IDENTIFICATION OF THE CIR1 DISEASE RESISTANCE GENE IN ARABIDOPSIS THALIANA

BY ANASTASHIA DIENER

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SUPERVISOR: ROBERT INGLE

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ABSTRACT

Plants rely on an elaborate multi-layered defence system to perceive and effectively respond to disease causing pathogens. The defence-related *cir1* (*constitutively induced resistance 1*) mutant was first isolated in an effort to identify components of the *Arabidopsis thaliana* defence system essential for resistance against pathogens. The *cir1* mutant has previously been described as having increased resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and oomycete pathogen *Hyaloperonospora parasitica* Noco2 and was shown to constitutively express salicyclic acid-, jasmonic acid/ethylene- and reactive oxygen intermediate-responsive genes. Genetic analysis and mapping studies of the mutation revealed that it is recessive and may be encoded by one of eight genes located within a 309.10 kb region on the lower arm of chromosome four.

Further characterisation of the resistance properties of the mutant demonstrated that *cir1* exhibits increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (AvrB). T-DNA insertion mutant lines were obtained for each of the eight candidate genes. These mutant lines were analysed to identify candidates which could phenocopy characteristics of the *cir1* mutant. The *at4g11100* T-DNA insertion mutant line displayed high basal levels of the defence-related PR-1 and EDS1 proteins and increased resistance to the avirulent *Pseudomonas syringae* pv. *tomato* DC3000 (AvrB). Furthermore, it has been demonstrated through *Pseudomonas syringae* pv. *tomato* DC3000 (AvrB) bacterial assays and PR-1 Western blot analysis that complementation of the *at4g11100* mutant with the pFAST-G01::*AT4G11100* construct rescued the mutant phenotype. A restored phenotype was also observed following PR-1 Western Blot analysis of the complemented *cir1* line. Surprisingly, sequence and expression analysis indicated that the *cir1* mutation was not the result of a point mutation or altered gene expression. Thus, although previous research has provided strong evidence for the role of *CIR1* as a negative regulator of resistance, the genetic basis of the mutation and the mechanisms by which this regulation is achieved has yet to be determined.

LIST OF ABBREVIATIONS

°C	degree Celsius
µ	micro
APS	Ammonium persulphate
At	*Arabidopsis thaliana*
Avr	avirulence
bp	base pair(s)
C	Carboxy-terminal
CC	coiled-coil
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
cir1	constitutively induced resistance 1 mutant
Col-0	*Arabidopsis thaliana* ecotype Columbia
DEPC	diethylpyrocarbonate
dH_2O	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ET	ethylene
ETI	effector-triggered immunity
g	gram(s)
g	gravity constant (9.81 ms-1)
GFP	green fluorescent protein
h	hour(s)
HR	hypersensitive response
JA	jasmonic acid
kb	kilobase(s)
KB	King's broth

kDa	kiloDalton(s)
l	litre(s)
LB	Luria Bertani
LRR	leucine-rich repeat
LUC	firefly luciferase reporter gene
PR-1::LUC	Col-0 plants harbouring a *PR-1::LUC* gene cassette
m	milli
M	molar
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
Mb	megabase(s)
min	minute(s)
mm	millimetre(s)
mRNA	messenger ribonucleic acid
N	amino-terminal
NADPH	nicotine adenine dinucleotide phosphate (reduced form)
NBS	nucleotide binding site
ng	nanogram(s)
nm	nanometer(s)
OD	optical density
P	probability
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PR	pathogenesis-related
PRR	pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato* DC3000
PTI	PAMP-triggered immunity
R	resistance
RLK	receptor-like kinase

RLU	relative light units
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second(s)
siRNA	small interfering ribonucleic acid
T-DNA	transfer deoxyribonucleic acid
TIR	*Drosophila* Toll and human interleukin-1 receptor
U	unit(s)
UTR	Untranslated region
UV	ultraviolet
V	Volt
v/v	volume per volume
w/v	weight per volume

TABLE OF CONTENTS

PLAGIARISM DECLARATION ..... **I**

ACKNOWLEDGEMENTS ..... **II**

ABSTRACT ..... **III**

LIST OF ABBREVIATIONS ..... **IV**

LIST OF FIGURES ..... **X**

LIST OF SUPPLEMENTARY FIGURES ..... **XI**

LIST OF TABLES ..... **XII**

CHAPTER 1 : LITERATURE REVIEW **1**

1.1 *Arabidopsis thaliana* as a model organism 1

1.2 The plant immune system 3

1.2.1 PAMP/DAMP perception 7

1.2.2 Downstream signalling events of PTI 9

1.2.3 Pathogen virulence through the suppression of PTI 11

1.2.4 Pathogen effector perception 13

1.2.5 R protein-mediated signalling 15

1.3 Negative regulators in the plant defence network 25

1.4 The identification of a novel defence related mutant, *cir1* 27

1.4.1 Characterization of the *cir1* mutant 27

1.4.2 Candidate analysis 28

1.5 Specific project objectives 35

CHAPTER 2 : MATERIALS AND METHODS **36**

2.1 Plant Material and Growth Conditions 36

2.2 Microbial Strains and Plant Infection 36

2.2.1 Escherichia coli 36

2.2.2 Agrobacterium tumefaciens 36

2.2.3 Pseudomonas syringae 38

2.2.4 Botrytis cinerea 39
2.3 RNA Manipulation 39
2.3.1 RNA Isolation from *Arabidopsis* 39
2.3.2 Electrophoresis of RNA 40
2.3.3 cDNA synthesis 40
2.3.4 Quantitative real-time PCR 40
2.4 DNA Manipulation 41
2.4.1 Genomic DNA Isolation from *Arabidopsis* 41
2.4.2 Amplification of DNA 42
2.4.3 Electrophoresis of DNA 45
2.4.4 Gel extraction of DNA 45
2.4.5 DNA Sequencing and Analysis 45
2.4.6 Cloning Techniques 46
2.5 *Arabidopsis* transformation 48
2.5.1 Plant preparation 48
2.5.2 Transformation of competent *A. tumefaciens* cells 49
2.5.3 Preparation of transformed *A. tumefaciens* 49
2.5.4 Floral dip 50
2.5.5 Isolation of positive plant transformants 50
2.5.6 Luciferase imaging of *Arabidopsis* 51
2.6 Western Blotting 51
2.6.1 Total protein isolation from *Arabidopsis* 51
2.6.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) 51
2.6.3 Western Blot Analysis 52
2.6.4 Chemiluminescence detection 53
2.7 Statistical analyses 53
CHAPTER 3 : RESULTS AND DISCUSSION 54
3.1 Pathogen susceptibility analysis of the *cir1* mutant 54
3.2 Identification of T-DNA insertion lines for each candidate gene 55
3.3 Isolation of a homozygous *at4g11130* SALK line 58

3.4 Susceptibility analyses of T-DNA insertion lines to infection 58
3.4.1 *AT4G11120*, *AT4G11130* and *AT4G11160* are unlikely candidates for *CIR1*.....62
3.4.2 The variable nature of the *cir1* mutant 62
3.5 In-vivo analysis of *PR-1*::luciferase activity in T-DNA insertion lines.....65
3.5.1 Establishment of *PR-1::LUC* transgenic *Arabidopsis* 65
3.5.2 LUC activity varies between, independently generated, transgenic *Arabidopsis* T_2 lines.....66
3.6 *PR-1* protein accumulation in T-DNA insertion lines 67
3.7 EDS1 protein accumulation in T-DNA insertion lines 70
3.8 Characterization of the *at4g11100* T-DNA insertion mutant.....72
3.8.1 Susceptibility analyses of the *at4g11100* mutant.....72
3.9 Complementation of the *cir1* mutant.....75
3.9.1 Generation of the pFAST-G01::*AT4G11100* transgenic *Arabidopsis*.....75
3.9.2 Identification of a complementing line for the *at4g11100* mutation based on *Pseudomonas syringae* assays.....76
3.9.3 Identification of a complementing line for the *cir1* and *at4g11100* mutation based on PR-1 Western blot analysis.....79
3.10 Investigating the molecular basis of the *cir1* mutation.....81
3.10.1 Sequence analysis of the *AT4G11100* gene in the *cir1* mutant 81
3.10.2 *AT4G11100* isoform expression analysis.....82
CHAPTER 4 : CONCLUSIONS..... 85
CHAPTER 5 : REFERENCES..... 87
CHAPTER 6 : SUPPLEMENTARY FIGURES..... 110

LIST OF FIGURES

FIGURE 1-1: A SIMPLIFIED SCHEMATIC REPRESENTATION OF THE DIFFERING RESPONSES OF THE PLANT HOST TO PATHOGENS.. 6
FIGURE 1-2: SCHEMATIC OVERVIEW OF SOME OF THE SA SIGNALLING NETWORK COMPONENTS IN *ARABIDOPSIS* DEFENCE AGAINST BIOTROPHIC ATTACK. 20
FIGURE 1-3: SIMPLIFIED SCHEMATIC DIAGRAM DEPICTING THE ROLE OF TRANSCRIPTION FACTORS IN THE DEFENCE NETWORK INVOLVING THE JA AND ET SIGNALING PATHWAYS. 24
FIGURE 3-1: SUSCEPTIBILITY ANALYSIS OF THE *CIR1* MUTANT TO INFECTION BY *P. SYRINGAE*. 56
FIGURE 3-2: *BOTRYTIS CINEREA* DETACHED LEAF INFECTION ASSAY OF *CIR1*.. 57
FIGURE 3-3: GENE STRUCTURE OF THE *CIR1* CANDIDATE GENES. 59
FIGURE 3-4: PRIMER DESIGN FOR THE PCR BASED SCREENING METHOD USED TO IDENTIFY HOMOZYGOUS *AT4G11130* MUTANT LINES.. 60
FIGURE 3-5: PCR SCREENING FOR HOMOZYGOUS *AT4G11130* T-DNA INSERTION MUTANTS.. 61
FIGURE 3-6: SUSCEPTIBILITY ANALYSIS OF THE *AT4G11120* MUTANT TO INFECTION BY *P. SYRINGAE.* 63
FIGURE 3-7: SUSCEPTIBILITY ANALYSIS OF THE *AT4G11160 AND AT4G11130* MUTANT TO INFECTION BY *P. SYRINGAE.*. 64
FIGURE 3-8: HIGHLY VARIABLE LUC ACTIVITY DETECTED IN T-DNA INSERTION MUTANT LINES OF *ARABIDOPSIS* TRANSFORMED WITH THE *PR-1::LUC* TRANSGENE.. 68
FIGURE 3-9: WESTERN BLOT ANALYSIS INVESTIGATING PR-1 PROTEIN ACCUMULATION IN *CIR1*, COL-0, *EDS1*, *PR-1::LUC* AND ALL THE T-DNA INSERTION MUTANT LINES.. 69
FIGURE 3-10: WESTERN BLOT ANALYSIS INVESTIGATING EDS1 PROTEIN ACCUMULATION IN *CIR1*, COL-0, *EDS1*, *PR-1::LUC* AND ALL THE T-DNA INSERTION MUTANT LINES. 71
FIGURE 3-11: AT4G11100 EXPRESSION IN THE AT4G11100 T-DNA INSERTION MUTANT LINE. 73
FIGURE 3-12: SUSCEPTIBILITY ANALYSIS OF THE *AT4G11100* MUTANT TO AVIRULENT *P. SYRINGAE (AVRB)* INFECTION.. 74
FIGURE 3-13: IDENTIFICATION OF PFAST-GO1::*AT4G11100* AND PFAST-G01 FLUORESCENT SEED. 77
FIGURE 3-14: SUSCEPTIBILITY ANALYSIS OF PFAST-G01::*AT4G11100* TRANSFORMED *AT4G11100* T_1 LINES.. 78
FIGURE 3-15: WESTERN BLOT ANALYSIS OF PFAST-G01::AT4G11100 TRANSFORMED *CIR1* AND *AT4G11100* T_1 LINES.. 80
FIGURE 3-16: AMPLIFICATION OF THE FULL LENGTH TRANSCRIPT OF THE SPLICE VARIANTS AT4G11100.1 AND AT4G11100.2. 83
FIGURE 3-17: BASAL RELATIVE EXPRESSION OF *AT4G11100.1* IN *CIR1*.. 84

LIST OF SUPPLEMENTARY FIGURES

SUPPLEMENTARY FIGURE 1: SUSCEPTIBILITY ANALYSIS OF THE *AT4G11110 AND AT4G11140* MUTANT TO INFECTION BY *P. SYRINGAE.*. 110
SUPPLEMENTARY FIGURE 2: SUSCEPTIBILITY ANALYSIS OF THE *AT4G11150 AND AT4G11170* MUTANT TO INFECTION BY *P. SYRINGAE.* 111
SUPPLEMENTARY FIGURE 3: *SAC*I AND *XBA*I TEST DIGEST OF THE PFGC5941::*PR-1::LUC*::OCS PLASMID.. 112
SUPPLEMENTARY FIGURE 4: PRIMERS DESIGNED TO AMPLIFY THE *PR-1::LUC*::OCS WERE USED IN A COLONY PCR REACTION ON SELECTED *A. TUMEFACIENS* COLONIES TO IDENTIFY POSSIBLE TRANSFORMANTS. .. 112
SUPPLEMENTARY FIGURE 5: *ECORI* DIGEST OF THE PCR8::AT4G11100 PLASMID. 113
SUPPLEMENTARY FIGURE 6: *PSTI* DIGEST OF THE PFAST-G01::AT4G11100 PLASMID.. 113

LIST OF TABLES

TABLE 1-1: DESCRIPTION OF EIGHT *CIR1* CANDIDATE GENES ON CHROMOSOME FOUR 29
TABLE 2-1: *ARABIDOPSIS* STRAINS AND TRANSGENIC LINES USED IN THIS EXPERIMENT.. 36
TABLE 2-2: PRIMERS AND PCR CONDITIONS USED IN THIS STUDY 43
TABLE 2-3: SEQUENCING PRIMERS USED IN THIS STUDY 46
TABLE 2-4: VECTORS USED IN THIS STUDY 49

CHAPTER 1: LITERATURE REVIEW

With the increasing global concern over food production, a greater emphasis is being placed on efforts to increase crop yields. To meet the 2009 Declaration of the World Summit on Food Security target by 2050, the average annual production of food needs to increase by 44 million metric tonnes per year (Tester & Langridge, 2010). Major obstacles to achieving this goal include pathogens and pests such as bacteria, fungi, viruses, nematodes and insects, which claim 10-16% of the global harvest (Chakraborty & Newton, 2011). Conventional methods which rely on the use of pesticides and fungicides can be ineffective due to their short-lived nature and adverse side-effects. An alternative approach is the use of plant breeding programs which rely on the principles of Mendelian genetics to produce pathogen/pest resistant varieties. However, in both methods monogenic plants with little to no genetic diversity often succumb to disease when selective pressure on the pathogen results in the evolution of strains with the ability to overcome host resistance. The need for a solution which increases disease tolerance to a range of pathogens without affecting beneficial soil microbes has encouraged research into the production of transgenic plants (Liu et al, 2005). Recent advances in genetic engineering have identified several key strategies for improving resistance in transgenic plants. Such advances include the introduction of functional resistance genes from a donor plant into an unrelated host plant, detoxification of pathogen virulence factors, expression of antimicrobial peptides and modification of signalling pathways (Collinge et al, 2008; Wally & Punja, 2010). Future progress in this field will not be possible without elucidating the mechanisms behind plant immunity and improving our knowledge of plant-pathogen interactions.

1.1 *Arabidopsis thaliana* as a model organism

Arabidopsis thaliana (*Arabidopsis*), an angiosperm in the mustard family (Brassicaceae), was originally adopted not only for its utility in molecular biology but for its use towards answering fundamental questions concerning the structural and functional properties common to most eukaryotes. In 2000 the *Arabidopsis* Genome Initiative (AGI), a

multinational collaboration, sequenced 115.4 megabases (mb) of the 125 mb *Arabidopsis* ecotype Columbia (Col-0) genome (The Arabidopsis Genome Initiative, 2000). This was largely facilitated by the plant's short generation time (approximately 6 weeks), small size, large number of seed produced and relatively small nuclear genome (Meinke, 1998). Based on the high quality sequence data, microarray gene expression data and biochemical knowledge the Col-0 accession ecotype was generally accepted as the reference genotype (The Arabidopsis Genome Initiative, 2000; Zimmermann et al, 2004).

Research on *Arabidopsis* has also further enhanced our knowledge of plant-microbe interactions allowing us to dissect the molecular mechanisms underlying disease resistance and plant susceptibility. As with all good model organisms, the insight gained through *Arabidopsis* pathology has been applicable to a range of other plant pathosystems (Nishimura & Dangl, 2010). For example, complete genome sequences of *Oryza sativa* (rice) and *Arabidopsis* revealed significant homology (approximately 70%) between these genomes (Paterson et al, 2005). Furthermore, a high degree of synteny was observed between *Arabidopsis* and the soybean, *Medicago truncatula* (Mudge et al, 2005). In tomato, the relative inefficiency and labour intensivity of current approaches to the functional annotation of genes has led researchers to look towards *Arabidopsis* (Mysore et al, 2001). Indeed, the introduction of *Arabidopsis* NPR1, a protein required for broad spectrum resistance to a wide range of pathogens, into a tomato cultivar, conferred enhanced resistance to *Ralstonia solanacearum*, *Fusarium oxysporum* f. sp. l*ycopersici*, *Stemphylium solani* and *Xanthomonas campestris* pv. *vesicatoria* (Lin et al, 2004).

The analysis of numerous disease resistant or enhanced susceptibility mutants has greatly contributed to the identification of key components of the signal transduction networks involved with defence. Forward genetic and reverse genetic approaches, including RNA interference (RNAi), are just a few of the techniques that have further aided in the analysis of plant gene function. In the forward genetics approach, large scale mutagenesis of wild-type seed by a chemical mutagen such as ethyl methane sulfonate (EMS) or irradiation is required to produce mutant collections with single nucleotide changes or deletions in the genome. The technique is particularly useful when null mutations in a given gene result in lethality as chemical mutagenesis has the ability to produce an allelic series where strong, intermediate and weak alleles of a given gene all exist in the same population. Following

phenotypic analysis, characterization of the gene underlying the mutation may provide insight into its biological function. One of the major drawbacks of this approach is identifying the DNA alteration responsible for the mutation. The identification of a single mutation in a large genome was once considered to be impractical; however, although costly, recent advances in next generation sequencing has improved its feasibility (Østergaard & Yanofsky, 2004; Pesaresi, 2007; Alonso & Ecker, 2006). Reverse genetics is an alternative approach which starts with the gene of interest and ends with the analysis of the corresponding mutant. The advent of sequence-indexed insertion mutant database such as the Salk Institute Genomic Analysis Laboratory (SIGNAL, http://signal.salk.edu/cgi-bin/tdnaexpress) has greatly enabled this approach (Alonso et al, 2003). In addition to these approaches, RNAi allows for the quantitative reduction of expression of any particular gene or groups of genes and is particularly useful in the characterisation of genes where null mutations result in lethality (Small, 2007).

1.2 The plant immune system

Plants have evolved an array of sophisticated defence mechanisms to detect and respond to pathogens to prevent the establishment of disease. Disease causing pathogens can be classified on the basis of their lifestyle. Biotrophs derive their nutrition from living host tissue while necrotrophs proliferate on dead or dying plant matter, ultimately destroying host cells (Glazebrook, 2005). Hemibiotrophs combine both methods depending on the stage of their life cycle, often having an necrotrophic phase preceded by an initial biotrophic phase (Glazebrook, 2005). As a consequence, plants have evolved several lines of defence to deal with both non-cultivar specific (non-host) and cultivar-specific pathogens outlined in Figure 1-1 (Prell & Day, 2001; Gabriel & Rolfe, 1990).

Non-host resistance is a common form of defence against a broad spectrum of pathogens, relying on both constitutive defences and inducible responses to impede pathogen colonization (Ingle et al, 2006; Nürnberger & Lipka, 2005). Constitutive defences such as the waxy cuticle, rigid cell walls, antimicrobial enzymes and antimicrobial secondary metabolites provide the first line of defence (Mysore & Ryu, 2004; Thordal-Christensen, 2003). In the event that the pathogen manages to access the interior of the plant, by

entering through wounds or natural openings and penetrate the cell wall, they are then detected by host pattern recognition receptors leading to the induction of basal defences (Nürnberger & Lipka, 2005). Induction of these defences are activated upon the detection of general elicitors such as flagellin and elongation factor (EF-Tu) which are associated with both pathogenic and non-pathogenic microbes (Kunze et al, 2004; Gómez-Gómez & Boller, 2002). These pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) are highly conserved and as a consequence, are recognised in a non-cultivar specific manner by the host's transmembrane pattern recognition receptors (PRRs), outlined in Figure 1-1A (Parker, 2003; Gómez-Gómez & Boller, 2002). This process of non-self recognition is referred to as PAMP-triggered immunity (PTI) and constitutes one arm of the two-armed innate immune system (Nürnberger & Brunner, 2002; Parker, 2003; Nürnberger & Lipka, 2005).

A successful pathogen would therefore require some means of evading PTI in order to successfully colonise the host plant. Some pathogens utilize type III secretion systems (TTSS) to deliver effectors (predominantly proteins) into the plant cell, thereby enhancing their virulence by circumventing host immunity, manipulating host metabolism, and also by promoting the leakage of nutrients required for growth of the pathogen (see Figure 1-1B), (Jones & Dangl, 2006; Bender et al, 1999). The selective pressure imposed by these pathogens on plants has resulted in the co-evolution of plant resistance (R) genes, which through the recognition of pathogen virulence factors induce effector-triggered immunity (ETI), (Katagiri et al, 2002; Nürnberger & Lipka, 2005). Current research involving the isolation and identification of functional *R* genes revealed that despite the vastly different modes of pathogen colonization and the wide variety of pathogen taxa analysed, only five classes of proteins are encoded by the known plant *R* genes (Jones & Dangl, 2001). NBS-LRR proteins form part of the most predominant class of these proteins and are characterized by a nucleotide binding site (NBS) and a leucine rich repeat domain (LRR). NBS-LRR proteins can be further divided based on structural variation at the N-terminus. The TIR-NBS-LRR sub-family contains an N-terminal domain which shares homology with the Toll and interleukin-1 receptor (TIR) from animal immune systems, while the CC-NBS-LRR sub-family contains a putative coiled coil (CC) domain (Jones & Dangl, 2001; Gómez-Gómez, 2004). NBS-LRR mediated recognition of an effector, whether direct or indirect, usually triggers the

hypersensitive response (HR), characterised by programmed cell death (PCD) at the site of infection limiting the pathogens access to water and nutrients (see Figure 1-1C), (Greenberg & Yao, 2004). Pathogen perception may result in the development of resistance to secondary infection by a wide variety of virulent pathogens in distal tissues termed Systemic Acquired Resistance (SAR), (Ryals et al, 1996; Durrant & Dong, 2004). ETI represents the second arm of the innate immune system, and although reports have suggested a genetic overlap in these responses it is pertinent to note that ETI elicits a rapid, amplified and much more prolonged response than PTI. In fact, it has been proposed that the *R* gene-mediated layer of immunity was adapted from the pre-existing PTI defence machinery during the evolution of plant innate immunity (Jones & Dangl, 2001; Mysore & Ryu, 2004; Tsuda & Katagiri, 2010).

The *Arabidopsis-Pseudomonas syringae* interaction is one of particular importance as research in this area has contributed vastly to our understanding of the mechanisms underlying pathogen recognition and the associated signalling cascade (Katagiri et al, 2002). In this interaction resistance in the plant host is dependent upon the virulence-enhancing effector complement of the pathogen strain. Recognised effectors are termed avirulence (Avr) proteins and their interaction termed incompatible due to the subsequent resistance achieved by the host. The inability to recognise these virulence determinants renders the plant host susceptible in what is known as a compatible interaction (Katagiri et al, 2002). For example, *P. syringae pv. tomato* DC3000 (AvrB) is considered to be an avirulent pathogen because the plant host has the necessary R protein (RPM1) to recognize the invading AvrB protein thus activating ETI. Conversely, in *A. thaliana* the virulent strain *P. syringae* pv *tomato* DC3000 (*Pst* DC3000) maintains its virulence by overcoming the host's basal defences without activating ETI (Katagiri et al, 2002). Although it is possible for some R proteins in the susceptible plant to weakly interact with virulence determinants this is evidently insufficient to completely stop virulent *Pst* DC3000 infection (Jones & Dangl, 2006).

The main objective of this study was to identify the gene responsible for the enhanced disease resistance phenotype of the *Arabidopsis* mutant, *constitutively induced resistance 1* (*cir1*), (Murray et al, 2002). How this resistance phenotype has been achieved still remains unclear; however, key components of *cir1*-mediated resistance have been identified.

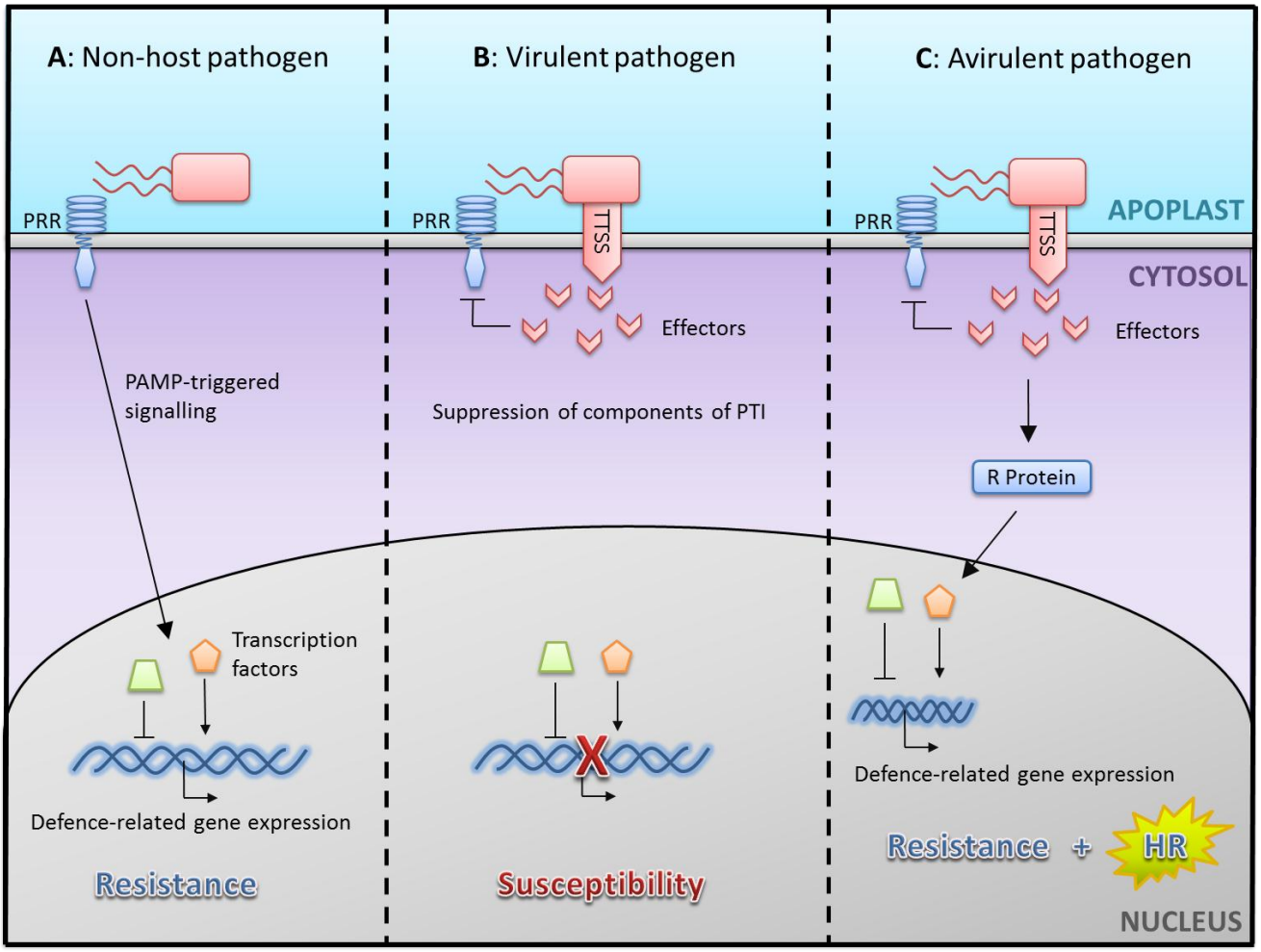

Figure 1-1: A simplified schematic representation of the differing responses of the plant host to pathogens. (A) A non-host pathogen enters the plant. The detection of a bacterial PAMP by the corresponding PRR activates downstream PAMP-triggered signalling events and regulatory transcription factors. These transcription factors act to mediate defence-related gene expression, which confers resistance against pathogen colonisation. (B) Virulent pathogens have evolved TTSS, which deliver effectors into the cytosol. These effectors function to suppress or interfere with components of PAMP-triggered signalling, rendering the plant susceptible to disease otherwise known as effector triggered susceptibility. (C) Certain plant cultivars have evolved R proteins, which directly or indirectly detect the activity of specific effectors, thus rendering the pathogen avirulent. This specific recognition activates effector-triggered signalling events which results in a rapid and more amplified defence response, including the HR.

The role of these components in plant defence will be discussed in this review. This review aims to highlight the complex nature of the two-branched *Arabidopsis* innate immune system to provide a basis for understanding of the molecular mechanisms underlying disease resistance.

1.2.1 PAMP/DAMP perception

The ability to discriminate a broad variety of potential pathogens from self, with a limited supply of receptors, is of paramount importance to the plant innate immune system. PTI is initiated upon the recognition of PAMPs by the cognate PRRs and its induction is associated with an array of defence mechanisms (Felix & Boller, 2003; Felix et al, 1999; Kunze et al, 2004). PAMPs such as flagellin, cold shock protein, lipopolysaccharides (LPS) and EF-Tu fulfil an indispensable role in the biology of the pathogen and as a result cannot be altered without the elimination of that function (Medzhitov & Janeway, 2000; Ingle et al, 2006; Felix & Boller, 2003; Kunze et al, 2004). Most known PRRs are receptor-like kinases (RLKs) carrying a predicted signal peptide, an extracellular leucine rich repeat (LRR), a transmembrane domain and an intracellular serine/threonine kinase domain (Gómez-Gómez & Boller, 2000).

One such receptor is FLAGELLIN SENSING 2 (FLS2) which perceives the flg22 epitope, a highly conserved stretch of 22 amino acids from the N-terminus of flagellin (Gómez-Gómez & Boller, 2000). The role of this epitope as a potent elicitor of defence was confirmed in studies where plants pre-treated with flg22 prior to infection with virulent *Pst* DC3000 displayed decreased growth of the pathogen when compared to control plants not treated with flg22 (Zipfel et al, 2004). Conversely, the decreased growth of the pathogen was not observed in *fls2* mutants indicating the need for functional FLS2 to detect and respond to flg22. Furthermore, the presence of additional perception systems were demonstrated when, following the pre-treatment of *fls2* mutant plants with crude bacterial extract, presumably carrying PAMPs other than flagellin; plants infected with *Pst* DC3000 exhibited decreased bacterial growth (Zipfel et al, 2004).

Another well characterized receptor is the EF-TU RECEPTOR (EFR), which perceives the EF-Tu epitope, elf18 (Zipfel et al, 2006). EF-Tu plays an essential role protein translation and

is one of the most abundant and conserved proteins in bacteria. EFR was first identified during a screen for LRR-RLK genes whose expression was induced by flg22 (Zipfel et al, 2006). Microarray experiments revealed that FLS2 and EF-Tu prompted the transcription of a shared set of RLKs, which suggests that a shared set of receptor genes may be activated by different PAMPs. Moreover, the role of EFR in PAMP-induced defence against *Agrobacterium* infection was demonstrated when *efr* mutants exhibited higher transformation efficiency than the wild-type controls. These claims were further validated after treatment with flg22 and elf18 almost completely abolished the transformation in wild-type plants (Zipfel et al, 2006).

A recent study has revealed that the PRRs require the association of another RLK to function. Both FLS2 and EFR have been shown to associate with the BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) following treatment with flg22 and elf18, respectively. BAK was first identified as a co-receptor of the brassinosteroid receptor (BRI1) and belongs to a cluster of five somatic embryogenesis-related kinases (SERKs), (Chinchilla et al, 2007; Aker & de Vries, 2008). The functional importance of this partnership was made apparent when *Arabidopsis bak1* mutants showed a reduced sensitivity to flg22 and to a lesser extent, elf18. The difference in responsiveness of the bak1 mutation on elf18 suggests that EFR may interact with other members of the SERK cluster (Chinchilla et al, 2007). While the exact function of BAK1 in receptor complexes has yet to be determined, it is considered to have a supporting involvement in signal transduction rather than in ligand perception. Additionally, the weak in-vitro autophosphorylation activity of FLS2 alone suggests that the sequential transphosphorylation, observed in BRI1/BAK1 complexes, is a mechanism for full FLS2 activation (Wang et al, 2008).

In addition to PAMPs, plants can recognise modified endogenous host molecules released upon microbial attack. These damage-associated molecular patterns (DAMPs) include polysaccharides released from plant cell walls (i.e oligo-α-galacturonides) and some endogenous peptides including Pep1 (Lotze et al, 2007; Hückelhoven, 2007; Huffaker et al, 2006). Upon the recognition of Pep1 by its cognate receptor, PEP RECEPTOR 1 (PEPR1), the signalling initiated leads to an increase in cytosolic calcium (Ca^{2+}) and the subsequent increase in defence gene expression (Qi et al, 2010; Krol et al, 2010). The limited redundancy noted with flg22 and elf18 perception is also noted with this DAMP as both

PEPR1 and its homologue PEPR2 are sensitive to Pep1 (Krol et al, 2010). It is only in the double mutant *pepr1/pepr2* that perception is abolished (Krol et al, 2010). This highlights the strong parallels between PAMP and DAMP perception and suggests that recognition may broadly converge on similar downstream signalling pathways.

1.2.2 Downstream signalling events of PTI

PAMP/DAMP perception and the ensuing defence responses are largely mediated by signal transduction cascades. The particular component of the complex defence system activated following recognition is dependent upon the individual recognition event, the signalling route and the secondary messengers employed. Altered Ca^{2+} levels and callose reinforcement of the cell wall are some of the early responses following PAMP perception (Nürnberger et al, 2004; Zhao et al, 2005; Donofrio & Delaney, 2001).

Membrane depolarization due to an increased influx of protons (H^+) and Ca^{2+}, and the associated efflux of potassium (K^+), anions and nitrate, represents one the earliest responses to PAMP/DAMP perception (Nürnberger et al, 2004; Boller & Felix, 2009; Wendehenne, 2002). How this achieved is still unclear; however it is has been proposed that a PAMP induced increase in cytoplasmic Ca^{2+} might aid in the opening of other membrane channels or through the activation of calcium dependent protein kinases (Lecourieux, 2002; Blume et al, 2000; Boller & Felix, 2009). While transient Ca^{2+} plays a pivotal role in defence induction, a more sustained level facilitates the oxidative burst and in some cases HR (Grant et al, 2000; da Cunha et al, 2006).

The oxidative burst is another early response and is primarily marked by the rapid and transient production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), at the attempted site of invasion (Apel & Hirt, 2004). The molecular machinery implicated in the oxidative burst may vary but likely mechanisms include the plasma membrane bound NADPH oxidase, a cell wall peroxidase, extracellular germin-like oxalate oxidases or apoplastic amine oxidases (Apel & Hirt, 2004; Torres et al, 2005; Mittler et al, 2004; Hückelhoven, 2007). The production of ROS is thought to contribute to defence directly, through its antimicrobial activity, and indirectly, by triggering cell wall cross-linking. Intriguingly, the production of ROS has been reported to act as a secondary messenger

further amplifying downstream defence responses including the induction of defence related genes such as *GLUTATHIONE S-TRANSFERASE 1* (*GST1*) and *PATHOGENESIS-RELATED* (*PR*) genes (Dardick & Ronald, 2006; Apel & Hirt, 2004; Grant et al, 2000).

To date, relatively little is known about the underlying molecular mechanisms linking receptor activation to signal transduction. The MAPK signalling cascade plays a significant role by transducing a recognition event into intracellular signalling event in eukaryotic cells (Colcombet & Hirt, 2008). These cascades rely on the sequential transfer of phosphate groups from MAPK kinase kinase (MAPKKK) to MAPK Kinase (MAPKK), and then to a MAPK to regulate resistance following PRR activation (Nakagami et al, 2005). For example, upon flg22 perception MEKK1 (a MAPKKK) phosphorylation activates the MAPKs, MPK3/6, through MKK4/5 (MAPKKs) activation, which acts positively on PTI (Asai et al, 2002; Nicaise et al, 2009). In addition, MEKK1 also activates MPK4 through MKK1/2 activation which acts negatively on PTI in the FLS2 mediated pathway (Suarez-Rodriguez et al, 2007; Nicaise et al, 2009). The activation of the MAPK cascade in turn activates defence-related gene expression through WRKY transcription factor activity (Asai et al, 2002). The WRKY group of plant transcription factors are characterised by a conserved WRKYGQK peptide sequence constituting the WRKY DNA binding domain. This domain interacts with the W-box (C/TTGACC/T) promoter element found in many defence genes (Ciolkowski et al, 2008; Yamasaki et al, 2005). WRKYs such as WRKY29 and its functional homolog WRKY22 have been implicated in the regulation of expression of *PR-1* and *PR-5* (Asai et al, 2002).

In addition to its well documented roles in cultivar-specific resistance (which will be discussed later), the plant signalling hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are considered crucial components of PTI (Pozo et al, 2005; Van Loon et al, 2006; Thatcher et al, 2005). For instance, genetic studies in which SA accumulation was impaired in *Arabidopsis* resulted in susceptibility to the non-host pathogens *Uromyces vignae* (cowpea rust fungus) and *Blumeria graminis* (barley powdery mildew), (Mellersh & Heath, 2003; Zimmerli et al, 2004). More recently, Tsuda and colleagues utilized *Pst* DC3000 *hrcC*, a bacterial strain with defects in the TTSS, to demonstrate the importance of SA accumulation in PTI (Tsuda et al, 2008). In their experiments, flg22 treatment of *Arabidopsis* mutants compromised in SA synthesis failed to induce PTI (Tsuda et al, 2008). Increasing evidence suggests that JA and ET play a more prominent role than SA in plant immunity

against non-host pathogens. Microarray gene expression profiling revealed that infection by the oomycete non-host pathogens *Phytophthora infestans* and *B. graminis* was associated with JA/ET activation (Huitema et al, 2003; Zimmerli et al, 2004). Interestingly, the activation of JA/ET related defences was not evident in *Arabidopsis* in response to virulent *Erysiphe cichoracearum*; however, ectopic activation of JA/ET did increase resistance against this pathogen signifying that the active suppression of JA/ET pathway facilitates infection (Zimmerli et al, 2004). The SA and JA/ET signalling pathways will be discussed in more detail later on in this review.

1.2.3 Pathogen virulence through the suppression of PTI

Pathogens have developed several strategies to induce susceptibility in an otherwise tolerant host. The complex cell wall obstructs the entry for most pathogens, resulting in the colonization of the intercellular space outside this barrier. Cell wall degrading enzymes and cell wall permeable toxins allow these non-invasive pathogens to bypass the cell wall of the plant thereby acquiring access to nutrients and water (Badel et al, 2002; Agrios, 1997). Perhaps the most well-known and efficient means of overcoming this barrier is through the secretion of effector proteins, by the TTSS, into the apoplast or directly into the cell during infection (Wolpert et al, 2002; Hauck et al, 2003; Bender et al, 1999; Jin & He, 2001). Effector proteins and toxins are thought to enhance pathogen virulence through the suppression of components of plant immunity such as HR, hormone signalling and defence-related gene expression (Nomura et al, 2005).

AvrPto and AvrPtoB are unrelated *Pst* DC3000 effector proteins which were identified as suppressors of PTI upstream of the PAMP-induced MAPK activation. Investigation into the structural and biochemical properties of these effectors revealed them as protein kinase inhibitors and E3 ubiquitin ligase-like proteins, respectively (Abramovitch et al, 2003; Xiang et al, 2008; Janjusevic et al, 2006). AvrPto is thought to contribute to pathogen virulence by not only binding to the kinase domains of the FLS2 and EFR RLKs, but also to their "co-receptor" BAK1 (Xiang et al, 2008; Shan et al, 2008). AvrPto inhibits autophosphorylation of the FLS2 and EFR RLKs, but not BAK1, suppressing the MAPK cascade and its associated defence outputs. In addition, AvrPto has also been implicated in the suppression of cell wall-

based defences, as TTSS defective *Pst* DC3000 mutants proliferate in transgenic AvrPto expressing *Arabidopsis* when compared to wild-type plants. Furthermore, these transgenic plants exhibited a severely impaired deposition of callose in response to TTSS defective *Pst* DC3000 (Hauck et al, 2003; Nomura et al, 2005). AvrPtoB's ability to suppress HR-associated PCD has been demonstrated in *Nicotiana benthamiana* and *Saccharomyces cerevisiae*. Further analysis of the protein revealed that PCD inhibition appeared to be associated with the C-terminal domain of AvrPtoB which displayed striking similarities to the U-box and RING-finger subunits of ubiquitin E3 ligases (Janjusevic et al, 2006). In *Arabidopsis,* the modular AvrPtoB protein was shown to interact with FLS2 and BAK1 through its N-terminus, an association further enhanced by flg22 treatment. This interaction enabled the polyubiquitination and subsequent degradation of FLS2 through the inherent E3 ligase activity of the C-terminus (Göhre et al, 2008).

Additional *P. syringae* effectors implicated in the suppression of PTI are AvrRpm1, AvrB and AvrRpt2, which are thought to interact with and modify numerous host proteins in an effort to enhance pathogen virulence (Zhou & Chai, 2008). AvrRpt2, in particular, has been shown to alter the plant's auxin physiology thereby promoting pathogen virulence in the host (Chen et al, 2007b). All three of these effectors have been shown to interact and modify plasma membrane located RPM1-INTERACTING PROTEIN 4 (RIN4), a modulator of stomatal aperture during pathogen invasion (Liu et al, 2009; Mackey et al, 2002).

In addition to the TTSS, phytotoxins also make a significant contribution to pathogen virulence through the suppression of plant defences. Several pathovars of *P. syringae* produce coronatine (COR), a chlorosis inducing polyketide. COR enhances pathogen virulence by promoting bacterial proliferation and lesion formation during infection (Bender et al, 1999). COR has been reported to act as a structural and functional analogue of one or more jasmonates which in turn activates JA/wound response genes (Weiler et al, 1994; Kunkel & Brooks, 2002; Laurie-Berry et al, 2006). This results in the suppression of SA-mediated defences required for resistance against *P. syringae,* as JA and SA are mutually antagonistic pathways (Kunkel & Brooks, 2002; Zhao et al, 2003). Thus it appears that *Pst* DC3000 uses multiple virulence systems to impair plant defences.

1.2.4 Pathogen effector perception

Plants have evolved a defence strategy to counteract the effector-mediated suppression of PTI. This strategy is based on the R protein-mediated recognition of these virulence enhancing effectors. The direct or indirect recognition of these avirulence proteins by its corresponding polymorphic intracellular immune receptor (typically NBS-LRR proteins) in the plant triggers an immune response far more effective than PTI. The simplistic gene for gene model of recognition, introduced by Flor in the 1940s, is based on a direct receptor-ligand type interaction between R and Avr proteins (Jones & Dangl, 2001). Despite research efforts only a few cases of direct binding between R proteins and pathogen effectors have been documented to date (Dodds et al, 2006; Deslandes et al, 2003; Jia et al, 2000). These findings brought about the notion that instead of a direct interaction, R proteins monitor the integrity of common host targets by indirectly recognizing "pathogen-induced modified self", which later became known as the "Guard hypothesis" (Jones & Dangl, 2001; Van der Biezen & Jones, 1998). In this model, R proteins effectively monitor key host targets for effector induced modification or degradation. The Guard model assumes that the pathogen enhances its virulence through the effector mediated manipulation of the "guardee" (host target) when the R protein is not present (Van der Biezen & Jones, 1998).

As mentioned earlier, AvrPto interacts with the kinase domains of PRRs, FLS2 and EFR, to suppress plant immunity. PTO, a Ser/Thr kinase with structural properties similar to that of the PRR RLKs, has also been shown to directly interact with the AvrPto protein (Xing et al, 2007). It appears that the AvrPto-FLS2/EFR and AvrPto-PTO interactions share similar sequence requirements suggesting that PTO has evolved to compete with FLS2 and EFR for AvrPto binding (Xiang et al, 2008; Zipfel & Rathjen, 2008). In addition, fractionation experiments have revealed that PTO interacts constitutively with the N-terminal of the PRF NBS-LRR protein, maintaining it in an inactive state (Mucyn et al, 2006). Research has shown that the conformational change of PTO upon AvrPto binding releases the negative regulation of PRF and is necessary for the activation of ETI in resistant *Solanum lycopersicum* (tomato) plants (Balmuth & Rathjen, 2007; Xing et al, 2007; Mucyn et al, 2006). When considering the Guard hypothesis as a model, PTO could be viewed as the virulence enhancing target of effector AvrPto and PRF as the "guard" perceiving these pertubations.

With no known role in the basal defences of *Arabidopsis*, PTO appears to be surprisingly dispensible as AvrPto contributes to virulence even in its absence (Chang et al, 2000). Conversely, in *Arabidopsis* plants lacking FLS2 this virulence is diminished suggesting that the PRRs are in fact the operative targets of virulence (Xiang et al, 2008). Emerging evidence supports the alternative theory in which PTO has evolved as a "decoy" RLK to attract AvrPto from its operative virulence targets, the FLS2 and EFR RLKs (Van der Hoorn & Kamoun, 2008; Oh & Martin, 2011). In the Decoy model, the classic guardee or decoy may have evolved from the effector target following a gene duplication event or may have been acquired independently through target mimicry. In addition, manipulation of the classic guardee or decoy has no consequence on pathogen fitness (Van der Hoorn & Kamoun, 2008).

Another well characterised example of host recognition that supports both the Guard and Decoy models involves RIN4. The plasma membrane bound RIN4 is a negative regulator of plant immunity as the over-expression and absence of RIN4 is characterized by reduced and enhanced PTI responses, respectively (Kim et al, 2005b). RIN4 is also "guarded" by the NBS-LRR R proteins, RPM1 and RPS2. In terms of the guard hypothesis, effectors AvrRpm1 and AvrB induce the phosphorylation of RIN4, a modification presumed to activate RPM1-mediated ETI (Mackey et al, 2002). A third effector, the cysteine protease AvrRpt2, activates RPS2-mediated ETI by cleaving RIN4 resulting in its subsequent degradation (Kim et al, 2005a). In the guard model, manipulation of the guarded effector target should promote pathogen virulence in the absence of the R protein guard. While several plausible theories exist including the possibility that the manipulation of RIN4 control over H^+-ATPase activity and stomatal opening may promote pathogen virulence, the exact mechanisms detailing how this achieved remains unclear (Liu et al, 2009). While these findings are consistent with the Guard model, the Decoy model cannot be disregarded as a definitive link between RIN4 manipulation and the enhancement of pathogen virulence has not yet been established (Van der Hoorn & Kamoun, 2008). This is further supported by the findings that AvrB, AvrRpm1 and AvrRpt2 retain their virulence functions in *rin4* mutant plants and in AvrRpt2 mutants defective in RIN4 degradation (Belkhadir et al, 2004; Lim & Kunkel, 2004). Thus it appears that RIN4 may not be the operative target of AvrRpm1 and AvrRpt2 or they might have multiple targets (Van der Hoorn & Kamoun, 2008). Defining the role of RIN4 as either a decoy or guardee depends, to a large extent, on the effect RIN4 has on pathogen virulence.

Recently, a *Pst* DC3000 effector was shown to abrogate AvrRpt2-induced ETI through the in-vitro interference of AvrRpt2-induced RIN4 modification. The $HopF2_{PTO\ DC3000}$ (HopF2) effector is mono-ADP-ribosyl-transferase and has a demonstrated ability to interfere with AvrRpt2-mediated cleavage through the ribosylation of RIN4. Consistent with the Guard model, HopF2 manipulation of RIN4 promoted *P. syringae* virulence; however, in this particular example HopF2 interaction with RIN4 was found to be direct and did not result in R protein-mediated ETI (Wilton et al, 2010). As the target of multiple effectors, including AvrPto, RIN4 must play an essential role in plant immunity other than extending the recognition repertoire of plants (Luo et al, 2009). However, further evidence is required to support this hypothesis.

The selection pressure imposed by successful host recognition drives the evolution of microbial variants which can evade host detection. Typically these variants have either lost or altered the effectors recognized by the plant host's immune system. However, it is likely that these effector residues have no functional role as their loss would result in a substantial loss of fitness for the pathogen, unless the pathogen's existing effector repertoire compensates for this potential loss. In an alternative strategy, microbial variants acquire novel effectors which suppress ETI triggered by other effectors. As a consequence, *R* genes subjected to these selective pressures co-evolve alongside its effector complement resulting in new microbial variants capable of overcoming ETI and new plant genotypes capable of re-introducing ETI (Thomma et al, 2011; Jones & Dangl, 2006).

1.2.5 R protein-mediated signalling

The manipulation of multiple key host targets greatly enhances the effector's contribution to virulence; however, the detection of only one of these events is sufficient to induce an immune response (Belkhadir et al, 2004; Jones & Dangl, 2006). The final outcome for the plant is largely dependent on the amount of defence events activated and the strength at which each of these responses contribute to overall resistance. R protein signalling involves several pathways which later converge to activate a similar set of defences. The TIR-NBS-LRR subset of R protein mediates ETI through the action of EDS1 and its interacting partners PAD4 and SENESCENCE ASSOCIATED GENE 101 (SAG101), (Feys et al,

2005). Another major subset of R protein, the CC-NBS-LRR type, mediates ETI through the plasma membrane associated protein NON SPECIFIC DISEASE RESISTANCE 1 (NDR1), (See Figure 1-2). For example, NDR1 mediates the activation of CC type proteins RPM1 and RPS2 through its physical interaction with RIN4, however much of the downstream signalling events have yet to be elucidated (Aarts et al, 1998; Day et al, 2006).

Although the distinct recognition mechanisms used in PTI and ETI suggest that these systems are separate, emerging evidence has demonstrated that they are not, but instead function as part of an integrated system. While there are considerable overlaps between the downstream defence signalling responses triggered by PTI and ETI, ETI responses appear to be more prolonged and robust. For example, R protein-mediated ROS accumulation is biphasic with an initial low amplitude phase (characteristic of that induced during PTI), followed by a prolonged high magnitude phase (Torres et al, 2006; Tsuda & Katagiri, 2010). The MAPK cascade is another shared feature which also exhibits a more prolonged response when activated through ETI than PTI (Tsuda & Katagiri, 2010; Underwood et al, 2007). Furthermore, the Ca^{2+} influx induced upon PAMP perception is also induced following effector recognition as sustained cytosolic Ca^{2+} levels are a requirement for HR and the oxidative burst (Grant & Loake, 2000). In addition, both of the PTI and ETI responses have the ability to produce the signalling hormones SA, JA and ET which in turn mediate these shared defence signalling pathways. Cross talk and synergisms between these signalling networks act, to an extent, to differentiate biotrophic from necrotrophic pathogen attack. Genetically, JA and ET are believed to act synergistically against necrotrophic attack while their interaction with SA is thought be antagonistic; however, emerging evidence suggests that these relationships are not that simple. Instead the interplay between SA, JA and ET is shown to consist of both positive and negative regulatory interactions, which ultimately function to determine the optimal response against potential invasion (Kunkel & Brooks, 2002; Schenk et al, 2000; Glazebrook et al, 2003; Pieterse et al, 2012).

1.2.5.1 SA-dependent signalling

SA is a key regulatory hormone implicated in a multitude of biotic and abiotic responses. Its role in plant innate immunity is well established, particularly in R protein-mediated resistance and as an inducer of local and systemic defence responses (Loake & Grant, 2007). Perhaps the most convincing line of evidence supporting SA's role as a crucial component of the defence signalling network comes from experiments that altered endogenous SA levels through the expression of the bacterial *nahG* in *Arabidopsis*. *Arabidopsis* plants expressing *nahG*, a SA hydroxylase which rapidly degrades SA to catechol, were blocked in the defence-related gene expression of *PR-1*, *PR-2* and *PR-5,* necessary for resistance against the virulent *P. syringae* and *Hyaloperonospora parasitica* (Delaney et al, 1994). SA's role in the signalling network has been defined through the analysis of several *Arabidopsis* mutants defective in SA perception and response, such as *SA induction deficient 2 (sid2), enhanced disease susceptibility 5 (eds5), non-expressor of PR-1 (npr1;* formerly *nim1), eds1 and phytoalexin deficient 4 (pad4),* (Wildermuth et al, 2001; Nawrath, 2002; Cao et al, 1994; Parker et al, 1996; Jirage et al, 1999)*. Arabidopsis SID2* encodes an isochorismate synthase necessary for the defence-associated accumulation of SA, as *sid2* mutants exhibit a dramatic reduction in SA (Wildermuth et al, 2001). The residual SA is thought to be produced through the phenylalanine pathway or by EDS5-mediated transportation of intermediates required for SA synthesis (Wildermuth et al, 2001; Nawrath, 2002; Mou et al, 2003; Falk et al, 1999; Jirage et al, 1999). Moreover, SA activates the expression of the SA-biosynthesis genes *EDS5* and *SID2* in a positive feedback loop (Wildermuth et al, 2001; Feys et al, 2001; Verberne et al, 2000). The activation of SA signalling and concomitant induction of SAR leads to the accumulation of PR proteins. PR proteins have long been considered the molecular marker for SAR and although the function of many of these proteins remain unclear, several have been associated with antimicrobial activity (Van Loon & Van Strien, 1999; Durrant & Dong, 2004).

Two key genes essential for activating SA accumulation in response to certain SA-inducing stimuli are *EDS1* and its interacting partner *PAD4*. These lipase-like proteins are believed to act upstream of SA as *PR-1* induction was rescued in *eds1* and *pad4* mutants, following the exogenous application of SA. Furthermore, the prevalence of a SA-mediated positive feedback loop has become evident as SA treatment induces the enhanced

expression of *EDS1* and *PAD4* in wild-type plants (Falk et al, 1999; Feys et al, 2001; Jirage et al, 1999; Zhou et al, 1998). The potentiating activities of the EDS1 PAD4 protein complex along with an additional partner SAG101 has been associated with HR development; however, HR was abolished in *eds1* mutants but not in *pad4* indicating that EDS1 is required for the initial accumulation of SA and HR development, and that its association with PAD4 further amplifies these defence responses (Feys et al, 2001).

SA signalling is mediated through two mechanisms, the defence protein NPR1-dependent pathway and the much less understood NPR1-independent pathway. When SA levels are low NPR1 exists in its inactive oligomeric form in the cytoplasm; however, increasing SA results in the breakage of the disulfide linkages between the oligomers, which then migrate as active monomers into the nucleus via nuclear pore proteins such as MOS (MODIFIER OF *snc1*) (Mou et al, 2003; Cheng et al, 2009; Monaghan et al, 2010). Nuclear localised NPR1 interacts with the TGA class of basic leucine zipper transcription factors activating defence gene expression in a SA-dependent manner. As regulators of SAR, TGAs 2, 5 and 6 are required for the activation of *PR-1* along with the de-repression of its negative regulator *SUPPRESSOR OF NPR1 INDUCIBILE 1* (SNI1) outlined in Figure 1-2 (Li et al, 1999; Zhang et al, 2003b). The importance of NPR1 in regulating signalling downstream of SA has been highlighted in several studies where plants expressing a mutant form of NPR1 were incapable of inducing SA-dependent SAR (Delaney et al, 1995; Tada et al, 2008). Moreover, overexpression of NPR1 enhanced resistance in *Arabidopsis,* although this was not associated with the constitutive activation of defence-related SAR marker gene expression (Cao et al, 1998; Friedrich et al, 2001).

Interactions between the SA and JA signalling pathways are believed to be mutually antagonistic, although instances of neutral or synergistic interactions have been described (Mur et al, 2006). Studies suggest that the relative concentration, timing and sequence of initiation of SA and JA all dictate the final defence outcome of the SA-JA interaction (Leon-Reyes et al, 2010; Koornneef et al, 2008). The predominantly antagonistic relationship between SA and JA is thought provide the plant with a mechanism to prioritize one pathway over the other. Depending on the lifestyle and sequence of the invader(s), preference would be given to the most appropriate signalling pathway. For example, induction of the SA signalling pathway by *Pst* DC3000 suppressed JA/ET- responsive gene expression and *A.*

brassicicola resistance (Spoel et al, 2007). Analysis of the *npr1* mutant of *Arabidopsis* revealed that the negative effect of SA on JA required functional cytosolic NPR1 (Spoel, 2003; Leon-Reyes et al, 2009). Other key players in SA-JA cross talk include MPK4, identified as a negative regulator of SA signalling and positive regulator of JA signalling (Petersen et al, 2000). Generally TGAs are considered to function as essential regulators of SA-responsive gene expression, however more recent evidence implicates these transcription factors in SA-JA crosstalk as the triple mutant *tga2/5/6* and quadruple mutant *tga2/3/5/6* were blocked in the SA-mediated inhibition of JA-responsive gene expression (Kesarwani et al, 2007; Ndamukong et al, 2007). Consistent with this theory, an additional report has implicated TGAs in the activation of JA/ET responsive gene expression in the absence of a SA stimulus (Zander et al, 2010).

1.2.5.2 JA-dependent signalling

JA and its derivatives, collectively called jasmonates (JAs), are important plant signalling molecules that regulate biotic and abiotic stress responses, in addition to their role in growth and development (Pozo et al, 2005). While HR-mediated PCD is relatively effective against biotrophs and hemibiotrophs, it cannot restrict the growth in the case of necrotrophs and may in fact aid in its virulence (Govrin & Levine, 2000; Glazebrook, 2005). Generally JA-dependent responses not associated with PCD are thought to provide a more effective, alternative immune response to necrotrophic attack (McDowell & Dangl, 2000).

JAR1 encodes a JA-amino acid synthetase necessary for the conjugation of JA with other amino acids such as isoleucine (Staswick & Tiryaki, 2004). In the nucleus, JA and its derivatives are bound by CORONATINE INSENSITIVE 1 (COI1), a receptor which is an essential component of all known JA-dependent responses and signalling (Xie et al, 1998; Feys et al, 1994). COI1 encodes a 16 LRR protein containing a F-box motif necessary for its role in protein ubiquitination (Xie et al, 1998). In its active state COI1 associates with other proteins in the SKP1-Cullin-F-Box complex (SCF^{coi1}) and functions as an E3 ubiquitin ligase, specifically recruiting JA-repressor proteins for degradation by the 26S proteasome (Xie et al, 1998; Turner et al, 2002).

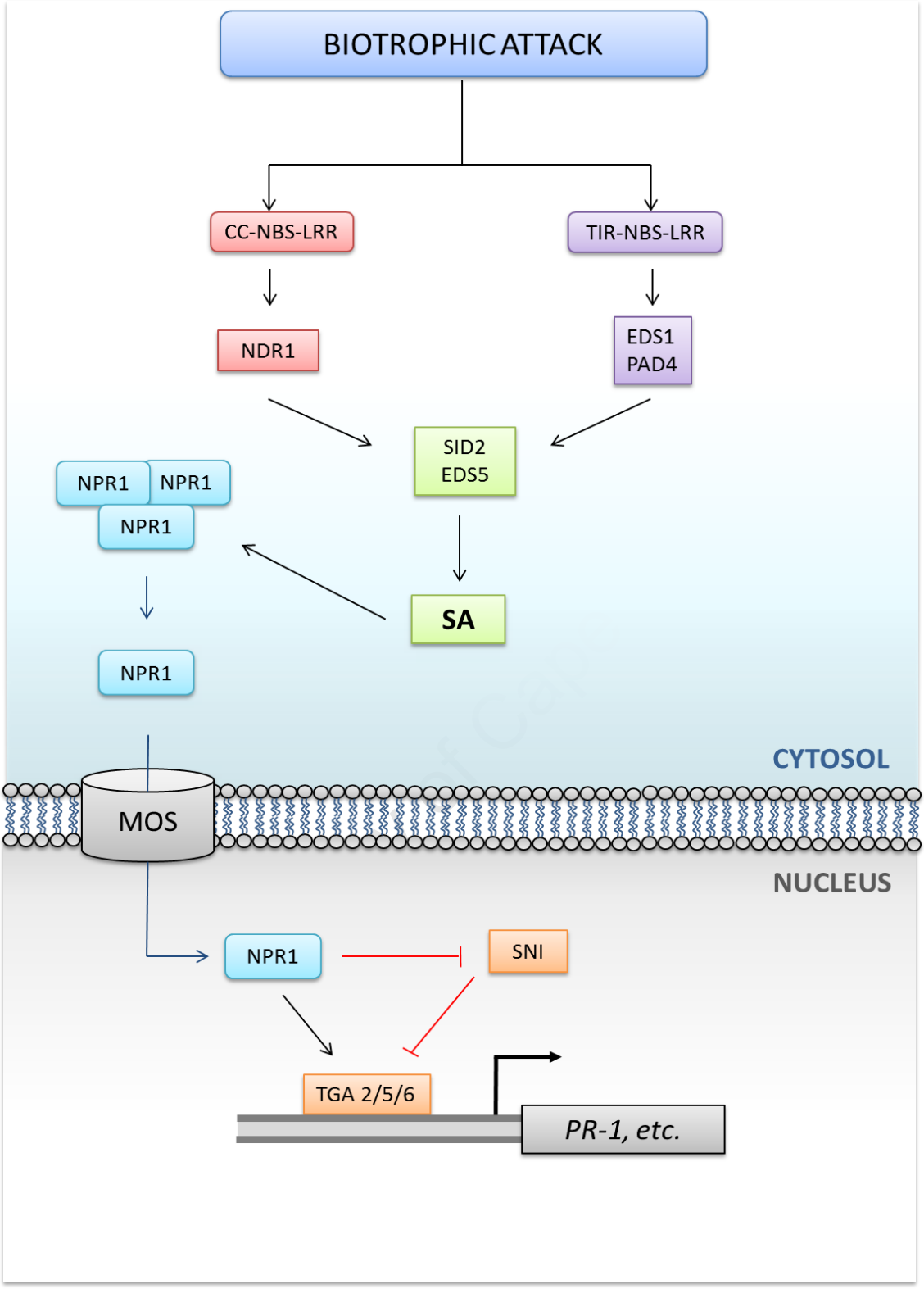

Figure 1-2: Schematic overview of some of the SA signalling network components in *Arabidopsis* defence against biotrophic attack. Black arrows indicate activation, red arrows inhibition and blue lines signify movement. Detection of the pathogen activates R protein-mediated signalling. At this point the signalling is dependent on the type of NBS-LRR protein activated. CC-NBS-LRR proteins signal through NDR1 whereas TIR-NBS-LRR proteins signal through EDS1 and PAD4. R protein-mediated signalling is associated with the activation of SA biosynthesis through regulatory activities of EDS5 and SID2. The subsequent accumulation of SA leads to the reduction of

the disulphide linkages in the NPR1 oligomeric complex releasing the NPR1 monomers. The NPR1 monomers are translocated into the nucleus where they interact with the TGA transcription factors to activate defence-related gene expression such as *PR-1*, presumably by countering the inhibitory effect of SNI.

These repressor proteins are members of the jasmonate ZIM-domain (JAZ) protein family, which negatively regulate JA-responsive genes through their interaction with the JA-regulatory transcription factor MYC2 (Chini et al, 2007; Thines et al, 2007). MYC2, encoded by *JASMONATE INSENSITIVE 1* (*JIN1*), is induced by JA in a COI1-dependent manner and repressed by the JAZ proteins JAZ1 and JASMONATE INSENSITIVE 3 (JAI3), (Lorenzo et al, 2004). In JA-stimulated cells, the SCF^{coi1}-dependent proteasome degradation of the JAZ proteins enables the MYC2-mediated activation of JA responsive genes (Chini et al, 2007; Memelink, 2009). This constitutes the MYC branch of the JA signalling pathway (see Figure 1-3). The MYC branch is associated with, but not limited to, wounding and herbivory, and usually culminates in the activation of expression of the JA-responsive marker gene *VEGETATIVE STORAGE PROTEIN* (*VSP*), (Dombrecht et al, 2007; Lorenzo et al, 2004; Kazan & Manners, 2012). A second branch, termed the ERF branch, is mediated by ETHYLENE RESPONSE FACTOR (ERF1) and OCTADECANOID-RESPONSIVE ARABIDOPSIS 59 (ORA59) members of the APETALA2/ETHYLENE RESPONSE FACTOR (APA2/ERF) family of transcription factors. Activation of the ERF branch is marked by the JA-responsive *PLANT DEFENSIN 1.2* (*PDF1.2*), and is generally believed to impart resistance against necrotrophs (Pré et al, 2008; Lorenzo et al, 2003; Mcgrath et al, 2005; Berrocal-Lobo et al, 2002). Intriguingly, this ERF1/ORA59-mediated upregulation of *PDF1.2* also required the concomitant activation of the ET signalling pathway signifying a role for ERF1 and ORA59 as principle integrators of the JA/ET signalling pathways (Lorenzo et al, 2003; Pré et al, 2008). The synergistic relationship between ET and JA will be discussed in more detail in the next section.

Much of the evidence implicating JA in the plant defence response has come from genetic analysis of mutants defective in JA sensitivity (*jar1*), perception (*coi1* and *jar1*) or synthesis (*fatty acid desaturase* [*fad3/fad7/fad8*] triple mutant), (Berger, 2002; Antico et al, 2012). These mutants displayed enhanced susceptibility to numerous pathogens including the fungal *Alternaria brassicicola*, *Botrytis cinerea* and *Pythium sp.,* and the bacterium

Erwinia carotovora (Thomma et al, 1998; Norman-Setterblad et al, 2000; Staswick et al, 1998).

Traditionally, the JA signalling pathway has been linked to immune responses following necrotrophic challenge. In contrast, several reports provide evidence for the contribution of this pathway to resistance against biotrophs. For example, *jar1* mutants infected with the fungal biotroph, *Golovinomycetes cichoracerum,* displayed an enhanced susceptibility when compared to wild-type plants (Fabro et al, 2008). Furthermore, the constitutive JA signalling mutant *constitutive expressor of vsp1* (*cev1*) exhibited an increase in resistance to *P. syringae pv. maculicola* (Ellis et al, 2002). Interestingly, JA has also been implicated in resistance against the virulent *Pst* DC3000 as pathogen growth in the *jar1* mutant was found to be much higher than that observed in wild-type plants (Pieterse et al, 1998).

1.2.5.3 ET mediation of SA and JA-dependent signalling

ET is a gaseous phytohormone involved in the mediation of a myriad of physiological and developmental processes including seedling emergence, leaf and flower senescence, fruit ripening and organ abscission. In addition, ET has a well-established role as an early response to pathogen perception leading to the induction of several defence responses (Van Loon et al, 2006). Surprisingly, ET has also been implicated as a virulence factor of fungal and bacterial pathogens as *P. syringae glycinea* mutants defective in ET production were impaired in their ability to multiply in leaves of soybean (Weingart et al, 2001). *ETHYLENE INSENSITIVE 2* (*EIN2*) has been identified as a central component of the ethylene signalling pathway as it is the only loss of function mutant to exhibit complete insensitivity to ethylene (Roman et al, 1995; Chen & Bleecker, 1995). In the absence of a stimulus, EIN2 is repressed by the Raf-like protein kinase, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of ethylene signalling (Figure 1-3). This negative relationship is maintained by a family of receptors (ETR1, ETR2, ERS1, ERS2, EIN4), which perceive ethylene (Clark et al, 1998; Chang et al, 1993; Sakai et al, 1998; Hua et al, 1998). Ethylene binding inactivates the receptor-CTR1 complex releasing its negative effect on ethylene signalling (Alonso et al, 1999; Kieber et al, 1993). In ET-stimulated cells, EIN2 activation induces the expression of *ERF1* (and possibly *ORA59*) through its downstream signalling components, the transcription factors EIN3 and EIN3-LIKE 1 (EIL1), (Solano et al, 1998; Guo & Ecker, 2004). Recently, it was

demonstrated that under un-induced conditions these transcription factors are held in an inactive state by the JAZ proteins and the co-repressor HISTONE DEACETLYASE 6 (HDA6), (Zhu et al, 2011). Thus, it appears that in addition to EIN2, the COI-dependent degradation of JAZ proteins is necessary for ERF1- (and possibly ORA59)-mediated activation of defence-related gene expression (Figure 1-3). Consistent with this notion, over-expression of *ERF1* and *ORA59* in *coi1* mutants and *ERF* in *ein2* mutants constitutively activated *PDF1.2* gene expression (Lorenzo et al, 2003; Pré et al, 2008). Surprisingly, ET does not act synergistically to all aspects of the JA signalling pathway as it has been shown to antagonize the MYC branch of immunity. This antagonistic relationship is thought to facilitate the prioritization of the defence signalling network towards JA- or ET-dependent defence pathways mediating resistance to necrotrophic attack (Anderson et al, 2004; Pieterse et al, 2012).

The relationship between SA and ET appears to be quite complex as both positive and negative regulatory interactions occur between the components of these pathways; however, they are less defined and supported by a limited set of data (Kunkel & Brooks, 2002). For example, *PR-1* gene expression does not require functional ET; however, its presence was shown to potentiate the SA-dependent induction of this gene in *Arabidopsis*. Conversely, basal *PR-1* expression in the *ein2* mutant was found to be significantly elevated (Lawton et al, 1994). ET is also believed to play a role in SA-JA signalling cross-talk. Recent evidence has emerged that although ET may potentiate NPR1-dependent SA responsive *PR-1* expression, it circumvents the role of NPR1 in the suppression of the JA response by SA (De Vos et al, 2006; Leon-Reyes et al, 2009). As a consequence, JA-dependent resistance was antagonized by SA in a NPR1-independent manner when *A. brassicicola* infection generated a higher level of endogenous ET (Leon-Reyes et al, 2009). Thus it appears that ET regulates the NPR1 dependency of SA-JA antagonism, presumably in an effort to prioritize JA and ET signalling pathways over the SA pathway (Leon-Reyes et al, 2009). Such a system may function to focus NPR1-dependent responses where they are needed most, such as the SA-dependent activation of *PR-1* expression. Moreover, it is believed to play a pivotal role in plant immunity against multi-attacker interactions (Pieterse et al, 2012).

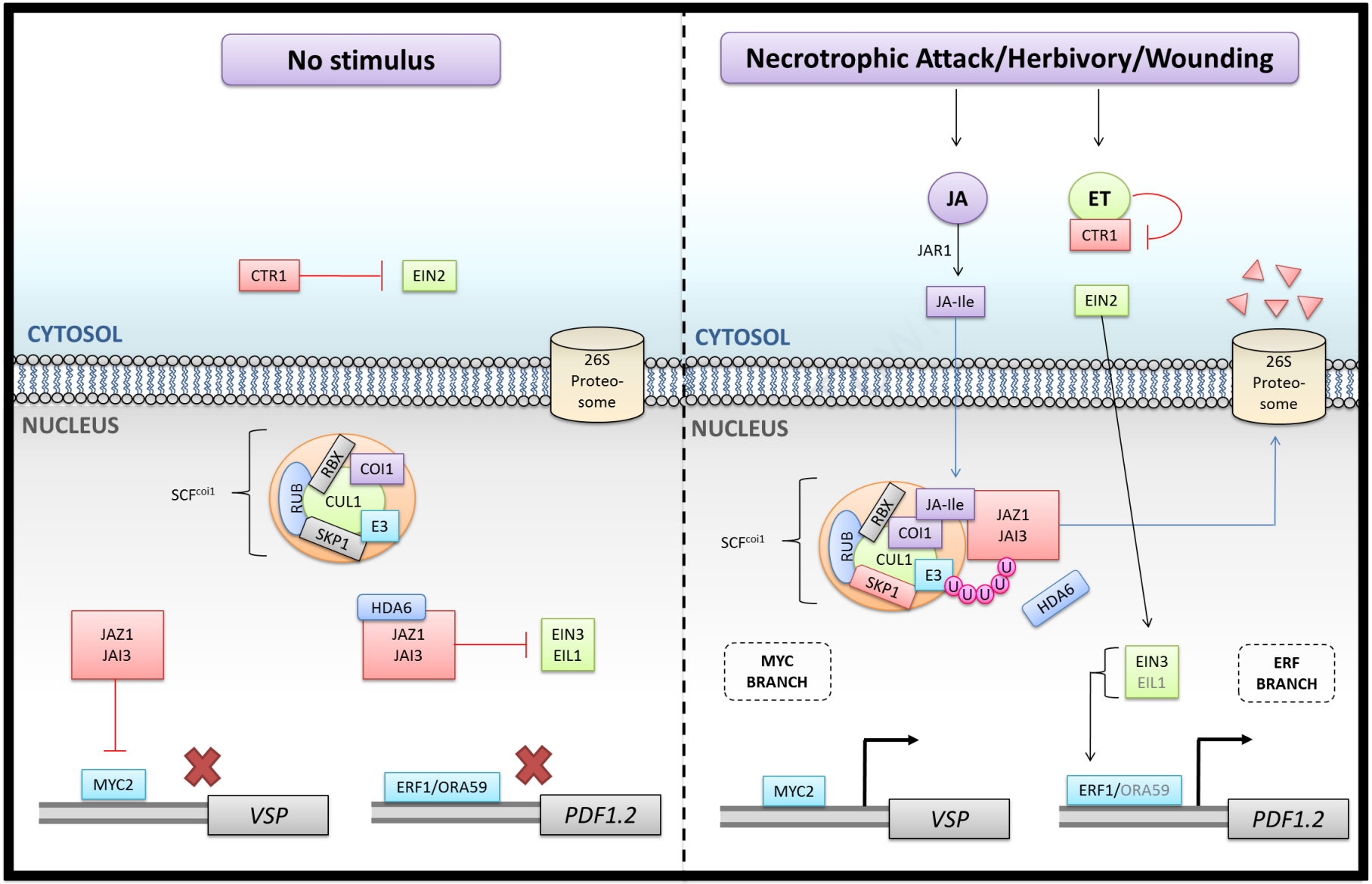

Figure 1-3: Simplified schematic diagram depicting the role of transcription factors in the defence network involving the JA and ET signaling pathways. Black arrows indicate activation, red arrows inhibition and blue lines signify movement. In the absence of a stimulus, CTR1 maintains EIN2 in an inactive state while the JAZ proteins, JAZ1 and JAI3, serve as negative regulators of MYC2 and recruit HDA6 in their repression of the EIN3/EIL1 transcription factors. Necrotrophic attack, herbivory or wounding triggers JA biosynthesis. Following synthesis, JA derivatives modified by the JA conjugate synthase (JAR1), such as jasmonyl isoleucine (JA-Ile),

migrate to the nucleus where they are bound by the COI1 receptor in the SCF^{coi} complex. This interaction drives the ubiquitination of JAZ proteins resulting in their subsequent degradation by the 26S proteosome. In the MYC branch, derepression of MYC2 results in the increased transcription of JA-responsive genes which in turn, have the ability to activate or repress various functions in the defence pathway. Traditionally, the MYC branch is activated in response to herbivory or wounding and is associated with increased *VSP* expression, although MYC2 has been reported to be involved in priming *Arabidopsis* defence systems (Pozo et al., 2008). The ERF branch is associated with responses to necrotrophic attack and is marked by increased *PDF1.2* expression. In the ERF branch, the JA-dependent degradation of JAZ repressors relieves EIN3 and EIL1 from repression. In addition, increased ET levels inactivates CTR1 resulting in the EIN2-dependent accumulation of EIN3 and EIL1 in the nucleus. EIN3 activates the expression of targets such as the ERF1 (and possibly ORA59) transcription factors responsible for the increased *PDF1.2* expression.

1.3 Negative regulators in the plant defence network

Plant defence responses are regulated through a complex and interconnected network of signalling pathways. Cross-talk between these pathways involves both synergistic and antagonistic interactions, regulated by low-molecular-weight signalling molecules such as SA, JA, ET, NO and ROS. Several excellent reviews provide a summary of how these pathways influence one another to fine-tune their overall defence outputs (Kunkel & Brooks, 2002; Jonak, 2002; Koornneef & Pieterse, 2008; Glazebrook, 2005; Bostock, 2005).

Numerous studies have identified negative regulators at key points in the plant defence signalling network permitting a tight regulation of cellular activation through a variety of mechanisms. Negative regulators at the early stages of immunity include the kinase-associated protein phosphatase (KAPP) and the plant U-box E3 ubiquitin ligase (PUB) triplet, PUB 22/23/24, of *Arabidopsis*. KAPP binds to the kinase domain of a subset of RLKs including FLS2, hindering its autophosphorylation, whereas the PUB triplet functions through E3 enzymatic activity to remove activators of PTI (Trujillo et al, 2008; Gómez-Gómez et al, 2001). The proposed functions of these regulators are as moderators of the immediate PTI response, down-regulating its output once pathogen challenge has subsided to avoid the adverse effects associated with the activation of constitutive defences (Trujillo et al, 2008). In addition, several regulators of SAR have been identified including *SNC1* (*SUPPRESSOR OF NPR1, CONSTITUTIVE 1*) and *SNI1* (*SUPPRESSOR OF NPR1, INDUCIBLE 1*), which revolve around the direct or indirect manipulation of NPR1 (Li et al, 2001; Li et al, 1999; Dong, 2004). Interestingly, NPR1 is involved in a negative feedback loop with SA as higher levels of SA were observed in infected *npr1* plants than in infected wild-type plants (Shah et al, 1997).

Under un-infected conditions *R* genes are generally maintained at low levels, possibly through post-transcriptional RNA silencing, while R protein mRNAs are subjected to high rates of turnover and alternative splicing (Yi & Richards, 2007; Palma et al, 2010). This initial regulatory mechanism acts as safeguard against autoimmune responses. Mediation of this aspect of plant immunity involves *ACCELERATED CELL DEATH 11 (ACD11)*, which negatively regulates R proteins triggering strong PCD, and *SUPPRESSOR OF RPS4-RLD (SRFR1)*, which negatively regulates R protein accumulation (Palma et al, 2010; Li et al, 2010).

The detrimental effects of the constitutive activation of defences have been demonstrated in an array of *Arabidopsis* mutants. Stunted growth, curly leaves and the spontaneous development of HR-like lesions are a few of the consequences associated with un-controlled SA accumulation, SAR and defence-related gene expression (Mauch et al, 2001; Durrant & Dong, 2004; Kunkel & Brooks, 2002; Glazebrook, 2005; Lorrain, 2003). Hence, negative regulators tighten the plants control over its immune system, possibly acting as a mechanism by which plants evaluate the cost to fitness of their responses so that they may amplify defence signals only when necessary (Tsuda & Katagiri, 2010; Sato et al, 2010).

While SA, JA and ET all contribute positively to immunity, the simultaneous up-regulation of all three of these signalling pathways has yet to be documented. In addition, the prevalence of mutually negative regulatory relationships between SA and JA provide evidence for the existence of a "sector switching" mechanism in plants (Sato et al, 2010; Katagiri & Tsuda, 2010). A similar relationship was observed between SA and the early MAMP-triggered (EMT) signalling sectors. Contrary to previous reports of SA induction following PAMP perception, Sato and colleagues demonstrated the existence of a mutually antagonistic relationship between SA and EMT and reasoned that these signalling pathways were also subjected to the sector switching phenomenon (Sato et al, 2010). Their argument for this counterintuitive theory was governed by two main factors. Firstly, while the activation of EMT does induce SA accumulation, this occurs much later on in the defence signalling timeline. Secondly, the mutual inhibition of either sector depends on the strength of SA signalling (Sato et al, 2010; Katagiri & Tsuda, 2010; Pritchard & Birch, 2011). This implies that although both signalling sectors are activated following PAMP perception, SA activation is maintained at a lower amplitude by the suppression activities of the highly

activated EMT sector. Should the EMT sector become impaired through the effector suppression of a well-adapted pathogen, the subsequent derepression of the SA sector would trigger the necessary responses, at the same time supressing the ineffective EMT sector.

This highlights the importance of mutual antagonism in carefully balancing resistance outputs, by evaluating and employing an adequate level of protection, while minimizing the associated costs. Plants regulate their early defence outputs through mutual inhibition between SA and EMT as it would be beneficial for plants not to employ too strong of a response at this point in the defence timeline. When more potent responses are required the SA, JA and ET sectors prevail. However, rather than acting as a functionally redundant system, inter sector inhibition promotes the action of one sector while the others serve as a backup (Tsuda et al, 2009; Katagiri & Tsuda, 2010; Pritchard & Birch, 2011).

1.4 The identification of a novel defence related mutant, *cir1*

Murray and colleagues sought to identify components of the signalling network involved in the establishment of disease resistance by identifying regulators of *PR* expression (Murray et al, 2002). In a forward genetics approach the *cir1* (*constitutively induced resistance 1*) mutant was identified following a screen for *Arabidopsis* mutants with altered *PR-1* gene expression patterns (Murray et al. 2002). In this experiment a novel *Arabidopsis* line (termed *PR-1::LUC*) was generated in the Col-0 background with the *PR-1* promoter transcriptionally fused to a firefly luciferase (LUC) reporter gene. Following EMS mutagenization, the *PR-1::LUC* transgenic plants were screened via LUC imaging to identify any mutants which mis-expressed *PR-1* following infection with the avirulent *Pst* AvrB. A modified and increased distribution of LUC activity was observed in the *cir1* mutant in comparison with the Col-0 and *PR-1::LUC* lines following infection.

1.4.1 Characterization of the *cir1* mutant

Northern blot analysis revealed the constitutive expression of several defence-related genes in the *cir1* mutant such as the SA-dependent *PR-1*, *PR-2* and *PR-5*, and the JA/ET-

dependent *PDF1.2* (Murray et al, 2002). *Cir1* also exhibits constitutive expression of *GST1*, a gene associated with the oxidative burst-stimulated development of HR; but did not display sporadic lesion formation placing it downstream of HR activation (Murray et al, 2002; Grant & Loake, 2000). In addition, *cir1* was shown to constitutively accumulate EDS1 protein. Furthermore, the increase in EDS1 protein levels corresponded with enhanced resistance to biotrophic and hemi-biotrophic pathogens (Carstens, 2008). Indeed, the *cir1* mutant exhibits enhanced resistance against the virulent pathogens *Pst* DC3000 and *H. parasitica* Noco2 (Murray et al, 2002). While *cir1* mediated resistance to both pathogens was determined to be SA- and NPR1-dependent, resistance to *Pst* DC3000 also required functional *JAR1* and *EIN2* (Murray et al, 2002). Epistasis analysis further revealed that *cir1* functions upstream of *npr1*, *ein2*, *jar1*, *coi1*, *eds1* and *pad4* mutations, as functional versions of these genes are required for the activation of defence-related gene expression and the subsequent resistance it confers (Murray et al, 2002; Carstens, 2008). In addition, gene expression profiling experiments of the *cir1* mutant successfully identified two novel components of innate immunity against *Pst* DC3000 in *Arabidopsis*, WRKY53 and the nematode resistance protein-like HSPRO2 (Murray et al, 2007).

Collectively these results implicate *CIR1* in the regulation of multiple defence signalling pathways. Hence, it is conceivable that *CIR1* negatively regulates immunity through the suppression of SA-, JA-, ET- and ROS-dependent defence-related gene expression.

1.4.2 Candidate analysis

First pass mapping experiments and segregation analysis determined that *cir1* is located on the lower arm of chromosome four and is a recessive mutation (Murray et al, 2002). More comprehensive fine mapping narrowed down the location of *CIR1* to a 309.10 kb region containing eight annotated genes (see Table 1-1), (Carstens, 2008). These eight genes will be discussed in more detail in the following sections.

Table 1-1: Description of eight *CIR1* candidate genes on chromosome four

Gene	Description
AT4G11100	Uncharacterized protein
AT4G11110	Member of the SPA (suppressor of *phyA-105*) protein family (SPA2)
AT4G11120	Putative translation elongation factor Ts (EF-Ts)
AT4G11130	RNA-directed RNA polymerase (RDR2)
AT4G11140	Cytokinin response factor 1 (CRF1)
AT4G11150	Vacuolar H^+-ATPase subunit E isoform 1 (VHA-E1/TUFF)
AT4G11160	Translation initiation factor IF-2-like protein
AT4G11170	Putative disease resistance protein (TIR-NBS-LRR class)

1.4.2.1 At4g11100: Uncharacterized protein

AT4G11100 encodes an uncharacterised protein that has only recently been implicated in plant immunity. An initiative examining the genetic interaction network of *Arabidopsis* has had promising results in its production of a proteome-wide binary protein–protein interaction map termed the *Arabidopsis* interactome version 1 (AI-1), (Arabidopsis Interactome Mapping Consortium, 2011). To generate this map 8000 open reading frames of protein-coding genes were prepared. The proteins encoded by these genes were analysed of all pairwise combinations through an improved, high-throughput, binary interactome mapping pipeline based on the yeast two-hybrid system. According to the interactome map AT4G11100 interacts with GSTU1 (*AT2G29490*) from the Tau class of *GSTs* (*GSTUs*), (Arabidopsis Interactome Mapping Consortium, 2011). GSTs have well documented roles in signalling and in the response to pathogen infection and plant hormones (Chen et al, 2007a; Moons, 2005); however, the high prevalence of functional redundancy in this gene

family hinders the identification of individual gene function (Dixon & Edwards, 2010; Alfenito et al, 1998). Hence, the role of this interaction is not fully understood.

Additional interactors of AT4G11100 have been identified through Dangl and colleagues' plant pathogen interaction network generated using 552 immune and pathogen proteins, in addition to that used in AI-1, as well as the same yeast two hybrid–based pipeline of AI-1 (Mukhtar et al, 2011). In this network, AT4G11100 was proposed to interact with two LRR-containing RLKs, AT3G50230 belonging to the CLAVATA 1 (CLV1)-like receptor kinase family and AT2G36570 encoded by the *Arabidopsis* ortholog of *Glycine max* LRR RLK 1 (GmLRK1), (Duarte et al, 2006; Kim et al, 2009; Mukhtar et al, 2011). Typically proteins encoded by the *CLV1* and *GmLRK1* genes are thought to function in fundamental developmental processes such as meristem maintenance and cell elongation, thus it remains unclear how AT4G11100 and its interacting partners would function in plant defence (Jeong et al, 1999; Kim et al, 2009).

1.4.2.2 At4g11110: Member of the SPA (suppressor of phyA-105) protein family (SPA2)

Arabidopsis has evolved several photoreceptors which monitor the ambient light environment, adjusting growth and development accordingly in a process known as photomorphogenesis. *AT4G11110* encodes the SPA1-RELATED 2 (SPA2) protein, which acts as a potent inhibitor of photomorphogenesis, possibly through its interaction with a known suppressor COP1 (Kim et al, 2002; Laubinger et al, 2004). In darkness, COP1 negatively regulates photomorphogenesis by promoting the ubiquitination and degradation of positively acting light signalling intermediates (Osterlund et al, 2000; Saijo et al, 2003). In the light, photoreceptors are thought to suppress the SPA2/COP1 complex releasing its inhibition on activators of the light response; however, how this is achieved still remains unclear. More recent evidence suggests that SPA2 functionality is hyper-inactivated by light (Balcerowicz et al, 2011). To date, no information regarding SPA2's role in plant immunity is available.

1.4.2.3 At4g11120: Putative translation elongation factor Ts

ELONGATION FACTOR Ts (EF-Ts) functions as a nucleotide exchange factor that facilitates the exchange of EF-Tu bound GDP for GTP. This exchange plays a fundamental

role in the elongation phase of protein synthesis in the plastids and mitochondria of plants (Fu et al, 2012). *AT4G11120* encodes a putative translation EF-Ts, first identified in an effort to define mitochondrial localized proteins in *Arabidopsis. AT4G11120* was assigned its function based on sequence comparison (Millar et al, 2001). No publications to date implicate *AT4G11120* in plant immunity.

1.4.2.4 At4g11130: RNA-directed RNA polymerase (RDR2)

RNA silencing provides a system for the transcriptional and post-transcriptional regulation of gene expression and the suppression of transposon activity (Baulcombe, 2004). These RNA silencing mechanisms are facilitated by the production of small RNAs such as microRNAs (miRNAs) or small-interfering RNAs (siRNAs) which direct sequence specific regulation through RNA cleavage, translational inhibition or chromatin modification (Willmann et al, 2011). *AT4G11130* encodes the RNA-DIRECTED RNA POLYMERASE 2 (RDR2), which along with DICER-LIKE 3 (DCL3) are involved in the biosynthesis of siRNAs from heterochromatic loci (Xie et al, 2004). These 24-nucleotide siRNAs recruit silencing components to form transcriptionally silent heterochromatin through chromatin modification events (Xie et al, 2004).

It has been suggested that a particular siRNA, nat-siRNAATGB2, derived from an overlapping pair of natural antisense transcripts (NATs) is specifically induced following avirulent *Pst* DC3000 (avrRpt2) infection (Borsani et al, 2005; Katiyar-Agarwal et al, 2006). This induction was shown to be both *RPS2*- and *NDR1*-dependent and resulted in *RPS2*-mediated ETI through the suppression of a negative regulator of the *RPS2* resistance pathway (Katiyar-Agarwal et al, 2006). Another report describes the negative regulation of *R* genes through the generation of endogenous siRNAs at the *RPP4* locus, which confers resistance to *P. syringae* and *H. parasitica* (López et al, 2011).

1.4.2.5 At4g11140: Cytokinin response factor 1

Cytokinin response factors (CRFs) are members of the AP2/Ethylene response factor (ERF) family of transcription factors (Nakano et al, 2006). The AP2/ERF proteins act as regulators of numerous developmental processes and also play a key role in the response to various biotic stresses (Gu, 2002; Gutterson & Reuber, 2004). Several members of the ERF

sub-family of AP2/ERF transcription factors have been demonstrated to enhance disease resistance in transgenic plants (Berrocal-Lobo et al, 2002). Moreover, mounting evidence implicates members of the ERF sub-family in the JA and ET signalling pathways as well as in disease resistance (Park et al, 2001; Mcgrath et al, 2005; Gutterson & Reuber, 2004; Berrocal-Lobo et al, 2002).

AT4G11140 encodes CRF1, a member of group four of the ERF sub-family of AP2/ERF transcription factors (Rashotte et al, 2006; Nakano et al, 2006). CRF proteins are regulated by cytokinin, an essential plant hormone for growth and development, and have been shown to affect leaf and cotyledon development in loss of function studies. Intriguingly, observed phenotypic differences between *crf* mutants and cytokinin receptor mutants suggest that CRF may also function in other signalling or developmental pathways (Rashotte et al, 2006).

An example of a CRF that has a function in *Arabidopsis* disease resistance against *Pst* DC3000 has been reported (Liang, 2010). *CRF5* expression was shown to be induced following *Pst* DC3000 infection while the over-expression of *CRF5* in transgenic plants resulted in the up-regulation of *PR* genes and enhanced pathogen resistance. Further analysis revealed a transcriptional activator domain on the C-terminus of the protein responsible for the activation of GCC element-containing *PR* genes. Thus, it appears that the signal transduction pathways necessary for cytokinin signalling and pathogen defence are closely associated (Liang, 2010).

1.4.2.6 At4g11150: Vacuolar H^+-ATPase subunit E isoform 1 (VHA-E1/TUFF)

The tight regulation of pH is crucial to all biological systems. In eukaryotic cells, maintaining endomembrane compartments at pH level relatively lower than that of the cytosol is necessary for numerous biological processes including membrane trafficking, protein degradation and the transport of small molecules across membranes (Forgac, 2007). To transport protons into the endomembrane compartments, eukaryotes have evolved ATP-dependent proton pumps, the most common of which is the vacuolar H^+-ATPase (V-ATPase), (Forgac, 2007; Dettmer et al, 2010; Dettmer et al, 2006). V-ATPases are multi-subunit complexes consisting of a membrane anchored V_0 domain involved in H^+ transport and a peripheral V_1 domain responsible for ATP hydrolysis (Nishi & Forgac, 2002). The V_0 domain

consists of an assembly of up to six subunits (a, c, ć , ć, d and e), while the V_1 domain is comprised of 8 subunits (A-H), (Nishi & Forgac, 2002). It is not unusual for V-ATPase subunits to be encoded by multiple genes, as this facilitates the formation V-ATPase complexes with different enzymatic and regulatory properties (Kawamura et al, 2000; Dettmer et al, 2010).

AT4G11150 is one of three differentially expressed genes encoding subunit E of the V_1 domain (VHA-E1/TUFF) and is important in upholding a functional secretory system required for normal embryogenesis (Strompen et al, 2005; Dettmer et al, 2010). Jones and colleagues reported a decrease in *TUFF* protein in response to *Pst* AvrRpm1 challenge, however, the mechanism by which it occurs and the purpose of this decrease remains unclear (Jones et al, 2006). It is possible that the *TUFF* protein may be directly or indirectly modified by AvrRpm1 or downstream components of this defence pathway, however the absence of *AT4G11150* is characterized by growth inhibition due to defective vesicle trafficking not evident in the *cir1* mutant (Jones et al, 2006; Strompen et al, 2005; Dettmer et al, 2006). Interestingly, a well known regulator of immunity, RIN4, has been shown to govern stomatal aperture through the regulation of plasma membrane H^+-ATPase activity during pathogen attack (Liu et al, 2009).

1.4.2.7 At4g11160: Translation initiation factor IF-2-like protein

To date very little information exists on *AT4G11160*, however it has been assigned a putative function, as a mitochondrial IF2, based on protein domain similarity to the prokaryotic translation initiation factor (IF2), (Miura et al, 2007). In eukaryote protein synthesis, IF proteins facilitate translation initiation by assembling the ribosomal subunits and initiator tRNA (met-tRNA) at the correct start codon on the mRNA (Dever, 2002). A plant ortholog of IF2, eukaryotic initiation factor 5B (eIF5B), was shown to perform a similar function suggesting the conservation of function between homologous IF and eIF proteins (Pestova et al, 2000).

Translation initiation factors with roles other than that observed in ribosome-mediated translation process have been identified before (Gaussand et al, 2011; Hopkins et al, 2008; Karniol et al, 1998). eIF5A is a highly conserved protein believed to exist as three isoforms, each encoded by a separate gene in a multi-gene family (Feng et al, 2007). Specifically, the

eIF5A-2 isoform was recently found to regulate *Pst* DC3000-induced PCD in *Arabidopsis,* suggesting a role for this eIF in the pathogenesis signalling network (Hopkins et al, 2008).

In addition to its role in transcription regulation, the eIF3 protein complex interacts with the COP9 signalsome, a multi-subunit complex involved in protein ubiquitination and turnover under a variety of biological conditions (Von Arnim, 2003; Karniol et al, 1998). Such biological conditions include hormone signalling and pathogen defence (Von Arnim, 2003).

To date, a specific role for AT4G11160 protein in pathogen defence has not yet been described.

1.4.2.8 At4g11170: Putative disease resistance protein (TIR-NBS-LRR class)

Computational analysis of the predicted protein domains of At4g11170 revealed the presence of a N-terminal TIR domain, a conserved NBS domain and the C-terminal LRR domain, providing strong evidence that it encodes a R protein of the TIR class. As described earlier, R proteins initiate ETI upon the specific recognition of pathogen avirulence products (Jones & Dangl, 2001). Previous research has demonstrated that mutations in the *R* gene may in some instances lead to constitutive expression of certain defence-related genes, although it is highly unlikely that these mutated *R* genes would function as negative regulators of resistance.

Snc1 and *ssi4* (*suppressors of npr1-5–based SA insensitivity*) are *R* gene mutants which are similar to *cir1* in that they have been identified as constitutive expressors of *PR* genes and confer resistance against bacterial and oomycete pathogens (Zhang et al, 2003a; Shirano et al, 2002). Furthermore, both mutants had mutations rendering their respective *R* gene constitutively active even in the absence of pathogen challenge (Zhang et al, 2003a; Shirano et al, 2002). Additionally, like *cir1*, *snc1* was also mapped to chromosome four and did not display sporadic lesion development (Zhang et al, 2003a; Li et al, 2001). In contrast, *snc1* and *ssi4* are dominant, gain of function mutations whereas *cir1* is recessive and thus likely to be a loss of function mutation characteristic of EMS mutagenesis (Zhang et al, 2003a; Shirano et al, 2002; Murray et al, 2002).

1.5 Specific project objectives

To date the identity of *CIR1* remains elusive but through genetic mapping and functional cloning studies likely *CIR1* candidates have been reduced to one of eight genes on the lower arm of chromosome four of *Arabidopsis* (Carstens, 2008). The main objective of this study was to identify the gene responsible for the *cir1* phenotype by evaluating all eight candidates using various genetic techniques. As *cir1* is a loss of function mutation, assays investigating the phenotypes of T-DNA insertion mutants of each of the candidate genes were undertaken. The underlying hypothesis behind this reverse genetics approach is that a null T-DNA insertion mutant in the *CIR1* gene would phenocopy the *cir1* mutant exhibiting enhanced resistance to *P. syringae* and constitutive defence-related gene expression.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

The *Arabidopsis thaliana* seeds used in this experiment were of the Columbia (Col-0) ecotype unless otherwise stated. The various transgenic lines (Table 2.1) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC) (Scholl et al, 2000), unless otherwise indicated. For all experiments, seeds were hydrated in 0.1% (w/v) agar before they were sown on a 1:1 mixture of peat (Jiffy Products, International AS, Norway) and vermiculite. Thereafter, the pots were covered with cling film to maximize humidity and placed under fluorescent light (80 – 100 µmol photon/sec/m^2) with 16-h light/ 8-h dark cycle at 22°C. The covers were removed within a week, exposing the soil and seedlings to a relative humidity of 55%.

2.2 Microbial Strains and Plant Infection

2.2.1 Escherichia coli

2.2.1.1 Culture conditions

E. coli XL1 blue, DH5α and One Shot® cells were cultured in Luria-Bertani (LB) media (Sambrook et al, 1989) either on plates (1.5 % w/v agar) or in liquid culture at 37°C overnight.

2.2.2 Agrobacterium tumefaciens

2.2.2.1 Culture conditions

Agrobacterium tumefaciens strain GV3101 (Holsters et al, 1980) was routinely cultured in LB medium containing rifampicin (150 µg/mL) and gentamycin (15 µg/mL) at 28°C in the dark.

Table 2-1: *Arabidopsis* strains and transgenic lines used in this experiment. The location of the T-DNA insertion was determined by the Salk Institute Genomic Analysis Laboratory database (http://signal.salk.edu/cgi-bin/tdnaexpress).

Strains/ Lines	Description	T-DNA Insertion site	Source
PR-1::LUC	*PR-1::LUC* transgenic line (Columbia ecotype)	n/a	(Murray et al, 2002)
cir1	*constitutively induced resistance 1* (*PR-1::LUC* transgenic line background)	n/a	(Murray et al, 2002)
eds1	*enhanced disease susceptibility 1* (Landsburg erecta ecotype)	n/a	(Aarts et al, 1998)
SALK_062847C	*at4g11100* T-DNA insertion mutant	Promoter	NASC (Alonso et al, 2003)
SALK_083331C	*at4g11110* T-DNA insertion mutant	Exon	NASC (Alonso et al, 2003)
SALK_009832C	*at4g11110* T-DNA insertion mutant	Intron	NASC(Alonso et al, 2003)
SALK_007854C	*at4g11120/at4g11130* T-DNA insertion mutant	3' UTR	NASC (Alonso et al, 2003)
SALK_142463C	*at4g11120* T-DNA insertion mutant	Promoter	NASC (Alonso et al, 2003)
SALK_059661	Segregating *at4g11130* T-DNA insertion mutant	Exon	NASC (Genotyped in this study)
SALK_038489C	*at4g11140* T-DNA insertion mutant	Promoter	NASC (Alonso et al, 2003)
SALK_019365C	*at4g11150* T-DNA insertion mutant	Promoter	NASC (Alonso et al, 2003)
SALK_128966C	*at4g11160* T-DNA insertion mutant	Exon	NASC (Alonso et al, 2003)
SALK_023944C	*at4g11170* T-DNA insertion mutant	Intron	NASC (Alonso et al, 2003)
SALK_091592C	*at4g11170* T-DNA insertion mutant	Exon	NASC (Alonso et al, 2003)

2.2.2.2 Competent cell preparation

A single colony of *A. tumefaciens* was inoculated into 10 mL LB media supplemented with the appropriate antibiotics and incubated with shaking at 28°C overnight. The following day 2 mL of the overnight culture was transferred to 50 mL fresh LB media with antibiotic selection and incubated with shaking at 28°C until the OD_{600} reached 0.5–1.0. The culture was chilled on ice before the cells were harvested by centrifugation at 3000 x *g* for 5 min at 4°C in a J2-21 Beckman centrifuge (Beckman Coulter, Inc., CA, USA). The resulting pellet was resuspended in 1 mL ice cold 20 mM $CaCl_2$. Aliquots of 100 µl were then immediately frozen in liquid nitrogen and stored at -80°C.

2.2.3 Pseudomonas syringae

2.2.3.1 Culture conditions

Laboratory strains of virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Whalen et al, 1991) and avirulent *P. syringae (*AvrB*)* (personal communication; Barbara Kunkel, Department of Biology, Washington University in St. Louis) were cultured in King's Broth (KB) medium (King et al, 1954) supplemented with 50 µg/mL Rifampicin for *Pst* DC3000 and 50 µg/mL rifampicin plus 20 µg/mL tetracycline at 30°C overnight. Solid media included 1.5% (w/v) agar.

2.2.3.2 Arabidopsis infection assay

Overnight cultured cells were centrifuged, washed and diluted in 10 mM $MgCl_2$ to a final OD_{600} of 0.002, which corresponds to 1 x 10^6 colony forming units (c.f.u)/mL (Katagiri et al, 2002). Four-week-old soil grown plants were infected with either the bacterial suspension or 10 mM $MgCl_2$ (mock-infection) by pressure infiltration of the abaxial surface of the leaf. Three to four leaves per plant and three plants per *Arabidopsis* line were infected. Infected and mock-infected plants were covered with cling film and left under normal growth conditions for up to 3 days. Infected leaves were harvested at 4 h (Day-0) and 48 h (Day-2) post-inoculation for analysis of bacterial growth. Uniform leaf disks (0.5 cm^2) taken from each of the three leaf samples, per plant, were ground in 10 mM $MgCl_2$. A dilution series of

the bacterial suspension was spotted on KB medium plates supplemented with appropriate antibiotics, followed by an incubation period of two days at 30°C. After two days the number of colonies were recorded for each sample and expressed as a bacterial titre (colony forming units/cm^2) on a log scaled graph.

2.2.4 Botrytis cinerea

2.2.4.1 Culture conditions

B. cinerea isolated from pepper (Grant et al, 2003) was maintained on sugar free apricot halves incubated at 25°C in the dark until sporulation had occurred (14 days post inoculation).

2.2.4.2 Arabidopsis infection assay

Spores were collected and counted using a haemocytometer (Denby et al, 2004). Thereafter the spore concentration was adjusted to 5 x 10^3 spores/mL in half strength grape juice. Leaves from 4-5 week old plants were excised and placed on trays filled with 1% (w/v) agar. Ten microliters of the spore suspension was drop inoculated onto the middle of the leaves and the tray was covered to maintain high humidity conditions. As a control, separate batches of leaves for each line were drop-inoculated with half strength grape juice only. The trays were kept at room temperature and were imaged 72 h, 96 h and 120 h post inoculation. For the experiment 10 leaves from the same developmental stage was taken from 10 different plants. Lesion size was measured using the ImageJ software (Rasband, W.S., U.S. National Institutes of Health, Bethesda, Maryland, USA) and was expressed as lesion area in mm^2.

2.3 RNA Manipulation

2.3.1 RNA Isolation from *Arabidopsis*

Approximately 100 mg of leaf tissue was homogenized in an extraction buffer based on the Tri-Reagent (0.1 M sodium acetate (pH 5.2), 0.8 M guanidine thiocyanate, 0.4 M

ammonium thiocyanate, 5% (v/v) glycerol, 38% (v/v) phenol (pH 4)). RNA was extracted according to the TRIzol reagent protocol (Invitrogen, Carlsbad, USA). RNA was quantified on a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Wilmigton, USA) at 260 nm where an OD_{260} of 1 is equal to 40 µg RNA/mL.

2.3.2 Electrophoresis of RNA

The integrity of the RNA was assessed on a RNA formaldehyde-agarose denaturing gel (1.2% (w/v) agarose, 1 X MOPS pH 7 (0.4 M MOPS, 0.1 M sodium acetate, 10 mM EDTA), 2.25% (v/v) formaldehyde). Before electrophoresis, 0.2 volumes of RNA sample application buffer (4 X MOPS, 2.7% (v/v) formaldehyde, 30.8% (v/v) formamide and 0.01 mg/mL EtBr) was added to the RNA sample, heated at 65°C for 5 min and snap cooled on ice. Following electrophoresis the gel was visualized on a Gel Doc™ XR UV transilluminator (Bio-Rad Laboratories, UK) and photographed as a loading control between RNA samples.

2.3.3 cDNA synthesis

Prior to cDNA synthesis, RNA was treated with DNase I (Fermentas, Ontario, Canada) as instructed by the manufacturer. A maximum of 2.5 µg RNA was used in the synthesis of cDNA which was performed according to the manufacturer's protocol (Invitrogen Corporation, California, USA) with the following modifications: Half the required enzyme (Superscript™ III) was used and the reaction was carried out as follows: cDNA synthesis was performed at 42°C for 2 h followed by heat inactivation step at 72°C for 15 min. Successful cDNA synthesis was confirmed in a standard PCR reaction with the *IMPL2* (*INOSITOL-MONOPHOSPHATASE LIKE 2; At4g39120*) primers listed in Table 2-2 at 1.5 mM $MgCl_2$.

2.3.4 Quantitative real-time PCR

Quantitative real time PCR was performed using the Rotorgene 6000 (Corbett Life Science Pty. Ltd., Sydney, Australia). The 2 x SensiMix™ SYBR® No-Rox kit (Bioline Ltd., London, UK), which contains the SYBR® green dye, dNTPs, stabilizers and enhancers, was used according to the manufacturer's protocol with the following modification: 10 µl

reaction volumes were used. Each 10 µl reaction consisted of template cDNA, 1 x SensiMix™ SYBR® No Rox and 100 nM of each primer. The amplification conditions used consisted of an initial step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, primer annealing according to the specific primer melting temperatures for 15 sec and elongation at 72°C for 15 sec. The primer sequence, their optimum annealing temperatures and their preferred cycling conditions are listed in Table 2-2. All PCR reactions were performed in triplicate with three biological repeats for each sample.

Expression analysis was carried out using the Rotorgene 6000 software using pooled cDNA to generate a standard curve for the housekeeping gene and gene of interest. The housekeeping gene utilized was *UBP12 (AT5G06600; UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 12)* as its expression is constitutive and constant under the conditions examined (Czechowski et al, 2005). Melt curve analysis confirmed the presence of a single, gene-specific cDNA product. Standard curves for both the housekeeping gene and gene of interest had a correlation efficiency (R^2) of 0.989. The two standard curve quantification method was used to calculate relative gene expression.

2.4 DNA Manipulation

2.4.1 Genomic DNA Isolation from *Arabidopsis*

Genomic DNA was extracted from leaf samples based on the plant DNA mini-preparation technique (Dellaporta et al, 1983) with the following modifications: 100 mg of leaf tissue was homogenized in 1.5 mL extraction buffer (100 mM Tris-HCl (pH 8), 50 mM EDTA (pH 8), 500 mM NaCl, 10 mM β-mercaptoethanol) and then heated at 65°C for 10 min. The rest of the procedure was carried at 4°C unless otherwise stated. After the addition of 500 µl of 5 M potassium acetate, samples were shaken vigorously and incubated at 4°C for 20 min. Samples were then centrifuged for 20 min and the resulting supernatant transferred to 1 mL isopropanol. DNA was precipitated at -20°C for up to 2 h. Following centrifugation for 15 min, DNA pellets were dissolved in 700 µl re-suspension solution (50 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8)) and centrifuged again for 5 min. The resulting supernatant was mixed with 75µl of 3 M sodium acetate (pH 5.2) and 500 µl isopropanol, and placed on ice for 15 min.

The DNA was pelleted by centrifugation, washed with 80% ethanol and dissolved in 100 µl TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)) with RNase (2 µg/mL). All centrifugation steps were done at 14 000 rpm at 4°C unless otherwise stated. DNA was quantified by applying 2 µl of undiluted DNA directly onto the Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmigton, USA) and its absorbance at 260 nm determined.

2.4.2 Amplification of DNA

The sequence specific primers used in this study can be found in table 2.2 along with their sequence information, the optimal annealing temperature and the preferred cycling conditions for polymerase chain reaction (PCR) amplification. A template ranging from 100 - 200 ng of genomic DNA or cDNA was utilized in each reaction. A typical reaction consisted of 1 X thermophilic DNA polymerase PCR buffer (without $MgCl_2$), 0.2 µM of each primer, 0.2 mM dNTPs (Fermentas, Ontario, Canada) and an appropriate concentration of $MgCl_2$. A magnesium titration indicated that most primers worked optimally at 2.5 mM $MgCl_2$. All amplifications were performed with Taq DNA polymerase (Fermentas, Ontario, Canada). All PCR reactions were carried out in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA) for 35 cycles.

2.4.2.1 High fidelity PCR for cloning

Velocity DNA polymerase (Bioline Ltd., London, UK) was utilized to amplify up insert fragments with a low error rate for cloning purposes. These amplifications were prepared according to the conditions recommended in the manufacturer's protocol with the following cycling conditions: DNA was initially denatured at 98°C for 2 min followed by 35 cycles (98°C for 30 s, 60°C for 30 s and 72°C for 30 s/Kb) and a final elongation of 72°C for 4-10 min. In some cases touchdown PCR cycling conditions had to be implemented to reduce non-specific amplification of DNA. In this particular reaction the annealing temperature is lowered by 1°C every second cycle to a "touchdown" annealing temperature thereby enriching for the correct product.

Table 2-2: Primers and PCR conditions used in this study

Primer name	Primer sequence	Ta (°C)	Cycling conditions
Screening SALK lines			
LP30F	5'-CTGATCGCGAGATTTCAGTTC-3'	56	15 s denaturation, 30 s annealing, 1 min elongation
RP30R	5'-AGAAGATTGGAGCAAGCTTCC-3'		
LBB1.3R	5'-ATTTTGCCGATTTCGGAAC-3'		
003F	5'-ACCGAATATCCGCTTCGAT-3'		
Expression analysis			
IMPL2F	5'-GCCATATGGCTTCAAACTCAAAAC-3'	60	15 s denaturation, 30 s annealing, 1 min elongation
IMPL2R	5'-GCTCTAGATCAATGCCACTCAAGTGAC-3'		
Cloning – Standard high fidelity PCR amplification of *At4g11100*			
INSRT00F	5'- TGTGACTGCAGAAATTACGAA - 3'	55	15 s denaturation, 30 s annealing, 1 min 30 s elongation
INSRT00R	5'- TGCCACGGAACTTTCTTATTA - 3'		
Cloning – Touchdown PCR amplification of *At4g11100*			
GCBF	5'- GCGCGGATCCACCAATCGGTCCTAACACTT - 3'	61*	15 s denaturation, 30 s annealing, 1 min 30 s elongation*
GCNR	5'- GCGCGCGGCCGCCAGAACCTTAACACCAGCAG - 3'		
Isoform analysis			
At4g11100.1F	5'- CGATTACGTTATCATTAACCAAC - 3'	55	15 s denaturation, 30 s annealing, 30 s elongation
NCG002F	5'- ATTTCGATTTCCACAATCTG - 3'	55	
NCG00R	5'- TCTGTTGCATCTGAAATGTT - 3'	55	

Colony PCR			
pFGC5941F	5’ - GTTGCCATGTCCTACACG - 3’	55	15 s denaturation, 30 s annealing, 4 min elongation
pFGC5941R	5’ - TTTACAACGTGCACAACAGA - 3’		
Quantitative real-time PCR			
UBP12F	5’ - TGGAGTTGTCGAAGCAGCACACT - 3’	60	15 s denaturation, 15 s annealing, 15 s elongation
UBP12R	5’ - CTTGGGTTGCTGGGAATAGC - 3’		
At4g11100.1F	5’ - CGATTACGTTATCATTAACCAAC - 3’	54	15 s denaturation, 15 s annealing, 15 s elongation
At4g11100.1R	5’ - TAAACCCAAACCAGGATTG - 3’		

* Refer to paragraph *2.4.2.1* below

Optimal results were obtained when a starting temperature of 65°C was reduced in 1°C increments at every second cycle for 10 cycles until a "touchdown" temperature of 60°C was reached. Thereafter, the reaction proceeded as normal for the next 25 cycles.

2.4.2.2 Colony PCR

Colony PCR reactions were performed in the same manner as described in *2.4.2* with the only exception being the replacement of a purified DNA template by a single bacterial colony.

2.4.3 Electrophoresis of DNA

DNA samples were separated on a 1.5% (w/v) agarose gel (with 0.016 µg/mL ethidium bromide) alongside a 1 Kb size marker (O' Gene Ruler ™ DNA ladder, Fermentas, Ontario, Canada). The gels were electrophoresed in 1 X TAE buffer (40mM Tris, 1mM EDTA, 0.11% (v/v) glacial acetic acid) for 1.5 - 3 h. DNA was visualized on a long wavelength (365 nm) UV transilluminator to minimize damage to the DNA during the gel excision process.

2.4.4 Gel extraction of DNA

DNA was purified from the gel slice using the Wizard SV Gel and PCR Clean up system (Promega Corporation, Madison, USA) according to the manufacturer's protocol.

2.4.5 DNA Sequencing and Analysis

DNA was sequenced on a ABI3730xl DNA analyser (Applied Biosystems, Foster City, USA) at the Central Analytical Facility (Stellenbosch, South Africa) or at Macrogen (Seoul, South Korea). Sequenced data (Table 2.3) was analysed using DNAMAN software (Version 4.13, Lynnon BioSoft, Quebec, Canada) and Chromas software (Version 2.01, Technelysium Pty Ltd, Queensland, Australia).

Table 2-3: Sequencing primers used in this study

Primer name	Primer sequence
Sequencing of *AT4G11100*	
00F	5'- AGGACAAATCAGAGAAGAAGATG - 3'
002F	5'- GGCAGGAACAGAGGAAACT - 3'
002R	5'- CTCTTTCTTCTTCCCCAACA - 3'
Vector specific primers	
GW1 (pCR8)	5' - GTTGCAACAAATTGATGAGCAATGC - 3'
GW2 (pCR8)	5' - GTTGCAACAAATTGATGAGCAATTA - 3'

2.4.6 Cloning Techniques

2.4.6.1 Blunt ending of DNA

Approximately 3-4 µg of DNA were blunt ended using 5 U of T4 DNA polymerase (Fermentas International Inc., Ontario, Canada) in 1 X Tango buffer (Fermentas International Inc., Ontario, Canada) with 2mM of each dNTP. The reaction mix was incubated at room temperature for 25 min followed by heat inactivation of the enzyme at 70°C for 20 min.

2.4.6.2 Ligation of DNA

Vector::insert ratio's ranging from 1:1 to 1:3 were used to determine the optimal ligation conditions. The amount of insert required was calculated according to the equation described below:

$$\frac{\text{Amount of vector (ng) X size of insert (Kb)}}{\text{Size of vector (Kb)}} \text{ X insert:vector molar ratio = Amount of insert (ng)}$$

A typical ligation reaction consisted of insert and vector DNA in a 1 X T4 DNA Ligase buffer (Fermentas International Inc., Ontario, Canada) or a 1 X Rapid Ligation Buffer (Promega Corporation, Madison, USA) with 3 – 5 U of T4 DNA Ligase. In each case the ligation was performed according to the manufacturer's protocol and incubated overnight at 4 °C.

2.4.6.3 TA cloning

To add the 3' A-overhangs necessary for TOPO TA cloning® (Invitrogen, Carlsbad, USA), gel purified PCR product was A-tailed for 30 min at 70°C in reaction consisting of 1 X thermophilic DNA polymerase PCR buffer, 0.2 µM dATP and 1 U of Taq DNA polymerase. The A-tailed product was then used in TOPO cloning reaction in which the Topoisomerase I activity of the pCR®8 (Invitrogen, Carlsbad, USA) entry vector facilitated the ligation of the PCR insert into the vector. The TOPO cloning reaction was carried out according to the manufacturer's instruction.

2.4.6.4 LR Gateway Cloning

The *att*L-containing pCR®8 facilitates the transfer of insert from the entry vector into a *att*R-containing destination vector through LR recombination. LR recombination was performed according to the manufacturer's protocol with the following modifications: To reduce background colonies the pCR8®::*At4g11100* entry vector was linearized with XbaI and the ratio of entry vector to destination vector used was 1:3. In addition, 1 µL of LR Clonase™ II enzyme was used and the reaction incubated at 25 °C overnight.

2.4.6.5 Transformation of competent E. coli cells

Competent cells of either the DH5α, XL1 or One Shot® strain of *E. coli* were thawed on ice before the addition of 50 – 100 ng of DNA. The mixture was left on ice for 30 min, heat shocked at 42°C for 45 s and snap cooled on ice for 2 min. Following the addition of 900 - 950 µl of LB media, cells were incubated at 37°C for 60 – 90 min with shaking. Approximately 100 µl of each transformation culture was plated in duplicate on LB agar with the appropriate antibiotics before being incubated overnight at 37°C.

2.4.6.6 Isolation of plasmid DNA

High quality plasmid DNA was isolated using the Wizard Plus DNA Purification System (Promega Corporation, Madison, US) according to the manufacturer's instructions.

2.4.6.7 Restriction endonuclease digestion of DNA

The digestion of plasmid DNA or PCR product typically required 5–10 U of restriction enzyme supplied by Fermentas International Inc. (Ontario, Canada). Enzyme reactions were carried out at 37°C for 2–4 h, unless otherwise stated, according to the manufacturers recommended conditions. In general, 1 U of enzyme per µg of DNA was utilized with an enzyme volume of no more than 10% of the total reaction volume.

2.4.6.8 Glycerol stocks

All positive transformants harbouring a vector of interest (Table 2-4) were incorporated into glycerol stocks for storage. In this procedure 680 µL of freshly grown overnight culture was added to 320 µL of pre-sterilized 50 % glycerol in a 1.5 mL eppendorf, flash frozen in liquid nitrogen and stored at -70°C.

2.5 *Arabidopsis* transformation

This protocol is based on the floral dip method of Clough and Bent (Clough & Bent, 1998).

2.5.1 Plant preparation

The first inflorescence shoots were removed to encourage the growth of more inflorescences. Plants were clipped several more times to obtain the maximum number of young flower heads with minimal silique formation. Additionally, plants were fertilized regularly with 0.14% (w/v) Phosphrogen (Bayer CropScience Group, Hertfordshire, UK) to maintain healthy growth.

Table 2-4: Vectors used in this study

Vector	Resistance	Transformation	Origin
pFGC5941	Kanamycin (50 µg/mL) Glufosinate ammonium	*E .coli* XL1 blue	*Arabidopsis* Biological Resource Centre (http://abrc.osu.edu/)
p7LUC	Ampicillin (100 µg/mL)	n/a	(Murray et al, 2002)
pGEM®-T Easy	Ampicillin (100 µg/mL)	*E. coli* DH5α	(Promega Corporation, Madison, USA)
pCR®8/GW/TOPO®	Spectinomycin (100 µg/mL)	*E. coli* One Shot®	(Invitrogen, Carlsbad, USA)
pFAST-G01	Streptomycin (50 µg/mL)	*E. coli* One Shot®	(Shimada et al, 2010)

2.5.2 Transformation of competent *A. tumefaciens* cells

DNA amounting to no more than 25% of the total volume of cells was added immediately to frozen *A. tumefaciens* competent cells of strain GV3101. These cells were heat shocked and thawed at 37°C for 5 min followed by the addition of 900 µl of LB media. The resulting transformation culture was incubated at 30°C for 6 h with shaking and plated in duplicate on LB agar with the appropriate antibiotics. The plates were incubated at 30°C for 2-3 days until the appearance of colonies. Successful transformants were identified through colony PCR and were incorporated into glycerol stocks.

2.5.3 Preparation of transformed *A. tumefaciens*

Successfully transformed *A. tumefaciens* were streaked onto selective LB agar and incubated at 30°C for 2 days. A single colony from this streak was cultured in 5 mL of LB with

the appropriate antibiotics for 2 days at 30°C. On the second day, the 5 mL culture was used to inoculate 500 mL of LB containing antibiotics which was then shaken at 30°C overnight. The cells were then harvested by centrifugation at 3500 X *g* for 15 min at room temperature. The resulting pellet was resuspended in a 250 mL solution of 5% (w/v) sucrose containing 0.05% (v/v) silwett L-77 surfactant (Lehle Seeds, Round Rock, USA).

2.5.4 Floral dip

Plants were inverted and their inflorescence submerged in the *A. tumefaciens* suspension for 5 s (Clough & Bent, 1998). Plants were then placed on their side in tissue-paper lined trays, covered with clingfilm and left overnight. The following day plants were placed upright and were watered from below to prevent loss of the *A. tumefaciens* (Clough & Bent, 1998).

2.5.5 Isolation of positive plant transformants

T_1 seeds harvested from pFGC5941::*PR-1::LUC* transgenic plant lines were hydrated in 0.1% (w/v) agar, sown evenly in the same pot of soil and left to grow under standard conditions. After 5 days the seedlings were sprayed with 0.015% (w/v) Basta (glufosinate ammonium) which was repeated twice more with 2-3 day intervals. Healthy plants which could be clearly distinguished from moribund seedlings were transferred to new pots.

T_1 seed collected from pFAST-G01::*AT4G11100* and pFAST-G01 transgenic plant lines were visualized under a Nikon Epifluorescent microscope (Nikon corporation, Tokyo, Japan) following excitation by blue light (450-490nm). A barrier filter (520nm) was used to separate fluorescence from background light. The pFAST-G01 vector contains a fusion construct where the GFP gene is under the control of the *OLE1* promoter, the most abundant oleosin in *Arabidopsis* seed (Shimada et al, 2008). For this reason, successfully transformed seed emitting green fluorescence were carefully selected using a wet toothpick.

2.5.6 Luciferase imaging of *Arabidopsis*

Four week old plants were pre-sprayed with a luciferin solution (1 mM luciferin (Promega Corporation, Madison, USA), 0.1% Triton X-100) twice a day, two days before imaging to reduce the background activity of accumulated luciferase (Millar et al, 1992). Plants were sprayed once more before imaging in the Xenogen Lumina in vivo Imaging System (Caliper Life Science, MA, USA). Bioluminescence was captured for 30 s using the GFP emission filter to avoid saturated pixels on the resulting image. Living Image v4.1 software (Caliper Life Sciences, MA, USA) was used to determine the counts per cm^2 by dividing the total bioluminescence counts by the measurable area. The measured area was kept the same for each plant and for each line.

2.6 Western Blotting

2.6.1 Total protein isolation from *Arabidopsis*

Total protein was isolated from leaf tissue as described by Ingle et al. (2005), with the following modifications: Leaf tissue was macerated in 400 µl of ice cold extraction buffer (0.5 M Tris–HCl, pH 7.5, 10 mM EDTA, 1% (v/v) Triton X-100, 2% (v/v) β-mercaptoethanol) to concentrate the protein. Following the Bradford assay, 40 mg of each protein sample was boiled in 5 x SDS sample application buffer (100 mM Tris-HCl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol-blue) for 10 min before being loaded onto the SDS-PAGE gel.

2.6.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on 12 or 15% (v/v) polyacrylamide gels prepared from a 40% (w/v) acrylamide/bisacrylamide (29:1 v/v) stock solution (Sigma-Aldrich Inc., St Louis, USA) using the Mini-PROREAN® 3 system (BioRad Laboratories, Inc. Hercules, USA). The separating gel consisted of 375 mM Tris-HCl (pH 8.8), 0.2% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS) and 0.05% (v/v) TEMED. It was overlaid with a stacking gel

comprised of 4% (v/v) acrylamide/bisacrylamide, 125 mM Tris-HCl (pH 6.8), 0.2% (w/v) SDS, 0.09% (w/v) APS and 0.1% (v/v) TEMED. Denatured protein samples and a pre-stained molecular weight marker (PageRuler™ Prestained Protein Ladder; Fermentas lnternational Inc., Ontario, Canada) were electrophoresed in a running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS) at 60 V for 30 min. Thereafter, the voltage was increased to its maximum (200 V) until the dye front had run off the gel.

2.6.3 Western Blot Analysis

Proteins were transferred at 15 V overnight onto nitrocellulose membranes (Schleicher and Schuell BioScience, Dassal, Germany) using the Mini Trans-Blot® Cell blotting apparatus (BioRad Laboratories, Inc. Hercules, USA) as per manufacturer's instructions. The transfer buffer used consisted of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Membranes were stained with Ponceau S (0.1% (w/v) Ponceau S in 5% acetic acid) for 10 min at room temperature and washed with dH_2O to obtain a clear background. Membranes were imaged prior to western blotting to determine if samples were equally transferred.

2.6.3.1 PR-1 blot incubation conditions

Membranes were blocked overnight at 4°C in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 8) containing 10% (w/v) skim milk powder. Thereafter, the PR-1 primary antibody was added as a 1:1000 dilution in TBS-T buffer with 10% (w/v) milk powder and incubated with gentle agitation for 2 h at room temperature. The membranes were washed 3 times for 10 min each with TBS-T buffer at room temperature. The primary antibody-antigen conjugates were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG (a gift from Nicola Illing, MCB Department, UCT) secondary antibody diluted 1:5000 in TBS-T buffer containing 10% (w/v) milk powder. The secondary antibody incubation period occurred at room temperature for 1 h followed by the membrane wash described before.

2.6.3.2 EDS1 blot incubation conditions

Membranes were blocked for 2 h at room temperature in TBS-T buffer containing 2% (w/v) skim milk powder. Primary EDS1 antibody was added as a 1:400 dilution in TBS-T buffer with 2% (w/v) milk powder and incubated with gentle agitation overnight at 4°C. Membranes were washed and incubated with secondary antibody as described above with the following modification: the secondary antibody was diluted in TBS-T buffer with 2% (w/v) skim milk powder.

2.6.4 Chemiluminescence detection

A homemade ECL assay was employed to detect chemiluminescence as described by Durrant and Fowler (1994). Detection was performed by exposing the membrane to photographic film for 1-5 min (Biomax light film; Kodak, USA).

2.7 Statistical analyses

One way ANOVA analyses of all data were carried out using Statistica 10 (StatSoft. Inc., USA) to determine if the plant host's genotype affects the bacterial titre/lesion area. Raw data were transformed prior to analysis using natural log values for bacterial titres (Figures 3-1, 3-6, 3-7, 3-12, 3-14, and Supplementary Figures 1 and 2) and square root values for the lesion area (Figure 3-2) to ensure homogeneity of variance and normality of error.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Pathogen susceptibility analysis of the *cir1* mutant

The *cir1* mutant has been identified as a constitutive expresser of both SA and JA/ET-dependent defence genes including *PR-1* and *PDF1.2* (Murray et al, 2002). Moreover, the mutant exhibits increased resistance against virulent microbial pathogens such as *Pst* DC3000 and *H. parasitica* Noco2 (Murray et al, 2002). To further investigate the pathogen resistance profile of *cir1*, the mutant along with the wild-type Col-0 and *PR-1::LUC* (the genetic background for *cir1*) plants were tested for resistance to the biotrophic pathogen *P. syringae* and the necrotrophic *B. cinerea*.

In the *P. syringae* assay, bacterial growth was quantified in response to both the avirulent *P. syringae* pv. *tomato* AvrB (*Pst* AvrB) and the virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) strains. It has been previously determined that *P.syringae* growth reaches a maximum value three days following infiltration with a bacterial suspension of OD600=0.002 (Whalen et al. 1991). However, in these experiments it was empirically determined that the *cir1* disease resistance phenotype is most evident 48 h post-inoculation (Figure 3-1 A and B).

Resistance to *P. syringae* is largely mediated by SA-dependent responses; although, the extent to which bacterial titres were reduced differed between the isogenic strains. While the *cir1* mutant displayed a significantly decreased susceptibility to both *Pst* AvrB and *Pst* DC3000 in comparison to the control lines 48 h post inoculation, the fold difference observed in growth between *cir1* and Col-0 was much greater in response to *Pst* AvrB (10-fold difference) than *Pst* DC3000 (Figure 3-1). Based on its distinct resistance profile, avirulent *Pst* AvrB was employed for further disease severity analyses.

In the *B. cinerea* assay, single excised leaves from 15 different plants were drop inoculated with a *B. cinerea* spore suspension and lesion development was used as an indication of that particular plant line's susceptibility to the pathogen (Ferrari et al, 2003; Govrin & Levine, 2000; Denby et al, 2004). Leaves drop-inoculated with half strength grape

juice without the spore suspension displayed no lesion development (results not shown). Lesion development in *cir1* leaves were found to be significantly lower than that of the wild-type Col-0 line 3 days post-inoculation (Figure 3-2). However, no significant difference was observed between *cir1* and its background line *PR-1::LUC*, therefore this assay was found to be unsuitable for further disease severity analyses of the candidate *CIR1* genes.

3.2 Identification of T-DNA insertion lines for each candidate gene

A considerable amount of effort has gone into characterising the *cir1* mutant (Murray et al. 2002; Carstens 2008). This has highlighted several defining features of the mutant in comparison to wild-type *Arabidopsis,* such as elevated expression of certain defence genes, including *PR-1* and *EDS1*, both in uninfected *cir1* plants and following pathogen attack relative to Col-0 plants (Carstens, 2008; Murray et al, 2002). These properties combined with the mutant's resistance capabilities to both avirulent and virulent *P. syringae* are consistent with a role for *CIR1* in PTI and ETI. The *CIR1* gene has been mapped to a region of chromosome IV containing eight protein-coding genes (Carstens 2008). In order to determine which one of these genes encodes *CIR1*, homozygous T-DNA insertion mutants for all eight candidate genes were analysed to determine whether any of them phenocopied the *cir1* mutant. During this investigation the Salk Institute Genomic Analysis Laboratory database (http://signal.salk.edu/cgi-bin/tdnaexpress) was queried to identify T-DNA insertion mutant(s) for each individual candidate gene (Alonso et al, 2003). Lines in which the T-DNA disrupted the promoter or coding region were preferred and up to two lines were chosen for further analyses (Figure 3-3).

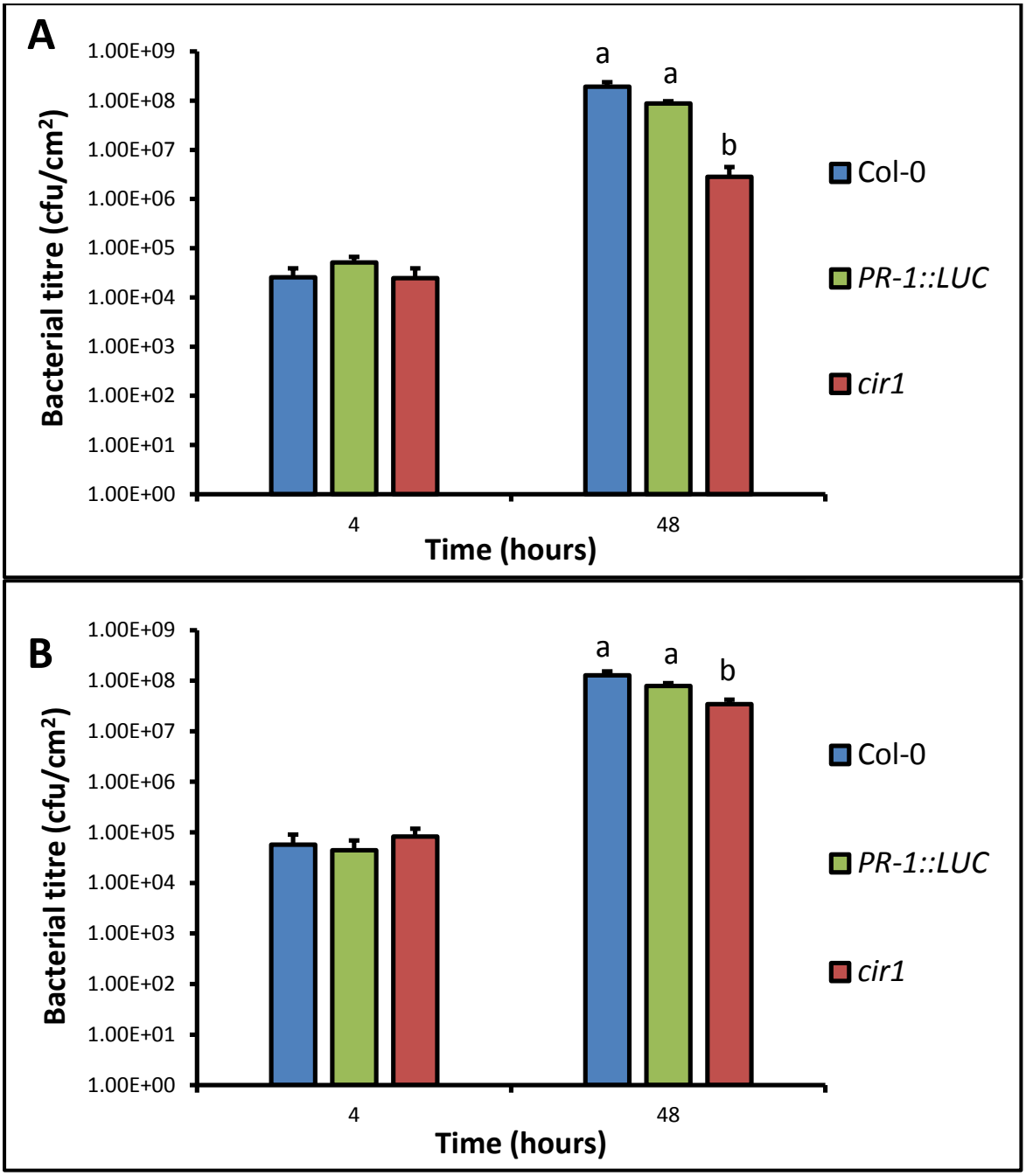

Figure 3-1: Susceptibility analysis of the *cir1* mutant to infection by *P. syringae*. Four to five week old soil grown Col-0 (negative control), *PR-1::LUC* (negative control) and *cir1* plant lines were assayed in response to avirulent *Pst* AvrB (A) and virulent *Pst* DC3000 (B). Bars represent the average bacterial titre line at 4 h and 48 h post-inoculation (hpi). Error bars represent the standard error of the mean (n = 5). Bacterial titre is defined as colony forming units (c.f.u.) per cm^2. One way ANOVA anaylsis revealed a significant effect of host genotype on bacterial titres 48 hpi for both assay (A) $p < 0.001$ and (B) $p = 0.004$. Letters denote significant ($P < 0.05$) differences in mean bacterial titres between the different lines as determined by Fisher's LSD (Least significant difference) post hoc analysis. No significant difference in bacterial titres were noted between lines at 4 hpi for both (A) $p = 0.718$ and (B) $p = 0.291$.

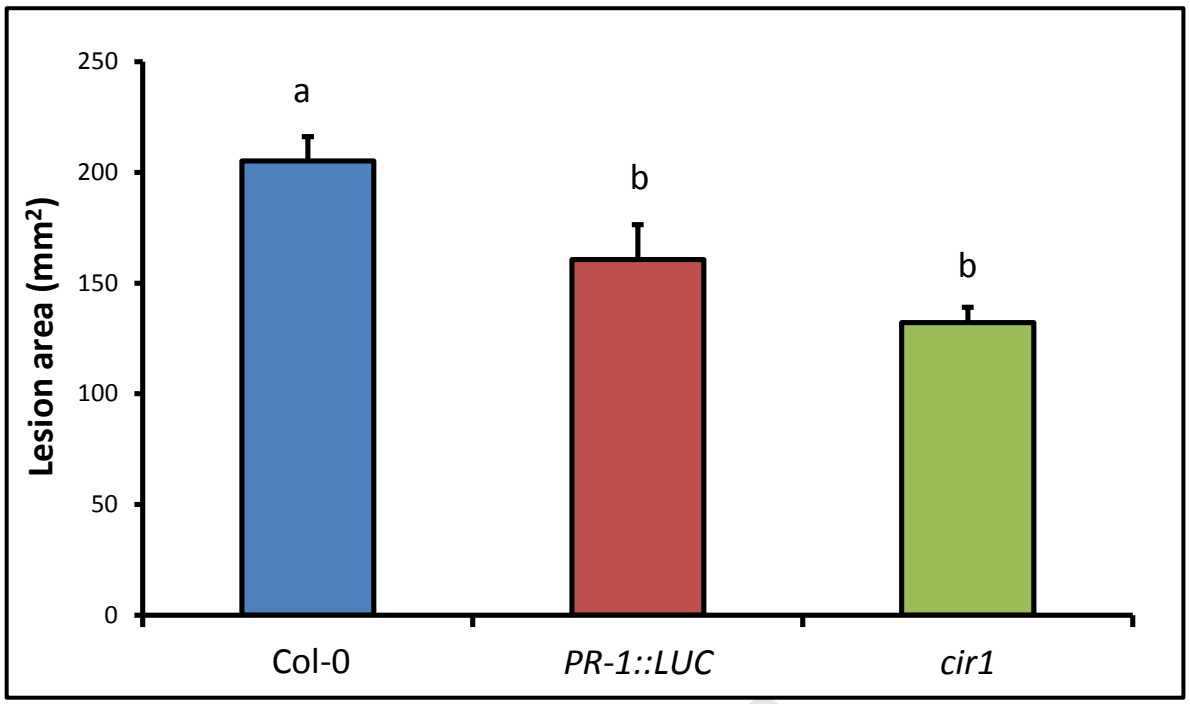

Figure 3-2: *Botrytis cinerea* detached leaf infection assay of *cir1*. Four to five week old soil grown *cir1*, Col-0 and *PR-1::LUC* leaves were drop inoculated with *B. cinerea* spores. Lesion area (mm^2) was measured 3 days post inoculation. The values represent the average lesion area calculated from three separate experiments. Error bars represent the standard error of the mean (n = 15). One way ANOVA analysis revealed a significant effect of host genotype ($p < 0.001$) on lesion area. Letters denote significant ($P < 0.05$) differences in lesion sizes between the different lines as determined by Fisher's LSD (Least significant difference) post hoc analysis. Data shown are representative of an experiment performed three times.

3.3 Isolation of a homozygous *at4g11130* SALK line

Homozygous T-DNA insertion lines were available for all of the candidate genes with the exception of *At4g11130*. For this reason, it was necessary to subject the segregating T_3 *SALK_059661* seed obtained from NASC to successive rounds of selection for the T-DNA insertion, to identify homozygous individuals. Screening for a selectable marker associated with the T-DNA insertion such as the *NPT II* (Kanamycin resistance) gene was not recommended due to the potential silencing of the gene after several generations of growth (Østergaard & Yanofsky, 2004). Instead, a PCR screening method was utilized with primers designed to discriminate between mutant and wild-type *At4g11130* alleles (Østergaard & Yanofsky, 2004). Primers used to amplify the wild-type allele were LP30 forward and RP30 reverse from Table 2-2. The mutant allele was amplified through the use of the RP30 primer and a T-DNA specific forward primer LBB1.3 (Table 2-2). The screening process depicted in Figure 3-4 involved two separate PCRs which facilitated the identification of homozygous wild-type, homozygous mutant or heterozygous individuals in the segregating population. To bulk up on seed, segregating T_3 *SALK_059661* seed was sewn onto soil and allowed to self-fertilize. Seed (T_4 generation) was harvested separately from each parental line. An initial screen of individuals from the T_4 progeny revealed two lines which were potentially homozygous for the T-DNA insertion (Figure 3-5A). One of these lines was selected for further analysis. Additional PCR screening confirmed homozygosity in the T_5 progeny of the selected line (Figure 3-5B).

3.4 Susceptibility analyses of T-DNA insertion lines to infection

To identify any candidates which phenocopy the *cir1* mutant, homozygous T-DNA insertion lines were analysed for enhanced resistance against the virulent *Pst* DC3000 and avirulent *Pst* AvrB strains. A simultaneous large scale assay on all the T-DNA insertion lines, including the appropriate controls, to both strains of *P. syringae* was not considered feasible. Instead, each line and its corresponding controls were assayed separately.

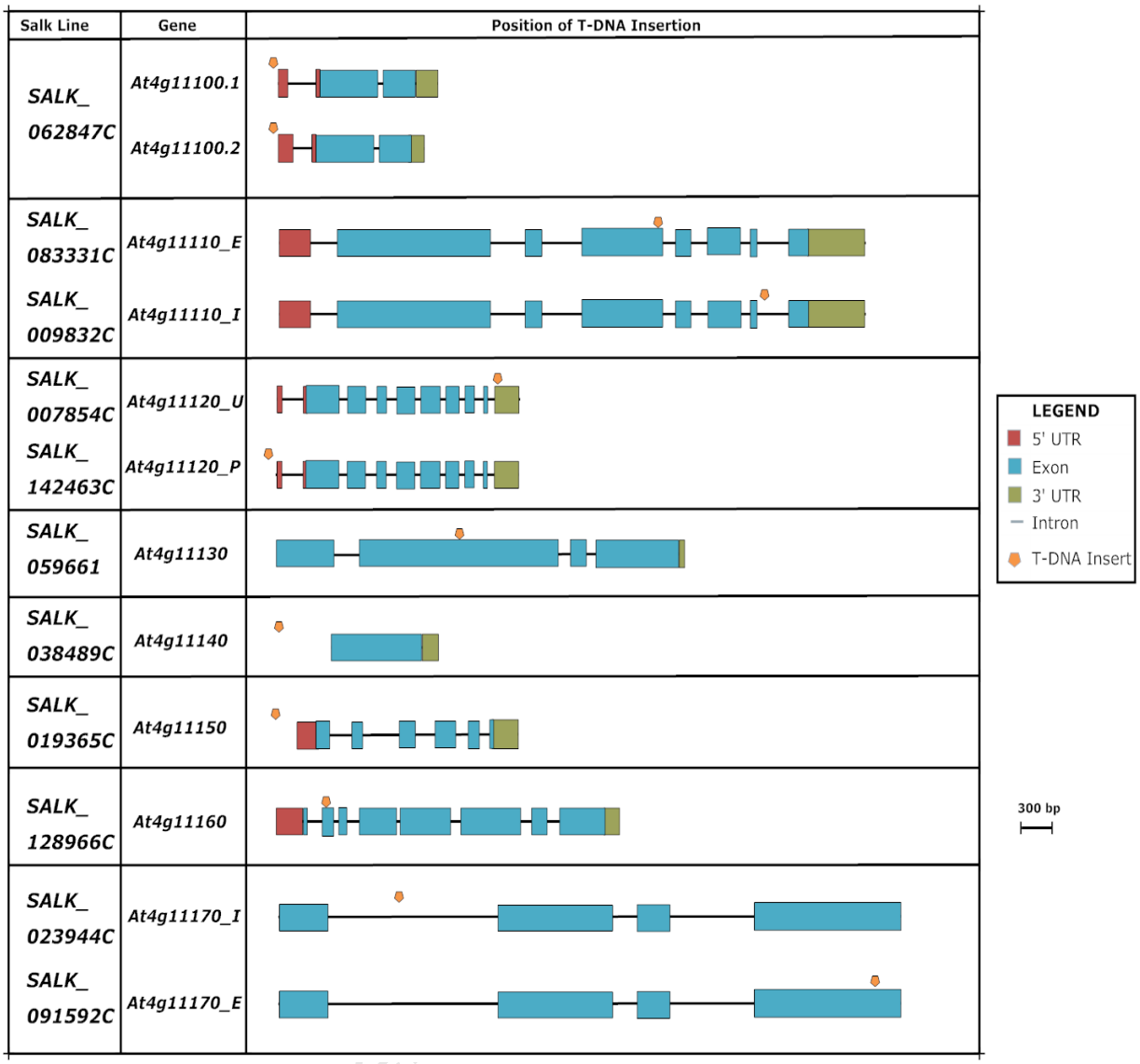

Figure 3-3: Gene structure of the *CIR1* candidate genes. The annotated sequence was obtained from TAIR (www.*Arabidopsis*.org) and used to design a schematic representation of each candidate gene. For each line obtained the approximate location of the T-DNA insertion is displayed, as determined from T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). For genes with more than one SALK T-DNA insertion line an additional letter denotes the predicted location of the insert whether it be in the (E) exon, (I) intron, (P) promoter or (U) 3' UTR.

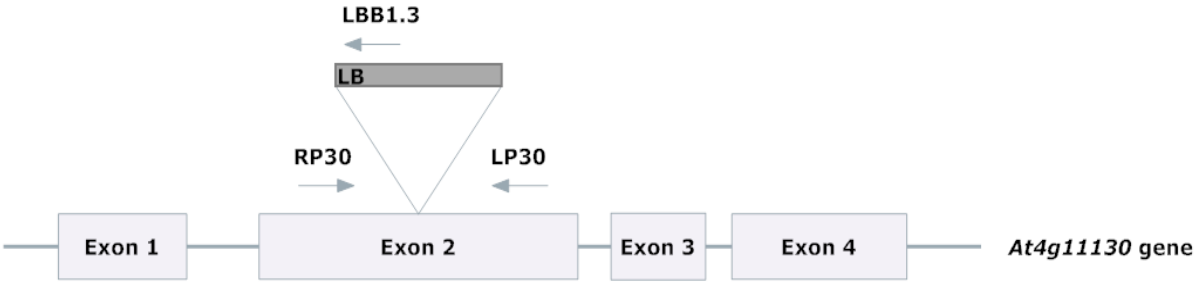

Figure 3-4: Primer design for the PCR based screening method used to identify homozygous *at4g11130* mutant lines. The predicted site of the T-DNA insertion is within exon 2 of *At4g11130* according to T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). Exons are indicated by boxes while primers and the direction of their amplification are displayed as arrows. The T-DNA insert, denoted by its left border (LB) and right border (RB), runs in the opposite direction to the gene and is flanked by primers RP30 and LP30, designed to amplify the wild-type copy of the *At4g11130* gene. The presence of the mutant allele is detected when a PCR containing the LBB1.3 reverse and RP30 primers results in the amplification of a product.

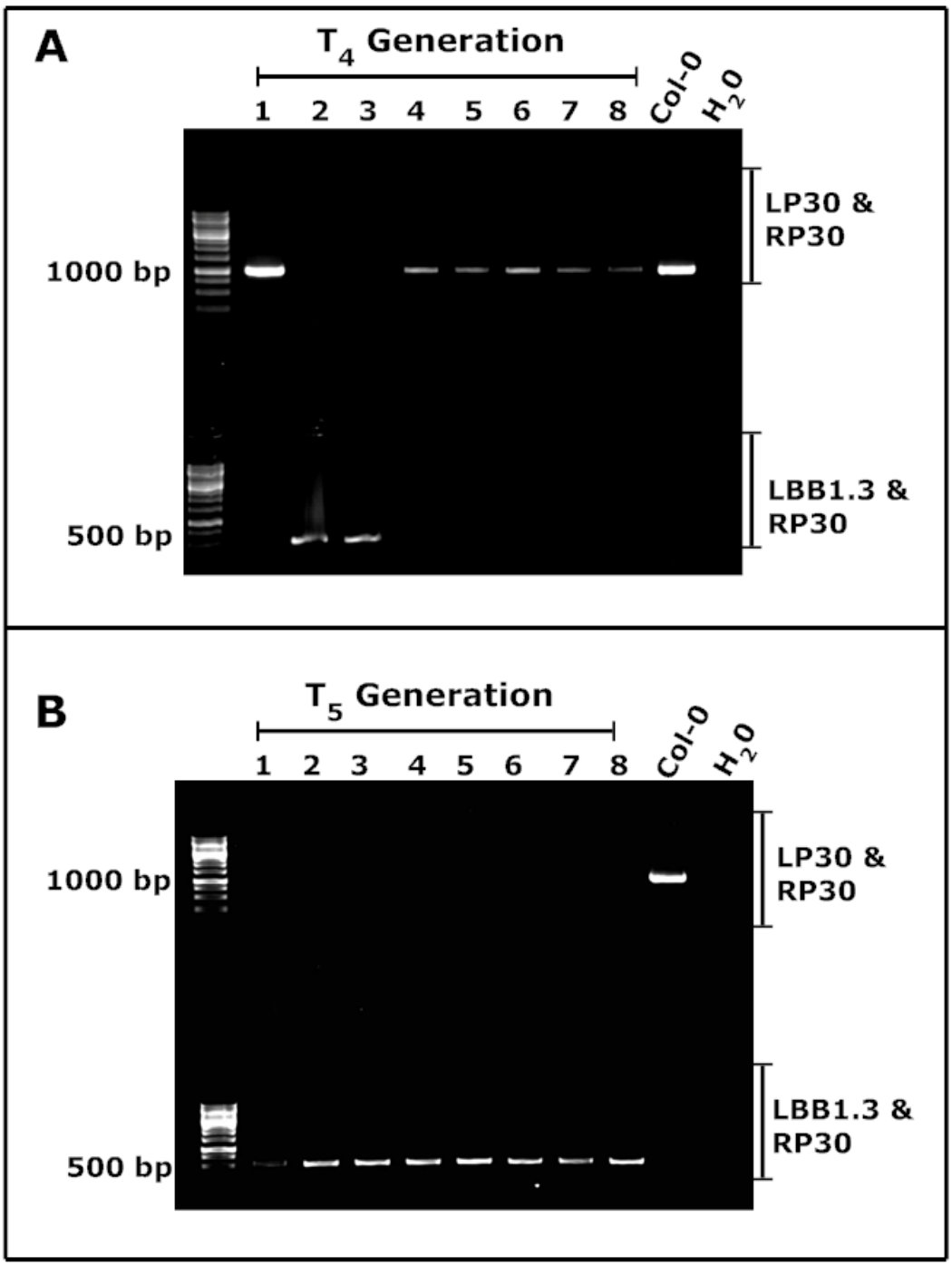

Figure 3-5: PCR screening for homozygous *At4g11130* T-DNA insertion mutants. Individuals from the T_4 generation (A) were screened for homozygous *at4g11130* T-DNA insertion mutants. Samples 2 and 3 were identified as possible homozygous lines for the T-DNA insertion. The T_5 generation (B) attained from self-fertilization of sample 2 plants were screened once more to confirm homozygosity of the progeny. Col-0 genomic DNA was used as a positive control confirming that the gene-specific LP30 and RP30 primers were able to amplify product in wild-type individuals. Water was used as a negative control in all PCRs. A band of 1021 bp represents the wild type allele while a band of around 520 bp represents the mutant allele.

3.4.1 *AT4G11120*, *AT4G11130* and *AT4G11160* are unlikely candidates for *CIR1*

SALK_142463C plants were subjected to infection by avirulent (Figure 3-6A) and virulent (Figure 3-6B) strains of *P. syringae*. The *SALK_142463C* line is a homozygous mutant for the *at4g11120* gene and is defective in its role as a PUTATIVE ELONGATION FACTOR Ts (EF-Ts), (Benichou et al, 2003). The *cir1* mutant displayed a greater than 100-fold less growth of the *Pst* AvrB pathogen than both *PR-1::LUC* and Col-0 wild-type lines, 48 h post inoculation (Figure 3-6A). In the same assay the *at4g11120* mutant exhibited a bacterial titre which differed significantly from that of Col-0, but not from that of *PR-1::LUC*. Similar results were obtained in response to *Pst* DC3000 where the bacterial titre was observed to be approximately 10-fold lower in the *cir1* mutant than in both Col-0 wild-type and *at4g11120* mutant lines. The *PR-1::LUC* control was omitted in Figure 3-6B due to insufficient data points. In *Pst* DC3000 assays on the *SALK_128966C* line (Figure 3-7A), the *At4g11160* insertion mutant, a 100-fold difference between the *cir1* mutant and Col-0 wild-type line is once again evident. While the difference in bacterial titres between *cir1* and Col-0 was found to be significant, post hoc analyses revealed that the *at4g11160* mutant had a bacterial titre which was not significantly different from that of the *PR-1::LUC* and Col-0 wild-type control lines. Similarly, the putative *at4g11130* T-DNA insertion mutant displayed a bacterial titre which did not differ significantly from that of the wild-type control lines following *Pst* AvrB infection (Figure 3-7B). Furthermore, the *cir1* mutant had a significantly lower (>10-fold) level of growth in leaves of infected plants when compared to the *at4g11130* T-DNA insertion mutant and wild-type control lines. These results suggest that the *at4g11120*, *at4g11130* and *at4g11160* mutant genotypes have no effect on host resistance to infection and as such are unlikely candidates for *cir1*.

3.4.2 The variable nature of the *cir1* mutant

While assays were performed on all the T-DNA insertion lines, the variability associated with the *cir1* mutant control hindered the identification of any significant changes in bacterial titres. The variable nature of *cir1* has been highlighted before during characterisation studies on the mutant (Carstens, 2008).

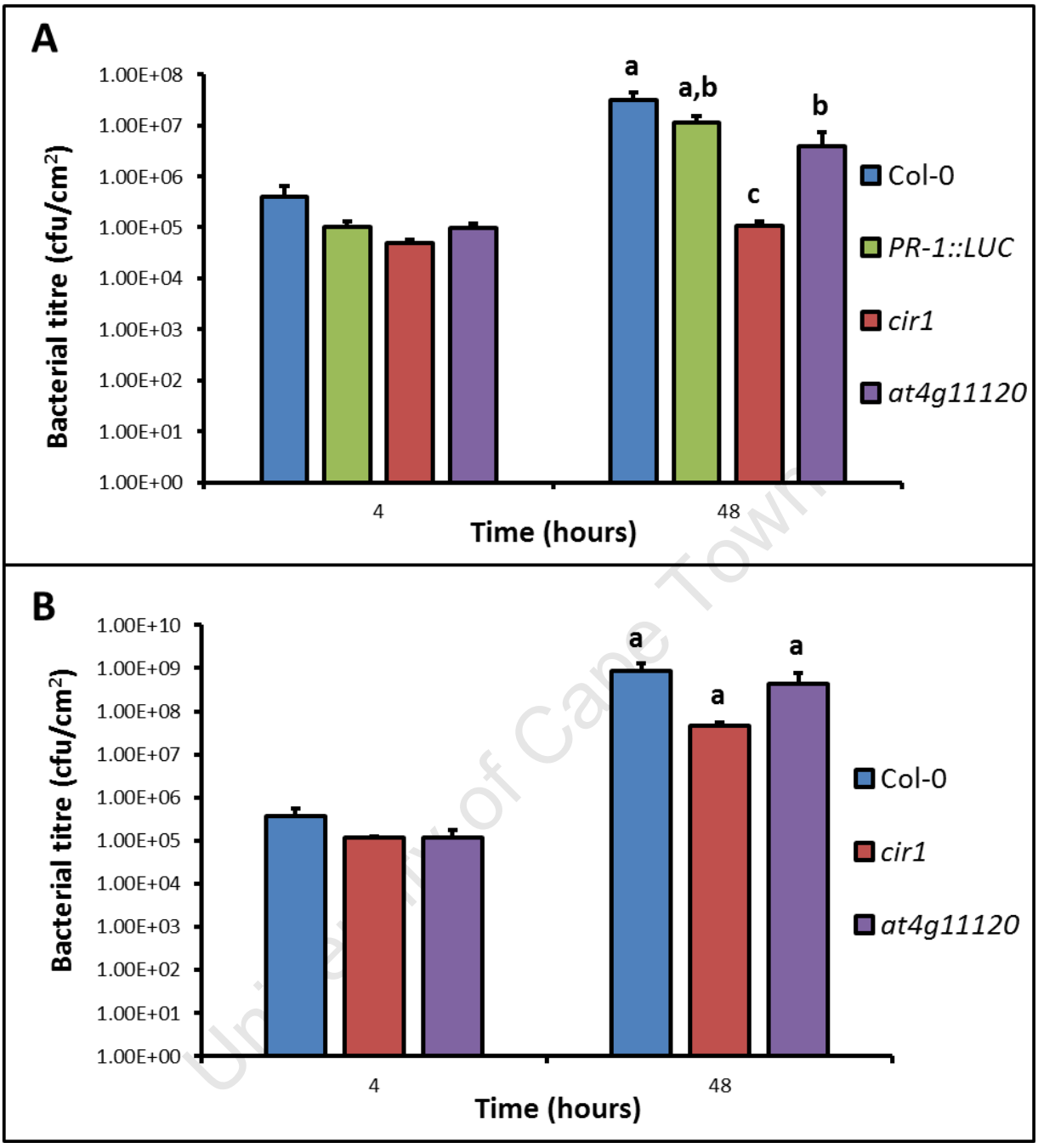

Figure 3-6: Susceptibility analysis of the *at4g11120* mutant to infection by *P. syringae.* Four week old soil grown Col-0 (negative control), *PR-1::LUC* (negative control), *cir1* (positive control), and *at4g11120* (*SALK_142463C*) plant lines were assayed in response to avirulent *Pst AvrB* (A) and virulent *Pst DC3000* (B). Bars represent the average bacterial titre line at 4 h and 48 h post-inoculation (hpi). Error bars represent the standard error of the mean (n = 3). Bacterial titre is defined as colony forming units (c.f.u.) per cm^2. One way ANOVA anaylsis revealed a significant effect of host genotype ($p = 0.001$) on bacterial titres at 48 hpi in (A). Letters denote significant ($P < 0.05$) differences in mean bacterial titres between the different lines as determined by Fisher's LSD (Least significant difference) post hoc analysis. No significant difference in bacterial titres were noted between lines at 48 hpi for (B) $p = 0.117$ and at 4 hpi for both (A) $p = 0.275$ and (B) $p = 0.263$).

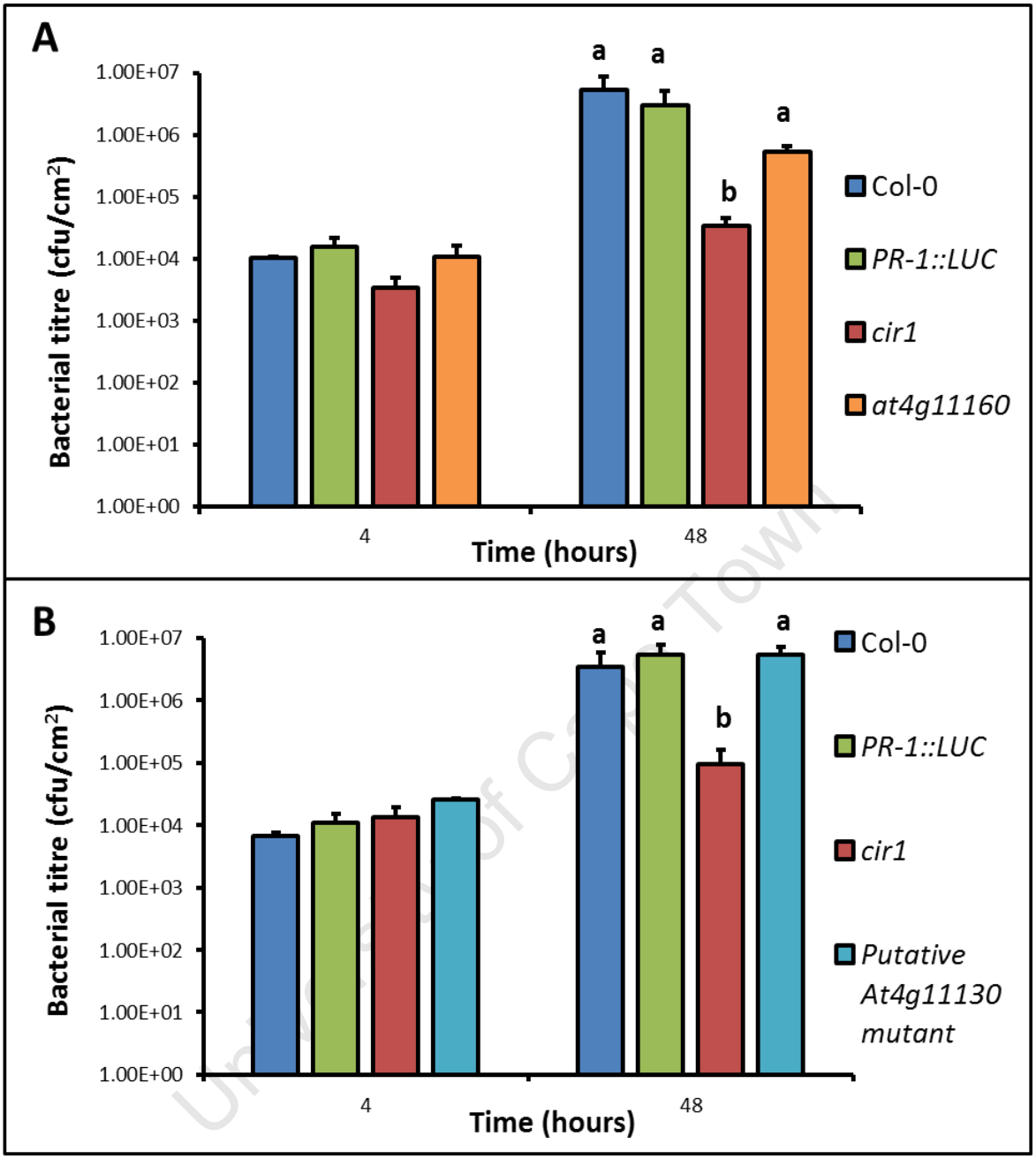

Figure 3-7: Susceptibility analysis of the *at4g11160 and at4g11130* mutant to infection by *P. syringae.* Four week old soil grown Col-0 (negative control), *PR-1::LUC* (negative control), *cir1* (positive control), *at4g11160* (*SALK_128966C*) and the putative *at4g11130* T-DNA insertion mutant plant lines were assayed. *at4g11160* lines were assayed in response to virulent *Pst DC3000* (A) while putative *at4g11130* T-DNA insertion mutant lines were assayed in response to avirulent *Pst AvrB* (B). Bars represent the average bacterial titre in three plants per line at 4h and 48 h post-inoculation. Error bars represent the standard error of the mean (n = 3). Bacterial titre is defined as colony forming units (c.f.u.) per cm^2. One way ANOVA anaylsis revealed a significant effect of host genotype on bacterial titres at 48 hpi in (A) p = 0.019 and (B) p = 0.002. Letters denote significant (P < 0.05) differences in mean bacterial titres between the different lines as determined by Fisher's LSD (Least significant difference) post hoc analysis. No significant difference in bacterial titres were noted between lines at 4 hpi for both (A) p = 0.070 and (B) p = 0.103).

Chemically induced mis-sense mutations, such as those produced by EMS, may result in mutants with conditional phenotypes. These phenotypes are either sensitive to changes in environmental conditions or dependent on the developmental stage of the mutant (Bowman et al, 1989). Furthermore, previous research has documented several constitutive defence mutants with the aforementioned conditional phenotype. For example, growth conditions associated with a high relative humidity suppress the enhanced disease resistance phenotype of the constitutive defence mutants *cpr22, ssi4* and *slh1*, presumably through its suppression of SA accumulation (Yoshioka et al, 2001; Zhou et al, 2004; Noutoshi et al, 2005). In these instances the *cir1* mutant did not differ significantly from the Col-0 and *PR-1::LUC* wild-type control lines (Supplementary Figures 1 and 2). As a result, no conclusive information could be obtained for the *at4g11110*, *at4g11140*, *at4g11150* and *at4g11170* mutants.

3.5 In-vivo analysis of *PR-1*::luciferase activity in T-DNA insertion lines

Due to the variability of the *cir1* pathogen susceptibility phenotype, a new approach had to be adopted to identify the putative candidate, based on the characteristic properties previously observed in the *cir1* mutant. Previous research has shown that the *cir1* mutation exhibits constitutive expression of the *PR-1::LUC* transgene resulting in high basal levels of luciferase activity (Murray et al, 2002). Moreover, Carstens (2008), based the selection of homozygous *cir1* mutants on the characteristically high luciferase levels obtained from the same transgenic construct. It can therefore be hypothesized that the transformation of each of the T-DNA insertion mutants with the *PR-1::LUC* transgene should produce knock-out lines with assayable luciferase activity. Such an assay could identify any knock-out lines with a similar *PR-1* expression profile to *cir1*, that being constitutive expression even in the absence of pathogen challenge (Murray et al, 2002).

3.5.1 Establishment of *PR-1::LUC* transgenic *Arabidopsis*

Construction of a suitable vector with a plant selection marker other than kanamycin was necessary as the T-DNA insertion mutant lines already possessed the *NPT II* marker. At

the time of this experiment it was not possible to obtain the original vector utilized by Murray et al. (2002). The pFGC5941 vector was therefore selected based on its kanamycin and BASTA selection markers into which the original *PR-1::LUC* transgene (maintained in p7LUC) would be ligated. Vectors p7LUC and pFGC5941 (Table 2-4) were linearized by their unique *Sac*I and *EcoR*I restriction sites respectively, blunt ended and digested with *XbaI*. As a result, the *PR-1::LUC::OCS* chimeric sequence was released from plasmid p7LUC as a 2.5 Kb fragment, which was later ligated into pFGC5941 replacing the endogenous CaMV35S promoter. Following transformation, a test digest was performed on the extracted plasmid using the *Sac*I and *Xba*I sites (Supplementary Figure 3). Following transformation into *A. tumefaciens* strain GV3101, a colony PCR was performed with the pFGC5941F and pFGC5941R primers (Table 2-2) to identify successful clones harbouring the *PR-1::LUC::OCS* (Supplementary Figure 4). Successful clones were later sequenced with the same primers to confirm the presence of the transgene and that no mutations had been introduced. Homozygous T-DNA insertion mutant lines were transformed with *A. tumefaciens* clones harbouring the *PR-1::LUC* transgene using the floral dip method described in 2.5. For each *PR-1::LUC* transformed T-DNA insertion mutant line, seed was collected from four plants resulting in four independent lines for the *PR-1::LUC* insertion. T_1 individuals which had survived the BASTA selection procedure were then imaged, in-vivo, and their LUC activity quantified. T_2 seed was harvested from these individuals. Transgenic *PR-1::LUC* lines could not be obtained for the *at4g11130* mutant.

3.5.2 LUC activity varies between, independently generated, transgenic *Arabidopsis* T_2 lines.

The *PR-1::LUC* transgene expression was evaluated through the in-vivo quantification of LUC activity. Transgenic T_1 plants were examined for high basal LUC activity similar to that observed in the *cir1* mutant. The LUC activity quantified in T_2 plants was found to be highly variable between independently generated transgenic lines, hampering the drawing of comparisons across different T-DNA insertion mutant lines (Figure 3-8). The mean LUC activity calculated from T_2 plants from each T-DNA insertion background was determined to be much higher than that observed in the original *PR-1::LUC (1)* plants obtained from

Murray et al. (2002). Furthermore, plants from the *PR-1::LUC (2)* transgenic line generated in this study displayed high basal LUC activity comparable to that of *cir1* (Figure 3-8). High basal LUC levels in transgenic plants, from a wild-type background, suggest that the reporter gene construct in these lines is not accurately reporting endogenous *PR-1* activity, unlike the original *PR-1::LUC* lines generated by Murray et al. (2002). This high degree of variability in transgene expression may be attributed to differences in its chromosomal location or the presence of multiple copies of transgene in the genome (Wakimoto, 1998; Wallrath, 1998). These issues can be remedied by performing additional experiments to detect single copy transgenic lines, to confirming homozygosity of the said single copy transgene and finally to determine the exact site of insertion.

While these experiments may seem trivial, isolating a single copy with a *PR-1::LUC* transgene of known location, for all 10 available T-DNA insertion mutant lines would be a huge undertaking. As a result no further tests were performed on these transgenic lines. Instead, emphasis was placed on finding a simpler, more feasible method of identifying any T-DNA insertion mutants with unusually high basal *PR-1* expression comparable to that of *cir1.*

3.6 *PR-1* protein accumulation in T-DNA insertion lines

Upon the successful acquisition of a PR-1 antibody from Dr Katherine Denby (University of Warwick), further investigation into constitutive expression of *PR-1* in the *cir1* mutant was undertaken. Based on previous studies investigating the *PR-1* expression profile of the *cir1* mutant (Murray et al, 2002), it can be theorized that constitutive *PR-1* mRNA levels would translate into a greater level of PR-1 protein accumulation. If this is the case in the *cir1* mutant, a putative candidate could be identified based on similar PR-1 protein accumulation properties. The results of the Western blot analysis investigating PR-1 protein accumulation are presented in (Figure 3-9). What is immediately evident in these results is the high basal accumulation of the PR-1 protein in *cir1* mutant. This level of accumulation was not observed in the wild-type Col-0 and *PR-1::LUC* controls, which is in agreement with previous research documenting the constitutive expression of endogenous *PR-1* in *cir1* (Murray et al, 2002; Carstens, 2008).

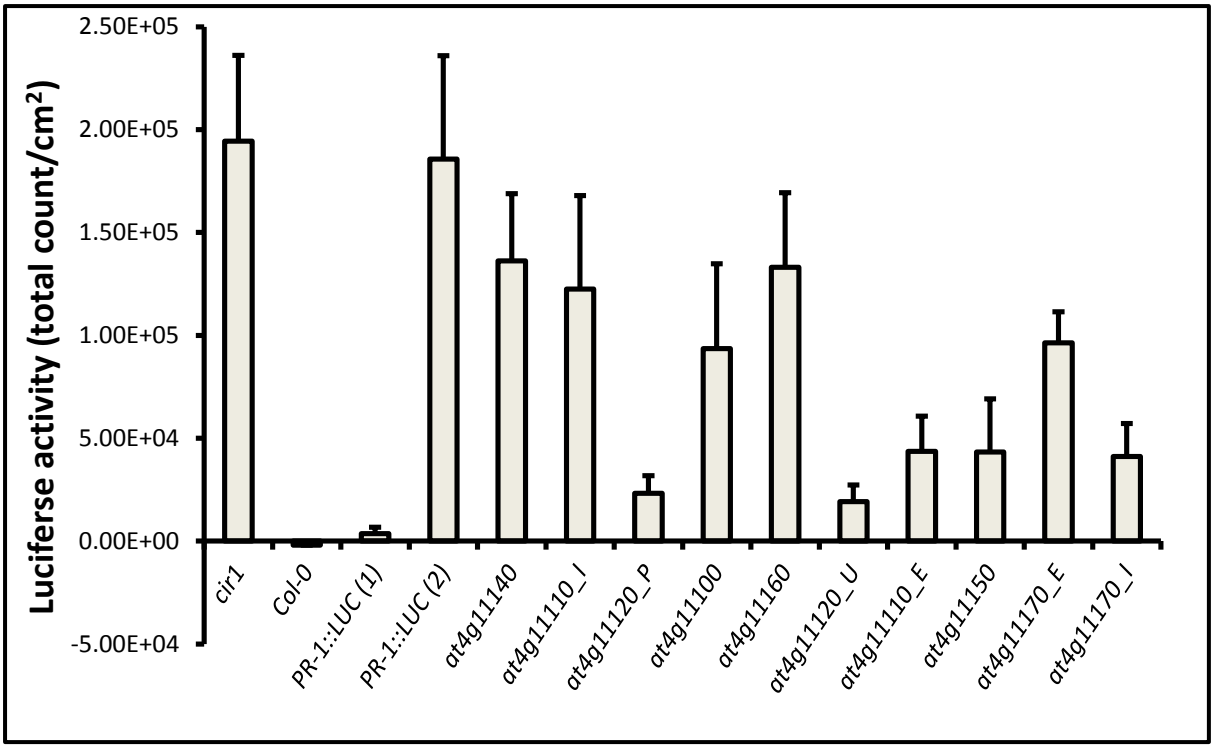

Figure 3-8: Highly variable LUC activity detected in T-DNA insertion mutant lines of *Arabidopsis* transformed with the *PR-1::LUC* transgene. Four week old T_1 *PR-1::LUC* T-DNA insertion mutant lines were imaged and their in vivo basal LUC activity measured. These lines were compared to the *cir1* mutant, Col-0 wild-type and original *PR-1::LUC (1)* wild-type controls. An additional *PR-1::LUC (2)* line was generated in this study as a wild-type control. During in-vivo image analysis each plant was held in a 2.07E+01 cm^2 pot. Total LUC activity was normalised to the area of the pot for each plant. Bars represent the average LUC activity obtained from four plants after normalization. Error bars represent the standard error of the mean.

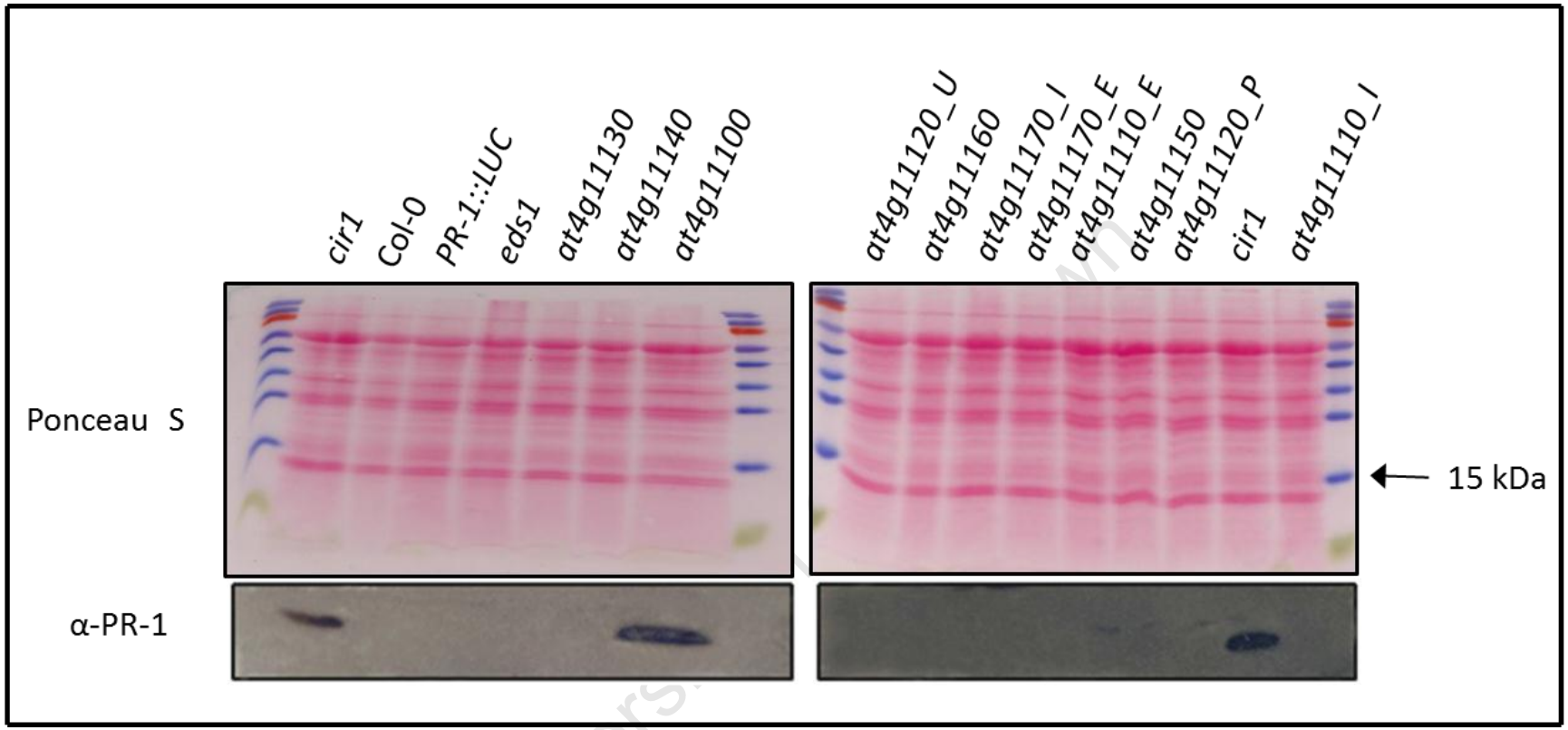

Figure 3-9: Western blot analysis investigating PR-1 protein accumulation in *cir1*, Col-0, *eds1*, *PR-1::LUC* and all the T-DNA insertion mutant lines. PR-1 protein accumulation was examined in *cir1* (positive control), Col-0 (Negative control), PR-1::LUC (negative control), *eds1* and all the T-DNA insertion mutants including the putative *at4g11130* mutant genotyped in this study. Due to the limited number of wells available two separate SDS-PAGE gels were utilized simultaneously. In each case a *cir1* control was included. Equal loading and total transfer of the protein was determined by Poncaeu S staining of the membrane. A band of 10 kDa, which corresponds to the predicted size of the PR-1 protein, was detected following the addition of PR-1 antiserum.

PR-1 accumulation in the *eds1* mutant was not observed. Furthermore, the high level of PR-1 accumulation noted in the *cir1* mutant was also observed in the mutant defective in *At4g11100* expression, but not in any of the other T-DNA insertion lines.

3.7 EDS1 protein accumulation in T-DNA insertion lines

Previous research has shown that *cir1* mediated resistance to biotrophic pathogens (i.e. *Pst* DC3000 and *Pst* AvrB) depends on functional EDS1 and PAD4 proteins (Carstens, 2008). Furthermore, a three times greater level of EDS1 protein accumulation in *cir1* was observed in both infected and uninfected plants, when compared to the Col-0 wild-type line (Carstens, 2008). To determine if EDS1 expression could be used as an additional marker of the *cir1* phenotype, both uninfected *cir1* and *at4g11100* mutants were examined in an EDS1 Western blot (antibody kindly provided by J. E. Parker, Max-Planck Institute for Plant Breeding Research). T-DNA insertion mutants were also analysed to identify any other mutants with altered EDS1 accumulation, not associated with high PR-1 accumulation under non-infection conditions. Western blot analysis reveals an EDS1 accumulation in both the *cir1* and *at4g11100* mutants not observed in the *PR-1*::LUC and Col-0 wild-type lines (Figure 3-10). A faint band was observed in the putative *at4g11130* mutant however the result was considered to be negligible as the level of EDS1 accumulation in this mutant was much lower than that of *cir1* and was not associated with an increased PR-1 protein accumulation. No accumulation of EDS1 was observed any of the other lines including the *eds1* mutant. The constitutive expression of *PR-1* in the *cir1* and *at4g11100* noted previously might therefore be attributed to constitutive *EDS1* expression in uninfected plants. This is consistent with previous findings which has shown that EDS1 acts upstream of SA-dependent *PR-1* mRNA accumulation (Falk et al, 1999).

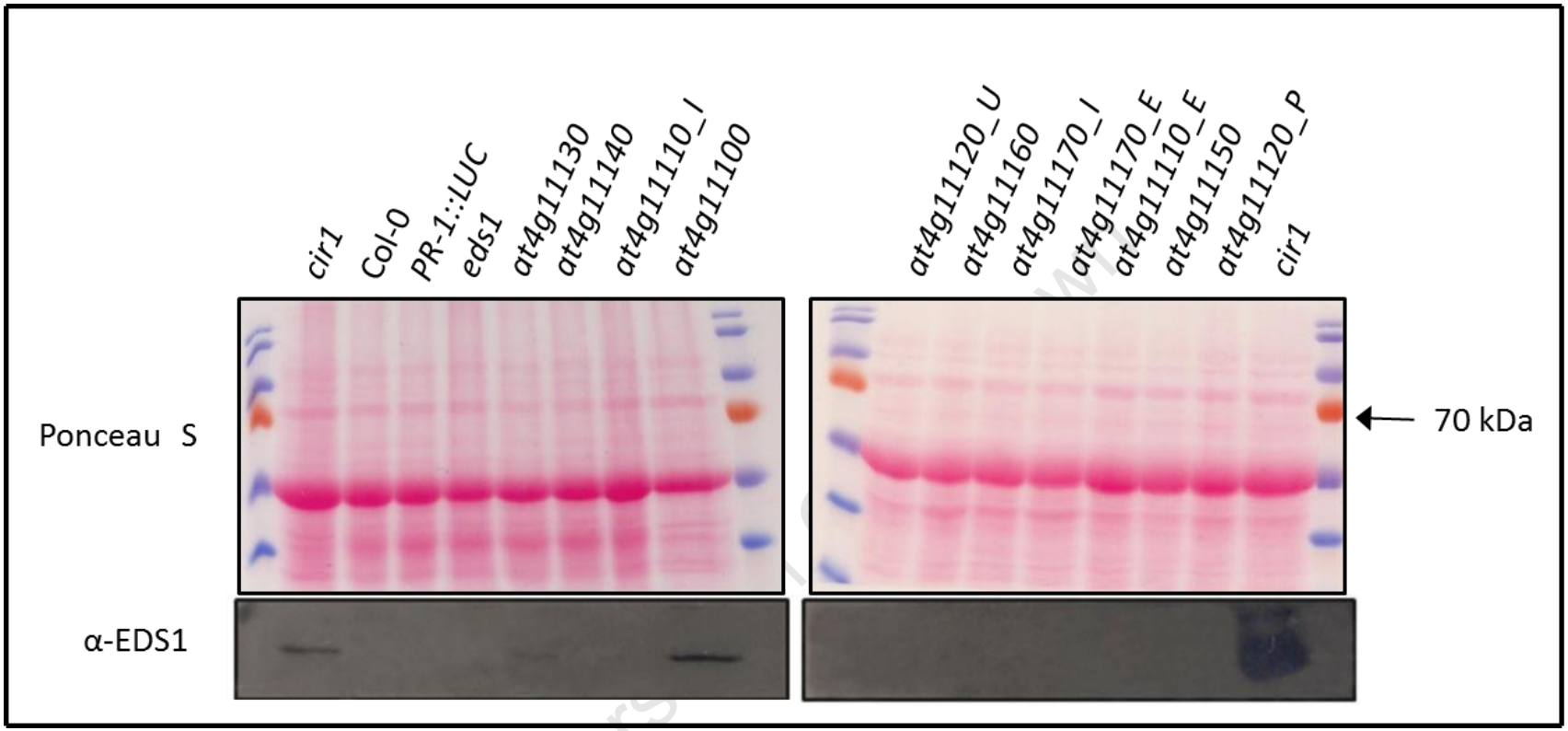

Figure 3-10: Western blot analysis investigating EDS1 protein accumulation in *cir1*, Col-0, *eds1*, *PR-1::LUC* and all the T-DNA insertion mutant lines. EDS1 protein accumulation was examined in *cir1* (positive control), Col-0 (Negative control), *eds1* (negative control), *PR-1::LUC* (negative control) and all the T-DNA insertion mutants including the putative *at4g11130* mutant genotyped in this study. Due to the limited number of wells available two separate SDS-PAGE gels were utilized simultaneously. In each case a *cir1* control was included. Equal loading and total transfer of the protein was determined by Poncaeu S staining of the membrane. A band of 70 kDa, which corresponds to the predicted size of the EDS1 protein, was detected following the addition of EDS1 antiserum

3.8 Characterization of the *at4g11100* T-DNA insertion mutant

Given that the *at4g11100* phenocopies the *cir1* mutant in terms of constitutive accumulation of PR-1 and EDS1 proteins, confirmation of the T-DNA insertion site, and whether the line was a true knockout was required. Sequencing results from a fragment amplified using the T-DNA specific LBB1.3 primer and the sequence specific 003F (Table 2-2) confirmed that the insert was in the promoter region 12 bp upstream of the 5' UTR, a location matching that predicted by TAIR.

Further investigation into *AT4G11100* on the TAIR database revealed two splice variants (Figure 3-11) associated with the gene. Primers specific to *AT4G11100.1* (NCG00F and NCG00R) and *AT4G11100.2* (NCG002F and NCG00R) isoforms were designed to amplify the full transcript from the *at4g11100* mutant, and the two wild-type controls *PR-1::LUC* and Col-0 (Figure 3-11). Expression analysis revealed that the *at4g111000* T-DNA insertion mutant was a true null mutant for both splice variants as no gene expression was observed in the *at4g11100* mutant when compared to the wild-type *PR-1::LUC* and Col-0 lines.

3.8.1 Susceptibility analyses of the *at4g11100* mutant

While the *at4g11100* mutant demonstrated high basal PR-1 and EDS1 protein levels similar to that observed in the *cir1* mutant, its effect on host resistance to infection had yet to be determined. It has been found through previous susceptibility analyses that significant differences in the resistance capability of plants were much more pronounced in response to the avirulent strain of *P. syringae (Pst* AvrB*),* (Figure 3-1A).

As a consequence, the *at4g11100* mutant was investigated in its response to avirulent pathogen challenge alongside the *cir1*, Col-0 and *PR-1::LUC* control lines. The results presented in Figure 3-12 demonstrate a 10-fold less growth of pathogen in the *at4g11100* mutant than in both *PR-1::LUC* and Col-0 wild-type lines. This significant reduction of bacterial growth was also observed in *cir1* mutant and no difference was noted between *cir1* and *at4g11100*.

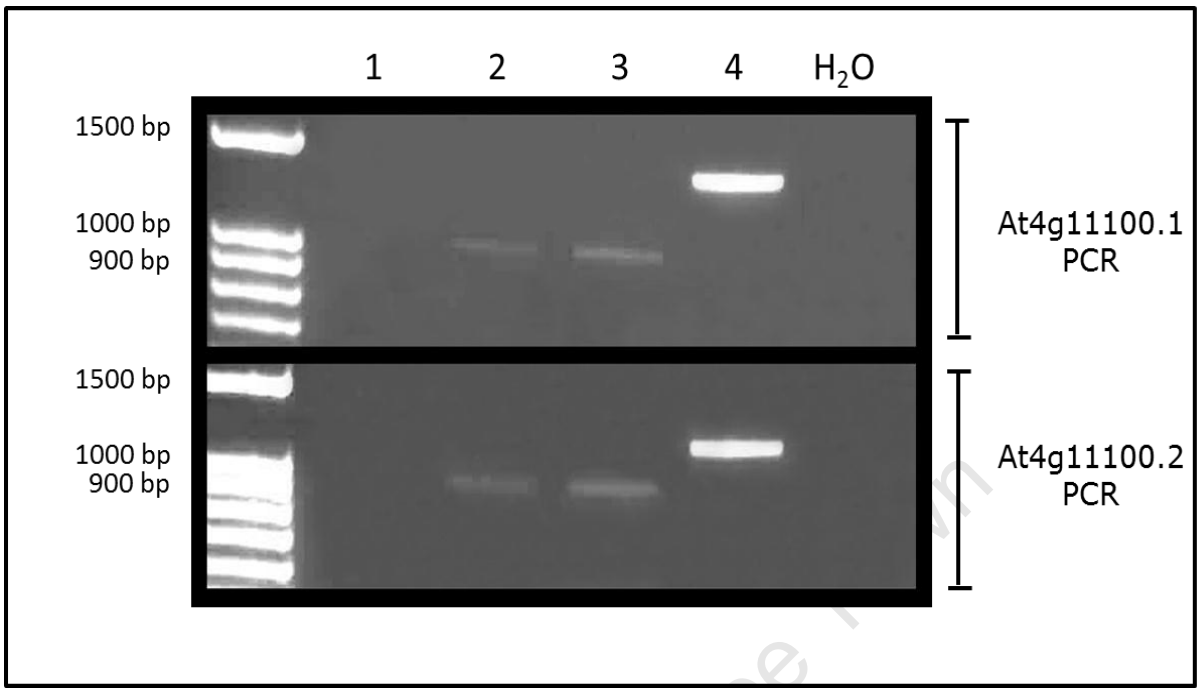

Figure 3-11: At4g11100 expression in the at4g11100 T-DNA insertion mutant line. Isoform specific primers were used to determine *AT4G11100.1* and *AT4G11100.2* expression in the *at4g11100* T-DNA insertion mutant (Lane 1), *PR-1::LUC* (Lane2) and Col-0 (Lane3), following RT-PCR. Lane 4 contains the genomic equivalent of that isoform and a no template control was included to ensure that no contamination was present. A band of 1000 bp represents the splice variant *AT4G11100.1* while a band of 1383 bp represents the genomic equivalent. A band of 948 bp represents the splice variant *AT4G11100.2* while a band of 1159 represents its genomic equivalent.

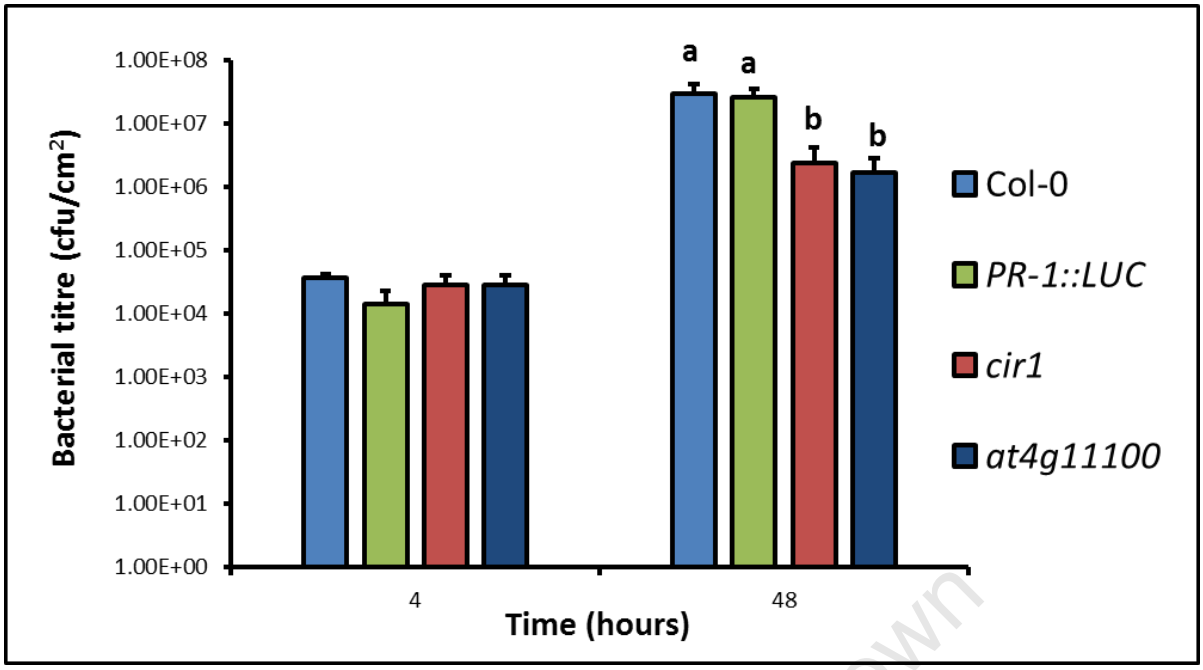

Figure 3-12: Susceptibility analysis of the *at4g11100* mutant to avirulent *P. syringae (AvrB)* infection. Four week old soil grown Col-0 (negative control), *PR-1::LUC* (negative control), *cir1* (positive control) and *at4g11100* mutant lines were assayed. Bars represent the average bacterial titre in three plants per line at 4h and 48 h post-inoculation. Bacterial titre is defined as colony forming units (c.f.u.) cm^2. Error bars represent the standard error of the mean (n = 5). One way ANOVA anaylsis revealed a significant effect of host genotype ($p = 0.001$) on bacterial titres at 48 hpi. Letters denote significant ($P < 0.05$) differences in mean bacterial titres between the different lines as determined by Fisher's LSD (Least significant difference) post hoc analysis. No significant difference in bacterial titres were noted between lines at 4 hpi ($p = 0.518$).

3.9 Complementation of the *cir1* mutant

3.9.1 Generation of the pFAST-G01::*AT4G11100* transgenic *Arabidopsis*

To confirm the role of *AT4G11100* as the *CIR1* gene, a complementation test was carried out. In this approach, wild-type *AT4G11100* (from *PR-1::LUC* genomic DNA) including approximately 1.5 Kb upstream of the gene was amplified using the GCBF and GCNR primers listed in Table 2-2 according to the method described in 2.4.2.1. The resulting product was TA cloned into the pCR8® entry vector. A test digest using the unique *EcoRI* sites was used to identify positive transformants (Supplementary Figure 5). Furthermore, these positive transformants had their coding regions sequenced using the GW1 and GW2 primers listed in Table 2-3 to ensure that no mutations were present. The *AT4G11100* insert was then transferred into the pFAST-G01 expression vector through LR recombination.

Following transformation into DH5α cells, to which negative selection was applied through the empty vector's ccdB domain, positive transformants were identified in a *PstI* test digest following plasmid extraction (Supplementary Figure 6). Following sequence analysis of the coding region, the pFAST-G01::*AT4G11100* expression vector was transformed into *A. tumefaciens* strain GV3101. Both the *cir1* and *at4g11100* mutant lines were transformed with the pFAST-G01::*AT4G11100* construct in an effort to complement the mutant phenotype. The controls for these lines were *cir1* and *at4g11100* transformed with the empty pFAST-G01 to ensure that the vector alone was not responsible for any differences observed in the phenotype. In addition, Col-0 and *PR-1::LUC* were also transformed with the empty pFAST-G01 vector in order to determine if the vector has an effect on the wild-type control lines.

The FAST (fluorescent accumulating seed technology) expression vector was chosen to avoid the potentially adverse effects of herbicide or antibiotic selection on the *Arabidopsis* lines. Instead, this technology utilises a fluorescent screenable marker under the control of the Oleosin promoter, located on the oil body membrane of the seed (Shimada et al, 2010). As a result, transgenic T_1 seed possessing the OLE1-GFP screenable marker could be identified from non-transformed seed under a fluorescence microscope. While variation in fluorescence was evident, fluorescent seed could be clearly distinguished from non-

fluorescent seed and only strongly fluorescent seeds were selected for further analyses (Figure 3-13).

To identify a complementing line from independently generated *pFAST-G01::At4g11100* transgenic lines, it was necessary to investigate whether the transformed *cir1* and *at4g11100* mutant lines exhibited a restored wild-type phenotype. The current study has highlighted several defining features of the mutation, that being increased resistance to *Pst* AvrB and constitutively high levels of PR-1 and EDS1 proteins. Thus complemented *cir1* and *at4g11100* plants transformed with pFAST-G01::*AT4G11100* construct should display reduced resistance to *Pst* AvrB, as well as a loss of constitutively high (or reduced) PR-1 protein levels.

3.9.2 Identification of a complementing line for the *at4g11100* mutation based on *Pseudomonas syringae* assays

An initial bacterial assay was carried out on the heterozygous T_1 generation of transformed *cir1* and *at4g11100* plants to determine the effect of pFAST-G01::*AT4G11100* on both mutants resistance to *Pst* AvrB (Figure 3-14). Due to the recessive nature of the *cir1* mutation, it could be postulated that one copy of the dominant wild-type allele would be enough to complement the mutation. Three plants per individual T_1 line were infected with *Pst* AvrB and the severity of the infection was determined at 48 h post inoculation. Based on the results from previous bacterial assays, no significant difference in bacterial titres has been observed at 4 h post inoculation between *cir1* and both the Col-0 and *PR-1::LUC* control lines (See 3.1). Therefore, the bacterial titres of infected plants were only determined at 48 h post inoculation. Both the *cir1* + pFAST-G01 and *at4g11100* + pFAST-G01 lines displayed significantly less growth of the pathogen than the Col-0 + pFAST-G01 and *PR-1::LUC* + pFAST-G01 lines. This indicates that transformation with the empty pFAST-G01 vector did not affect the disease phenotype of the *cir1* and *at4g11100* mutants. In contrast, the *at4g11100* + pFAST-G01::*AT4G11100* (1 and 2) lines exhibited a titre which differed significantly from that of the *cir1* + pFAST-G01 (1 and 2) and *at4g11100* + pFAST-G01 (1 and 2) lines but not that of the Col-0 + pFAST-G01 (1 and 2) and *PR-1::LUC* + pFAST-G01 (1 and 2) lines.

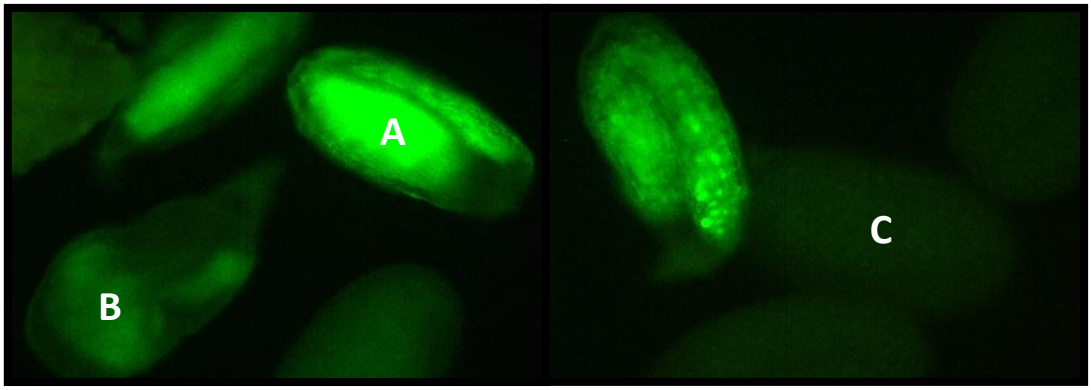

Figure 3-13: Identification of pFAST-GO1::*AT4G11100* and pFAST-G01 fluorescent seed. T_1 seed harvested from Col-0, *cir1*, *PR-1::LUC* and *at4g11100* T_0 lines were screened for GFP fluorescence under a fluorescent microscope. Both the *pFAST-G01::At4g11100* and *pFAST::G01* containing lines had strongly fluorescent (A), moderately fluorescent (B) and non-fluorescent (C) seed.

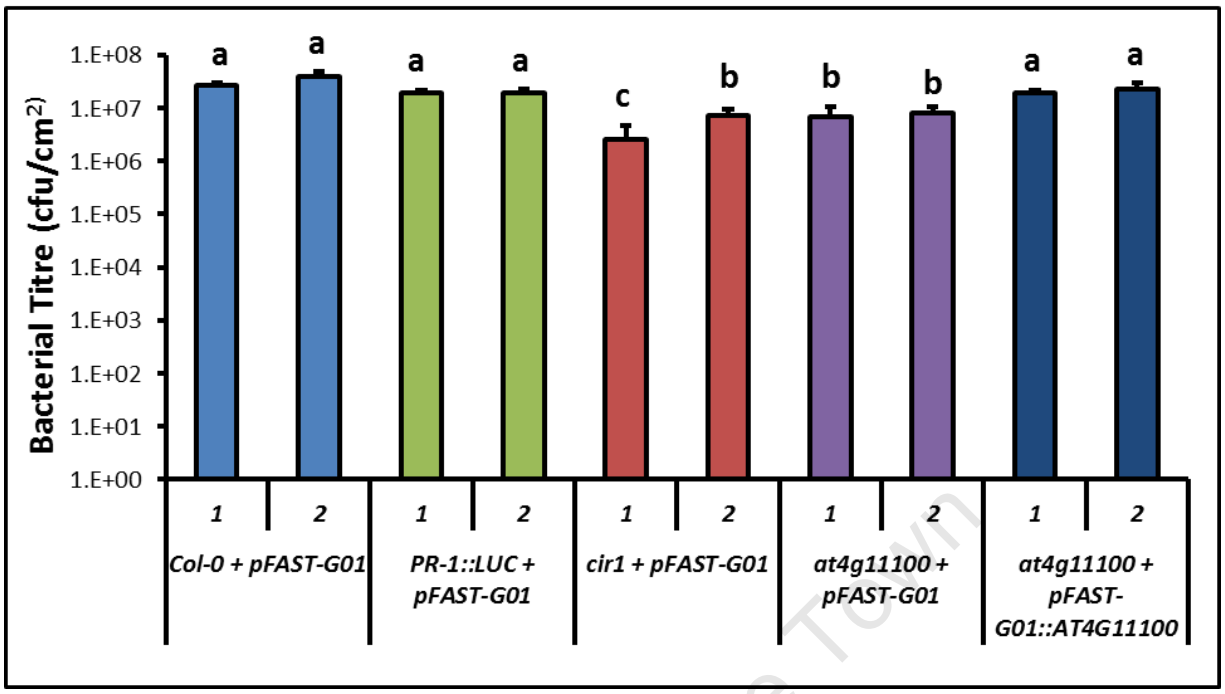

Figure 3-14: Susceptibility analysis of pFAST-G01::*AT4G11100* transformed *at4g11100* T_1 lines. Four week old soil grown *at4g11100* plants transformed with the pFAST-G01::*AT4G11100* vector were assayed in response to avirulent *Pst* AvrB. Col-0 and *PR-1::LUC* plants transformed with the empty vector (pFAST-G01) were utilized as negative controls while *cir1* and *at4g11100* plant lines transformed with the same empty vector were used as positive controls. Numbers on the X-axis represent the independent T_2 lines per transformed plant line. Bars represent the average bacterial titre 48 h post-inoculation (hpi). Error bars represent the standard error of the mean (n = 3). Bacterial titre is defined as colony forming units (c.f.u.) per cm^2. One way ANOVA anaylsis revealed a significant effect of host genotype ($p < 0.001$) on bacterial titre. Letters denote significant ($P < 0.05$) differences in mean bacterial titres between the different lines as determined by Fisher's LSD (Least significant difference) post hoc analysis.

This loss of enhanced resistance to *Pst* AvrB in both T_1 *at4g11100* + pFAST-G01::*AT4G11100* lines demonstrates that these two lines behave similarly to the wild-type control lines. Based on these results it appears that the pFAST-G01::*AT4G11100* transgene restored the *at4g11100* mutants phenotype to that of a susceptible phenotype associated with wild-type lines. Transformed *cir1* + pFAST-G01::*AT4G11100* plants could not be obtained at the time of the *Pst* AvrB assay as a majority of the seed failed to germinate and the few that did developed at a much slower rate than the other transformed lines.

3.9.3 Identification of a complementing line for the *cir1* and *at4g11100* mutation based on PR-1 Western blot analysis

To confirm that the pFAST-G01::*AT4G11100* construct complemented the *at4g11100* mutation as suggested by the previously determined loss of resistance to *Pst* AvrB in both *at4g11100* + pFAST-G01::*AT4G11100* lines, the effect of the construct on PR-1 protein accumulation was investigated. In addition, the effect of the pFAST-G01::*AT4G11100* construct on the PR-1 protein level of the *cir1* mutant, which had not been examined previously, also required further investigation. Only one line (Line 1) from the same T_1 individuals mentioned in 3.9.2 was used in this investigation; however, it is important to note that the protein extracts, examined in this assay, were from leaves excised from the plant prior to infection with *Pst* AvrB. In Figure 3-15, the level of PR-1 protein accumulation observed in Col-0 + pFAST-G01 and *PR-1::LUC* + pFAST-G01 lines were negligible in comparison to the PR-1 levels noted in the cir1 + pFAST-G01 and at4g11100 + pFAST-G01 lines. This suggests that the *cir1* and *at4g11100* lines transformed with the empty pFAST-G01 vector still maintain high basal levels of PR-1 protein accumulation. More importantly, a greatly reduced level of PR-1 protein accumulation was noted in the *cir1* + pFAST-G01::*AT4G11100* and *at4g11100* + pFAST-G01::*AT4G11100* lines. While a complete loss of *PR-1* expression was not noted, it may be reasonable to believe that a co-dominant relationship exists between the two alleles of these heterozygous T_1 individuals and that only homozygous recessive individuals would exhibit the complete loss of *PR-1* expression.

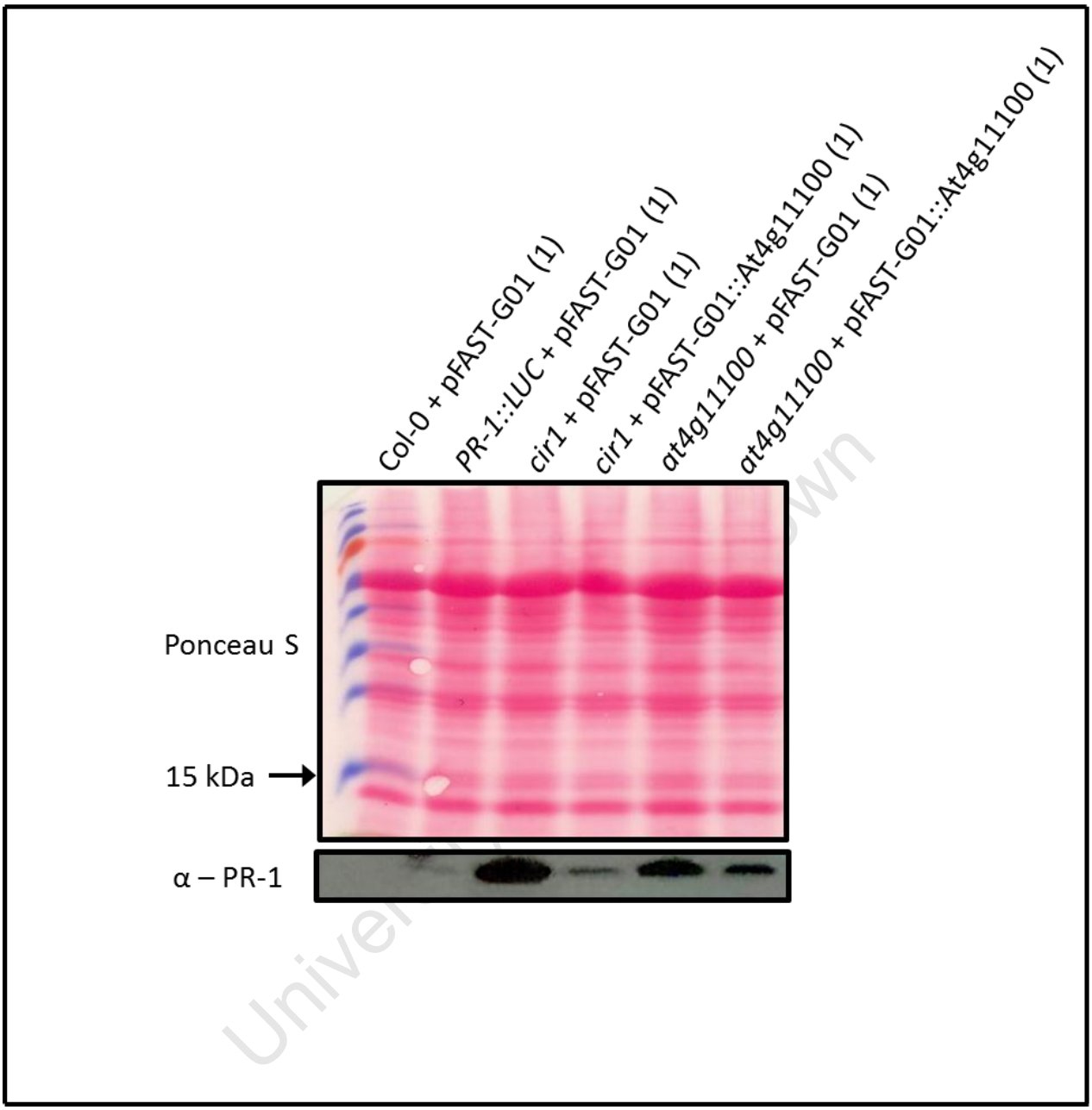

Figure 3-15: Western blot analysis of pFAST-G01::At4g11100 transformed *cir1* and *at4g11100* T_1 lines. PR-1 protein accumulation was examined in *cir1* and *at4g11100* lines transformed with the pFAST-G01::At4g11100 construct. Col-0 and *PR-1::LUC* plants transformed with the empty vector (pFAST-G01) were utilized as negative controls while *cir1* and *at4g11100* plant lines transformed with the same empty vector were used as positive controls. Numbers in brackets represent the independent T_2 lines per transformed plant line. Equal loading and total transfer of the protein was determined by Poncaeu S staining of the membrane. A band of 10 kDa, which corresponds to the predicted size of the PR-1 protein, was detected following the addition of PR-1 antiserum.

The loss of resistance to *Pst* AvrB in *at4g11100* + pFAST-G01::*AT4G11100* lines combined with the large reduction in PR-1 protein accumulation in both the *at4g11100* + pFAST-G01::*AT4G11100* and *cir1* + pFAST-G01::*AT4G11100* lines provide strong evidence that both the *at4g11100* and *cir1* mutations have been complemented in these T_1 lines.

3.10 Investigating the molecular basis of the *cir1* mutation

3.10.1 Sequence analysis of the *AT4G11100* gene in the *cir1* mutant

Further investigation of the *cir1* mutant was necessary to determine the mutation responsible for the constitutive expression of EDS1 and PR-1 observed in Western blot analysis, and the enhanced level of resistance noted in response to *P. syringae* (AvrB). Western blot and disease susceptibility analyses on the available T-DNA insertion mutant lines have identified a mutant line, *at4g11100*, which phenocopies *cir1*. These results collectively implicate the wild-type function of this previously uncharacterized protein in the *Arabidopsis* defence signalling pathway. As a consequence, sequence analysis of the *AT4G11100* gene in the *cir1* mutant would be essential for the identification of the mutation responsible for the resistance phenotype. Such a modification, typical of EMS mutagenesis, would involve a non-synonomous point mutation.

The entire coding region of *AT4G11100* was amplified from *cir1* and *PR-1::LUC* genomic DNA in a high fidelity PCR reaction using the INSRT00 primers listed in Table 2-2. The resulting product was cloned into the pGEM®-T Easy vector system (Promega Corporation, Madison, US) and transformed into DH5α cells as described in 2.4.6.5. Plasmid DNA extracted from successful transformants was sequenced using the 00F, 002F and 002R primers listed in Table 2-3.

Surprisingly, no mutations were noted in the coding region. Thus it is possible that the mutant phenotype may not be the result of a mutant or truncated protein but a change in gene regulation.

3.10.2 *AT4G11100* isoform expression analysis

To investigate whether or not the resistance phenotype present in *cir1* can be attributed to altered gene expression, *cir1*, Col-0 and *PR-1::LUC* were tested for *AT4G11100* expression. Three different cDNA samples were obtained per plant line. An initial PCR reaction was performed to determine if the expression of both isoforms could be detected in all three lines. Isoform specific primers were once again used to amplify the full transcript of both isoforms from the three biological repeats of each line. In this reaction the forward primers NCG00F and NCG002F, specific to the 5' UTR of *AT4G11100.1* and *AT4G11100.2* respectively, were used in conjunction with the same NCG00R reverse primer located in the 3' UTR of the gene. If abolition of *AT4G11100* expression is responsible for the resistance phenotype noted in cir1, no expression would be observed in the *cir1* mutant. This however was not the case. Instead, both isoforms were detected in the *cir1*, Col-0 and *PR-1::LUC* lines following a standard PCR reaction (Figure 3-16). Thus it is possible that it is not a knockout of *AT4G11100* expression but a knock-down in expression accounting for the resistance phenotype observed in *cir1*.

Quantitative real-time PCR analysis was performed to determine the basal expression levels of the splice variant *AT4G11100.1* in *cir1*, *PR-1::LUC* and Col-0. The sequence specific primers designed to amplify the housekeeping gene (*UBP12*) and gene of interest (*AT4G11100.1*) are listed in Table 2-2. In each case one primer of the pair was designed across an exon-exon/UTR-UTR junction to eliminate putative genomic DNA contamination. The two standard curve method was used and relative expression was obtained by normalising the expression of *AT4G11100.1* to *UBP12*. While *cir1* appeared to exhibit a lower level of *AT4G11100.1* expression than Col-0 and *PR-1::LUC*, there was no statistically significant difference (Figure 3-17). Although it should be noted that the data presented here are preliminary and require additional replicates to provide conclusive results. The basal expression levels of splice variant *AT4G11100.2* were also investigated in *cir1*, *PR-1::LUC* and Col-0; however, the identification of any differences in expression was hindered by inefficient primer design. Additional *AT4G11100.2* primers were designed in the limited region where the *AT4G11100.2* sequence differed from *AT4G11100.1* but none produced a quantifiable product.

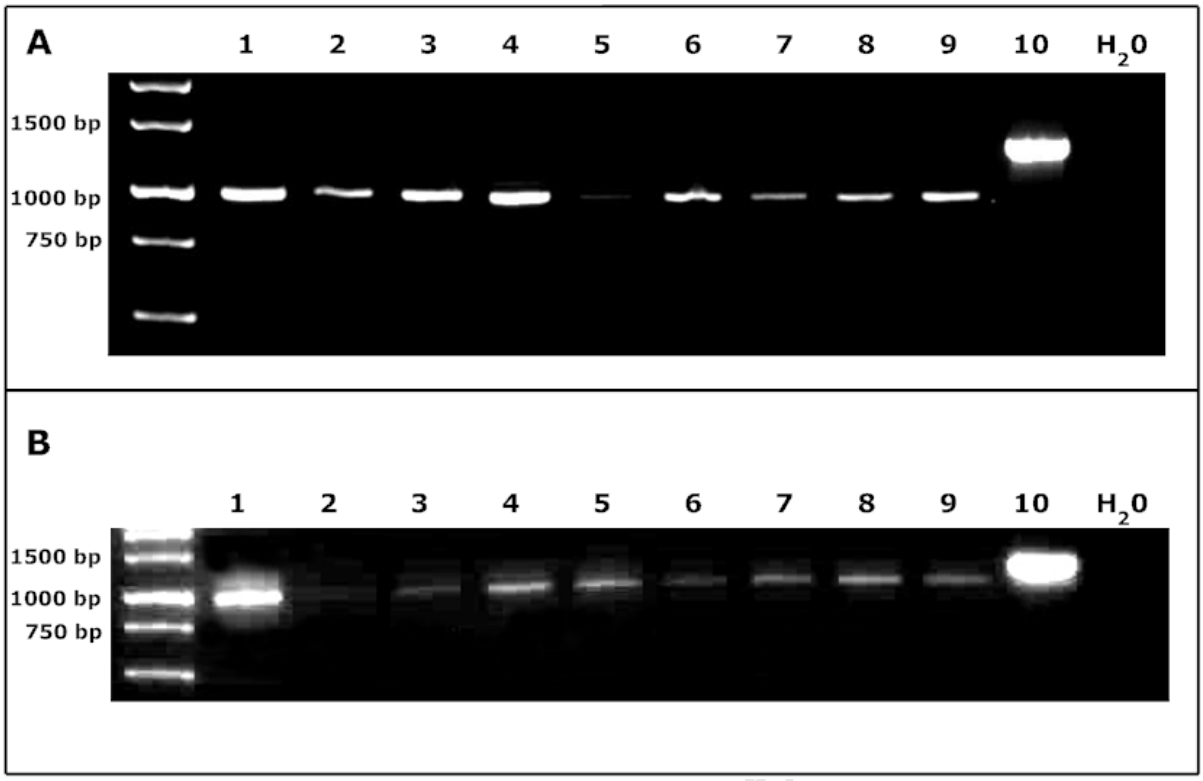

Figure 3-16: Amplification of the full length transcript of the splice variants At4g11100.1 and At4g11100.2. Isoform specific primers were used to amplify (A) *AT4G11100.1* and (B) *AT4G11100.2* from *cir1* (Lanes 1-3), Col-0 (Lanes 4-6) and *PR-1::LUC* (Lanes 7-9) cDNA samples. Lane 10 contains the genomic equivalent of that isoform and a no template control was included to ensure that no contamination was present. In (A) a band of 1000 bp represents the splice variant *AT4G11100.1* while a band of 1383 bp represents the genomic equivalent. In (B) a band of 948 bp represents splice variant *AT4G11100.2* while a band of 1159 represents its genomic the equivalent.

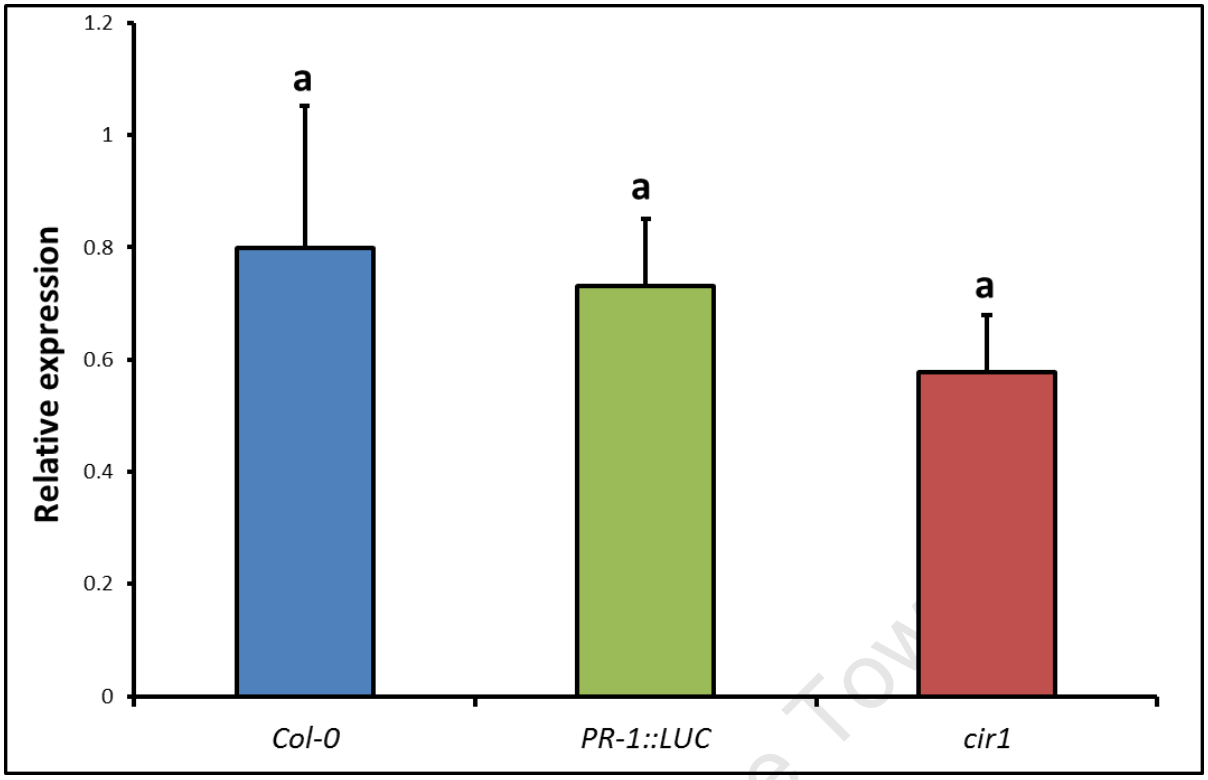

Figure 3-17: Basal relative expression of *AT4G11100.1* in *cir1.* Total RNA was extracted from leaves of uninfected Col-0, *PR-1::LUC* and *cir1* plants to determine the basal transcript levels of *AT4G11100* through qPCR analysis. Bars represent the relative expression acquired by normalizing expression to that of *UBP12* (*AT5G06600; UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 12*). Error bars represent the standard error of the mean (n = 3). One way ANOVA anaylsis revealed no significant effect of host genotype (p=0.692) on relative gene expression.

CHAPTER 4: CONCLUSIONS

Plants are sessile organisms and as a result have evolved a highly complex and integrated system of defence against disease causing phytophathogens. Compelling evidence suggests that this system is tightly regulated to either focus defence responses against a particular invader and/or to employ the most efficient defence response at minimal fitness cost to the host. *CIR1* has been proposed to encode a negative regulator of *Arabidopsis* defence signalling, as its absence is characterized by enhanced disease resistance and the constitutive expression of defence-related genes including *PR-1*, *PDF1.2* and *GST1* (Murray et al, 2002).

Cir1 has been described as having heightened resistance against the virulent pathogens *Pst* DC3000 and *H. parasitica* Noco2 (Murray et al, 2002). The results reported here demonstrate that in addition to conferring resistance against virulent *P. syringae* DC3000, the *cir1* mutation also confers resistance against the avirulent *Pst* AvrB (Figure 3-1A). Previous reports have only implicated *cir1* in basal resistance against virulent pathogens which was considered to be associated with the PTI response. The findings here suggest that in addition to its involvement in PTI, *cir1* may also be involved in the ETI response. The growth of *B. cinerea* was also determined to be significantly reduced in *cir1* leaves compared to Col-0 suggesting that the mutation also confers resistance against this necrotroph (Figure 3-2).

A considerable amount of effort has gone into mapping and characterising *CIR1* and its role in disease resistance. This project's main objective was to identify *CIR1* from the eight possible candidates by screening *Arabidopsis* T-DNA insertion mutants for characteristics similar to that of the *cir1* mutant. The results here describe *at4g11100,* a mutant line with a T-DNA insertion in the promoter region, as a candidate with enhanced resistance to *Pst* AvrB (Figure 3-12) and a high basal level of the defence-associated proteins PR-1 and EDS1 (Figures 3-9 and 3-10), phenocopying the *cir1* mutant. Surprisingly, sequence and expression analysis of the *cir1* mutant collectively could not identify a non-synonymous point mutation or a significant change in basal *AT4g11100.1* gene expression responsible for observed resistance phenotype (Figure 3-17). Thus, the genetic basis of *cir1* phenotype remains unclear; however, complementation assays have demonstrated that the constitutive

expression of *PR-1* in the *cir1* mutant is abolished by complementation with the wild-type *AT4G11100* gene (Figure 3-15). Additional experiments are required to determine whether the increased resistance to *Pst* AvrB exhibited by *cir1* is also abrogated.

It is important to note the results from the aforementioned experiments are preliminary and require confirmation, particularly through the characterisation of an additional *at4g11100* T-DNA insertion mutant. While the results of this study suggest that the resistance phenotype observed in *cir1* may be attributed to a disrupted *AT4G11100* promoter region, characterisation of an additional mutant with a T-DNA insertion located within the coding region of *AT4G11100* may provide further insight into the genetic basis of the *cir1* mutation. A yeast two-hybrid assay utilizing *CIR1* or *AT4G11100* as bait against an *Arabidopsis* cDNA library created from infected *P. syringae* or *B. cinerea* leaf tissue, may be used to identify interacting partners of *CIR1* or *AT4G11100* during pathogen challenge. Furthermore, this technique could also be used to confirm the recently reported interaction between *AT4G11100* and two LRR-containing RLKs (*AT3G50230* and *AT2G36570*), (Mukhtar et al, 2011). Further examination of these RLKs would also be necessary to determine if they are in fact involved in defence and may also prove to be useful in determining if they are negatively regulated by *CIR1* or *AT4G11100.* In addition, by investigating the transient expression of the fluorescent *CIR1* fusion protein through confocal microscopy it may be possible to infer the sub-cellular localisation of the *CIR1* protein under induced and non-induced conditions.

In conclusion, this study provides strong evidence for the role of *AT4G11100* as *CIR1*, and thus identifies this gene as a novel component of innate immunity in *Arabidopsis*. To date, the *AT4G11100* protein has not yet been characterised, therefore speculation into its molecular function is not possible at this point. The novel findings presented here contribute to our current understanding of the *Arabidopsis* disease resistance signalling network.

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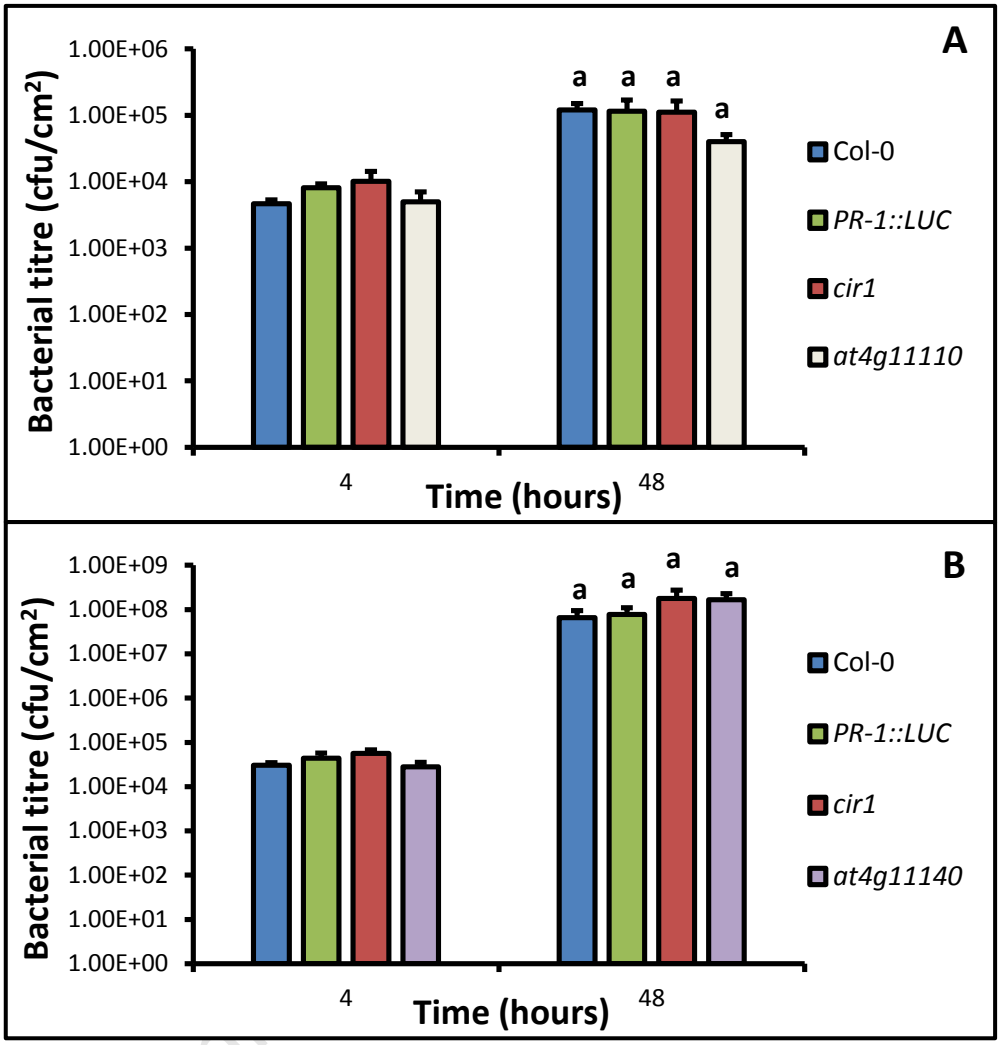
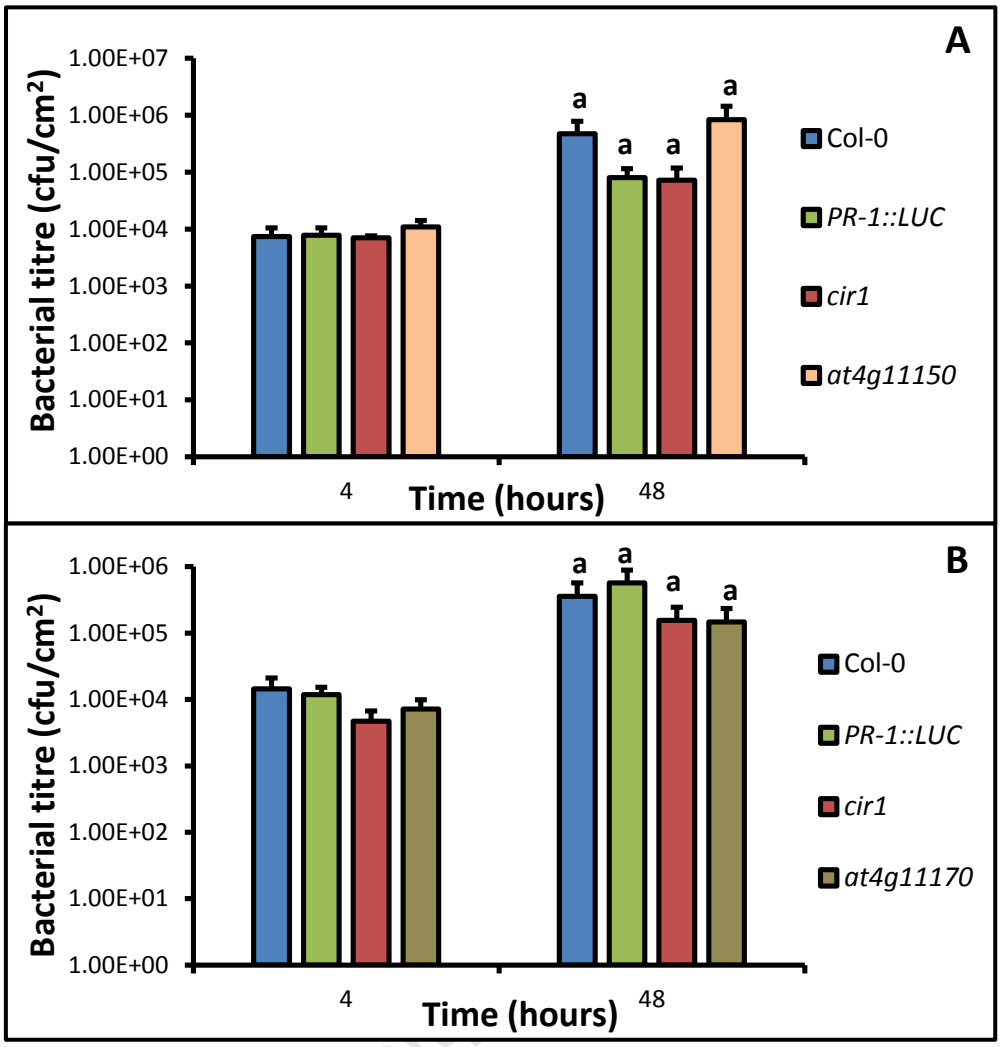
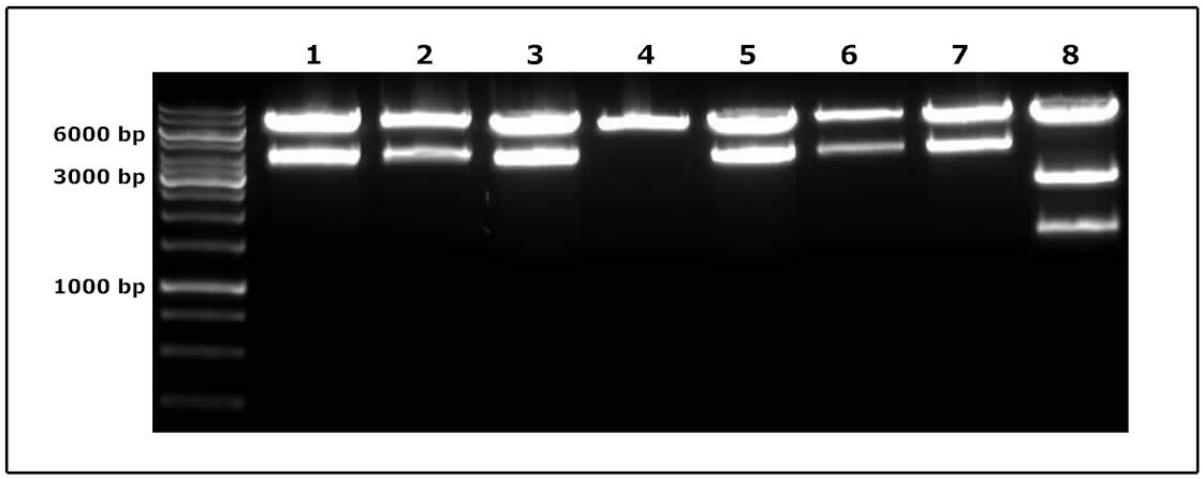
CHAPTER 6: SUPPLEMENTARY FIGURES



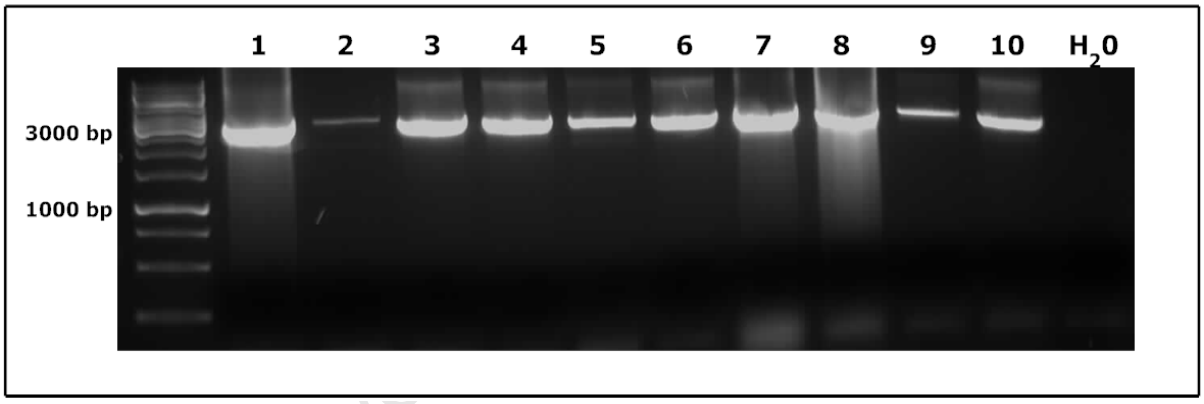
Supplementary Figure 1: Susceptibility analysis of the *at4g11110 and at4g11140* mutant to infection by *P. syringae.* Four week old soil grown Col-0 (negative control), *PR-1::LUC* (negative control), *cir1* (positive control), *at4g11110* and the *at4g11140* T-DNA insertion mutant plant lines were assayed. *at4g11110* lines were assayed in response to avirulent *Pst AvrB* (A) while *at4g11140* T-DNA insertion mutant lines were assayed in response to virulent *Pst DC3000* (B). Bars represent the average bacterial titre in three plants per line at 4h and 48 h post-inoculation. Error bars represent the standard error of the mean (n=3). Bacterial titre is defined as colony forming units (c.f.u.) per cm^2. One way ANOVA anaylsis revealed no significant effect of host genotype at 48 hpi for both assays (A) p = 0.463 and (B) p = 0.372.



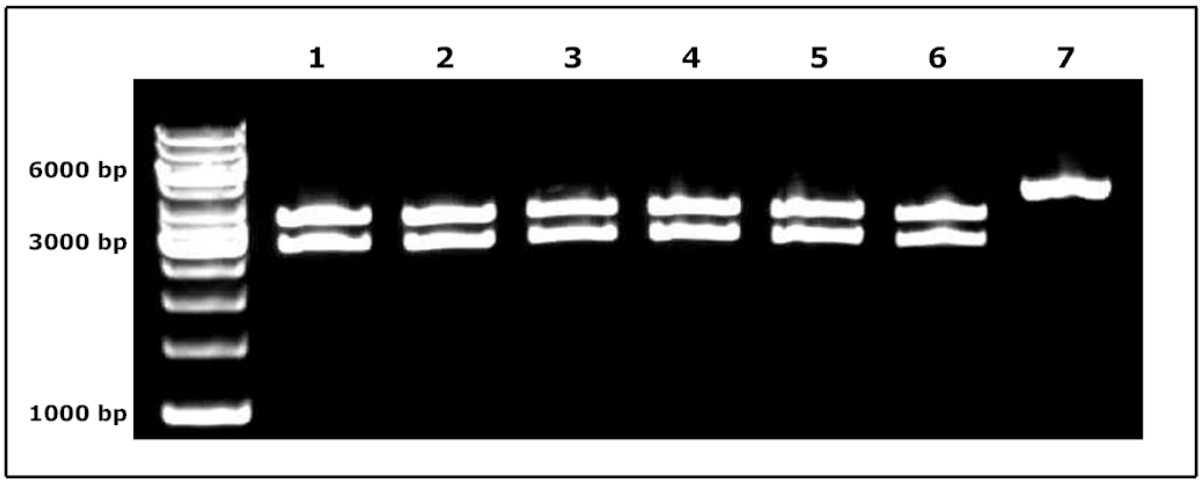
Supplementary Figure 2: Susceptibility analysis of the *at4g11150 and at4g11170* mutant to infection by *P. syringae*. Four week old soil grown Col-0 (negative control), *PR-1::LUC* (negative control), *cir1* (positive control), *at4g11150* (A) and the *at4g11170* (B) T-DNA insertion mutant plant lines were assayed. *Both* lines were assayed in response to avirulent *Pst AvrB*. Bars represent the average bacterial titre in three plants per line at 4h and 48 h post-inoculation. Error bars represent the standard error of the mean (n=3). Bacterial titre is defined as colony forming units (c.f.u.) per cm^2. One way ANOVA anaylsis revealed no significant effect of host genotype at 48 hpi for both assays (A) p = 0.502 and (B) p = 0.243.



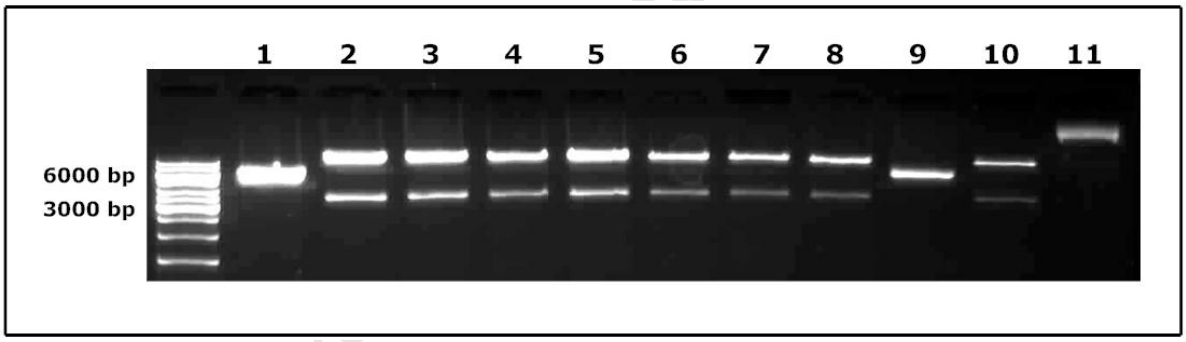
Supplementary Figure 3: *Sac*I and *Xba*I test digest of the pFGC5941::*PR-1::LUC*::OCS plasmid. Plasmid DNA was extracted from selected colonies approximately 24 h after the *E. coli* was transformed. A test double digest with *SacI* and *XbaI* restriction enzymes was performed to identify successful transformants. Lane 4 contains re-ligated vector (7372 bp) while lane 8 contains undigested vector. Lanes 1-3 and 5-7 contain a band representing both the vector (7372 bp) and the insert (4275 bp).



Supplementary Figure 4: Colony PCR of successful *A. tumefaciens* transformants Primers designed to amplify the *PR-1::LUC*::OCS were used in a colony PCR reaction on selected *A. tumefaciens* colonies to identify possible transformants. Lanes 1-10 represent the selected colonies from which the *PR-1::LUC*::OCS (4275 bp) could be amplified. Water was used as a negative control in lane 11.



Supplementary Figure 5: *EcoRI* digest of the pCR8::At4g11100 plasmid. Plasmid DNA was extracted from selected colonies approximately 24 h after the *E. coli* was transformed. A test digest with the *EcoRI* restriction enzyme was performed to identify successful transformants. Lane 7 contains undigested vector while lanes 1-6 contain a band representing the pCR8 vector (2799 bp) and a 3530 bp band representing both the At4g11100 insert (3450 bp) and 80 bp of vector sequence flanking the insert.



Supplementary Figure 6: *PstI* digest of the pFAST-G01::At4g11100 plasmid. Plasmid DNA was extracted from selected colonies approximately 24 h after the *E. coli* was transformed. A test digest with the *PstI* restriction enzyme was prformed to identify successful transformants. Lane 11 contains undigested vector while lanes 2-8 and lane 10 contain bands representative of the pFAST-G01::At4g11100 construct.