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 npr1-1* 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 plants' 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.,* 1998). 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 A*vr*

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*

al., 1980). 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 (H_2O_2) and superoxide molecules (O_2^-), 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 O_2^-, 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 *lsd1* 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. syringea* 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 signalling 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 (Ferrrari *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), *lsd (*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) / 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-signalling 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; Despres *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 hypothesised 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 play a minor role in local *B. cinerea* resistance at the site of infection (Ferrrari *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* (*hrl1)* 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 increased 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/JA 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, 20 mM $MgCl_2$, 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,

20 mM $MgCl_2$, 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) bromophenol 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 actetate, 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'-CTGCAGACTCATACACTCTGG-3' Reverse primer 5'-TATGTACGTGTGTATCGATGATC-3'; *PDF1.2* Forward primer 5'-ACGCGTCCGATACATTGAA-3' Reverse primer 5'-

CACAGCATTTAGCACCAAAG-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 300μCi; 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 mMsodium 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 *cir3*x*Ler* 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'-GTGACTAGTGGAAGACGCCAA-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) (Konieczy & Ausbel, 1993) markers obtained from the *Arabidopsis* Information Resource database website (http://www.arabidopsis.org). DNA from Columbia (Col-0), Landsberg (L*er*) 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 F_3 *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 F_2 *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 seperated 100 bp, 150 bp and 200 bp products for *NPR1* and 150 bp, 200 bp and 300 bp bands for *npr1-1*

1- Aminocyclopropane-1carboxylate (ACC) Treatment

Stratified seed of *cir3*, *ein2* and F_2 *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). M_2 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 F_1 *cir3 x PR-1::LUC* plants was performed. F_1 *cir3 x PR-1::LUC* displayed higher luciferase activity than PR-1::LUC but not to the same extent as *cir3* (Figure 1). However, F_1 progeny of *cir3* crossed to Col-0, 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 L*er* plants, an ecotype that does not contain the transgene or the genetic mutation. The F_1 generation was analysed for high luciferase activity to confirm that the cross had worked (Results not shown). F_1 plants were allowed to self-fertilize and the resulting F_2 progeny were assayed for luciferase activity. F_2 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

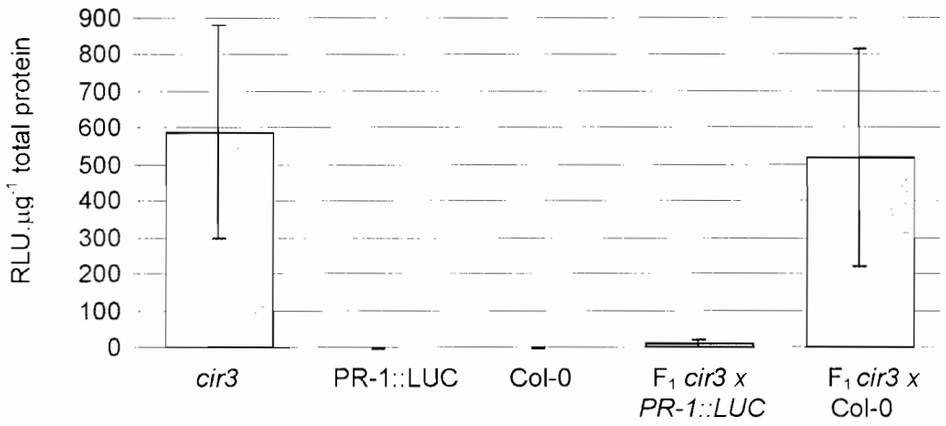


Figure 1. Luciferase activity is dominant in *cir3*. Four-week old *cir3*, PR-1::LUC, Col-0, F_1 *cir3 x PR-1::LUC* and F_1 *cir3 x* Col-0 were assayed for luciferase activity. Relative light units (RLU) is a measure of luciferase activity and averages from at least 10 plants are represented in the graph. 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 F_2 plants displaying low luciferase activity. Twenty-nine F_2 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

Chromosome & Marker Location	Sample size	Columbia (Col)	L. erecta (Ler)	Heterozygote (Het)	Rf (%)
(I) nga 280 80 cM	50	5	7	13	46
(II) nga 168 73 cM	56	4	14	10	50
(III) Bgl1 75 cM	58	0	11	18	32
(III) nga 6 81 cM	56	1	12	15	31
(IV) T20K18 50 cM	56	5	8	15	45
(V) nga 106 33 cM	50	6	6	13	50

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

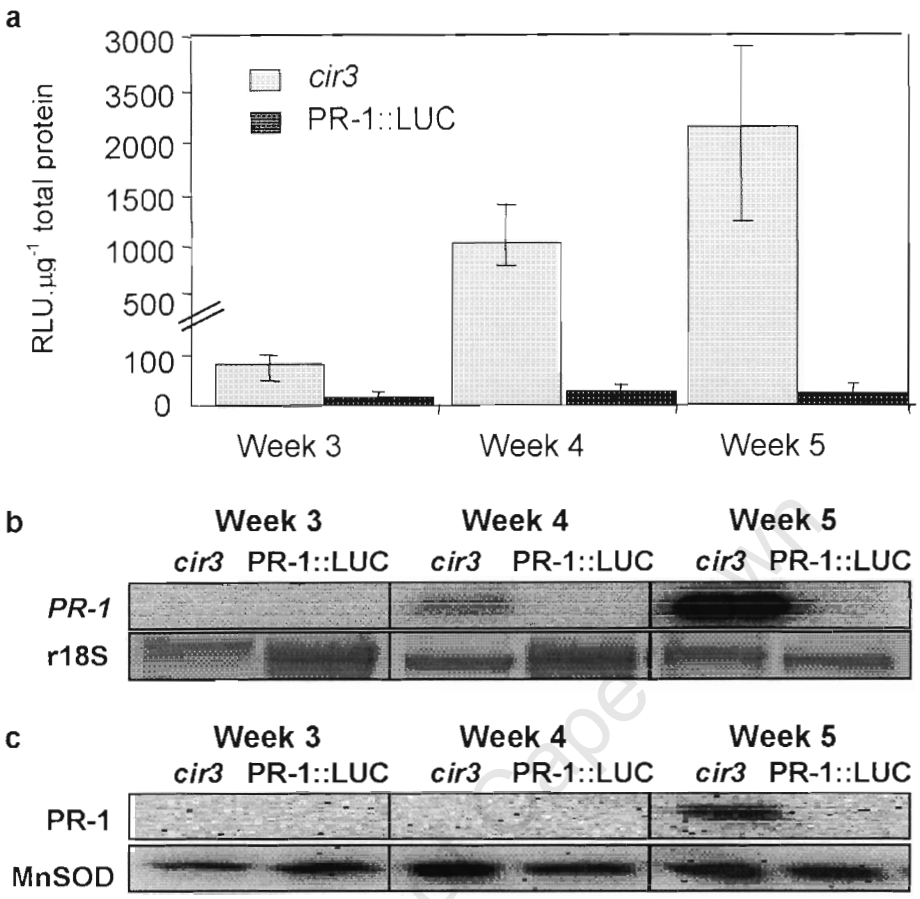


Figure 2. *cir3* displays increased PR-1::LUC *activity, PR-1* mRNA and PR-1 protein expression. (a) Luciferase assay of 3, 4 and 5-week old *cir3* and PR-1::LUC. RLU: Relative light units. Values presented are averages from five plants and error bars indicate standard error between values at the 95% 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.

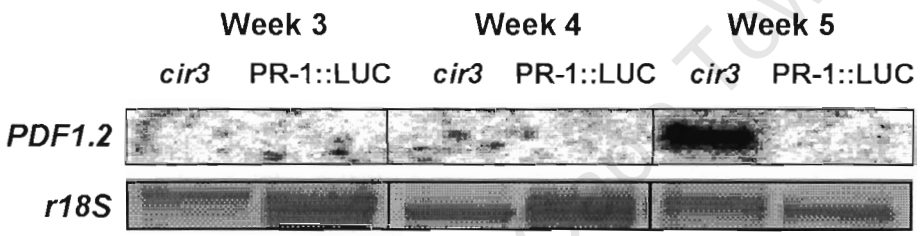


Figure 3. *cir3* displays hallmarks of an active Eth/JA acid pathway. Total RNA from 3, 4 and 5-week old *cir3* and PR-1::LUC plants was analyzed for *PDF1.2* mRNA expression. *r18S* indicates methylene blue staining of the 18s rRNA to indicate loading of total RNA. The experiment was repeated with similar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

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

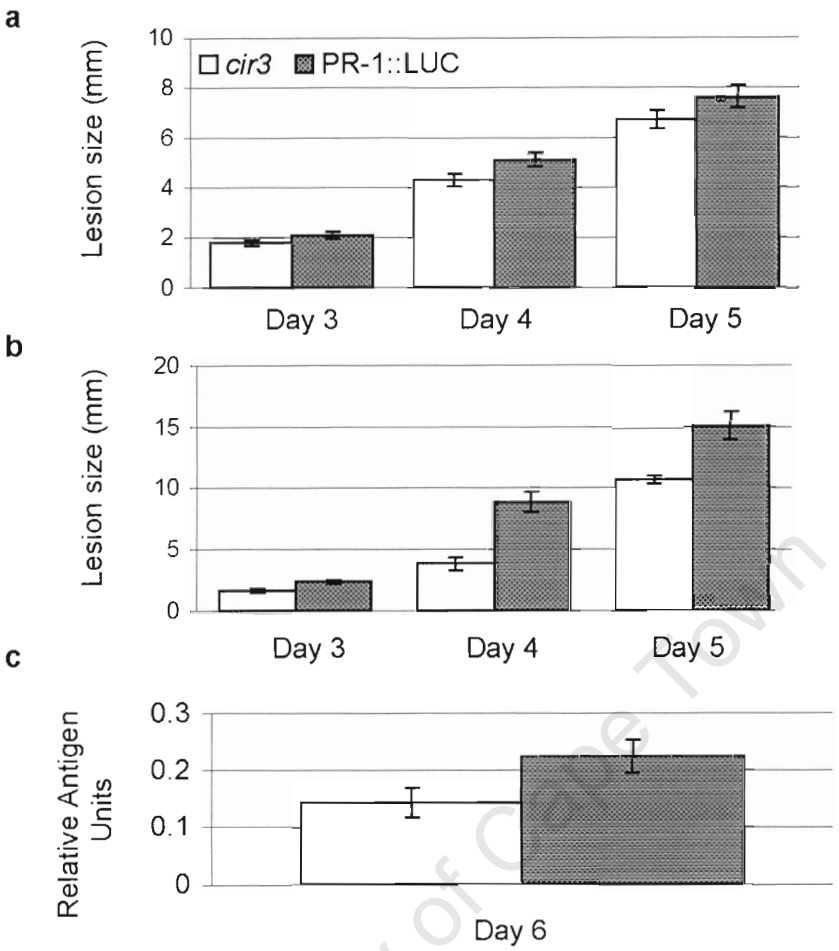


Figure 4. *cir3* shows increased resistance to *B. cinerea infection*. (a) Detached leaves of 4-week old *cir3* and PR-1::LUC were inoculated with *B. cinerea* spores and the lesion diameter measured 3, 4 and 5 days later. **(b)** Detached leaves of 5-week old *cir3* and PR-1::LUC were inoculated with *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 *cir3xPR-1::LUC* was performed to determine if *B. cinerea* resistance is also inherited as a dominant trait. F_1 *cir3xPR-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 cichoracearum* (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 x* 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 x PR-1::LUC* plants is unknown but F_1 *cir3 x 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-*

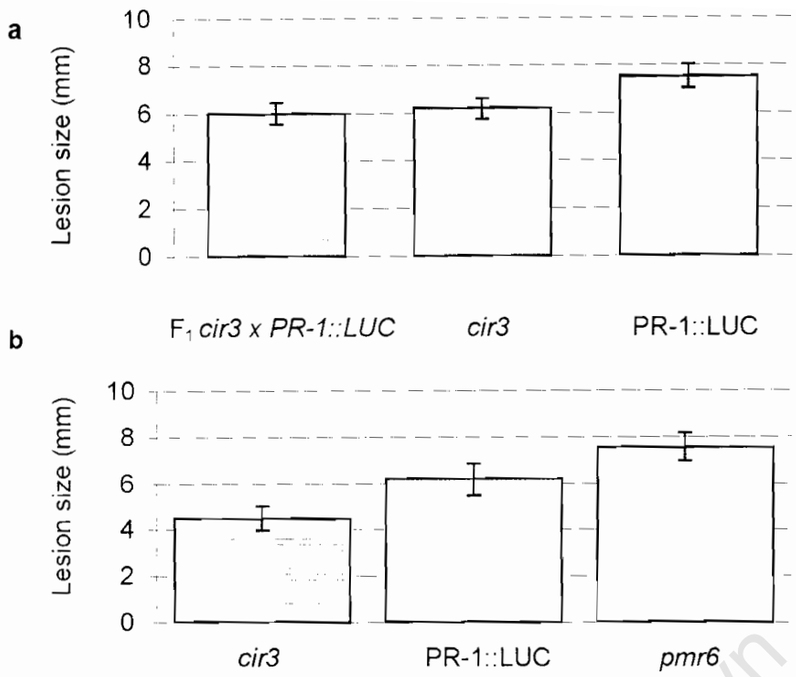


Figure 5: *cir3* resistance to *B. cinerea* is dominant and cir3 is not allelic to *pmr6*. **(a)** Four-week old F_1 *cir3 x PR-1::LUC*, *cir3* and PR-1::LUC plants were inoculated with *B. cinerea* spores and the lesion diameter measured 5 days later. **(b)** Detached leaves of 4-week old *cir3*, PR-1::LUC 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). Its 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 *cir3*-mediated *Botrytis cinerea* resistance

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 pathays.

Transgenic nahG plants are used extensively to determine whether a specific mutation is dependent on SA accumulation as *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 catechol (Delaney *et al.,* 1994; Gaffney *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 *PR-1*, in response to pathogen infection or SA treatment (Cao *et al.,* 1994, 1997; Kinkema *et al.,* 2000). Mutation of the *NPR1* gene causes susceptibility to biotrophic pathogen attack and inability to induce expression of *PR*-genes but does not affect resistance to *B. cinerea* infection (Cao *et al.,* 1994, Ferrari *et al.,* 2003). *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, 1984; 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 F_1 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 F_1 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 F_1 *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 F_1 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 NlaII restriction site in the *NPR1* sequence is removed by the point mutation. Thirty-nine 4-week old F_2 *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 *npr1-1* homozygous (χ^2= 0.08, P= >0.5) (Table 4.1). A luciferase assay was performed concurrently on the 39 4-

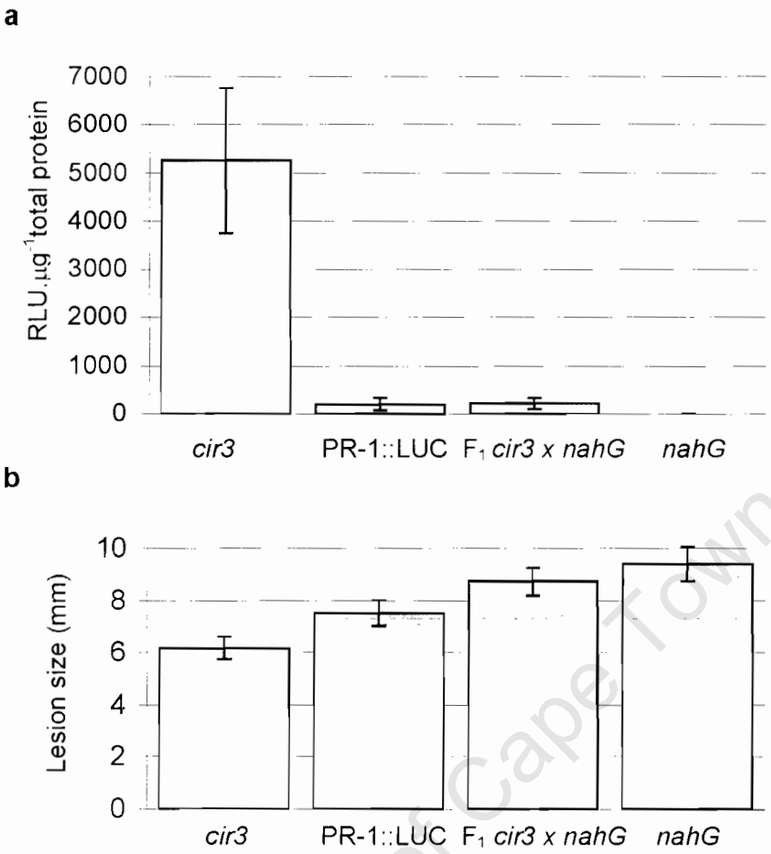


Figure 1. *PR-1::LUC* activity and *B. cinerea* resistance in *cir3*is SA dependent. (a) Luciferase assay of 4-week old *cir3, PR-1::LUC,* F_1 *cir3 x nahG* and *nahG. NahG* does not contain the *PR-1::LUC* transgene and acts as a negative control. F_1 *cir3 x nahG* plants displayed luciferase activity comparable to *PR-1::LUC* plants. **(b)** Detached leaves of 4-week old *cir3, PR-1::LUC,* F_1 *cir3 x nahG* and nahG were inoculated with *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 ¼ (9) are *npr1-1* (*nn*) homozygous and ¾ (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.

	cir3 control	F_2 *cir3 x npr1-1*	F_2 *cir3 x npr1-1*	*npr1-1* control
Inferred Genotype from PCR based screen	*NN*	*NN, Nn* 30	*nn* 9	*nn*
Luciferase activity (RLU.µg-1 total protein	3873 (± 2374)	252 (± 164)	5.6 (± 3.15)	-3.25 (± 2.35)

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*

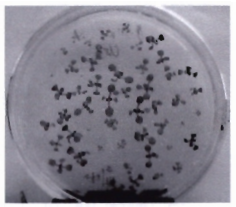
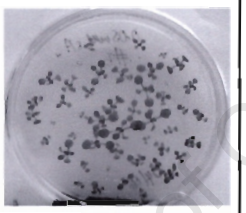
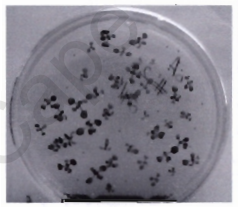
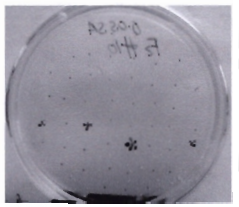
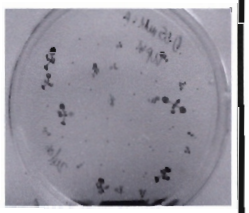
	cir3 control	3 Representative lines from the 9 *npr1*-1 homozygous F_2 *cir3 x npr1-1* lines			*npr1-1* control
a *NPR1-1* genotype	*NPR1-1/NPR1-1*	*npr1-1/npr1-1*	*npr1-1/npr1-1*	*npr1-1/npr1-1-*	*npr1-1/npr1-1*
b Luciferase Activity (RLU.μg^{-1} total protein)	*3875 ± 2373*	*13.9*	3.3	3.5	-4 ± 3
c SA phenotype					
Inferred Genotype	***cir3cir3 NPR1-1NPR1-1***	***cir3cir3 npr1-1npr1-1***	***CIR3cir3 npr1-1npr1-1***	***CIR3CIR3 npr1-1npr1-1***	***CIR3CIR3 npr1-1npr1-1***
Expected # of lines		**2.25**	**4.5**	**2.25**	
Observed # of lines		**1**	**5**	**3**	
χ^2 test 1.91 P= >0.1					

Figure 2. Increased *PR-1::LUC* expression of *cir3* is *NPR1*-dependent. PCR analysis (a) and luciferase assay (b) of 4-week old F_2 *cir3 x npr1-1* plants and controls indicate that loss of *NPR1-1* in *cir3* results in low *PR-1::LUC* expression. Three representative n*pr1-1* homozygous F_3 *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.

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 (χ^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 F_3 *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 F_3 *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 F_2 *cir3 x ein2* lines that were homozygous for the *ein2 mutation,* 150 F_2 *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.

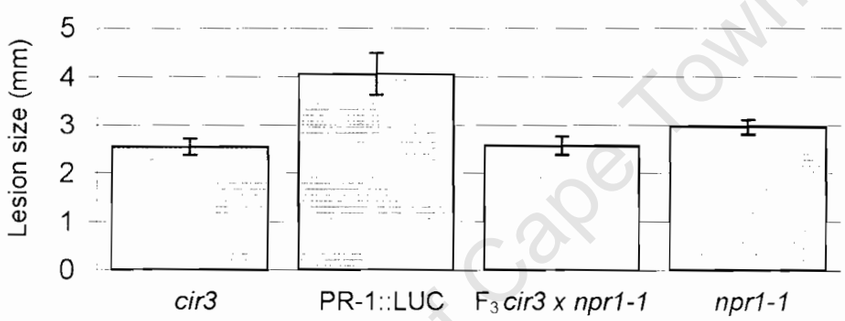


Figure 3. The role of *NPR1-1* mediated *cir3* resistance to *B. cinerea* is inconclusive. Detached leaves of 4-week old *cir3, PR-1::LUC,* F_3 *cir3 x npr1-1* and *npr1-1* were inoculated with *B. cinerea* spores and the lesion diameter was measured 5 days later. Values presented are averages from at 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 F_2 *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 signalling is not required for the increased PR-1::LUC activity of *cir3* as 17 lines displayed high luciferase activity (χ^2= 0.07, P= >0.5). Ten F_2 *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 F_3 *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 F_3 *cir3*x*ein2* double mutants (Figure 4).

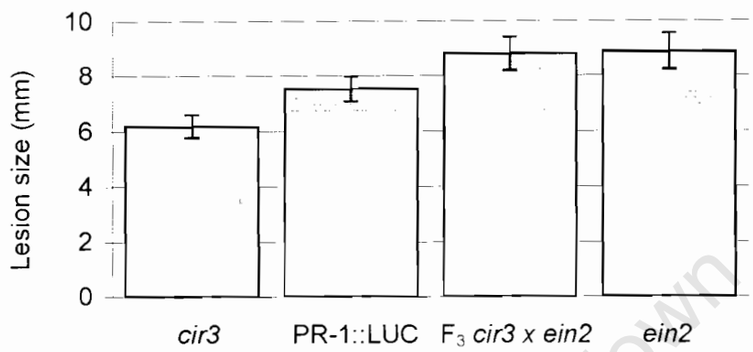


Figure 4. A functional Ethylene signaling pathway is needed for *B. cinerea* resistance in *cir3*. Detached leaves of 4-week old *cir3, PR-1::LUC,* F_3 *cir3* x *ein2* (*cir3/-*; *ein2/ein2)* and *ein2* were inoculated with *B. cinerea* spores and the lesion diameter was measured 5 days later. Values 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.,* 1996; 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 Meyer & 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 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_2O_2 production promoting infection to occur (van Wees & Glazebrook, 2003). Increased H_2O_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 (Meyerowitz, 1989).

A plants' 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 form 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

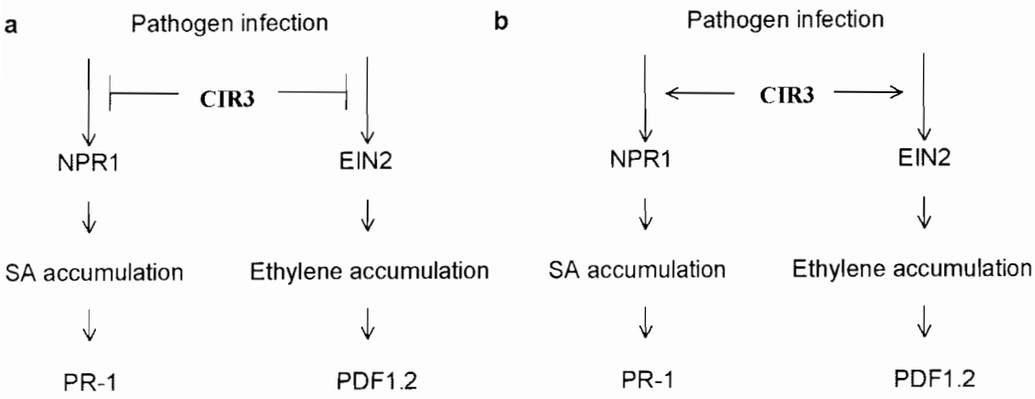


Figure 1. Schematic diagrams suggesting alternative roles of the wildtype *CIR3* gene product in the defence signalling pathway. a) The wildtype *CIR3* gene product acts as an inhibitor of the defence signalling pathway and lack of CIR3 protein could allow the defence signalling pathway to be constitutively activated. However, the dominant nature of the mutant phenotype also suggests that a threshold level of the CIR3 protein is needed to give an effective inhibition response. b) The wildtype *CIR3* gene product acts as an activator of the defence signalling pathway and mutating one of the *CIR3* regulatory sequences, such as the promoter, may result in over-expression of *CIR3* thereby constitutively activating the defence signalling pathway.

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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