CHAPTER 3
MICROARRAY ANALYSIS OF THE *ARABIDOPSIS THALIANA CIR1* (CONSTITUTIVELY INDUCED RESISTANCE 1) MUTANT REVEALS CANDIDATE DEFENCE RESPONSE GENES AGAINST *PSEUDOMONAS SYRINGAE* PV *TOMATO* DC3000.

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3.1. Abstract

Microarray expression profiling on the model plant *Arabidopsis thaliana* has contributed to the elucidation of plant defence responses and resistance against disease. An *Arabidopsis* mutant, *cir1* (constitutively induced resistance 1), previously showed enhanced resistance to the pathogenic biotrophic bacterium *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000. It was hypothesised that induced or repressed genes in *cir1* may play a role in conferring resistance against this pathogen. This study investigated differential gene expression in wild type and *cir1* plants without pathogen challenge using a custom 500-probe microarray, biased towards defence-response and signalling genes, to identify transcripts, which may be required for resistance in *cir1*. Several genes were found to be induced in *cir1* at a significance threshold of $-\log_{10}(p)$ equal to 3 ($p < 0.001$) using a mixed model ANOVA approach. The induction of the genes encoding AtACP1 (sodium inducible calcium binding protein), AtP2C-HA (protein phosphatase 2C), AtGSTF7 (glutathione S transferase), tryptophan synthase beta-like and AtPAL1 (phenylalanine ammonia lyase 1) and the repression of AtEREBP-4 (ethylene response element binding protein 4) and HFR1 (long hypocotyl in far-red 1) in *cir1* correlates with publicly available microarray data which shows the same genes differentially expressed in a similar manner in *Arabidopsis* plants infected with *Pst*. This observation supports our hypothesis that these genes contribute to disease resistance in *cir1*.

3.2. Introduction

Plants, being sessile, have evolved a battery of defence response genes to protect themselves from biotic and abiotic stresses. These may be preformed or induced responses. If preformed defences such as physical barriers or antimicrobial compounds are overcome, the invading pathogen will encounter induced defences which rely on initial pathogen recognition and the triggering of signalling cascades involving the signalling molecules salicylic acid (SA), jasmonates including jasmonic acid (JA) and methyl jasmonate (MeJa), and ethylene (ET) (reviewed in Thatcher et al., 2005).

These signalling cascades lead to the expression of pathogenesis related (PR) proteins, peroxidases, proteinase inhibitors and the production of antimicrobial secondary compounds to elicit defence against the invading pathogen. If the pathogen is able to overcome the host induced defences, the plant becomes diseased and the interaction is termed compatible. However, not all plant-pathogen encounters result in disease. The vast majority of resistant interactions are the result of non-host resistance, which involves the induction of a basal defence system following recognition of pathogen-associated molecular patterns (PAMPs) by the plant (reviewed in Jones and Dangl, 2006). An interaction that results in disease (compatible) also triggers a basal defence mechanism, however, this response is ineffective in curbing the pathogen. There is evidence of the repression of basal defence genes by pathogen effectors as a strategy to mediate susceptibility (Jones and Dangl, 2006). A second type of resistance employed by plants is cultivar-specific resistance, which involves the recognition of an avirulence (avr) gene product in the pathogen by the corresponding resistance (R) gene product in the plant, consequently resulting in no disease (Nimchuk et al., 2003). A feature of this gene-for-gene resistance, termed an incompatible interaction, is the hypersensitive response (HR), a localised area of cell death, which prevents further spread by the pathogen (Greenberg, 1997), and the establishment throughout the plant of systemic acquired resistance (SAR) to a broad range of virulent pathogens (Ryals et al. 1996). Recent work has indicated that similar components required for signal transduction are employed by both non-host and R-avr mediated resistance (Navarro et al., 2004, reviewed in Ingle et al., 2006). Tao et al. (2003) observed that although signal transduction mechanisms in compatible and incompatible interactions are qualitatively similar, there is a distinct quantitative difference; the responses in the incompatible interaction reach higher levels earlier than in the compatible interaction.

Most information regarding the plant defence response has been made possible by research on the model plant *Arabidopsis thaliana*. The availability of the entire genome sequence has facilitated faster map-based cloning of genes and has provided information for the production of microarrays. The latter technology has been used extensively in *Arabidopsis* to find co-ordinately expressed genes during pathogen attack and abiotic treatments (Maleck et al., 2000; Chen et al., 2002; Mahalingham et al., 2003; Tao et al., 2003). Several platforms for microarrays exist: Affymetrix GeneChip® on silicon wafers and cDNA or long oligonucleotide microarrays on glass slides. The advantage is that thousands of genes can be screened in a single experiment. However, microarrays remain an expensive technology especially if Affymetrix GeneChip® whole-genome arrays are being used. One strategy to reduce costs is to develop custom microarrays with genes predicted or known to be involved in a particular biological process. This allows researchers to increase the level of replication per microarray slide, and make valuable conclusions from the data even though a small subset of genes is represented. This has been demonstrated in the development of a custom programmed cell death (PCD) microarray for Arabidopsis containing approximately 100 cDNAs representing genes previously implicated or hypothesised to play a role in PCD and known animal PCD genes (Swidzinski et al., 2002); and the use of a 150 cDNA microarray to analyse the plant response to mechanical wounding, insect feeding and water-stress (Reymond et al., 2000). Another example is the use of a custom set of defence response genes from *Arabidopsis* to investigate the response to lipopolysaccharide, a PAMP (Zeidler et al., 2004).

The data generated by *Arabidopsis* Affymetrix microarrays are publicly available to the *Arabidopsis* research community allowing one to generate and test hypotheses *in silico* before embarking on wet-lab experiments (Berger, 2004). This strategy also contributes to cost and time saving as suitable candidate genes can be identified prior to gene function studies. Several *Arabidopsis* databases are available which allow access to microarray data: The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org/), Nottingham Arabidopsis Stock Centre's microarray database (NASCArrays, http://arabidopsis.info/) and the Stanford Microarray Database (SMD, http://genome-www5.stanford.edu/). In addition, databases such as GENEVESTIGATOR (Zimmermann et al., 2004) and DRASTIC--INSIGHTS (Database Resource for Analysis of Signal Transduction In Cells, Button et al., 2006) provide researchers with tools which facilitate gene mining in order to make important biological inferences from microarray data. The use of these databases in hypothesis testing

was recently demonstrated by the identification of a novel gene, flavin dependent monoxygenase 1 (*FMO1*), whose gene product is required for the development of SAR in systemic tissue (Mishina and Zeier, 2006). *FMO1* was initially identified as being up-regulated in *Arabidopsis* leaves inoculated with avirulent and virulent strains of the bacterial pathogen *P. syringae* pv *maculicola* based on microarray data from NASC (NASCARRAYS-59: impact of type III effectors on plant defence responses) and TAIR (TAIR-ME00331: response to virulent, avirulent, type III secretion system-deficient and non host bacteria) databases.

Mutants in *Arabidopsis* have been widely used in the study of disease resistance (Murray et al., 2002a). The *Arabidopsis cir1* (constitutively induced resistance 1) mutant was selected based on a screen of transgenic *Arabidopsis* plants containing a chimeric *PR-1:: luciferase* (*PR-1::LUC*) gene fusion (Murray et al., 2002b). The mutant *cir1* showed enhanced expression of *PR-1* in the absence of pathogen challenge and super-induction of the *PR-1* gene following attempted *P. syringae* pv *tomato* DC3000 (*Pst*) (*avrB*) infection. In addition, *cir1* showed constitutive expression of "marker" genes of the SA signalling pathway (*PR-2*, *PR-5*), JA/ET signalling pathway (*PDF1.2*) and the oxidative burst (*AtGSTF6*). *Cir1* had a similar level of resistance to *Pst* and *Hyaloperonospora parasitica* NOC02 as wild type *Arabidopsis* plants exhibiting SAR after exogenous application of SA (Murray et al., 2002b). The function of CIR1 is unknown, although the mutation was mapped to the lower arm of chromosome 4. Murray et al. (2002b) propose that the wild type CIR1 protein is a negative regulator of disease resistance.

Here, the gene expression profiles of the mutant *cir1* and its background, the transgenic line *PR-1::LUC* (hereafter referred to as *luc2*) was investigated, to determine which defence response genes are affected by the *cir1* mutation. A customised 500-probe EST microarray biased towards genes involved in plant defence and signalling was used. Our microarray experiments identified seven genes that were differentially expressed in *cir1* compared to *luc2* (induced: *AtACP1*, *AtP2C-HA*, *AtGSTF7*, tryptophan synthase beta-like and *AtPAL1;* repressed: *AtEREBP-4, HFR1*) that showed similar gene expression patterns in Col-0 plants challenged with *Pst* (*avrB*) at an early time point and Col-0 plants challenged with *Pst* at a later time-point based on publicly available microarray data. These genes could possibly contribute to *cir1*-mediated resistance against this pathogen.

3.3. Materials and Methods

Plant Growth

Homozygous seeds of the *PR1::LUC* transgenic line, *luc2* and *cir1* (Murray et al., 2002b) were sown on Jiffy Disks (Jiffy Products International, Norway) and maintained under controlled environmental conditions at 25^{o}C under a 16hr photoperiod light/ 8 hour dark under 100 PAR (Photosynthetic Available Radiation). Five-week-old plants were harvested by cutting the leaf material and freezing in liquid nitrogen.

Microarray experiments

Experimental Design

The experimental design was a direct comparison between *cir1* and *luc2* (Naidoo et al., 2005). Three biological replicates were performed i.e. the experiment was repeated on three different occasions with plants grown under the same conditions. A technical replicate and dye-swap replicate within each biological replicate was included. In total, 12 slides were used.

Array Design

Arabidopsis cDNA clones, referred to as the L35 collection, were purchased from Mendel Biotechnology (California, USA). The collection was assembled from a collection of EST (expressed sequence tag) libraries from different organs of *Arabidopsis* ecotype Col-0, which had not been subjected to any treatment, and cloned into the pZipLox vector. The mRNA sources were 1) 7 day germinated etiolated seedlings; 2) tissue culture grown roots; 3) rosettes of staged plants half with a 24 hour light cycle, half on 16 hour light, 8 hour dark; 4) stems, flowers and siliques of staged plants half with a 24 hour light cycle, half on 16 hour light, 8 hour dark. Approximately 500 clones were selected from the L35 collection based on their annotations and previous data which implicates these genes as putative cell signalling, defence or stress response genes owing to their regulation under conditions of either abiotic stress (e.g. drought, cold, salinity, wounding); biotic stress (e.g. insect, bacterial, fungal, viral, herbivore) or chemical treatment (e.g. SA, methyl jasmonate, JA, ethylene, abscisic acid (ABA), hydrogen peroxide). Also, some genes predicted to be involved in plant defence response and signalling were included such as kinases. The *PR-1* (At2g14610), *PR-2* (At3g57260), *PR-5* (At1g75040), *AtGSTF6* (At1g02930) and *PDF1.2* (At5g44420) genes were not available in the L35 collection and were thus added to the 500 set from a different source (Murray et al., 2002b). Redundant clones were identified by performing BLASTN or BLASTX comparisons within the 500 selected clone-set. These clones were not removed

prior to the preparation of the microarray. It was determined that the microarray contained cDNAs that represented approximately 300 unigenes after redundant cDNAs were identified. The gene ontologies for the 300 unigenes were assigned using the gene ontology tool available on TAIR and compared to the gene ontologies for the entire *Arabidopsis* genome. Table 3.1 indicates the functional categorisation for the selected genes represented as (A) a percentage relative to the 300 unigenes and (B) as a percentage of the genes with the same ontology for all annotated genes in the whole genome (approximately 29 000 genes). The latter percentages represent significant enrichment for each category if one bears in mind that the 300 unigenes represent 1% of the *Arabidopsis* genome.

Clones were PCR amplified using vector specific primers (5'-CGCTCTAGAGGATCCAAGCTTACGT-3' and 5'-ACCGGTCCGGAATTCCCGGGTCGAC-3') and the products purified using the Multiscreen® PCR Purification Plate (Millipore, Molsheim, France). Sequence verification of a random selection of clones was performed after re-racking for the spotting procedure to confirm that the clones and their respective positions corresponded. The amplicons were diluted to a final concentration of 125 ng/ul in 50% DMSO for spotting. The DNA was spotted onto Corning® Gap II slides (Corning Inc., New York, USA) in duplicate using the Generation III Arrayer (Molecular Dynamics Inc., Sunnyvale, CA, USA) at the ACGT Microarray facility (http://microarray.up.ac.za). Following spotting the slides were allowed to dry at 45-50% relative humidity overnight and spotted DNA was then bound to the slides by UV cross-linking at 250mJ for 3 min. Included on the array were DNA spots of the artificial spiking controls Lucidea™ Universal ScoreCard™ (Amersham Biosciences), which were used as hybridization controls. Negative controls containing no DNA were also spotted on the array.

Table 3.1. Selected Gene Ontology of 300 unigenes from the custom 500 probe array, representing 1% of the Arabidopsis genome, indicated as (A) a percentage relative to the 300 unigenes and (B) as a percentage of the corresponding gene ontology for all annotated genes within the Arabidopsis genome (approximately 29000 genes).

Gene Ontology Category	Functional Category	A	B
Biological Process	Signal transduction	5.8%	15%
	Response to abiotic or biotic stimulus	3.4%	18%
	Response to stress	3.2%	15%
	Electron transport or energy pathways	1.4%	4%
Molecular Function	Kinase activity	5.4%	28%
	Transcription factor activity	2.0%	16%
	Nucleic acid binding	1.7%	3%
	Receptor binding activity	1.1%	3%

Samples

Six to eight individual plants were pooled from a trial for each labelling experiment.

For the preparation of targets, RNA was isolated from harvested Arabidopsis leaves using TRI Reagent® (Sigma, St Louis, Missouri, USA), according to the manufacturer's protocol for large-scale extraction. The RNA was thereafter further purified using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, California). mRNA was isolated using the Oligotex® mRNA Mini Kit (Qiagen). RNA yield was determined by measuring absorbency at 260nm using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). The CyScribe™ Post Labeling Kit (GE Healthcare Ltd, UK, Buckinghamshire) was used for incorporation of amino-allyl dNTPs during the cDNA synthesis from 500 ng mRNA per sample and subsequent addition of cyanine 3 (Cy3) or cyanine 5 (Cy5) labels. The corresponding spiking RNA samples (Lucidea™ Universal ScoreCard™, Amersham Biosciences) were also added to the Arabidopsis mRNA samples during this reaction. The cDNA was purified using the PCR purification kit (Qiagen) before and after dye-coupling with Cy3 or Cy5 dyes.

Hybridisations

Prior to hybridisation, slides were pre-treated in a solution of 1% BSA, 0.2% SDS and 3.5 X SSC at 65^{o}C for 15 min. Slides were washed in distilled water (Sigma) and dried using high pressure nitrogen. The hybridisation solution, consisting of 50% formamide, 25% Amersham

hybridisation buffer (GE Healthcare Ltd) and the Cy3 and Cy5 labelled targets were heated to 95°C and then cooled on ice. The hybridisation solution was added to the slides under a clean coverslip and then allowed to hybridise overnight at 42°C in a HybUP hybridization chamber (NB Engineering, Pretoria, South Africa). The slides were washed in a solution of 1.0 X SSC, 0.2% SDS for 4 min at 42°C, followed by two washes in 0.1 X SSC, 0.2% SDS for 4 min at 42°C, and three washes in 0.1 X SSC for 1 min at room temperature. The slides were dipped in distilled water a few times before being dried with high pressure nitrogen, and scanned using the Genepix™ 4000B scanner (Axon Instruments, Foster City, CA, USA).

Data Analysis

Data was captured using ArrayVision™ version 6 (Imaging Research Inc., GE HealthCare Life Sciences, USA). A grid was overlaid and spots with poor morphology were flagged from the analysis. Data was analysed using a mixed model analysis of variance (ANOVA) in the statistical program SAS® version 8.2 (SAS® Institute Inc., Cary, North Carolina, USA.) according to the method of Wolfinger et al. (2001) which essentially performs a global normalisation. Briefly, the normalisation model that was used was as follows:

$y_{gijks} = \mu + D_k + T_i + A_j + (TA)_{ij} + \varepsilon_{gijks}$ where μ represents the overall mean value, D is the dye effect, T is the main effect for treatments, A is the main effect for arrays and TA is the interaction between arrays and treatments and ε is the random error. The assumptions that were made were that the effects $A_j, (TA)_{ij}, \varepsilon_{gij}$ are normally distributed with zero means and variance components σ^2_A, σ^2_{TA} *and* σ^2_ε respectively, and the latter named effects are independent both across their indices and with each other, and μ is a fixed effect. The data was Bonferroni adjusted to correct for multiple testing. The processed microarray data is available as supplementary data at the following website (to be determined in discussion with the Editor).

Northern blot analysis

Northern blot analysis was performed using the DIG-Easy Hybridisation system (Roche Molecular Biochemicals, Germany). Briefly, 20 μg of *cir1* and *luc2* plant RNA, resolved on a denaturing formaldehyde gel, was transferred to a nylon membrane via downward capillary blotting overnight and UV cross-linked at 120 mJ for 3 min. The probes were created by amplifying the insert from the corresponding bacterial clones of *AtGSTF6* (At1g02930, cloned into pBluescript® II SK(+) vector, Stratagene, USA, California) and dehydrin

(At5g66400, available from the Mendel L35 collection) using vector specific primers in the presence of DIG dNTP's. Hybridisation was allowed to proceed overnight at 60°C. Washes and detection were performed according to the DIG non-radioactive nucleic acid labelling and detection system (Roche Molecular Biochemicals). Hybridisation signals were quantified using the densitometry function of the Versadoc™ imaging system (Bio-Rad Laboratories, Inc., CA, USA).

Quantitative reverse-transcription PCR (qRT-PCR)

Two-step quantitative reverse-transcription PCR (qRT-PCR) was performed using a LightCycler instrument (Version 1.2, Roche Diagnostics GmbH, Germany). PCR primers were designed to each target in PrimerDesigner v5 (Scientific & Educational Software, Cary, North Carolina, USA). The primer pair for the *LUC* gene was forward 5'-ACCCGAGGGGGATGATAAAC-3' and reverse 5'-AGAGACTTCAGGCGGTCAAC-3'. The primer pair designed for *AtACP1* was forward 5'-AGACGGAGATGGGAGACTGA-3' and reverse 5'-AGTTGGAAATGTGCGGTGT-3' while the primer pair for *AtEREBP-4* was forward 5'- GAACCATCACCAACCAATCC-3' and reverse 5'-GTCCCAAGCCAGATCCTACA-3'. Primers for *PR-1* (At2g14610) and *AtSERK4* (At2g13790) were selected from the purchased primer library for *Arabidopsis* Pathogen-inducible Genes (Sigma). Two micrograms of total DNaseI-treated and column-purified RNA extracted from *cir1* and *luc2* plants were reverse transcribed into first strand cDNA using ImpromII Reverse Transcriptase (Promega, USA, Madison, Wisconsin) according to manufacturer's instructions. The LightCycler FastStart DNA Master^{PLUS} SYBR Green I system (Roche) was used for qRT-PCR starting in a standard 20 µl reaction as recommended by the manufacturer. All PCR reactions were performed in duplicate and a biological replicate was also included. This biological replicate was derived from an independent trial that had not been subjected to microarray expression profiling. Relative quantification was performed with the LightCycler software (version 3.5.3, Roche) using the Second Derivative Maximum method. For normalizing expression levels, the primers for the assumed house-keeping genes cap binding protein 20 (At5g44200) and actin 2 (At3g18780) from the *Arabidopsis* pathogen-inducible gene set (Sigma) was used. Cycling consisted of a 95°C activation step for 10 min, 40 cycles of 95°C, annealing temperature specific for each primer combination and an extension of 72°C for 2 min. Melting curve analysis and agarose gel electrophoresis of the qRT-PCR products was performed to confirm that the individual qRT-PCR products

corresponded to a single homogenous cDNA fragment of expected size. The products were also sequenced to confirm their identity.

Data comparison and Hypothesis testing

We compared the expression profiles of genes found to be differentially expressed in *cir1* with publicly available microarray data for *Pst* (*avrB*) and *Pst* at early (6 hours post inoculation) and late (24 hours post inoculation) time points in *Arabidopsis* ecotype Columbia plants. The data was obtained from NASCArrays (Experiment Reference Number: NASCARRAYS-120, AtGenExpress: response to virulent, avirulent, typeIII-secretion system deficient and nonhost bacteria). The data was normalised according to the Affymetrix MAS 5.0 scaling protocol. The triplicate experiments per time-point were averaged and compared to the mock inoculation at the respective time point. A $\log_2$ value greater than 0.75 was considered up-regulated while a log2 value less than -0.75 was considered down-regulated. Intermediate values were considered unchanged. Additional expression data of Arabidopsis genes induced during incompatible interactions with *Pst* (*avrRpt2*) generated by Maleck et al. (2000), Glombitza et al. (2004) and De Vos et al. (2005) was accessed from DRASTIC—INSIGHTS (Button et al., 2006).

3.4. Results

The cir1 mutant displays the characteristic constitutive expression of LUC, PR-1 and AtGSTF6.

Previously it had been shown that *cir1* displays constitutive expression of *PR* genes (including *PR-1* and *GST1*) and high levels of luciferase activity (Murray et al., 2002b). In order to confirm that the *cir1* mutation was stable under growth conditions at the University of Pretoria, the expression of marker genes, previously shown to be up- regulated in *cir1*, was tested. Quantitative PCR analysis showed that *LUC* and *PR-1* are constitutively expressed in *cir1* compared to *luc2* and Northern blot analysis confirmed the expected expression pattern of *AtGSTF6* (also called *GST1*) as reported by Murray et al. (2002b) (Figure 3.1).

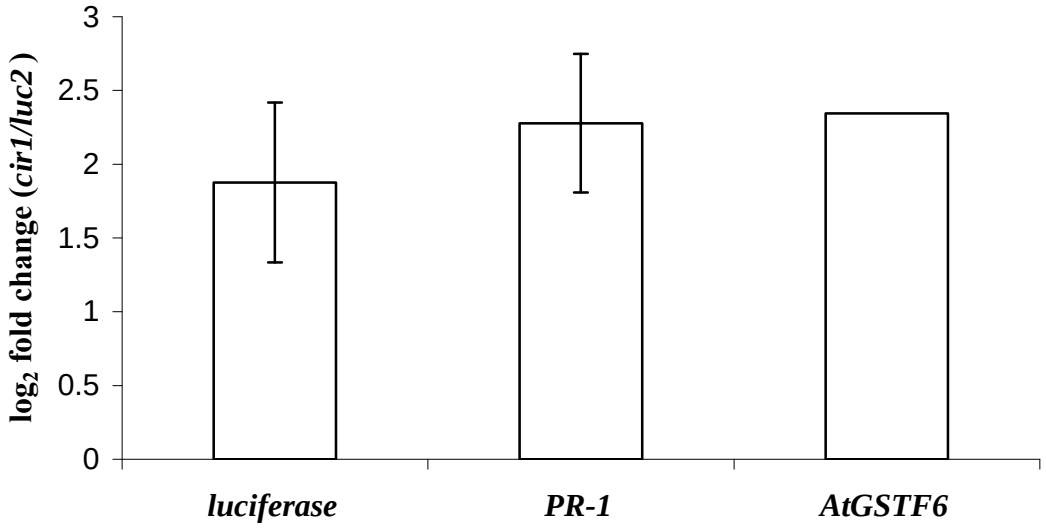

Figure 3.1. Expression of defence marker genes in *cir1* and *luc2* plants. Expression is represented by qRT-PCR data for *PR-1* and the luciferase reporter gene, while Northern blot analysis data from a single experiment, quantified by densitometry, is represented for *AtGSTF6*. Error bars represent the standard deviation of replicate experiments. The experiments were repeated with similar results.

Expression profiling of cir1 and luc2

Transcript levels of selected genes in leaves of *cir1* and *luc2* plants were directly compared using a set of 12 custom glass slide microarrays spotted with 500 probes corresponding to defence response and signalling genes. The microarray data were subjected to analysis using a mixed model analysis of variance (modified from Wolfinger et al., 2001) in the statistical program SAS v8.2. The null hypothesis for every gene was that there is no significant difference in expression between *cir1* and *luc2*. The resulting data was displayed as a "Volcano" plot, shown in Figure 3.2. Fifteen genes were regarded as differentially expressed in *cir1* compared to *luc2* at a p-value of $-\log_{10}(p)$ equal to 3 (this corresponds to a 1 in 1000 possibility of being incorrect by rejecting the null hypothesis) and a $\log_2$ fold change greater than 0.75 or less than –0.75 (Table 3.2). This threshold corresponds to a fold change of 1.7 and was selected so that subtle changes in expression could also be included for genes with low expression as in the case of Thilmony et al. (2006) who used a minimum fold change threshold of 1.5 and Truman et al. (2006) who used a minimum fold change threshold of 1.8. This represents approximately 5% of the genes screened. Some of the genes were represented

twice as a different cDNA probe of the same gene (results not shown). This provided further confidence that the results were reproducible.

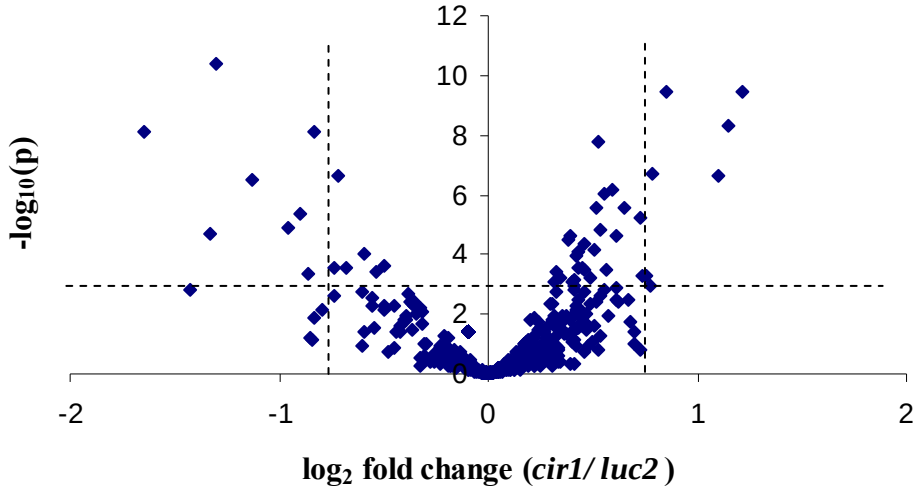

Figure 3.2. A volcano plot generated in SAS v8.2 of microarray data comparing expression in *cir1* and the *luc2*. Those ESTs with a p-value greater than $-\log_{10}(p)=3$ ($p=0.001$) and a $\log_2$ fold change greater than 0.75 or less than –0.75 were selected as differentially expressed.

Table 3.2. Genes differentially induced in the mutant *cir1* compared to the transgenic background *luc2* as revealed by microarray analysis (p<0.001)[*].

AGI Number	Gene Name	Full Name	Expression	Fold Change
At1g72770	AtP2C-HA	Protein phosphatase 2C	UP	2.1
At5g49480	AtACP1	Calcium-binding protein, salt inducible / calmodulin	UP	2.3
At1g02920	AtGSTF7	Glutathione-S-transferase -11	UP	2.2
At2g13790	AtSERK4	Leucine rich repeat protein kinase protein	UP	1.8
At2g37040	AtPAL1	Phenylalanine ammonia lyase	UP	1.7
At5g38530	tryptophan synthase β-like	Tryptophan synthase beta-like	UP	1.7
At3g61200	thioesterase	Thioesterase family protein	UP	1.7
At5g66400	dehydrin	Dehydrin (AtRAB 18 YSK group)	DOWN	5.6
At5g61600	AtEREBP-4	Transcription factor, AP2/ethylene response binding protein	DOWN	4.0
At5g60390	EF1-α	Elongation factor 1 alpha	DOWN	2.7
At3g16460	jacalin lectin	Jacalin lectin family	DOWN	2.5
At3g58500	AtPP2A-4	serine/threonine protein phosphatase PP2A-4 catalytic subunit	DOWN	2.5
At1g02340	HFR1	BHLH like protein long hypocotyl in far-red 1 (HFR1)	DOWN	1.9
At5g05410	AtDREB2A	DRE-binding protein	DOWN	1.8
At1g18710	AtMYB47	Myb family transcription factor	DOWN	1.8

[*]Statistical significance determined using a mixed model analysis of variance (ANOVA) according to Wolfinger et al. (2001).

The 500-probe microarray included the defence response genes *PR-1*, *PR-2*, *PR-5*, *PDF1.2* and *AtGSTF6*, previously shown to be up-regulated in *cir1*, as positive controls (Murray et al., 2002b). Probes corresponding to these genes were prepared independently for microarray spotting since they were not available in the L35 collection. However, these clones proved to be poor sources of microarray probes as the resulting spots were of consistently poor quality.

Confirmation of microarray data was carried out using complementary expression analysis techniques. The expression of three up-regulated (*AtGSTF7, AtACP1* and *AtSERK4*) and two down-regulated genes (dehydrin and *AtPP2A-4*) were confirmed using qRT-PCR or Northern blot analysis. Figure 3.3 shows the expression profiles for the selected genes in *cir1* relative to the expression in *luc2*. The expression pattern for the genes obtained from microarray analysis is also indicated. The amplitude of expression is higher in the qRT-PCR results in each case however it is important to note that the trend of expression is similar.

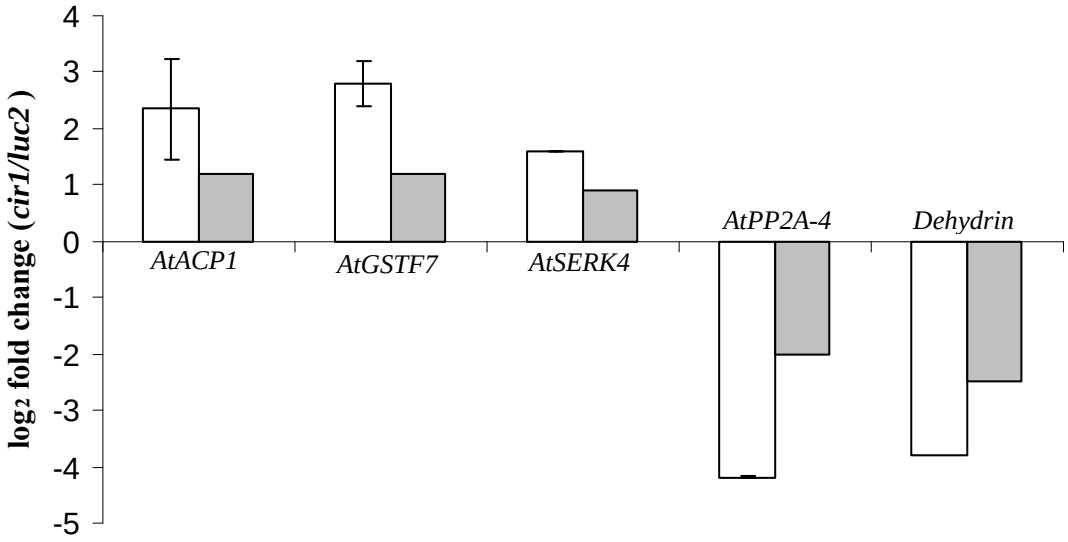

Figure 3.3. Confirmation of differential expression in *cir1* versus *luc2* plants observed in microarray studies. qRT-PCR results are shown for *AtACP1*, *AtGSTF7*, *AtSERK4* and *AtPP2A-4* (white bars). Northern blot analysis was performed for the dehydrin gene and quantified by densitometry (the result from a single experiment is displayed). The expression ratio for each gene in the microarray experiment is indicated as dark bars. The error bars represent the standard deviation of replicate experiments. The experiments were repeated with similar results.

Differentially regulated genes in cir1 are differentially regulated in a similar manner in Arabidopsis following Pst challenge.

Tao et al. (2003) demonstrated that gene expression profiles in a resistant interaction increased to higher levels earlier than in a susceptible interaction. Thus it would be expected that genes required for resistance against *Pst* in *cir1* would be induced/repressed to higher levels earlier in a resistant interaction with *Pst* compared to a susceptible interaction with *Pst*. The expression of these *cir1*-differentially expressed genes was compared to public microarray data from a *Pst* infiltration experiment in which compatible and incompatible interactions were studied (NASCARRAYS-120). The latter data shows induction of *PR-1*, *PR-2*, *AtPAL1, AtP2C-HA*, *AtACP1* and jacalin lectin, and repression of *AtEREBP-4* during an incompatible interaction with *Pst* (*avrB*) (Table 3.3). Other microarray studies investigating the same type of interaction show the induction of *PR-1, PR-2, PR-5*, *AtGSTF6*, *AtPAL1* (De Vos et al., 2005), *AtGSTF7* (Glombitza et al., 2004), jacalin lectin family protein (De Vos et al, 2005) and *AtDREB2A* (De Vos et al., 2005) and the repression of *AtEREBP-4* (De Vos et al., 2005) and *AtSERK4* (Maleck et al., 2000) during *Pst (avrRpt2)* challenge at late time points i.e. >12hours (Table 3.3).

Cir1 responsive genes show three distinct patterns of expression that match the expression patterns in response to *Pst* in the above datasets: 1) genes which are induced early during an incompatible interaction and later during a compatible interaction (*PR-2, AtP2C-HA, AtACP1, AtPAL1* and tryptophan synthase beta-like); 2) genes which are induced or repressed during both compatible and incompatible interactions at early and late time-points (*PR-1* and *HFR1* respectively) and 3) genes which are repressed early on during an incompatible interaction and remain unchanged or induced later during a compatible interaction (*AtEREBP4*) (Table 3.3).

Table 3.3. Expression pattern of genes differentially regulated in *cir1* in Col-0 plants challenged with *Pst* (data derived from publicly available microarray experiments: NASCARRAY-120[1] and Maleck et al., 2000[2]; De Vos et al., 2005[2]; Glombitza et al., 2004[2] which were compared to mock-inoculations).

Gene Name	GO Biological Process	*cir1**	COMPATIBLE		INCOMPATIBLE		
			Pst 6hpi[1]	*Pst* 24hpi[1]	*Pst*(*avrB*) 6hpi[1]	*Pst*(*avrB*) 24hpi[1]	*Pst* (*avrRpt2*)[2]
PR-1	SAR, defence response						
PR-2	SAR, response to cold						
PR-5	Regulation of anthocynin biosynthesis, SAR, response to UV-B.						
PDF1.2	Jasmonic acid and ethylene, insect & wound response						X
AtGSTF6	Response to water deprivation andoxidative stress.		X	X	X	X	
AtGSTF7	Toxin catabolism		X	X	X	X	
AtP2C-HA	Protein amino acid dephosphorylation						X
AtACP1	Hyperosmotic salinity response						X
AtPAL1	Phenylpropanoid biosynthesis, wounding & oxidative stress						
tryptophan synthase □-like	Tryptophan biosynthesis						X
AtSERK4	Protein amino acid phosphorylation						
thioesterase	Unknown						X
AtEREBP-4	Regulation of transcription						
HFR1	Signal transduction						X
jacalin lectin	Response to cold						
AtDREB2A	Response to water deprivation						
dehydrin	Response to ABA stimulus, cold acclimation, water deprivation.						X
AtMYB47	Jasmonic acid stimulus, response to salt stress						X
AtPP2A-4	Protein amino acid dephosphorylation		X	X	X	X	X
EF1-α	Translational elongation		X	X	X	X	X

*expression compared to *luc2*

X No Data Repressed No Change Induced

3.5. Discussion

Basal defences, which are employed unsuccessfully in susceptible plants, and R-avr interactions activate similar gene responses. However, it has been shown in some cases that the speed at which these responses are activated determines whether the interaction between pathogen and plant will result in disease or no disease (Jones and Dangl, 2006; Tao et al., 2003).

Cir1 has been shown to be resistant to the bacterial pathogen *Pst.* Therefore the current study investigated whether the expression of selected genes in *cir1* is similar to that observed during *Pst* challenge in Col-0 plants. Tao et al. (2003) demonstrated that the expression pattern of genes in a resistant interaction increased to higher levels earlier than in a suceptible interaction. Based on this premise, it was hypothesised that genes which are required for defence against *Pst* in *cir1* would be induced/repressed at an early time-point during an incompatible interaction and induced/repressed only later in a compatible interaction or remain unchanged. The induced genes, which match this profile, are: *PR-1, PR-2, AtACP1, AtP2C-HA, AtPAL1* and tryptophan synthase beta-like (Table 3.3). It has also been demonstrated that *PR-5*, *AtGSTF6* and *AtGSTF7* are induced during an incompatible interaction with *Pst* (De Vos et al., 2005; Glombitza et al., 2004, Table 3.3). Therefore, out of a total of 12 genes up-regulated in *cir1*, 9 genes are either induced early during an incompatible interaction and/or at a later time point following a compatible interaction with *Pst*. In addition, *AtEREBP4*, which is down-regulated in *cir1*, was repressed during the incompatible interaction with *Pst* (*avrB*) (Table 3.3). These expression profiles in *cir1* provide clues as to the mechanism of resistance against *Pst* in this plant. One could speculate that constitutive expression of these genes in *cir1* may prime the plant defence response against *Pst.*

How does this occur? Many of the genes up-regulated in *cir1* are well-known defence-related genes. For example, *PR-1*, *PR-2* and *PR-5* are produced in response to pathogen attack via the SA signalling pathway. The role of these genes in plant defence has been demonstrated in mutants compromised in SAR: non-expressor of *PR* genes 1 (*npr1*) or constitutive expressor of *PR* genes (*cpr1*) (Bowling et al., 1994; Cao et al., 1994). *PR-1* and *PR-2* were also among the 117 genes induced specifically by the Type III Secretion System (TTSS) effector proteins of *Pst* (Hauck et al., 2003). The biochemical property of PR-1 is not known while the PR-2 protein (1,3 β-glucanase) has antifungal activity and hydrolyses 1,3 β-glucan polymers

present in fungal cell walls. *PR-5* encodes an anti-fungal thaumatin-like protein, which is so-named due to the sequence similarity of the protein with an intensely sweet tasting protein isolated from the fruits of the West African rain forest shrub *Thaumatococcus danielli* (Cornelissen et al., 1986). SA accumulation following pathogen infection is through the action of two enzymes: phenylalanine ammonia lyase (PAL) and isochorismate synthase 1 (ICS1), although ICS1 is thought to play the major role (Wildermuth, 2001; Durrant and Dong, 2004). *AtPAL1* is up-regulated in *cir1* (Table 3.2) and is also induced in response to elicitors or during several incompatible interactions including *H. parasitica* (Edwards et al., 1987; Davis and Ausubel, 1989; Hahlbrook and Scheel, 1989; Mauch-Mani and Slusarenko, 1996). *AtPAL1* expression is induced 4hrs after challenge with avirulent *Pst* (*avrRpm1*) but is not significantly changed at the same time-point during *Pst* or *Pst hrp*$^-$ infection suggesting that early induction of *AtPAL1* may be a consequence of the recognition of the *avr* gene product (Truman et al., 2006). PAL activity provides the precursors for lignin biosynthesis and thus may provide further protection from infecting pathogens by lignification of the cell wall in *cir1*. *Cir1* displays high levels of SA accumulation and constitutive expression of *ICS1* (Murray and Denby, unpublished results). PAL1 and ICS1 may therefore both contribute to SA accumulation in *cir1*. The accumulation of SA has been demonstrated as necessary for *cir1*-mediated resistance against *Pst*, since *cir1 nahG* plants, which convert SA to inactive catechol, show wildtype susceptibility (Murray et al., 2002b).

Cir1 also displays constitutive expression of *Pst*-inducible genes involved in early defence signalling responses. An early response to pathogen attack is the transient changes in the ion permeability of the plasma membrane and the increase in the amount of cytosolic Ca^{2+} ions, which may be elicitor-derived or released from internal stores and mediate down-stream defence reactions (Blume et al., 2000; Grant et al., 2000, Xu and Heath, 1998). *AtACP1* is a calmodulin-related protein (Jang et al., 1998). Calmodulin proteins bind Ca^{2+} and are involved in decoding the Ca^{2+} signatures and transducing signals by activating specific targets and pathways (Snedden and Fromm, 2001). It is speculated that downstream responses to Ca^{2+} signalling may be an important component of resistance to *Pst* as it has been demonstrated that there is an increase in cytoplasmic calcium in response to *Pst* (*avrRpm1*) infection in Col-0 plants (Grant et al., 2000).

Another early defence response observed in *cir1* is the accumulation of glutathione-S-transferase (GST) proteins (Table 3.2, Fig.1). GSTs are involved in the detoxification of both

endogenous and xeno-biotic compounds (Marrs, 1996; Armstrong, 1997; Hayes and McLellan, 1999) including reactive oxygen intermediates (ROIs) produced following an incompatible plant:pathogen interaction. GST enzymes scavenge ROIs, maintaining ROI homeostasis in plant cell compartments (Mittler et al., 2004). *AtGSTF6* and *AtGSTF7* are induced by MeJA (von Rad et al., 2005), SA and ET (Glombitza et al., 2004) and following infection with *H. parasitica* (Maleck et al., 2000; Rairdan et al., 2001). GST accumulation following pathogen attack may therefore be an important requirement for defence against a wide range of pathogens. AtP2C-HA is a member of the plant protein phosphatase 2C family, which act as regulators of various signal transduction pathways (Rodriguez, 1998). In particular, *AtP2C-HA* is implicated in regulating ABA signalling (Rodriguez et al., 1998). The induction of *AtP2C-HA* during *Pst* challenge in Col-0 (Table 3.3) is consistent with a role for ABA signalling in the regulation of defence against this pathogen (Mohr and Cahill 2003, 2006). In addition, Melotto et al. (2006) provided evidence of a role for ABA in effecting stomatal closure in response to both virulent and avirulent *Pst*.

Tryptophan synthase is part of the tryptophan pathway and tryptophan is a precursor for several compounds including the major phytoalexin camalexin which is an antimicrobial secondary compound involved in defence against infection (Tsuji et al., 1992; Paxton et al., 1994). *AtSERK4* is up-regulated in *cir1* but down-regulated following *Pst* infection (Table 3.3). *AtSERK4* is up-regulated in response to flg22 (a peptide of the bacterial PAMP flagellin) treatment but is not induced under *Pst* infection, which suggests that the pathogen is able to suppress this response (Navarro *et al*., 2004). This is in accordance with the findings of Thilmony et al. (2006), which identifies *AtSERK4* as a PAMP-induced gene that is not induced in response to *Pst*. The up-regulation of *AtSERK4* in *cir1* suggests that this component of PAMP-induced basal defences may be activated in *cir1* prior to pathogen invasion and may thus be responsible for the resistance phenotype of *cir1* against *Pst*. It can be speculated that the up-regulation of *AtSERK4* results in the production of a transcription factor leading to the down-stream production of *PR* proteins responsible for overcoming the pathogen. Transcript profiling of *cir1* using whole genome microarrays and subsequent comparison to the 96 core basal defence response genes described by Truman et al. (2006), would be necessary to determine whether other PAMP-inducible genes are up-regulated in *cir1*.

Although *PDF1.2* is up-regulated in *cir1* (Murray et al. 2002), it is repressed in wild-type plants following both virulent and avirulent *Pst* infection (Table 3.3). *PDF1.2* is induced by the accumulation of both JA and ET (Penninckx et al., 1996, 1998). Suppression of *PDF1.2* at later time points may reflect the accumulation of SA following *Pst* infection, which inhibits JA and ET through a negative cross-talk mechanism. This cross-talk mechanism appears to be uncoupled in *cir1* as both SA-dependent and JA/ET-dependent genes are expressed to high levels (Murray et al., 2002b). Interestingly the *AtMYB47* and *HFR1* genes, which are induced by MeJa treatment (Yanhui et al., 2006; McGrath et al., 2005; De Vos et al., 2005), are suppressed in *cir1* (Table 3.3), probably by SA-dependent cross talk. *AtEREBP-4,* which is down-regulated in *cir1,* belongs to the AP2/ERF domain family of transcription factors, which binds to the GCC box promoter elements of pathogen responsive genes *PDF1.2, Thi 2.1, PR-4* (Zhou et al., 1997; Manners et al., 1998).

Gene discovery studies usually focus on those genes which respond positively in the organism of interest i.e., are up-regulated. Down-regulated genes are equally interesting as repression may have knock-on or direct effects to obtain a desired phenotype. Thus, the expression of down-regulated *cir1* genes following *Pst* infection were investigated. However, a clear correlation was not observed. Half of the genes down-regulated in *cir1* were up-regulated following both compatible and incompatible *Pst* infection. These included the dehydrin and *AtDREB2A* genes, which are both induced by wounding and water stress (Stintzi et al., 2001; Cheong et al., 2002). Wright and Beattie (2004) suggest that there is a greater water stress in incompatible interactions than in compatible interactions with *Pst* owing to the lower water potentials in the former interaction during the HR. This is in accordance with the observation in Table 3.3 showing the induction of the dehydrin and *AtDREB2A* genes during *Pst* (*avrB*) challenge. No HR is observed in *cir1* even upon pathogen challenge with *Pst* (Murray et al., 2002b), which may account for the repression of dehydrin and *AtDREB2A* in *cir1*. The biological role of these two genes in *cir1* is unclear, as *cir1* plants did not display increased sensitivity to drought stress (results not shown).

Relatively few genes were differentially expressed in *cir1* in our study. The most probable reason for this is that the custom microarray did not contain all defence response genes in Arabidopsis. An additional reason for this may be that expression in *cir1* was compared to expression in its transgenic background *luc2* without pathogen challenge as it was hypothesised that genes required for resistance in *cir1* would be constitutively expressed. It is

also possible however, that some genes required for *cir1* resistance would only be induced upon pathogen challenge.

In conclusion, by using a combination of a subset of customised *Arabidopsis* genes, and publicly available microarray data, genes implicated in defence have been identified in *cir1*. Further studies on *cir1* should highlight important genes required for both basal and gene-for-gene resistance to *Pst*. Transcript levels in *cir1* have been measured; however gene function studies are necessary to investigate the role of the genes *in vivo*. Over expression and knock-out experiments employing RNAi or crosses with T-DNA mutants of the respective genes would be the next step in determining if they are required for CIR1-mediated resistance to *Pst*.

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CHAPTER 4
HOST TRANSCRIPT PROFILING IN *ARABIDOPSIS THALIANA* ECOTYPE COL-5 DURING INFECTION WITH THE *EUCALYPTUS* ISOLATE OF *RALSTONIA SOLANACEARUM*, K (BCCF 401).

This chapter has been written in the format of an article for the Journal of Functional Plant Biology. The initial infection trials between *R. solanacearum isolate* BCCF 402 and *Arabidopsis thaliana* ecotype Col-5 were performed by Joanne Weich (2004). I performed the subsequent microarray work including RNA isolations, hybridisations, data analysis, bioinformatics analysis and qRT-PCR validations.

4.1 Abstract

Ralstonia solanacearum, the causal agent of bacterial wilt, affects several plant species and results in devastating crop losses worldwide. This soil borne vascular pathogen also infects the tree species *Eucalyptus* in Congo and South Africa. The compatible interaction between *Arabidopsis thaliana* ecotype Col-5 and the *Eucalyptus* isolate K (BCCF 401) was selected for further molecular characterisation of the plant defence response during *Ralstonia* infection using microarray analysis. A screen of 5000 *Arabidopsis thaliana* ESTs revealed approximately 120 genes differentially regulated by *R. solanacearum* infection at a significance threshold of $p<0.03$ (Bonferroni corrected). Marker genes of the methyl jasmonate/ ethylene defence response pathways, PR-3 and PR-4, were up-regulated while PR-5, a marker of the salicylic acid defence signalling pathway, was down-regulated. The 120 genes differentially expressed during *R. solanacearum* infection showed similar expression profiles during infection induced by compatible and incompatible interactions with *Pseudomonas syringae* pv. tomato (*Pst*) and a compatible interaction with *Botrytis cinerea*. Comparative expression profiles also suggested a role for Abscisic Acid in Col-5 during *R. solanacearum* infection of Col-5. The basal defence responses in Col-5 against *R. solanacearum* infection were investigated by comparing the expression data to that during treatment with the pathogen associated molecular patterns (PAMPs) flg22 and lipopolysaccharide, and the Type Three Secretion System deficient *Pst hrp*$^-$ mutant. A subset of the genes which were induced by PAMPs were repressed by *R. solanacearum* infection, and vice versa, suggesting that these genes may be repressed or induced, respectively, by specific *R. solanacearum* effectors. Together, this research represents the first expression profiling experiment between *R. solanacearum* and a susceptible host.

4.2 Introduction

Plants respond to pathogen attack via an integrated set of defences, which may be constitutive or induced (Thatcher et al., 2005). Pathogens that are able to overcome constitutive antimicrobial compounds and structural barriers encounter an induced response that is triggered by the recognition of pathogen-derived elicitors, which may be general (e.g. PAMPs) or effectors, which are race specific (e.g. avr proteins). The elicitors and effectors are perceived by receptors located either at the cell surface or inside the cell (Dardick and Ronald, 2006). Pathogen recognition by the plant results in a series of signalling cascades that involve the signalling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). There is a tendency for plants to rely on the JA and ET signalling pathways for resistance against necrotrophic pathogens and on the SA signalling pathway for resistance against biotrophic pathogens (Thomma et al., 1999). The activation of signalling components eventually leads to the expression of plant defence and protection genes such as pathogenesis related (PR) proteins, glutathione-S-transferases (GST), peroxidases, proteinase inhibitors and the production of secondary antimicrobial compounds and even the cross-linking of cell wall proteins (Thatcher et al., 2005).

Many authors have demonstrated the suitability of Arabidopsis microarrays for the study of plant-pathogen interactions including responses to insect, fungal, viral and bacterial pathogens (Reymond et al., 2000; Zwiesler-Vollick et al., 2002; Narusaka et al., 2003; Marathe et al., 2004). However, most research has focused on the resistant interaction. Susceptible interactions have only recently received attention in microarray studies (for example, Dowd et al., 2004; Thilmony et al., 2006). The value of investigating a susceptible interaction lies in the finding of Tao et al. (2003) who demonstrated that, in the interaction between Arabidopsis and *Pseudomonas syringae*, the gene expression changes that occur in a resistant interaction (incompatible) are similar to those in a susceptible interaction (compatible), only the amplitude of expression is higher earlier on in a resistant interaction than in a susceptible one. Indeed, a similar phenomenon was found to be responsible for the difference in resistance and susceptibility to *Verticillium dahliae* in cotton species; the difference in susceptibility being associated with the timing and intensity of certain gene expression changes (Bell, 1994). Similar expression analyses support the view that the compatible and incompatible responses share similar transcriptional expression profiles (Katagiri and Glazebrook, 2003; Thilmony et al., 2006).

A susceptible plant also responds to general elicitors (i.e. PAMPs such as bacterial lipopolysaccharides (LPS), flagellin, cold-shock protein and elongation factor Tu, and fungal glucan and chitin) to mediate a basal defence response. However these responses are insufficient to prevent disease onset (Jones and Dangl, 2006). The 22 amino acids found on the N-terminus of flagellin, the subunit of the bacterial surface structure flagellum, is conserved in several bacterial pathogens and is able to induce a defence response in plants to a higher level than flagellin itself (Felix et al., 1999). LPS from Gram-negative bacteria induces an oxidative burst and the production of antimicrobial enzymes in pepper and tobacco (Newman et al., 2000; Meyer et al., 2001). The pretreatment of plants with LPS results in the enhancement of the plant's defence response to subsequent pathogen challenge and LPS was able to potentiate the expression of *PR* genes upon subsequent bacterial inoculation (Newman et al., 2000). In some cases, bacterial PAMPs may not be detected by the host e.g. although *R. solanacearum* pathogen possesses functional flagellin, it is not responsible for the activation of a defence response in Arabidopsis (Pfund et al., 2004). Arabidopsis plants challenged with the wild-type and aflagellate *R. solanacearum* strains of isolate K60 showed similar disease levels regardless of whether they contained the flagellin receptor FLS2 or not. Microarray expression profiling in Arabidopsis has shown that PAMPs such as flg22 and LPS induce basal defence responses and that the responses induced by both these PAMPs overlap to some extent (Zeidler et al., 2004). Experiments on Arabidopsis plants challenged with mutants of bacterial pathogens deficient in the Type Three Secretion System (TTSS) pathway *(hrp$^-$)* and wild type bacterial pathogens suggest that specific effectors are able to suppress host basal defences, which are otherwise induced by PAMPs, to cause disease (Thilmony et al., 2006; Truman et al., 2006).

Ralstonia solanacearum is considered one of the most important plant pathogenic bacteria, causing bacterial wilt disease on a broad range of hosts including potato and the tree species *Eucalyptus*. The pathogen enters the host via root wounds or sites of secondary root emergence and moves towards the xylem vessels where it multiplies and spreads (Salanoubat et al., 2002). The root cortex and vascular parenchyma are colonised and cell walls are disrupted as a result of the extracellular products such as extracellular polysaccharide (EPS1), which facilitates the spread of the pathogen through the vascular system, and several plant cell wall-degrading enzymes, which result in the accumulation of cellular debris. This effectively destroys the plants vascular system. Once the plants water-uptake system is compromised, the plants wilt completely (Genin and Boucher, 2002).

In Southern Africa, *R. solanacearum* poses a threat to the forestry industry as the disease was detected in *Eucalyptus* plantations in South Africa and Uganda (Coutinho et al., 2000; Roux et al., 2001). The presence of the pathogen in *Eucalyptus* plantations is a cause for concern as *Eucalyptus* is increasingly clonally propagated (Coutinho et al., 2000). The *Eucalyptus* isolate K (BCCF 401) from South Africa (Fouch-Weich et al., 2006) was also able to cause disease on Arabidopsis ecotype Col-5 (Weich, 2004). The use of Arabidopsis as a host against the *R. solanacearum* pathogen was previously demonstrated by Deslandes et al. (1998) who showed that the tomato isolate of *R. solanacearum* (GMI1000) was pathogenic on ecotype Col-0 and did not cause disease on ecotype Nd-1. Figure 4.1 shows the disease index for each bacterial strain-ecotype combination from a single trial eighteen days after inoculation with the *R. solanacearum* isolate BCCF 401 or GMI1000 (Weich, 2004). Similar disease indices were obtained for subsequent trials (Weich, 2004). Col-5 was susceptible to both GMI1000 and BCCF 401, however GMI1000 was more virulent, causing severe wilt symptoms on Col-5 earlier than the *Eucalyptus* isolate BCCF 401. Ecotype Nd1, in comparison to Col-5, showed resistance to strains GMI1000 and BCCF 401.

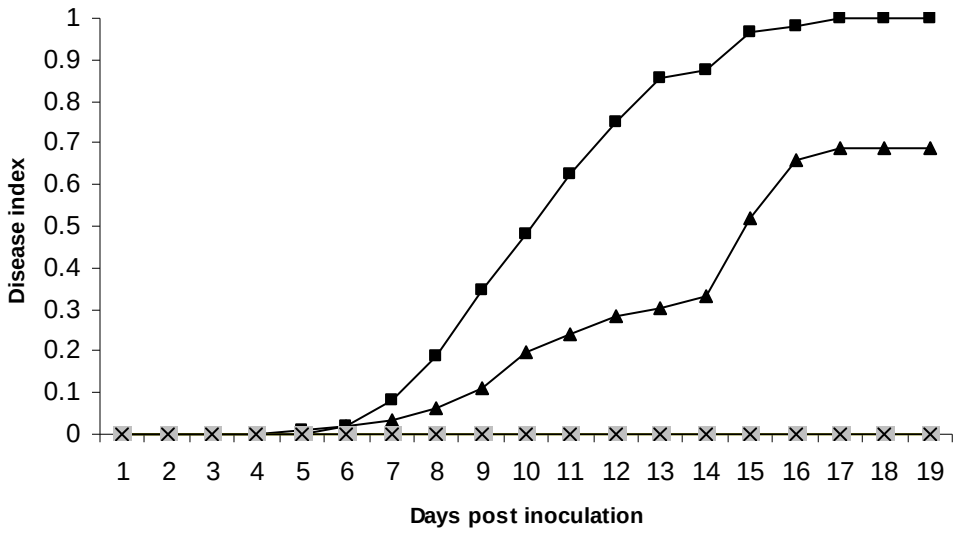


Figure 4.1. Disease index for Arabidopsis ecotypes infected with strains of *R. solanacearum.* Ecotype Col-5 infected with *R. solanacearum* ecotype GMI1000 (■), ecotype Col-5 infected with isolate BCCF 401 (▲), ecotype Nd-1 after challenge with GMI1000 (■) and ecotype Nd-1 after challenge with BCCF 401(X). The disease index was calculated based on data from 14 individual plants over 20 days. Replicate infection experiments yielded similar results. Data from Weich (2004).

Based on the susceptibility observed in Col-5 to *R. solanacearum* BCCF 401, this interaction was investigated in a microarray experiment profiling the expression of approximately 20% of the Arabidopsis genome. The aim was to determine the gene expression changes that take place in the plant during *R. solanacearum* infection. Subsequently, bioinformatics comparisons using publicly available data were performed to address the following questions: 1) does *R. solanacearum* infection induce an expression profile that is consistent with the trend for a necrotrophic pathogen, 2) can the signalling pathway(s) important for defence be predicted and 3) is there evidence for basal defence responses in Col-5 against *R. solanacearum* BCCF401? The motivation for investigating basal defence expression in Col-5 against the pathogen is two-fold; to determine host genes possibly targeted by specific effectors and to identify genes, which could be targeted for genetic manipulation to improve host resistance against the pathogen.

4.3 Materials and Methods

4.3.1 Plant material

Seeds of Arabidopsis ecotype Col-5 were obtained from The Nottingham Arabidopsis Stock Centre (NASC, www.arabidopsis.info) and sterilized with 70% ethanol, 1.5% sodium hypochlorite and washed in sterile distilled water. Seeds were germinated on Murashige and Skoog (Murashige and Skoog, 1962) medium for two weeks under 16 hr day conditions. The plants were transferred to Jiffy pots (Jiffy France, Lyon, France) and grown for four weeks under 16 hr light, 25ºC-26ºC, 50% relative humidity and 300-350 lum/sqf. The plants were watered with a solution of Feedall® (Aquasol (Pty) Ltd, Potchefstroom, SA) once a week.

4.3.2 Inoculations

R. solanacearum isolate BCCF 401 or GMI1000 was grown on solidified Bacto-agar Glucose Triphenyltetrazolium chloride (BGT) media at 28ºC for 48 hr. Colonies that displayed a virulent phenotype (mucoid) were transferred to liquid B media and incubated overnight at 28^{o}C.

Inoculations were performed according to Deslandes et al. (1998). Briefly, the Jiffy pots containing the Arabidopsis plants were cut horizontally through the middle to wound and expose the roots and soaked in a solution of bacteria ($1x10^{8}$ cfu/ml) for 30 min. Control plants were soaked in a solution of the media without any bacteria. The plants were placed on moist vermiculite and maintained at 26ºC, 60%-70% humidity and 16 hr day length. The plants were rated on a scale from zero (no disease) to 4 (100% wilted/dead plants) according to the method of Deslandes et al. (1998), where wilt symptom 1 is descriptive of plants showing less than 25% of the leaves wilted; symptom 2, less than 50% of the leaves wilted; symptom 3, more than 50% to 75% of the leaves wilted and symptom 4, 76%-100% of the plant is wilted to dead. The data was used to calculate the Disease Index using the formula, $DI = [\sum(n_i \times v_i) / (V \times N)]$, where DI = Disease Index; n_i = number of plants with respective disease rating; v_i = disease rating (0, 1, 2, 3 or 4); V = the highest disease rating (4); and N = the number of plants observed (Winstead and Kelman, 1952). The disease index is shown in Figure 4.1.

The aerial parts of plants displaying wilt symptom 1-2 (termed early wilt) and wilt symptom 3-4 (termed late wilt) were harvested (discarding the roots). Similarly, the aerial parts of control plants showing no wilt symptoms at the respective time-points were harvested. Eight-

twelve plants were harvested for each biological replicate experiment. The experiment was performed twice.

4.3.3 RNA isolation

Total RNA was isolated from control and infected tissue using TriReagent (Sigma, Aldrich) according to the manufacturer's instructions and further purified using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, California). mRNA was isolated using the OligoTex mRNA Isolation Kit (Qiagen). RNA yield was determined by measuring absorbency at 260 nm, using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA).

4.3.4 Microarray experiments

Corning Gap II slides consisting of 7200 Arabidopsis cDNA elements (from the Mendel Biotechnology L35 collection) were purchased from the University of Cape Town, South Africa (capar). The identities of the array elements are included in Appendix A. Probes were labelled using 500 ng mRNA per labelling reaction using the Amerham Indirect Labelling Kit. cDNA was purified prior to dye-coupling using the Qiagen PCR purification kit (Qiagen) and again after labelling. Prior to hybridisation, slides were pre-treated in a solution of 0.2% BSA, 0.2% SDS and 3.5X SSC at 65^{o}C for 15 min. Slides were washed in Sigma water and dried using compressed air. The hybridisation solution, consisting of 50% formamide, 25% Amersham hybridisation buffer (GE Healthcare Ltd.) and the Cy3 and Cy5 (Amersham Biosciences) labelled probes were heated to 95^{o}C and then cooled on ice. The hybridisation solution was added to the slides under a clean coverslip and then allowed to hybridise overnight at 42^{o}C in a HybUP hybridisation chamber (NB Engineering, Pretoria, South Africa). The slides were washed in a solution of 1.0 X SSC, 0.2% SDS for 4 min at 42^{o}C, followed by two washes in 0.1 X SSC, 0.2% SDS for 4 min at 42^{o}C, and three washes in 0.1 X SSC for 1 min at room temperature. The slides were dipped in MilliQ water a few times before being dried using compressed air and scanned using the Axon GenePix 400B Scanner (Axon Instruments, Foster City, CA, USA).

Data was captured using GenePix (v 2.0). A grid was overlaid and spots with poor morphology were flagged from the analysis. The experimental design was a direct comparison for each symptom comparing the wilted samples with non-wilted samples. The dye assignments were reversed in a subsequent experiment and a biological replicate was

performed. In total, per symptom (early wilt or late wilt) 4 slides were hybridised. Figure 4.2 shows the experimental design that was used.

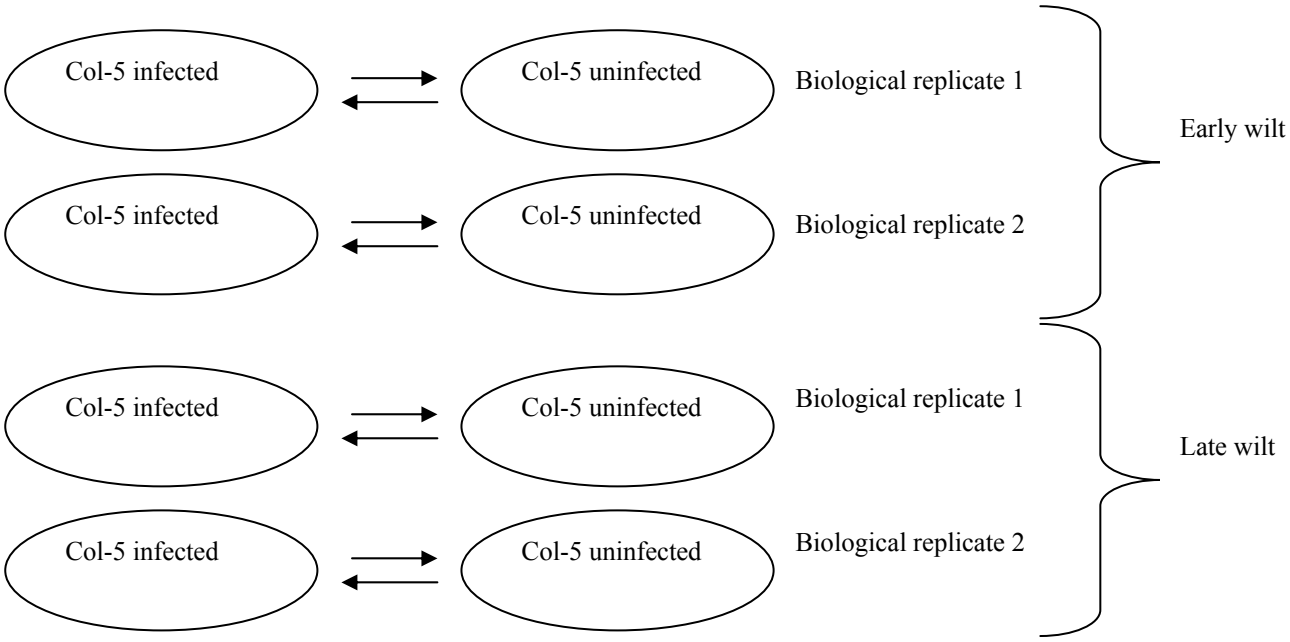


Figure 4.2 Experimental Design employed in microarray expression profiling between Col-5 plants infected with *R. solanacearum* BCCF 401 and Col-5 plants that were uninfected at two time-points: early wilt and late wilt. Each oval represents a sample. The arrows represent a slide and the head of the arrow represents a sample labelled with the Cy5 dye while the tail of the arrow represents a sample labelled with the Cy3 dye (Naidoo et al., 2005).

Gene expression data were normalized and significant gene expression differences identified using the mixed model ANOVA approach of Wolfinger et al. (2001) as described in Chapter 3, section 2.2.5. The data was adjusted for multiple testing using the Bonferroni correction and volcano plots were generated for both wilting conditions. Those genes with a $\log_2$ fold change greater than 0.75 and less than -0.75 with a $-\log_{10}P>1.5$ ($p<0.03$) were selected as differentially expressed in response to the infection. The normalised microarray data is available as supplementary data in a MIAME compliant format at the following website: http:// www.bi.up.ac.za:8080/base2.

4.3.5 Quantitative reverse-transcription PCR (qRT-PCR)

Two-step quantitative reverse-transcription PCR (qRT-PCR) was performed using a LightCycler instrument (Version 1.2, Roche Diagnostics GmbH). PCR primers were designed

using Primer Designer version 4 (Scientific & Educational Software, Cary, North Carolina, USA). Primer sequences are as follows: PR-3 (At3g12500) forward 5'GACTGCTCAGCCTCCCAAAC3' and reverse 5'ATACGATCGGCGACTCTCCC3'; Sip1 (At3g57520) forward 5'CGATAACCGTTCTCCAACAG3' and reverse 5'AAAGTCAAGCCCAACCTC3'; TAT (At5g53970) forward 5'TTCCTCGCATCGACCAGAAG3' and reverse 5'AGTTGCATCTGCTGCAAACG3'; OEC23 (At1g06680) forward 5'CAACAATGCAGTGGCAACAG3' and reverse 5'GCTTGTGCTTTGCAGATGTC3'. PR-4 selected from the purchased Primer library for Arabidopsis Pathogen-inducible Genes (Sigma, St Louis, Missouri, USA). Two micrograms of total DNaseI-treated and column-purified RNA extracted from wilted and control plants were reverse transcribed into first strand cDNA using ImpromII reverse transcriptase (Promega, Madison, WI) according to manufacturer's instructions. The LightCycler FastStart DNA Master^{PLUS} SYBR Green I system (Roche) was used for real-time PCR starting in a standard 20 µl reaction as recommended by the manufacturer. All PCR reactions were performed in duplicate and a biological replicate was included. Relative quantification was performed with the LightCycler software (version 3.5.3, Roche) using the Second Derivative Maximum method. For normalizing expression levels, the Sigma inducible primer pair (Cap Binding Protein (CBP) 20) or the elongation factor-1-alpha-related GTP binding protein factor (W43332, At1g18070.1, forward 5'TGCGGTTGTCGAGGAGTGGTG3' and reverse 5'AACCCGAAAGCCGTCTCCTG3') were used. Cycling consisted of a 95^{o}C activation step for 10 min, 40 cycles of 95^{o}C, annealing temperature specific for each primer combination and an extension of 72^{o}C for 2 min. Data acquisition was performed between 72^{o}C and 80^{o}C. Melting curve analysis and agarose gel electrophoresis of the qRT-PCR products were performed to confirm that the individual qRT-PCR products corresponded to a single homogenous cDNA fragment of expected size.

4.3.6 Gene ontologies

Gene ontologies (GO) for the 5000 unigenes represented on the cDNA microarray were determined using GOSlim (www.arabidopsis.org) at level 1 for categories: biological process, molecular function and cellular component. Gene ontologies were determined for genes differentially regulated by infection by using FatiGO (http://fatigo.bioinfo.cipf.es/). The list of Atg AGI locus identifiers of those genes which were found to be a) up regulated during early wilt, b) down regulated during early wilt, c) up regulated during late wilt and d) down regulated during late wilt, were entered into the program. The GO was determined for the

category biological process and the level was set to 5. Only those GOs with more than 1 gene per GO are represented. Over-represented GO terms in the category biological process was determined using GOStat (Beißbarth and Speed, 2004) by comparing to the GO terms of the 5000 unigenes represented on the microarray and to that of the whole genome. A χ^2 Test or a Fischer's Exact Test was used to approximate the p-value that represents the probability that the observed number of counts of each GO term could have resulted by randomly distributing this GO term between the tested group and the reference group. The error rate inadvertently generated by multiple testing was controlled using the Holm correction.

4.3.7 Data comparison and hypothesis testing

Arabidopsis Affymetrix microarray data, centered around a mean of 1000, was downloaded from NASCArrays (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl.). Experiments were: NASCARRAYS-120 (*Pst* experiments), NASCARRAYS-167 (*Botrytis cinerea* infection), NASCARRAYS-172 (ACC, ethylene precursor treatment), NASCARRAYS-174 (MeJA treatment), NASCARRAYS-176 (ABA treatment), NASCARRAYS-192 (SA treatment) and NASCARRAYS-137 and NASCARRAYS –141 (for drought stress and control treatments respectively). Replicate data was averaged and the $\log_2$ fold change was calculated for each gene relative to the control in each experiment in Microsoft Excel. Those genes, which were found to be differentially expressed during late wilt after *R. solanacearum* BCCF 401 infection (128 genes) were selected for analysis under different biotic and abiotic stress conditions. The log2 fold expression ratios of these 128 genes were extracted from the Microsoft Excel Spreadsheet. Data was available for only 120 of the 128 genes. The combined data for the 120 genes were clustered hierarchically using Manhattan distance and complete linkage in The Institute for Genomic Research (TIGR) Multi-experiment viewer (TIGR MeV v2.2).

PAMP data for flg22 and LPS treatment was downloaded from NASCARRAYS-121 and the gene expression data for the 120 genes (of the 128 genes found to be differentially expressed during late wilt) were extracted and computed in Microsoft Excel. Data for *Pst hrp*$^-$ treatment and *Pst* DC3000 treatment from NASCARRAYS-120 were similarly extracted. Replicate data within each dataset was averaged and $\log_2$ fold change was calculated for each gene at each treatment and time-point relative to the relevant controls. Data for LPS at 4 hr, flg22 at 4 hr, *Pst hrp*$^-$ at 24 hr and *Pst* DC3000 at 24 hr were compared to *R. solanacearum* BCCF 401 infection profiles for the 120 selected genes in TIGR MeV. Late time-points were selected to

facilitate comparability to the late wilt expression profile induced by *R. solanacearum*. Genes were clustered manually into several clusters based on the criteria that:

1) the genes were induced by PAMPs (either flg22, LPS or other PAMPs represented by induction by *Pst hrp*$^-$ treatment),
2) induced during *Pst* DC3000 infection and induced by *R. solanacearum* infection (cluster I, 13 genes), repressed by PAMPs, repressed during *Pst* DC3000 infection and repressed during *R. solanacearum* infection (cluster II, 9 genes),
3) genes, which are induced by PAMPs, induced by *Pst* DC3000 but repressed during *R. solanacearum* infection (cluster III, 10 genes) and
4) genes which are PAMP-repressed, and *R. solanacearum* induced (cluster IV, 6 genes).

The accepted threshold for an up-regulated gene was a $\log_2$ fold change greater than 0.75 and less than -0.75 for a down-regulated gene.

4.4 Results

4.4.1 R. solanacearum infections

The Arabidopsis ecotype Col-5 was infected with Eucalyptus isolate BCCF 401 and the plants observed every day for 20 days. Figure 4.3 shows wilt symptoms on Col-5 after infection. Col-5 showed wilting symptoms approximately 10 days after inoculation with the pathogen with wilt symptom 0.5 beginning as early as 5 days (Figure 4.3 B). Wilt symptom 1 to 2 was observed 7 to 10 days after infection (Figure 4.3 C, D; early wilt) while wilt symptom 3 to 4 was observed 15 to 20 days post infection (Figure 4.3 E, F; late wilt).

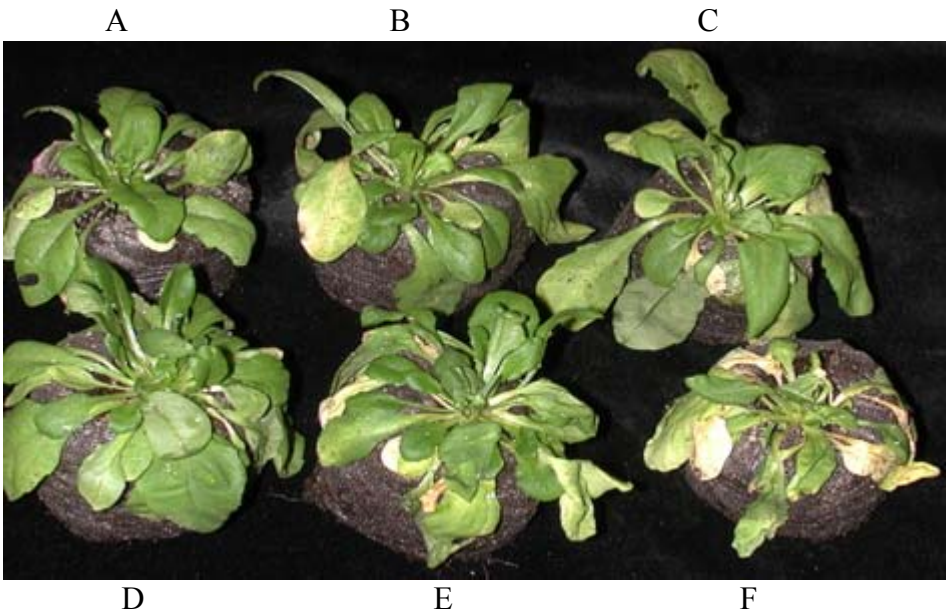


Figure 4.3 Wilt symptoms on Col-5 inoculated with $1X10^8$ cfu/ml of *R. solanacearum* isolate K using a root-inoculation method. The control plants were inoculated with a suspension of media and water. A: healthy control plant, no wilt symptom; plant showing wilt symptom B: 0.5; C: 1; D: 2; E: 3 and F: 4.

4.4.2 Expression profiling of Col-5 after infection with R. solanacearum isolate BCCF 401.

Aerial parts of Col-5 plants infected with *R. solanacearum* and showing wilt symptom 1 or 2 (early wilt) were harvested and subjected to microarray gene expression profiling in comparison to uninfected plants. Microarray expression profiling was also carried out on Col-5 plants infected with *R. solanacearum* and showing wilt symptom 3 or 4 (late wilt). The cDNA microarray slides used for profiling contained 5000 unigenes representing approximately 20% of the Arabidopsis genome. Table 4.1 shows the Gene Ontologies of the genes represented on the cDNA microarray slide as a percentage of that GO in the whole genome. The cDNA microarray does not contain any bias of GO terms although it does

contain 28% and 29% of genes annotated as response to abiotic or biotic stimulus and response to stress respectively relative to the whole genome (Table 4.1).

Table 4.1 Gene Ontologies for 5000 unigenes represented on the Arabidopsis cDNA microarray used for expression profiling of the susceptible interaction between *R. solanacearum* BCCF401 and Col-5.

GO Category	Description	% of genes relative to the whole genome
Cellular Component	other cellular components	17
	other membranes	17
	other intracellular components	32
	other cytoplasmic components	31
	chloroplast	27
	nucleus	24
	plastid	27
	mitochondria	44
	ribosome	34
	cytosol	31
	plasma membrane	25
	cell wall	18
	ER	18
	Golgi apparatus	18
	extracellular	12
Molecular Function	other molecular functions	12
	other enzyme activity	19
	hydrolase activity	21
	other binding	20
	transferase activity	23
	protein binding	22
	DNA or RNA binding	18
	transporter activity	22
	transcription factor activity	16
	kinase activity	21
	nucleotide binding	16
	structural molecule activity	24
	nucleic acid binding	22
	receptor binding or activity	14
Biological Process	other biological processes	18
	other metabolic processes	23
	other cellular processes	23
	protein metabolism	17
	response to abiotic or biotic stimulus	28
	cell organization and biogenesis	25
	transport	27
	response to stress	29
	developmental processes	19
	transcription	26
	signal transduction	19
	electron transport or energy pathways	21
	DNA or RNA metabolism	18

The expression data was analysed in SAS v8.2 using a mixed model ANOVA (Wolfinger et al., 2001) and "Volcano plots" were generated. Based on these volcano plots, the number of genes significantly up and down-regulated at a significance level of $p<0.03$ (Bonferroni corrected) and a $\log_2$ fold change >0.75 or <-0.75 were counted and are represented in Figure 4.4. The table of differentially regulated genes is available in Appendix B.

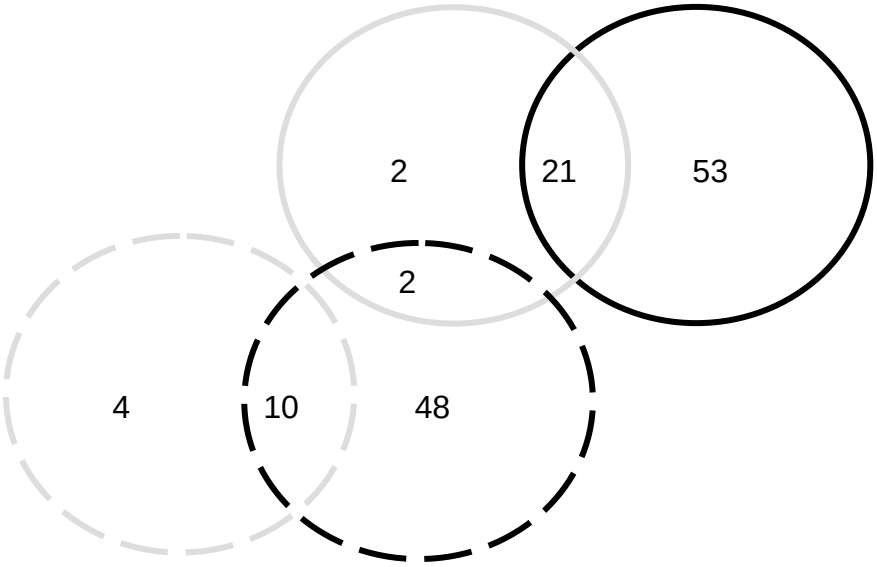


Figure 4.4. Venn diagram representing up-regulated (solid lines) and down-regulated (dashed lines) gene sets in early (grey circles) and late wilt stages (black circles) in response to *R. solanacearum* BCCF 401 infection. Genes were selected following mixed model ANOVA analysis and only those genes considered significantly up and down regulated ($\log_2$ fold change >0.75 or <-0.75 respectively; p-value <0.03) are represented.

The expression of some genes changed dramatically in response to *R. solanacearum* BCCF 401 infection, for example: pathogen-responsive alpha-dioxygenase (At3g01420) which was up-regulated 22-fold by *R. solanacearum*, osmotin 34 (At4g11650), which was up-regulated 19-fold and Lipid Transfer Protein 3 (LTP3) (At5g59320) which was up-regulated 14-fold during the late wilt time-point respectively. The most repressed genes were Pathogen and Circadian Controlled 1 (PPC1) (At3g22231), which was down-regulated approximately 3-fold, GATA type zinc finger domain containing protein (At3g54810), which was down-regulated approximately 3-fold and an ethylene response factor subfamily gene (At2g44840) which is approximately 2-fold down-regulated during late wilt stages in response to *R. solanacearum* BCCF 401 infection. The two genes that were induced during early wilt in

response to the pathogen and that were then repressed at the late wilt stage are a putative clathrin coat assembly protein (At1g47830) and an unknown expressed protein (At1g51670).

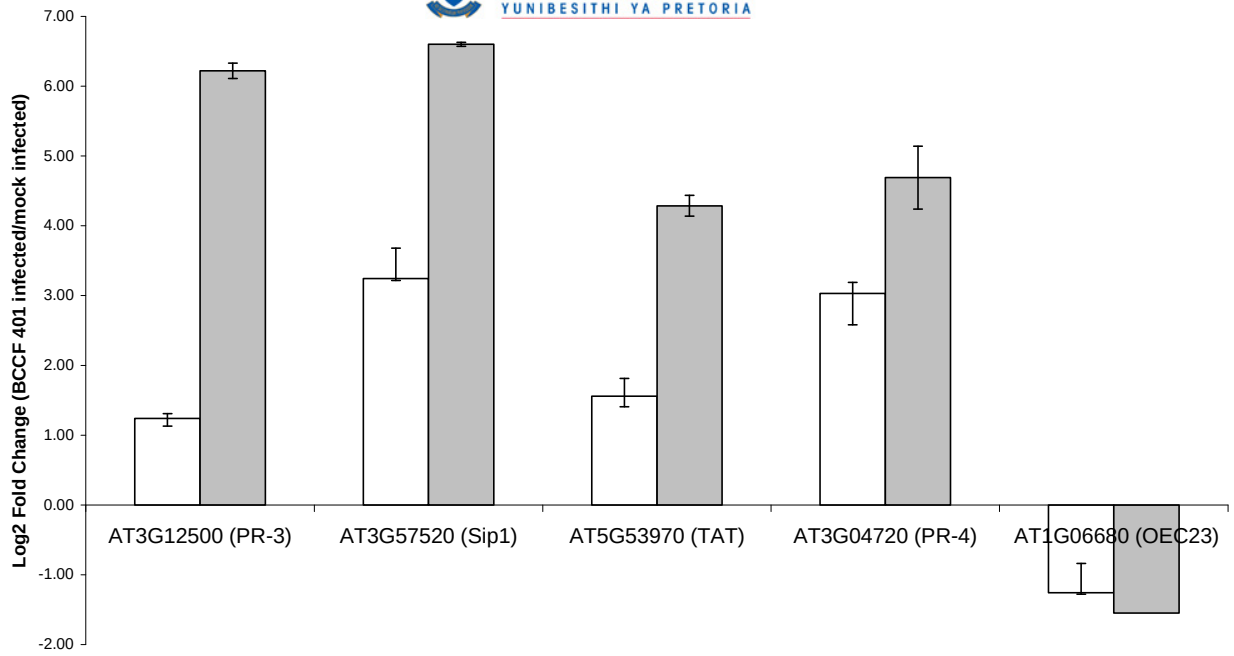
4.4.3 *Confirmation of Microarray Data*

qRT-PCR analysis was performed on selected genes with low p-values and their expression profiles compared to microarray data to confirm that these genes were truly differentially regulated by BCCF 402 infection. Figure 4.5 a and b show the expression ratio for five selected genes: basic endochitinase (PR-3), Seed imbibition protein homologue (Sip1), Tyrosine amino transferase (TAT), pre-hevein like protein (PR-4) and oxygen evolving complex 23 (OEC23), in the qRT-PCR experiments and the microarray experiments respectively.

The qRT-PCR results match the expression patterns for each of the genes tested. In most cases, the expression is higher than that obtained with the microarray data. This may relate to the higher sensitivity of the qRT-PCR technique in determining expression levels. The qRT-PCR data was normalised to Cap Binding Protein 20 (At5g44200) and to the elongation factor-1-alpha-related GTP binding protein factor (At1g18070), which appeared to be expressed constitutively in microarray experiments (fold change = 1, and p value= 0.000315, late wilt expression profile) and showed constitutive expression in most biotic stress conditions tested based on Affymetrix microarray data available on GENEVESTIGATOR (Zimmermann et al., 2004). In all cases tested, normalisation using either the Cap Binding Protein 20 gene or At1g18070, produced similar results (results not shown). The qRT-PCR data supports the microarray data confirming that the microarray results are of good quality and representative of gene expression values.

PR-3 and PR-4 are markers of the JA/ ET response and are induced at both time-points after *R. solanacearum* infection. A marker of the SA response pathway (PR-5) is not differentially expressed during early wilt but is repressed during late wilt. PR-1, another marker of the SA response pathway, is absent from the microarray. The up-regulation of the JA/ ET responsive marker genes PR-3 and PR-4 and the repressed SA-responsive marker gene PR-5 during during *R. solanacearum* infection suggests that JA/ET responses may be induced in response to infection and that SA responses may be repressed by the infection.

A



B

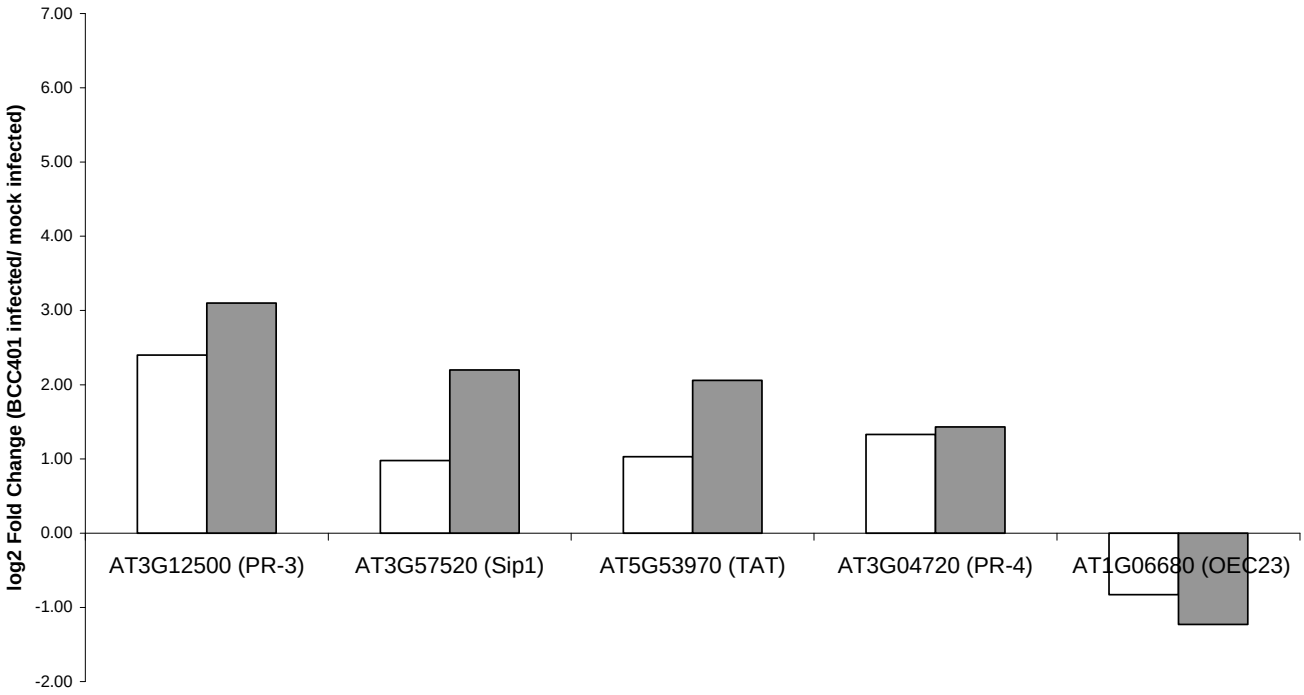


Figure 4.5 Expression data for selected Arabidopsis genes during *R. solanacearum* BCCF 401 infection relative to mock-inoculations at the same time-points. A) qRT-PCR results and B) microarray results. The gene expression for the up-regulated genes basic endochitinase (PR-3), Seed imbibition protein homologue (Sip1), Tyrosine amino transferase (TAT) and pre-hevein like protein (PR-4) and for the down-regulated gene oxygen evolving complex 23 (OEC23), is shown. Empty bars represent expression levels during early-wilt infection stages while grey bars represent expression levels during late-wilt infection stages. In the case of qRT-PCR data, the data from at least three technical replicates are indicated. Results from a second biological replicate were similar. The mean expression ratios of the five genes from the four replicate microarray experiments are represented in B).

4.4.5 Gene ontologies indicate R. solanacearum induces biotic and abiotic stress responses in Col-5.

Gene ontologies for the differentially regulated gene groups (induced during early wilt, induced during late wilt, repressed during early wilt and repressed during late wilt) were determined for the category biological process in FatiGO at level 5 (Al-Shahrour et al., 2004). Figure 4.6 shows these results.

The GOs provide clues as to the type of processes the genes are involved in. One of the processes indicated by up-regulated genes is the response to water deprivation, which would be expected for plants undergoing wilting due to *R. solanacearum* infection (Figure 4.6 A and C). During *R. solanacearum* infection, the xylem of the plant becomes clogged with bacteria and bacterial debris reducing the plants ability to take up water and thus wilting ensues (Genin and Boucher, 2002). Thus it may be expected that the plant would undergo an abiotic-type stress which would be water deprivation, as well as a biotic stress in the form of *R. solanacearum* infection which secretes cell wall degrading enzymes and effectors directly into the plant cell (reviewed in Hikichi et al., 2007). Several up-regulated genes are also involved in the defence response against virus, fungi and in the innate immune response (Figure 4.6 A, C). The phenotypic symptoms observed in Col-5 during the late wilt stage (wilt symptoms 3 to 4, Figure 4.2) correlate well with the biological processes which are transcriptionally down-regulated at the late wilt stage (Figure 4.6 D). For example, the down-regulation of processes such as reproductive structure development and organisation of anatomical structure may coincide with a break-down of the plant's anatomical structure during wilting caused by BCCF 401. In addition, at the late wilt stages (Figure 4.6 D) genes involved in the photosynthetic light reaction are repressed. This may be correlated with the decline in photosynthesis in leaves showing wilting symptoms as a result of bacterial wilt infection.

More genes appear to be responding during the late wilt time-point compared to the early wilt in Col-5 (Figure 4.3). The higher number of differentially expressed genes at the late wilt stage is a reflection of the wilt symptoms and cellular damage incurred by the pathogen attack. This explanation is in accordance with the various GOs indicating damage described in Figure 4. 5D and during this wilt stage, the symptoms on Col-5 are severe (almost 60% of the plant is wilted).

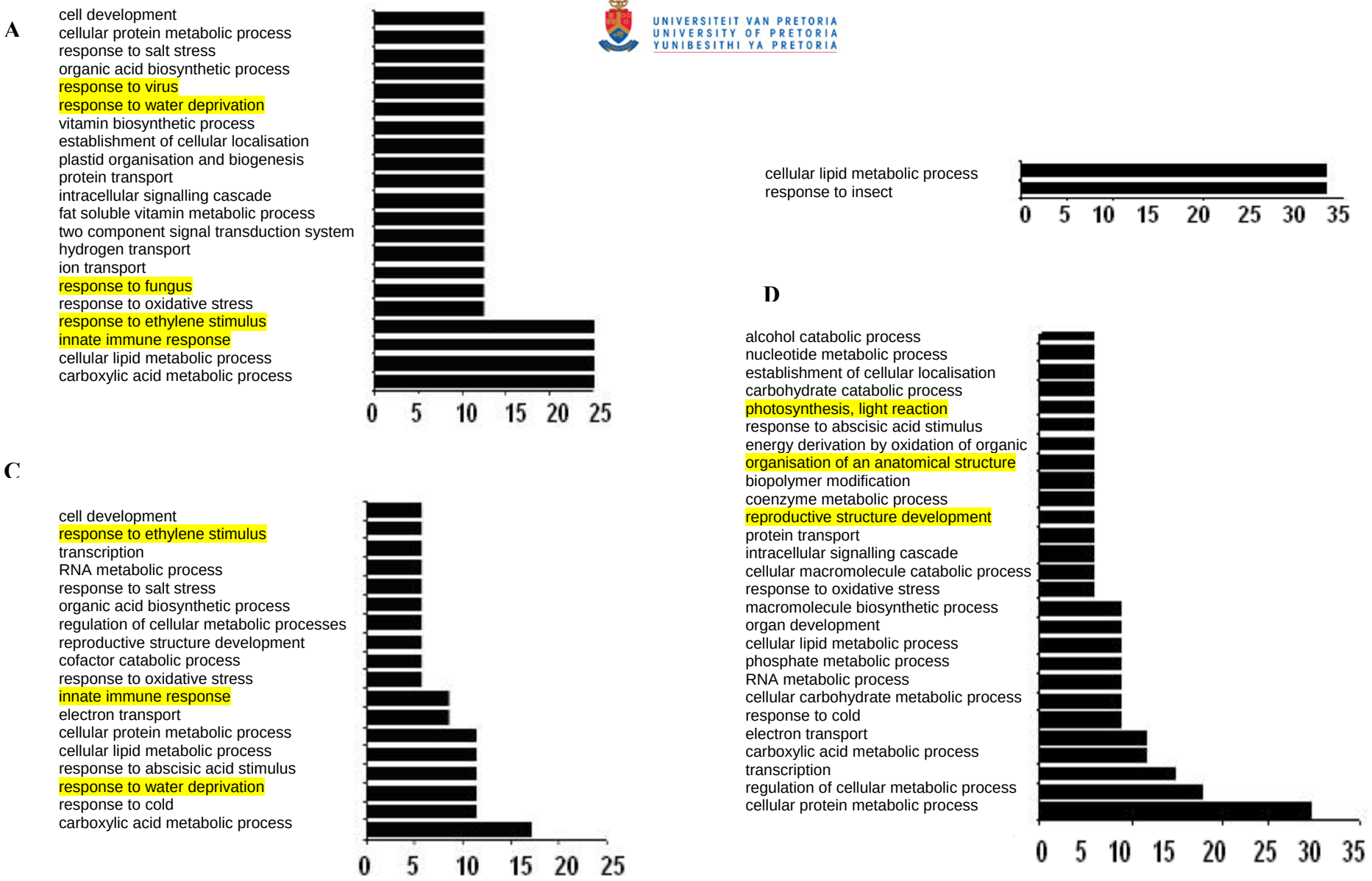


Figure 4.6 GO, biological process, categorization of Arabidopsis genes differentially regulated after inoculation with *R. solanacearum* BCCF 401 using FatiGo at level 5. A) up-regulated at early wilt (27 genes), B) down regulated at early wilt (14 genes), C) up-regulated at late wilt (69 genes), D) down regulated at late wilt (59 genes). Percentages indicate the total number of genes in the cluster with a particular ontology. Only categories with more than 5% of the total number of genes present in the cluster are shown.

Over-represented GO terms in each of the up-regulated and down-regulated clusters were investigated for the category biological process in comparison to 1) the 5000 unigenes represented on the microarray and 2) the whole Arabidopsis genome (approximately 30 000 genes) using GOStat. Significantly over-represented GO terms ($p<0.05$; Holm corrected) were obtained for up-regulated genes induced during early wilt by BCCF 401. These GO terms are listed in Table 4.2 alongside their corresponding p-values and % representation in the gene set.

Table 4.2 Over-represented GO terms in the category biological process for early-wilt up-regulated genes in comparison to the 5000 unigenes represented on the microarray and to the whole genome using GOStat.

Gene Ontology	Relative to 5000	p-value	Relative to genome	p-value
response to other organism	45%	0.00002	3.22%	0.0008
response to ethylene stimulus	25%	0.00645	1.49%	0.0538
innate immune response	25%	0.01011	1.90%	0.0158
response to wounding	18%	0.00979	1.32%	0.0487
response to water deprivation	14%	0.03756	0.49%	0.0018
jasmonic acid and ethylene-dependent systemic defence response	50%	0.00122	1.26%	0.0272
lipid metabolic process	25%	0.04916	4.53%	0.0218

This data provides further evidence (in addition to Figure 4.6) that both biotic (reponse to other organism) and abiotic (response to water deprivation) stress responses are induced by *R. solanacearum* infection (Table 4.2). The plant responds to the pathogen attack by inducing an innate immune response, which appears to be mediated by the jasmonic acid and ethylene defence pathway (Table 4.2).

4.3.5 Comparative expression profiling of Arabidopsis genes differentially expressed during R. solanacearum infection, under various biotic and abiotic stress treatments.

Following the observation that *R. solanacearum* induces genes involved in response to biotic and abiotic stress, the expression profiles of the 128 genes found to be differentially regulated during late wilt were compared to the expression profiles of these genes in Col-0 under biotic stress treatments with virulent and avirulent *Pseudomonas syringae* (*Pst*) and the necrotrophic pathogen *Botrytis cinerea,* and under abiotic stress conditions (drought stress in shoot tissue at 0.25, 0.5, 1 and 12 hr after treatment). Expression data was available for 120 of the 128 differentially regulated genes. Comparisons were also made to hormone treatment with MeJA, SA, ABA and the ethylene precursor ACC in Col-0 at various time-points to determine whether the signalling pathways involved in the response against *R. solanacearum* could be

predicted from the expression profiles of the 120 genes. Hierarchical clustering was performed on the combined data set for which the log2 ratios were computed relative to the respective controls. Figure 4.7 A and B shows the results of hierarchical clustering of the 120 genes across the various experiments using Manhattan distance and complete linkage.

The clustering results suggest that the expression profile induced by *R. solanacearum* matches that of *Pst* DC3000 and *Pst avrRpm1* infection at 24hr (Figure 4.7 A). Similarly, *B. cinerea* infection after 48 hr produces a similar expression profile in Col-0 to that of *R. solanacearum* infection. *B. cinerea* is a necrotrophic pathogen while *Pst* is considered a hemi-biotroph, (Toth and Birch, 2005). The similar expression profile induced by *R. solanacearum* and by *B. cinerea* and *Pst* during the necrotrophic phase (24hr) is consistent with the suggestion that *R. solanacearum* is a necrotrophic pathogen. It would be expected that the gene expression pattern induced by *R. solanacearum* would match a pattern of expression similar to that induced by the compatible *P. syringae* interaction rather than the incompatible *Pst* interaction. Pearson correlation coefficients do confirm that this is the case (0.52 for *R. solanacearum* vs *Pst DC3000* and 0.49 for *R. solanacearum* vs *Pst AvrRpm1*).

The expression profile induced by BCCF 401 is most similar to that of ABA treatment 3 hr after treatment in Col-0 (Figure 4.7 B). Although PR-3 and PR-4, marker genes for the MeJA/ET signalling pathways were up-regulated in Col-5 during *R. solanacearum* infection (Figure 4.5), a comparison to the expression profiles of MeJA and ACC treated Col-0 plants at various time-points do not support a clear role for the MeJA or ethylene pathways in response to *R. solanacearum* pathogen attack as expression profiles do not match. Instead, many of the 120 genes responding to *R. solanacearum* are similarly regulated by ABA treatment at 3 hrs (Figure 4.7 B). Based on the expression profiles, it can be predicted that the ABA signalling pathway is operating in response to *R. solanacearum* infection.

During *R. solanacearum* infection, wilting does occur. This is reminiscent of wilting that is observed during drought stress. The response to water deprivation seen in the GOs (Figure 4.6) suggests that the transcriptional response in *R. solanacearum* would be similar to that during drought stress. However this is not the case (Figure 4.7 B). This could also be attributed to the manner in which these drought stress experiments were performed which would not allow for direct comparability (Kilian et al., 2007). The AtGenExpress drought experiments were conducted on plants grown on MS medium and subjected to a 10% loss of

dry weight while wilting induced by *R. solanacearum* results in a far more severe drought-stress phenotype.

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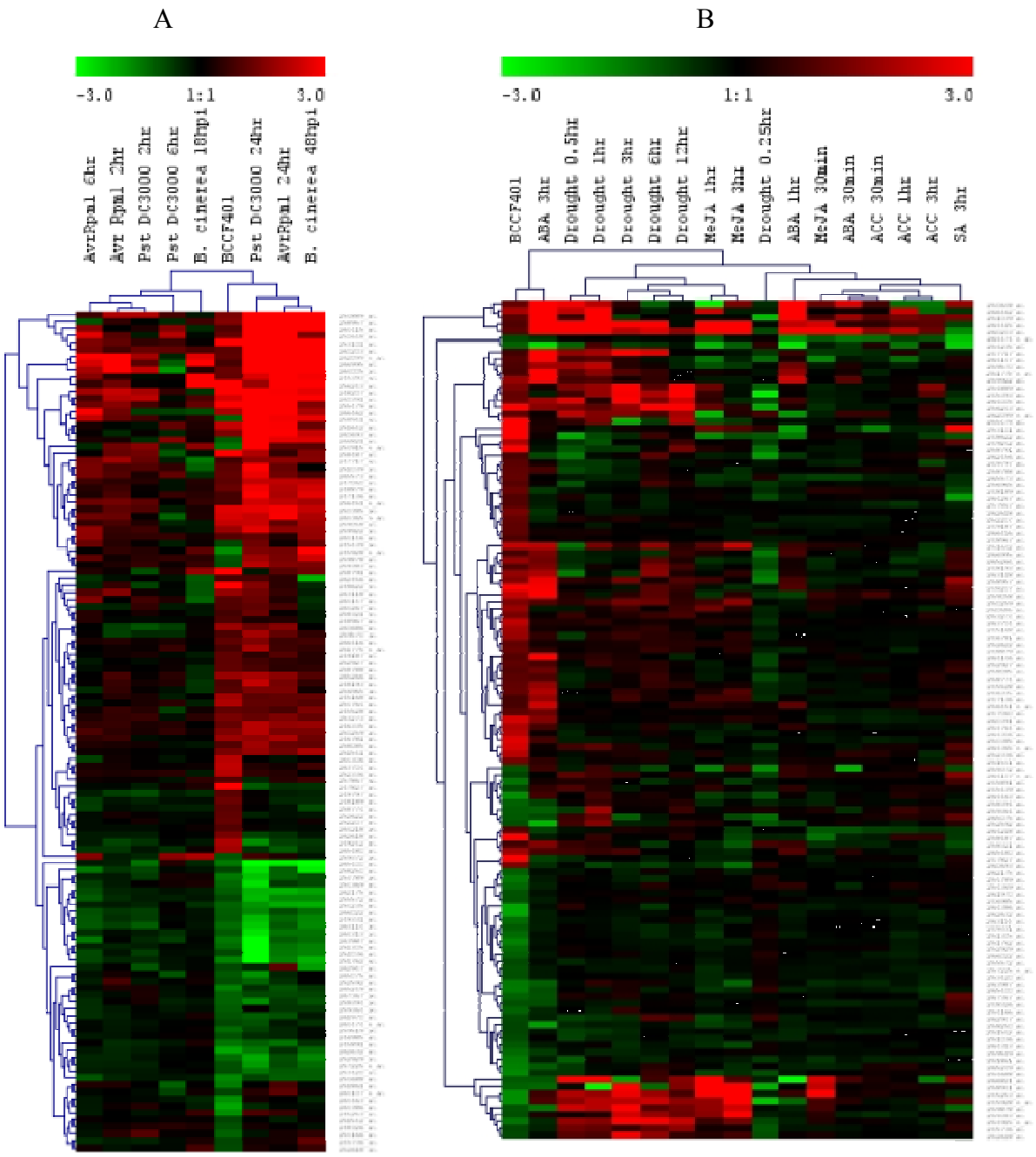

Figure 4.7 Hierarchical clustering (complete linkage, Manhattan distance) of the 120 Arabidopsis genes that are differentially regulated during *R. solanacearum* infection 7 days after infection across various different conditions: (A) *Pst and B. cinerea* infection (B), drought stress and hormone treatment (MeJA, ACC, ABA, SA) at the indicated time-points relative to their controls (expressed as log2 fold change).

4.3.6 Basal defence responses in Col-5 against R. solanacearum infection.

Basal defences are often induced in compatible interactions, however, these defences may be described as a weak form of immunity, ineffective in preventing disease (Jones and Dangl, 2006). We looked for evidence for basal defence responses in Col-5 during *R. solanacearum* infection by performing a bioinformatics comparison to PAMP-induced responses in Col-0 treated with flg22 and LPS from *Pst* DC3000 (NASCARRAYS-121), and with *Pst hrp*$^-$ and *Pst* DC3000 (NASCARRAYS-120) for the 128 genes shown to be induced during *R. solanacearum* infection during late wilt. Data was available for 120 of these genes. Of the 120 genes, a subset (38) met the criteria of basal defence response genes described in the materials and methods. Figure 4.8 shows the comparison between these expression profiles as an expression matrix generated in TIGR MeV (v2.2) for the 38 selected genes.

It appears that some basal defences are induced by BCCF 401 infection since some of the 128 genes are also induced under conditions where basal defence are known to operate e.g. during *Pst* DC3000 infection, *Pst hrp*$^-$ infection, flg22 and LPS treatment (Figure 4.8 cluster I). The response to these treatments are indicative of a basal defence response against the pathogen and as such could be a weak form of PAMP Triggered Immunity or PTI (Jones and Dangl, 2006) (Figure 4.8 cluster I). Similarly, those genes that are repressed during *R. solanacearum* infection, repressed during *Pst* DC3000 infection and repressed by PAMPs (flg22, LPS, *hrp*$^-$), would be indicative of PTI (Figure 4.8 cluster II) against *R. solanacearum*. Cluster I contains genes such as LTP3 (AT4G02380), glycosyl hydrolase family protein 51 (AT4G34180) and cytochrome P450 81F1 (AT4G37430). Cluster II contains genes involved in photosynthesis such as plastocyanin-like domain-containing protein (AT2G42690), ribulose bisphosphate carboxylase small chain 3B (AT4G12880), and two kinases: Leucine-rich repeat family protein / protein kinase family protein (AT3G15850) and putative mitogen-activated protein kinase (MPK3) (AT3G55800).

There is indirect evidence to suggest that *R. solanacearum* effectors may be responsible for targeting the basal defence response as some genes which are repressed by PAMPs (*hrp*$^-$ treatment, flg22 and/ or LPS) and are induced by *Pst DC3000* (which contains effectors), are similarly induced by *R. solanacearum* (Figure 4.8, cluster III). This may be indicative of common *R. solanacearum*, *Pst DC3000* effector targets that mediate effector triggered susceptibility (ETS) (Figure 4.8 III). Genes represented in cluster IV (Figure 4.8), can be considered specific *R. solanacearum* effector targets as they are induced during *Pst hrp*$^-$

infection and are thus PAMP – induced genes but are also induced during *Pst DC3000* infection and are repressed during *R. solanacearum* infection. Table 4.3 lists genes, which are potentially *R. solanacearum* effector-targets in Col-5. Defence-related genes such as PR-3 and osmotin are possible effector targets which are induced by bacterial effectors while vegetative storage protein 2 (VSP2) and PR-5 are potentially down-regulated by effectors (Cluster III and IV, Table 4.3).

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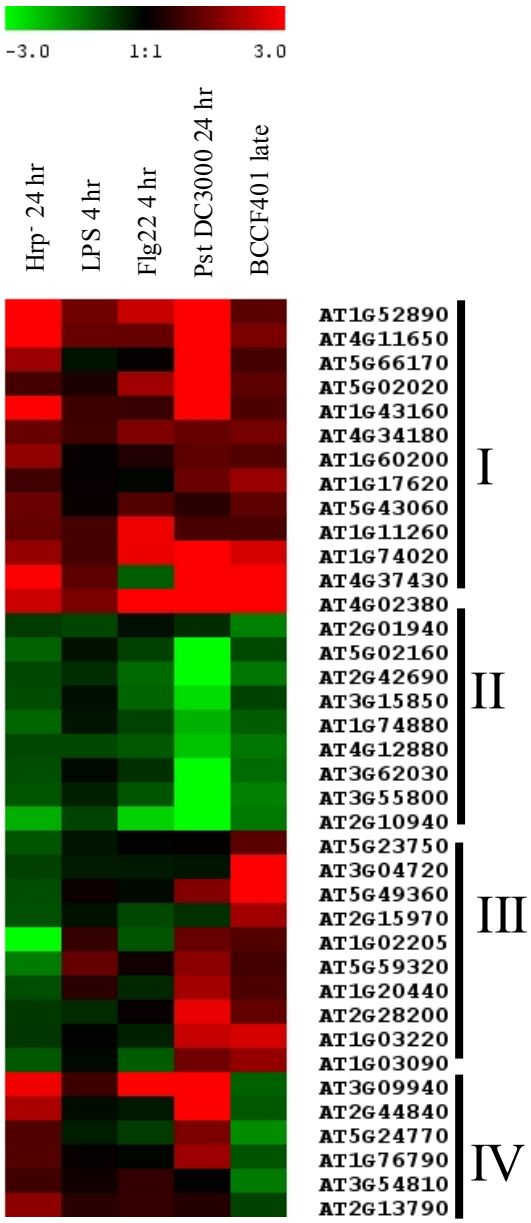

Figure 4.8 Arabidopsis genes showing basal defence response against *R. solanaceearum* in comparison to *Pst* DC3000 and PAMP-induced genes. Cluster I are genes induced by PAMPs and effectors, cluster II are genes which are repressed by PAMPs and effectors, cluster III are genes which are repressed by PAMPs but induced by effectors and cluster IV are genes which are induced by PAMPs but repressed by effectors.

Table 4.3 Arabidopsis genes, which are potential targets for *R. solanacearum* effectors derived from comparisons between PAMP-induced expression profiles and pathogen-induced profiles (Figure 4.8).

Cluster	Description
III	similar to polyphosphoinositide binding protein Ssh1p
	Osmotin-like protein (OSM34)
	Basic endochitinase PR-3
	Proton-dependent oligopeptide transport (POT) family protein
	CER1 protein
	H+-transporting two-sector ATPase
	UVB-resistance protein UVR8 (Arabidopsis thaliana)
	branched-chain alpha-keto acid dehydrogenase E1 beta subunit (DIN4)
	Extracellular dermal glycoprotein, putative / EDGP
	Glucose transporter (STP1)
IV	Fatty acid desaturase family protein
	Thaumatin PR-5
	Vegetative storage protein 2 (VSP2)
	ERF (ethylene response factor) family protein
	Expressed protein
	Leucine-rich repeat family protein

4.4. Discussion

We investigated the defence response in Arabidopsis ecotype Col-5 against *R. solanacearum* during a susceptible interaction using microarray expression profiling of 5000 unigenes and obtained 41 genes differentially regulated during early wilt induced by *R. solanacearum* infection and 128 genes differentially regulated during the late wilt stage. Bioinformatics comparisons were performed with the objective of determining whether *R. solanacearum* infection induces an expression profile that is consistent with that of a necrotrophic pathogen, what signalling pathways may be involved in the response against the pathogen, whether basal defence responses are induced by pathogen infection and further, whether gene targets of *R. solanacearum* effectors can be predicted.

The induction of the marker genes for JA/ET, PR-3 and PR-4 by *R. solanacearum* was shown by microarray analysis and qRT-PCR (Figure 4.5) This is in accordance with Hirsch et al. (2002) who observed an induction of these marker genes (PR-3 and PR-4) in response to *R. solanacearum* strain K60 infection in leaves of the susceptible ecotype Col-0. Wilt symptoms were delayed in ethylene insensitive mutants in response to virulent strains of *R. solanacearum* (Hirsch et al., 2002). Ethylene was suggested to be involved in the wilting response and not *R. solanacearum* resistance as homozygous *ein2-1* plants in a resistant background (Nd1) remained resistant to a virulent *R. solanacearum* strain (Hirsch et al., 2002). *R. solanacearum* is also capable of producing plant-like hormones such as ethylene (Freebain and Buddenhagen, 1964). This may be a strategy by the pathogen to promote disease as in the case of the bacterial toxin coronatine from *P. syringae*, which is a mimic of the hosts' MeJA involved in defence signalling (Bender et al., 1999; Staswick et al., 2005). The MeJA signalling pathway antagonises the SA pathway, which is important for defence against the pathogen.

It has been suggested that plant defence responses are tailored to the attacking pathogen. In Arabidopsis, resistance to biotrophic pathogens tends to rely on salicylic acid dependent, JA/ ET independent responses while resistance to necrotrophic pathogens is more reliant on JA and ET dependent, SA- independent responses (Thomma et al., 1999). Thus, these expression profiles could be used to classify pathogen as biotrophs or necrotrophs (Oliver and Ipcho, 2004). Despite the induction of the JA/ET marker genes PR-3 and PR-4 and the repression of the SA-marker gene PR-5 in response to *R. solanacearum*, clustering does not reveal high correlation between profiles induced by *R. solanacearum* infection and profiles induced by JA

and ET treatment or a negative correlation with expression profiles induced by SA treatment for the 120 Arabidopsis genes (figure 4.7 A). However the expression profiles generated by *R. solanacearum*, *B. cinerea* and *P. syringae* are highly similar for the 120 genes investigated. *B. cinerea* is a classic necrotrophic pathogen while *P. syringae* may be considered a hemi-biotroph (Glazebrook, 2005; Toth and Birch, 2005) which begins its life cycle as a biotroph and switches to a necrotrophic type of pathogenesis. Twenty four hours after infection, *Pst* would possibly have entered a necrotrophic mode of pathogenesis, deploying effectors to suppress host defences. The similar expression profiles suggest that *R. solanacearum* is a necrotrophic pathogens. Further motivation that *R. solanacearum* is a necrotroph is that the pathogen produces several cell wall degrading enzymes (Allen et al., 1991). This criteria has been used previously to classify necrotrophs (reviewed in Oliver and Ipcho, 2004).

Based on the expression profiles in Figure 4.7, it can be predicted that the ABA signalling pathway is operating in response to *R. solanacearum* infection. The role of abscisic acid in plant defence has been suggested to be both positive (e.g. against *Pythium irregulare* in Arabidopsis, Adie et al., 2007) or negative (e.g. against *Fusarium oxysporum*, Anderson et al., 2004). Pathogens such as *Botrytis* are also capable of producing ABA and are thought to enhance host susceptibility by manipulating host defences (Marumo et al., 1982). Therefore the up-regulation of ABA responsive genes in the host may not necessarily be due to the plant. There is no current evidence to support the hypothesis that *R. solanacearum* produces ABA to promote susceptibility. The secondary cell wall mutants *irx1* (*ir*regular *x*ylem 1), *irx3* and *irx5,* which carry a mutation in the *AtCesA8, AtCesA7 and AtCeSA8* genes respectively, confer enhanced resistance to *R. solanacearum* GMI1000 independently of SA, JA and ethylene (Hernandez-Blanco et al., 2007). Comparative transcript profiling of the former mutants showed the constitutive induction of ABA-responsive genes suggesting a role for ABA signalling in conferring disease resistance against *R. solanacearum*. Furthermore, ABA mutants (*abi1-1*, *abi2-1*, and *aba1-6*) were more susceptible to the pathogen. The induction of ABA-responsive genes observed in the susceptible interaction with Col-5 and BCCF 401 suggests that ABA signalling alone may not be sufficient to provide resistance against *R. solanacearum*. It is also possible that ABA signalling is induced by wilting caused by infection and is therefore not involved directly in resistance but could contribute by delaying the eventual collapse of the plant (reviewed in Zhang et al., 2006).

Basal defences are often not sufficient to protect plants from pathogens as effectors are able to directly suppress host responses (He et al., 2006; Truman et al., 2006). Several genes have been identified with an increase in expression during *R. solanacearum* infection or *Pst* DC3000 infection compared to PAMP-induced responses by *Pst hrp*$^-$ and flg22 or LPS. This suggests that the genes are potential targets of specific *R. solanacearum* effectors, which manipulate genes at the transcript level repressing the plant defence system. The genes described in Table 4.3 are potential biotechnology targets, which if repressed (cluster III) or induced (cluster IV) may enhance resistance against *R. solanacearum*.

Although the flg22 region of *R. solanacearum* shows a high degree of amino acid similarity to the flg22 region of several other *Pseudomonas* species (shown in appendix C), *R. solanacearum* flagellin from isolate K60 is not a major elicitor of defence responses in Arabidopsis (Pfund et al., 2004). It is possible that *R. solanacearum* has other PAMPs, which would elicit a similar defence response as has been shown for flg22 in Col-0. Thus, the flg22-induced gene expression in Col-5 was used as a marker for PAMP-induced expression in Col-5 against *R. solanacearum*. The enzyme responsible for the production of lipopolysaccharide (lipopolysaccharide heptosyltransferase) in *Pst* DC3000 is similar to that found in *R. solanacearum* GMI1000 (40% amino acid identity, appendix D). The LPS from both sources was also shown to induce a NO burst in Arabidopsis (Zeidler et al., 2004). This suggests that *R. solanacearum* produces a type of LPS capable of eliciting similar basal defence responses to that of *Pst* DC3000.

One consideration for the approach that has been undertaken, which uses expression data from Arabidopsis treated with *Pst* and PAMPs to determine effector targets by *R. solanacearum* in Col-5, is that effectors may be quite specific. An example of this is the work of He et al. (2006) who demonstrated the specific suppression of PAMP-induced responses by the effectors AvrPto and AvrPtoB from *P. syringae* in Arabidopsis protoplasts. This suppression occurs upstream of the MAPK signalling cascade at the plasma membrane. AvrRpt2 or AvrRpm1, effectors with known virulence effects, did not suppress early PAMP-specific gene activation or MAPK signalling, suggesting that effector proteins may block the PAMP-induced defence response in different ways (He et al., 2006). It is also possible that genes which have been described as effector targets in the current study may not be targets per se but down-stream effects of the manipulation of targets by pathogen effectors.

R. solanacearum contains several putative effectors (Cunnac et al., 2004a; Cunnac et al., 2004b; Occhialini et al., 2005; Angot et al., 2006; Meyer et al., 2006). One well-characterised effector in strain GMI1000 is PopP2, which interacts with RRS1-R (Deslandes et al., 1998), while Cunnac et al., (2004b) identified 48 putative effectors in GMI000. Each of these putative effector genes was disrupted individually in mutant GMI1000 strains and used to challenge the resistant *M. truncatula* line F83005.5 (Vailleau et al., 2007). In all cases, no susceptible phenotype was observed suggesting that none of the candidate effectors alone are required for resistance. *R. solanacearum* strain UW551, which belongs to race 3, biovar 2 has only 6 or 7 effectors apparently "missing" compared to GMI1000 and three effectors: RRSL00326, RRSL01019, and RRSL03923, are unique to UW551 (Mukaihara et al., 2004; Gabriel et al., 2006). BCCF 401 belongs to the same race and biovar as GMI1000 (race 1, biovar 3), thus the two pathogens may share common effectors.

A logical approach to understanding the basal defence response in Col-5 against *R. solanacearum* would be to challenge the plants with TTSS-deficient *hrp* mutants of BCCF 401 and with wild-type BCCF 401. Expression profiling of genes responding to the pathogen should then be conducted using whole-genome microarrays. This would provide evidence of the suppression or induction of specific gene targets by *R. solanacearum* BCCF 401 effectors.

An alternative approach to identify canididate genes, which could be required to improve defence against *R. solanacearum*, would be to identify genes which remain constitutively expressed during *R. solanacearum* infection but whose expression changes under other conditions (e.g. resistant interactions). This comparison was made for 85 genes that were constitutively expressed during early wilt and late wilt time points in comparison to the uninfected Col-5 plants ($p<0.05$, log2 fold changes >-0.75 and <0.75). An interesting gene, that was identified as not responding during the incompatible interaction with *R. solanacearum* but downregulated during an incompatible interaction with *Pst*, was an auxin responsive protein (At5g43700). Navarro et al. (2006) indicated that decreasing auxin signalling can increase resistance to bacterial pathogens. Thus, this type of comparison may be useful to identify further candidate genes to enhance resistance against *R. solanacearum*.

In summary, several differentially regulated genes in Col-5 responding to *R. solanacearum* infection have been identified. Comparative expression profiling analysis reveals that the expression profile generated by *R. solanacearum* infection is suggestive of a necrotrophic

pathogen and supports a role for ABA signalling in the response to the pathogen. Evidence for basal defence responses in Col-5 against *R. solanacearum* and gene expression patterns, which is hypothesised to be effector targeted, have been observed. The hypotheses generated from the transcription profiling data would have to be validated at the gene function level i.e. using knock-out technology or over expression in the future. In this study, expression profiling has been conducted on 20% of the Arabidopsis genome. Screening of the entire Arabidopsis genome would provide a well-rounded view of the overall gene responses to the pathogen and would allow for the comparison of available whole-microarray data on an equal footing.

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