plant rapidly accumulates signalling molecules in response to pathogen recognition through the gene-for-gene interaction. Signalling molecules accumulate at a slower rate during compatible *P. syringae* and *B. cinerea* interactions. Signalling molecules are responsible for establishing systemic resistance in distal parts of the plant to provide broad-spectrum defence to secondary infections caused by a compatible *P. syringae* interaction whereas they play a role in limiting the spread of *B. cinerea* growth to systemic tissue of the infected plant. The three major signalling molecules involved in induction of defence responses are salicylic acid (SA), ethylene (Eth) and jasmonic acid (JA) (Glazebrook, 2001). General defence responses employ the activity of all three signalling molecules but activation of a specific defence pathway in response to a specific type of pathogen is usually governed by either SA-dependent or Eth/JA-dependent signaling pathways. Induction of the SA-dependent pathway is
mainly correlated with inducing resistance to both virulent and avirulent biotrophic pathogens, whereas the Eth/JA-dependent defence pathway is induced by necrotrophic pathogen attack (Glazebrook, 2001; Pieterse et al., 2001). The network of defence signalling is a complex one as molecular components from both SA and Eth/JA pathway have been shown to exhibit cross-talk with each other and therefore these signalling molecules are not restricted to defence against only one type of pathogen (Glazebrook et al., 2003).

The SA-dependant defence pathway has been studied more extensively. SA signalling is not only vital for establishing broad-spectrum resistance to secondary biotrophic pathogen infection called systemic acquired resistance (SAR) but has also been shown to play a role in conferring local resistance to both biotrophic and B. cinerea infection (Ryals et al., 1996; Ferrari et al., 2003). Accumulation of SA is critical for local resistance to P. syringae and B. cinerea infection and establishing SAR in incompatible P. syringae and other biotrophic interactions. The importance of SA accumulation is displayed by the increased pathogen susceptibility of Arabidopsis harbouring the Pseudomonas putida salicylate hydroxylase gene, nahG, which converts SA to catechol. The transgenic nahG plants display increased susceptibility to infection by B. cinerea and both virulent and avirulent forms of P. syringae. A distinction between the role of SA accumulation in necrotrophic and biotrophic interactions is the fact that the loss of SA only affects local resistance to B. cinerea whereas both local and
systemic *P. syringae* resistance is lost (Gaffney et al., 1993; Delaney et al., 1994; Ferrari et al., 2003).

Two pathways have been implicated for producing high levels of endogenous SA in response to pathogen attack. SA can be synthesized as a by-product of the phenylpropanoid metabolism pathway or via the action of isochorismate synthase (ICS) that converts chorismate to SA (Shah, 2003). Disruption of molecular components from either of these pathways has a detrimental effect on defence signaling. The SA induction deficient (*sid2*) gene encodes a putative ICS enzyme, which is expressed at the site of pathogen infection (Wildermuth et al., 2001). Mutations in the *sid2* gene result in enhanced susceptibility to both virulent and avirulent biotrophic pathogen attack but not to the same extent as expression of *nahG* (van Wees & Glazebrook, 2003). A difference in *B. cinerea* resistance profiles of *nahG* and *sid2* were also observed indicating that the action of *nahG* may cause additional undesired effects in disease resistance signalling (Nawrath & Metraux, 1999; van Wees & Glazebrook, 2003; Ferrari et al., 2003). *B. cinerea* resistance was unaffected by the *sid2* mutation indicating that resistance to *B. cinerea* was independent of SA synthesis via the ICS-dependant pathway (Ferrari et al., 2003). However, accumulation of SA synthesised from the phenylpropanoid pathway is vital for local *B. cinerea* resistance as WT plants treated with an inhibitor of the phenylpropanoid pathway displayed increased susceptibility to *B. cinerea* infection (Govrin & Levine, 2002; Ferrari et al., 2003).
SA synthesis and accumulation is notably critical for certain aspects of defence responses to particular pathogens. The role of SA signalling in response to biotrophic pathogen infection has been extensively studied but research is still underway to determine why SA accumulation is needed for local B. cinerea resistance. A vast number of studies have shown that a plant’s response of increasing endogenous SA levels in both local and distal tissue to biotrophic pathogen attack correlates with the induction of pathogenesis-related (PR) genes such as PR-1 (Ward et al., 1991). A few of these PR proteins have been shown to exhibit anti-microbial activity and it is hypothesised that the presence of these proteins could play a role in preventing secondary pathogenic infection (Uknes et al., 1992). Mutations in the Arabidopsis genome which render the plant either unable to accumulate or perceive SA increases susceptibility to biotrophic pathogen infection and lower PR-1 expression. These mutants include non-expressor of PR1 (npr1, Cao et al., 1994), enhanced disease susceptibility (eds1, eds5, eds16, Glazebrook et al., 1996), non-disease resistant 1 (ndr1, Shapiro & Zhang, 2001), phytoalexin deficient 4 (pad4, Glazebrook & Ausubel, 1994; Jirage et al., 1999), and sid2 (Nawrath & Metraux, 1999; Wildermuth et al., 2001).

Conversely, there are mutants that display increased SA levels and constitutive defence gene expression that display increased resistance to pathogen attack, such as: constitutive expessor of PR genes (cpr, Bowling et al., 1994), dnd (Yu et al., 1998), Isd (Dietrich et al., 1994) and acd mutants (Greenberg & Ausubel, 1993; Greenberg, 1997). Exogenous treatment of plants with SA or one of its synthetic analogues, benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
(BHT) i 2,6-dichloroisonicotinic acid (INA), mimics the SAR constitutive mutants as treatment results in expression of PR genes and establishment of SAR in the absence of pathogen infection (Friedrich et al., 1996).

Numerous studies have failed to pinpoint the mobile signal involved in inducing PR-1 expression and establishing systemic resistance in distal parts of the plant once SA has accumulated. What has been established is that there are two SA-dependent defence-signaling pathways; one that utilizes the NPR1 gene and the other, which is NPR1-independent. A mutation of the NPR1 gene results in increased susceptibility to biotrophic pathogen infection and the inability to express PR-1 or establish SAR in response to SA treatment or pathogen infection (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996). The NPR1 protein contains ankyrin repeats and displays homology to a mammalian transcriptional regulator involved in immune responses suggesting a conserved evolutionary role in defence (Cao et al., 1997; Ryals et al., 1997). The NPR1-dependent defence pathway is thought to be established under reducing conditions as recent studies have shown that binding of SA inhibits the activities of several antioxidants (Durner & Klessig, 1995; Slaymaker et al., 2002). Under non-reducing conditions, NPR1 protein monomers are found associated with each other in a large complex in the cytoplasm (Mou et al., 2003; Després et al., 2003). A link between initiation of gene transcription and ROI production or SA accumulation in response to pathogen attack was always suggested but never revealed (Lamb & Dixon, 1997). It could be hypothesized that ROI and SA
production causes changes in the redox state of the cell allowing the NPR1 oligomer to disaggregate into monomers (Kinkema et al., 2000). These NPR1 monomers translocate to the nucleus but are unable to induce expression of PR genes directly as they do not contain a DNA-binding domain (Kinkema et al., 2000). Yeast-two hybrid experiments and in planta assays have shown a physical interaction between NPR1 and TGA transcription factors (Despres et al., 2000; Zhang et al., 1999; Fan & Dong, 2002). There are ten TGA factors in Arabidopsis and these TGA factors display some form of functional redundancy (Zhang et al., 2003). Three of these factors, namely TGA2, TGA5 and TGA6 have been shown to interact with NPR1 in yeast two hybrid assays whereas two, TGA1 and TGA4, only interact with NPR1 in planta after SA treatment. A plant producing a mutagenised TGA2 protein, displays a similar enhanced disease susceptible phenotype as npr1 (Fan & Dong, 2002). The tga2 tga5 tga6 triple mutant displayed all but one of the npr1 mutant phenotypes, as it was not more susceptible to virulent pathogens (Zhang et al., 2003). Therefore, other TGA factors could be playing a role in resistance to different pathogens. TGA factors have been shown to recruit to the PR-1 promoter in vivo but it is not certain as to the exact role these factors play in regulating its expression as the promoter of PR-1 contains both positive and negative regulatory sequences (Maleck et al., 2000). WRKY factors are also regulators of PR-1 expression as they are able to bind to the numerous W-boxes found in the PR-1 promoter (Maleck et al., 2000). The WRKY family also consists of numerous functionally redundant members and it is therefore difficult to isolate one specific factor (Eulgem et al., 2000).
There has however been suggestion of WRKY70 playing a positive role in PR-1 expression, as over expression of WRKY70 causes constitutive PR-1 expression (Li et al., 2004).

The SA- NPR1 dependent defence-signalling pathway is vital for activating both local and systemic response to *P. syringae* infection, but its involvement in defence against *B. cinerea* infection remains unclear. As mentioned earlier, accumulation of SA through the PAL pathway seems to pay a minor role in local *B. cinerea* resistance at the site of infection (Ferrari et al., 2003). However, numerous studies suggest that SA- NPR1-dependent signalling plays no role in induction of both local and systemic resistance to *B. cinerea* infection. Mutation of the NPR1 gene does not affect *B. cinerea* resistance (Ferrari et al., 2003) and PR-1 expression is not critical for *B. cinerea* resistance because even though *B. cinerea* induces PR-1 expression in wildtype plants, induction of systemic resistance in distal parts of the plant is not observed (Govrin & Levine, 2002; Ferrari et al., 2003).

The enhanced resistance phenotype of the suppressor of SA-insensitivity (ssi1 & ssi2) mutants is not compromised in an *npr1* mutant background, indicating the presence of an NPR1-independent SA pathway (Shah et al., 2000). The SA-NPR1 independent pathway is also activated in the *cpr5*, *cpr6* and hypersensitive response-like lesions 1 (hr11) mutants (Clarke et al., 2000; Devadas et al., 2002).
In addition to NPR1-dependent PR-1 expression, plants also produce phytoalexins such as camalexin via NPR1-independent mechanisms in response to both biotrophic and necrotrophic pathogen infection. Camalexin is an antimicrobial compound that requires signals from the NPR1 independent SA pathway to accumulate (Zhao & Last, 1996). Mutants that are unable to produce phytoalexins, such as the pad mutants, display increased susceptibility to both necrotrophic and biotrophic pathogen attack (Glazebrook et al., 1997b). Pad3 has a mutation in a cytochrome P450 monooxygenase enzyme and displays increased susceptibility to pathogen attack (Glazebrook et al., 1997b, Zhou et al., 1999). The pad3 mutants show decreased susceptibility to local B. cinerea infection, as they are unable to synthesize camalexin (Ferrari et al., 2003). These findings are supported by results obtained by Denby et al. (2004) which show a correlation between camalexin levels and B. cinerea susceptibility in a wide range of Arabidopsis ecotypes. Camalexin displays anti-fungal activity in vitro (Ferrari et al., 2003) but increased camalexin production is not the complete answer to B. cinerea local resistance as some mutants with normal or elevated levels of camalexin are still susceptible to infection (Ferrari et al., 2003). However, camalexin could be involved in establishing systemic resistance to B. cinerea infection as the rate of death of pad mutants showed an inverse correlation with the levels of camalexin produced (Ferrari et al., 2003).

Defence signalling via accumulation of Eth and JA hormones is initiated in response to a different set of pathogens and is different to the SA-dependent
SAR response as it is not activated by R-AVR interactions, is independent of SA accumulation, and does not result in expression of PR genes (Pieterse et al., 1998; Knoester et al., 1999). Eth and JA accumulate in response to some compatible interactions, such as infection by virulent strains of *P. syringae*, and necrotrophic pathogens such as *B. cinerea*. It is not known how the Eth/JA defence signalling pathway is activated as the R-AVR interaction does not play a role in initiating these signalling pathways but it could be hypothesised that the breakdown products resulting from pathogen entry act as elicitors of the Eth/JA defence pathway (Bailey, et al., 1990; Pieterse et al., 1998; Knoester et al., 1999).

The importance of Eth and JA defence signalling in response to biotrophic pathogen attack was elucidated through studies of plants containing mutations that disrupt Eth/JA signalling pathway. Plants with disrupted Eth/JA signalling pathways are able to establish an SAR in response to avirulent biotrophic pathogens but are more susceptible to certain virulent biotrophic pathogen attack (Bent et al., 1992; Feys et al., 1994; Ton et al., 1999; Kloek et al., 2001). This indicates that SA, Eth and JA signalling is required for general defence response to pathogens that are not recognised by the host plant. Eth and JA defence signalling is not involved in the SA-dependent SAR response activated by avirulent biotrophic pathogen attack (Lawton et al., 1995).
Eth and JA defence signalling has also been shown to be crucial for activating a resistance response to necrotrophic pathogen attack. Disruption of the Eth signalling pathway through the ethylene insensitive 2 (ein2) and ethylene response factor 1 (erf1) mutations or the JA signalling pathway via jasmonic acid resistant 1 (jar1) and coronatine insensitive 1 (coi1) mutations results in increased susceptibility to necrotrophic pathogens such as B. cinerea (Ferrari et al., 2003). Wildtype plants also induce expression of the antifungal peptide PLANT DEFENSIN 1.2 (PDF1.2) in response to B. cinerea infection (Penninckx et al., 1996; Zimmerli et al., 2001). Ein2, erf1, jar1 and coi1 are unable to express PDF1.2 after pathogen attack indicating that both the Eth and JA pathways have to be activated for expression of this gene (Penninckx et al., 1996, 1998). PDF1.2 expression is the hallmark of an activated Eth/JA pathway in view of the fact that nahG plants are able to accumulate PDF1.2 in response to necrotrophic pathogen attack indicating that the expression of PDF1.2 is independent of SA (Penninckx et al., 1996, 1998).

Activation of defence gene expression via the ethylene-signalling pathway has been partly elucidated by exploiting the triple response phenotype to identify plants with aberrant ethylene signalling (Guzman & Ecker, 1990). Cloning and epistatic analysis of various ethylene-signalling mutants has revealed a signal transduction pathway, from receptors all the way to transcription factors (Stepanova & Ecker, 2000; Wang et al., 2002). The membrane bound ETR1 protein is one of five known ethylene receptors (Wang et al., 2002) and a
mutation in one of these receptors results in an ethylene-insensitive phenotype, as the plant is unable to detect accumulation of ethylene (Wang et al., 2002). Increased susceptibility to B. cinerea infection is displayed by etr1-1 mutants, indicating that perception of ethylene that has accumulated in response to the pathogen is needed for a full resistance response (Ferrari et al., 2003). ETR1 has been shown to interact with CTR1, a Raf-like protein that when mutated causes a constitutive ethylene response phenotype (Clark et al., 1998). In contrast to CTR1’s negative regulatory role on the ethylene pathway, the membrane protein EIN2 is hypothesised to be a positive regulatory protein of ethylene signalling as ein2 mutants are insensitive to ethylene (Guzman & Ecker, 1990) and act upstream of CTR1. Mutation of the ein2 gene results in increased resistance to B. cinerea and virulent P. syringae but maintains a wildtype response to avirulent pathogen attack indicating that Eth signalling is not involved in both local and systemic induced resistance (Thomma et al., 1999). Identification of EIN3, a transcription factor acting downstream of both EIN2 and CTR1 revealed the existence of nuclear regulatory mechanisms to activate gene expression (Chao et al., 1997, Solano et al., 1998). EIN3 has been shown to bind the promoter of ERF1 and induce its expression (Solano et al., 1998). ERF1 expression is induced after B. cinerea infection of wildtype plants and transgenic plants overexpressing ERF1 display increased resistance to B. cinerea infection, which suggests that the ERF1 transcription factor may play a role in activating transcription of a range of genes whose products have anti-fungal properties (Berrocal-Lobo et al., 2002).
B. cinerea attack does not activate the SAR pathway in Arabidopsis due to the lack of a R-AVR interaction but does activate the less-understood Eth/JA ISR resistance response (Govrin & Levine, 2002; Penninckx et al., 1996). B. cinerea enters the host plant discreetly without inducing the plant to rapidly express defence genes and secondary metabolites which would be to the pathogen's detriment. The host plant produces camalexin at the onset of B. cinerea infection to limit growth but mutations that cause excessive amounts of camalexin to be produced do not always confer complete resistance against B. cinerea infection (Veronese et al., 2004). This indicates that the SA-dependent accumulation of camalexin is only a local general defence response to curb pathogen infection (Zhao & Last, 1996). Slight resistance to B. cinerea infection has also been demonstrated by over-expressing certain components in the Eth/JA defence pathway such as ERF1 or treating a plant with Eth or JA but the ultimate key to unlocking the door to complete B. cinerea resistance has not yet been found (Berrocal-Lobo et al., 2002; Thomma et al., 1999).

It has been hypothesized that resistance to B. cinerea will require a suite of genes, all involved in partial resistance, as modification of a single gene will be insufficient. Quantitative trait loci (QTL) have been shown to provide partial resistance to B. cinerea infection (Denby et al., 2004). The genes underlying these QTL have not yet been identified, however four loci in Arabidopsis termed BOTRYTIS SUSCEPTIBLE (BOS) have been shown to be important for B.
cinerea resistance (Mengiste et al., 2003; Veronese et al., 2004). Bos1 mutant plants displayed increased susceptibility to two necrotrophic pathogens, B. cinerea and Alternaria brassicicola, but did not differ from wildtype plants when infected with biotrophic pathogens (Mengiste et al., 2003). Mutation of the BOS2 gene conferred increased susceptibility to B. cinerea but retained wildtype resistance profiles to A. brassicicola and biotrophic pathogen infection. The bos3 mutation, however, has disrupted both SA and Eth/IA pathways, as it displayed susceptibility to both necrotrophic pathogens but resistance to biotrophic pathogen attack. The mutation with the widest range of disease profiles, displaying susceptibility to both necrotrophic pathogens and an avirulent pathogen, was bos4 (Veronese et al., 2004).

PDF1.2 expression was reduced in bos2, 3 and 4 in a manner that suggests that the levels of PDF1.2 expression were inversely proportional to the extent of B. cinerea susceptibility (Veronese et al., 2004). However, bos1 susceptibility was independent of PDF1.2 expression as bos1 displayed wildtype levels of PDF1.2 (Mengiste et al., 2003). The varied PR-1 expression patterns displayed by 3 of the bos mutants, namely bos2, 3 and 4, correlated to the respective disease resistance profile against biotrophic pathogen infection (Veronese et al., 2004). This is again in contrast to bos1 where the high level of PR-1 expression was uncoupled from resistance as bos1 displayed a wildtype resistance profile to biotrophic pathogen infection (Mengiste et al., 2003). Camalexin levels were also uncorrelated to B. cinerea resistance profiles as high levels of camalexin in bos3
mutants reiterate the fact that the fungicidal properties of camalexin are not the sole answer to providing *B. cinerea* resistance (Veronese et al., 2004). The diverse disease profiles and defence-gene expression exhibited by these four mutants imparts more evidence for the hypothesis that a consortium of genes needs to be activated to provide an effective *B. cinerea* resistance response.

*cir3* was initially isolated for displaying altered SAR phenotypes but further characterisation of *cir3* will hopefully provide another link between the SA, Eth/JA pathways as it displays activation of SA and Eth/JA defence signalling pathways.
Chapter 2
Materials and Methods

Plant Material and Growth Conditions

PR-1::LUC transgenic Arabidopsis, ecotype Col-0, plants were generated as described (Murray et al., 2002) and cir3 plants were isolated from an ethylmethane sulfonate (EMS) mutagenised PR-1::LUC line (Murray et al., 2005). Sterilised seeds were stratified for 2 days at 4°C and single-seeded onto plant nutrient (PN, Haughn & Somerville, 1986) agar plates. Plants were grown under 24 hour light conditions in 100% humidity. Seeds were stratified for 2 days and then germinated on peat pellets (Jiffy-7, AS Jiffy Products LTD, Norway) and grown in 16 hour light and 8 hour dark conditions at 24°C and 50% relative humidity.

Luciferase Assay

A crude protein extraction was performed by homogenizing leaf tissue in extraction buffer (0.1 M sodium phosphate buffer, pH 7.2, 5 mM 1,4-Dithiothreitol (DTT)). Background luminescence of the crude protein was measured by adding 100 μl assay buffer (60 mM Tris-HCl pH 8, 26 mM MgCl₂, 20 mM DTT, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM adenosine 5’-triphosphate (ATP)) to 20 μl crude protein extract and a reading was obtained 20 seconds later using a Luminometer (Luminoskan, TL-Plus, Labsystems). Luciferase activity was measured for 20 seconds after adding 100 μl luciferase buffer (60 mM Tris pH 8, 100 μM luciferase).
20 mM MgCl₂, 20 mM DTT, 2 mM EDTA, 1 mM luciferin (Biosynth, Switzerland). The background reading was subtracted from the luciferase reading to determine the relative light units (RLU). The protein concentration of each sample was measured using the Bradford assay (Bradford, 1976) and either a bovine serum albumin (BSA) or a γ-β-globulin standard curve was used for quantification.

**Western Blot Analysis**

Total protein was extracted from leaf material by grinding it in 250 μl extraction buffer (1X Tris Buffered Saline (TBS), 1 mM EDTA pH 7) containing three protease inhibitors: 2 mM phenyl methylsulfonyl fluoride (PMSF), 2 mM Benzamide, 10 mM ε-amino Caproic acid) (Zhao & Last, 1995). The extracted protein was quantified using the Bradford assay (Bradford, 1976) and a BSA standard curve and then combined with an equal volume of 2X sodium dodecyl sulphate (SDS) sample application buffer (20% (v/v) glycerol, 4% (v/v) SDS, 0.0025% (v/v) bromochloro blue, 0.12 M Tris-HCl, pH 6.8) and boiled for 3 - 5 minutes. For PR-1 protein detection, 15 μg of protein was loaded onto the gel matrix. Protein samples were on a separated on a 12% (w/v) SDS-polyacrylamide gel (PAG) (Laemmli, 1970), electro-blotted and transferred to nitrocellulose membrane with a pore size of 0.2μM (Protran™, Schleicher & Schuell, Germany) and stained with 0.1% (w/v) Ponceau S, 5% (v/v) acetic acid. Primary antibodies were raised in rabbit (Kliebenstein et al., 1999) and an alkaline-phosphatase goat-anti-rabbit IgG (Boehringer Manheim, Germany) probe was used at a dilution of 1:250 and 1:1000 respectively to detect binding.
The blot was visualized using chemiluminescence (Durrant & Fowler, 1994) and the Gene genome software. Membranes were stripped and probed with manganese superoxide dismutase (MnSOD) antisera to demonstrate loading and transfer of total proteins.

**Northern Blot Analysis**

Total RNA was isolated from leaf tissue by homogenizing it in 500 μl Solution D (4M guanidinium thiocyanate, 25 mM sodium citrate pH7, 0.5% (w/v) Sarkosyl, 0.7% (v/v) β-mercaptoethanol. RNA was extracted with an equal volume of water saturated phenol, pH 4, 0.5 M sodium acetate, pH 5.2 and chloroform: isoamyl (49:1) solution. RNA was precipitated on ice with an equal volume of isopropanol and 0.8 M sodium citrate/1.2 M NaCl. The RNA pellet was washed in 75% ice-cold ethanol, air-dried and resuspended in deionised formamide.

Spectrophotometric quantification at 260 nm of the total RNA was performed. 10 μg total RNA was electrophoresed in a denaturing formaldehyde-agarose gel, transferred via capillary action to Hybond-N hybridization membrane (Amersham-Pharmacia, Piscataway, NJ) and stained with methylene blue (0.3 M sodium acetate (pH 5.5-7), 0.03% (w/v) methylene blue) to allow visualization of the RNA on the membrane. The PR-1 and PDF1.2 DNA was amplified from genomic DNA using gene-specific primers

- **PR-1 Forward** primer 5’-CTGCAGACTCATACTCTGG-3’
- **PR-1 Reverse** primer 5’-TATGTACGTGTGTA TCGATGATC-3’
- **PDF1.2 Forward** primer 5’-ACGCGTCCGATACATTGAA-3’
- **PDF1.2 Reverse** primer 5’-
CACAGCATTAGCACCAAAG-3'), and gel purified using the Wizard® SV Gel & PCR clean-up system (Promega) according to the manufacturers protocol. The cDNA probes were radiolabelled with $^{32}$CTP (Specific activity 300±Ci; Amersham, UK) using the Megaprime™ DNA Labelling System (Amersham Biosciences, UK). Hybridisation of the probed membrane and subsequent washing were performed as described (Church and Gilbert, 1984). The membrane was incubated in 6X SSPE (0.9 M sodium chloride, 58 mM sodium dihydrogen phosphate, 6 mM EDTA, pH 7.4), 5XDenharts solution, 1% (w/v) SDS, 100 µg.ml$^{-1}$ heat denatured salmon sperm and probed with the $^{32}$P-labelled cDNA fragment. Washing was performed at low stringency (6XSSPE, 0.1% (v/v) SDS at 55°C for 30 minutes; 2XSSPE, 0.1% (v/v) SDS at 55°C for 30 minutes) Probe intensity was visualized using a Phosphorimager (Molecular Imager® FX, Bio-Rad).

Mapping Analysis, DNA Extraction, and PCR conditions

Segregating $F_2$ cir3xLer plants displaying low luciferase activity but containing the PR-1: LUC transgene were selected for mapping of the CIR3 gene. DNA was extracted from leaf tissue using the super quick DNA extraction buffer (Edward et al., 1991). The DNA pellet was resuspended in Tris EDTA (TE) and treated with 1/10 volume 10 mg.ml$^{-1}$ ribonuclease (Rnase) for 10 minutes at room temperature.
Luciferase gene specific primers, 5'-GTGACTAGTGGAAGACCCAA-3' sense and 5'-AACGAGCTCTGAATACAGTTAC-3' anti-sense were used to detect the PR-1:LUC transgene. PCR conditions were: 20 minutes at 96°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, 1 minute at 72°C, and 5 minutes at 72°C using Taq polymerase (Pluthero, 1993).

Mapping analysis was done using PCR-based single sequence-length polymorphic (SSLP) (Bell & Ecker, 1994) and cleaved amplified polymorphic sequences (CAPS) (Konieczny & Ausbel, 1993) markers obtained from the Arabidopsis Information Resource database website (http://www.arabidopsis.org). DNA from Columbia (Col-0), Landsberg (Ler) and a combination of the two, heterozygote (Het.), was used as a positive control. A water control (i.e. PCR reaction with no DNA) was included to detect contamination of reagents. To determine linkage, the recombination frequency (Rf) was calculated: Rf = ((#Col*2) + # Het.) / # Chromosomes.

Disease-Resistance Assay using Botryris cinerea inoculations and Monoclonal Antibody-based Immunoassay

The B. cinerea pepper isolate, obtained from Dr G. Loake at the University of Edinburgh, was maintained on apricot halves and leaf drop inoculations were performed as in Denby et al. (2004). In brief, 4µl of the spore suspension was placed on either attached leaves of whole plants or on excised leaves on 0.8% water agarose medium and kept at 100% humidity to allow infection to occur.
The diameter of the dark grey disease lesions was measured 5 days after infection.

To quantify *B. cinerea* fungal growth infected leaf material was ground in 3 ml phosphate buffered saline (PBS) and assayed by enzyme-linked immunosorbent assay (ELISA) using a genus-specific monoclonal antibody, BC12.CA4, as previously described (Dewey et al., 2000). Dessert wine (a gift from F. M. Dewey) was used as a source of *B. cinerea* antigen. Wine was diluted in PBS and a standard curve was constructed to enable absorbance readings to be expressed as relative antigen units.

**Selection of npr1 using Salicylic Acid (SA) Treatment and PCR Selection**

SA Treatment: Stratified seed of *cir3, npr1-1*, and *F3 cir3xnpr1-1* was germinated on PN agar containing 0.05 mM SA (Cao et al., 1997, Zhang et al., 2003).

NPR1-1 PCR Selection: DNA from *F2 cir3xnpr1-1, cir3* and *npr1-1* plants was extracted as before and used for PCR analysis using *NPR1* gene-specific primers as described by Li et al. (1999). PCR conditions were: 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C, 1 minute at 72°C, and 10 minutes at 72°C using Taq polymerase (Pluthero, 1993). An aliquot of each sample was checked for the presence of a 770 bp product by gel electrophoresis of a 1% (w/v) agarose gel in 1X Tris-Acetate EDTA (TAE) buffer and digested with *NlaIII* overnight at 37°C. Electrophoresis of the digested PCR
products on a 4% agarose gel separated 100 bp, 150 bp and 200 bp products for
NPR1 and 150 bp, 200 bp and 300 bp bands for np1-1

1. Aminocyclopropane-1-carboxylate (ACC) Treatment

Stratified seed of cir3 ein2 and F2 cir3xein2 was germinated on PN agar
containing 10 μM ACC (Sigma, USA) in the dark for 5 days and screened for the
triple response phenotype (Guzman & Ecker, 1990).
Chapter 3

Characterisation of cir3, a novel defence related mutant

Isolation and investigation of molecular components underlying the disease resistance pathway in the model plant, *Arabidopsis thaliana*, has been underway since the 1980’s (reviewed by Dong, 1998, 2001). Wildtype plants are susceptible to virulent pathogens and elicit a resistance response to avirulent pathogen attack. Wild type plants of different ecotypes have been shown to display different resistance phenotypes to the same pathogen (Dong et al., 1991; Whalen et al., 1991; Dangl et al., 1992). This indicates that the resistance response is genetically controlled as the slight differences in genetic information between ecotypes forms the basis of the different resistance profiles to a certain pathogen.

Rigorous genetic analysis of mutagenised *Arabidopsis* plants displaying altered resistance phenotypes has been fundamental in ascertaining the defence-signalling pathway leading to a resistance response (Glazebrook & Ausubel, 1997a; Dong, 1998; Thomma et al., 2001). Various techniques have been used to create genetic mutations in *Arabidopsis* plants as its small genome size and the availability of its entire genomic sequence (The *Arabidopsis* Genome Initiative, 2000) make it an ideal system to scrutinize genes involved in activating the defence signalling response. Chemically mutagenised or T-DNA transformed *Arabidopsis* plants are screened for increased or decreased pathogen resistance.
by comparing the rate of infection between the wildtype and mutant line. Transgenic Arabidopsis plants harbouring reporter gene constructs under the control of a known defence gene promoter have been used to determine when and where a specific defence gene is expressed (Cao et al., 1994; Murray et al., 2002). To discover other genes that affect the expression of the defence gene in question, researchers create random genetic mutations in these transgenic plants and look for altered expression of the reporter gene construct (Murray et al., 2002). The spatial expression of the endogenous gene is inferred by assaying for reporter activity. Luciferase, one such reporter, can be assayed without harming or killing the plant.

The constitutive induced resistance (cir) mutations were isolated using the above screening strategy (Murray et al., 2002). A transgenic line (PR-1::LUC) containing the firefly luciferase reporter gene under the control of the tobacco PR-1a promoter was mutagenised with ethyl methyl sulfonate (EMS). M2 plants were screened for altered reporter gene activity in the absence of pathogen attack (Murray et al., 2002). Four mutant lines displaying increased expression of the reporter gene, PR-1::LUC, were identified. Three of the mutations were found to be recessive and one was dominant (Murray et al., 2005).

The recessive mutant cir1 was characterised further for an altered resistance phenotype to various pathogen infections and for expression of additional defence-related genes (Murray et al., 2002). Cir1 exhibited increased resistance
to infection by the oomycete pathogen, *Peronospora parasitica* Noco2, and the bacterial pathogen *P. syringae pv. tomato* (Pst) DC3000 in an SA-dependent manner (Murray *et al.*, 2002). *Cir1* also displayed constitutive expression of other defence related genes such as *PDF1.2*, *PR-2*, *PR-5* and *GST1* (Murray *et al.*, 2002). The aim of this research was to characterise the dominant *cir3* mutation and determine how CIR3 fits into the defence-signalling pathway.

**Results**

To confirm that the increased luciferase activity displayed by *cir3* is a dominant phenotype, a luciferase assay of 4-week old F₁ *cir3 x PR-1::LUC* plants was performed. F₁ *cir3 x PR-1::LUC* displayed higher luciferase activity than PR-1::LUC but not to the same extent as *cir3* (Figure 1). However, F₁ progeny of *cir3* crossed to Col-O, a wildtype plant line that does not contain the PR-1::LUC transgene, did display luciferase activity to the same degree as *cir3* (Figure 1).

To determine the genetic position of CIR3, *cir3* pollen was used to fertilize Ler plants, an ecotype that does not contain the transgene or the genetic mutation. The F₁ generation was analysed for high luciferase activity to confirm that the cross had worked (Results not shown). F₁ plants were allowed to self-fertilize and the resulting F₂ progeny were assayed for luciferase activity. F₂ plants displaying low luciferase activity were earmarked for the mapping population. To confirm that the low luciferase activity observed in these lines was due to the presence of
Figure 1. Luciferase activity is dominant in cir3. Four-week old cir3, PR-1::LUC, Col-0, F1 cir3 x PR-1::LUC and F1 cir3 x Col-0 were assayed for luciferase activity. Relative light units (RLU) is a measure of luciferase activity and averages from at least 10 plants are represented in the graph. Error bars indicate standard error. Experiments were repeated twice with similar results.

A homozygous wildtype CIR3 genotype and not due to the absence of the reporter gene, PCR analysis using PR-1::LUC specific primers was performed on DNA extracted from the F2 plants displaying low luciferase activity. Twenty-nine F2 plants that displayed low luciferase activity and contained the PR-1::LUC transgene were used in the mapping population. First pass mapping of CIR3 using single sequence-length polymorphisms (SSLP) (Bell & Ecker, 1994) and co-dominant cleaved amplified polymorphic sequence (CAPS) (Konieczy & Ausubel, 1993) markers indicated that the wildtype CIR3 gene was situated on the lower arm of chromosome 3, approximately 30cM from the Bgl1 and nga6 markers (Table 3.1). Markers on chromosomes 1, 2, 4 and 5 showed recombination frequencies of approximately 50%, indicating no linkage.
Table 3.1: First pass mapping indicates that CIR3 is on the lower arm of Chromosome 3. The sample size refers to the number of chromosomes analysed. Rf:

Recombination frequency is calculated: ((# Col X 2) + # Het) / Sample size

<table>
<thead>
<tr>
<th>Chromosome &amp; Marker Location</th>
<th>Sample size</th>
<th>Columbia (Col)</th>
<th>L. erecta (Ler)</th>
<th>Heterozygote (Het)</th>
<th>Rf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) nga 280 80 cM</td>
<td>50</td>
<td>5</td>
<td>7</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>(II) nga 168 73 cM</td>
<td>56</td>
<td>4</td>
<td>14</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>(III) BglI 75 cM</td>
<td>58</td>
<td>0</td>
<td>11</td>
<td>18</td>
<td>32</td>
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<tr>
<td>(III) nga 5 81 cM</td>
<td>56</td>
<td>1</td>
<td>12</td>
<td>15</td>
<td>31</td>
</tr>
<tr>
<td>(IV) T20K18 50 cM</td>
<td>56</td>
<td>5</td>
<td>8</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>(V) nga 106 33 cM</td>
<td>50</td>
<td>6</td>
<td>6</td>
<td>13</td>
<td>50</td>
</tr>
</tbody>
</table>

Initial luciferase assays have shown that 4-week old cir3 plants display high reporter gene activity. To determine whether expression of the transgene is affected by the age of the plant, unchallenged 3, 4 and 5-week old cir3 and PR-1::LUC plants were assayed for luciferase activity. Luciferase activity was much higher in cir3 than PR-1::LUC at all ages and increased in an age dependent manner (Figure 2). The luciferase activity of PR-1::LUC plants was negligible in comparison to cir3. SA has been shown to induce the expression of PR-1 in Arabidopsis (Ward et al., 1991; Ryals et al., 1996) and exogenous SA treatment of PR-1::LUC caused induction of high luciferase activity to the same extent as uninoculated 5-week old cir3 (N. Adams, Honours Thesis 2002).
When a reporter construct is used to determine the expression profile of a gene of interest, it does not always obey the same regulatory pathways that the endogenous gene is subjected to. The reporter gene or its product may not contain sequences that direct it for transcriptional or translational regulation. To determine if the PR-1::LUC reporter gene gives an accurate reflection of the expression patterns of the endogenous PR-1 mRNA and PR-1 protein, tissue for RNA and protein isolation was harvested from the same 3, 4 and 5-week old plants that were used for the luciferase assay. PR-1::LUC activity mirrors the age dependent expression of endogenous PR-1 mRNA (Figure 2b) and PR-1 protein (Figure 2c) as the highest expression is seen in 5-week old cir3 plants. The endogenous expression of PR-1 mRNA in cir3 could be undergoing additional regulation in comparison to the reporter gene construct or the luciferase assay could be more sensitive, as slight PR-1 mRNA accumulation only appears in 4-week old cir3 plants. It is also probable that post-translational regulation of PR-1 protein is occurring in cir3 or the western blot analysis is not as sensitive as the luciferase assay, as the PR-1 protein is not prevalent in 3 and 4-week old cir3 plants.

PR-1 is a classic marker of the SA mediated defence-signalling pathway. The components of the plant defence pathway activated by ethylene (Eth) and jasmonic acid (JA) signalling have not been as well characterized as those in the SA defence pathway. However, it is clearly documented that expression of the anti-fungal gene Plant Defensin 1.2 (PDF1.2) is a hallmark of an activated Eth/JA
Figure 2. *cir3* displays increased PR-1::LUC activity, PR-1 mRNA and PR-1 protein expression. (a) Luciferase assay of 3, 4 and 5-week old *cir3* and PR-1::LUC. RLU: Relative light units. Values presented are averages from five plants and error bars indicate standard error between values at the 95% confidence level. (b) Total RNA isolated from unchallenged 3, 4 and 5-week old *cir3* and PR-1::LUC plants was probed for PR-1 expression. r18S indicates methylene blue staining of the 18s rRNA to indicate loading of total RNA. (c) Total protein from unchallenged 3, 4 and 5-week old *cir3* and PR-1::LUC was probed with PR-1 anti-sera. The membrane was re-probed with anti-sera against MnSOD to indicate loading. All experiments were repeated at least three times with similar results.
defence-signalling pathway in response to necrotrophic pathogen attack (Penninckx et al., 1996; Thomma et al., 1998, 1999). To determine whether PDF1.2 is expressed in unchallenged cir3, RNA from the 3, 4 and 5-week old plants that was used for PR-1 expression analysis was used for PDF1.2 expression analysis. Increased expression of PDF1.2 was only seen in 5-week old cir3 in comparison to PR-1 (Figure 3). The cir3 mutation has therefore activated both the SA and Eth/JA defence signalling pathways.

![Figure 3. cir3 displays hallmarks of an active Eth/JA acid pathway. Total RNA from 3, 4 and 5-week old cir3 and PR-1::LUC plants was analyzed for PDF1.2 mRNA expression. r18S indicates methylene blue staining of the 18s rRNA to indicate loading of total RNA. The experiment was repeated with similar results.](image)

Cir3 did not display increased resistance to infection by biotrophic pathogens (Murray et al., 2005) even though it displayed hallmarks of an activated SA-mediated signalling pathway. Therefore, as it also displayed an activated Eth/JA signalling pathway, which is important for resistance to necrotrophic pathogens, cir3 was challenged with B. cinerea spores. This would give an indication as to
Figure 4. cir3 shows increased resistance to *B. cinerea* infection. (a) Detached leaves of 4-week old cir3 and PR-1::LUC were inoculated with *B. cinerea* spores and the lesion diameter measured 3, 4 and 5 days later. (b) Detached leaves of 5-week old cir3 and PR-1::LUC were inoculated with *B. cinerea* spores and the lesion diameter measured 3, 4 and 5 days later. (c) Five-week old cir3 and PR-1::LUC were inoculated with *B. cinerea* spores and 6 days post infection discs of infected tissue were used in an ELISA assay. Relative antigen units are a measure of *B. cinerea* growth and values represent the difference between Relative antigen units of infected and uninfected leaf material. Averages were calculated using 3 similar sized leaves from at least 5 plants and error bars indicate standard error. All experiments were repeated with similar results.
whether the increased PDF1.2 expression of cir3 plays a role in increasing resistance to a necrotrophic pathogen. Detached leaves of 4 and 5-week old cir3 and PR-1::LUC plants were inoculated with spores of B. cinerea isolated from pepper (previously used in Denby et al., 2004) and incubated at high humidity for infection to occur. Two methods were used to establish how cir3 responds to B. cinerea infection. Firstly, the diameter of the water soaked lesion that had formed on the leaf surface was measured 3, 4 and 5 days post inoculation, as an indicator of the extent of infection. Secondly, to determine the growth of B. cinerea in planta, discs of infected leaf tissue were harvested 6 days post infection for an enzyme-linked immunosorbent assay (ELISA) using a genus specific monoclonal antibody, BC12.CA4 (Dewey et al., 2000). The discs were of equal size and encompassed the entire lesion. Depending on the extent of necrosis, the disc did include some healthy tissue around the lesion.

Lesion measurement of 4 and 5-week old plants 3, 4 and 5 days post infection indicated that cir3 was more resistant to B. cinerea infection than PR-1::LUC wildtype (Figure 4a, b). A larger difference between cir3 and PR-1::LUC B. cinerea resistance was seen at 5-weeks which correlates with increased defence gene expression at this age. In order to produce larger lesions on PR-1::LUC, B. cinerea grew more in PR-1::LUC than cir3 as indicated by an ELISA assay of infected leaf discs from 5-week old cir3 and PR-1::LUC (Figure 4c).
To establish if both increased luciferase expression and increased resistance to *B. cinerea* is caused by a single mutation of the *Cir3* gene, *B. cinerea* resistance of *F*$_1$ *cir3*×*PR-1::LUC* was performed to determine if *B. cinerea* resistance is also inherited as a dominant trait. *F*$_1$ *cir3*×*PR-1::LUC* plants were as resistant as *cir3*, indicating that the *cir3* resistance phenotype is dominant (Figure 5a).

A dominant mutation of the *Powdery Mildew Resistant 6 (PMR6)* gene causes an increased resistance phenotype to another necrotrophic pathogen, *Erisyphe cichoreacearum* (Vogel et al., 2002) and *pmr6* has been shown to reside on the lower arm of chromosome III. To determine if *cir3* and *pmr6* display similar *B. cinerea* phenotypes, *B. cinerea* resistance of 4-week old *pmr6* and *cir3* plants was tested using the detached leaf method. *Pmr6* displayed a slightly increased susceptibility to *B. cinerea* infection (Figure 5b) in comparison to *PR-1::LUC* plants.

**Discussion**

The *PR-1::LUC* activity displayed by *F*$_1$ *cir3*×*Col-0* plants demonstrates clearly that the increased *PR-1::LUC* activity conferred by the *cir3* mutation is segregating as a dominant trait (Figure 1). The reason for partial silencing of the *PR-1::LUC* transgene in *F*$_1$ *cir3*×*PR-1::LUC* plants is unknown but *F*$_1$ *cir3*×*PR-1::LUC* still displays significantly higher luciferase activity than *PR-1::LUC*. Contrary to previous reports (Jorda and Vera, 2000), the *PR-1::LUC* transgenic line has been shown to be a reliable *PR-1* mRNA expression marker as *PR-
Figure 5: cir3 resistance to B. cinerea is dominant and cir3 is not allelic to pmr6.

(a) Four-week old F1 cir3 x PR-1::LUC, cir3 and PR-1::LUC plants were inoculated with B. cinerea spores and the lesion diameter measured 5 days later. (b) Detached leaves of 4-week old cir3, PR-1::LUC and pmr6 were inoculated with B. cinerea spores and the lesion diameter was measured 5 days later. Averages were calculated from 3 similar sized leaves from 5 plants and error bars indicate standard error. Experiments were repeated twice with similar results.

PR-1::LUC expression accurately mirrored the expression of the endogenous PR-1 gene (Figure 2). The luciferase protein is not very stable and therefore the increased luciferase activity over time is probably due to increased expression and not accumulation (Van Leeuwen et al., 2000).
PR-1 expression was shown to increase in an age-dependent manner (Figure 2) along with another defence related gene, PDF1.2 (Figure 3), supporting the hypothesis of developmentally controlled defence-mechanisms (Kus et al., 2002). Numerous studies have shown that healthy, uninfected Tobacco and Arabidopsis plants express defence genes as they get older or start to senesce (Fraser, 1981; Butt et al., 1998; Quirino et al., 1999). It is therefore possible that cir3 may simply have accelerated development leading to early PR-1 expression. However, cir3 plants did not show any visual signs of early senescence and looked similar to PR-1::LUC. Kus et al. (2002) describe age-related resistance (ARR), whereby wildtype plants acquire resistance to P. syringae, as they get older. Mutants that were unable to accumulate SA, such as sid2, did not display ARR, whereas npr1-1 did display ARR indicating that ARR requires SA accumulation but is independent of PR-1 expression (Kus et al., 2002). The lack of correlation between PR-1 expression and ARR in Col-0 observed by Kus et al. (2002) also suggests that cir3 mediated resistance is not an early ARR response. Interestingly, other mutants showing expression of defence genes in the absence of pathogen infection, such as cpr5 and cpr6, also displayed increased expression of defence genes with age (Bowling et al., 1997, Clarke et al., 1998). It is possible that cir3 and other constitutive defence mutations activate a signalling component that is only expressed in older plants. In this scenario, increased PR-1 expression observed in 5-week old cir3 plants is due to constitutive activation of a signaling component that is not expressed in 2-week old plants.
The antagonistic relationship documented between certain points of the SA and JA defence signalling pathways (Pieterse et al., 2001; Spoel et al., 2003) is not displayed by the cir3 mutation, as hallmark gene expression of both signalling pathways was apparent. Activation of both defence pathways is not unknown as the cpr6 mutant also displays both PR-1 and PDF1.2 expression in the absence of pathogen attack (Bowling et al., 1997; Clarke et al., 1998). The increased PR-1 expression conferred by cir3 was uncoupled from resistance, as cir3 was not more resistant to biotrophic pathogen infection (Murray et al., 2005). It could be hypothesised that the cir3 mutation does not activate a critical subset of genes involved in resistance to biotrophic pathogen attack. Reduced lesion development and B. cinerea growth of cir3 in comparison to PR-1::LUC indicated that the activated Eth/JA pathway could be playing a role in resistance to B. cinerea. The role of PDF1.2 in cir3 resistance to B. cinerea must be treated with caution, as expression of PDF1.2 is not visible in 4-week old cir3 plants, yet cir3 displays resistance to B. cinerea at this age. This could be due to low sensitivity of the RNA hybridisation protocol allowing low levels of PDF1.2 in 4-week old cir3 to be undetected. However, many reports have shown that pathogen resistance does not rely on the function of a single gene but the partial activities of many (Ferrari et al., 2003) and other Eth/JA dependent genes involved in B. cinerea resistance could be up regulated in cir3.
Resistance to *B. cinerea* and increased PR-1::LUC expression both segregate as dominant traits in *cir3* suggesting that both phenotypes are caused by the same mutation. Further studies would be needed to confirm that a single mutation is responsible for both phenotypes as *cir3* was mutagenised with EMS, which creates numerous random mutations. Concurrent luciferase and *B. cinerea* assays of F$_2$ *cir3* x PR-1::LUC plants would be needed to determine if the phenotypes segregate together. An alternative but more time consuming approach would be to introduce the wildtype gene, once it has been cloned, into the mutant and determine if both phenotypes were rescued.

It was hypothesized that the *PMR6* gene found on chromosome III could be allelic to *CIR3*, as *PMR6* is the also found on the lower arm of chromosome III and *pmr6* displays increased resistance to a necrotrophic pathogen, *Erysiphe cichoracearum* (Vogel et al., 2002). Detached leaf assays of 4-week old *pmr6*, *cir3* and PR-1::LUC were performed to determine if *cir3* and *pmr6* displayed similar *B. cinerea* resistance phenotypes, which would indicate that they might be allelic. *Pmr6* displayed a larger average lesion diameter than both *cir3* and PR-1::LUC, indicating increased susceptibility to *B. cinerea* infection (Figure 6b). The difference in *B. cinerea* resistance between *cir3* and *pmr6*, along with a considerable difference in their genetic locations, suggests that they are not allelic. *PMR6* is found at approximately 7cM whereas first pass mapping of the *CIR3* gene suggests that it could be more than 40cM from that location.
Therefore it is hypothesized that CIR3 defines a novel defence related gene involved in *B. cinerea* resistance.
Chapter 4

Mechanism of \textit{cir3}-mediated \textit{Botrytis cinerea} resistance

\textit{Cir3} expresses hallmarks of both activated SA and Eth/JA pathways. The SA and Eth/JA defence signalling pathways display some interaction and interplay between the signalling components is still currently being dissected (Kunkel & Brooks, 2002). To determine if a phenotype displayed by a mutant is dependent on SA, Eth or JA signalling, researchers make use of mutations that are known to affect these pathways.

Transgenic \textit{nahG} plants are used extensively to determine whether a specific mutation is dependent on SA accumulation as \textit{nahG} encodes a salicylate hydroxylase protein. This renders the plant unable to accumulate SA in response to pathogen attack as the salicylate hydroxylase converts SA to catechoi (Delaney \textit{et al}., 1994; Gaffney \textit{et al}., 1993). SA accumulation induces downstream defence signalling, which can occur via two ways: NPR1-dependent or NPR1-independent. NPR1 is a transcription factor with ankyrin repeats and has been shown to translocate to the nucleus to activate defence gene expression, such as \textit{PR-1}, in response to pathogen infection or SA treatment (Cao \textit{et al}., 1994, 1997; Kinkema \textit{et al}., 2000). Mutation of the \textit{NPR1} gene causes susceptibility to biotrophic pathogen attack and inability to induce expression of \textit{PR}-genes but does not affect resistance to \textit{B. cinerea} infection (Cao \textit{et al}., 1994, Ferrari \textit{et al}., 2003). \textit{NPR1} is hypothesized to regulate cross...
talk between SA and JA defence pathways but further studies have to be conducted to establish this interaction (Spoel et al., 2003).

The Eth/JA defence pathway is commonly activated by necrotrophic pathogen attack (Thomma et al., 1998, 1999). Ethylene is produced in response to a wide range of stimuli and is vital in protecting the plant from environmental stresses (Yang & Hoffman, 1994; Wang et al., 2002). Enzymes involved in the biosynthetic pathway of ethylene production in a plant are known but further investigation is needed to determine how they are regulated (Yang & Hoffman, 1984, Wang et al., 2002). 1-Aminocyclopropane-1-carboxylate (ACC) synthase converts ACC to ethylene and is thought to be the key regulatory step in the pathway (Apelbaum et al., 1981). Treatment with ACC results in the plant producing increased levels of ethylene. Germination of wildtype plants on medium containing ACC in the absence of photosynthesis gives rise to the triple response, i.e. the inhibition of root/hypocotyl elongation, apical hook tightening and swelling of the hypocotyl (Guzman and Ecker, 1990). Ethylene insensitive 2 (ein2) is a recessive mutation isolated from a screen of plants displaying an altered triple response phenotype. Ein2 did not display the triple response phenotype when grown on ACC in the dark and instead displayed the phenotype of plants grown in air (Guzman & Ecker, 1990). It is unknown as to how ein2 disrupts the ethylene-signalling pathway. EIN2 is a metal-transporter protein hypothesized to be involved in signalling or a receptor of ethylene production (Alonso et al., 1999). ein2 plants display a normal SAR pathway but increased
susceptibility to *B. cinerea* infection (Thomma et al., 1999). Ethylene defence signalling also works hand in hand with another plant hormone JA as accumulation of both hormones is needed for the expression of the defence gene, PDF1.2. *PDF1.2* expression is correlated with *B. cinerea* infection and therefore confirms the need for concomitant activation of both Eth and JA pathways for a defence response against necrotrophic pathogen attack (Penninckx et al., 1996).

Both the SA and Eth:JA pathways have been implicated in defence signalling at the primary site of *B. cinerea* infection but establishing systemic resistance to *B. cinerea* is only dependent on Eth:JA signalling (Ferrari et al., 2003). This phenomenon has been demonstrated by infecting plants that are unable to perceive or accumulate SA, Eth or JA with *B. cinerea* (Ferrari et al., 2003). Double mutant analysis of cir3 crossed to plant lines with defective SA or Eth signalling will place cir3 mediated *B. cinerea* resistance in the context of the SA-dependent and Eth:JA-dependent pathways.

**Results**

Exogenous SA treatment or removal of SA via the action of salicylate hydroxylase causes respectively the induction or suppression of *PR-1* expression in *Arabidopsis* (Ward et al., 1991; Delaney et al., 1994; Gaffney et al., 1993). To determine whether the increased *PR-1:: LUC* activity of cir3 was SA dependent, cir3 pollen was used to fertilize Col-O harbouring the *nahG* transgene. Thirteen
4-week old F₁ progeny were analysed for both luciferase activity and the presence of the PR-1::LUC transgene, as both the cir3 mutation and the nahG phenotype segregate in a dominant manner. It was found that accumulation of SA is needed for cir3 luciferase activity, as the F₁ progeny exhibited luciferase activity of the same magnitude as PR-1::LUC (Figure 1a). A study done by Ferrari et al. (2003) suggests that SA is needed for local but not systemic resistance to B. cinerea attack. To determine whether cir3 resistance to B. cinerea is SA dependent, detached leaves of 4-week old F₁ cir3 x nahG were infected with B. cinerea spores. Lesion diameter measurements 5 days post infection indicated that cir3 resistance to B. cinerea is SA dependent as resistance to B. cinerea is lost in the F₁ progeny (Figure 1b).

To determine whether cir3 SA-dependent PR-1::LUC activity and resistance to B. cinerea infection were NPR1- dependent or independent, cir3 x npr1-1 double mutants were isolated and characterised. NPR1 translocates to the nucleus following SA treatment and induces the expression of PR-1 (Kinkema, et al., 2000). A single point mutation caused by EMS resulted in the npr1-1 mutation (Cao et al., 1994). A PCR-based CAPS marker screen can detect polymorphisms between NPR1 and npr1-1 as an NlaI restriction site in the NPR1 sequence is removed by the point mutation. Thirty-nine 4-week old F₂ cir3 x npr1-1 plants were analysed for the npr1-1 mutation using the PCR-based CAPS markers screen. As expected for recessive inheritance of the npr1-1 mutation.
approximately a quarter of the population was \textit{npr1-1} homozygous ($\chi^2 = 0.08, P = >0.5$) (Table 4.1). A luciferase assay was performed concurrently on the 394-

![Figure 1. PR-1::LUC activity and \textit{B. cinerea} resistance in \textit{cir3} is SA dependent. (a) Luciferase assay of 4-week old \textit{cir3}, PR-1::LUC, \textit{F\textsubscript{1}} \textit{cir3} x \textit{nahG}, and \textit{nahG}. \textit{nahG} does not contain the \textit{PR-1::LUC} transgene and acts as a negative control. \textit{F\textsubscript{1}} \textit{cir3} x \textit{nahG} plants displayed luciferase activity comparable to PR-1::LUC plants. (b) Detached leaves of 4-week old \textit{cir3}, PR-1::LUC, \textit{F\textsubscript{1}} \textit{cir3} x \textit{nahG}, and \textit{nahG} were inoculated with \textit{B. cinerea} spores and the lesion diameter was measured 5 days later. Values presented on both graphs are averages from at least five plants using 3-4 leaves from each plant. Error bars indicate standard error between values at the 95\% confidence level.]}
week old $F_2$ cir3 x npr1-1 plants and the 9 plants that were homozygous for the npr1-1 mutation were also found to display low luciferase activity (Table 4.1). This, together with the cir3 x nahG data, indicated that the increased luciferase activity of cir3 is dependent on a functional NPR1-dependent SA signalling pathway.

Table 4.1: npr1-1 homozygous $F_2$ cir3xnpr1-1 plants display low PR-1::LUC activity. PCR analysis of 39 $F_2$ cir3xnpr1-1 plants indicate that \( \frac{1}{4} (9) \) are npr1-1 (nn) homozygous and \( \frac{3}{4} (30) \) of the population were NPR1-1 homozygous (NN) or heterozygous (Nn). The average luciferase activity from 5 plants of each control (cir3, npr1-1) is indicated. The low averages of luciferase activity from the 9 npr1-1 homozygous $F_2$ cir3 x npr1-1 plants indicate that cir3 reporter gene activity is NPR1-1 dependent. The average luciferase activity displayed by the 30 N/-$F_2$ cir3 x npr1-1 plants was significantly higher than the npr1-1 control plants.

<table>
<thead>
<tr>
<th>Inferred Genotype from PCR based screen</th>
<th>cir3 control</th>
<th>$F_2$ cir3 x npr1-1</th>
<th>$F_2$ cir3 x npr1-1</th>
<th>npr1-1 control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NN</td>
<td>NN, Nn</td>
<td>nn</td>
<td>nn</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3873</td>
<td>252</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(± 2374)</td>
<td>(± 164)</td>
<td>(± 3.15)</td>
</tr>
</tbody>
</table>

npr1-1 plants are unable to grow beyond the cotyledonous stage and display bleaching when grown on PN agar containing high concentrations of SA (Cao et al., 1997, Zhang et al., 2003). $F_3$ seed from each of the 9 npr1 homozygous lines was collected and plated on PN agar containing 0.5 mM SA to verify the npr1-1
Figure 2. Increased PR-1::LUC expression of cir3 is NPR1-dependent. PCR analysis (a) and luciferase assay (b) of 4-week old F2 cir3 x npr1-1 plants and controls indicate that loss of NPR1-1 in cir3 results in low PR-1::LUC expression. Three representative npr1-1 homozygous F3 cir3 x npr1-1 and control lines were germinated on PN agar containing 0.05 mM SA (c). The presence of the cir3 mutation reverses the SA sensitivity of npr1-1 and therefore the cir3 genotype can be inferred.

<table>
<thead>
<tr>
<th></th>
<th>cir3 control</th>
<th>3 Representative lines from the 9 npr1-1 homozygous F2 cir3 x npr1-1 lines</th>
<th>npr1-1 control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a</strong> NPR1-1 genotype</td>
<td>NPR1-1/NPR1-1</td>
<td>npr1-1/npr1-1</td>
<td>npr1-1/npr1-1</td>
</tr>
<tr>
<td><strong>b</strong> Luciferase Activity (RLU µg⁻¹ total protein)</td>
<td>3875 ± 2373</td>
<td>13.9</td>
<td>3.3</td>
</tr>
<tr>
<td><strong>c</strong> SA phenotype</td>
<td>cir3cir3 NPR1-1/NPR1-1</td>
<td>cir3cir3 NPR1-1/npr1-1</td>
<td>CIR3cir3 npr1-1/npr1-1</td>
</tr>
</tbody>
</table>

Inferred Genotype

<table>
<thead>
<tr>
<th>Expected # of lines</th>
<th>Observed # of lines</th>
<th>χ² test</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td>cir3cir3</td>
<td>2.25</td>
<td>1</td>
<td>1.91</td>
</tr>
<tr>
<td>cir3cir3 NPR1-1/npr1-1</td>
<td>4.5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>CIR3cir3 npr1-1/npr1-1</td>
<td>2.25</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>CIR3CIR3 npr1-1/npr1-1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SA sensitivity phenotype. Seedlings from all 9 lines were expected to show a 100% bleached phenotype but a segregation of the npr1-1 SA sensitivity phenotype was seen. Three lines displayed the SA sensitive phenotype conferred by the npr1-1 mutation, 5 lines displayed a segregation of the SA sensitivity phenotype in a 3:1 ratio of SA-insensitive to SA-sensitive plants and 1 line displayed 100% insensitivity (Figure 2). These results are consistent with a 1:2:1 segregation ratio ($\chi^2 = 1.91$, P > 0.1) that would be expected if the dominant cir3 rescued the npr1-1 phenotype. The cir3 mutation segregates in a dominant manner and therefore three-quarters i.e. approximately 7 of the 9 npr1-1 homozygous lines should also contain the cir3 mutation in a heterozygous or homozygous state. The distinction between lines heterozygous and homozygous for cir3 is made apparent by the 5 lines that display seedlings segregating for SA sensitivity and 1 line displaying 100% SA-insensitivity. The 3 lines that displayed the npr1-1 SA phenotype are hypothesized to contain the wildtype CIR3 gene. The cir3 x npr1-1 line that displayed 100% growth on SA was used for B. cinerea assays as it is likely to contain both mutations in a homozygous state. However, results obtained from a detached leaf assay of 4-week old F3 cir3 x npr1-1 to determine whether cir3 resistance to B. cinerea is NPR1 dependent or independent were inconclusive as both npr1-1 and cir3 were significantly more resistant than PR-1::LUC and cir3 x npr1-1 was not significantly different to cir3 or npr1-1(Figure 3).
To determine if the increased luciferase activity and *B. cinerea* resistance displayed by *cir3* was dependent on a functional ethylene pathway, *cir3* was investigated in a dysfunctional ethylene-signalling background and crossed to *ein2*. To isolate *F₂ cir3 x ein2* lines that were homozygous for the *ein2* mutation, 150 *F₂ cir3 x ein2* seed were screened for a triple response phenotype by germination on PN agar containing 10 μM ACC in the dark. One quarter of the seedlings were expected to display the triple response phenotype conferred by the recessively inherited *ein2* mutation presuming that the *cir3* mutation did not affect the *ein2* phenotype.

Figure 3. The role of *NPR1-1* mediated *cir3* resistance to *B. cinerea* is inconclusive. Detached leaves of 4-week old *cir3, PR-1::LUC, F₃ cir3 x npr1-1* and *npr1-1* were inoculated with *B. cinerea* spores and the lesion diameter was measured 5 days later. Values presented are averages from at least five plants using 3-4 leaves from each plant. Error bars indicate standard error between values at the 95% confidence level.

Twenty-nine seedlings displaying the triple response phenotype and hence *ein2* homozygous, were transplanted to soil and allowed to grow. A leaf from each of
the 4-week old ein2 homozygous F2 cir3 x ein2 lines was harvested for a luciferase assay to determine if the increased luciferase activity of cir3 was dependent on a functional ethylene signalling pathway. The cir3 mutation and PR-1::LUC transgene are both dominantly inherited therefore 9/16 of the ein2 homozygous lines are expected to contain at least 1 copy of cir3 and PR-1::LUC. A high luciferase to low luciferase ratio of 9:7 for two segregating dominant traits controlled by single genes (cir3 and PR-1::LUC) was observed indicating that ethylene signaling is not required for the increased PR-1::LUC activity of cir3 as 17 lines displayed high luciferase activity ($\chi^2 = 0.07$, $P > 0.5$). Ten F2 cir3 x ein2 lines displaying the highest luciferase activity were allowed to self-fertilize and set seed. Both cir3 and PR-1::LUC segregate as dominant traits making it difficult to find a triple homozygous mutant. Ten 4-week old plants from each of the 10 F3 cir3 x ein2 lines were assayed for luciferase activity to identify a line that displayed 100% high luciferase activity, indicative of cir3 and PR-1::LUC being homozygous. Two of the ten lines displayed high luciferase activity for all 10 plants tested. Due to the dominant inheritance of cir3 and PR-1::LUC these two lines were only hypothesised to be homozygous lines as a bigger sample number of plants would be needed to confidently affirm the homozygous nature. The line that displayed the highest luciferase activity profile was chosen for B. cinerea assays. To ensure that the B. cinerea assay was performed on plants that were cir3 mutant, 3 leaves and 1 leaf from each of the 4-week old double mutant plants were concurrently harvested for a detached leaf B. cinerea assay and luciferase assay respectively to correlate the B. cinerea assay with plants that
displayed a cir3 high luciferase phenotype. Lesion size measurements were collected from plants that displayed high luciferase activity (cir3/-; ein2/ein2). The cir3 resistance to B. cinerea is dependent on a functional ethylene-signalling pathway as the cir3-mediated resistance was lost in the F3 cir3xein2 double mutants (Figure 4).

Figure 4. A functional Ethylene signaling pathway is needed for B. cinerea resistance in cir3. Detached leaves of 4-week old cir3, PR-1::LUC, F3 cir3 x ein2 (cir3/-; ein2/ein2) and ein2 were inoculated with B. cinerea spores and the lesion diameter was measured 5 days later. Values presented are averages from at least five plants using 3-4 leaves per plant. Error bars indicate standard error between values at the 95% confidence level.

Discussion

To establish which signalling pathways are involved in the increased PR-1::LUC activity and B. cinerea resistance displayed by cir3, double mutant analysis of cir3 with SA and Eth mutants was performed. The presence of both the nahG transgene and npr1 mutation resulted in loss of the increased PR-1::LUC expression of cir3, indicating that the increased PR-1::LUC expression is
coupled to the increased levels of SA displayed by cir3 (Murray et al., 2005). Failure to accumulate or perceive SA through the action of nahG and the npr1 mutation respectively, results in decreased expression of PR-1 and increased susceptibility to biotrophic pathogen infection (Gaffney et al., 1993; Cao et al., 1994; Delaney et al., 1994). Therefore, the requirement for accumulation of SA and a functional NPR1-dependent SA signalling pathway to induce constitutive expression of PR-1 in cir3 is expected. PR-1 expression has been shown to be independent of the Eth defence-signalling pathway (Pieterse et al., 1995; 1998) and, as expected, the increased luciferase activity of cir3 is unaffected in an ein2 background.

Increased resistance to B. cinerea is, however, lost when there is no SA accumulation or Eth signalling in cir3. These results correlate with findings by Ferrari et al. (2003), which show that PR-1 expression is uncoupled from B. cinerea resistance. The role of SA in B. cinerea defence has been studied for several years with contradictory results. Treating plants with SA or one of its analogues conferred increased systemic resistance to B. cinerea (De Meyar & Hofte, 1997; Murphy et al., 2000). Studies by Govrin & Levine, (2002) and Ferrari et al. (2003) indicate that SA plays a minor role in resistance to B. cinerea infection which contrasted with studies by Thomma et al. (1999) which show that loss of SA accumulation and signalling had no effect on B. cinerea resistance. Ferrari et al. (2003), however, concluded that SA is necessary for local not systemic B. cinerea resistance which would explain why Thomma et al. (1999)
did not see a difference between SA mutants and wildtype plants, as they were analysing systemic *B. cinerea* resistance. The conflicting results obtained thus far could also be due to isolate specificity of *B. cinerea* as different isolates were used in each study. Previous reports indicate that nahG and *npr1-1* displayed different resistant phenotypes to local *B. cinerea* infection which correlate to results found in this study (Ferrari *et al.*, 2003). The small effect of the *npr1-1* mutation on *B. cinerea* resistance was more pronounced when other defence mechanisms such as ethylene signalling was impaired (Ferrari *et al.*, 2003). nahG is more susceptible to *B. cinerea* infection and when *cir3* is in a nahG background, it loses resistance to *B. cinerea*, indicating that, local resistance is dependent on SA accumulation. The decreased resistance to *B. cinerea* could be due to SA depletion but reports by Govrin & Levine (2002) cautioned the research community to carefully interpret data collected from experiments that relied on the nahG transgene as a SA scavenger. The by-product of SA degradation, catechol, has been shown to interfere with resistance pathways and may be a misguided exploration of SA-dependence (van Wees & Glazebrook, 2003). Wildtype plants treated with catechol were more susceptible to *P. parasitica* infection as accumulating catechol results in H$_2$O$_2$ production promoting infection to occur (van Wees & Glazebrook, 2003). Increased H$_2$O$_2$ production could also facilitate *B. cinerea* infection as recent studies have shown a positive correlation between ROI production and the rate of *B. cinerea* infection (Govrin & Levine, 2000). Ferrari *et al.* (2003) indicated that *npr1-1* did not show an altered resistance phenotype to *B. cinerea* infection in comparison to wildtype plants but
could still be playing a minor role as the double mutant npr1-1 x ein2, was more susceptible than ein2. In this study, however, npr1-1 displayed increased resistance to B. cinerea compared to the PR1::LUC parental line. These contradicting results could either be due to the use of different isolates of B. cinerea as previous reports have shown that resistance can be isolate specific (Denby et al., 2004) or due to the actual npr1-1 parent line not being used for this assay. It is possible that PR1::LUC and Col differ in their susceptibility to this pathogen. As there was no significant difference in B. cinerea resistance between cir3 and npr1-1, the role of NPR1-1 in cir3 resistance to B. cinerea infection could not be determined.

The results of cir3 double mutant analysis therefore reiterate the importance of SA in local resistance to B. cinerea and support the hypothesis that SA accumulation is important for establishing basal defence against B. cinerea by activating a subset of defence genes. CIR3 is hypothesised to function upstream of SA accumulation as removal of the high levels of SA displayed by cir3 results in loss of both its phenotypes.

Previously the ethylene insensitive phenotype of ein2 mutants was shown to result in increased susceptibility to B. cinerea infection indicating a role for ethylene signalling in B. cinerea resistance (Thomma et al., 1999, Ferrari et al., 2003). In addition reports have also shown that pretreating plants with ethylene conferred increased resistance to B. cinerea (Diaz et al., 2002). The need for
effective ethylene signalling is once again indicated by the increased susceptibility of cir3 x ein2 mutants to B. cinerea infection. Cir3 is therefore also acting upstream of ein2 and may be activating additional defence genes other than PDF1.2 that are also dependent on ethylene signalling, as previous reports indicate that expression of PDF1.2 does not always correlate with increased B. cinerea resistance (Ferrari et al., 2003). These double mutant analyses demonstrate that resistance to B. cinerea in cir3 is dependent on both SA and Eth signalling pathways. This supports the hypothesis that the activation of different subsets of defence genes via the activation of both SA and Eth signaling pathways leads needed for complete resistance to B. cinerea and future studies will analyse systemic resistance to B. cinerea infection in the cir3 double mutants.

Loss of NPR1 function results in increased SA toxicity of germinating seedlings (Cao et al., 1997, Zhang et al., 2003). The underlying mechanism for this phenotype has not been studied. It could be hypothesized that NPR1 has a dual function, to induce an anti-oxidant pathway to prevent oxidative stress brought on by high levels of SA (Chen et al., 1993) and to initiate defence responses through induction of PR-1 expression. Mutation of the cir3 gene reverses the toxic effects of SA in npr1 mutants. CIR3 acts upstream of salicylic acid and hence NPR1, and it could be hypothesized that the CIR3 protein is acting as a negative regulator of genes involved in combating oxidative stress the cell. Therefore, the inability of cir3 x npr1-1 seedlings to induce expression of anti-oxidant genes through the NPR1-1 signalling pathway, is rescued by the increased expression.
of antioxidant genes conferred by the cir3 mutation allowing cir3 x npr1-1 seedlings to germinate on media containing SA. The heightened expression of GST1, which encodes a protein with antioxidant properties, in cir3 plants suggests that the absence of cir3 could be allowing the induction of anti-oxidant genes to combat the ROI produced as a result of high SA levels (Murray et al., 2005).

In conclusion, local resistance to B. cinerea infection displayed by cir3 relies on signaling components from both the SA and Eth response pathways. It would be interesting to determine if the SA and Eth-mediated cir3 resistance response is effective against various B. cinerea isolates, which would suggest that cir3 is necessary for broad-spectrum B. cinerea resistance. Future studies will determine whether systemic resistance to B. cinerea is induced by the cir3 mutation.
Chapter 5

Concluding Remarks

Plant-pathogen interactions have been studied for many years and initial studies used economically important hosts such as soybean, tomato and bean plants (Keen, 1990). In the 1990's researchers began using Arabidopsis as a host plant even though the plant pathogens that they were interested in were not found to naturally infect Arabidopsis. Therefore there will always be limits to studying Arabidopsis-pathogen interactions, as it is not possible to deal with every facet of a biological plant-pathogen system. However, Arabidopsis has proven to be an ideal model plant as its small size, short life cycle, and small genome makes it easy to maintain and genetically manipulate in the laboratory (Meyерowitz, 1989).

A plant's ability to defend itself was suggested to be under genetic control by the gene-for-gene hypothesis put forward by Flor in 1971. Initial studies of the Arabidopsis-P. syringae system also displayed hallmarks of a gene-for-gene interaction, paving the way for additional Arabidopsis-pathogen systems to be explored. The discovery of numerous genes involved in defence signalling pathways have been possible through analysis of Arabidopsis carrying genetic mutations (Ausubel et al., 1995). Both forward and reverse genetic techniques have been used to discover genes involved in defence. Arabidopsis plants have been transformed with reporter genes under the control of defence genes promoters from other plant species (Murray et al., 2002) or genes hypothesized
to be involved in defence, such as MPK6, have been knocked out to determine its specific role in defence (Menke et al., 2004). Bioinformatics has also played a significant role in finding new defence gene candidates in Arabidopsis by performing BLAST searches of defence-related genes from other plant species such as the Mlo gene from Barley that is involved in powdery mildew resistance (Devoto et al., 1999). Arabidopsis has also been mutated using various forms of mutagenesis with the aim of knocking out the function of a gene and causing an altered resistance phenotype. Cloning of these genes lead to the discovery of new genes involved in defence (Glazebrook et al., 1997a). Reverse genetic approaches can also identify genes that have more than one function and therefore a correlation between developmental signalling pathways and defence related alterations could be found. Recent technological developments has allowed the introduction of a functional genomics approach to identifying numerous genes involved in defence. The use of microarray technology enables large scale gene discovery and is likely to have significant impact in plant defence field as it is a well-established hypothesis that a concert of genes and not the action of a single gene is needed for finding a complete answer to a constitutive resistance response phenotype.

The novel defence related gene, cir3 forms a part of the concert of genes needed for establishing the ultimate resistance response as it is involved in both the SA and Eth signalling pathway, upstream of NPR1 and EIN2 (Figure 1). Elucidating the role of the wildtype CIR3 gene in defence signalling is
complicated as the mutant cir3 phenotype is dominant. Cir3 PR-1::LUC activity and B. cinerea resistance has still to be placed in context with JA signalling and future experiments will characterise cir3 x jar1 double mutants. B. cinerea resistance does display some isolate specificity and it would be interesting to determine cir3's resistance profile to a range of B. cinerea isolates. If cir3 displays resistance to all isolates tested it could be hypothesised that cir3...
upregulates a set of genes needed for broad-spectrum resistance to *B. cinerea* infection. Once cir3's isolate specific resistance profile has been established it would be interesting to correlate differences in resistance responses, if any, are mediated via SA, Eth and JA signalling by determining isolate specificity of the double mutants. The ability to identify cir3 homozygous plants from SA-insensitive cir3 x npr1 lines will make the identification of an ideal mapping population more efficient. Future studies will focus on cloning the CIR3 gene to elucidate its ultimate function in the complex defence resistance pathway.
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