

Characterization of tolerance to bacterial wilt in the model plant *Arabidopsis*

by

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Declaration

I, Jane Bredenkamp declare that the dissertation, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Preface

Bacterial wilt caused by *Ralstonia solanacearum* is a disease that affects a wide range of economically important plant species. The lack of genetic tools and pathosystem models available in natural hosts, including *Eucalyptus*, prevents in depth molecular study in many host plants (Naidoo *et al.*, 2011). The extensive genomic resources readily available for the model organism *Arabidopsis thaliana* allow for in depth genetic and molecular investigation of plant-pathogen interactions (Nishimura and Dangl, 2010). *Arabidopsis* has been found to be a host of numerous fungal, viral and bacterial pathogens as well as oomycetes and nematodes (Deslandes *et al.*, 1998; Nishimura and Dangl, 2010). Several *Arabidopsis* R genes have also been identified that are effective against a variety of pathogens highlighting its capability as a pathosystem tool (Deslandes *et al.*, 1998). Continued investigation into the area of plant-pathogen interactions will greatly aid biotechnological efforts to improve plant resistance to pathogens and subsequently prevent economic losses of important crop species.

Previously, a pathosystem was described between the model organism *Arabidopsis* and a *Eucalyptus* isolate of *R. solanacearum* BCCF402. In this pathosystem, Kil-0 was concluded to be “resistant” to *R. solanacearum* BCCF402 as Kil-0 plants did not develop any bacterial wilt symptoms. Accession Be-0 was susceptible to BCCF402 as Be-0 plants showed wilting as early as 3 days after inoculation. However, in a recent study (Weich, 2004), a plate counting technique used to quantify bacterial numbers of BCCF402 in *Arabidopsis* accessions showed that the bacterial load of *R. solanacearum* in accession Kil-0 was only one order of magnitude less than the concentration of bacteria found in susceptible accession Be-0 (Weich, 2004). This unexpected result suggested that the Kil-0 - *R. solanacearum* interaction could be one of tolerance instead of resistance. This study focused on confirming and characterizing the tolerant interaction between model plant *Arabidopsis* and the bacterial wilt pathogen *R. solanacearum* BCCF402. To further

understand the tolerance response to BCCF402, a mCherry-based tool was developed to visualize BCCF402 *in planta*.

Chapter 1 is a literature review which discusses the pathogen *R. solanacearum* virulence strategies, Arabidopsis defence responses and the outcomes that occur when a pathogen infects a host plant. The tolerant, resistant and susceptible plant – pathogen interactions are described in detail. Molecular techniques to quantify and visualize the pathogen are also discussed. The literature review serves as an introduction to the study.

Chapter 2 discusses the material and methods utilized in the study.

Chapter 3 provides the results obtained in the study that characterizes the tolerant interaction as well as the development and utilization of mCherry-tagged BCCF402.

Chapter 4 represents a discussion of the results. Potential molecular mechanisms and genetic determinants of tolerance are discussed as well as possible future work.

References are provided at the end of the discussion.

Oral and poster presentations as well as publications pertaining to this work are listed below:

Oral and Poster presentations:

- Bredenkamp J, Naidoo S, Berger DK. A quantitative PCR assay for the accurate quantification of *Ralstonia solanacearum* in Arabidopsis plants. Paper presented at: Northern Branch Symposium of the Southern African Society for Plant Pathology; 31 Aug 2012; Pretoria, SA.
- Bredenkamp J, Naidoo S, Berger DK. Challenging the dogma: gene-for-gene tolerance, not resistance, to bacterial wilt in Arabidopsis. Poster session presented at: 47th Congress of the Southern African Society for Plant Pathology; 23 – 26 Jan 2011; Kruger National Park, SA.
- Bredenkamp J, Naidoo S, Berger DK. Challenging the dogma: gene-for-gene tolerance, not resistance, to bacterial wilt in Arabidopsis. Paper presented at: 38th Conference of the South African Association of Botanists; 15 – 18 Jan 2012; Pretoria, SA.

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Abstract

Ralstonia solanacearum, the causal agent of bacterial wilt disease, has been found to affect numerous economically important plants. Understanding the molecular basis of resistance, tolerance and susceptibility of plants to pathogens such as *R. solanacearum* is a major goal of molecular plant pathologists. Prior to this study it was thought that Arabidopsis accession Kil-0 shows gene-for-gene “resistance” to an African *Eucalyptus* isolate of *R. solanacearum*, BCCF402. However, a subsequent preliminary study indicated that Kil-0 may exhibit “tolerance” which is defined as the plant’s ability to support high pathogen numbers without displaying disease symptoms or a reduction in host fitness. The aim of this study was to determine if Kil-0 was tolerant to *R. solanacearum* BCCF402. The bacterial load of *R. solanacearum* was quantified in accessions Kil-0 and Be-0 using dilution plating and quantitative PCR methods. The *cytC* gene region was used to quantify *R. solanacearum* in *Arabidopsis* plants and the amount of bacterial DNA was normalized to “alien” DNA that was spiked into each sample. High bacterial concentrations of BCCF402 were found in Kil-0 but plants exhibited no wilting symptoms. Additionally, Kil-0 plants inoculated with BCCF402 showed no significant reduction in fitness compared to control Kil-0 plants. In contrast, high bacterial numbers and severe disease symptoms were observed in the susceptible Be-0 plants, whereas Nd1 plants contained a low number of bacteria and no disease symptoms indicative of a resistance response. These results illustrated that Kil-0 is tolerant to *R. solanacearum* isolate BCCF402. A tool for the visualization of *R. solanacearum* in *Arabidopsis* plants was designed. *R. solanacearum* isolate BCCF402 was tagged with two mCherry-containing plasmids under the constitutive expression of the *tac* promoter. The expression levels of mCherry were suitable for successful visualization *in planta*. BCCF402 cells transformed with the mCherry-containing plasmids were not affected in terms of virulence or disease progression compared to wildtype BCCF402 cells. A plasmid loss of 30-35% was observed in mCherry-tagged BCCF402 cells at later stages of *Arabidopsis* infection. mCherry-tagged BCCF402 was successfully visualized in Kil-0 leaves at early infection stages.

Chapter 1

Literature review

**Investigating the outcomes of pathogen virulence
strategies and host defence responses in the
Arabidopsis thaliana – *Ralstonia solanacearum*
interaction**

1.1 Introduction

Bacterial wilt is a plant disease of worldwide economic importance. *Ralstonia solanacearum*, the causal agent of bacterial wilt, is a major pathogen in the study of bacterial virulence strategies. The complete genome sequence of *R. solanacearum* isolate GMI1000 has been particularly useful in elucidating its pathogenicity determinants (Salanoubat *et al.*, 2002; Poueymiro and Genin, 2009). *R. solanacearum* is classified under the phylum β -Proteobacteria and the genus *Ralstonia* (Genin and Boucher, 2002). The pathogen has a broad host range, infecting over 200 host species (Schell, 2000). *R. solanacearum* has some interesting characteristics such as being a soil-borne, gram-negative bacterium that infects the host plant through root wounds or natural openings at the emergence of secondary root growth (Genin and Boucher, 2002; Turner *et al.*, 2009). The bacteria multiply in the root vascular tissue to eventually colonize the xylem vessels and vascular system (Genin and Boucher, 2002). *R. solanacearum* secretes exopolysaccharides and enzymes that result in cell damage. The accumulation of extracellular product and damaged tissue disrupt the flow of water up the xylem resulting in plant death (Genin and Denny, 2012). Symptoms characteristic of this disease include root rot, brown discoloration of the xylem and blue to black streaks on the wood of hosts (Roux *et al.*, 2001).

The model organism, *Arabidopsis thaliana*, is often used in the study of plant-pathogen interactions due to the extensive amount of genomic resources available. *Arabidopsis* was found to be a host of the phytopathogen *R. solanacearum* (Deslandes *et al.*, 1998). GMI1000, a *R. solanacearum* strain isolated from tomato, caused disease symptoms on *Arabidopsis* accession Col-5 but was found to cause a resistant response on accession Nd1. Resistance in Nd1 against *R. solanacearum* was found to be conferred by a single recessive gene (Deslandes *et al.*, 1998). It was determined that Nd1 resistance was conferred by the recessive *RRS1-R* allele (Deslandes *et al.*, 2002). *RRS1-R* confers resistance by recognizing the PopP2 (*Pseudomonas* outer protein P2) effector of the *R. solanacearum* pathogen (Deslandes *et al.*, 2003). PopP2 and *RRS1-R* interacts

physically in the nucleus where PopP2 auto-acetyltransferase activity occurs to elicit a defence response (Tasset *et al.*, 2010).

When a pathogen infects a plant, three interactions can occur; susceptibility, resistance and tolerance. Susceptibility occurs when a pathogen colonizes the plant extensively resulting in symptom development (Robb, 2007). Resistance is defined as a host's ability to restrict the amount of pathogen proliferation and colonization (Kover and Schaal, 2002). Tolerance traits, on the other hand, result in the plant's ability to withstand high numbers of bacteria with regards to plant fitness and yield (Kover and Schaal, 2002). While resistant and susceptible interactions have been studied extensively, there has been very little investigation of the tolerance response to plant pathogens. Further study and characterization of tolerance mechanisms are needed to shed some light on this phenomenon.

1.2 *R. solanacearum* as a phytopathogen

R. solanacearum is an economically important phytopathogenic bacteria, causing lethal wilting diseases on a huge number of plant species (Genin and Denny, 2012). Phylogenetic analysis has revealed extensive genetic diversity and this group of closely related organisms is generally referred to as the *R. solanacearum* species complex (RSSC) (Prior and Fegan, 2005). This gram negative, soil-borne bacterium is found worldwide and is especially endemic in tropic and sub tropic climates but has been observed in the more temperate regions of Western Europe (Genin and Boucher, 2002). *R. solanacearum* has a large host range and causes bacterial wilt on more than 200 host species belonging to over 50 botanical families (Schell, 2000). *R. solanacearum* infects Solanaceous plants such as tomato and tobacco, leguminous plants such as groundnut and French bean and has been known to cause Moko disease in monocotyledonous hosts such as banana (Genin and Boucher, 2002). *R. solanacearum* infection usually results in the death of its host and is known for causing huge economic losses in

agriculture and forestry industries around the world (Coutinho *et al.*, 2000; Roux *et al.*, 2001). In South Africa, *R. solanacearum* was found to afflict the agronomically important forestry species, *Eucalyptus*, in 1997 (Coutinho *et al.*, 2000). Bacterial wilt caused by *R. solanacearum* was initially observed in *Eucalyptus* plantations in Brazil and China in the early 1980's and has also been reported in the Democratic Republic of Congo (DRC), Uganda, Australia and Venezuela (Coutinho *et al.*, 2000; Roux *et al.*, 2001; Gan *et al.*, 2004).

Coutinho and colleagues reported the first evidence of bacterial wilt caused by *R. solanacearum* on *Eucalyptus* plantations in South Africa in 2000 (Coutinho *et al.*, 2000). The *E. grandis* x *E. camaldulensis* hybrid displayed wilting symptoms and tree death was caused by *R. solanacearum* biovar 3 race 1. This has the potential to have a negative effect on the forestry industry in South Africa making control management of bacterial wilt on *Eucalyptus* a priority. Control of bacterial wilt on different hosts has been largely unsuccessful to date. The extensive diversity of the pathogen's virulence strategies has hampered attempts to breed for resistance and management is minimal. At present, pathogen-free seed, crop rotation and the use of a few known resistant and tolerant plant lines are the main control strategies employed (Coutinho *et al.*, 2000; Genin and Boucher, 2002; Ramesh *et al.*, 2009).

1.2.1 Phylogenetic classification of the *R. solanacearum* species complex

The diverse strains of the *R. solanacearum* species complex (RSSC) were originally separated into races and biovars. *R. solanacearum* was classified into 5 races based on their host range (Villa *et al.*, 2005) and 6 biovars grouped according to their ability to metabolize various disaccharides and alcohol carbohydrates (Castillo and Greenberg, 2007). While these systems proved useful for many years, their lack of a genetic basis proved insufficient to represent the diversity of the *R. solanacearum* species complex.

These systems were replaced by the far more efficient phylotype scheme for classifying *R. solanacearum* (Prior and Fegan, 2005).

A number of molecular approaches have been utilized to perfect our classification of the diversity of *R. solanacearum* (Prior and Fegan, 2005). Restriction fragment length polymorphism (RFLP) analysis and DNA sequence analysis of the 16S rDNA sequence and the *hrp* gene region provided an initial basis of *R. solanacearum* classification (Cook *et al.*, 1989; 1994; Poussier and Luisetti, 2000). As the number of analyses increased, it became clear that the diversity of *R. solanacearum* could be divided into four major groups termed phylotypes (Prior and Fegan, 2005). The new hierarchical classification scheme was proposed based on phylogenetic sequence analysis of the 16S-23S internal transcribed spacer (ITS) region, endoglucanase, *hrpB* and *mutS* genes (Prior and Fegan, 2005).

The phylotypes generally relate to the geographic origin of the strains within each phylotype. Phylotype I include strains that originate chiefly from Asia and generally include strains belonging to biovars 3, 4 and 5. Tomato isolate GMI1000 and *Eucalyptus* isolate BCCF402 are biovar 3 strains belonging to this phylotype. Phylotype II consists of strains belonging to biovars 1 and 2 from America as well as the Race 3 potato pathogen and the Race 2 banana pathogen (Prior and Fegan, 2005). Phylotype III consists of isolates mainly originating from Africa and surrounding islands. Phylotype II has two subclusters, IIA and IIB. Phylotype IV strains are from Indonesia, Japan and Australia and include *Ralstonia syzygii* and the blood disease bacterium (BDB) which are species closely related to *R. solanacearum* (Prior and Fegan, 2005). The phylotyping scheme proved to have a number of advantages over the race and biovar systems. This new system should allow for an accurate prediction of the biological and evolutionary properties of *R. solanacearum* strains as well as providing information on the geographic origin of RSSC strains (Prior and Fegan, 2005).

The grouping of the *R. solanacearum* isolates into four phylotypes was supported by a number of studies. A comparative genomic hybridization analysis was performed to investigate the gene distribution among 18 *R. solanacearum* strains representative of the RSSC. A GMI1000 microarray was used in this study and the hierarchical clustering of the *R. solanacearum* strains was consistent with the phylotype system (Guidot *et al.*, 2007). A comparison of the genome sequences of six *R. solanacearum* strains produced the same four phylotypes (Remenant *et al.*, 2010; 2011). A phylogenetic analysis of strains representative of the RSSC indicated that *R. solanacearum* contains four evolutionary lineages that diverged many years ago due to geographic isolation that corresponded to the four phylotypes (Castillo and Greenberg, 2007). A similar, more recent multilocus sequence analysis found the same phylotype groups as well as the occurrence of recombination events both within and between phylotypes. Interestingly, the study further defined eight clades that could be superimposed on the existing four phylotypes (Wicker *et al.*, 2012).

1.2.2 Genomes of *R. solanacearum*

R. solanacearum genomes are organized into a bipartite system consisting of two circular structures; the larger chromosome and the smaller megaplasmid (Salanoubat *et al.*, 2002). The combined genome sequence was determined to be on average 5.8 Mb in size (Salanabout *et al.*, 2002; Genin and Denny, 2012). Both of these structures contain essential house-keeping genes as well as pathogenicity-related genes which indicate an ancient co-evolution of the two replicons (Guidot *et al.*, 2007; Genin and Denny, 2012). To date, 11 *R. solanacearum* genomes have been sequenced (Genin and Denny, 2012) including the genome of *R. solanacearum* strain GMI1000 that was completed in 2002 (Salanoubat *et al.*, 2002). These 11 sequenced genomes provide a good illustration of the diversity in the *R. solanacearum* species complex.

Functional characterization shows that the chromosome contains the majority of housekeeping and basic survival genes (Salanabout *et al.*, 2002). The megaplasmid,

however, contains a large amount of genomic islands that may encode strain-specific advantages in different environments and life-styles and in the pathogenesis of various host species (Genin and Denny, 2012). Genomic islands are regions that may have been obtained through lateral gene transfers (Remenant *et al.*, 2010; 2011). A gene distribution study among 18 different *R. solanacearum* strains supported this observation (Guidot *et al.*, 2007). Based on comparative genomic hybridization (CGH) using a microarray developed from the genome sequence of *R. solanacearum* strain GMI1000, it was shown that 63% of megaplasmid genes are variable from one strain to another (Guidot *et al.*, 2007). Variable genes were shown to be grouped into genomic islands (Guidot *et al.*, 2007). Pathogenicity related functions encoded by the megaplasmid include the type 3 secretion system (T3SS) including effectors, type 4 pilus biosynthesis, flagellar motility, hydrolytic enzymes and genes that play a role in exopolysaccharide (EPS) biogenesis (Salanoubat *et al.*, 2002; Genin and Denny, 2012).

Sequencing of the GMI1000 genome has shown that the *R. solanacearum* genome has the potential for plasticity and has resulted in the designation of alternative codon usage regions (ACUR) which represents approximately 7% of the genome (Genin and Boucher, 2002). The ACUR's have been shown to contain a large amount of genes with a G+C content that is lower than the 67% average (Salanoubat *et al.*, 2002). These regions that especially correspond with transposable elements and prophage-associated regions and some constitute genomic islands which could have originated through horizontal gene transfers (Salanoubat *et al.*, 2002; Genin and Denny, 2012). The ACUR's also contain pathogenicity related genes such as those encoding type 3 effector proteins as well as several duplicated open reading frames (ORF's) of unknown function (Genin and Boucher, 2002).

Many *R. solanacearum* genomic islands are associated with prophages or transposon-like elements (Remenant *et al.*, 2011). Prophages are important features in driving the evolution and emergence of pathogenic varieties of many soil borne microbes due to their abundance in soil and their effectiveness as vectors of horizontal gene transfer (Genin

and Denny, 2012). Several genes that are involved in the plant-pathogen interaction are associated with prophages. The *R. solanacearum* Type III effector *PopP2* gene is linked to a prophage that is inserted in different genomic locations in the three *R. solanacearum* strains that contains *PopP2* (GMI1000, Po82 and CMR15) (Genin and Denny, 2012).

1.3 *R. solanacearum* virulence strategies

1.3.1 Mode of *R. solanacearum* invasion

R. solanacearum can live for a very long time in soil by associating with plant debris or asymptomatic hosts (Genin and Boucher, 2002). The process of *R. solanacearum* infection has been described in tomato (Vasse *et al.*, 1995), petunia (Zolobowska and Van Gijsegem, 2006) and *Medicago truncatula* (Vailleau *et al.*, 2007; Turner *et al.*, 2009). *R. solanacearum* colonization in Arabidopsis roots during a compatible interaction between the Arabidopsis ecotype Col-0 and *R. solanacearum* strain GMI1000 has also been established (Digonnet *et al.*, 2012). Generally, the bacterium enters the roots of its host plant through natural openings at the emergence of secondary root growth. *R. solanacearum* may also penetrate the roots via sites of physical wounding created by insects and nematodes (Schell, 2000). In Arabidopsis, *R. solanacearum* is able to penetrate between the root epidermal cells particularly at the root apex (Digonnet *et al.*, 2012).

The bacteria then move through the intracellular spaces of the root cortex cells to reach the xylem vessels. Upon infection, plasmolysis as well as pectin degradation was observed throughout the root cortex. These pectin degradation products, called oligogalacturonides, may induce a plant defence response against *R. solanacearum* (Nakaho *et al.*, 2000; Digonnet *et al.*, 2012). In Arabidopsis, *R. solanacearum* appears to gain entry into the vascular system via the pericycle cells located at the xylem poles. *R. solanacearum* infection results in pericycle cell death allowing spread of the bacteria in

the xylem (Digonnet *et al.*, 2012). At this stage, the bacteria proliferates extensively ensuring effective colonization of the vascular system. *R. solanacearum* is able to spread from vessel to vessel to the upper part of the plant through degradation of the pit membranes (Nakaho *et al.*, 2000). The combination of a large amount of *R. solanacearum* cells, their secreted exopolysaccharides and plant debris block the flow of water that travels up the xylem vessels to the aerial parts of the plant, resulting in wilting symptoms and eventual plant death (Genin and Boucher, 2002).

In *Medicago truncatula*, *R. solanacearum* infection was observed in a susceptible and a resistant line (Turner *et al.*, 2009). *R. solanacearum* was able to penetrate the xylem vessels of the susceptible line but this occurrence was absent in the resistant line (Turner *et al.*, 2009). This indicates that certain mechanisms are in place that restricts the penetration of bacteria into the xylem vessels and subsequently prevents the spread of bacteria to aerial tissue in the resistant plants (Turner *et al.*, 2009). In resistant tomato lines, the pit membranes that divide xylem vessels appeared thicker than the membranes of susceptible cultivars, preventing bacterial spread (Nakaho *et al.*, 2000; Digonnet *et al.*, 2012). Therefore, the structure of the xylem vessel may play a role in determining a resistant or susceptible outcome in the plant – *R. solanacearum* interactions.

1.3.2 General *R. solanacearum* virulence factors

Ralstonia solanacearum secretes a variety of extracellular proteins and cell wall degrading enzymes that enhance the pathogen's ability to overcome the host plant's defences (Schell, 2000). One of the most essential determinants of virulence for *R. solanacearum* is its ability to secrete EPS during the infection process to aid pathogen colonization. An EPS-deficient mutant strain of *R. solanacearum* was unable to penetrate the xylem vessels of tomato plants suggesting a role for EPS in assisting the invasion of bacteria into the xylem vessels (Vasse *et al.*, 1995). Alternatively, EPS may be involved in reducing the plant's defence response (Genin and Boucher, 2002). The *R. solanacearum* isolate GMI1000 is known to employ three EPS compounds. EPS1 is an acidic molecule

and makes up 90% of all EPS molecules used, however, EPS3 and EPS4 are also involved in the infection strategy. A study by Araud-Razou *et al.* (1998) was performed to address the role of EPS1 during infection of the tomato root with *R. solanacearum*. The mutant *R. solanacearum* strains that were incapable of producing major EPS were unable to invade xylem vessels and induce plant defence mechanisms. Thus, the EPS1 polymer was necessary for tomato root infection and vascular colonization in tomato roots (Araud-Razou *et al.*, 1998). Interestingly, EPS induces the expression of the ethylene and salicylic acid signalling pathways in a resistant tomato cultivar but not in the susceptible tomato line (Milling *et al.*, 2011).

The type 2 secretion system (T2SS) is also important for *R. solanacearum* virulence. A host of approximately thirty extracellular proteins including cell wall degrading enzymes (CWDE's) are transported over the plant's outer membrane by this system (Liu *et al.*, 2005; Hikichi *et al.*, 2007). The numerous bacterial CWDE's includes one β -1,4-endoglucanase (Egl), one endopolygalacturonase (PehA), two exopolygalacturonases (PehB and PehC), one β -1,4-cellobiohydrolase (CbhA) and a pectin methylesterase (Pme) (Hikichi *et al.*, 2007). *R. solanacearum* mutants that are deficient in all six of these CWDE's were less virulent on plant hosts such as tomato. While EPS is believed to be the major factor involved in pathogen virulence, these six cell wall degrading enzymes may quantitatively and significantly contribute to virulence (Liu *et al.*, 2005). However, it is believed that the other twenty four proteins that are also transported via the type 2 secretion system play a substantial role in pathogen virulence (Poueymiro and Genin, 2009).

Motility plays a significant role in pathogen virulence. Both flagellar-driven swimming and pilus-driven twitching motility contribute to virulence. A hypermotile *motN* mutant showed a significant reduction in *R. solanacearum* virulence on tomato when inoculated using a soil soak method (Meng *et al.*, 2011). These *motN* mutant bacteria, however, exhibited a wild-type virulent phenotype when they were introduced directly into the tomato plant xylem (Meng *et al.*, 2011). This method and others indicate that directed swimming

motility is important for *R. solanacearum* virulence at the initial stages of host root location and infection (Tans-Kersten *et al.*, 2001; Yao and Allen, 2006; Meng *et al.*, 2011). Polar type 4 pili have been shown to contribute to virulence on tomato by driving twitching motility of the bacterium (Kang *et al.*, 2002). Type 4 pili can be divided into a number of subclasses. Both type 4a and type 4b pili have been shown to contribute to virulence of *R. solanacearum* (Wairuri *et al.*, 2012).

R. solanacearum appears to also be able to detoxify ROS *in planta* (Brown and Allen, 2004). Knockout of *acrA* and *dinF* may prevent the synthesis of components of multidrug efflux pumps reducing virulence due to an inability to dispose of toxic compounds the pathogen may encounter (Brown *et al.*, 2007). Evidence for the synthesis and use of phytohormones by *R. solanacearum* for disease progression in host plants has begun to emerge. The pathogen produces and secretes ethylene in Arabidopsis where ethylene production is controlled by the HrpG regulon (Valls *et al.*, 2006). *R. solanacearum* derived ethylene is able to manipulate host defence responses and can therefore subdue plant resistance and promote virulence (Valls *et al.*, 2006). It is known that ethylene signalling is involved in wilt disease symptom development (Hirsch *et al.*, 2002). Auxin and cytokinin may also contribute to virulence but this mechanism is not fully understood (Genin and Denny, 2012).

1.3.3 Regulation of *R. solanacearum* virulence

R. solanacearum virulence pathway pathogenicity factors are regulated by a complex, inter-connected network that responds to environmental stimuli such as plant signals and bacterial density as well as internal triggers (Genin and Denny, 2012). At the centre of this complex regulatory network is the PhcA regulon. PhcA is a LysR-type family transcriptional regulator that is able to respond to the cell density of bacteria in the surrounding environment to cycle the phenotypic state of the pathogen from one of survival to a state of virulence. Levels of PhcA are modulated by the autoinducer 3-hydroxy palmitic acid methyl ester (3-OH PAME) which is a quorum-sensing signal

(Fig 1.1) (Schell, 2000). PhcB is a methyltransferase protein that appears to play a role in the synthesis of 3-OH PAME. In a situation of high cell density, such as colonization in the vascular system at later stages of infection, PhcS and PhcR form a two-component system that reacts to the high concentration of 3-OH PAME in the surrounding environment thereby increasing levels of PhcA (Fig 1.1 A). PhcS and PhcR reduce levels of PhcA in the absence of 3-OH PAME. In the presence of 3-OH PAME, PhcS appears to phosphorylate PhcR, abolishing its function as a repressor of PhcA production (Schell, 2000). PhcA in turn regulates the expression of virulence factors such as EPS and CWDE's (Fig 1.1 A). This regulatory system is encoded by the *phcBSR* operon (Genin and Denny, 2012). *prhIRJ* expression is repressed by PhcA and this results in the suppression of HrpB-regulated genes (Yoshimochi *et al.*, 2009). During the initial stages of infection, however, when *R. solanacearum* is present in the soil and in the beginning stages of *in planta* colonization, the bacterial density is low, PhcA production is suppressed and invasion and survival factors are activated. The expression of HrpB is induced in response to plant signals and not repressed by high PhcA levels leading to the activation of the T3SS (Fig 1.1 B).

Additional secondary regulatory systems make up the Phc network. PhcA and the VsrAD two-component system are required to induce the transcription of *XpsR*. Both *XpsR* and *VsrC* promote EPS gene expression (Fig 1.1 A). PhcA suppresses the expression of the PehSR two-component system encoded by the *pehSR* operon. PehS and PehR are responsible for the expression of *pehA* as well as the *fli* genes and *pilA* which play a role in swimming and twitching motility (Fig 1.1 B) (Genin and Denny, 2012).

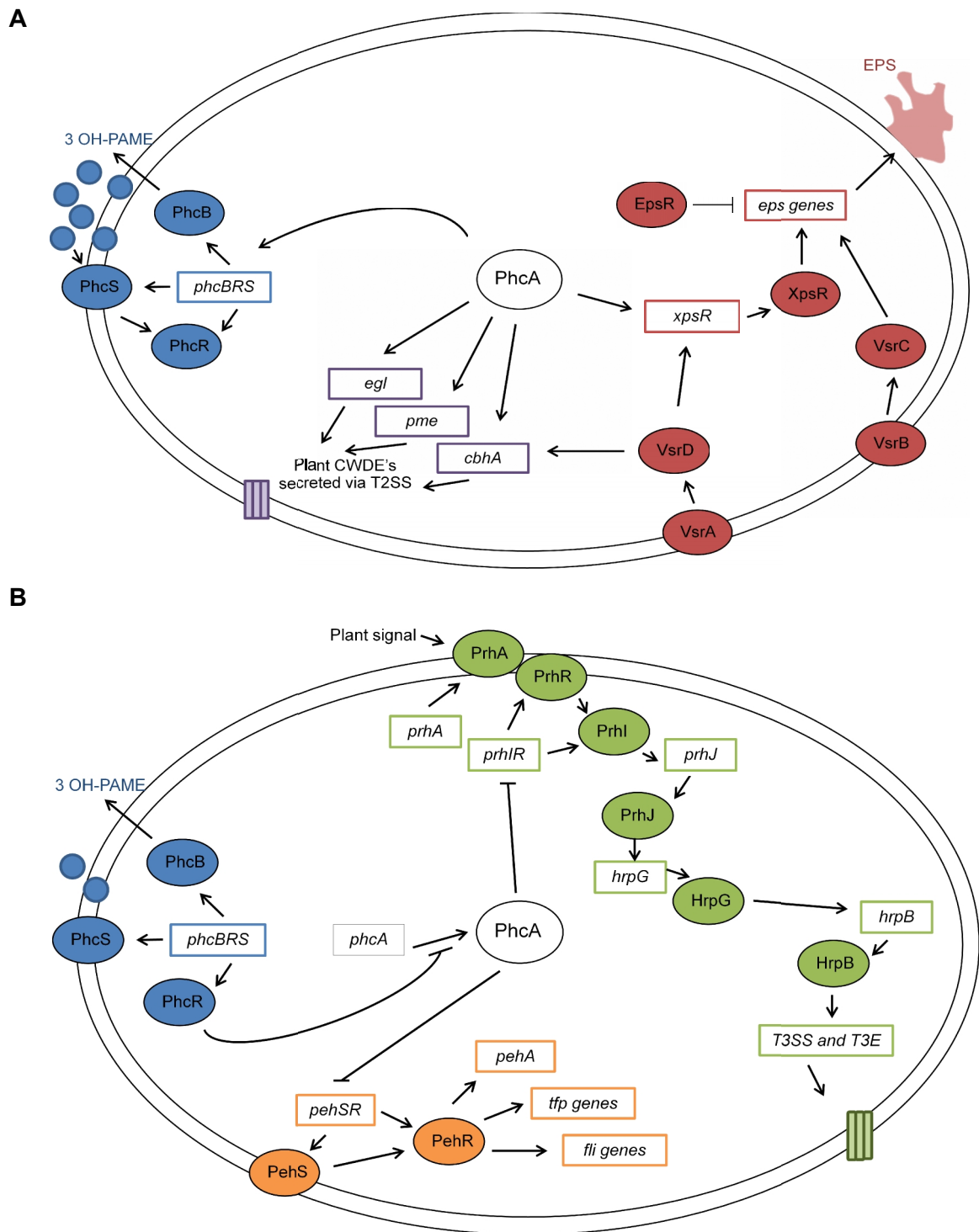


Figure 1.1. The *R. solanacearum* virulence and pathogenicity regulatory networks (Adapted from Genin and Denny, 2012). **A.** Traits that are positively regulated by PhcA. **B.** Traits that are negatively regulated by PhcA. Blunt end connections represent transcriptional or post-transcriptional repression.

1.3.4 The Type 3 secretion system

R. solanacearum deploys its type 3 secretion system machinery to deliver type 3 secreted effectors (T3E) or virulence proteins directly into plant host cells. The hypersensitive response and pathogenicity (*hrp*) genes encode the components and effectors of the T3SS and elicits a hypersensitive response on non-host plants (Poueymiro and Genin, 2009). The T3SS forms a pore complex that spans the inner and outer membranes of the bacterium. A Hrp pilus is formed that extends to the plant cells (Büttner and Bonas, 2006). The function of the T3SS is to deliver T3E proteins into plant cells. Once inside the plant cells, the effectors target plant mechanisms to suppress plant defences and obtain nutrients (Genin and Boucher, 2002). The HrpB regulon is a transcriptional regulator that controls the expression of the T3SS and T3E (Cunnac *et al.*, 2004). The *hrp_{II}* box (TTCG-n16-TTCG) element identified in the promoters of the HrpB-regulated genes is responsible for HrpB-dependent gene expression (Cunnac *et al.*, 2004). A study revealed that HrpB controls the regulation up to 180 genes in media (Occhialini *et al.*, 2005).

During pathogenesis, HrpB is activated downstream of the Prh regulatory pathway (Fig 1.1 B). The Prh cascade consists of the outer membrane receptor PrhA (Yoshimochi *et al.*, 2009) as well as the PrhIRJ proteins (Genin and Denny, 2012). Upon physical interaction with the plant cell, the PrhA receptor perceives the plant cell signal and activates the prh regulatory cascade which includes the membrane protein PrhR and transcriptional activators PrhI, PrhJ, HrpG and HrpB (Büttner and Bonas, 2006; Hikichi *et al.*, 2007; Yoshimochi *et al.*, 2009). The nature of the plant signal recognized by PrhA is not known, but it may be a nondiffusible molecule that is a component of the plant cell wall. Expression of the *hrp* genes are then induced by the HrpB regulon (Fig 1.1 B) (Hikichi *et al.*, 2007).

1.3.5 Type 3 effectors of *R. solanacearum*

Phytopathogenic bacteria T3E's contribute to the repression of host defence responses (Poueymiro and Genin, 2009). The T3SS appears to be involved in many different parts of the bacterial infection process including the colonization and proliferation of the pathogen within the vascular system (Poueymiro and Genin, 2009). To date, 74 T3E have been revealed from *R. solanacearum* strain GMI1000. A further 20 have been added due to genomic sequencing of other *R. solanacearum* strains (Molk2, UW551 and IPO1609). A large number (>30) of the T3E's that have been identified to date exist in all sequenced *R. solanacearum* strains (Genin and Denny, 2012). *R. solanacearum* strains encode T3E's belonging to several gene families, each consisting of 3-7 members. Interestingly, nearly half of all known T3E's appear to be specific to the RSSC (Genin and Denny, 2012).

One such family of T3E that contribute to the virulence of *R. solanacearum* on host plants are the *GALA* genes. The *GALA* effectors have a leucine-rich repeat (LRR) and an F-box domain. They are employed by *R. solanacearum* to suppress host defence by subduing or mimicking host cellular components and to obstruct the ubiquitin/proteasome pathway (Angot *et al.*, 2006). *R. solanacearum* effectors interact with the plant SKP1-like proteins that form part of SCF complexes. *GALA*'s recruit plant proteins for SCF-type ubiquitination and subsequent degradation (Angot *et al.*, 2006). The *GALA* family of effectors contribute significantly to virulence and this was confirmed when the knocking out of all 7 *GALA* genes significantly decreased virulence of GMI1000 on *Arabidopsis* and tomato lines. T3E have also been indicated to contribute to host range. Indeed, *GALA7* seemed to be important for GMI1000 infection and disease progression on *Medicago truncatula* (Angot *et al.*, 2006).

Certain effectors can be detected by host plants. Plants have evolved resistance (R) proteins that recognize pathogen effectors or "avirulence" proteins and trigger a T3E-dependent defence response (Poueymiro and Genin, 2009). This type of recognition

often leads to localized cell death known as the hypersensitive response (HR). According to Hu *et al.* (2008), an HR was not observed in *Arabidopsis* roots during an *R. solanacearum* infection when PopP2 recognition and plant resistance induction was mediated by RRS1-R (Hu *et al.*, 2008). Three *R. solanacearum* “avirulence” proteins have been characterized to date including AvrA, PopP1 and PopP2. The GMI1000 AvrA protein activates a HR in tobacco species (Carney and Denny, 1990). PopP1 is essential for pathogenicity on petunia and PopP2 on *Arabidopsis* (Lavie *et al.*, 2002, Deslandes *et al.*, 2003).

PopP2 belongs to the YopJ/AvrRxv family of effectors. When this *R. solanacearum* GMI1000 avirulence protein is recognized by the R protein RRS1-R in *Arabidopsis* ecotype Nd1, a resistant defence response is activated (Deslandes *et al.*, 2003). RRS1-R is a member of the TIR-NB-LRR class of R proteins and it possesses a C-terminal WRKY domain. PopP2 and RRS1-R colocalize in the plant nucleus where these two proteins interact physically for the induction of an RRS1-R-mediated resistance response (Deslandes *et al.*, 2003; Tasset *et al.*, 2010). RRS1-R requires both a physical interaction with PopP2 as well as PopP2 auto-acetyltransferase activity for defence activation to occur (Tasset *et al.*, 2010). PopP2 was found to physically associate with the host component, RD19, which is an *Arabidopsis* cysteine protease. The PopP2 - RD19 complex is formed in the plant nucleus where it is needed for RRS1-R-mediated resistance (Bernoux *et al.*, 2008).

1.4 *Arabidopsis* as a model for phytobacteriology

Arabidopsis is one of the most extensively studied plants with diverse ecotypes collected from all over the world (Glazebrook *et al.*, 1997). The *Arabidopsis* genome of accession Col-0 was sequenced in 2000, promoting genetic studies using this model organism. The genome contained 125 megabases of sequence consisting of approximately 30 000 genes (TAIR, version 8) encoding for proteins in more than 11 000 families

(www.arabidopsis.org). Arabidopsis is an annual plant that grows to approximately 20-25 cm tall and is a member of the mustard family (Brassicaceae). There are numerous advantages of using Arabidopsis as a model plant organism (Meinke *et al.*, 1998). Extensive genomic resources of Arabidopsis are readily available. Its short generation time (6-8 weeks) makes Arabidopsis ideal for lab experimentation. It can be self-pollinated to produce thousands of seed per plant, allowing mutant lines to be produced with ease. Arabidopsis is also easily transformed by *Agrobacterium*-mediated transformation (Meinke *et al.*, 1998).

Pathosystems have been invaluable in the study of plant-pathogen interactions. *Pseudomonas syringae* is a gram-negative bacterium that afflicts a large number of hosts. Different strains of *P. syringae* are known for their high host specificity (Nishimura and Dangl, 2010). One of the first pathogens found to infect *Arabidopsis thaliana* was the bacterial pathogen *Xanthomonas campestris* pv. *campestris* which marked the beginning of pathogen interaction studies. The discovery that another pathogen, the obligate biotroph powdery mildew (*Erysiphe cichoracearum*), infects Arabidopsis has been important in the genomics era (Nishimura and Dangl, 2010). Powdery mildew is host-adapted and it has proven important to compare their virulence strategies to those pathogens of wider host range. These three Arabidopsis pathosystems have significantly furthered the discovery of disease resistance mechanisms and pathways (Nishimura and Dangl, 2010).

The number of pathogens that infect the model organism Arabidopsis has resulted in an attempt to classify pathogens into generalized groups. Arabidopsis pathogens have been classified into necrotrophs, biotrophs and less commonly, hemibiotrophs (Oliver and Ipcho, 2004). In summary, biotrophs obtain nutrients from living host cells and are mediated by the SA-dependent signalling pathways. Necrotrophs kill host tissue to obtain their energy and are controlled by the JA and ET-dependent signalling pathways. Hemibiotrophs are pathogens that live initially in a biotrophic manner followed by a necrotrophic lifestyle (Hammond – Kosack and Parker, 2003; Oliver and Ipcho, 2004).

Although these groups are used frequently by plant pathologists, many pathogens are classified as having more than one mode of infection. The classification of pathogens has evolved over the years but it is clear that plant pathogens employ various and diverse infection strategies to obtain nutrients from their hosts (Oliver and Ipcho, 2004). These range from physical attacks “brute force” attacks using cell wall degrading enzymes that result in damage and death of host plant cells. More subtle “stealth” mechanisms include the manipulation and repression of plant defence responses through the secretion of effectors via the T3SS (Toth and Birch, 2005).

1.5 Plant immunity

Plants encounter a variety of pathogens in their natural environment including bacteria, fungi, viruses, nematodes and oomycetes (Pieterse *et al.*, 2009). Plants have developed two approaches to recognize and respond to pathogen attack (Jones and Dangl, 2006). On the outer cell surface of the host plant cell, pattern recognition receptors (PRR's) are able to detect conserved components of microbial pathogens called pathogen associated molecular patterns (PAMP's). PAMP's are generally essential constituents found in a large group of phytopathogens such as flagellin for bacteria, chitin for fungi, glycoproteins and liposaccharides. Recognition of PAMP's by the plant cell leads to signal transduction and elicitation of a basal resistance called PAMP-triggered immunity (PTI). Phytopathogens in return are able to deliver effector proteins directly into the host cell and these intracellular effectors often interfere with PTI. Effectors enhance the virulence of the pathogen and help the pathogen overcome basal resistance or PTI (Pieterse *et al.*, 2009).

Plants evolved a second mechanism of pathogen recognition, where intercellular plant receptors known as resistance (R) proteins perceive the virulence effector molecules and initiate an effector-triggered immunity (ETI). A co-evolutionary arms race exists between the plant and pathogen whereby the pathogen evolves new, variable and expendable effectors to subdue the plant defence and the plant evolves R proteins to detect them

(Dodds and Rathjen, 2010). Interestingly, PTI and ETI induce similar defence responses, but ETI elicits a stronger and faster response often resulting in a hypersensitive response (Dodds and Rathjen, 2010).

The inability of pathogens to infect their host plant is referred to as the “incompatible” or resistant interaction, which can be divided into two classes, namely non-host resistance and host-specific resistance (Hammond-Kosack and Parker, 2003; Thatcher *et al.*, 2005). Non-host resistance involves resistance factors developed against all races of a particular pathogen (Thatcher *et al.*, 2005). This type of immunity depends on both preformed structures as well as a basal defence activated through the perception of the host to microbial elicitors (Gimenez-Ibanez and Rathjen, 2010). In contrast, host-specific resistance involves host receptors that recognize effectors of the invading pathogen to elicit a resistant or tolerance response and are dependent upon a particular pathogen race (Thatcher *et al.*, 2005). A “compatible” or susceptible response, however, is when the invading pathogen can effectively colonize and cause disease on the host plant. The pathogen does this by actively suppressing host defences (Gimenez-Ibanez and Rathjen, 2010).

PTI is active against non-specific pathogens and induces a non-host resistance, whereas ETI is effective against strain-specific pathogens (Dodds and Rathjen, 2010). Investigating the plant-pathogen interaction requires an integrated approach with studies focusing on how the plant immune system is able to perceive and induce an appropriate defence response against pathogens together with studies on how phytopathogens are able to manipulate and subvert the defence response through the use of effectors to eventually cause disease (Dodds and Rathjen, 2010).

1.5.1 Phytopathogen recognition by pattern recognition receptors to initiate PTI

Pattern recognition receptors (PRR's) can belong to one of two main classes, transmembrane receptor kinases (RK) and transmembrane receptor-like proteins (RLK). PRR's generally contain an extracellular LRR domain that serves as the recognition component and an intracellular kinase domain (Jones and Dangl, 2006). The Arabidopsis genome contains approximately 610 members of the RK gene family and 57 members of the RLK class of receptors (Dodds and Rathjen, 2010).

One of the most widely understood recognition events of PAMP's by PRR's is in the case of bacterial flagellin. Flagellin is a component of the bacterial flagella, the organ of motility (Gimenez-Ibanez and Rathjen, 2010). Arabidopsis receptor kinase Flagellin Sensing 2 (FLS2) perceives the 22 amino acid peptide FL22 which forms part of a conserved flagellin domain (Zhou and Chai, 2008) and downstream signalling is activated. Many PRR's interact with Brassinosteroid Insensitive 1-Associated Kinase-1 (BAK1), a LRR receptor kinase to induce a defence response (Dodds and Rathjen, 2010). BAK1 plays a central role in plant defence. BAK1 belongs to the somatic embryogenesis receptor kinase (SERK) family of receptors and is also referred to as SERK3 (Dodds and Rathjen, 2010). BAK1 appears to have an indirect role in PTI activation. After PAMP perception, the FLS2 receptor immediately dimerizes with BAK1 and both proteins in this complex are phosphorylated resulting in signal transduction. BAK1 probably also plays a role in the recognition of other PAMP's by associating with additional PRR's in the LRR-receptor kinase family (Dodds and Rathjen, 2010; Schulze *et al.*, 2010).

Botrytis-induced kinase 1 (BIK1), a cytoplasmic protein kinase, has been shown to mediate signalling of the FLS2-BAK1 complex. The function of BIK1 in plant immunity is not fully understood however defence signalling via the FLS2-BAK1 complex may be mediated directly by BIK1. It appears that dual transphosphorylation between the FLS2/BAK1 complex and the interacting molecule BIK1 leads to certain conformational

changes which finally results in the phosphorylation and dissociation of BIK1 from the FLS2/BAK1 complex and subsequent induction of the PTI signalling cascade (Lu *et al.*, 2010; Wang, 2012; Belkhadir *et al.*, 2012; Muthamilarasan and Prasad, 2013). Defence responses are mediated by the well characterized mitogen-activated protein kinases (MAPK's) in a MAP kinase signalling pathway (Dodds and Rathjen, 2010).

One of the components of plant immunity that has been investigated extensively is MAPK signalling. A MAPK cascade which is triggered by PRR recognition of PAMP molecules typically consist of MAPK kinase kinases (MAPKKK) which phosphorylate MAPK kinases (MAPKK), which in turn activates MAPK's downstream. These pathways result in the activation of numerous regulatory molecules including transcription factors (Asai *et al.*, 2002). MAPK signalling plays a central role in both PTI and ETI. One well-established kinase signalling example, is the MAPK cascade that is stimulated as a result of flagellin receptor recognition. It consists of the MAPKK's, MKK4 and MKK5, that transfer a signal to downstream MAPK's, MPK3 and MPK6, and ultimately leads to the induction of WRKY transcription factors and defence gene expression (Asai *et al.*, 2002). An alternative cascade which is simultaneously activated by flg22 recognition consists of MEKK1, MKK1, MKK2 and MPK4 with MEKK1 acting at the beginning of the pathway (Suarez-Rodriguez *et al.*, 2007; Gao *et al.*, 2008).

1.5.2 Effector recognition

Perception of effectors is mediated by a group of recognition proteins commonly known as resistance (R) proteins. The largest class of R proteins are intracellular receptors that consist of a central nucleotide binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain (Rafiqi *et al.*, 2009). R genes confer resistance to a variety of pathogens such as fungi, oomycetes, bacteria, viruses and insects (Rafiqi *et al.*, 2009). The plant R proteins can be further divided into two subclasses based on their variable N-terminal domain. R proteins can contain a Toll interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain at the N-terminal end (Caplan *et al.*, 2008; Dodds and Rathjen, 2010).

1.5.2.1 Direct and indirect recognition strategies

R proteins can perceive pathogen effectors through either direct or indirect strategies. In direct recognition, the R protein receptors bind physically to the effector molecule triggering an immune signalling response (Fig 1.2 A). Yeast two-hybrid (Y2H) assays have been used to describe direct recognition such as the interaction between the rice CC-NB-LRR Pi-ta protein and the *Magnaporthe grisea* effector AvrPita (Jia *et al.*, 2000). The flax TIR-NB-LRR L and M proteins recognize and bind to the *Melampsora lini* fungal effectors AvrL567 and AvrM, respectively (Dodds *et al.*, 2004; Catanzariti *et al.*, 2010).

In an indirect recognition mechanism, host accessory proteins are perceived and altered by pathogen effectors and these are perceived by the R protein (Dangl and Jones, 2001). Accessory proteins may be molecules that pathogen effectors target and manipulate to promote virulence or they may be mimics of effector targets that plants have evolved to act as decoys for effector modification (Muthamilarasan and Prasad, 2013). Three variations exist that describe these mechanisms. In the “guard” model, R proteins guard an accessory protein (guardee) that is perceived or altered by a pathogen effector (Fig 1.2 B). This model describes the Arabidopsis “guardee” protein, RIN4 (RPM1 interacting protein 4). RIN4 interacts exclusively with R proteins RPM1 and RPS2 (Resistance to *Pseudomonas Syringae* 2) to form active complexes (van der Hoorn and Kamoun, 2008; Dodds and Rathjen, 2010).

Arabidopsis RIN4 is targeted by three effectors from *P. syringae* which results in the induction of RPM1 and RPS2 and subsequent immune signalling. The cysteine protease effector AvrRpt2 degrades RIN4 which activates RPS2, while the AvrRPM1 and AvrB effectors promote phosphorylation of RIN4, which then activates the RPM1 immune response (Caplan *et al.*, 2008; Rafiqi *et al.*, 2009). However, to coincide with the “guard” model, RIN4 is required to be a target of pathogen virulence. It is proposed that RIN4 is an inhibitor of defence signalling and so pathogen effectors potentially target RIN4 and

enhance its function in the inhibition of the plants defence response (Caplan *et al.*, 2008; Rafiqi *et al.*, 2009).

In the decoy model, the accessory protein is modified by an effector resulting in R protein recognition. However, in this case, the accessory protein is a mimic of an effector virulence target acquired through evolution (Fig 1.2 C). The R protein Prf constitutively associates with the accessory “decoy” protein Pto kinase in tomato. Pto kinase is highly related to specific domains contained in AvrPto and AvrPtoB effector targets, FLS2 and CERK1 (Zipfel and Rathjen, 2008; Gimenez-Ibanez *et al.*, 2009). Thus, the *P. syringae* effector AvrPto is able to disrupt the Pto – Prf complex which allows Prf to activate a defence response (Mucyn *et al.*, 2009; Dodds and Rathjen, 2010). The bait-and-switch model has also been described. An effector initially associates with the accessory or “bait” protein, and this interaction facilitates subsequent direct recognition of the effector by the R protein to induce defence responses (Fig 1.2 D) (Collier and Moffett, 2009; Dodds and Rathjen, 2010).

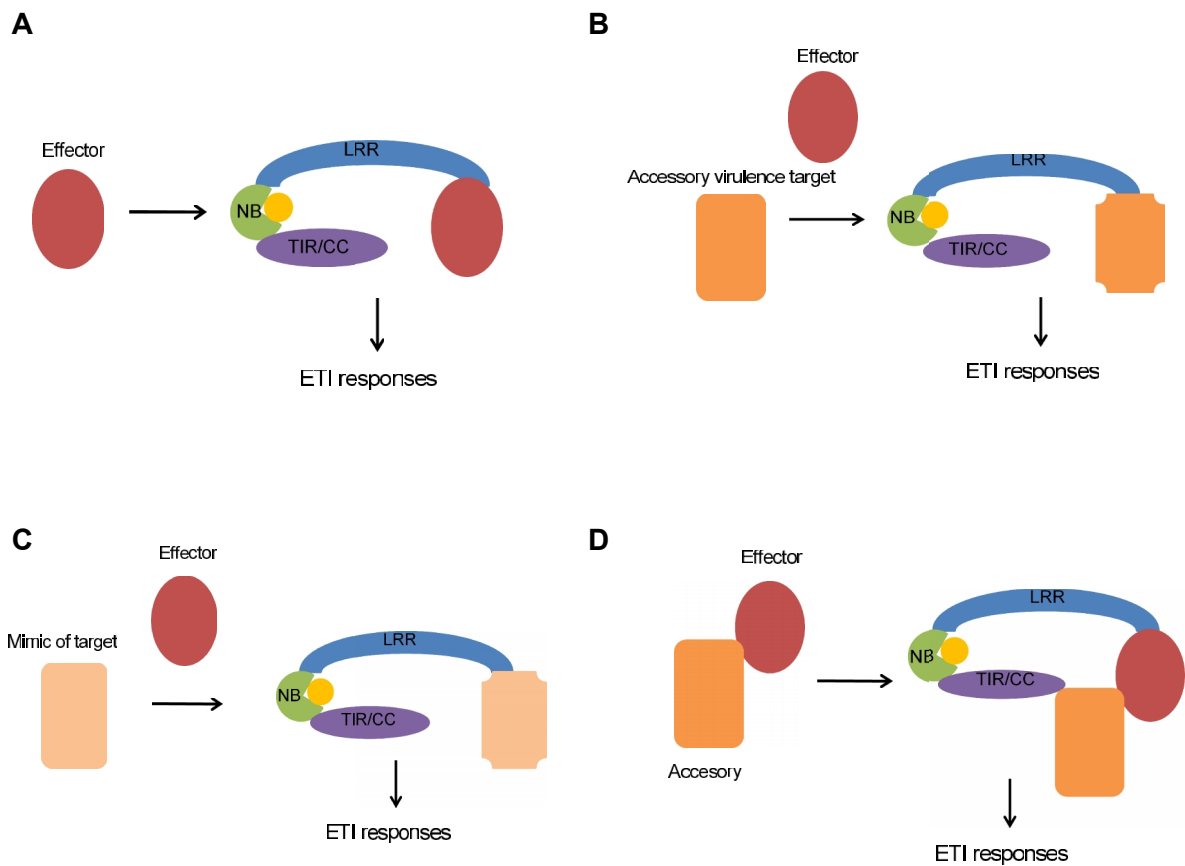


Figure 1.2. **Direct and indirect effector recognition strategies (Adapted from Dodds and Rathjen, 2010).** R proteins can perceive pathogen effectors through direct and indirect modes. **A.** In the direct model, R proteins interact physically with effector molecules (blue, green, yellow and purple) to induce defence responses. **B.** In the guard model, R proteins perceive accessory proteins that have been altered by a pathogen effector to induce defence responses. The accessory molecule in this case is a virulence target of the pathogen effector. **C.** In the decoy model, R proteins perceive accessory proteins that have been altered by a pathogen effector to induce defence responses. The accessory molecule in this case is a mimic of an effector virulence target. **D.** In the bait-and-switch model, an effector associates with an accessory protein before direct binding by the R protein and defence induction.

While these conceptualized models provide a basis of the mode of effector recognition, it is clear that they do not sufficiently represent all mechanisms of effector perception (Dodds and Rathjen, 2010). Other unique recognition mechanisms also exist. Effector AvrPto utilizes its E3 ligase activity to target specific plant defence proteins for ubiquitination and degradation, such as the tomato protein Fen. Pto kinase together with its interacting component Prf recognizes and phosphorylates AvrPto which in turn inactivates its E3 ligase degradation ability. The host plant is therefore able to disrupt the pathogen's disease development (Ntoukakis *et al.*, 2009). There have also been cases that have been reported recently where two R proteins are necessary for the perception of specific pathogen effectors and defence signalling activation. The R genes *RPS4* and *RRS1* (Resistance to *Ralstonia solanacearum* 1), are organized in a tandem composition within the major recognition gene complex MRC-J on chromosome 5 in Arabidopsis (Narusaka *et al.*, 2009). RSP4 has been shown to recognize the effector AvrRsp4 of *P. syringae* and RRS1 confers resistance to *R. solanacearum* by perceiving effector PopP2. New evidence has demonstrated that both proteins need to exist for dual pathogen effector perception of these effectors and for the induction of defence responses against the fungus *Colletotrichum higginsianum* (Narusaka *et al.*, 2009). There are numerous other cases of a tandem configuration in the Arabidopsis genome where two R proteins are required to induce resistance (Dodds and Rathjen, 2010).

1.5.2.2 R protein activation

Generally, R proteins are conserved nucleotide-controlled molecular switches that translate varied pathogen signals into an appropriate defence response. The process of cycling from a state of R protein inhibition to a state of R protein activation needs to be firmly controlled to limit the unnecessary use of plant resources (Takken and Tameling, 2009). R proteins may be autoinhibited, where intramolecular forces between the domains of the R protein restrain the protein in an inactive form until an effector or accessory protein disrupts it. The NB-ARC (nucleotide binding adapter shared by APAF-1, R proteins and CED-4) domain present in most R proteins consists of the NB domain as well as the ARC1 and ARC2 subdomains (Takken and Tameling, 2009). An interaction between the

N-terminal part of the LRR domain and the ARC2 subdomain are vital in holding R proteins in an inactive state. Pathogen effector recognition may result in an exchange of ADP (ADP-bound “OFF” state) for ATP (ATP-bound “ON” state) resulting in the disruption of the LRR-ARC interaction and the activation of plant immune signalling (Takken and Tameling, 2009). ATP hydrolysis restores the R protein to its ADP-bound “OFF” state (Takken and Tameling, 2009).

1.5.3 Immune signalling pathways and downstream responses

Various induced responses and cellular events are initiated during PTI and ETI after pathogen recognition. These include the drastic influx of calcium ions into the cytoplasm, the production of reactive oxygen species (ROS), mitogen-activated protein (MAP's) kinase signalling, the strengthening of the cell wall at sites of infection through the production of callose and lignin, expression of pathogenesis-related (PR) proteins and often a hypersensitive response (Pieterse *et al.*, 2009). Investigating the signalling responses that are induced downstream of PAMP or effector recognition is important in understanding the complete immune signalling network (Dodds and Rathjen, 2010).

1.5.3.1 ETI signalling pathways

Only a small number of signalling proteins have been discovered in the effector-triggered immune response. Enhanced disease susceptibility 1 (EDS1) interacts with all TIR-NB-LRRs to elicit ETI signalling and the non-race-specific disease resistance 1 (NDR1) interacts with the CC-NB-LRR class of R proteins to induce the ETI pathway (Zhou and Chai, 2008). There has been a distinct lack of success in identifying signalling components in the ETI pathway. However, it has been demonstrated that some R proteins relocate to the nucleus upon effector recognition and associate with components in the nucleus to elicit defence responses (Zhou and Chai, 2008). The tobacco N protein, barley MLA10 protein and Arabidopsis RPS4 and RRS1-R proteins localize to the plant nucleus upon recognition (Deslandes *et al.*, 2003; Wirthmueller *et al.*, 2007; Caplan *et al.*, 2008).

Of interest will be to determining the importance of R protein nuclear directed relocalization in signalling induction.

1.5.3.2 Hormone signalling

Hormone signalling is a crucial component in the complex network of plant-pathogen interactions. The salicylic acid (SA) and jasmonic acid (JA) – ethylene (ET) hormone pathways act downstream of ETI and PTI and are central regulators of defence gene expression and immune signalling (Bari and Jones, 2009). These pathways often interact with one another through crosstalk, resulting in antagonistic or cooperative interactions between the individual pathways (Derksen *et al.*, 2013). The SA-dependent and JA/ET-dependent signalling pathways generally act antagonistically, with SA induced in response to biotrophs and the JA/ET pathway involved in resistance against necrotrophic pathogens. These pathways result in the expression of certain pathogenesis related genes (PR) that often act as specific markers for hormone signalling pathway induction. There is also a substantial amount of overlap between these pathways (Tsuda *et al.*, 2009). Major progress has been made recently in understanding the role of SA, JA and ET as well as other hormones such as abscisic acid (ABA), auxin, cytokinins and gibberellic acid and signalling pathways in the plant defence response (Bari and Jones, 2009; Pieterse *et al.*, 2009). A number of papers have been published recently that extensively review the complex network of interacting signalling pathways and their role in the resistance to pathogens (Pieterse *et al.*, 2012; Cui and Luan, 2012; Denance *et al.*, 2013; Derksen *et al.*, 2013).

1.6 The Compatible Interaction

Phytopathogenic microorganisms have evolved numerous virulence strategies such as toxins, enzymes, hormones, polysaccharides and T3E's to survive in diverse external environments as well as within host plants. For a phytopathogen to invade the host plants

they come into contact with, they must overcome numerous pre-existing structural barriers and antimicrobial precursors to persist and thrive in host plants. Pathogens also have to overcome the plant cell wall and to suppress host defence responses to obtain nutrients they require from the plant. Bacteria utilize their T3SS to penetrate both the plant cell wall and plasma membrane and deliver effectors directly into the cytoplasm of plant host cells (Gohre and Robatzek, 2008). Once the pathogen has overcome these structural and chemical obstacles, plants are able to recognize pathogenic bacteria and induce defence responses such as PTI and ETI. Pathogen effectors manipulate and inhibit plant defences at the levels of perception, signalling and the defence response in both the PTI and ETI pathways (Zhou and Chai, 2008).

1.6.1 Bacterial type 3 effectors suppress PTI, ETI and manipulate hormone signalling

PTI is one of the signalling cascades that are targeted for bacterial manipulation. Numerous studies have shown that several components of PTI are suppressed by bacterial effectors (Zhou and Chai, 2008). AvrPto is a kinase inhibitor that associates directly with several PAMP receptors, including FLS2 to repress induced defence responses and promote susceptibility to the pathogen (Xing *et al.*, 2007; Xiang *et al.*, 2007). Another bacterial effector that manipulates regulatory components of the hosts PTI response is HopAI1. HopAI1 targets Arabidopsis MAP kinases, MPK3 and MPK6, to inhibit PTI signalling (Zhang *et al.*, 2007). RAR1, a negative regulator of PTI, is required for AvrB-dependent inhibition of PTI (Shang *et al.*, 2006). AvrB promotes pathogen virulence by suppressing flg22-induced deposition of callose in the host plant (Shang *et al.*, 2006). AvrB has also been shown to induce jasmonate signalling in Arabidopsis (He *et al.*, 2004). HopUI displays ADP-ribosyltransferase (ADP-RT) activity and is able to ADP-ribosylate several Arabidopsis host proteins at specific arginine residues. Biochemical analysis demonstrated that HopUI was able to ribosylate the Arabidopsis RNA-binding protein GRP7. GRP7 has been shown to contribute to plant immunity. When HopUI specifically ribosylates GRP7 at two arginine residues, its RNA-binding ability is

inhibited and pathogen susceptibility in the plant is increased (Fu *et al.*, 2007; Feng and Zhou, 2012).

AvrPtoB has evolved an interesting mechanism to suppress ETI in tomato host plants. The AvrPtoB “avirulence” effector is recognized by Fen kinases in the tomato host to elicit an ETI response. The carboxy terminal domain of AvrPtoB is an E3 ubiquitin ligase. When Fen perceives and interacts with AvrPtoB, it is targeted for ubiquitination and subsequent degradation. ETI induction is therefore blocked in the tomato plant (Rosebrock *et al.*, 2007). HopMI interacts with Arabidopsis proteins and appears to disrupt them using its proteasome/degradation pathway. One of these proteins, AtMIN7 is important for vesicle trafficking. AtMIN7 appears to play a role in defence only in the presence of other effectors which suggests that it is involved in ETI. AtMIN7 interference by HopMI appears to inhibit ETI (Nomura *et al.*, 2006; Feng and Zhou, 2012).

Another manner of host manipulation discovered involves effector molecules that function as transcription factors to elicit the expression of genes that promote pathogen virulence. *Xanthomonas* spp. encode a host of AvrBs3/PthA effectors that consist of a nuclear-localization sequence (NLS), an acidic transcriptional activation domain, and a DNA binding leucine-rich repeat domain (da Cunha *et al.*, 2007). The AvrBs3 effector from *X. campestris* pv. *vesicatoria* binds directly to a particular promoter element in the *upa20* gene in the nucleus of pepper plants to induce its expression which may act to increase the susceptibility of the host to pathogen infection and colonization (da Cunha *et al.*, 2007; Kay *et al.*, 2007; Zhou and Chai, 2008).

A few studies have demonstrated bacterial effectors can alter plant hormone production to promote pathogen virulence. AvrPtoB has been shown to activate ethylene-biosynthesis genes in tomato which promoted *P. syringae* infection and disease severity (Cohn and Martin, 2005). In Arabidopsis, AvrPtoB enhanced pathogen virulence and growth by manipulating the production of abscisic acid (ABA) (Truman *et al.*, 2006; de Torres-Zabala

et al., 2007). Another *P. syringae* effector, AvrRpt2, was shown to promote auxin production in *Arabidopsis* to enhance susceptibility to this pathogen (Chen *et al.*, 2007).

1.7 The Tolerant Interaction

When a phytopathogen infects a host plant, three different interactions can occur. They are resistance, susceptibility and tolerance. Resistance is broadly defined as a state in which pathogen colonization is very limited (Robb, 2007). Host traits that result in resistance are those that limit pathogen contact with the host and those that reduce pathogen growth *in planta* (Kover and Schaal, 2002). Susceptibility occurs when the pathogen spreads extensively through the plant and results in disease development and often plant death (Robb, 2007). The interplay between plant recognition, signalling and immune induction and the pathogen's ability to suppress host defences will result in one of these outcomes. Tolerance, in general, is defined as the host plant's ability to sustain or tolerate pathogen infection without a reduction in plant fitness (Kover and Schaal, 2002). Tolerant and resistant interactions have been well studied in terms of plant-insect interactions (Strauss and Agrawal, 1999; Leimu and Koricheva, 2006; Schmidt and Baldwin, 2009). By comparison, the importance of tolerance in plant-pathogen interactions has been the focus of little investigation and important questions regarding its mechanisms and evolution are still not clearly understood (Gao *et al.*, 2009).

1.7.1 Defining and measuring tolerance

The concept of tolerant plant-pathogen interactions have been seen in the literature for many years (Robb, 2007). One of the first comprehensive definitions of tolerance was stated by J. F. Schafer (Schafer, 1971). He said that "Tolerance may be defined as the capacity of a cultivar resulting in **less yield** or **quality** loss relative to **disease severity** or **pathogen development** when compared with other cultivars or crops". This definition

highlights some key aspects of tolerance. Certain parameters of quantification such as plant yield or level of disease need to be determined for tolerance (Robb, 2007). Also, it included both “tolerance to disease” and “tolerance to pathogen development” in the definition. This reduced the uncertainty surrounding the literature at the time, where either one or the other was studied. This definition also demonstrated the importance of determining tolerance through comparison, i.e. the reduction in yield must be compared in different cultivars or crops when a consistent amount of disease development or pathogen numbers *in planta* occurs (Robb, 2007).

Clarke (1986) endeavoured to expand on the terminology of tolerance. Clarke put forward three parts to tolerance i) tolerance of the parasite is “the ability of a plant to endure the effects of levels of parasitic infection, which, if they occurred at equivalent levels in other plants of the same or of similar species, would cause greater damage”; ii) tolerance of disease is “the ability of a plant to endure the effects of levels of disease, which, if they developed to equivalent levels in other plants of the same or of similar species, would cause greater impairment of growth or yield”; iii) overall tolerance is “the ability of a plant to endure the effects of levels of parasitic infection and disease, which, if they occurred at equivalent levels in other plants of the same or of similar species, would cause greater impairment of growth or yield”. A pathogen can be defined as a microorganism that is capable of causing disease on a host. A parasite is an organism that lives in or on another organism or host and benefits by exploiting the host for resources, often at the other’s expense. Clarke’s definition identified two additional aspects of tolerance. First that in tolerance, the level of reduction on plant yield or growth is dependent on both tolerance to the parasite as well as tolerance to disease but often one contributes more to overall tolerance than the other. Also, individual plants may be found to be tolerant (Robb, 2007).

Once we have an understanding of the terminology of tolerance, we are able to see which aspects need to be measured accurately to determine tolerance (Robb, 2007). Following these definitions, three aspects need to be quantified i.e. parasite biomass, disease symptom severity and yield reduction of host. Plant growth is often measured using stem

height, rosette diameter or fresh weight (Veronese *et al.*, 2003; Dietrich *et al.*, 2005; Robb *et al.*, 2007). Yield can be quantified using the number of fruits or mass of seed produced (Diener and Ausubel, 2005; Robb, 2007). Disease severity is often measured by scoring of disease symptoms or determining the amount of leaf tissue that display symptoms (Robb *et al.*, 2007). Quantification of pathogen biomass *in planta* is often problematic but plating out of plant tissue or whole plant material on selective media to determine the number of colony forming units (CFU's) per gram of ground plant tissue, is especially useful in small plants such as *Arabidopsis*. A quantitative PCR assay for identification and quantification is an accurate method for determining pathogen biomass. Primers based on pathogen-specific regions and internal controls provide a means for standardization to ensure this is a reliable method of pathogen quantification (Robb, 2007).

1.7.2 Evolution of tolerance

The evolution of parasite virulence and defence mechanisms is a subject of great importance to plant biology and agricultural studies. Research in this area has resulted in a substantial amount of mathematical and empirical theory (Carval and Ferriere, 2010). Host-parasite evolutionary models demonstrate that how virulent a parasite is depends upon trade-offs between the need to exploit host responses for rapid parasite reproduction (within-host reproduction) so that the parasite can be transmitted successfully from one host to another and the need to prevent plant mortality as a result of the parasite infection so that parasite infection and growth in the host can be prolonged (within-host survival) (Frank, 1996; Alizon *et al.*, 2009; Carval and Ferriere, 2010). The host has evolved two methods to protect itself from parasite attack. In resistance, the host prevents parasite development and infection by allocating resources into defence responses that result in a reduction in the fitness of the parasite. In tolerance, the host restricts the negative effects of infection and allocates resources into mechanisms that reduce the damage the parasite may cause (Boots, 2008; Carval and Ferriere, 2010).

Most models to date have focused on the coevolution of resistance with virulence or the coevolution of tolerance with virulence. The difference between resistance and tolerance

in terms of coevolution with virulence is important to understand. Tolerance does not have an adverse effect on the fitness of parasites. This means that if a parasite invades a tolerant host it will remain infectious for a longer period of time and this will elevate the pervasiveness of the parasite and its disease in a crop field (Carval and Ferriere, 2010). However, selection of resistance will reduce the fitness of the parasite and thus limit the occurrence of parasite infection. Quantitative models have been established to determine the cost and benefits for selection of tolerance (Miller *et al.*, 2005). The evolution of tolerance can drive elevated virulence (Restif and Koella, 2003; Miller, 2006). This has implications in agriculture and the application of tolerant crops to manage disease. Parasite selection in response to plant tolerance may result in an increased prevalence of the pathogen with a decreased, but still a considerable level of the disease occurring in crop plants in the area (Miller, 2006). To obtain a complete picture of host-parasite evolution, host-pathogen models that investigate plant-pathogen interactions need to include coevolution of virulence, resistance and tolerance in one unified model and to take into account ecological, epidemiological and environmental factors (Carval and Ferriere, 2010).

Recent studies have discovered that tolerance is a common outcome upon pathogen infection of host plants. Genetic variation in tolerance has also been observed in some studies (Kover and Schaal, 2002). A study on the natural variation of 19 *Arabidopsis* accessions inoculated with *P. syringae* indicate that the accessions vary in terms of tolerance and that tolerance significantly affects plant-pathogen coevolution (Kover and Schaal, 2002).

1.7.3 Mechanisms and genetic determinants of tolerance

The genetic control of tolerance has received little attention. Existing knowledge is that resistance in plant-pathogen interaction is a quantitative trait conferred by a single resistance (R) gene, whereas tolerance is conferred in a polygenic manner (Barret *et al.*, 2009). A recent study provided evidence for the involvement of an *R* gene in a tolerance

response in *Arabidopsis* against *P. syringae* strain DC3000 expressing the avirulence gene *AvrRpm1*. Isogenic lines and natural accessions of *Arabidopsis* where disease and fitness differences could be attributed directly to the presence of the *Rpm1* gene, provided novel evidence that an *R* gene could affect both tolerance and resistance (Roux *et al.*, 2010). *Arabidopsis Rpm1* mediates resistance at intermediate inoculum levels where there were significant differences in disease symptoms between resistant (contains the *Rpm1* allele) and susceptible (does not contain the *Rpm1* allele) accessions (Roux *et al.*, 2010). However, a fitness advantage of 46 – 77% was observed at inoculum levels where there was no significant difference in disease symptoms between the susceptible and resistant plants. This indicated that *Rpm1* was involved in a tolerance response. A potential mechanism for how *Rpm1* plays a role in tolerance is that *Rpm1* activates certain response pathways that require the reallocation of resources when *Arabidopsis* is infected at high doses which may cause a higher yield or growth rate of infected lines (Roux *et al.*, 2010). Tolerant plants infected with a virulent pathogen may be able to redirect resources or induce mechanisms that can increase plant fitness to the pathogen.

There have only been a few studies investigating the mechanisms of tolerance. Chen *et al.* (2004) showed that *Verticillium* tolerant “Craigella” tomatoes were effectively able to trap the pathogen in primary sites or locations in the stems of plants due to the plant’s vascular coating response, however were not able to eliminate fungal colonization that normally takes place in the resistant and susceptible interaction. Therefore, the pathogen multiplied to a very high concentration in the tolerant plant but *Verticillium* colonization was contained in initial sites and therefore did not result in symptom expression (Chen *et al.*, 2004). The reduction of ethylene production or sensitivity in some studies indicates that the lack of symptom development in tolerant plants can be a result of a reduction in ethylene biosynthesis after pathogen infection (Robison *et al.*, 2001; Mansoori and Smith, 2005).

An interesting pathosystem involving an interaction between the soil-borne vascular fungal pathogen *Verticillium dahliae* and the model plant *Arabidopsis* has been the focus of some

molecular investigation. A *V. dahliae* tolerance gene (*VET1*) was found to promote host tolerance (Veronese *et al.*, 2003). The authors proposed that the susceptibility seen in the *Arabidopsis* accession Col-0 was associated with accelerated life-cycle traits and senescence-like symptoms elicited by *V. dahliae* infection. Tolerance in the C-24 accession was the result of a fungal-induced interference of the shift from plant growth to flowering resulting in the interruption of chlorotic symptom development (Veronese *et al.*, 2003). Similar fungal numbers were observed in the susceptible and tolerant lines at 28 dpi. Thus, senescence-like symptom progression seen in the susceptible Col-0 accession was not associated with the high amount of fungal colonization *in planta*. The mild chlorotic symptoms in tolerant C-24 plants that also contained a high fungal load were associated with the biosynthesis of ethylene or ET responses (Veronese *et al.*, 2003).

Veronese *et al.* (2003) suggest that this discrepancy between extensive pathogen colonization and lack of symptom development may be explained by the possibility that symptoms can result from pathogen-induced signals that interfere and disrupt plant growth. Changes such as leaf senescence are then perceived as disease symptoms. Host symptoms may, therefore, be a result of signals activated by the pathogen that are used to exploit the host. Host tolerance may be a result of the host's ability to prevent the pathogen from manipulating its normal development using pathogen-induced signals (Veronese *et al.*, 2003).

A microarray study of the susceptible and tolerant tomato interaction with the fungal pathogen *V. dahliae* suggested that the tolerant interaction may be the result of active suppression of host gene expression. A number of genes which may be associated with defence or wounding such as free radical scavenging and programmed cell death were down-regulated in the tolerance response (Robb *et al.*, 2007). Those processes that contribute to the development of wilt symptoms such as leaf senescence and cell death appear to be suppressed in the tolerant interaction. Symptom responses in the susceptible interaction may, therefore, also be defensive as they may represent host responses designed to limit pathogen success (Robb *et al.*, 2007). The tolerant plants

were also taller than the uninoculated controls with the most likely mechanism being the reduction of ethylene biosynthesis (Robb *et al.*, 2007). Inoculation of *ein2-1* (ethylene – insensitive mutant) *Arabidopsis* plants with virulent *R. solanacearum* strains resulted in delayed wilt symptom development but supported high bacterial concentrations (Hirsch *et al.*, 2002). This phenotype is similar to a tolerance response. Ethylene, therefore, appears to be involved in disease symptom development (Hirsch *et al.*, 2002). Microarray studies investigating the susceptible *R. solanacearum* - *Arabidopsis* interaction and wilt disease development have also been performed (Hu *et al.*, 2008; Naidoo *et al.*, 2011). The activation of ethylene signalling pathway marker genes was observed in this compatible interaction (Naidoo *et al.*, 2011). Further differential gene expression studies with the focus on tolerant interactions would help to elucidate the potential mechanisms of tolerance.

1.7.4 Tolerance to insect herbivory

Generally, insect herbivores cause mechanical damage on plant tissues during feeding. In response, plants are able to defend against insect attack by perceiving the tissue damage caused by insect herbivory as well as recognizing certain compounds in insect oral secretions. Following this perception plants activate a number of direct and indirect defence responses (Howe and Jander, 2008). Direct mechanisms include the secretion of toxins and defence compounds that deter insect herbivory. Indirect mechanisms include volatile production upon herbivore attack that facilitates intra-plant communication between damaged and undamaged plant tissues as well as attracting natural predators of herbivores (Howe and Jander, 2008). Plants also activate induced defence responses at the site of plant damage and often systemically throughout the plant to confer resistance against herbivory. The jasmonic acid signalling pathway plays a central role in the activation of defences in response to surface tissue damage. These defences all contribute to insect immunity (Howe and Jander, 2008).

Plants are also able to tolerate insect damage by inducing tolerance responses that reduce the effect of plant tissue damage on plant fitness (Howe and Jander, 2008). A number of recent empirical studies have investigated physiological plant responses that occur after insect damage in order to identify mechanisms of tolerance to herbivore damage (Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999; Stowe *et al.*, 2000; Tiffin, 2000). Research has also been performed that focuses on evolutionary and environmental aspects that affect tolerance (Tiffin, 2000). Understanding the potential mechanisms of tolerance to insect herbivory will assist in the elucidation of the evolutionary and ecological role of tolerance in insect-plant interactions (Tiffin, 2000). Mechanisms that affect plant fitness include resource allocation (Baldwin and Preston, 1999; Hochwender *et al.*, 2000), increases in photosynthetic activity (Schmidt and Baldwin, 2009), compensatory growth and activation of dormant meristems (McNaughton, 1983; Stauss and Agrawal, 1999; Stowe *et al.*, 2000), storage of reserves in protected tissues (Schwachtje *et al.*, 2006) and phenological patterns (Marquis, 1988).

Some important discoveries concerning the genes and pathways induced in insect tolerance (Schwachtje *et al.*, 2006; Schmidt and Baldwin, 2009) and the evolutionary and ecological dynamics of insect tolerance (Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999) have been made recently. Previously it was thought that tolerance to herbivory was highly correlated to resource availability, where tolerance to insect damage is elevated under rich resource conditions. However, recently it is becoming clear that the relationship between resource availability and tolerance is more complex than previously thought (Fornoni, 2011). Multiple factors of herbivory and the availability of resources will determine levels of tolerance under different environmental conditions (Wise and Abrahamson, 2007). In terms of the evolution, tolerance to herbivory was found to be under natural selection as it is a heritable trait. While early models predicted that tolerance and resistance were mutually exclusive and thus a trade off existed between these two defence mechanisms, recent predictions state that the interaction between tolerance and resistance is likely to be dependent on a number of factors including plant genotype, resource allocation, reproductive costs and the environment (Stowe *et al.*, 2000; Nunez-Farfan *et al.*, 2007).

The genetic control of tolerance to herbivory has also been the focus of some investigation. Schwachtje *et al.* (2006) demonstrated rapid alterations in the allocation of sugars in *Nicotiana attenuata* following stimulated herbivore attack and damage. They found that GAL83, a β -subunit of a SnRK1 (SNF1-related kinase) kinase complex was down-regulated in response to herbivore attack, and subsequent silencing of the gene resulted in the increased reallocation of sugars to the plant roots. They further determined a fitness benefit of this sugar reallocation to plant roots, as attacked GAL83-silenced *N. attenuata* plants utilized their stored sugars to prolong reproductive ability allowing plant to tolerate insect herbivory (Schwachtje *et al.*, 2006).

1.8 The Arabidopsis - *R. solanacearum* pathosystem

Arabidopsis is a model organism in the study of the plant defence network and has been shown to be a host of *R. solanacearum* (Yang and Ho, 1998). As described previously, an Arabidopsis – *R. solanacearum* pathosystem was developed by Deslandes *et al.* (1998). *R. solanacearum* strain GMI1000, isolated from tomato, was able to infect and cause complete wilting of Arabidopsis ecotype Col-5. The Nd1 ecotype was found to be resistant to GMI1000. In this study, the major determinant of resistance to GMI1000 in ecotype Nd1 was mapped to a single recessive locus, *RRS1* (Deslandes *et al.*, 1998). In Arabidopsis, two alleles, recessive *RRS1-R* and dominant *RRS1-S*, were determined to confer resistance and susceptibility, respectively, to the bacterial wilt pathogen, *R. solanacearum* (Deslandes *et al.*, 2002).

The N terminal of the *RRS1* proteins contain domains found in the plant Toll-IL-1 receptor-nucleotide binding site leucine-rich repeat (TIR-NB-LRR) class of R proteins. The C-region terminal has characteristics of the WRKY motif suggesting a regulatory role in the expression of the signalling pathways resulting in resistance or susceptibility (Deslandes *et al.*, 2002). The resistance mediated by *RRS1-R* is partially dependent on

salicylic acid and NDR1, indicating the induction of similar signalling pathways to those induced in effector-triggered immunity (Deslandes *et al.*, 2002).

For *RRS1-R* to confer resistance to *R. solanacearum*, a specific *Avr* gene must be present in the pathogen's repertoire of effectors to interact with *RRS1-R* in an incompatible interaction. The avirulence gene in *R. solanacearum* was found to be PopP2, a T3E belonging to the YopJ/AvrRxv effector family (Deslandes *et al.*, 2003) Yeast two-hybrid analysis was used to detect the physical interaction between PopP2 and the *RRS1-R* protein (Deslandes *et al.*, 2003). It was further demonstrated that *RRS1-R* activates a resistance response by recognizing a nuclear complex formed between PopP2 and the interacting protein RD19 (Deslandes *et al.*, 2003, Bernoux *et al.*, 2008).

A new Arabidopsis – *R. solanacearum* pathosystem was established by Fouché-Weich (Weich, 2004). This pathosystem was developed by screening the responses of seven Arabidopsis ecotypes against eight different *Eucalyptus* and potato isolates of *R. solanacearum* (Fouché-Weich *et al.*, 2006). In this pathosystem, Kil-0 was proposed to be “resistant” to an African *Eucalyptus* isolate of *R. solanacearum*, BCCF402, whereas Be-0 was determined to be the susceptible ecotype (Naidoo *et al.*, 2011). A recent study confirmed that the “resistance” phenotype in ecotype Kil-0 is conferred by the recessive *RRSI-R* allele and that the Kil-0 *RRSI-R* protein recognizes the *R. solanacearum* BCCF402 avirulence protein PopP2 to activate a defence response mediated by the defence signalling network (van der Linden, 2010).

Interestingly, the bacterial load determined in previous studies on this pathosystem showed that *R. solanacearum* concentration in resistant ecotype Kil-0 was only one order of magnitude less than the determined concentration of bacteria found in the susceptible ecotype Be-0 (1×10^{12} colony forming units of bacteria per gram fresh weight). This unexpected result suggested that the Kil-0 - *R. solanacearum* interaction could be one of

tolerance instead of resistance (Weich, 2004). Further investigation of this finding is one of the topics of the current MSc dissertation.

1.8.1 Signalling pathways and responses activated against *R. solanacearum*

1.8.1.1 The Resistant Interaction

Ethylene responsive factors (ERFs) play a pivotal role in modulating the induction of ethylene-dependent defence responses. The tomato ERF protein known as TSRF1 (tomato stress-responsive factor 1) was shown to be induced when plants were treated with ethylene and salicylic acid and when infected with *R. solanacearum* strain BJ1057 (Zhang *et al.*, 2004). TSRF1 physically interacts with GCC box elements and the overexpression of *TSRF1* in tomato or tobacco induced the expression of *PR* genes that contained these GCC box elements in their promoter sequences. The overexpression of TSRF1 consequently increased resistance to *R. solanacearum*. TSRF1 is, therefore, involved in plant resistance to *R. solanacearum* strain BJ1057 (Zhang *et al.*, 2004).

Overexpression of *TSRF1* in tobacco induced ABA biosynthesis which subsequently elicited the production of ethylene (Zhou *et al.*, 2008). However, when ABA levels are elevated such as after the exogenous treatment of ABA, TSRF1 binding to the GCC box in *PR* genes is disrupted which enhances the susceptibility of tobacco plants to pathogen invasion (Zhou *et al.*, 2008). TSRF1 has also been shown to bind to the CEI/GCC element found in the promoters of ABA-responsive genes (Zhang *et al.*, 2008). Treatment of tobacco plants with ABA enhanced the expression of ABA-responsive genes (Zhang *et al.*, 2008). The biocontrol agent *Pythium oligandrum* (PO) is able to induce resistance to *R. solanacearum* through the induction of the JA-dependent signalling pathway (Hase *et al.*, 2008).

1.8.1.2 The Susceptible Interaction

Arabidopsis ecotype Col-5 was shown to be susceptible to *R. solanacearum* isolate BCCF401 (Naidoo *et al.*, 2011). The compatible defence responses against isolate BCCF401 were studied using differential gene expression analysis (Naidoo *et al.*, 2011). This study demonstrated that the majority of differential gene expression transpired at the later stages of wilt disease development (Naidoo *et al.*, 2011). Marker genes of the JA/ET-dependent pathway, *PR-3* and *PR-4*, were up-regulated during this experiment and the SA signalling pathway was inhibited at later time points in the compatible Col-5 - *R. solanacearum* interaction (Naidoo *et al.*, 2011). An increased susceptibility to *R. solanacearum*, indicated by the development of wilt symptoms, was observed in resistant Nd1 plants homozygous for the *NahG* gene (encodes salicylate hydroxylase, which converts salicylic acid into inactive catechol) after inoculation with the pathogen (Deslandes *et al.*, 2002), suggesting that SA is at least partially involved in resistance to *R. solanacearum*.

The role of ET in *Arabidopsis* against *R. solanacearum* was described (Hirsch *et al.*, 2002). In this study, ethylene played a role in the progression of wilt symptoms in *Arabidopsis* against *R. solanacearum*. An ethylene insensitive mutant, *ein2-1* was shown to delay the development of wilt symptoms in the susceptible ecotype Col-0 after inoculation with GMI1000 (Hirsch *et al.*, 2002). *R. solanacearum* is able to produce ethylene which can be utilized by the pathogen to increase pathogenesis and disease susceptibility in the host. This can be compared to the production of coronatine in *P. syringae*, which mimics a component of the JA signalling pathway. SA signalling, which is involved in resistance against *P. syringae*, is antagonized as a result (Grant and Jones, 2009).

Screening for mutants that prevent the development of disease upon *R. solanacearum* infection was performed. Three *Arabidopsis* cellulose synthase (CESA) genes were discovered, namely *CESA4/IRREGULAR XYLEM5 [IRX5]*, *CESA7/IRX3*, and

CESA8/IRX1 (Hernandez-Blanco *et al.*, 2007). Studies have shown that *irx* mutants with impaired CESA proteins have an improved resistance against *R. solanacearum* (Hernandez-Blanco *et al.*, 2007). In contrast, ABA mutants (*abi1-1*, *abi2-1* and *aba1-6*) showed an enhanced susceptibility to the pathogen (Hernandez-Blanco *et al.*, 2007). Mutant plants impaired in CESA genes showed an increased expression of ABA-responsive defence-related genes (Hernandez-Blanco *et al.*, 2007). These results indicate that ABA plays an important role in the plant defence against *R. solanacearum*.

The ABA signalling pathway was activated in susceptible ecotype Col-5 in response to *R. solanacearum* strain GMI1000 in a genome microarray experiment (Naidoo *et al.*, 2011). This was in agreement with Hu *et al.* (2008) who showed an activation of genes that play a role in ABA biosynthesis and signalling in susceptible Arabidopsis plants after infection with GMI1000 at a later stage of infection (Hu *et al.*, 2008). Therefore, Naidoo *et al.* (2011) proposed that ABA promotes resistance to *R. solanacearum* at early stages of infection, but it enhances susceptibility during the later stages of infection (Naidoo *et al.*, 2011).

In the above findings, several signalling pathways appear to play a role in the Arabidopsis – *R. solanacearum* interaction. SA was shown to be involved in plant defence against *R. solanacearum* in Arabidopsis, tomato and tobacco. In Arabidopsis, ET promotes the progression of wilt symptoms but it enhances resistance to *R. solanacearum* in tomato. ABA may play different roles in different stages of infection in Arabidopsis but it has been shown to only promote disease progression in tomato and tobacco.

1.9 Fluorescent tagging: a tool in the study of phytobacteriology

The use of autofluorescent proteins is a well-established tool in the visualization and localization of pathogen populations *in planta*. An understanding of pathogen colonization and progression will give us insight into pathogen virulence strategies and potential mechanisms of plant defence. Fluorescent proteins are live cell imaging markers that have become invaluable in cell biology and biotechnology (Shaner *et al.*, 2004). Autofluorescent proteins have become invaluable in the study of plant-pathogen interactions, biosensor development, biofilm formation, microbial behaviour and microbe gene expression in living cells in real time (Larrainzar *et al.*, 2005). Autofluorescent techniques have the capability of direct *in situ* visualization of tagged phytopathogens at the single cell level. Many recent studies have also developed dual labelling strategies for the simultaneous visualization of different microbial populations and different physiological processes (Lugtenberg *et al.*, 2002). For example, Bloemberg *et al.* (2000) was able to visualize three microbial populations simultaneously by using three *P. fluorescens* WCS 365 populations tagged with three different autofluorescent proteins (Bloemberg *et al.*, 2000). Of particular interest is the investigation of plant colonization by microbes (Lugtenberg *et al.*, 2002; Lagopodi *et al.*, 2002).

Visual observation of the pathogens mode of infection and colonization has been performed through the constitutive expression of green fluorescent protein (GFP) (Vailliau *et al.*, 2007). It was determined that the *R. solanacearum* isolate GMI1000 entered the root of the legume *M. truncatula* at the point of emergence of secondary root growth. After two to four days post inoculation, pathogen colonization moved up the vascular system to the aerial part of the plant in a compatible interaction (Vailliau *et al.*, 2007). The compatible interaction between *Arabidopsis* and *Ralstonia solanacearum* GMI1000 was investigated by means of immunolabelling studies using *R. solanacearum*-specific Rsol-I antibodies (Digonnet *et al.*, 2012). Confocal microscope analysis revealed that the pathogenic bacterium penetrates the xylem vessels by degrading certain components of the plant's cell wall using its arsenal of CWDE's. The pathogen proliferated extensively in

the xylem vessels to result in symptom development (Digonnet *et al.*, 2012). A recent study has also concluded that mCherry is a useful tagging reporter construct in the localization of bacterial species (Lagendijk *et al.*, 2010).

Autofluorescent proteins can also be used as reporters to monitor gene expression and protein localization in real time and with living cells. Autofluorescent expression can be visualized and quantified in whole cultures using fluorometry techniques or in individual cells and subcellular compartments via confocal microscopy (Lorang *et al.*, 2001). Deslandes *et al.* (2003) utilized GFP and RFP fusions to determine whether the Arabidopsis R protein RRS1 and the *R. solanacearum* Avr protein PopP2 colocalize in the Arabidopsis plant nucleus. This study showed that a PopP2::GFP fusion protein is specifically targeted to the plant nucleus. Additionally, using an RRS1::RFP fusion together with the PopP2::GFP protein, the study demonstrated that in the presence of PopP2, the RRS1 fusion protein was detected in the Arabidopsis plant nucleus indicating that the PopP2 protein is necessary for visualization of RRS1 in the nucleus (Deslandes *et al.*, 2003).

1.10 Quantitative (Real Time) PCR

An accurate estimate of the pathogen biomass *in planta* is often required in determining and measuring tolerance, susceptibility and resistance in plant-pathogen interactions. Culturing approaches where plant tissue is plated out onto a selective media have been used extensively in the past, however, they are often labour-intensive and time-consuming (Martin *et al.*, 2000). Quantitative PCR (qPCR) is a reliable and sensitive method that produces accurate and consistent results and is commonly used for the quantification of pathogen biomass and detection of pathogen progression in host plants (Martin *et al.*, 2000; Llorente *et al.*, 2010). qPCR is a rapid, sensitive method to detect and quantify target pathogen DNA in a variety of environments such as plant tissues, soil and water

samples and is useful in the study of plant pathogen and disease diagnostics (Schaad and Frederick, 2002), epidemiology and plant-pathogen interactions (Schena *et al.*, 2004).

There are many studies that have been reported on the development of a SYBR Green-based qPCR assay for the quantification and early detection or monitoring of phytopathogen growth in their respective host plants. These studies assess the development of pathogen growth and disease progression of numerous biotrophic and necrotrophic fungi and bacteria (Atallah *et al.*, 2007; Jiménez-Fernández *et al.*, 2010; Baumgartner *et al.*, 2010; Llorente *et al.*, 2010; Korsman *et al.*, 2012). Examples include monitoring *Phytophthora infestans* growth in early potato infection (Llorente *et al.*, 2010), the quantification and detection of *Cercospora* spp. in infected maize lines (Korsman *et al.*, 2012) and the development of a qPCR assay to examine growth and development of a number of well-known fungal and bacterial phytopathogens in the model organism *Arabidopsis* (Brouwer *et al.*, 2003). A study by Lievens *et al.* (2006) demonstrated the use of qPCR to detect and quantify a number of tomato pathogens in plant and soil samples. Early, accurate detection and identification of these economically important phytopathogens is important for effective plant disease management (Lievens *et al.*, 2006).

Weller *et al.* (2000) were able to detect *R. solanacearum* in potatoes using a Taqman assay at a detection level of 100 cells per ml. When the fluorescence breached an arbitrary threshold value (Threshold Cycle, C_T) they were able to determine that the target DNA of the bacteria was present. They were also able to identify *R. solanacearum* within one day of receiving samples by using the Taqman assay.

1.11 Conclusion

In this literature survey, we have summarized evidence that tolerance in plant – bacterial interactions can be conferred by a single *R* gene in a gene-for-gene interaction. A potential tolerance response was observed in *Arabidopsis* accession Kil-0 to *R. solanacearum* BCCF402, isolated from *Eucalyptus*. The literature survey was focussed on the study of plant-pathogen interactions, in particular the *Arabidopsis* – *R. solanacearum* interaction. Important sections concentrating on the virulence strategies of phytopathogen *R. solanacearum* and plant immunity were reviewed. The compatible and tolerant plant – pathogen interactions were also described in detail. The aims of this particular MSc study were to determine whether *Arabidopsis* accession Kil-0 is tolerant to *R. solanacearum* BCCF402. An additional aim was to develop a fluorescent protein tool where *R. solanacearum* BCCF402 will be tagged with mCherry for the visualization of bacteria cells in *Arabidopsis* plants.

Chapter 2

Material and methods

2.1 *R. solanacearum* strains, plasmids and media

R. solanacearum isolate BCCF402 was cultured on solidified Bacto-agar Glucose Triphenyltetrazolium chloride (BGT) media at 28 °C and virulent mucoid colonies were grown in liquid B medium (Deslandes *et al.*, 1998). Antibiotics were added at the following concentrations when required: Gentamycin 50 µg/ml, tetracycline 16 µg/ml and rifampicin 50 µg/ml. The *R. solanacearum* strains and plasmids used in this study are listed in Table 2.1. Growth curves were obtained by diluting an overnight culture to an OD₆₀₀ of 0.2 in 100 ml of B medium containing the appropriate antibiotics. Cultures were grown for 12 hours at 28 °C on a rotational shaker. Regular spectrophotometer readings were taken at 30 minute intervals.

R. solanacearum isolate BCCF402 was transformed with plasmids pMP7604 and pMP7605 containing the mCherry gene (Lagendijk *et al.*, 2010). Transformation was accomplished using a modified calcium chloride competent cell preparation and heat shock method (Ausubel *et al.*, 1997). Selected BCCF402 strains were cultured overnight at 28 °C and 1 ml of BCCF402 overnight culture was inoculated into 100 ml B medium. Additionally, 1 ml of 2 M MgCl₂ was added to the culture. The culture was grown at 28 °C in a shaking incubator until an OD₆₀₀ of 0.4 - 0.6 was achieved. The culture was chilled on ice for 1 hour 30 minutes and then transferred into pre-chilled 50 ml Falcon tubes. Centrifugation of cells was performed at 5000 rpm at 4 °C for 5 minutes. The pelleted cells were gently re-suspended in 16.6 ml ice-cold 0.1 M CaCl₂ solution and left on ice for 1 hour 30 min. Following a second centrifugation, cells were re-suspended in 1ml of ice-cold CaCl₂ solution containing 15 % glycerol over 1 hour. Aliquots of competent cells were flash frozen in liquid nitrogen and stored at -80 °C until needed for heat shock. For the heat shock method, 100 µl of competent cells were added to a 15 ml Falcon tube. The cells were mixed with approximately 10 ng of plasmid DNA in a 10-25 µl solution. The solution was mixed gently and stored on ice for 20 minutes. The solution was heat shocked at 42 °C for 90 seconds in a water bath. The solution was cooled on ice for 2 minutes and then transferred to 1 ml B medium where it was incubated at 28 °C for 1 hour

while shaking. The medium was streaked (100 μ l) onto BGT medium containing appropriate antibiotics. The transformed cells, re-named BCCF402_4 (pMP7604) and BCCF402_5 (pMP7605), were selected on BGT plates containing a final concentration of 16 μ g/ml tetracycline and 50 μ g/ml gentamycin, respectively.

Rifampicin mutants were prepared as described by Naidoo *et al.* (2011). Rifampicin mutants of *R. solanacearum* BCCF402_5 strain were prepared by reviving BCCF402_5 cells from -80 °C glycerol storage by streaking on BGT medium. Individual BCCF402_5 mucoid colonies were grown overnight in 5 ml of B medium at 28 °C. The overnight culture was centrifuged for 1 minute at 3000 rpm, to remove all the supernatant. The resulting pellet was spread onto BGT medium containing 50 μ g/ml rifampicin and the plates were incubated at 28 °C for two days. Single mucoid colonies were infiltrated on tobacco leaves to test for virulence. Overnight culture of single mucoid colonies was centrifuged to remove the supernatant. The pellet was re-suspended in 1 ml sterile distilled water. Approximately 250 μ l was infiltrated into a *Nicotiana tabacum* cv. Petit havana leaf as described by Weich (2004). Plants were observed for a hypersensitive response (HR) for 7 days. Water was used as a negative control. Colonies that produced a HR were stored at -80 °C in 15 % glycerol. Rifampicin resistant strains were designated BCCF402_5^{rif}. BCCF402^{rif} strains were prepared previously by Weich (2004).

Table 2.1. Bacterial strains and plasmids utilized in this study

Bacterial strains and plasmids	Characteristics	Reference
<i>R. solanacearum</i> strains		
BCCF402	Wildtype, isolated from <i>Eucalyptus</i> sp. Race 1; Biovar 3; Phylotype I	Fouché-Weich <i>et al.</i> , 2006
BCCF402 ^{rif}	Rifampicin mutant of BCCF402	Weich, 2004
BCCF402 ^{hrp}	<i>hrp</i> mutant of BCCF402	Weich, 2004
BCCF402_4	BCCF402 containing pMP7604	This study
BCCF402_5	BCCF402 containing pMP7605	This study
BCCF402_5 ^{rif}	Rifampicin mutant of BCCF402_5	This study
Plasmids		
pMP7604	pMP6031 derivative containing mCherry gene under control of the <i>tac</i> promoter, Tet ^R	Lagendijk <i>et al.</i> , 2010
pMP7605	pBBR1MCS-5 derivative containing mCherry gene under control of the <i>tac</i> promoter, Gent ^R	Lagendijk <i>et al.</i> , 2010

Tet^R: tetracycline resistance, Gent^R: gentamycin resistance

2.2 Growth of *Arabidopsis* accessions

Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0 were obtained from the Nottingham *Arabidopsis* Stock Centre (<http://www.arabidopsis.info>). *Arabidopsis* seeds were sterilized by washing with 70 % ethanol followed by washing with 1.5 % sodium hypochlorite for 30

minutes. Seeds were rinsed three times with distilled water and then re-suspended in 0.1 % (w/v) agarose. The re-suspended seeds were placed onto plates containing MS (Murashige and Skoog, 1962) medium for germination. Seeds were incubated at 25 °C for 2 days to allow for vernalization. The plates were covered in foil and incubated at room temperature for 24 hours. Seedling growth was promoted by placing plates in a growth chamber under 16 hr light/8 hr darkness at 20 °C for two weeks. The plantlets were grown and maintained further on Jiffy pots (Jiffy France, Lyon, France) in a growth chamber at a temperature of 22 °C under 16 hr light, 25 to 30 % relative humidity and 300-350 lum/sqf for four weeks. Plants were watered once per week with a solution of 2.5g/l Multifeed (Plaaskem [Pty] Ltd., Johannesburg, SA).

2.3 Bacterial inoculations

Arabidopsis plants were root inoculated according to Deslandes *et al.* (1998). *R. solanacearum* strains used in this study were grown first on plates containing BGT medium at 28 °C for 2 days. Mucoïd bacteria was transferred into liquid B medium and incubated with agitation at 28 °C overnight. *R. solanacearum* strains were diluted to a final concentration of 1×10^8 CFU/ml for the inoculation of *Arabidopsis* plants. Six week old *Arabidopsis* plants were wounded for inoculation by cutting horizontally approximately 2 cm from the bottom of the jiffy pots. The wounded roots were placed in the bacterial suspension and allowed to soak for 30 minutes. The control plants were placed in liquid B media without any bacteria. The plants were transferred to moist vermiculite ensuring that the leaves were not touching the sides of the tray. The plants were placed in a growth chamber and maintained at 26 °C, 50 % relative humidity, 16 hr light and 500 lum/sqf light intensity.

After inoculation, the plants were monitored for wilting symptom development. Plants were scored on a scale from 0 (no disease) to 5 (completely wilted or dead). The disease index (DI) was determined using the formula: $DI = [\sum(n_i \times v_i)/(V \times N)]$, where n_i = number of

plants with respective disease rating; v_i = disease rating (0, 0.5, 1, 2, 3, 4 or 5); V = highest disease rating (5); and N = the number of plants observed (Winstead and Kelman, 1952).

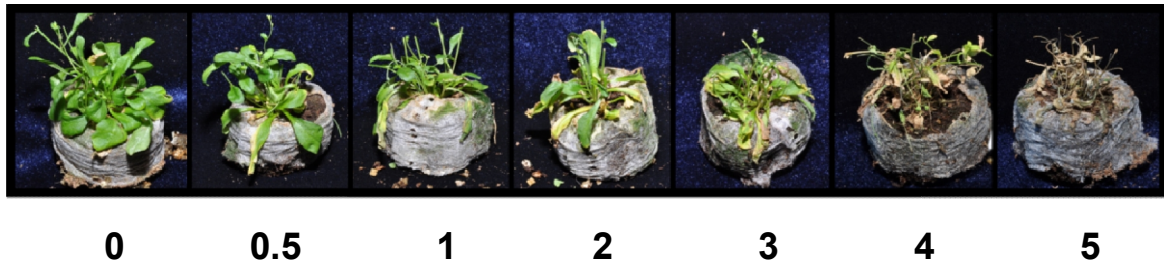


Figure 2.1. **Photographic images representing scoring for disease symptoms for Arabidopsis plants.** Arabidopsis plants are scored from 0 (no disease) to 5 (completely wilted).

R. solanacearum BCCF402^{nif} and BCCF402_5^{nif} cells were quantified *in planta* using a dilution plating method described by Deslandes *et al.* (1998). After inoculation (0, 4, 8, 12, 16 days post inoculation) the aerial parts of the plant were harvested. Three to four plants were harvested for each biological replicate and 3 or 4 biological replicates were prepared for each infection trial. The aerial plant material harvested for each biological replicate was pooled together and weighed. The plant material was surface sterilized for 3 minutes in 70 % ethanol and then rinsed three times in distilled water. A mortar and pestle was utilized to grind aerial tissue. Sand was used to aid grinding. Before grinding, a volume of sterile water 3 x the fresh weight of the plant material was added to the plant tissue in the mortar. A 10 x dilution series was prepared using the ground plant material as the starting solution. A volume of 100 μ l of each dilution was plated onto BGT plates containing 50 μ g/ml rifampicin for selection of *R. solanacearum* BCCF402^{nif} cells. BGT plates contained 50 μ g/ml rifampicin and 50 μ g/ml gentamycin for selection of *R. solanacearum* BCCF402_5^{nif} cells. Plates were stored at 28 °C for 2 days to allow growth of colonies. Colonies were counted and colony forming units per gram fresh weight (CFU/g fresh weight) was calculated.

2.4 Quantitative PCR amplification

The amount of *R. solanacearum* DNA in infected *Arabidopsis* plants was measured using quantitative PCR. Total DNA was extracted from *Arabidopsis* plants infected with *R. solanacearum* strain BCCF402 and diluted to a final concentration of 10 ng/μl. The primers RsoISF and RsoISR (Table 2.2) were designed to amplify a fragment of the *R. solanacearum* Cytochrome c1 peptide (Kang *et al.*, 2007). A spiked internal control of “alien” DNA contained on a plasmid was used to normalize gene quantification. The primers CPR1_2F and CPR1_2R (Table 2.2) were used to amplify a fragment of the *Cercospora zae-maydis cytochrome P450 reductase* gene (GenBank accession numbers AF448828 and FG242129) designated *cpr1* (Korsman *et al.*, 2012). The primers UBQ10F and UBQ10R (Table 2.2) were designed to amplify a fragment of the *Ubiquitin 10* gene from *Arabidopsis* (Genbank accession number AT4G05320). All primers were shown to be specific to their target DNA. The *cpr1* fragment had been cloned into the pJET1.2/blunt vector from the CloneJET™PCR Cloning Kit (Fermentas Inc., Hanover, USA) and this was renamed pJET-CPR1-Cz (Appendix A). An amount of 20 pg of the pJET-CPR1-Cz vector DNA containing the *cpr1* gene was added to all samples before DNA extraction. The amount of vector DNA (20 pg) added to each sample at the beginning of DNA extraction was determined in such a way that it took approximately 20 PCR cycles for the amplification of vector DNA to cross the threshold of background fluorescence (Ct value).

Quantitative PCR amplifications were performed in a total volume of 10 μl on a LightCycler® 480 instrument (Roche Diagnostics Corp., Basel, Switzerland). Each reaction contained DNA template (1 μl) or water in the non-template controls, the LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics Corp.), 0.5 μM of each primer and sterile distilled water. A 384-well qPCR plate was used with 3 replicates of each dilution. The cycling conditions included 1 cycle of pre-incubation at 95 °C for 5 min; 45 cycles of PCR at 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 15 sec; 1 cycle of melting curve analysis at 95 °C for 5 sec, 65 °C for 1 min and 95 °C continuously; and

lastly a single cooling cycle at 40 °C for 10 min. Absolute (2nd derivative max) quantification was used to analyze runs.

The Cytochrome C1 standard curve and the Ubiquitin 10 standard curve were constructed using known standards that contained *R. solanacearum* DNA and Arabidopsis DNA in concentrations that mimic the natural infection in the plants and when combined together, yield a final concentration of 10 ng/μl. A 10-fold dilution series of vector DNA of known concentrations (1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg and 0.01 pg) was used to construct the plasmid *cpr1* standard curve. The amount of DNA from unknown samples was determined using the relevant standard curves. The amount of *R. solanacearum* CytC DNA quantified was normalized to the amount of plasmid *cpr1* DNA quantified from infected plant tissue.

Table 2.2. Oligonucleotide primers utilized in this study

Primer name	Sequence (5'-3')	Annealing temperature (°C)	Amplicon length (bp)
RsolSF	GTTGTCCAGTGCGCTGTGGT	60	256
RsolSR	GTATGGCCTGCATGGCCTGT	60	256
CPR1_2F	TGAACTACGCGCTCAATG	54	164
CPR1_2R	TCTCTCTTGGACGAAACC	54	164
UBQ10F	CTGCGTGGAGGTATGCAGAT	64	227
UBQ10R	CGCAGGACCAAGTGAAGAGT	64	227
mCherry_Start	ATGGTGAGCAAGGGCGAGGA	55	711
mCherry_Stop	TTACTTGTACAGCTCGTCAA	55	711
mCherryF	AGGACGGCGAGTTCATCCTAC	54	-
mCherryR	TAGATGAACTCGCCGTCCTG	54	-

2.5 Biomass and seed fitness parameters

After *Arabidopsis* accessions Kil-0 and Be-0 were infected with *R. solanacearum* isolate BCCF402, whole plant tissue was harvested at 16 days post inoculation to evaluate the biomass yield of accessions challenged with BCCF402 compared to those that were mock-inoculated. Plant material from three biological replicates of 10 plants each was harvested. Plant material was placed in a 36 °C oven for 24 hours to dry plant tissue and the dry weight was obtained.

The seed yield was determined by collecting all the seed produced from accessions Kil-0 and Be-0. Seed from three biological replicates of 3 plants each were collected according to Weigel and Glazebrook (2002) for approximately four weeks after BCCF402 infection or until plant senescence. Once the seed had been collected, one thousand seeds of each accession were counted. The one thousand seeds were weighed to obtain a measurement of the mass of 1000 seeds (mg). The total seed was weighed and the total number of seed per accession was determined. Seeds were tested for their germination ability. Seed collected from accessions Kil-0 and Be-0 were placed onto MS medium and allowed to germinate as described previously. Three biological replicates of one hundred seeds each were evaluated. Once the formation of leaves and roots had been observed, the percentage germination rate of each accession was determined.

2.6 DNA isolations

Genomic DNA was isolated from *Arabidopsis* and *R. solanacearum* based on the cetyl-trimethyl-ammonium bromide (CTAB) extraction method described by Lukowitz *et al.* (2000). A small amount of plant tissue (1-3 rosette leaves) was crushed in 300 µl of 2 X CTAB (2% [w/v] cetyl-trimethyl-ammonium bromide, 1.4 M NaCl, 100 mM

Tris HCl pH 8.0, 20 mM ethylene diamine tetraacetic acid [EDTA]) with a pestle. The tissue was incubated at 65 °C for 1 hour. The tissue was allowed to cool and 300 µl of chloroform was added and vortexed thoroughly. The mixture was centrifuged at 13000 rpm for 3 minutes. The upper phase was transferred to a new reaction tube. 300 µl of 2-Propanol was added to the reaction tube and mixed well. The mixture was centrifuged again at 13000 rpm for 5 minutes. The supernatant was removed while being careful to leave the pelleted DNA. The pellet was washed with 500 µl of 70 % ethanol and centrifuged at 13000 rpm for 5 minutes. The ethanol was carefully removed and the pellet was allowed to dry. The dry DNA was re-suspended in 40 µl of TE (10 mM Tris HCl pH 8.0, 1 mM EDTA) buffer. The same protocol was used to extract DNA from *R. solanacearum* overnight culture using 2 ml as the starting material. Plasmid DNA was isolated using the Invisorb Spin Plasmid MiniTwo Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. A starting amount of 2 ml of *R. solanacearum* overnight culture containing plasmid DNA was placed in a reaction tube. A volume of 40 µl of distilled water was used to re-suspend plasmid DNA.

2.7 PCR amplifications and sequencing

R. solanacearum BCCF402_4 and BCCF402_5 strains were screened with PCR using the mCherry_Start and mCherry_Stop primers (Table 2.2) to verify the presence of the mCherry gene contained on the pMP7604 and pMP7605 plasmids. This primer pair had an annealing temperature of 55 °C.

PCR amplifications of the *cpr1* gene using CPR1_2F and CPR1_2R primers (annealing temperature of 54 °C) and the *cytC* gene region using RsoISF and RsoISR primers (annealing temperature of 60 °C) were performed with *R. solanacearum*, Arabidopsis and plasmid DNA (pJET-CPR1-Cz) (Korsman *et al.*, 2012) as templates to determine whether primers are specific to their DNA targets.

Each PCR was performed in 0.2 ml tubes and contained 1 X Taq reaction buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μM of each primer and 1 U of Taq DNA polymerase (BIOTAQ DNA polymerase; Bioline Ltd., London, UK). 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 seconds, an annealing step (temperatures dependent on primer pairs are indicated in Table 2.2), an elongation step at 72 °C for 40 seconds. A final extension at 72 °C for 5 min and a final hold at 4 °C were also included. PCR amplification fragments were analyzed by electrophoresis on a 1 % (w/v) agarose gel.

Sequencing of the mCherry gene and its promoter region from plasmids pMP7604 and pMP7605 was performed using external primers mCherry_Start and mCherry_Stop and internal primers mCherryF and mCherryR (Table 2.2). The primers generated four overlapping sequences. Each cycle sequencing reaction was performed in 0.2 ml reaction tubes and resulted in a final reaction volume of 10 μl. Sequencing was performed on a ABI PRISM 3100 Automated DNA sequencer (Applied Biosystems, Carlsbad, USA) using the Big Dye Terminator Cycle sequencing reaction kit v3.1 (Applied Biosystems, Carlsbad, USA). The PCR program consisted of an initial step at 96 °C for 1 min, 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 min, followed by a holding step of 4 °C. An ethanol precipitation method was used, where 3 M NaOAc and 100 % cold ethanol was added to 10 μl of PCR product. The reaction was centrifuged at 14000 rpm for 30 min. The supernatant was removed. The pellet was washed with 70 % ethanol and then centrifuged at 14000 rpm for 15 min. The supernatant was removed again and the air dried pellet was sequenced.

2.8 Restriction enzyme digestions of mCherry plasmids

The mCherry plasmids pMP7604 and pMP7605 were restriction enzyme digested to confirm the presence of the mCherry fragment. Single enzyme digestions were performed

using BamHI and NcoI (Fermentas Inc., Hanover, USA). Double digestions using NcoI and Sall and sequential double digestions using NcoI and BglII (Fermentas Inc., Hanover, USA) restriction enzymes were also performed. Single and double digestion reaction mixtures contained 8 μ l of isolated plasmid DNA, 1.5 μ l of 10 X Buffer H (Fermentas Inc., Hanover, USA) and 0.5 μ l of single digestion enzymes (BamHI or NcoI) or 0.5 μ l of each enzyme in the double digestion (NcoI and Sall) made up to a total volume of 15 μ l. The reactions were incubated at 37 °C for 1 to 3 hours to allow for complete digestion. The enzyme was inactivated by incubation at 65 °C for 30 min. In the sequential double digestion, enzyme BglII was first used to digest 8 μ l of plasmid DNA at 37 °C for 1 hour. The reaction contained 1.5 μ l of 10 X Buffer M (Fermentas Inc., USA), 0.5 μ l of BglII made up to a final volume of 15 μ l. After the 1 hour incubation, 2.5 μ l 10 X Buffer H and 0.5 μ l NcoI was added to the reaction and made up to a total volume of 25 μ l. The reactions were incubated at 37 °C for another hour for total digestion. Digested fragments were analyzed by electrophoresis on a 1 % (w/v) agarose gel.

2.9 Quantification of mCherry fluorescence

The fluorescence levels of mCherry in *R. solanacearum* strains BCCF402_4 and BCCF402_5 was quantified using a Fluoroskan Ascent®FL machine (Thermo Fisher Scientific, Waltham, USA). BCCF402_4 and BCCF402_5 cells were cultured overnight in B liquid medium at 28°C. Each overnight culture was normalized to an OD₆₀₀ of 0.8. Overnight cultures of *R. solanacearum* strains were centrifuged at 13000 rpm for 2 min to pellet cells. All supernatant B medium was removed to prevent background fluorescence and cells were re-suspended in 300 μ l of distilled water. Cells were transferred to black 96-well plates. Fluorescence was quantified by excitation with 530 nm and by measuring the emission at 635 nm. Three separate mCherry transformation events of each strain were prepared for fluorometry analysis. Three replicates of each of these *R. solanacearum* strains were performed.

2.10 Microscopy

Bacterial cells of mCherry-tagged strains were studied using a Zeiss Axiovert 200 light microscope (Zeiss, Oberkochen, Germany) equipped with a Zeiss Filterset number 15 with an excitation filter of 546nm and a long-pass emission filter of 590 nm. Images were captured using an AxioCam MRc5 camera (Zeiss). Cells were fixed in 80 % glycerol for visualization. *R. solanacearum* strain, BCCF402_5^{mf} inoculated into Arabidopsis plants were studied using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany) fitted with either a 63 x or 20 x objective lens. Leaves were dissected until only the large central vein remained and then cross sectioned to open up the xylem. BCCF402_5^{mf} cells in the plant tissue were excited with a Helium-Neon laser at 543 nm. Images of the plant material were acquired using a 560 nm long-pass filter. Image acquisition and analysis was performed using Carl Zeiss Laser Scanning Systems LSM 510 software, version 3.2. Analysis was performed at the Electron Microscope (EM) Unit at the University of Pretoria.

2.11 Statistical analysis

Statistical analyses were performed using Microsoft Excel 2007 (T-tests; $p < 0.05$, means, standard errors) and GraphPad Prism software (GraphPad Software Inc., La Jolla, USA). (1-way ANOVA; $p < 0.05$ with Tukey pairwise distribution; $p < 0.05$).

Chapter 3

Results

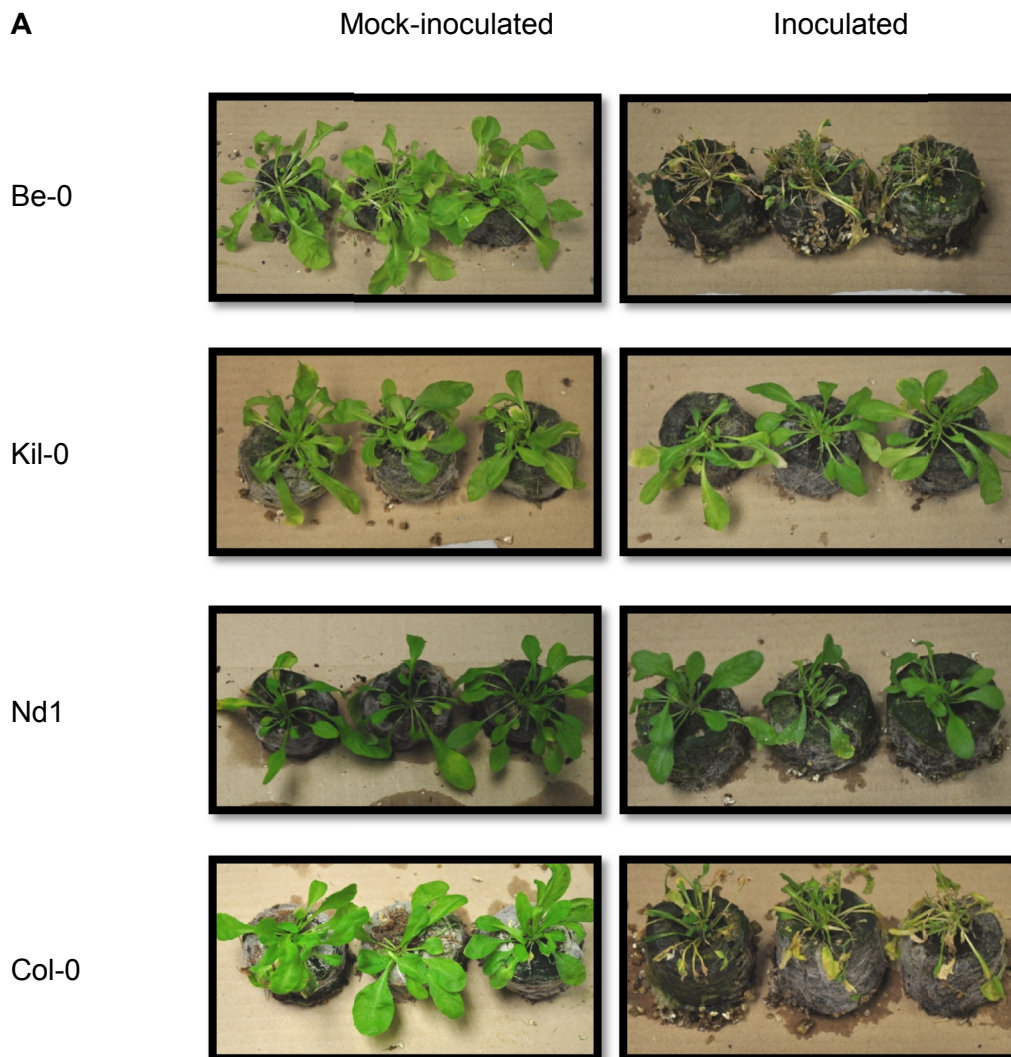
3.1 Arabidopsis accession Kil-0 is tolerant to *R. solanacearum* isolate BCCF402

For this study we have defined tolerance as the plant's ability to withstand higher numbers of bacteria with regards to plant fitness and yield without developing significant disease symptoms (Kover and Schaal, 2002). Resistance is the plant's ability to restrict pathogen colonization and results in low bacterial numbers and no disease symptoms (Kover and Schaal, 2002). Previous studies have identified a potential tolerant interaction between *Arabidopsis* accession Kil-0 and the *Eucalyptus* isolate of *R. solanacearum* known as BCCF402 (Weich, 2004). The tolerant interaction was indicated by high bacterial numbers observed in Kil-0 plants even though there was an absence of disease symptom development (Weich, 2004). This was compared to the high bacterial numbers but severe disease symptoms observed in the susceptible accession Be-0 when infected with the same *R. solanacearum* isolate BCCF402 (Weich, 2004). In order to determine if Kil-0 is tolerant to BCCF402, the severity of disease symptoms was monitored, bacterial load was quantified and plant fitness parameters were measured.

3.1.1 Disease symptom development of *Arabidopsis* accessions Kil-0, Be-0, Nd1 and Col-0 when inoculated with *R. solanacearum* BCCF402^{rif}

Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0 were root inoculated with *R. solanacearum* BCCF402^{rif}, and plants were monitored for symptom development. Accession Be-0 developed severe disease symptoms with plants wilting as early as 3 days post inoculation (dpi). Wilting symptoms developed rapidly until most plants were dead by 14 dpi (Fig 3.1 A and 3.1 B). Figure 3.1 A shows that Kil-0 plants, when inoculated with *R. solanacearum* BCCF402^{rif}, remained symptom free by 14 dpi and the disease index (DI) scores shows that no disease symptoms developed throughout the trial (Fig 3.1 B). Multiple replicate independent experiments were performed and the results

were consistent for each of these trials. In a small number of Kil-0 plants, wilting symptoms were seen at later stages of BCCF402^{rif} infection. Accession Nd1 appears to elicit a symptomless resistant response to BCCF402^{rif} as expected. Accession Col-0 developed severe disease symptoms with plants dying by 14 dpi consistent with a susceptible response. Control plants of accessions Kil-0, Be-0, Nd1 and Col-0 were mock-inoculated with liquid B medium not containing any *R. solanacearum* BCCF402^{rif} bacterial cells and did not develop any wilting symptoms during the infection trial (Fig 3.1 A).



B

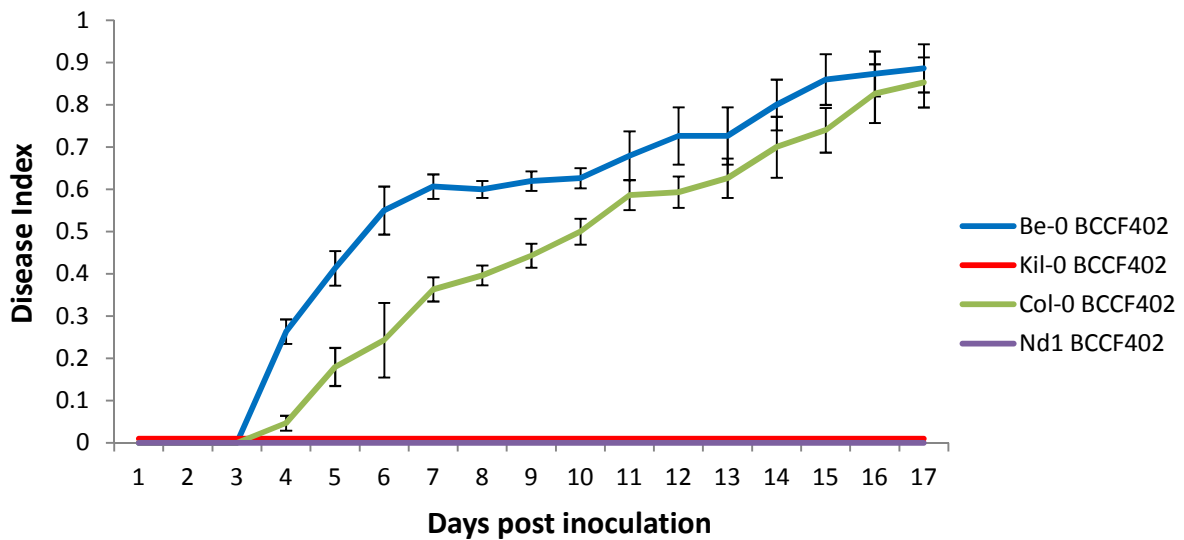


Figure 3.1. **Inoculation of Arabidopsis accessions with *R. solanacearum* isolate BCCF402^{rif}**. A: Photographs of plants representing disease symptom severity of Arabidopsis accessions Be-0, Kil-0, Nd1 and Col-0 at 14 dpi when inoculated with BCCF402^{rif} as well as when mock-inoculated. B: Progression of disease symptoms of Arabidopsis accessions Be-0, Kil-0, Nd1 and Col-0 when inoculated with BCCF402^{rif}. Error bars indicate standard error and were determined using disease index scores of a total of 30 plants per accession (3 replicates of 10 plants each).

3.1.2 *In planta* bacterial quantification of *R. solanacearum* isolate BCCF402^{rif} in Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0

In planta bacterial quantification was performed using two separate methods, dilution plating and quantitative PCR. Root inoculations were performed on Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0. Aerial parts of the plants were harvested at a number of time points, namely 4, 8, 12 and 16 days post inoculation (dpi). A total of 20

plants (4 biological replicates of 5 plants each) were harvested for each accession at every time point. The harvested plant material was pooled and then divided in half for future use in the dilution plating and quantitative PCR experiments. In an initial experiment, it was shown that grinding all plant material in liquid nitrogen using a mortar and pestle before dividing the ground material for the separate dilution plating and qPCR experiments resulted in the most accurate results (data not shown).

Bacterial numeration using the dilution plating method showed an exponential increase in *R. solanacearum* BCCF402^{rif} in aerial parts of Be-0 over time resulting in a concentration of approximately 1×10^{12} colony forming units per gram fresh weight (CFU/g fresh weight) at 16 days post inoculation (dpi) (Fig 3.2). However in accession Kil-0, despite plants remaining symptomless throughout the trial, BCCF402^{rif} bacterial numbers reached 1×10^{11} CFU/g fresh weight at 16 dpi, only one order magnitude less than the number of bacteria quantified in the susceptible accession Be-0 (Fig 3.2). The high bacterial numbers and lack of symptoms in Kil-0 is indicative of a tolerant response. Bacterial numbers in accession Nd1 remained at a consistent concentration of 1×10^7 to 1×10^8 CFU/g fresh weight (Fig 3.2). This is expected from the resistant accession and bacterial numbers were notably lower than in Be-0 and Kil-0. Accession Col-0 also achieved a high bacterial load consistent with a susceptible response (Fig 3.2).

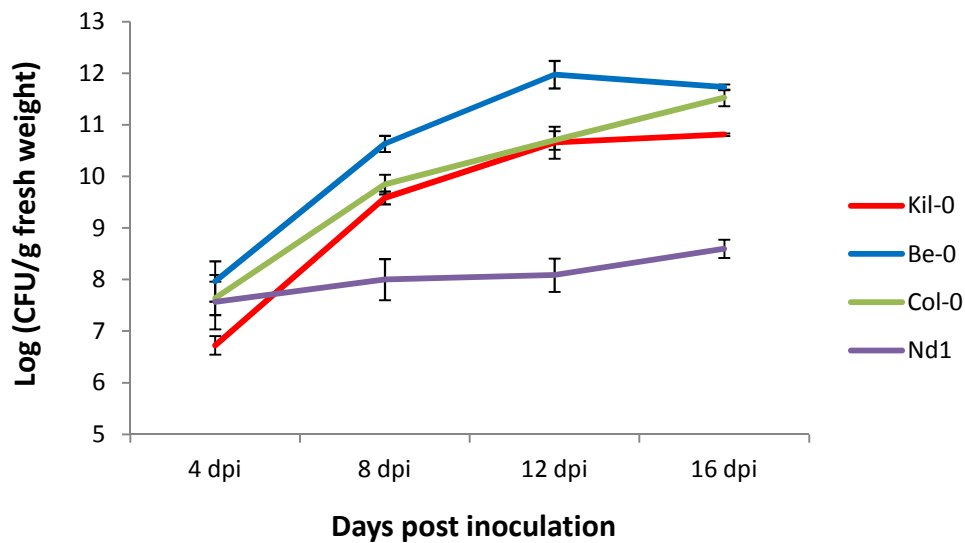


Figure 3.2. **Bacterial numeration of *R. solanacearum* BCCF402^{rif} in the aerial parts of *Arabidopsis* accessions Kil-0, Be-0, Nd1 and Col-0 after root inoculation.** Error bars indicate standard error and were analyzed for 4 replicates of 5 plants each.

3.1.3 *In planta* bacterial quantification of *R. solanacearum* BCCF402^{rif} in *Arabidopsis* using quantitative PCR

The accurate quantification of *R. solanacearum* biomass *in planta* to evaluate the severity of pathogen infection in *Arabidopsis* accessions Kil-0, Be-0, Nd1 and Col-0 was determined using a qPCR assay. We developed a quantitative PCR assay to measure the amount of *R. solanacearum* DNA in *Arabidopsis* plants based on the amplification of the Cytochrome c1 gene region. This method ensures an additional reliable quantitative measurement of pathogen biomass in plants.

3.1.3.1 Design of a quantitative PCR assay for the quantification of *R. solanacearum* DNA in planta

We hypothesized that it would prove effective to normalize the amount of pathogen DNA quantified to an internal control of “alien” DNA rather than to host DNA. In susceptible accessions, pathogen biomass is overestimated as a result of host DNA degradation that occurs due to necrosis of the host tissue. Normalization of pathogen DNA using this “alien” internal control rather than to host DNA avoids the effects of this host tissue necrosis during disease progression. A fungal gene, the *Cercospora zeina* Cytochrome P450 reductase (*cpr1*) gene, was the “alien” DNA chosen for the qPCR assay (Korsman *et al.*, 2012). A plasmid (pJET-CPR1-Cz, Appendix A) containing a fragment of the *cpr1* gene was added during the DNA extraction step and pathogen biomass was normalized based on the plasmid DNA rather than host DNA.

To assess the specificity of the primers for the amplification of the *cpr1* and *cytC* gene, a PCR was performed evaluating the primers in the presence of DNA from *R. solanacearum*, Arabidopsis and the pJET-CPR1-Cz plasmid as well as in a no-template control (Fig 3.3). No amplification occurred when the *R. solanacearum* *cytC* gene specific primer pair, RsolSF and RsolSR (Kang *et al.*, 2007), was used in the presence of Arabidopsis (Fig 3.3 lane 2) and pJET-CPR1-Cz plasmid DNA (Fig 3.3 lane 3) but a band of 256 bp was observed in the presence of *R. solanacearum* DNA (Fig 3.3 lane 1). Amplification of the fungal *cpr1* gene, using specific primers CPR1_2F and CPR1_2R, occurred only in the presence of plasmid DNA, producing a 164 bp fragment (Fig 3.3 lane 6). BLAST analyses comparing the *cpr1* and *cytC* gene specific primers to the genome sequences of Arabidopsis, *R. solanacearum* and *C. zeina* showed that primers were specific to their target DNA (data not shown).

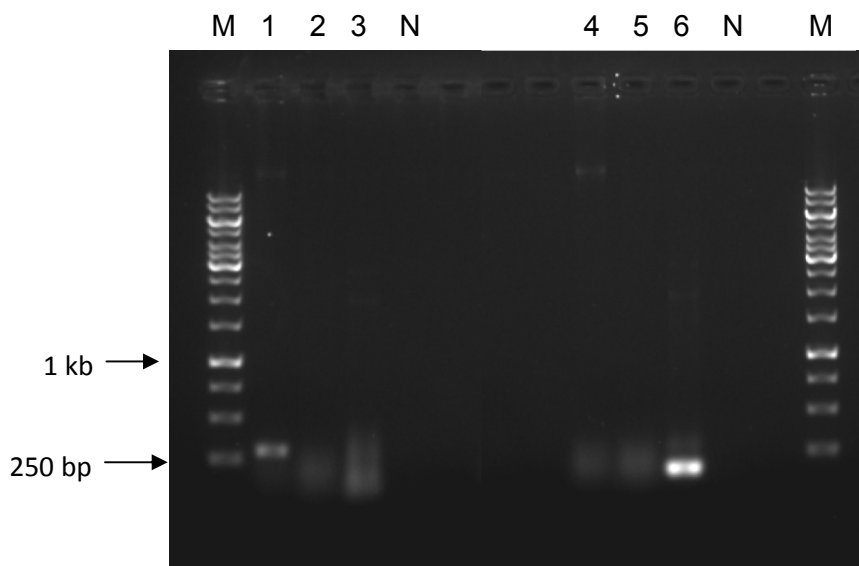


Figure 3.3. **PCR amplification of the *cytC* and *cpr1* gene specific primers using *R. solanacearum*, Arabidopsis and pJET-CPR1-Cz plasmid DNA as templates.** M: 1 kb molecular marker. Lanes 1 to 3: PCR products of reactions using the RsolSF and RsolSR primer pair and containing the following as DNA templates: lane 1: *R. solanacearum* DNA, lane 2: Arabidopsis Be-0 DNA, lane 3: plasmid DNA. Lanes 4 to 6: PCR products of reactions using the CPR1_2F and CPR1_2R primer pair and containing the following as DNA templates: lane 4: *R. solanacearum* DNA, lane 5: Arabidopsis Be-0 DNA, lane 6: plasmid DNA. N: no-template controls. PCR products were analyzed by electrophoresis using a 1 % agarose gel.

To determine the amount of plasmid DNA that must be added to each sample undergoing DNA extraction, three dilutions were tested in such a way that when spiking the plant samples with pJET-CPR1-Cz plasmid DNA at the beginning of the DNA extraction protocol and after re-suspending DNA samples in 40 μ l of distilled water it took approximately 20 PCR cycles for the amplification of plasmid DNA to cross the threshold of background fluorescence (Ct value). The amounts of 1.2 pg, 12 pg and 120 pg were added to the beginning of a DNA extraction containing 5 ng of *R. solanacearum* DNA and approximately 0.05 g of Arabidopsis Be-0 plant tissue. DNA extraction took place and a

qPCR assay was performed. According to Figure 3.4, the amount of 12 pg of plasmid DNA resulted in an average Ct value of 25 cycles. The amount of 1.2 pg of plasmid DNA resulted in an average Ct value of 30.6 cycles while the amount of 120 pg of plasmid DNA resulted in an average Ct value of 23 cycles. Plasmid DNA that crosses threshold fluorescence after 25 cycles will be easily extrapolated using the *cpr1* standard curve (Fig 3.5 C). A concentration of 20 pg of plasmid DNA would provide adequate DNA after DNA extraction for normalization of *R. solanacearum* DNA (Fig 3.4).

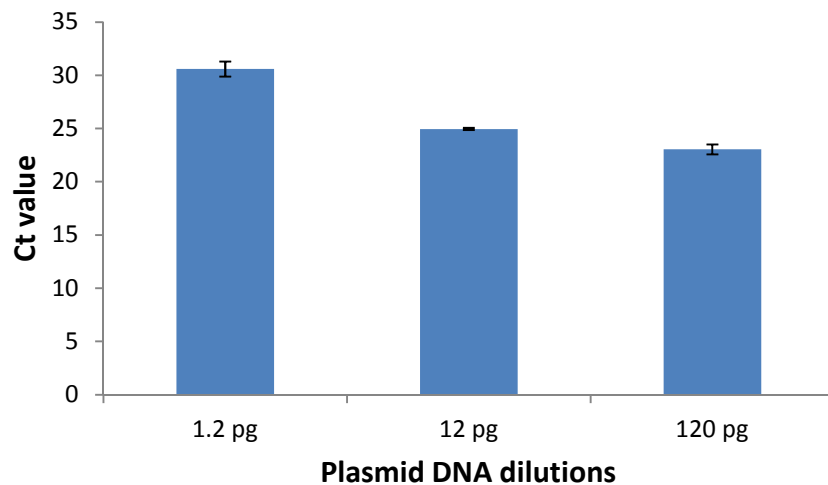


Figure 3.4. **Amounts of plasmid DNA optimal for the normalization of *R. solanacearum* DNA.** Three different amounts of pJET-CPR1-Cz plasmid DNA were added to Arabidopsis plant tissue in the first step of the DNA extraction protocol. The threshold cycle after the qPCR run, using CPR1_2F and CPR1_2R primer pair, was measured. Assays were performed for 3 biological replicates to determine standard errors indicated by error bars on graph.

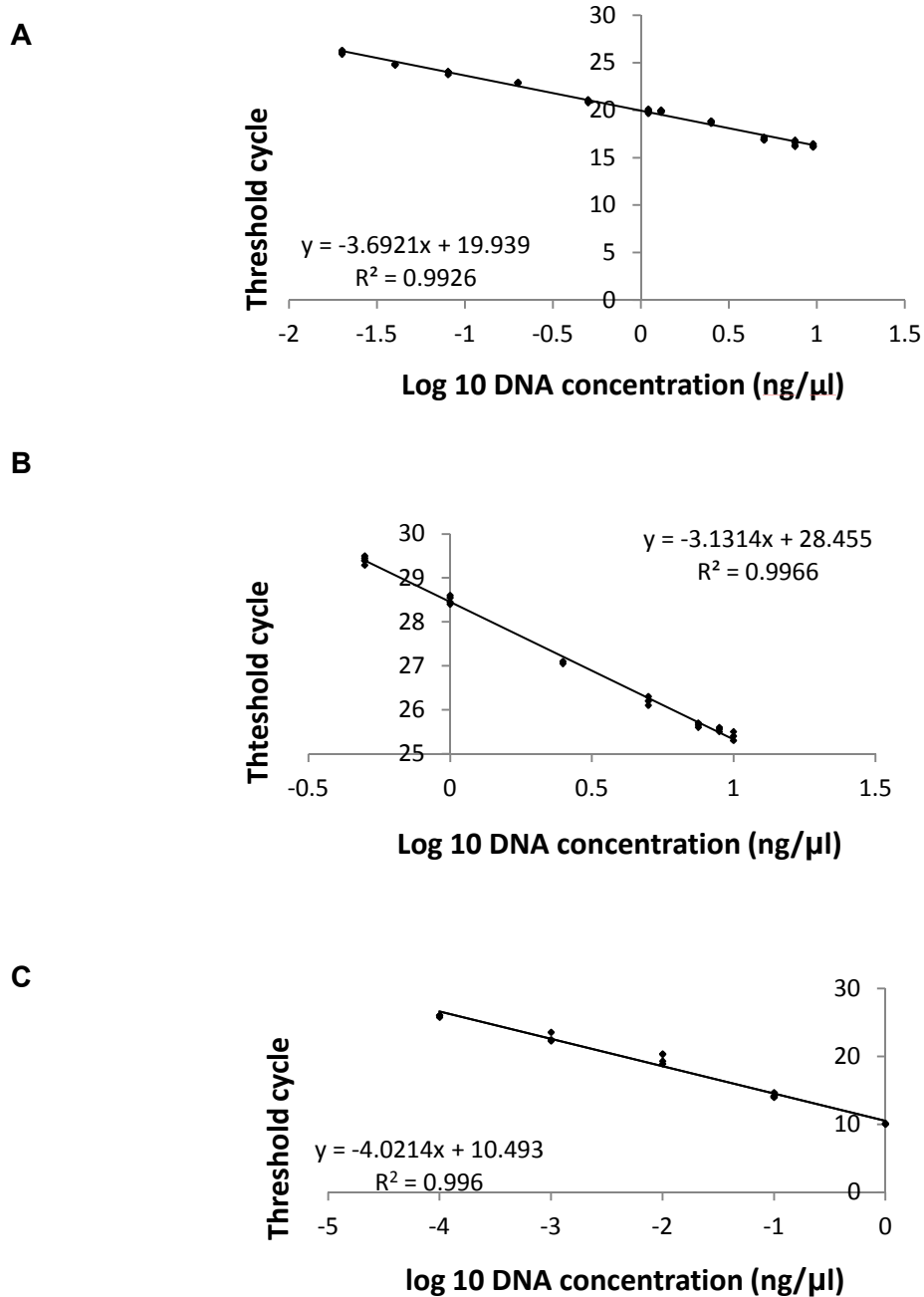


Figure 3.5. **Standard curves used for the qPCR assay to quantify *R. solanacearum* in *planta*.** Graphical illustration of A: *cytC* amplification using RsoISF and RsoISR primer pair, B: *Ubq10* amplification using UBQ10F and UBQ10R primer pair, C: *cpr1* amplification using CPR1_2F and CPR1_2R primer pair. Figures A and B represent standard curves of *R. solanacearum* DNA and Arabidopsis DNA that were combined together to yield a final concentration of 10 ng/μl. Figure C represents a standard curve for *cpr1* amplification using serial dilutions of pJET-CPR1-Cz plasmid DNA.

3.1.3.2 Evaluating plasmid DNA as an internal control for measuring *R. solanacearum* biomass

To test the efficiency of using pJET-CPR1-Cz plasmid DNA as the internal control, Arabidopsis accessions Kil-0 and Be-0 were inoculated with *R. solanacearum* BCCF402. Plant material (3 biological replicates of 3 plants each) was harvested at 12 days post inoculation (dpi). qPCR assays using *ubq10*, *cytC* and *cpr1* specific primers were performed on DNA extracted from the same plant material. Standard curves were generated according to the Materials and Methods (Fig 3.5 A, B and C). An amount of 20 pg of plasmid DNA was added to the beginning of each DNA extraction. Pathogen DNA was normalized to either plasmid DNA or host DNA for comparison.

The ratio of pathogen DNA to plant DNA shows a large difference in Kil-0 plants compared to Be-0 plants (Fig 3.6 A). However, the ratio of pathogen DNA to plasmid DNA shows a much smaller difference between the Kil-0 and Be-0 plants (Fig 3.6 B). This result indicates that there is a small difference in the pathogen biomass in Kil-0 plants compared to Be-0 plants when normalized to plasmid DNA. When pathogen DNA is normalized to plant DNA, an overestimation of pathogen biomass in Be-0 may be the cause of the large ratio difference between the Kil-0 and Be-0 plants. The ratio of pathogen DNA to plasmid DNA can be compared to the plate counting method at 12 dpi from figure 3.2 (Fig 3.6 C).

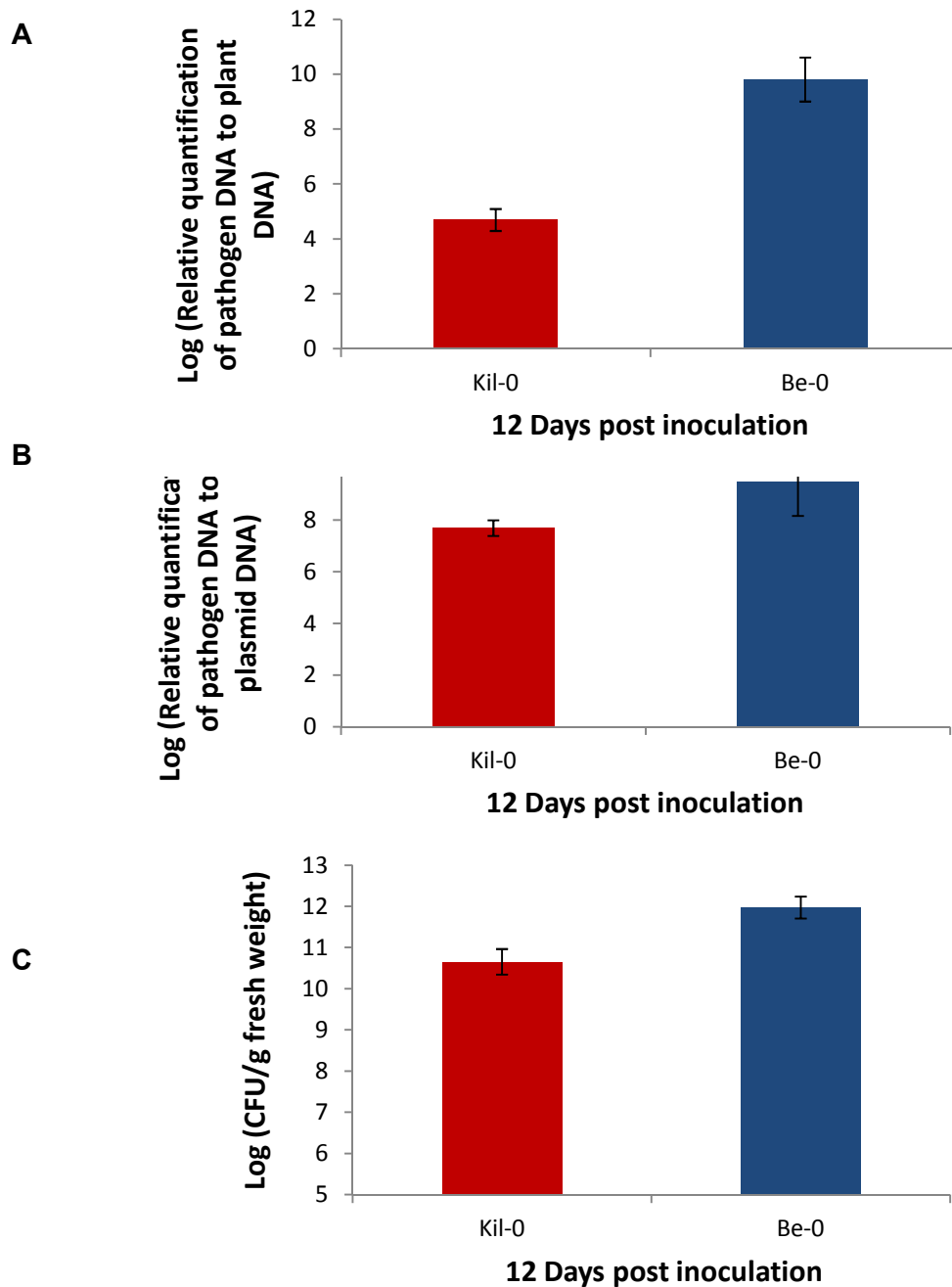


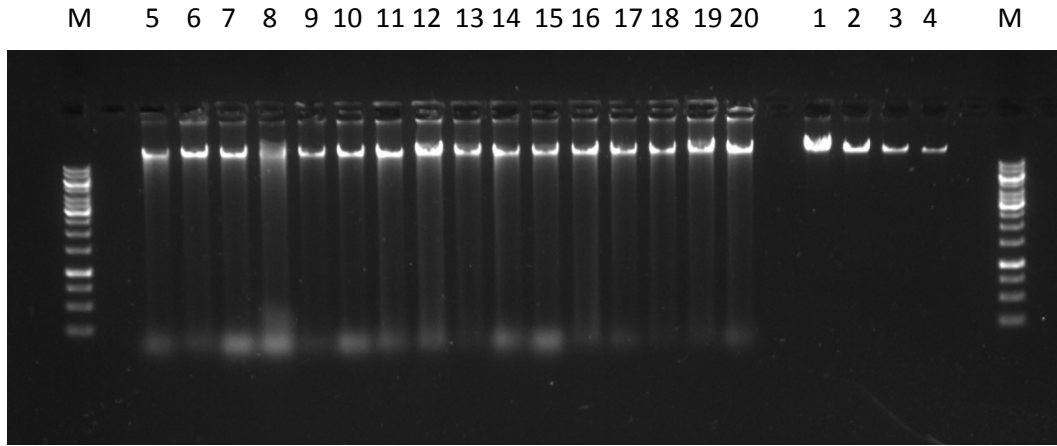
Figure 3.6. Comparison of bacterial numeration of *R. solanacearum* BCCF402^{rif} in *Arabidopsis* accessions Kil-0 and Be-0 and qPCR graphs where *R. solanacearum* DNA was normalized to plant DNA or to plasmid DNA at 12 dpi. DNA extracted from *Arabidopsis* accession Kil-0 and Be-0 was subjected to the qPCR assay to quantify *R. solanacearum* DNA normalized to A: *Arabidopsis* DNA or B: plasmid DNA C: DNA extracted from *Arabidopsis* accession Kil-0 and Be-0 was subjected to plate counting. Error bars indicate standard error.

3.1.3.3 qPCR quantification of *R. solanacearum* DNA in infected Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0

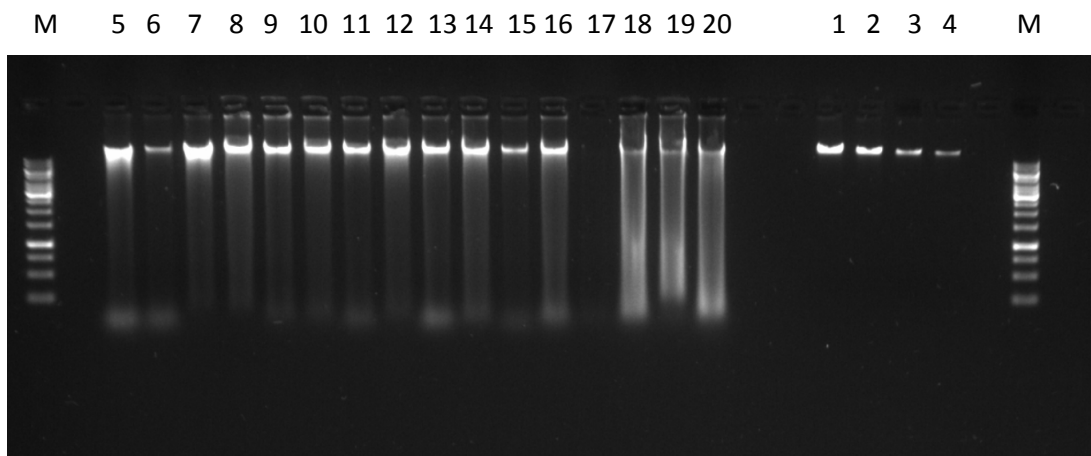
Amplification of the *cytC* gene region by quantitative PCR (qPCR) was undertaken in order to assess *R. solanacearum* biomass in inoculated Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0. DNA extractions (Fig 3.7 A to D) were performed on whole aerial tissue of accessions Kil-0, Be-0, Nd1 and Col-0 and subjected to the qPCR assay using *cytC* and *cpr1* gene specific primers. An amount of 20 pg of pJET-CPR1-Cz plasmid DNA was added at the start of each DNA extraction. RNase was also added to remove RNA from the DNA samples. High quality DNA was obtained from accessions Col-0 and Nd1 (Fig 3.7 A and B). Lambda standards were used to determine the amount of genomic DNA isolated. DNA samples were diluted to 10 ng/ul and qPCR assays were performed. Be-0 and Kil-0 samples were performed on one qPCR run and Col-0 and Nd1 samples were performed on a separate qPCR run. The amount of *R. solanacearum* DNA in each sample was determined from the *cytC* standard curve (Fig 3.5 A) and normalized to the amount of plasmid DNA determined from the *cpr1* standard curve (Fig 3.5 B).

The bacterial load of *R. solanacearum* was quantified in Kil-0 and Be-0 accessions using the qPCR assay. A large amount of bacterial DNA was observed in the susceptible accession Be-0 with a similarly high amount of bacterial DNA found in accession Kil-0 at 12 and 16 dpi (Fig 3.8 A). These results were comparable to the high concentrations of bacteria measured in these accessions using the standard dilution plating method (Fig 3.2). The amount of pathogen DNA quantified in accessions Nd1 showed a larger difference to the amount of pathogen DNA found in Col-0 (Fig 3.8 B).

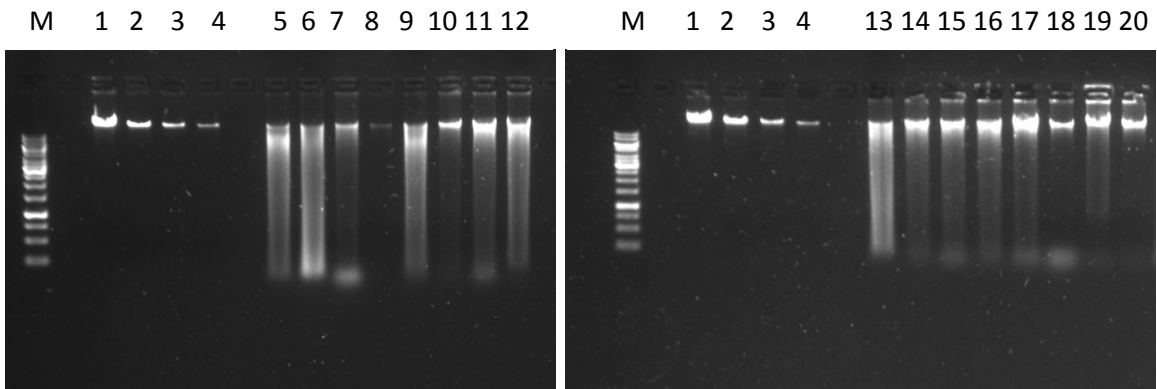
A



B



C



D

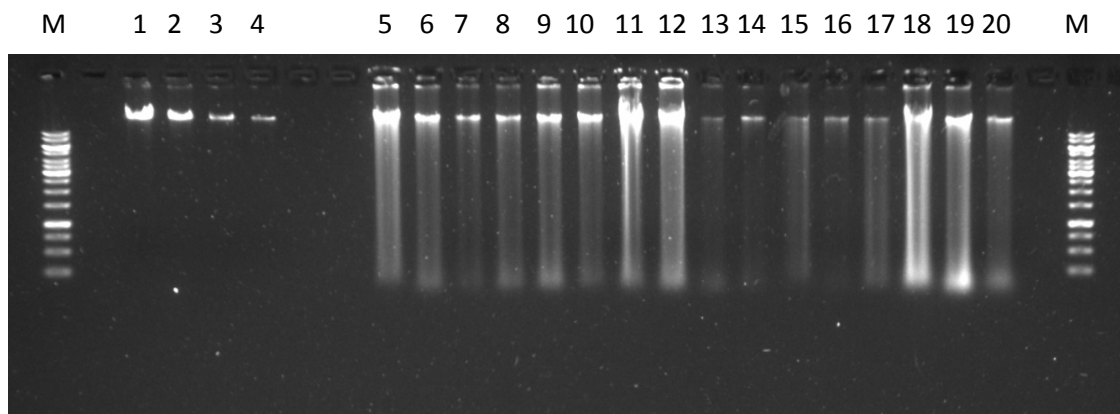


Figure 3.7. **Genomic DNA isolated from Arabidopsis accessions Kil-0, Be-0, Nd1 and Col-0 plants.** A: DNA isolated from accession Nd1, B: DNA isolated from accession Col-0, C: DNA isolated from accession Kil-0, D: DNA isolated from accession Be-0. M: 1kb Molecular ladder. Lanes 1 to 4 of each photograph: Lambda standards, lane 1: 100 ng, lane 2: 50 ng, lane 3: 25 ng, lane 4: 12.5 ng. Lanes 5 to 20 of each photograph (A, B, C, D): genomic DNA isolated from 4 biological replicates at 4 (lanes 5 – 8), 8 (lanes 9 – 12), 12 (lanes 13 – 16) and 16 (lanes 17 – 20) days post inoculation. The DNA was analyzed by electrophoresis through a 1 % (w/v) agarose gel.

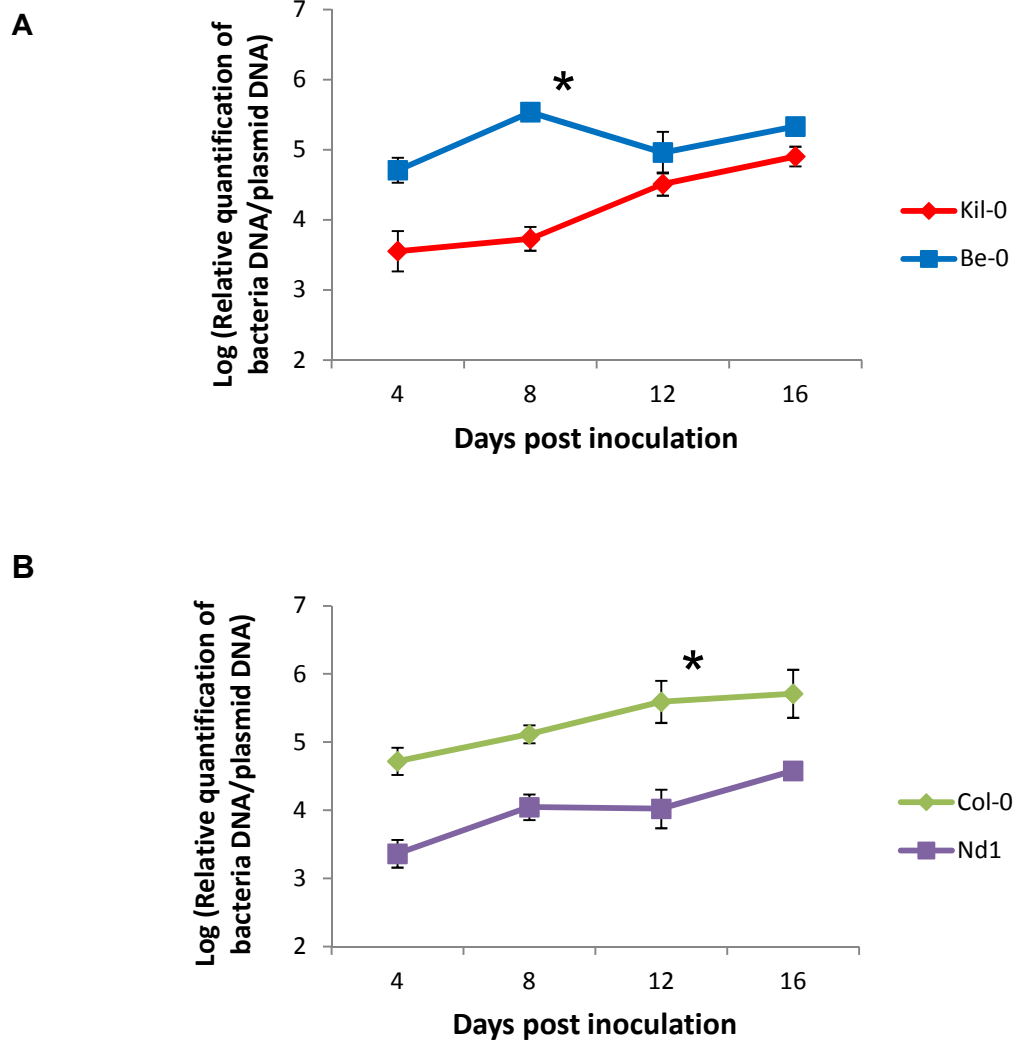


Figure 3.8. *R. solanacearum* quantification in *Arabidopsis* accessions Kil-0, Be-0, Nd1 and Col-0 using qPCR normalized to plasmid DNA. A: *R. solanacearum* DNA quantified in accessions Kil-0 and Be-0, B: *R. solanacearum* DNA quantified in accessions Col-0 and Nd1. Error bars indicate standard errors. Asterisks indicate significant differences (1-Way ANOVA; $p < 0.05$, Tukey pairwise comparison; $p < 0.05$).

3.1.4 Arabidopsis accession Kil-0 does not show a reduction in fitness after infection with *R. solanacearum* BCCF402^{rif}

In order to determine if Arabidopsis accession Kil-0 is tolerant to *R. solanacearum* isolate BCCF402^{rif} we need to observe that there is not a significant reduction in fitness despite the high bacterial numbers that are present in Kil-0 plants and the lack of disease symptoms. The parameters that were measured that represent fitness were plant biomass yield, seed yield and seed viability of accessions Kil-0 and Be-0 inoculated with BCCF402^{rif} compared to mock-inoculated plants of the same accessions (Barth *et al.*, 2003).

The dry weight of accession Kil-0 was not significantly less in plants inoculated with *R. solanacearum* BCCF402^{rif} compared to plants that were mock-inoculated (T-test; $p < 0.05$). The dry weight of the susceptible accession Be-0 when inoculated with BCCF402^{rif} was significantly reduced compared to mock-inoculated Be-0 plants (Fig 3.9 A). In accession Kil-0, there was no significant difference in the number of seed produced by plants inoculated with BCCF402^{rif} compared to mock-inoculated plants. The germination yield of Kil-0 inoculated plants was also not significantly different from the germination yield of the mock-inoculated plants with both germination yields remaining high (Fig 3.9 B and C). In contrast, accession Be-0 inoculated with BCCF402^{rif} did not produce seed as the plants died before seed developed. Mock-inoculated Be-0 plants produced a similar number of seed per plant as the Kil-0 control plants and these control Be-0 plants germinated at a rate that was similar to Kil-0 control plants. Of note is the observation that Kil-0 plants inoculated with BCCF402^{rif} produced a greater amount of seed per plant compared to the mock-inoculated Kil-0 plants, though the result was not significant (Fig 3.9 B).

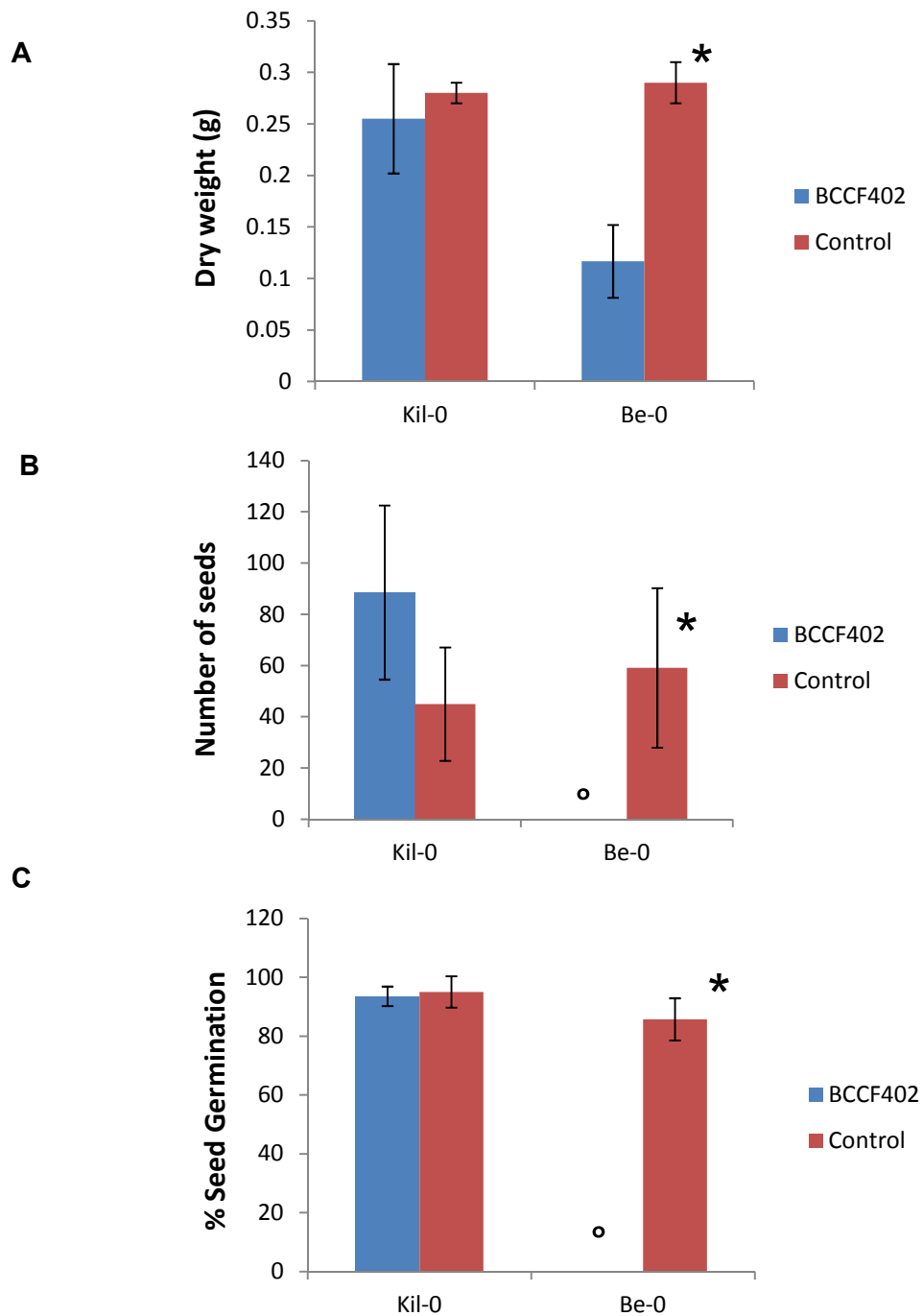


Figure 3.9. **Fitness parameters of Arabidopsis accessions Kil-0 and Be-0 when inoculated with *R. solanacearum* BCCF402^{rif}.** A: Dry weight measured at 16 dpi from 3 replicates of 10 plants each, B: Seeds were counted from 3 replicates of 3 plants each, C: % seed germination. Bars on graphs are an indication of standard errors. Asterisks indicate significant differences (Student's T-test, $p < 0.05$). The circle indicates that no seed was produced as the plants died before flowering and seed production.

3.2 Visualization of *R. solanacearum* BCCF402 *in planta* using a mCherry fluorescent protein tag

The red-range fluorescent protein mCherry was used to tag *R. solanacearum* strain BCCF402 for the visualization and localization of bacterial cells in *Arabidopsis* roots and leaves. The aim of this work was to develop a molecular tool by tagging *R. solanacearum* BCCF402 with mCherry that is expressed at a level that allows visualization *in planta* and can be maintained throughout an infection trial. This will contribute to the understanding of the progression of the *R. solanacearum* infection in the *R. solanacearum* – *Arabidopsis* interaction.

3.2.1 Tagging *R. solanacearum* BCCF402 with mCherry

R. solanacearum BCCF402 was transformed with plasmids pMP7604 and pMP7605 (obtained from Universiteit Leiden, the Netherlands, Legendijk *et al.*, 2010) which contained the *mCherry* gene under the control of the *tac* promoter using a heat shock method (Fig 3.11). Transformed cells were selected on BGT plates containing appropriate antibiotics (16 µg/ml tetracycline for pMP7604, 50 µg/ml gentamycin for pMP7605) and were incubated at 28 °C for 2 days. Single colonies from each transformation event were selected, stored at -80 °C in glycerol and used in following studies. *R. solanacearum* BCCF402 strains transformed with plasmids pMP7604 and pMP7605, renamed BCCF402_4 and BCCF402_5 respectively underwent several tests to confirm the presence of mCherry plasmids in the BCCF402 strains.

In order to verify that pMP7604 and pMP7605 were introduced into BCCF402, PCR amplifications were performed using primers mCherry_Start and mCherry_Stop which are specific for the mCherry fragment. Plasmid DNA was successfully isolated from overnight

cultures of BCCF402_4 and BCCF402_5 that were incubated at 28 °C and contained the appropriate antibiotics. The concentration and purity of the plasmid DNA was measured using the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The 260/280 ratio was above 1.9 for all samples. The DNA extracted ranged from 50-100 ng/μl (data not shown). The mCherry fragment was successfully amplified from plasmid DNA. A single band at a size of 711 bp representing the full mCherry fragment was observed for BCCF402_4 and BCCF402_5 as expected. The negative control yielded no amplification product (Fig 3.10)

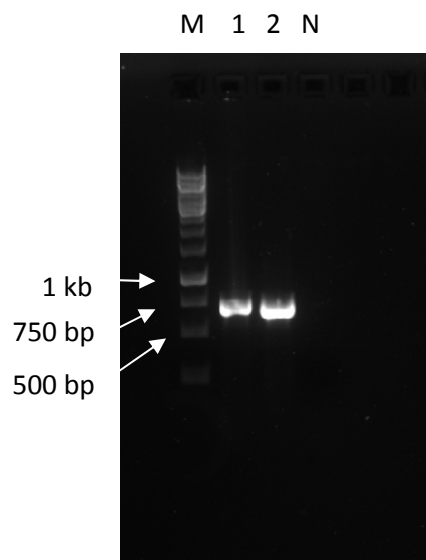


Figure 3.10. **PCR amplification of mCherry fragment from plasmids pMP7604 and pMP7605 using primer pair mCherry_Start and mCherry_Stop.** M: 1 kb molecular marker, lane 1: PCR product from pMP7604, lane 2: PCR product from pMP7605, N: negative water control. PCR products were analyzed using electrophoresis on 1.5 % agarose gel.

In order to confirm the plasmid structure of pMP7604 and pMP7605 and to verify the presence of the mCherry fragment, restriction enzyme digestion of the plasmids was performed. Plasmids were isolated from *R. solanacearum* strains BCCF402_4 and BCCF402_5 and restriction enzyme digestion was performed using various enzymes identified from restriction enzyme maps designed using Genbank sequences of the pMP7604 backbone, pME6031 (AF118811); the pMP7605 backbone sequence pBBR1MCS-5 (U25061), mCherry fragment sequence (AY678264.1) and the *tac* promoter sequence (E03904.1) (Fig 3.11 A and 3.11 B). Plasmids were digested with single enzymes BamHI and NcoI (Fig 3.11 C and 3.11 D). Double digestions were also performed using NcoI and Sall as well as NcoI and BglII in a sequential double digestion. Plasmid pMP7604, when digested with BamHI was expected to produce two fragments of 8310 bp and 834 bp in size (Fig 3.11 C lane 1). Single digestion with NcoI was also expected to result in two fragments, 8778 bp and 366 bp in size (Fig 3.11 C lane 2). The restriction enzyme NcoI proved especially helpful as it was a site that was present within the mCherry fragment. The banding pattern when digested with this enzyme would confirm the presence of the mCherry fragment. The double digestion of pMP7604 with NcoI and Sall was expected to produce four fragments of 6436 bp, 2342 bp, 324 bp and 42 bp in size (Fig 3.11 C lane 3). Digestion with NcoI and BglII was expected to produce three fragments of 8761 bp, 366 bp and 17 bp in size (Fig 3.11 C lane 4).

Restriction enzyme digestion of pMP7605 with BamHI was expected to produce two fragments of 4768 bp and 834 bp in size (Fig 3.11 D lane 1) while digestion with NcoI was expected to produce 4615 bp and 987 bp size fragments (Fig 3.11 D lane 2). Digestion of pMP7605 with NcoI and Sall was expected to produce three fragments of 4615 bp, 568 bp and 419 bp in size (Fig 3.11 D lane 3). pMP7605 digested with NcoI and BglII was expected to produce three fragments of 4042 bp, 987 bp and 573 bp in size (Fig 3.11 D lane 4). All banding patterns coincide to what is expected according to the restriction maps except for the smaller sized fragments (42 bp and 17 bp) that were most likely too small to be seen on the agarose gel.

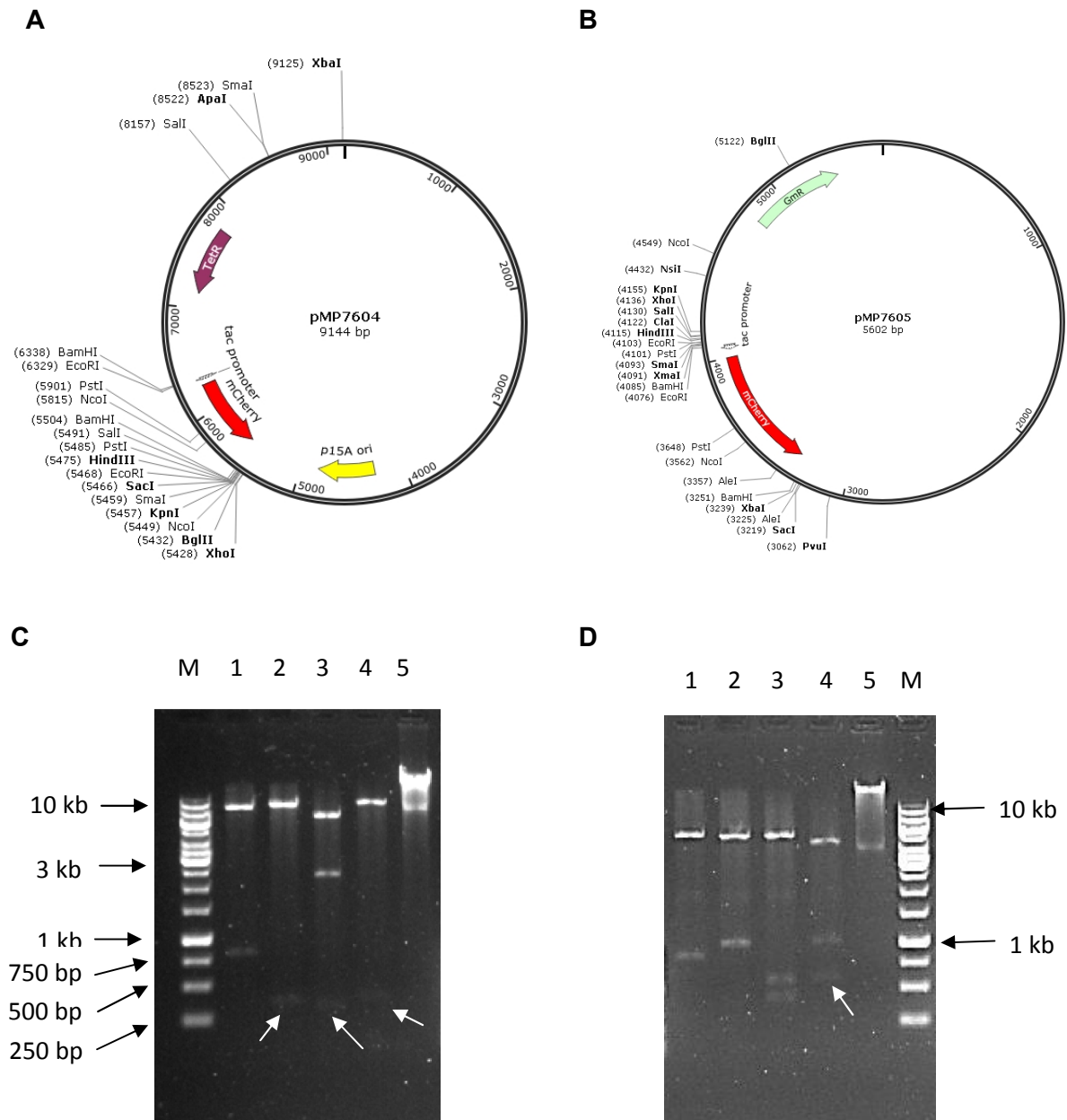


Figure 3.11. **Restriction enzyme mapping of pMP7604 and pMP7605.** A: Plasmid map of pMP7604. B: Plasmid map of pMP7605. C and D: Restriction enzymes digestion of plasmids of C: pMP7604 and D: pMP7605. M: 1 kb molecular marker, lane 1: BamHI, lane 2: NcoI, lane 3: NcoI and SalI, lane 4: NcoI and BglII, lane 5: uncut plasmid. PCR products were analyzed using electrophoresis on 1.5 % agarose gel.

The full length mCherry fragment including the *tac* promoter was sequenced from pMP7604 and pMP7605 isolated from *R. solanacearum* BCCF402 strains. Sequencing was performed using external and internal primers to achieve a complete mCherry sequence spanning the promoter region. Primers mCherry_Start, mCherry_Stop, mCherryF and mCherryR were used (Table 2.2). The consensus sequence matches perfectly with the mCherry sequence from Genbank except for one mismatched base in the *tac* promoter region (Genbank E03904.1) of both pMP7604 and pMP7605 (Fig 3.12). This mutation was present in the -35 consensus sequence of the promoter region and therefore could affect mCherry expression levels.

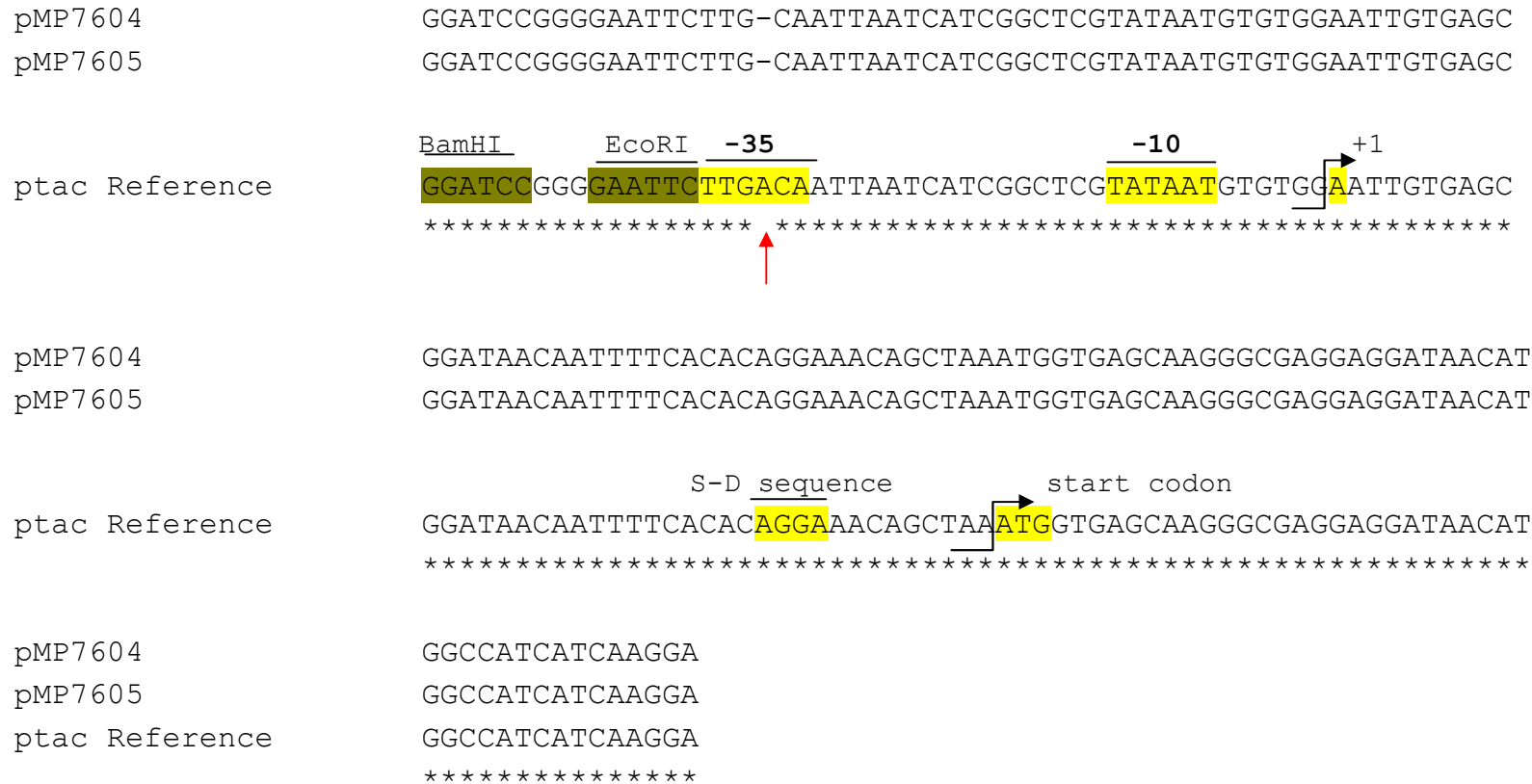


Figure 3.12. **Sequence comparison of *tac* promoter for pMP7604, pMP7605 and Genbank reference sequence (E03904.1).** Green shading indicates restriction sites, yellow shading indicates consensus sequences namely the -35 consensus sequence, the -10 Pribnow box (TATA box), the Shine-Dalgarno sequence as well as transcriptional and translational start sites. The asterisks indicate conserved bases and the red arrow indicates a sequence difference.

3.2.2 Analysis of mCherry expression levels in *R. solanacearum* BCCF402_4 and BCCF402_5

An analysis of the expression levels of mCherry produced in *R. solanacearum* BCCF402_4 and BCCF402_5 was performed in order to assess the brightness of the different strains. To measure the amount of mCherry produced by the BCCF402_4 and BCCF402_5 strains, fluorescence was quantified using fluorometry and *R. solanacearum* cells were visualized using light microscopy. *R. solanacearum* BCCF402_5 strains produced the highest fluorescent levels compared to the wildtype *R. solanacearum* BCCF402 strain according to the fluorometric analysis (Fig 3.13 A). This was considerably more than the expression levels of mCherry in the BCCF402_4 strains. Both BCCF402_4 and BCCF402_5 strains produced more mCherry than the wildtype BCCF402 strain despite having a mutation in the -35 consensus sequence in the *tac* promoter region

Rifampicin mutants of BCCF402_5 were prepared and the amount of fluorescence was compared to the BCCF402_5 strains. Rifampicin resistant strains were generated to enable the precise re-isolation of bacterial strains from *Arabidopsis* and to quantify bacterial counts by dilution plating on selective media. The rifampicin resistant BCCF402_5^{rif} strains were generated. A high fluorescent level of mCherry was produced in the BCCF402_5^{rif} strain compared to the wildtype BCCF402 strain (Fig 3.13 B).

In order to determine whether the fluorescence detected by the fluorometer will be sufficient for visualization of *R. solanacearum* cells under a microscope, light microscopy was performed. Overnight BCCF402_5 and BCCF402_5^{rif} bacterial cells were suspended in 80 % glycerol and fluorescence microscopy was performed. mCherry production was visualized using fluorescence microscopy (Fig 3.14).

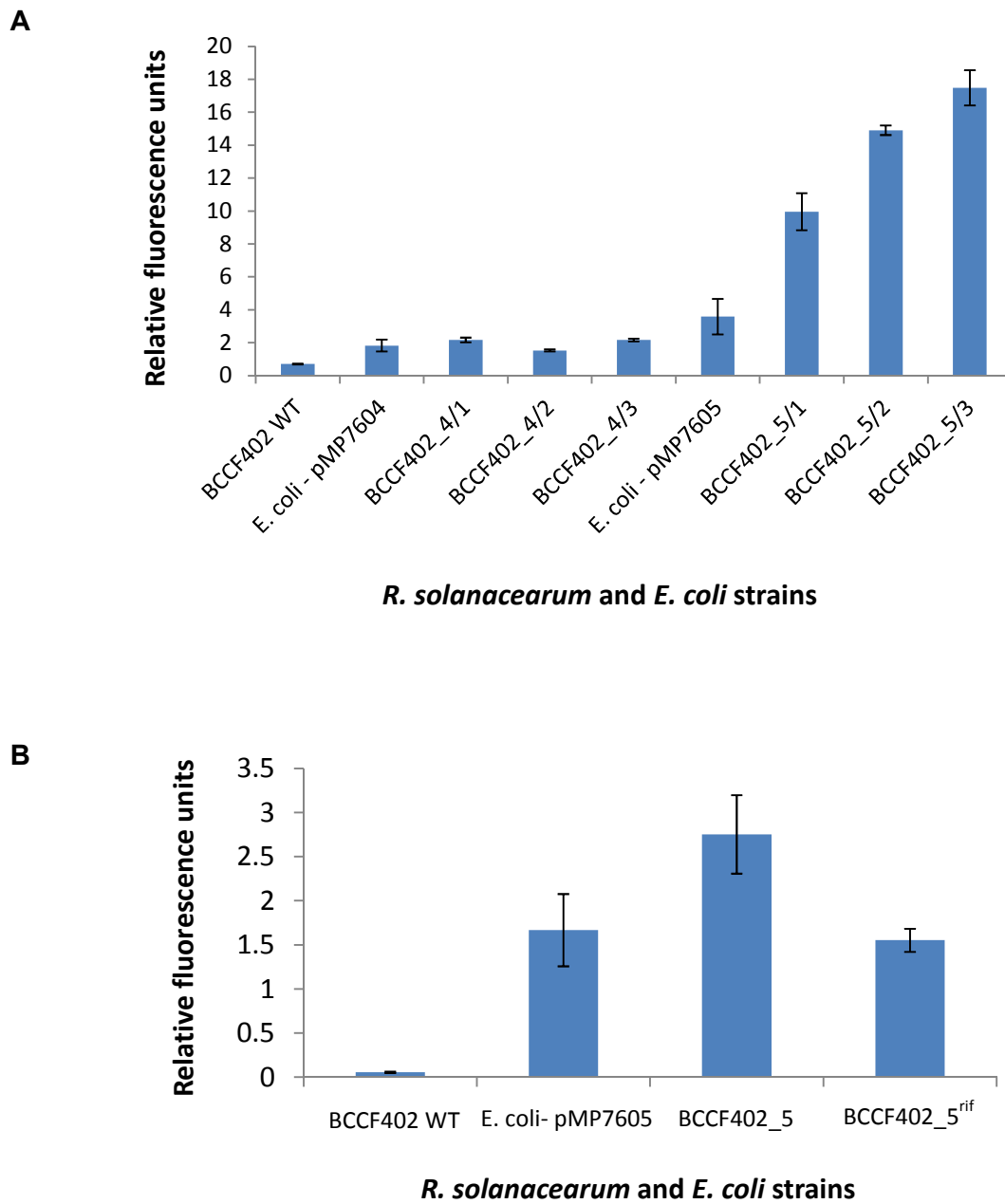


Figure 3.13. **Analysis of mCherry production in *R. solanacearum* BCCF402_4 and BCCF402_5 strains.** A and B: Fluorescence of mCherry produced by BCCF402_4 and BCCF402_5 strains were quantified using a fluorometer with an excitation wavelength of 530 nm and an emission wavelength of 635 nm. Error bars indicate standard errors determined from three independent replicates.

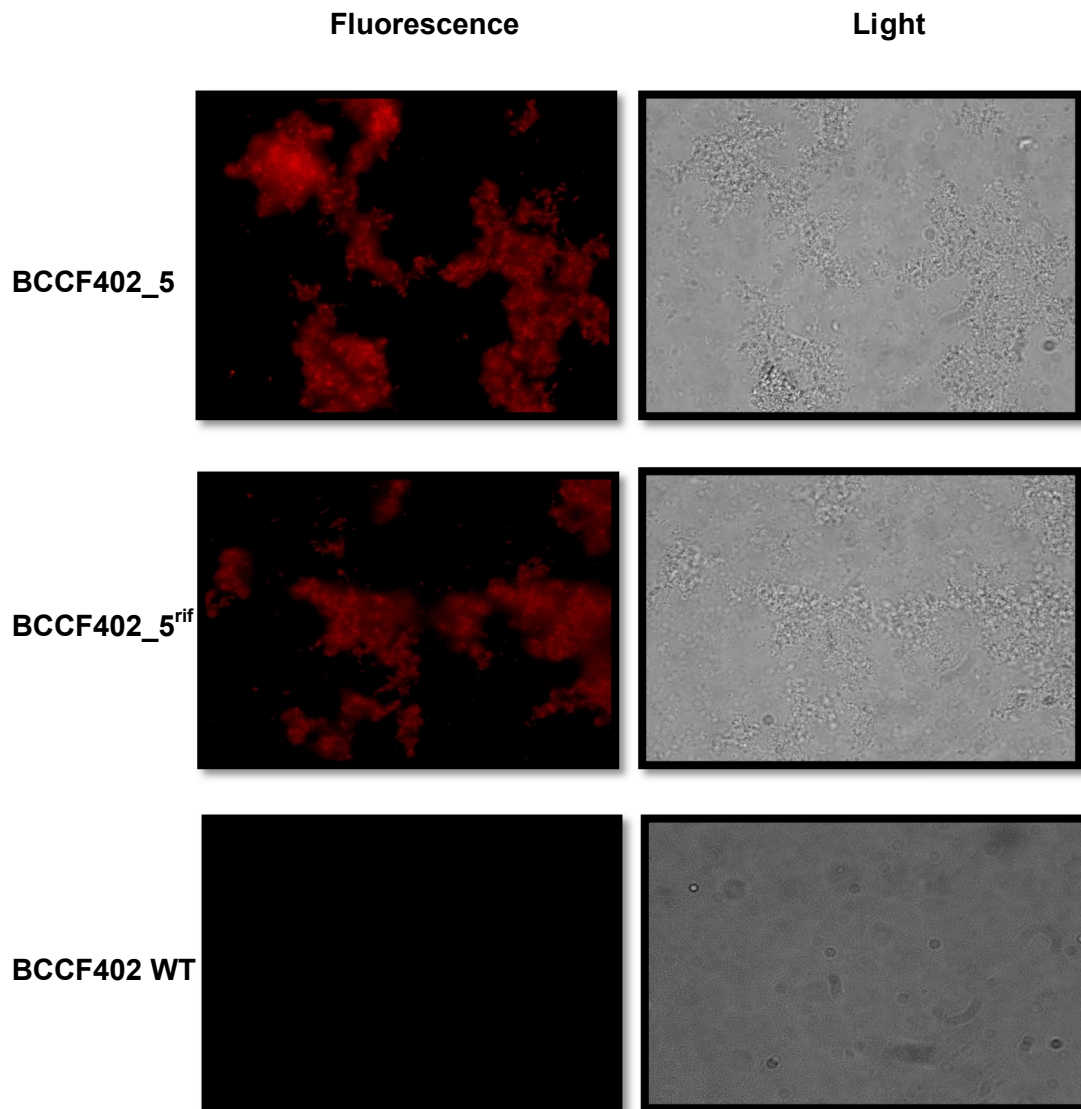


Figure 3.14. **Microscopic images of *R. solanacearum* BCCF402_5 and BCCF402_5^{rif} strains.** Light Microscopic analysis of BCCF402 wildtype, BCCF402_5 (containing pMP7605) and BCCF402_5^{rif} strains. Images on the left were made by fluorescence light microscopy using an excitation filter of 546 nm and an emission filter of 560 nm to obtain fluorescing image. The corresponding images on the right were acquired using light microscopy.

3.2.3 Growth rate, virulence and stability

The presence of plasmids and the expression of mCherry were evaluated for their effect on *R. solanacearum* strains in terms of virulence, growth rate and disease progression. Growth of BCCF402_4 and BCCF402_5 strains in liquid B medium was monitored. B media contained 16 µg/ml tetracycline for BCCF402_4 cells and 50 µg/ml of gentamycin for BCCF402_5 cells. Spectrophotometer OD₆₀₀ readings were taken every half an hour. Growth of *R. solanacearum* strains were very similar (Fig 3.15 A). *R. solanacearum* BCCF402_5 and BCCF402_5^{rif} strains were shown to be virulent because of their ability to cause a hypersensitive response on tobacco leaves (Fig 3.15 B). Tobacco cv. petit havana leaves were infiltrated with an overnight culture of BCCF402_5 and BCCF402_5^{rif} cells. Negative water and *hrp* mutant controls did not develop a hypersensitive response on tobacco (Fig 3.15 B).

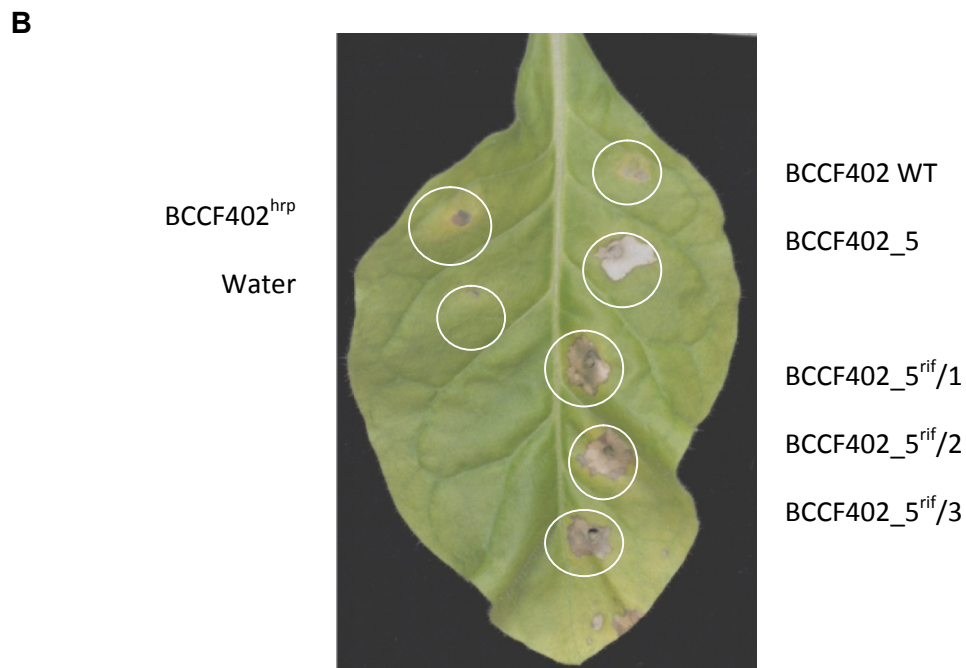
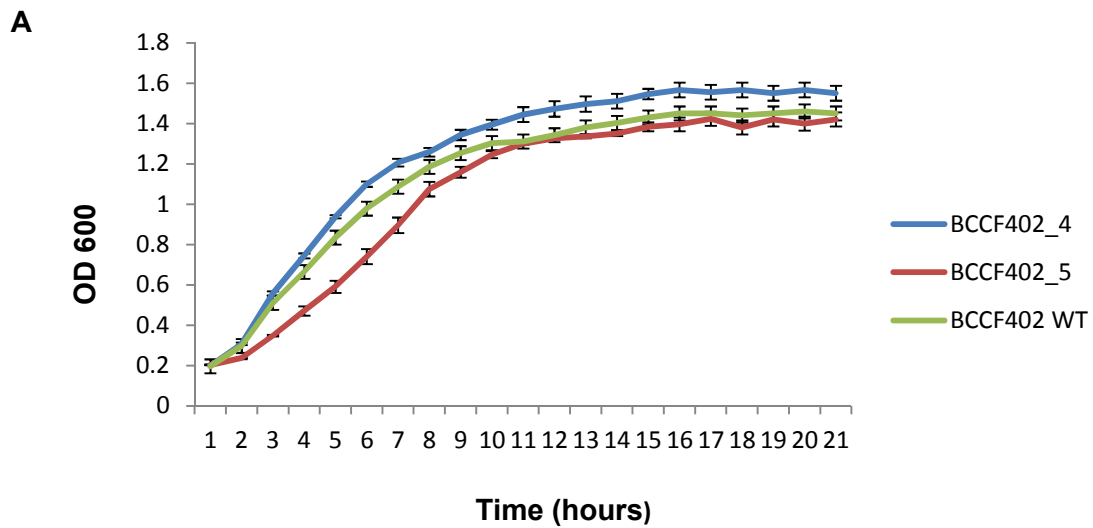


Figure 3.15. **Growth rate analysis and virulence of *R. solanacearum* strains.** A: Growth of the BCCF402_4 and BCCF402_5 strains compared to wildtype *R. solanacearum* BCCF402 strain. Spectrophotometer (OD₆₀₀) readings were taken in half hour intervals. Bars on graph indicate standard errors determined from three replicate readings. B: The presence of the hypersensitive response on the nonhost tobacco cv. petit havana with BCCF402_5 and BCCF402_5^{rif} strains. Negative water and *hrp* mutant controls are present.

Arabidopsis accessions Kil-0 and Be-0 were inoculated with *R. solanacearum* strain BCCF402_5^{rif}/1 and 2 to determine whether plant disease symptom development (Fig 3.16) and bacterial growth *in planta* (Fig 3.17) is adversely affected by the presence of the mCherry plasmid pMP7605 in BCCF402_5 cells. Arabidopsis accession Be-0 showed very similar disease symptom progression when inoculated with two *R. solanacearum* BCCF402_5^{rif} strains compared to the isolate BCCF402^{rif} (Fig 3.16). The *R. solanacearum* BCCF402 strains did not result in the development of severe disease symptoms in the Be-0 accession. In previous experiments, Be-0 plants were completely wilted by approximately 14 days post inoculation (dpi). Accession Kil-0 did not develop any disease symptoms when inoculated with *R. solanacearum* BCCF402^{rif} and BCCF402_5^{rif} strains (data not shown).

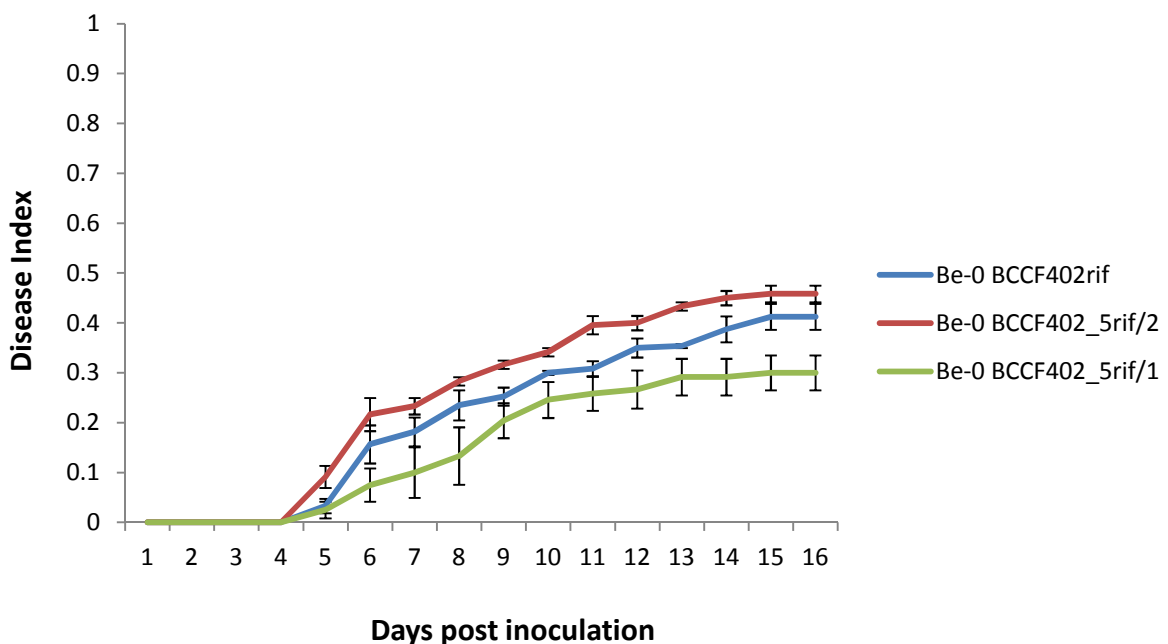


Figure 3.16. **Progression of disease symptoms in Arabidopsis accession Be-0 when inoculated with BCCF402^{rif} and BCCF402_5^{rif} strains.** Errors bars on graph represent standard errors which were determined from 3 replicates of 10 plants each.

Arabidopsis accessions Be-0 and Kil-0 were inoculated with two *R. solanacearum* strains (BCCF402_5^{rif}/1 and BCCF402_5^{rif}/2). Bacterial counting was performed to determine whether *in planta* bacterial numbers were affected due to the introduction of mCherry plasmid pMP7605 into *R. solanacearum* strains. Arabidopsis accession Be-0 inoculated with *R. solanacearum* BCCF402^{rif} did not contain the high bacterial numbers seen in previous infection trials (Fig 3.17 A and B). This result corresponds to the disease index (DI) scores, where the degree of the bacterial infection was not as severe as observed previously (Fig 3.16). Be-0 plants infected with the BCCF402_5^{rif}/1 and 2 strains have considerably lower bacterial numbers than the BCCF402^{rif} strain at 8 dpi. BCCF402_5^{rif} bacterial numbers then appear to increase rapidly until reaching numbers that are similar to the BCCF402^{rif} strain at 12 dpi (Fig 3.17 A and B).

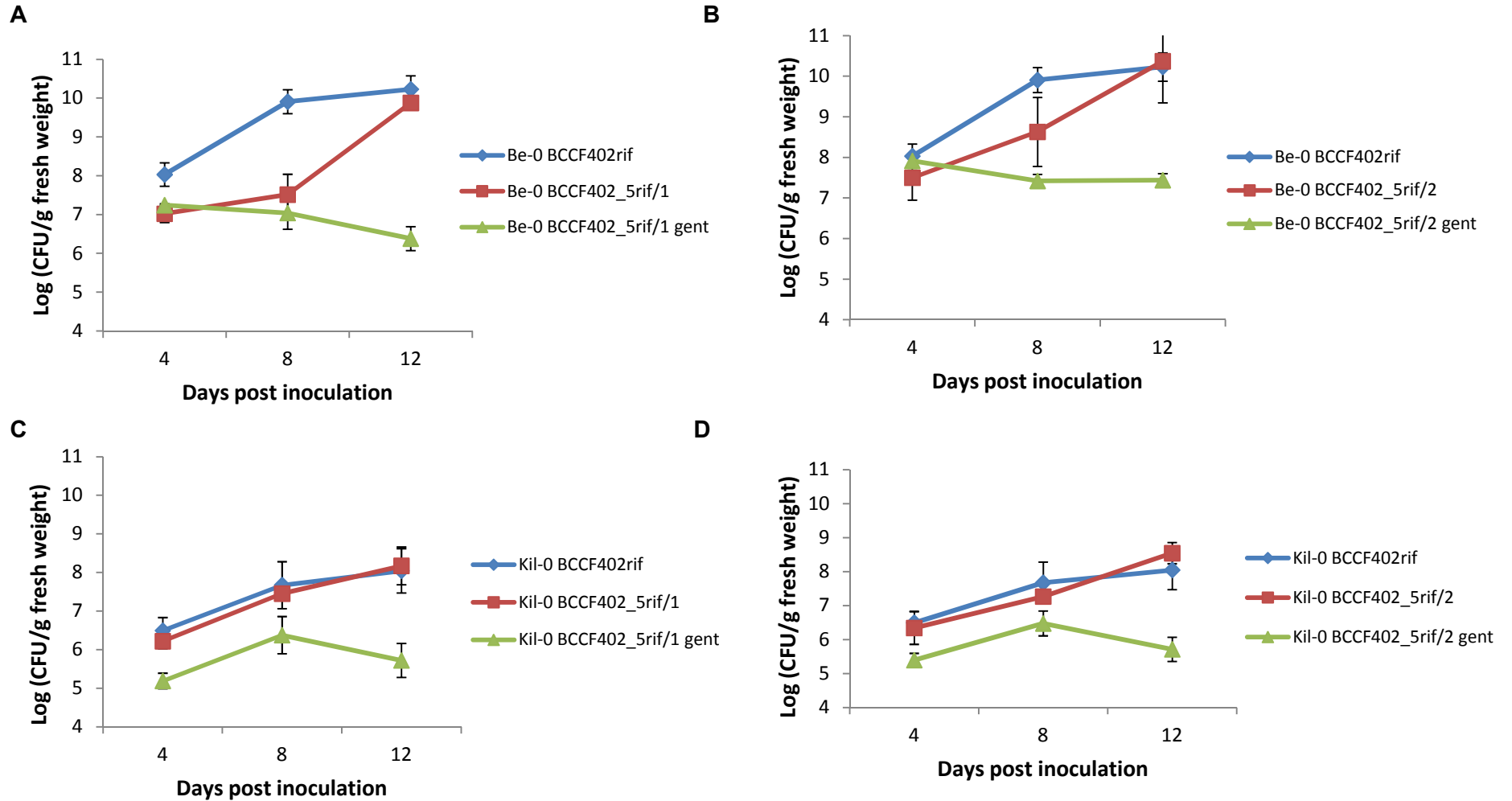


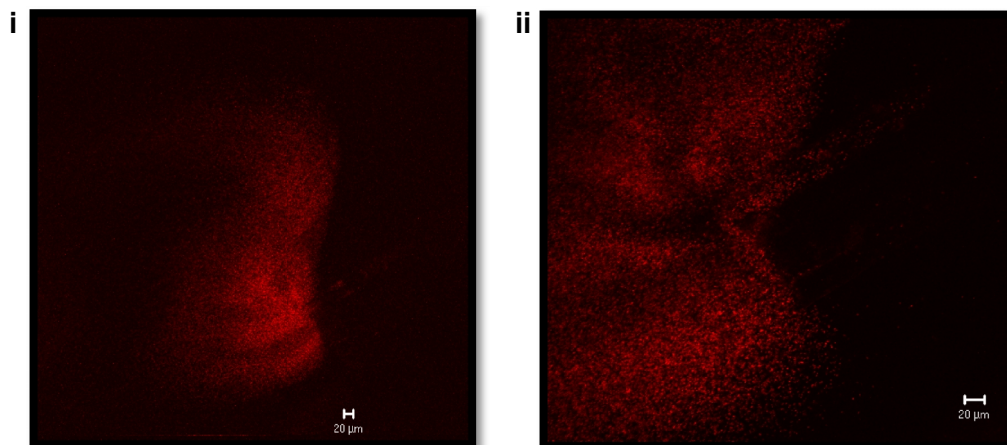
Figure 3.17. Bacterial numeration of *R. solanacearum* BCCF402_5^{rif} strains in Arabidopsis accessions Be-0 and Kil-0 after root inoculation. Error bars indicate standard errors which were determined from 3 replicates of 3 plants each.

The stability of the plasmid was tested by selecting for bacterial cells that have lost the plasmid over time by selecting on BGT plates containing 50 ug/ml rifampicin and 50 ug/ml of gentamycin (referred to as BCCF402_5^{rif} gent in Fig 3.17) by only picking up those bacterial cells that still contain the plasmid over time. The same samples were also selected on BGT plates just containing 50 ug/ml rifampicin. This shows a comparison of all the cells that started off with the plasmid but picks up on all the cells that were present in Arabidopsis plants including those that have lost the plasmid and those that still contain the plasmid i.e. number of cells that should have plasmid if it was not lost over time. BCCF402_5^{rif} cells plated onto gentamycin containing plates (selects only for those bacteria that still contain the plasmid) show a notable decrease in pathogen numbers over time in Be-0 plants (Fig 3.17 A and B). However, no significant differences were seen at 8 dpi. In Kil-0 plants, the BCCF402_5^{rif}/1 and 2 strains plated onto gentamycin and rifampicin containing plates, shows numbers increasing at 8 dpi and then decreasing at 12 dpi (Fig 3.17 C and D). It is interesting to note that the trends of the BCCF402_5^{rif} strains 1 and 2 when plated on plates that either do or do not contain 50 µg/ml gentamycin are similar in both Kil-0 and Be-0 plants.

3.2.4 Visualization of mCherry-tagged *R. solanacearum* BCCF402_5^{rif} in Arabidopsis leaves

Confocal microscopy was performed on accession Kil-0 leaves infected with *R. solanacearum* BCCF402_5^{rif}. Xylem contents were released into the water suspension when dissecting making it difficult to observe mCherry-tagged BCCF402_5^{rif} cells within Arabidopsis xylem tissue (Fig 3.18 A i and ii). *R. solanacearum* BCCF402_5^{rif} cells were visualized in Arabidopsis leaves (Fig 3.18 B i - vi). *R. solanacearum* wildtype strain BCCF402^{rif} served as a negative control. Fluorescence was not detected for the wildtype BCCF402^{rif} strain (Fig 3.18 C i - iv).

A



B

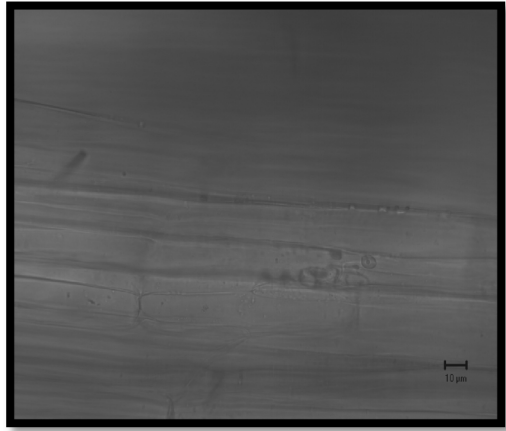
Fluorescence

Light

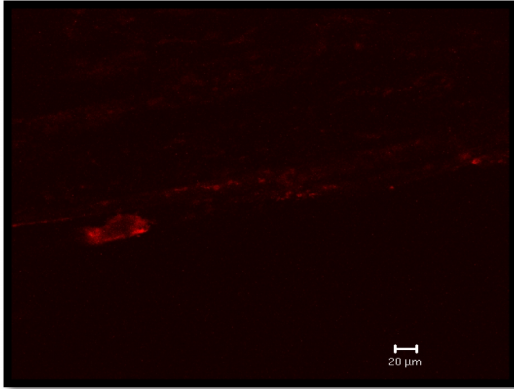
i



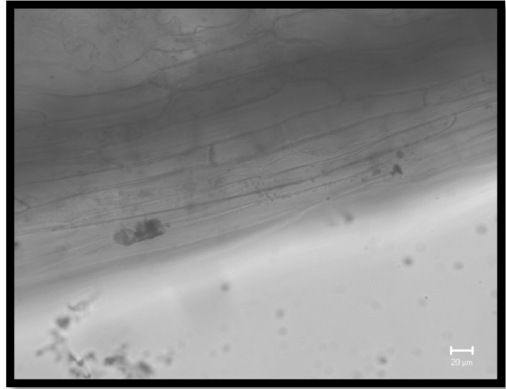
ii



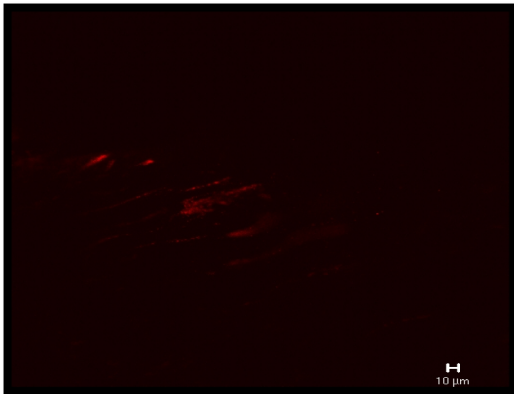
iii



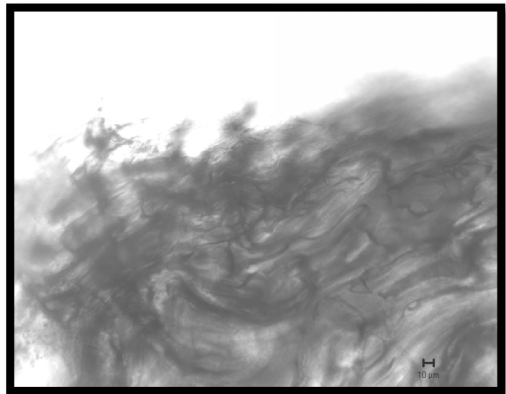
iv



v



vi



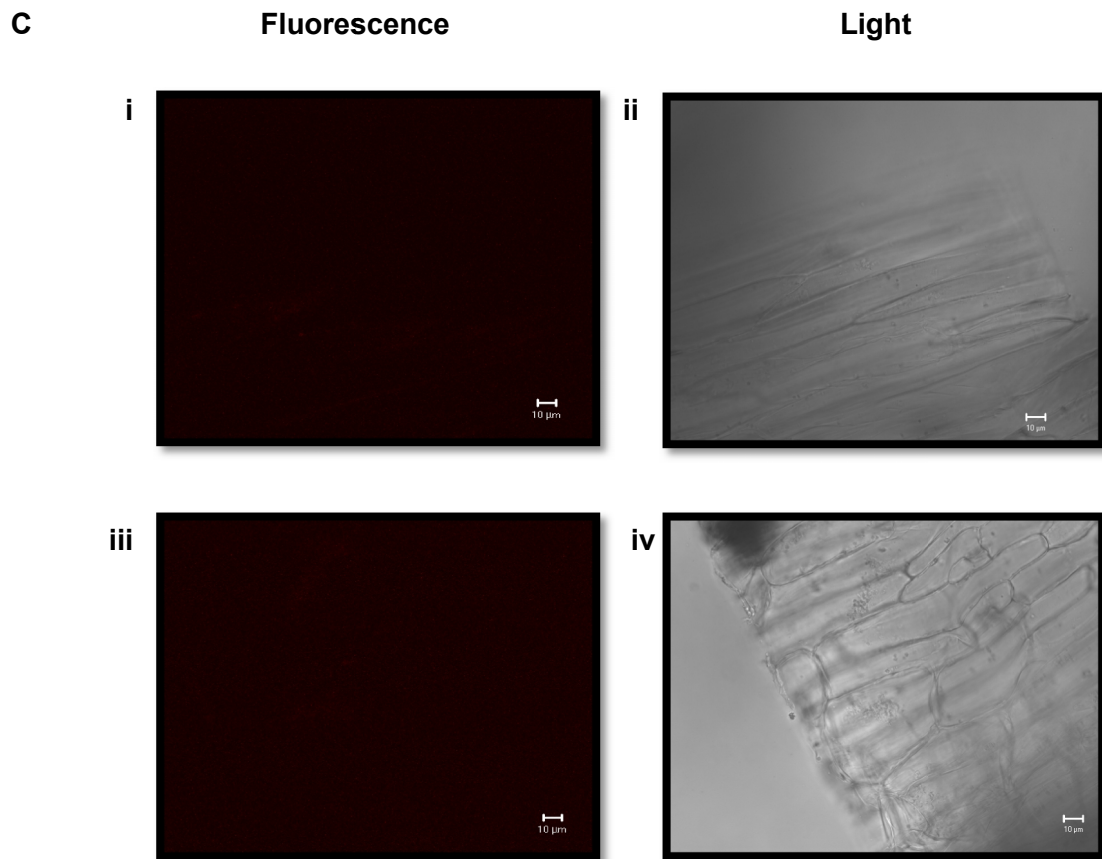


Figure 3.18. **Confocal microscopy analysis of the leaves of Arabidopsis accession Kil-0 after inoculation *R. solanacearum* BCCF402_5^{rif} at 20 dpi.** A and B: Arabidopsis leaf colonization visualized using a Zeiss LSM 510 META confocal microscope. BCCF402_5^{rif} cells in the plant leaf tissue were excited with a Helium-Neon laser at 543 nm. Images of the plant material were acquired using a 560 nm long-pass filter. B: Images on the left were made by fluorescence microscopy and the corresponding images on the right by normal light microscopy, C: Arabidopsis accession Kil-0 leaves when inoculated with BCCF402^{rif} wildtype as a negative control. Images on the left were obtained from fluorescence microscopy and the corresponding images on the right by normal light microscopy.

Chapter 4

Discussion

4.1 The tolerant *R. solanacearum* – *Arabidopsis* interaction

Bacterial wilt caused by *R. solanacearum* is a disease of great interest because it affects many economically important plant species. Much research has been done to investigate the resistant and susceptible interactions between phytopathogens and their plant hosts, however, understanding the tolerance responses against plant pathogens has been the focus of little investigation. Tolerance is defined as the plants ability to withstand high bacterial numbers without displaying disease symptoms and without a reduction in plant fitness (Kover and Schaal, 2002). Such observations were made in *Arabidopsis* accession Kil-0, suggesting a case of tolerance in Kil-0 to *R. solanacearum* BCCF402, a strain isolated from *Eucalyptus*. We approached the hypothesis of tolerance by addressing whether i) high bacterial numbers were present in Kil-0 plants ii) whether an absence of wilting symptoms were observed in Kil-0 plants and iii) whether Kil-0 plants were not significantly affected in plant fitness when inoculated with BCCF402.

Several results indicate that accession Kil-0 was tolerant to BCCF402. Kil-0 plants exhibited no wilting symptoms throughout the infection trial, however high bacterial numbers were observed *in planta* when using the dilution plating method for bacterial enumeration (Fig 3.1 and 3.2). This was in contrast to the susceptible accession Be-0 that supported high bacterial numbers *in planta* but developed severe wilt symptoms when inoculated with BCCF402. In a small number of Kil-0 plants, wilting symptoms were seen at later stages of BCCF402^{mf} infection. This is most likely due to nonreproducible physiological differences of individual plants (Fig 3.1 B). The resistant accession Nd1 did not develop any wilting symptoms and did not support a high bacterial load with bacterial numbers being notably lower than in Be-0 and Kil-0 when inoculated with the same *R. solanacearum* BCCF402 strain (Fig 3.1 and 3.2). Accession Col-0 developed severe disease symptoms and a high bacterial load consistent with a susceptible response (Fig 3.1 and 3.2). These results were consistent with the responses elicited when accessions Nd1 and Col-5, a glabrous derivative of Col-0, were infected with *R. solanacearum* isolate GMI1000 (Deslandes *et al.*, 1998). The root wounding inoculation method resulted in rapid

initial uptake of bacteria through the xylem and this resulted in relatively high bacterial numbers at early time points (4 dpi).

Additionally, Kil-0 plant fitness demonstrated by plant biomass yield, seed number and seed germination measurements was not significantly reduced when inoculated with BCCF402 compared to mock-inoculated Kil-0 plants (Fig 3.9 A – C). These data showed that accession Kil-0 was tolerant to BCCF402 and this was consistent with our working definition resulting in no reduction in fitness despite the high numbers of bacteria found *in planta*. Be-0 was confirmed to be susceptible to BCCF402. Be-0 plant fitness, however, was significantly affected when inoculated with *R. solanacearum* BCCF402 strain compared to mock-inoculated Be-0 plants signifying a susceptible interaction. Of interest in this MSc is the observation that Kil-0 plants inoculated with BCCF402^{rif} produced a greater amount of seed per plant compared to the mock-inoculated Kil-0 plants, though the result was not statistically significant (Fig 3.9 B). Tolerant plants infected with a virulent pathogen may be able to redirect resources or induce mechanisms that can increase plant fitness to the pathogen (Roux *et al.*, 2010).

Determining the amount of pathogen biomass *in planta* can be especially problematic in biological systems. A simple approach is plating out of tissue onto media containing appropriate selective antibiotics. Arabidopsis is small enough that whole plant tissue can be ground and plated out to calculate the number of CFU's (colon forming units) per gram of plant tissue (Robb, 2007). This method has the disadvantage of being a labour-intensive and lengthy process. Advantages are that only living cells are recovered with this approach and it has been shown to produce accurate and consistent results in the *R. solanacearum* – Arabidopsis pathosystem (Deslandes *et al.*, 1998; Weich, 2004; Van der Linden, 2010). PCR – based assays are considered the most reliable approach for pathogen biomass estimates *in planta* or in soil. qPCR allows fast, dependable, cost-effective and accurate identification and quantification of phytopathogens (Martin *et al.*, 2000). Furthermore, qPCR – based detection techniques are less inconsistent and more sensitive than the culturing method (Llorente *et al.*, 2010).

Numerous recent studies have developed qPCR assays to detect and quantify pathogens in plant species, such as *C. zeina* in maize (Korsman *et al.*, 2012), *V. dahliae* in potato lines (Atallah *et al.*, 2007), *Fusarium oxysporum* in chickpea cultivars (Jiménez-Fernández *et al.*, 2010) as well as a number of bacterial pathogens in Arabidopsis (Brouwer *et al.*, 2003). These studies have shown the reliability of using such an approach for pathogen biomass measurements. In most of these studies, the pathogen biomass was standardized to host plant DNA. However, as shown in Eshraghi *et al.* (2011), the standardization of the amount of pathogen DNA to plant DNA resulted in an overestimation of pathogen load at the later stages of infection where plant necrosis results in the degradation of host DNA. An overestimation of *Phytophthora cinnamomi* load *in planta* was observed in the necrotic susceptible plants when compared to the resistant plants when plant DNA was used for standardization (Eshraghi *et al.*, 2011). Furthermore, latter authors demonstrated the validity of using an internal control of foreign DNA (in this case the mouse *ScFvB1* gene) contained on a plasmid added during DNA extraction to overcome this problem and accurately quantify the pathogen biomass (Eshraghi *et al.*, 2011).

In this particular MSc study, a qPCR tool to quantify *R. solanacearum* in Arabidopsis plants was developed. This method ensured an additional reliable quantification measurement of pathogen biomass in plants other than dilution plating. Primers that were specific to the *R. solanacearum* Cytochrome c1 peptide gene (*CytC*) were designed to quantify *R. solanacearum* DNA concentrations *in planta*. An internal control of “alien” fungal DNA contained on a plasmid (pJET-CPR1-Cz, Appendix A) was used to normalize *R. solanacearum* DNA quantities to obtain a relative measurement of *R. solanacearum* DNA in different Arabidopsis samples. In susceptible accessions, pathogen biomass is often overestimated as a result of plant DNA degradation that occurs as a result of host tissue necrosis, especially at the later stages of disease progression. The use of an internal control for the standardization of pathogen DNA measurements rather than host DNA will prevent this overestimation of pathogen biomass. The *C. zeina* cytochrome P450 reductase (*cpr1*) gene was utilized as the “alien” internal control gene (Korsman *et al.*, 2012).

The amount of pJET-CPR1-Cz plasmid DNA to be spiked into each DNA extraction was determined. The three amounts of plasmid DNA (1.2 pg, 12 pg and 120 pg) to be tested were determined in such a way that when spiking plant samples with plasmid DNA before the DNA extraction method takes place, it will take approximately 20 PCR cycles for the amplification of plasmid DNA to cross the threshold of background fluorescence (Ct value). A plasmid amount of between 12 pg and 120 pg was determined to be sufficient plasmid DNA to be accurately amplified using qPCR technique but small enough plasmid amount so as to not overload the PCR reaction or the qPCR system (Fig 3.4). A final amount of 20 pg was decided to be adequate for spiking in plant samples as an internal control.

The effectiveness of using plasmid DNA as an internal control for measuring *R. solanacearum* biomass was evaluated. It was demonstrated that adding 20 pg of pJET-CPR1-Cz plasmid DNA during Arabidopsis sample DNA extraction and subsequent normalization, resulted in a more accurate pathogen biomass measurement than when the same *R. solanacearum* DNA concentration was normalized to host Arabidopsis DNA, especially in the susceptible interactions where there is a high degree of necrosis (Fig 3.6). The DNA quantification method based on normalizing to plasmid DNA (Fig 3.6 B) was more accurate than normalizing to plant DNA (Fig 3.6 A), because pathogen biomass differences between the Kil-0 and Be-0 accessions estimated from this method was more similar to the results obtained from the plate counting method (Fig 3.6 A).

The pJET-CPR1-Cz plasmid used in our study, that contained *cpr1* as the internal control, had no homology to either the host or *R. solanacearum* pathogen genomes. Similarly, no amplification occurred when *R. solanacearum* *cytC* gene specific primer pair, RsoIF and RsoIR, was used in the presence of Arabidopsis and pJET-CPR1-Cz plasmid DNA (Fig 3.3). Therefore, the primers used for the amplification of the *cpr1* and *cytC* genes were found to be specific for their respective DNA templates (Fig 3.3). The CPR1_2F and CPR1_2R primers that amplify the *cpr1* fragment were also shown to be specific to *C. zeina* DNA when amplified in the presence of other fungal DNA and therefore it was

unlikely that any fungal endophytes were quantified using these primers (Korsman *et al.*, 2012).

A qPCR quantification of *R. solanacearum* DNA in inoculated Kil-0, Be-0, Nd1 and Col-0 leaves was performed in order to assess *R. solanacearum* biomass in Arabidopsis accessions. It is of interest to note that DNA isolated from accessions Kil-0 and Be-0 showed some degradation (Fig 3.7). However, most samples do still have an intact high molecular weight band. This should allow for relative quantification of *R. solanacearum* and plasmid DNA if one assumes each type of DNA is degraded to the same extent. Fig 3.8 A showed a high amount of bacteria DNA found in Kil-0 at 12 and 16 dpi compared to the large amount of bacteria DNA observed in susceptible accession Be-0 at these time points. These results were comparable to the high bacterial load found in Kil-0 and Be-0 using the dilution plating methods (Fig 3.2). This was consistent with the hypothesis that accession Kil-0 is tolerant to BCCF402 and Be-0 is the susceptible accession. The larger difference in the concentration of bacteria observed in Nd1 versus Col-0 (Fig 3.8 B) confirmed that Col-0 is susceptible to BCCF402 and Nd1 is resistant to BCCF402. These results verified that the qPCR assay was an effective method for quantifying *R. solanacearum* biomass in infected Arabidopsis plants. This qPCR assay will be a useful tool in future studies of the Arabidopsis – bacterial wilt pathosystem.

Tolerance in plant-pathogen interactions is considered to be a polygenic trait (Barret *et al.*, 2009). However, work done by Liesl van der Linden in her Masters dissertation showed that the “resistance” phenotype in Kil-0 to *R. solanacearum* BCCF402 was conferred by the single recessive gene, *RRS1*, in a gene-for-gene model of defence (Van der Linden, 2010). Previous studies showed that the recognition of PopP2 effector by *RRS1*-R and their subsequent physical interaction are required for resistance in accession Nd1 to *R. solanacearum* GM11000 (Deslandes *et al.*, 2002; Deslandes *et al.*, 2003). The “resistant” phenotype in Kil-0 was also dependent on PopP2 recognition and induction of defence responses (Van der Linden, 2010). This MSc study, however, further showed that the Kil-0 phenotype was indicative of tolerance rather than resistance. Therefore,

tolerance in Kil-0 is conferred by the single *RRS1* gene. The results of these two studies combined suggest that the recognition of PopP2 by RRS1 elicits a tolerant response in Kil-0 when infected with *R. solanacearum* BCCF402, and a resistant response in Nd1.

The Kil-0 *RRS1* gene may be involved in conferring tolerance in this system. RRS1-R is an atypical R protein that contains the TIR-NB-LRR domains and a C-terminal WRKY domain (Deslandes *et al.*, 2002). In studies involving the Nd1 resistant response, RRS1-R has been proposed to function as a negative regulator of plant defence responses either through its WKRY domain or through the action of additional transcription factors (Deslandes and Rivas, 2012). PopP2 contains a catalytic triad that is important for its enzymatic ability. RRS1-R is able to recognize PopP2 auto-acetyltransferase activity and this may be necessary for the regulation of defence-related gene expression (Deslandes and Rivas, 2012). Activation of *RRS1-R*-mediated defence responses requires RD19 (Responsive to Dehydration19). RD19 is an Arabidopsis cysteine protease which is normally localized in the plant vacuole-associated vesicles. In the presence of the effector PopP2, RD19 is relocalized to the plant nucleus where the proteins interact physically and this complex is recognized by RRS1-R to activate plant resistance responses (Bernoux *et al.*, 2008). Elicitation of plant resistance may also be the result of PopP2 inhibiting or disrupting RD19. RD19 may be targeted by PopP2, and this modification is recognized by RRS1-R to activate the expression of defence genes.

The PopP2 perception and interaction of additional different host components may explain the tolerance phenotype in Kil-0. The *RRS1-R* allele in tolerant plants may not recognize the PopP2 effector as effectively or in the same manner as in resistant plants and this could result in the expression of different defence genes. The structure of the RRS1 protein may also be a factor in the induction of tolerance responses of Kil-0 to BCCF402. The full length coding sequence of the RRS1 protein from accessions Kil-0 and Nd1 were compared (Van der Linden, 2010). A high level of identity (99 %) was observed between the two RRS1 proteins. There are only 8 amino acids that are different between the Kil-0 and Nd1 protein with the Kil-0 RRS1 protein having 5 amino acids less at the 3' end (Van

der Linden, 2010). This may correspond to differences in the level of PopP2 recognition and perception or the specificity and strength of gene activation or suppression. It is probable that activation of defence responses downstream in the signalling pathway play a role in tolerance.

4.2 mCherry as a tool in the visualization of BCCF402

R. solanacearum BCCF402 was tagged with mCherry for the reliable visualization of bacterial cells in Arabidopsis plants. The visual localization and progression of BCCF402 bacterial colonization in specific Arabidopsis plant tissue or regions was important in this particular MSc study to determine potential mechanisms of tolerance. Few studies have addressed the potential mechanisms of the tolerant interaction. However, some studies do show that in the tolerance response, some of the host's usual resistance defence responses are induced, but others are not. Chen *et al.* (2004) showed that *Verticillium* tolerant "Craigella" tomatoes were effectively able to trap the pathogen in primary sites or locations in the stems of plants due to the plant's vascular coating response, however were not able to eliminate fungal colonization that normally takes place in the resistant interaction. Therefore, the pathogen multiplied to a very high concentration in the tolerant plant but *Verticillium* colonization was contained in initial sites and therefore did not result in symptom expression (Chen *et al.*, 2004). This may be comparable to our study, where *R. solanacearum* may be localized in specific cells and therefore unable to cause disease, but still proliferates to a very high concentration in the tolerant plant. Additionally, quantification of fluorescing cells in Arabidopsis accessions using microscopy quantification techniques can be a useful tool in determining pathogen biomass measurements *in planta*.

Fluorescent proteins are valuable markers for the live imaging of biological processes and microbial populations. A host of several new monomeric red fluorescent protein variants have been described recently. mCherry is considered an extremely bright and photostable

option (Shaner *et al.*, 2004). mCherry has recently been utilized in a number of studies (Hillson *et al.*, 2007; Lewenza *et al.*, 2008; Malone *et al.*, 2009; Lagendijk *et al.*, 2010). We have introduced plasmids pMP7604 and pMP7605 containing mCherry under the constitutive expression of the *tac* promoter into *R. solanacearum* BCCF402. The stability of the plasmids and the expression levels of mCherry in *R. solanacearum* BCCF402 were evaluated. The effect of the plasmids or mCherry expression on the growth and virulence of BCCF402 *in planta* were also measured. The *R. solanacearum* BCCF402 strains transformed with pMP7604 and pMP7605 underwent several tests to confirm the presence of the mCherry plasmids in these strains. PCR analysis verified that pMP7604 and pMP7605 were successfully introduced into BCCF402 as the mCherry fragment was successfully amplified from BCCF402 plasmid DNA after the plasmid DNA was extracted from overnight cultures of BCCF402_4 and BCCF402_5 (Fig 3.10). The plasmid structure of pMP7604 and pMP7605 as well as the presence of the mCherry fragment in pMP7604 and pMP7605 was confirmed using restriction enzyme digestions (Fig 3.11). All banding patterns coincide to what is expected according to the restriction maps except for the smaller sized fragments (42 bp and 17 bp) that were most likely too small to be seen on the agarose gel. Restriction analysis of plasmids pMP7604 and pMP7605 confirmed that mCherry was present. The two vectors were chosen for their selective ability and for the ease in which they are introduced into bacterial strains. High expression levels are also expected due to the high copy number plasmids. Interestingly, the backbone of pMP7605, (pBBRMCS-5 – derived from the cloning vector pBBR) is expected to have a higher copy number than the backbone of pMP7604 (pME6031 - containing the pVS1 replicon) (Lagendijk *et al.*, 2010).

The expression levels of mCherry produced in *R. solanacearum* BCCF402_4 and BCCF402_5 were measured in order to assess the brightness of the different strains using fluorometry (Fig 3.13 A). Light microscopy was performed to determine if fluorescence is sufficient for visualization of *R. solanacearum* cells under a microscope (Fig 3.14). Higher levels of mCherry production were consistently observed in BCCF402 cells transformed with pMP7605 than cells transformed with pMP7604 (Fig 3.13 A). The intensity of fluorescence coincides with the different copy numbers of the plasmids utilized

in this study. The large amount of fluorescence detected in the BCCF402_5 strains suggested that *R. solanacearum* BCCF402 strains harbouring pMP7605 would be the best strains for future microscopy experiments. Light microscopy showed that BCCF402 tagged with pMP7605 can be used for visualization of bacterial cells (Fig 3.14). The amount of fluorescence produced from rifampicin mutants of BCCF402_5 (named BCCF402_5^{rif}) was also evaluated (Fig 3.13 B) and the virulence of BCCF402_5^{rif} strains was examined (Fig 3.15 B). A high fluorescent level of mCherry was produced in the BCCF402_5^{rif} strain compared to the wildtype BCCF402 strain (Fig 3.13 B) and BCCF402_5^{rif} strains virulence was confirmed by their ability to cause a hypersensitive response on tobacco (Fig 3.15 B). These results indicated that BCCF402_5 cells were successfully expressed mCherry at a high enough level to allow for visualization with microscopy. A mutation in the -35 consensus sequence of the *tac* promoter was found in pMP7604 and pMP7605 when the full length mCherry fragment and *tac* promoter was sequenced (Fig 3.12). Studies have shown that changes or mutations in the -10 consensus sequence, the -35 consensus sequence and in the spacing (bp) between these two sequences can have an effect on the expression activity of a bacterial promoter, most often a negative one (Mulligan *et al.*, 1985; Dickson *et al.*, 1989; Mathews and Sriprakash, 1994). This mutation could therefore affect mCherry expression levels. Thus, it was important to evaluate the expression levels of mCherry from pMP7604 and pMP7605. In our study, tagged cells were well visualized using microscopy techniques despite this mutation, indicating that mCherry levels were sufficiently expressed.

mCherry tagged *R. solanacearum* strains were evaluated in terms of their growth rate, virulence and stability. The growth curves of mCherry-tagged BCCF402 strains were not adversely affected by the presence of the plasmid or the expression of mCherry on growth of the *R. solanacearum* strains in media compared with the wildtype *R. solanacearum* strain BCCF402 (Fig 3.15 A). Virulence of transformed strains and pathogen growth *in planta* when introduced into Kil-0 and Be-0 also was not negatively affected due to mCherry compared to BCCF402 without mCherry (Fig 3.16 and 3.17). The *R. solanacearum* BCCF402 strains did not result in the development of severe disease symptoms in the Be-0 accession (Fig 3.16). This may be due to non-optimal growth room

conditions. Kil-0 plants inoculated with BCCF402^{rif} and BCCF402_5^{rif}/1 and 2 strains, show a similar bacterial concentration which suggest that the plasmids do not affect *R. solanacearum* strains in terms of pathogen colonization or growth in Kil-0 plants (Fig 3.17 C and D).

BCCF402_5 cells experienced a 6-14 % loss of the plasmid pMP7605 at 8 days post inoculation (dpi) and a plasmid loss of 30-35 % was observed at 12 dpi during an infection trial. *R. solanacearum* cells lose their plasmids as the *in planta* trial progresses which may affect the viability of using mCherry plasmids for *in planta* visualization. However, mCherry is suitable for visualization at early time points in the infection. These data indicate i) *R. solanacearum* cells containing mCherry plasmid pMP7605 are not affected in terms of growth in Kil-0 plants but growth is delayed in accession Be-0, ii) the pMP7605 plasmid was not stable and *R. solanacearum* cells lost the plasmid over time due to lack of selective pressure. This was most likely due to lack of antibiotic selection, indicating a limitation of using plasmid tagged BCCF402 *in planta* (Fig 3.17).

Plasmid stability is determined by a balance between the benefit provided by a selective advantage of certain genes on the host bacterial cell and the cost of the metabolic or energy burden as a result of plasmid replication and expression of often constitutively regulated genes (McLouchlin, 1994). Heeb *et al.* (2000) developed a set of pVS1-p15A shuttle vectors that were shown to be completely stable in *Pseudomonas fluorescens* when infected in wheat plant roots for more than 1 month making these type of vectors invaluable (Heeb *et al.*, 2000). The stability of a plasmid that was derived from one of shuttle vectors was investigated in liquid media with no antibiotic selection pressure. *Pseudomonas putida* strains carrying pMP7604 (containing the pVS1 replicon) showed no loss of plasmid over approximately 30 generations. The stability of *P. putida* strains carrying a different pMP7605 plasmid (derived from a pBBR vector) showed a 3% loss of plasmid at day 3 (Lagendijk *et al.*, 2010). The mCherry-tagged BCCF402 strains can be used in localization studies that take place at the early stages of *R. solanacearum* infection. However, confocal microscopy did show that BCCF402 was well visualized *in*

planta at later stages of infection as a large number of bacteria were still present (Fig 3.18). These results indicated that mCherry-tagged *R. solanacearum* BCCF402_5 strains can be visualized *in planta*. Bacterial cells transformed with mCherry plasmid pMP7605 are well suited for visualization and can be used as a tool to localize *R. solanacearum* *in planta*. In future this visualization tool will be used to quantify bacterial numbers in plants, localize cells in specific plant tissues and to observe the pathogen at different stages of infection.

4.3 Future work

To further elucidate the role the *RRS1* gene plays in conferring tolerance in the Kil-0 phenotype, knockout and overexpression of the *RRS1* gene could be performed. To accurately phenotype the *RRS1* knockout and overexpression lines, the plants will be inoculated with virulent *R. solanacearum* strain BCCF402. Disease scoring, pathogen biomass measurements using plate counting and qPCR, and fitness parameters will be evaluated to determine the strength of the tolerance or susceptible response.

Further characterization of the tolerant response in Arabidopsis accession Kil-0 to BCCF402 will be the focus of future research. We hypothesize that differences in the downstream responses may result in a resistant or tolerant outcome. In future, a comprehensive understanding of this plant-pathogen interaction will require a complete description of the transcriptome of the tolerant Arabidopsis accession. The experiment could be set up as follows. A description of the transcriptome of a tolerant and susceptible Arabidopsis defence response against *R. solanacearum* will be obtained using RNA Sequencing (RNA-seq) analysis. Arabidopsis Kil-0 plants will be inoculated with BCCF402 in a tolerant interaction and with *R. solanacearum* BCCF402^{PopP2} strain in a susceptible interaction. Data analysis and differential gene expression analysis will be performed to obtain the significantly up-regulated or down-regulated candidate genes. Over-expression

lines and knockdown lines will then be constructed of differentially regulated candidate genes to determine the contribution of the candidate genes to the tolerance response of *Arabidopsis* Kil-0 against *R. solanacearum* BCCF402.

In order to determine downstream candidate genes that may contribute in the tolerant outcome of *Arabidopsis* accession Kil-0 to *R. solanacearum* isolate BCCF402, a quantitative trait loci (QTL) mapping approach would be effective in identifying genes that underlie tolerance traits. To perform the QTL mapping method, *Arabidopsis* accessions Kil-0 (tolerant) and Nd1 (resistant) will be crossed to produce F1 seeds. A heterozygous F1 plant will be self-fertilized. F2 seeds will then be grown and each plant will be self-fertilized. Recombinant inbred lines (RILs) will be derived from the F2 seeds. The F2 generation and RILs will be phenotyped and genotyped. To accurately phenotype the F2 and RIL seeds, the plants will be inoculated with virulent *R. solanacearum* strain BCCF402. Disease scoring, pathogen biomass measurements using plate counting and qPCR, and fitness parameters will be evaluated to determine a tolerant or resistant interaction. The F2 and RIL seeds will also be extensively genotyped. A set of markers equally spaced over the *Arabidopsis* genetic map will be selected for the RIL Kil-0/Nd1 map. QTL analysis to identify and locate QTL will be carried out using Windows QTL Cartographer 2.5 (<http://statgen.ncsu.edu/qtlcart/WQTLCart.htm>). LOD statistics will be used to determine the significance of the QTL. Once the F2 and RIL population has been used for QTL mapping, subsequent molecular identification of responsible genes will be carried out (Balasubramanian *et al.*, 2009; Keurentjies *et al.*, 2007; Loudet *et al.*, 2002).

In this MSc study, there was reduced plasmid stability during plant infections due to a lack of antibiotics present to maintain the plasmids. The mCherry plasmid was lost over time which may limit its use at later stages of plant infection. Transposons can be used for the stable integration of mCherry plasmids in the *R. solanacearum* genome but have the drawback of only being present as one copy per cell which results in lower expression of mCherry proteins in comparison with the multicopy plasmids. Legendijk *et al.* (2010) made use of a bacterial transposon Tn7 vector called pBK-miniTn7 plasmid to clone mCherry

and the *tac* promoter into this plasmid to obtain a transposon vector (pMP7607) for integration into the bacterial genome (Lagendijk *et al.*, 2010). Sequence and fluorometry analysis of this vector showed that mutations in the promoter and *tac* promoter sequence resulted in little or no production of the mCherry protein. This plasmid was therefore not used in our study.

However, the transposon vector, pMP7605, containing a fully functional mCherry gene could be transformed into *R. solanacearum* strain BCCF402 using quadripartite mating. Conjugation of pMP7607 could be accomplished by mixing donor *E. coli* DH5 α containing pMP7607, helper *E. coli* containing pRK2013, helper *E. coli* containing pUX-BF13 and the recipient *R. solanacearum* strain BCCF402. The mixture will be spotted out on LB agar plates with no antibiotics. The plates will be incubated overnight at 37 °C. The spot will then be scraped off and resuspended in liquid LB media followed by a serial dilution. The resuspended media will be plated out on LB plates containing antibiotics for the selection of the *R. solanacearum* strain and the mCherry containing plasmid. Colonies will be tested for the presence of the plasmid and mCherry fragment.

The tolerant interaction can be studied in further detail using a mCherry stable *R. solanacearum* strain. The tolerant interaction would be visualized in Arabidopsis Kil-0 plants when inoculated with mCherry-tagged BCCF402. A BCCF402^{PopP2} mutant strain transformed with mCherry would result in a susceptible interaction during Kil-0 plant infection. The infection and colonization process would then be observed using microscopy techniques. The differences in the infection and vascular colonization progression during a tolerant and susceptible interaction could then be directly compared. In this way, crucial differences in the stages of the infection process and plant responses induced as a result of these two different interactions would not be attributed to the background genetics of different Arabidopsis accessions. A similar study was performed that involved the use of a number of GMI1000 mutant strains that were disrupted in regulatory genes that play a role in the type 3 secretion system and pathogen virulence. The phenotypes of each of these mutant strains were visualized in tomato root infections

using microscopy techniques to determine differences in their vascular colonization process. Their role in *R. solanacearum* pathogenicity was therefore determined (Vasse *et al.*, 2000).

4.4 Conclusion

In conclusion, this study has performed further phenotyping and qPCR evidence for a case of tolerance in *Arabidopsis* accession Kil-0 to the bacterial wilt pathogen *R. solanacearum* BCCF402. The tolerance response was conferred by a single *R* gene, *RRS1-R*, in a gene-for-gene interaction. Additional investigation of downstream responses, potential molecular mechanisms and biochemical interactions using methods such as differential gene expression arrays and localization studies will help to further characterize this phenomenon. This discovery is important for the disease management of economically important crop species, including *Eucalyptus*, where *RRS1-R* used for resistance may in fact result in tolerant interaction where crop plants may be asymptomatic but still harbour the pathogen. In the context of agriculture, a tolerant interaction will allow extensive proliferation of the pathogen in an agricultural environment that possibly includes other susceptible plant species.

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Appendix A

pJET-CPR1-Cz

