Analysis of *Arabidopsis* plants transformed with *AtPRX34* for defence against bacterial wilt

by

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DECLARATION

I, Therése C. de Castro, hereby declare that this dissertation, which is hereby submitted for the degree *Magister Scientiae* at the University of Pretoria, contains my own independent work and has not been previously submitted by me for any degree at this or any other tertiary institute.

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Preface

The development of resistant plant varieties against various pathogens requires an adequate understanding of resistant and susceptible plant-pathogen encounters. To this end, plant defence studies have been conducted on model organisms like *Arabidopsis thaliana*. These studies identified an ongoing evolutionary arms race between plants and pathogens. Certain plants can produce an effective defence response to protect themself against invaders with either preformed or induced mechanisms.

The soilborn pathogen, *Ralstonia solanacearum,* invades various economically important plants resulting in bacterial wilt disease. The pathogen is globally distributed with sporadic occurrence on *Eucalyptus* in South Africa. *Eucalyptus* is planted for pulp production and as mining timber. An African pathosystem between *Arabidopsis* and African-*Ralstonia* isolates serves as a model for further investigations. Transcript profiling on the resistant *Arabidopsis* ecotype, Kil-*0* (Killean), identified *peroxidase 34* (*AtPRX34*) as a candidate defence response during pathogen challenge with *R. solanacearum*.

The large group of peroxidases in *Arabidopsis* contain very similar protein domains and structures, which complicate the classification of individual peroxidases functions. *AtPRX34* forms part of the class III peroxidase group that contains two catalytic cycles responsible for various functions *in planta*, including plant defence. The mechanisms for protecting the plant can either be cell wall thickening, production of a reactive oxygen species or function as an antimicrobial metabolite. *AtPRX34* is a secreted peroxidase that is implicated in a number of different abiotic and biotic conditions. Silencing of both *AtPRX34* and *AtPRX33* resulted in plants showing enhanced symptom development against fungal and bacterial pathogens due to a diminished oxidative burst. This gene is also induced under aluminium, phosphate and ozone stress and implicated in root elongation.

The **aim of this M.Sc.** study was to determine the role of *AtPRX34* in resistance against *R. solanacearum* isolated from *Eucalyptus* trees in order to support the gene expression profile data that implicated *AtPRX34* as a candidate gene involved in enhanced resistance in *A. thaliana* ecotype, Kil-*0,* against isolate CK. This was accomplished by investigating the overexpression of *AtPRX34* in susceptible ecotypes, Be-*0* (Bensheim) and Col-*0* (Columbia), and in Kil-*0*.

Chapter 1 of this dissertation is a review of the literature published to date regarding peroxidase involvement in plant defence focusing on defence response in *A. thaliana* against the pathogen, *R. solanacearum.* In this chapter, the induced defence response that produces secondary signalling phytohormones after the recognition of pathogen associated

molecular patterns and avirulence proteins by pattern recognition receptors or resistant proteins, were highlighted. The different physiological roles of peroxidases and their putative role in plant defence were summarized.

AtPRX34 CDS was cloned into a constitutive expression vector followed by floral dip transformation into three *Arabidopsis* ecotypes. High exogenous *AtPRX34* expression levels with marginal increases in the peroxidase activity were detected for these transgenic lines. In **Chapter 2**, the hypothesis that the susceptible transgenic *AtPRX34* lines should improve protection against the invading pathogen was investigated. Only two of the six lines screened indicated a significantly reduced wilt development compared to their respective wildtypes. Be-*0* overexpression *AtPRX34* line (BOE5) suggested a possible role for this peroxidase in limiting pathogen spread in early infection stages. The Col-*0* overexpression line (COE3) maintained its protection against the pathogen for 11 days. To investigate the overexpression of *AtPRX34* in Kil-*0*, the symptom development under both pathogen and temperature stress was analysed. It has been previously documented that resistance in Kil-*0* becomes compromised under high temperatures. Both transgenic lines (KOE17 and KOE32) showed significantly reduced wilt development compared to Kil-*0*, implicating a possible strategy to tolerate high temperature and pathogen challenges.

The findings presented in this dissertation represent the outcomes of a study undertaken from February 2008 to August 2010 in the Department of Genetics, University of Pretoria, under the supervision of Dr. S. Naidoo. The following poster was generated based on preliminary results obtained in this M.Sc. study:

De Castro, T.C., Berger, D.K., Myburg, A.A. and Naidoo, S. 2010. Investigating a candidate *Arabidopsis* defence response gene, *peroxidase 34* (*AtPRX34*), against *Ralstonia solanacearum* from *Eucalyptus* trees. Congress of the South African Genetics Society, 9-10 April, Bloemfontein, South Africa.

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ABBREVIATIONS

ACT2	Actin 2
AtPRX34	*Arabidopsis thaliana* peroxidase 34
Be-*0*	Bensheim ecotype *0*
BCCF	Bacterial Culture Collection FABI
BOE1	Be-*0* overexpressing *AtPRX34* in transgenic event 1
BOE5	Be-*0* overexpressing *AtPRX34* in transgenic event 5
BOE6	Be-*0* overexpressing *AtPRX34* in transgenic event 6
CBP20	CAP Binding Protein 20
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
Col-*0*	Columbia ecotype *0*
COE3	Col-*0* overexpressing *AtPRX34* in transgenic event 3
COE4	Col-*0* overexpressing *AtPRX34* in transgenic event 4
COE12	Col-*0* overexpressing *AtPRX34* in transgenic event 12
dNTP	Deoxyribonucleotide triphosphate
dpi	Days post inoculation
ENDO	Endogenous peroxidase 34
EXO	Exogenous (transgene) peroxidase 34
EF1	Elongation Factor 1α
EF1GTP	Elongation Factor 1α GTP-binding protein
Kil-*0*	Killean ecotype *0*
KOE17	Kil-*0* overexpressing *AtPRX34* in transgenic event 17

KOE32	Kil-*0* overexpressing *AtPRX34* in transgenic event 32
OB	Oxidative burst
ORF	Open reading frame
PCR	Polymerase chain reaction
PR	Pathogenesis related
Prxs	Class III Peroxidases
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative PCR
T-DNA	Transfer DNA
TUB4	Tubulin 4
UBQ5	Ubiquitin 5

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CHAPTER 1

LITERATURE REVIEW

The involvement of class III peroxidase in plant defence focusing on defence against the pathogen, *Ralstonia solanacearum*

1.1. Introduction

Studying plant-pathogen interaction is important to understand the pathogen and to provide resistance to the plants against a variety of pathogens in the long-term. Plant-pathogen interactions are complicated and investigating their interaction may involve examining the plant and the pathogen separately before studying the interaction.

The model organism, *Arabidopsis thaliana,* can be used to investigate plant-pathogen interactions as the plant is vulnerable to various pathogens. A large number of studies investigating the *Arabidopsis* plant defence response have provided evidence of a signalling-cascade network in the plant to provide adequate defence. This cascade may be launched by the recognition of pathogen-associated molecular patterns (PAMPs) or avirulence (AVR) proteins, leading to the activation of downstream secondary phytohormone responses for adequate defence induction against the invading pathogen.

The pathogen, *Ralstonia solanacearum,* is the causal agent of bacterial wilt in economically important plants. The completion of the genome sequence of the tomato isolate, GMI1000, and geranium isolate, UW551, provided new insights into the pathogen (Denny, 2000; Salanoubat et al., 2002). The genome sequences provided candidate virulence and pathogenicity factors required for general pathogen infection and for specific host interactions.

In 1997, a new disease developed on *Eucalyptus* trees in the KwaZulu Natal province of South Africa (Coutinho et al., 2000). The pathogen caused wilting, death of stems and reduced plant growth. Studies conducted identified the pathogen as *R. solanacearum*. This pathogen induced wilting on *Eucalyptus* and was first reported in 1980 in Brazil, followed by reports world wide (Coutinho et al., 2002; Coutinho et al., 2000; Xu et al., 2009). The pathogen infects cuttings, preventing the vegetative propagation of hybrids of *E. grandis* X *E. camaldulensis* (GC) for pulp and paper production in South Africa.

The presence of *R. solanacearum* in South Africa, at present, is not a major concern, with only sporadic occurrences (Coutinho et al., 2000; Fouche-Weich et al., 2006; Roux et al., 2001; Xu et al., 2009). The identification of resistant *Eucalyptus* is needed for effective disease investigation. It is of value to investigate *R. solanacearum* on a model organism to identify candidate defence response genes for future genetic manipulation of *Eucalyptus* trees or as markers in breeding programs. These genetically modified trees may provide resistance to *Ralstonia solanacearum* and other pathogens. The investigation of the *R. solanacearum – A. thaliana* interaction serves as a model plant-pathogen interaction.

To date, a number of different *R. solanacearum - A. thaliana* plant-pathogen interactions have been investigated. These will be reviewed and specific emphasis given to

the experiment conducted on the African *R. solanacearum* - *A. thaliana* pathosystem. Stemming from the latter research, several candidate genes possibly involved in defence against *R. solanacearum* were identified by microarray analysis. One such candidate is *peroxidase 34* (*AtPRX34*, fold change >2 and *p*-value <0.05) (Naidoo, 2008).

Peroxidases are a large family with similar protein structures and conserved amino acid domains. The promoter regions between the peroxidases are highly diverse (Cosio and Dunand, 2009). The *cis*-elements may be responsible for tissue specific and differential expression between highly similar peroxidases (Kumari et al., 2008; Sasaki et al., 2007; Valério et al., 2004). Redundancies between peroxidases themselves make correct classification of the roles of peroxidases difficult (Passardi et al., 2006b).

The current interest in class III peroxidases (Prxs) is evident from the number of recently published reviews on this topic. A recent review lists all the *AtPRXs* functions identified to date and provides current information on the mechanisms of the Prxs enzyme in performing each function (Cosio and Dunand 2009). Plant defence (Almargo 2009) and lignification (Marjamaa et al., 2009) specific reviews have also been published that discuss possible involvement of Prxs in these processes. The role of *AtPRX34* in plant defence still remains to be clarified.

1.2. *Arabidopsis thaliana*

Arabidopsis thaliana is one of the model organisms used in plant research and provides the necessary features for genetic studies, which in turn may generate information about economically and agriculturally important plants such as *Eucalyptus*. The completion of the *A. thaliana* genome project in 2000 resulted in significant progress in genetic studies by making phenotype to gene identification easier. This resulted in identifying functions for parts of the genome sequence (Somerville, 2006; Wilson, 2000). Some advantages of *A. thaliana* as a model organism in genetic studies are: 1) it is a diploid organism, 2) it is easily grown and has a short generation time (five to six weeks), which facilitate growth in laboratory growth rooms, 3) the plants can be easily transformed by *Agrobacterium*, 4) these plants can generate up to 10 000 seeds per plant, 5) plants can self-pollinate and can be cross-pollinated and 6) *A. thaliana* is genetically diverse with a variety of available ecotypes. The adaptation of *Arabidopsis* to facilitate growth and survival in different habitats is responsible for generating genetic diversity in these plants (Beemster et al., 2002) that is exploited by researchers.

1.3. *Ralstonia solanacearum*

Ralstonia solanacearum is a β-proteobacterium pathogen that causes wilting in economically important plants and has a global distribution (Denny, 2000; Salanoubat et al., 2002). *R. solanacearum* has a wide host range and affects more than 200 plant species, causes devastating crop loss (Denny, 2000; Salanoubat et al., 2002). The pathogen is a soil-borne bacterium that invades the host through wounded roots, colonizes the vascular tissue and subsequently spreads through the plant (Salanoubat et al., 2002). Secreted exopolysaccharides (EPS) and accumulative bacterial numbers block the xylem vessels, hindering water movement from the roots to the aerial parts of the plant, resulting in the classic drooping of the leaves (Figure 1.1).

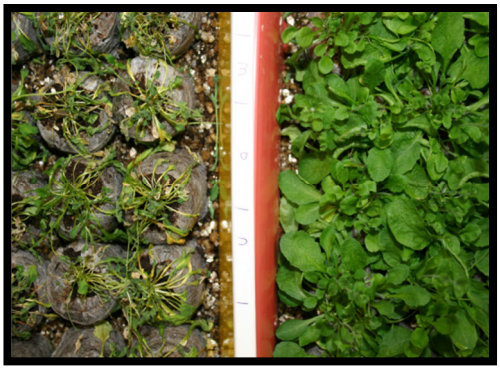

Figure 1.1. *Arabidopsis* plant showing leaf wilting due to water blockage by *Ralstonia solanacearum* isolate CK (leftside) and health Arabidospsis plants (rightside).

R. solanacearum is classified based on three different classification targets namely, race (five), biovar (six) and phylotype (four) (Buddenhagen et al., 1962; Hayward, 1991; Villa et al., 2005). The race classification system is based on host range, where race 2 and 3 contain a narrow host range and race 1 contains a wide host range. This classification method is poorly structured and not taxonomically relevant (Hayward, 1964; Villa et al., 2005). The biovar method is based on biochemical properties of *R. solanacearum* and classified on the acidification of media during metabolism of six carbohydrates. The phylogenetic classification system was determined by targeting the *16S rDNA*, *endoglucanase* (*elg*), *hypersensitive responsive and pathogenicity B* (*hrpB*) and *16S to 23S internal transcribed spacer region* (Castillo and Greenberg, 2007; Villa et al., 2005). The phylotypes reflect the different geographic origin of the bacteria. Asia is represented by phylotype 1, America - phylotype 2, Africa and Indonesia - phylotype 3 and Japan and Australia - phylotype 4 (Castillo and Greenberg, 2007).

The completion of the genome sequence of *R. solanacearum* isolate GMI1000 (race 1, biovars 3 and phylotype 1) isolated from a tomato cultivar provided new insights into the pathogenicity of this pathogen. The genome is divided into a 3.7 Mb chromosome and 2.1 Mb megaplasmid. The genome revealed candidate pathogenesis genes involved in the type II secretion system (T2SS), the type III secretion system (T3SS), EPS biosynthesis, hormone production and adhesion or surfaces proteins. Knockouts of these candidate genes in the bacterium have been used to determine the effect on invasion, colonization and pathogenicity and identify important *R. solanacearum* specific pathogenicity and virulence factors (Denny, 2000; Poueymiro and Genin, 2009; Salanoubat et al., 2002); selected mutant studies will be discussed below. The genome sequence of *R. solanacearum* strain UW551 (race 3, biovar 2 and phylotype 2) from a geranium cultivar was compared to the genome sequence of GMI1000. This comparison led to the discovery of strain specific genes involved in pathogenicity and virulence (Denny, 2000; Salanoubat et al., 2002).

R. solanacearum produces a variety of extracellular proteins to assist in infecting the host plant. The most important secreted molecules are the exopolysaccharides (EPS). EPS deficient mutant studies suggested that the function of these EPS was to block the vascular system that affected water transport. An alternative hypothesis suggested that EPS may prevent the detection of the pathogen by reducing the plant's defence mechanism (Araud-Razou et al., 1998; Denny, 2000). *R. solanacearum* also produces additional extracellular enzymes to degrade the plant cell wall. These include polygalacturonases (PglA, PehB and PehC), endoglucanase (elg) and pectin methylesterases (Salanoubat et al., 2002).

R. solanacearum contains a number of different *hrp* genes that encode for the T3SS. Multiple signalling pathways regulate the virulence factors of the bacteria. The main regulator protein is PhcA, which activates and represses various virulence genes (Brumbley et al., 1993; Genin et al., 2005). This virulence factor negatively regulates the T3SS at the level of HrpG protein. A network of signaling-regulators that respond to environmental stimuli, host cell detection and bacterial density regulate PhcA (Salanoubat et al., 2002). Previously, 48 candidate type III effector genes in *R. solanacearum* isolate GMI1000 were identified (Cunnac et al., 2004; Vailleau et al., 2007) and recently, 74 putative type III effector genes have been proposed from GMI1000, where 28 of these proteins are actively secreted by the T3SS (Poueymiro and Genin, 2009). A T3SS-deficiency mutant in the *R. solanacearum* strain, OE1-1 (isolated from tobacco) showed reduced virulence due to its loss in ability to colonize the intercellular space of its host (Kanda et al., 2003). Mutant studies of OE1-1 identified the importance of the T2SS in systemic infection with transmission electron microscopy indicating the absence of the T2SS-deficient mutant in the

xylem vessels (Tsujimoto et al., 2008). This mutant retains the ability to produce ESP and to colonize the intercellular spaces of tobacco plants. *R. solanacearum* isolate GMI1000 contains a large number of possible type III effector genes, which can all function in altering plant defense responses. The studies conducted on OE1-1 also indicated that the other secretion systems can be involved in the colonization of the plant. A clear understanding of the complexity of the pathogen is important to provided adequate protection for the plant.

1.3.1. *Eucalyptus* as a host

Eucalyptus plantations and *R. solanacearum* infections are found across the globe with various climate conditions (tropical, subtropical and warm temperature areas, Elfstrand et al., 2001). Wilting on *Eucalyptus* was first reported in 1980 in Brazil, followed by reports in China, Indonesia, Taiwan, Thailand, Vietnam, Australia, Venezuela, South Africa and Uganda (Coutinho et al., 2002; Coutinho et al., 2000; Xu et al., 2009). These bacterial strains are all race 1 type with either biovar 1 (Brazil) or biovar 3 (China, Taiwan, Australia and Venezuela). Various symptom development is found on infected *Eucalyptus* trees, namely wilting of the leaves, leaf drop, root rot, reduced growth rate, death of stems and brown discolouration of xylem (Coutinho et al., 2000; Roux et al., 2001). *Eucalyptus* species susceptible to *R. solanacearum* includes *E. camaldulensis, E. citriodora, E. grandis, E. leizhou, E. pellita, E. propinqua, E. saligna, E. urophylla* and *E. grandis* (Elfstrand et al., 2001).

The bacterial wilt disease was first reported in the KwaZulu Natal province of South Africa in 1997 on *Eucalyptus* trees (Coutinho et al., 2000). Studies conducted identified the pathogen as *R. solanacearum*. The pathogen infects ramets and prevents the vegetative propagation of GC. Currently, occurrences are sporadic and only affects GC hybrids located in regions with high temperature conditions, namely Kwambanambi or Matubatuba in South Africa (Coutinho et al., 2000). *R. solanacearum* currently has an impact in plantations of the Democratic Republic of the Congo, Uganda and China (Coutinho et al., 2000; Fouche-Weich et al., 2006; Roux et al., 2001; Xu et al., 2009).

1.4. Plant defence responses

Plants utilize either preformed or induced responses or a combination of both to protect themselves from invasive pathogens. The preformed response includes antimicrobial compounds and structural barriers. The plants' first defence mechanism is the outside epidermal layer of the plant, which prevents pathogen entry. The cell wall components provide a physical barrier between the pathogen and the cell (Anderson et al.,

2005). Some pathogens can successfully overcome these barriers and invade the plant by penetrating the surface layer or stomata or by exploiting wounded tissue.

Plant-pathogen interactions may be divided into two classes namely, host-specific recognition and non-host resistance (Ingle et al., 2006). In host-specific recognition, the host receptors or proteins recognise the invading pathogen, which results in either a compatible or incompatible reaction (Hammond-Kosack and Parker, 2003). A compatible response is when the host is susceptible to the invading pathogen, i.e. it fails to detect the invading pathogen. An incompatible reaction is when the host shows tolerance or resistance to the pathogen, i.e. the host survives the attack. The non-host specific resistance is where resistance factors are developed against all the strains of a specific pathogen (Thatcher et al., 2005). Biotrophic pathogens infect plants but do not cause plant death, as biotrophs use the plants' nutrients for its own survival (Gupta et al., 2000; Pieterse et al., 2009). Nectrophic pathogens like *R. solanacearum* cause spots and vascular wilting resulting in the death of the plant due to the secretion of toxins, ESP and cell-wall degrading enzymes (Pieterse et al., 2009; Thatcher et al., 2005).

Pathogens and plants are continuously involved in an evolutionary arms race, where either the pathogen develops additional virulence factors to cause disease, or the plant counteracts this by providing adequate protection (pathogen recognition or activation of defence signalling) against the pathogen (recently reviewed in Boller and Felix, 2009; Ingle et al., 2006; Jones and Dangl, 2006; Nishimura and Dangl, 2010).

1.4.1. Detection of PAMPs

Plants and the pathogens process a number of different mechanisms to insure their own survival. The plant's Pattern Recognition Receptors (PRR) are located in the cell membrane with a region extracellularly exposed to recognise the Microbial- or Pathogen Associated Molecular Patterns (MAMPs or PAMPs) produced by the pathogen (Ingle et al., 2006). This is responsible for providing the plant with a basal immunity and can provide the plant with broad-spectrum resistance against pathogens containing similar PAMPs (Lacombe et al., 2010). The PRR contains a Leucine-Rich Receptor domain (LRR) that recognizes small segments of the pathogen, the elicitors (Chisholm et al., 2006).

A known example of this PAMP triggered immunity (PTI) is the host FLAGELLIN-SENSING 2 (FLS2) receptor (Boller and Felix, 2009) that recognises the pathogen elicitor, flg22 (22 amino acids at the N-terminus of the flagellin protein) and generates an effective defence response through the activation of the Mitogen-Activated Protein Kinase (MAPK) cascade (He et al., 2006; Jones and Dangl, 2006). These short segments (20-22 amino

acid of the elicitor) are essential for protein function and altering these N-terminus sequences to evade detection by the host can alter the protein function (Ingle et al., 2006). It is worth mentioning that even though some pathogens have evolved to alter their flagellin sequences resulting in not detection via FLS2, they are still detected by the plant due to presence of other PAMPs. Three known pathogens, namely *Agrobacterium tumefaciens*, *R. solanacearum* and *Pseudomonas syringae* altered their flg22 segment to avoid detection. Other pathogens can still evade detection by producing specific effectors to block PTI (Jones and Dangl, 2006).

Another well-known PTI recognition system is the Elongation Factor Tu (EF-Tu) from various bacterial pathogens and is recognized by EF-Tu Receptor (EFR) found in *Arabidopsis* and *Brassicaceae* (Boller and Felix, 2009; Zipfel, 2008). Conserved 18 amino acids from the N-terminal of the protein (elf18) is sufficient to trigger a defence response in these plants (Lacombe et al., 2010). Various other PRRs have been identified that detect various pathogens including; bacterial detection receptors (XA21), fungal detection receptors (CeBiP, CERK, EIX1 and EIX2) and EIX1, and EIX2 that can also detect oomycete PAMPs (Lacombe et al., 2010).

1.4.2. Detection of effectors

The pathogen counteracts PTI by using the T3SS to inject effectors inside the cell (Friedman and Baker, 2007), referred to as effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Two R protein models have been proposed for recognition of effector proteins. In the classic model, the R proteins recognise the AVR proteins directly (Chisholm et al., 2006). If either the host or pathogen lacks one of these proteins the plant cannot produce an effective defence response or may result in delayed defence activation. In the guard model, the effectors target specific proteins in the signalling cascade to block the basal defence response. R proteins guard these effector targets for any changes to ensure the activation of basal defence (Chisholm et al., 2006).

R proteins are classified into five distinct groups based on their LRR domains. The major class is the nucleotide-binding site LRR (NBS-LRR) class, which can be divided into either N-terminal homology to Toll and Interleukin-1 receptors (TIR-NBS-LRR), and the leucine-zipper (LZ-NBS-LRR) or coiled coil motif classes (CC-NBS-LRR; Hammond-Kosack and Parker, 2003; Jones and Dangl, 2006; Tameling and Joosten, 2007). These domains are responsible for the recognition of the effector proteins of the pathogen. Mutant analyses have identified two definite defence-signalling pathways and another is still unknown. The first is the NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) and avrPphB

SUSCEPTIBLE 2 (PBS2) required R protein resistance and the second is the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) required R proteins for plant resistance (Glazebrook, 2001). The R-AVR protein recognition activates an effective and rapid defence response in the plant through the MAPK cascade, reactive oxygen species (ROS) and hypersensitive response (HR; Glazebrook, 2001).

RIN4 is a negative regulator of the basal defence in *A. thaliana* and is monitored by RPM1 and RPS2 (two R proteins) when infected with *P. syringae*. The avrRpm1 and avrB phosphorylates the RIN4 protein which results in a decrease of basal defence response, known as effector-triggered susceptibility (Mackey et al., 2002). RPM1 monitors the RIN4 for phosphorylation and when detected induces a rapid defence response, known as effector-triggered immunity (ETI). These plants are resistant to *P. syringae* infection. An AVR protein, avrRpt2, degrades RIN4 and indirectly decreases the RPM1 protein, which leads to susceptibility of the host (ETS). The plant retaliates by producing a new R gene, namely RPS2, which detects the degradation of RIN4 and leads to host resistance (ETI). This is an example of plant-pathogen interaction that illustrates the continuous evolutionary arms race (Reviewed in Ingle et al., 2006; Jones and Dangl, 2006).

1.4.3. Defence signalling

The recognition of an invasive pathogen by the plant induces a number of different defence responses to protect the plant and prevent the spread and early colonization of the pathogen. These responses may lead to the activation of secondary pathways involving the signalling phytohormones, namely, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), gibberellic acid (GA), auxin, brassinosteriods (BS) and cytokinins (CK; Pieterse et al., 2009). The first four phytohormones will be discussed in more detail as evidence for GA, auxin, BS and CK in plant defence is limited (For a recent review see Bari and Jones, 2009; Pieterse et al., 2009).

a) Early defences

Plant cells overexpress hydroxyproline-rich cell wall glycoproteins (HPRGs) during the primary response. These HPRGs block the vascular vessels and prevent pathogen spread. The plant's initial reaction is to open ion channels and to phosphorylate proteins that result in the initiation of a signal transduction cascade followed by the production of antimicrobial metabolites and proteins (Fry, 2004; Passardi et al., 2005; Thatcher et al., 2005). The signal cascade induces the oxidative burst (OB) and HR, which prevent spread of the pathogen.

ROS is produced by plasma-membrane bound NADPH oxidases and cell wall-bound peroxidases in the apoplast and can lead to HR, which can subsequently result in cell death. All plants produce a primary OB against invading pathogens and only resistant hosts produce a secondary OB at a later time (Low and Merida, 1996). Superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO^-) all contribute to the HR. H_2O_2 causes lignification of the cell wall through cross-linking and both H_2O_2 and NO^- can induce the expression of defence associated genes, phenylalanine ammonia-lyase (PAL), pathogenesis related (PR) genes and glutathione *S*-transferase (GST) genes (Bi et al., 1995; Delledonne et al., 1998). Balance levels between ROS and NO^- determine the effectiveness of subsequent cell death. The early defence pathway usually attempts to prevent the spread of the pathogen and activate the systemic acquired defence response. Secondary hormone pathways (SA, JA and ET) are induced by specific pathogens to provide an improved defence response.

b) Systemic defences

The inducible response is due to the recognition of the pathogen by the host, leading to subsequent induction of a network-signalling cascade (Thatcher et al., 2005). Posttranscriptional modifications of target proteins in the cell generate this specific signalling response to the invading pathogen. Modification mechanisms used by the cell are phosphorylation, ubiquitination, sumoylation, *S*-nitrosylation and glycosylation (Stulemeijer and Joosten, 2008). A secondary signalling cascade to prevent pathogen invasion amplifies the early signalling pathway. This is a complex network of interactions that is regulated by transcription factors (TF) and plant hormones (Balbi and Devoto, 2008; Hammond-Kosack and Parker, 2003).

The phytohormone, SA, plays an essential role in plant defence and systemic acquired resistance (Recently reviewed in Lu, 2009). SA signalling activates PR-1, β-1,3-glucanase 2 (BGL2), thaumatin-like proteins (PR-5) and chitinases (CHIB) that contributes to plant resistance (Uknes et al., 1993) via the regulatory protein NONEXPRESSOR OF PR GENES 1 (NPR1; Pieterse et al., 2009). Mutant studies conducted on the biotroph, *Hyaloperonospora parasitica* and necrotrophs, *Alternaria brassiciola* and *Botrytis cinerea,* determined that the SA pathway is required for defence against biotrophs and that the JA/ET pathway is required for necrotrophs (Thomma et al., 1998). A defined SA pathway has been determined via mutant studies.

The presence of PAD4 and EDS1 signalling proteins are required for accumulation of SA. PAD is similar to triacylglycerol lipase, which again is similar to EDS. This lipase enzyme is responsible for the breakdown of triacylglycerols to glycerols and fatty acids. *Pad*

mutants show an attenuated PR response resulting in low SA accumulation, whilst *eds* mutants shows no PR production. Both SA INDUCTION DEFICIENT 2 (SID2) and EDS5 are also required for SA accumulation later in the signalling pathway (Glazebrook, 2001; Thatcher et al., 2005). SID2 is a putative isochorismate synthase believed to catalyse chorismate to SA (Wildermuth et al., 2001). *Sid* mutants have low SA accumulation with no PR expression (Glazebrook, 2005). Yeast two-hybrid studies identified the importance of the NPR1 ankyrin repeat domain, which is responsible for direct protein-protein interaction (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000). NPR1 interacts with the basic region/ leucine zipper (bZIP) transcription factor (TGA) and WKRY transcription factor to express defence related genes (Anderson et al., 2005).

Jasmonates regulate different important plant developmental processes (Balbi and Devoto, 2008; Turner et al., 2002). The phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are also responsible for defence signalling (Recently reviewed in Browse, 2009). The *coronatine insensitive 1* (*coi1*) mutant indicated that necrotrophs primarily activate the JA/ET pathway for an effective defence response (Balbi and Devoto, 2008; Thatcher et al., 2005). The JA pathway activate the expression of defensin (PDF1.2) and thionin (Thi2.1) for resistance (Glazebrook, 2005). Mutant studies conducted in *A. thaliana* identified a variety of components in the JA pathway (Hammond-Kosack and Parker, 2003).

The *A. thaliana* mutants *fad* (ineffective fatty acid desaturase), *dad1*, *dde1*, *opr3* are deficient in the biosynthesis of JA. The proteins, COI1, JASMONIC ACID RESISTANT 1 (JAR1) and MPK4 are needed downstream of JA accumulation to activate a MYB transcription factor to induce defence related genes (Balbi and Devoto, 2008; Glazebrook, 2005). JAR is a protein similar to adenylate-forming enzyme believed to control JA metabolism. The COI1 protein is an important receptor in JA signalling and the SCF^{COI1} is required for all JA processes (Browse, 2009; Katsir et al., 2008).

Ethylene is a plant hormone involved in various physiological processes and is activated by both abiotic and biotic stimuli. ET activates the nuclear-localized TF, ETHYLENE INSENSITIVE 3 (EIN3), which in turn induces the ETHYLENE RESPONSE FACTOR 1 (ERF1) to activate the defence related genes (Glazebrook, 2005; Thatcher et al., 2005). The GCC box genes are expressed and PDF1.2, CHIB and THI2.1 are produced to defend the host against an invading pathogen. The JA and ET pathways work synergistically to produce PDF1.2 for resistance (Anderson et al., 2005; Glazebrook, 2001).

Abscisic acid is another plant hormone that was recently implicated in plant defence (Recently reviewed in Cutler et al., 2010). ABA plays a crucial role in plant development and, like ET, is involved in abiotic and biotic stress responses (Zhou et al., 2008). The

overexpression of an ERF, namely TOMATO STRESS-RESPONSIVE FACTOR 1 (TSRF1) in tobacco plants induces the expression of ABA and ethylene (Zhou et al., 2008). It has been discovered that this ERF provides significant resistance in tobacco and tomato plants against *R. solanacearum* (Zhang et al., 2004).

1.5. *Arabidopsis* and *R. solanacearum* interaction studies

The presence or absence of a recognition system between plants and pathogens usually determines the disease outcome on the plants. The investigation for discovering a R specific gene providing resistance in *A. thaliana* against *R. solanacearum* isolate GMI1000 started with locus mapping of the cross between Col-*0* (Columbia; susceptible) and Nd-1 (Niederlenz, Germany; resistant) which identified a specific region that segregates as a simple inherited recessive trait (Deslandes et al., 1998). The Resistance to *R. solanacearum* 1 – resistance (RRS1-R) and Resistance to *R. solanacearum* 1 – susceptible (RRS1-S) genes were respectively isolated from ecotypes, Nd-1 and Col-*0*. The RRS1-R gene is the first known R gene discovered that contains a WRKY domain, which implies that the R protein plays a regulatory role in plant defence (Deslandes et al., 2002; Lahaye, 2004). Transgenic Nd-1/*ndr1* (*non-race-specific disease resistance 1)* and Nd-1/ *NahG* (contains salicylate hydroxylate that converts SA into inactive catechol) plant's bacterial numbers were compared to Col-*0* and Nd-1 plants infected with GMI1000. The results indicated that the transgenic plants showed higher bacterial numbers compared to Nd-1, but still less than Col-*0*. This specific R protein recognition is salicylic acid and NDR1 dependent, but SA may not be the sole signalling components due to later disease development in transgenic Nd-1 plants (Deslandes et al., 2002).

A split-ubiquitin two-hybrid system in yeast identified the type III effector that interacts with the RRS1 protein namely PopP2 (Deslandes et al., 2003). This interaction occurred in both Col-*0* and Nd1. This interaction was localized in the cell nucleus (Deslandes et al., 2003). The investigation of a *sensitive to low humidity 1* (*slh1*) mutant, a gain in function mutant in No-*0* (Nossen, Germany) plants, indicated that the SA defence pathway was overexpressed. Map-based cloning of SLH1 identified it as a resistance-like protein, which is identical to RRS1-R. The sequencing of the *shl1* mutants R gene revealed the insertion of a stop codon in the WRKY domain of the protein. Interestingly, the *slh1* mutant had this R-AVR defence response constitutively activated (Noutoshi et al., 2005).

A pathosystem was established by Weich (2004) between *A. thaliana* ecotypes and African *R. solanacearum* strains from Congo, Uganda and South Africa; K (BCCF 401), CK (BCCF 402), CC (BCCF 403) and 27B (BCCF 427). These strains were isolated from

various *Eucalyptus* trees in the above-mentioned areas. This study identified susceptible *A. thaliana* ecotypes like Be-*0* (Bensheim, Germany) and resistant ecotypes like Kil-*0* (Killean, United Kingdom). There was only one order of magnitude difference in the bacterial load in the aerial parts of the resistant ecotype, Kil-*0* compared to the susceptible, Be-*0* plants (Weich, 2004).

The resistant interaction was exploited to investigate the expression profile in Kil-*0* to identify candidate defence response genes against *R. solanacearum* strain BCCF402 (Naidoo, 2008). The mRNA transcripts of Kil-*0* plants infected with BCCF 402 were compared with uninfected (mock) Kil-*0* plants by whole-genome microarray analysis. Genes that were differentially regulated at seven days post inoculation was established at a fold change greater than 1.65. Transcriptome analysis identified 13 differentially expressed genes (Naidoo, 2008), including *lipid transfer protein 3* (*AtLTP3*; At5G59320), *peroxidase 34* (*AtPRX34*; At3G49120), *tropinone reductase* (*AtSAG13*; At2G29350), *avirulence-induced* gene (*AtAIG1*; At3G28940), *translation initiation factor* (*AtSUI1*; At5G54940), *SKP1 interacting partner 5* (*AtSKP5*; At3G54480) and an “expressed protein” (At3G11770).

Reverse transcription quantitative real-time PCR (RT-qPCR) was implemented to confirm the microarray results and to determine if there was differential expression of these genes in Kil-*0* and Be-*0*. The RT-qPCR expression ratios indicated that *AtLTP3*, *AtAIG1* and *AtPRX34* were differentially up regulated in Kil-*0* compared to Be-*0* four days post inoculation (dpi), with Be-*0* showing late up regulation for all three of these genes (seven dpi), indicating that the increased levels of these proteins at four dpi may play some contributing role in resistance against *R. solanacearum*. *AtSAG13* was differentially upregulated seven dpi, which was not seen in Be-*0* (Naidoo, 2008).

Bioinformatic analysis was conduced on these four genes using GeneVestigator v3 (Zimmermann et al., 2005). The expression profiles of these differentially expressed genes during *R. solanacearum* infection were similar to the expression profile during *P. syringae* infections (Naidoo, 2008) except for *AtLTP3*, which did not show any specific trend. A student *t*-test analysis was conducted to determine the significance of the difference between an avirulent (avrRpm1) and a virulent (DC3000) interaction. Both *AtAIG1* and *AtSAGS12* showed significant (p<0.01) upregulation six hours post inoculation and *AtPRX34* showed a significant up regulation at both 6 hours (p<0.1) and 24 hours (p<0.01) post inoculation.

All four of these genes have been implicated in plant defence responses in the past and it will be interesting to determine their involvement in plant defence. *AtPRX34* (Intapruk et al., 1994) is a worthy candidate for future analysis to determine its involvement in plant resistance. This peroxidase is a cell wall-bound, secreted class III peroxidase, which

accumulates in the roots of the plant (Passardi et al., 2006a). Passardi et al., (2006) indicated that it could possibly be an element of SA but not ABA signalling due to the discovery of six SA specific responsive elements (W-box, TTGACC) upstream of this gene. Transgenic knockdowns of *AtPRX34* in Col-*0* resulted in enhanced susceptibility to a variety of pathogens *(Golovinomyces orontii, B. cinerea, P. syringae* DC3000 and *P. syringae* pv *maculicola*; Bindschedler et al., 2006). This resulted in the identification of *At*PRX34 and *At*PRX33 being essential for plant OB (Bindschedler et al., 2006) that plays a very important role in basal plant defences. In their proposed ROS model, the apoplast peroxidase is responsible for the initial rapid OB, which activates the NADPH for a plasma-membrane secondary OB. *R. solanacearum* infects the roots of the plant and eventually enters the secondary xylem vessels (Salanoubat et al., 2002). AtPRX34 may play an important role in hindering entry via the cell wall or provides an effective ROS (Bindschedler et al., 2006).

1.6. Peroxidases

Peroxidases are a large superfamily that is further divided into smaller groups; the non-animal peroxidase is of importance (Welinder, 1992), with three different classes (Table 1.1). These peroxidases contain a common structure consisting of a heme group formed from Fe^{III} and protroporphyrin IX that is capable of reducing H_2O_2 using different donor substrates (Passardi et al., 2006a). Class I intracellular peroxidases are located in chloroplasts, mitochondria, peroxysomes and cytoplasm (Jiménez et al., 1998; Teixeira et al., 2004). Class II peroxidases are solely produced by fungi and the only heme-containing peroxidase that can degrade lignin debris (Martínez et al., 2005; Passardi et al., 2005; Piontek et al., 2001). Class III secreted peroxidases are a complex group with multiple roles usually located in either the vacuole or the cell wall (Almagro et al., 2009; Matsui et al., 2003; Neuhaus, 1996; Passardi et al., 2005; Welinder, 1992). This group is implicated in multiple physiological roles due to the oxidation of different donor substrates by utilizing H_2O_2 (Table 1.2).

Table 1.1. Overview of the three non-animal heme peroxidases with information on their three-dimensional structure and generalized function.

Non-animal peroxidase	Basic information	Three-dimensional structure	Subdivided peroxidases	Basic Function	Reference
Intracellular Class I	All living organisms except green algae	Contains no Ca^{2+}, no disulfide bridges and no signal peptide	CCP (EC 1.11.1.11) APX (EC 1.11.1.5) CP (EC 1.11.1.6)	Scavenger of H_2O_2 and O_2^- by oxidizing substrates. Affinity for ascorbate and detoxifying H_2O_2	(Erman and Vitello, 2002; Obinger et al., 1999; Passardi et al., 2005; Shigeoka et al., 2002; Skulachev, 1998)
Class II	Produced only by fungi	Four disulfide bridges, two Ca^{2+} glycosylated sites and contains a signal peptide	Mn (EC 1.11.1.13) Lignin (EC 1.11.1.14) Versatile (EC 1.11.1.16)	Degradation of lignin in the soil. Oxidation of variety of molecules including various pollutants	(Martínez et al., 2005; Passardi et al., 2005; Piontek et al., 2001; Ruiz-Duenas et al., 2001)
Secreted Class III	Plant peroxidase	Four disulfide bridges, two Ca^{2+} glycosylated sites and contains a signal peptide	EC 1.11.1.7	*In vitro* able to oxidize various phenolic compounds and *in vivo* still unclear. Multifunctional due to the peroxidative and hydroxylic cycles	(Hiraga et al., 2001; Kawano, 2003; Passardi et al., 2005)

CCP, Cytochrome *c* Prxs; APX, Ascrobate Prxs; CP, Catalase Prxs; Mn, Manganese Prxs; H_2O_2, hydrogen peroxide; O_2^-, superoxide.

Table 1.2. Overview of the different physiological processes in which peroxidases are involved and which of the two cycles are responsible for producing that specific function. The two enzymatic cycles chemical reactions were simplified.

Physiological processes	**Function**	**H_2O_2 and reactive radical levels**	**Cycle**
Germination	Seed protection	Generating reactive radicals and H_2O_2 to reduce pathogen attack	Hydroxylic, H_2O_2 = OH• +OH•O_2 or HOO^-
Cellular growth and cell wall loosening	Cell elongation	Generating ROS and controlling H_2O_2 levels	Hydroxylic, H_2O_2 = OH• +OH•O_2 and peroxidative XH +H_2O_2 = X• +H_2O
	Cell wall loosening	Reactive radicals can cleave polysaccharides (pectin and xyloglucan)	Hydroxylic, H_2O_2 = OH• +OH•O_2 or HOO^-
Cell wall reinforcement	Create physical barrier; cell wall stiffening	Formation of diferulic linkage between polysaccharides-bound lignins or polysaccharides	Peroxidative, XH +H_2O_2 = X• +H_2O
Diferulic bonds			
Extensins		Create rigid extensin cross-linking that blocks cell wall loosening in primary cell wall	
Lignification		Cross-linking of three *p*-hydroxycinnamyl alcohols to form lignins	
Suberisation		Cross-linking of phenolic monomers of the primary cell wall	
Senescence	Involvement in cell death	O_2^- and H_2O_2 induction and development of senescence stage	Hydroxylic, H_2O_2 = OH• +OH•O_2 or HOO^-

1.6.1. Class III peroxidases

Secreted Class III peroxidases (Prxs, EC1.11.1.7) are a complex group with multiple gene members in plants. The reason for these numerous gene duplications during evolution are still unclear, but are believed to be the result of plants adapting to terrestrial life, which contains elevated oxygen concentrations. The *A. thaliana* genome contains 73 different family members (Welinder, 1992) and *O. sativa* contains 138 (Passardi et al., 2004a). A Class III Prxs database: PeroxiBase is available (http://peroxidase.isb-sib.ch) and contains all the nucleotide and protein sequence information from different organisms to facilitate in the classification and identification of Prxs (Bakalovic et al., 2006; Passardi et al., 2007).

The Prxs contain very similar protein structures with certain conserved amino acid residues and can contain a signalling extension and/or a Ca^{2+} binding site (Nielsen et al., 2001; Østergaard et al., 2000; Welinder et al., 2002). The similarities between the structure and the complexity of the two catalytic activities imply that specialized regulatory control is required for the different Prxs (Passardi et al., 2005). Phylogenetic analysis of the promoter regions of Prxs in *A. thaliana* revealed high diversity among these regions (Cosio and Dunand, 2009), indicating that different *cis*-elements may be responsible for spatial and temporal expression of an individual peroxidase (Prx) in response to different stimuli (Kumari et al., 2008; Sasaki et al., 2007; Valério et al., 2004).

Identifying the function of a Prx with the current available techniques is difficult. Classifying the newly discovered Prx protein is not a straightforward process and is made more complex by post-transcriptional regulation that produced multiple proteins from one gene (Gabaldón et al., 2007; Laugesen et al., 2007). The main problem is identifying the function *in planta* because all Prxs have the same catalytic ability, *in vitro*. Overexpression or knockout approaches do not always produce informative results and the large group of Prxs complicate gene silencing methods (Cosio and Dunand, 2009). Targeting regions for silencing is complicated due to the high sequence similarity among members, usually resulting in silencing of numerous closely related genes. Knockout is not always constructive because closely related family members can catalyse the knockout Prx function, resulting in no phenotypic differences (Cosio and Dunand, 2009).

Redundancies between Prxs themselves cause problems with correct classification of the roles for a specific Prx (Passardi et al., 2006b). A recent review by Cosio and Dunand (2009) indicated the discrepancy between two *A. thaliana* Prxs, *AtPRX33* and *AtPRX34*. Experiments conducted indicate multiple different roles for these Prxs. Briefly summarized: both Prxs were implicated in basal plant defence by producing an initial OB (Bindschedler et al., 2006) and both promote root elongation (Passardi et al., 2006b). *AtPRX34* was also implicated in aluminium stress (Richards et al., 1998), phosphate starvation (Hammond-

Kosack and Parker, 2003), ozone stress (Ludwikow et al., 2004) and in *P. syringae* infection (Mohr and Cahill, 2007). These results can indicate a possible role for *AtPRX34* in general stress signalling. Further analysis will need to be implemented to better understand the role of these two Prxs and to answer some of the following questions: 1) how can *AtPRX34* be important in plant defence, root elongation and other abiotic stresses, 2) why is *AtPRX33* not implicated in the same abiotic stresses, and 3) are there different functions for the same Prx gene at different developmental stages of plant life?

1.6.2. Defence role of class III peroxidases

Plants protect themselves against pathogen attack by activating a number of defence mechanisms that may be preformed or induced (Section 1.3). The possible involvement of Prxs in plant defence will be discussed based on cell wall modifications, production of ROS and reactive nitrogen species (RNS) and their involvement in systemic signalling. It is known that Prxs have a role in both necrotophic and biotrophic pathogen invasion (Van Loon et al., 2006).

a) Cell wall reinforcement

Cell wall modifications occur in normal cells and are activated in cells upon pathogen attack (Cosio and Dunand, 2009). Prxs catalyse processes in the cell wall resulting in a stronger physical barrier to limiting pathogen entry and spread. Prxs are involved in four different reinforcement mechanisms namely: lignification, suberization, extensins cross-linking and diferulic bond-formation.

Lignification occurs in secondary cell walls (Barceló, 1995; Irshad et al., 2008; Yokoyama and Nishitani, 2006) and is the cross-linking of three monolignins (*p*-coumaryl, coniferyl and sinapyl alcohol) by Prxs and H_2O_2 dependent reactions that form part of the phenylpropanoid biosynthesis pathway (Barceló, 1995). Suberization is the same process as lignification but only occurs in primary cell walls (Bernards et al., 2004; Keren-Keiserman et al., 2004).

In yellow lupine tissue studies showed that soluble sugars can inhibit the infection of *Fusarium oxysporum* (Morkunas et al., 2004; Morkunas et al., 2005). An increase in peroxidase activity to pyrogallol, syringaldazine and guaiacol in embryo axes infected and supplemented with sucrose indicated an early involvement in lignification and a possible role in phenolic oxidation against *F. oxysporum* (Morkunas and Gmerek, 2007).

As mentioned previously, plant cells overexpress HPRGs during the early defence response to block the vascular vessels which result in preventing the spread of the

pathogen (Fry, 2004; Passardi et al., 2005; Thatcher et al., 2005). Extensins are a well-known family of HRGPs with a structural motif repeat in its backbone (Fry, 2004). Extensins block the vascular vessels via a Prxs and H_2O_2 dependent process, which make the cell wall harder for the pathogen to penetrate (Fry, 2004; Passardi et al., 2005). Ferulic acid bond-formation involves a diferulic linkage between polysaccharide-bound lignins or polysaccharides in the primary cell wall (Ralph et al., 2004) via Prxs and H_2O_2 dependent processes. This strengthens the cell wall and decreases its susceptibility to pathogen attack (Grabber et al., 1998).

A newly identified barley class III Prx, HvPrx40, accumulates in the epidermal cells upon *Blumeria graminis* f.sp. *hordei* (*Bgh*) infections. Overexpression of this Prx conferred enhanced resistance against *Bgh* and silencing of this Prx showed opposite results. These two experiments complement each other. Further experiments indicated that this Prx is not involved in the production of H_2O_2 at the site of fungal penetration (Johrde and Schweizer, 2008), indicating that this Prx is involved in another Prx-mediated defence mechanism.

b) Reactive oxygen species and reactive nitrogen species

ROS and RNS create a toxic environment for the pathogen by producing large amounts of free radicals (Bindschedler et al., 2006; Cosio and Dunand, 2009; Delannoy et al., 2003) and are also involved in activating the secondary signalling pathway to induce the production of PR genes (Cosio and Dunand, 2009; Laloi et al., 2004). Prxs form part of the PR-protein 9 subfamily that increase and accumulate during pathogen attack (Passardi et al., 2005; Van Loon et al., 2006).

ROS produce an OB that can lead to HR production in plants, preventing pathogen spread and colonization. The system responsible for generating ROS in plants is still unknown. Four different enzymes can possibly produce ROS, namely: 1) plasma membrane NADPH oxidases (Desikan et al., 1996), 2) cell wall Prxs (Bindschedler et al., 2006; Bolwell et al., 2002; Choi et al., 2007; Kawano, 2003), 3) polydiamine oxidases (Angelini and Federico, 1989) and 4) oxalate oxidases (Zhang et al., 1995). Prxs are mainly involved in ROS detoxification because the production of free radicals in the hydroxylic cycle is a slow process (Almagro et al., 2009) but experiments conducted on pea leaves treated with a fungal elicitor produced a ROS from extracellular Prxs (Kawano, 2003; Kiba et al., 1997; Kiba et al., 1996), indicating in certain plant-pathogen interactions, Prx-dependent ROS can be generated.

RNS can also be produced by Prxs activity by generating free NO^-, which is toxic to the cells and can lead to programmed cell death (Neill et al., 2003; Van Camp et al., 1998). NO^- is a substrate of Prxs and detoxifies it in plant tissues (Glover et al., 1999). NO^- can also

irreversibly bind to Prxs heme-group (Yonetani et al., 1972) which inhibits any further catalytic activity of the enzyme (Barceló, 1995; Ferrer and Ros Barceló, 1999; Ischiropoulos et al., 1996).

Overexpression of a class III Prx, *TaPrx103,* in wheat conferred increased resistance when infected with the powdery mildew fungus, *B. graminis* f.sp *tritici* (*Bgt*; Christensen et al., 2004; Schweizer et al., 1999). Interestingly, this Prx is naturally expressed in the inner leaf of wheat and by expressing this Prx in the epidermal layer, the transgenic plants showed enhanced leaf resistance to *Bgt* with no visible yield loss (Altpeter et al., 2005). Microarray studies indicated that the transgenic wheat showed no significant difference in gene expression compared to wildtype, with only a marginal difference in the infected transgenic tissue. The transgenic wheat plants with the tissue-specific Prx expression in their epidermal cells, generate H_2O_2 and the cells better responded to programmed cell death, which is thought to be responsible for the increased resistance in the leaves against *Bgt* (Schweizer, 2008). This raises the question: Why haven't the wheat cultivars evolved to express *TaPrx103* in its epidermis?

Five different French Bean Peroxidases (FBP) expression levels were investigated after treatment with an elicitor created from *Colletotrichum lindemuthianum*. FBP1 was induced within three hours after treatment and was mainly expressed in the roots of the plant (Blee et al., 2001). Transgenic *A. thaliana* plants expressing the antisense of this FBP1 showed enhanced susceptibly to *P. syringae*, *G. orontii*, *B. cinerea* and *F. oxysporum*. Microarray experiments conducted on these transgenic *Arabidopsis,* indicated that two glutathione peroxidases (At1G63460 and At3G63080), which are not cell wall-associated and *AtPRX34* transcript levels were reduced. *AtPRX33* and *AtPRX34* have 75% homology between each other and *AtPRX33* was not presented on the microarray slide. Further RT-qPCR analysis indicated that both *AtPRX34* and *AtPRX33* were targeted by the antisense construct. The experiment concluded that in *A. thaliana,* a Prx-dependent oxidative burst is required for resistance against the above mentioned fungal and bacterial pathogens (Bindschedler et al., 2006).

c) Involvement in signalling pathways

Prxs play a part in inducing secondary signalling pathways but also react to the secondary metabolites themselves. The exact mechanisms of these interactions are not currently understood. There is evidence that Prxs respond to a variety of secondary metabolites: in *Catharanthus roseus* (Madagascar periwinkle) seedlings treated with SA, JA, ET, ABA and GA exhibited induced Prxs activity (El-Sayed and Verpoorte, 2004), in grapevine Prx expression levels respond to MeJA treatment (Repka et al., 2004), in *Panax*

ginseng (Korean ginseng) suspension culture Prxs activity respond to SA and MeJA (Ali et al., 2006) and in peanut seedlings, JA increased Prxs protein levels (Kumari et al., 2006). Application of SA on white clover induced the expression of a class III Prx, *TrPrx2* in roots within 48 hours, indicating a possible role of this Prx in defence (Crockard et al., 1999). SA also acts as a Prx substrate (Chen et al., 1993; Rutter et al., 1983) and generated free radicals from the Prx dependent reaction (Kawano and Muto, 2000) in tobacco.

1.7. Functional studies in *Arabidopsis*

Agrobacterium tumefaciens is a useful tool to transform *A. thaliana* due to the bacterium's ability to transfer DNA into the plant's genome. Large collections of T-DNA insertion lines are available from the *Arabidopsis* consortium (http://signal.salk.edu or www.tmri.org). The flanking regions of most of these mutants have been sequenced and can be screened via PCR due to their known insert positions. The problem with these mutants is that not all of them result in knockdown of the target gene and not all of them showed a phenotypic difference (Britt and May, 2003).

Various vectors are available to create the required transgenic event. Naming a select few: 1) A gene can be constitutively overexpressed, 2) expressed under the control of a tissue-specific promoter, 3) expressed under the control of an inducible promoter, 4) tagged with a fluorescent label and 5) silenced by antisense or hairpin constructs (Karimi et al., 2007). The insertion event into the plants genome is random and multiple inserts can occur.

The random insertion of the T-DNA can result in a decrease of the vector expression when inserted into heterogenous regions of the genome and can disrupt gene expression of endogenous genes when inserted close to or into the gene. The vector expression can also be enhanced or suppressed when the insertion event occurs close to *cis*-elements. The expression methods (constitutive, induced or tissue-specific) can affect endogenous genes and protein regulation, for example homology between a transgene and endogenous gene can result in post-transcriptional silencing of these genes. Silencing methods can result in false targeting or degradation of the silencing construct (Earley et al., 2006). The science of functional genetics is not perfect and that is why accurate control strategies are necessary to ensure that the correct conclusions are made. This requires that more than two transgenic insertion events be examined to account for any positional or silencing effects.

1.8. Conclusion

The development of resistant plant varieties has been recognised as a strategy to curb disease incidences of various pathogens. To this end, plant resistance against various pathogens have been studied in the model plant *A. thaliana*. We can exploit the current African *R. solanacearum* - *A. thaliana* pathosystem to gain valuable information to increase resistance in other non-model hosts, like *Eucalyptus*. Thereafter, the knowledge obtained can be applied in this non-model host to try and improve resistance against *R. solanacearum*. Genetic manipulation of a susceptible host could improve defence against various pathogens by using a broad-spectrum defence response gene. The genetic manipulation of downstream-induced genes like *AtPRX34* may provide a resistant or tolerant phenotype against a broad spectrum of pathogens.

AtPRX34 forms part of the secreted Class III peroxidases, which consist of two reaction cycles namely the peroxidative and hydroxylic cycles. The peroxidative cycle is responsible for oxidizing different substrates in the presence of H_2O_2 and the hydroxylic cycle is responsible for producing ROS and free radicals (Passardi et al., 2005; Passardi et al., 2004b). These two reaction cycles are responsible for implicating peroxidase involvement in a number of different defence responses, ranging from germination to programmed cell death. *AtPRX34* has a cell wall signalling extension (Nielsen et al., 2001; Østergaard et al., 2000; Welinder et al., 2002) and is mostly expressed in the roots where it is involved in root elongation by monitoring H_2O_2 levels (Passardi et al., 2005). In the proposed *AtPRX34* and *AtPRX33* generating ROS model, these apoplast Prxs are responsible for the first rapid OB, which activates the plasma-membrane NADPH for a second larger OB (Bindschedler et al., 2006). These protect the plant by activating basal defence responses against invading pathogens. *AtPRX34* is a candidate defence response gene to provide resistance in susceptible ecotypes due to the supporting evidence from literature and the RT-qPCR results indicating significant earlier activation of *AtPRX34* in Kil-*0* compared to Be-*0* four days post inoculation (Naidoo, 2008).

R. solanacearum infects the roots of the plant and eventually enters the secondary xylem vessels where EPS excreted by the pathogen and cell wall debris block water movement to aerial parts of the plant resulting in drooping of the leaves (Salanoubat et al., 2002). A number of different hypotheses can be generated for the putative involvement of *AtPRX34* in protecting *A. thaliana* plants against *R. solanacearum*. *AtPRX34* may play an important role in hindering cell wall entry by reinforcing the secondary cell wall and neighbouring fibres or provide an effective ROS to prevent pathogen colonization and spread by creating a toxic and unfavourable environment (Bindschedler et al., 2006).

In this study we investigated the putative defence role of *AtPRX34* in *A. thaliana* against *R. solanacearum*. The *AtPRX34* gene was isolated from the resistant ecotype, Kil-*0* followed by the constitutive overexpression of the gene in both susceptible ecotypes, Be-*0* and Col-*0,* and resistant ecotype, Kil-*0,* to assess the potential peroxidase function in conferring resistance against *R. solanacearum* by assessing the phenotypes of the infected transgenic plants. In parallel, a purchased knockdown line (SALK_051769) in the *AtPRX34* gene of the susceptible ecotype, Col-*0,* was investigated to determine if the knockdown line increases susceptibility to *R. solanacearum*.

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CHAPTER 2

Phenotypic analysis of *Arabidopsis thaliana* plants overexpressing *AtPRX34* infected with *Ralstonia solanacearum* isolate BCCF402

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Chapter 2 was prepared in the format of a manuscript for publication in a research journal (e.g. *Forest Pathology*). I generated all the plant materials and analysed all of the relevant data related to this study. Dr. Christine Olivier (Department of Genetics, University of Pretoria) provided necessary advice and support for the experimental completion of the peroxidase analysis of the transgenic and untransformed plants. Prof. Fourie Joubert (Department of Bioinformatics, University of Pretoria) constructed the three dimensional protein structure of the mutated *AtPRX34* protein. Project leader, Dr. S. Naidoo conceived the idea for this project from her PhD experiments and supervised the experiments and analyses for the completion of this project. Dr. S. Naidoo and Prof. A.A. Myburg provided the funding for this project and edited the manuscript. Prof D.K. Berger edited this manuscript and provided expert advice on critical aspects of the research.

2.1. Introduction

Ralstonia solanacearum is responsible for bacterial wilt disease in more than 200 different plant species and causes devastating crop losses in economically important plants. The pathogen colonizes the xylem vessels in the vasculature of the roots, blocking the flow of water to the leaves which results in wilting (Denny, 2000; Salanoubat et al., 2002).

Eucalypts belong to the family Myrtaceae and are the second most commonly planted forest trees in the world. In South Africa, *Eucalyptus* trees are used for pulp production and as mining timber. *R. solanacearum* wilt on *Eucalyptus* was first reported in 1980 in Brazil followed with reports worldwide from China, Taiwan, Australia, Central Africa (Uganda and Democratic Republic of Congo) and Venezuela (Coutinho et al., 2002; Coutinho et al., 2000; Fouche-Weich et al., 2006; Roux et al., 2001; Xu et al., 2009). In Central Africa, bacterial wilt can result in a mortality rate of 50% in a single *Eucalyptus* plantation (Roux et al., 2001). The strain CK (Congo Kissoko, BCCF402) was isolated from *E. grandis* trees. This strain was first discovered on *Eucalyptus* trees in the KwaZulu Natal province of South Africa in 1997 (Coutinho et al., 2000). It caused wilting and death of ramets preventing the vegetative propagation of the hybrids of *E. grandis* X *E. camaldulensis* (GC). Currently, *R. solanacearum* is not a major pathogen in South Africa and shows only a sporadic occurrence.

The model organism, *Arabidopsis thaliana* has in many aspects provided glimpses into the complex relationship that exists between a pathogen and its host. It has been established with experiments that *A. thaliana* is a host for *R. solanacearum*. A previous resistant interaction: *A. thaliana* (ecotype Nd-1, Niederlenz, Germany) and *R. solanacearum* (tomato strain GMI1000) identified a single recessive gene, Resistance to *R. solanacearum* 1 (*RRS1-R*) responsible for resistance (Deslandes et al., 2002; Deslandes et al., 1998). The RRS1-R protein interacts with the popP2 (RSc0868) protein from the bacteria to induce an effective defence response (Deslandes et al., 2003). In the compatible interaction of GMI1000 and Col-*0* (Columbia) the allele associated with susceptibility was defined as *RRS1-S*. The bacterial load in the compatible interaction was extremely high (10^{11} to 10^{12} cfu/gram of fresh weight) with low numbers (10^{5} to 10^{6} cfu/gram of fresh weight) in the incompatible interaction. An African pathosystem with the Congolese *Eucalyptus* strain CK identified a susceptible *A. thaliana* ecotype, Be-*0* (Bensheim, Germany) and resistant ecotype, Kil-*0* (Killean, United Kingdom). Be-*0* developed wilting symptoms four days post inoculation (dpi) and died 14 dpi while Kil-*0* remained wilt-free 14 dpi (Weich, 2004).

This resistant interaction was exploited to identify putative defence response genes against strain CK, BCCF402 (Naidoo, 2008). The mRNA transcripts of infected plants were compared with mock treated plants by whole-genome microarrays derived from Col-*0*

cDNA. It was hypothesised those transcripts that are expressed in Kil-*0* but not in Be-*0* or which are induced earlier in Kil-*0* compared to Be-*0* are potential candidate defence response genes. A single gene was differentially expressed in Kil-*0* one dpi and only 12 genes were upregulated seven dpi with a fold change greater than 1.65 (Naidoo, 2008; adjusted *p*-value< 0.01). Some of these genes have been implicated in plant defence responses in the past and it would be interesting to determine their involvement in defence. *AtPRX34* (At3G49120; Intapruk et al., 1994) was one of the identified candidate defence response genes and with reverse transcription quantitative real-time PCR (RT-qPCR) the transcript levels of *AtPRX34* were determined to be upregulated in Kil-*0* compared to Be-*0* four dpi, with Be-*0* showing late upregulation seven dpi, which suggested that the production of this protein at four dpi may be essential for resistance against *R. solanacearum* (Figure 2.1, Naidoo, 2008).

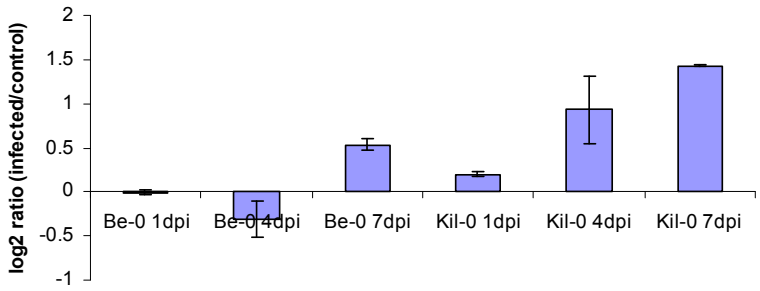


Figure 2.1. The expression profile of *AtPRX34* in Be-*0* and Kil-*0* plants after infection with *R. solanacearum* isolate BCCF402 as determined by RT-qPCR for 1, 4 and 7 dpi. The error bars represent the standard deviation between technical replicates (Naidoo, 2008).

AtPRX34 forms part of the secreted Class III peroxidases (Prxs, EC1.11.1.7) with two catalytic enzyme activities namely the peroxidative (oxidation of substrates in the presence of hydrogen peroxide) and hydroxylic cycles (production of ROS and free radicals) (Passardi et al., 2005; Passardi et al., 2004). These enzyme reactions implicate peroxidases in three possible roles in plant defence: 1) thickening of the physical barrier by lignification, suberization, extensions and ferulic bond formation (Cosio and Dunand, 2009), 2) production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bindschedler et al., 2006) and 3) induction by phytohormones as antimicrobial metabolites (El-Sayed and Verpoorte, 2004). The identification of individual Prxs functions are complicated by the large gene family. *A. thaliana* contains 73 members (Welinder, 1992) with high similarity among the members. For example, AtPRX34 is 95% identical to AtPRX33 at the protein level (Passardi et al., 2006), but they show expression differences under various conditions (Cosio and Dunand, 2009).

Analysis of their promoter regions indicated that *AtPRX34* contains W-box elements commonly implicated in salicylic acid activation and *AtPRX33* contains abscisic acid responsive elements, however both contain various abiotic responsive *cis*-elements (light, temperature, auxin and a root elongation motif) (Passardi et al., 2006). This could possibly explain why both genes have been implicated in root elongation after knockdown and overexpression studies in *A. thaliana* (Passardi et al., 2006). However it doesn't explain why *AtPRX34* is highly induced under aluminium stress (Richards et al., 1998), decreased under phosphate starvation (Hammond-Kosack and Parker, 2003), upregulated with ozone stress (Ludwikow et al., 2004) and in *Pseudomonas syringae* infection the gene was upregulated (Mohr and Cahill, 2007). Silencing of both *AtPRX34* and *AtPRX33* by introducing the antisense of *French Bean Peroxidase 1* (*FBP1*) in Col-*0* resulted in enhanced disease development when infected with various pathogens (*Golovinomyces orontii*, *Botrytis cinerea*, *P. syringae* DC3000 and *P. syringae* pv *maculicola*) resulting from a diminished oxidative burst (OB) in these transgenic plants (Bindschedler et al., 2006). The authors proposed a model which suggested that the first OB was produced by the apoplastic Prxs and the larger second OB was produced by plasma-membrane NADPH, implicating *AtPRX34* and *AtPRX33* in producing ROS and free radicals responsible for generating the initial OB.

This study investigated the potential involvement of *AtPRX34* in plant defence against *R. solanacearum* strain CK in *Arabidopsis thaliana*. The aim was to assess the disease response of the overexpression *AtPRX34* transgenic lines after infection with *R. solanacearum* and determine if the increase in *AtPRX34* transcript levels reduce the susceptibility to the susceptible ecotypes, Be-*0* and Col-*0.* The study consisted of the isolation of *AtPRX34* from the resistant ecotype, Kil-*0*, followed by the construction and transformation of a constitutive overexpression vector into the susceptible ecotypes, Be-*0* and Col-*0* and the resistant ecotype, Kil-*0*, followed by RT-qPCR analysis of the endogenous and transgene *AtPRX34* levels and a peroxidase assay to analyse enzyme activity in these transgenic lines.

2.2. Materials and Methods

2.2.1. Bioinformatic analysis

Bioinformatic analysis was conduced on *AtPRX34*, probe ID 252291_s_at using the GeneVestigator v3 tool (Zimmermann et al., 2005) for the investigation of *AtPRX34* expression profiles in response to various biotic stresses. The following microarray data were used for analysis: AT-106 (*P. syringae*), AT-108 (*Phytophthora infestans*), AT-161

(*Alternaria brassicicola*), AT-147 (*B. cinerea*), AT-128 (EF-Tu) and AT-107 (LPS, Flg22, HrpZ, EF-Tu and NPP1). Student *t*-tests were used to determine the statistical significance of the differential expression levels of this gene compared to the control treatments ($p<0.05$) in each microarray data set.

2.2.2. Plant material and growth conditions

A. thaliana ecotypes presented in this study were Kil-*0*, Be-*0* and Col-*0*. Seed stocks were obtained from the Nottingham Arabidopsis Stock Centre (NASC; www.arabidopsis.info). The mutant, SALK_051769, was purchased from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org; Alonso et al., 2003) and had the T-DNA insert in the promoter region of *AtPRX34* in the Col-*0* background.

Seeds were surface sterilized with 70% ethanol for five minutes, 10% bleach containing 0.1% Triton-X was added to the seed for 15 minutes and the seeds were rinsed with distilled water. Seeds were re-suspended in 0.1% (w/v) agar and dispersed on minimal 0.8% (w/v) agar containing 1mM KNO_3. Excess moisture was allowed to evaporate overnight before plates were incubated at 22°C under long day conditions (16 hours light / eight hours dark) for one to two weeks. Seedlings were removed from the agar with forceps and planted on Jiffy® pots (Jiffy Products International AS, Norway). Trays containing the Jiffy® pots were covered with cling wrap and incisions were cut into the cling wrap approximately four days later. Plants were watered three times per week. The seedlings were grown at 25-26°C under long day conditions with a light intensity of 300-500 lum/sqf and a relatively humidity of 50% to 55% for approximately three weeks. The conditions were monitored by the HOBO® data logger (Onset computer Corporation, Bourne, USA).

2.2.3. Bacterial strains and growth conditions

Escherichia coli cell lines used in this study were DH5α and DB3.1. DH5α cells were transformed with the constructed vectors and stored in 70% glycerol stock. DB3.1 cells were used to propagate the empty pMDC32 destination vector (Curtis and Grossnikklaus, 2003). Cell lines were cultured on Luria-Bertani (LB) media (1% Tryptone, 0.5% Yeast extract, 1% NaCl, and 1.5% Agar) for 16 hours at 37°C with 100 µg/ml spectinomycin for recombinant pCR8®/GW/TOPO® and 50 µg/ml kanamycin for recombinant pMDC32. The propagation of the empty destination vector required an additional 20 µg/ml chloramphenicol.

Agrobacterium tumefaciens strain LBA4404 was cultured in yeast extract peptone (YEP) medium (1% yeast extract, 1% bacto-peptone and 0.5% sodium chloride) at 28°C for three days containing 50 µg/ml rifampicin, 30 µg/ml streptomycin and 50 µg/ml kanamycin, which was specific for the recombinant destination vector.

2.2.4. RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from infected leaves and stems of Kil-*0* and Be-*0* plants with the QIAzol™ Lysis reagent (Qiagen, Valencia CA, USA) following the manufacturer's instructions. RNA yield was determined by measuring absorbance at 260 nm using a Nanodrop® ND-100 Spectrophotometer (Nanodrop Technologies, USA). The extracted samples were digested with RNase-free DNaseI (Roche, Basel, Switzerland) to remove genomic DNA contamination. Residual DNaseI was inactivated by heating the reaction at 65°C for ten minutes and removed from the sample using the RNeasy® Plant MinElute™ Cleanup Kit (Qiagen) following the manufacturer's guidelines.

Five micrograms of RNA was incubated with 0.5 µl of RNasin RNase Inhibitor (Promega, Wisconsin, USA) and 0.5 µg/µl of oligo dT at 70°C for ten minutes, followed by first-strand cDNA synthesis using Improm-II Reverse Transcriptase (Promega) according to the manufacturer's instructions. A single cycle of 25°C for ten minutes, 42°C for 60 minutes and 70°C for 15 minutes was performed. The cDNA samples were then tested for genomic DNA contamination by using an intron-spanning primer pair (AtUBQ10_F, ATTCTCAAAATCTTAAAAACTT and AtUBQ10_R, TGATAGTTTTCCCAGTCAAC) that amplifies a fragment of the *A. thaliana Ubiquitin 10* (*AtUBQ10*; At4G05320) gene. The reaction conditions were 0.4 µM of each primer, 0.4 mM dNTPs, 0.4 U Excel High Fidelity Taq (Southern Cross) and 1X Buffer with a final reaction volume of 20 µl and the cycle conditions were denaturation at 94°C for one minute, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and elongation at 72°C for 60 seconds followed by a final extension cycle at 72°C for five minutes. The PCR products were analysed using agarose gel electrophoresis. Amplification of an 850 bp fragment indicated the presence of genomic DNA contamination and a fragment size of 545 bp indicated the presence of a cDNA product.

2.2.5. Vector construction

2.2.5.1. Cloning the coding sequence of *AtPRX34* into the pCR8®/GW/TOPO® vector

The complete coding sequence of *AtPRX34* was amplified from Kil-*0* cDNA using the following primers: AtPRX34_F, CCTCCAGCTTTGACCCGTTT and AtPRX34_R,

TCTTGTAGCCACATATTGG. An 1196 bp fragment was amplified using the following reaction conditions: 0.4 µM of each primer, 0.4 mM dNTPs, 2.5 U TaKaRa Ex Taq (Separations) and 1X Buffer with a final reaction volume of 20 µl. The cycle conditions were: denaturation at 94°C for two minutes, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 59°C for 30 seconds and elongation at 72°C for 90 seconds followed by a final elongation cycle at 72°C for 30 minutes. The PCR product was purified using the Nucleospin Extract II Kit (Separations, Machery-Nagel, Düren, Germany) according to the manufacturers' guidelines.

The purified PCR product was cloned into the pCR8®/GW/TOPO® vector (Invitrogen, Appendix A1) and transformed into One Shot® chemically competent cells (Invitrogen) according to the manufacturer's instructions. Colony PCR was performed with a vector-specific primer pair: M13_F_29 (CACGACGTTGTAAAACGAC) and M13_R_narrow (GGAAACAGCTATGACCATG) to confirm the presence of the *AtPRX34* CDS and with a combination of a vector-specific and gene-specific primer pair (M13_F_29 and AtPRX34_R) to determine the orientation of the insert. The reaction conditions mentioned in section 2.2.4 were used and the cycle conditions were: denaturation at 94°C for one minute, 30 cycles of denaturation at 94°C for 20 seconds, primer annealing at 60°C for 30 seconds and elongation at 72°C for three minutes followed by a final elongation cycle at 72°C for five minutes

pCR8®/GW/TOPO®-*AtPRX34* (Appendix A2) plasmid was isolated from three positive colonies according to the manufacturer's instructions using the GeneJet Plasimd Miniprep Kit (Fermentas, Ontario, Canada). The purified plasmid DNA was used as template for DNA sequencing to confirm the correct sequence and orientation of the fragment with the above-mentioned primers. DNA sequencing was performed by MacroGen (Maryland, USA) and analysed with Vector NTI Advance 9.1 Software (Invitrogen).

2.2.5.2. Construction of the plant *AtPRX34* expression vector

The destination vector used in this study was a Gateway® enabled vector, pMDC32 (Appendix A3; Curtis and Grossniklaus, 2003). The *AtPRX34* fragment was LR-cloned from pCR8®/GW/TOPO®-*AtPRX34* entry vector into pMDC32 destination vector by using Gateway LR Clonase II Enzyme mix (Invitrogen; Figure 2.2) and cloned into One Shot® chemically competent cells. Positive colonies were selected on selective media containing 50 µg/µl of kanamycin. Positive transformed colonies were confirmed using colony PCR with the same vector-specific and gene-specific primer combination and PCR conditions as mentioned in section 2.2.5.1. pMDC32-*APRX34* (Appendix A4) plasmid DNA was isolated

from individual colonies using the GeneJet Plasmid Miniprep Kit (Fermentas) and used as a template for DNA sequencing to verify that the correct insertion event occurred.

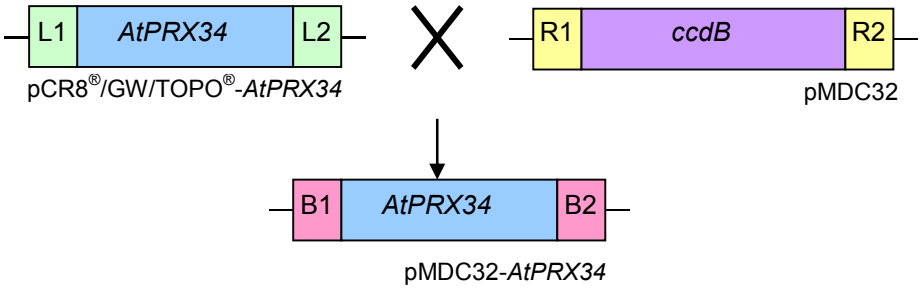


Figure 2.2. Diagram of the Gateway vector system illustrating the complementary recombination facilitated by Gateway LR Clonase II Enzyme mix (Invitrogen). The LR Clonase II enzyme mix enables a fragment (*AtPRX34*) flanked with *att*L sites to be transferred into a destination vector (pMDC32) containing *att*R sites, producing an expression clone with *att*B sites (Karimi et al., 2007).

2.2.6. Transformation of competent *Agrobacterium tumefaciens*

Frozen LBA4404 competent cells were transformed with 500 ng of recombinant pMDC32-*AtPRX34* vector DNA by exposing the mixture of cells and vector to heat shock at 37°C for five minutes. The mixture was then cooled rapidly on ice. A 900 µl of yeast extract peptone (YEP) medium was added and the culture was incubated at 28°C for four hours with shaking (250 rpm) followed with centrifugation at 6450 x *g* for three minutes. The supernatant was removed and the pellet was re-suspended in the remaining YEP media. The cells were plated out on 15% (w/v) YEP agar plates containing 50 µg/ml rifampicin, 30 µg/ml streptomycin and 50 µg/ml kanamycin and incubated at 28°C for three days. *Agrobacterium* colonies transformed with pMDC32-*AtPRX34* were confirmed by performing a colony PCR with the same vector-specific and gene-specific primer combination and cycle conditions as mentioned in section 2.2.5.1.

2.2.7. Transformation of *A. thaliana* ecotypes

A positively identified pMDC32::*AtPRX34 Agrobacterium* colony was used to inoculate five ml of YEP medium containing the appropriate antibiotics. The culture was incubated at 28°C for two days with agitation. Two millilitres of the overnight culture was used to inoculate 200 ml YEP containing the same antibiotics and incubated at 28°C for two days with shaking. Cells were pelleted by centrifugation at 2576 x *g* for 15 minutes and re-suspended in 5% (w/v) sucrose with an OD_{600} measurement of 0.8 to 1 (Adjusted with 5%

(w/v) sucrose if required). The surfactant, Silwett L-77 (LETHEL, Round Rock, Texas, USA), was added to the transformation culture to a concentration of 0.05%.

A modified version of the Clough and Bent (1998) floral dip method was used to transform wildtype *A. thaliana* Be-*0*, Kil-*0* and Col-*0* plants. Secondary inflorescence stems of the plants were immersed in the transformation medium for approximately 60 seconds. The excess bacterium was shaken off and the plants were placed on their sides in trays. The trays were covered and kept moist overnight. The following day the plants were placed upright again. The floral dip process was repeated a week later with the same plants. Transgenic generation 1 (T1) seeds were harvested from the infected siliques (Clough and Bent, 1998).

2.2.8. Selection of transgenic *Arabidopsis* plants

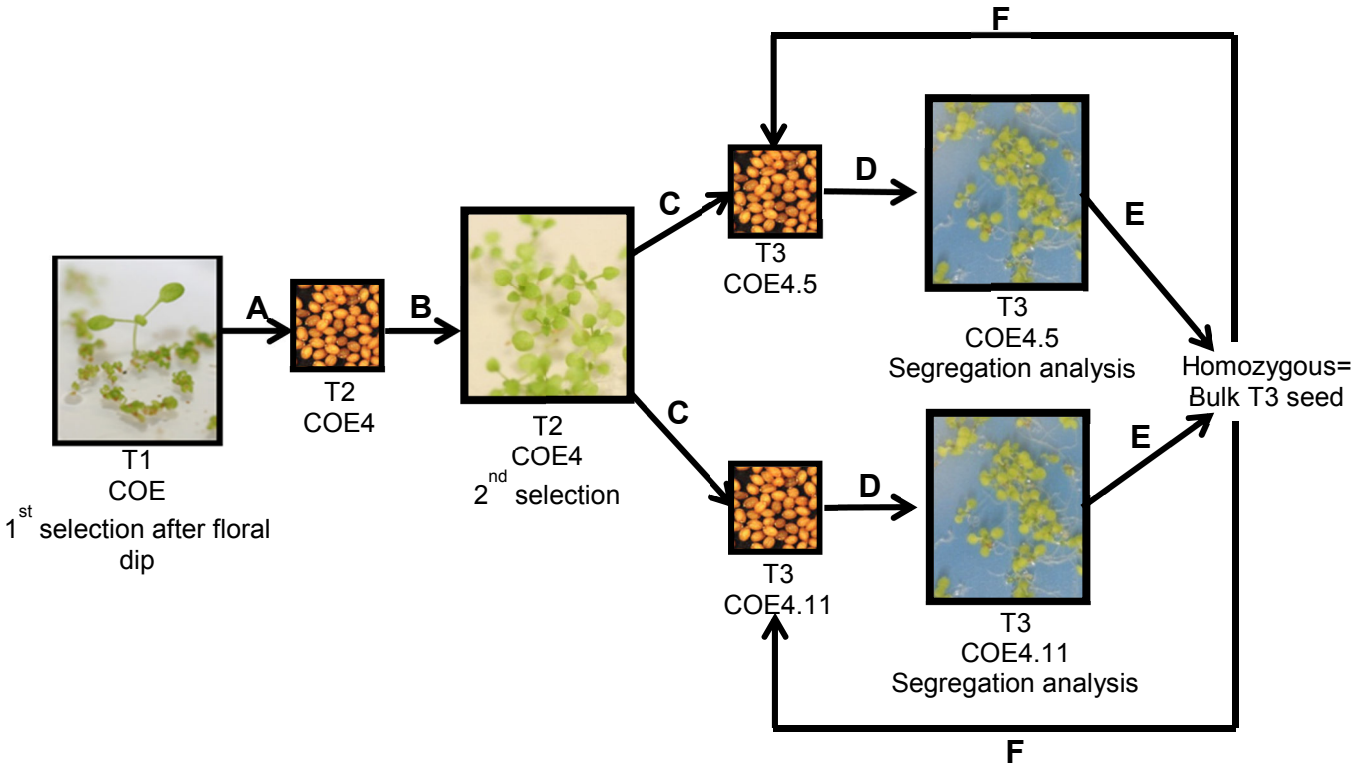
2.2.8.1. *Arabidopsis* seed selection

One hundred microlitre of T1 seeds were surface sterilized as described in section 2.2.2 and sown on minimal agar containing 20 mg/L hygromycin B and 100 mg/L cefotaxime. Seedlings were considered positive transgenic lines when roots developed into the antibiotic agar. Rooted seedlings were transferred to Jiffy® pots after a week. The seedlings were allowed to self-pollinate and T2 seed were collected and selected in the same way as T1 seed. The seedlings were named according to the background in which they were transformed, the construct used and designated with a specific numbers. The first number represents an individual transgenic line identified at the T2 generation for a specific event and the second number represent individual lines identified at T3 generation for a specific event, for example BOE1.1 (Be-*0* overexpression of *A. thaliana PRX34* in transgenic event 1 for both T2 and T3), KOE1.1 (Kil-*0*) overexpression of *AtPRX34* in transgenic event 1 for both T2 and T3) and COE1.1 (Col-*0* overexpression of *AtPRX34* in transgenic event 1 for both T2 and T3) as illustrate in Figure 2.3.

2.2.8.2. PCR analysis of pMDC32-*AtPRX34* transgenic lines

Genomic DNA was extracted from antibiotic resistant T1, T2 and T3 plants using the NucleoSpin II Plant DNA Extraction Kit (Machery-Nagel, Düren, Germany) following the manufacturer's instructions. A vector-specific (pMDC32_B_R, TGATAATCATCGCAAGACCGGCAAC) and gene-specific (AtPRX34_F) primer pair was used to screen for positive transgenic plants (Appendix A4). The PCR had an initial denaturation step of 94°C for two minutes, followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C and elongation for 90 minutes at 72°C with a final elongation step of five minutes at 72°C. The reaction conditions as mentioned in

section 2.2.4 were used and produced a 1373 bp fragment.**Figure 2.3.** Illustration of the transgenic generation and the bulking of the T3 seeds after segregation analysis. The seeds collected from the floral dip



flowers were denoted as T1 seeds. These T1 seeds were germinated on selective media. Seeds were collected from individual plants that survived the selection process (A) and denotated as T2 seeds. These seeds were germinated on selective media (B) and again individual plants that survived the selection process seeds (T3) were collected (C). A subsample of the T3 seeds were analysed for homozygosity (D) and if the Chi-DIST analysis indicated that multiple lines (COE4.5 and COE4.11) were homozygous (E), the remaining T3 seeds were bulked (F) and this bulk was called COE4.

2.2.8.3. Homozygosity selection

Firstly, an effective homozygosity screening method was developed by using Be-*0*, Col-*0*, BOE1 and a homozygous transgenic in the Col-*0* background (confirmed and provided by Ms Marja O'Neill, MSc at University of Pretoria) and were plated on 1% Murashige and Skoog media (1 g/l MS salt, 30 g/l sucrose and 8 g/l agar, pH 5.9) without or with different concentration of hygromycin B (50 µg/µl, 100 µg/µl and 150 µg/µl). Secondly, homozygosity was screened by surface sterilization of the T3 seeds from individual plants as described in section 2.2.2, followed by sowing of approximately a 100 seeds per plate (1% MS media with 50 µg/µl hygromycin B). After a week the number of plants that grew with root development and the number of plants that started to grow but died because of selection were documented. In Microsoft Office Excel 2007, the Chi-DIST (*p*-value) values were calculated by hypothesizing that the transgenic line was homozygous when 99% of

the plants survive and only 1% die. The outcome of the same transgenic lines being hemizygous were also tested, by hypothesizing that 75% of the T3 plants would survive and 25% would die.

2.2.9. Reverse transcription quantitative PCR analysis of transgenic plants

Four-week-old plants of the different ecotypes (Be-*0*, Kil-*0* and Col-*0*) and different T3 homozygous plants (BOE1, BOE5, BOE6, COE3, COE4, COE12, KOE17, KOE32) were used to prepare samples for RT-qPCR. Three biological replicates for each individual line were collected and three separate plants were bulked per biological rep. RNA was isolated from the aerial parts of the plants with the NucleoSpin II RNA Plant Kit (Machery-Nagel, Düren, Germany) following the manufacturer's instructions. The rest of the sample preparation was previously discussed in section 2.2.4.

RT-qPCR analysis was according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Primers used for RT-qPCR were designed using Primer Designer 4 v4.20 (Sci Ed Central, Cary, North Carolina, USA) to amplify short fragments between 170 bp and 300 bp and synthesized by Whitehead Scientific (Cape Town, South Africa; Table 2.1). One microliter of 1:10 diluted cDNA was used with 5 µl of LightCycler® 480 SYBR Green I Master mix 2 x concentration (Roche, Mannheim, Germany) and 0.5 µl each of the primers with a final PCR reaction volume of 11 µl and performed on the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, GmbH, Basel, Switzerland). RT-qPCR cycle conditions included a pre-incubation step to activate the FastStart Taq at 95°C for five minutes and an amplification step with 45 cycles of 95°C for ten seconds, 62°C for ten seconds and 72°C for 15 seconds with a single acquisition at the end of each cycle. This was followed by melting curve analysis with one cycle of 95°C for five seconds, 65°C for one minute and 95°C continuously with ten acquisitions per 1°C and a cooling step of 40°C for ten seconds. The samples were analysed in triplicate. Absolute quantification /2nd derivate Max software on the LightCycler® 480 was used to obtain the crossing point values for each sample. The crossing point data was imported into qBASE*plus* v1.0 for relative quantification and normalization (Hellemans et al., 2007) and final analyses were conducted in Microsoft Office Excel 2007. Significance was determined by a two-tailed Student *t*-test ($p<0.05$ and $p<0.01$) between the three biological replicates of the transformed and untransformed plants.

Table 2.1. RT-qPCR primers for target and reference genes used in the analysis of the transgenic plants.

Gene Name	AGI number	Sequence forward 5'-3'	Sequence reverse 5'-3'	Product size (bp)
ENDO	At3G49120	GGAGGCAATGAATAGGATGG	ATATGTATTCTTGTAGCCAC	177
EXO	At3G49120	TCGAGGCGCGCCAAGCTATC	AGGACATGACCTATCGTAGA	295
AtCBP20	At5G44200	AGCTCGTGGATTACGGTACT	TTCTCCGGTCTCATGTCACT	238
AtEF1GTP	At1G18070	AAGCACCGGAAGACTTGA	CATCCACCTGACCACTAAGA	279
AtUBQ5	At3G62250	GGTGGTGCTAAGAAGAGGAA	TCGATCTACCGCTACAACAG	273
AtACT2	At3G18780	TCATACGGTCAGCGATACCT	CATCACGATTGGTGCTGAGA	242
AtEF1	At1G07920	GTTGAGATGCACCACGAGTC	TCCTTACCAGAACGCCTGTC	284
AtTUB4	At5G44340	GAGCGAACAGTTCACAGCTA	GCTGCTTGCTTACACAGCTT	242
18S	18S rRNA	AGTAAGCGCGAGTCATCAGC	TCCTTCCGCAGGTTCACCTA	214

Amplification efficiencies were determined for each primer pair by generation a serial dilution (1:2, 1:10, 1:20 and 1:50) of cDNA isolated from KOE32 biological replicate 2. The primer sequences are indicated in Table 2.1. The *18S rRNA* (*18S*) target was used for the internal run calibration to normalize between different runs. Six possible reference targets [*CAP binding protein 20* (*AtCBP20*), e*longation factor 1α-related GTP binding protein* (*AtEF1GTP*), *actin 2* (*AtACT2*) and *ubiquitin 5* (*AtUBQ5*), *elongation factor 1α* (*AtEF1*) and *tubulin 4* (*AtTUB4*)] were tested to determine which showed stable expression level between the different samples and could be used for normalization of the target genes. Transcript abundance levels of the targets of interest, endogenous *peroxidase 34* (ENDO) and transgene *peroxidase 34* (EXO) were normalized to the raw transcript abundance levels of *AtEF1* and *AtTUB4*.

2.2.10. Analysis of peroxidase activity in transgenic plants

The same four-week-old frozen plant material that was used to prepare RNA samples for RT-qPCR was used to extract crude protein. The proteins of three biological replicates of each plant line were extracted by grinding 100 mg of aerial parts of the plant in 100 mM Tris-HCl buffer (pH 7.2) containing 5 mM 2-mercarptoethanol and 0.25 M sucrose. The samples were centrifuged at 14000 x *g* for five minutes and the supernatant was collected as the soluble protein fraction (cytosolic proteins). The pellet was re-suspended in 20 mM Tris-HCl buffer (pH 7.2) containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ and shaken end-over-end at 4°C overnight. The supernatant was collected as the

peripheral bound protein fraction (ionically membrane-bound proteins) after centrifugation at 14000 x *g* for five minutes (Bindschelder et al., 2006). The protein concentration for each sample was determined by using the Bradford assay (Bradford, 1976).

A serial dilution of that sample was generated in Tris buffered saline pH 7.2 (TBS, 25 mM Tris and 150 mM NaCl). The absorbance was measured at 595 nm on the Multiskan Ascent plate reader (Thermo Labsystems, AEC Amersham) with the addition of 50 µl of the Bradford reagent (Coomassie® Brilliant Blue G-250 dye, Sigma, USA). A standard curve for bovine serum albumin (BSA) was generated by measuring the absorbance at 595 nm using a serial dilution of 10 mg/ml solution of BSA (Bradford, 1976).

The peroxidase activity was determined using a serial dilution of 0.4 mg/ml soluble and 0.26 mg/ml bound protein extracted by measuring the oxidation of *o*-phenylenediamine dihydrochloride (OPD, Sigma, Missouri, USA) in 10 mM sodium citrate buffer pH4.5 and 0.1 mM H_2O_2 at 450 nm within one minute. The oxidation of the substrate was measured as the increase in absorption from a colourless to an orange solution (Figure 2.4). The peroxidase activity in the protein sample was expressed as absorbance (nm) per minute per protein concentration (µg/ml).

Figure 2.4. Illustration of the catalytic activity of peroxidases to oxidize the substrate, *o*-phenylenediamine dihydrochloride (colourless solution) to 2,3-diaminophenazine (orange solution) in sodium citrate buffer with H_2O_2. A: Extracted soluble protein fraction, B: Extracted bound protein fraction, C: Positive control and D: Negative control.

2.2.11. Plant-pathogen challenges

2.2.11.1 Growth and maintenance of biological material

Seed of the different ecotypes (Be-*0*, Kil-*0* and Col-*0*) and different transgenic plants (BOE1, BOE5, BOE6, COE3, COE4, COE12, KOE17, and KOE32) were surface sterilized with 70% ethanol, 1.5% sodium hypochlorite and rinsed three times with distilled water. The seeds were re-suspended in 1% liquid MS and seed-agar solution was dispersed on solid

MS media by dripping drops of the solution containing approximately 5 seeds evenly over the plate. The plates were sealed with Parafilm and wrapped in foil before being incubated at 4°C for two days. The plates were further incubated at room temperature for 24 hours where after the foil was removed and the seeds on the plates were grown at 22°C for another two weeks under long day conditions. The seedlings were planted on jiffy® pots and grown as previously described for four weeks. Plants were watered three times a week with a solution of Multifeed Classic (http://www.plaaskem.co.za/) with removal of any inflorescence stems during the experiment.

Glycerol stocks of *R. solanacearum* isolate CK (BCCF402) were streaked out onto Bacto-agar Glucose Triphenyltetrazolium chloride media (BGT, 10 g/l peptone, 1 g/l yeast extract, 15 g/l bacto-agar, 4 ml of 1.25% triphenyltetrazolium and 25 ml of 20% glucose) and incubated at 28°C for 48 hours. Six loops of mucoid (displaying a virulent phenotype) bacteria per plate were transferred into 200 ml of liquid B media (10 g/l peptone, 1 g/l casmino acid, 1 g/l yeast extract and 25 ml of 20% glucose). Inoculum was incubated overnight at 28°C with agitation (250 rpm) (Deslandes et al., 1998).

2.2.11.2 Plant infections

The concentration of the overnight culture was determined by taking a measurement at 600 nm. An OD_{600} of one is estimated to be $1X10^9$ cfu/ml. The overnight culture was diluted to a concentration of $1X10^8$ cfu/ml with distilled water and the same dilution factor was used to dilute B media for the control inoculum. The bottom part (2 cm) of the Jiffy® pot was cut horizontally through the middle to wound and expose the roots of the plants. Cut Jiffy® pots were soaked in trays containing 500 ml of diluted bacteria solution or 500 ml of diluted B media for 30 minutes and placed on moist vermiculite. The growth conditions were the same as mentioned previously (Section 2.2.2) and constantly monitored with the data logger for two weeks. During this period the plants were scored based on the development of classic wilting symptoms (Deslandes et al., 1998) (Table 2.2).

The disease score was used to generate a disease index of the progression of disease symptoms of the plant. Average disease rating was calculated by the following formula: Average disease rating = $\sum v_i$ / N and disease index by the following formula: Disease index = $[\sum (n_i \text{ X } v_i) / (V \text{ X } N)]$ (Deslandes et al., 1998), where n_i = number of plants with respective disease score, v_i = disease score (0, 1, 2, 3, 4, 5), V = highest possible disease score (5) and N = the total number of plants used for scoring disease symptoms (Winstead and Kelman, 1952). In this experiment, the plant-pathogen trials were repeated three times with 12 plants each and the average disease index was calculated.

Table 2.2. Disease scale for scoring wilting development of *A. thaliana* plants infected with *R. solanacearum.*

Disease rating	Disease symptoms
0	No disease
0.5	First leaf show wilting symptoms
1	More than one leaf wilts and < 25% of leaves show wilting
2	25% - 50% of leaves show wilting
3	50% - 75% of leaves show wilting
4	75% - 100% of leaves show wilting
5	Plant death

2.3. Results

2.3.1. *AtPRX34* expression analysis under various biotic conditions

The expression profile of *AtPRX34* levels were investigated to determine if *AtPRX34* levels were increased during biotic stresses and to hypothesize a possible role for *AtPRX34* in plants defence. The expression profile for the following conditions was investigated: pathogen challenges (*P. syringae*, *P. infestans*, *A. brassicicola* and *B. cinerea*) and during PAMPs recognition. The PAMPs includes bacterial produced; LPS (Lipopolisaccharides which are cell surface molecules), Flg22 [Flagellin 22 recognised by Flaggellin-sensing 2 receptor (FLS2)], HrpZ (Hypersensitive reaction and pathogenicity Z) and EF-Tu [Elongation Factor-Tu recognised by EF-Tu Receptor (EFR)] and oomycete produced NPP1 (Necrosis-inducing Phytophthora Protein 1).

These results are summarized in Table 2.3. *P. infestans*, *P. syringae* AvrRpm1 and *B. cinerea* induced a fold change larger than 1.65 for *AtPRX34*. This threshold was used because this was the same cut-off value used in the microarray experiment that identified *AtPRX34* as a possible candidate gene for plant defence response (Naidoo, 2008). *AtPRX34* transcript levels were significantly induced in most of the pathogen challenges except in *A. brassicicola*. After recognition of some of the PAMPs (LPS, HrpZ and NPP1), the *AtPRX34* expression level was significantly induced (p<0.05). These results suggest that *AtPRX34* is induced during biotic stresses in several systems.

Table 2.3. Summary of the expression profile of *AtPRX34* under various conditions: AT-106, AT-108, AT-161, AT-147, AT-128 and AT-107.

Array identity	Conditions	Description of conditions	Fold changes	T-test results
Pathogen challenges				
AT-106	*P. syringae*	Time points: 2hr, 6hr and 24hr. Compared $MgCl_2$ treatment to DC3000, respectively	1.12 0.19 1.61	0.01* 0.49 0.01*
		Time points: 2hr, 6hr and 24hr. Compared $MgCl_2$ treatment to AvrRpm1, respectively	0.99 0.75 1.91[a]	0.06 0.06 0.01*
AT-108	*P. infestans*	Time points: 6hr, 12hr and 24hr compared to water control, respectively	3.31[a] 1.49 1.99[a]	0.01* 0.01* 0.01*
AT-161	*A. brassicicola*	48hr compared to $MgSO_4$ treatments	0.58	0.33
AT-147	*B. cinerea*	Time points: 18hr and 48hr compared to untreated, respectively	1.76[a] 1.79[a]	0.04* 0.01*
PAMPs				
AT-107	Flg22	Time points: 1hr and 4hr compared to water treatments, respectively	0.52 0.39	0.08 0.19
AT-107	FLS	Time points: 1hr and 4hr compared to water treatments, respectively	1.48 -0.72	0.01* 0.13
AT-107	HrpZ	Time points: 1hr and 4hr compared to water treatments, respectively	0.60 1.27	0.04* 0.01*
AT-107	NPP1	Time points: 1hr and 4hr compared to water treatments, respectively	0.88 1.41	0.02* 0.01*
AT-128	EF-Tu	Time points: 0 min, 30 min and 60 min. 0 min was taken as control	-0.08 0.06	0.43 0.75

[a] - Fold change > 1.65.

* - Displaying a significant difference from the control treatments (p<0.05, Student's t-test).

2.3.2. Construction of *AtPRX34* overexpression vector

The complete open reading frame of *AtPRX34* was amplified from Kil-*0* cDNA, which was synthesised from extracted RNA. The amplified product was analysed using agarose gel electrophoresis (Figure 2.5) and the purified fragment was cloned into the T/A entry vector, pCR8®/GW/TOPO® (Invitrogen). This ligation mixture was used to transform One

Shot® (Invitrogen) chemically competent cells. Various colonies were screened for the insertion of the pCR8®/GW/TOPO®-*AtPRX34* construct and the orientation of the fragment was determined by colony PCR. The amplified products were analysed by agarose gel electrophoresis (Figure 2.6 A) with the expected fragment sizes for the vector-specific primers being 1245 bp and for the vector-specific and gene-specific primer combination, 1224 bp.

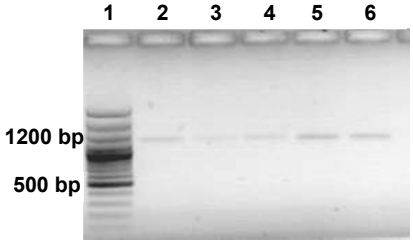


Figure 2.5. A 1.5% (w/v) agarose gel electrophoresis of an 1196 bp fragment indicating the ORF of *AtPRX34* amplified from Kil-*0* cDNA using AtPRX34_F and AtPRX34_R primers. Lane 1 is a 100 bp DNA size standard (GeneRuler™ 100 bp Plus DNA Ladder, Fermentas) and lane 2 to 6 contains amplified *AtPRX34* products.

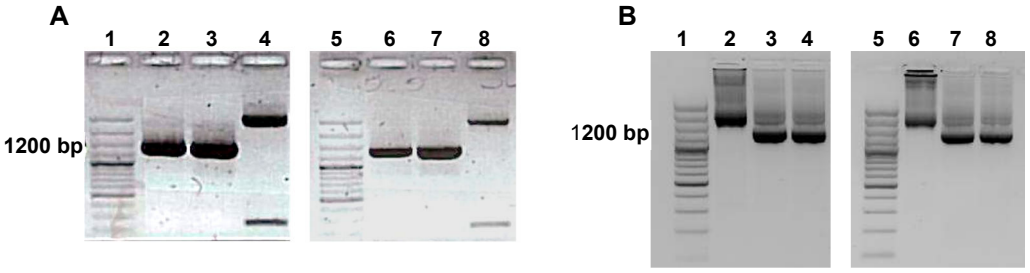


Figure 2.6. A 1.5% (w/v) agarose electrophoresis of colony PCR products amplified using two primer combinations. A. Colony PCR products amplified from pCR8®/GW/TOPO®-*AtPRX34* in *E. coli* cells. Lane 1 and 5 contain a 100 bp DNA size standard, lane 2 to 4 indicate primer pair, M13_F_29 and M13_R_narrow (1245bp) and lane 6 to 8 primer pair, M13_F_29 and AtPRX34_R (1224bp). Lane 4 and 8 indicate amplification of an empty vector. B. Colony PCR products amplified from pMDC32-*AtPRX34* in *A. tumefaciens* cells. Lane 1 and 5 contain a 100 bp DNA size standard, lane 2 to 4 indicate primer pair, M13_F_29 and M13_R_narrow and lane 6 to 8 primer pair, M13_F_29 and AtPRX34_R. Lane 2 and 6 indicate amplification of a vector with the incorrect fragment insert.

The sequences of three clones: 2, 5 and 8, were analysed by aligning them to the Col-*0*, *AtPRX34* sequence obtained from the TAIR website (http://www.arabidopsis.org) and the alignment indicated that all three clones contained base-pair mutations which could be the result of mutations occurring due to PCR-induced errors during amplification from the cDNA and/or due to differences between the two ecotypes, Col-*0* and Kil-*0*. Some of these mutations led to amino acid substitutions (Figure 2.7). In the figure, substitutions at position 29 and 270 indicate possible ecotype differences, because all three clones contain these

mutations and the variation is only seen between the ecotypes. Other substitutions also occurred within the sequences: a nonsense mutation occurring in clone 2 at position 137. Clone 8 contained a missense mutation caused by cDNA amplification, resulting in the change from a leucine to a proline. Both these amino acids contain a non-polar hydrophobic side-chain. Three-dimensional protein construction of clone 8 sequence indicated that this mutation is unlikely to affect the structure of the *AtPRX34* protein (Appendix B1, constructed by Prof. Fourie Joubert) and that the mutation occurred away from the active sites of the peroxidase (Appendix B2). As such, clone 8 was used in subsequent procedures.

The pCR8®/GW/TOPO® entry vector contains *att*L sites which flanks the T/A cloning site and pMDC32 contains *att*R sites which flanks *ccdB* gene. The *AtPRX34* fragment from pCR8®/GW/TOPO®-*AtPRX34* was transferred to pMDC32 and the *ccdB* gene was transferred from pMDC32 into pCR8®/GW/TOPO® by LR Clonase II Enzyme mix, simultaneously (Figure 2.2). The *ccdB* gene is lethal in DH5α cell line and this results in no pCR8®/GW/TOPO®-*ccdB* recombinant colony growth. The pMDC32 destination vector contains a double 35S CaMV promoter responsible for constitutive expression of the inserted gene and contains an antibiotic resistant gene, hygromycin.

The *AtPRX34* fragment from the selected pCR8®/GW/TOPO®-*AtPRX34* construct was transferred into pMDC32 by the LR reaction and transformed into One Shot® chemically competent cells. Colony PCR was performed as previously mentioned, followed by sequence analysis of the open reading frame. The pMDC32-*APRX34* construct contained the same amino acid substitutions as mentioned, indicating no additional substitutions occurred (Appendix B3). The transformation of the pMDC32-*APRX34* construct into *A. tumefaciens* LB4404 cells was confirmed by the colony PCR. The expected fragment sizes for the vector-specific primers (1245 bp) and for the vector-specific and gene-specific primer combination (1224 bp) were obtained (Figure 2.6 B).

2.3.3. Selection of transgenic *Arabidopsis* plants

Agrobacterium tumefaciens cells containing pMDC32-*AtPRX34* were used to transform *A. thaliana* ecotypes; Be-*0*, Kil-*0* and Col-*0* by using a modified version of the floral dip method (Clough and Bent, 1998). The T1 seeds were sown on minimal media containing hygromycin and cefotaxime to identify positively transformed plants (Figure 2.8). Three rooted individual T1 seedlings were transferred to jiffy pots and allowed to self-pollinate for each ecotype. The T2 and T3 seeds were screened with the same protocol.

```
                        10         20         30         40         50         60
AtPRX34_Col-0       MHFSSSSTSSTWTILITLGCLMLHASLSAAQLTPTFYDRSCPNVTNIVRETIVNELRSDP
AtPRX34-Clone 2     MHFSSSSTSSTWTILITLGCLMLHASLSTAQLTPTFYDRSCPNVTNIVRETTVNELRSDP
AtPRX34-Clone 5     MHFSSSSTSSTWTILITLGCLMLHASLSTAQLTPTSYDRSCPNVTNIVRETIVNELRSDP
AtPRX34-Clone 8     MHFSSSSTSSTWTILITLGCLMLHASLSTAQLTPTFYDRSCPNVTNIVRETIVNELRSDP

                        70         80         90        100        110        120
AtPRX34_Col-0       RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA
AtPRX34-Clone 2     RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA
AtPRX34-Clone 5     RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA
AtPRX34-Clone 8     RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA

                       130        140        150        160        170        180
AtPRX34_Col-0       CPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTLPQLKA
AtPRX34-Clone 2     CPRTVSCADMLTIAAQ*SVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTLPQLKA
AtPRX34-Clone 5     CPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTLPQLKA
AtPRX34-Clone 8     CPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTPPQLKA

                       190        200        210        220        230        240
AtPRX34_Col-0       SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP
AtPRX34-Clone 2     SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP
AtPRX34-Clone 5     SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP
AtPRX34-Clone 8     SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP

                       250        260        270        280        290        300
AtPRX34_Col-0       LNGNRSALVDFDLRTPTVFDNKYYVNLKERKGLIQSDQELFSSPNATDTIPLVRAYADGT
AtPRX34-Clone 2     LNGNRSALVDFDLRTPTVFDNKYYVNLKEQKGLIQSDQELFSSPNATDTIPLVRAYADGT
AtPRX34-Clone 5     LNGNRSALVDFDLRTPTVFDNKYYVNLKEQKGLIQSDQELFSSPNATDTIPLVRAYADGT
AtPRX34-Clone 8     LNGNRSALVDFDLRTPTVFDNKYYVNLKEQKGLIQSDQELFSSPNATDTIPLVRAYADGT

                       310        320        330        340        350        360
AtPRX34_Col-0       QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
AtPRX34-Clone 2     QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
AtPRX34-Clone 5     QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
AtPRX34-Clone 8     QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
```

Figure 2.7. Alignment of the predicted amino acid sequence of pCR8®/GW/TOPO®-*AtPRX34* (three clones) to the Col-*0* *AtPRX34* sequence obtained from the TAIR website (http://www.arabidopsis.org), indicating that all three pCR8®/GW/TOPO®-*AtPRX34* clones contained amino acid substitutions compared to Col-*0*. The black shadings indicate sequence identity and the white shadings indicate amino acid differences among sequences. Position 29 and 270 indicate ecotype differences between the Col-*0* and Kil-*0* ORF.

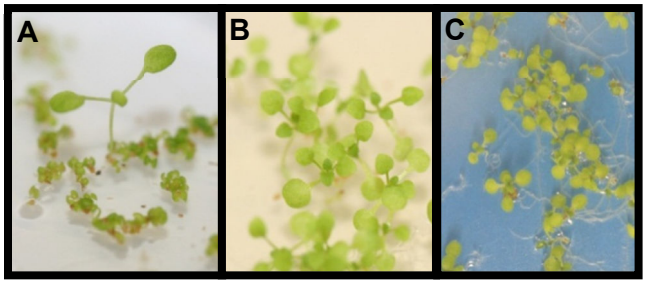


Figure 2.8. Hygromycin selection of *A. thaliana* seeds transformed with pMDC32-*AtPRX34*. A. T1 seeds screened after floral dip transformation. The black arrow indicates a transgenic T1 plant surrounded with germinated but non-transgenic plants. B. Hygromycin selection of T2 plants with more seedlings surviving compared to T1. C. T3 selected on hygromycin plates with visible root development.

Leaf material from antibiotic resistant T1, T2 and T3 lines were used to extract genomic DNA for PCR analysis to confirm the insertion of the pMDC32-*AtPRX34* T-DNA fragment into the genome of these lines. Because all these plants contain endogenous *AtPRX34*, a vector- and gene-specific primer combination was used to confirm T-DNA insertion. The amplified product was analysed using agarose gel electrophoresis (Figure 2.9). The correct fragment size of 1408 bp was obtained. This was absent in the untransformed plants.

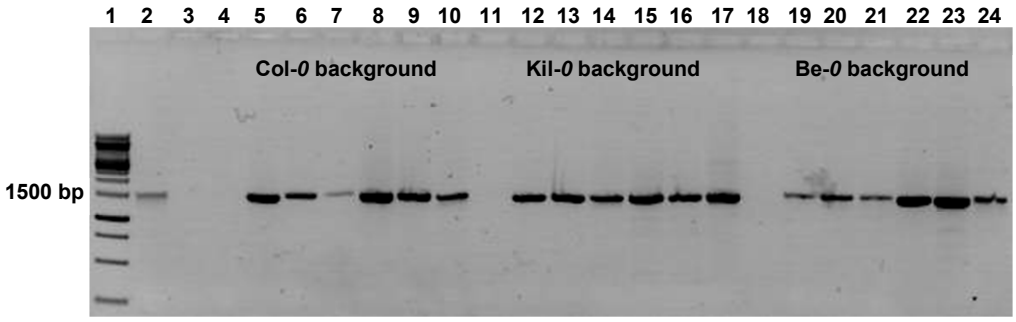


Figure 2.9. A 1.5% (w/v) agarose gel electrophoresis of the PCR products amplified from integrated pMDC32-*AtPRX34* into plant genomic DNA using primer pairs, AtPRX34_F and pMDC32_B_R generating product size of 1408bp. Lane 1 contains a 1kb DNA size marker, lane 2 contains plasmid DNA from pMDC32-*AtPRX34* (positive control), lane 3 is template-less PCR (negative control), lane 4, 11 and 18 are wildtypes: Col-*0*, Kil-*0* and Be-*0* respectively (negative control), lane 5 to 10 are Col-*0* lines (COE3, 4, 8, 11, 12 and 13), lane 12 to 17 are Kil-*0* lines (KOE17, 21, 26, 32, 33 and 40) and lane 19 to 24 are Be-*0* (BOE1, 2, 4, 5, 6 and 7).

Homozygous transgenic lines are necessary for further experiments and the correct screening for these transgenic lines was essential. The ecotype, Be-*0* did not grow effectively on minimal media and this made testing segregation difficult. MS media contains more nutrients for Be-*0* growth, which resulted in better plant growth. Various concentration of hygromycin (50 µg/µl, 100 µg/µl and 150 µg/µl) in MS media was tested to identify a suitable concentration for homozygosity analysis (Figure 2.10). Fifty micrograms per microliter was identified as the best concentration for testing segregation analysis and was used in subsequent experiments.

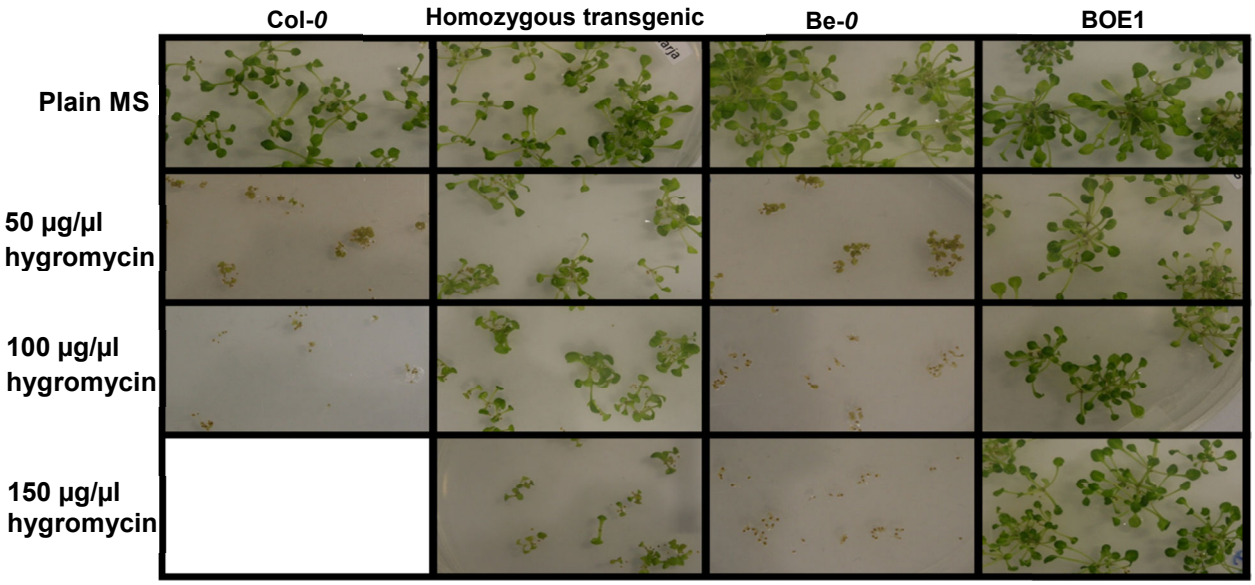


Figure 2.10. Assessing different hygromycin concentrations (50 µg/µl, 100 µg/µl and 150 µg/µl) for identifying a concentration for further segregation analysis. Col-*0* and a homozygous transgenic line provided by Ms Marja O'Neill in Col-*0* background served as a negative and positive control, respectively with assessment of the Be-0 and BOE1 line. All the seedlings survived on the MS media without any antibiotics and both the wildtypes seedlings died on all the hygromycin selected media. The homozygous transgenic seedlings struggled to grow on 100 µg/µl and 150 µg/µl hygromycin plates. The BOE1 line thrived on all three concentrations.

The T3 lines were screened for homozygosity by recording the number of seedlings that grew and the number of seedlings that died. The null hypothesis for a transgenic line to be homozygous was 99% of the seedling would survive and only 1% of the seedling would die. The Chi-DIST function in Excel was used which indicates the likelihood that the observed results fit best with the expected results. A small Chi-DIST value indicates that the hypothesis can be rejected and a large Chi-DIST value indicate that the transgenic line is more likely to be homozygous. Various overexpression events for all three ecotypes were screened (Table 2.4) and the individual plants per transgenic event were kept separate until homozygosity for each line was identified. Three homozygous lines for Be-0 and Col-*0* and two for Kil-*0* were generated and named: BOE1 (from BOE1.10), BOE5 (from BOE5.4 and BOE5.7), BOE6 (from BOE6.11), COE3 (from COE3.3), COE4 (from COE4.5 and COE4.11), COE12 (from COE12.1), KOE17 (from KOE17.9) and KOE32 (from KOE32.5).

Table 2.4. Chi-DIST analysis of various transgenic lines (T3) to determine the T-DNA segregation pattern and identify homozygous lines. High Chi-DIST values indicate that the hypothesis can't be rejected and low Chi-DIST values the hypothesis can be rejected.

Seed name	Chi-DIST for homozygosity[1]	Chi-DIST for hemizygosity[2]
BOE1.10	0.125	1.22E-18
BOE5.4	0.215	1.09E-12
BOE5.7	0.392	9.14E-18
BOE6.11	0.49	2.41E-15
COE3.3	0.237	3.94E-11
COE4.5	0.248	3.28E-11
COE4.11	0.032	7.35E-10
COE12.1	0.259	9.13E-11
KOE17.4*	2.99E-34	0.007
KOE17.9	0.029	1.47E-6
KOE32.5	0.60	3.28E-13

[1]- Null hypothesis: 99% of seedlings survived and 1% of seedlings could die.

[2]- Null hypothesis: 75% of seedlings survived and 25% of seedlings could die.

*- Indicate a transgenic line that was still segregating.

2.3.4. Reverse transcription quantitative PCR (RT-qPCR) analysis of transgenic plants

Total RNA was extracted from four-week-old plants from the previously mentioned homozygous T3 overexpression lines and from the three ecotypes. The extracted samples were analysed using agarose gel electrophoresis (Figure 2.11) and the concentrations ranged from 110 ng/µl to 550 ng/µl. First strand cDNA was synthesised for all the samples and screened for genomic DNA contamination by using an intron spanning primer pair targeting *AtUBQ10* (Figure 2.11). The presence of an 850 bp fragment indicated residual genomic DNA and a 545 bp amplicon indicated synthesised cDNA.

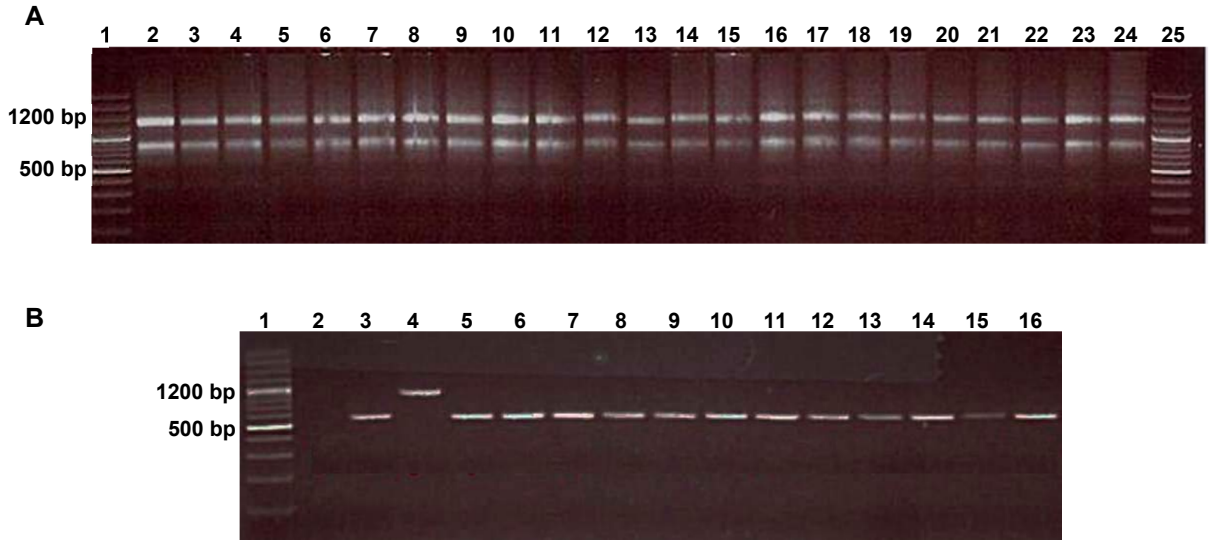


Figure 2.11. A 1.5% (w/v) agarose gel electrophoresis of RNA quality and amplified product of the *AtUBQ10* primers for a subset of the samples analysed. A. Total RNA was extracted from wildtypes, overexpression *AtPRX34* transgenic lines and SALK_051769 material. Lane 1 and 25 contain a 100 bp DNA size standard, lane 2- 4 contain isolated RNA from Be-0, lane 5- 7 are Kil-*0*, lane 8- 10 are Col-*0*, lane 11- 13 are SALK_051769, lane 14- 16 are BOE1, lane 17- 19 are BOE5, lane 20- 22 are BOE6, lane 23 and 24 are COE3. B. The PCR primers span an intron of the *AtUBQ10* generating product sizes of 850bp for gDNA and 545bp for cDNA. Lane 1 contain a 100bp DNA size marker, lane 2 is temple-free PCR (negative control), lane 3 is known cDNA (positive control), lane 4 is gDNA (control for gDNA contamination), lane 5- 7 indicates isolated RNA from Be-0, lane 8- 10 are Kil-*0*, lane 11- 13 are Col-*0*, lane 14- 16 are SALK_051769.

Relative quantitative transcript abundance analysis was performed to evaluate the expression level of *AtPRX34*, targeting both transgene (exogenous) and endogenous expression. The exogenous transcript levels were analysed to determine if the pMDC32-*AtPRX34* transgene was being expressed and the endogenous transcript levels were evaluated to determine the affect of the transgene on the normal *AtPRX34* expression levels. To evaluate the endogenous and exogenous expression levels between different samples accurately, normalization between samples is required and becomes more reliable if multiple references are used (Hellemans 2007).

The complete RT-qPCR analysis was performed with three different software programs. Firstly, the LightCycler® 480 melting curve analyses were performed to determine if a single melting peak for each primer pair was obtained (Appendix C1). The amplified products for each primer pair was also analysed using agarose gel electrophoresis to confirm that a single melting peak represented a single fragment (Figure 2.12) and the RT-qPCR products were sequenced to confirm correct fragment amplification (data not shown). The crossing point values for each biological and technical replicate were also generated with the LightCycler® 480 absolute quantification software and transferred into the second

program, qBASE*plus* v1.0 for determining the relative quantification for the target genes. This program evaluates the PCR amplification efficiency, calculates the reference genes stability and indicates the reproducibility between the technical replicates analysed. After the relative quantity for each gene was determined, the data was imported into Microsoft Office Excel 2007 for histogram construction and statistical analysis.

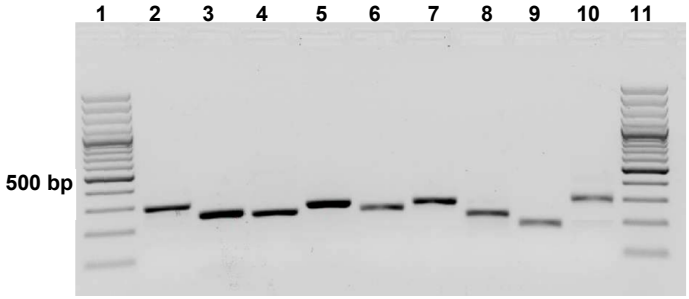


Figure 2.12. 1.5% Agarose gel electrophoresis of bulk RT-qPCR product of the reference and target specific primer pairs. Lane 1 and 11 are a 100 bp DNA size standard, lane 2 is *AtEF1GTP*, lane 3 is *AtCBP20*, lane 4 is *AtTUB4*, lane 5 is *AtEF1*, lane 6 is *AtACT2*, lane 7 is *AtUBQ5*, lane 8 is *18S*, lane 9 is ENDO and lane 10 is EXO.

The standard amplification curves for each gene were generated from a standard serial dilution (1:2, 1:10, 1:20 and 1:50) by calculating the PCR regression. The amplification efficiency was determined from the regression line for each primer pair with the highest standard error for the endogenous *AtPRX34* amplification at 0.07. This analysis takes the PCR amplification efficiency into account, while the R^2 values indicated correlation between the different dilution points (Table 2.5).

Normalization is required to correct for differences between sample cDNA quantity and quality. The stability for a single reference gene cannot be calculated, but multiple references can be evaluated with the qBASE normalization software. The software determines both the stability parameter (M) before normalization and the coefficient of variation (CV) after normalization of a specific reference gene compared to other selected reference genes. The threshold for these stability values were set at 0.5 and 25%, respectively and the lower the values, the more stable the expression of the reference gene. An ideal reference gene has a CV value of zero after normalization and the gene should show the same relative profile for all the samples analysed after normalization (Hellemans 2007). Various candidate references genes: *AtCBP20*, *AtEF1GTP*, *AtACT2*, *AtUBQ5*, *AtEF1* and *AtTUB4* were analysed with the normalization software (Appendix C2). *AtEF1* and *AtTUB4* exhibited the most constant expression levels across samples analysed (the transgenic lines and wildtypes), with an M value of 0.3 and CV of 10.5% (Appendix C3).

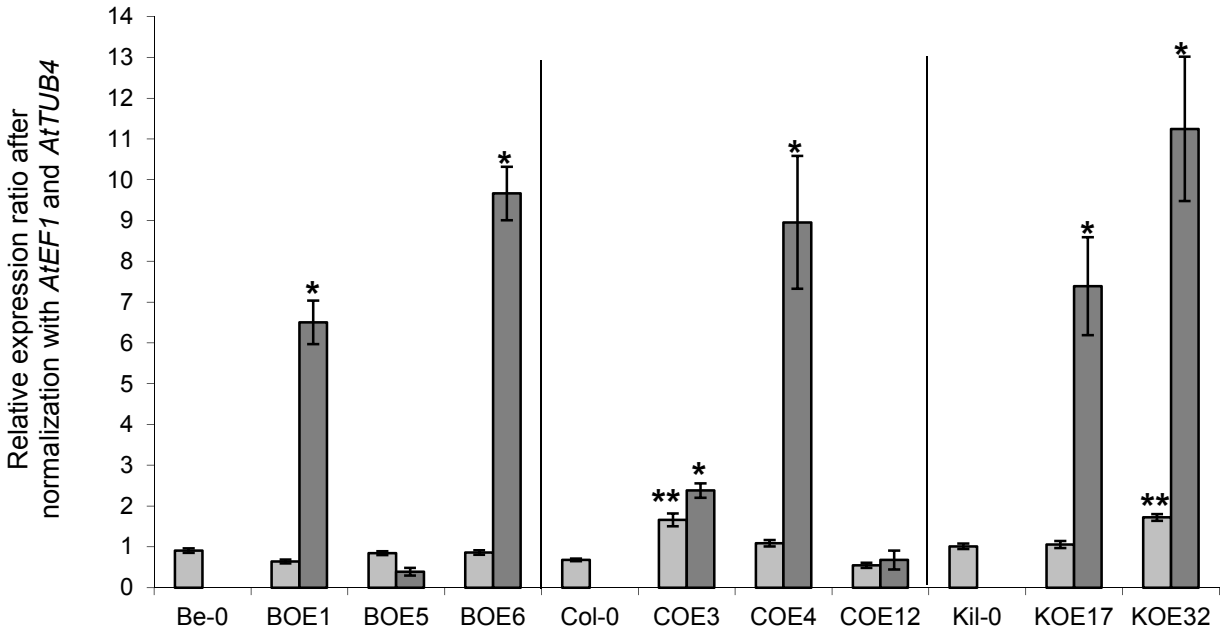
Table 2.5. Amplification efficiency of the reference and target specific primer pairs used for RT-qPCR analysis. The PCR efficiency was determined with linear regression analysis and the R^2 values indicated correlation between the serial dilution points.

Genes targets	Efficiency (E)	Standard error of the efficiency (SE)	R^2 value
AtEF1GTP	1.83	0.01	0.95
AtCBP20	2.42	0.01	0.98
AtTUB4	1.88	0.03	0.96
AtEF1	1.90	0.03	0.97
AtACT2	1.81	0.01	0.99
AtUBQ5	1.90	0.02	0.98
ENDO	2.14	0.07	0.94
EXO	1.79	0.02	0.99

The normalization factor was calculated by determining the geometric mean and standard deviation from *AtEF1* and *AtTUB4* values for each biological sample. This factor should be similar for each sample and when it is different it indicates variation in the starting quantities, problems with the PCR or the use of unstable reference genes (Derveax 2010). In experiments using different starting quantities, the normalization factors should not vary more than two to three fold among all the samples analysed. A fold difference of 1.5 between the normalization factors was detected in this experiment (data not shown). The expression levels of the target genes of interest (EXO and ENDO) were normalized with this calculated factor by dividing the crossing point value with the normalization factor of each sample. The technical reproducibility was determined by the software program for each biological sample by flagging the technical replicates with value differences larger than 0.5. Only nine samples resulted in technical variation larger than 0.5, which indicated a 97.01% technical reproducibility in this experiment.

The expression analysis of each transgenic *AtPRX34* line was compared to its respective wildtype control (Figure 2.13). High transgene expression was detected in BOE1, BOE6, COE4, KOE17 and KOE32 lines (p<0.01) with low exogenous transcript levels detected in BOE5 and COE12 and intermediate levels for COE3. The endogenous *AtPRX34* transcript levels were analysed to investigate the affect of overexpressing Kil-*0 AtPRX34* in these transgenic plants. BOE1 and COE12 had lower endogenous expression of *AtPRX34*, but not significantly low compared to their respective wildtypes. Unexpectedly, COE3 and KOE32 showed significantly (p<0.05) higher endogenous profiles compared to their respective wildtypes. The endogenous transcript profiles for BOE1, BOE5, BOE6,

COE4, COE12 and KOE17 showed no significant expression differences compared to the wildtypes, indicating that BOE1, BOE6, COE4 and KOE17 lines with exhibited high overexpression of *AtPRX34* levels had no affect on the endogenous levels. In all of the transgenic lines created, the overall *AtPRX34* transcript level was relatively higher



compared to their respective wildtype levels.

Figure 2.13. The relative expression profiles of exogenous and endogenous targets of *AtPRX34* after normalisation with *AtEF1* and *AtTUB4* for wildtypes: Be-*0*, Col-*0* and Kil-*0* and overexpression *AtPRX34* transgenic lines from uninfected four-week-old leaf material. The light grey bars indicate endogenous expression and dark grey bars indicate exogenous expression. The error bars represent the standard error of the biological replicates (n =3). A * indicates transcript abundance values which are significantly different from their respective wildtype controls (p<0.01, Student's t-test). A ** indicates transcript abundance values which are significantly different from their respective wildtype controls (p<0.05, Student's t-test).

2.3.5. Analysis of peroxidase activity in transgenic plants

Total proteins were extracted from the same frozen four week-old plant material used to extract total used to extract total RNA for RTqPCR analysis. Both soluble and peripheral bound protein fractions were extracted with three biological replicates for all the samples except for COE3. The amount of plant material sampled was only sufficient to generate two biological replicates of the COE3 line.

The concentration of each extracted protein fraction was unknown and needed to be determined to standardize the peroxidase analysis. The protein concentration of unknown samples was determined using a standard curve generated from a serial dilution of a known protein sample (BSA) by measuring the absorbance at 595 nm with addition of the Bradford reagent. Regression analysis was used to generate a standard protein concentration curve of bovine serum albumin with a R^2 value of 0.99 (Appendix D). This standard curve was used to determine the protein concentration of each protein fraction by generating a serial dilution of the sample with the addition of the Bradford reagent.

The soluble and peripheral bound fractions were diluted to a final concentration of 0.4 mg/ml and 0.26 mg/ml respectively. The total peroxidase activity was analysed to determine the effect of overexpressing *AtPRX34* in these plant lines. The peroxidase activity was determined by measuring the colour formation at 450 nm in the presence of a colourless OPD substrate (Figure 2.4).

The peroxidase activity level of each transgenic *AtPRX34* line was compared to its respective wildtype (Figure 2.14). The soluble fraction analysis indicated that only KOE32 showed a significant (p<0.01) increase in peroxidase activity compared to its wildtype. Both BOE6 and COE12 showed significantly decreased peroxidase activity compared to their respective wildtypes with a significance of p<0.05. On inspection, these two samples indicated very weak colour formation after adding OPD and H_2O_2. Both these fractions were repeated with similar results, which could possibly indicate poor peroxidase extraction or weak peroxidase activity. The other lines: BOE1, BOE5, COE3, COE4 and KOE17 showed no significant difference in their peroxidase activity profiles. COE3, COE4 and KOE17 lines showed large variation between their biological replicates.

The peripheral protein fraction peroxidase assay indicated increased peroxidase activity in KOE32 (p<0.01) and a weaker increase in the COE4 line (p<0.05). Lines BOE1, BOE5, BOE6, COE12 and KOE17 showed no significant difference compared to their respective wildtypes. No conclusions can be made for COE3 because one biological replicate indicated increased peroxidase activity while the other showed very low enzyme activity, resulting in a large standard error for this line.

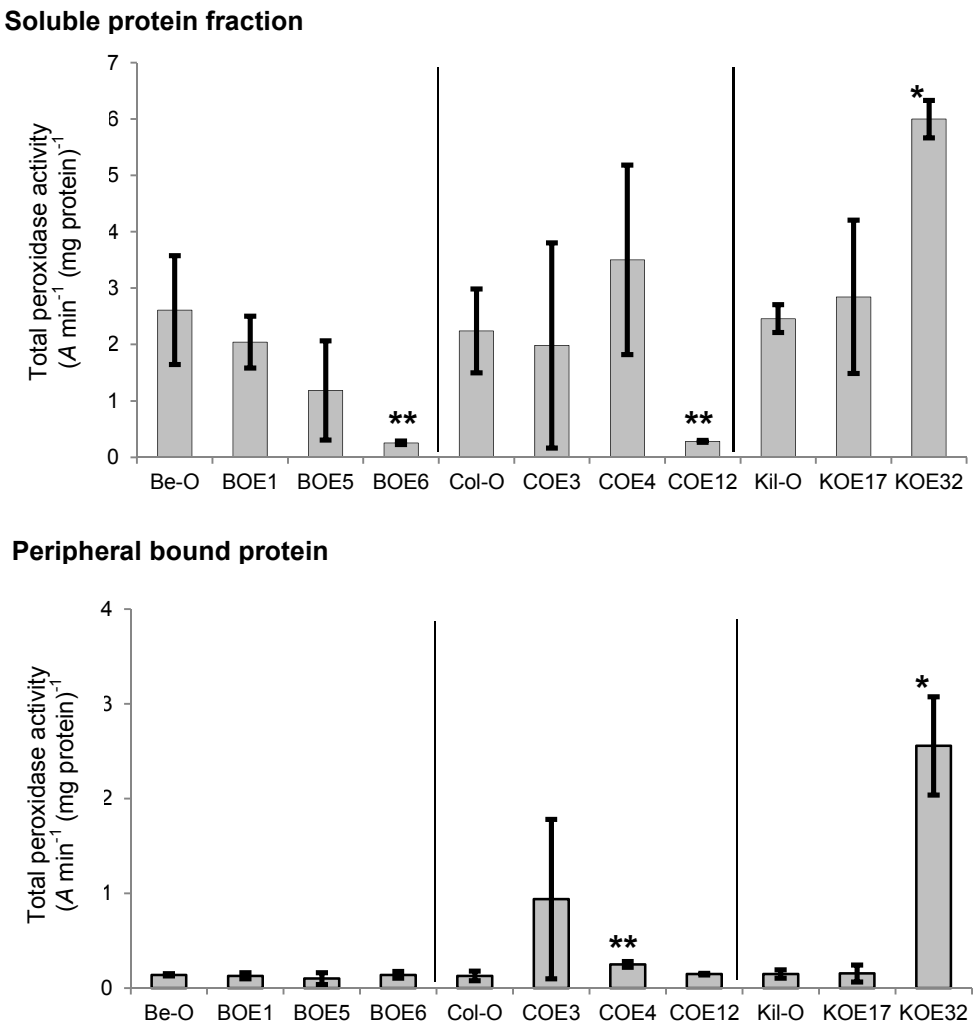


Figure 2.14. Total peroxidase activity of the overexpression *AtPRX34* lines and wildtypes for four-week-old leaf material**.** The peroxidase activity was determined by measuring the colour formation at the absorbance at 450 nm. Soluble and peripheral bound fractions were analysed. The y-axis indicates absorbance value per minute standardizing with the protein concentration (0.4 mg/ml for soluble and 0.26 mg/ml for peripheral proteins). The error bars represent the standard error of the biological replicates (*n* =3). A * indicates activity values, which are significantly different from the control ($p<0.01$, Student's *t*-test) and ** indicate activity values with a significance at ($p<0.05$, Student *t*-test).

2.3.6. Plant-pathogen challenges

Firstly, a bacterial concentration needed to be determined for the plant-pathogen infections that induced an effective disease progression for the susceptible ecotype, Be-0 (wilt symptom development at 4 dpi and plant death at 14 dpi). The trial was conducted by infecting both Be-*0* and Kil-*0* plants with $1X10^6$, $1X10^7$, $1x10^8$ and $4X10^8$ cfu/ml of *R. solanacearum* isolate CK (Appendix E1). Be-*0* plants infected with $1X10^8$ bacteria showed

the most severe disease progression compared to the other concentrations of bacterial inoculum used and 1X10^8 cfu/ml was used for the final plant-pathogen challenges (Figure 2.15).

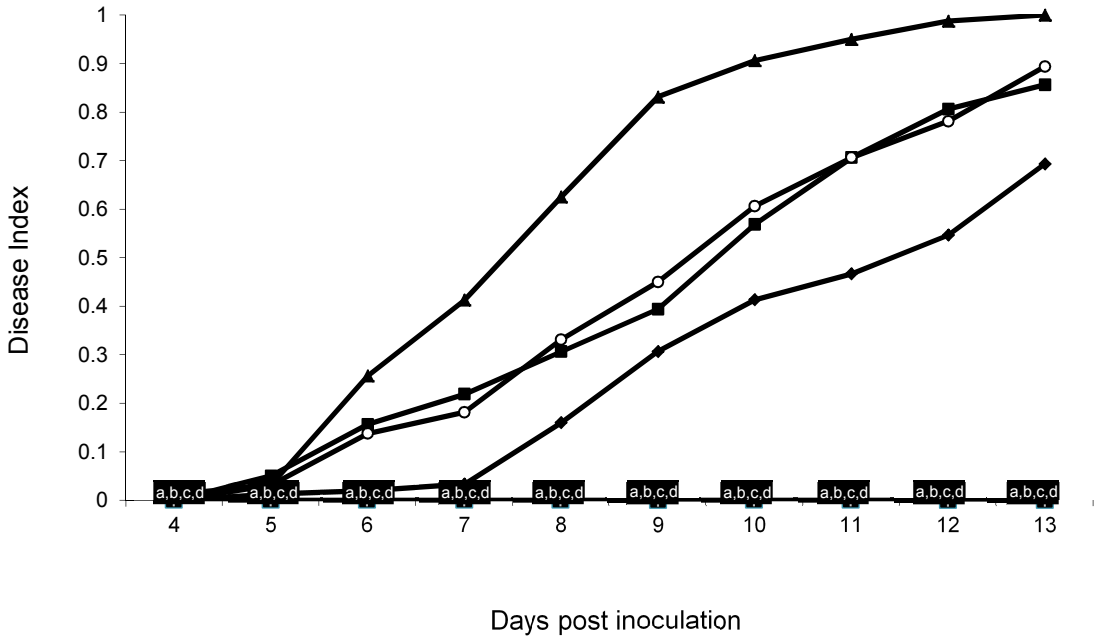


Figure 2.15. Disease progression in various *A. thaliana* ecotypes, Be-*0* and Kil-*0* after root inoculation with different concentration (1X10^6, 1X10^7, 1X10^8 and 4X10^8 cfu/ml) of *R. solanacearum* strain CK. Sixteen plants were used to calculate the disease index at each time point. Be-0 infected with 1X10^6 (♦), 1X10^7 (■), 1X10^8 (▲) and 4X10^8 (○) and Kil-0 infected with 1X10^6 (a), 1X10^7 (b), 1X10^8 (c) and 4X10^8 (d).

The wildtype lines and the transgenic *AtPRX34* lines were root inoculated with 1X10^8 cfu/ml of virulent *R. solanacearum* isolate CK. Pink mucoid colonies with white halos were selected for the inoculum because this indicated virulence of *R. solanacearum*. All the ecotypes and transgenic lines were also infected with sterile broth to serve as control. The plants were monitored for classic wilting symptoms (Table 2.2) for three weeks (Be-*0* plants, two weeks and Kil-*0* and Col-*0* for three weeks). The average disease index was generated for each plant line by using the disease indexes calculated from three separate plant-pathogen trials containing 12 plants each.

The aim of this study was to determine if overexpression of *AtPRX34* in two susceptible ecotypes, Be-*0* and Col-*0*, will enhance resistance against the bacterial pathogen *R. solanacearum* isolate CK. The BOE5 line had a significant (p<0.01) delay in disease development early on in the plant-pathogen interaction. This line and the other two BOE lines died after 14 days post inoculation (dpi) similar to the Be-*0* plants (Figure 2.16). The COE3 line exhibited a significant (p<0.01) delay in disease development from 11 dpi up

until the last day of monitoring symptoms. The other two lines, COE4 and COE12 showed similar symptom progression compared to Col-*0* (Figure 2.17).

Previous observations suggest that increasing the temperature results in resistance in Kil-*0* being compromised. In order to determine if the overexpression *AtPRX34* lines would afford protection in Kil-*0* under such conditions, the temperature in the growth room was increased to 31.5°C (Appendix E2). Temperature induced challenges showed that resistance in Kil-*0* was compromised and wilting symptoms became visible. KOE17 line had significantly (p<0.01) delayed wilting development compared to Kil-*0* from 16 dpi and KOE32 exhibited delayed symptom development from 19 dpi onwards under these high temperature conditions (Figure 2.18).

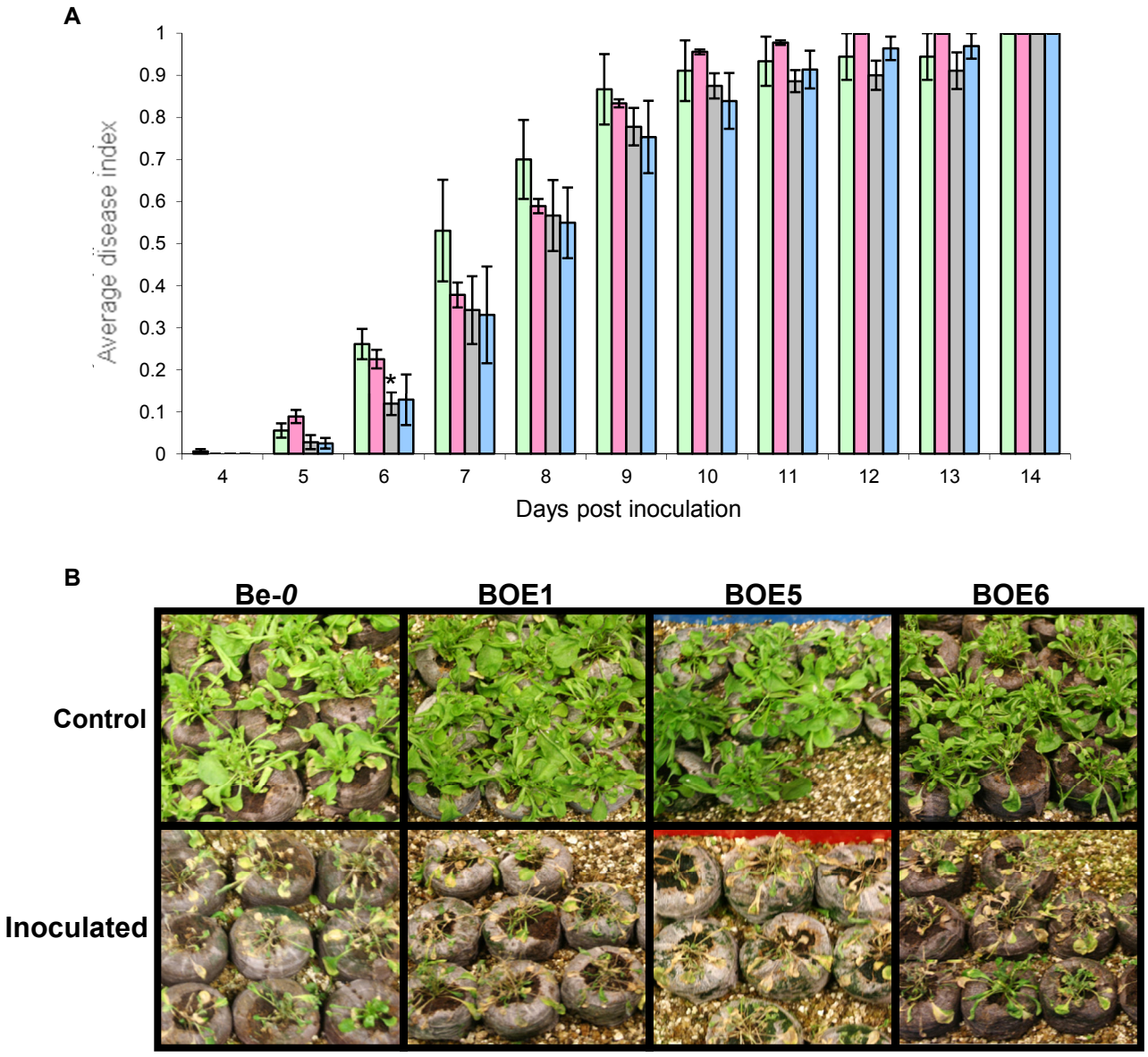


Figure 2.16. Disease symptoms on Be-*0* and *AtPRX34* overexpression lines after root inoculation with $1X10^8$ cfu/ml of *R. solanacearum* strain CK. Twelve plants were used to calculate the disease index at each time point and three replicates were performed (A). Light green, pink, grey and blue bars indicate Be-*0*, BOE1, BOE5 and BOE6, respectively. The error bars indicate the standard error calculated from the three replicate disease indexes. Asterisks indicate values significantly different (p<0.01, t-test) compared to the wildtype. Wilting symptoms clearly visible on Be-*0* and overexpression *AtPRX34* lines 12 dpi, indicating plant death of all the plants (B). The uninfected plants remained healthy.

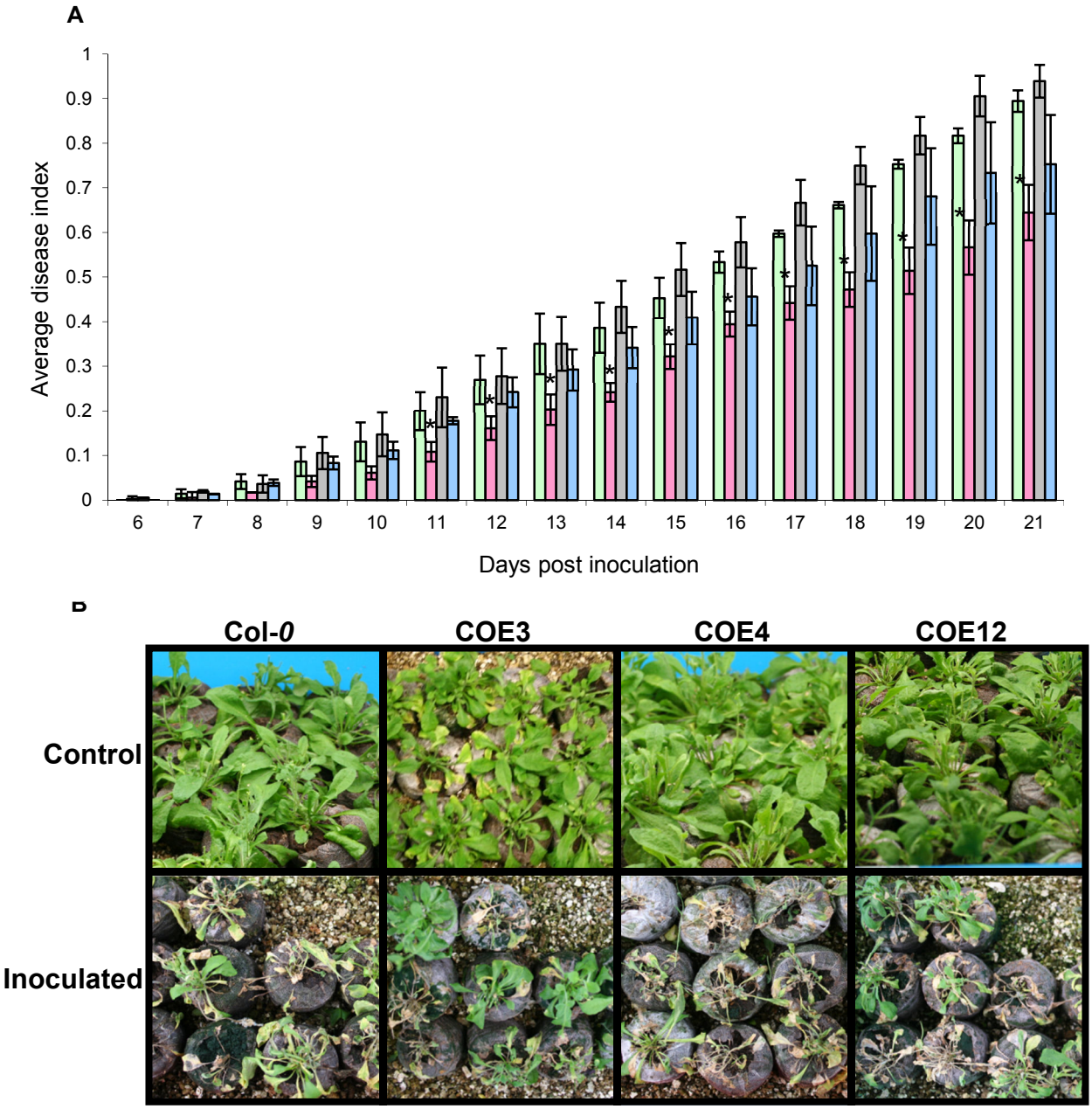


Figure 2.17. Disease symptoms on Col-*0* and *AtPRX34* overexpression lines after root inoculation with 1X10^8 cfu/ml of *R. solanacearum* strain CK. Twelve plants were used to calculate the disease index at each time point and three replicates were performed (A). Light green, pink, grey and blue bars indicate Col-*0*, COE3, COE4 and COE12, respectively. The error bars indicate the standard error calculated from the three replicate disease indexes. Asterisks indicate values significantly different (p<0.01, t-test) compared to the wildtype. Wilting symptoms visible on Col-*0* and overexpression *AtPRX34* lines 21 dpi, indicating various degree of symptom development (B). The uninfected plants remained healthy.

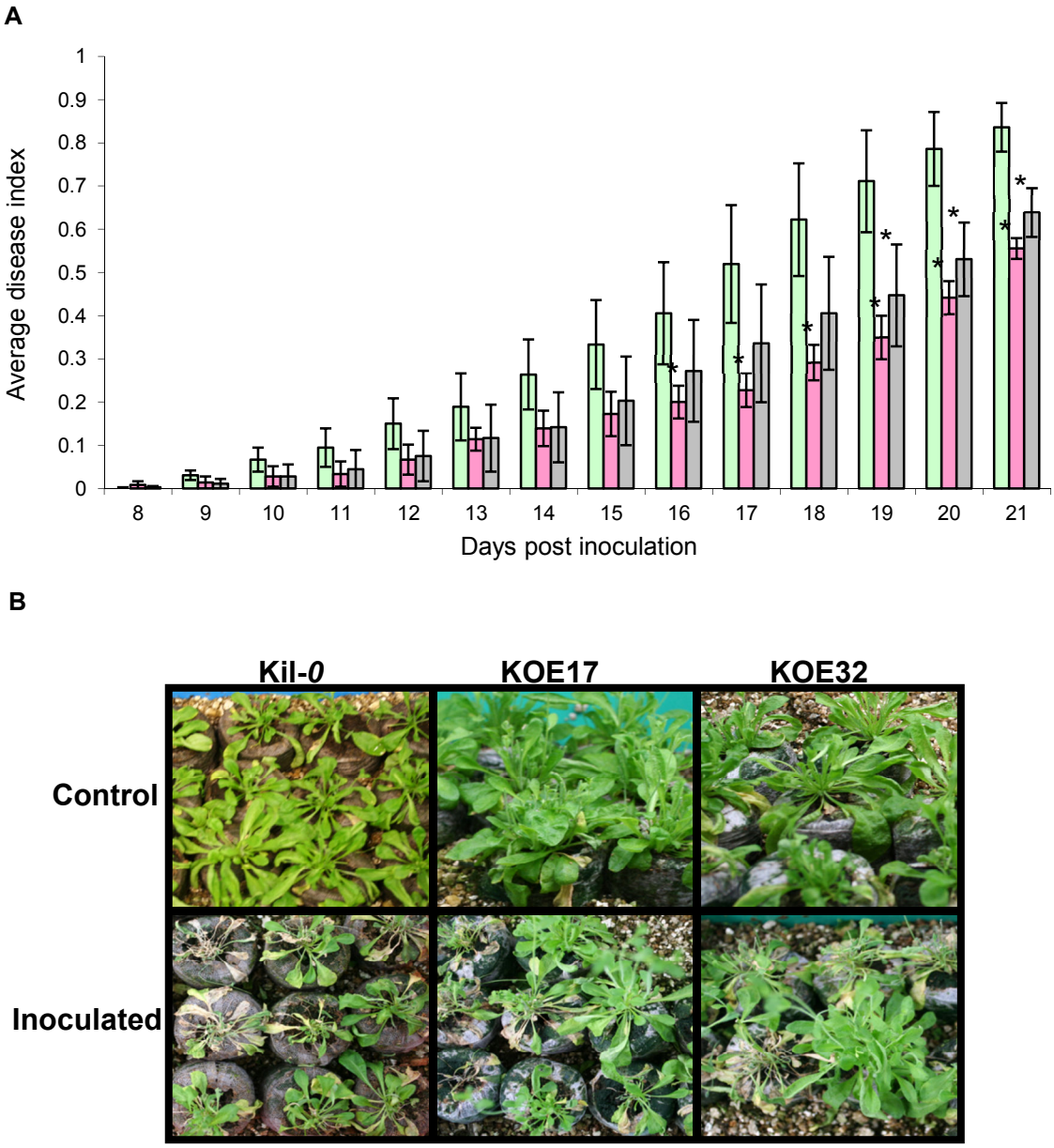


Figure 2.18. Disease symptoms on Kil-*0* and *AtPRX34* overexpression lines after root inoculation with 1X10^8 cfu/ml of *R. solanacearum* strain CK. Twelve plants were used to calculate the disease index at each time point and the experiment was repeated three times (A). Light green, pink and grey bars indicate Kil-*0*, KOE17 and KOE32, respectively. The error bars indicate the standard error calculated from the three disease indexes. Asterisks indicate values significantly different (p<0.01, t-test) compared to the wildtype. Wilting symptoms visible on Kil-*0* and overexpression *AtPRX34* lines 21 dpi, exhibiting minor wilt symptom development (B). The uninfected plants remained healthy.

2.4. Discussion

The expression profile of *AtPRX34* has led to a proposed role for this gene in defence against *R. solanacearum* (Naidoo, 2008) and it is suggested to be involved in either cell wall thickening (Cosio and Dunand, 2009), producing a ROS (Bindschedler et al., 2006) or antimicrobial metabolites (El-Sayed and Verpoorte, 2004). To support the gene expression profile, this study aimed to determine the role of *AtPRX34* in defence against *R. solanacearum* by testing the hypothesis that overexpression of *AtPRX34* in susceptible ecotypes would lead to reduction of disease severity against the pathogen. The study investigated the expression profile of *AtPRX34* in response to various pathogens using currently available GeneVestigator profiles (Zimmermann et al., 2005) and qualified *AtPRX34* as a candidate gene in defence. It was hypothesised that overexpression of *AtPRX34* in susceptible ecotypes will provide increased protection against *R. solanacearum*. To this end, transgenic overexpression lines of *AtPRX34* were constructed and tested for response to *R. solanacearum* challenge. The relative transcript levels of endogenous and exogenous *AtPRX34* and the total peroxidase activity for all generated transgenic lines were analysed. The disease response was assessed and two susceptible lines, BOE5 and COE3 showed delayed symptom development compared to their respective wildtypes from early time points and KOE17 and KOE32 showed delayed wilt development under high temperature conditions compared to the wildtype. The results of all the experiments, conducted in this study, are summarized in Table 2.6. In parallel, a purchased knockout line, SALK_051769, of the *AtPRX34* gene in the susceptible ecotype, Col-*0* was investigated to determine if the mutant increased susceptibility to *R. solanacearum.* Unfortunately, *AtPRX34* expression levels were not reduced in this SALK_051769 line and will only be discussed in Appendix F.

Table 2.6. Summarising all the results generated for the different experimental analysis conducted on the transgenic lines and their respective wild-types. The qRT-PCR results for both the endogenous and exogenous relative expression and the peroxidase activity determined for both the soluble and peripheral bound protein fraction are tabulated.

	qRT-PCR analysis		Peroxidase activity		*Ralstonia* infections
	endogenous expression	exogenous expression	Soluble protein	Pheripheral bound protein	Significance observed*
Be-*0* [a]					
BOE _1	slightly lower	very high	similar	similar	
BOE_5	similar	very low	similar	similar	6 dpi
BOE_6	similar	very high	much lower	similar	
Col-*0* [a]					
COE_3	slightly higher	slightly higher	similar	higher	11- 21 dpi
COE_4	slightly higher	very high	similar	slightly higher	
COE_12	slightly lower	very low	much lower	similar	
Kil-*0* [a]					
KOE_17	similar	very high	similar	similar	16- 21 dpi
KOE_32	slightly higher	very high	high	very high	19- 21 dpi

[a] - A transgenic line's results are compared to the result obtained for its respective wild-type.

* - The days post inoculation of the transgenic lines infected with *R. solanacearum* which indicated a significant difference compared to the wild-type are indicated.

2.4.1 Putative role of *AtPRX34* as revealed by bioinformatic analysis

The expression profiles of *AtPRX34* in publicly available data were investigated with bioinformatic analysis focusing on various biotic stresses. The available data was used to propose a possible role for *AtPRX34* in plant defence by determining if it was induced during PAMP-triggered immunity or during pathogen challenges. *AtPRX34* probe with a fold change >1.65 was only observed for the pathogen challenges, when infected with *P. infestans* at 6 hr and 24 hr, *B. cinerea* at 18 hr and 48 hr and *P. syringae* AvrRpm1 at 24 hr. None of the PAMP-triggered responses induced the *AtPRX34* transcript levels higher than the threshold of 1.65. FLS and NPP1 treatments did show fold changes of 1.48 and 1.41, respectively, indicating that *AtPRX34* transcript levels during these treatments were marginally induced. *AtPRX34* is differentially regulated during the compatible interaction of *P. infestans*, incompatible interaction of *P. syringae* AvrRpm1 and in *B. cinerea* and not remarkably induced by the different PAMP treatments, suggesting that *AtPRX34* is a downstream product of defence signalling. The evidence from the expression profiles suggest that *AtPRX34* in *P. suringae* was induced in both a resistant and susceptible interaction and during FLS and NPP1 treatment, showing that *AtPRX34* response to PAMPs and pathogen (virulent and avirulent) interactions. This could suggest that *AtPRX34*

is downstream activated when the plant encounters a foreign entity. This observation resulted in the hypothesis that increased levels of *AtPRX34* in susceptible lines may enhance resistance against pathogens.

Previous experiments identified that resistance in Nd-1 inoculated with GMI1000 is due to gene-for-gene resistance, the RRS1-R protein in *Arabidopsis* recognizing popP2 in *Ralstonia* (Deslandes et al., 2003; Deslandes et al., 2002; Deslandes et al., 1998). This raised the question if resistance in Kil-*0* is due to the RRS1-R protein interaction (Personal communication: Liesl van der Linden, MSc study, University of Pretoria). The F2 progeny generated by crossing Kil-*0* and Be-*0* identified a 1:3 segregation ratio after inoculation with *R. solanacearum* CK, suggesting that resistance in Kil-*0* is due to a single recessive gene. Genetic analysis indicated that the possible resistant-gene segregated with the RRS1-R gene locus and DNA sequence comparison revealed that Kil-*0* contained an allelic variant of RRS1-R. A *popP2* mutant developed wilting symptoms on Kil-*0* while its compliment mutant did not show any symptom development, suggesting that resistance in Kil-*0* is due to RRS1-R-like recognition in Kil-*0*. This is consistent with the available transcript data, which suggested that *AtPRX34* is induced downstream in the defence cascade, probably after the effecter molecule from the pathogen is recognized by the R gene from *Arabidopsis*. This is also supported by RT-qPCR analysis of *AtPRX34* which was two times higher induced four and seven dpi in Kil-*0* compared to Be-0 (Naidoo, 2008).

2.4.2. Conservation of *AtPRX34* sequence in different ecotypes and species

The CDS sequence of Kil-*0* contained three amino acid changes: Ala29Thr, Leu175Pro and Arg270Gln (Figure 2.7) compared to Col-*0*. Two of these substitutions (Ala29Thr and Arg270Gln) were identified in all three screened clones and could be the result of ecotype differences, with the other substitution possibly created during PCR amplification. Due to the possibility of ecotype differences in their CDS, the *AtPRX34* ortholog in Be-*0* was also sequenced (data not shown) and compared to that of Col-*0* and Kil-*0*. The *AtPRX34* ortholog of Be-*0* did not differ in sequence from that of Col-*0*, indicating that the amino acid changes were only present in Kil-*0*. Could these two changes possibly result in Kil-*0* containing an AtPRX34 enzyme with better catalytic activity? This is probably not the reason for the amino acid substitutions given that increased Kil-*0* *AtPRX34* transcript levels inserted into the Be-0 and Col-*0* backgrounds only resulted in marginal or no increases in peroxidase activity compared to their respective wildtypes (Figure 2.14).

A three-dimensional protein model of AtPRX34 containing the Leu175Pro substitution was constructed (Prof F. Joubert) from the X-ray structure of 1W4W

constructed from Horseradish peroxidase isoform C. The position where the mutation occurred indicated that it was unlikely to change the folding of the peroxidase. Unfortunately, 3D protein models do not indicate with certainty that the structure and activity of the protein will not be affected. Peroxidases contain various conserved sequences identified from crystallography and site-directed mutagenesis form Horseradish isoform C, which is important for structural formation and protein function (Mathe et al., 2010). These include the heme-binding site (distal: Arg38, Phe41 and His42; proximal: Asn70 and His 170), Ca^{2+} pectate binding motif [*Arabidopsis*: Lys[X]$_5$Lys[X]$_2$Lys (Dunand et al., 2002; Shah et al., 2004)], sites for cysteine and salt bridges (Lehtonene et al., 2009) and other single amino acids which are important for substrate activity (Mathe et al., 2010). The conserved amino acids do not occur at the Leu175Pro substitution site in the *AtPRX34* CDS (Appendix B2). These findings coupled with the detection of an increase in peroxidase activity of KOE32 compared to Kil-*0* (Figure 2.14), suggested that the AtPRX34 protein containing the Leu175Pro substitution was functional and able to catalyse substrates *in vitro*. Unfortunately, it is still possible that this substitution affected the function of the protein *in vivo* which could explain why the overexpression *AtPRX34* transgenic lines in Be-*0* and Col-*0* background did not show a strong effect in the disease development against the pathogen.

2.4.3. Expression levels of *AtPRX34* in transgenic lines

The relative transcript levels in the various transgenic lines and ecotypes were analysed to determine if the exogenous transgene was being overexpressed and if the expression of the transgene was affecting the endogenous *AtPRX34* transcript levels due to co-suppression from gene silencing. Using three biological replicates, where one replicate contained a bulk of three individual plants, technical variability was considered. The qBASE normalization software was used to determine the best set of reference genes to use for accurate normalization between samples (Hellemans et al., 2007).

A 35S CaMV promoter was used in this study to constitutively induce *AtPRX34*. This promoter is highly active and functions in various organs and developmental stages (Benfey et al., 1989), but high transcript levels can result in gene silencing (Elmayan and Vaucheret, 2003; Lindbo et al., 2003; Schubert et al., 2004; Vaucheret et al., 1998). Various inducible promoters have been created to solve these problems (Li et al., 2005; Roslan et al., 2001; Zuo et al., 2000). Transgenic plants with an inducible construct grow normally and the target gene is only induced when necessary.

A degree of variation was obtained for exogenous expression in the various transgenic lines with the highest relative expression value after normalization at 11.24 for KOE32 and the lowest value at 0.39 for BOE5. Transgene expression was detected in all eight transgenic lines, but *AtPRX34* was only being overexpressed in BOE1, BOE6, COE4, KOE17 and KOE32. Independent transgenic lines with the same T-DNA construct can show differences in transgene expression up to 100 fold (Schubert et al., 2004). This could be due to either the number of transgene integrations or the position of integration within the genome (Day et al., 2000; Peach and Velten, 1991). The more T-DNA inserted into the genome, the higher the level of gene expression and integrations into heterochromatin could result in low expression levels (Day et al., 2000; Peach and Velten, 1991). For this reason multiple transgenic events per construct is screened. All of the transgenic lines, except BOE5, expressed the exogenous *AtPRX34* transcripts higher than their respected endogenous counterpart.

Similar endogenous *AtPRX34* transcript profiles for Be-*0*, Col-*0* and Kil-*0* were identified (Figure 2.13), indicating that the resistant ecotype did not contain higher levels of *AtPRX34* compared to the susceptible ecotypes before pathogen infection. *AtPRX34* expression levels were induced faster in Kil-*0* plants compared to Be-0 at four and seven dpi (Figure 2.1, Naidoo, 2008), indicating that the recognition of the pathogen prompts the production of the defence response gene, *AtPRX34*.

The endogenous *AtPRX34* transcript levels in the overexpressing *AtPRX34* transgenic lines showed different expression patterns compared to their relative ecotypes, with either similar or different (down- or upregulated) expression profiles (Figure 2.13). Six lines: BOE1, BOE5, BOE6, COE4, COE12 and KOE17 exhibited no significant difference (p<0.01) in endogenous expression and two of these lines: BOE1 and COE12 did displayed a downregulation of the endogenous *AtPRX34* levels compared to their respective wildtypes. This could be due to co-suppression of the endogenous gene either through transcriptional gene silencing, which is heritable, or post-transcriptional gene silencing which is systemically acquired (Schubert et al., 2004; Vaucheret et al., 1998). Other studies have also identified that gene silencing correlated with the strength of the promoter used, occurred more frequently in homozygous lines compared to hemizygous lines and was usually triggered by a threshold concentration of the transgene (Elmayan and Vaucheret, 2003; Lindbo et al., 2003; Schubert et al., 2004; Vaucheret et al., 1998). The other two lines; COE3 and KOE32 surprisingly exhibited increase expression levels of the endogenous gene with 2.4 and 2 times higher expression compared to wild-types, respectively. Reasons for this increase in endogenous expression are unclear, but do not influence the outcome of this experiment provided that the total (exogenous and

endogenous) *AtPRX34* levels are increased to assess the disease response in reaction to high *AtPRX34* levels. Future work will entail the analysis of the endogenous *AtPRX34* transcript levels in these overexpressing *AtPRX34* transgenic lines during plant-pathogen infections.

2.4.4. Peroxidase activity in transgenic lines

It was necessary to determine if the transgenic lines exhibited increased AtPRX34 protein levels by measuring the total peroxidase enzyme activity *in vitro*. Currently, due to the high similarity between peroxidases (95% identity between *AtPRX34* and *AtPRX33* at amino acid level), a specific antibody for AtPRX34 is unavailable and Western blotting would not be informative. The relative peroxidase enzyme activity was determined for all transgenic lines and their respective ecotypes from crude protein extracts for both soluble and peripheral bound fractions from uninfected four-week-old leaf material. *A. thaliana* contains 73 different peroxidases (Welinder, 1992) which contribute to the peroxidase activity at any given time.

Increase peroxidase activity was measured for KOE32 in the soluble fraction and for both COE4 and KOE32 in the peripheral bound fraction. Five of the lines showed increased exogenous expression levels, but only two lines for the peripheral bound fraction and only one line exhibited an increase in peroxidase activity for the soluble fraction. Could this be due to inhibition of translation or post-translational control? An endogenous peroxidase-like gene, *spi2* was transformed into Norway spruce and the peroxidase activity found to be both similar and higher than levels in the untransformed plants (Elfstrand et al., 2001). Their relative mRNA levels suggested a degree of expression (2.5 to 5 for the various transgenic lines), with high mRNA levels not necessarily resulting in an increase in peroxidase activity (Elfstrand et al., 2001), indicating that there was possibly a threshold limit for the transgene levels. This finding was similar to the results found in the current study: the peroxidase activity in certain lines showed increase or similar activity compared to their respective ecotypes and in certain lines, high transcript abundance levels did not result in increased peroxidase activity. Another reasonable explanation for the increased mRNA transcript levels not resulting in an increased peroxidase activity may be due to the protein mutation within the *AtPRX34* construct resulting in a none functional or less function enzyme.

The COE3 transgenic line showed increased enzyme activity in both fractions, but due to the large variation among replicates, no definite conclusions could be made. Large variation among biological replicates has also been seen in a study where peroxidase activity was determined in liquid moss cultures, but differences in peroxidase activity between the two treatments were quite visible (Lehtonene et al., 2009).

The present study investigated peroxidase activity in a different approach to other studies which separately measured peroxidase activity and the ROS, or which separated the peroxidases by isoelectric focusing or SDS-PAGE (Bindschedler et al., 2006; Lehtonene et al., 2009). The relative peroxidase activity for each sample was determined by measuring the colour formation of 2,3-diaminophenazine from *o*-phenylenediamine dihydrochloride (OPD) at 450 nm (Hamilton et al., 1999; Mekler and Bystryak, 1992). The total peroxidase activity was a relative measurement because a standard curve for the end product could not be generated because neither 2,3-diaminophenazine nor a purified peroxidase was available to generate an accurate standard curve for the end product. The extinction coefficient of OPD required for the conversion of the absorbance value to a molar concentration could not be obtained. The absorbance was measured per minute per total protein used. Other studies used different substrates, but with similar conversion methods. Anionic peroxidase enzyme activity were measured in *A. thaliana* by using guaiacol and the activity was reported as 1 nM substrate per minute per mg of protein used (Lebedeva et al., 2003). Peroxidase activity was measured in Norway spruce by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS] as a substrate with the peroxidase activity unit in *A* min^{-1} (mg protein)$^{-1}$ (Elfstrand et al., 2001).

In experiments by Bindschedler, the antisense *FBP1* (*French bean peroxidase 1*) line in Col-*0* exhibited significantly lower peroxidase activity (about 50% less) in the peripheral bound fraction compared to Col-*0* (Bindschedler et al., 2006). The silencing target was determined with microarray analysis as either *AtPRX34* or two glutathione peroxidases (At1G63460 and At3G63080). The authors suggested that these glutathione peroxidases will not be present in the peripheral bound fraction and the likely reason for decreased peroxidase activity was the silencing of *AtPRX34*. Further RT-qPCR analyses that included *AtPRX33*, as this gene was not represented on the microarray slide (Affymetrix ATH1 GeneChip), suggested that the relative transcript levels of both genes were decreased. The antisense *FBP1* showed enhanced disease development when infected with various pathogens (*G. orontii*, *B. cinerea* and *P. syringea*) and this enhanced susceptibility was the result of a diminished OB in this transgenic line (Bindschedler et al., 2006). Overexpression lines for *AtPRX34* and *AtPRX33* were not investigated in their study. Future work will include assessing the disease response of the constructed overexpression *AtPRX34* lines in this study against the various pathogens used in their study followed by determining the OB and ROS levels within the constructed transgenic lines.

2.4.5. Assessment of disease response against *R. solanacearum*

Initially, various concentrations of *Ralstonia* inoculum were investigated to identify the bacterial concentration that produces the desired disease progression for Be-*0* and Kil-*0* (Be-*0* plants with their first wilt symptoms at four dpi and plant death occurring 14 dpi and Kil-*0* plants with no symptom development for 14 dpi). The aim of this study was to determine if the susceptible transgenic lines, overexpressing *AtPRX34*, show improved protection against *R. solanacearum*. Be-0, Col-*0* and their transgenic lines were inoculated with $1X10^8$ cfu/ml of *R. solanacearum* with root soaking and the witling symptoms were documented for two to three weeks. Only one line for each ecotype: BOE5, six dpi (Figure 2.16) and COE3 from 11 dpi to 21 dpi (Figure 2.17), showed a significant delay in symptom development compared to their respective ecotypes.

BOE5 was further investigated because of its marginal level of exogenous *AtPRX34* transcript levels. This line can be used to compare to the other lines with higher exogenous expression and determine if there is a threshold for *AtPRX34* expression within *Arabidopsis* plants. *AtPRX34* is a peroxidase enzyme which can have adverse effect on plant development. In this line, a delay in disease development was observed during an early time-point, suggesting a possible role for low levels of *AtPRX34* in limiting the pathogen during early stages of pathogen inoculation, but due to the severe susceptibility of Be-0, the marginally overexpression of *AtPRX34* was insufficient to provide further protection during later time-points. The inoculation of these severely susceptible lines with lower concentrations ($1X10^6$ cfu/ml or $1X10^7$ cfu/ml) of *R. solanacearum* may offer the lines with sufficient time to respond and provided better protection against the invading pathogen, generating a plant-pathogen interaction for further analyses to evaluate the effect of overexpressing *AtPRX34* in the Be-0 background during bacterial inoculations. BOE5 showed the lowest level of *AtPRX34* overexpression levels and no increase in peroxidase activity, suggesting that very high levels of the transcript may not be beneficial for the plants and that may be the reason as to why only one of the Be-*0* transgenic lines expressing very low levels of *AtPRX34* showed a significant effect on disease development. Another possibility is a pleiotrophic effect due to the insertion of *AtPRX34* construct within BOE5 resulting in the disruption of a gene or genes within the *Arabidopsis* genome which may be responsible for the earlier delay in disease phenotype.

In the COE3 line, a similar reduction was observed in the rate of early symptom-development, but the delay in disease development was maintained for the remainder of the experiment, suggesting a possible role of *AtPRX34* in Col-*0* in limiting the pathogen spread early on during infection and maintaining the protection against the pathogen. Both COE3 and COE12 showed moderated exogenous *AtPRX34* express transcripts, like BOE5 and

were also further investigated to establish if the peroxidase enzyme have any adverse effect on plant development. Col-*0* is less susceptible to *R. solanacearum* than Be-*0* and the disease symptoms were scored for 21dpi to obtain disease index values of 1, which correspond to plant death due to wilting. COE3 showed in one of its biological replicates an increase in peroxidase activity. The same possible threshold hypothesis cannot be true for Col-*0* transgenic lines, as COE12 line did not show reduced symptom development against the pathogen. It could be hypothesised that moderate transcript levels and moderate peroxidase activity provides COE3 with reduced diseased development.

The overexpression of *AtPRX34* in the susceptible lines was therefore inadequate to provide protection against *R. solanacearum*, with the exception of COE3. This phenotype was not confirmed by a number of different transgenic events. Multiple other T3 lines have been generated for Be-*0* and Col-*0* during this study but have not yet been screened for homozigosity or challenged with bacterial inoculum. Future work will involve the screening of more T3 homozygous lines for evaluating their disease response to *R. solanacearum* and determining if they confirm delay in disease development.

A previous observation suggested that the resistance in Kil-*0* is compromised when temperatures are increased. The aim was to determine if the resistant transgenic lines, overexpressing *AtPRX34*, show tolerance against *R. solanacearum* under high temperature stress. Kil-*0* and its transgenic lines were inoculated with $1X10^8$ cfu/ml of *R. solanacearum* with root soaking and the witling symptoms were documented for three weeks with a temperature increase to 31.5°C at the second dpi. Both lines, KOE17, from 16dpi and KOE32, from 19dpi (Figure 2.18) showed increased tolerance to the pathogen inoculation compared to Kil-*0*, implicating *AtPRX34* involvement in limiting pathogen spread in these transgenic lines.

These results are consistent with previous literature where analysis of the *AtPRX34* promoter region implicated the gene in both biotic and abiotic stress, with the inclusion of the *cis*-elements for temperature response (Lebedeva et al., 2003; Passardi et al., 2006). This implicates AtPRX34 in functioning in both thermoregulation and limiting pathogen invasion. Both lines have high transcript induction with only KOE32 showing a significantly strong peroxidase activity, arguing that high peroxidase activity might be ineffective in managing both temperature and pathogen invasion. A study conducted on barley identified that short heat pulses induced resistance against powdery mildew, *Blumeria graminis* f.sp *hordei*, due to endogenous peroxidase producing an OB and reinforcing the cell wall against the invading fungus (Vallelian-Bindschedler et al., 1998). A future prospect is to determine *AtPRX34* (endogenous and exogenous) levels in both infected and uninfected Kil-*0*

transgenic and wildtype material, to test if the endogenous gene is induced in response to temperature stress.

Various overexpression transgenic plants expressing a variety of plants peroxidases have been studied. The overexpression of a wheat *TaPrx103* gene showed increased resistance when infected with the powdery mildew fungus, *B. graminis* f.sp *tritici* (Christensen et al., 2004; Schweizer et al., 1999). The transgenic wheat plants generated H_2O_2 and responded better to programmed cell death (Schweizer, 2008). The sweetpotato overexpressing *swpa4* in tobacco plants showed significantly lower lesion development and increased resistance against the fungus, *Phytophthora parasitica* var *nicotianae* and resistance to insect feeding of *Spodoptera litura* in early developmental stages of the larvae, due to the increase in peroxidase activity, in both lignin and phenolic content and in H_2O_2 levels (Kim et al., 2008).

2.4.6. Conclusion and future prospects

The constitutive expression of *AtPRX34* in the susceptible *Arabidopsis* ecotypes did not universally increase tolerance against *R. solanacearum*. However, the rate of wilting development was marginally reduced at an early time point in one transgenic line and significantly reduced for a number of days in another transgenic line. Could *AtPRX34* play a defence role in early infection stages by limiting the pathogen? As part of this study, efforts are underway to investigate the bacterial load by performing plate counting and pathogen-specific qPCR in these transgenic lines compared to the untransformed ecotypes at very early time-points.

Another possible reason for this insufficient protection against this pathogen could be that the *AtPRX34* gene alone is not responsible for providing the resistance in the Kil-*0* ecotype. Other candidate defence response genes (*AtLTP3*, *AtSAG13*, *AtAIG*, *AtSUI*, *AtSKP5* and an “expressed protein”) were also discovered by the transcriptome analysis conducted on infected Kil-*0* plants (Naidoo, 2008) and overexpression of any combination of these in the current transgenic plants might provide improved protection against the pathogen, or could it be that the correct downstream induction (time and quantity) of *AtPRX34* after R gene recognition is necessary for plant defence?

The constitutive expression of *AtPRX34* in the resistant *Arabidopsis* ecotype under an increased temperature stress showed an increased tolerance to *R. solanacearum* invasion for the two of the transgenic lines, implicating the overexpression of *AtPRX34* in managing both abiotic and biotic stresses which is consistent with previous literature (Lebedeva et al., 2003; Passardi et al., 2006). As part of this study, the bacterial load in these transgenic lines compared to the untransformed ecotypes will be investigated to

determine if the tolerance to the pathogen under high temperature is due to limited pathogen spread.

The role of *AtPRX34* in *Arabidopsis* defence against *R. solanacearum* was investigated but certain questions still remain: what are the functions of *AtPRX34* in plant defence and does it play a role with *AtPRX33*? Could it possibly be due to the thickening of the cell wall or the production of an effective H_2O_2, leading to OB or possibly function as an antimicrobial metabolite, creating an unfavourable environment for the pathogen? Experiments are currently available to investigate all these different possibilities, but due to the redundancies between peroxidases family members, identifying an individual peroxidase function still remains complicated. Answers may come from further investigation of the constructed transgenic lines and their ecotypes.

2.5. References

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CHAPTER 3

Dissertation summary

Analysis of *Arabidopsis* plants transformed with *AtPRX34* for defence against bacterial wilt

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Submitted in partial fulfilment of the requirements of the degree ***Magister Scientiae***
Department of Genetics
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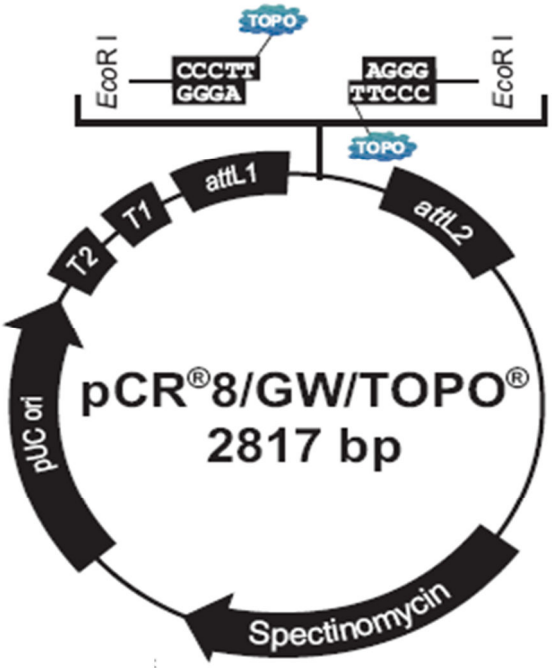
Ralstonia solanacearum is a soil-borne pathogen causing wilting in economically important plants and tree genera such as *Eucalyptus*. In previous research, the *Eucalyptus* derived *R. solanacearum* isolate BCCF402 was found to be highly pathogenic on *Arabidopsis thaliana* ecotype Bensheim (Be-*0*) and mildly pathogenic on Columbia (Col-*0*). However, ecotype Killean (Kil-*0*) remained resistant to pathogen challenge. A previous microarray study in the resistant ecotype, Kil-*0* revealed *peroxidase 34* (*AtPRX34*) as a candidate defence gene against *R. solanacearum*. Plant peroxidases have been identified as essential for oxidative burst, which plays an important role in basal defence. This functional genetic study aimed to determine the role of *AtPRX34* in resistance against the pathogen, *R. solanacearum*. The complete open reading frame of *AtPRX34* obtained from Kil-*0* was cloned into a constitutive expression vector, pMDC32 and transformed into Be-*0*, Col-*0* and Kil-*0* via *Agrobacterium*. Three transgenic events for both Be-*0* (BOE) and Col-*0* (COE), and two transgenic events for Kil-*0* (KOE) were recovered and analysed. Reverse transcription quantitative PCR (RT-qPCR) analysis targeting *AtPRX34* transgene expression indicated increased transcript levels for these transgenic lines, but peroxidase assays revealed increased enzyme activity for only two lines, COE4 and KOE32. Homozygous KOE, BOE and COE transgenic lines and the three untransformed ecotypes were challenged with *R. solanacearum*. BOE5 and COE3 showed reduced wilt development early on during the infection process, compared to the untransformed plants (p<0.01, t-test) while the other two BOE and COE transgenic lines showed no significant difference from

the untransformed plants. However, BOE5 did develop wilt symptoms and eventually died, while COE3 maintained a tolerant phenotype against the pathogen for the remainder of the trial. Under conditions of high temperature, when resistance in Kil-*0* is compromised, KOE17 and KOE32 lines showed reduced disease severity. For future work, the hypothesis that *AtPRX34* is involved in limiting the pathogen during early stages of infection will be tested by investigating the bacterial load in these overexpression lines at early time points after infection.

Appendixes

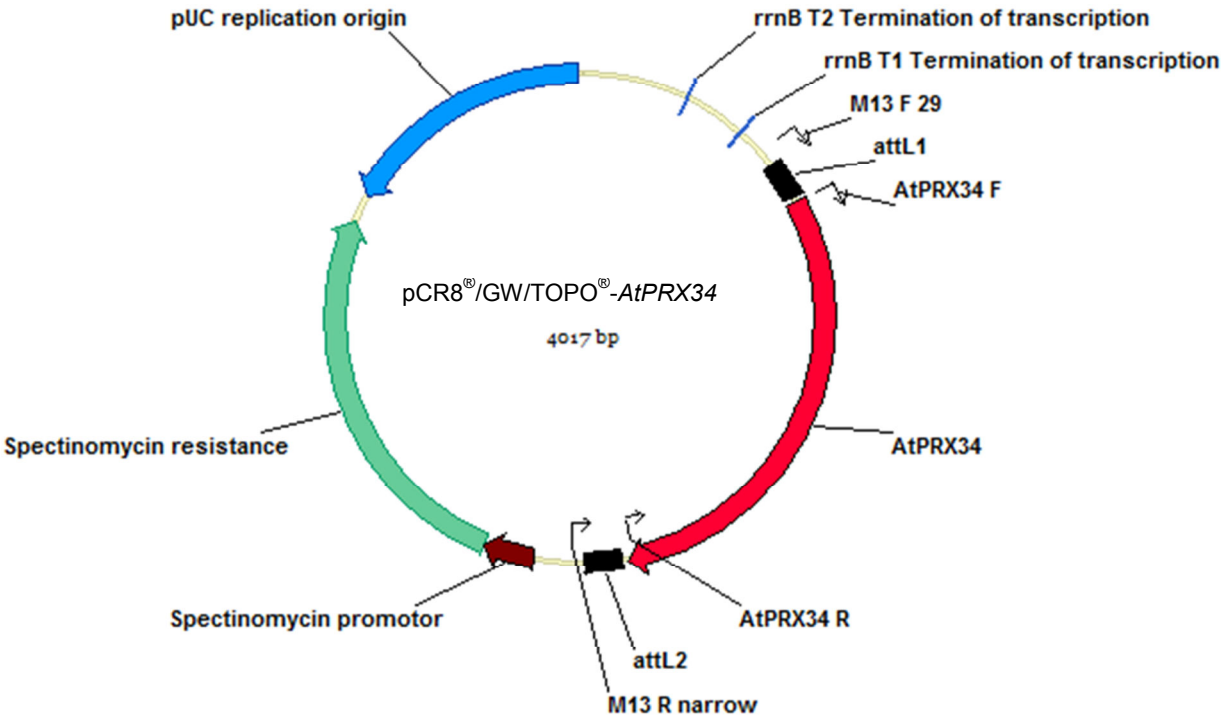
Appendix A1

Vector map of pCR8®/GW/TOPO®. The cloning site is flanked with *att*L sites for recombinant Gateway® enabled cloning. The plasmid provides *E. coli* cells with spectinomycin resistance and the plasmid contains the pUC origin of replication, which produces a high-copy in *E. coli* cells.



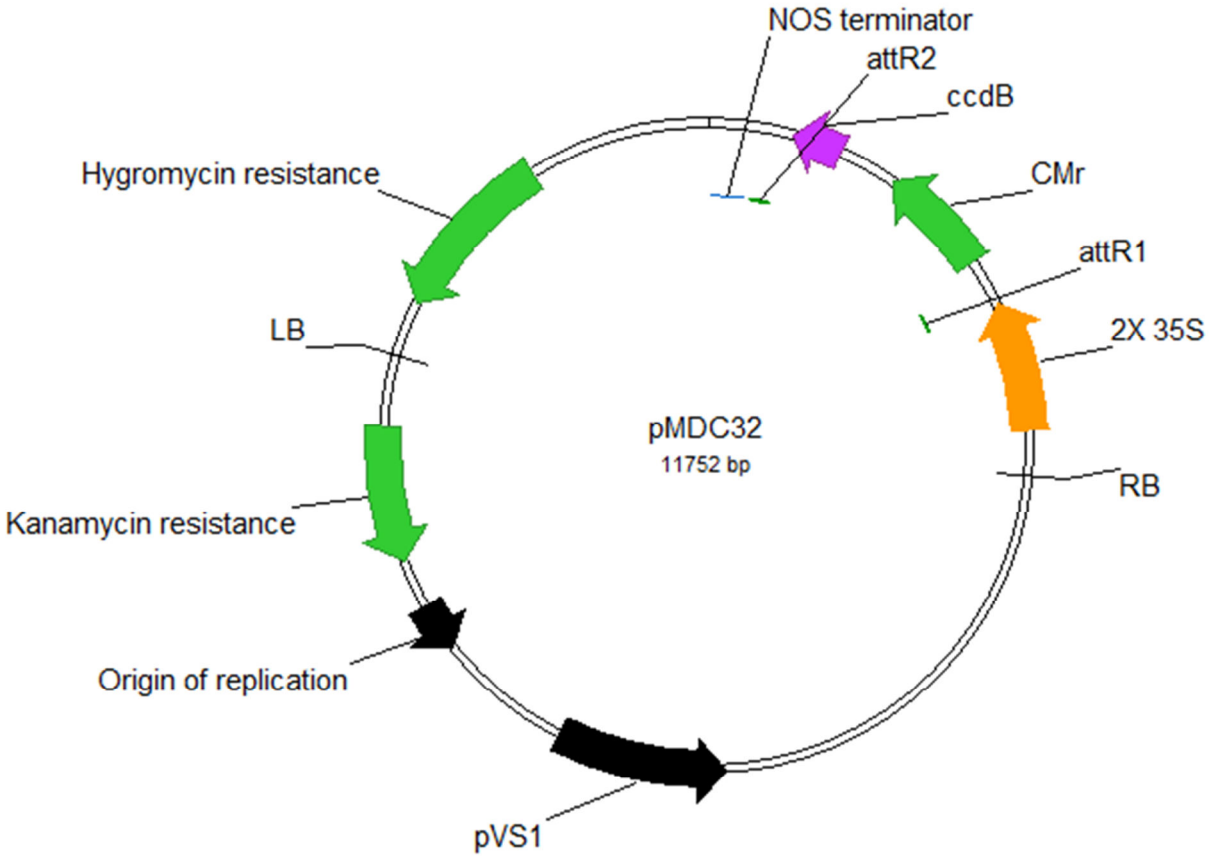
Appendix A2

The constructed vector map of pCR8®/GW/TOPO®-*AtPRX34* from cloning the ORF of *AtPRX34* into pCR8®/GW/TOPO® between the two *att*L sites. The primer binding sites for obtaining the ORF of *AtPRX34* (AtPRX34_F and AtPRX34_R) and the primer binding sites for screening the transformed *E. coli* cells (M13_F_29, M13_R_narrow and AtPRX34_R) are indicated with arrows.



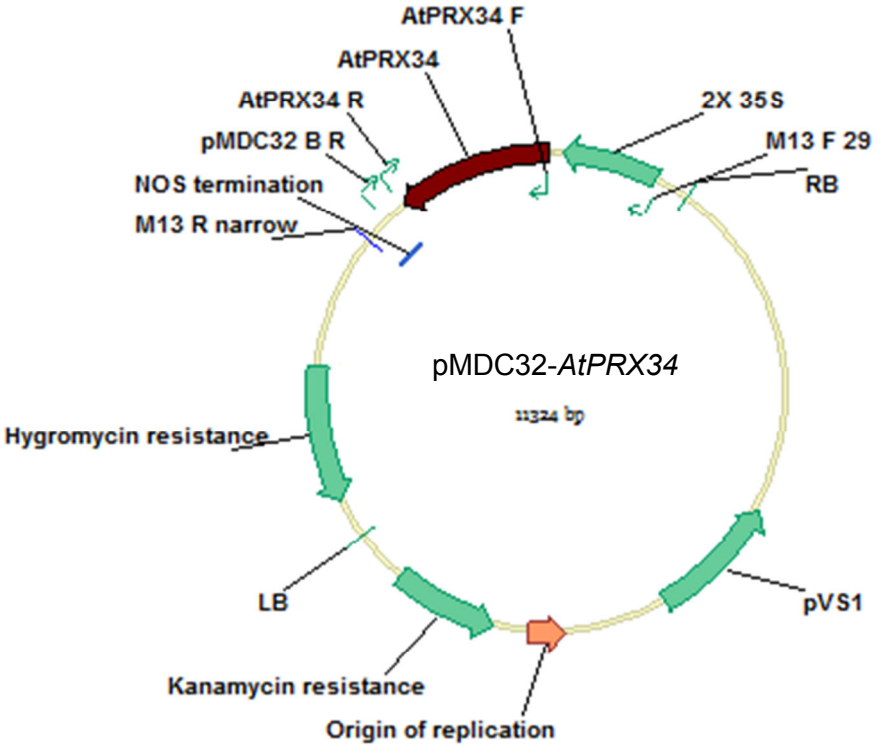
Appendix A3

Vector map of pMDC32. The vector contains *att*R sites for recombinant Gateway® enabled cloning with plasmid containing *att*L sites and these sites flank a *ccdB* gene, lethal in DH5α. The plasmid provides *E. coli* cells with kanamycin resistance and contains pBR322 origin of replication. The entry vector contains both left and right borders for integration into plants genome and will provide the plant with hygromycin resistance.



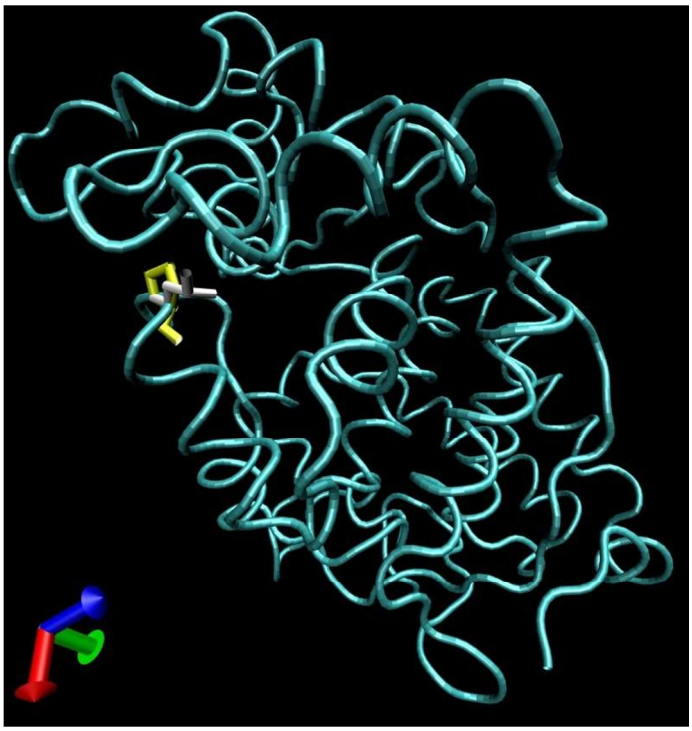
Appendix A4

The constructed destination vector map of pMDC32-*AtPRX34* from Gateway® enabled recombination from pCR8®/GW/TOPO®-*AtPRX34* into pMDC32. The primer binding sites for obtaining the ORF of *AtPRX34* (AtPRX34_F and AtPRX34_R), the primer binding sites for screening the transformed *E. coli* cells (M13_F_29, M13_R_narrow and AtPRX34_R) and the primer binding sites for screening transgenic *A. thaliana* plants (pMDC32_B_R and AtPRX34_F) are indicated with arrows.



Appendix B1

Three-dimensional protein structure of the leucine to proline substitution in *AtPRX34* (constructed by Prof. Fourie Joubert). The model was created based on the X-ray structure of 1W4W (http://www.pdb.org/pdb/explore/explore.do?structureId=1W4W) from Horseradish peroxidase C, with a sequence homology of 85% using the Modeller package in Accelrys Discovery Studio 2.5.5. The leucine and proline are indicated with white and yellow, respectively. The structure indicated that this mutation is unlikely to affect the folding of the *AtPRX34* protein and that the mutation occurred away from the active sites of the peroxidase.



Appendix B2

Alignment of the predicted amino acid sequence of *AtPRX34* CDS in Kil-*0* compared to Horseradish Peroxidase isoform C. The black shadings indicate sequence identity, the white shadings indicate amino acids differences among the sequences and the grey shading indicate the Leu175Pro substitution. A * indicates heme-binding sites, a + indicates *Arabidopsis* peroxidase Ca^{2+} pectate binding site, a 0 indicates Cys forming disulfide bridge sites, a ^ indicates Asp-Arg salt bridge sites and ~ shows two other amino acids important in plant peroxidases.

```
                            0                          *  ** 0    0
Horseradish       QLTPTFYDNSCPNVSNIVRDTIVNELRSDPRIAASILRLHFHDCFVNGCDASILLDNTTS
Kil-0             QLTPTFYDRSCPNVTNIVRETIVNELRSDPRIAASILRLHFHDCFVNGCDASILLDNTTS

                     ~     *                    0     0 ^
Horseradish C     FRTEKDAFGNANSARGFPVIDRMKAAVERSCPRTVSCADLLTIAAQQSVTLAGGPSWRVPL
Kil-0             FRTEKDAFGNANSARGFPVIDRMKAAVERACPRTVSCADMLTIAAQQSVTLAGGPSWRVPL

                   ^               ~                              *      0
Horseradish C     GRRDSLQAFLDLANANLPAPFFTLPQLKDSFRNVGLNRSSDLVALSGGHTFGKNQCRFIMD
Kil-0             GRRDSLQAFLELANANLPAPFFTPPQLKASFRNVGLDRPSDLVALSGGHTFGKNQCQFILD

                                            0                      +     +  +
Horseradish C     RLYNFSNTGLPDPTLNTTYLQTLRGLCPLNGNLSALVDFDLRTPTIFDNKYYVNLEEQKGL
Kil-0             RLYNFSNTGLPDPTLNTTYLQTLRGLCPLNGNRSALVDFDLRTPTVFDNKYYVNLKEQKGL

                     ~                                                     0
Horseradish C     IQSDQELFSSPNATDTIPLVRSFANSTQTFFNAFVEAMDRMGNITPLTGTQGQIRLNCRVV
Kil-0             IQSDQELFSSPNATDTIPLVRAYADGTQTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVV

Horseradish C     NSN
Kil-0             NSN
```

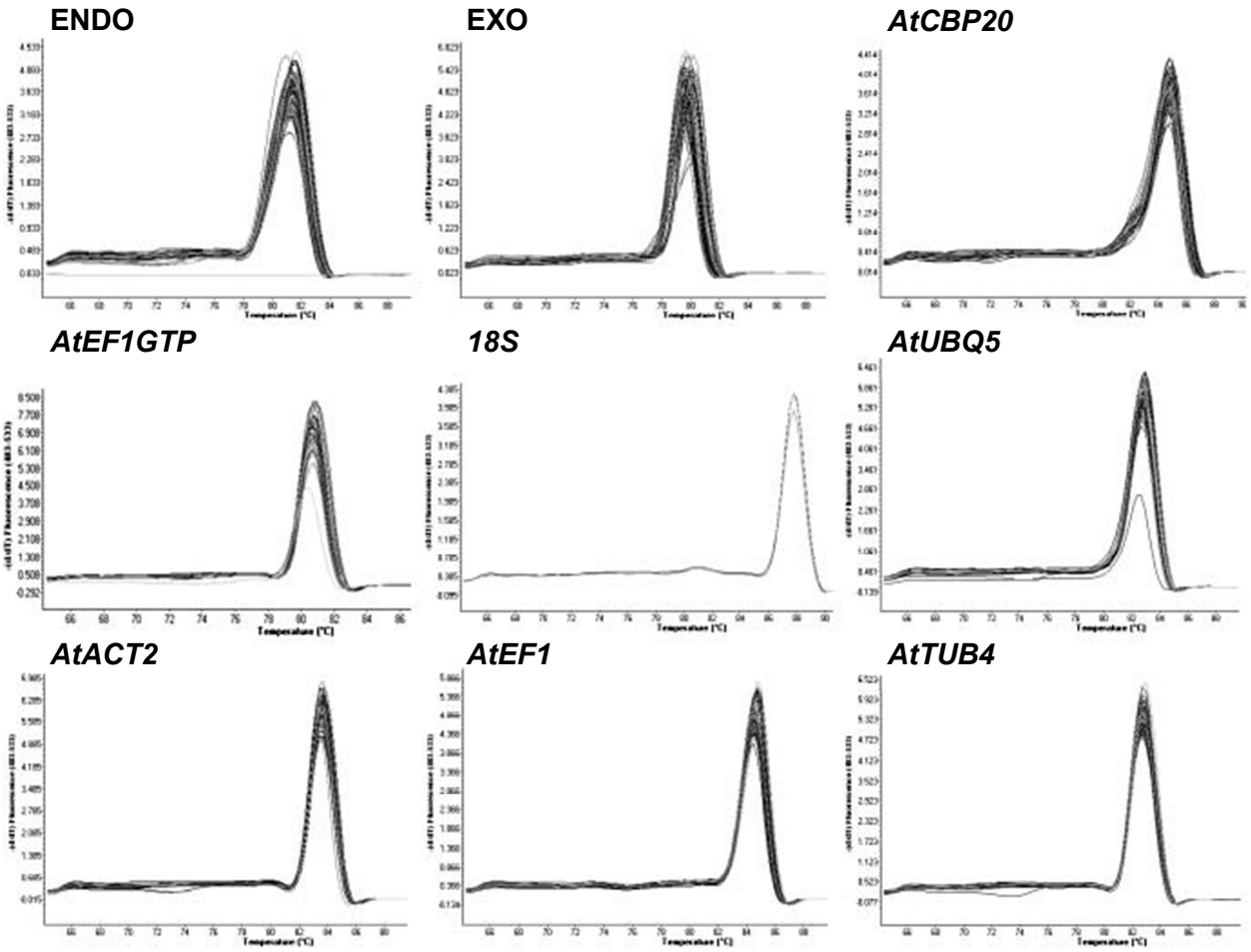
Appendix B3

Alignment of the predicted amino acid sequence of pMDC32-*APRX34* to the Col-*0*, *AtPRX34* sequence obtained from the TAIR website (http://www.arabidopsis.org) and the identified pCR8®/GW/TOPO®-*AtPRX34* clone 8, indicating that the pMDC32-*APRX34* construct contained the same amino acid substitutions in pCR8®/GW/TOPO®-*AtPRX34*. This demonstrates that no additional mutations occurred. The black shadings represent sequence similarities and the white shadings represent amino acid differences between sequences.

```
                        10        20        30        40        50        60
AtPRX34_Col-0     MHFSSSSTSSTWTILITLGCLMLHASLSAAQLTPTFYDRSCPNVTNIVRETIVNELRSDP
AtPRX34_Clone 8   MHFSSSSTSSTWTILITLGCLMLHASLSTAQLTPTFYDRSCPNVTNIVRETIVNELRSDP
AtPRX34_pMDC32    MHFSSSSTSSTWTILITLGCLMLHASLSTAQLTPTFYDRSCPNVTNIVRETIVNELRSDP

                        70        80        90       100       110       120
AtPRX34_Col-0     RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA
AtPRX34_Clone 8   RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA
AtPRX34_pMDC32    RIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVERA

                       130       140       150       160       170       180
AtPRX34_Col-0     CPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTLPQLKA
AtPRX34_Clone 8   CPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTPPQLKA
AtPRX34_pMDC32    CPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTPPQLKA

                       190       200       210       220       230       240
AtPRX34_Col-0     SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP
AtPRX34_Clone 8   SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP
AtPRX34_pMDC32    SFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLCP

                       250       260       270       280       290       300
AtPRX34_Col-0     LNGNRSALVDFDLRTPTVFDNKYYVNLKERKGLIQSDQELFSSPNATDTIPLVRAYADGT
AtPRX34_Clone 8   LNGNRSALVDFDLRTPTVFDNKYYVNLKEQKGLIQSDQELFSSPNATDTIPLVRAYADGT
AtPRX34_pMDC32    LNGNRSALVDFDLRTPTVFDNKYYVNLKEQKGLIQSDQELFSSPNATDTIPLVRAYADGT

                       310       320       330       340       350       360
AtPRX34_Col-0     QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
AtPRX34_Clone 8   QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
AtPRX34_pMDC32    QTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM*
```

Appendix C1

Melting curves of RT-qPCR products for each primer pair generated from the LightCycler® 480 software (Roche). X-axis indicates the temperature (°C) and y-axis indicates (d/dT) Fluorescence (483-533). The melting curves indicate the temperature at which the RT-qPCR product denatured and the fluorescent signal of the SYBR Green (Roche) is detected.



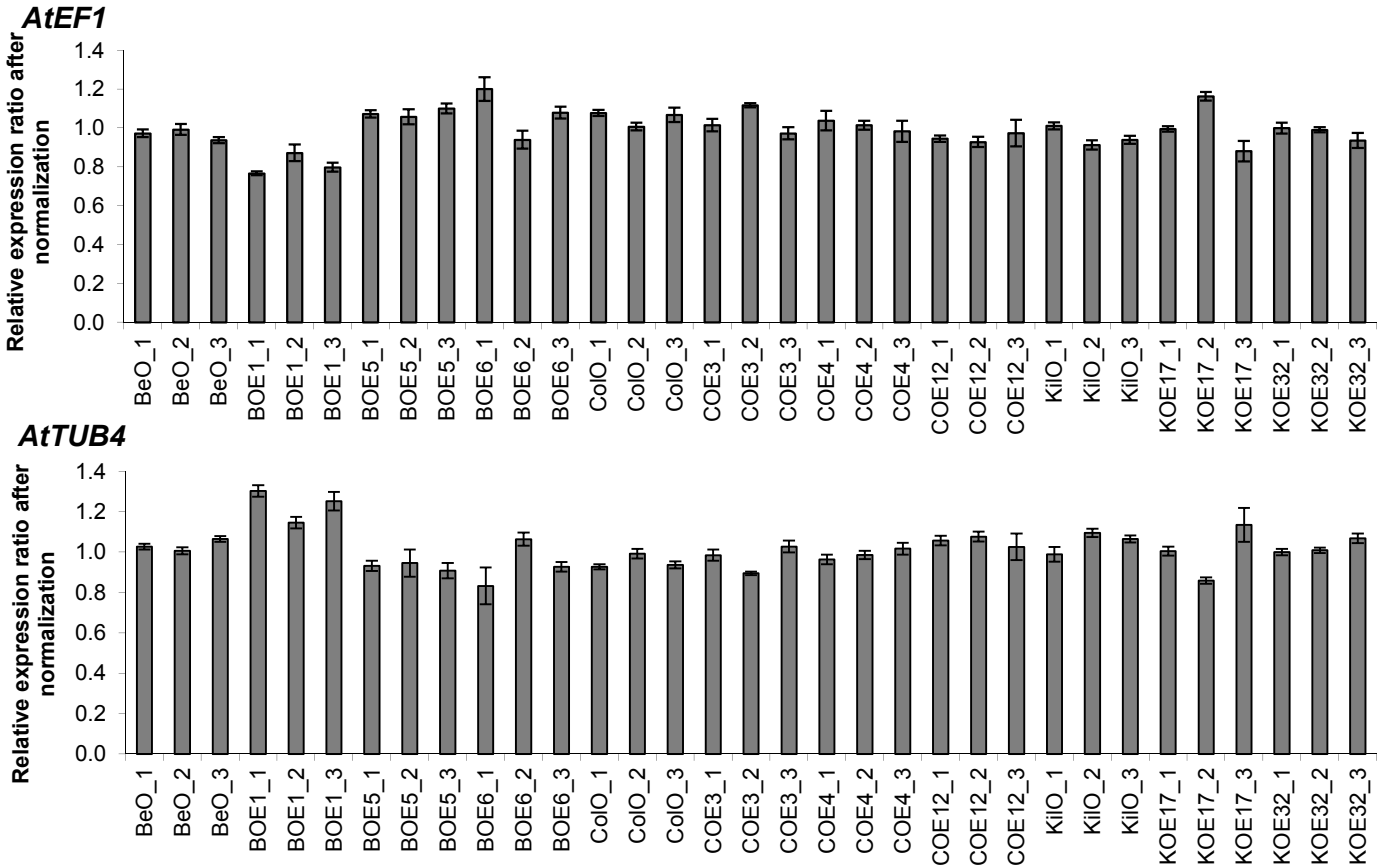
Appendix C2

References genes *AtCBP20*, *AtEF1GTP*, *AtACT2*, *AtUBQ5*, *AtEF1* and *AtTUB4* were analysed with the qBASE normalization software to determine the stability parameter (M) and the coefficient of variation (CV) for each gene. All the samples were used in the analysis (overexpression *AtPRX34* transgenic lines and wildtypes). The M value is listed first followed by the CV value. The other reference genes were compared to *AtEF1* and *AtTUB4*, separately (pairwise comparison) and to the combined reference genes, *AtEF1* and *AtTUB4* (three-way comparison). *AtCBP20* expression levels significantly differed between samples, which resulted in the software program being unable to calculate any values.

	AtTUB4	*AtACT2*	*AtCBP20*	*AtEF1GTP*	*AtUBQ5*
AtEF1	0.3; 10.5 %	0.68; 24%	Not available	1.43; 50.6%	0.84; 32%
AtTUB4		0.58; 20.5%	Not available	1.38; 48.7%	0.87; 33.5%
AtEF1 and AtTUB4		0.52; 20.7%	Not available	1.04; 43.7%	0.67; 27.6%

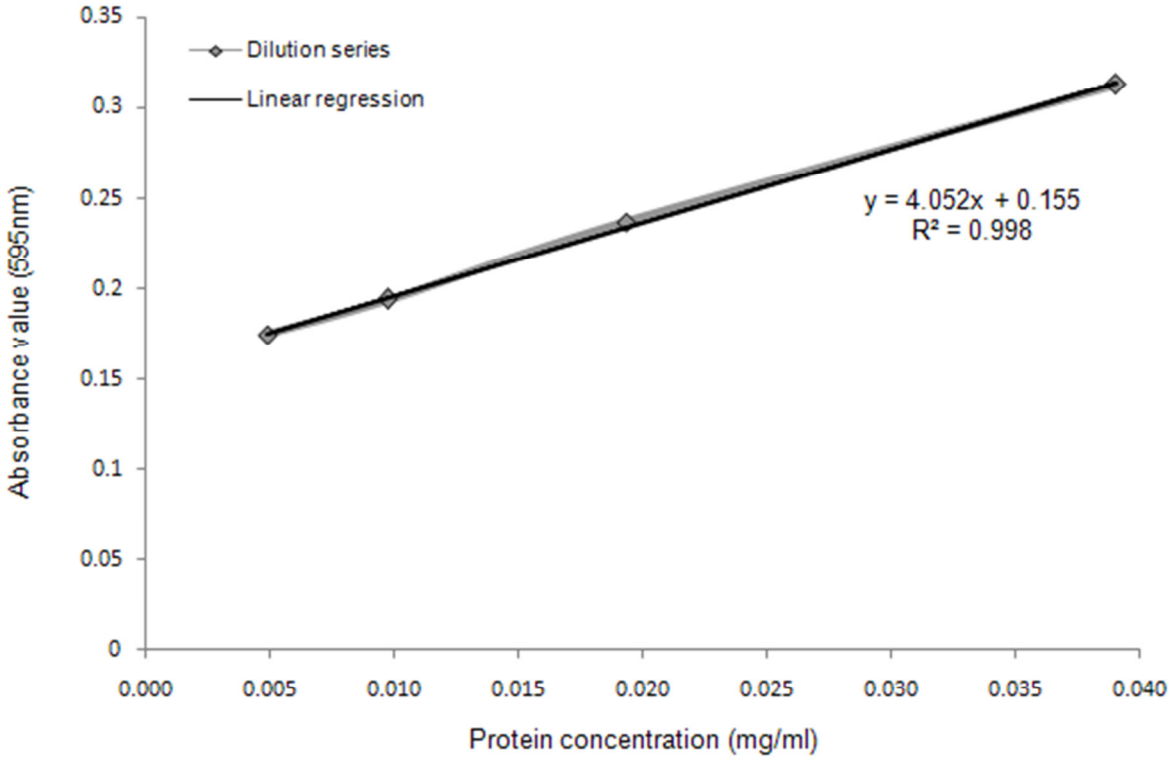
Appendix C3

The *AtEF1* and *AtTUB4* expression profiles after normalization with the factor calculated from both *AtEF1* and *AtTUB4* for all the samples analysed: overexpression *AtPRX34* transgenic lines and wildtypes. These constant transcript levels (M= 0.3 and CV= 10.5 %) were used to normalize endogenous and exogenous target samples. The error bars represent the standard error among the technical replicates.



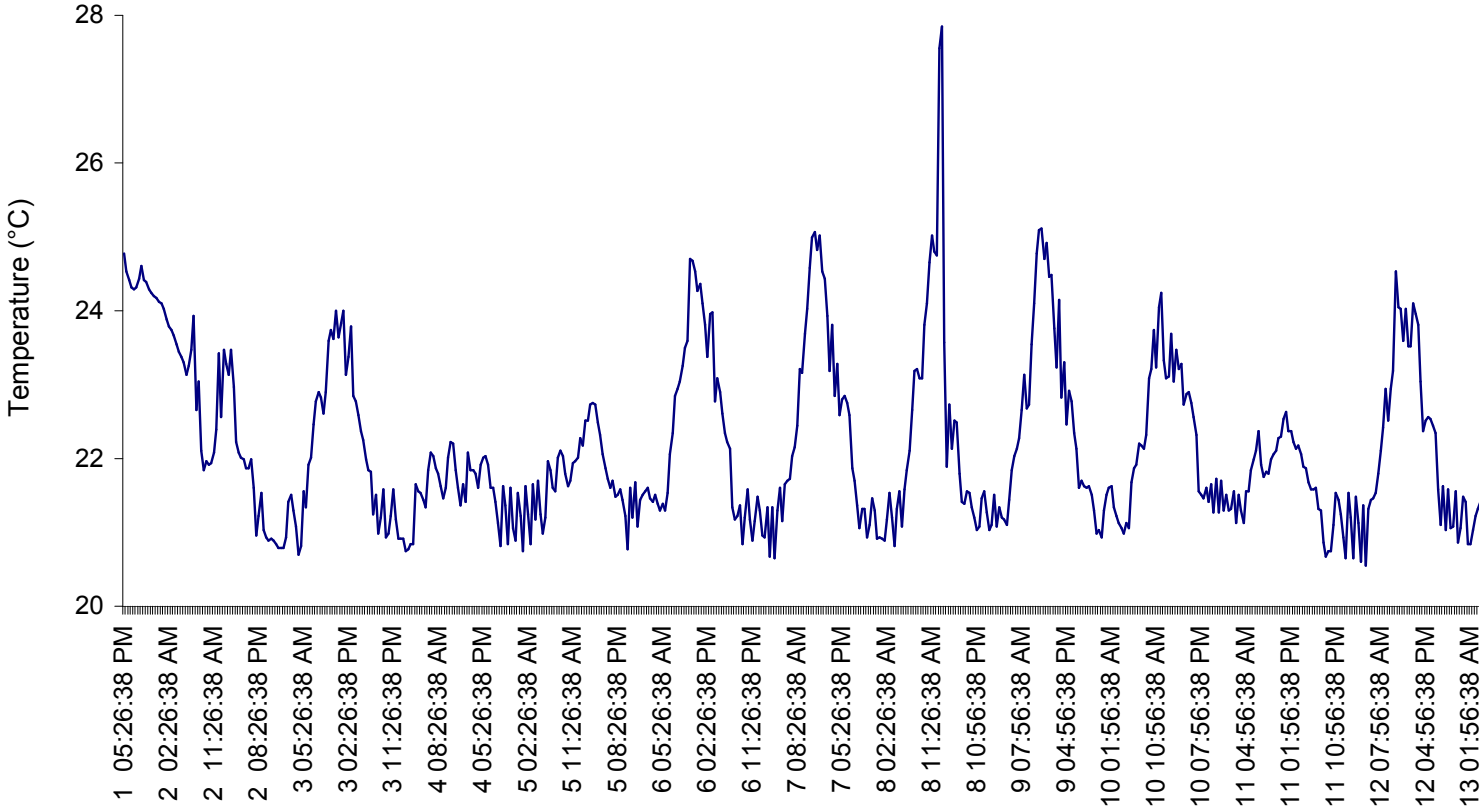
Appendix D

Linear regression trend of bovine serum albumin by measuring the blue-colour formation at an absorbance of 595 nm. The standard protein concentration curve can be used to determine protein concentrations of unknown samples. The slope and R^2 values are indicated for the graph.



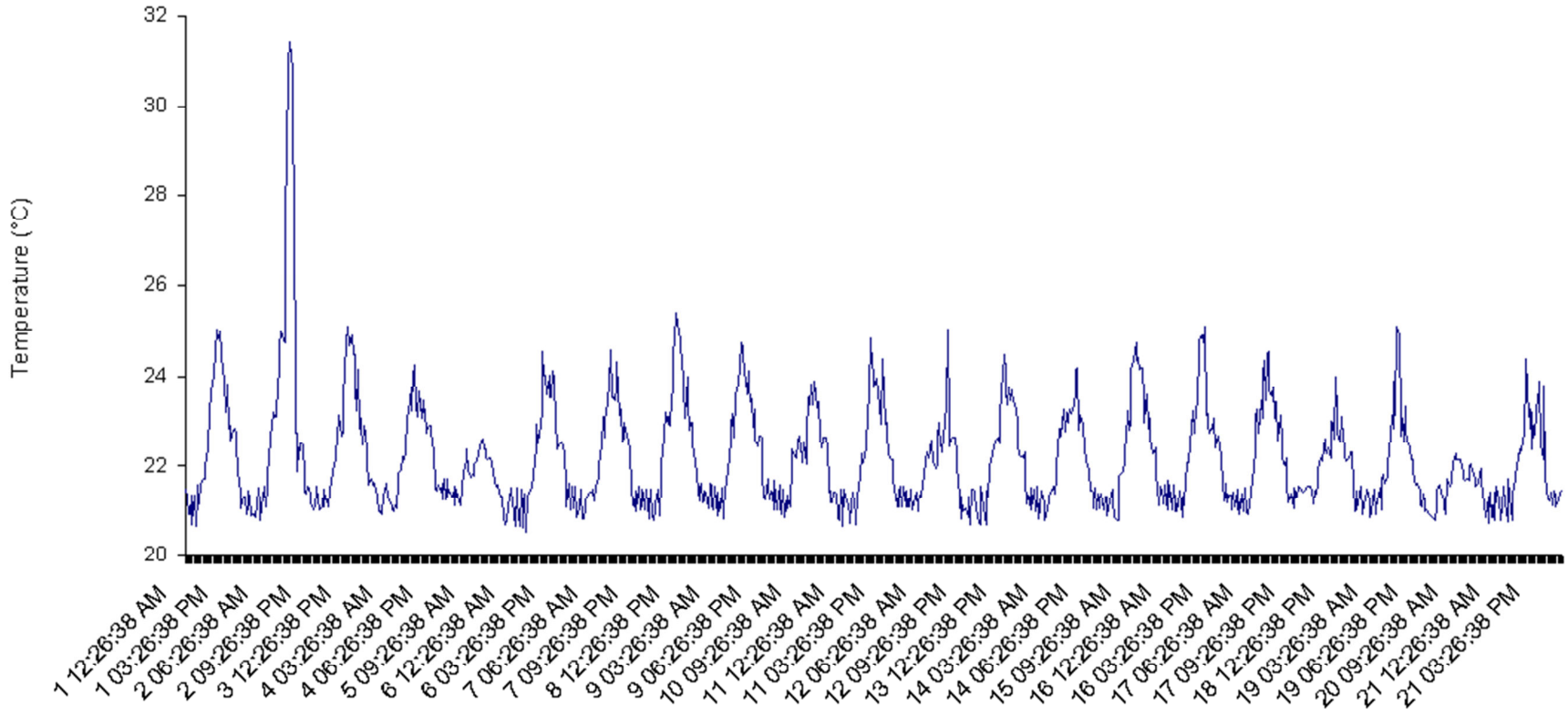
Appendix E1

Temperature readings during the experiment conducted on the different concentrations ($1X10^6$, $1X10^7$, $1X10^8$ and $4X10^8$ cfu/ml) of *R. solanacearum* for 13 days. The measurements were monitored with a HOBO® data logger (Onset computer corporation, Bourne, USA). The x-axis indicated: first the days post inoculation followed by the time the reading was taken.



Appendix E2

Temperature readings during the experiment conducted on the overexpression lines and ecotype challenged with *R. solanacearum* for 21 days. The measurements were monitored with a HOBO® data logger (Onset computer corporation, Bourne, USA). The x-axis shows the days post inoculation followed by the time the reading was taken. On the second day post inoculation, the temperature was increased to 31.5°C.



Appendix F: SALK_051769 analysis

A purchased SALK_051769 in the *AtPRX34* gene of the susceptible ecotype, Col-*0* obtained from Arabidopsis Biological Resource Center (http://www.arabidopsis.org) was investigated to determine if the mutant enhances susceptibility to *R. solanacearum.* Unfortunately, the *AtPRX34* levels in this mutant line were not knocked down.

Materials and methods

a) PCR analysis of SALK_051769 mutant

Genomic DNA was extracted as previously mentioned in section 2.2.8.2 from leaf material. The first primer pair: AtPRX34_PRO_F, TTAGTGGAGACGACCAAGAG and AtPRX34_EXO1 _R, AGGACATGACCTATCGTAGA span the T-DNA insert in the promoter region and the second primer pair: SALK_F, GCGTGGACCGCTTGCTGCAACT and AtPRX34_EXO1_R determine the correct insertion of the T-DNA into the *AtPRX34* promoter region (Figure 2.19). The PCR reaction conditions, as mentioned in section 2.2.4, were used and the cycle conditions had an initial denaturation step of 94°C for two minutes followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C and elongation for two minutes at 72°C, followed by a final elongation step of five minutes at 72°C. The first primer pair produces an 1191 bp fragment in the Col-*0* background, indicating the wildtype allele and the second primer pair produces a 597 bp fragment in the mutant line, indicating a T-DNA insertion allele. These two primer pairs were used to determine if the mutant lines are homozygous for the T-DNA insert and heterozygous or homozygous for the wildtype allele.

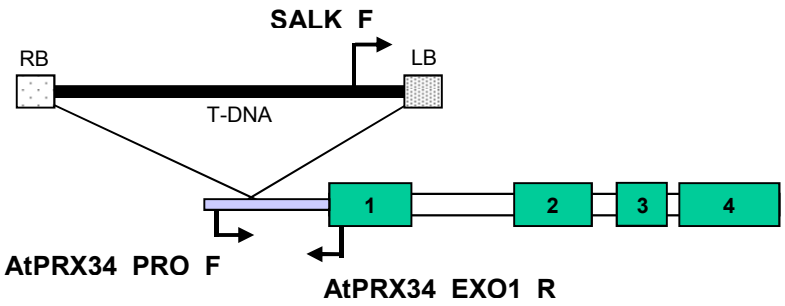


Figure 2.19. Schematic diagram of SALK_051769, indicating the insertion position of T-DNA fragment into the promoter of *AtPRX34*. The *AtPRX34* gene structure contains 4 exons and three introns. The primer binding sites for gene-specific primers (AtPRX34_PRO_F and AtPRX34_EXO1_R) and the T-DNA-specific primer (SALK_F) are indicated with arrows.

b) Reverse transcription quantitative PCR (RT-qPCR) of SALK_051769

RT-qPCR was carried out as previously mentioned in section 2.2.9 from four-week-old plants. The endogenous *AtPRX34* transcript levels were normalized to *AtEF1* and *AtTUB4*.

Results

a) PCR analysis of SALK_051769 mutant

The SALK_051769 mutants' genomic DNA was screened with two different primer pair combinations to identify if the mutants were homozygous or heterozygous for the T-DNA insert. The first primer pair spans the T-DNA insertion site, which will indicate the presence of the wildtype allele or the absence of the T-DNA insert in the promoter region and the second primer pair contains a T-DNA specific primer and *AtPRX34* specific primer that indicate the presence of the T-DNA into promoter. Mutants are homozygous for the T-DNA insert if a fragment size of 597 bp is obtained for the second primer combination and no amplification in the first PCR. Mutants are heterozygous when an 1191 bp and 597 bp fragments are amplified from the first and second PCR respectively. Two different SALK_051769 plants were screened and both a homozygous and a segregating mutant were identified (Figure 2.20).

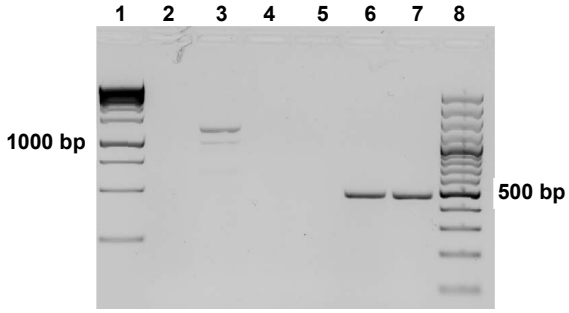


Figure 2.20. A 1.5% (w/v) agarose gel electrophoresis of the PCR products amplified from integrated T-DNA into plants genomic DNA of SALK_051769 using primer combinations: AtPRX34_PRO_F; AtPRX34_EXO1_R and SALK_F; AtPRX34_EXO1_R generating product sizes of 1191 bp and 597 bp, respectively. Lane 1 is a 1kb DNA size standard, lane 2 and 5 are template-free PCR (negative control), lanes 3, 4, 6 and 7 are SALK lines. Lane 2 to 4 are amplified products from the first primer pair and lane 5 to 7 are amplified products from the second primer pair and lane 8 is a 100 bp DNA size standard.

b) Reverse transcription quantitative PCR (RT-qPCR) of SALK_051769

The SALK-051769 endogenous levels were analysed with the overexpression *AtPRX34* transgenic lines and were normalized with the reference genes, *AtEF1* and

AtTUB4. The biological reproducibility was very low and indicated with separate bars (Figure 2.21). *AtPRX34* transcript levels did not indicated a knockout or even a knockdown of the gene compared to the Col-*0* wildtype, instead the opposite effect was detected. This RT-qPCR experiment was repeated with new cDNA and similar results were obtained (data not shown).

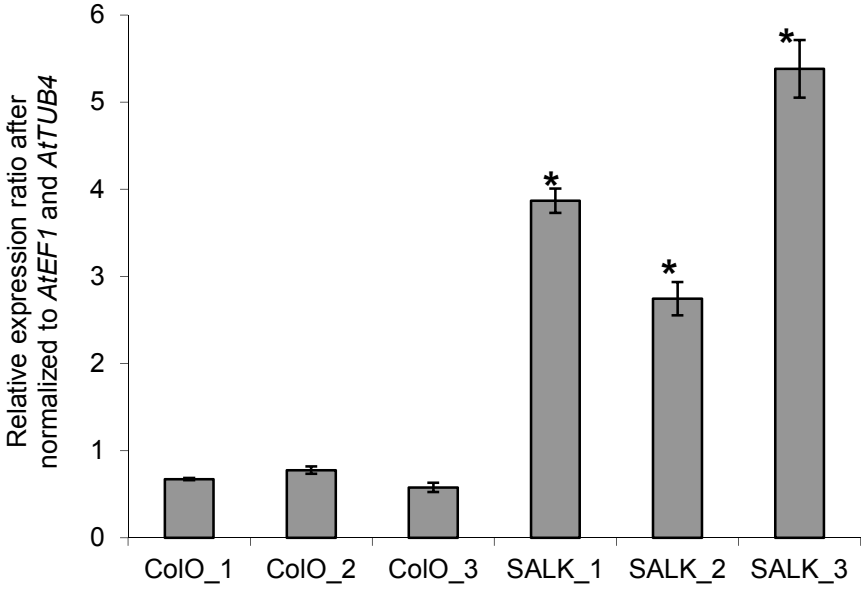


Figure 2.21: Relative expression profiles of the endogenous *AtPRX34* gene in the SALK_051769 and the Col-*0* wildtype. The error bars represent the standard error of the technical replicates (*n* =*3*). The SALK_051769 line for all three biological replicates exhibited significantly ($p<0.01$, Student *t*-test) increased expression compared to Col-*0*.

Discussion

The SALK_051769 in the *AtPRX34* gene of the susceptible ecotype, Col-*0* was to be investigated to determine if an *AtPRX34* mutant could enhance the susceptibility to *R. solanacearum.* The use of a susceptible line to investigate the effect of a defence response gene would have some concerns: 1) Would the effect of no *AtPRX34* transcript in a susceptible line be accurately investigated, 2) would a difference in wilt symptoms be detected between the mutant line and its wildtype and 3) could a conclusion be made from such a study? Unfortunately, the available T-DNA integrations have been made in a susceptible ecotype to *R. solanacearum*. The SALK_051769 line with the T-DNA integration into the promoter region of the target gene was the only obtainable *AtPRX34* mutant at the start of the study. Currently a large number of new mutant are available on Arabidopsis Biological Resource Centre (http://www.arabdopsis.org).

The *AtPRX34* levels in the purchased SALK_051769 line were not decreased by the T-DNA insertion into the promoter region of the gene. Surprisingly, the opposite effect was detected with an increase in *AtPRX34* transcript levels. This could be explained by a number of different possibilities, which need to be tested to confirm these hypotheses. The region from 200 bp (region of T-DNA integration) upwards in the promoter is not necessary for activation of the *AtPRX34* expression and the T-DNA could possibly enhance the transcription of this gene. Read-through of the T-DNA into the *AtPRX34* gene region cannot be excluded, because read-through of the left border of the constructs are quite frequent (Chilton and Que, 2003; Kim et al., 2003). It is also possible that the T-DNA is disrupting the transcription regulation elements, which is responsible for inhibiting transcription.

The solution to this dilemma is to silence *AtPRX34* in Kil-*0* plants and determine if these transgenic lines show enhanced susceptibility to *R. solanacearum*. This strategy was proposed and investigated for this MSc study, but unfortunately due to the high sequence similarity between peroxidases (Cosio and Dunand, 2009) obtaining a fragment large enough to induce silencing without targeting family members (73 member in *A. thaliana*, Welinder, 1992) are practically impossible.