The genetic basis of resistance in *Arabidopsis thaliana* ecotype Kil-0 against *Ralstonia solanacearum* isolate BCCF 402 from *Eucalyptus*

by

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Declaration

I hereby declare that this thesis is my own work and has not been submitted for any degree at any other university.

LE van der Linden

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Acknowledgements

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Preface

Bacterial wilt, caused by *Ralstonia solanacearum*, is an important plant disease that affects several hosts, such as tomato, potato, tobacco, banana and *Eucalyptus*. *Eucalyptus* is economically important for its wood, which is used for the manufacturing of paper, viscose and rayon and construction timber. *R. solanacearum* infection has been observed on *Eucalyptus* species in three African countries: in the KwaZulu Natal region of South Africa, in the Democratic Republic of Congo and Uganda. Only a small number of *Eucalyptus* plantations have been investigated in Africa and the severity of the disease may be underestimated.

In a previous study in our laboratory, the virulence of several *R. solanacearum* isolates obtained from the three countries mentioned above was tested against a number of *Arabidopsis thaliana* ecotypes to develop a new pathosystem. This pathosystem involved the two ecotypes Killean (Kil-0) and Bensheim (Be-0) and the Congo isolate BCCF 402. Be-0, the susceptible ecotype, shows severe wilt symptoms two weeks after root inoculation, while Kil-0, the resistant ecotype, remains healthy two weeks after inoculation. The aim of this study was to characterize the genetic basis of resistance in Kil-0 to the isolate BCCF 402. Resistance against *R. solanacearum* is a complex trait and we hypothesised that resistance in Kil-0 against isolate BCCF 402 is polygenic or due to a single gene.

This thesis consists of three chapters and the content of each chapter is described in a few words. Chapter 1 is a literature review, which discusses the soilborne pathogen *R. solanacearum*, the model plant *A. thaliana*, plant defense as well as techniques used to characterize resistance in a plant against a pathogen.

Chapter 2 characterizes the genetic basis of resistance in the *A. thaliana* ecotype Kil-0 to a *Eucalyptus* isolate of *R. solanacearum*. The characterization was conducted using several different techniques, including genetic crosses between the susceptible (Be-0) and resistant (Nd-1 (Niederzenz) and Kil-0) *A. thaliana* ecotypes, sequencing of the *RRS1* gene in Kil-0, mutation and complementation studies of the *popP2* gene in the *Eucalyptus* isolate BCCF 402 and the use of a co-dominant CAPS marker to determine if *RRS1* in Kil-0 is linked to resistance. Chapter 3 provides a summary of the results obtained in this study and a

comparison between the *A. thaliana-R. solanacearum* pathosystems. Possible future work is also discussed.

Abbreviations

ACUR	Alternate Codon Usage Regions
BCCF	Bacterial Culture Collection FABI
BGT	Bacto-agar Glucose Triphenyltetrazolium chloride
bp	base pairs
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpi	days post inoculation
EDTA	ethylenediamine tetraacetic acid
hr	hour
kb	kilobase
min	minute
MS	Murashige and Skoog media
ng	nanogram
ORF	open reading frame
PCR	polymerase chain reaction
PR	pathogenesis related
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
TTSS	Type III secretion system
µg	microgram

Chapter 1

Literature review

1.1 *Ralstonia solanacearum*

1.1.1 Bacterial wilt disease

Ralstonia solanacearum (previously known as *Pseudomonas solanacearum*) is the causal agent of bacterial wilt and it is a gram negative, aerobic rod classified in the β-subdivision of the Proteobacteria (Schell, 2000*). R. solanacearum* has a worldwide distribution and a large host range of more than 200 species in 50 families. *R. solanacearum* affects a wide variety of plants and these include economically important hosts, such as tomato, potato, tobacco, banana, cowpea, peanut, cashew, papaya and olive (Schell, 2000). Bacterial wilt was initially observed in *Eucalyptus* plantations in China and Brazil (Chao, 1982; Sudo *et al.,* 1983). Thereafter the disease has also been reported on *Eucalyptus* in Australia, South Africa, Uganda, Congo and Venezuela (Coutinho *et al.*, 2000; Roux *et al.*, 2001; Gan *et al*., 2004). Surveys were recently conducted in major *Eucalyptus* plantations in China and bacterial wilt was one of the most frequently encountered stem diseases (Zhou *et al.,* 2008). Forest industries are growing in Africa and this disease can cause serious problems under optimal conditions (Reddy *et al.,* 2002). There is no effective universal control method for bacterial wilt disease, although various approaches such as crop rotation; soil treatments and disease avoidance have been used to limit the disease (Ramesh *et al*., 2009). One of the most successful ways of reducing the occurrence of the disease is to combine preventative measures with the use of resistant cultivars (Poussier *et al*., 2002).

1.1.2 Classification and identification of *R. solanacearum*

R. solanacearum is a species complex and a large diversity of strains belongs to this complex (Prior and Fegan, 2005). The genetic diversity of strains of *R. solanacearum* needs to be characterized to identify groups of strains that have similar biological properties, evolutionary relationships or geographic origins. The information will help in improving breeding strategies for obtaining durable resistance to bacterial wilt in

economically important plants affected by this pathogen. *R. solanacearum* was originally classified into five races (related to host range) and six biovars (related to the ability to metabolize three sugar alcohols and three disaccharides), but these classifications schemes are not sufficient to cover the diversity of strains represented in the *R. solanacearum* species (Castillo and Greenberg, 2007).

RFLP analysis on the *hrp* gene region and 16S rRNA sequence analysis have also been used as a basis of classifying *R. solanacearum* (Cook *et al*., 1989; Cook *et al*., 1994; Poussier and Luisetti, 2000). The PCR-RFLP method was used to identify the causal agent of bacterial wilt from *Eucalyptus* plantations in the Democratic Republic of Congo (DRC), South Africa and Uganda and from potato fields in South Africa (Fouché-Weich *et al*., 2006). PCR-RFLP analysis was performed on the *hrp* gene to determine and group the biovars of the *R. solanacearum* strains. The *Eucalyptus* strains and one potato strain were identified as biovar 3 isolates and the other potato isolates were identified as biovar 2 isolates. This study confirmed that PCR-RFLP is a reliable tool for identification and classification of *R. solanacearum* (Fouché-Weich *et al*., 2006). In this particular MSc study we are using the *Eucalyptus* isolate BCCF 402 that was characterized as a biovar 3 isolate along with the other *Eucalyptus* strains.

A new hierarchical classification scheme based on DNA sequence analysis of the ITS region, endoglucanase, *hrpB* and *mutS* genes was proposed by Prior and Fegan (2005). The *R. solanacearum* species complex was divided into four phylotypes or genetic groups. Each phylotype relates to the geographic origin of the strains. Phylotype I consists of strains coming from Asia, Phylotype II consists of strains primarily from America, Phylotype III members are mainly from Africa and Phylotype IV strains are from Indonesia, Japan and Australia (including *Pseudomonas syzygii* and the blood disease bacterium (BDB)). Consistency of this phylotyping scheme was tested by sequencing more isolates from Asia (Villa *et al*., 2005). The 16S rDNA, endoglucanase and *hrpB* genes were partially sequenced and different levels of polymorphisms were

observed in each of these DNA regions. The strains were consistently divided into four clusters (phylotypes) as proposed by Prior and Fegan (2005). The study also demonstrated the distribution and diversity of the Asian strains, which are present in three of the four clusters. A GMI1000 microarray was used to investigated the distribution of the GMI1000 orthologous genes in other *R. solanacearum* strains and the hierarchical clustering of the strains was fully consistent with the classification into the four phylotypes (Guidot *et al*., 2007). The phylotyping scheme showed to have several advantages over the race and biovar classification systems (Prior and Fegan, 2005). The scheme reflects the evolutionary history of the organisms, it can differentiate among a large number of strains and the system provides valuable information about the geographical origin of the strains (Prior and Fegan, 2005).

1.1.3 Genome sequencing of *R. solanacearum*

Genome sequencing of *R. solanacearum* offers a starting point towards the functional analysis of pathogenicity determinants in the pathogen (Salanoubat *et al*., 2002). Molecular determinants responsible for the diversity in host range among the different strains can be identified (Genin and Boucher, 2002). The availability of sequence information makes it possible to use DNA microarrays to analyse the biodiversity among strains and to identify strain specific genes. The function of type III effectors and their targets in plant cells can be determined (Genin and Boucher, 2002). This information will be valuable in developing new methods to control the disease.

The genome sequence of GMI1000 (race 1, biovar 3, phylotype I)

R. solanacearum strain GMI1000 is a biovar 3, race 1 strain and it was isolated from tomato in French Guyana (Salanoubat *et al*., 2002). The whole-genome of the strain was sequenced by the random sequencing method and the genome was assembled into two circular molecules (a 3.7 Mb chromosome and a 2.1 Mb megaplasmid). The size of the genome is 5.8 Mb with a high G+C content of about 67% and it encodes about 5120 proteins (Genin and Boucher, 2002). The megaplasmid encodes genes that play a role in

the overall fitness of the bacterium and may give advantages in different environments (Salanoubat *et al.*, 2002). Examples are genes that play a role in flagellum biosynthesis, pathogenicity and catabolism of aromatic compounds (Genin and Boucher, 2002). The chromosome encodes all the basic mechanisms needed for the survival of the pathogen (Salanoubat *et al*., 2002). The megaplasmid also contains duplications of vital genes, such as a rDNA locus, three tRNAs, a second σ54 factor gene (*rpoN2*) and a second subunit α of DNA polymerase III (*dnaE2*) (Genin and Boucher, 2002). Enzymes responsible for the metabolism of small molecules, such as methionin biosynthesis, are only encoded on the megaplasmid. Thus, the megaplasmid might still be in the process of obtaining new functions through duplication or translocation of important genes from the chromosome (Genin and Boucher, 2002).

The *R. solanacearum* genome is complex and it has potential for plasticity (Genin and Boucher, 2002). The genome has a high number of transposable elements as well as Alternate Codon Usage Regions (ACURs) which corresponds to about 7% of the genome. The ACURs are usually associated with transposable elements or prophage sequences and these regions have a high number of genes with a G+C content that is significantly lower than the 67% average. The ACURs also contain several pathogenicity genes (such as genes encoding effector proteins translocated through the type III secretion pathway) and several duplicated open reading frames (ORFs) with an unknown function. The ACURs can be pathogenicity islands (PAI) acquired through horizontal transfer and these regions might be involved in a duplication/evolution process, contributing to genomic variation.

Several genes encoding proteins that may be involved in pathogenicity were identified in the GMI1000 genome (Genin and Boucher, 2002). *R. solanacearum* contains genes that are homologous to known bacterial effector genes which specify race/cultivar resistance in the other pathogens. Gene clusters involved in the synthesis of fimbrae/pili or large surface proteins related to haemagglutinins were also identified. Pili are

important for natural transformation competence as well as adherence to plant surfaces. Genes encoding new plant cell wall- degrading enzymes such as 1,4 β-cellobiosidase, genes involved in the synthesis of plant hormones as well as ORFs encoding quorum sensing systems were also identified in the *R. solanacearum* genome.

Genome sequence of *R. solanacearum* strain UW551 (race 3, biovar 2, phylotype II)

The *R. solanacearum* strain UW551 (race 3, biovar 2, phylotype II) was isolated from a diseased geranium and this strain is also pathogenic on tomato and potato (Gabriel *et al*., 2006). The UW551 strain is listed as a United States Department of Agriculture Select Agent (pathogens that can pose a risk to public health and safety). The R3B2 strains are able to cause symptoms on potato at temperatures as low as 16˚C. An 8x draft genome was obtained for this UW551 strain and 4454 protein coding open reading frames (ORFs) were identified. The genome also has 43 tRNAs, 5 rRNAs and 62% of the ORFs have a functional assignment. The UW551 genome was compared with the genome of strain GMI1000 and these two strains were 71% syntenic in gene organization. All the main classes of genes known to be involved in pathogenicity (such as plant-degradative enzymes, type III effectors and *R. solanacearum* ORFs known to be induced in plants) were highly conserved.

A total of 402 unique UW551 ORFs were identified and only a few appeared to be involved in pathogenicity and none were involved in cold tolerance (Gabriel *et al*., 2006). This suggests that pathogenic and cold adaptation variability within the species may be caused by regulatory genes, genes of unidentified function or genetic variation found among conserved genes. Genomic comparisons revealed a 22-kb region that is present in GMI1000, but absent from UW551 and this region encodes enzymes that are important for utilization of the three sugar alcohols that distinguish biovars 3 and 4 from biovars 1 and 2. A cluster of 38 prophage genes were identified in UW551 that were absent from GMI1000. This cluster was examined by polymerase chain reaction, among

a group of 58 strains from different races and biovars, and PCR analyses showed that this cluster was present in all R3B2 strains (Gabriel *et al*., 2006).

Gene distribution among different *R. solanacearum* strains

Comparative genomic hybridization (CGH) was used to investigate the gene distribution among strains of *R. solanacearum* (Guidot *et al*., 2007). A collection of 18 strains that are representative of the biodiversity of the *R. solanacearum* species were chosen for the study. Complete genome sequencing of the GMI1000 strain made it possible to develop a microarray with 5074 oligonucleotides that are representative of the 5120 predicted genes from GMI1000. This array was used to study the distribution of GMI1000 genes in distant *R. solanacearum* strains. Only 2690 (53%) of the genes represented on the array are present in all the *R. solanacearum* strains tested and this set of genes represents the *R. solanacearum* core genome. A list of 2338 variable genes have been identified within the *R. solanacearum* species and the variable genes showed to be clustered in genomic islands.

Some of the genomic islands are included in regions with an ACU and these genomic islands could have originated from the acquisition of foreign genes through lateral gene transfers (Guidot *et al*., 2007). The other genomic islands correspond to genes that have a similar base composition as the core genes and these genes could be ancestral genes lost by deletion in specific strains or the genes could originate from horizontal gene transfers. A large majority of the pathogenicity genes are part of the core genome and this indicates that *R. solanacearum* is an ancestral pathogen. Two sets of pathogenicity genes were variable from strain to strain and these are genes encoding hemagglutinin-related proteins (class of surface proteins required for adhesion to plant tissues) and type III secretion system (TTSS) effectors (Guidot *et al*., 2007).

1.1.4 Bacterial wilt disease in the plant host

Mode of invasion of *R. solanacearum*

R. solanacearum can survive in the soil for a long time by associating with plant debris or weed hosts that are symptomless carriers (Genin and Boucher, 2002). The pathogen infects potential hosts by first attaching to the root surface and forming microcolonies. The colonization of the bacteria happens especially at the root elongation zone and at sites of lateral root emergence due to root exudates or a weakened barrier at these sites (Schell, 2000). The intercellular spaces of the root cortex are subsequently invaded through natural infection sites or through physical wounds.

After invading the intercellular spaces of the root cortex, the bacteria accumulate around the stele and break into the xylem vessels at sites where the endodermis is compromised (Schell, 2000; Vailleau *et al.,* 2007). After colonization of the xylem vessels, the bacteria travel rapidly to the upper parts of the plant and can be detected throughout the stem. The plants show extensive wilting due to reduced sap flow caused by the presence of large amounts of *R. solanacearum* cells and their exopolysaccharide slime in the xylem vessels. The plants collapse and die from further degradation of vessels and surrounding tissues. *R. solanacearum* cells produced in the plant are released back into the ground by the roots or collapsed stems. The bacteria live saprophytically in the soil and will wait for the next potential host (Schell, 2000; Genin and Boucher, 2002).

Regulation of virulence in *R. solanacearum*

All the major secretion pathways described in Gram-negative bacteria are present in *R. solanacearum*, but the nature of the substrates for the majority of the pathways are unknown and their contribution to virulence have not been determined (Poueymiro and Genin, 2009). Bacterial wilt disease development is greatly dependent on the action of the Type II and Type III secretion pathways.

The Type III secretion system (TTSS) is used by gram-negative bacterial pathogens of animals and plants as protein secretion machinery to deliver effector proteins directly into the host cells (Tang *et al*., 2006). The hypersensitive response and pathogenicity (*hrp*) genes encodes the TTSS and confers the bacterium's ability to cause disease on host plants and to elicit a hypersensitive response on nonhost plants. The TTSS consists of about 20 different components and at least nine components are conserved among animal and plant pathogenic bacteria (Büttner and Bonas, 2006). The core structural design of the system is similar in the different bacterial species. The TTSS, which injects the effector proteins into plant cells, spans both bacterial membranes (inner and outer membranes) and is connected to an extracellular Hrp pilus and the TTS translocon. The translocon is inserted into the plasma membrane of the host plant (Büttner and Bonas, 2006).

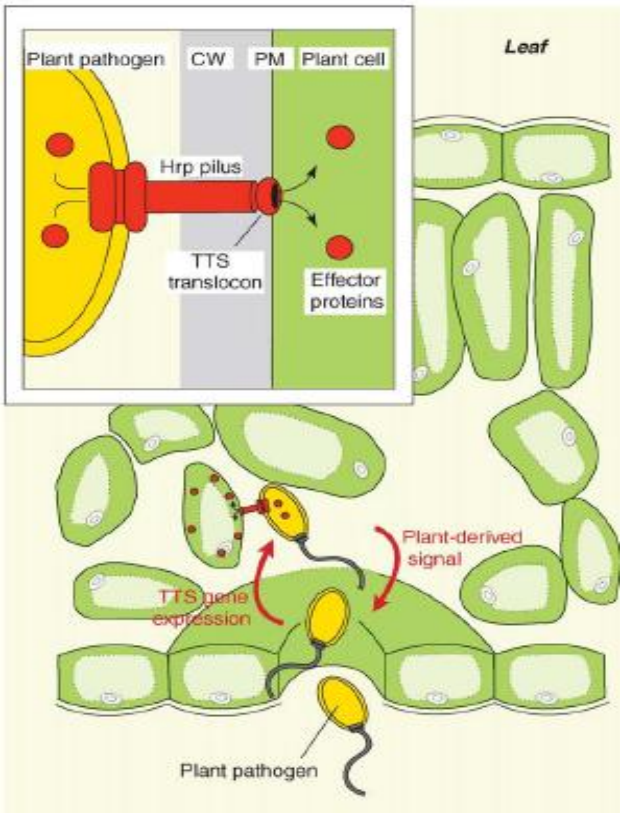

Figure 1.1. **The type III secretion system (TTSS) is used by gram-negative bacterial pathogens of plants as protein secretion machinery to deliver effector proteins directly into the host cells (Büttner and Bonas, 2006).** The bacteria can infect plants through natural openings such as stomata and multiply in the intercellular spaces. The TTSS is induced by an unknown plant derived signal and the system injects effector proteins into the plant cell. The enlarged view of the plant pathogen-interaction indicates that the secretion apparatus spans both bacterial membranes and is connected to an extracellular Hrp pilus and a TTS translocon. The translocon inserts in the plasma membrane of the plant. The TTSS and effectors are shown in red and the bacterial flagella are indicated in dark grey. Abbreviations: CW, cell wall; PM, plasma membrane.

R. solanacearum also has a type II secretion system (T2SS) that secretes several known virulence factors such as a consortium of plant cell wall-degrading enzymes (CWDEs)

(Hikichi *et al.,* 2007; Poueymiro and Genin, 2009). Some of the known CWDEs include a β-1,4-endoglucanase (EgI), an endopolygalacturonase (PehA), two exopolygalacturonases (PehB and PehC), and a pectin methyl esterase (Pme). The T2SS is involved in invasion of *R. solanacearum* into the xylem vessels, leading to systemic infection (Hikichi *et al.,* 2007; Poueymiro and Genin, 2009).

R. solanacearum has two infection stages: the bacterium first invades and multiples in the intercellular spaces of the roots along with invasion of the xylem vessels, while the later stage involves proliferation and production of exopolysaccharides (EPS) in the xylem vessels (Hikichi *et al*., 2007). Host sensing is important for the bacterial pathogens to infect their hosts (Tang *et al.,* 2006). Physical interaction between the bacteria and the plant cell is sensed by the outer membrane receptor PrhA (Hikichi *et al.,* 2007; Yoshimochi *et al.,* 2009) (Figure 1.2). This membrane protein has significant similarities with siderophore receptors and mutation in this protein was shown to disrupt contact-dependent type III gene induction, but not induction by minimal medium (Tang *et al.,* 2006). The chemical nature of the plant signal perceived by PrhA is unknown, but it can be a nondiffusible plant wall component that is resistant to protease and heat treatments. PrhA transmits the plant signal to a regulatory cascade, including the membrane protein PrhR and the transcriptional activators PrhI, PrhJ, HrpG and HrpB (Tang *et al.,* 2006; Hikichi *et al.,* 2007; Yoshimochi *et al.,* 2009) (Figure 1.2). HrpB then activates the expression of *hrp* and effector genes. The expression of several genes which encode proteins secreted through the type II secretion system is positively regulated by HrpB (Hikichi *et al.,* 2007). This suggests that expression of specific T2SS-secreted proteins is co-regulated with that of type III effectors (Figure 1.2).

PhcA is a LysR family transcriptional regulator and is at the center of this complex regulatory network, which controls the expression of pathogenicity factors in *R. solanacearum* (Hikichi *et al.,* 2007; Yoshimochi *et al.,* 2009). This regulatory network responds to environmental conditions, the presence of host cells and bacterial density.

The expression of PhcA is regulated in response to cell density by a quorum-sensing mechanism that involves the specific autoinducer molecule 3-hydroxy palmitic acid methyl ester (3-OH PAME) (Figure 1.2). During the early stages of infection when bacterial density is low, PhcA is not expressed. The expression of *hrpB* is induced in response to plant signals and *hrpB* activates the *hrp* regulon, which constructs the T3SS. The T2SS is also activated to facilitate movement of the bacteria into the xylem vessels (Hikichi *et al.,* 2007). At later stages of infection when the bacterial density is high, 3-OH PAME accumulates and this leads to the activation of PhcA and subsequent production of EPS in the xylem vessel as well as activation of potent CWDEs (Pme and cellulases). *prhIR* expression is repressed by PhcA and this result in suppression of *hrpB*-regulated genes (Yoshimochi *et al.,* 2009).

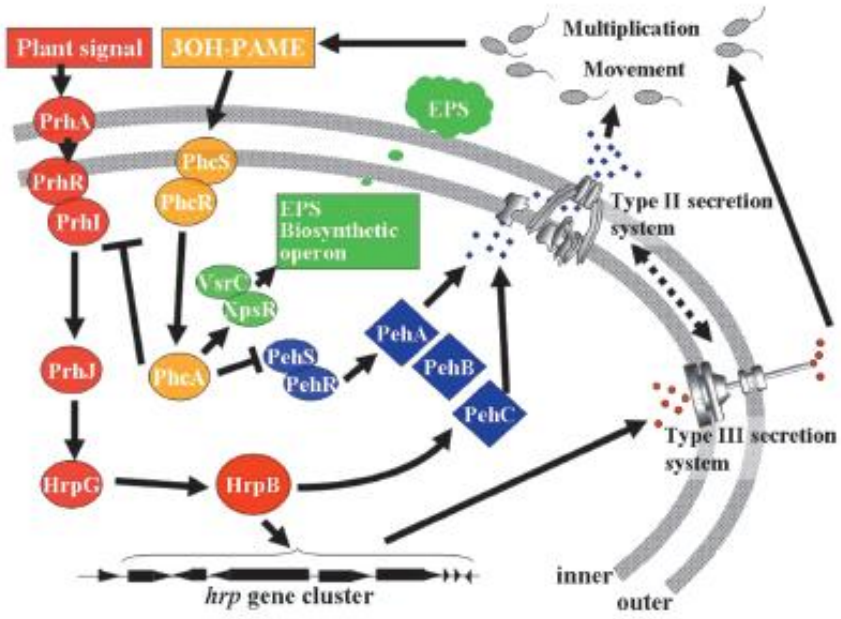

Figure 1.2. **The regulation of the virulence-related genes in *R. solanacearum*. Symbols are: →, positive regulation; ⊣, negative regulation; ↔, interactions (Hikichi *et al.*, 2007).**

Effectors secreted by *R. solanacearum*

The effectors secreted by *R. solanacearum* are all presumed to aid in virulence by stimulating or repressing various cellular functions in the host, but some of these effectors are recognized by cognate resistance proteins in certain host cultivars to trigger a defense response (Turner *et al.,* 2009). Several methods were used to identify the effectors from *R. solanacearum.* As mentioned previously, the HrpB protein controls the transcription of *hrp* genes, involved in the production of the TTSS, as well as effector genes (Cunnac *et al.,* 2004a). A hrp_{II} box (TTCGn16TTCG) element was identified in the promoters of HrpB-regulated genes that is responsible for the Hrp-B dependent activation of these genes. This property was utilized to search the genome sequence of the *R. solanacearum* isolate GMI1000 for promoters that contained the *hrpB*-responsive element (Cunnac *et al.,* 2004a). The search for this motif were not sufficient, because other effector genes were also identified as being part of the *hrpB* regulon, but they lacked a hrp_{II} box in their promoter region (Cunnac *et al.,* 2004b).

A subsequent study in isolate GMI1000 then used a 70-mer oligonucleotide-based DNA microarray that covered almost all of the genes in the strain to identify the complete set of genes that are under the control of the HrpB protein (Occhialini *et al.,* 2005). A total of 26 new candidate effector genes was added to the list of previously identified effectors. A transposon/promoter trap system was also used to identify effectors in isolate GMI1000 and thirty *HrpB* activated genes situated outside the *hrp* gene cluster were identified (Mukaihara *et al.,* 2004).

To date, more than 80 TTSS effectors from *R. solanacearum* were identified. This includes a list of 74 effectors from strain GMI1000 as well as a list of 20 new effectors obtained from the genomic sequences of other strains (Molk2; UW551 and IPO1609) (Poueymiro and Genin, 2009). Homologs of 45% of the effectors identified in GMI1000 are present in other bacterial pathogens of plants and animals, which indicates that more than half of the effectors in this isolate are *R. solanacearum* species specific.

Effectors that function as virulence factors to promote disease in the host plant

Bacteria make use of effectors to suppress the defense responses in plants (da Cunha *et al.,* 2007). A family of T3SS effectors (GALAs) that contributes to virulence of *R. solanacearum* on host plants were identified among the large repertoire of effectors secreted by *R. solanacearum* (Angot *et al*., 2006). A mutant *R. solanacearum* strain was developed in which all of the seven *GALA* effector genes have been deleted. The mutant was no longer pathogenic on *A. thaliana* and less virulent on tomato. A *GALA7* mutant strain also had reduced virulence on *Medicago tranculata* plants. These results indicate that the GALA effectors are required for disease on the host plants. The GALA effectors have a leucine-rich repeat and as well as an F-box domain (Angot *et al.,* 2006). These effectors are similar to a class of eukaryotic proteins called F-box proteins. The eukaryotic F-box proteins together with the Skp1 and Cullin 1 subunits, make up the SCF-type E3 ubiquitin ligase complex and regulate specific protein ubiquitinylation. The ubiquitin-tagged proteins are either modified or degraded by the 26S proteosome. This pathway is very important for many developmental processes in plants such as hormone signaling, flower development and stress responses (Angot *et al.,* 2006).

R. solanacearum GALA T3SS effectors interact with a subset of the 19 different *A. thaliana* Skp1-like proteins just like *A. thaliana* F-box proteins (Angot *et al*., 2006). GALAs are hypothesized to interfere with the host ubiquitin/proteosome pathway by recruiting plant proteins into SCF complexes (Poueymiro and Genin, 2009). The plant proteins are ubiquitinated and subsequently modified or degraded. These target proteins could be components of the plant defense response or effectors from the pathogen that have performed their function and need to be degraded (Poueymiro and Genin, 2009).

Type III effectors that trigger a defense response in the host plant

The presence or action of certain effectors can be detected by specific receptors (resistance proteins) in some host plants (Poueymiro and Genin, 2009). The recognition

of the pathogen by the host leads to a rapid localized programmed cell death called the hypersensitive response (HR). These effectors that trigger a defense response in the plants are known as avirulence factors and three *R. solanacearum* avirulence proteins have been characterized to date. The GMI1000 *avrA* gene product activates an HR response in several tobacco species and resistance against isolate GMI1000 in certain Petunia and *A. thaliana* ecotypes is due to the detection of the PopP1 or PopP2 effectors, respectively (Carney and Denny, 1990; Lavie *et al.,* 2002: Deslandes *et al.,* 2003).

The Pseudomonas outer protein P2 (PopP2) belongs to the YopJ/AvrRxv effector family and is the avirulence protein in isolate GMI1000 that triggers a specific resistance response in the *A. thaliana* ecotype Nd-1 upon recognition by the resistance protein RRS1-R (Deslandes *et al.,* 2003). RRS1-R is a member of the TIR-NBS-LRR class of R proteins and it possesses a C-terminal WRKY domain which is characteristic of some plant transcriptional factors. Yeast two-hybrid analysis showed that RRSI-R and PopP2 physically interacts and RRS1-R is targeted to the plant nucleus upon coexpression with PopP2 (Deslandes *et al.,* 2003).

RD19, an *A. thaliana* cysteine protease, was identified as a PopP2-interacting partner and is normally localized in the plant lytic vacuole (Bernoux *et al.,* 2008). PopP2 physically interacts with RD19 and relocalizes RD19 to the plant nucleus. The *A. thaliana rd19* mutant that is homozygous for both the *rd19* mutation and the *RRS1-R* gene showed loss of full resistance to the *R. solanacearum* isolate GMI1000. This result indicates that RD19 is needed for RRS1-R mediated resistance. RD19 showed increased expression during infection by *R. solanacearum* and no physical interaction between RD19 and RRS1-R was detected in the nucleus in the presence of PopP2 (Bernoux *et al.,* 2008). The function of RD19 is not yet known, but it could act as a transcription factor to induce plant defense responses as determined for other cysteine proteases (Bernoux *et al*., 2008). The contribution of PopP2 to virulence of *R. solanacearum* on host plants is

unknown, but different models can be hypothesized, in which RD19 is the main target of the effector (Poueymiro and Genin, 2009). PopP2 interacts with RD19 in the nucleus and PopP2 could either inhibit RD19 to suppress defense response in the plant or PopP2 requires an RD19-dependent modification to carry out its virulence function. In a resistant plant, RRS1-R can recognize these modifications in the RD19/PopP2 complex and activate defense responses through its WRKY domain (Poueymiro and Genin, 2009).

1.2 *A. thaliana* as a model system for dicotyledenous plants

A. thaliana is known as the model for research in dicotyledonous plants and it is a member of the mustard family (Cruciferae or Brassicaceae) (Meinke *et al.*, 1998). *A. thaliana* has several traits that make it an ideal model for basic research. It has a very short life cycle that is completed in six weeks and the flowers can self-pollinate to produce thousands of seeds. The plant has a very small size (mature plants are about 15 to 20 cm in height) that limits the requirement of many growth facilities and it can also be easily transformed by *Agrobacterium tumefaciens* (Meinke *et al.,* 1998).

The genome sequence of the *A. thaliana* was completed in 2000 and published in *Nature* (The Arabidopsis Genome Initiative, 2000). The genome of *A. thaliana* is relatively small (135 megabases) and it is organized into 5 chromosomes. Many papers that deal with *A. thaliana*-pathogen interactions have been published since the sequencing of the genome (Nishimura and Dangl, 2010). Information about *A. thaliana* such as genes, clones, markers, sequences, mutants and seed stocks can be obtained from The Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org).

Many virulent and avirulent bacterial, fungal and viral pathogens of *A. thaliana* have been identified and *A. thaliana* displays a variety of defense responses that were also described in other plants (Glazebrook *et al.,* 1997). Many mutations in *A. thaliana* have been described that cause a wide variety of specific defense-related phenotypes.

Analysis of these mutants provide insight into the complex signal transduction pathways leading to the activation of defense responses involved in protecting plants from pathogen attack (Glazebrook *et al.,* 1997).

1.3 Plant immunity

Plants encounter a wide variety of pathogenic microorganisms such as fungi, bacteria, viruses, nematodes and oomycetes in their natural environment (Bari and Jones, 2009; Pieterse *et al.,* 2009). These pathogens can be classified as necrotrophs, biotrophs and hemibiotrophs. Necrotrophs kill their host cells by producing phytotoxins and feed on the dead contents, whereas biotrophs obtain nutrients from living host tissues. Some pathogens display both of these lifestyles and are called hemibiotrophs (Pieterse *et al.,* 2009).

Plants have preformed physical and chemical barriers that prevent pathogen invasion and some of these barriers are dense epidermal layers, waxy cuticles on leaves, the lignin and pectin content of plant cell walls and antimicrobial peptides (Bari and Jones, 2009; Pieterse *et al.,* 2009). Many pathogens can overcome preformed defenses, but plants employ inducible defense responses to limit further pathogen attack. The inducible defense responses can be classified as basal defense or cultivar specific resistance and are activated upon pathogen recognition (Pieterse *et al.,* 2009).

Pattern-recognition receptors (PRRs) in plants can detect pathogen-associated molecular patterns (PAMPs) such as flagellin, chitin, glycoproteins and lipopolysaccharides (LPS) from pathogens. This recognition leads to downstream signal transduction and the activation of basal resistance or PAMP triggered immunity (PTI) (Pieterse *et al.,* 2009). Pathogens can overcome basal resistance in the plants by acquiring effector molecules to promote the virulence of the pathogen. The effectors are transported into the host cell to target signaling components of the basal defense

system. PTI in the plant is suppressed and this leads to effector-triggered susceptibility (ETS). Some members of a plant species can acquire resistance (R) proteins that recognizes the activity of the effector molecule to initiate a secondary immune response called effector-triggered immunity (ETI)(cultivar specific resistance). The outcome of the battle between the plant and the pathogen will depend on the balance between the capability of the pathogen to repress the defense response in the plant and the capacity of the host to detect the pathogen and to activate effective defense responses (Pieterse *et al.,* 2009). This interaction between PTI, ETS and ETI was nicely illustrated in the four-phased 'zigzag'model by Jones and Dangl (2006).

R proteins

Several R genes have been cloned that confer resistance to a variety of pathogens such as fungi, oomycetes, bacteria, viruses, nematodes and sucking insects (Rafiqi *et al.,* 2009). Most R genes are classified into one of two main classes and one of the classes encodes membrane bound proteins consisting of an extracellular leucine-rich repeat (LRR) domain with or without an intracellular kinase domain. The other class contains the largest group of plant R proteins that consist of a nucleotide binding domain (NB) and LRR domain and these proteins are intracellular (Rafiqi *et al.,* 2009).

The plant NB-LRR proteins can be further classified into two classes based on their variable amino termini (Caplan *et al.,* 2008). Most of the NB-LRR proteins contain a Toll interleukin 1 receptor homology (TIR) protein-protein interaction domain (TIR-NB-LRR) at the amino terminus and these proteins share structural and functional similarities to the TOLL immune receptor in *Drosophila* and Toll-like receptors (TLR) in mammals (Caplan *et al.,* 2008). The second class contains a coiled-coil (CC) protein-protein interaction domain (CC-NB-LRRs) instead of the TIR domain (Caplan *et al.,* 2008).

The plant NB-LRR proteins can recognize the effectors from pathogens either directly (Receptor-Ligand Model) through physical interaction with the effector or indirectly

(Guard-Model) by sensing modifications of host proteins (guardees) targeted by the effectors from the pathogen (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009). One of the first examples where recognition occurs through direct physical interaction is between the Pita CC-NB-LRR receptor in rice and the AVR-Pita effector from the fungus *Magnaporthe grisea* (Caplan *et al.,* 2008). The AVR-Pita effector is recognized by direct interaction with the LRR domain of Pita and recognition as well as resistance were abolished by a single amino acid substitution in the LRR domain. Another example of direct interaction was observed between the *A. thaliana* RRS1-R (TIR-NB-LRR-WRKY) protein and the *R. solanacearum* PopP2 effector (Deslandes *et al.,* 2003).

Two variations of indirect recognition of pathogen effectors by resistance proteins in plants exists (Caplan *et al.,* 2008). The NB-LRR protein from the host can be constitutively bound to the guardee host factor or the R protein binds to the guardee after the guardee is bound by the effector. The *A. thaliana* RPM1 interacting protein 4 (RIN4) is a guardee host factor that constitutively interacts with the CC-NB-LRR proteins RPM1 and RPS2 (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009) . RIN4 can be modified by three effectors from *Pseudomonas syringae* and this leads to activation of RPM1 and RPS2. RIN4 suppresses the plant's basal defense response and it is possible that the effectors are secreted by the pathogen to modify RIN4 to stabilize its ability to repress the host's immune response. The AvrRpt2 effector from *P. syringae* is a cysteine protease that cleaves RIN4 and this leads to activation of RPS2, while the AvrRMP1 and AvrB effectors promote phosphorylation of RIN4, which then activates the RPM1 immune response. Studies in tomato identified another host guardee, Pto kinase, that constitutively associates with the NB-LRR receptor Prf. Prf and Pto forms an preimmune complex that is in an inactive state and the AvrPto effector from *P. syringae* disrupts the inhibitory activity of Pto on Prf, which allows Prf to activate a defense response (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009).

Research in *Nicotiana glutinosa* identified a guardee that first interacts with the pathogen effector and is then recognized by the NB-LRR receptor (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009). The p50 effector from Tobacco mosaic virus (TMV) recruits the chloroplastic sulfurtransferase N receptor interacting protein 1 (NRIP1) from the chloroplast to the cytoplasm and nucleus through an unknown mechanism. Recent findings suggest that the p50 effector and the NRIP1 guardee forms a preimmune complex that is recognized by the N immune receptor to activate a defense response (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009).

Another mechanism of pathogen recognition was recently discovered and this mechanism involves effector molecules that act as transcription factors to induce the expression of non-NB-LRR resistance proteins (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009). The AvrBs3 effector binds to the promoter of the pepper *Bs3* resistance gene in the nucleus and induces its expression to confer resistance against *Xhantomonas campestris*. Cloning of the resistance gene showed that the differences between the resistant and susceptible alleles are in the promoter regions and the AvrBs3 effector specifically activates the expression of the resistant allele. The interaction between AvrBs3 and Bs3 is an example of the pathogen-host arms race at the transcriptional level. The pathogen secretes effector molecules that acts as transcription factors to induce the expression of specific genes that promote susceptibility. However, the host has acquired resistance proteins that contain the effector-targeted promoter elements. These resistance proteins are induced by the effectors to confer resistance against the pathogen (Caplan *et al.,* 2008; Rafiqi *et al.,* 2009).

ETI is usually accompanied by a cell death response, which is costly for the plant. Therefore, R proteins should be repressed in the absence of a pathogen and quickly activated when a pathogen attacks the plant (Takken and Tameling, 2009). R proteins are kept in an inactive state through intramolecular interactions between the domains of the R protein. The NB-ARC domain in R proteins consist of the NB, ARC1 and ARC2

domains and studies have shown that the N-terminal part of the LRR domain as well as the ARC2 subdomain play an essential role in keeping the protein in an inactive state. Disruption of the interaction between these two domains through mutations or domain swaps abolishes inhibition and constitutively activates the R protein (Takken and Tameling, 2009).

A model has been proposed for R protein activation where R proteins operate as nucleotide-controlled molecular switches (Fig. 1.3) (Takken and Tameling, 2009). The R proteins stay in an inactive ADP-bound "OFF" state when a pathogen is absent. The interaction between the N-terminal part of the LRR domain and the ARC2 domain is disrupted when a pathogen effector is recognized and a more open conformation (intermediate state) of the R protein is formed and ADP can be exchanged for ATP. ATP binding alters the interaction between the other domains and the R protein adopts an active conformation (ATP-bound "ON" state) to activate defense responses. ATP-hydrolysis changes the protein back to its ADP-bound "OFF" state (Takken and Tameling, 2009).

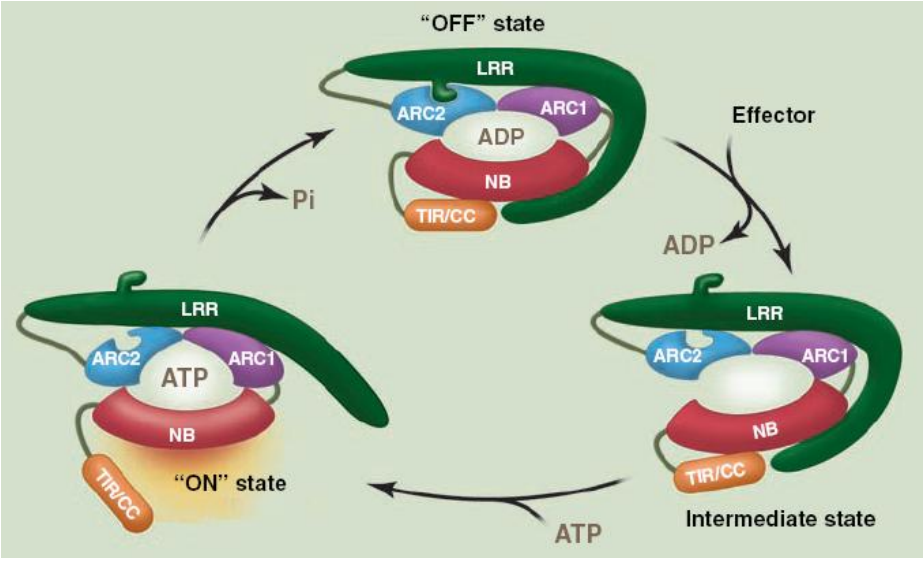

Figure 1.3. **A model for the activation of an R protein upon recognition of a pathogen effector (adapted from Takken and Tameling, 2009).**

Studies have shown that NB-LRR proteins such as RRS1-R, RPS4, MLA10 and N localizes to the nucleus in the plant upon pathogen recognition (Caplan *et al.,* 2008). RRS1-R contains its own WRKY transcription factor domain, whereas other immune receptors such as MLA and N directly interacts with transcription factors. The AVR_{A10} effector from *Blumeria graminis* activates the interaction between the CC domain of the barley MLA10 protein and the WRKY1 and WRKY2 transcription factors. N immune receptor from *N. glutinosa* interacts with squamosa promoter-like (SPL) transcription factors. Studies have shown so far that most of the transcription factors fused to or associated with NB-LRR proteins act as transcriptional repressors of the basal plant defense response. The *slh1* mutant of the Arabidopsis ecotype No-0 has a single amino-acid insertion in the WRKY domain of the *RRS1-R* gene and exhibits constitutively activated defense responses. Furthermore, the basal defense in barley plants is suppressed by the WRKY1 and WRKY2 transcription factors which interacts with the MLA10 receptor (Caplan *et al.,* 2008)

1.3.1 Defense responses induced by plants

Several defense response are activated after pathogen recognition and these include strengthening of the cell walls through the production of callose and lignin, the synthesis of antimicrobial secondary metabolites such as phytoalexins and the accumulation of antimicrobial proteins such as pathogenesis-related (PR) proteins that degrade fungal and oomycete cell walls (Thatcher *et al.,* 2005; Pieterse *et al.,* 2009).

A hypersensitive response (HR) is also activated after the recognition of effector molecules from the pathogen by R proteins from the host (Thatcher *et al.,* 2005; Pieterse *et al.,* 2009). The HR is the rapid death of cells at the site of pathogen invasion to confine the growth of the pathogen through the synthesis of antimicrobial compounds and the limitation of nutrients. The HR is specifically active against biotrophic pathogens, because this response favors the growth of necrotrophic

pathogens whose virulence strategy relies on the ability to kill host cells (Thatcher *et al.,* 2005; Pieterse *et al.,* 2009).

Hormones are produced by the plant and these small molecules are important for the regulation of plant growth, development and reproduction (Pieterse *et al.,* 2009). Plant hormones also play an important part in the regulation of the plant's defense response and evidence for this comes from studies performed in the model plants *A. thaliana* and *Nicotiana tabacum* (tobacco). Several mutants and transgenic lines were generated that were affected in the biosynthesis, perception and signal transduction of the hormones and individual components of each hormone signaling pathway were identified (Pieterse *et al.,* 2009). Major progress has been made in understanding the role of SA, JA and ET in plant defense responses and different pathways are regulated by these hormones. The signaling pathways and some of the main components in each pathway are mentioned briefly in the following section. Figure 1.4 illustrates the different signaling pathways as well as the crosstalk between the pathways. Other hormones such as abscisic acid, auxin and gibberellic acid also play a role in plant defense and are in included in figure 1.4. The roles of these hormones will be discussed later in the chapter.

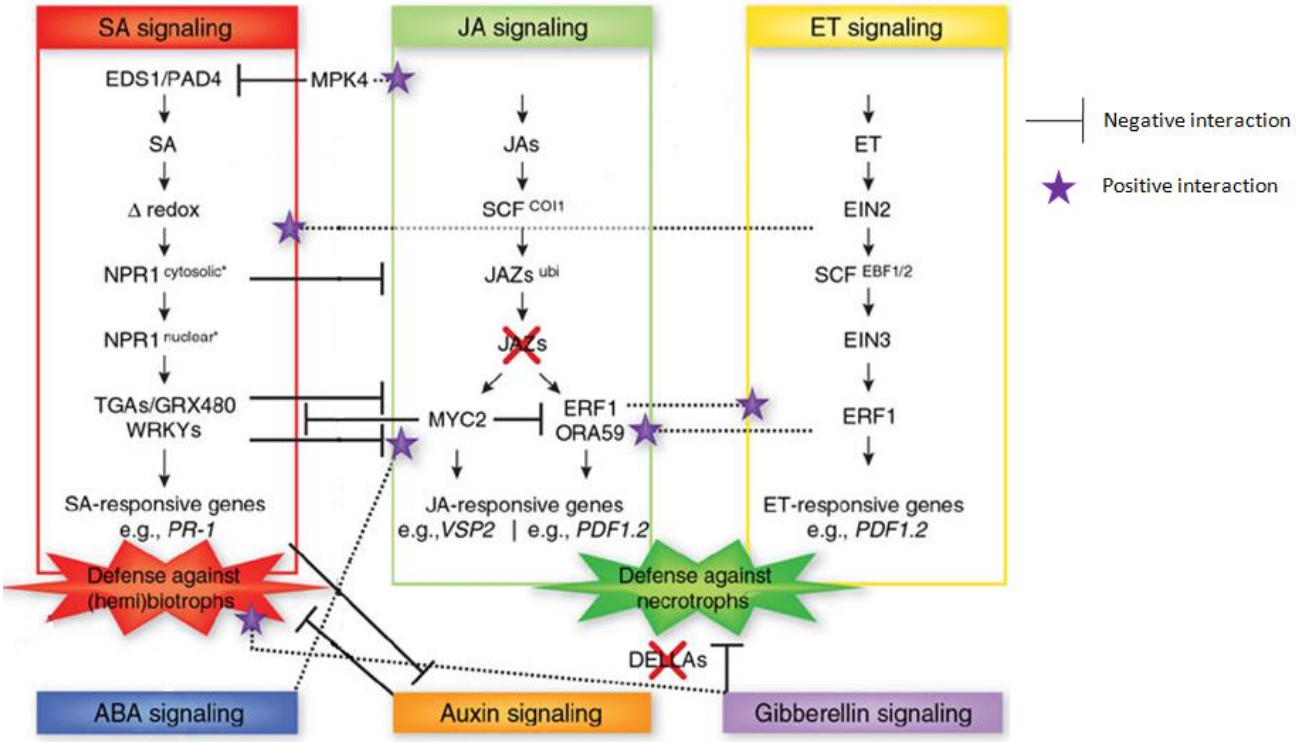

Figure 1.4. **Interaction between different hormones in the plant defense response.** Not all of the components of the different pathways are shown (adapted from Pieterse *et al.,* 2009).

1.3.1.1 Salicylic acid signaling

Defense responses against biotrophic (*Peronospora parasitica*) and hemi-biotrophic (*P. syringae*) pathogens are usually activated by SA and SA also plays an important role in the establishment of systemic defense responses such as systemic acquired resistance (SAR) (Bari and Jones, 2009). The *NPR1* (non-expressor of *PR* genes 1) gene encodes an ankyrin-repeat containing protein and this protein plays a key regulatory role in SA signaling (Thatcher *et al.,* 2005). NPR1 interacts with a subclass of basic region/leucine zipper (bZIP) transcription factors called TGAs. A change in the cellular redox potential is mediated by SA and the NPR1 oligomer in the cytosol is reduced to its active monomeric form (Pieterse *et al.,* 2009). The monomeric NPR1 translocates to the nucleus and enhances the binding of TGA transcription factors to SA responsive promoter elements in antimicrobial genes such as *PR-1*.

1.3.1.2 Jasmonic and Ethylene signaling

Pathogens with a necrotrophic lifestyle (*Botrytis cinerea*) are usually deterred by defense responses activated by JA and ET (Bari and Jones, 2009). The main components in the JA signaling cascade are coronatine insensitive 1 (COI1), the jasmonate resistant 1 (JAR1), the jasmonate ZIM-domain (JAZ) proteins as well as several transcription factors (Jasmonate insensitive 1/MYC2 (JIN1/MYC2), ethylene response factor 1 (ERF1) and ORA59). COI1 is an F-box protein involved in the SCF-mediated protein degradation by the 26S proteasome and the JAZ proteins forms a complex with the E3 ubiquitin ligase SCF^{COI1} complex to repress transcription of JA-responsive genes (Pieterse *et al.,* 2009). JAR1 is a JA amino acid synthetase and is responsible for the conjugation of isoleucine to JA (JA-Ile) (Bari and Jones, 2009). As soon as JA accumulates, JA-Ile is synthesized to bind to COI1 in the SCF^{COI1} complex and the JAZ proteins are ubiquitinated and degraded through the 26S proteasome (Pieterse *et al.,* 2009). JA responsive genes such as the plant defensin gene (*PDF1.2*) are subsequently activated by the transcription factors (MYC2, ERF1 and ORA59) in the signaling pathway.

Ethylene is detected by plasma membrane receptors such as ETR1 (ethylene insensitive) in the ethylene signaling pathway (Pieterse *et al.,* 2009). CTR1 (constitutive triple response) is a protein kinase that represses the membrane protein EIN2 (ethylene insensitive) in the absence of ethylene. Once ethylene is detected, downstream signaling through EIN2 is activated and the E3 ubiquitin ligase $SCF^{EBF1/2}$-dependent 26S proteosome degradation of transcription factors such as EIN3 (ethylene insensitive) is inhibited. EIN3 activates transcription factors such as ERF1, which binds to the GCC box promoter elements of defense genes such as *PDF1.2* (Pieterse *et al.,* 2009).

1.3.1.3 Interactions between the pathways regulated by SA, JA and ET

Plants encounter a wide variety of pathogenic and beneficial microorganisms and plants need regulatory mechanisms to adapt to changes in their complex environment (Pieterse *et al.,* 2009). Interactions between the different signaling pathways is a vital

tool for regulating defense responses against the different microorganisms (Bari and Jones, 2009). Several important proteins that regulate the cross talk between the three different pathways have been identified in *A. thaliana* and some of these components are briefly described below.

Crosstalk between SA and JA signaling pathways

The interaction between JA and SA signaling pathways are mostly antagonistic, but synergistic interactions were also reported (Bari and Jones, 2009). Research have shown that NPR1 in the SA pathway plays an important role in SA- mediated suppression of the JA signaling pathway (Bari and Jones, 2009; Pieterse *et al.,* 2009). *A. thaliana npr1* mutant plants were unable to repress JA-responsive genes. WRKY70 also acts as a regulator that mediates the expression of SA-dependent genes and represses JA-responsive genes (Bari and Jones, 2009). Constitutive expression of SA-responsive *PR* genes, suppression of the JA-responsive gene *PDF1.2* as well as resistance to the biotrophic pathogen *Erysiphe cichoracearum* was observed in transgenic *A. thaliana* plants overexpressing the *WRKY70* gene. The mitogen activated protein kinase 4 (MPK4) is another key protein that regulates the cross talk between the SA and JA pathways. MPK4 is a negative regulator of the SA pathway, because mutant *mpk4 A. thaliana* plants had high levels of SA, constitutive expression of SA-responsive *PR* genes and improved resistance to the *P. syringae* pv. *tomato*. In contrast, these *mpk4* mutants showed reduced expression of JA-responsive genes and susceptibility to the necrotrophic pathogen *Alternaria brassicicola* (Bari and Jones, 2009).

GRX480 is a glutaredoxin that is responsible for redox regulations of protein activities in different cellular processes (Bari and Jones, 2009). SA induces the expression of GRX480 and it interacts with the TGA transcription factors as well as NPR1. The expression of the JA regulated *PDF1.2* gene was repressed by GRX480 and its hypothesized that NPR1 from the SA pathway activates GRX480 and GRX480 forms a complex with TGA transcription factors and suppresses JA-responsive genes (Bari and Jones, 2009). The JA-

regulated transcription factor JIN1/MYC2 suppresses the SA signaling pathway during *P. syringae* DC3000 infection in *A. thaliana*. Higher levels of SA, up regulation of *PR* genes and improved resistance to *P. syringae* DC3000 was observed in the mutant *jin1* plants compared to the wild type plants (Bari and Jones, 2009).

Crosstalk between ET and JA signaling pathways

Synergistic interaction were mostly reported for the JA and ET signaling pathways (Bari and Jones, 2009; Pieterse *et al.,* 2009). The activation of the ET as well as the JA pathway is required for the expression of the *A. thaliana PDF 1.2* gene. The ERF1 and ORA59 are two transcription factors that unite the ET and JA signaling pathways and both are activated by the JA and ET pathway (Pieterse *et al.,* 2009). The *PDF1.2* gene was constitutively expressed by overexpressing the *ERF1* or *ORA59* in the JA-insensitive mutant *coi1* or *ERF1* in the ET-insensitive mutant *ein2*.

The JA-regulated MYC2 transcription factor plays an important role in regulating the expression of two classes of JA-responsive genes (Pieterse *et al.,* 2009). This transcription factor activates the expression of JA-responsive genes such as *VSP2* and *LOX2* and represses the expression of JA/ET-responsive genes such as *PDF1.2* that are expressed by ERF transcription factors. The ERF branch of the JA response is activated when both the ET and JA pathways are switched on, but the MYC2 branch of the JA response is activated when the ET signal is absent (Pieterse *et al.,* 2009). This differential JA response was observed in whole genome expression profiling of *A. thaliana* that were infected with the fungus *A. brassicicola* (which triggers the production of ET and JA) or infested with the insect *Frankliniella occidentalis* that stimulates the synthesis of JA but not ET (Pieterse *et al.,* 2009). The ERF and MYC2 transcription factors control the expression of different JA-responsive genes to activate the most optimal response against a specific attacker.

Crosstalk between ET and SA signaling pathways

ET improves the response of *A. thaliana* to SA and triggered enhanced expression of the *PR-1* gene (Pieterse *et al.,* 2009). The ET-insensitive mutant *ein2* blocked the positive effect of ET on the SA-induced expression of *PR-1* and this finding shows that the regulation of the SA pathway by ET is EIN2 dependent (Pieterse *et al.,* 2009).

1.3.1.4 The roles of other hormones in plant defense

Several other hormones such abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinin (CK) and brassinosteroids (BR) are produced by the plant that also play a role in plant defense. The role of these hormones in plant defense is less well studied and current findings on the role of ABA, auxin and GA will be discussed briefly below.

Abscisic acid

The role of ABA in plant defense is less straightforward than that of the other hormones (Ton *et al.,* 2009). ABA acts a positive regulator of defense in some plant-pathogen interactions, whereas it promotes susceptibility in other pathosystems. Several studies have reported the role of ABA in promoting disease resistance. Certain fungal and oomycete pathogens enter the cell wall of plants directly by applying mechanical force or through the help of cell-wall-degrading enzymes, while other fungi and bacteria enter the plant through natural openings such as wounds or stomata (Ton *et al.,* 2009). Plants prevent invasion by pathogens by rapidly closing their stomata upon recognition of the microbes. The recognition of pathogens was mimicked by applying PAMPs such as the flagellin derivative flg22 and LPS on *A. thaliana* ABA-deficient *aba3-1* plants. The mutant plants failed to rapidly close their stomata upon PAMP recognition and the mutants were also more susceptible to *P. syringae* (Bari and Jones, 2009; Ton *et al.,* 2009). Resistance in *A. thaliana* against *Leptosphaeria maculans* fungus depends on callose depositions and the *A. thaliana* ABA biosynthetic *aba1-3* as well the ABA-response *abi1-1* mutants had increased susceptibility to *L. maculans* as well as a reduced level of

callose deposition. However, bacterial flg22-induced callose deposition was suppressed in *A. thaliana* cotyledons pretreated with ABA (Ton *et al.,* 2009).

ABA also acts as a negative regulator of disease resistance. Two chemicals (1,2-benzisothiazol-3(2H)-one1,1-dioxide (BIT) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH)) were used to induce SAR in *A. thaliana* and pretreatment with ABA suppressed SA-dependent SAR in the *A. thaliana* plants (Ton *et al.,* 2009). The SA-inducible defenses activated by *P. syringae* DC3000 was also suppressed by ABA and these findings show that ABA acts as a suppressor of the SA-dependent defense responses. The MYC2 and the ERF branch of the JA signaling pathway act antagonistically on each other, as previously mentioned. Previous studies have shown that ABA suppresses resistance in *A. thaliana* to the necrotrophic fungus *Fusarium oxysporum* as well as expression of the JA/ET-responsive genes such as *PDF1.2*, PR4 (PATHOGENESIS RELATED PROTEIN 4) and LEC (LEAFY COTYLEDON) whose expression are activated by the ERF transcription factors (ERF branch of the JA signaling pathway). Resistance against the necrotrophic fungus *Pythium irregulare* is dependent on the JA signaling pathway and *A. thaliana* mutants deficient in ABA were more susceptible to *P. irregulare*. ABA also promoted the expression of defense-related genes through the MYC2 branch of JA-response pathway. These findings suggest that ABA plays a role in fine-tuning the JA-dependent defenses by acting as inhibitor of the ERF branch of the JA response pathway, which is involved in resistance against *F. oxysporum*, as well as a activator of the MYC2 branch of the JA response pathway that is required for resistance against *P. irregulare* (Ton *et al.,* 2009).

ABA has a complex role in regulating defense responses against various pathogens by suppressing or promoting disease and based on findings discussed above a model has been proposed where both possibilities are integrated (Ton *et al.,* 2009). ABA acts mostly as a positive regulator of early defense responses, such as stomatal closure and deposition of callose, to halt harmful pathogens. These early defense response can be

sufficient in preventing disease and the activation of later defense responses such as JA- and SA-dependent defenses will be unnecessary. ABA acts mostly as an inhibitor of later defense responses to prevent the plant from wasting energy on costly defense responses (Ton *et al.,* 2009). The model does not cover all finding about the role of ABA in disease resistance and the diversity of pathogens on hosts should be expanded to reveal the exact role of ABA in plant defense.

Auxin

Auxin plays a role in plant development and susceptibility to *P. syringae* was promoted by auxin (Pieterse *et al.,* 2009). Expression profiling of *A. thaliana* showed that SA inhibited the auxin signaling pathway and increased resistance to the (hemi)biotrophic pathogens such as *H. arabidopsidis* and *P. syringae*. The suppression of auxin signaling by SA seems to be an important part of SA-dependent resistance against (hemi)biotrophs (Pieterse *et al.,* 2009).

Gibberellins

GAs are hormones that assist in the growth of plants by stimulating the degradation of growth-repressing DELLA proteins (Pieterse *et al.,* 2009). Previous studies in *A. thaliana* demonstrated that DELLA proteins are positive regulators of the JA/ET-dependent defense response and promotes resistance to necrotrophic pathogens, but the proteins represses the SA-dependent defense response and promotes susceptibility to biotrophs (Bari and Jones, 2009). However, the degradation of DELLA proteins are triggered by GA and exogenous application of GA on *A. thaliana* enhanced susceptibility to *A. brassicicola* (necrotroph) and promoted resistance to *P. syringae* DC3000 (biotroph). These findings suggest that GAs regulate the SA-JA-ET network by controlling the degradation of DELLA proteins.

1.3.1.5 Systemic defense responses

Systemic acquired resistance (SAR) is a long-lasting systemic resistance against a wide range of pathogens such as bacteria, fungi and viruses (Thatcher *et al.,* 2005). During SAR, SA accumulates locally and systemically to express defense-related genes, such as *PRs*. Once a defense response is activated at the site of pathogen invasion, SAR is triggered in distal plant parts to prevent pathogen invasion in the undamaged tissues (Pieterse *et al.,* 2009). The significance of SA in SAR was confirmed by showing that transgenic and mutant plants impaired in SA signaling were unable to activate a SAR response or express *PR* genes (Thatcher *et al.,* 2005; Pieterse *et al.,* 2009). The mobile signaling molecule(s) that are transported throughout the plant to trigger SAR are not yet identified, but several studies have hypothesized that methyl-SA, JAs, a plastid glycerolipid-based factor and a lipid-transfer protein could be candidates as mobile signaling molecules (Pieterse *et al.,* 2009).

Induced systemic resistance (ISR) is another form of a systemic defense response that is activated by beneficial root colonizing microorganisms such as mycorrhizal fungi and rhizobacteria (Pieterse *et al.,* 2009). ISR is mostly regulated by the ET and JA signaling pathways and are effective against pathogens sensitive to these pathways, whereas SAR is mostly effective against biotrophic pathogens that are sensitive to SA (Pieterse *et al.,* 2009).

1.4 Plant defense response against *R. solanacearum*

Resistance against *R. solanacearum* (hemi-biotroph) is a complex trait and single-gene resistance (Deslandes *et al.,* 1998; Ho and Yang, 1999) as well as polygenic resistance (Godiard *et al.,* 2003; Vailleau *et al.,* 2007) in different hosts against bacterial wilt have been reported. Various responses against *R. solanacearum* in hosts such as *A. thaliana*, tomato and tobacco have been described, which will be discussed in the section below.

1.4.1 *Arabidopsis thaliana*

As described previously, RRS1-R confers resistance against *R. solanacearum* and activates a resistance response against GMI1000 by recognizing a nucleur complex formed between the PopP2 effector secreted from GMI1000 and the *A. thaliana* host target RD19 (Deslandes *et al.,* 2003; Bernoux *et al.,* 2008). Previous work by Deslandes *et al.* (1998) identified the *RRS1* locus and RRS1-R is the first R-gene identified that confers resistance against *R. solanacearum*. In this study a pathosystem was developed between isolate GMI1000 and the *Arabidopsis thaliana* ecotypes Nd-1 and Col-0. The two *A. thaliana* ecotypes were inoculated with isolate GMI1000 and Col-5 was susceptible to GMI1000 in a hrp-dependent manner, while Nd-1 was identified as the resistant ecotype remaining symptom-free 10 days post inoculation (dpi). Genetic analysis of crosses between Nd-1 and Col-5 revealed that resistance to isolate GMI1000 is due to a single recessive locus known as *RRS1* (Deslandes *et al.,* 1998).

Further work by Deslandes *et al.* (2002) identified dominant (*RRS1-S*) and recessive (*RRS1-R*) alleles of the *RRS1* gene from the susceptible ecotype Col-5 and the resistant ecotype Nd-1, respectively. The two RRS1 alleles encode highly similar TIR-NB-LRR-WRKY proteins. However, the RRS1 protein encoded by the *RRS1-S* allele is 90 amino acids shorter than RRSI-R in Nd-1 (Deslandes *et al.,* 2002).

A sensitive to low humidity 1 (*slh1*) mutant was characterized in the *A. thaliana* ecotype No-0 (Noutoshi *et al.,* 2005). Growth was normal for these mutant plants on agar plate medium, but when *slh1* plants were transferred to soil they displayed activation of a pathogen-independent defense response as well as HR-like cell death. The *SLH1* gene in ecotype No-0 is identical to the *RRS1-R* gene and the No-0 ecotype did show resistance to the *R. solanacearum* strain GMI1000. The *slh1* mutant contains a single amino acid insertion in the WRKY domain of the *SLH1* gene that causes a loss of WRKY domain DNA-binding activity. The findings from this study suggest that the WRKY domain of SLH1 may repress resistance signaling of SLH1 and the mutant slh1 protein may adopt a

conformation similar to that of the activated SLH1 (Noutoshi *et al.*, 2005). Two models were proposed for the function of the SLH1 protein. In the first model, the SLH protein functions as a transcriptional repressor of defense genes by the binding of the WRKY domain to the W-box in promoters of defense genes. After pathogen infection, the PopP2 effector interacts with SLH1 and the WRKY domain dissociates from the promoters and expression of the defense genes are induced. In the second model, the SLH WRKY domain can be a virulence target of effector products and function as a guardee as defined in the 'guard hypothesis' (Noutoshi *et al.*, 2005). The SLH1 protein is kept in an inactive state via intramolecular associations of the WRKY domain with the TIR-NB-LRR region of the resistance protein. After pathogen infection, the PopP2 effector targets the WRKY domain to modify it or cause its dissociation from the TIR-NB-LRR region and this leads to activation of the SLH1 protein.

The *A. thaliana RPS4* is the *R* gene that confers resistance to the *P. syringae* strain DC3000 possessing the *avrRps4* effector (Narusaka *et al.,* 2009). The *RPS4* gene as well as the *RRS1* gene are located within a cluster of disease-resistance loci on chromosome 5 identified as the multiple resistance complex J (*MRC-J*). This cluster also contains other *R* genes such as *RPP8* and unknown NB-LRR containing encoding genes. Findings from the study showed that both *RRS1* and *RPS4* function cooperatively in conferring resistance to *R. solanacearum* as the *rps4-21/rrs1-1* double mutant showed similar levels of susceptibility to *R. solanacearum* as the single mutants (*rrs1-1*, *rrs1-2* and *rps4-21* plants).

Previous work has identified a new pathosystem between the *A. thaliana* ecotypes Be-0 and Kil-0 and the *Eucalyptus* isolate BCCF 402 (Weich, 2004). Be-0, the susceptible ecotype, shows severe wilt symptoms two weeks after root inoculation, while Kil-0, the resistant ecotype, remains healthy two weeks after inoculation. Whole genome microarrays were used to study the interaction between Kil-0 and isolate BCCF 402 (Naidoo, 2008). Thirteen genes were found to be induced in Kil-0 in response to isolate

BCCF 402 and quantitative reverse-transcription PCR (qRT-PCR) was performed on ten of these genes to confirm the results obtained from microarray analysis and to determine the expression of these genes in Be-0 after inoculation with isolate BCCF 402.

The transcripts of the identified genes such as the lipid transfer protein 3 (LTP3), peroxidase (PRX34), tropinone reductase (SAG13), avirulence- induced gene (AIG), translation initiation factor (SUI), SKP1 interacting partner 5 (SKP5) and an "expressed protein" accumulated to higher levels earlier in Kil-0 than in Be-0 (Naidoo, 2008). Interesting roles for some of these genes are discussed. LTPs are peptides that have antimicrobial activity against pathogens and LTP3 were one of the genes that were constitutively up-regulated in the *A. thaliana irx* mutants that were resistant against isolate GMI1000 (Hernandez-Blanco *et al.,* 2007). Peroxidases such as PRX34 are important for the production of reactive oxygen species such as H_20_2 to defend the plant against pathogen attack (Naidoo, 2008). PRX34 is a cell-wall bound class III peroxidase and it is located mainly in the roots of *A. thaliana*. The SAG 13 gene is mainly used as a marker for programmed cell death, whereas AIG is an avirulence-induced gene that is induced early after infection with the *P. syringae* strain containing the *avrRpt2* effector.

Resistance in ecotype Nd-1 against isolate GMI1000 is SA- and NDR1-dependent as Nd-1 plants containing the *NahG* gene (encodes salicylate hydroxylase, which converts SA to catechol) developed wilt symptoms after inoculation with the pathogen and *ndr1/ndr1* double mutants also showed increased susceptibility (Deslandes *et al.,* 2002). The role of ET in *A. thaliana* against *R. solanacearum* was described by Hirsch *et al.* (2002). ET was shown to play a role in wilt symptom development. Ethylene insensitive plants (*ein2-1*) in a susceptible Col-0 genetic background showed delayed wilt symptom development after inoculation with isolate GMI1000 and the *PR-3* and *PR-4* ethylene-inducible transcripts accumulated in Col-0 infected plants. The transcript levels of these ethylene-responsive genes were much weaker in Nd-1 and *ein2-1* plants (Hirsch *et al.,* 2002).

A search for mutants unable to develop disease upon *R. solanacearum* infection was performed to identify genes that regulate the development of infections caused by the pathogen (Hernandez-Blanco *et al.,* 2007). Three *A. thaliana* CESA subunits (CESA4/IRREGULAR XYLEM5 [IRX5], CESA7/IRX3, and CESA8/IRX1) are needed for secondary cell wall formation and mutations in any of the three CESAs conferred improved resistance to *R. solanacearum*. ABA have a direct role in resistance to *R. solanacearum* as comparative transcript profiling of the *irx* mutants showed constitutive induction of ABA-responsive genes and ABA mutants (*abi1-1, abi2-1 and aba1-6*), defective in ABA-signaling, showed enhanced susceptibility to *R. solanacearum* (Hernandez-Blanco *et al.,* 2007). However, whole-genome microarray analysis showed that 40% of the genes induced during wilt disease development in the susceptible ecotype Col-5 after infection with GMI1000 were involved in ABA biosynthesis and signaling (Hu *et al.,* 2008). The transcripts of most of these disease-associated genes accumulated at later stages of infection. The susceptible interaction between the *A. thaliana* ecotype Col-5 and the *Eucalyptus* isolate BCCF401 were investigated using microarray expression profiling of 5000 unigenes and ABA was also shown to play a role in late susceptible responses to bacterial wilt (Naidoo *et al.,* 2010). Based on the findings from the studies described above it seems that ABA has a complex role in response to *R. solanacearum* infection. It is proposed that ABA play a different role during different stages of infection. ABA promotes resistance to *R. solanacearum* during the early stages of infection, whereas it enhances susceptibility during the later stages of infection (Naidoo *et al.,* 2010).

1.4.2 Tomato and Tobacco

The tomato ERF transcription factor, tomato stress-responsive factor 1 (TSRF1), was previously isolated by using the ethylene-responsive GCC box as a bait in the yeast one-hybrid system (Zhang *et al.,* 2004). The expression of TSRF1 was induced in tomato plants infected with the *R. solanacearum* strain BJI057 as well as treatment of the plants with ethylene or salicylic acid. TSRF1 physically interacts with the GCC box *in vitro* and

constitutive expression of *TSRF1* in tomato or tobacco stimulated the expression of GCC box containing *PR* genes as well as enhanced resistance to the *R. solanacearum* strain BJI057 (Zhang *et al.,* 2004).

Overexpression of *TSRF1* in tobacco increased the production of ABA and the higher levels of ABA further triggered the production of ethylene (Zhou *et al.,* 2008). However, ABA treatment of tobacco plants deactivated the binding of TSRF1 to the GCC box in *PR* genes and reduced resistance to the *R. solanacearum* strain BJI057. TSRF1 can also bind to an ABA-responsive element (CEI/GCC) and treatment of tobacco plants with ABA enhanced the binding of TSRF1 with the ABA-responsive element and increased the expression of ABA-responsive CEI/GCC containing genes (Zhang *et al.,* 2008).

The oomycete *Pythium oligandrum* is a biocontrol agent that colonizes the roots of crop species and treatment of tomato roots (*Solanum lycopersicum* cv. Micro-Tom and Moneymaker) with *P. oligandrum* mycelial homogenate reduced bacterial wilt symptoms caused by *R. solanacearum* isolate 8242 (Hase *et al.,* 2008). *P. oligandrum* mycelial homogenate treatment of the roots also induced an ET-dependent defense response in the tomato plants. The JA-responsive PR-6 protein was expressed in both tomato cultivars after treatment with the *P. oligandrum* mycelial homogenate and *P. oligandrum* induced resistance to *R. solanacearum* was reduced in *jai1-1* mutant tomato plants. These findings suggest that the JA-dependent signaling pathway is also important for *P. oligandrum* induced resistance against *R. solanacearum* (Hase *et al.,* 2008).

Findings from the studies described above have shed some light on the roles of signaling molecules in response to *R. solanacearum*. SA plays an overall role in resistance against *R. solanacearum*, whereas ET promoted susceptibility of *A. thaliana* to *R. solanacearum*, but enhanced resistance of tomato to the pathogen. It is proposed

that ABA have different roles during different stages of infection in *A. thaliana*, whereas it promoted wilt disease development in tomato and tobacco.

1.5 Genetic analysis of resistance against *R. solanacearum* in *A. thaliana*

1.5.1 Segregation analysis

A strategy for characterizing resistance against a pathogen is to identify a pathogen isolate that is virulent on one *A. thaliana* accession. This is then followed by screening other ecotypes for resistance against this particular isolate (Glazebrook *et al.,* 1997). The resistant accession is then crossed with the susceptible one to obtain the F_1 (the first filial generation) and F_2 progeny, which will be used for further analysis (Koornneef *et al.,* 2006). The F_1 hybrid is used to determine if resistance or susceptibility is dominant and it is selfed to obtain the F_2 generation. The F_2 progeny is inoculated with the pathogen and screened as resistant or susceptible to determine if resistance segregated as a single gene or more than one gene.

A 3:1 (resistant: susceptible or susceptible: resistant) ratio in the F_2 generation or 1:1 (resistant: susceptible) ratio in the backcross generation (F_1 plants crossed to the recessive parent) indicates monogenic inheritance (Koornneef *et al.,* 2006). Two different loci can also affect the outcome of one characteristic and a dihybrid cross involving these genes can produce a phenotypic ratio that is different from the classical 9:3:3:1 ratio for two genetically independent genes. These ratios depend on the epistatic relationship between the genes. Chi-square analysis is used to determine if data observed from the experiment fits any of the expected ratios (Koornneef *et al.,* 2006).

Genetic analysis of resistance in *A. thaliana* to *R. solanacearum* was performed in a few studies and two examples are mentioned. The leaves of two *A. thaliana* ecotypes were

inoculated with the Taiwanese *R. solanacearum* isolate Ps95 and clear differences between the susceptible and resistant accessions were observed (Ho and Yang, 1999). The susceptible ecotype N913 developed symptoms 3 to 5 dpi and were completely wilted 14 dpi. Wilt symptoms did not develop in any part of the resistant ecotype S96 by 14 dpi, but necrosis did develop around the inoculation site at 1 dpi and appeared to be an HR. Crosses were performed between ecotype S96 and N913 to obtain an F_2 segregating generation that was screened after inoculation with isolate Ps95. A 3:1 ratio of resistant to susceptible was obtained in the F_2 progeny after inoculation and it indicates that resistance in S96 to isolate Ps95 is due to a single dominant locus (Ho and Yang, 1999).

In another study, two ecotypes (Col-0 and Ler (Landsberg *erecta*)) were inoculated with the tomato isolate 14.25 (Godiard *et al.,* 2003). L*er* was characterized as the susceptible ecotype in which all of the rosette leaves were completely wilted within two weeks. The resistant ecotype Col-0 remained symptom-free two weeks after inoculation. The data obtained after screening the 14.25 inoculated F_2 population from a cross between the Col-0 and L*er* accessions was not consistent with a monogenic or digenic model and genetic analysis of Col-0 x L*er* recombinant inbred lines indicated that resistance to isolate 14.25 was determined by three loci designated as *QRS* (Quantitative Resistance to *R. solanacearum*) loci (Godiard *et al.,* 2003). *QRS1* and *QRS2* are located on chromosome 2, whereas *QRS3* is situated on chromosome 5.

1.5.2 Allelism test

The allelism test is used to determine if a gene is allelic to previously characterized genes (Koornneef *et al.,* 2006). If an *A. thaliana ecotype* has a similar phenotype (such as single recessive resistance to a particular pathogen) to a previously characterized ecotype, the two homozygous recessive plants should be crossed to determine if the new gene is allelic to the previously characterized gene. This process is known as complementation and it can only be used for phenotypes that display a recessive

nature. The phenotype of the F_1 hybrid is determined and if the genes are allelic, the F_1 progeny will have a similar phenotype as the parents. The F_1 progeny will show a different phenotype (susceptibility) if the traits are due to two different genes. The F_1 hybrids of two homozygous parents are not informative when the phenotypes are due to dominant genes and the F_1 progeny must be selfed to find recessive phenotypes, in the case of nonallelism, in the F_2 progeny (Koornneef *et al.,* 2006).

1.6 Cleaved Amplified Polymorphic Sequence (CAPS) markers and co-segregation analysis

Plants can be distinguished from one another by differences in their genetic material (DNA) and these differences are known as DNA polymorphisms (Semagn *et al.,* 2006). Molecular markers are DNA sequences found at specific sites in the genome that are passed on by the standard laws of inheritance from one generation to the next. Various molecular marker techniques have been developed to detect and exploit DNA polymorphisms in plants and these include methods such as restriction fragment length polymorphisms (RFLPs); cleaved amplified polymorphic sequences (CAPS); random amplified polymorphic DNA (RAPD) and microsatellites, but this section will mainly focus on CAPS markers.

CAPS is a PCR-based marker where specific sections of DNA is amplified by PCR with specific primers and these amplified fragments are then digested with restriction enzymes to reveal the polymorphism (Semagn *et al.,* 2006). CAPS markers need sequence information to design the specific primers. CAPS markers are co-dominant (homozygous and heterozygous individuals can be distinguished) and only small amounts of DNA are needed for CAPS analysis. CAPS markers are assayed using agarose gel electrophoresis (Semagn *et al.,* 2006). CAPS markers were previously developed within certain genes to determine if the gene could be responsible for a specific phenotype in the plant and one example is mentioned below.

Flesh color inheritance was studied in watermelon and results indicated that a single gene determines the color difference between canary yellow and red flesh (Bang *et al.,* 2007). It was proposed that the lycopene β-cyclase (*LCYB*) gene is responsible for determining the color difference and three SNPs were identified in the coding region of *LCYB* between canary yellow and red. A CAPS marker was developed from one of these SNPs and 80 individuals from an F_2 and BC population (the F_1 from a cross between canary yellow and red were either selfed or backcrossed to both parents to produce the F_2 and BC populations, respectively) was screened with the CAPS marker. The CAPS marker perfectly co-segregated with the flesh color phenotypes and these results strongly suggest that the *LCYB* gene is responsible for determining flesh color in watermelon (Bang *et al.,* 2007).

1.7 Targeted mutagenesis in bacteria

Several genomes of gram-negative bacteria have been sequenced and a large number of genes with an unknown function have been identified (Dale and Park, 2010). The function of a specific gene can be determined by inactivating the gene and the process is known as gene replacement, allelic replacement or gene knockout. The whole gene or a specific part is replaced with an inactivated gene through homologous recombination. An example of gene replacement through homologous recombination is illustrated in figure 1.5. An inactivated gene whose central part has been replaced by an antibiotic resistance gene (aminoglycoside phosphotransferase (*aph*) provides resistance to kanamycin) is cloned into a specific plasmid. The plasmid cannot replicate in the chosen host that contains the specific wild-type gene that needs to be inactivated. Once transformed in the specific host, selection for kanamycin resistance will isolate specific cells in which the functional gene is replaced by the mutated gene through homologous recombination. Homologous recombination occurs at the DNA regions which flank the antibiotic selection marker and these regions are the same as the regions of the specific functional gene in the host (Dale and Park, 2010). Genes interact in several ways and the inactivation of a gene can affect the expression of other genes, especially if the gene is

part of an operon. This possibility can be investigated by introducing a plasmid containing the wild-type gene into the mutant strain. The introduced plasmid will restore the original wild-type phenotype if the mutation in the specific gene is responsible for the observed effect. The process is known as complementation (Dale and Park, 2010).

The pCZ367 integrative plasmid was used to create a *popP2* mutant strain and to confirm that PopP2 is the avirulence protein recognized by RRS1-R in the resistant *A. thaliana* ecotype Nd-1 (Deslandes *et al.,* 2003). The 5' -internal part and the 3' terminal end of the *popP2* gene were cloned upstream and downstream, respectively, of a promoterless *lacZ* gene followed by a gentamycin resistance gene in the pCZ367 plasmid. The linearized pCZ367 plasmid containing the mutated *popP2* gene was transformed into isolate GMI1000 and the functional *popP2* gene was replaced with the inactivated *popP2* gene through homologous recombination. A functional *popP2* gene and its 5' promoter region was cloned in the broad-host-range vector pLAFR6 and transformed in the mutant strain to convert the mutant strain back to the wild-type strain (Deslandes *et al.,* 2003). The GMI1000 strain containing an inactivated *popP2* gene caused severe wilt symptoms on Nd-1 plants up to 5 days after inoculation, whereas inoculation of Nd-1 plants with the mutant strain complemented with a functional *popP2* gene reversed the virulent phenotype observed on Nd-1 plants to avirulent (Deslandes *et al.,* 2003).

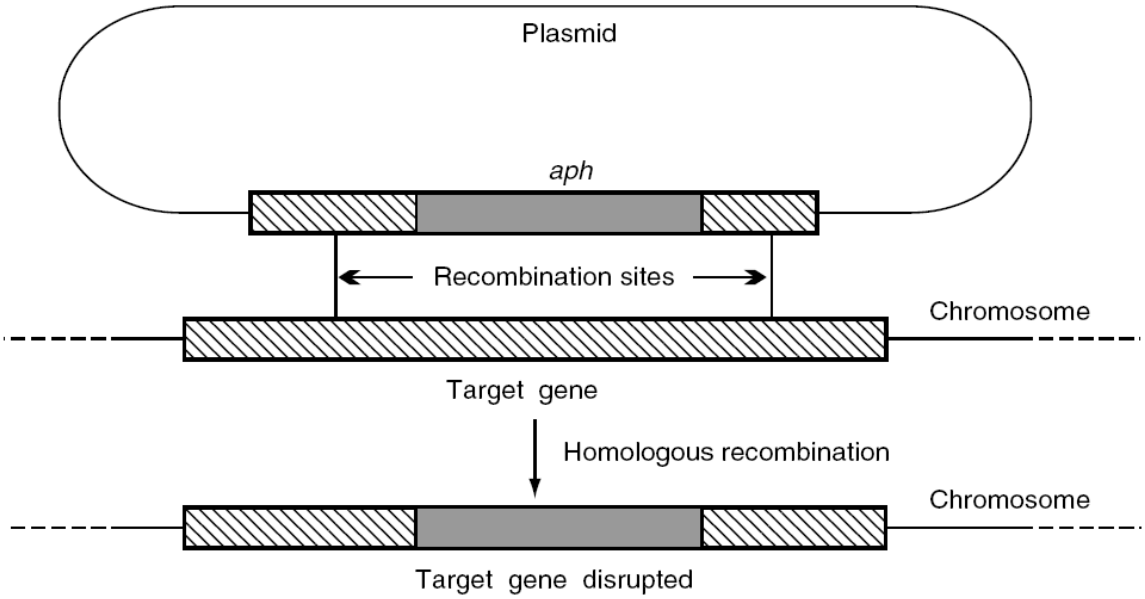

Figure 1.5. **Mutation of a target gene in a host strain through homologous recombination between the inactivated cloned gene and the specific wild-type gene on the chromosome (aminoglycoside phosphotransferase (*aph*) confers resistance to kanamycin) (Dale and Park, 2010).**

1.8 References

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Chapter 2

Characterization of the genetic basis of resistance in the *A. thaliana* ecotype Kil-0 against the *R. solanacearum* isolate BCCF 402 from *Eucalyptus*

2.1 Abstract

Ralstonia solanacearum, the causal agent of bacterial wilt disease, affects a wide range of economically important plants. Previous work identified a new pathosystem between a *Eucalyptus* isolate of *R. solanacearum* (BCCF 402) and the *Arabidopsis thaliana* ecotypes Bensheim (Be-0) and Killean (Kil-0). Be-0, the susceptible ecotype, shows severe wilt symptoms two weeks after root inoculation, while Kil-0, the resistant ecotype, remains healthy two weeks after inoculation. The aim of this study was to characterize the genetic basis of resistance in Kil-0 to the isolate BCCF 402. Genetic analysis of crosses between Kil-0 and Be-0 revealed that resistance to isolate BCCF 402 is due to a single recessive locus. This locus is likely to be the *RRS1* gene, since resistance co-segregates with the *RRS1* allele from Kil-0. Amino acid comparisons showed that Kil-0 contains an allelic variant of *RRS1* with the highest level of identity to *RRS1-R* from ecotype Nd-1 compared to Be-0 and Col-0. Previous work has shown that the *RRS1-R* gene in ecotype Nd-1 confers resistance against the *R. solanacearum* isolate GMI1000 by recognizing the PopP2 effector in GMI1000. This is one of the interactions that support the gene-for gene model of resistance to pathogens. The *popP2* gene in isolate BCCF 402 was sequenced and allelic differences was observed at the *popP2* locus between isolates GMI1000 and BCCF 402. This study also revealed that the *popP2* mutant of BCCF 402 failed to produce a resistant interaction with Kil-0 and avirulence was restored when Kil-0 was inoculated with the mutant complemented with a functional *popP2* gene. This suggests that the recognition between the *RRS1* allele in Kil-0 and PopP2 in BCCF 402 is necessary to confer resistance, supporting the gene-for-gene model in our pathosystem.

2.2 Introduction

R. solanacearum causes bacterial wilt and it is a gram negative, aerobic rod classified in the β-subdivision of the Proteobacteria (Schell, 2000). *R. solanacearum* has a worldwide distribution and a large host range of more than 200 species in 50 families. *R. solanacearum* infects a wide variety of plants and these include economically important hosts, such as tomato, potato, tobacco, banana, cowpea, peanut, cashew, papaya, olive and *Eucalyptus* (Schell, 2000). *R. solanacearum* infects its host mainly at the root elongation zone and at sites of lateral root emergence (Vailleau *et al.,* 2007; Bernoux *et al.,* 2008). The pathogen invades the intercellular spaces and crosses the endoderm to reach the xylem vessels where it multiplies and spreads into the aerial regions of the plant. The bacteria produce exopolysaccharides to block the vascular system of the plant and the obstruction of the vascular system leads to reduced water flow and wilting of the plant. *R. solanacearum* secretes up to 80 effectors into the host plant via the Type III secretion system (TTSS) that alter the host and suppresses its defense responses (Bernoux *et al.,* 2008).

Plants have acquired two lines of defense against pathogen attack and the first line of defense is known as basal or PAMP-triggered immunity (PTI) (Bernoux *et al.,* 2008; Block *et al.,* 2008). Conserved molecules known as pathogen-associated molecular patterns (PAMPs) such as flagellin and elongation factor Tu from bacteria are recognized by pattern recognition receptors (PRRs) in the host plant. The basal defense can be suppressed by microbes that secrete effectors with different virulence functions to enable the pathogens to cause disease. In the second line of defense, the host recognizes these effectors (avirulence factors [Avr]) or effector-induced changes through plant resistance (R) proteins. This type of defense is known as effector-triggered immunity (ETI) and it is often associated with a hypersensitive response (HR). Direct interaction between R and Avr proteins have been observed in only some pathosystems such as *Magnaporthe grisea*-rice (Oryza sativa), *Ralstonia solanacearum-A. thaliana*, *Melampsora lini*-flax (*Linum usitatissimum*) and tobacco mosaic virus (TMV)-tobacco (*Nicotiana tabacum*). Usually R proteins detect changes in host targets by an effector to trigger ETI and this indirect interaction is known as the guard hypothesis (Bernoux *et al.,* 2008).

A. thaliana is a widely accepted model for the study of plant-pathogen interactions and several *R* genes active against a number of pathogens have been identified (Deslandes *et al.,* 1998). A pathosystem was previously developed between two *A. thaliana* ecotypes (Nd-1 and Col-5) and the French Guyana strain GMI1000 from tomato. Complete wilting and high bacterial population levels were detected in the leaves of the susceptible ecotype Col-5 after root inoculation. No wilting and lower bacterial densities were detected in the resistant ecotype Nd-1. Genetic crosses between Nd-1 and Col-5 and subsequent pathogen challenges revealed a 1:3 segregation of resistance: susceptibility in the F_2 progeny, suggesting that resistance is caused by a single recessive gene. The resistance locus was identified as *RRS1* (recognition of *R. solanacearum*) and it is located on chromosome 5 (Deslandes *et al.,* 1998). *RRS1-R* is the allele in Nd-1 that confers resistance against GMI1000, while *RRS1-S* is the susceptible allele in Col-5 (Deslandes *et al.,* 2002). *RRS1-R* encodes a TIR-NBS-LRR R protein which contains a C-terminal WRKY domain characteristic of some plant transcriptional factors (Deslandes *et al.,* 2002).

The Pseudomonas outer protein P2 (PopP2) belongs to the YopJ/AvrRxv protein family and is the *R. solanacearum* Type III effector which interacts with RRS1-R in Nd-1 and initiates a defense response (Deslandes *et al.,* 2003). PopP2 and RRS1-R contain nuclear localization signals (NLS) and during infection both these two proteins are colocalized to the plant nucleus. The *A. thaliana* cysteine protease RESPONSIVE TO DEHYDRATION19 (RD19) was identified as a PopP2-interacting protein and its expression is activated during infection with the strain GMI1000 (Bernoux *et al.,* 2008). An *A. thaliana rd19* mutant in an *RRS1-R* genetic background developed wilting symptoms in response to GMI1000 infection. This result suggests that RD19 is necessary for RRS1-R mediated resistance. RD19 is generally situated in the plant lytic vacuole, but upon coexpression with PopP2 the protein is relocalized to the plant nucleus and physically interacts with PopP2. No physical interaction between RRS1-R and RD19 in the presence of PopP2 was detected in the nucleus. According to the results obtained it is proposed that during GMI1000 infection RD19 associates with PopP2 to form a nuclear complex that is recognized by RRS1-R and a defense response is activated (Bernoux *et al.,* 2008).

In a previous study, the virulence of several *R. solanacearum* isolates from *Eucalyptus* obtained from three African countries (Congo, Uganda and South Africa) was tested against a number of *A. thaliana* ecotypes to develop a new pathosystem (Weich, 2004). This pathosystem involved the two ecotypes Kil-0 and Be-0 and the Congo isolate BCCF 402. The ecotypes showed a clear differential response when inoculated with the *R. solanacearum* Congolese isolate. Isolate BCCF 402 caused severe wilt symptoms and killed Be-0 two weeks after inoculation, while Kil-0 was identified as the resistant ecotype remaining healthy two weeks after inoculation (Weich, 2004).

In this study we show that resistance in Kil-0 to isolate BCCF 402 is due to a single recessive locus. This locus is likely to be the *RRS1* gene, since resistance co-segregates with the *RRS1* allele from Kil-0. Amino acid comparisons showed that Kil-0 contains an allelic variant of *RRS1* with the highest level of identity to *RRS1-R* from ecotype Nd-1 compared to Be-0 and Col-0. Results from this study also showed that a *popP2* mutant of BCCF 402 failed to produce a resistant interaction with Kil-0. This suggests that the recognition between the *RRS1* gene in Kil-0 and PopP2 in BCCF 402 is necessary to confer resistance, supporting the gene-for-gene model in our pathosystem.

2.3 Materials and methods

2.3.1 Bacterial strains, plasmids and *A. thaliana* ecotypes

The plant material, bacterial strains and constructs utilized in this study are indicated in Table 2.1. *Escherichia coli* JM109 cells were cultured in Luria-Bertani (LB) medium wereas *R. solanacearum* strains were cultured on solidified Bacto-agar Glucose Triphenyltetrazolium chloride (BGT) media or in liquid B media as described by Weich (2004). Antibiotics were added at the following concentrations: Gentamycin 10ug/ml, tetracycline 10 ug/ml and ampicillin 100 ug/ml.

Table 2.1. **Materials utilized in this study.**

Materials	Characteristics	Supplier/reference
***A. thaliana* ecotypes**		
Nd-1	Medium rosette; leaves are weakly hairy Location: Niederzenz, Germany	The Nottingham Arabidopsis Stock Centre (NASC)
Kil-0	Hairy leaves; large rosette Location: Killean, United Kingdom	NASC
Be-0	Small leaves with serrate margin. Very large rosette Location: Bensheim, Germany	NASC
***R. solanacearum* strains**		
GM1000	Host plant: Tomato Geographical origin: French Guyana Race 1; Biovar 3	Boucher *et al.* (1985)
BCCF 402	Host plant: *Eucalyptus sp.* Geographical origin: Congo Race 1; Biovar 3	Fouché-Weich *et al.* (2006)
GRS100	GMI1000 *popP2* mutant strain	Deslandes *et al.* (2003)
GRS478/GRS479	BCCF 402 *popP2* mutant strains	Stéphane Genin (INRA-CNRS, Castanet Tolosan, Cedex, France)
Vectors		
pLAFR6	Broad-host-range vector containing transcriptional terminators flanking multilinker of pUC18, Tet^R	Huynh *et al.* (1989)
pLAFR6 ::*popP2*	A 2 kb HindIII-XbaI fragment containing the BCCF 402 *popP2* gene and its promoter was cloned into pLAFR6	This Study
popP2 mutant construct	PCZ367 integrative plasmid containing a disrupted *popP2* gene, Amp^R, $Gent^R$	Deslandes *et al.* (2003)

Amp^R: ampicillin resistance, Tet^R : tetracycline resistance, $Gent^R$: gentamycin resistance.

2.3.2 Growth and maintenance of *A. thaliana* ecotypes

The required amount of seeds were washed with 70% (v/v) ethanol and afterwards sterilized for 30 min in 1.5% (v/v) sodium hypochlorite. Seeds were rinsed three times with sterile distilled water and resuspended in 0.1% (w/v) agarose. The resuspended seeds were sown on MS (Murashige and Skoog, 1962) plates and germination was accelerated by incubating seeds at 4°C for 2 days (stratification). After stratification, the plates were covered with foil and incubated at 25°C for two days. Leaf and root development were promoted by growing the seedlings under 16 hr light/ 8 hr darkness in a growth chamber for two weeks. The plantlets were transferred to Jiffy pots (Jiffy France, Lyon, France) and grown for four weeks in a growth chamber maintained at a temperature of 22°C with 16 hr light, 25-30% relative humidity and 300-350 lum/sqf. The plants were watered four times (once a week) with a solution of 2.5g/l Multifeed® (Plaaskem (Pty) Ltd, Johannesburg, SA).

2.3.3 Cross-fertilization of *A. thaliana* ecotypes

Crossing of the *A. thaliana* ecotypes was performed according to Weigel and Glazebrook (2002). An illustration of the crosses performed between the ecotypes is provided in Figure 2.1. The most suitable flower from the female parent was chosen for the cross and crossing was performed under an optical microscope (Fig. 2.1 A). Forceps (Dumostar #5; Ladd Research Industries, Williston, Vermont, USA) were used to remove flowers with anthers that began to shed pollen onto the stigma to reduce the risk of self-fertilization. The same forceps were also used to remove the sepals, petals and immature anthers from the flower used for crossing, but the carpels were left intact (Fig. 2.1 B-D). A suitable anther from a mature flower of the parent plant was brushed on the stigma of the exposed carpel (Fig. 2.1 E-F). The female parent with the pollinated inflorescence was kept inside a plastic covering to prevent contamination with pollen from other *A. thaliana* plants. The siliques elongated after three days if the cross was successful (Fig. 2.1 G). The F_2 and F_3 progeny were obtained by selfing F_1 and F_2 progeny, respectively.

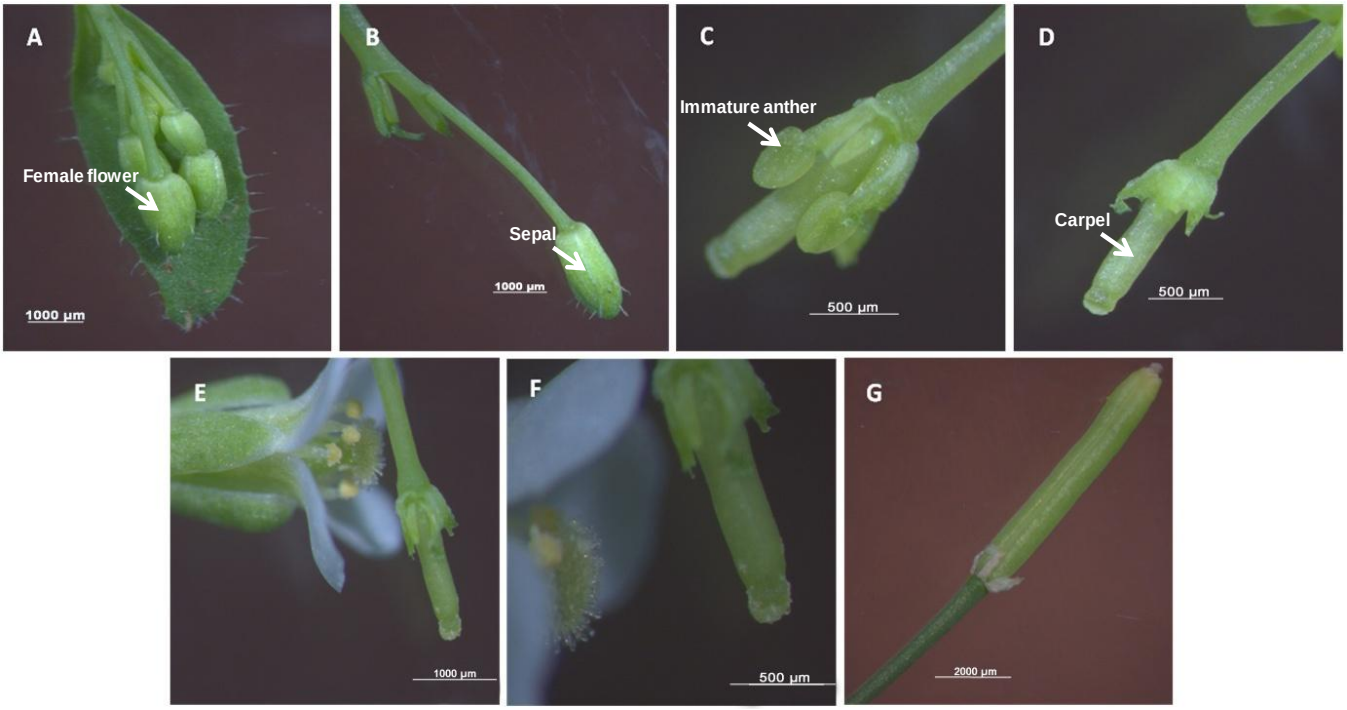


Figure 2.1. **Microscopic images illustrating the crosses performed between the *A. thaliana* ecotypes. A,** The most suitable flower on the female parent was identified. **B-D,** The sepals, petals as well as immature anthers were removed from the female flower, while the carpel was left intact. **E-F**, A mature anther from the male parent was brushed on the stigma of the exposed carpel. **G**, A silique emerged three days after a successful cross.

2.3.4 Bacterial inoculations

Root inoculations of the *A. thaliana* plants were performed according to Deslandes *et al.* (1998). *R. solanacearum* isolates were prepared for inoculation by first growing the strains on solidified Bacto-agar Glucose Triphenyltetrazolium chloride (BGT) media (Weich, 2004) at 28°C for 48 hrs. Mucoid colonies were inoculated in liquid B media (Weich, 2004) and incubated overnight at 28°C. *A. thaliana* plants (six weeks old) were prepared for inoculation by cutting 2 cm from the bottom of the Jiffy pot and the exposed wounded roots were soaked in a suspension of bacteria (10^8 cfu/ml) for 30 min. The plants were transferred to moist vermiculite in a growth chamber maintained at 26°C, 50% relative humidity, 16hr light/ 8 hr darkness and 500 lum/sqf light intensity. After inoculation, the plants were assessed daily for wilt symptom

development. The inoculated plants were rated on a scale from zero (no disease) to 5 (completely dead plants) (Weich, 2004). The data was then used to determine the disease index using the following formula: DI = $[\sum(n_i \times v_i)/(V \times N)]$ as described by Winstead and Kelman (1952). The formula was applied with slight modifications. DI = Disease Index; n_i = number of plants with respective disease rating; v_i = disease rating (0, 0.5, 1, 2, 3, 4 or 5); V = the highest disease rating (5); and N = the number of plants observed.

2.3.5 Genomic DNA isolations

A modification of the cetyl-trimethyl-ammonium bromide (CTAB) extraction procedure described by Lukowitz *et al*. (2000) was used to extract genomic DNA from *A. thaliana*. A single rosette leaf was crushed in a 1.5 ml reaction tube with 300 µl of 2 X CTAB buffer [2% (w/v) CTAB, 1.4 M NaCl, 100 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) pH 8.0, and 20 mM ethylene diamine tetraacetic acid (EDTA)] and a plastic pestle. The crushed tissue was incubated at 65°C for an hour and allowed to cool before adding 300 µl of chloroform. The plant debris was removed by centrifugation at 13 000 rpm for 5 min and the supernatant was transferred to a clean 1.5 ml reaction tube. Chromosomal DNA from the recovered supernatant was precipitated by adding 300 µl of isopropanol and pelleted by centrifugation at 13 000 rpm for 5 min. The pellet was rinsed with 500 µl of 70% (v/v) ethanol and air dried after centrifugation (13 000 rpm, 5 min). Sterile distilled water (40 µl) was added to dissolve the DNA pellet. The same method was used to isolate genomic DNA from *R. solanacearum* strains using 2 ml of bacterial culture as starting material.

2.3.6 RNA isolation and cDNA synthesis

Total RNA was isolated from frozen plant tissue using the QIAzol® lysis reagent (Qiagen, Valencia, California) according to the manufacturer's instructions. The extracted RNA was then further purified using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, California). The quality and the concentration of the RNA samples were determined by using a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The RNA integrity was evaluated by electrophoresis on a denaturing 1 % (w/v) agarose gel. cDNA was synthesized

from the clean RNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, California) according to the manufacturer's instructions and cleaned using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, California).

2.3.7 PCR amplifications

All PCR reactions were conducted in 0.2 ml tubes using a general PCR reaction mixture and amplification program unless stated otherwise. The reaction mixture contained 1 X Taq reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer and 1 U Taq DNA polymerase (BIOTAQ™ DNA polymerase, Bioline Ltd., London, England). The primer pairs used for amplification are indicated in Table A1 (Appendix A). The PCR cycling conditions included an initial denaturation step at 94°C for 5 min. This was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at (temperatures are indicated for each primer pair in Appendix A) for 30 sec and extension at 72°C for 1 min and 30 sec. A final extension step at 72°C for 7 min and a 4°C hold were also included.

The C-terminal region of the *RRS1* gene in ecotypes Kil-0 and Be-0 was amplified using the RRS1F-925 and the RT3 primer pairs (Appendix A) to obtain a 983 bp PCR product. This was the size of the amplified product expected for Nd-1. One hundred nanograms of genomic DNA was used as template for PCR amplifications and an initial denaturation step at 95°C for 3 min as well as a shorter extension step of 1 min at 72°C were used in the PCR cycling conditions.

Recombinant colonies were screened with colony PCR, using the M13F and M13R primer pairs (Appendix A), to ensure that the colonies contained the desired fragments. The colonies were used as templates and a higher concentration (0.2 mM) of each primer was used in the PCR reaction.

Synthesized cDNA was screened for genomic DNA contamination by PCR using the Ubiquitin forward and reverse primers (Appendix A). Fifty nanograms of cDNA was used a template in the PCR reactions.

In order to clone and sequence the full length *RRS1* gene from ecotypes Kil-0 and Be-0, four primer pairs were designed from the full length *RRS1* cDNA sequence of the *A. thaliana* ecotype Nd-1. The primers were used to generate four overlapping PCR fragments from Kil-0 and Be-0 cDNA. The primers and the sizes of the PCR products expected are shown in Table A1 (Appendix A). One hundred nanograms of cDNA was used as template in the PCR reactions and two independent PCR amplicons were generated from each primer pair for each ecotype.

The two BCCF 402 *popP2* mutant strains (GRS478 and GRS479; four clones of each) were screened with PCR to verify that the strains contained the disrupted *popP2* gene. One hundred nanograms of genomic DNA from each of the eight clones were screened with the POPP2 5'FLANKF and LACZR as well as the GENTF and POPP2 3'FLANKR primer pairs (Appendix A). An initial denaturation step at 94°C for 3 min as well as a shorter extension step of 1 min at 72°C were used in the PCR cycling conditions.

The POPP2FLF and the POPP2FLR primers (Appendix A) were used to amplify the full-length *popP2* gene and its promoter region from isolate BCCF 402 genomic DNA. One hundred nanograms of genomic DNA was used as template for PCR amplification and two independent PCR products were generated for the *popP2* gene and cloned into the PTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas Inc., USA) as described below.

The GRS478(6) strain transformed with the pLAFR6::*popP2* construct were screened with the pLAFR6 specific primers (PLAFFOR and PLAFREV; Appendix A) to determine if the strain contained the pLAFR6 cosmid harbouring the functional *popP2* gene.

2.3.8 Ligations and transformation

All PCR products were purified before cloning using the QIAquick PCR Purification Kit (Qiagen, Valencia, California). The clean PCR products were cloned into the pTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas Inc., USA), according to the manufacturer's instructions. The full length *popP2* gene and its promoter were cloned into the linearized pLAFR6 cosmid using T4 DNA ligase (Fermentas Inc., USA) according to the manufacturer's instructions.

The preparation of competent *E. coli* JM109 cells by the calcium chloride-mediated method as well as cell transformation using as a heat shock treatment, were performed as previously described (Sambrook and Russell, 2006).

The pLAFR6::*popP2* construct was transformed into the *R. solanacearum* GRS478(6) strain by electroporation. A modification of the procedure described by Allen *et al.* (1991) was used to prepare GRS478(6) electrocompetent cells. One milliliter of overnight culture was inoculated into 100 ml of B media containing 10 µg/ml gentamycin and grown at 28°C with vigorous shaking. The mid-log phase cells were transferred to prechilled 50 ml Falcon tubes and incubated on ice for 20 min. After incubation on ice, the cells were washed successively in 1, 0.5, 0.1 and 0.01 volumes of ice cold 10% glycerol. The pellets were pooled after washing and the cells (100 µl) were aliquoted into sterile ice cold microcentrifuge tubes. The competent cells were stored at -80°C until needed for electroporation. The cuvettes (0.1 cm electrode gap) (Bio-Rad, Richmond, California) were placed at -20°C for 1 hour before electroporation. One hundred nanograms of DNA was added to the cells and the strain was transformed by electroporation with an electroporator (Eppendorf, Hamburg, Germany) at a capacitance of 10 microfarads and an electric field of 12.5kV/cm. The cells were immediately added to 1ml of B media and agitated for 3 hours. The transformed cells (100 µl) were plated on BGT plates containing 10 µg/ml gentamycin and 10 µg/ml tetracycline.

Plasmid or Cosmid DNA was isolated using the Invisorb® Spin Plasmid Mini Kit Two (Invitek, Berlin, Germany).

2.3.9 Restriction enzyme digestions

Genomic DNA from *A. thaliana* plants was screened with the *RRS1* CAPS marker by amplifying the C-terminal region of the *RRS1* gene with the RRS1F-925 and RT3 primer pairs as described above and digesting the PCR products overnight at 37°C using LweI (Fermentas Inc., USA). The reaction mixtures included 20 µl of PCR product, 2µl of 10 X Buffer Tango™ (Fermentas Inc., USA) and 1.5µl of LweI (10 u/µl) in a total volume of 32 µl. The enzyme was inactivated by

incubation at 65°C for 20 min. The digested products were analyzed by electrophoresis on a 2% (w/v) agarose gel for genotyping of individuals.

The PTZ57R/T-*popP2* vector was double digested with HindIII and XbaI (FastDigest® restriction enzymes, Fermentas Inc., USA) to subclone the full-length *popP2* gene and its promoter into pLAFR6. The reactions contained 1 µg PTZ57R/T-*popP2* plasmid or 1 µg pLAFR6 cosmid, 1 X FastDigest® Buffer and 1 FastDigest® Unit (FDU) of each enzyme. The reaction mixture was made up to 20 µl using sterile distilled water. The samples were incubated at 37°C for 1 hour and the enzymes were inactivated by heating the samples for 10 min at 80°C. The digested fragments were seperated on a 1% (w/v) agarose gel and the *popP2* gene with its promoter was recovered from the gel using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, California).

2.3.10 Sequencing

All sequencing reactions were performed at Inqaba Biotec™ (Pretoria, South Africa) and Macrogen (Rockville, MD, USA) using the M13 primers. An additional sequencing primer (POPP2INR 5'AGTGACAGGGAGAGTATC3') was designed to sequence the internal region of the full-length *popP2* gene and its promoter.

2.3.11 Statistical analysis

Statistical analysis were performed using Microsoft Excel 2007 (means, standard deviations and Chi-Square analysis) and R (Wilcoxon Rank Sum Test) (Bauer, 1972).

2.4 Results

2.4.1 Resistance in Kil-0 against isolate BCCF 402 is due to a single recessive gene.

Previous work identified a pathosystem between the *R. solanacearum* isolate BCCF 402 and the *A. thaliana* ecotypes Be-0 and Kil-0 (Weich, 2004). Be-0, the susceptible ecotype, shows severe wilt symptoms two weeks after root inoculation, while Kil-0, the resistant ecotype, remains healthy two weeks after inoculation. In order to determine if resistance in Kil-0 against isolate BCCF 402 is polygenic or due to a single-gene, the F_1, F_2 and F_3 progeny from a cross between Kil-0 and Be-0 was tested for their response to isolate BCCF 402.

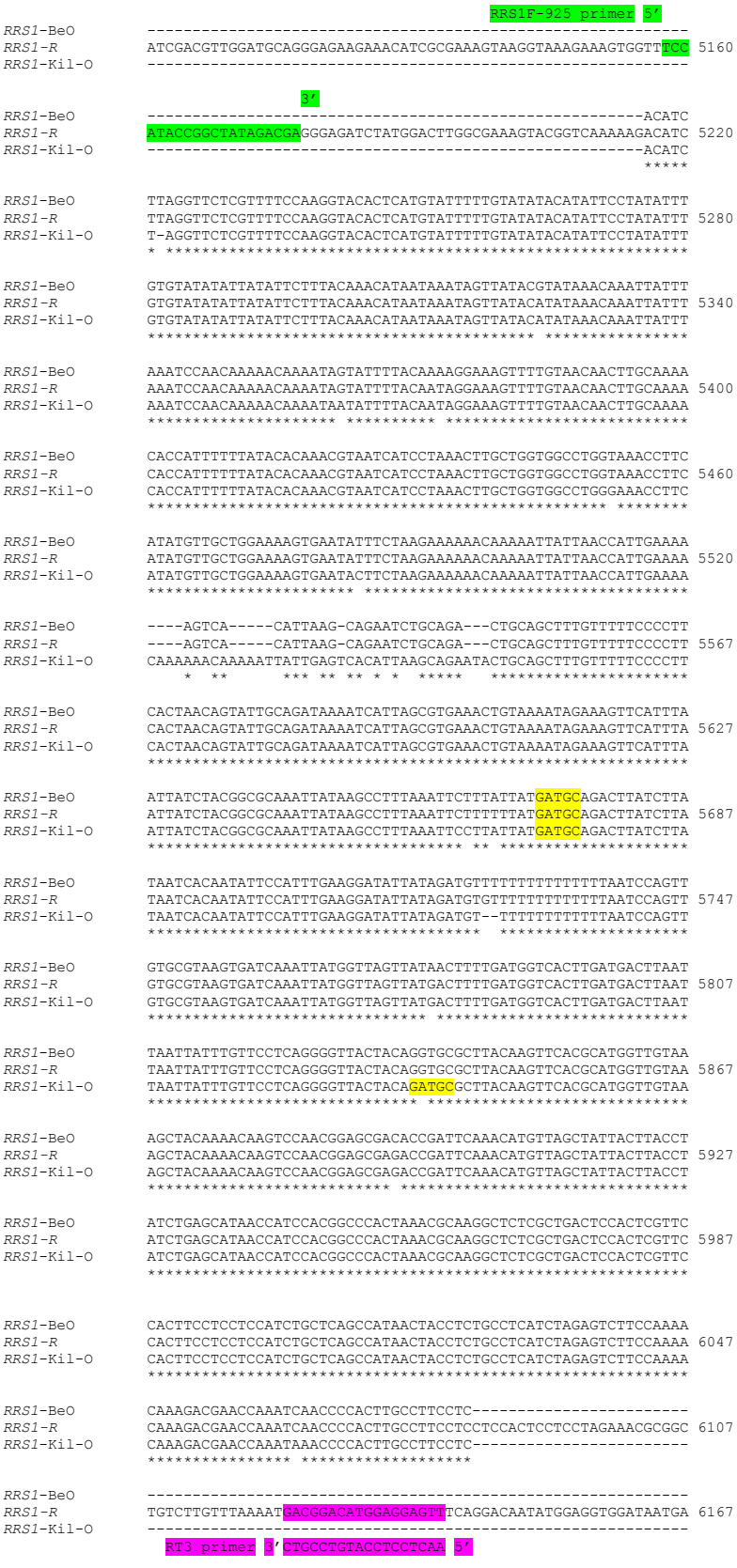
2.4.1.1 Development of a Cleaved Amplified Polymorphic Sequence (CAPS) marker to screen the F_1 progeny from a cross between Kil-0 and Be-0.

One of the purposes of developing a CAPS marker within the C-terminal region of *RRS1* gene was to screen the F_1 progeny from a cross between Kil-0 and Be-0 to confirm that the crosses were successful. The RRS1F-925 and the RT3 primers were designed from the C-terminal region of the *RRS1-R* genomic DNA sequence of ecotype Nd-1. The primers were used to generate PCR products from the genomic DNA of the *A. thaliana* ecotypes Kil-0 and Be-0. The cloned PCR products were sequenced with the M13F and M13R primers at Macrogen (Rockville**,** MD**,** USA). Differential restriction sites were identified between the alleles using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/).

Figure 2.2 A illustrates the genomic DNA sequence alignment of the C-terminal region of the *RRS1* gene in ecotypes Kil-0 and Be-0 and Nd-1. The *RRS1-R* allele was included in the alignment to give an indication of the region of the *RRS1* gene that was amplified with the RT3 and RRS1F-925 primer pairs. The Kil-0 allele of *RRS1* has two LweI restriction sites and the Be-0 allele has only one restriction site. LweI digestion of the *RRS1* PCR products would yield a 516 bp, a 313 bp and a 164bp fragment for the Kil-0 allele and a 504 bp and 479 bp fragment for the Be-0 allele (Fig. 2.2 B). The CAPS marker was tested on the parent plants before screening the progeny plants. The C-terminal region of the *RRS1* gene from Kil-0 and Be-0 was amplified with the RT3 and RRS1F-925 primers and digested with LweI. As expected, LweI digestion of the

RRS1 PCR products yielded three fragments for Kil-0 allele (a 516 bp, a 313 bp and a 164bp fragment), whereas two fragments were observed for the Be-0 allele (a 504 bp and 479 bp fragment) (Fig. 2.2C).

A



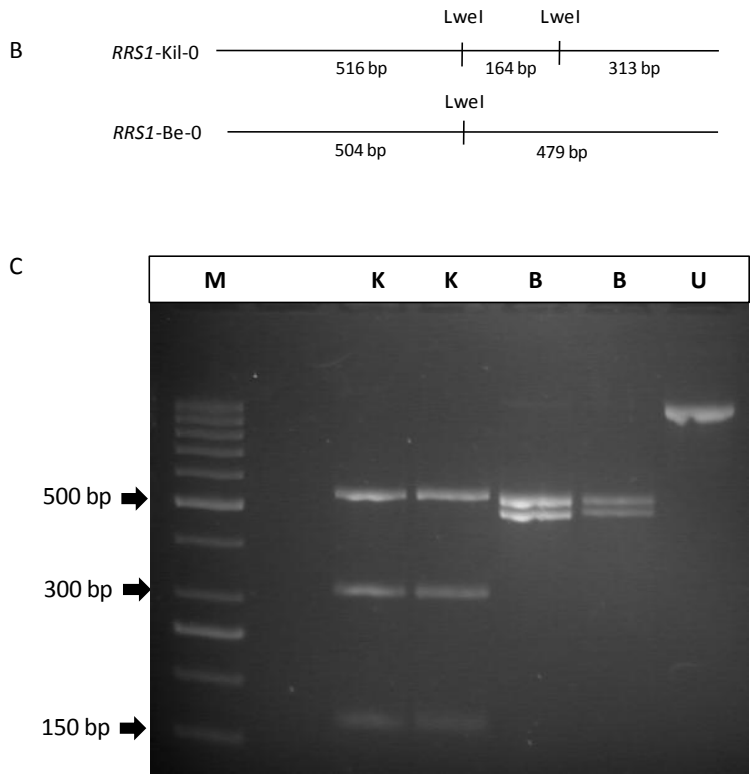


Figure 2.2. **Development of a CAPS marker to screen the F_1 progeny from a cross between Kil-0 and Be-0.** (A) A comparison of the genomic DNA sequences of the C-terminal region of the *RRS1* gene in ecotypes Kil-0, Be-0 and Nd-1. The alignment shows the DNA amplified with the RT3 and RRS1F-925 primer pairs. The recognition sites for LweI are shown in yellow. The nucleotide sequence of the *RRS1-R* gene from Nd-1 is available in GenBank (accession number AX103684) and the numbers to the right show the positions on the *RRS1-R* gene. (B) A schematic diagram illustrating LweI digestion of the C-terminal region of the *RRS1* gene. (C) LweI digestion of the *RRS1* PCR products from Kil-0 and Be-0. M: 50 bp DNA marker (Fermentas Inc., USA); lane K: Kil-0; lane B: Be-0; lane U: undigested *RRS1* PCR product from *A. thaliana* genomic DNA. Digested products were analyzed by electrophoresis on a 2% (w/v) agarose gel in 1X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

2.4.1.2 Screening of heterozygous F_1 progeny with the CAPS marker

The F_1 progeny was screened with the *RRS1* CAPS marker to confirm that the crosses performed between Kil-0 and Be-0 were successful. Genomic DNA was isolated from the F_1 progeny and the C-terminal region of the *RRS1* gene was amplified using the RT3 and RRS1F-925 primers. The *RRS1* PCR products were digested afterwards with LweI.

Isolation of genomic DNA from heterozygous F_1 plants

Genomic DNA was successfully isolated from 32 F_1 plants and the concentration and purity of the DNA for the different samples were measured with the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The 260/280 ratio was above 1.8 for all of the samples and the elevated ratios were in part due to RNA (as seen on gel). The amount of nucleic acid extracted from the different F_1 samples ranged from a minimum of 4 µg up to a maximum of 60 µg per rosette leaf. The F_1 progeny DNA were also visualized on a 1% (w/v) agarose gel to assess the quality of the DNA and to confirm the presence of RNA (Fig. 2.3, lane 1 to 32). The F_1 samples had a single intact band of genomic DNA near the wells of the agarose gel and residual RNA was observed in all of the samples. The samples were not treated with RNAse as the RNA did not interfere with the restriction analysis. DNA extraction was repeated on one of the F_1 samples (Fig. 2.3, lane 27) which was initially unsuccessful (data not shown). Good quality DNA was previously isolated from the parent plants, Kil-0 and Be-0, (data not shown).

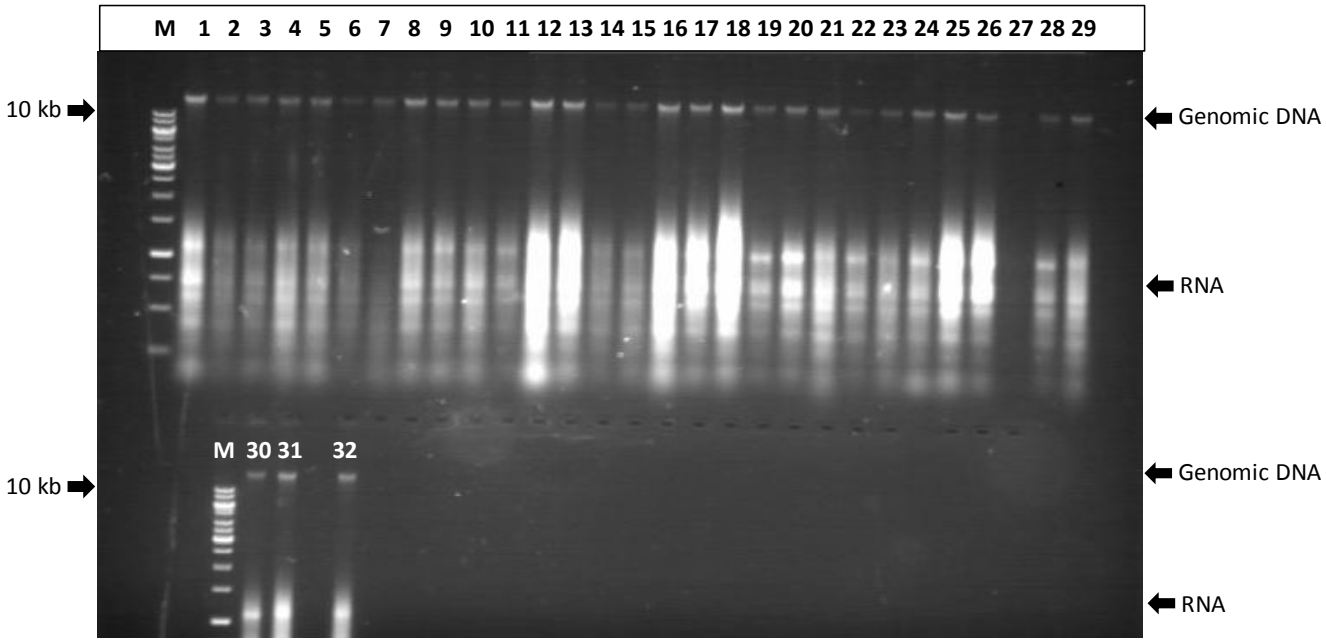


Figure 2.3. **Genomic DNA isolated from F_1 progeny plants.** M: 1 kb molecular marker (Fermentas Inc., USA); lanes 1 to 32: Genomic DNA isolated from F_1 progeny plants. The DNA were analyzed by electrophoresis through a 1% (w/v) agarose gel in 1X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

PCR amplification of the C-terminal region of the *RRS1* gene in the F_1 plants

The C-terminal region of the *RRS1* gene was successfully amplified from the DNA of the 32 F_1 progeny (Fig. 2.4, lanes 1 to 32) and parent plants (Fig. 2.4, lanes K and B). All of the F_1 plants and parent plants had the expected 983 bp PCR product. Two of the F_1 plants (Fig. 2.4, lanes 14 and 15) did not have any PCR products and PCR was repeated on these two samples before restriction analysis (data not shown). The negative control yielded no amplification product, as expected (Fig. 2.4, lane N).

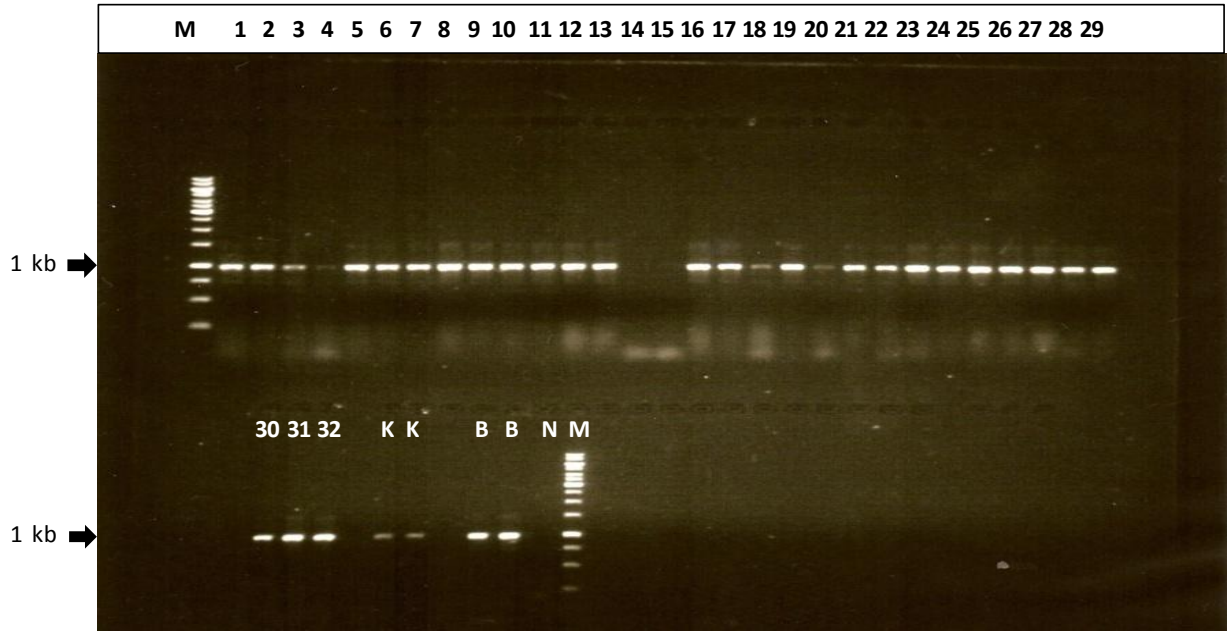


Figure 2.4. **PCR amplification of the C-terminal region of the *RRS1* gene from F_1 progeny and parent plants using the primer pair, RT3 and RRS1F-925.** M: 1kb DNA marker (Fermentas Inc., USA); lanes 1 to 32: PCR products amplified from F_1 progeny genomic DNA; lanes K and B: PCR products amplified from Kil-0 and Be-0 genomic DNA, respectively; lane N: negative water control. PCR products were analyzed by electrophoresis through a 1% (w/v) agarose gel in 1X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

LweI digestion of the *RRS1* PCR amplicons

Four of the F_1 plants grew poorly and therefore they were removed from the study. The *RRS1* PCR products for the parents were digested to serve as controls and the PCR products from Kil-0 digested with LweI yielded three fragments (a 516 bp, a 313 bp and a 164bp fragment) (Fig. 2.5 A and B, lane K). Two fragments were observed for the Be-0 PCR products digested with LweI (a 504 bp and a 479 bp fragment) (Fig. 2.5 A and B, lane B). Restriction analysis of the *RRS1* PCR products from the F_1 progeny confirmed that the crosses performed between Kil-0 and Be-0 were successful (Fig. 2.5 A and B, lanes 1 to 28). All of the F_1 progeny had the Kil-0 and Be-0 alleles for the *RRS1* gene.

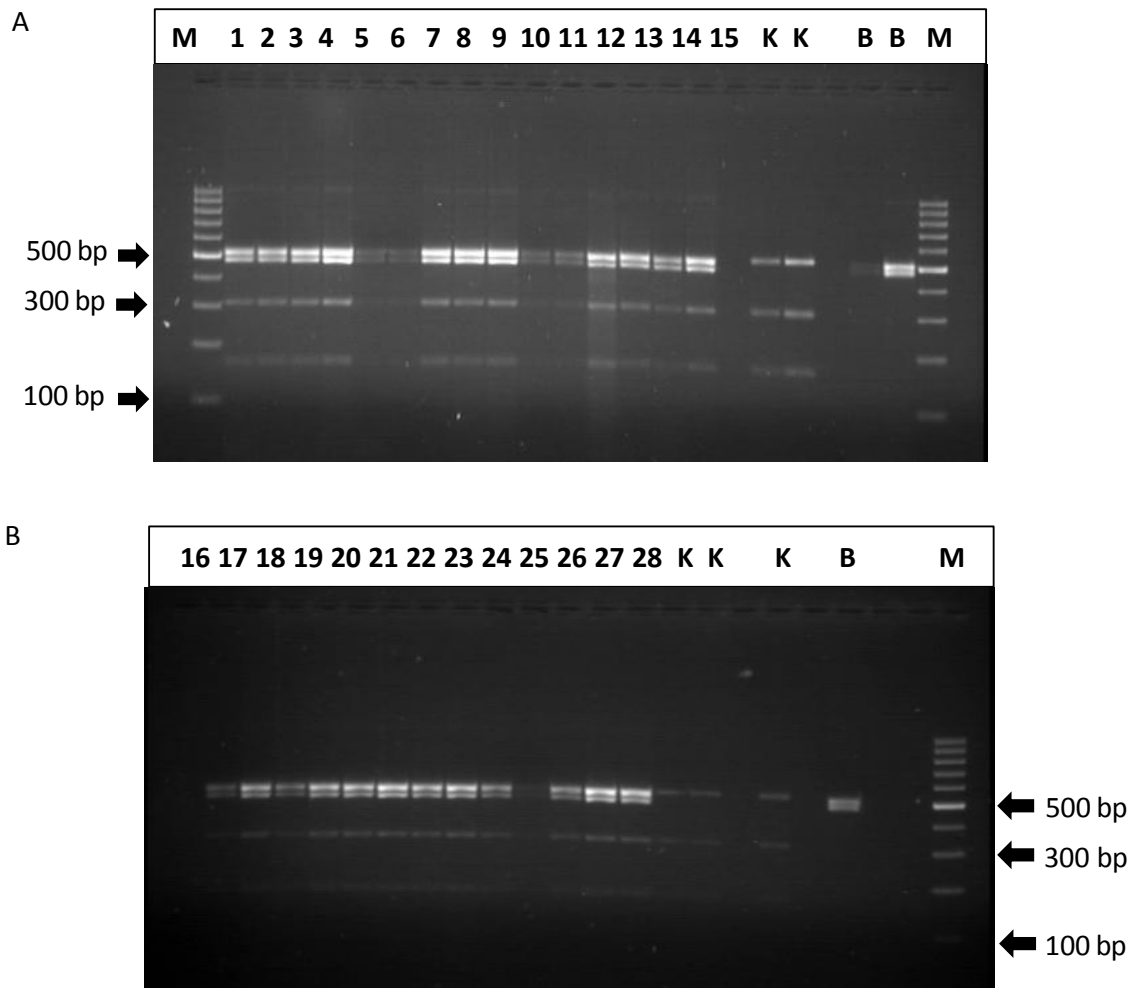


Figure 2.5. **Digestion of the *RRS1* PCR products from the F_1 progeny, Kil-0 and Be-0 with LweI.** M: 100bp DNA marker (Fermentas Inc., USA); lanes 1 to 28: F_1 PCR products digested with LweI; lane K: Kil-0 PCR products digested with LweI; B: Be-0 PCR products digested with LweI. Digested products were analyzed by electrophoresis on a 2% (w/v) agarose gel in 1X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

2.4.1.3 Inoculation of the F_1 progeny from a cross between Kil-0 and Be-0 with the *R. solanacearum* isolate BCCF 402

To investigate if resistance in Kil-0 against isolate BCCF 402 is dominant or recessive, the F_1 progeny was tested for their response to isolate BCCF 402. F_1 plants were obtained from crosses between the resistant ecotype Kil-0 and the susceptible ecotype Be-0 as illustrated in Figure 2.1. All of the F_1 plants were heterozygous for the *RRS1* gene (Fig. 2.5 A and B). The parent plants and F_1 plants were inoculated by cutting and soaking the roots in a bacterial suspension of the *R. solanacearum* strain BCCF 402. Kil-0 remained healthy 14 days post inoculation (dpi), while BCCF 402 caused severe wilt symptoms and killed Be-0 14 dpi (Fig. 2.6 A and B). The 18 F_1 plants showed delayed wilt symptom development, but most were completely dead at 14 dpi except for four of the F_1 plants (Fig. 2.6 A and B). The reason for the delayed wilt symptom development in the F_1 plants is perhaps due to the low inoculation levels received by the F_1 plants. The four F_1 plants which appear healthy in Figure 2.6 A showed wilt symptoms at 14 dpi and eventually died at 17 dpi, at which time the parent, Kil-0, remained healthy. The results showed that the F_1 progeny was susceptible to isolate BCCF 402 and that resistance in Kil-0 against isolate BCCF 402 is recessive.

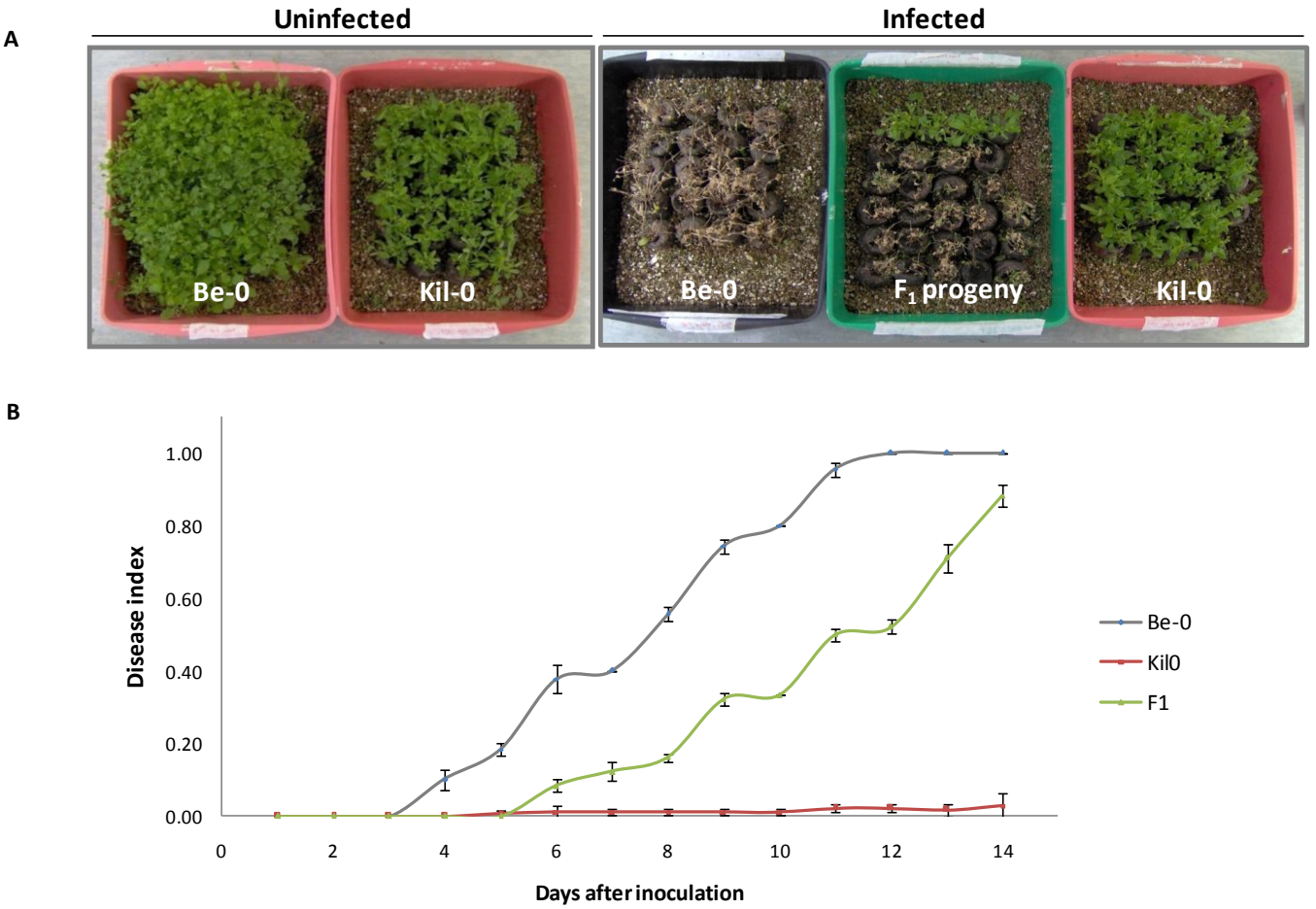


Figure 2.6. **Inoculation of the F_1 progeny from a cross between Kil-0 and Be-0 with the *R. solanacearum* isolate BCCF 402.** A: Phenotypic responses of the parent plants (Kil-0 and Be-0) and the F_1 progeny to isolate BCCF 402 14 dpi. B: Progression of disease symptoms on the parent plants (Be-0 and Kil-0) and the F_1 progeny 14 dpi with isolate BCCF 402. The inoculated plants were scored on a scale from zero (no disease) to 5 (completely dead plants). The data was used to calculate the disease index. Means and standard deviations were analyzed from disease index values of a total of 18 plants per ecotype or F_1 progeny (three replicates of 6 plants each).

2.4.1.4 Inoculation of the F_2 and F_3 progeny with isolate BCCF 402

To investigate if resistance in Kil-0 against isolate BCCF 402 is polygenic or due to a single-gene, the F_2 progeny was tested for their response to isolate BCCF 402. The F_2 segregating population

was obtained from the selfed F_1 progeny and the F_2 progeny was inoculated with isolate BCCF 402. The F_2 progeny were either characterized as having a Kil-0 phenotype (resistant) or a Be-0 phenotype (susceptible) after inoculation with strain BCCF 402. The resistant and susceptible plants were then counted. Two separate inoculation trials were performed and a 1:3 segregation ratio (resistant: susceptible) was observed in the F_2 progeny (Table 2.2). Chi-Square analysis is used to find out how the observed value of a given phenomena is significantly different from the expected value (http://www2.lv.psu.edu/jxm57/irp/chisquar.html.) The *P* value is the probability that the difference of the observed from that expected is due to chance alone. High *P*-values were obtained for the calculated χ^2 values and it means there is a very high probability that any deviation from expected is due to chance only. The 1:3 ratio of resistant to susceptible plants suggests that a recessive resistance locus is present in ecotype Kil-0 and is absent in ecotype Be-0.

Table 2.2**. Segregation analysis of *R. solanacearum* isolate BCCF 402 resistance in the F_2 progeny from crosses between ecotypes Kil-0 (resistant) and Be-0 (susceptible).**

		Number of plants						
Trial	Cross	Resistant	Susceptible	Total	Expected ratio (R:S)	Observed ratio (R:S)	$\chi2^a$	*P*
1	Kil-0 × Be-0	74	215	289	1:3	1:2.9	0.06	0.9>*P*>0.7
2	Kil-0 × Be-0	92	295	387	1:3	1:3.2	0.31	0.7>*P*>0.5

[a] χ^2 values were calculated for a segregation ratio of 1 resistant : 3 susceptible plants.

The results obtained for the F_2 progeny as discussed above were verified by the phenotypic analysis of the F_3 progeny from 6 resistant F_2 lines inoculated with isolate BCCF 402. The F_3 progeny had a similar phenotype as Kil-0 after inoculation with BCCF 402 (Fig. 2.7). Kil-0 started showing minor wilt symptoms during the trial and this was probably due to high temperatures in the growth room (28°C instead of 26°C). These symptoms were also observed in Kil-0 in previous trials with high temperatures (Weich, 2004).

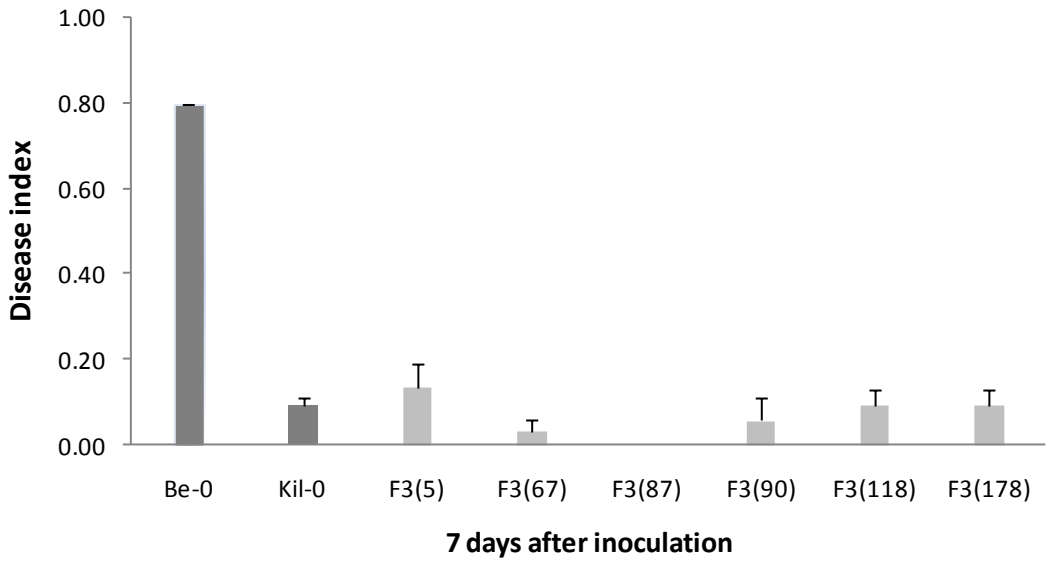


Figure 2.7. **Disease index of the parent plants (Be-0 and Kil-0) and the F_3 progeny from 6 resistant F_2 lines 7 dpi with the *R. solanacearum* isolate BCCF 402.** The inoculated plants were scored on a scale from zero (no disease) to 5 (completely dead plants) (Weich, 2004). The data was used to determine the disease index. Means and standard deviations were analyzed from disease index values of a total of 24 plants per ecotype or F_3 progeny (three replicates of 8 plants each).

2.4.2 The recessive *RRS1* allele in Kil-0 is linked to resistance against isolate BCCF 402.

Genetic analysis of crosses between Kil-0 and Be-0 revealed that resistance to isolate BCCF 402 is due to a single recessive locus. Previous work by other authors showed that resistance to the tomato isolate GMI1000 of *R. solanacearum*, in a cross between *A. thaliana* ecotypes Col-5 and Nd-1, also segregated as a simply inherited recessive trait, and *RRS1-R* was identified as the major determinant of resistance against this isolate (Deslandes *et al*., 2002). To determine if the *RRS1* allele in Kil-0 is linked to resistance, the co-segregation of the *RRS1* allele with the resistant or susceptible phenotypes was investigated by screening the F_2 individuals from a cross between Kil-0 and Be-0 with the *RRS1* CAPS marker. The F_2 individuals were inoculated

with isolate BCCF 402 and scored for disease development. Leaf tissues from individual F_2 plants were collected and genomic DNA was isolated from each F_2 plant using the CTAB method. The C-terminal region of the *RRS1* gene in the F_2 plants was amplified using the RT3 and RRS1F-925 primers and the PCR products were digested with LweI.

Representative images of the F_2 progeny and parent plants, that were screened with the *RRS1* CAPS marker, are shown in Figure 2.8 and the results are described briefly. As mentioned previously, digestion of the *RRS1* PCR amplicon from Kil-0 yielded a 516 bp; a 313 bp and a 164 bp fragment (Fig. 2.8 A and B, lane K), whereas digestion of the *RRS1* PCR product from Be-0 yielded a 504 bp and a 479 bp fragment (Fig. 2.8 A and B, lane B). Ten F_2 plants that were scored as susceptible had either a combination of Kil-0 and Be-0 alleles or only the Be-0 allele for the *RRS1* gene (Fig. 2.8 A, lanes 1 to 10). Ten F_2 plants that were scored as resistant had only the Kil-0 allele for the *RRS1* gene (Fig. 2.8 B, lanes 11 to 20). The only exception was one of the resistant F_2 individuals that had both the Kil-0 and Be-0 alleles for the *RRS1* gene and this could have been due to incorrect scoring of the phenotype of the individual (Fig. 2.8 B, lane 13). Not all of the F_2 samples are shown in the figures below (see Appendix B). Fourteen of the forty-eight F_2 plants, that were scored as resistant, had the Kil-0 allele whereas sixteen susceptible F_2 plants had the Be-0 allele and seventeen susceptible F_2 plants had a combination of Kil-0 and Be-0 alleles for the *RRS1* gene. These results show that the resistant and susceptible phenotypes cosegregated with the CAPS marker.

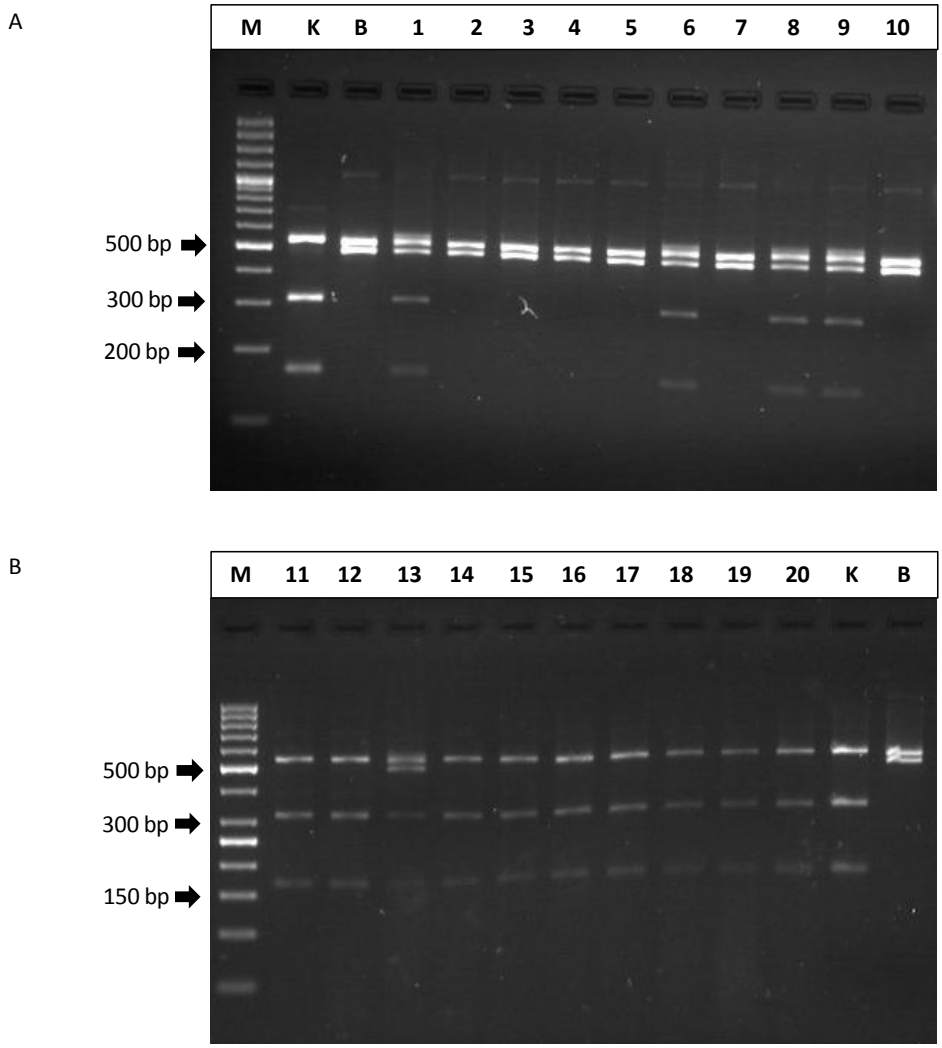


Figure 2.8. **LweI digestion of the *RRS1* PCR products from the F_2 progeny of a cross between Kil-0 and Be-0.** A: lanes 1 to 10: LweI digestion of the *RRS1* PCR products from F_2 progeny that was scored as susceptible; M: 100bp DNA marker (Fermentas Inc., USA). B: lanes 11 to 20: LweI digestion of the *RRS1* PCR products from F_2 progeny that was scored as resistant. M: 50bp DNA marker (Fermentas Inc., USA). For both A and B: lane K: LweI digestion of the Kil-0 *RRS1* PCR product; lane B: LweI digestion of the Be-0 *RRS1* PCR product. The digested PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel in 1X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

2.4.3 Kil-0 contains an allelic variant of *RRS1* with the highest level of identity to *RRS1-R* from ecotype Nd-1 compared to Be-0 and Col-0

The locus in Kil-0 conferring resistance against isolate BCCF 402 is likely to be the *RRS1* gene, since resistance co-segregates with the *RRS1* allele from Kil-0. To investigate if resistance to the *R. solanacearum* strains is due to alleles of one gene (*RRS1*) or of different genes, the F_1 progeny from a cross between Kil-0 and Nd-1 was tested for their response to the *R. solanacearum* strains BCCF 402 and GMI1000. To investigate if Kil-0 contains the *RRS1-R* gene or an allele of *RRS1*, the full length coding sequence of *RRS1* in Kil-0 was determined and compared to the *RRS1-R* coding sequence in Nd-1.

2.4.3.1 Testing allelism of resistance to isolates BCCF 402 and GMI1000 in ecotypes Kil-0 and Nd-1

Screening of the F_1 progeny from a cross between Kil-0 and Nd-1 with the *RRS1* CAPS marker

The F_1 progeny was screened with the *RRS1* CAPS marker to confirm that the crosses performed between Kil-0 and Nd-1 were successful. Genomic DNA was isolated from the F_1 progeny and the C-terminal region of the *RRS1* gene was amplified using the RT3 and RRS1F-925 primers. The *RRS1* PCR products were digested afterwards with LweI. The *RRS1* PCR products from the parents were also digested to serve as controls. The PCR product from Kil-0 digested with LweI yielded three fragments (a 516 bp, a 313 bp and a 164bp fragment), as expected (Fig. 2.9 A, lane K). Two fragments were observed for the Nd-1 PCR product digested with LweI (a 504 bp and a 479 bp fragment) (Fig. 2.9 A, lane N). The Nd-1 and Be-0 alleles have only one LweI recognition site (Fig. 2.2 A) and digestion of the Be-0 *RRS1* PCR product yielded the same two fragments as Nd-1 (Fig. 2.9 A, lane B). All of the F_1 progeny had the Kil-0 and Nd-1 alleles for the *RRS1* gene and these results confirm that crosses performed between Kil-0 and Nd-1 were successful (Fig. 2.9, lane 1 to 15).

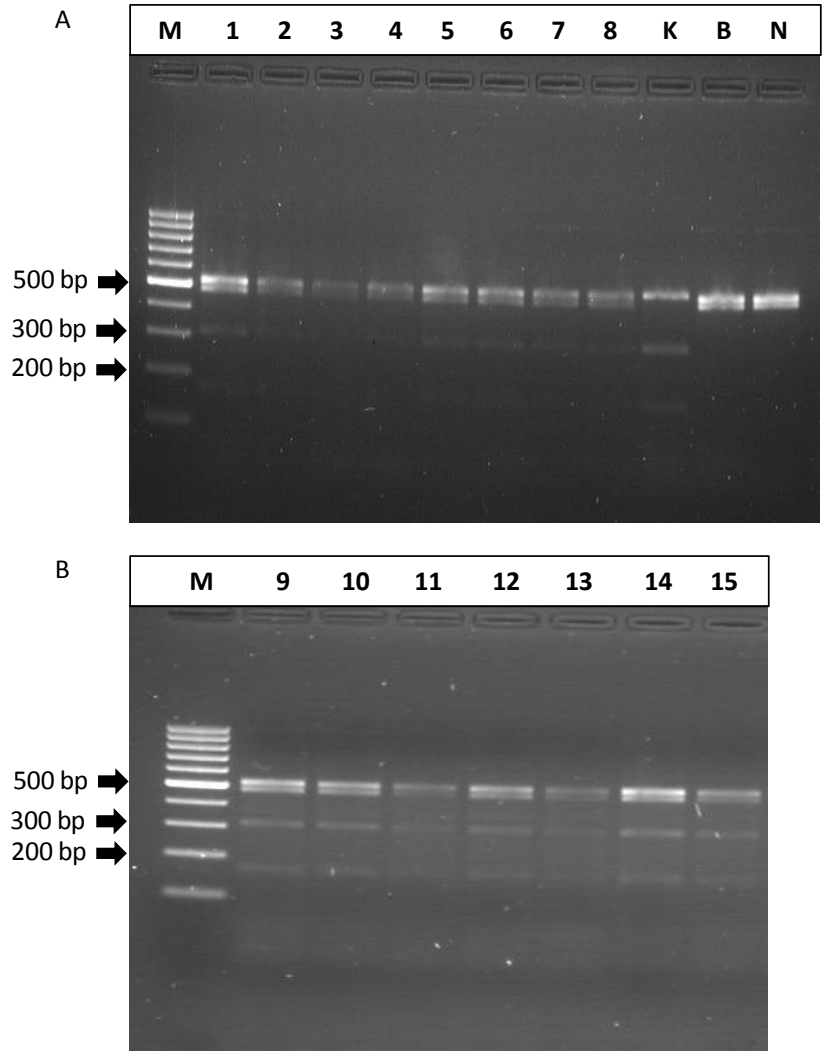
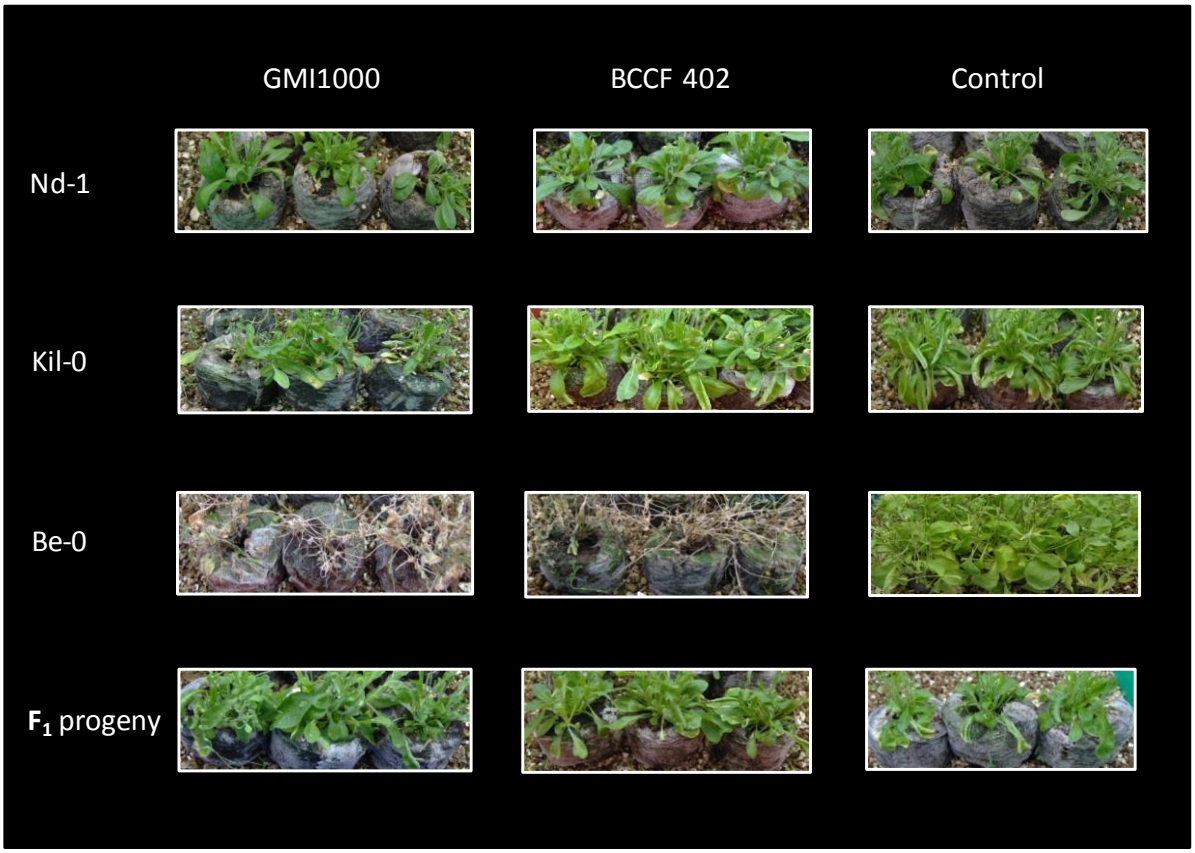


Figure 2.9. **LweI digestion of the *RRS1* PCR products from the heterozygous F_1 progeny of a cross between Kil-0 and Nd-1.** M: 100bp DNA marker (Fermentas Inc., USA); lanes 1 to 15: LweI digestion of the *RRS1* PCR products from the heterozygous F_1 progeny of a cross between Kil-0 and Nd-1; lane K: LweI digestion of the Kil-0 *RRS1* PCR product; lane B: LweI digestion of the Be-0 *RRS1* PCR product; lane N: LweI digestion of the Nd-1 *RRS1* PCR product. The digested PCR products were analyzed by electrophoresis through a 2% (w/v) agarose gel in 1 X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

Inoculation of the F_1 progeny and parents with the *R. solanacearum* isolates BCCF 402 and GMI1000

To determine whether the resistance gene present in Kil-0 is *RRS1*, the F_1 progeny from a cross between Kil-0 and Nd-1 was inoculated with isolates BCCF 402 and GMI1000. The parent plants as well as the susceptible ecotype Be-0 were inoculated with the same strains to serve as controls. Nd-1 was resistant to both strains and remained symptom-free 14 dpi, while the susceptible ecotype Be-0 showed severe wilt symptoms to each of the two strains and died 14 dpi (Fig. 2.10 A and B). Kil-0 remained healthy to isolate BCCF 402, but showed intermediate symptoms to isolate GMI1000 14 dpi (Fig. 2.10 A and B). The F_1 progeny from a cross between Kil-0 and Nd-1 remained healthy to each of the two strains 14 dpi (Fig. 2.10 A and B). These results indicate that the resistance gene in Kil-0 is allelic to *RRS1* and resistance in Kil-0 to isolate BCCF 402 is not due to a different gene.

A



B

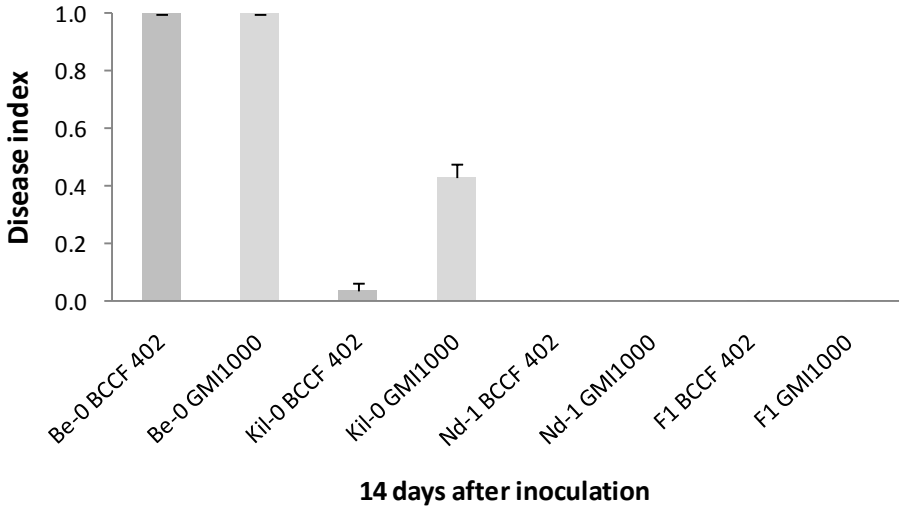


Figure 2.10. **Inoculation of the parent plants and F_1 progeny from a cross between Kil-0 and Nd-1 with the BCCF 402 and GMI1000 isolates.** A: Phenotypic responses of the parent plants and F_1 progeny to the BCCF 402 and GMI1000 strains 14 dpi. B: Disease index of the parent plants and F_1 progeny after inoculation with the BCCF 402 and GMI1000 strains. Infected plants were rated on a scale from zero (no disease) to 5 (completely dead plants). The data was then used to determine the disease index. Means and standard deviations were calculated from disease index values of a total of 15 plants per ecotype or F_1 progeny (3 replicates of 5 plants each).

2.4.3.2 Sequencing of the *RRS1* gene in the *A. thaliana* ecotypes Kil-0 and Be-0

The full-length *RRS1* gene was sequenced by first isolating RNA from *A. thaliana* leaf tissue. cDNA was synthesized from the clean RNA and four primer pairs, designed from the full length *RRS1* cDNA sequence of the *A. thaliana* ecotype Nd-1, were used to amplify the full-length coding sequence of the *RRS1* gene in Kil-0 and Be-0 (Appendix A). Two independent PCR amplicons for each fragment from each ecotype were cloned. The M13 primers were used to sequence two independent clones for each fragment from each ecotype.

RNA isolation from the leaf tissue of the *A. thaliana* ecotypes Kil-0 and Be0 and screening of synthesized cDNA with the ubiquitin primers.

A large-scale RNA extraction procedure was used to obtain RNA from the *A. thaliana* leaf tissue. The quality and the concentration of the RNA samples were determined by using a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The measured 260/280 and the 260/230 ratios were close to 2 and this indicates that high quality RNA was isolated. The amount of RNA extracted from the samples ranged from as low as 21 µg up to a maximum of 36 µg. The integrity of the RNA was also assessed on a 1% (w/v) formaldehyde denaturing agarose gel (Fig. 2.11 A, lanes 1 to 4). Intact RNA was isolated from all of the samples. All of the samples had clear 18S and 28S ribosomal RNA (rRNA) bands. The 28S rRNA bands are about twice as intense as the 18S rRNA bands and the RNA samples also had several smaller ribosomal RNAs from the chloroplast confirming the presence of high quality RNA.

cDNA was synthesized from total cellular RNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, California, USA) as recommended by the manufacturer. The ubiquitin primers were used to screen the cDNA for DNA contamination by PCR. The primers cover a 300-bp intron region at the 5' UTR of the ubiquitin 10 gene (At4g05320). The control genomic DNA sample showed the expected 850 bp product (Fig. 2.11 B, lane 4), while a smaller fragment (550 bp) was amplified from the intronless cDNA (Fig. 2.11 B, lanes 1 to 3).

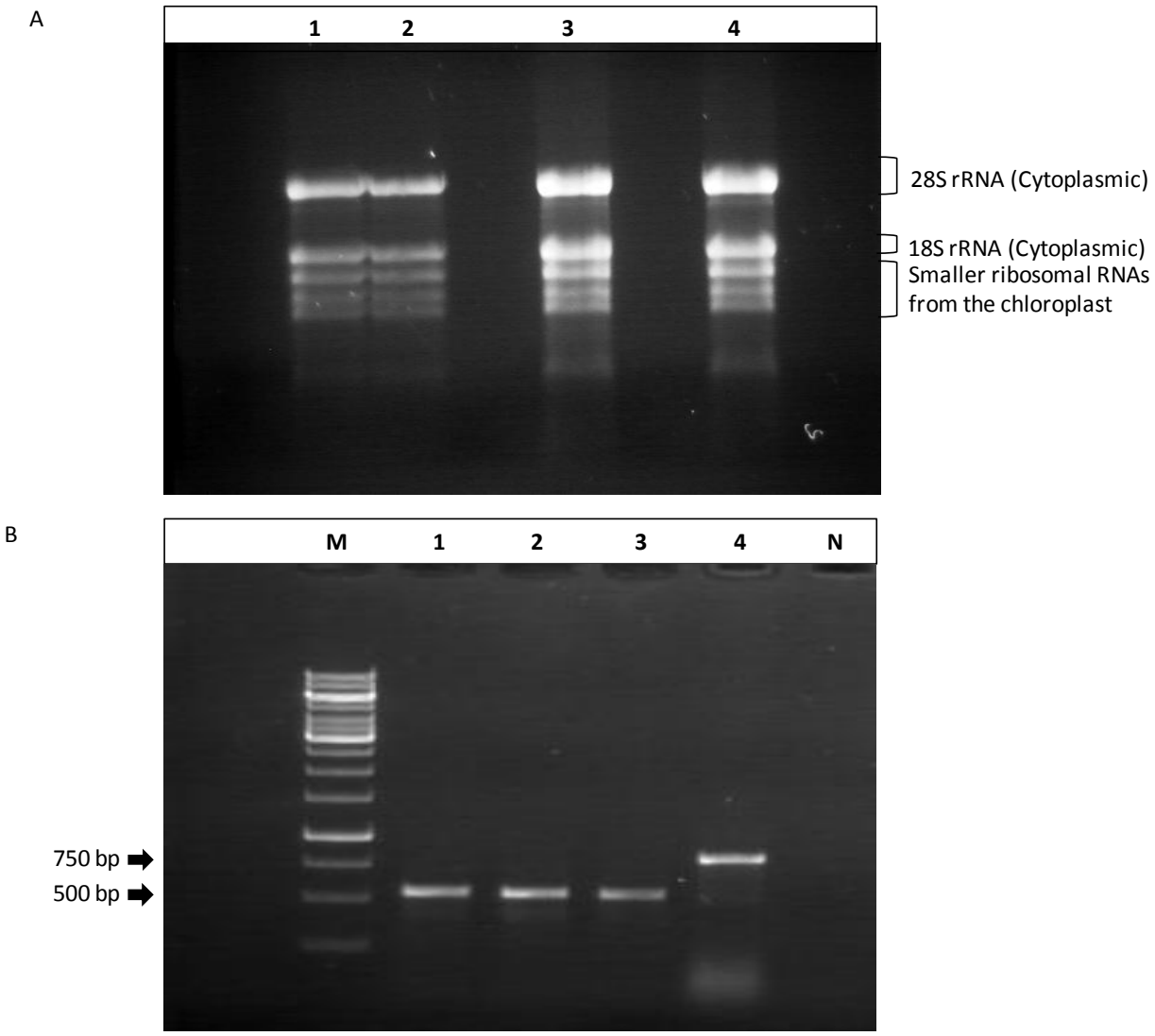


Figure 2.11. **RNA isolation from *A. thaliana* leaf tissue and PCR analysis of the synthesized cDNA with the ubiquitin primers.** A: RNA isolated from *A. thaliana* leaf tissue. Lanes 1 and 2: RNA from ecotype Nd-1; lanes 3 and 4: RNA from ecotypes Kil-0 and Be-0, respectively. RNA (500 ng) was evaluated on a 1% (w/v) denaturing agarose gel. B: Ubiquitin PCR on synthesized cDNA; M: 1kb molecular marker (Fermentas Inc., USA); Lanes 1 to 4: PCR products of reactions containing the following as template: lanes 1-3: cDNA from ecotypes Nd-1, Kil-0 and Be-0, respectively; lane 4: *A. thaliana* control genomic DNA; lane N: negative water control. PCR products were analyzed by electrophoresis through a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8) containing 0.5µg/ml ethidium bromide.

PCR amplification and cloning of the full length *RRS1* gene from Kil-0 and Be-0 cDNA

The full-length *RRS1* gene in Kil-0 and Be-0 was sequenced from synthesized cDNA. Four primer pairs were designed from the full length *RRS1* cDNA sequence of the *A. thaliana* ecotype Nd-1 (Appendix A). Figure 2.12 illustrates the primers that were used to generate the four overlapping fragments (C1, C2, C3 AND C3M) from Kil-0 and Be-0 cDNA.

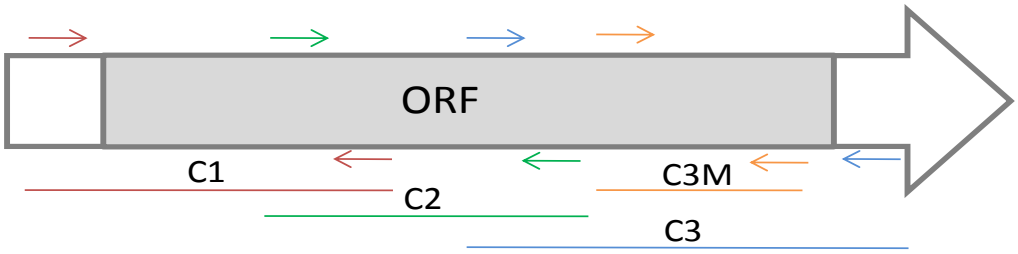


Figure 2.12. **Schematic illustration of the *RRS1-R* gene in Nd-1.** Primer pairs (coloured arrows) and their corresponding fragments (coloured lines) are indicated (red: RRS1-C1 primers and C1 fragment; green: RRS1-C2 primers and C2 fragment; blue: RRS1-C3 primers and C3 fragment and orange: RRS1-C3M primers and C3M fragment). Arrow head indicates the direction of transcription. Grey region indicates the coding region or open reading frame (ORF) and white regions are untranslated regions.

Two independent PCR amplicons for each fragment from each ecotype were cloned. The insert sizes were verified with colony PCR using the universal M13F and M13R primers before isolating plasmid DNA (data not shown). Two independent clones for each fragment from each ecotype were selected for sequencing. The clones obtained for Be-0 were BC1-2, BC1-3, BC3-1, BC3-3, BC2-1, BC2-3, BC3M-1 and BC3M-2 and the clones selected for Kil-0 were KC1-2, KC1-3, KC3-2, KC3-3, KC2-1, KC2-3, KC3M-1, and KC3M-2. The plasmid DNA was isolated from the colonies and the inserts were screened with the gene-specific primers before sequencing (Appendix A). The inserts for the C1 and C3 clones were screened with the RRS1-C1 and RRS1-C3 primers, respectively. Amplification yielded a 1552 bp product for the C1 clones (clones BC1-2, KC1-2, KC1-3, Fig. 2.13 A, lanes 1 to 3, respectively) whereas a 1698 bp product was obtained

for the C3 clones (clones BC3-1, BC3-3, KC3-2, KC3-3, Fig. 2.13 A, lanes 4 to 7, respectively). The BC1-3 clone was also screened with the gene-specific primers before sequencing (data not shown). The RRS1-C2 and RRS1-C3M primers were used to amplify the inserts of the C2 and C3M clones, respectively. PCR amplification yielded a 1152 bp product for the C3M clones (clones KC3M-1, KC3M-2, BC3M-1 and BC3M-2, Fig. 2.13 B lanes 1 to 4, respectively) and a 1396 bp product was obtained for the C2 clones (clones KC2-1, KC2-3, KC2-5, BC2-1, BC2-3 and BC2-5, Fig. 2.13 B lanes 5 to 10, respectively). Faint bands were observed for the PCR products of KC3M-2 and the BC3M-2 clones (Fig. 2.13 B, lanes 2 and 4, respectively) and PCR was repeated on the two samples (data not shown). The extra C2 clones (KC2-5 AND BC2-5) were not sequenced. The negative controls yielded no amplification products, as expected (Fig. 2.13 A and B, lanes N).

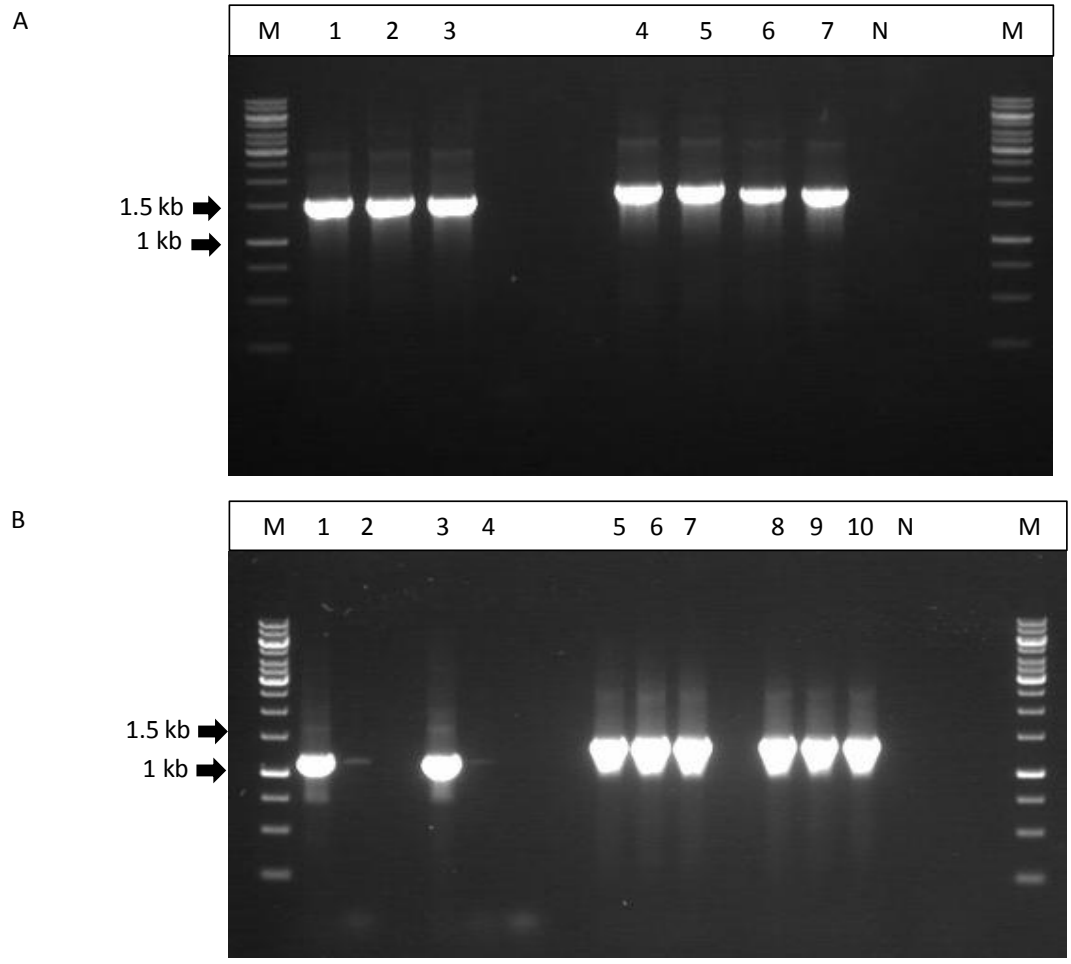


Figure 2.13. **PCR analysis of the *RRS1* cDNA clones with gene-specific primers.** A: PCR screening of the C1 and C3 clones with the RRS1-C1 and RRS1-C3 primers, respectively. M: 1kb molecular marker (Fermentas Inc., USA); lanes 1 to 7: PCR products of reactions containing the following as template: lanes 1 to 3 plasmid DNA from clones BC1-2, KC1-2 and KC1-3, respectively; lanes 4 to 7: plasmid DNA from clones BC3-1, BC3-3, KC3-2 and KC3-3, respectively; lane N: negative water control. B: PCR screening of the C2 and C3M clones with the RRS1-C2 and RRS1-C3M primers, respectively. M: 1kb molecular marker (Fermentas Inc., USA); lanes 1 to 10: PCR products of reactions containing the following as template: lanes 1 to 4: plasmid DNA from clones KC3M-1, KC3M-2, BC3M-1 and BC3M-2, respectively; lanes 5 -10: plasmid DNA from clones KC2-1, KC2-3, KC2-5, BC2-1, BC2-3 and BC2-5, respectively; lane N: negative water control. PCR products were analyzed by electrophoresis through a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8) containing 0.5µg/ml ethidium bromide.

Sequencing and analysis of the predicted protein sequences of the *RRS1* gene from ecotypes Kil-0 and Be-0

The *RRS1* coding sequences were obtained by sequencing two independent clones for each fragment from each ecotype using the universal M13F and M13R primers. The RRS1 protein in Kil-0 and Be-0 was sequenced and compared to the RRS1 protein sequences in Nd-1 and Col-0 to determine if the ecotypes contain different alleles of the same gene (Fig. 2.14). The amino acid sequences of the RRS1 proteins were analyzed and the NH_2 termini contained domains general to the Toll-IL-1 receptor–nucleotide binding site–leucine-rich repeat (TIR-NBS-LRR) class of R proteins. The C-terminal regions contained a nuclear localization signal (NLS) (not shown) and a WRKY domain.

The RRS1 proteins from the different ecotypes vary in their length. The Col-0 (RRS1-S) protein is 90 amino acids shorter, while the RRS1 protein from Be-0 is 23 amino acids shorter than RRS1-R from Nd-1. RRS1 protein from Kil-0 was 5 amino acids shorter than RRS1-R, but the protein had the highest level of identity (98.9%) to RRS1-R (Table 2.3, grey blocks). The deletions at the C-terminal end did not eliminate the WRKY motifs. Amino acid comparisons of the RRS1 proteins showed that Kil-0 contains an allelic variant with the highest level of identity to the RRS1-R protein in ecotype Nd-1 known to confer resistance against isolate GMI1000.

Table 2.3. **Percentage similarities and identities between RRS1 protein sequences.** The grey blocks represent percentage identities between the RRS1 sequences, whereas the white blocks represent percentage similarities between the RRS1 sequences.

Ecotypes	**Kil-0**	**Be-0**	**Col-0**	**Nd-1**
Kil-0	x	97.3	91.9	98.9
Be-0	97.6	x	93.3	97.3
Col-0	92.4	93.7	x	91.8
Nd-1	99.1	97.5	92.1	x

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```
       1                                                                                                  100
Kil0   MTNCEKDEEFVCISCVEEVRYSFVSHLSEALRRKGINNVVVGVDSDDLLFKESQAKIEKAGVSVMVLPGNCDPSDVWLDKFAKVLECQRNNKDQAVVSVL
Nd-1   MTNCEKDEEFVCISCVEEVRYSFVSHLSEALRRKGINNVVVDVDIDDLLFKESQAKIEKAGVSVMVLPGNCDPSEVWLDKFAKVLECQRNNKDQAVVSVL
Be-0   MTNCEKDEEFVCISCVEEVRYSFVSHLSEALRRKGINNVVVDVDIDDLLFKESQAKIEKAGVSVMVLPGNCDPSEVWLDKFAKVLECQRNNKDQAVVSVL
Col-0  MTNCEKDEEFVCISCVEEVRYSFVSHLSEALRRKGINNVVVDVDIDDLLFKESQAKIEKAGVSVMVLPGNCDPSEVWLDKFAKVLECQRNNKDQAVVSVL
       101                                                                                                200
Kil0   YGDSLLRDQWLSELDFRGLSRIHQSRKECSDSILVEEIVRDVYETHFYVGRIGIYSKLLEIENMVNKQPIGIRCVGIWGMPGVGKTTLAKAVFDQMSSAF
Nd-1   YGDSLLRDQWLSELDFRGLSRIHQSRKECSDSILVEEIVRDVYETHFYVGRIGIYSKLLEIENMVNKQPIGIRCVGIWGMPGIGKTTLAKAVFDQMSSAF
Be-0   YGDSLLRDQWLSELDFRGLSRIHQSRKECSDSILVEEIVRDVYETHFYVGRIGIYSKLLEIENMVNKQPIGIRCVGIWGMPGIGKTTLAKAVFDQMSSAF
Col-0  YGDSLLRDQWLSELDFRGLSRIHQSRKECSDSILVEEIVRDVYETHFYVGRIGIYSKLLEIENMVNKQPIGIRCVGIWGMPGIGKTTLAKAVFDQMSSAF
       201                                                                                                300
Kil0   DASCFIEDYDKSIHEKGLYCLLEEQLLPGNDATIMKLNSLRDRLNSKRVLVVLDDVCNALVAESFLEGFDWLGPGSLIIITSRDKQVFRLCGINQIYEVQ
Nd-1   DASCFIEDYDKSIHEKGLYCLLEEQLLPGNDATIMKLSSLRDRLNSKRVLVVLDDVCNALVAESFLEGFDWLGPGSLIIITSRDKQVFRLCGINQIYEVQ
Be-0   DASCFIEDYDKSIHEKGLYCLLEEQLLPGNDATIMKLSSLRDRLNSKRVLVVLDDVCNALVAESFLEGFDWLGPGSLIIITSRDKQVFRLCGINQIYEVQ
Col-0  DASCFIEDYDKSIHEKGLYCLLEEQLLPGNDATIMKLSSLRDRLNSKRVLVVLDDVRNALVGESFLEGFDWLGPGSLIIITSRDKQVFCLCGINQIYEVQ
       301                                                                                                400
Kil0   GLNEKEARQLFLLSASIKEDMGEQNLHELSVRVISYANGNPLAISVYGRELKGKKKLSEMETAFLKLKRRPPFKIVDAFKSSYDTLSDNEKNIFLDIACF
Nd-1   GLNEKEARQLFLLSASIMEDMGEQNLHELSVRVISYANGNPLAISVYGRELKGKKKLSEMETAFLKLKRRPPFKIVDAFKSSYDTLSDNEKNIFLDIACF
Be-0   GLNEKEARQLFLLSASIKEDMGEQNLHELSVRVISYANGNPLAISVYGRELKGKKKLSEMETAFLKLKRRPPFKIVDAFKSSYDTLSDNEKNIFLDIACF
Col-0  GLNEKEARQLFLLSASIKEDMGEQNLQELSVRVINYANGNPLAISVYGRELKGKKKLSEMETAFLKLKRRPPFKIVDAFKSTYDTLSDNEKNIFLDIACF
       401                                                                                                500
Kil0   FQGENVNYVIQLLEGCGFFPHVEIDVLVDKCLVTISENRVWLHKLTQDIGREIINGETVQIERRRRLWEPWSIKYLLEYNEHKANGEPKTTFKRAQGSEE
Nd-1   FQGENVNYVIQLLEGCGFFPHVEIDVLVDKCLVTISENRVWLHKLTQDIGREIINGETVQIERRRRLWEPWSIKYLLEYNEHKANGEPKTTFKRAQGSEE
Be-0   FQGENVNYVIQLLEGCGFFPHVEIDVLVDKCLVTISENRVWLHKLTQDIGREIINGETVQIERRRRLWEPWSIKYLLEYNEHKANGEPKTTFKRAQGSEE
Col-0  FQGENVNYVIQLLEGCGFFPHVEIDVLVDKCLVTISENRVWLHKLTQDIGREIINGETVQIERRRRLWEPWSIKYLLEYNEHKANGEPKTTFKRAQGSEE
       501                                                                                                600
Kil0   IEGLFLDTSNLRFDLQPSAFKNMLNLRLLKIYCSNPEVHPVINFPTGSLHSLPNELRLLHWENYPLKSLPQNFDPRHLVEINMPYSQLQKLWGGTKNLEM
Nd-1   IEGLFLDTSNLRFDLQPSAFKNMLNLRLLKIYCSNPEVHPVINFPTGSLHSLPNELRLLHWENYPLKSLPQNFDPRHLVEINMPYSQLQKLWGGTKNLEM
Be-0   IEGLFLDTSNLRFDLQPSAFKNMLNLRLLKIYCSNPEVHPVINFPTGSLHSLPNELRLLHWENYPLKSLPQNFDPRHLVEINMPYSQLQKLWGGTKNLEM
Col-0  IEGLFLDTSNLRFDLQPSAFKNMLNLRLLKIYCSNPEVHPVINFPTGSLHSLPNELRLLHWENYPLKSLPQNFDPRHLVEINMPYSQLQKLWGGTKNLEM
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       601                                                                 700
Kil0   LRTIRLCHSHHLVDIDDLLKAENLEVIDLQGCTRLQNFPAAGRLLRLRVVNLSGCIKIKSVLEIPPNIEKLHLQGTGILALPVSTVKPNHRELVNFLTEI
Nd-1   LRTIRLCHSQHLVDIDDLLKAENLEVIDLQGCTRLQNFPAAGRLLRLRVVNLSGCIKIKSVLEIPPNIEKLHLQGTGILALPVSTVKPNHRELVNFLTEI
Be-0   LRTIRLCHSQHLVDIDDLLKAENLEVIDLQGCTRLQNFPAAGRLLRLRVVNLSGCIKIKSVLEIPPNIEKLHLQGTGILALPVSTVKPNHRELVNFLTEI
Col-0  LRTIRLCHSHHLVDIDDLLKAENLEVIDLQGCTRLQNFPAAGRLLRLRVVNLSGCIKIKSVLEIPPNIEKLHLQGTGILALPVSTVKPNHRELVNFLTEI
       701                                                                 800
Kil0   PGLSEASKLERLTSLLESNSSCQDLGKLICLELKDCSCLQSLPNMANLDLNVLDLSGCSSLNSIQGFPRFLKQLYLGGTAIREVPQLPQSLEILNAHGSC
Nd-1   PGLSEASKLERLTSLLESNSSCQDLGKLICLELKDCSCLQSLPNMANLDLNVLDLSGCSSLNSIQGFPRFLKQLYLGGTAIREVPQLPQSLEILNAHGSC
Be-0   PGLSEASKLERLTSLLESNSSCQDLGKLICLELKDCSCLQSLPNMANLDLNVLDLSGCSSLNSIQGFPRFLKQLYLGGTAIREVPQLPQSLEILNAHGSC
Col-0  PGLSE---LERLTSLLESNSSCQDLGKLICLELKDCSCLQSLPNMANLDLNVLDLSGCSSLNSIQGFPRFLKQLYLGGTAIREVPQLPQSLEILNAHGSC
       801                                                                 900
Kil0   LRSLPNMANLEFLKVLDLSGCSELETIQGFPRNLKELYFAGTTLREVPQLPLSLEVLNAHGSDSEKLPMHYKFNNFFDLSQQVVNDFFLKALTYVKHIPR
Nd-1   LRSLPNMANLEFLKVLDLSGCSELETIQGFPRNLKELYFAGTTLREVPQLPLSLEVLNAHGSDSEKLPMHYKFNNFFDLSQQVVNDFFLKALTYVKHIPR
Be-0   LRSLPNMANLEFLKVLDLSGCSELETIQGFPRNLKELYFAGTTLREVPQLPLSLEVLNAHGSDSEKLPMHYKFNNFFDLSQQVVNDFFLKALTYVKHIPR
Col-0  LRSLPNMANLEFLKVLDLSGCSELETIQGFPRNLKELYFAGTTLREVPQLPLSLEVLNAHGSDSEKLPMHYKFNNFFDLSQQVVNDFLLKTLTYVKHIPR
       901                                                                1000
Kil0   GYTQELINKAPTFSFSAPSHTNQNATFDLQPGSSVMTRLNHSWRNTLVGFGMLVEVAFPEDYCDATDVGISCVCRWSNKEGRSCRIERNFHCWAPGKVVP
Nd-1   GYTQELINKAPTFSFSAPSHTNQNATFDLQPGSSVMTRLNHSWRNTLVGFGMLVEVAFPEDYCDATDVGISCVCRWSNKEGRSCRIERNFHCWAPGKVVP
Be-0   VYTQELINKAPTFSFSAPSHTNQNATFDLQPGSSVMTRLNHSWRNTLVGFGMLVEVAFPEDYCDATDVGISCVCRWSNKEGRSCRIERNFHCWAPGKVVP
Col-0  GYTQELINKAPTFSFSAPSHTNQNATFDLQSGSSVMTRLNHSWRNTLVGFGMLVEVAFPEDYCDATDVGISCVCRWSNKEGRSCRIERKFHCWAPWQVVP
       1001                                                               1100
Kil0   KVRKDHTFVFSDVNMRPSTGEGNDPDIWAGLVVFEFFPINQQTKCLNDRFTVTRCGVRVINVATGNTSLENISLVLSLDPVEVSGYEVLRVSYDDLQEMD
Nd-1   KVRKDHTFVFSDVNMRPSTGEGNDPDIWAGLVVFEFFPINQQTKCLNDRFTVTRCGVRVINVATGNTSLENISLVLSLDPVEVSGYEVLRVSYDDLQEMD
Be-0   KVRKDHTFVFSDVNMRPSTGEGNDPDIWAGLVVFEFFPINQQTKCLNDRFTVTRCGVRVINVATGNTSLENISLVLSLDPVEVSGYEVLRVSYDDLQEMD
Col-0  KVRKDHTFVFSDVNMRPSTGEGNDPDIWAGLVVFEFFPINQQTKCLNDRFTVRRCGVRVINVATGNTSLENIALVLSLDPVEVSGYEVLRVSYDDLQEMD
       1101                                                               1200
Kil0   KVLFLYIASLFNDEDVDFVAPLIAGIDLDVSSGLKVLADVSLISVSSNGEIVMHSLQRQMGKEILHGQSMLLSDCESSMTENLSDVPKKEKKHRESKVKK
Nd-1   KVLFLYIASLFNDEDVDFVAPLIAGIDLDVSSGLKVLADVSLISVSSNGEIVMHSLQRQMGKEILHGQSMLLSDCESSMTENLSDVPKKEKKHRESKVKK
Be-0   KVLFLYIASLFNDEDVDFVAPLIAGIDLDVSSGLKVLADVSLISVSSNGEIVMHSLQRQMGKEILHGQSMLLSDCESSMTENLSDVPKKEKKHRESKVKK
Col-0  KVLFLYIASLFNDEDVDFVAPLIAGIDLDVSSGLKVLADVSLISVSSNGEIVMHSLQRQMGKEILHGQSMLLSDCESSMTENLSDVPKKKKKHSESRVKK
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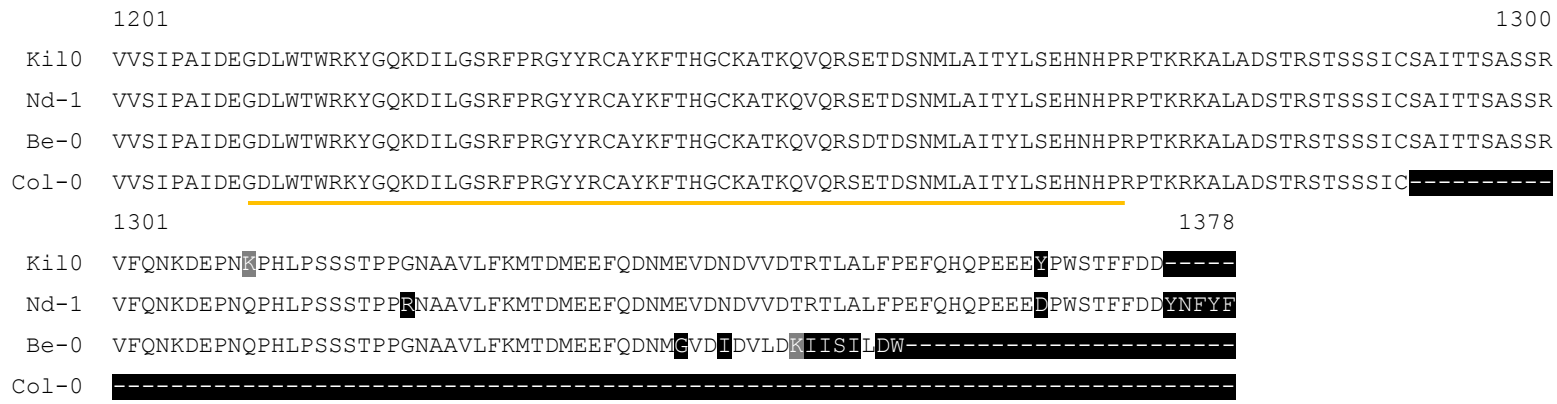


Figure 2.14. **Sequence comparison of the RRS1 proteins in Kil-0 and Be-0 with the previously characterized RRS1 proteins in Nd-1 and Col-0.** Identical and conserved amino acids are black letters on white background. Weakly similar amino acids are white letters on grey background and non-similar amino acids are white letters on black background. The underlined regions indicate the different conserved domains (red: TIR domain, blue: NBS domain, green: LRR domain and yellow: WRKY domain).

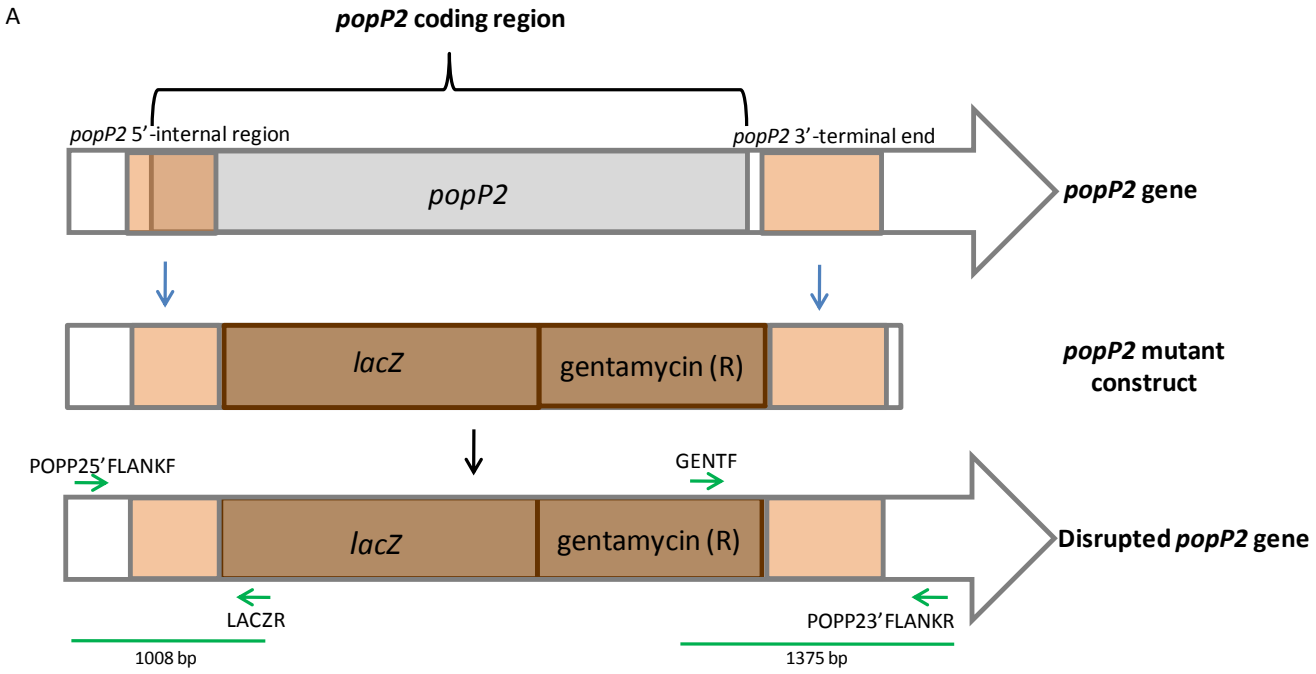
2.4.4 PopP2 is the avirulence determinant in *R. solanacearum* isolate BCCF 402 recognized by RRS1 in Kil-0 to initiate a defense response

Genetic analysis of crosses between Kil-0 and Be-0 revealed that resistance to isolate BCCF 402 is due to a single recessive locus. This locus is likely to be the *RRS1* gene, since resistance co-segregates with the *RRS1* allele from Kil-0. Previous work has shown that the *RRS1-R* gene in ecotype Nd-1 confers resistance against the *R. solanacearum* isolate GMI1000 by recognizing the PopP2 effector in GMI1000 (Deslandes *et al.,* 2003). To determine whether *RRS1* in Kil-0 is the locus conferring resistance against isolate BCCF 402, the resistant Kil-0 plants were challenged with a BCCF 402 *popP2* mutant strain to determine if PopP2 is the avirulence protein recognized by RRS1 in Kil-0. The absence of the PopP2 avirulence protein will abolish resistance in Kil-0 if RRS1 is the locus conferring resistance in Kil-0 by interacting with the PopP2 effector from isolate BCCF 402. Kil-0 plants were also challenged with the BCCF 402 *popP2* mutant strain complemented with a functional *popP2* gene to confirm that the result observed on Kil-0 plants after inoculation with the BCCF 402 *popP2* mutant strain was due to a disrupted *popP2* locus.

2.4.4.1 Genomic DNA isolation and PCR screening of BCCF 402 *popP2* mutant strains

Two BCCF 402 *popP2* mutant strains (GRS478 and GRS479; four clones of each strain) were produced by introducing the pCZ367 integrative plasmid containing a disrupted *popP2* gene (Appendix C) into the BCCF 402 strain via natural transformation (Stéphane Genin, INRA-CNRS, Castanet Tolosan, Cedex, France). Genomic DNA was isolated from each of the eight clones and the deletion of the *popP2* locus was confirmed by screening the BCCF 402 *popP2* mutant strains with PCR. Gene replacement was confirmed by screening the mutant strains and wild type strains with two primer pairs (Appendix A and Fig. 2.15 A). The POPP25'FLANKF and LACZR primers amplified a 1008 bp product, while a 1375 bp product was obtained with the POPP23'FLANKR and the GENTF primers. The results for one of the clones (GRS478 (6)) from one of the mutant strains are shown. The GRS478(6) mutant strain contained the 1375 bp as well as the 1008 bp product (Fig. 2.15 B and C, lanes 1 to 3 and lanes 10 to 11, respectively). Both PCR products were also obtained for the GRS100 mutant strain (GMI1000 *popP2* mutant

strain) that was included as a positive control (Fig. 2.15 B and C, lanes 4 to 6 and 12 to 13). No amplification products were detected for the untransformed BCCF 402 strain, as expected (Fig. 2.15 B and C, lanes 7, 8, 14 and 15). The *popP2* mutant construct (Fig. 2.15 B and C, lanes 9 and 16) and negative water control (Fig. 2.15 B and C, lane N) also yielded no amplification products. PCR analysis confirmed that the GRS478(6) clone contained an inactivated version of the *popP2* gene.



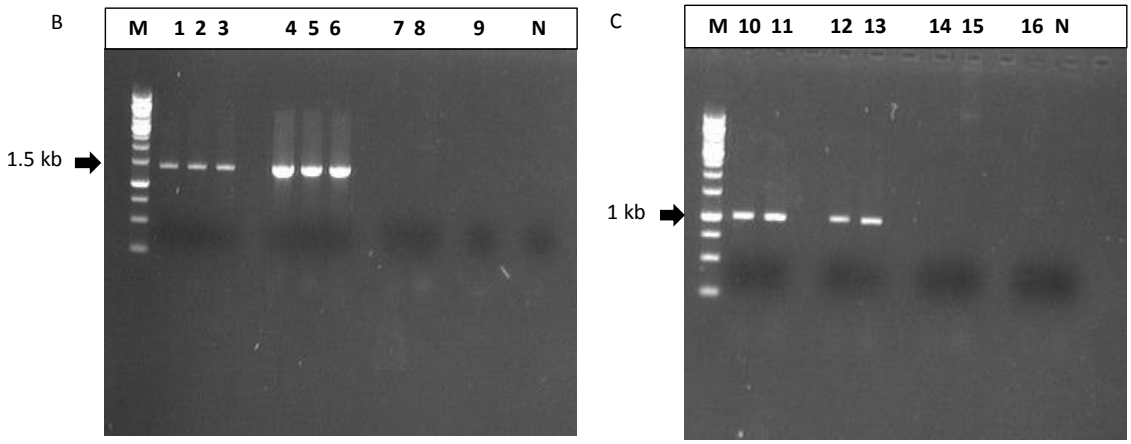


Figure 2.15. **Schematic illustration of the disruption of the *popP2* gene through homologous recombination and PCR screening of the BCCF 402 *popP2* mutant strains.** A: Disruption of the *popP2* gene by allelic replacement. Homologous recombination between the inactivated *popP2* gene in the *popP2* mutant construct and the target *popP2* gene in strain BCCF 402. The grey region indicates the coding region of *popP2* and the orange regions and blue arrows indicates the sites where homologous recombination occurs (5'-internal region and 3'-terminal end). The arrow heads indicate the direction of transcription. The 5'-internal part and 3'-terminal end regions flank the promoterless *lacZ* gene and a gentamycin resistance cassette in the *popP2* mutant construct. Gene replacement was confirmed by using two primer pairs (POPP25'FLANKF and LACZR as well as the POPP23'FLANKR and the GENTF primers). The green arrows and green lines indicate the primer pairs and their corresponding fragments (the sizes of the fragments are indicated below the green lines). B: PCR screening of the mutant and BCCF 402 untransformed strains with the POPP23'FLANKR and the GENTF primers. C: PCR screening of the mutant and BCCF 402 untransformed strains with the POPP25'FLANKF and LACZR primers. M: 1kb molecular marker (Fermentas Inc., USA); Lanes 1 to 16: Amplification products of reactions containing the following as template: lanes 1 to 3 and 10 to 11: genomic DNA from strain GRS478(6); lanes 4 to 6 and 12 to 13: genomic DNA from strain GRS100; lanes 7 to 8 and 14 to 15: genomic DNA from the untransformed BCCF 402 strain; lanes 9 and 16: plasmid DNA from the *popP2* mutant construct; lane N: negative water control. PCR products were analyzed by electrophoresis through a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8) containing 0.5µg/ml ethidium bromide.

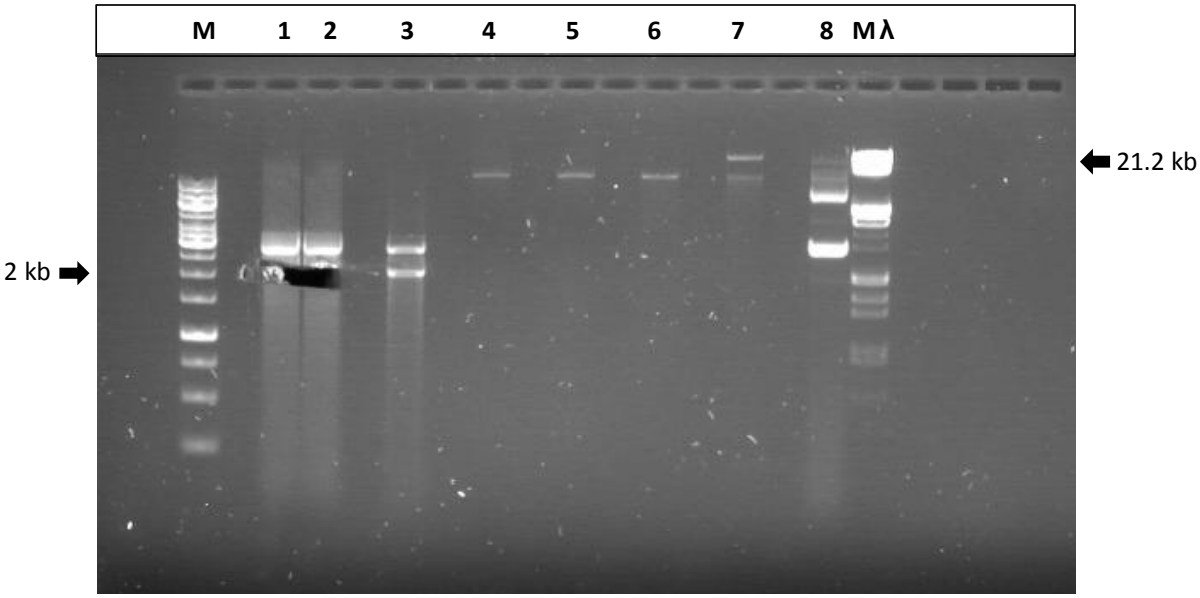
2.4.4.2 Complementation of the BCCF 402 *popP2* mutant strain with the wild-type *popP2* gene.

Preparation of the BCCF 402 *popP2* gene and its promoter for subcloning into the broad-host-range vector pLAFR6

Genomic DNA was isolated from the BCCF 402 wild-type strain using the 2% CTAB method and the full-length *popP2* gene and its promoter region was amplified from the DNA with the POPP2FLF and the POPP2FLR primers (Appendix A). The primers were designed from the genomic DNA sequence of the *R. solanacearum* isolate GMI1000 (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi). Two independent PCR products were generated for the *popP2* gene and cloned into the PTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas Inc., USA). Two independent clones were sequenced and one of the clones were chosen for further analysis. Sequence comparison of the PopP2 proteins in isolates GMI1000 and BCCF 402 showed that there are amino acid differences between the proteins (Appendix D).

The double digestion of the PTZ57R/T-*popP2* vector as well as the broad-host-range vector pLAFR6 are illustrated in Figure 2.16 A. The isolation of the full length *popP2* gene and its promoter after HindIII/XbaI digestion of the PTZ57R/T-*popP2* vector yielded a fragment of ~2kb, as expected (Fig. 2.16 A, lanes 1 to 3). The broad-host-range pLAFR6 cosmid was also double digested with the same enzymes and yielded a linearized fragment of ~21kb, as expected (Fig. 2.16 A, lane 6). The pLAFR6 cosmid was also digested separately with HindIII and XbaI and a linearized fragment of ~ 21kb was obtained for both enzymes (Fig. 2.16 A, lanes 4 and 5, respectively). This result confirmed that both enzymes digested the cosmid. Undigested pLAFR6 cosmid and PTZ57R/T-*popP2* vector was also included in the gel to compare against the digested fragments (Fig. 2.16 A, lanes 7 and 8, respectively). After digestion, the full length *popP2* gene and its promoter was recovered from the gel and subcloned into the linearized pLAFR6 cosmid. The pLAFR6::*popP2D1* construct (Fig. 2.16 B) was transformed into competent *E. coli* JM109 cells as described before. The recombinant colonies were screened with PCR for the correct fragments using the popP2FLF and POPP2FLR primers as described previously.

A



B

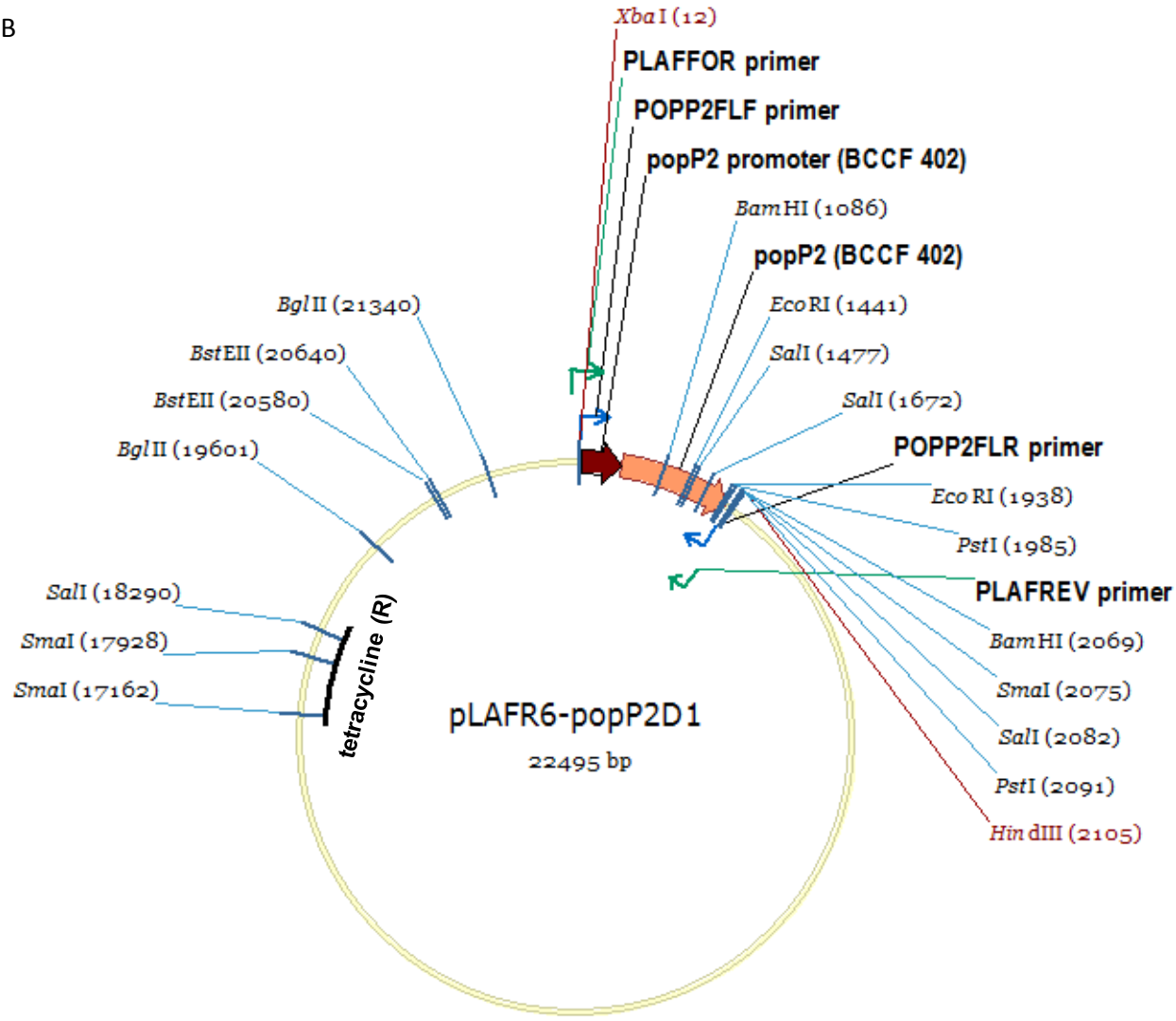


Figure 2.16. **HindIII/XbaI double digestion of the PTZ57R/T-*popP2* vector and pLAFR6 cosmid as well as a graphical representation of the pLAFR6::*popP2D1* construct.** A: HindIII/XbaI double digestion of the PTZ57R/T-*popP2* vector and pLAFR6 cosmid. M: 1kb molecular marker (Fermentas Inc., USA); Mλ: λ EcoRI/HindIII DNA ladder; lanes 1 to 3: PTZ57R/T-*popP2* vector digested with HindIII and XbaI; lane 4: pLAFR6 digested with HindIII; lane 5: pLAFR6 digested with XbaI; lane 6: pLAFR6 digested with HindIII and XbaI; lane 7: Undigested pLAFR6; lane 8: Undigested PTZ57R/T-*popP2* vector. Digested fragments were separated on a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8) containing 0.5µg/ml ethidium bromide. B: Plasmid map of pLAFR6::*popP2D1*. The primer binding sites, selected restriction enzyme recognition sites as well as the tetracycline resistance locus are indicated.

Transformation of the pLAFR6::*popP2D1* construct into the mutant strain GRS478(6) and screening of the recombinant colonies with pLAFR6 specific primers

The pLAFR6::*popP2D1* construct (Fig. 2.16 B) was isolated from *E. coli* JM109 cells using the Invisorb® Spin Plasmid Mini Kit Two (Invitek, Berlin, Germany). The pLAFR6::*popP2D1* construct was transformed into the mutant strain GRS478(6) via electroporation and recombinant colonies were screened with the pLAFR6 specific primers (Appendix A). PCR was performed on thirty GRS478(6) colonies and all of the colonies yielded a 2.3 kb amplified product, as expected (Fig. 2.17, lanes 1 to 30). All of the colonies contained the pLAFR6::*popP2D1* construct and one of the recombinant colonies were chosen for the inoculation study. GRS478(6) transformed with the empty pLAFR6 cosmid and 10 ng of pLAFR6 cosmid were also included as positive controls. PCR amplification yielded a product of 249 bp for both controls, as expected (Fig. 2.17, lane 31 and 32). No PCR product was detected for the negative water control (Fig. 2.17, lane N).

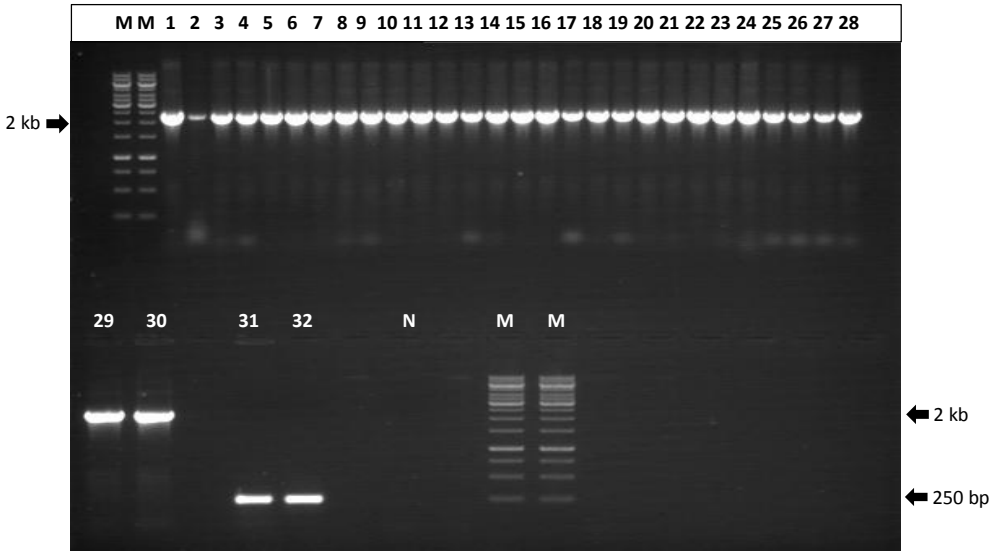
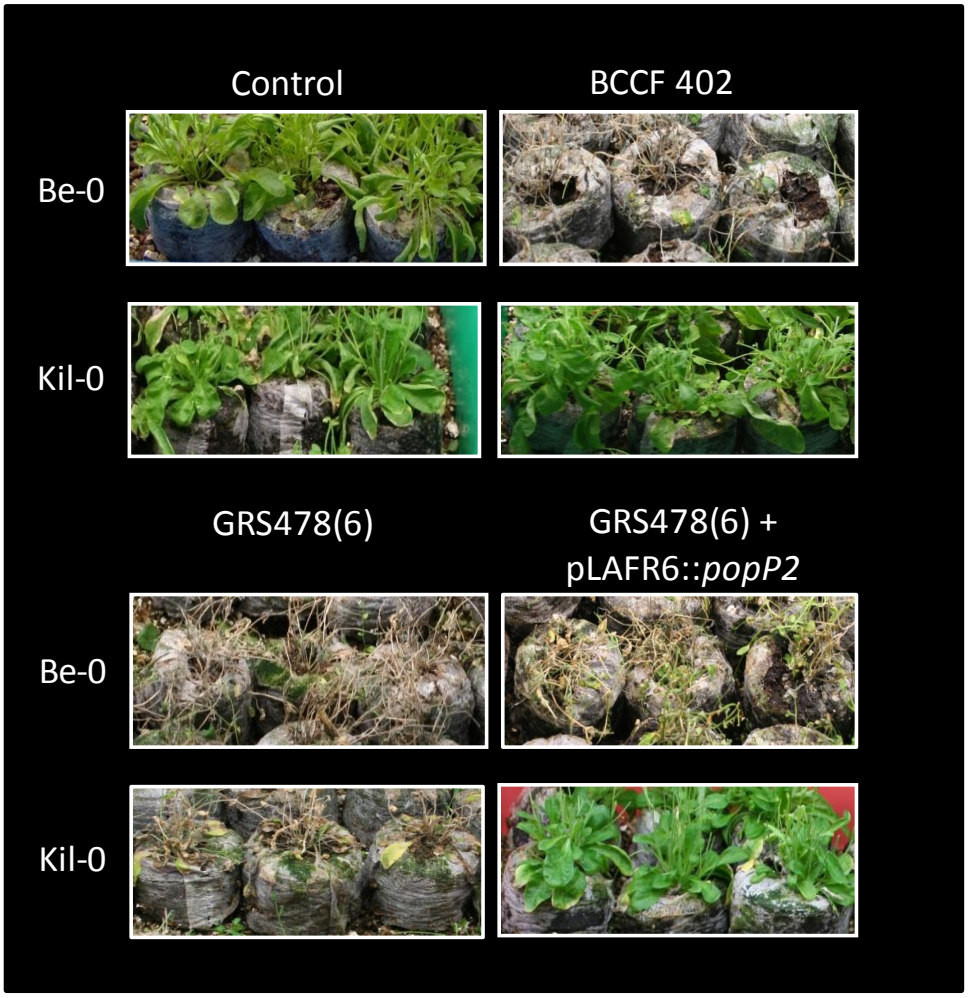


Figure 2.17. **PCR screening of GRS478(6) colonies transformed with the pLAFR6::*popP2D1* construct.** M: 1kb molecular marker (Fermentas Inc., USA); lanes 1 to 30: GRS478(6) colonies transformed with the pLAFR6::*popP2D1* construct; lane 31: GRS478(6) colony transformed with the empty pLAFR6 cosmid; lane 32: pLAFR6 cosmid; lane N: negative water control. Amplified products were analyzed by electrophoresis through a 1% (w/v) agarose gel in 1 X TAE buffer (pH 8) containing 0.5µg/ml ethidium bromide.

2.4.4.3 Challenging Kil-0 and Be-0 plants with the wild-type BCCF 402, the GRS478(6) (BCCF 402 *popP2* mutant) strains and the GRS478(6) strain complemented with a functional *popP2* gene.

The resistant *A. thaliana* ecotype Kil-0 was challenged with a *popP2* mutant strain to determine whether the *popP2* gene in isolate BCCF 402 behaves like a classical avirulence gene which triggers a defense response in Kil-0 harboring the matching resistance gene (*RRS1*). Kil-0 plants inoculated with the wild-type strain BCCF 402 remained healthy 19 dpi (Fig. 2.18 A and B). The *popP2* mutant (GRS478(6)) strain caused severe symptoms and killed Kil-0 19 dpi. Avirulence was restored when Kil-0 was inoculated with the GRS478(6) strain complemented with the pLAFR6 cosmid harboring a functional *popP2* gene (pLAFR6::*popP2D1* construct) (Fig. 2.18 A and B). Be-0 on the other hand, remained susceptible when challenged with each of the three different strains (Fig. 2.18 A and B). Uninoculated control plants remained healthy throughout the trial (Fig. 2.18 A). These results illustrate that PopP2 is the avirulence protein in isolate BCCF 402 recognized by RRS1 in Kil-0 to initiate a defense response.

A



B

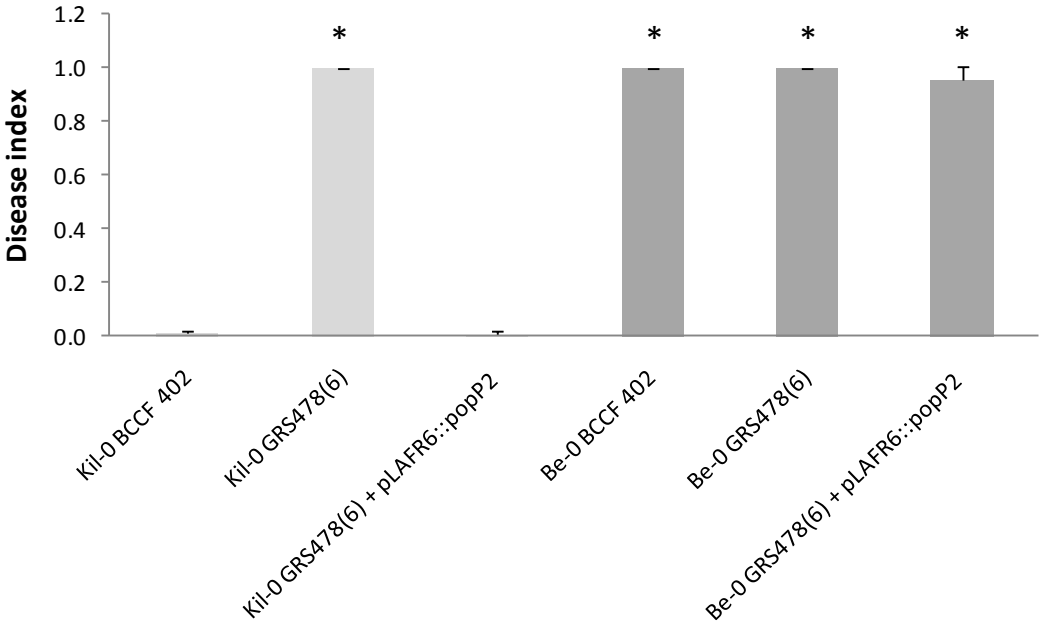


Figure 2.18. **Inoculation of Kil-0 and Be-0 with the BCCF 402, the GRS478(6) and the GRS478(6) + pLAFR6::*popP2* strains.** A: Phenotypic responses of Be-0 and Kil-0 to the BCCF 402, the GRS478(6) and the GRS478(6) + pLAFR6::*popP2* strains 19 dpi. B: Disease index of Kil-0 and Be-0 plants after inoculation with the BCCF 402, GRS478(6) and the GRS478(6) + pLAFR6::*popP2* strains 19 dpi. Inoculated plants were rated on a scale from zero (no disease) to 5 (completely dead plants). Means and standard deviations were calculated from disease index values of a total of 40 plants per ecotype (4 replicates of 10 plants each). Asterisks indicate statistically significant difference from resistant plants (plants that remained healthy after pathogen inoculation) (Wilcoxon Rank Sum Test, $P < 0.05$).

2.5 Discussion

A. thaliana was used in this study to analyze the mechanism of resistance against *R. solanacearum*. Previous work identified a pathosystem between a *Eucalyptus* isolate of *R. solanacearum* (BCCF 402) and the *A. thaliana* ecotypes Be-0 and Kil-0 (Weich, 2004). The susceptibe ecotype (Be-0) displayed severe wilt symptoms two weeks after root inoculation, while the resistant ecotype (Kil-0) remained healthy two weeks after inoculation. The overall aim of this study was to characterize the genetic basis of resistance in Kil-0 to the isolate BCCF 402 and the results obtained from the study are discussed.

2.5.1 Resistance in Kil-0 against isolate BCCF 402 is due to a single recessive gene.

One of the objectives of this study was to determine if resistance in Kil-0 against isolate BCCF 402 is polygenic or due to a single gene. Genetic crosses between Be-0 and Kil-0 and subsequent pathogen challenges revealed a 1:3 segregation of resistance: susceptibility in the F_2 progeny, suggesting that resistance was caused by a single recessive gene (Table 2.2). Resistance against *R. solanacearum* is a complex trait and single-gene resistance (Deslandes *et al.,* 1998; Ho and Yang, 1999) as well as polygenic resistance (Godiard *et al.,* 2003) in different hosts against bacterial wilt have been described. Plant disease resistance conferred by a single (*R*) gene is known as qualitative resistance, whereas resistance mediated by multiple genes or quantitative trait loci (QTLs) is referred to as quantitative disease resistance (QDR) (Poland *et al.,* 2009). Resistance provided by multiple genes is incomplete and each of the genes makes an additive contribution to the overall disease resistance in the plant. QDR is generally more durable than *R*-gene-mediated resistance, because pathogen variants that overcome QRLs (a locus with an effect on QDR) achieve only a minor advantage. *R*-genes are reasonably easy to manipulate and high levels of resistance are achieved by these genes. However, *R*-genes lack durability in some systems (especially against pathogens that have a high evolutionary potential) and are not available against certain diseases such as diseases caused by necrotrophic pathogens (Poland *et al.,* 2009). Methods have been proposed that use *R*-genes to confer durable resistance such as stacking of *R*-genes (Bent and Mackey, 2007). The goal of this

method is to have more than one *R*-gene in every individual plant against a pathogen. Growth of the pathogen is restricted even if one of the individuals of a pathogen is not detected by one of the *R*-genes. *R*-genes in combination with pesticides are also used to increase the durability of disease control (Bent and Mackey, 2007).

In bacterial, fungal and viral plant-pathogen interactions several recessively inherited *R*-genes have been reported such as the barley *mlo*, rice *xa5*, *A. thaliana edr1* and *eIF4E* from many crops (Hammond-Kosack and Kanyuka, 2007). The role in plant disease resistance of only a few of the recessive resistance genes have been characterized and the roles of *mlo* and *eIF4E* will be discussed briefly. Naturally occurring and chemically induced mutations in the barley and the *A. thaliana mlo* gene provides broad-spectrum resistance to several races of powdery mildew fungi (Hammond-Kosack and Kanyuka, 2007). The MLO proteins are located in the plasma membrane and contains seven transmembrane domains. The MLO protein operates as a suppressor of plant defense and or programmed cell death where it colocalizes and physically interacts with ROR2/PEN1. ROR2/PEN1 are syntaxins that function as mediators of the fusion of vesicles with the plasma membrane. It is proposed that MLO participates in vesicle-associated processes at the plant plasma membrane and the fungus exploit it for its own growth advantage (Hammond-Kosack and Kanyuka, 2007). MLO is characterized as a negative regulator of plant defense that is activated by pathogen effectors to suppress the defense response in the plant (Pavan *et al.,* 2010).

Most of the recessive resistances against viruses in the family *Potyviridae* are due to mutations in isoforms of the plant translation initiation factor 4E (eIF4E) (Pavan *et al.,* 2010). eIF4E is a component of the translation initiation complex and it interacts with a small viral protein VPg (viral protein genome linked). The exact role of eIF4E in the potyvirus infection cycle is unknown, but mutations in eIF4E could prevent interaction between VPg and the translation initiation complex (Pavan *et al.,* 2010). The lack of this interaction could result in the inability of the virus to complete its life cycle and effectively infect the plant. eIF4E is characterized as a susceptibility factor that is needed by the pathogen for its growth and reproduction.

The genes that are required for susceptibility against pathogens are known as susceptibility genes (*S* genes) and these genes can be categorized as negative defense regulators or susceptibility factors (plant factors that are needed by the pathogen for its growth and development) as discussed above (Pavan *et al.,* 2010). *S* genes can be genetically classified as dominant genes and their impairment would lead to recessive resistance. Thus absence of these genes would prevent pathogens to reproduce or suppress the defense responses in the plant. Resistance mediated by mutations in the barley *Mlo* and the pepper *eIF4E* genes are durable and are still used in the field after more than 30 and 50 years from their introduction in agriculture, respectively (Pavan *et al.,* 2010). Thus, the elimination of plant *S* genes provides an alternative breeding strategy for durable resistance against pathogens.

2.5.2 The recessive *RRS1* allele in Kil-0 is linked to resistance against isolate BCCF 402.

Genetic analysis of crosses between Kil-0 and Be-0 revealed that resistance to isolate BCCF 402 is due to a single recessive locus. Previous work by other authors showed that resistance to the tomato isolate GMI1000 of *R. solanacearum*, in a cross between *A. thaliana* ecotypes Col-5 and Nd-1, also segregated as a simply inherited recessive trait, and *RRS1-R* was identified as the major determinant of resistance against this isolate (Deslandes *et al*., 2002). The next objective of the study was to determine if the *RRS1* allele in Kil-0 is linked to resistance. BCCF 402 inoculated F_2 individuals from a cross between Kil-0 and Be-0 were screened with the *RRS1* CAPS marker. The resistant and susceptible phenotypes cosegregated with the CAPS marker and this indicates that *RRS1* and/or a closely linked gene could be controlling resistance or susceptibility to isolate BCCF 402 (Fig. 2.8. A and B). Many of the *R* genes are present in clusters and there may not be recombination in the cluster. An extensive cluster of disease resistance loci known as the multiple resistance complex J (*MRC-J*) was identified on chromosome 5 of *A. thaliana* (Narusaka *et al.,* 2009). The cluster contains well-characterized genes such as *RPS4*, *RRS1* and *RPP8* as well as unknown NB-LRR-protein encoding genes. Findings from the study indicated that both *RRS1* and *RPS4* function cooperatively in conferring resistance to *R. solanacearum* strain 1002 as the *rps4-21/rrs1-1* double mutant showed similar levels of

susceptibility to *R. solanacearum* as the single mutants (*rrs1-1*, *rrs1-2* and *rps4-21* plants) (Narusaka *et al.,* 2009). Future work would involve in determining whether *RPS4* is also required for resistance against isolate BCCF 402.

2.5.3 Kil-0 contains an allelic variant of *RRS1* with the highest level of identity to *RRS1-R* from ecotype Nd-1 compared to Be-0 and Col-0

The F_1 progeny from a cross between Kil-0 and Nd-1 was tested for their response to the *R. solanacearum* strains BCCF 402 and GMI1000 to determine if resistance to the strains is due to alleles of one gene (*RRS1*) or of different genes. The results from the study indicated that the resistance gene in Kil-0 is allelic to *RRS1* and resistance in Kil-0 to isolate BCCF 402 is not due to a different gene (Fig. 2.10 A and B).

The Nd-1 accession displayed a higher level of wilt resistance than the Kil-0 accession. Nd-1 was resistant to both strains (GMI1000 and BCCF 402), while Kil-0 showed intermediate symptoms to isolate GMI1000 and remained healthy to isolate BCCF 402 (Fig 2.10 A and B). There are three possible explanations for the difference observed in resistance between the two ecotypes. Kil-0 may contain weaker alleles of other proteins downstream of the signaling pathway that are targets of effectors in GMI1000. Isolate GMI1000 could have acquired these effectors that are absent from isolate BCCF 402 or BCCF 402 could have lost them. The F_1 progeny from a cross between Kil-0 and Nd-1 was resistant to both isolates (Fig 2.10 A and B) and the progeny could have obtained the stronger allele from Nd-1 for the proteins downstream in the signaling pathway to confer resistance against GMI1000. The other possible explanation is that Kil-0 contains a weaker *RRS1* allele that activates a weaker defense response. The *RRS1* gene encodes a TIR-NBS-LRR R protein which contains a C-terminal WRKY domain (Deslandes *et al.,* 2002). TIR domain plays a role in downstream signaling and the NBS domain is important for ATP binding and exhibits ATPase activity (Deslandes *et al.,* 2002). The *RRS1* gene in Kil-0 was sequenced as described below and amino acid differences were observed in both of these domains between the RRS1 proteins in Kil-0 and Nd-1. The *RRS1* gene in Kil-0 could activate a weaker defense response or could be affected in binding of ATP. However, Kil-0 is resistant to isolate BCCF 402 and resistance was mediated by the *RRS1* gene

that recognizes the PopP2 effector. The F_1 progeny was also resistant to both isolates and this indicate that both RRS1 proteins are functional and are able to mediate a defense response against pathogen attack. Thus, the susceptibility of Kil-0 to isolate GMI1000 could be due to weaker alleles of proteins downstream in the signaling pathway. A third explanation could be due to different PopP2 proteins present in isolate GMI1000 and BCCF 402 (Appendix D) and the *RRS1* allele in Kil-0 may not recognize the PopP2 protein in GMI1000 effectively and a weak defense response is activated. However, the F_1 progeny was resistant to both isolates and these results indicate that the PopP2 proteins from both isolates are recognized by the RRS1 proteins and an effective defense response is activated.

The RRS1 protein in Kil-0 and Be-0 was sequenced and compared to the RRS1 protein sequences in Nd-1 and Col-0 to determine if the ecotypes contain different alleles of the same gene. The amino acid sequences of the RRS1 proteins were analyzed and the NH_2 termini contained domains general to the TIR-NBS-LRR class of R proteins (Fig. 2.14). The leucine-rich repeat (LRR) domain mediates protein-protein and protein-ligand interactions and plays an important role in specific recognition of pathogen derived elicitors (Deslandes *et al.,* 2003; Caplan *et al.,* 2008; Collier and Moffett, 2009). A nucleotide-binding (NBS) domain is also present in the proteins. The NBS domain is important for ATP binding and exhibits ATPase activity. The TIR domain has homology to *Drosophilla* Toll and to the human interleukin-1 receptor and plays a vital role in downstream signaling and pathogen recognition. The C-terminal regions contained a nuclear localization signal (NLS) (not shown) and a WRKY domain. The WRKY domain could be responsible for regulating the expression of signaling pathways leading to resistance or susceptibility, whereas the NLS might assist in targeting the proteins to the nucleus (Deslandes *et al.,* 2003; Caplan *et al.,* 2008; Collier and Moffett, 2009).

The *RRS1* genes differ in the position of their stop codons (Fig. 2.14). The Col-0 (RRS1-S) protein is 90 amino acids shorter, while the RRS1 protein from Be-0 is 23 amino acids shorter than RRS1-R from Nd-1. RRS1 protein from Kil-0 was 5 amino acids shorter than RRS1-R, but the protein had the highest level of identity (98.9%) to RRS1-R (Table 2.3, grey blocks). The deletions at the C-terminal end did not eliminate the WRKY motifs. The importance of RRS1-R in

resistance was emphasized by structure-functional analysis of two susceptible homozygous intragenic recombinant lines (Deslandes *et al.,* 2002). A chimaeric *RRS1* gene, whose nucleotide sequence matches the RRS1-R up to the stop codon of RRS1-S, was transformed into one of the lines. Susceptibility of the transgenic line to *R. solanacearum* isolate GMI1000 illustrated that the 90 amino acids at the C-terminal end of the *RRS1-R* gene are essential for the function of the protein in disease resistance (Deslandes *et al.,* 2002). The chimaeric RRS1 gene in the other line contained the WRKY domain of *RRS1-R* and the TIR-NBS-LRR domains of *RRS1-S* and this protein did not function as a resistance protein. These results indicated that the other domains of RRS1-R are also important for resistance. Yeast-two hybrid or structure-functional analysis should reveal if the amino acid differences in Kil-0 and Be-0 as well as the truncations at the C-terminal end has an effect on the function of the proteins. Amino acid comparisons of the RRS1 proteins showed that Kil-0 contains an allelic variant to the RRS1-R protein in ecotype Nd-1.

Allelic differences was also observed at the *popP2* locus between the *R. solanacearum* isolates GMI1000 and BCCF 402 (Appendix D). Based on the results reported above and a previous study showing a direct physical interaction between the RRS1 proteins from *A. thaliana* and the PopP2 protein from isolate GMI1000 (Deslandes *et al.,* 2003), it is suggested that both the *RRS1* locus and the *popP2* locus may be under diversifying selection. R genes represent a flexible component of the plant defense system and the coevolution of *R* and effector genes may be controlled by the molecular basis of effector recognition in R-mediated resistance (Catanzariti *et al.,* 2010). Effectors from pathogens can be detected by the host through a direct physical interaction with R proteins and these effectors can alter their binding sites to avoid recognition without affecting their function. The *R* genes have to evolve to obtain new recognition specificities to detect the pathogens. This specific situation was illustrated in the flax rust pathosystem where sequencing analysis at the *R* and effector loci provided evidence for strong diversifying selection (Catanzariti *et al.,* 2010).

R genes that encode proteins which detect effector perturbation on host proteins (guard hypothesis) were mostly found to be conserved and not under diversifying selection (Catanzariti *et al.,* 2010). These effectors are recognized indirectly and can only prevent

detection by becoming non-functional. The *RPS5* gene from *A. thaliana* displays minimal allelic variability and it detects the *P. syringae* pathogen through proteolytic cleavage of the protein kinase PBS1 by the AvrPphB effector (Bent and Mackey, 2007).

Despite the fact that different alleles were reported for both of the proteins from the hosts (Kil-0 and Nd-1) and the pathogens (GMI1000 and BCCF 402), a high level of identity was observed between the alleles from both proteins (98.9% for the RRS1 proteins and 98.8% for the PopP2 proteins). The differences observed in the RRS1 protein from Kil-0 may not affect the structure of the protein and the PopP2 effector from isolate BCCF 402 is effectively recognized to activate a defense response.

2.5.4 PopP2 is the avirulence determinant in *R. solanacearum* isolate BCCF 402 recognized by RRS1 in Kil-0 to initiate a defense response

Previous work has shown that the *RRS1-R* gene in ecotype Nd-1 confers resistance against the *R. solanacearum* isolate GMI1000 by recognizing the PopP2 effector in GMI1000 (Deslandes *et al.,* 2003). To determine whether *RRS1* in Kil-0 is the locus conferring resistance against isolate BCCF 402, the resistant Kil-0 plants were challenged with a BCCF 402 *popP2* mutant to determine if PopP2 is the avirulence protein recognized by RRS1 in Kil-0. Inactivation of the *popP2* gene in isolate BCCF 402 made the strain virulent on ecotype Kil-0 and this result demonstrated that PopP2 is the avirulence protein in isolate BCCF 402 which is recognized by RRS1 in Kil-0 to initiate a defense response (Fig. 2.18 A and B).

Based on results obtained from previous studies, a model is proposed for the activation of *RRS1-R*-mediated defense responses in ecotype Nd-1 against isolate GMI1000 (Noutoshi *et al.,* 2005; Bernoux *et al.,* 2008; Poueymiro and Genin, 2009). RD19 is an *A. thaliana* cysteine protease, which is normally localized in the plant lytic vacuole. RD19 is required for RRS1-R-mediated resistance in Nd-1 and the function of RD19 in plant defense is unknown, but it could act as a transcription factor to induce plant defense responses as mentioned for other plant cysteine proteases. Both RRS1-R and RD19 relocalize to plant nucleus in the presence of PopP2 and PopP2 interacts with both proteins. The function of PopP2 in promoting bacterial virulence

is not defined, but it can inhibit RD19 and disrupt the expression of defense genes or RD19 modifies PopP2 to activate it. It is proposed that a protein complex is formed in the nucleus between RD19 and PopP2 due to the alteration or inhibition of RD19 by PopP2. This complex is recognized by RRS1-R or RRS1-R recognizes the modification of PopP2 induced by RD19.

RRS1-R could function as a negative regulator of the plant defense response. This hypothesis was formulated in a study where a mutation in the WRKY domain of RRS1-R disrupted the DNA binding activity of this domain to the W-box and this lead to the constitutive activation of the salicylic acid dependent defense responses in the *slh* mutant. It is proposed that the nuclear complex formed between PopP2 and RD19 is recognized by the wild type RRS1-R and the interaction of RRS1-R with this complex brings about dissociation of the WRKY domain from the DNA, resulting in transcriptional activation of defense response genes.

The recessive nature of the RRS1-R protein in heterozygous plants from a cross between Nd-1 and Col-0 suggests that the RRS1-S protein could be a dominant repressor of the function of the RRS1-R protein (Deslandes *et al.,* 2002). The *RRS1* genes (*RRS1-S* and *RRS1-R*) encode highly similar proteins and it is suggested that they compete for bacterial or plant components that are required for pathogen recognition or the activation of signal transduction pathways (Deslandes *et al.,* 2003). The WRKY domain in both proteins are intact and both proteins are able to bind to the PopP2 effector. However, the amino acid differences present in the NH_2-terminal domain as well as the truncation of the C-terminal domain of RRS1-S could affect the structure and function of the RRS1-S protein and RRS1-S cannot activate a plant defense response (Deslandes *et al.,* 2002).

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Chapter 3

Summary, Concluding discussion and Future work

3.1 Summary

R. solanacearum is a soilborne, vascular pathogen infecting several hosts including many economically important plants (Deslandes *et al.,* 2002). Previous work has revealed a novel pathosystem between a *Eucalyptus* isolate of *R. solanacearum* (BCCF 402) and the *A. thaliana* ecotypes Be-0 and Kil-0 (Weich, 2004). Isolate BCCF 402 caused severe wilt symptoms and killed Be-0 two weeks after root inoculation, while Kil-0 was identified as the resistant ecotype remaining healthy two weeks after inoculation.

The aim of this study was to characterize the genetic basis of resistance in Kil-0 to the isolate BCCF 402. Genetic crosses were performed between Be-0 and Kil-0 and the F_1 progeny was inoculated with isolate BCCF 402. All of the tested F_1 plants were susceptible to the pathogen. The F_2 population was obtained from the selfed F_1 progeny and a 1:3 segregation ratio (resistant: susceptible) was observed in the F_2 progeny after pathogen challenge. These results suggest that resistance is caused by a single recessive locus. The results were verified by the phenotypic analysis of the F_3 progeny from resistant F_2 lines inoculated with isolate BCCF 402. All of the inoculated plants were resistant to the pathogen. This single recessive locus is likely to be the *RRS1* gene, since resistance co-segregates with the *RRS1* allele from Kil-0. To investigate if resistance to the *R. solanacearum* strains is due to alleles of one gene (*RRS1*) or of different genes, the F_1 progeny from a cross between Kil-0 and Nd-1 was tested for their response to the *R. solanacearum* strains BCCF 402 and GMI1000. The F_1 progeny remained healthy to each of the two strains. These results indicate that the resistance gene in Kil-0 is allelic to *RRS1* and resistance in Kil-0 to isolate BCCF 402 is not due to a different gene.

Amino acid comparisons showed that Kil-0 contains an allelic variant of *RRS1* with the highest level of identity to *RRS1-R* from ecotype Nd-1 compared to Be-0 and Col-0. Previous work has shown that the *RRS1-R* gene in ecotype Nd-1 confers resistance against the *R. solanacearum* tomato isolate GMI1000 by recognizing the PopP2 effector (Deslandes *et al.,* 2003). This is one of the interactions that support the gene-for gene model of resistance to pathogens (Flor, 1971). In this study it was shown that the *popP2* mutant of BCCF 402 failed to produce a resistant interaction with Kil-0 and avirulence was restored when Kil-0 was inoculated with the mutant complemented with a functional *popP2* gene. This suggests that

the recognition between RRS1 in Kil-0 and PopP2 in BCCF 402 is necessary to confer resistance, supporting the gene-for-gene model in our pathosystem.

3.2 Comparisons between the *A. thaliana-R. solanacearum* pathosystems

A. thaliana have been widely used as a model to study resistance against bacterial wilt. Single-gene as well as polygenic resistance in *A. thaliana* against *R. solanacearum* have been described (Deslandes *et al.,* 1998; Ho and Yang, 1999; Godiard *et al.,* 2003). As reported above, two pathosystems have identified the single recessive *RRS1* gene as a main determinant of resistance to *R. solanacearum*. RRS1 in ecotype Ws-0 has also recently shown to confer resistance to the *R. solanacearum* strain *Rs*1002 by recognizing the pathogen determining factor PopP2 (Narusaka *et al.,* 2009). These results indicate that RRS1 may be used as a general component of wilt resistance by resistant ecotypes to recognize the PopP2 effector from *R. solanacearum* strains and prevent disease.

Several differences and similarities were noticed between the *A. thaliana* pathosystems with pathogens from other hosts. The Col-5 and Be-0 ecotypes displayed susceptibility to the race 1 biovar 3 strains (BCCF 402 and GMI1000) (Deslandes *et al.,* 1998; Weich, 2004). These two ecotypes also contained truncated *RRS1* alleles. The Nd-1 accession displayed a higher level of wilt resistance than the Kil-0 accession. Nd-1 was resistant to both strains (GMI1000 and BCCF 402), while Kil-0 showed intermediate symptoms to isolate GMI1000 and remained healthy to isolate BCCF 402.

There are three possible explanations for the difference observed in resistance between the two ecotypes. Kil-0 may contain weaker alleles of other proteins downstream of the signalling pathway that are targets of effectors in GMI1000. Isolate GMI1000 could have acquired these effectors that are absent from isolate BCCF 402 or BCCF 402 could have lost them. The F_1 progeny from a cross between Kil-0 and Nd-1 was resistant to both isolates and the progeny could have obtained the stronger allele from Nd-1 for the proteins downstream in the signalling pathway to confer resistance against GMI1000.

The other possible explanation is that Kil-0 contains a weaker *RRS1* allele that activates a weaker defense response. The *RRS1* gene encodes a TIR-NBS-LRR R protein which contains a

C-terminal WRKY domain (Deslandes *et al.,* 2002). TIR domain plays a role in downstream signalling and the NBS domain is important for ATP binding and exhibits ATPase activity (Deslandes *et al.,* 2002). Amino acid differences were observed in both of these domains between Kil-0 and Nd-1 and the *RRS1* gene in Kil-0 could activate a weaker defense response or could be affected in binding of ATP. However, Kil-0 is resistant to isolate BCCF 402 and resistance was mediated by *RRS1* gene that recognizes the PopP2 effector. The F_1 progeny from a cross between Kil-0 and Nd-1 was also resistant to both isolates and this indicates that both RRS1 proteins are functional and are able to mediate a defense response against pathogen attack. Thus, the susceptibility of Kil-0 to isolate GMI1000 could be due to weaker alleles of proteins downstream in the signaling pathway.

A third explanation could be due to different PopP2 proteins present in isolate GMI1000 and BCCF 402 and the *RRS1* allele in Kil-0 may not recognize the PopP2 protein in GMI1000 effectively and a weak or delayed defense response is activated. There are four amino acid differences between the two PopP2 proteins (Appendix D). However, the F_1 progeny from a cross between Kil-0 and Nd-1 was resistant to both isolates and these results indicate that the PopP2 proteins from both isolates are recognized by the RRS1 proteins and a effective defense response is activated.

3.3 Future work

Previously, the interaction between *R. solanacearum* and *A. thaliana* was studied by focussing on defense responses in the leaf and stem tissue against the pathogen (Hu *et al.,* 2008). Since the pathogen is applied to the root tissue, it was hypothesized that most of the transcriptional changes required for resistance would occur in the root tissue of the plant. In this study we have shown that resistance is mediated by a recessive *RRS1* allele in Kil0 and isolate BCCF 402 contains the *popP2* avirulence gene that is recognised by the *RRS1* allele in Kil-0. This gene-for-gene interaction could be used to study the root specific responses in Kil-0 against *R. solanacearum*. A bacterial-free approach that allows the activation of a resistance response in a very precise manner could be followed. *A. thaliana* Kil-0 plants can be transformed with the *popP2* effector gene under the control of an inducible promoter. The roots of the plants will be sprayed with the inducer to trigger the expression of *popP2*. PopP2 will be recognized by RRS1 in the plant and this will activate defense signaling. Root

samples will be collected at different time points after application of the inducer to perform analysis. The analysis of the root tissue involves transcript profiling of marker genes of the SA and JA/ET pathways by qRT-PCR as well as the measurement of phytohormone levels.

Kil-0 and Nd-1 displayed different responses after inoculation with isolates GMI1000 and BCCF 402. Nd-1 was resistant to both strains (GMI1000 and BCCF 402), while Kil-0 showed intermediate symptoms to isolate GMI1000 and remained healthy to isolate BCCF 402. These differences observed between Kil-0 and Nd-1 could be due to a weaker *RRS1* allele present in Kil-0 that activates a weaker defense response. Kil-0 may also contain weaker downstream signalling components that are targets of effectors in GMI1000. Isolate GMI1000 could have acquired these effectors that are absent from isolate BCCF 402 or BCCF 402 could have lost them. Techniques such as RNAi or VIGS (Burch-Smith *et al.,* 2004) could be used to knock-down the *RRS1* allele from Kil-0 and the functional *RRS1-R* gene from Nd-1 with its promoter can be introduced into the mutant Kil-0 line. Resistance to isolate GMI1000 will be restored if the difference observed is due to a weaker *RRS1* allele in Kil-0. The genome of isolate BCCF 204 could be sequenced and possible effectors that are present in GMI1000 and absent from isolate BCCF 402 could be identified.

The *Eucalyptus grandis* genome is being sequenced by the US Department of Energy (DOE) Joint Genome Institute (JGI) and a preliminary 8X draft assembly of the genome is available on the public *Eucalyptus* Genome Database (http://eucalyptusdb.bi.up.ac.za/). The availability of the genome sequence will make it possible to search for the orthologue of the *RRS1* gene. The role of RRS1 in resistance against *R. solanacearum* can be determined in *Eucalyptus* by inoculating resistant species such as *Eucalyptus citriodora* or *Eucalyptus exserta* with the *R. solanacearum popP2* mutant strains (BCCF 402 and GMI1000 mutant strains) (Ran *et al.,* 2005). *Eucalyptus grandis* trees under 2 years old are vulnerable to the disease (Ran *et al.,* 2005) and older trees can be challenged with the mutant strains. The *Eucalyptus* plants should remain resistant to the wild-type strains, but will be susceptible to the mutant strains if PopP2 is recognized by RRS1 to initiate a defense response.

A previous study showed that both *RRS1* and *RPS4* from the *A. thaliana* ecotype Ws-0 function cooperatively in conferring resistance to the *R. solanacearum* strain 1002 (Narusaka *et al.,* 2009). Future work would involve to determine if RRS1 and RPS4 function

together in Kil-0 to confer resistance against isolate BCCF 402. Single (*rrs1* or *rps4*) and double (*rrs1/rps4*) mutant *RRS1* and *RPS4* Kil-0 plants can be inoculated with isolate BCCF 402 and susceptibility will indicate if these genes provide resistance to the pathogen. The *rrs1* and *rps4* T-DNA tagged mutants were generated in the Ws-0 ecotype and can be introduced into the Kil-0 accession by crossing Kil-0 with the mutant Ws-0 plants. Knockdown techniques such as VIGS or RNAi can also be used to generate the mutant plants (Burch-Smith *et al.,* 2004).

The PopP2 effector belongs to the YopJ/AvrRxv family and its members share structural similarities with the C55 peptidase family of the CE clan of cysteine proteases (Bernoux *et al.,* 2008). *Yersinia pestis* contains the YopJ effector that has deubiquitinating and acyltransferase activities that are necessary for the death of infected macrophages and for the suppression of host proinflammatory responses (Bernoux *et al.,* 2008). The contribution to bacterial virulence of PopP2 is unknown and it should be determined whether PopP2 also has similar enzyme activities. The YopJ family also have partial structural similarity with the yeast Ubiquitin-Like Protease 1 (ULP1) and effectors such as PopP2 from this family may function as SUMO (small ubiquitin-like modifier) proteases to disrupt the regulation of the SUMO pathway (Hotson and Mudgett, 2004; Lahaye, 2004). PopP2 physically interacts with RRS1 as well as RD19 and induces their relocalization into the plant nucleus (Bernoux *et al.,* 2008). Further work would involve looking for conserved SUMO attachment sites on these targets of PopP2 (RD19 and RRS1) to predict a possible role for PopP2 as a SUMO protease.

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Appendix A

Table A1. **Primers used for PCR amplifications**

Purpose	Sequence forward 5'-3'	Sequence reverse 5'-3'	Annealing temperature (°C)	Product size (bp)
PCR amplification of the C-terminal end of the *RRS1* gene	RRS1F-925 (TCCATACCGGCTATAGACGA)	RT3 (AACTCCTCCATGTCCGTC)	60	983
PCR screening of recombinant colonies	M13F (GTTTTCCCAGTCACGACGTTG)	M13R (TGACCGGATAACAATTCACACAG)	55	-
PCR screening of cDNA for genomic DNA contamination	UBIQF (ATTTCTCAAAATCTTAAAAACTT)	UBIQR (TGATAGTTTTCCCAGTCAAC)	58	Genomic: 850 cDNA: 550
PCR amplification of the full length *RRS1* gene	RRS1-C1F (ATGAAAAAGCATCGTCGGCATA)	RRS1-C1R GAAGGTTGTTTTAGGTTCTCCA	63	1552
	RRS1-C2F (CATGTTGAAATTGATGTCCTTG)	RRS1-C2R (CGTTGACCACTTGTTGAGATAG)	62	1396
	RRS1-C3F (CCAACTTCCTTTAAGCCTAGAG)	RRS1-C3R (CTGAAAATCATTGACACTGCAA)	60	1698
	RRS1-C3MF (GACTACTGTGATGCTACAGATG)	RRS1-C3MR (GTCATTATCCACCTCCATATTG)	60	1152
PCR screening of BCCF 402 mutant strains	POPP2 5'FLANKF (CGGGGATTGAGATACGTTGTGAC)	LACZR (AAGGGGGATGTGCTGCAAGG)	60	1008
	GENTF (CCTCGATCAGTCCAAGTGGCC)	POPP2 3'FLANKR (GCTCAACGACAAGGCAAGC)	60	1375
PCR amplification of the full-length *popP2 gene* and its promoter	POPP2FLF (GAGGGTGGTCGTAATGGTTG)	POPP2FLR (CTGGTTTGTGAGTTGTTGTC)	60	2046
PCR screening of GRS478(6) colonies transformed with the pLAFR6::*popP2* construct.	PLAFFOR (GGCATTCTTGGCATAGTG)	PLAFREV (GCTGACGGCTATCACCAT)	58	2300

Appendix B

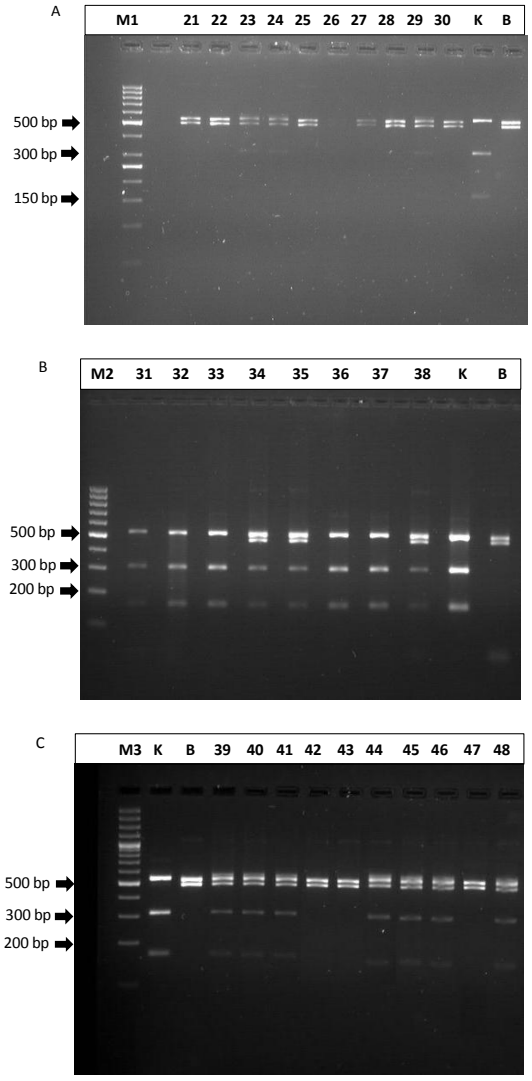
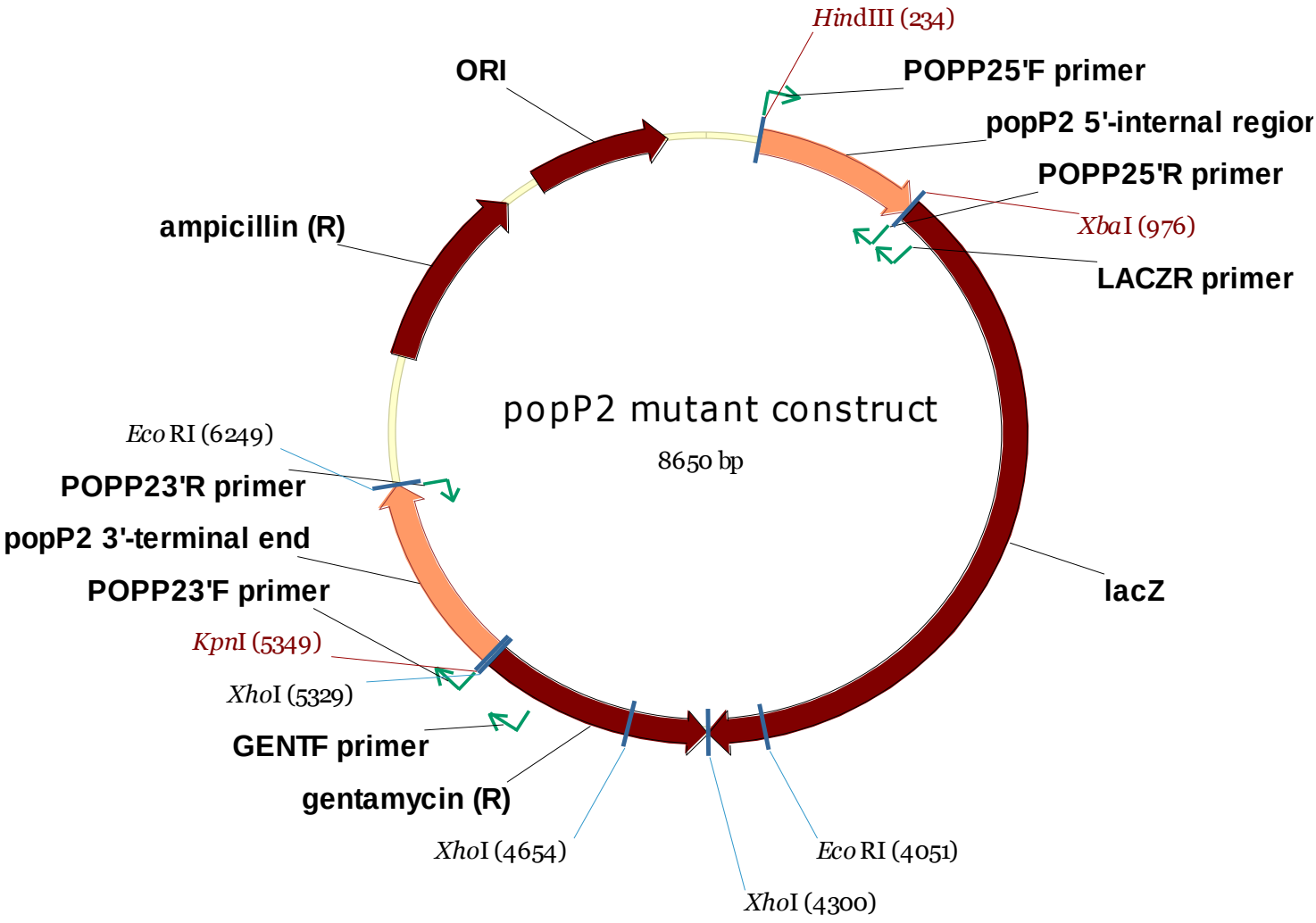


Figure B. **LweI digestion of the *RRS1* PCR products.** M1: 50bp DNA marker (Fermentas Inc., USA); M2: 100bp DNA marker (Fermentas Inc., USA); M3: 100bp DNA marker (Fermentas Inc., USA). A, B and C: lanes 21 to 30, 34, 35, 38 and 39 to 48: LweI digestion of the *RRS1* PCR products from the F_2 progeny that were scored as susceptible; Restriction analysis was repeated on sample 26 (Data not shown). B: lanes 31 to 33 and 36 and 37: LweI digestion of the *RRS1* PCR products from F_2 progeny that were scored as resistant. For A and B and C: lane K: LweI digestion of the Kil-0 *RRS1* PCR product; lane B: LweI digestion of the Be-0 *RRS1* PCR product. The digested PCR products were analyzed by electrophoresis through a 2% (w/v) agarose gel in 1X TAE buffer (pH 8) containing 0.5 ug/ml ethidium bromide.

Appendix C

***popP2* mutant construct**



Appendix D

```
          1                                                                                                   100
BCCF 402  MKVSSANAGVSASSADNTSARPSQTSADTTALGRRRRAPEDAPGSPPARRQRQDSPEDSAQTMFRRAGMTALPPSPATSEHVPLLDNRPTLERMGVDHPL
 GMI1000  MKVSSANAGVPASSADNTSARPSQTNADTTPLGRRRRAPDDAPGSPPARRQRQDSPEDSAQTMFRRAGMTSLPPSPATSEHVPLLDNRPTLERMGVDHPL
          101                                                                                                 200
BCCF 402  PGRTWYETGHTTASLADRTSTASAAQVASSSRSAGPATAARPQPTRTSAGQQATVDRLRTQVTGFLSGALGKLQALSAQNMDPELAQFRVLDVDRAIMPL
 GMI1000  PGRTWYETGHTTASLADRTSTASAAQVASSSRSAGPATAARPQPTRTSAGQQATVGRLRTQVTGFLSGALGKLQALSAQNMDPELAQFRVLDVDRAIMPL
          201                                                                                                 300
BCCF 402  LIVAENARNPGLNLVPLHMDMAEDEEVRTQPPMAGSRHIAEFVASARPGRYRAVIDDGSHTRAADIRKDASGTSVIVVDPLRKEKDESAYVDYADNVNME
 GMI1000  LIVAENARNPGLNLVPLHMDMAEDEEVRTQPPMAGSRHIAEFVASARPGRYRAVIDDGSHTRAADIRKDASGTSVIVVDPLRKEKDESAYVDYADNVNME
          301                                                                                                 400
BCCF 402  FGEHAKCAFIPVDIQKSFFDCRILSLSLALKMHDKDDAFAAFHETLRNGGDPSHHVSRAQQTEELGATLVLDGAPLVDARMMKHGQAASSVSRYLGNHPE
 GMI1000  FGEHAKCAFIPVDIQKSFFDCRILSLSLALKMHDKDDAFAAFHETLRNGGDPSHHVSRAQQTEELGATLVLDGAPLVDARMMKHGQAASSVSRYLGNHPE
          401                                                                                             488
BCCF 402  QSTVPVNKRNETLGERTTRHLVKRKVRNRADSEGRVTSGETKEITFSNSVEQKRIALLNRAASYVNSAPPPVVMRMAKLLQDSLLDTN
 GMI1000  QSTVPVNKRNETLGERTTRHLVKRKVRNRADSEGRVTSGETKEITFSNSVEQKRIALLNRAASYVNSAPPPVVMRMAKLLQDSLLDTN
```

Figure D. **Sequence comparison of the PopP2 proteins in isolates GMI1000 and BCCF 402.** Identical and conserved amino acids are black letters on white background. Weakly similar and non-similar amino acids are white letters on black background.