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Response of Potato to coronafacic acid
a virulence factor in
Pectobacterium

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Science

at

Lincoln University

by

Pavithra Coimbatore Ramakrishnan

Lincoln University

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Abstract
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Pectobacterium atrosepticum (*Pba*) and *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbr*) are necrotrophic bacterial plant pathogens that cause blackleg disease on potato stems during the growing season and soft rot of tubers post-harvest. Coronafacic acid (CFA), encoded by the *cfa* gene cluster, is one among many pathogenicity determinants that have been identified in *Pba* SCRI1043 and *Pbr* NZEC1. CFA is a component of coronatine (COR), which in the hemibiotrophic pathogen *Pseudomonas syringae* functions as a molecular mimic of Jasmonic Acid (JA) during pathogenicity on host plants to suppress the Salicylic Acid (SA) signalling pathway. The SA pathway is essential for defence against *P. syringae*, and other hemibiotrophic and biotrophic pathogens.

Studies on *Arabidopsis thaliana* and tomato have identified that genes related to JA signalling and wound response are differentially transcribed upon infection with *P. syringae* carrying the *cfa* gene cluster. Studies to date on *Pba* have confirmed the influence

of CFA in pathogenicity on potato plants, but its mode of action in *Pectobacterium* spp. still remains unclear.

In this study, a model plant-pathogen system was developed for studying the interaction of *Pectobacterium* spp. with potato. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was then used to investigate the expression of target genes upon infection with *Pbr* NZEC1 and a CFA knockout mutant, identified from the previous studies in tomato and *A. thaliana* using COR. As an alternative approach, Illumina-based RNA sequencing (RNA seq) was used to investigate the global transcription of potato in response to this pathogen to identify novel genes differentially expressed in this pathosystem.

Although qRT-PCR showed no significant differential expression of candidate genes, RNA seq identified the differential expression of a multitude of genes upon infection with *Pbr* NZEC1 and the CFA knockout mutant. Of particular note were the large proportion of differentially expressed genes related to ethylene (ET) biosynthesis and the JA pathway. These pathways are essential for production of defensin, which is central to plant defence against necrotrophic pathogens such as *Pectobacterium*. The research also provided new information on the isoforms of these defence related genes that are active in tubers providing novel insights into how potato may respond to tuber infection in general.

Keywords: *Pectobacterium*, coronafacic acid, RNA sequencing, ethylene biosynthesis and signalling, Jasmonic acid.

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Chapter 1 Introduction

1.1 Potato production and development of disease resistance

Potatoes are considered to be the fourth most important staple food source after wheat, rice and corn and are grown worldwide (<http://faostat.fao.org/>). In New Zealand, around 10,670 hectares of arable land is used to grow potatoes. They are grown for the table, for processing into other potato products and as seed tubers. In 2010, total potato exports were worth \$100 million of which the frozen potato product accounted for 83% of the total value. The seed potato industry in New Zealand is small with just over 1,100 hectares used to grow seed potatoes. However, seed tubers are also exported to tropical countries like Sri Lanka where they are difficult to produce (<http://www.potatoesnz.co.nz/Overview/Our-Industry/Industry-profile.htm>).

Seed tubers are usually processed as highly regulated crops to keep them free of pathogens, yet diseases such as common scab, powdery scab, potato tuber moth and soft rot continue to reduce tuber quality and yield. The main causative agents of soft rot and stem rot (blackleg) in temperate regions are soft rot *Erwinias* (SREs). To date, no chemical control measures have been identified; hence the control of SREs relies mainly on crop management practices. The use of breeding for development of resistance to diseases has proven an alternative approach to overcome the economic losses caused by these pathogens. Breeding for resistant plants can be enhanced by knowledge of plant defence responses to pathogens.

1.2 Mechanisms of plant defence

Plant pathogens use diverse life strategies to infect a host. Since plants lack an adaptive immune system, their defence to such pathogens is mediated primarily by the innate immunity of each cell and on the systemic signals emitted from the site of infection (Ausubel, 2005; Dangl & Jones, 2001). Two branches of plant defence in response to microbial attack have been recognised. The first involves transmembrane pattern recognition receptors (PRRs) that respond to pathogen or microbial-associated molecular patterns (PAMPs or MAMPs) (Zipfel & Felix, 2005). The second system uses the polymorphic Nucleotide binding leucine rich repeat (NB-LRR) protein products that are encoded by the *R* gene (Dangl & Jones, 2001).

Jones and Dangl (2006) presented a “zigzag” model of plant defence, in which plant defence to microbial pathogens was divided into four phases. Phase 1 of this model involves the PAMP-triggered immunity (PTI) which is produced when PRRs recognise PAMPs or MAMPs. In phase 2, a successful pathogen enters the host, effectors are deployed which interfere with the PTI resulting in effector triggered susceptibility (ETS). In phase 3, if an effector is recognised specifically by one of the NB-LRR proteins it results in effector-triggered immunity (ETI). ETI is represented as an amplified PTI response which results in a hypersensitive cell death response (HR). In the final phase, the pathogen suppresses the ETI response either by discarding the specific effector or by obtaining or activating a new effector (Jones & Dangl, 2006). Activation of PRR and NB-LRR results in elicitation of defence-associated signalling in the host; resulting in crosstalk between the pathways involved in signalling. Salicylic acid (SA) (resistance against biotrophic pathogen), Jasmonic acid (JA) and Ethylene (ET) (defence against necrotrophic

pathogen) are the primary signalling molecules associated with defence against microbes (Glazebrook, 2005).

1.2.1 SA mediated defence response

The formation of necrotic lesions by a pathogen is associated with the activation of an integrated set of localised and systemic responses which includes cell wall rigidification, synthesis of phytoalexins and accumulation of pathogenesis-related proteins (PRs). Thus, the activation of defence genes extends systemically, conferring broad spectrum resistance to pathogens in uninfected plant parts (C. M. J. Pieterse & van Loon, 1999). Previous studies have shown that SA is a key component of local defence and for establishment of such systemic acquired resistance (SAR) (Durrant & Dong, 2004). *Arabidopsis thaliana* mutants impaired in production of SA (*sid2*, *pad4*, *eds1*, *eds4*, *eds5*), transgenic plants that fail to accumulate elevated levels of SA (via expression of SA degrading enzyme *nahG*), and plants that carry mutations in *Non Expression of PR1 (NPR1)*, exhibit enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* and fungal pathogens *Peronospora parasitica* and *Erysiphe*, suggesting that the SA-dependent pathway is essential for defence against these pathogens (Feys & Parker, 2000; Glazebrook, et al., 1996; Reuber, et al., 1998). *PR* gene activation is not always SA-dependent; however, the *PR* genes activated upon pathogen attack are not always similar to the *PR* genes activated by exogenous application of SA or its functional analogue (C. M. J. Pieterse & van Loon, 1999). Furthermore, expression of pathogen mediated *PR* genes is not affected in transgenic *nahG* tobacco plants when challenged with *Pectobacterium carotovorum* indicating that the defence response to this pathway is SA independent (Vidal, et al., 1997). Further studies have identified that, SA independent *PR* gene activation involves the action

of JA and ET suggesting that JA and ET play an important role in regulating the plant defence system too (C. M. J. Pieterse & van Loon, 1999).

1.2.2 JA mediated defence response

JA plays a vital role in plant growth and development mediating growth inhibition, senescence, tendril coiling, flower development, leaf abscission and plant response to abiotic and biotic stress (Wasternack, 2007). JA is also responsible for tuber development in potato, yam and bulb development in onion. JA is derived from the fatty acids linoleic or linolenic acid via the octadecanoid pathway. The conversion of linolenic acid to 12-oxo-phytodienoic acid is a multi-step enzymatic process involving lipoxygenase (*lox*), allene oxide synthase (*aos*), allene oxide cyclase (*aoc*) and 12-oxo-phytodienoate reductase 3 (*12-opr3*) (Figure 1.1) (Schaller, 2001). Desaturation of the fatty acids is brought about by fatty acid desaturase (*fad*) which catalyses the conversion of dienoic to trienoic fatty acid. In *A. thaliana* three different genes encoding fatty acid desaturase have been identified (*fad3*, *fad7* and *fad8*). An *Arabidopsis thaliana* triple mutant ($\Delta fad3 fad7 fad8$) produces very low levels of trienoic fatty acid and is impaired in JA synthesis, and hence exhibits enhanced susceptibility to a variety of pathogens including the bacterial plant pathogen *P. carotovorum* (Norman-Setterblad, et al., 2000), and the fungal pathogens *Alternaria brassicicola*, *Botrytis cinerea* and *Pythium* spp. (Leon & Sanchez-Serrano, 1999). Activation of the JA dependent defence pathway results in the accumulation of several defence related proteins which include *plant defensin 1.2* (*PDF 1.2*), *thionin 2.1*, *hevein-like protein* and *chitinase B* (Wasternack, 2007).

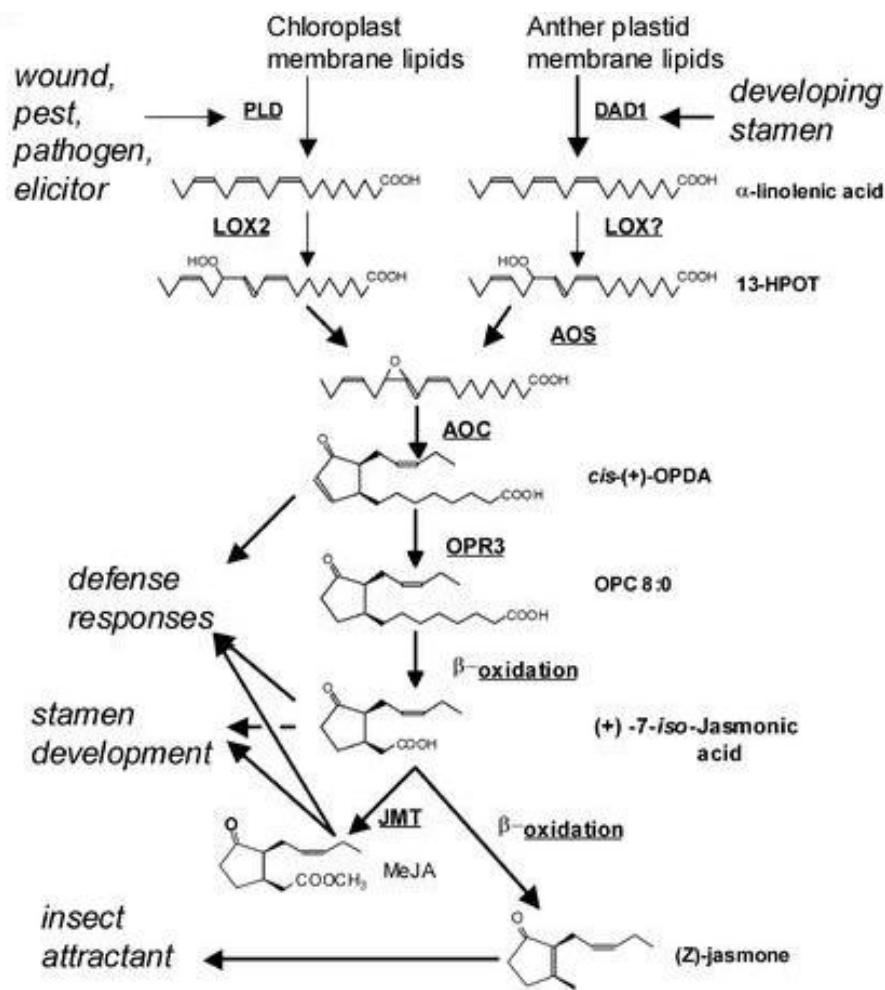


Figure 1.1: Jasmonic acid biosynthesis (Stintzi & Browse, 2000)

1.2.3 ET mediated defence response

ET, being a diffusible phytohormone, plays a critical role in integrating developmental signals such as seed germination, fruit ripening, abscission and senescence and response to biotic and abiotic stress (Abeles, 1973). Adverse biotic and abiotic stress induces ET synthesis, which in turn regulates a wide range of genes involved in wound signalling and pathogen defence. ET is synthesized from the amino acid methionine to S-adenosyl-L-methionine (SAM) by the enzyme S-adenosyl methionine synthase. SAM is then converted into 1-aminocyclopropane-1-carboxylic-acid (ACC), a reaction that is catalysed by ACC

synthase (ACS). ACC is the immediate precursor of ET, and therefore ACS determines the rate of ET production. Thus, the regulation of this enzyme is crucial for the control of ET biosynthesis. The final step in ET biosynthesis involves the action of the enzyme ACC oxidase (ACO), which converts ACC into ET (Wang, et al., 2002) (Figure 1.2).

ET mediated plant defence depends on the type of pathogen attacking the plants as well as the plant species. Plants deficient in ET signalling can show either increased susceptibility or increased resistance. In *A. thaliana*, ET insensitive 2 (*EIN2*) mutants develop only minimal disease symptoms when challenged with *P. syringae* pv. *tomato* or *Xanthomonas campestris* pv. *campestris* (Bent, et al., 1992). However, these mutants show enhanced susceptibility to *B. cinerea* indicating that the ET mediated signalling pathway is essential for defence against necrotrophic pathogens (Thomma, et al., 1999).

Studies in *A. thaliana* have identified that the response of the *EIN2* mutant to pathogen attack is generally parallel to the pattern observed in the jasmonate response mutant (*jar1-1*) and coronatine insensitive 1 (*coi1*) mutants. Activation of *PDF 1.2* in *A. thaliana* by *A. brassicicola* has been shown to be blocked in *EIN2* mutants similar to the *coi1* mutant, indicating that an intact JA and ET pathway is required for *PDF 1.2* production (Kunkel & Brooks, 2002). These observations suggest that ET and JA interact and positively regulate the expression of certain defence genes such as *PDF 1.2* (Wang, et al., 2002).

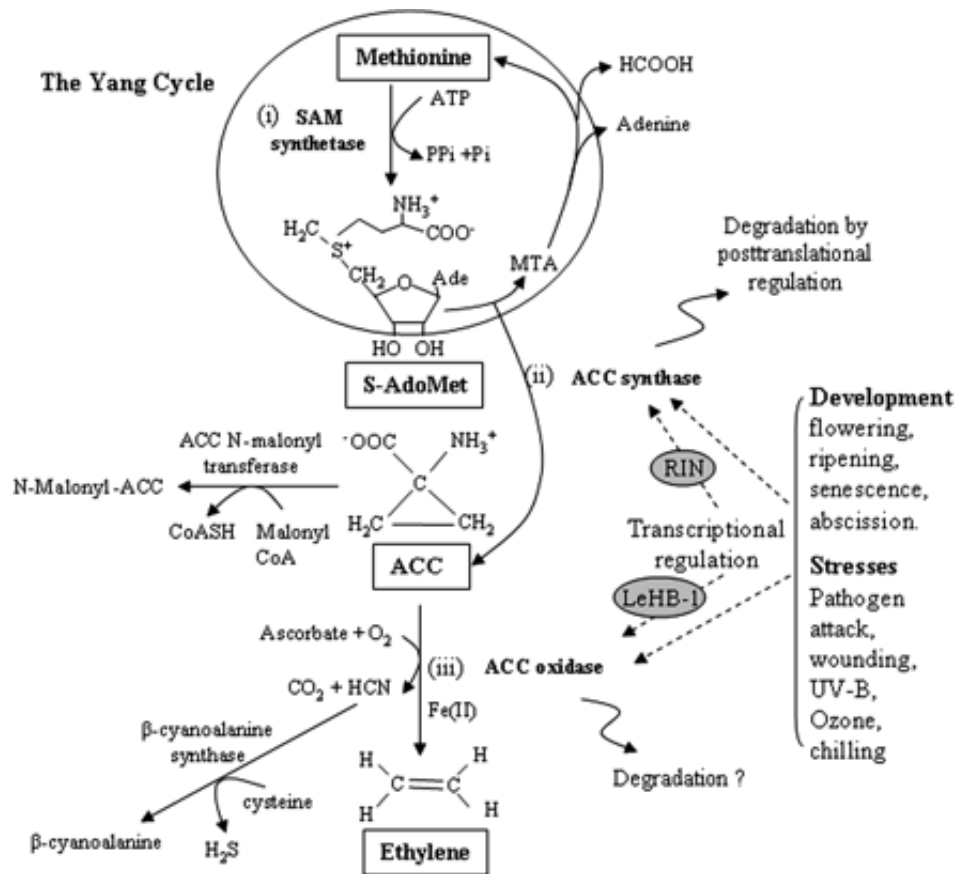


Figure 1.2: ET biosynthesis (Lin, et al., 2009)

1.2.4 Interactions between defence pathways

The three pathways described above are clearly overly simplified as JA, SA and ET signalling involves a complex signalling network in which they positively and negatively regulate their interactions to establish SAR. The studies conducted in *A. thaliana* that provide evidence for the crosstalk between the three defence pathways are summarised in Figure 1.3. *Arabidopsis thaliana* mutants *crp5* and *crp6* constitutively express pathogen related genes *PR-1* and *PDF 1.2* in the absence of a pathogen. These mutants also carry a mutation in the *npr1* gene, which is required for the activation of *PR* genes downstream of SA. This indicates that the activation of *PR-1* is a SA-mediated NPR1 independent response. When a mutation in the *EIN2* gene was introduced into *crp5* and *crp6*, *PR-1* gene

expression was abolished confirming the existence of an interaction between ET and the SA-dependent NPR1 independent pathway (C. M. J. Pieterse & van Loon, 1999).

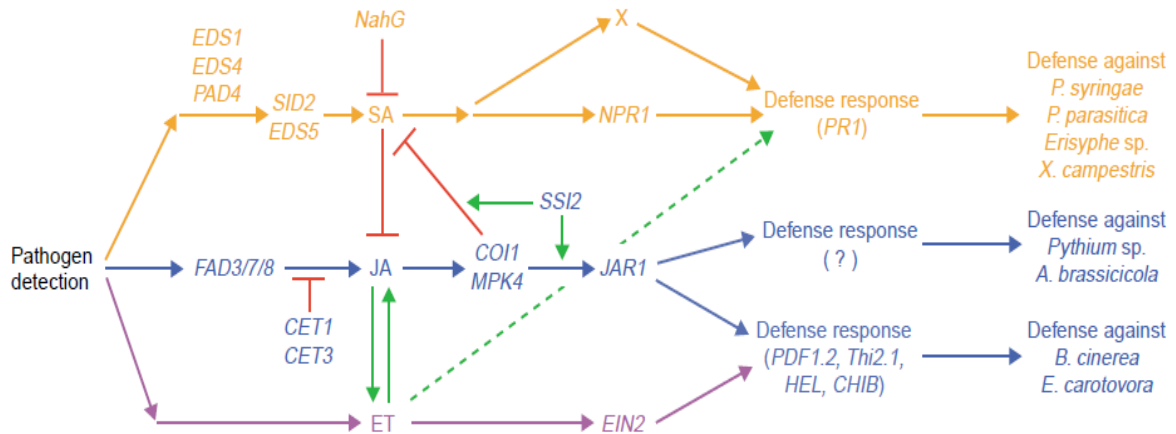


Figure 1.3: Working model of SA, JA and ET pathogen defence pathways and their interactions in *A. thaliana* (Kunkel & Brooks, 2002).

In summary, studies have shown that plant-microbe interactions and defence signalling pathways are capable of differentially expressing different defence pathways or a combination of pathways based on the type of infection they are exposed to. The resistance conferred by the SA-dependent pathway is considered to be directed primarily towards biotrophic (and hemibiotrophic) pathogens whereas the JA/ET pathway appears to play a more important role in resistance against necrotrophs (Glazebrook, 2005).

1.3 The Soft Rot Erwinias

SREs are necrotrophic, gram negative, rod-shaped, facultative anaerobes that cause diseases on a variety of crops (Perombelon, 1992). They are members of the

Enterobacteriaceae, which includes both plant and animal pathogens such as *Erwinia amylovora*, *Escherichia coli*, *Salmonella* spp., and *Yersinia* spp. Although the genus name *Erwinia* has been used to describe SREs, based on 16s rDNA sequence and their ability to grow at 39°C taxonomic revision has grouped them within the genera *Pectobacterium* and *Dickeya* (Hauben, et al., 1998). Furthermore, based on DNA–DNA hybridization, phenotypic characterisation and serological reactions four *Pectobacterium* subspecies have been reclassified into *Pectobacterium atrosepticum* (*Pba*), *Pectobacterium carotovorum* (*Pcc*), *Pectobacterium betavasculorum*, and *Pectobacterium wasabiae* (synonyms; *Erwinia carotovora* subsp. *atroseptica*, *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *betavasculorum*, and *Erwinia carotovora* subsp. *wasabiae* respectively) (Gardan, et al., 2003).

Pba, *Pcc*, and *Dickeya* spp. are the major SREs that cause disease on potatoes. *Dickeya* isolates previously placed under the single species *Erwinia chrysanthemi* have been reclassified into six species. *Dickeya dadantii*, *Dickeya dianthicola* and *Dickeya zae* are most frequently associated with disease on potatoes (Samson, et al., 2005). More recently, a new subspecies *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbr*) has been identified, which causes blackleg disease on potato plants in Brazil and South Africa (Duarte, et al., 2004; van der Merwe, et al., 2010). This pathogen has also been isolated from potato plants in New Zealand (Andrew Pitman, personal communication).

Pba has a narrow host range and is restricted mainly to potato plants in temperate regions. It does not survive in soil longer than a year, unless contained in diseased tubers. *Pba* is

responsible for blackleg disease of potato plants during the growing season and soft rot of tubers post-harvest (Perombelon, 2002; Perombelon & Kelman, 1980; Sledz, et al., 2000). In the field, blackleg symptoms are expressed when the pathogen predominates in the rotting mother tuber, invades the stem and multiplies in the xylem vessels under favourable environmental conditions. The stem of the diseased plant typically shows a black to light brown decay that extends from an inch to more than a foot from the soil surface. The leaves of the infected plant tend to roll upward, become yellow, wilt, and often die (Perombelon & Kelman, 1980).

Pcc has a wide host range affecting crops in subtropical and temperate regions. *Pcc* multiplies and persists in the root zones of host and non-host crops and weed species, remaining dormant until the environmental conditions are favourable for causing disease (Perombelon, et al., 1985). *Pcc* causes soft rot of tubers in potato crops. Studies to identify the pathogens associated with blackleg of potato plants have also led to isolation of *Pcc* strains from lesions. Initially the isolation of *Pcc* from the blackleg lesions was thought to be due to cross contamination or opportunistic infection. However, further studies revealed that some *Pcc* strains are capable of causing blackleg in up to 50% of potato plants in temperate conditions (De Haan, et al., 2008).

1.4 Pathogenicity determinants of *Pectobacterium*

1.4.1 Plant cell wall degrading enzymes

The ability of *Pectobacterium* strains to infect plants is due in large part to the production of a wide range of Plant Cell Wall Degrading Enzymes (PCWDEs). Comparative genomics on *Pba*, *Pcc* and *Pbr* have revealed that all three species encode orthologous PCWDEs including ten pectate lyases (Pel), one pectin lyase (Pnl), four polygalacturonases (Peh), two cellulases and one rhamnogalacturonase (Glasner, et al., 2008). Genome sequencing of *Pba* SCRI1043 has led to the identification of at least 20 putative PCWDEs in this strain (Bell, et al., 2004). Of all the PCWDEs identified in *Pba* SCRI1043, pectinases are believed to be the most important exoenzymes. They break down the pectin in the middle lamella of plant cell walls causing tissue and cell damage leading to cell leakage. Many pectinases such as Pel, Pnl, pectin methyl esterase (Pme) and Peh have been implicated in pathogenesis and have been identified in multiple forms (isoenzymes). Pel are the main pectinases involved in pathogenesis, although their number vary between species, subspecies and strain (Toth, et al., 2003).

1.4.2 Secretion systems

In gram negative bacteria, secretion systems are involved in transport of numerous proteins into the host including those associated with the virulence. Plant-pathogenic bacteria encode several secretion systems including the Type 1 secretion system (T1SS), T2SS, T3SS, T4SS, T5SS, T6SS and T7SS. The genome of *Pba* SCRI1043 encodes a T1SS,

T2SS, T3SS, T4SS and T5SS. The T2SS (the OUT system) in *Pectobacterium* is required for the secretion of PCWDEs (Pel, Cel and Peh). Secretion through the T2SS in *Pectobacterium* is a two-step process involving the Sec or Tat export machinery, which transports the secreted protein into the periplasm and then into the exterior through the T2SS apparatus (Coulthurst, et al., 2008). *DsbA* is a thiol-disulfide oxidoreductase which catalyses the formation of disulphide bonds between pairs of cysteine residues in target proteins. *DsbA* is required for the disulphide bonding of both the resident periplasmic protein and the protein that is secreted into the periplasmic space. In *Pectobacterium* T2SS and *DsbA* activity is required for full virulence on potato tubers (Shevchik, et al., 1995).

The T3SS is capable of secreting proteins into the extracellular space and translocating proteins into the host cell. The *hrp* genes encode T3SS apparatus that mediate the transfer of these effector proteins into the eukaryotic host cell. The T3SS is often required for the pathogen to grow and cause disease on susceptible plants, but can elicit defence responses in resistant plants (Galan & Collmer, 1999; Greenberg & Vinatzer, 2003). Comparative genomics has identified that the T3SS secretion system is conserved in *Pba*, *Pcc* and *Pbr* (Gardan, et al., 2003). Mutation studies have been conducted to identify the role of the *hrp* gene cluster in the *Pba*-potato interaction. Mutants with Tn5 insertions in *hrcC*, *hrcV*, *hrpN* and *dspE/A* were tested for their pathogenicity on potato stems relative to wild-type strain *Pba* SCRI1039. Disease lesions were reduced considerably in plants inoculated with the mutant strains. This data suggests that the T3SS and effector, *dspE/A* are involved in pathogenicity of *Pba* on potato (Holeva, et al., 2004).

The T4SS is required for plasmid conjugation and is also capable of secreting proteins to the host cell. Unlike T3SSs, they are present in different locations in the genome of *Pba*, *Pbr* and *Pcc*. In *Pcc*, only a remnant of the T4SS is present, however in *Pba* the T4SS is involved in the virulence of the pathogen on potato (Bell, et al., 2004). The T5SS includes an autotransporter and two partner secretions, which play an important role in the pathogenicity of *Dickeya* spp. and several other plant pathogenic bacteria (Glasner, et al., 2008). In addition to secretion systems, other determinants associated with virulence of *Pectobacterium* include agglutination and cell attachment factors, siderophores, detoxifying genes (*MsrA* and *Sap*), and phytotoxins (Bell, et al., 2004).

1.4.3 Coronafacic acid (CFA)

Pba SCRI1043 produces CFA, which is a component of coronatine (COR), a phytotoxin of *P. syringae* (Bender, et al., 1999). In *Pba* SCRI1043, CFA is encoded by a 22 kb biosynthetic gene cluster (Figure 1.4). It comprises nine genes (*cfa1-cfa8B*) that are required for the synthesis of the polyketide as well as a gene for the synthesis of coronafacic ligase (CFL), which mediates ligation of CFA to coronamic acid (CMA) in *P. syringae* (Bender, et al., 1999). However, *Pba* SCRI1043 lacks the *cma* genes, which suggests COR is not produced by *Pectobacterium*. Inactivation of *cfa6* and *cfa7* in *Pba* SCRI1043 causes a significant reduction in the pathogenicity of this bacterium on potato plants (Bell, et al., 2004). Pathogenicity studies have also shown that closely related *Pcc* strains that lack the CFA gene cluster fail to cause blackleg disease in potato plants (Pitman, et al., 2008). Though the CFA biosynthetic cluster plays a role in the development of blackleg disease on potato plants, there is no information on whether *Pectobacterium*

produces other related CFA conjugates as observed in *P. syringae* (Toth, et al., 2006). Regardless, the presence of a CFA cluster in numerous SREs isolated from potato plants infected with blackleg disease suggests that the CFA gene cluster is important for virulence on potato stems (Slawiak & Lojkowska, 2009).

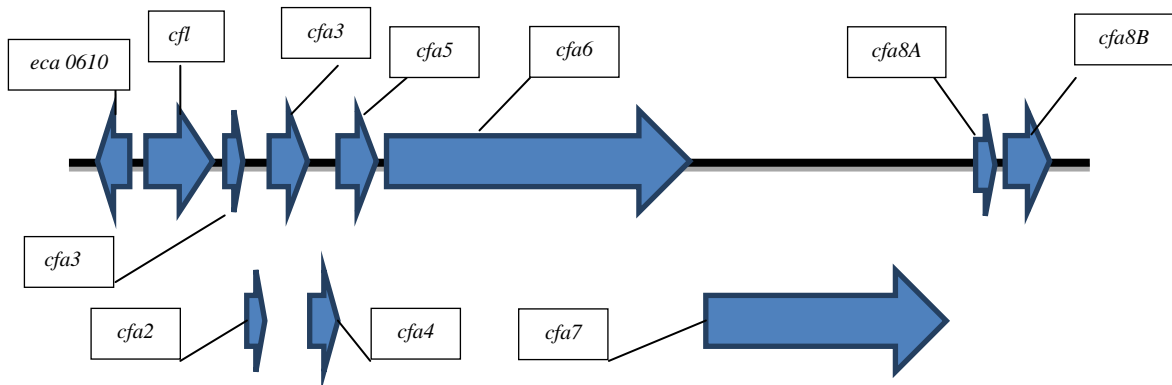


Figure 1.4: Organisation of the CFA biosynthetic cluster in *Pba* SCR11043.

1.5 CFA in other plant pathogenic bacteria

Recent genome sequencing of *Streptomyces scabies* 87-22 has identified a region similar to the CFA cluster present in *Pba* SCR11043 and *P. syringae* (Bignell, et al., 2010). *Streptomyces* spp. cause scab on economically valuable root and tuber crops including potato (Loria, et al., 2006). In *S. scabies*, the CFA cluster is encoded by eight genes (*cfa1-cfa8*), and is expressed under conditions that induce thaxtomin production, the key virulence factor in *S. scabies* (King & Calhoun, 2009). The CFA mutant shows reduced virulence on potato, but still causes extensive stunting of the roots and shoots as well as chlorosis and eventual death of the host plant (Bignell, et al., 2010). These data suggest the

CFA-like cluster is not essential for pathogenicity but contributes to the severity of the disease induced by *S. scabies*.

In *P. syringae*, CFA is encoded by nine genes (*cfa1-cfa9*) as well as the genes encoding CMA. To identify the genes involved in COR synthesis, mutation studies were carried out in *P. syringae* pv. *tomato* DC3000 (Brooks, et al., 2004). Mutants with Tn5 insertions in *cfa6* (DB4G3) and *cmaA* (AK7E2) and a *cmaA cfa6* double mutant (DB29) were used for the study. These knockouts failed to produce detectable amounts of CFA and CMA resulting in impaired COR production and showed a reduced ability to elicit disease symptoms in *A. thaliana* (Brooks, et al., 2005a). The conservation of CFA and CFA-like biosynthetic clusters in different plant pathogenic bacteria suggests that these toxins are important in many host pathogenic interactions.

1.6 The role of COR in pathogenicity of *P. syringae*

There is little knowledge about CFA in *Pba* SCRI1043 and its role in pathogenicity on potatoes. However, in *P. syringae* COR is structurally related to the plant hormone JA as well as other active derivatives like Jasmonoyl-Isoleucine (JA-Ile). COR functions as a molecular mimic of JA-Ile by promoting COI1-JAZ interactions and activating JA signalling; in turn suppressing the SA signalling pathway that is crucial for plant defence against biotrophic pathogens like *P. syringae* (Katsir, et al., 2008; Melotto, et al., 2008). These data suggest COR manipulates plant hormone signalling to enhance the susceptibility of a host to bacterial infection.

To assess whether COR and/or CFA function as jasmonate analogues, expression of several jasmonate responsive genes was monitored in plants inoculated with the wild-type strain *P. syringae* pv. *tomato* DC3000 or COR biosynthetic mutants. RNA blot analysis revealed that the jasmonate responsive genes *Atcor1*, (encodes for chlorophyllase), *lox2* (encodes for Lipoxygenase and is induced on treatment with JA or wounding) and *PDF 1.2* were strongly induced 24 hours (h) after inoculation with wild-type. However, *cmaA*, *cfa6* and *cmaA cfa6* mutants did not show significant induction of transcriptional expression. Studies have also shown that the intact COR molecule but not the precursor CFA, function to stimulate JA signalling during infection of *A. thaliana* (Brooks, et al., 2005a). These data suggest that COR acts as a jasmonate analogue and plays a vital role in expression of disease in *A. thaliana* by *P. syringae*.

Though previously described studies have identified the effects of COR in *A. thaliana*, studies to understand the response of tomato to *P. syringae* pv. *tomato* DC3000 and the COR-defective strain DB29 have also identified genes involved in the JA pathway (*lox*, *aos*, and *12-opr3*) as well as wound inducible genes proteinase inhibitor (*pi*) and polyphenol oxidase (*ppo*) that were up-regulated in a COR-dependent manner during pathogenesis. The expression of the JA pathway genes was induced within 12 h of inoculation, but not in seedlings inoculated with mutant strain DB29 (Uppalapati, et al., 2008).

1.7 Aim of this study

COR initiates a response in the JA pathway that leads to the suppression of SA production, demonstrating that interactions between plant defence pathways is important in triggering an appropriate response to pathogen attack. Research to understand the influence of CFA in manipulating host defence responses have been undertaken in *P. syringae*, a hemibiotrophic pathogen. Though CFA is a component of COR, it is usually COR which is responsible for biasing host defence system during infection. Thus, given the absence of CMA, the necrotrophic lifestyle of SREs, and their interaction on a different host system, the aim of this study was to explore the transcriptional response of potato to *Pectobacterium* encoding the CFA gene cluster. Furthermore, given the availability of the potato genome (The Potato Genome Sequencing Consortium, 2011), through this study we also hoped to gain a better insight into the host defence system in potato, which could lead to novel strategies for combating SREs and other pathogens in the future.

Chapter 2

Materials and Methods

2.1 Bacterial strains

Pectobacterium strains and *Escherichia coli* (Table 1) were cultured in Luria Bertani (LB) medium (Miller, 1972) at 28°C for 24 h and at 37°C for 16 h, respectively. Where appropriate, cultures were grown with antibiotics at the following concentrations: 50 µg ml⁻¹ kanamycin (Km); 170 µg ml⁻¹, chloramphenicol (Chl); 100 µg ml⁻¹, ampicillin (Amp); 20µg ml⁻¹, tetracycline (Tet). For long term storage, equal volumes of an overnight culture were mixed with 20% glycerol and stored at -80°C.

Table 2.1: List of strains used in this study.

Strains	Description	Source	Antibiotic Resistance
<i>Pectobacterium</i> strains			
<i>Pba</i> SCRI1043	Wild-type strain that causes blackleg	Bell et al., 2004	
<i>Pba</i> SCRI1043 Δ <i>cfa8</i>	Mutant strain of <i>Pba</i> SCRI1043 carrying <i>Tn5</i> insertion in <i>cfa8</i> gene	Toth unpublished *	Km
<i>Pba</i> SCRI1043 Δ <i>cfa7</i>	Mutant strain of <i>Pba</i> SCRI1043 carrying inactivated <i>cfa7</i> gene	This study	Chl
<i>Pbr</i> NZEC1	NZ strain that causes soft rot of tubers and blackleg disease	Pitman et al., 2008	
<i>Pbr</i> NZEC1 Δ <i>cfa7</i>	Mutant strain of <i>Pcc</i> NZEC1 carrying inactivated <i>cfa7</i> gene	Panda unpublished ⁺	Chl
<i>Escherichia coli</i>			
TOP 10	Competent cells used for cloning genes	Invitrogen	
JM109	Competent cells carrying recombinant plasmid	Panda unpublished ⁺	Km and Chl
s17-1 λ -pir + pNJ5000	Helper strain used to mobilize plasmid	Invitrogen	Tet

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2.2 Media and solutions

All the recipes for media and solutions used in this study are listed below (Table 2.2).

Table 2.2: List of media and solutions used in this study.

Component	Amount
Minimal Glucose Media (1 L)	
50x Phosphate buffer	20.0 ml
Ammonium sulphate (10% (w/v))	10.0 ml
Magnesium sulphate (1M)	400 µl
20% Glucose	10.0 ml
Agar	16.0 g
Minimal High Sucrose Media (1 L)	
50x Phosphate buffer	20.0 ml
Ammonium sulphate (10% (w/v))	10.0 ml
Magnesium sulphate (1M)	400 µl
50% Sucrose	200.0 ml
Agar	16.0 g
50X Phosphate buffer (500 ml)	
Di potassium phosphate (K_2HPO_4)	350.0 g
Potassium phosphate (KH_2PO_4)	100.0 g
Borate lysis buffer (200 ml)	
Ethylene glycolbis (β - aminoethyl ether)-N-N'-tetraacetic acid	2.3 g
Sodium boratedecahydrate (Borax)	15.25 g
Sodium dodecyl sulphate (SDS)	2.0 g
Sodium deoxycholate	1.0 g
Polyvinylpyrrolidone (Mr 40,000) (2% (w/v))*	0.4 g/20 ml
Dithiothreitol (DTT)*	31.0 mg/ 20 ml
Lithium chloride extraction buffer (30 ml)	
Tris-HCl 1 M, (pH 8)	2.5 ml
Lithium chloride (LiCl) 4 M	0.6 ml
Ethylenediaminetetraacetic acid (EDTA) 0.5 M	0.5 ml

SDS 20 % (w/v)	1.25 ml
CTAB buffer (50 ml)	
CTAB	1.0 g
Tris-HCl 1M (pH 8)	2.5 ml
EDTA 0.5 (pH 8)	1.0 ml
Sodium chloride (NaCl)	4.09 g
MOPS buffer pH 7.0 (1 L)	
3-(N-morpholino)propanesulfonic acid (MOPS)	83.7 g
Sodium acetate (NaOAc) 50 mM	13.6 g
EDTA 0.5 M, (pH 8)	3.7 g
Formaldehyde gel Loading buffer (10 ml)	
Bromophenolblue solution	16 µl
EDTA 0.5 M, (pH 8.0)	80 µl
Formaldehyde 12.3 M	720 µl
100% glycerol	2.0 ml
Formamide	3.8 ml
10X MOPS buffer	4.0 ml
5 X Tris/Borate/EDTA buffer (TBE) (1 L)	
Tris Base	54.0 g
Boric acid	27.5 g
EDTA	20.0 ml

*Added to the buffer just before use

2.3 RNA extraction

Several techniques for extraction of RNA were compared in this study; below are the generic protocols for each of them.

2.3.1 RNA extraction using the RNeasy Plus Mini Kit

RNA from infected potato tubers sampled around the point of infection was isolated using the RNeasy Plus Mini Kit (Qiagen). Briefly, for each inoculation, 50 mg of tuber material was ground in liquid nitrogen using a mortar and pestle. To avoid the action of RNase, 10 μ l of β -mercaptoethanol (β -ME) was added to 1 ml of RLT buffer (Qiagen), and 600 μ l of the resulting RLT buffer was added to the mortar and pestle and transferred into microfuge tubes. Cells were disrupted by vigorous vortexing. The mixture was incubated in a 70°C water bath for 10 min with vigorous shaking before the lysate was transferred to a spin column and centrifuged for 2 min at 14000 rpm. 0.5 volumes of ethanol (96-100%) was added to the supernatant, before total RNA was isolated according to the manufacturer's instructions. In addition to the on-column DNase digestion included in the manufacturer's protocol, the RNA samples were also subjected to further treatment with 1 unit of TURBO DNase (TURBO DNA-freeTM kit, Ambion) for 30 min.

2.3.2 RNA extraction using hot borate lysis buffer

Cores of potato tubers, sampled around the point of infection using a 6mm Unicore were frozen using liquid nitrogen and stored at -80°C. Sodium deoxycholate and polyvinylpyrrolidone were added to the borate lysis buffer (Wan and Wilkins 1994) on the day of the extraction and the buffer was incubated at 80°C in the water bath for 15-30 min. Approximately 2 g of each sample was ground in liquid nitrogen using a mortar and pestle, before 1 ml of hot borate lysis buffer was added to the sample and was transferred into a Falcon tube. The lysate was mixed using a vortex and then centrifuged at 10,000 rpm for 5 min to pellet the debris from the clear lysate. 200 μ l of the clear lysate was transferred into

a clean microfuge tube and the remaining 800 μ l was stored at -80°C and was re-extracted when necessary. 10 μ l of Proteinase K was added to the lysate and was mixed briefly using a vortex. Finally, total RNA was extracted using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's instructions, followed by treatment with 1-2 U of Turbo DNase (TURBO DNA-freeTM kit, Ambion) for 30 min.

2.3.3 RNA extraction using lithium chloride precipitation

Total RNA from freeze-dried potato tubers was isolated using a lithium chloride precipitation method. For each sample, 7 ml of extraction buffer was added into a 15 ml falcon tube, followed by an addition of 7 ml of phenol (pH 4.6-5.6). The resulting extraction buffer was incubated in a 80°C water bath for 15-30 min. At the same time, approximately 2 g of a freeze dried tuber was ground in liquid nitrogen using a mortar and pestle prior to the addition of 14 ml of hot extraction buffer to the sample. The sample mixture was transferred into a 15 ml Falcon tube and mixed using a vortex for 2-5 s. 10 ml of sterile distilled water (SDW) was added to the samples and the content was once again vortexed for 1-2 min followed by the addition of 15 ml of chloroform: isoamyl alcohol (24:1). After repeated vortexing for 1-2 min, the samples were centrifuged at 4°C for 20 min at 14000 rpm. The upper aqueous layer was transferred into a 50 ml Falcon tube containing equal volumes (16 ml) of 4M LiCl previously incubated at -20°C . The content was mixed thoroughly by shaking and the tube was incubated at -80°C overnight. After incubation, the tubes were thawed on ice and were centrifuged at 4°C at 14000 rpm for 40 min. The supernatant was discarded and the pellet was resuspended in 5 ml SDW, 0.1 volumes of 3M sodium acetate (NaOAc) (pH 5.2), and 3 volumes of 100% ethanol. The

samples were incubated at -80°C for at least 1 h (can also incubate for several days). After incubation, the samples were centrifuged at 4°C at 14000 rpm for 40 min. Ethanol was discarded and the pellets were air dried. Total RNA was resuspended in 500 µl of RNase free water. The RNA was then purified and genomic DNA contamination was removed using the SV total RNA isolation kit (Promega) according to manufacturer's instructions.

RNA concentration and purity was analysed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The A260/280 ratios provide information regarding the protein contamination in the sample. It works on the principle that nucleic acid displays an absorbance at 260 nm whereas the protein shows absorbance at 280 nm. Sufficiently pure samples should have a ratio of 1.9-2. RNA integrity was determined using a Bioanalyser 2100 (Agilent Technologies). RNA samples of good quality have a RNA Integrity Number (RIN) ≥ 7 , whereas a RIN < 7 indicates significant RNA degradation.

2.3.4 Gel electrophoresis of RNA

RNA isolated using the previously described techniques was visualized by gel electrophoresis in a denaturing gel. The gels were prepared by heating 1 g of agarose in 72 ml of water; after cooling at 60°C, 10X MOPS buffer and formaldehyde were added to give a final concentration of 1X and 2.2 M, respectively. RNA samples (100-500 ng) were prepared with 2 µl of 5X MOPS running buffer, 3.5 µl of formaldehyde and 10 µL of formamide to a final volume of 20 µl, adjusted with DECP treated water. The samples were incubated at 65°C followed by chilling on ice and subsequent centrifugation for 5 s. 2

μ l of formaldehyde gel buffer was added to the sample for electrophoresis. Along with the samples, 5 μ l of HyperLadder I (Bioline) was used as a molecular weight standard. The gel was run in 1X MOPS buffer. After electrophoresis, the gel was stained in ethidium bromide (0.5 μ g/ ml in 0.1 M ammonium acetate) for 30-45 min and was visualised using a UV transilluminator. Gel images were recorded using the Versadoc gel documentation system.

2.4 DNA extraction

2.4.1 Isolation of genomic DNA

DNA from potato plants and tubers was extracted using the CTAB extraction method (Doyle, 1990). In a microfuge tube, approximately 90-140 mg of plant material was homogenised using a micropestle. The lysate was incubated at 50°C overnight with 550 μ l of 2X CTAB buffer. After incubation, 550 μ l of chloroform: isoamyl alcohol (29:1) was added to the lysate and mixed by inversion for 5 min, followed by centrifugation at 8000 rpm for 13 min. About 400 μ l of supernatant was transferred into a clean microfuge tube containing 0.2 volumes of 2 M NaCl (4% PEG). The content was vortexed and incubated at 4°C for 2 h followed by centrifugation at 13000 rpm for 10 min. Approximately 500 μ l of the supernatant was transferred to a clean microfuge tube and equal volumes of 100% isopropanol was added and mixed by inversion. The mixture was incubated at -20°C for 30 min. After centrifugation at 13000 rpm for 10 min the supernatant was discarded. To the pellet, 300 μ l of 70% ethanol was added and centrifuged at 13000 rpm for 15 min. The supernatant was discarded and the pellet was air dried. 50 μ l of SDW was used to resuspend the pellet. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.4.2 Isolation of plasmid DNA

Plasmid DNA for enzymatic digestion and for quantitative real-time RT-PCR (qRT-PCR) was isolated from *E. coli* cultures using a QIAprep Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions. Briefly, cells from an overnight culture were harvested by centrifugation at 1600 rpm for 5 min. The cells were resuspended in 250 µl of buffer P1 and then P2. To the content, 350 µl of buffer N3 was added and mixed thoroughly by inversion. The tubes were centrifuged at 13000 rpm for 10 min. The content was transferred into a QIAprep mini spin column (Qiagen) and the plasmid DNA was eluted using elution buffer.

2.4.3 Restriction endonuclease digestion

Plasmid preparations were digested using restriction enzyme. Reaction mixtures were prepared with appropriate buffer, 1 µg of plasmid DNA and 5 units of enzyme. Nuclease free water was used to make up the reaction to 50 µl. The reaction mix was gently vortexed and incubated at 37°C for 16 h. The resulting digest was visualised by electrophoresis on a 1% gel.

2.4.3.1 Gel electrophoresis of DNA

PCR products, restriction enzyme-digested DNA or plasmid DNA were mixed with bromophenol blue tracking dye and separated on a 1-2% (w/v) agarose gels, prepared and

run in 1X TBE buffer containing $1 \mu\text{g ml}^{-1}$ ethidium bromide. Electrophoresis of DNA was generally carried out for 1 h at 80 V. Along with the DNA samples, 5 μl of HyperLadder IV or HyperLadder I (Bioline) was used as a DNA size standard. Following electrophoresis, the DNA was visualized in a UV transilluminator. Gel images were recorded using a Versadoc gel documentation system.

2.4.4 DNA purification from agarose gels

DNA fragments extracted from an agarose gel were purified using a QIAquick Gel extraction kit (Qiagen). Briefly the DNA fragments from the gel were excised, placed in a centrifuge tube, and weighed. Following this, three volumes of QC buffer were added. The resulting mixture was incubated in a heat block for 10 min at 50°C (until the gel was dissolved). One gel volume of isopropanol was then added and mixed thoroughly before the content was transferred into a QIAquick spin column, and the DNA was eluted using the manufacturer's instructions.

2.5 Polymerase Chain Reaction (PCR)

In this study, PCR was used to screen colonies to confirm the identity of recombinant clones. qRT-PCR was also used to quantify the differential expression of host genes upon infection with bacterial strains. The primers used in this study are listed in Table 2.3.

2.5.1 Standard PCR protocol

For amplification of genomic DNA, Platinum *Taq* DNA polymerase (Invitrogen) was used. Reactions were set up in 50 µl volumes, each reaction mixture containing 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTP mixture, 1.5 µl of 50 mM MgCl₂, 300 nM of each primer, 0.2 units of *Taq*, and 1-5 µl of DNA (100-500 ng). For preparation of multiple PCR reactions, a master mix was prepared to ensure consistency. Thermal cycling was performed in a Biorad thermal cycler. PCR cycling conditions were as follows: 94°C for 1 min; followed by 30 cycles of 94°C (20 s), 60°C (20 s), and 68°C (20 s) (the elongation time is set based on the principle 1 min to amplify 1 kb of the target sequence); and a final cycle of 68°C (2 min).

2.5.2 cDNA synthesis

cDNA was synthesised from RNA samples using a SuperScript Vilo cDNA synthesis kit (Invitrogen). The Superscript enzyme mix includes the SuperScript III RT RnaseOUT recombinant Ribonuclease Inhibitor. 5X VILO reaction mix contains the random primers, MgCl₂ and dNTPs. Reactions were carried out in a 20 µl volume containing 4 µl of 5 X VILO reaction mix, 2 µl of 10 X SuperScript enzyme mix, 2 µg of RNA and nuclease free water to bring the volume to 20 µl. The reaction mix was incubated at 25°C for 10 min followed by 42°C for 60 min. The reaction was terminated at 85°C for 5 min.

2.5.3 Quantitative Real-time RT-PCR

To quantify the relative expression of potato genes under different conditions, Sybr Green based q-RT PCR was used. The Applied Biosystems StepOne Plus™ was used for real time fluorescence detection of PCR products and the results were analysed with Applied Biosystems StepOne software V2.1. For each qRT-PCR reaction, a 1 in 5 dilution of prepared cDNA was used as a template. Reactions were carried out in 11 µl containing 5.5 µl of Sybr Green master mix, 100-300 nM of each primer, nuclease free water to bring the volume up to 10 µl and 1 µl of prepared cDNA as template (1 in 5 dilutions). qRT-PCR reactions were performed in triplicate. The cycling conditions were 95°C for 3 min; followed by 40 cycles of 95°C for 20 s, 58°C - 60°C for 20 s, 72°C for 20 s with data capture during the extension phase of each cycle. Melt curve analysis was undertaken for all reactions to confirm the amplification of the appropriate product. The amplification efficiency of each assay was determined using a plasmid containing a single copy of each target gene. A 10X dilution of the recombinant plasmid (pPCR1) was prepared and used as template for qRT-PCR to generate standard curves for each reaction, by plotting relative DNA concentration versus log(Ct) value (Ct value is the cycle at which the fluorescence rises beyond the background level). All plate runs included a positive (recombinant plasmid) control and no-template control as a negative.

To estimate the slope and constant, linear regression of Ct values for the standards on the log₁₀ Copy number (CN) were carried out using equation 1 given below. From these regressions, the efficiency factor (Eff) was calculated using equation 2. The quantity for each sample was calculated using equation 3

Equation 1: $Ct = \text{Const} + \text{Slope} * \log_{10} \text{CN}$

Equation 2: $\text{EFF} = 10^{(-1 / \text{Slope})}$

Equation 3: $\text{Quantity} = 10^{(Ct - \text{Const} / \text{Slope})}$

Before further analysis, the mean Ct of the triplicates for each sample was calculated. To analyse the ratios of quantities, the mean Cts for each sample were converted into the ratio of the quantity of the test gene to the quantity of the reference gene (EFL) using the estimated calibration parameters (described previously). The \log_{10} of these ratios were analysed with analysis of variance. Differences between the mean \log_{10} ratio for treatment (mutant-treated, mock-inoculated, non-inoculated) and the mean for wild-type (Wt) treated were calculated and transformed, to give ratios between each treatment and the wild-type treatment (R_{wt}). The transformed least significant difference (LSD) was then calculated to give a least significant ratio (LSR); which is the smallest ratio significantly greater than 1. ($1/\text{LSR}$ gives the largest ratio for which the ratio is significantly smaller than 1). All analyses were carried out with GenStat (GenStat Committee, 2011b).

Table 2.3: List of primers used in this study.

Gene Name	Primer Name	Accession Number	Tm	Primer concentration (nM)	Primer sequence
Plant gene					
1-aminocyclopropane-1-carboxylate synthase (<i>acc synthase</i>)	Acc syn 147-F	PGSC0003DMB000000147:	60°C	300	AGCCACAAATGATGGTCATGGAGAAA ACACCATGAGGATTTTGTGTTGGATGA AGTTCAAGGTCGGGGTGGTCT
	Acc syn 147-R	1139221-1142430			
	Acc syn 445-F	PGSC0003DMB000000445:	60°C	300	GCCGGAGCCGTAGATTCGCC
	Acc syn 445-R	109190-116382			
1-aminocyclopropane-1-carboxylate oxidase (<i>acc oxidase</i>)	Acc oxd 087-F	PGSC0003DMB000000087:	60°C	300	AACACAAGTGTTCCTTTCCGGTGGA ATCAACAACCTTCAGACATGCGGGGT GCAGCTCGTGAATCAGCACTACG
	Acc oxd 087-R	454193-458140			
	Acc oxd 372-F	PGSC0003DMB000000372:	60°C	300	TGTGCTTTCCAGTCTGTGTTGCT
	Acc oxd 372-R	88546-90395			
Fatty acid desaturase (<i>fad</i>)	FAD 604-F	PGSC0003DMB000000604:	60°C	300	CCTGTGTTTGCATGGTGACGACG CCAATGGGTAGATGACACCGTTGGT TGGGTAGATGACACCGTTGGTCT AAGCAGTGTGGAAATGGTGACGACG GGATTGCTCAAGGTTGTGTTGGGG
	FAD 604-R	170564-172436			
	FAD 992-1F	PGSC0003DMB000000992:	60°C	300	AGACCAACGGTGTGTCATCTACCCA
	FAD 992-1R	26391-27525			
	FAD 992-2F	PGSC0003DMB000000992:	60°C	300	AGACCAACGGTGTGTCATCTACCCA
FAD 992-2R	41520-42636				
Lipoxygenase (<i>lox</i>)	Lox 147-F	PGSC0003DMB000000147:	60°C	300	GCTAATCGATTCGTGTACCACCATCA ACTGTGGGGCGATTTGGGAGG ACCACCACAGCCAGTTTTACCTCA
	Lox 147-R	2367-7111			
	Lox 309-F	PGSC0003DMB000000309:	60°C	300	AGCGGTGTGCTGGATACACC
	Lox 309-R	661391-666497			

Allene oxide cylase (<i>aoc</i>)	Aoc 423-F	PGSC0003DMB000000423: 308213-309445	60°C	300	TTGCTAAAGGGGACAAGATCTCCGA
	Aoc 423-R				GTGTCTATGAGCTCAATGAACGTGACC
Apetala 2 (<i>ap2</i>)	Ap2-F	PGSC0003DMB000000623: 152070-155371	60°C	300	GGTCCGAGTTCGTGACTCAGCA
	Ap2-R				GGATCCACAGACGGATCCACCCA
Defensin	Def-1F	PGSC0003DMB000000729: 16788 17956	60°C	300	TGATAGTGGAGGCCGAAGACTTGCGA
	Def-1R				AACCGCTCCGTACTGCAAACGA
	Def-2F	PGSC0003DMB000000300: 12862-14682	60°C	300	ATGAGCGGTGGTGACAGAGGC
	Def-2R				CAGTCTGGCACACGGAGGCG
Jasmonic amino acid conjugating enzyme (<i>Jaace</i>)	Jaace-F	PGSC0003DMB000000069: 1114169-1121512	60°C	300	AGCCAGTTCTGGATCAGGCTTGAGTA
	Jaace-R				TGTTGCAAATATAAGGGACGGAGTCC T
Poly phenol oxidase (<i>ppo</i>)	PPO-1F	PGSC0003DMB000000535: 143786-45786	60°C	300	TTCGGTGACTTTGGTCACGTGTT
	PPO-1R				CGCTTTGCCATATTGGAATTGGGACC
	PPO-2F	PGSC0003DMB000000083: 826611-828969	60°C	300	ACTTGGAGCTCTGTATTTGGGAAACC
	PPO-2R				CCTGACGACGATCCACGTAGCTT
PR gene (uncharacterised)	PR-F	PGSC0003DMB000000682: 89022-89882	60°C	300	TCACAAGCTCAAACTCTCCCCAAG
	PR-R				GCTGCTAGCCTATTGTCCCATGTCA

S-adenosylmethionine synthase 1 (<i>sams1</i>)	SAM-F	PGSC0003DMB000000008: 2142265-2144759	60°C	300	TGCCTAGAACAAGACCCAGAGAGCA
	SAM-R				CCACCTTCGCCTTGGTTGTGA
WRKY-4	WRKY4-F	PGSC0003DMB000000104: 587735- 590190	60°C	300	AGCGATGTTTCAGTGACCCAAGC
	WRKY4-R				TGGATAGCAGTTAGGACGGGACATCA
Elongation factor α (<i>efl \alpha</i>)	EFL-F	PGSC0003DMB000000044: 1359183-1361000	60°C	300	ATTGGAAACGGATATGCTCCA
	EFL-R				TCCTTACCTGAACGCCTGTCA
Isochromate synthase (<i>ics</i>)	ICS-F	PGSC0003DMB000000180: 940429-950662	60°C	300	TCCATGGCCAATGCAGGGCT
	ICS-R				TGTCGATGAATGGATGCCAAGGCG
Glycerol kinase (<i>gk</i>)	GK-F	PGSC0003DMB000000026: 577296-580319	60°C	300	TGGCTGTTGGATAATGTGGAGGGGT
	GK-R				TCTCCACACCTCCTGTTAGGTTCCA
Proteinase inhibitor (<i>pi</i>)	PI-F	PGSC0003DMB000000119: 296840-298274	60°C	300	ACCCGAACAACAGTTGATGCATATTCC
	PI-R				GCGATTTCGAACATGCAGACGCC
Allene oxide synthase (<i>aos</i>)	Aos-F	PGSC0003DMB000000017: 903857-905687	58°C	100	TGGTCCCAGGTGGCATGTTCG
	Aos-R				GGCGACTATGGGTTGCCGGG

12-oxophytodienoate reductase (<i>12-opr3</i>)	12-OPR3-F 12-OPR3-R	PGSC0003DMB000000272:594 289-599242	60°C	300	TGCCTGCTTCGGTTTGCCCA AGGAGCTGATCGTGTAGGCGT
Bacterial gene					
CFA7	CFA7-F CFA7-R	Eca0516-0614	60°C	300	CAATTAGACGCAGCCAGACAG TGAGTGTATTCCCACGTCCA

Note - The primers were designed using genome sequence for “Summer Delight”. The Summer Delight contigs were assembled against the Double Monoploid (DM) potato genome sequence ("Genome sequence and analysis of the tuber crop potato," 2011). The consensus sequence was extracted and the primers were designed using Geneious Pro 5.6.

2.6 Bacterial transformation

For cloning purposes, plasmids were introduced into chemically competent TOP10 *E. coli* cells using one shot chemical transformation as per the manufacturer's instructions (Invitrogen, Australia). Briefly, 2 µl of plasmid DNA was mixed into a vial containing 100 µl of Top10 competent cells on ice, followed by incubation for 5 to 30 min. The cells were then heat-shocked for 30 s at 42°C without shaking (heat shock treatment) and transferred back onto ice. 250 µl of Super Optimal broth with catabolite repression (provided in the kit) was immediately added to the transformation mix and the vials incubated at 37°C for 1 h at 200 rpm in a shaking incubator. Approximately 10-50 µl of the resulting culture was spread onto an agar plate (LB) containing appropriate antibiotic selection and the plates were then incubated at 37°C overnight. Transformants were obtained by picking single colonies, which were subsequently screened for the recombinant plasmid by PCR.

2.6.1 Allelic exchange mutagenesis

Pectobacterium strains were transformed with recombinant plasmids using tri-parental mating (Goldberg & Ohman, 1984). Overnight cultures of wild-type *Pectobacterium* strains, the donor *E. coli* JM109 containing the recombinant plasmid and the helper strain s17-1 λ-pir + pNJ5000 (used to mobilize the cloning vector) were grown in 5 ml of LB with appropriate antibiotics. 1 ml of the culture was pelleted and washed with the same amount of LB without any antibiotics. 10 µl of each culture were then mixed together and spotted onto the surface of an LB agar plate, prior to incubation at 25°C. After approximately 24 h incubation, the spot was scraped and streaked on to minimal media containing glucose, Km and Chl and incubated at 25°C for a further 3-4 days. Resistant

single colonies were picked and grown in 5 ml LB with no selection. Overnight cultures were diluted in 1X phosphate buffer (1:100). 100 µl of the dilution was spread on to minimal medium containing sucrose and incubated at 25°C for 4-5 days. Single colonies resistant to sucrose were streaked onto LB agar plates containing Chl. Each growing colony resistant to Chl was screened by PCR for homologous recombination resulting in the insertion of recombinant fragment into the target gene. PCR products were run on 1% gel to confirm that the gene of interest had been mutated.

2.7 Pathogenicity Assays

2.7.1 Soft rot assay

Bacterial cultures were grown overnight in LB broth at 200 rpm in a shaking incubator at 28°C. Cells were harvested by centrifugation at 1600 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in the same volume of 10 mM MgCl₂. Inoculum concentration was adjusted to 10⁶ colony forming unit (CFUs ml⁻¹); by measuring the optical density at 600nm (OD₆₀₀= 0.8) using a spectrometer and by changing the volume accordingly with 10 mM MgCl₂. Prior to inoculation, potatoes were washed in tap water and air-dried. Using a cork borer, uniform bores were then made in the tubers (approximately 3 mm diameter by 10 mm deep) and 10 µl of the overnight inoculum was inoculated into the hole using a pipette (equivalent to 10⁶ cells per inoculation site). The tuber plug was replaced and sealed using petroleum jelly. Tubers were placed in a plastic container layered with paper towels soaked in water (to maintain humidity) and incubated in a growth chamber at 22°C for one week. Potato tubers of different cultivars (cvs), even in size, were inoculated with each bacterial strain. As a control, tubers of each cv were

inoculated with 10 mM MgCl₂. After incubation, tubers were weighed, the rotten tissue was then removed by washing and the tubers were re-weighed after blotting dry with a paper towel. Susceptibility to soft rotting was assessed by calculating the weight of tissue lost after removing the rotten tissue (Wright, et al., 1991).

The percentage of weight loss (i.e. $100 \times (\text{initial wt} - \text{final wt}) / \text{final wt}$) was analysed using a binomial generalized linear model approach (McCullagh & Nelder, 1989), with a logit link. Differences between cvs and strains, and other contrasts were assessed with F-tests within the analysis of deviance conducted as part of the analysis. The analyses were carried out with GenStat (GenStat Committee, 2011b).

2.7.2 Blackleg assay

Potato tubers of different cvs were allowed to sprout at room temperature for approximately 2 weeks. The sprouted tubers were potted in sterile potting mix and the plants were grown for approximately three weeks in a controlled growth chamber, with a 16 h photoperiod at 22°C and 80% humidity. When they reached 20-25 cm in height, stems of seven plants of each cvs were inoculated with an overnight culture of each strain. For controls, three plants were inoculated with 10 mM MgCl₂. The stem under the second fully expanded leaf was inoculated with 10 µl of each bacterial strain (equivalent to 10⁶ cells per inoculation site) using a 10 µl pipette, the wound was then sealed using petroleum jelly. Disease symptoms were assessed by measuring the length of the lesion every day for a period of 10 days.

2.7.3 *In planta* growth curve assays

Overnight bacterial cultures were used for inoculation of potato stems and tubers. As described previously 10 μ l of overnight culture (containing approximately 10^6 cells) were inoculated into each stem and tuber. The bacterial population in each plant was measured 1 hour post inoculation (hpi), and 1, 2, 3, 4, 6, 8; 10 days post inoculation (dpi). For tubers, cell counts were measured up to 7 dpi. Each time point was represented by 5 replicates for plants and 3 replicates for tubers. The stem and tubers were sampled around the inoculation site, and were homogenised using a sterile micropestle. A 100 μ l aliquot of the lysate was resuspended using 900 μ l of sterile water and a serial dilution of each replicate was prepared and plated on LB agar plates. Plants inoculated with 10 mM $MgCl_2$ were used as a control. Plants and tubers were incubated as described previously. The CFUs in each plate were counted and the data for different treatments was analysed statistically.

The data for all counted plates was included in the analysis, with data for both strains analysed in a single analysis. The standard approach for counts is a Poisson generalized linear model (GLM, McCullagh & Nelder, 1989). However, for this experiment, there were multiple plates per tuber, and also for a given tuber, each plate was for a different serial dilution. Therefore, the data was analysed using an extension of the Poisson GLM that allowed adjustments for individual tubers to be included (as a random effect) and also an adjustment for the dilution factor for each plate. The adjustment included the dilution factor as a parameter-less offset (McCullagh & Nelder, 1989); that is, the count per plate is the underlying count for the tuber, divided by the dilution factor. The counts were therefore analysed with a Poisson-gamma hierarchical generalized linear model (Lee, et al., 2006),

with logarithmic links, and an offset of $\log(1/\text{dilution})$. Times were included as a fixed effect and tubers as a random effect. In addition, the random variation was estimated separately for wild-type and mutant inoculated tubers. Random and fixed effects were assessed using X^2 tests of changes in the likelihood of dropping the term as implemented in GenStat's HGRTEST and HGFTEST procedures (GenStat Committee, 2011a). The analysis was carried out with GenStat (GenStat Committee, 2011b).

2.8 RNA sequencing and bioinformatic analysis

RNA sequencing was performed by Axseq Technologies: four samples were run in one lane of Illumina HiSeq 2000 with 100 bp paired-end sequencing. The nucleotide sequence retrieved from sequencing was analysed using bioinformatics tools. In Table 2.4 the software used for each analysis and their descriptions are listed.

The first step in RNA sequencing analysis was to check for the quality of the raw data which includes applying a quality cut-off threshold and removing adapter sequences. Less than one percentage of the raw reads were removed by this quality control step because the remaining sequence was too short (less than 50 nucleotides) for further processing. In addition, 14 bases from the 5' end of reads were removed to eliminate the last traces of adapter burn in.

The main aim of the study was to identify the genes that are significantly differentially expressed in response to *Pectobacterium* and in particular the presence of the CFA cluster.

This was achieved by first mapping the filtered reads from each library separately to the potato reference genome (The Potato Genome Sequencing Consortium, 2011) using TopHat tool (<http://tophat.cbcb.umd.edu/>). The mapped reads were pooled according to treatments and then analysed using Cufflink (<http://cufflinks.cbcb.umd.edu/>). This program assembles the aligned reads to transcripts, and estimates their abundance by measuring the transcript abundance in Fragment per Kilobase of exon per Million fragments mapped (FPKM), which is analogous to RPKM (<http://cufflinks.cbcb.umd.edu/howitworks.html>). Cuffdif was used to analyse the differential expression of the transcriptomes in each treatment. Cuffdif uses cufflinks transcript quantification engine to calculate gene and transcript expression levels in multiple treatments and tests them for significant differences. The observed difference in gene expression was tested for significance using the statistical test provided in Cufflink. Student's t-Test (two tailed) was used to identify differentially expressed genes. Cufflink uses t-test to calculate the p value of the observed change, this helps in looking for genes with statistically significant changes in absolute expression. The subsequent results were tabulated in a table that included a FPKM value for each gene in each treatment library. To identify the differentially expressed genes each treatment was compared with the other three treatments.

Table 2.4 : List of Bioinformatic tools used for analysis.

Tool	Description	URL
Fastqc	Read quality visualization	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
FastqMcf	Read clipping	http://code.google.com/p/ea-utils/wiki/FastqMcf
Tophat	Aligning reads to the genome	http://tophat.cbc.umd.edu/
Cufflinks	Determining FPKM values	http://cufflinks.cbc.umd.edu/
Mapmap	Visualization	http://mapman.gabipd.org/
Python scripting	Extract data	
Geneious Pro	Primer design	http://www.geneious.com/

Chapter 3

Development of an experimental model for transcriptional studies involving potato and *Pectobacterium*

3.1 Summary

SREs belonging to the genus *Pectobacterium* are causative agents of blackleg infection in potato stems, seed piece decay and soft rot of tubers during storage. CFA has been identified as an important virulence factor involved in the pathogenicity of *Pectobacterium* on potato. In this chapter, the susceptibility of four potato cvs was tested using blackleg and soft rot assays. The aggressiveness of different *Pectobacterium* strains and their respective CFA mutants were also compared to establish an appropriate experimental model for studying the interactions between potato and SREs. “Iwa” and “Summer Delight” were identified to be susceptible to soft rot infection by both *Pba* SCRI1043 and *Pbr* NZEC1. In addition, *Pbr* NZEC1 was identified to be an aggressive pathogen when compared with *Pba* SCRI1043.

3.2 Background

Different cvs of potato differ in their susceptibility to *Pectobacterium* species. Potato tubers from breeding lines have been continuously tested for susceptibility to *Pectobacterium* to assist in breeding for resistant potato lines. To identify the difference in susceptibility of New Zealand potato breeding lines to soft rot, tubers of 23 cvs were assessed for their susceptibility to *Pba* over five seasons (Wright, et al., 1991). Eighteen

cvs were compared with that of “Iwa”, “Ilam Hardy”, “Rua”, “Stormont enterprise” (susceptible) and breeding line 065/27 (resistant) for which the response to *Pba* had previously been characterised. It was observed that although potato lines differed in susceptibility they remained consistent between seasons (Wright, et al., 1991). Breeding line 1463.1, “Rua” and “Bison” were consistently very susceptible while “Kaipara”, 064/18 and 065/27 were least susceptible to soft rot infection by *Pba*. In a more recent attempt to characterize the susceptibility of New Zealand cvs based on the specific gravity of their tubers, “Ilam Hardy” was found to be highly susceptible, whereas “Summer Delight” and “Karakā” were shown to be relatively resistant to soft rot infection by *Pba* when compared with other breeding lines (Wright, et al., 2005).

Previous studies to optimize an inoculation method for a soft rot assay have also shown the variability between cvs to soft rot infection. The susceptibility of potato tubers to soft rot by *Pba* and *Pcc* were assessed over a period of three years using both a tuber inoculation and tuber slice method (Lapwood, et al., 1984). Certain cvs were consistently more susceptible to soft rot infections by *Pectobacterium* than other cvs. “Klondyke” and “Manna” were highly susceptible and “Drayton” remained resistant over the period.

Research has also shown that different enterobacterial strains have different virulence on stems and tubers of potato. Studies to evaluate the aggressiveness of *Pba*, *Pcc*, and *Ech* in causing soft rot and stem rot disease have identified *Pcc* as an aggressive causative agent of soft rot infections when compared to *Pba* and *Ech*. However, certain cvs differed in susceptibility to soft rot and blackleg when challenged with the same strain, which strongly indicates that there are differences in resistance mechanisms in tubers and in plants

(Hidalgo & Echandi, 1982). Subsequent studies have also shown *Pbr* to be an aggressive pathogen when compared to *Pba* (Duarte, et al., 2004).

The main aim of the research in this chapter was to establish an appropriate combination of both potato cv and pathogen to investigate the influence of CFA on the host response to *Pectobacterium*. In order to identify a susceptible cv, cvs most relevant to the New Zealand breeding program and for which genetic information was readily available were selected. Given these criteria, three cvs “Iwa”, “Summer Delight”, “Karaka” and breeding line “1021/1” were selected for this study. Though “Iwa” is very old and is no longer used in the breeding programme, it has been well characterised to be susceptible to soft rot and blackleg infections. Breeding line “1021/1” shows high resistance to cold induced sweetening and is important for the breeding programme, whereas “Karaka” and “Summer Delight” are well adapted to growing in different climatic conditions and are also used as parents in the breeding programme (Jeanne Jacobs, personal communication). Of these cvs the genome sequence for all except “Iwa” is readily available (Jeanne Jacobs, personal communication).

We also compared the aggressiveness of *Pbr* NZEC1 and *Pba* SCRI1043 and their respective *cfa* mutants to identify a suitable strain for subsequent transcriptional studies. A strain that causes sufficient disease symptoms was required. Furthermore, a difference in the aggressiveness of the wild-type and its CFA mutant was necessary to study the role of CFA in potato.

3.3 Results

3.3.1. Selection of cvs and strains for transcriptional studies

3.3.1.1 Soft rot assay

Four cvs (“Iwa”, “Summer Delight”, “Karakā” and breeding line “1021/1”) were screened for their susceptibility to *Pba* SCRI1043 and *Pbr* NZEC1 using tuber assays as described in Section 2.7.1. To identify cvs with the greatest difference in susceptibility to the wild-type strains and their respective CFA mutants, for each cv seven tubers were inoculated with *Pba* SCRI1043, *Pba* SCRI1043 Δ *cfa8*, *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7*. In addition, three tubers per cv were inoculated with MgCl₂ as a mock-inoculated negative control. Tubers were incubated for seven days under humid conditions and the susceptibility to soft rotting was calculated by measuring the percentage weight loss.

Average percentage weight loss caused by *Pbr* NZEC1 was 10.4 % across all cvs, significantly higher ($p < 0.001$) than that caused by *Pba* SCRI1043 (3.8%) (Figure 3.1, Table 3.1). On average, however the percentage of tuber weight loss varied between the cvs ($p < 0.001$ for overall differences between cvs). The greatest percentage in weight loss in tubers inoculated with *Pbr* NZEC1 was recorded for “Summer Delight” and the least for “Karakā” (percentage weight loss for “Summer Delight” was 8.1, “1021/1” was 5.6, “Iwa” was 4.8 and “Karakā” was 2.8) (Table 3.1).

For tubers inoculated with *Pba* SCRI1043, the difference in weight loss was fairly similar for all the cvs ($p=0.213$). However, the greatest percentage of weight loss in tubers inoculated with *Pba* SCRI1043 was again observed for “Summer Delight” and the least for “Iwa” (“Summer Delight” 5.5, “1021/1” 5.2, “Iwa” 2.8 and “Karaka” 2.7). The negative control showed no symptoms and weight loss for the mock-inoculated controls was negligible (<0.5% for all cvs).

The amount of tissue macerated by the wild-type strains *Pbr* NZEC1 and *Pba* SCRI1043 was higher than that macerated by the respective CFA mutants. For tubers inoculated with *Pbr* NZEC1, the percentage of weight loss was significantly higher for the wild-type (10.4%) than that of the *cfa7* mutant (4.4%) ($p<0.001$). For *Pba* SCRI1043, the percentage weight loss for tubers inoculated with the *cfa8* mutant (3.0%) was lower than that for the wild-type (3.8%), but this difference was not statistically significant ($p=0.213$).

Though the *Pba* SCRI1043 Δ *cfa8* mutant had been used for experiments because it was readily available it did not strongly influence the pathogenicity of *Pba*. The *cfa8* gene encodes for PaaI thioesterase. PaaI thioesterase is a tetrameric acyl-CoA thioesterase responsible for phenylacetic acid degradation in bacteria. The *cfa7* gene, which has previously been shown to be involved in pathogenicity of *Pba* (Bell, et al., 2004), encodes for a type 1 polyketide synthase (PKSs) which is involved in the biosynthesis of CFA (Rangaswamy, et al., 1998). As a result, given the lack of effect in the *cfa8* mutant, a *cfa7* mutant was generated for further pathogenicity studies.

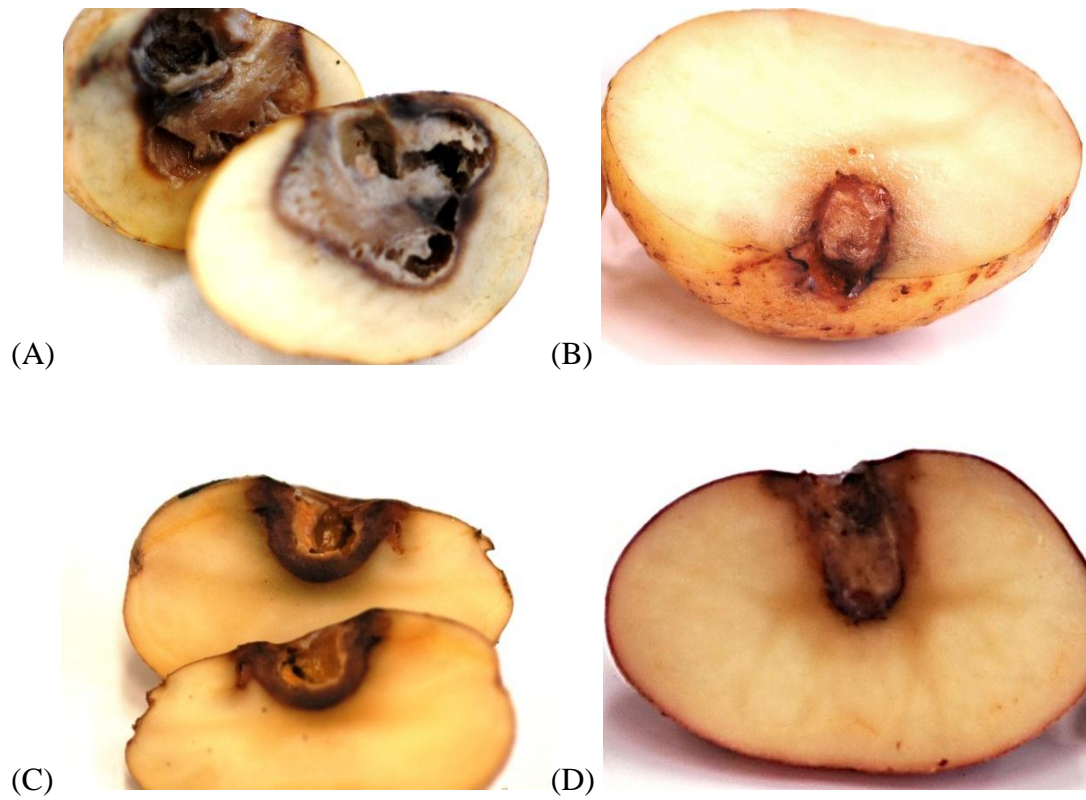


Figure 3.1: Soft rot on tubers (“Summer Delight”) caused by (A) *Pbr* NZEC1, (B) *Pbr* NZEC1 Δ *cfa7* mutant, (C) *Pba* SCRI1043 or (D) *Pba* SCRI1043 Δ *cfa8*.

Table 3.1: Percentage loss in tuber weight due to soft rot caused by *Pbr* NZEC1 and *Pba* SCRI1043 and their respective CFA mutants.

Note: 95% confidence limits are shown in brackets.

Cultivar	Treatment	Negative Control	Tuber weight loss (%)	
			<i>Pbr</i> NZEC1 or <i>Pbr</i> NZEC1 Δ <i>cfa7</i>	<i>Pba</i> SCRI1043 or <i>Pba</i> SCRI1043 Δ <i>cfa8</i>
“1021/1”	Control	0.0 (0.0,17.4)		
	WT		8.6 (6.2,11.8)	5.2 (3.4,7.9)
	Mutant		5.2 (3.4,7.9)	3.7 (2.2,6.1)
“Iwa”	Control	0.4 (0.0,4.7)		
	WT		11.8 (9.2,15.0)	2.8 (1.6,4.8)
	Mutant		2.0 (1.0,3.9)	2.7 (1.5,4.8)
“Karaka”	Control	0.1 (0.0,3.2)		
	WT		5.4 (3.7,7.9)	1.7 (0.9,3.4)
	Mutant		2.7 (1.6,4.7)	1.5 (0.7,3.2)
“Summer Delight”	Control	0.2 (0.0,4.6)		
	WT		15.7 (12.6,19.5)	5.5 (3.7,8.1)
	Mutant		7.5 (5.4,10.4)	4.1 (2.7,6.2)

3.3.1.2 Construction of *Pba* SCRI1043 Δ *cfa7*

To produce a *cfa7* mutant of *Pba* SCRI1043, *Pba* SCRI1043 was transformed with pK18mobsacB (Km^R) (Schäfer, et al., 1994) carrying a non-functional copy of the *cfa7* gene (Chl^R), as described in Section 2.6.1. Growth on media containing sucrose was used to select for mutants of *Pba* SCRI1043, in which the suicide vector had been lost and the wild-type copy of *cfa7* had been replaced by the non-functional copy via allelic exchange. CFA7 primers designed to amplify the *cfa7* gene (Table 2.3) were used to confirm the allelic exchange by PCR. A PCR product of approximately 400 bp was amplified from *Pba*

SCRI1043, characteristic of the presence of a functional *cfa7* gene in this strain (Figure 3.2). However, colonies resistant to sucrose and Chl failed to amplify the fragment indicative of the absence of a functional *cfa7* gene.

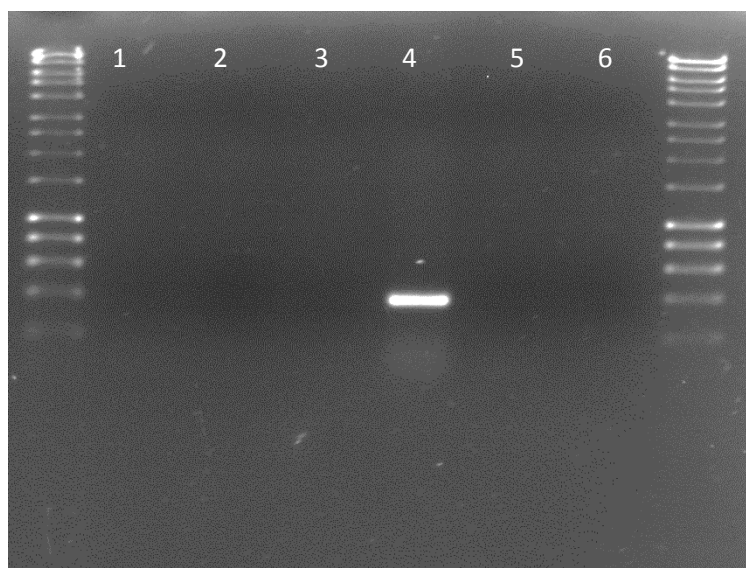


Figure 3.2: Gel image showing confirmation of allelic exchange in *Pba* SCR1043 resulting in inactivation of *cfa7*. Lane 1 to 3, *Pba* colonies resistant to sucrose and Chl; Lane 4, *Pba* SCR1043; Lane 5, *Pcc* NZEC1 Δ *cfa7*; Lane 6, No template control.

The susceptibility of potato tubers to *Pba* SCR1043 and the resulting mutant *Pba* SCR1043 Δ *cfa7* was subsequently compared by conducting soft rot assays on all the previously tested cvs. Though percentage of weight loss varied between cvs ($p < 0.001$), the difference was not as marked as when tubers were inoculated with *Pbr* NZEC1 (Figure 3.3). However, the average percentage weight loss for tubers inoculated with wild-type (6.1%) was substantially higher than that of the mutant (1.5%). In this experiment, the percentage of weight loss for tubers inoculated with *Pba* SCR1043 was greater for

“Karaka” and least for “Summer Delight” (“Karaka” 7.3, “Iwa” 7.8, “1021/1” 5.4, “Summer Delight”. 4.6) (Table 3.2). The fold change in weight loss of tubers inoculated with wild-type or mutant was however greatest in “Iwa” and “Summer Delight” (5.57 and 5.1 fold reduction, respectively) when compared to the remaining cvs (~3.2 fold reduction).

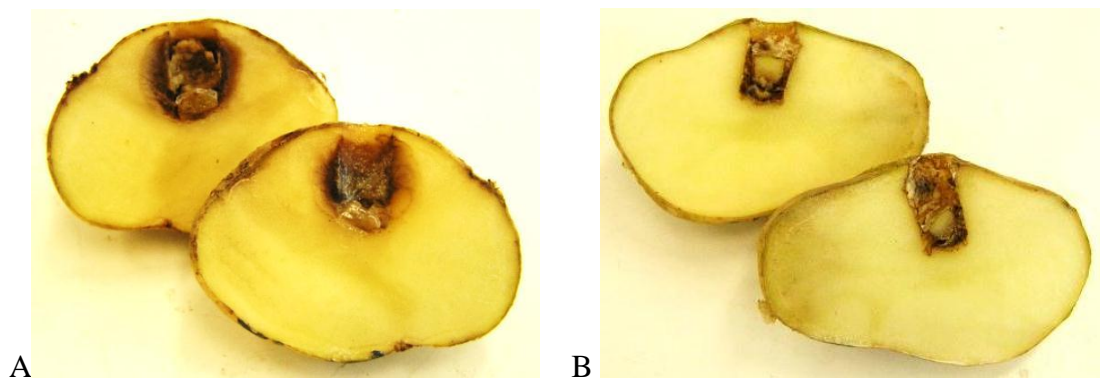


Figure 3.3: Lesions on tubers (“Summer Delight”) caused by (A) *Pba* SCRI1043 or (B) *Pba* SCRI1043 Δ *cfa7*.

Table 3.2: Percentage loss in tuber weight due to soft rot caused by *Pba* SCRI1043 and a mutant in *cfa7* biosynthesis.

95% confidence limits are shown in brackets.

Cultivar	Treatment	Tuber weight loss (%)
“1021/1”	Control	1.2 (0.5,3.1)
	<i>Pba</i> SCRI1043	5.4 (4.2,7.0)
	<i>Pba</i> SCRI1043 Δ <i>cfa7</i>	1.5 (1.0,2.3)
“Iwa”	Control	0.9 (0.3,2.7)
	<i>Pba</i> SCRI1043	7.8 (6.1,10.0)
	<i>Pba</i> SCRI1043 Δ <i>cfa7</i>	1.4 (0.8,2.6)
“Karaka”	Control	0.6 (0.2,1.6)
	<i>Pba</i> SCRI1043	7.3 (5.7,9.3)
	<i>Pba</i> SCRI1043 Δ <i>cfa7</i>	2.3 (1.5,3.4)
“Summer Delight”	Control	0.5 (0.2,1.2)
	<i>Pba</i> SCRI1043	4.6 (3.4,6.1)
	<i>Pba</i> SCRI1043 Δ <i>cfa7</i>	0.9 (0.5,1.6)

3.3.1.3 Selection of time points for RNA extraction

From the soft rot assays, *Pbr* NZEC1 was identified as an aggressive pathogen when compared with *Pba* SCRI1043 (Table 3.1). Further analysis also confirmed that overall “Summer Delight” was the most susceptible cv to both *Pbr* and *Pba* and showed the greatest difference in its response to wild-type and mutants unable to produce CFA. Hence, we decided to study the plant-microbe interaction in “Summer Delight” using *Pbr* NZEC1 and its respective CFA mutant. To identify a suitable time point to study the transcriptional response of this potato cv to *Pbr*, the growth dynamics of *Pbr* NZEC1 and the corresponding *cfa* mutant were assessed *in planta*.

To assess growth of *Pbr* in tubers, 15 tubers of “Summer Delight” were inoculated with either *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7* as mentioned in Section 2.7.1. At each time point (1 hpi, 1, 2, 4 and 6 dpi), three tubers were sampled around the site of inoculation to establish the number of CFUs per ml of tuber suspension. As a control, tubers were mock-inoculated with MgCl₂ were sampled at appropriate time points.

The pattern of growth as estimated by mean CFUs over time varied between *Pbr* NZEC1 and *Pbr* NZEC1 Δ *cfa7* ($p < 0.001$ for the strain by time interaction). This was primarily because of the higher count at the last assessment for *Pbr* NZEC1 in comparison to *Pbr* NZEC1 Δ *cfa7* (Figure 3.4). However, the estimated CFUs for both *Pbr* NZEC1 and *Pbr* NZEC1 Δ *cfa7* increased from 1 hpi to 2 dpi (considered log phase) (Figure 3.4). For *Pbr* NZEC1, the estimated CFUs then remained constant, whereas for *Pbr* NZEC1 Δ *cfa7* CFUs decreased from 2 dpi to 6 dpi. No bacterial contamination was identified in agar plates incubated with mock-inoculated tuber suspension.

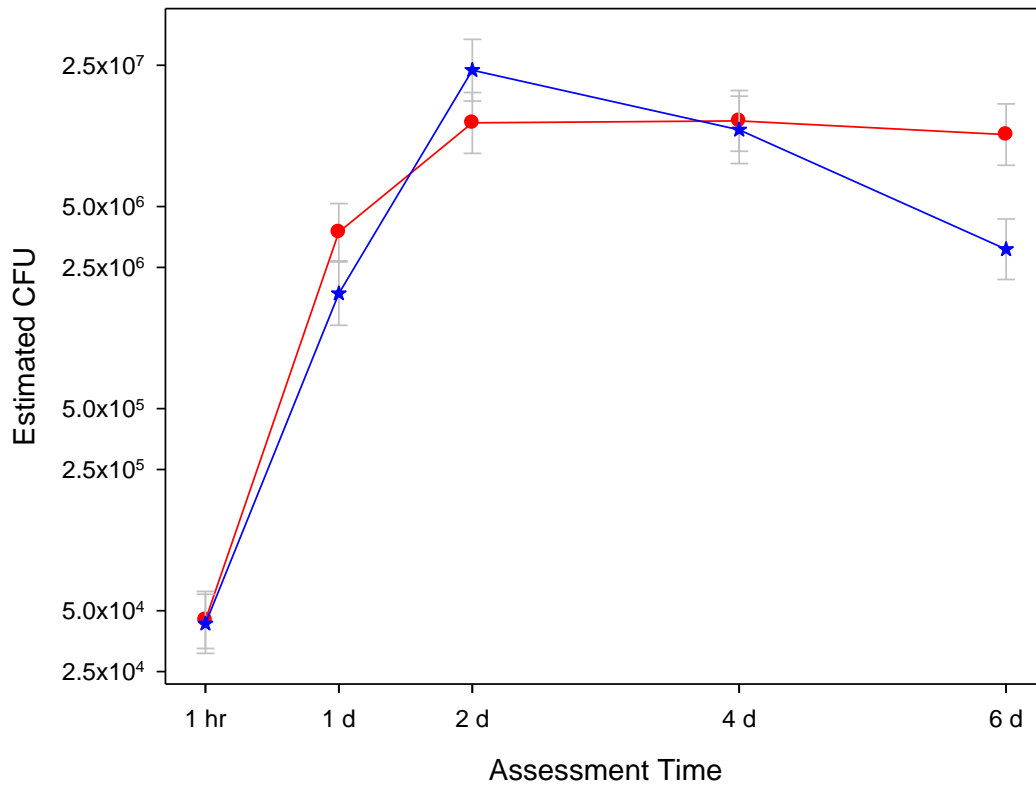


Figure 3.4: Growth of *Pbr* NZEC1 (red dot) and *Pbr* NZEC1 Δ *cfa7* (blue star) in potato tubers (“Summer Delight”) as shown by the estimated CFUs at each time point. Vertical bars represent approximately 95% confidence limit.

3.3.1.4 Blackleg and growth curve assays in potato stems

Blackleg assays were carried out to compare the susceptibility of stems of different cvs to *Pba* SCRI1043, *Pbr* NZEC1 and their respective mutants. For each cv 10 plants were inoculated with *Pba* SCRI1043, *Pbr* NZEC1, *Pba* SCRI1043 Δ *cfa7* or *Pbr* NZEC1 Δ *cfa7*. As a mock inoculation control three plants were inoculated with 10 mM MgCl₂ as described in Section 2.7.2. Unfortunately, attempts to quantify the virulence of these strains in potato stems using all four cvs resulted in no significant disease symptoms.

To understand why disease symptoms were not observed an *in planta* growth curve of *Pbr* NZEC1 using “Summer Delight” was established over a 10 day period. This provided insights into the growth dynamics of *Pbr* NZEC1 and whether growth inhibition was responsible for the lack of symptoms. Thirty five potato plants of “Summer Delight” were inoculated with *Pbr* NZEC1 as mentioned in Section 2.7.2 and then incubated at 90% humidity in a growth chamber. At 1 hpi and 1, 2, 4, 6, and 8 dpi five plants were sampled along the site of inoculation. As a mock inoculation control, three plants inoculated with 10 mM MgCl₂ were sampled at the alternate time points. A growth curve analysis was carried out as described in Section 2.7.3.

Estimated mean CFU varied significantly between the assessment times ($p < 0.001$ for an overall test). The major difference was between 1 hpi and the later times, with CFUs at 1 hpi lower than the other counts. After this point *Pbr* NZEC1 appeared to increase in number reaching levels in the plant of approximately 2.75×10^5 . Only minor differences were observed between the other time points ($p = 0.057$), principally relating to the low count at day 2. However, no plants showed any disease symptoms.

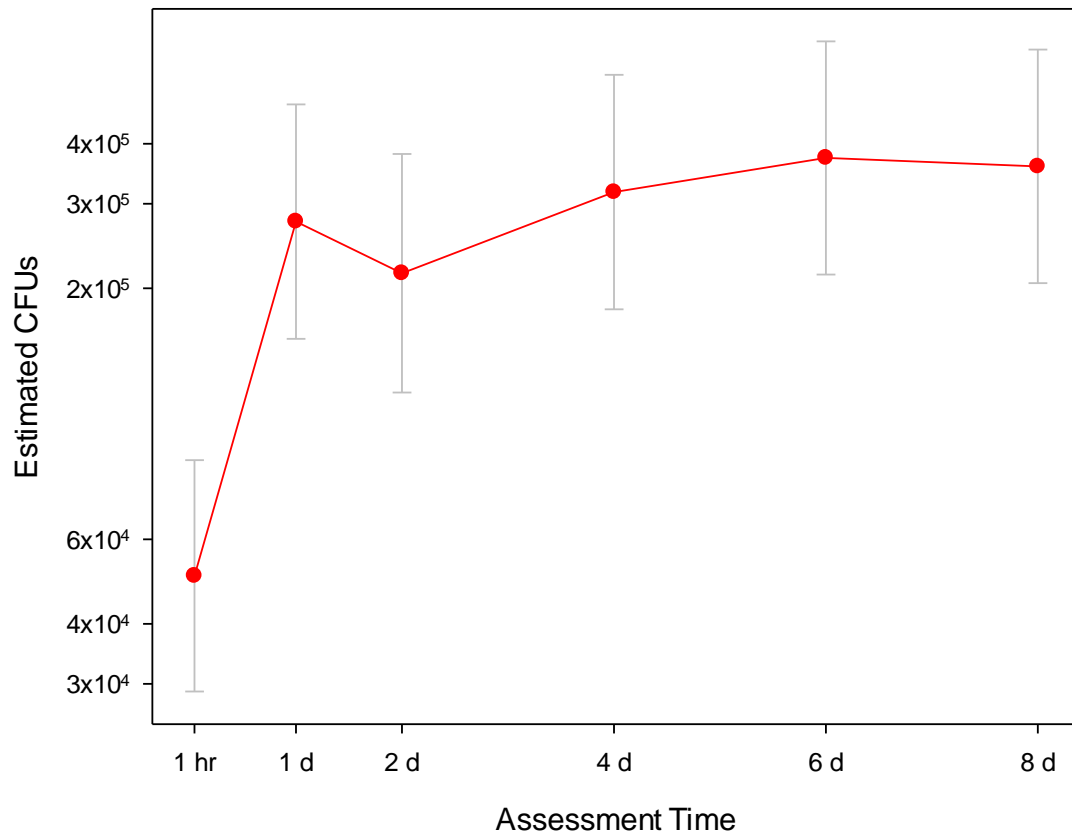


Figure 3.5: Growth of *Pbr* NZEC1 in plant stems (“Summer Delight”) as shown by estimated CFUs at each time point. Vertical bars represent approximately 95% confidence limit

3.4 Discussion

In this chapter, pathogenicity tests were conducted to identify a cv susceptible to *Pectobacterium* infection that would enable a comparison of pathogenicity of wild-type strains of *Pectobacterium* and their respective CFA mutants. The soft rot assay showed that of the four cvs tested “Summer Delight” and “Iwa” have greatest susceptibility to *Pbr*

NZEC1 and *Pba* SCRI1043. In contrast, “Karaka” generally showed little infection and thus was considered to be more resistant (Table 3.1). These data corresponded with the results of the previous study on New Zealand cvs and their susceptibility to soft rot infection in which “Iwa” was highly susceptible and “Summer Delight” was relatively susceptible to soft rot infections when compared to cv Karaka (Wright, et al., 2005).

Weight loss caused by infection of tubers by *Pba* SCRI1043 was not as evident as the weight loss caused by the *Pbr* NZEC1. This suggested that *Pbr* NZEC1 may be a more aggressive seed pathogen than *Pba* SCRI1043. *Pbr* was identified as an aggressive tuber pathogen in South Africa causing blackleg disease and soft rot of tubers (van der Merwe, et al., 2010). Pathogenicity assays also identified *Pbr* in Brazil to be more aggressive than *Pba* under lab conditions (Duarte, et al., 2004). Other studies using different strains of *Pbr*, *Pcc* and *Pba* have identified *Pbr* and *Pcc* to be more aggressive than *Pba* (Marquez-Villavicencio, et al., 2011).

The soft rot assays showed that the weight loss caused by *Pbr* NZEC1 was significantly higher when compared with its respective CFA7 mutant. “Iwa” and “Summer Delight” showed the maximum difference in weight loss between wild-type *Pbr* NZEC1 and the respective CFA mutant. However, tubers treated with *Pba* SCRI1043 Δ *cfa8* showed no significant difference in weight loss when compared to the wild-type strain. CFA8, a putative thioesterase, is located in the 3' end of the CFA transcript and mediates the release of free CFA (Zhao, et al., 2003). However, lack of reduction in disease lesions by CFA8 mutants suggests that this gene is not essential for production of a functioning CFA or for

the pathogenicity of *Pba*. In contrast, expression of *cfa7* (type1 PKS) is required for the biosynthesis of CFA and in turn the pathogenicity of *Pectobacterium* (Bell, et al., 2004).

Pathogenicity tests using *Pba* SCRI1043 Δ *cfa7* showed a significant difference between *Pba* SCRI1043 and the CFA mutant on all cvs (Table 3.2). Although the weight loss for all the cvs showed that “Karaka” was most susceptible and “Summer Delight” to be resistant to soft rot infections by *Pba* SCRI1043 strain, the maximum difference in the weight loss between the wild-type and CFA mutant was observed in “Summer Delight”. The shift in the susceptibility of the cvs to *Pectobacterium* infection apparent in Table 3.1 and Table 3.2 was possibly due to differences in physiological age of tubers. Though we tried to control this variation by using tubers grown from the same field, since the age of the tubers were not known, it was difficult to control the variation. Other studies have also reported the influence of tuber age and physiology on the susceptibility of tubers to infection by *Pectobacterium* strains (Marquez-Villavicencio, et al., 2011). This result confirms that more mature tubers are more susceptible to soft rot infection by *Pectobacterium*.

Unfortunately, the experiments conducted to study the susceptibility of the four cvs to blackleg failed to show consistent symptomology on all plants. Yet in a separate experiment using plants of “Summer Delight” inoculated with *Pba* SCRI1043, symptoms became visible within five days of infection (data not shown). The maturity of the plants appeared to affect the susceptibility of the plant to blackleg. The cvs showed significant difference in growth rate. Emergence and growth of “Iwa” and “Summer Delight”, the most susceptible cvs to *Pectobacterium* were much quicker than the other cvs. “Karaka” which is known to have greater resistance to *Pectobacterium* infection showed very late

emergence, taking 6-8 weeks to reach 20 cm height. Thus, susceptible cvs might have reached sufficient maturity to resist infection by the time they were used in these experiments. Certainly it was also observed that with maturity the stem of “Summer Delight” plants turned rigid and woody. Therefore, in subsequent experiments we would conduct assays separately on the different cvs to establish a susceptible cv for studying interactions associated with blackleg.

Analysis of growth of *Pbr* NZEC1 *in planta* identified fluctuations in growth patterns of the bacterium. Though there was a significant increase in growth of *Pbr* NZEC1 early after inoculation, inoculum concentrations of 10^5 failed to produce any disease symptoms during the experiment. Furthermore, inoculum levels did not continue to rise instead staying relatively constant. These results suggested that the mature plants showed some resistance to *Pectobacterium*. Perhaps these cvs are more susceptible to soft rot than blackleg. Previous studies have identified that potato lines when challenged with the same strain show differences in susceptibility to blackleg and soft rot of tubers (Hidalgo & Echandi, 1982). These data suggest that the defence mechanism in tubers might be different to that in the upper part of the plant. Subsequent blackleg assays will be conducted individually on all the cvs with identical physiological characteristics rather than with identical physical age. Inoculum concentration will also be optimised, so further blackleg susceptibility assays can be carried out for transcriptional studies.

Given that *Pectobacterium* usually enters the plant via tubers and having identified the lack of aggressiveness of *Pba* SCRI1043 on potato tubers, we decided to use *Pbr* NZEC1 and potato tubers (“Summer Delight”) to study the transcriptional response of potato to SREs.

To establish appropriate time points to extract RNA we conducted a growth curve of *Pbr* and its respective CFA knockout in tubers. Six days after inoculation there was a significant difference in growth rate of the two strains. The growth of wild-type and CFA mutants of *Pbr* NZEC1 increased significantly between 1hpi and 1dpi, indicative of the active log phase. Since the growth curve of both *Pbr* NZEC1 and its CFA mutant showed similar growth dynamics at 24 hpi and were in the log phase, RNA was extracted from plants at 24 hpi. Previous plant-microbe interaction studies have identified the log phase as a suitable time point to study the host defence mechanism (Brooks, et al., 2005a; Uppalapati, et al., 2008).

3.5 Conclusion

In this study, we confirmed the aggressiveness of *Pbr* NZEC1 and the influence of the CFA gene cluster in the virulence of the *Pectobacterium*. Due to the susceptibility of “Summer Delight” and the aggressiveness of *Pbr* NZEC1 this plant pathogen combination was chosen for further transcriptional studies. From the growth curve of *Pbr* NZEC1 in tubers we decided to isolate RNA at 24 hpi, which would provide optimal parameters to study early interactions between the plant and the microbe.

Chapter 4

The use of qRT-PCR to study differential gene expression in potato in response to *Pectobacterium*

4.1 Summary

The influence of *Pectobacterium* on expression of host defence genes has been studied in this chapter using qRT-PCR. Furthermore, to understand the role of CFA in manipulating host defence, the transcriptional expression was compared in response to wild-type strains of *Pectobacterium* and their respective mutants. Homologues of genes previously shown to be influenced by COR during *P. syringae* infections on tomato were studied. However, no significant difference in expression level of target genes was observed in response to *Pbr* and the CFA mutant.

4.2 Background

Studies of transcription require sensitive, precise and reproducible measurement for specific mRNA sequences, which enables the accurate quantification of temporal and spatial patterns of gene expression (Peirson, et al., 2003). However, classical approaches like northern blotting and RNase protection assays are not suitable in many cases for studying gene expression owing to their low sensitivity, requirement of high concentrations of starting RNA template and the extensive time taken to undertake the procedure. The advent of qRT-PCR has led to these approaches being superseded (Peirson, et al., 2003).

qRT-PCR is an advanced technique for reliable quantification of low-copies of mRNA in biological samples (Tichopad, et al., 2004). The sensitivity, accuracy and practical ease of this approach support the use of qRT-PCR in studying differential gene expression. The basic approach of this technique is that the intensity of the fluorescence signal emitted is proportional to the quantity of transcript produced in the sample (Heid, et al., 1996). The work flow of qRT-PCR includes sample acquisition, RNA extraction, cDNA synthesis, experimental setup, data normalisation and analysis. qRT-PCR has become a common choice for the quantification of gene expression (Freeman, et al., 1999; Ginzinger, 2002) and is also recommended for validation of microarray data as well as other techniques used to study global changes in RNA transcripts.

Although qRT-PCR is widely used for gene expression studies, a reliable normalisation of the qRT-PCR data using a reference gene is critical for an accurate relative quantification of gene expression. Suitable reference genes are constitutively expressed or “housekeeping” genes whose expression remains unaltered under various experimental conditions (Nicot, et al., 2005). The reference gene is used as an internal control to normalize the variation in the cDNA concentration between reactions and the differences in Real Time (RT) efficiency. Given the lack of universal genes with consistent expression, it is necessary to evaluate reference gene expression for an experimental system. Several stable reference genes have been identified under different experimental conditions for many plants including potato (Nicot, et al., 2005).

RNA extraction is the crucial step in gene expression analysis and can affect the efficiency of quantification substantially. Primary concerns related to RNA extraction include inconsistency in RNA yields from various tissues and the purity of extracted RNA. An optimized RNA procedure should provide high quality RNA with minimal degradation and no contamination with proteins or genomic DNA. Many conventional extraction procedures including those that use hot borate buffer, sodium dodecyl sulfate, lithium chloride, and Trizol have been used for RNA extraction from leaves, stems and flowers (Li, Wang, Sun, & Li, 2011). However, RNA extraction from potato tubers can be difficult owing to the high concentration of polyphenols and polysaccharides (Kumar, et al., 2007; Logemann, et al., 1987; Luo, et al., 2011). Hence, optimization of RNA extraction protocols is crucial for transcriptional studies in potato tubers.

Potato tubers (“Summer Delight”) and *Pbr* NZEC1, identified in the last chapter as a suitable cv-strain model were used for the transcriptional experiments in this study. As a prerequisite for expression studies, RNA extraction from potato tubers was optimized and reference genes for internal control of qRT-PCR were established. qRT-PCR was used to quantify the expression of homologues of the genes identified in previous studies in tomato using *P. syringae* to examine CFA dependent differential expression of host defence genes in potato.

4.3 Results

For transcription studies, nine potato tubers (“Summer Delight”) were inoculated with either *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7* as described in Section 2.7.1. As a mock-

inoculated control, three tubers were inoculated with 10 mM MgCl₂. Tissue from the tubers was sampled from around the site of inoculation at 24 hpi. Three non-inoculated tubers were also sampled as negative controls. The samples were immediately frozen using liquid nitrogen and stored at -80°C.

For each treatment, RNA extracted from three tubers were pooled together to represent one biological replicate. Treatments with the *Pbr* NZEC1 and its CFA mutant were represented by three biological replicates with one biological replicate for mock-inoculated and non-inoculated control.

Total RNA extraction was compared from the tuber samples using three different methods as described in Section 2.3 to obtain a sufficient quality and quantity of total RNA for downstream analysis. RNA quality was assessed using spectrophotometric methods, gel electrophoresis, and Agilent 2100 Bioanalyzer as described in Section 2.3.

4.3.1 RNA extraction from potato tubers

A difference in the yield and purity of total RNA extracted from potato tubers was observed depending on the method used for extraction. Total RNA from each potato tuber was first extracted using the RNeasy Plus Mini kit. To confirm the integrity of the RNA and DNA contamination in the samples, each sample was run on a denaturing agarose gel. Total RNA of all samples extracted using the RNeasy Plus Mini kit displayed smearing (Figure 4.1) suggesting degradation of the RNA extracted using this technique. The

A₂₆₀/₂₈₀ ratio in samples extracted using the RNeasy Plus Mini kit ranged from (1.88 to 1.95), which confirms low protein contamination. Finally, the spectrophotometry showed that the yield of total RNA extracted using the RNeasy Plus Mini kit was low (~78.25 ng/μl). Hence, an alternative method for RNA extraction was tested.

To improve quality and yield, total RNA from tubers was extracted using a hot borate method. Potato tuber samples previously frozen using liquid nitrogen were treated with hot borate buffer prior to extraction with the RNeasy Plus Mini kit to effectively reduce the polysaccharide and polyphenol contamination. Total RNA from all samples on a denaturing gel showed multiple fragments (data not shown), which is indicative of significant RNA degradation. The RNA extracted was then tested for protein contamination using NanoDrop. The ratio remained consistent for all the samples (~1.9), suggesting much less protein contamination when compared to RNA samples extracted using RNeasy Plus Mini kit. The average yield from this method of RNA extraction was (93 ng/μl) also higher than the concentration of RNA extracted using an RNeasy Plus Mini kit alone. RNA integrity was further analysed using the Agilent 2100 Bioanalyzer RNA LabChip (Figure 4.2). Unfortunately, the average RNA Integrity Number (RIN) for four samples tested was 4.6, which is indicative of RNA degradation (Table 4.1).

As a result of apparent degradation of the RNA samples using borate treatment, RNA extraction was performed using a LiCl precipitation method. Although this method of RNA extraction is laborious and time consuming the yield of RNA (822 ng/μl) was significantly higher than the amount obtained using the other two methods (Table 4.1). Interestingly, we observed that the LiCl precipitation method showed higher protein

contamination (A260/280 ratio ~1.55 to 1.8). However, protein levels were significantly reduced after a clean-up using the SV RNA extraction kit (Promega). RNA samples were analysed on a denaturation gel (data not shown) which showed distinct 28S and 18S ribosomal RNA bands. The integrity was confirmed using Bioanalyser (Figure 4.2). The average RIN value for eight samples extracted using the LiCl precipitation method was 7.8 (Table 4.1), which suggests very little or no RNA degradation. Hence, total RNA extracted using this protocol was used for downstream transcriptional analysis.

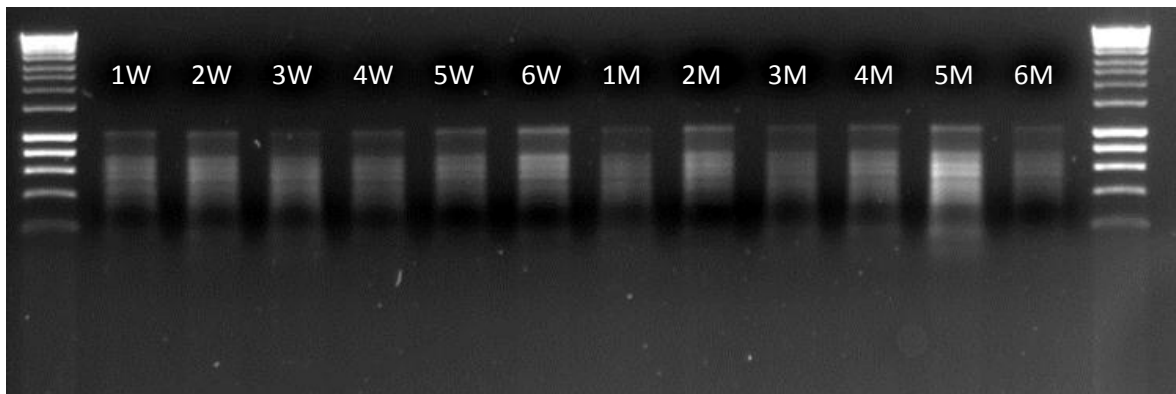


Figure 4.1: Gel electrophoresis image of RNA samples extracted from potato tubers (“Summer Delight”) using the RNeasy Plus Mini kit.

Note: Samples 1W to 6M represent the technical replicates of each pooled sample inoculated with wild-type and mutant strains.

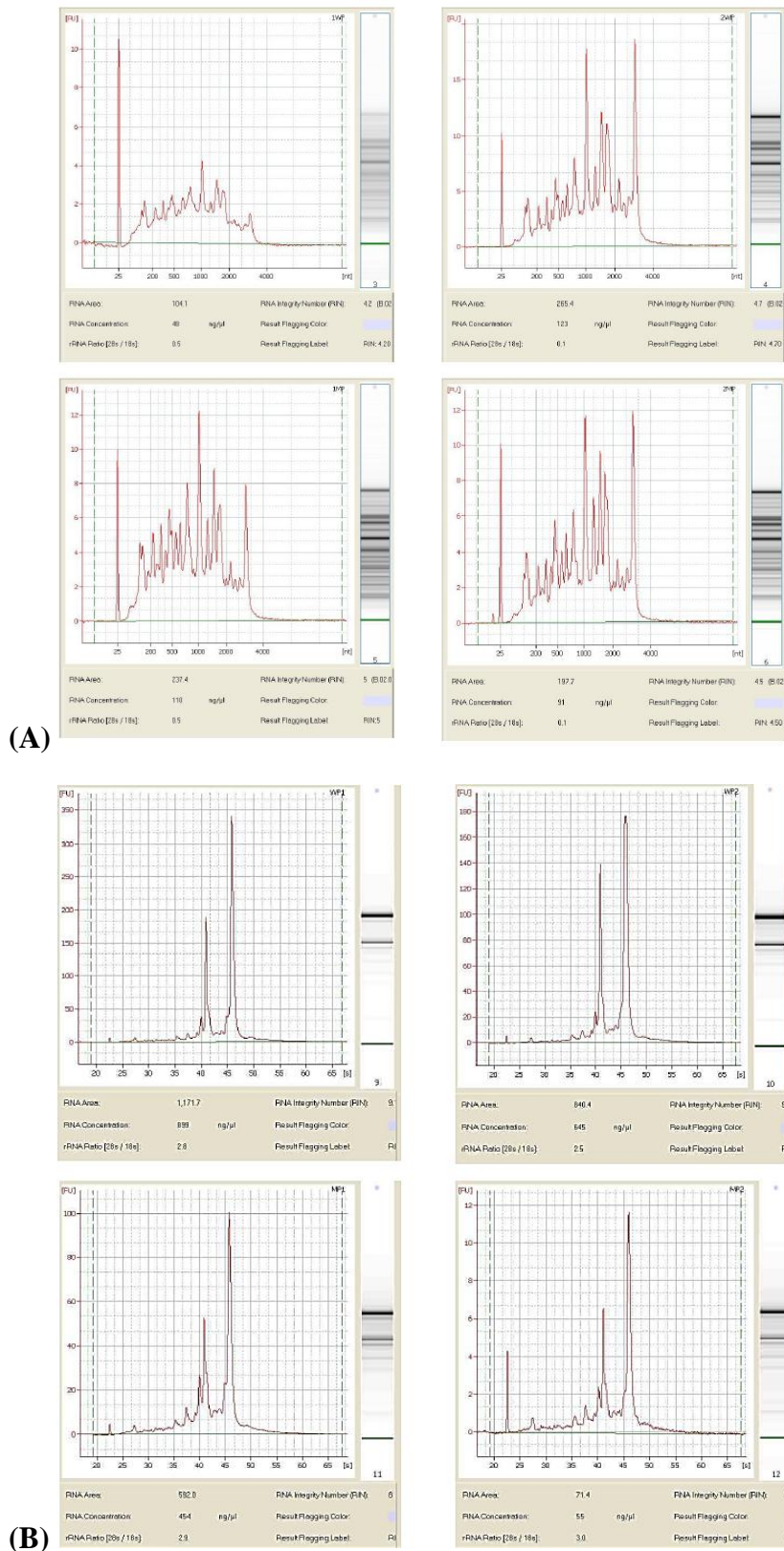


Figure 4.2: Bioanalyzer results of RNA samples extracted using (A) hot borate lysis buffer and, (B) the LiCl precipitation method.

Table 4.1: Total RNA concentration and RIN value for RNA samples extracted using the RNeasy Plus Mini kit, hot Borate lysis buffer and the LiCl extraction buffer.

Note : Samples not tested for RNA integrity are represented by N/A

Sample Name	RNeasy Plus Mini kit		hot Borate buffer		LiCl precipitation	
	RNA Concentration (ng/μl)	RIN value	RNA Concentration (ng/μl)	RIN value	RNA Concentration (ng/μl)	RIN value
1WP	66	N/A	48	4.2	520	8
2WP	70	N/A	123	4.7	640	7.8
3WP	N/A	N/A	N/A	N/A	2052	7.5
1MP	80	N/A	110	5	1720	7.2
2MP	97	N/A	91	4.5	276	7.5
3MP	N/A	N/A	N/A	N/A	232	8.4
IMGP	N/A	N/A	N/A	N/A	580	8.7
1NIP	N/A	N/A	N/A	N/A	556	7.9
Average	78.25	N/A	93	4.6	822	7.8

4.3.2 Selection of an internal control for qRT-PCR

To identify a stable reference gene, transcription of candidate genes previously described as suitable for use in potato (Nicot, et al., 2005) was compared under four conditions using qRT-PCR. The seven genes encoding β tubulin, elongation factor (*efl* α), actin, cyclophin, heat shock protein (*hsp20.2*), adenine phosphor ribosyl transferase (*aprt*) and cytoplasmic ribosomal protein *l2* were tested for their constitutive expression in tubers treated with *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7* as well as mock-inoculated and non-inoculated control tubers. Transcription of all genes was detected using qRT-PCR (Figure 4.3). The

transcription level of each gene varied between treatments. The expression of all genes was detected at 15-20 Ct, except β -tubulin and actin which were detected at 25 Ct. The *hsp20.2* and *actin* gene showed the maximum standard deviation (0.78 and 0.72 respectively) across treatments indicating that transcription of these genes fluctuates most under different conditions. In contrast, *efla* showed the least standard deviation amongst samples (0.09) confirming the results of Nicot *et.al* (2005), which showed *efla* to be the most stable of all candidate internal controls.

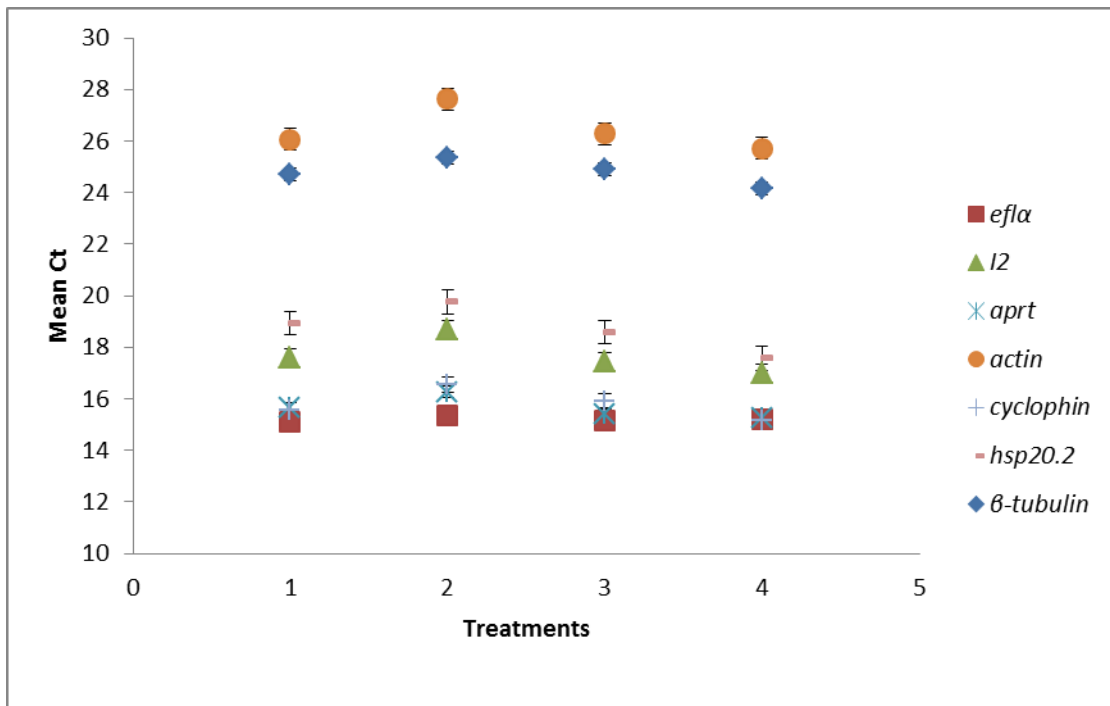


Figure 4.3: Mean Ct values for housekeeping genes in different treatments. Bars show +/- standard deviation. The transcription of *efla* remained consistent in all treatments.

4.3.3 Expression analysis of target genes in potato (“Summer Delight”)

4.3.3.1 Selection of target genes

To identify the influence of CFA on the host defence system upon infection by *Pbr* NZEC1, candidate potato genes were selected based on previous literature on differential expression in tomato and *A. thaliana* to COR (Table 4.2). Homologous genes to lipoxygenase (*lox2*), allene oxide synthase (*aos*), 12-oxo-phytodienoate reductase 3 (*12-opr3*), protease inhibitor (*pi*), poly phenol oxidase (*ppo*) glycerol kinase (*gk*) and isochromate synthase (*ics*) were used as target genes in this study.

Table 4.2: Host defence genes differentially expressed in tomato in response to COR and the homologous potato genes used for qRT-PCR in this study.

Gene Name	Tomato gene ID	Potato gene ID	Response to COR in Tomato and <i>A. thaliana</i>
<i>lox2</i>	AK320013.1	PGSC0003DMG400031856	Up-regulated
<i>aos</i>	AF230371.1	PGSC0003DMG400001149	Up-regulated
<i>12-opr3</i>	AK321579.1	PGSC0003DMG400030890	Up-regulated
<i>pi</i>	NM_001247732	PGSC0003DMG400009268	Up-regulated
<i>ppo</i>	AC232778.1	PGSC0003DMG400022430	Up-regulated
<i>gk</i>	AC235806.1	PGSC0003DMG400014144	Up-regulated
<i>ics</i>	NM_001247865.1	PGSC0003DMG400033038	Constitutive expression

4.3.3.2 Primer optimisation for qRT-PCR

Primer concentrations for qRT-PCR analysis were optimized for each gene by performing reactions with each primer at different concentrations including 100 nM, 200 nM, 300 nM and 400 nM. The optimal annealing temperature for each primer pair was then determined using temperature gradient qRT-PCR. The optimized primer concentrations and annealing temperature for each target sequence are described in Table 2.3.

4.3.3.3 Development of a standard curve for relative quantification of target genes

For relative gene quantification, standard curves were developed to calculate amplification efficiencies and regression values for each qRT-PCR. For this purpose, pPCR1 carrying a single copy of each target gene as well as the *efla* gene was commercially synthesised (Genscript, U.S.A) (Appendix A). A 10-fold serial dilution of pPCR1 starting at 500,000 copies / μ l was used to create a standard curve.

A standard curve was used for each target in each qRT-PCR reaction. Using the standard curve, the efficiency for all qRT-PCR reactions was shown to be ≥ 1.86 and the R^2 was greater than 97.4. The amplification efficiency and regression value for each gene is listed in Table 4.4.

Table 4.3: Amplification efficiency and regression value (R^2) for each target gene.

Gene Name	Efficiency	R2
<i>efla</i>	1.92	99.7
<i>lox</i>	1.97	99.5
<i>aos</i>	1.81	97.1
<i>12-opr</i>	1.86	97.4
<i>pi</i>	1.95	99.2
<i>ppo</i>	1.94	98.9
<i>gk</i>	1.91	98.9
<i>ics</i>	1.93	97.4

4.3.3.4 Transcriptional analysis of target genes

No significant difference ($p < 0.05$) was observed between the back-transformed ratios for any gene in response to *Pbr* NZEC1, *Pbr* NZEC1 Δ *cfa7* and the mock-inoculated control. The ratios for the non-inoculated control were not significantly different from other treatments for any gene either. However, non-significant differential expression ($p > 0.05$) between treatments was observed for several genes.

JA related target genes

Transcription of the *lox2* gene showed no significant difference between the tubers inoculated with wild-type or the mutant treatments. However, there was a non-significant decrease ($p > 0.05$) in the transcription of *lox2* in both the wild-type and mutant-treated samples when compared to the mock-inoculated and non-inoculated controls. The mean

log₁₀ ratio (normalized to *eflα*) for *lox2* gene expression in the tubers treated with *Pbr* NZEC1 and *Pbr* NZEC1Δ*cfa7* was 5.94x10⁻³ and 8.82x10⁻³, respectively, was lower than the mock-inoculated and non-inoculated controls (1.65x10⁻² and 1.70x10⁻², respectively) (Figure 4.3).

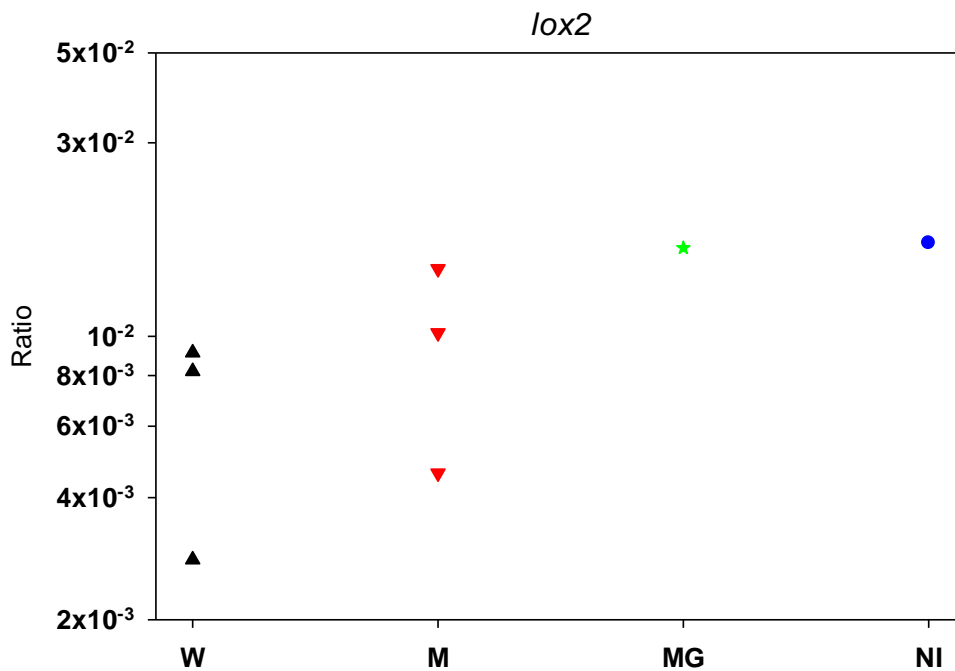


Figure 4.4: Ratio of target gene expression for *lox2* normalised to *eflα* in potato tubers inoculated with either *Pbr* NZEC1 (W) or *Pbr* NZEC1Δ*cfa7* (M), or mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 0.61 for *lox2*.

Transcription of *aos* remained similar in tubers treated with *Pbr* NZEC1 and the CFA7 mutant. However, relative expression of *aos* was non-significantly higher ($p > 0.05$) in wild-type and the mutant-treated and mock-inoculated control when compared with the non-inoculated control. The mean log₁₀ ratio for *aos* gene expression in *Pbr* NZEC1, *Pbr*

NZEC1 Δ *cfa7* and mock-inoculated control was 1.26×10^{-1} , 1.30×10^{-1} and 2.09×10^{-1} respectively, whereas the ratio for non-inoculated control was 8.00×10^{-2} (Figure 4.5).

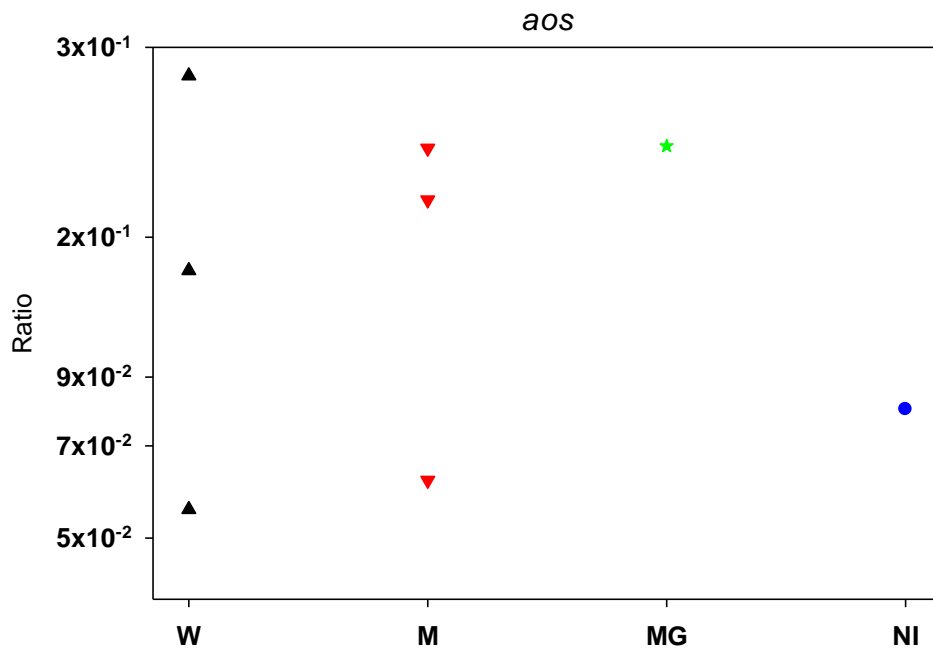


Figure 4.5: Ratio of target gene expression for *aos* normalised to *efla* in potato tubers inoculated with either *Pbr* NZEC1 (W) or *Pbr* NZEC1 Δ *cfa7* (M), or mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 0.71 for *aos*.

The mean log₁₀ ratio in *I2-opr* for the wild-type, mutant-treated and mock-inoculated samples were 1.93×10^{-1} , 3.30×10^{-1} and 3.71×10^{-1} . No significant difference was observed in the mean log₁₀ ratio of *I2-opr3* in these treatments. However, the transcription of *I2-opr3* was significantly ($p < 0.05$) repressed in wild-type treated samples when compared to the non-inoculated control (7.36×10^{-1}) (Figure 4.6).

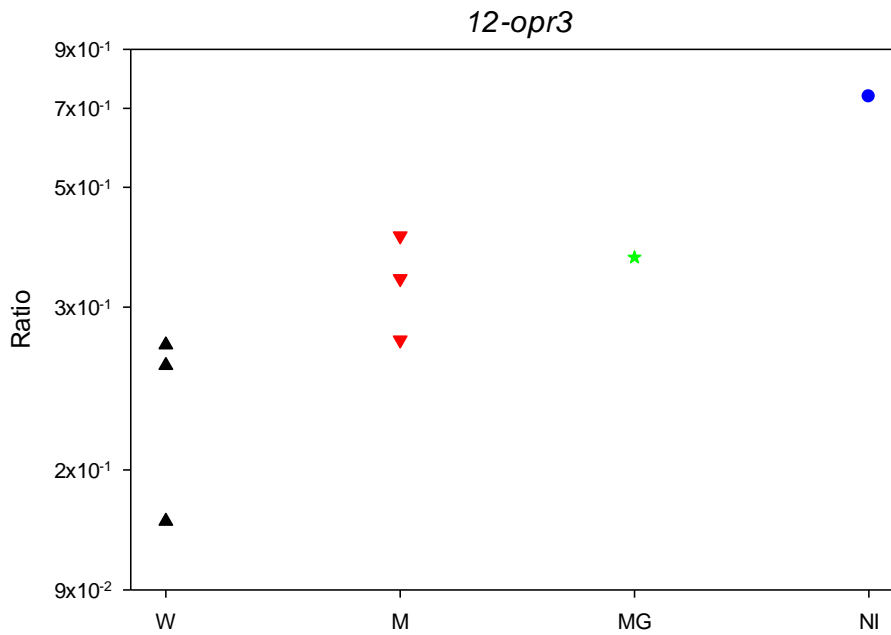


Figure 4.6: Ratio of target gene expression for *12-opr3* normalised to *eflα* in potato tubers inoculated with either *Pbr* NZEC1 (W) or *Pbr* NZEC1 Δ *cfa7* (M), or mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 0.33 for *12 opr3*.

Wound related target genes

Relative expression of *pi* showed no significant difference between the wild-type and mutant-treated samples. However, non-significant differential expression ($p > 0.05$) was observed between both mock-inoculated and non-inoculated controls and the pathogen-treated samples. The mean log₁₀ ratio for wild-type (1.99×10^{-2}) and mutant (2.45×10^{-2}) treated samples was lower than the mock-inoculated (1.08×10^{-1}) and non-inoculated samples (8.59×10^{-2}) (Figure 4.7).

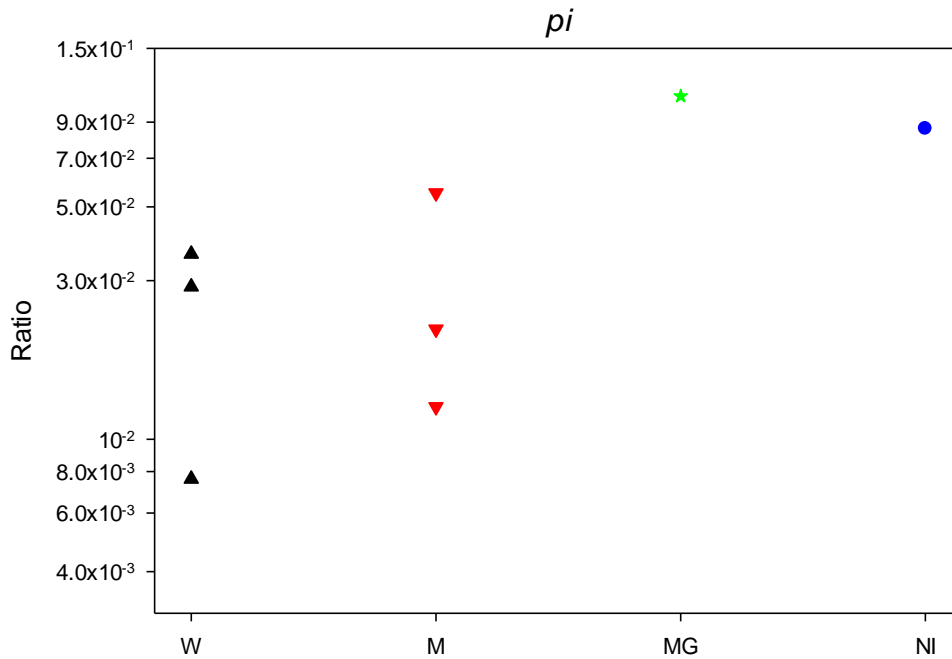


Figure 4.7: Ratio of target gene expression for *pi* normalised to *efla* in potato tubers inoculated with *Pbr* NZEC1 (W) *Pbr* NZEC1 Δ *cfa7* (M), or mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 0.78 for *pi*.

The relative transcription of *ppo* in *Pbr* NZEC1 Δ *cfa7* was lower than the expression in *Pbr* NZEC1, mock-inoculated sample and non-inoculated control. Mean log₁₀ fold ratios for CFA mutant and *Pbr* NZEC1 wild-type samples were 5.87×10^{-3} and 1.35×10^{-2} respectively (Figure 4.7). However, no difference in transcription was observed between mock-inoculated (3.19×10^{-2}) and non-inoculated control (2.08×10^{-2}).

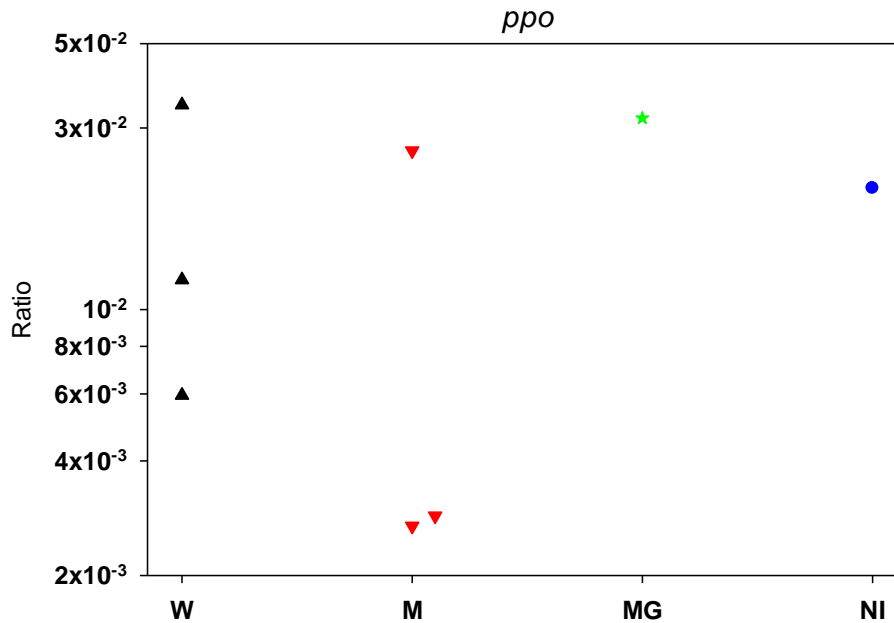


Figure 4.8: Ratio of target gene expression for *ppo* normalised to *eflα* in potato tubers inoculated with *Pbr* NZEC1 (W) *Pbr* NZEC1Δ*cfa7* (M), or mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 1.09 for *ppo*.

SA related target genes

To monitor the effect on the SA pathways during infection with *Pectobacterium*, transcription of *ics* and *gk* was measured in RNA samples. Gene expression of *ics* showed no significant difference between the wild-type and mutant-treated samples. However, the relative transcripts in the pathogen treated samples were lower compared to the mock-inoculated and non-inoculated controls ($p > 0.05$). The mean log₁₀ fold ratio for wild-type (7.78×10^{-3}) and mutant (6.36×10^{-3}) were lower than the mock-inoculated (1.12×10^{-2}) and non-inoculated control (1.24×10^{-2}) (Figure 4.9).

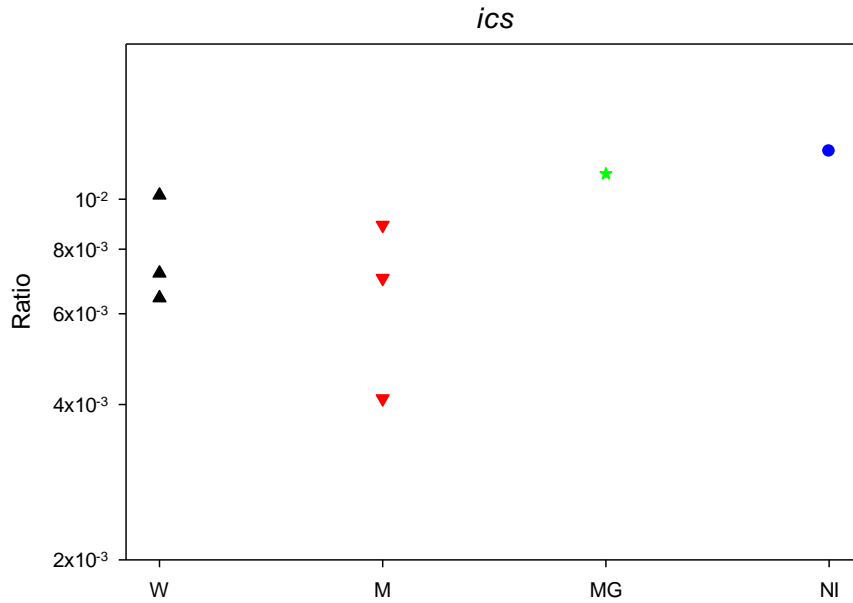


Figure 4.9: Ratio of gene expression for *ics* normalised to *eflα* in potato tubers inoculated with *Pbr* NZEC1 (W) *Pbr* NZEC1Δ*cfa7* (M), or in mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 0.32 for *ics*.

The relative expression of *gk* showed no significant difference between pathogen-treated mock-inoculated and non-inoculated control samples. The mean log₁₀ fold ratio for wild-type, and the mutant-treated was 5.41×10^{-2} , 3.36×10^{-2} respectively, which showed no significant difference when compared to the non-inoculated control (2.94×10^{-2}) (Figure 4.10). The transcription of *gk* was non-significantly induced ($p > 0.05$) in response to the mock-inoculated control (6.99×10^{-2}) when compared to the non-inoculated control.

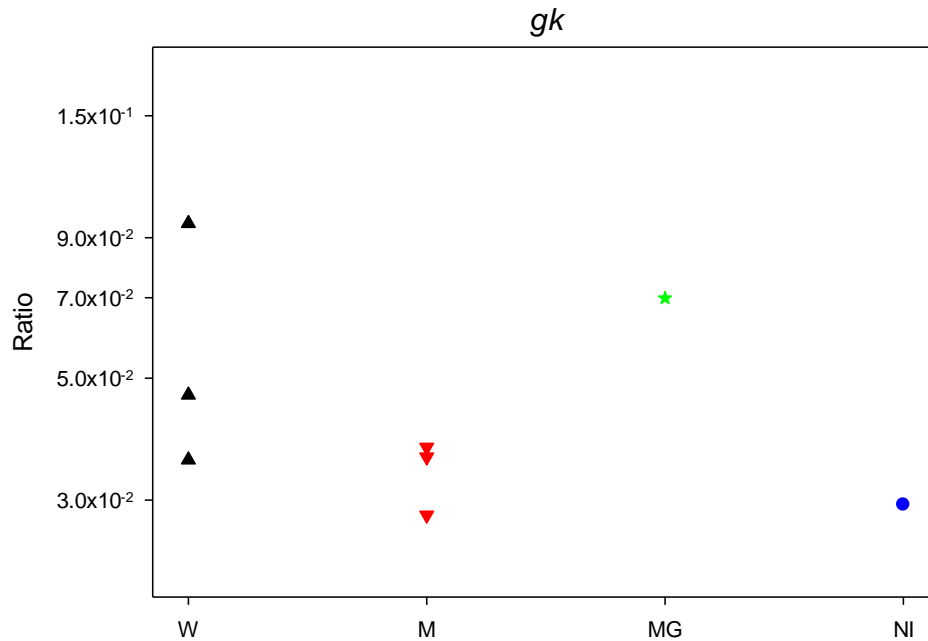


Figure 4.10: Ratio of gene expression for *gk* normalised to *efl α* in potato tubers potato inoculated with *Pbr* NZEC1 (W) *Pbr* NZEC1 Δ *cfa7* (M), or mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 0.37 for *gk*.

4.4 Discussion

Differences were observed in the quantity and quality of RNA obtained using the three extraction protocols compared in this study. The hot borate buffer extraction technique has previously been recommended for RNA extraction from tubers (Luo, et al., 2011), but failed to produce the quality and quantity of RNA necessary for downstream application in this study. Similar results were observed from RNA extraction using the RNeasy Plus Mini kit, which showed extensive denaturing of RNA. However, the lithium chloride precipitation method proved most effective for extracting RNA from potato tubers.

Agarose gel electrophoresis of RNA produced using this technique showed distinct 28S and 18S ribosomal RNA bands, the A260/280 ratio ranged between 1.8 and 2.0, which is optimal for RNA extraction and the RIN values obtained from Bioanalyser were greater than 7 (Figure 4.2 and Table 4.1). A value of above 7 is commonly considered to represent RNA with high integrity. Having confirmed the quality of RNA extracted using this technique was of a suitable standard for downstream analysis, this RNA was used for further transcriptional studies. The lithium chloride-based RNA precipitation protocol has been reported as an effective protocol to extract RNA from various sources of plant material rich in polysaccharides and phenols including grapevine (Tattersall, et al., 2005), lily bulb (X. Li, et al., 2011), and tomato (H. M. Wang, et al., 2009). Lithium chloride based techniques supersede other techniques described previously for extraction of total RNA from potato tubers.

Accurate quantification of transcripts using relative qRT-PCR requires the use of an appropriate internal control and specific PCR conditions. A suitable internal control shows minimal changes in expression in comparison to the gene of interest, which may show significant differential expression over the period of an experiment (Dean, et al., 2002). In this study, the genes tested by Nicot *et al.*, (2005) were tested for their expression levels under the experimental conditions used in this study. Small differences in expression levels for some genes were observed between treatments. The *actin* and *hsp 20.2* gene showed the greatest variability. In contrast, expression of *efla* gene was consistent in all treatments. The stability of *efla* in these experiments was consistent with the previous study by Nicot *et al.*, 2005, in which *efla* was the most stable of all candidate housekeeping genes in potato. Furthermore, *efla* has been proven to be an effective internal control for use with

other plants including *Nicotiana benthamiana* (Dean, et al., 2002) and *Brassica juncea* (Chandna, et al., 2012). As a result, *efla* was used as an internal control for transcriptional studies in potato in this study.

Transcription of genes related to the JA pathway and the SA pathway were studied in response to infection of potato with *Pbr* NZEC1, which carries the CFA gene cluster as well as a mutant in which CFA had been inactivated. Interestingly, expression patterns for these genes were not consistent with those previously observed in tomato upon infection with a *P. syringae* strain producing COR. Expression studies in tomato in response to *P. syringae* strains producing COR identified COR dependent induction of jasmonate responsive genes (Brooks, et al., 2005a; Uppalapati, et al., 2005; Zhao, et al., 2003). Expression of *lox2*, a JA biosynthesis gene was induced within 24 h of infection with the COR producing *P. syringae* strain. However, *lox2* expression in potato showed no significant difference in transcription when exposed to wild-type or the CFA mutant. The lack of differential transcription of *lox2* in potato could be because of different isoforms being active in different organs in this host. Previous studies have characterized three isoforms of *lox* in potato (Royo, et al., 1996). *lox1* is expressed in roots and tubers, *lox2* in leaves and *lox3* in leaves and roots. We originally planned to study the interaction in stem which may have shown induction of *lox2*. However, our studies in tubers validate the lack of expression of *lox2* in tubers and suggest it is not active against *Pectobacterium*. Recent access to genome sequence for “Summer Delight” has revealed multiple genes annotated as *lox* in potato. In potato tubers, the *lox1* gene is involved in the production of the large amounts of 9-hydroperoxides (and a lower amount of 13-hydroperoxides) from linolenic and linoleic acid. This contrasts to the situation in leaves, where *lox2* and *lox3* have higher

affinity towards 13-hydroperoxides (Lulai, et al., 2011; Royo, et al., 1996). Studying transcription of the other isoforms may identify those expressed in tubers and that respond to infection by *Pectobacterium*, providing a better understanding of whether the JA pathway plays a role in defence in tubers.

Transcriptional profiling in tomato also identified a significant up-regulation of other JA biosynthesis genes including *aos* and *12-opr3* (Uppalapati, et al., 2005). However, similar results were not observed in our study on potato. Though the transcription of *aos* varied between the pathogen treated samples and the non-inoculated control, no significant fold change was detected. In contrast, *12-opr3* transcription was suppressed by 3-fold in wild-type treated tubers when compared with the non-inoculated control. However, no significant difference was observed between mutant treatment and the controls. To date, only one isoform of *12-opr3* has been identified in potato, which suggests that unlike in tomato *12-opr3* is not induced during infection of potato by *Pectobacterium*. Similar to *lox*, organ specificity of *aos* isomers have been observed in potato (Stumpe, et al., 2006). To date three isomers *aos1*, *aos2* and *aos3* have been identified in potato, of which expression of *aos3* is specific to tubers and stolons. Furthermore, *aos3* is distinguished from its counterparts by high substrate specificity to 9-hydroperoxides of linolenic acid when compared to 13-hydroperoxides (Stumpe, et al., 2006) (Figure 4.11). However, 9-hydroperoxide is not involved in the synthesis of JA. This suggests that as an alternative to JA other oxylipin products might be involved in defence signalling in potato tubers. Previous studies to identify the pattern and dynamics of oxylipins in potato leaves have identified increased expression of colnelenic acid (divinyl ester) during infection with *Phytophthora infestans*, thus rendering resistance to late-blight disease (Weber, et al.,

1999). However, oxylipins produced in tubers and their function in defence still remains unknown.

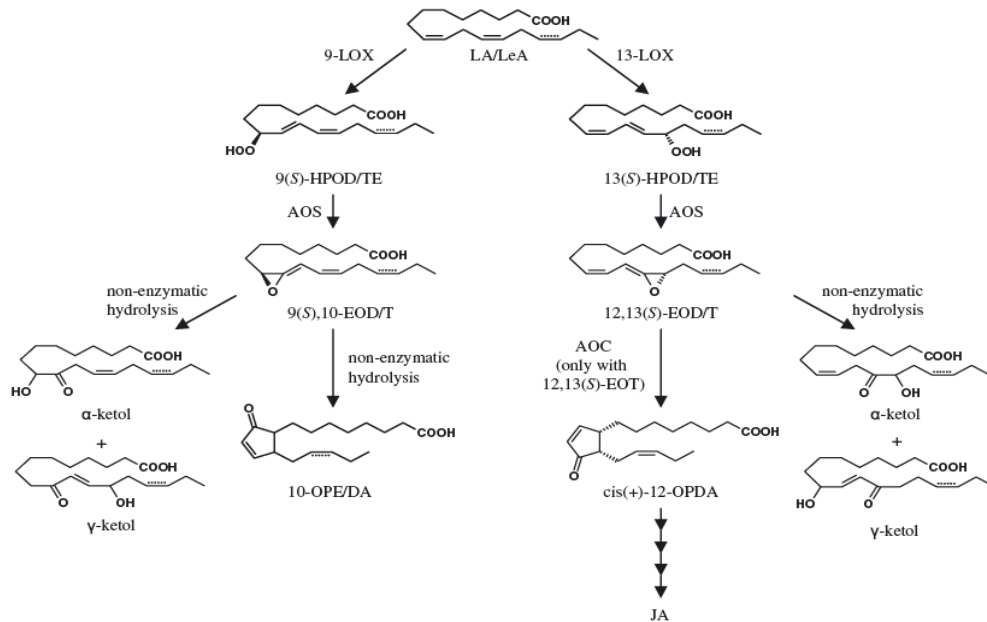


Figure 4.11: JA biosynthesis in plants (Stumpe, et al., 2006).

Studies to identify COR dependent gene responses have also shown the induction of JA and wound responsive genes encoding PI and PPO (Zhao, et al., 2003). However, no significant difference in expression was observed for these genes in response to *Pbr* NZEC1 or the CFA7 mutant in potato. To date, 15 isomers of *pi* and nine isomers of *ppo* have been identified in potato (Potato Genome Sequencing Consortium, 2011). Isomers active in tubers are not known and thus the absence of induction may be due to studying an isoform that is not induced in response to *Pectobacterium* in tubers.

Previous studies have shown COR activates the JA pathway which in turn results in suppression of the SA defence pathway that limits the virulence of *P. syringae* (Brooks, et al., 2005a). Therefore, the transcription of *ics* and *gk* were studied in potato to identify the role of CFA in manipulating SA-mediated host defence to *Pectobacterium*. Although COR manipulates SA-mediated host defence in tomato, the *ics* gene responsible for SA biosynthesis was constitutively expressed in all treatments. In contrast, the expression of *gk* was induced 24 hpi with *P. syringae* and its COR mutants (Brooks, et al., 2005a). No significant difference was observed in the transcription of *ics* or *gk* in response to *Pbr* NZEC1 or CFA mutant. The lack of expression of *ics* and *gk* is possibly due to the necrotrophic lifestyle of *Pectobacterium*. This is consistent with previous studies which have confirmed SA dependent host defence against hemibiotrophic and biotrophic pathogens (Kunkel & Brooks, 2002; C. Pieterse, et al., 2006; C. M. J. Pieterse & van Loon, 1999).

Several explanations exist for our inability to detect the effect of CFA on the expression of the defence related genes investigated in this study. It is important to note that our experiments were designed to study differential expression in both stems and tubers. Due to a lack of blackleg symptoms in stems and the inability to identify a suitable cv the transcriptional profiling was conducted only in tubers. Thus, the lack of similar expression patterns for these defence genes in potato is possibly due to factors such as variable isoforms for the same genes and organ specificity of the isoforms. However, it is also possible that CFA might target a different host pathway in potato tubers. The catalytic activity of *lox* and substrate specificity of *aos* in tubers to produce 9-hydroperoxides suggest the involvement of other oxylipins such as colnelenic acid might act as inducers of

different signalling pathways (Weber, et al., 1999). Further studies are required to dissect the JA biosynthesis pathway in tubers.

4.5 Conclusion

The qRT-PCR results indicate that the genes investigated in this study may not be appropriate markers to examine pathogen defence in potato tubers. Therefore, to gain a better understanding of host defence systems in potato tubers and their response to CFA, global transcription profiling using RNA-seq might be a more useful approach; this technique assists in the differentiation of multiple gene isoforms and their expression.

Chapter 5

Transcriptional response of Potato to *Pectobacterium* encoding CFA

5.1 Summary

RNA sequencing was used to conduct global transcriptional profiling on potato (“Summer Delight”) during infection with *Pbr* NZEC1 or the CFA mutant. The results from the RNA-seq were validated using qRT-PCR. It was identified that infection with *Pbr* NZEC1 induced the differential expression of multiple defence pathways including ET biosynthesis and signalling, auxin, abscisic acid and other transcription factors involved in defence response. This has provided insight into the underlying host defence mechanisms involved during potato-*Pectobacterium* interactions.

5.2 Background

The transcriptome is the complete set of transcripts in a cell. The main aim of transcriptomics is to categorise all species of RNA including the mRNA, non-coding RNA and small RNAs to determine the structure of each gene and to quantify the changing expression level of its transcript under different conditions (Tang, et al., 2009).

Both hybridization and sequence based techniques have been developed to quantify the transcriptome. Hybridization approaches such as ‘Microarrays’ provide high throughput and are relatively inexpensive, however the high background levels owing to cross hybridization, limited dynamic range of detection and their reliance on existing knowledge of the genome being studied limit the use of this technique (Barrett & Kawasaki, 2003). In contrast to the use of microarrays, the sequence-based approach directly determines the cDNA sequence in a sample. However, using Sanger sequencing to sequence cDNA libraries provides low throughput and is very expensive. The high costs usually prevent analysing more than a portion of the transcripts and as a consequence the analysis is not quantitative and the isoforms may not be detected. These disadvantages limit the use of microarray and traditional sequencing technology in quantitative analyses of transcriptomes (Z. Wang, et al., 2009).

The recent development of high-throughput nucleotide sequencing provides millions of reads per single sequencing run. This can be applied to transcriptome sequencing (RNA-seq) (Morozova & Marra, 2008). The abundance of sequence obtained using this technique provides several advantages including the ability to detect novel transcripts and transcript isoforms, the ability to map transcripts to the genome and quantification of transcripts. Unlike microarrays, RNA-seq does not limit detection of transcripts to those that correspond to the genome sequence, providing an alternative approach for transcriptomics of non-model organisms that are yet to have their genomes sequenced (Grabherr, et al., 2011). Furthermore, RNA-seq does not have any upper limits and hence it has a large dynamic range over which transcripts can be detected (Z. Wang, et al., 2009). As a consequence, RNA sequencing technology has all but replaced microarrays and traditional

sequencing techniques and is in the process of revolutionizing transcriptome analyses (Z. Wang, et al., 2009).

The basic principle of RNA-seq is that the frequency with which a certain RNA molecule in a given RNA mixture is sequenced is proportional to the number of copies of this RNA molecule in the mixture. The workflow of RNA sequencing involves converting the total population of RNA within a sample into a cDNA fragment library with adapters. Each library is then sequenced to obtain raw reads. The reads may be single end or paired end based on the sequencing platform and approach. Depending on the sequencing technology used, the reads may vary from 30-400 nucleotides in length. These raw reads can be aligned to a reference genome, a set of reference transcripts or assembled into a set of transcripts to generate a genome scale transcription map. This provides both a transcriptional structure and expression level for each gene (Z. Wang, et al., 2009).

Given the theoretical advantages of RNA-seq, this technology was used to conduct a pilot study to examine the response of potato to *Pectobacterium* infection. The main aim was to provide evidence of host defence systems involved in response to infection by *Pectobacterium* in potato. A comparison of *Pbr* NZEC1 and its CFA mutant was also performed to identify the role of CFA in manipulating host defence gene expression likely to render the plant susceptible to soft rot.

Transcriptional changes were evaluated in the potato tuber ("Summer Delight") 24 hpi with *Pbr* NZEC1 or the respective CFA mutant. For this purpose, RNA samples were

extracted. The cDNA synthesis, sequencing libraries and actual sequencing was performed by Axseq technologies as described in Section 2.8. Primary data generation from the RNA-seq results was performed primarily by Dr Mark Fiers, The New Zealand Institute for Plant and Food Research.

5.3 Results

5.3.1 Processing RNA seq data

The general work flow of RNA seq analysis is illustrated below (Figure 5.1). The raw data from Illumina was provided in a FASTQ format, which was subsequently subjected to a quality check and trimming as described in (Section 2.8). After trimming, an average of ~46 million reads with a read length of at least 86 nucleotides was obtained from each treatment. Using the trimmed sequences, differentially expressed genes were identified from the Cuffdiff analysis (Section 2.8) and results were tabulated. An example of the differences in gene expression patterns for different treatments is shown in the RNA-seq plot for *Pbr* NZEC1 and the CFA mutant. By comparing the different treatments we were able to identify genes significantly differentially expressed in response to wild-type and the CFA mutant (Figure 5.2).

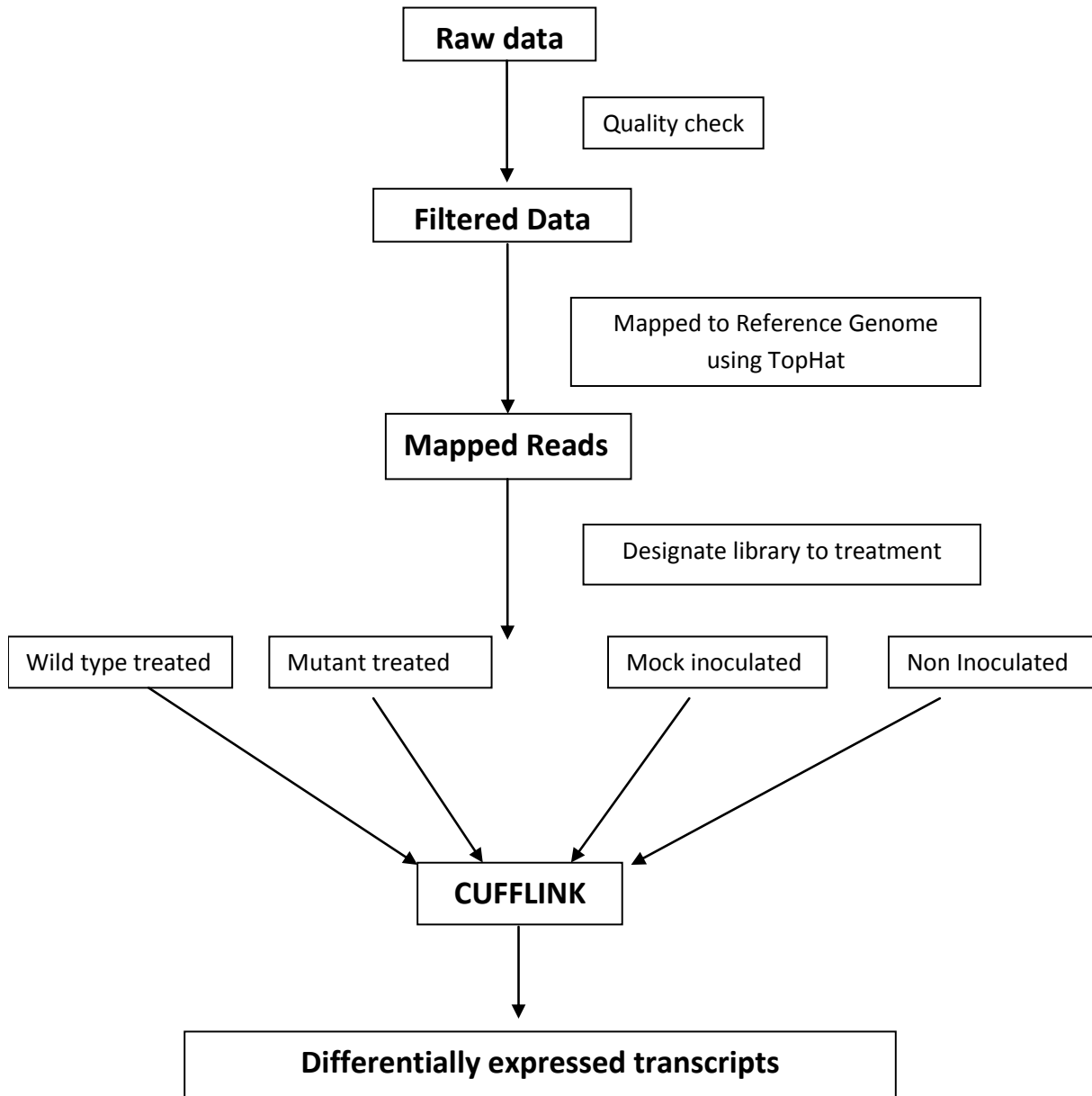


Figure 5.1: Workflow for RNA seq data analysis.

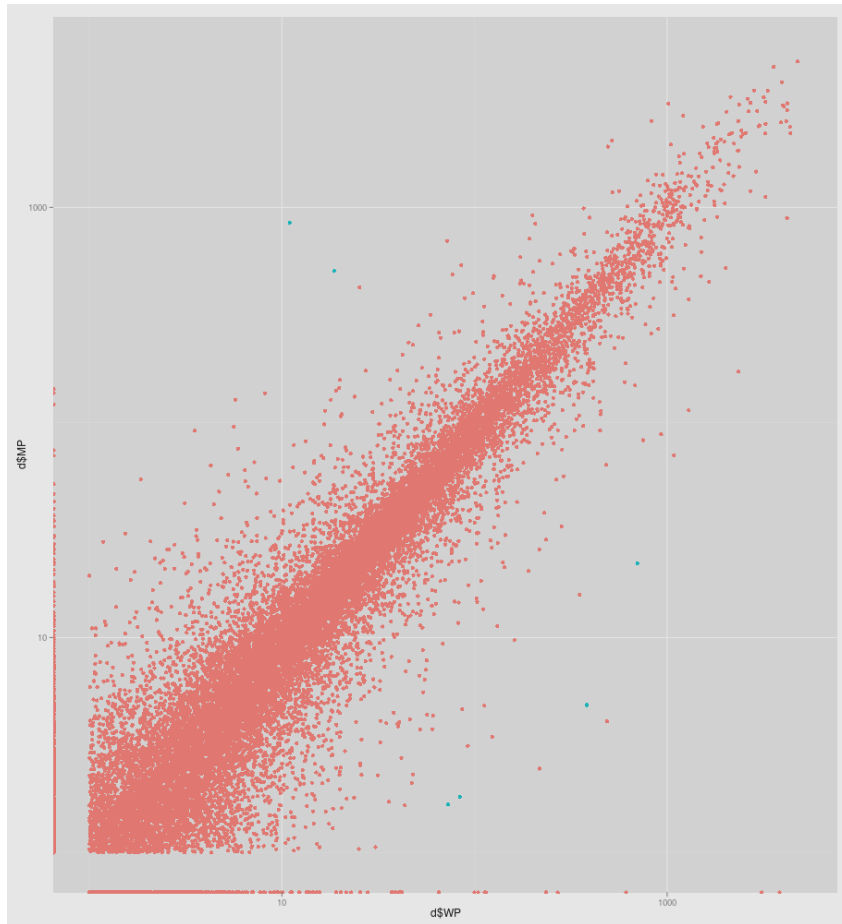


Figure 5.2: RNA-seq plot showing FPKM values for potato 24 hpi with wild-type (ds WP) or CFA mutant (ds MP). The genes shown in this plot represent those identified by Cuffdif analysis. Genes showing non-significant differential expression are plotted in red whereas genes showing significant differential expression ($p \leq 0.05$) are plotted in blue.

5.3.1.1 Differential expression in potato using RNA-seq

Differential gene expression in potato tubers treated with *Pbr* NZEC1 and *Pbr* NZEC1 Δ *cfa7* or in mock-inoculated and non-inoculated control tubers was compared using a Venn diagram (Figure 5.3). The fold change between genes expressed in the non-inoculated control and the wild-type treated, mutant-treated and mock-inoculated sample were calculated and a complete list of genes differentially expressed in these treatments is presented in Appendix B.

When log 2-fold differential expression relative to the non-inoculated control was used as a threshold ($p \leq 0.05$), a total of 1275, 590 and 263 genes were differentially expressed in potato in response to inoculation with either wild-type, the CFA mutant, or in the mock-inoculated control, respectively (Figure 5.3). Only 50 of these differentially expressed genes were identified as common between the three treatments. A total of 321 genes were differentially expressed both in response to *Pbr* NZEC1 and the CFA mutant, whereas 33 and 50 genes were co-induced or repressed in response to both wound and wild-type or wound and mutant, respectively. In contrast, 871, 169, and 130 genes were identified as differentially expressed only in response to *Pbr* NZEC1, *Pbr* NZEC1 Δ *cfa7* or mock-inoculation, respectively. As a response to *Pbr* NZEC1, 599 genes were induced and 272 genes were down-regulated, whereas 107 genes were up-regulated and 62 genes were down-regulated in potato tubers in response to the CFA mutant.

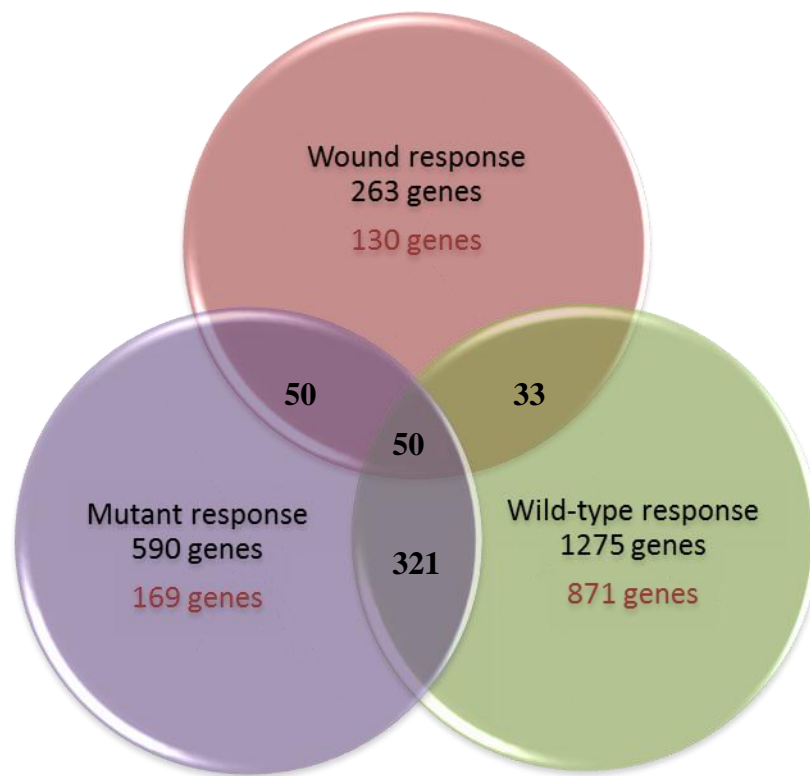


Figure 5.3: Numeric representation of differentially expressed genes in potato tubers in response to *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7* and in mock-inoculated control using Venn diagram. The number of genes in red represents the number of genes expressed only in response to particular treatment.

5.3.1.2 Gene Ontologies and visualization using Mapman

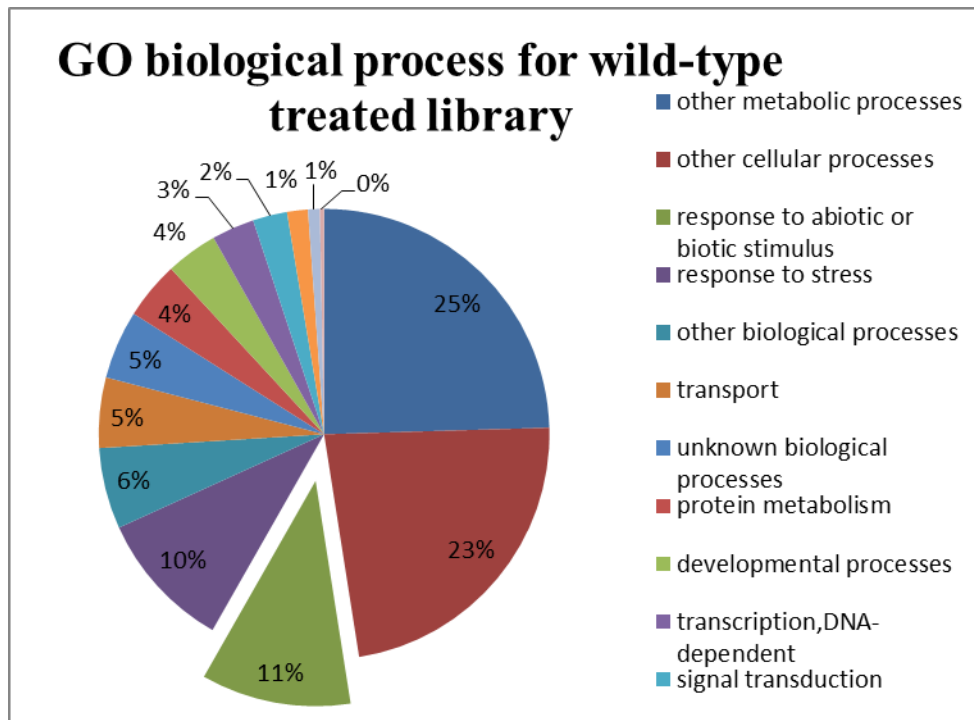
Differentially expressed genes were divided into gene categories to establish whether specific functional categories were over represented in each treatment. Gene Ontology (GO) categories were used for this purpose. Since the GO for the genes in the potato genome was yet to be assigned, GO categories for the model plant *A. thaliana* were used (<http://www.arabidopsis.org/tools/bulk/go/index.jsp>). The *A. thaliana* gene ids of differentially transcribed genes were identified using custom blast (NCBI Blast 2.2.8); the

best blast hit with an E-value cut-off of 1E-10 was used as a threshold. GO provides ontology of defined terms representing gene products, dividing them into three main categories: cellular component (part of cell or its extracellular environment), molecular function (activities of gene at molecular level such as binding or catalysis) and biological process (set of molecular events with a defined beginning and end). Approximately 40% of genes expressed only in response to *Pbr* NZEC1, *Pbr* NZEC1 Δ *cfa7* or mock-inoculated control were involved in biological processes. Of these, 11% of the genes induced or repressed in response to *Pbr* NZEC1 were predicted to respond to biotic and abiotic stress whereas only 8% of genes differentially expressed in response to *Pbr* NZEC1 Δ *cfa7* or the mock-inoculated control were thought to be associated with biotic and abiotic stress (Figure 5.4). The remaining 60% of genes were not assigned to biological processes, which could either be due to the lack of close homology of potato to *A. thaliana* or because the *A. thaliana* homology was not assigned to the biological process category.

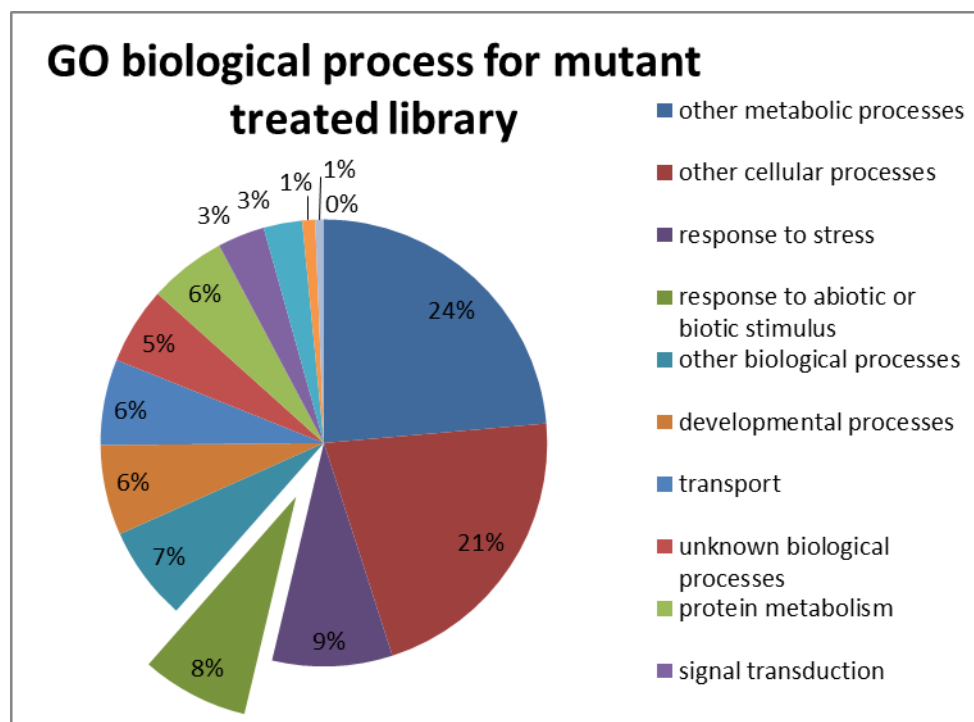
In addition to categorizing the differentially expressed genes using GO, genes were also visualised in Mapman. Mapman is a visualization tool that displays large RNA data sets in diagrams, dividing genes into categories such as of ‘biotic and abiotic stress’, ‘primary and secondary metabolic pathway’ and other processes. Hence, to visualize the differentially expressed genes in all treatments Potato GO mapping was used (<http://mapman.gabipd.org/web/guest/mapmanstore>). Consistent with the gene ontology results, genes involved in biotic stress were differentially expressed in response to *Pbr* NZEC1 and the CFA mutant (Figure 5.5). Genes involved in ET biosynthesis were regulated in response to both *Pbr* NZEC1 and *Pbr* NZEC1 Δ *cfa7*. However, the relative levels of expression varied between treatments. Furthermore, inoculation with *Pbr* NZEC1

resulted in differential expression of genes in different pathways including ET signalling, auxin, abscisic acid synthesis, cell wall degradation, proteolysis and secondary metabolite synthesis as well as transcription factors (Figure 5.5). No significant regulation of the defence pathways was observed in response to the mock-inoculated control (Figure 5.5).

(A)



(B)



(C)

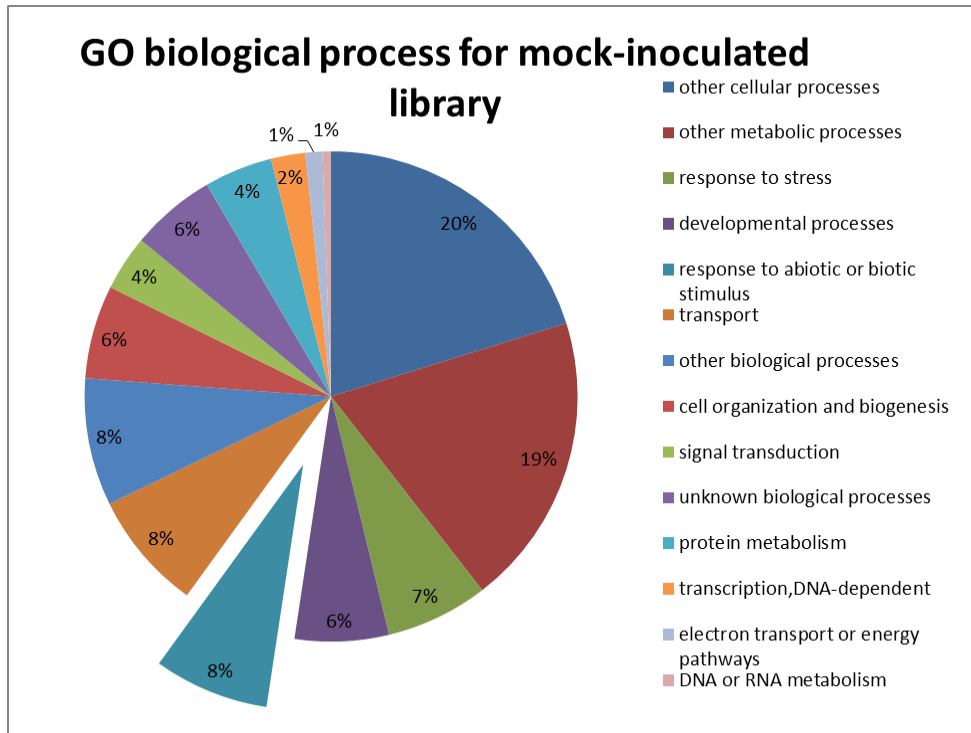
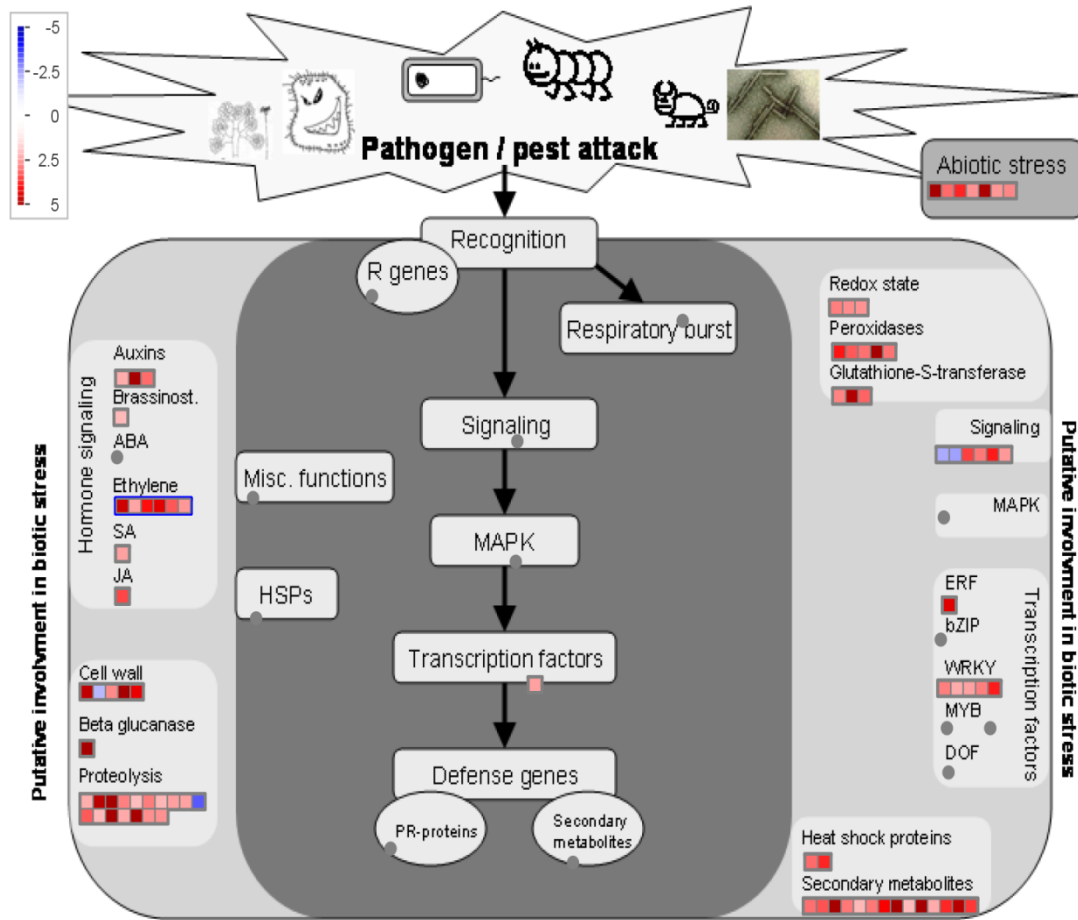
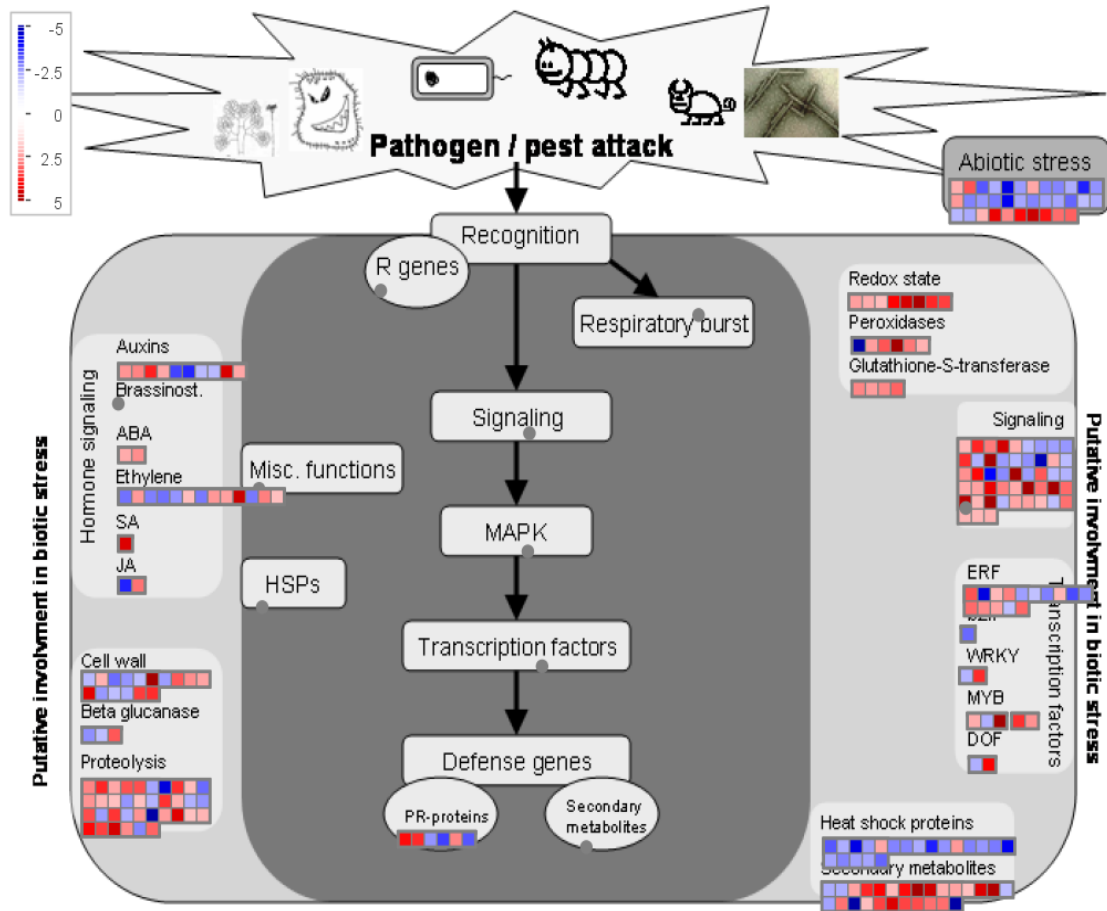


Figure 5.4: Functional categorization (GO) for genes predicted to be involved in biological processes in response to A) *Pbr* NZEC1, B) *Pbr* NZEC1 Δ *cfa7* or C) mock-inoculation.

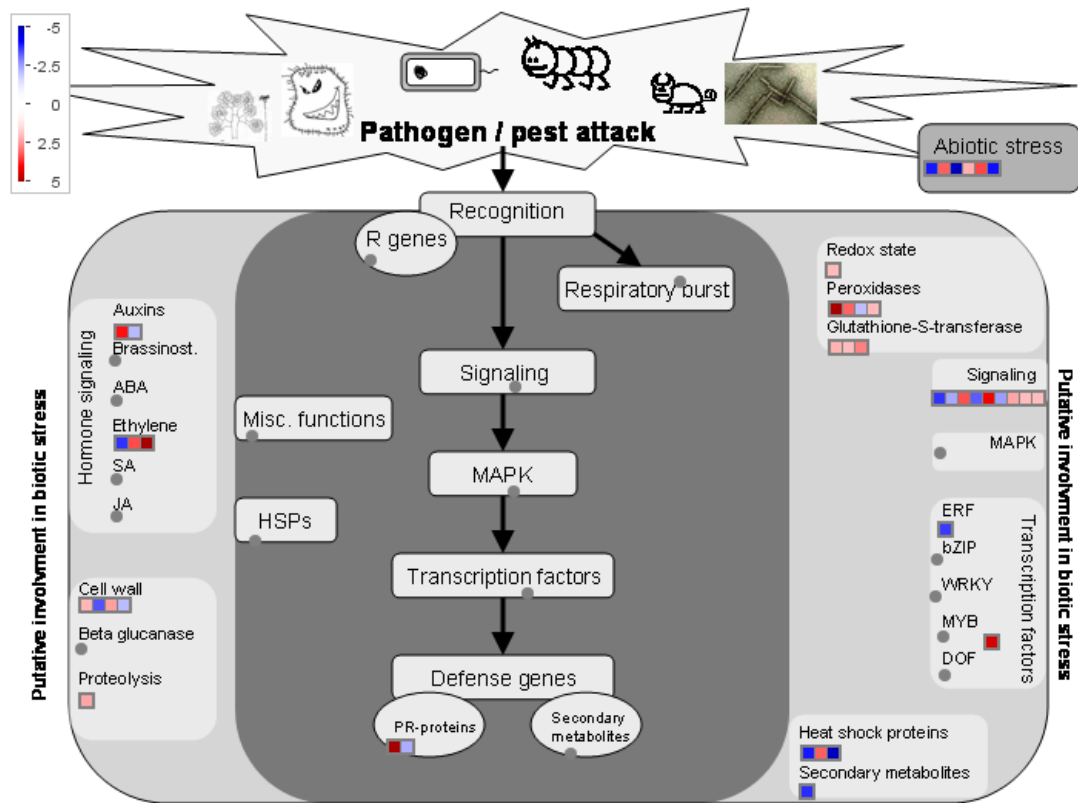
A)



B)



C)



D)

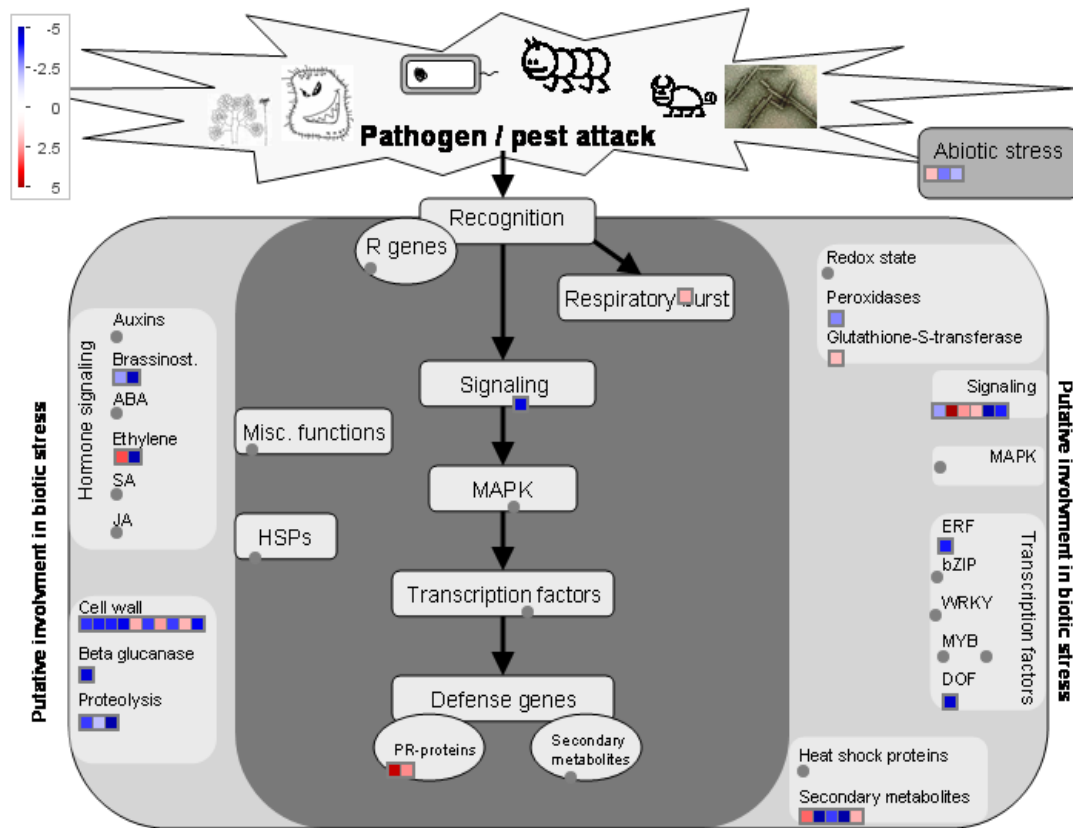


Figure 5.5: Differentially expressed genes as visualized in Mapman using GO mapping for potato. Genes expressed in response to A) both wild-type and CFA mutant, B) wild-type only, C) only mutant or D) mock-inoculated only.

5.3.2 Transcriptional profiling of potato tubers (“Summer Delight”) treated with *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7* and in mock-inoculated tubers

The response of potato to wild-type *Pectobacterium* and to the CFA mutant was diverse and not all the gene categories are covered in this thesis. Only the effect of *Pectobacterium* and the CFA mutant on biological processes related to host defence and pathogenicity are discussed in this chapter. To understand the response of host defence pathways, differentially expressed genes in response to wild-type and the CFA mutant were classified

into functional groups related to ET signalling, JA/wound responsive and other transcription factors (which include disease responsive factors).

Table 5.1: List of selected genes significantly induced (>log-2 fold) or significantly repressed (>log-2 fold) in potato tubers 24 hpi with *Pbr* NZEC1 or *Pbr* NZEC1 Δ *cfa7* as well as in mock-inoculated tubers.

Note: Non-inoculated control was used to normalize differentially expressed genes. Non-significant interactions are provided in brackets. When value is not present the data did not qualify the quality control.

Gene Name	Locus	Log 2-fold change		
		<i>Pbr</i> NZEC1	<i>Pbr</i> NZEC1 Δ <i>cfa7</i>	Mock- inoculated
JA biosynthesis and signalling				
<i>fad</i>	PGSC0003DMB000000566: 67702-68990	3.96	(-0.42)	(0.61)
<i>fad</i>	PGSC0003DMB000000566: 44346-45710	2.56	(-0.78)	(0.25)
<i>fad</i>	PGSC0003DMB000000604: 170564-172436	4.30	(-1.23)	(0.03)
<i>fad</i>	PGSC0003DMB000000992: 26391-27525	3.33	-3.46	(-0.93)
<i>fad</i>	PGSC0003DMB000000992: 41520-42636	2.44	-3.16	(-0.75)
<i>fad</i>	PGSC0003DMB000000566: 50892-52434	2.61	(-0.62)	(-0.32)
<i>fad</i>	PGSC0003DMB000000566: 69633-70788	2.42	(0.65)	(-0.10)
<i>lox</i>	PGSC0003DMB000000060: 163071-166930	(1.22)	-2.33	(-0.68)
<i>lox</i>	PGSC0003DMB000000071: 1530574-1533001	(1.28)	(-1.56)	-2.06
<i>aos</i>	PGSC0003DMB000000633: 88317-90353	(-1.98)	(-0.62)	(0.31)
<i>aoc</i>	PGSC0003DMB000000423: 308213-309445	-3.77	(-0.49)	(1.16)
<i>12-opr3</i>	PGSC0003DMB000000272: 594289-599242	(-1.90)	(-0.80)	(0.43)

<i>des</i>	PGSC0003DMB000000555: 182-1935	6.77	4.77	3.54
<i>Jaace</i>	PGSC0003DMB000000069: 1114169-1121512	2.38	(1.58)	
MYC2	PGSC0003DMB000000048: 220507-221970	-4.02	(-1.33)	(1.42)

Ethylene Biosynthesis and Signalling

<i>sams</i>	PGSC0003DMB000000277: 811890-814269	2.11	(1.05)	(0.13)
<i>acs</i>	PGSC0003DMB000000147: 1139221-1142430	(1.91)	(0.15)	(0.57)
<i>aco</i>	PGSC0003DMB000000372: 88546-90395	4.34	3.80	(1.52)
<i>aco</i>	PGSC0003DMB000000093: 376312-387061	-2.85	(-0.96)	(0.80)
<i>aco</i>	PGSC0003DMB000000099: 1488286-1490004	-2.10	-3.63	(-1.21)
<i>aco</i>	PGSC0003DMB000000150: 729161-730898	2.06	(-0.22)	(0.23)
<i>aco</i>	PGSC0003DMB000000087: 454193-458140	-4.45	(1.06)	(0.88)
<i>aco</i>	PGSC0003DMB000000174: 936818-938747	2.40	2.26	(-0.43)
<i>EIL2</i>	PGSC0003DMB000000499: 89962-94045	(1.4)	(0.64)	(-0.4)
<i>ERF1</i>	PGSC0003DMB000000095: 1070918-1071672	3.28	(-1.71)	-3.78
<i>ERF5</i>	PGSC0003DMB000000271: 153711-154449	2.58	(0.93)	(-0.56)
<i>ERF10</i>	PGSC0003DMB000000130: 832068-832979	2.46	(0.70)	(-0.27)
<i>defensin</i>	PGSC0003DMB000001004: 25927-27795	4.36	(0.02)	(-0.20)

Wound responsive

<i>pi</i>	PGSC0003DMB000000119: 321982-322681	-3.12		
<i>pi</i>	PGSC0003DMB000000119: 296840-298274	(-1.50)	(-0.35)	(-0.32)
<i>pi IIa</i>	PGSC0003DMB000000244: 683644-684339	-2.11	(-0.35)	(-0.55)
<i>pi type-2 CEVI57</i>	PGSC0003DMB000000400: 517510-518303	(-1.60)	(0.80)	(1.30)
<i>ppo</i>	PGSC0003DMB000000535: 143786-145786	4.29	4.50	2.03
<i>PR1</i>	PGSC0003DMB000000682:	9.19	9.80	4.39

	89022-89882			
<i>PRI</i>	PGSC0003DMB000000682: 122154-122493	7.93	8.38	3.63
Other Transcription factors				
WRKY transcription factor	PGSC0003DMB00000028:2 12350-215394	2.31	-3.94	
WRKY transcription factor 4	PGSC0003DMB000000104: 587735-590190	-2.69	(-0.43)	(1.71)

5.3.2.1 JA responsive signalling

Genes involved in JA biosynthesis were differentially expressed in response to *Pbr* NZEC1 but not the CFA mutant. For example, four genes annotated as *fad* (including 2 splice variants for three FAD annotated genes) involved in the early stages of JA biosynthesis were significantly up-regulated in response to *Pectobacterium* and were significantly down-regulated in CFA mutant, however, no significant induction was observed in response to mock-inoculated control (Table 5.1).

Twenty one genes annotated as *lox* were identified during the analysis. Seven genes showed differential expression in response to one or more treatments although the differences were not significant (at $p=0.05$). Two *lox* genes showed non-significant induction (log 1.2 fold change) in response to wild-type whereas expression of these genes was repressed in response to CFA mutant and mock-inoculated control (Table 5.1).

Interestingly, other genes involved in the JA biosynthetic pathway (*aos*, *aoc*, *12-opr3*) were down-regulated in response to wild-type. No significant expression was observed in response to the CFA mutant or mock-inoculated control (Table 5.1). The divinyl ether

synthase (*des*) gene is involved in the conversion of 9-hydroperoxide to colneleic and colnelenic acid. This gene was highly up-regulated in response to wild-type, and to a lesser extent in response to the CFA mutant and in mock-inoculated tubers.

Jasmonate ZIM - domain protein1, essential for the activation of MYC2, (a JA responsive transcription factor) was not induced in response to *Pbr* NZEC1 (Appendix B). Furthermore, transcription of MYC2 was significantly down-regulated in *Pbr* NZEC1 treated samples, with no significant change for the CFA mutant and the mock-inoculated control (Table 5.1).

5.3.2.2 ET signalling pathway

Differential expression of genes involved in the ET biosynthesis pathway was observed in response to *Pbr* NZEC1. The *sams* and *acs* genes involved in the synthesis of ACC (the immediate precursor of ET) were up-regulated in response to the *Pbr* NZEC1. However, no significant differential expression was observed in response to the CFA mutant or the mock-inoculated (Table 5.1).

Multiple genes annotated as *aco*, involved in the oxidation of ACC to ET, were identified in this analysis. Of the 16 genes, 11 were differentially expressed in response to the various treatments. In particular, six *aco* genes showed significant differential expression in response to *Pbr* NZEC1, of which three genes were up-regulated and three were down-regulated (Table 5.1). In response to the CFA mutant only three of these genes were

significantly differentially expressed, of which two were up-regulated and one was down-regulated. No significant change in expression of *aco* genes were recorded in the mock inoculated controls.

Genes involved in ET signalling were also induced in response to *Pbr* NZEC1. EIN3-Like2 (EIL2) (Non-significant induction), and ET response factors (ERF1, ERF5 and ERF10) (Table 5.1), were all induced in wild-type treated tubers. In contrast, no significant induction of these genes was observed in response to the CFA mutant or the mock inoculated control. In fact, ERF1 was repressed in response to CFA mutant and the mock-inoculated control. Consistent with the up-regulation of ERF1 in response to *Pbr* NZEC1, the transcription of the gene encoding defensin was also up-regulated, but failed to show a significant difference in response to the CFA mutant.

5.3.2.3 Wound response gene expression

In this study, *pi* was significantly repressed in response to both *Pbr* NZEC1 and the CFA mutant (Table 5.1). This is in contrast, to studies that showed JA or the precursors of the JA biosynthesis pathway induced the expression of *pi* in tomato (Farmer & Ryan, 1992).

PPO is involved in catalysing the oxygen dependent oxidation of phenols to quinones during the wound response. Expression of *ppo*, is also involved in the plant defence against both biotic and abiotic stress. Transcription of *ppo* was highly induced in response to both *Pbr* NZEC1 and the CFA mutant and in the mock-inoculation control. The transcription of

PR1 was also highly induced in response to wild-type and the CFA mutant, and to a lesser extent in mock-inoculated control.

5.3.2.4 Other transcription factors

Expression of a number of transcription factors was affected in tubers treated with *Pbr* NZEC1, *Pbr* NZEC1 Δ *cfa7* or upon mock-inoculation. A gene annotated as WRKY-4 was down-regulated in response to *Pbr* NZEC1 whereas the transcription of WRKY-4 was up-regulated in the mock-inoculated control.

Transcription of a second WRKY transcription factor was significantly up-regulated in response to *Pbr* NZEC1 and significantly repressed in the CFA mutant (Table 5.2). The WRKY transcription factor up-regulated in response to *Pbr* NZEC1 remains to be annotated and hence the significance of this finding remains uncertain. However, the differential expression of this uncharacterized WRKY (PGSC0003DMB00000028:212350-215394) is of considerable note for further studies on *Pectobacterium*-potato disease interactions, as it shows a significant response to the phytotoxin CFA.

5.3.3 qRT-PCR verifies the differential expression in response to *Pbr* NZEC1 and its respective CFA7 mutant

The differential expression of ET and JA biosynthesis genes identified using RNA-seq was validated using qRT-PCR. For this purpose the cDNA from tissues treated with *Pbr*

NZEC1, the CFA mutant, the mock-inoculated and the non-inoculated control was synthesised as mentioned in Section 2.5.1.2.

5.3.3.1 Primer optimization and standard curve for qRT-PCR

Gene specific primers (Table 2.3) were designed for a subset of genes. Primers were designed using Geneious Pro 5.6. The primers were designed using the genome sequence of “Summer Delight”. The “Summer Delight” contigs were assembled against the Double Monoploid (DM) potato genome sequence (The Potato Genome Sequencing Consortium, 2011). The exons and the introns were visualised in Geneious Pro 5.6. The primers were designed to amplify within the exons of each gene. The optimal primer concentration and the annealing temperature for each qRT-PCR were standardised for each gene as described in Section 4.3.3.2. For relative gene quantification, standard curves were developed to calculate amplification efficiencies and regression values for each qRT-PCR. For this purpose, plant genomic DNA was used to generate a standard curve. A 10-fold serial dilution of the genomic DNA starting at 100 ng/μl was used.

For 13 out of 16 genes analysed, the qRT-PCR results corresponded well with the RNA-seq results. For example, the mean log₁₀ fold ratio for the *fad* (PGSC0003DMB000000604: 170564-172436) gene was 6.51×10^{-2} in response to the wild-type which was significantly induced when compared to the response to the CFA mutant (3.23×10^{-3}) (Figure 5.5). The mean back transformed ratios for all the genes analysed using qRT-PCR are listed in Appendix C.

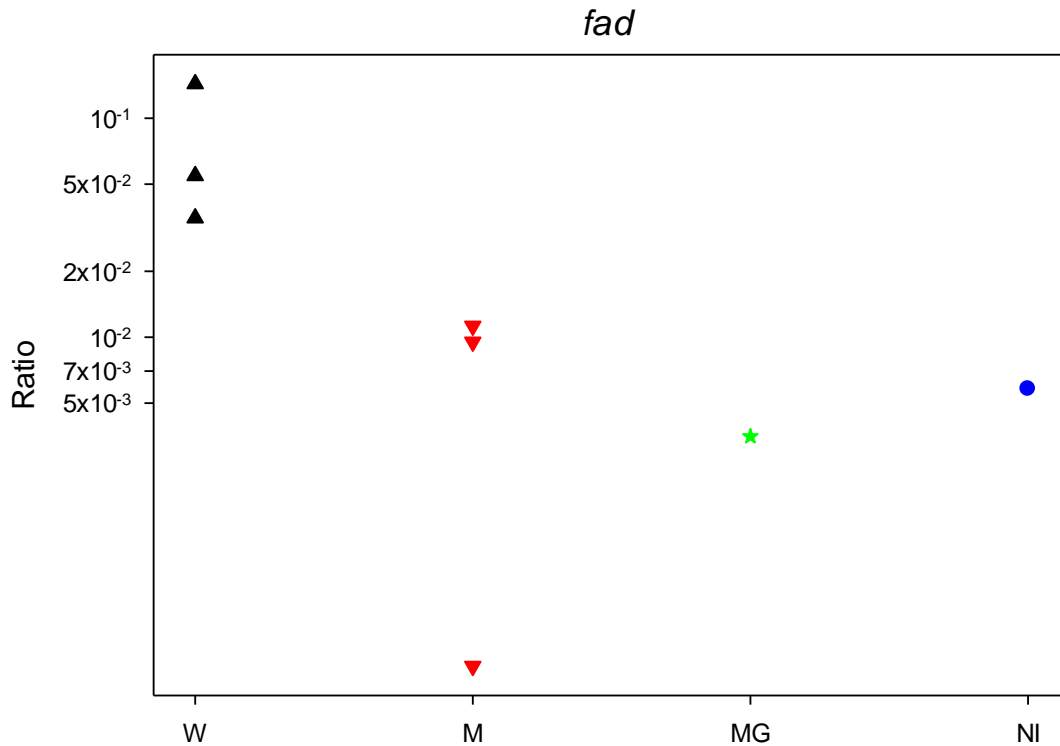


Figure 5.6: Ratio of target gene expression for *fad* in potato tubers inoculated with either *Pbr* NZEC1 (W) or *Pbr* NZEC1 Δ *cfa7* (M), or in mock-inoculated (MG) and non-inoculated (NI) tubers. LSD between W and M on the transformed scale was 1.49 for *fad*.

5.4 Discussion

RNA sequencing from total RNA was successfully used to identify genes differentially expressed in potato tubers in response to infection by *Pectobacterium* thus avoiding the expense and the bias associated with DSN treatment. Previously, DSN treatments have been used to remove abundant transcripts to facilitate the quantification of less abundant transcripts. Some studies have shown the effective removal of prokaryotic rRNA by DSN treatment while preserving the original relative abundance of mRNA (Yi, et al., 2011). However, as DSN treatment is expensive the ability to use total RNA both reduces the cost and the possible bias from RNA processing.

The reliability of results depends on the length of the reads and depth of the sequencing. The longer the read, the more likely it is to identify the transcript it originated from, whereas the 'depth' defines the dynamic range of the transcript profile that can be extracted from the data. Illumina sequencing provides up to 125 million reads per RNA sample library per lane on a flow cell on a HiSeq2000 of up to 100 bp in length (Axeq, personal communication). Consistent with this, the number of reads and the read lengths obtained in this study met the criteria required for downstream analysis. Furthermore, because of the dynamic range of the RNA-seq we were able to detect the less abundant transcripts and to identify multiple isoforms, which could prove useful for further studies.

Pbr NZEC1 and the CFA mutant mediated transcriptional changes were higher when compared to the mock-inoculated control. Approximately, 17% of the genes were modulated by both *Pbr* NZEC1 and its CFA mutant. However, there was very little overlap between *Pbr* NZEC1 and *Pbr* NZEC1 Δ *cfa7* with the mock-inoculated control. Further visualization of transcriptional changes using Mapman showed that many of the genes involved in biological processes were related to different biotic stress related pathways.

Several indicators of wound response, including PIs and PPO were induced in response to the mock-inoculated control. The *ppo* gene was induced in response to both *Pbr* and the CFA mutant in comparison to the mock-inoculated control. Previous studies have identified a significant increase in the transcription of *ppo* in response to infection by *P. syringae* in tomato plants. It was also observed that transgenic tomato plants that over-express potato PPO were highly resistant to infection by *P. syringae* (L. Li & Steffens, 2002). The genes encoding PI were significantly repressed in response to *Pbr* NZEC1.

Previous studies have identified the up-regulation of *pi* in response to the hemibiotrophic pathogens (C. M. J. Pieterse, et al., 1998). Hence the down-regulation of PIs could be a *Pectobacterium* mediated defence response.

The PR1 and the WRKY transcription factors involved in SA-mediated defences were differentially expressed in response to *Pbr* NZEC1. In our study, we also found that PR1 was induced by *Pbr* NZEC1 and the CFA mutant. However, it was induced to a lesser extent in the mock inoculated, suggesting PR1 can be induced by both *Pectobacterium* and wounding. A small number of putative WRKY transcriptional factors were differentially expressed in response to *Pbr* NZEC1. In addition, studies in potato in response to *Pba* and *P. infestans* has identified *St*-WRKY1 to be induced during the interaction (Dellagi, et al., 2000). In *A. thaliana* WRKY70 has been identified to be involved in the SA mediated defence response. WRKY70 represses the activity of JA/ET signalling pathway by inducing PR genes (J. Li, et al., 2006). WRKY4 transcription factor was down-regulated in response to wild-type *Pbr* NZEC1. These data are consistent with previous studies in which over expression of WRKY4 suppressed the expression of *PR1* (in our study PR1 was induced) and consequently resulted in susceptibility of *A. thaliana* to biotrophic infection by *P. syringae* (Lai, et al., 2008) while mutation in WRKY4 caused susceptibility of *A. thaliana* to necrotrophic fungal infection by *Botryis*. The down-regulation of WRKY4 and the up-regulation of PR1 in potato tubers in response to infection by *Pbr* suggests host mediated defences are initiated against the necrotrophic infection by *Pectobacterium*, even in a susceptible reaction.

Transcriptional studies in tomato and *A. thaliana* in response to COR suggested that COR antagonizes the SA mediated pathway by up-regulating the JA pathway. Indeed, a series of studies utilizing a number of COR and CFA biosynthetic mutants have determined the same (Brooks, et al., 2005b; Uppalapati, et al., 2007; Zhao, et al., 2003). However, a previous study using purified COR on tomato leaves did not observe SA-mediated defence suppression (Uppalapati, et al., 2005). Studies to identify the response of tomato to purified CFA also identified that CFA induces the transcription of ET biosynthesis genes but failed to show a significant effect on the JA pathway. These data are consistent with the limited expression of genes involved in JA biosynthesis in potato in response to *Pbr* NZEC1.

Genes encoding FAD and LOX, involved in the initial desaturation of fatty acids and biosynthesis of the initial JA precursors 13-hydroperoxide and 9-hydroperoxide were induced in response to *Pbr* NZEC1 but not the CFA mutant. Consistent with our previous speculation in Chapter 4, tuber specific expression of several genes annotated as *lox* was detected whereas others were not induced. The role of each in synthesis of 13-hydroperoxide and 9-hydroperoxide is unknown but the organ specific induction of several genes may be a consequence of their substrate specificity and the abundance of 9-hydroperoxides in tubers (Geerts, et al., 1994). Furthermore, genes involved in the synthesis of JA from 13-hydroperoxide were found to be down-regulated, including *aos*, *aoc* and *12-opr3*. This could possibly be due to the abundance of 9-hydroperoxides, thereby depleting the substrate (13-hydroperoxide) required for JA synthesis.

Instead, the gene encoding DES, involved in the catalyses of 9-hydroperoxides to colneleic and colnelenic acid was highly induced in pathogen treated samples. This gene was also

induced to a lesser extent in mock-inoculated samples. The results from this analysis suggest that *des* might be wound responsive; however, the log fold change observed in *Pbr* NZEC1 and in the CFA mutant treated samples suggest the involvement of *des* in pathogen-mediated defence in tubers. Similar results have been observed in potato leaves during infections with *P. infestans* (Weber et al., 1999) and *P. syringae* (M. Stumpe et al., 2001). During infection by *P. infestans* expression of colneleic and colnelenic acid was highly induced in resistant potato cv Matilda when compared to the susceptible cv Bintje, which suggests an anti-microbial property of colneleic and colnelenic acid (Weber, et al., 1999) and the potential activity of *des* in response to *Pbr* NZEC1. Furthermore, the results from this study suggest the existence of a JA independent defence response against *Pectobacterium* that utilises *fad* and other initial precursors in the JA biosynthesis pathway.

Previous studies have identified that the JA responsive pathway is essential for plant defence against both microbial and insect stress (Avanci, et al., 2010). In *A. thaliana*, mutation in MYC2 resulted in the repression of wound and insect induced defence response genes. Consequently, the MYC2 *A. thaliana* mutants showed susceptibility to insect stress (Dombrecht, et al., 2007). In contrast, a MYC2 mutation lead to induced expression of the JA-dependent pathogen induced defence response (Anderson, et al., 2004). These results suggest that MYC2 acts as a positive regulator of the JA mediated defence response in order to activate the defence response against insects and at the same time negatively regulates the pathogen mediated JA defence response. In addition, it was observed that ERF1 and MYC2 are mutually antagonistic at the transcriptional level (Dombrecht, et al., 2007). Consistent with these data, we observed the induction of ERF1

and the repression of MYC2 in potato tubers in response to *Pectobacterium* (Figure 5.5). Given that the JA biosynthetic pathway does not seem to be active in tubers, the down regulation of MYC2 may be due to the induction of ERF1 as part of the ET signalling pathway.

ET biosynthesis was significantly up-regulated in response to *Pbr* NZEC1 but not in the CFA mutant. In particular, the enzymes (*sams* and *acs*) involved in the synthesis of ACC were induced in response to wild-type (Figure 5.6). The various *aco* genes involved in the biosynthesis of ET from ACC showed different expression profiles in the presence of *Pbr* NZEC1 and the CFA mutant. Three were differentially expressed in the presence of either wild-type or the CFA mutant (2 up-regulated and 1 down-regulated), while two were down-regulated and one was up-regulated in the presence of the wild-type but not the mutant. ACOs are encoded by multigene families and previous studies have identified that the ACO encoding genes are differentially expressed in response to different biotic and abiotic stresses (Broekaert, et al., 2006). Studies have also shown the up-regulation of *aco* in response to CFA and in response to COR (Uppalapati, et al., 2005) and that the virulence and the avirulence of *P. syringae* induced the expression of specific *aco* genes (*aco1* and *aco2*), involved in the biosynthesis of ET. The results of the current study in potato tubers also suggest that differential expression of *aco* genes contributes to the regulation of ET biosynthesis. However, further studies are required to analyse the factors regulating the expression of *aco* in response to *Pectobacterium* in potato, and to categorise these genes based on their role in ET biosynthesis and their response to stress.

Consistent with the induction of ET biosynthesis, several genes involved in ET signalling including ERFs, EIN3 and EILs were up-regulated only in response to wild-type. Recent studies have identified ERF1 as an immediate target for EIN3 and EILs. ERF1 belongs to a large family of ET response element binding proteins (EREBPs) that binds to GCC-box, a promoter motif present in defence genes induced by pathogen and ET (Guo & Ecker, 2004; Solano, et al., 1998). It is speculated that ERF1 could also be involved in regulation of the JA-mediated defence response. The GCC-box required for ERF1 binding in the defence gene promoter is also a JA responsive element (Guo & Ecker, 2004). Thus, ERF1 might be an essential transcription factor for both the ET and JA signalling pathway, and both signalling pathways are required simultaneously for the induction of ERF1 and defensin synthesis. Previous studies have identified the associated induction of defensin by both ET and JA signalling pathway (Penninckx, et al., 1998), which suggest that ET and JA signalling is essential for an active host defence mechanism against the necrotrophic pathogen. However, the notion of parallel induction of JA and ET pathway is not coherent with the present results. This is validated with the induction of the defensin gene in response to *Pbr* NZEC1 but not in *Pbr* NZEC1 Δ *cfa7* nor the mock-inoculated control.

The apparent lack of JA biosynthesis in tubers and the synthesis of colneleic and colnelenic acid suggest that these compounds might activate a defence response that is independent of JA biosynthesis. However, further studies are required to quantify the synthesis of these compounds in tubers and to confirm their anti-microbial property against *Pectobacterium*. Furthermore, additional work is necessary to establish whether this alternative pathway links into defensin production.

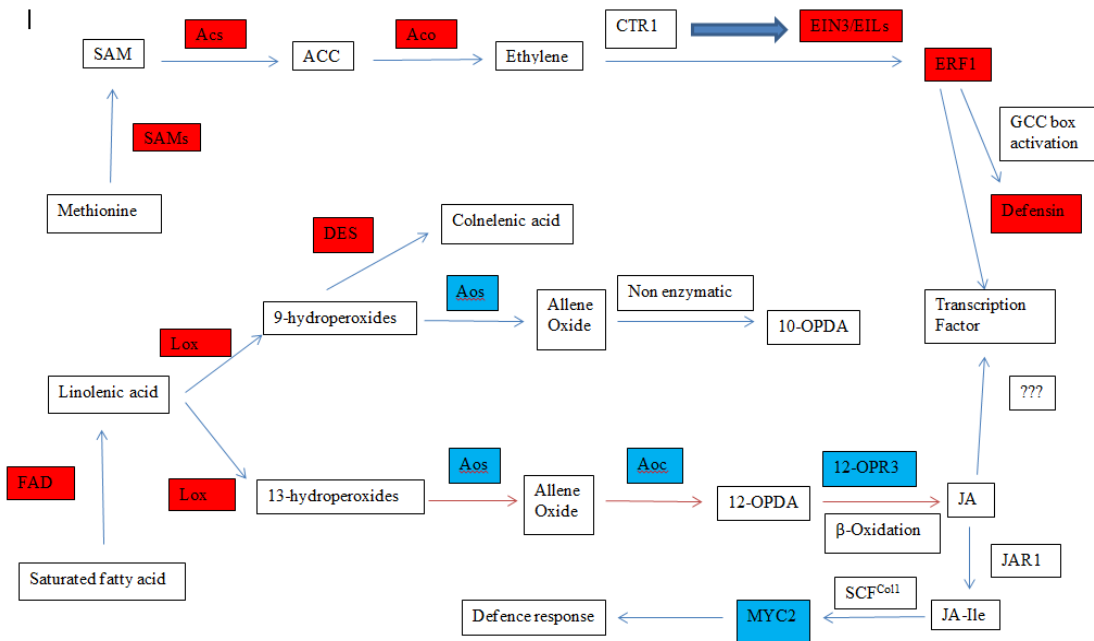


Figure 5.7: Diagrammatic representation of the JA and ET biosynthesis pathway and their possible crosstalk. The enzymes in the red box are up-regulated in response to the wild-type and in blue box are down-regulated in response to mutant.

Finally, plant defence responses against biotic and abiotic stresses are tuned to defence against a particular stress. It is speculated that the host defence response observed in this study is probably a resistance mechanism employed by the host against *Pectobacterium* infection, regardless of the fact the plant eventually succumbs to disease. However, it is likely that RNA isolation at 24 hpi is not suitable to study the influence of CFA. The CFA mediated host defence response could take place during the early stages of infection and hence early time points like 6 and 12 hpi could be used for further studies to identify CFA mediated manipulation of host defence responses.

5.5 Conclusion

RNA-seq analysis using total RNA was sufficient to reveal the induction of ET signalling and partial induction of the JA pathway in response to *Pbr* NZEC1 at 24 hpi. Key genes involved in these pathways were differentially expressed, a summary of which is shown in Fig 5.7. Thus, RNA-seq provides a cost-effective alternative to microarrays for the study of transcription in potato and of the plant's interaction with *Pectobacterium*. However, further studies are required to confirm the presence of colneleic and colnelenic acid *in planta* in response to *Pbr* NZEC1 and their possible role in defence. To gain a better understanding regarding the role of CFA, transcriptional profiling during the early stages of infection will be carried out in further studies preferably with purified CFA.

Chapter 6

General discussion

6.1 Final Conclusion

Transcriptional studies in tomato during infection by *P. syringae* (a hemibiotrophic) and or upon addition of purified COR (CFA is a component of COR) have identified the COR mediated differential expression of defence genes. As a consequence it has been speculated that COR mediated activation of the JA signalling pathway antagonises SA mediated signalling, which is essential for defence against biotrophic and hemibiotrophic infection by *P. syringae*. Mutation studies in the necrotroph *Pba* SCRI1043 have identified CFA as an important virulence factor involved in the pathogenesis of *Pba* on potato stems (Bell, et al., 2004). However, the alternative pathogenic lifestyle and the absence of CMA suggest that this virulence factor might behave differently in *Pectobacterium*. To date, the transcriptional response of potato to *Pectobacterium* infection remains unclear.

The work in this thesis was conducted to expand the knowledge available on the *Pectobacterium*-potato interaction. Overall it was hoped that the results from this study would provide an insight into the targets in the host that could be of importance for future resistance breeding programs and also to provide a greater understanding of the unrealized complexity of the *Pectobacterium*-potato interaction.

Initially, the research involved identifying a suitable cultivar-pathogen model to study the transcriptional response. It was hypothesised that different cultivars differ in their

susceptibility to *Pectobacterium* and that different species of *Pectobacterium* have different aggressiveness on potato stems and tubers. Consistent with this hypothesis, differences in the susceptibility to soft rot infection was observed between the four cultivars used in this study. Furthermore, “Iwa” and “Summer Delight” were susceptible to soft rot infection by both *Pba* SCRI1043 and *Pbr* NZEC1. In addition, *Pbr* NZEC1 was identified to be an aggressive pathogen when compared to *Pba* SCRI1043. Blackleg assays on all four cultivars failed to produce any consistent symptomology. It has been postulated that mature plants are more resistant to blackleg infection. Unfortunately, we performed a single experiment using plants of the same age, yet they appeared to have very different physiological ages. Hence, to confirm this hypothesis and to identify a suitable cultivar for studying the influence of *Pectobacterium* on stems, in future experiments individual cultivars will be examined for their susceptibility to blackleg infection by both *Pba* SCRI1043 and *Pbr* NZEC1.

Given the lack of blackleg symptoms on all cultivars and *Pectobacterium* being a seed borne pathogen, further studies were carried out in tubers. Though “Iwa” was highly susceptible for soft rot infection, the genome sequence was not available, hence “Summer Delight” and *Pbr* NZEC1 were used as a model cultivar-pathogen combination to study the transcriptional response of the host to *Pectobacterium* and CFA.

The transcriptional response to CFA was studied using total RNA extracted from potato tubers (“Summer Delight”) 24 hpi with *Pbr* NZEC1 and *Pbr* NZEC1 Δ *cfa7*. The use of total RNA for downstream applications made the process quicker, more economical and introduced less potential bias than using processing techniques such as DSN treatment. To

extract optimum amounts of RNA for downstream analysis, different extraction techniques were evaluated, however, RNA extracted using the lithium chloride precipitation method provided the optimum quantity and quality of RNA.

Initially, the transcriptional response was studied by targeting JA related genes differentially expressed in response to COR during infection by *P. syringae* using qRT-PCR. However, qRT-PCR on target genes failed to show any significant differential transcription in response to infection by *Pectobacterium*. Since previous studies in potato have identified multiple isoforms of several JA biosynthetic genes (e.g. *lox* and *aos*) as well as their organ and substrate specificity in potato plants, it was speculated that their presence could well have been the reason behind the lack of differential expression. In this study, transcriptional profiling using qRT-PCR was due to be conducted in both stems and in tubers in response to both blackleg and soft rot infection. However, due to the lack of symptoms in the stem in response to infection by *Pba* and *Pbr*, qRT-PCR was carried out only in tubers in response to soft rot infection.

No previous studies in potato have shown the transcriptional response to *Pectobacterium* and CFA. Thus, given we used genes differentially expressed in tomato in response to a biotrophic pathogen, it could also be that a different host response was elicited in potato against a necrotrophic infection by *Pectobacterium*. As a result, to identify the defence signalling pathways involved in response to *Pectobacterium* and to identify the possible isomers involved in JA biosynthesis in tubers, global transcription profiling in response to *Pectobacterium* infection was carried out using RNA-seq.

Downstream analysis of the RNA-seq results identified significant differential expression of genes in response to the wild-type when compared with the mutant and the mock-inoculated control. In fact, mock-inoculation altered the expression of very few genes (a good internal control).

Infection with *Pbr* NZEC1 was observed to affect multiple biological processes including the JA, ET and auxin signalling pathways as well as MYB and WRKY transcription factors. Consistent with the previous qRT-PCR results, the genes involved in JA biosynthesis (*aos*, *12-OPR3*) failed to show significant differential expression. However, as speculated, organ specific differential expression of *lox* was identified. In addition, the expression of *des* involved in the synthesis of colneleic acid was significantly induced in response to the pathogen and to a lesser extent the mock-inoculated control, suggesting it may have a role as both an antimicrobial and in wound response. Further studies are required to quantify the synthesis of this compound during *Pectobacterium* infection and how the composition of the 13 and 9-hydroperoxides in tubers influences the response to *Pectobacterium*. However, the lack of induction of several downstream JA biosynthesis genes suggests a JA independent defence response in tubers in response to *Pectobacterium* infection.

In contrast, ET biosynthesis and signalling was induced in response to wild-type infection. Consistent with up-regulation of ET signalling, the expression of the defensin gene was also induced in response to the *Pbr* NZEC1 but not the CFA mutant or the mock-inoculated.

Further studies are required to understand the role of CFA in mediating defence responses. Furthermore, the data from this analysis were likely more of an indirect downstream consequence of CFA rather than a direct effect. With this in mind, transcription profiling will be carried out during the early stages of infection and using purified CFA.

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Appendix A

A.1 Sequence of pPCR1 used for generating standard curve

AAGTTCCTTACCTGAACGCCTGTCAATCTTGGTCAAGATCTCAGCAAACCTTGAC
AGCAATGTGGGAAGTGTGGCAGTCGAGCACTGGTGCATATCCGTTTCCAATCT
GAAAATCAAAAGCTAGATGGGTTGAAATGTGGGGCTATCTTAAGAAGATTAGC
CATCATTTTGGAGTACCAATACCCTCCGAAAAAGGGTGATGCTGTAAACATGAT
ATGGAGGGAGGTGAAGCACAAGCTTCCTGCTAAAAATACCCGGCGACTATGG
GTTGCCGGGTATTGGTCCATGGAAAGATAGGCTTGATTACTTTTACAATCAAG
GGAAAGACGAATTTTTTCGAATCAAGAGTAGTGAAATACAAATCAACTATATTC
AGAACGAACATGCCACCGGGACCATTCATTTCTTCTAAAAAATGCAAAGGGTG
GAAGATGACAGCAGGCTTAAAGGTTTCTCCGATTTACACAACCTTTTTCTCTT
TGAAATGCCATCACGGCAAGAAGCAGCCAGAGTCAAGGCGGGCGATTGGTTTT
GAAAAGACGTTCCATGGCCAATGCAGGGCTAGAACTGCAGGCCAAAGTTCGA
GTTTCAACGGTTCCAATAGGATCTCTTGAATCGCCTTGGCATCCATTCATCGAC
AATGAAAAAAGATGAGTCCATGGGAGTTCCTGGAGCTTTCATAATCAAGAATT
ACCACCACAGCCAGTTTTACCTCAGGACAGTCGTTTTAGAAAGATGTTCTCTGGAC
ATGGTGAACCTTCATTTTGTGTGCAATTCATGGGTGTATCCAGCACACCGCTACA
AAAATGCCTGCTTCGGTTTGCCCATATGCTACGTATCGTGGCTGTGTTACATGA
AGATAGGCAAGCTTGAACCAGAATGGAGTTGGATTTTGTTTAGTCTTTCAAC
AACTGCTAAGCCTAGGCTGAGTGGATTAGAGTCCATGGCATCAAGATGATCTA
TTGCTGGTGAACTCTAACGCCTACACGATCAGCTCCTATTGCTGAGAAAACA
ATGAGCGGTGGTGCAGAGGCAAGAACCTGCGAGTCACAGAGCCACAGTTTCA
AGGGGCCATGTGTTGGCGATACCAACTGCGCCTCCGTGTGCCAGACTGAAGGA
AAAGTGCATTGAAGCTTCTGTGGCTGTTGGATAATGTGGAGGGGTTAAATGAA
GCTGTTAAAAAAGGAGATGCCATCTTTGGAACCTATCGACACCTGGTTAATTTG
GAACCTAACAGGAGGTGTGGAGAAAAAAAACCCGAACAACAGTTGATGCATA
TTCCATCAACTTTTTTGGTTCCTAAATATGGGCAAATTCCAAAGTCAATTCGAG
GGTCACATTCTTTGGCGTCTGCATGTTTCGAAATCGCTTAAAACTGATGAAGCAA
CTTCCTGACGACGATCCACGTAGCTTCAATCAACAAGCAAATGTTTCATTGTGCT
TATTGTGATGGTGCTTATGATCAACTAGGTTTCCCAAATACAGAGCTCCAAGTT
CATTCTTCTTAAAA

Appendix B

Appendix B is on the enclosed CD

Note: N/A represent non-statistically significant fold change.

Appendix C

Table C 1: Back-transformed mean ratios (\log_{10} Ratios) for the 16 genes in response to *Pbr* NZEC1, CFA mutant, mock-inoculated and non-inoculated control validated using qRT-PCR

Gene Name	In response to			
	<i>Pbr</i> NZEC1	<i>Pbr</i> NZEC1 Δ <i>cfa7</i>	Mock- inoculated	Non- inoculated
<i>fad 992-1</i>	6.79x10 ⁻²	1.58x10 ⁻²	5.12x10 ⁻³	4.27x10 ⁻³
<i>fad 992-2</i>	2.38x10 ⁻¹	2.63x10 ⁻²	1.41x10 ⁻²	0.20x10 ⁻¹
<i>fad 604</i>	6.51x10 ⁻²	3.23x10 ⁻³	3.52x10 ⁻³	5.81x10 ⁻³
<i>lox</i>	1.08x10 ⁻¹	7.93x10 ⁻²	5.73x10 ⁻²	4.83x10 ⁻²
<i>aoc</i>	7.43x10 ⁰	4.08x10 ⁰	2.87x10 ¹	7.98x10 ⁰
<i>sams</i>	3.10x10 ¹	8.31x10 ⁰	1.19x10 ⁰	1.87x10 ⁰
<i>acs1</i>	1.69x10 ⁰	2.63x10 ⁻¹	3.29x10 ⁻¹	2.20x10 ⁻¹
<i>acs2</i>	2.09x10 ⁻¹	2.48x10 ⁻²	1.14x10 ⁻²	2.50x10 ⁻²
<i>aco 0087</i>	6.43x10 ⁻¹	5.53x10 ⁻¹	1.66x10 ⁰	1.02x10 ⁰
<i>aco 037</i>	2.36x10 ⁻¹	3.83x10 ⁻²	7.13x10 ⁻²	3.98x10 ⁻²
<i>defensin</i>	5.45x10 ⁻³	7.74x10 ⁻⁴	6.03x10 ⁻⁴	6.51x10 ⁻⁴
<i>jaace</i>	1.85x10 ⁰	3.58x10 ⁻¹	3.38x10 ⁻¹	2.81x10 ⁻¹
<i>ap2</i>	1.96x10 ⁻¹	7.52x10 ⁻¹	3.71x10 ⁰	1.99x10 ⁰
<i>poly</i>	1.74x10 ⁻²	4.21x10 ⁻³	1.89x10 ⁻²	2.60x10 ⁻²
<i>PR</i> <i>uncharacterised</i>	2.50x10 ⁻²	6.95x10 ⁻³	4.04x10 ⁻²	2.11x10 ⁻³
<i>warky</i>	1.06x10 ⁻¹	1.69x10 ⁻¹	4.57x10 ⁻¹	2.88x10 ⁻¹