A Eucalyptus bacterial wilt isolate from South Africa is pathogenic on Arabidopsis and manipulates host defences

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Summary

Ralstonia solanacearum, the causal agent of bacterial wilt, has been reported from Eucalyptus plantations in at least three countries in Africa. The lack of genomics resources in Eucalyptus species led us to develop and study a pathosystem between a previously characterized South African isolate and the model plant *Arabidopsis thaliana*. *Ralstonia solanacearum* BCCF401 isolated from a *Eucalyptus grandis* × *Eucalyptus camaldulensis* hybrid was shown to cause disease on *A. thaliana* ecotype Col-5. Arabidopsis genomics tools were exploited to investigate gene expression changes during wilt disease development, and thereby develop hypotheses that can be tested in Eucalyptus once genomics resources are available. Transcriptome analysis using 5000 *A. thaliana* ESTs was performed and revealed 141 genes that were differentially regulated by *R. solanacearum* infection (at a significance threshold of $p < 0.03$; Bonferroni corrected). A software tool 'Rank Correlation Comparer' was developed to compare expression profiles with Arabidopsis Affymetrix NASCArray data. High correlations were observed between the response of Arabidopsis plants to both Eucalyptus (BCCF401) and tomato (GMI1000) isolates of *R. solanacearum*, as well as to *Pseudomonas syringae*, *Botrytis cinerea* and treatment with abscisic acid. Basal defence responses in Col-5 in response to *R. solanacearum* were investigated by comparing the expression data following *R. solanacearum* infection to data after treatment with the Pathogen Associated Molecular Patterns (PAMP) flg22 and lipopolysaccharide, and the Type Three Secretion System deficient *Pseudomonas syringae* pv. *tomato 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. We hypothesize that these genes represent targets of *R. solanacearum* effectors. The pending release of the

Eucalyptus genome sequence will enable orthologues to be identified and these hypotheses to be tested in Eucalyptus trees.

1 Introduction

Ralstonia solanacearum is considered to be one of the most important plant pathogenic bacteria, causing bacterial wilt disease on a broad range of hosts including woody plants such as Eucalyptus, Casuarina, mulberry and olive trees (Coutinho et al. 2000; Xu et al. 2009). Eucalyptus species have been reported as a host for the pathogen in Brazil, China, Taiwan, Australia and Venezuela (Coutinho et al. 2000; Xu et al. 2009). In Africa, *R. solanacearum* poses a threat to the forestry industry as the disease was detected in Eucalyptus plantations in South Africa, Uganda and the Democratic Republic of Congo (Coutinho et al. 2000; Roux et al. 2001; Fouché-Weich et al. 2006). The severity of the disease in Africa may be underestimated as a limited number of Eucalyptus plantations have been surveyed. The pathogen infects both cutting and ramets (individuals which originate vegetatively from a single ancestor), preventing the vegetative propagation of hybrids of *Eucalyptus grandis* × *Eucalyptus camaldulensis* commonly used for pulp and paper production in South Africa (Coutinho et al. 2000). The presence of the pathogen in Eucalyptus plantations is a cause for concern as Eucalyptus is increasingly clonally propagated (Coutinho et al. 2000). It is possible that entire plantations could be lost due to susceptibility of the clone to a particular pathogen.

Eucalyptus plantations are most susceptible to bacterial wilt within the first 2 years of growth, and most commercially grown hybrids are susceptible (Ran et al. 2005a,b). Selection and breeding for resistance is the most attractive long term solution, although glasshouse experiments have indicated that treatment with biocontrol agents (*Pseudomonas* spp.) or salicylic acid can suppress disease development in the susceptible species *Eucalyptus urophylla* (Ran et al. 2005a,b).

Observations of the infection process using microscopy have revealed aspects of bacterial wilt disease progression, particularly in the tomato pathosystem (Vasse et al. 2000). 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, 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 plant's water-uptake system is compromised, the plants wilt completely (Genin and Boucher 2002b).

Ongoing molecular studies are revealing elements of pathogenicity mechanisms of *R. solanacearum* (Poueymiro and Genin 2009). The genome sequence of the tomato isolate of *R. solanacearum* (GMI1000) revealed a battery of putative virulence factors/effectors encoded on pathogenicity islands characterized by Alternate Codon Usage Regions and similarity to Type Three Secretion System (TTSS) effectors (Genin and Boucher 2002b;

Salanoubat et al. 2002). Subsequently, a range of functional studies have revealed host-specific and broad spectrum *R. solanacearum* effectors in studies of tomato, tobacco, lettuce, pepper, cotton and *A. thaliana* (Poueymiro and Genin 2009; Wroblewski et al. 2009).

Arabidopsis thaliana has been adopted as a model system to study various plant–pathogen interactions as a result of the lack of genomic and pathosystem resources in natural hosts. The use of *A. thaliana* as a host for 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-5 and did not cause disease on ecotype Nd-1. Subsequent expression studies of the susceptible interaction between Col-5 and GMI1000 using whole-genome microarray analysis revealed that the tomato pathogen induces abscisic acid (ABA) and pathogen-associated molecular pattern (PAMP) responses (Hu et al. 2008).

Studies in *A. thaliana* have been very useful in describing the integrated set of plant defences, and many of these constitutive or induced responses may be conserved between many species (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 race specific (e.g. effectors, such as 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). Activation of these defence pathways are also observed in susceptible plants; however, at a later stage and/or to a lower intensity than during a resistant interaction (Tao et al. 2003).

A basal defence response also develops in susceptible plants treated with general elicitors [i.e. PAMPs such as bacterial lipopolysaccharides (LPS), flagellin, cold-shock protein and elongation factor Tu, as well as fungal glucan and chitin]. However, these responses are insufficient to prevent disease onset (Jones and Dangl 2006). LPS from Gram-negative bacteria induce 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). Flg22, a 22 amino acid peptide found at the N-terminus of flagellin, the subunit of the bacterial surface structure flagellum, is able to induce a defence response in plants to a higher level than flagellin itself (Felix et al. 1999). In some cases, bacterial PAMPs may not be detected by the host, e.g. although *R. solanacearum* possesses 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 TTSS pathway (*hrp*$^-$) and wild-type bacterial pathogens suggest that specific effector proteins from the pathogen are able to suppress host basal defences, which are otherwise induced by PAMPs, to cause disease (Thilmony et al. 2006; Truman et al. 2006). This is reinforced by studies demonstrating that some effectors can indeed suppress basal immunity, for example *hopPtoM* and *avrE* genes of *P. syringae* encode suppressors of salicylic acid mediated basal immunity (Debroy et al. 2004).

Our current study builds on previous work in which *R. solanacearum* was reported for the first time from *E. grandis* × *E. camaldulensis* plantations in South Africa (Coutinho et al. 2000). Subsequently, the same isolate (BCCF401), as well as Eucalyptus isolates from two other African countries (Uganda and Democratic Republic of Congo) were confirmed to be biovar 3 (Fouché-Weich et al. 2006). Importantly, although they are both biovar 3, the Eucalyptus isolate BCCF401 from South Africa in this study is distinct from the well-characterized tomato isolate GM1000 from French Guyana based on PCR-RFLP and AFLP analysis (Fouché-Weich et al. 2006). In the current study, we have characterized the plant response to this Eucalyptus isolate BCCF401 using an *A. thaliana* pathosystem. Genomics resources were limited for Eucalyptus, and therefore we chose to use Arabidopsis as a model system, which would facilitate translation of the results to the tree host once the annotated Eucalyptus genome sequence is released (planned for 2010, Myburg et al. 2008).

We have shown that the Eucalyptus *R. solanacearum* isolate BCCF401 was pathogenic on *A. thaliana* ecotype Col-5. Based on the observed susceptibility, 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 infection. We used bioinformatics tools to compare our data with publicly available microarray data, which illustrated that there was substantial overlap in the response of Arabidopsis plants to *R. solanacearum* isolates from Eucalyptus and tomato. However, we also obtained evidence for manipulation of basal defence responses, and isolate-specific responses which we hypothesize are targets of host-specific effectors. Our data provide useful leads for future work to characterize the response in Eucalyptus trees, which will be greatly facilitated by the release of the annotated Eucalyptus genome sequence (Myburg et al. 2008). Orthologues to Arabidopsis genes that are putative targets of *R. solanacearum* BCCF401 effectors can then be identified using phylogenomic approaches (Cao et al. 2008).

2 Materials and methods

2.1 Plant material

Seeds of Arabidopsis ecotype Col-5 were obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://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 (1962) medium for 2 weeks under 16 h day conditions. The plants were

transferred to Jiffy pots (Jiffy France, Lyon, France) and grown for 4 weeks under 16 h light, 25–26°C, 50% relative humidity and 200–250 μmoles/m^2/s. The plants were watered with a solution of Feedall® [Aquasol (Pty) Ltd, Potchefstroom, SA, USA] once a week.

2.2 Bacterial isolates and growth media

Ralstonia solanacearum isolates BCCF401 from *E. grandis* × *E. camaldulensis* clones in South Africa (Coutinho et al. 2000; Fouché-Weich et al. 2006) or GMI1000 (Boucher et al. 1985; Deslandes et al. 1998) were grown on solidified Bacto-agar Glucose Triphenyltetrazolium chloride (BGT) media at 28°C for 48 h. Colonies that displayed a virulent phenotype (mucoid) were transferred to liquid B media (Boucher et al. 1985) and incubated overnight at 28°C. To prepare the rifampicin mutant, two colonies of BCCF401 were picked from a fresh culture (16–24 h) on BGT medium and subjected to overnight incubation in B broth. After centrifugation, the pellet was spread on BGT medium containing rifampicin (50 μg/ml) and glucose (0.5%). The plates were incubated for 3 days at 28°C. Single rifampicin resistant wild-type like, mucoid colonies were selected and overnight broth cultures were prepared (named BCCF401*).

Preparation of the BCCF401 *hrp*$^-$ mutant involved the extraction of genomic DNA from *hrp*$^-$*R. solanacearum* strain GMI1402, a derivative of GMI1000 carrying a disruption in the *hrc*S (*hrp*-conserved) gene (Arlat et al. 1992). The receiver strain BCCF401 was grown for 3 days in minimal media [one-quarter strength M63 (Maniatis et al. 1982), with a final concentration of 2% glycerol], on a rotary shaker at 30°C. When an optical density of 1 was obtained, the bacterial growth solution was placed on a nitrocellulose filter on B medium without glucose and incubated with 0.3 μg/μl total genomic DNA of GMI1402. Incubation was carried out at 30°C for 2–3 days. The bacterial growth was collected by quick centrifugation of the nitrocellulose filter using 1 ml of sterile distilled water. The filter was removed and the remaining suspension mixed by vortexing. One hundred microlitres of this suspension was streaked onto a selection plate of BGT medium containing 50 μg/ml kanamycin. Incubation followed at 30°C for 2–3 days. The *hrp*$^-$ mutants that were no longer capable of inducing a hypersensitive response on tobacco were chosen (data not shown).

2.3 Bacterial inoculations and disease scoring

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 (1×10^8 cfu/ml) for 30 min. Control plants were soaked in a solution of liquid B media without any bacteria. The plants were placed on moist vermiculite and maintained at 26°C, 60–70% humidity and 16 h day length.

The plants were rated on a scale from 0 (no disease) to 4 (100% wilted/dead plants) according to the method of Deslandes et al. (1998), where wilt symptom 0.5 was descriptive of plants showing less than 12% of the leaves wilted; symptom 1, less than 25% of the leaves wilted; symptom 2, less than 50% of the leaves wilted; symptom 3, 50–75% of the leaves wilted and symptom 4, 76–100% of the plant was wilted/dead. The data were 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).

Bacterial enumerations were carried out as described by Deslandes et al. (1998) with selection for *R. solanacearum* BCCF401* and GMI1000 on 50 µg/ml rifampicin and BCCF401 *hrp*$^{-}$ mutant on 50 µg/ml kanamycin.

2.3 RNA isolation

Total RNA was isolated from aerial parts of 8–12 control and infected plants per biological replicate using TriReagent (Sigma Aldrich, St Louis, Missouri, USA) according to the manufacturer's instructions and further purified using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). 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, DE, USA).

2.4 Microarray experiments

The experimental design consisted of a direct comparison between control and inoculated samples at different times after inoculation with the bacteria (early and late). Two biological experiments were performed and a technical replicate (with reversion of the dye assignments) was carried out for both of these. Four slides per symptom (early wilt or late wilt) were hybridised. Figure S1 shows the experimental design that was used. 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 NCBI GEO database (accession no. GSE19178). Labelling, hybridisations, scanning and data capture were conducted as described in Naidoo et al. (2007).

Gene expression data were normalized and significant gene expression differences identified using the mixed model anova approach of Wolfinger et al. (2001). The data were corrected 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 or less than −0.75 with a $-\log_{10}p > 1.5$ ($p < 0.03$) were selected as differentially expressed in response to the infection. Microarray data have been deposited in the NCBI GEO database (GSE19178).

2.5 Reverse transcription quantitative PCR

Two-step reverse-transcription quantitative PCR (RT-qPCR) was performed using a LightCycler instrument (Version 1.2; Roche Diagnostics GmbH, Mannheim, Germany). PCR primers were designed using Primer Designer version 4 (Scientific & Educational Software, Cary, NC, 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* primers were from the purchased Primer library for Arabidopsis Pathogen-inducible Genes (Sigma). Two micrograms of total DNaseI-treated and column-purified RNA extracted from treated and control plants were reverse transcribed into first strand cDNA using ImpromII reverse transcriptase (Promega, Madison, WI, USA) according to manufacturer's instructions. The LightCycler FastStart DNA Master^{PLUS} SYBR Green I system (Roche) was used for real-time PCR in a 20 µl reaction. 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 *Cap Binding Protein* (CBP) *20 gene* (Sigma) or the *elongation factor-1-alpha-related GTP binding protein factor* (W43332, At1g18070.1, forward 5'TGCGGTTGTCGAGGAGTGGTG3' and reverse 5'AACCCGAAAGCCGTCTCCTG3') were used. The *elongation factor-1-alpha-related GTP binding protein factor* gene was expressed constitutively in microarray experiments (log_2-fold change $\sim$ 0, and p-value = 0.0003, 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. 2005). In all cases tested, normalisation using either the *CBP 20* gene or *elongation factor-1-alpha-related GTP binding protein factor* gene produced similar results (results not shown). Cycling consisted of a 95°C activation step for 10 min, 40 cycles of 95°C for 30 s, annealing temperature specific for each primer combination and an extension of 72°C for 2 min. Data acquisition was performed between 72°C and 80°C. Melting curve analysis and agarose gel electrophoresis of the RT-qPCR products were performed to confirm that the individual RT-qPCR products corresponded to a single homogenous cDNA fragment of expected size. All amplicons were sequenced to confirm that the correct gene was targeted.

2.6 Gene ontologies

Over-represented GO terms in the category biological process were 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 (TAIR7 release). A chi-squared 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.

2.7 Bioinformatics comparison of expression data with NASCArrays data

2.7.1 Rank correlation comparer

The microarray expression data for selected genes from early wilt and late wilt stages of infection with BCCF401 was compared with the NASCArrays Arabidopsis Affymetrix database to identify experiments with similar expression profiles. The tool Rank Correlation Comparer (RCC) was developed for this purpose within the web-based 'MicroArray Data Interface for Biological Annotation' (MADIBA) (Law et al. 2008). Affymetrix experimental data from NASCArrays were stored within the MADIBA database (http://www.bi.up.ac.za/MADIBA/). In each NASCArrays experiment, the ratio of the

expression level in the test case to the expression level in the corresponding control case (no treatment or 0 h time-point) was calculated and log2-transformed. The median of all replicate values was calculated. The expression of the submitted genes across each experiment was extracted and ranked in order of expression ratios. A Spearman rank correlation coefficient between the submitted data and each experiment stored in the database was calculated using RCC (http://www.bi.up.ac.za/MADIBA/organisms.php).

2.7.2. Comparison with basal defences

Microarray expression data for 120 of 134 genes that were differentially expressed in *A. thaliana* in response to *R. solanacearum* BCCF401 were extracted from selected experiments in NASCARRAYS. These experiments represented responses to the Pathogen Associated Molecular Patterns (PAMPs) flg22 and lipopolysaccharide (LPS), and to a non-virulent *Pst hrp*$^-$ mutant, virulent *Pst* DC3000 and *R. solanacearum* GMI1000. Replicate data within each NASCARRAYS dataset was averaged, and $\log_2$-fold change was calculated for each gene at each treatment and time-point relative to the relevant controls using Microsoft® Excel 2002 (Microsoft, Redmond, WA, USA). TIGR MultiExperiment Viewer (MeV) (Saeed et al. 2003) was used to cluster the complete dataset. Basal defence response genes were selected if they were induced/repressed by PAMP treatments (flg22, LPS or other PAMPs represented by *Pst hrp*$^-$ treatment) or virulent pathogens (*Pst* DC3000, *R. solanacearum* BCCF401, or *R. solanacearum* GMI1000). The accepted threshold for induced and repressed genes was a $\log_2$ fold change greater than 0.75 and less than −0.75, respectively.

3 Results

3.1 Eucalyptus isolate of *Ralstonia solanacearum* is virulent on *Arabidopsis thaliana*

The bacterial wilt isolate BCCF401 from an *E. grandis × E. camaldulensis* plantation in KwaZulu Natal, South Africa (Coutinho et al. 2000; Fouché-Weich et al. 2006) was able to cause disease on *Arabidopsis thaliana* ecotype Col-5. Susceptibility was confirmed in at least three independent experiments. Col-5 plants showed wilt symptoms approximately 10 days after inoculation. Wilt symptom 1–2 was observed 7–10 days after infection (Fig. 1a(ii) and (iii); early wilt) while wilt symptom 3–4 was observed 15–20 days post-infection (Fig. 1a(iv) and (v); late wilt). Disease index scores showed that *R. solanacearum* GMI1000, an isolate from tomato (Boucher et al. 1985) was more virulent than BCCF401 on *A. thaliana* ecotype Col-5 (Fig. 1b). Ecotype Nd1, in comparison to Col-5, was resistant to both GMI1000 and BCCF401 (Fig. 1b). Inoculation of Col-5 with a *hrp*$^-$ strain of BCCF401 resulted in no disease development (Fig. 1c; Fig. S2). Bacterial numbers reached 1×10^{11} in Col-5 plants infected with BCCF401 but remained at 1×10^{7} in Col-5 plants inoculated with the *hrp*$^-$ strain of BCCF401 and in the control-resistant interaction between ecotype Nd-1 and BCCF401 (Fig. 1c; Fig. S2). This suggests that disease development is *hrp*-dependent, requires multiplication of the bacteria, and is not simply due to a toxin from the pathogen.

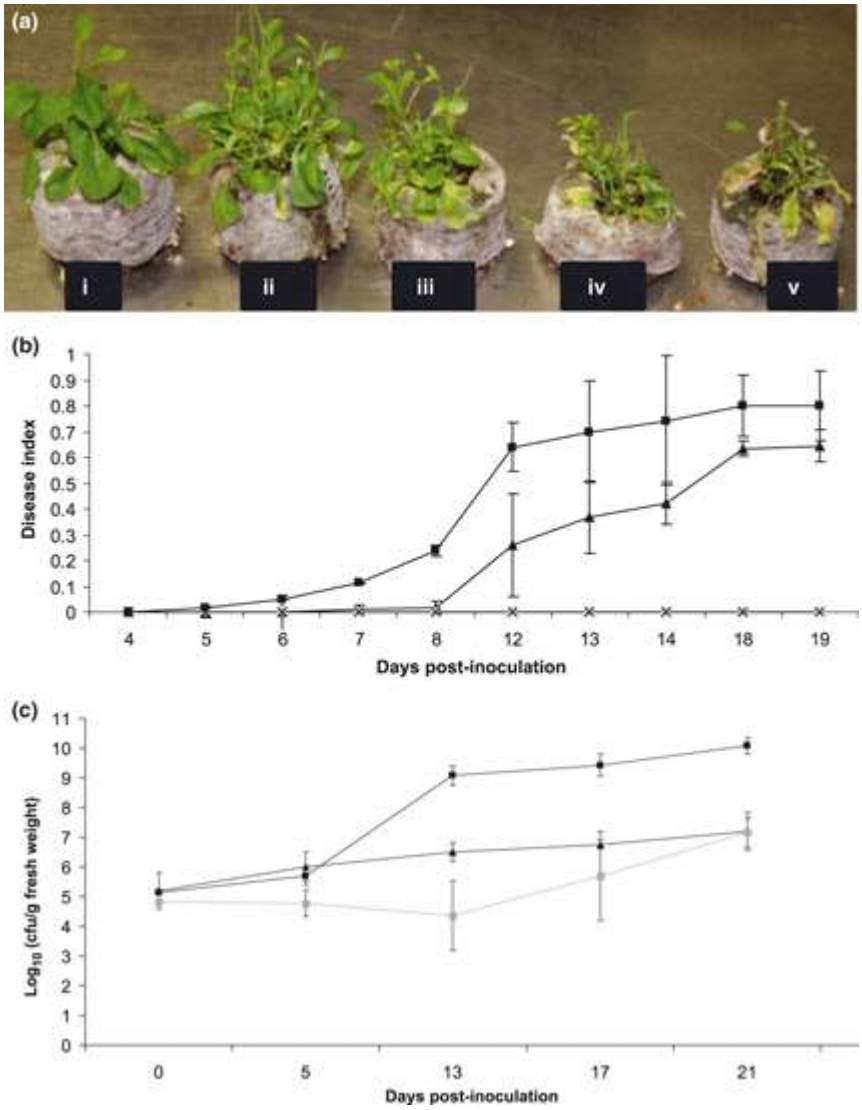

Figure 1. Susceptibility of *Arabidopsis thaliana* ecotypes to *Ralstonia solanacearum*. (a) Wilt symptoms on Col-5 inoculated with 1×10^8 cfu/ml of *R. solanacearum* BCCF401 using a root-inoculation method. The control plants were inoculated with a suspension of media and water.(i) Healthy control plant, no wilt symptom; plant showing wilt symptom 1 (ii), 2 (iii), 3 (iv) and 4 (v). (b) Disease index for Arabidopsis ecotypes infected with isolates of *R. solanacearum.* Ecotype Col-5 inoculated with *R. solanacearum* GMI1000 (■), ecotype Col-5 inoculated with BCCF 401 (▲), ecotype Nd-1 after challenge with GMI1000 (■) and ecotype Nd-1 after challenge with BCCF 401(×). The disease index for each treatment was calculated based on data from 14 individual plants over 20 days. Replicate infection experiments yielded similar results. (c) Bacterial numbers in Arabidopsis ecotypes inoculated with *R. solanacearum.* Ecotype Col-5 infected with *R. solanacearum* BCCF 401* (■), ecotype Col-5 infected with the hrp$^-$ mutant of BCCF 401 (▲) and ecotype Nd-1 inoculated with *R. solanacearum* isolate BCCF 401* (■). Bacterial counts were calculated on nine plants per time-point. *R. solanacearum* BCCF 401* is a spontaneous rifampicin resistant mutant of *R. solanacearum* BCCF 401 used to ensure that only *R. solanacearum* colonies were counted.

3.2 Eucalyptus and tomato isolates of *R. solanacearum* elicit similar expression profiles in Arabidopsis

cDNA microarray expression profiling of *A. thaliana* Col-5 plants inoculated with *R. solanacearum* BCCF401 and sampled at early wilt and late wilt stages separately was carried out in replicate experiments. A mixed model anova analysis (Wolfinger et al. 2001;

Adie et al. 2007) revealed 141 genes that were significantly up or down-regulated using stringent selection criteria (log_2-fold change >0.75 or <−0.75; $p < 0.03$ – Bonferroni corrected) (Fig. 2; Table S1). A greater proportion of genes were differentially expressed at the late wilt stage (72%) (Fig. 2). The cDNA microarray slides used for profiling contained 5000 Unigenes obtained from EST collections, representing approximately 20% of the Arabidopsis genome. There was no significant overrepresentation of GO terms on the microarray although 28% and 29% of genes on the microarray are annotated as responsive to abiotic/biotic stimulus and to stress, respectively, relative to the whole genome (Table S2). Although *R. solanacearum* is a root pathogen, in this study, aerial parts of the plant (leaf and stem) were selected for analysis as the root tissues of the plants were difficult to obtain from the Jiffy pots and at the later wilt symptoms, roots became damaged by infection and were insufficient for microarray profiling experiments. It has previously been shown that leaf inoculations of *R. solanacearum* in Arabidopsis illicit similar wilting symptoms to that observed by root inoculation (Deslandes et al. 1998).

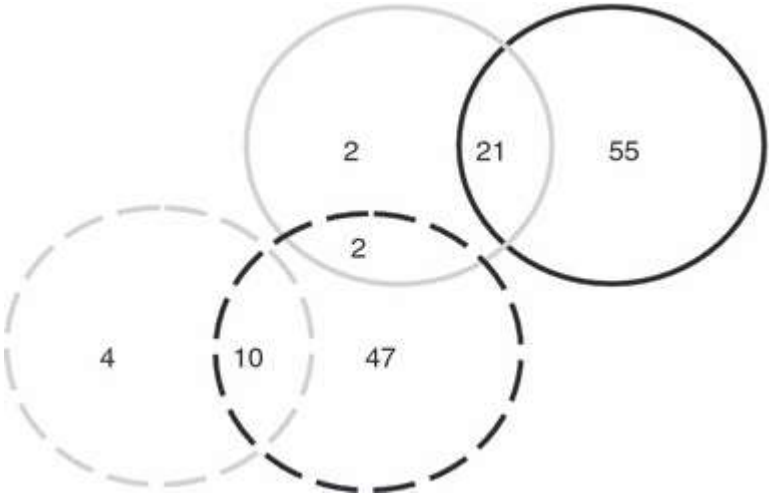

Figure 2. Venn diagram representing up-regulated (solid lines) and down-regulated (dashed lines) gene sets in early (grey circles) and late wilt stages (black circles) in response to *Ralstonia solanacearum* BCCF 401 infection. Genes were selected following mixed model anova analysis. Only those genes considered significantly up- and down-regulated are represented (log_2 fold change > 0.75 or <−0.75, respectively; p-value < 0.03).

Microarray expression data from the susceptible *A. thaliana–R. solanacearum* BCCF401 interaction were compared with data from other *A. thaliana* treatments. The RCC tool was developed for this purpose (http://www.bi.up.ac.za/MADIBA/organisms.php). Late wilt expression ratios for all genes that showed consistent expression across replicates (218 genes; anova analysis, $p < 0.03$) were submitted to RCC for comparison to expression data from Arabidopsis NASCArrays. Experiment comparisons are carried out in RCC based on the ranks of expression ratios, rather than the expression ratios themselves, to better account for differences in the microarray platforms. The highest correlation during late wilt responses (0.71) was obtained with expression data from infection of *A. thaliana* Col-5 plants with the *R. solanacearum* isolate GMI1000 from tomato (Table 1). The correlation between the early wilt responses in Col-5 by BCCF401 and GMI1000 was 0.6 (data not shown). This suggests conservation of virulence strategies by *R. solanacearum* isolates from different hosts. Top ranking correlations were also shown to compatible interactions with *Pseudomonas syringae* and *Botrytis cinerea* (Table 1). Interestingly, there were also high

correlations with two independent ABA treatments (Table 1), lending support to previous reports that ABA is involved in the plant response to bacterial wilt (Hernandez-Blanco et al. 2007). Correlation was also observed with expression data from the resistant response of Arabidopsis plants to *R. solanacearum* GMI1000, which may reflect similarities between the early resistant response and late susceptible responses, as has been observed in other plant–pathogen interactions (Tao et al. 2003).

Table 1. NASCArrays experiments with expression profiles similar to the late wilt expression profile induced by *R. solanacearum* BCCF401 infection in Arabidopsis.

NASCARRAY reference	Experiment name	Experiment description	Control	p-value	Rank correlation[1]
447	Marco_Col-1000-D3	Compatible interaction *Rsol GMI1000* t = 8 days	t = 0	1.05E–31	0.71
447	Marco_Nd-DeltaPopP2-D3	Compatible interaction *Rsol GMI1000* t = 8 days	t = 0	3.96E–24	0.64
447	Marco_Col-1000-D1	Compatible interaction *Rsol GMI1000* t = 5 days	t = 0	1.32E–21	0.61
120	AtGen_A-13_23-1_REP1_ATH1	Compatible interaction *Pst DC3000* t = 24 h	Untreated	3.83E–20	0.59
447	Marco_Nd-DeltaPopP2-D1	Compatible interaction *Rsol GMI1000* t = 5 days	t = 0	3.07E–19	0.58
120	AtGen_A-25_26-1_REP1_ATH1	Incompatible interaction *Pst avrRpm1* t = 24 h	Untreated	1.71E–17	0.56
176	RIKEN-GODA21A	ABA treatment t = 3 h	Untreated	4.94E–15	0.52
447	Marco_Nd-1000-D1	Incompatible interaction *Rsol GMI1000* t = 5 days	t = 0	2.42E–14	0.51
447	Marco_Nd-1000-D3	Incompatible interaction *Rsol GMI1000* t = 8 days	t = 0	2.31E–13	0.49
167	BC482-1	Compatible interaction *Botrytis cinerea* t = 48 h	Untreated	2.69E–13	0.49
57	Okamoto_gpa1-treated	ABA treatment t = 3 h	Untreated	1.07E–11	0.46

[1]The highest rank correlation results from the RCC Tool (http://www.bi.up.ac.za/MADIBA/organisms.php) are indicated.

GOstat analysis for the category biological process (Beißbarth and Speed 2004) of the gene lists indicated that both biotic (response to other organisms; innate immune response, JA and ET-dependent defence responses) and abiotic (response to water deprivation) stress responses were induced by *R. solanacearum* infection during early (Table 2) and late wilt (data not shown). A significant number of genes had common GO terms and were identified as over-represented GO terms ($p < 0.05$; Holm corrected) relative to the 5000 unigenes on the microarray, and to the whole Arabidopsis genome (Table 2). Each percentage indicates the proportion of that GO term which is found in the up-regulated gene set relative to the proportion of that GO term found on the 5000 unigene microarray or in the whole genome.

Table 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		Relative to genome	
	Percentage	GOstat p-value	Percentage	GOstat p-value
Response to other organism	45	0.00002	3	0.0008
Response to ethylene stimulus	25	0.006	2	0.05
Innate immune response	25	0.01	2	0.02
Response to wounding	18	0.01	1	0.05
Response to water deprivation	14	0.04	1	0.002
Jasmonic acid and ethylene-dependent systemic defence response	50	0.001	1	0.03
Lipid metabolic process	25	0.05	5	0.02

Consistent with the GOstat analysis, some markers associated with the JA/ET responses such as the pathogenesis-related protein genes *PR-3* and *PR-4* (Samac et al. 1990; Potter et al. 1993) were induced at both time-points after *R. solanacearum* infection and confirmed by RT-qPCR (Fig. 3a; Table S1). A marker of the SA signalling pathway (*PR-5*) was not differentially expressed during early wilt but was repressed during late wilt (Table S1). *PR-1*, another marker of the SA response pathway, was absent from the microarray. These data suggest that the JA/ET defence pathway may be induced and the SA defence pathway may be repressed by the infection.

In addition to *PR-3* and *PR-4*, RT-qPCR was carried out on a selection of genes (*seed imbibition protein homologue – Sip1, Tyrosine amino transferase – TAT, oxygen evolving complex 23 – OEC23*) that showed significant differential expression at both wilt stages ($p < 0.03$) by microarray analysis. The independent RT-qPCR technique showed the same expression profiles, thus validating the microarray data (Figs 3a,b).

Several water deprivation genes were induced during bacterial wilt infection in Col-5 plants. These include those encoding dehydrin family proteins: *response to dehydration 17* (rd17) and *rd19* (At1g20440 and At4g39090), cold-regulated genes *COR78* and *COR413* (At5g52310 and At2g15970), *late embryogenic abundant protein 5* (At4g02380) and a *NAC transcription factor* (At1g52890). A protease inhibitor, named *Arabidopsis thaliana drought repressive 4* (*ATDR4*; At1g73330) was repressed during both wilt stages. According to GENEVESTIGATOR, transcripts of *ATDR4* decline to below detection levels in response to progressive drought stress. Responses similar to water deprivation would be expected for plants undergoing wilting due to *R. solanacearum* infection. During *R. solanacearum* infection, the xylem of the plant becomes clogged with bacteria and bacterial debris, which reduces the plant's ability to take up water and thus wilting ensues (Genin and Boucher 2002b). Thus, wilt disease would result from water deprivation, as well as from the 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).

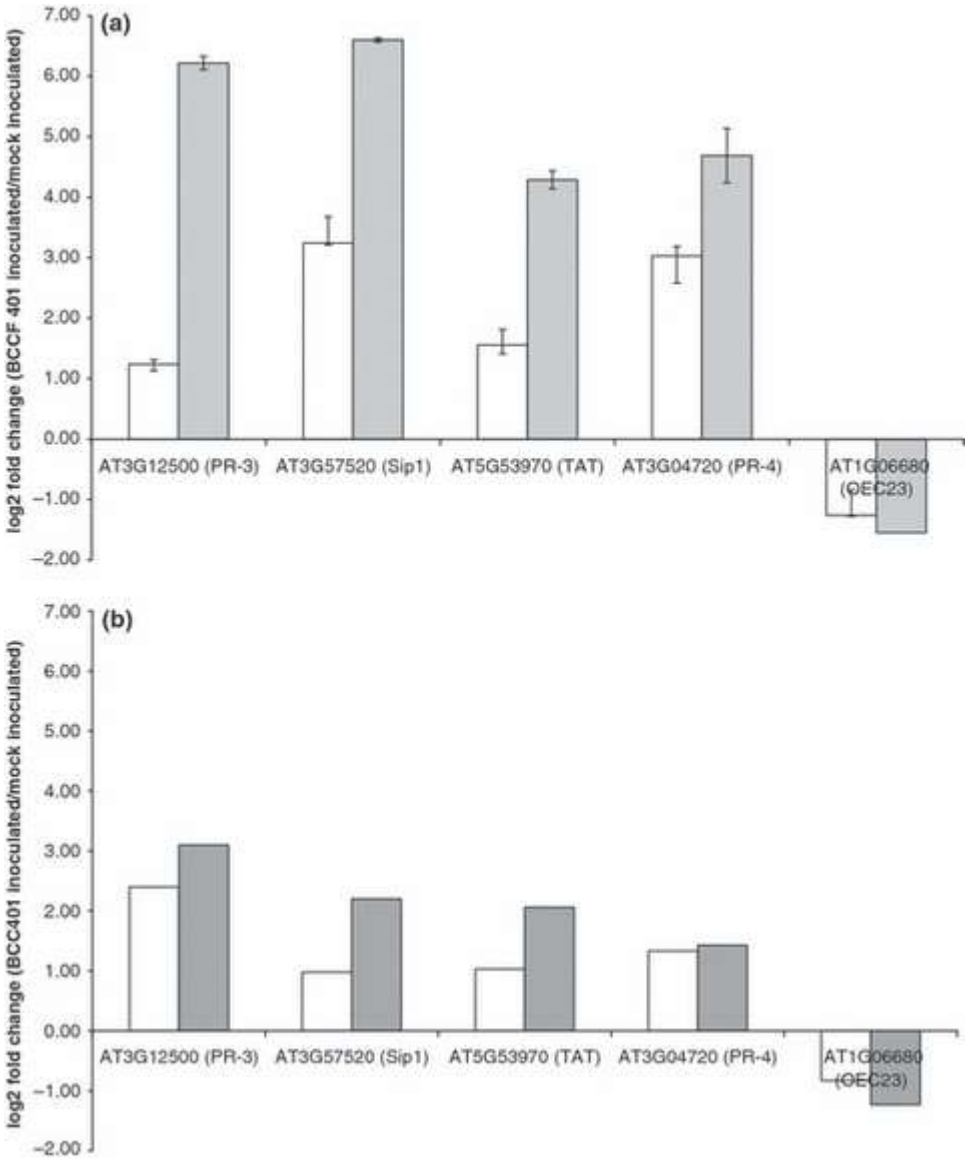

Figure 3. Expression data for selected *Arabidopsis thaliana* genes after *Ralstonia solanacearum* BCCF 401 inoculation relative to mock-inoculations at the same time-points. (a) RT-qPCR results and (b) microarray results. Gene expression ratios for the up-regulated genes *basic endochitinase (PR-3)*, *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)* are 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 RT-qPCR experiments, the data from at least three technical replicates are indicated. Results from a second biological replicate experiment were similar. The mean expression ratios of the five genes from the replicate microarray experiments are represented in (b).

3.4 Evidence for manipulation of host defence responses by *R. solanacearum* from Eucalyptus

Basal defences are often induced in the late stages of compatible interactions. These defences may be described as a weak form of immunity, ineffective in preventing disease (Jones and Dangl 2006). We investigated basal responses by performing bioinformatics comparisons 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

134 genes shown to be differentially regulated during *R. solanacearum* infection. Data were available for 120 of these genes. A subset (38) of the 120 genes met the criteria of basal defence response genes as being induced/repressed by PAMPs and/or virulent pathogens, and were clustered into groups I–VI (Fig. 4).

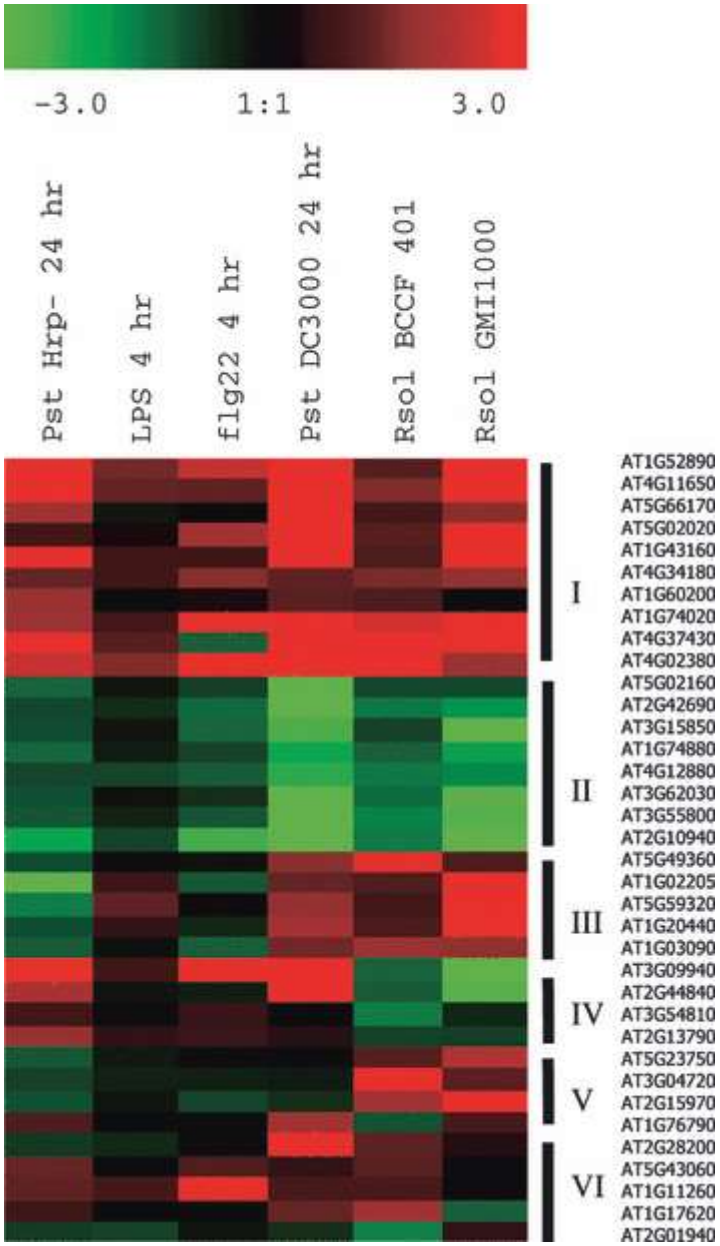

Figure 4. Heat map of *Arabidopsis thaliana* genes showing basal defence response profiles against *R. solanacearum*, *Pst* DC3000 and PAMPs. Green, black and red indicate down-regulated, unchanged and up-regulated genes respectively. Cluster I are genes induced by PAMPs and effectors, cluster II are genes which are repressed by PAMPs and effectors, cluster III are genes which are repressed by PAMPs but induced by effectors, cluster IV are genes which are induced by PAMPs but repressed by *R. solanacearum* effectors, cluster V are genes that are repressed by PAMPs but induced by *R. solanacearum* effectors and cluster VI are genes which are repressed or induced by *R. solanacearum* GMI1000-specific effectors. Expression data was obtained from NASCArrays and this study.

Some basal defences were induced by BCCF401 infection since some of the 38 genes are also induced under conditions where basal defence are known to operate, e.g. during flg22

and LPS treatment, *Pst hrp*$^-$ infection, and *Pst* DC3000 infection (Fig. 4 cluster I). This can be considered a weak form of PAMP Triggered Immunity or PTI (Jones and Dangl 2006) (Fig. 4 cluster I). Similarly, those genes that are repressed during *R. solanacearum* infection (GMI1000 and BCCF401), during *Pst* DC3000 infection as well as by PAMPs (flg22, LPS, *hrp*$^-$), would be indicative of PTI against *R. solanacearum* (Fig. 4 cluster II). 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).

Cluster III (Fig. 4) represents genes that could be targeted by effectors from *Pst DC3000* or *R. solanacearum* that change the expression pattern mediated by PAMPs (*hrp*$^-$ treatment, and/or flg22). Such effectors could be common to both types of pathogen or distinct effectors which target the same protein. Genes in cluster IV (Fig. 4) can be considered specific *R. solanacearum* effector targets as they are induced during *Pst hrp*$^-$ infection (i.e. they are PAMP-induced genes) and are also induced during *Pst DC3000* infection, but are repressed during *R. solanacearum* GMI1000 and BCCF401 infection. Defence-related genes such as *PR-3* (AT5G49360; cluster III) are possibly targets that are induced by bacterial effectors, while *PR-5* is potentially down-regulated by *R. solanacearum* effectors (AT2G44840; cluster IV). Cluster V represents genes that may be responding to *R. solanacearum* effectors. Cluster VI represents genes that are induced by either PAMPs and *Pst* DC3000 or *R. solanacearum* BCCF401 but repressed by GMI1000. Cluster VI also contains genes that are repressed by PAMPs, *Pst* DC3000 and *R. solanacearum* BCCF401 but induced by GMI1000. Cluster VI may represent responses that are specific to GMI1000.

4 Discussion

4.1 *Arabidopsis thaliana* ecotype Col-5 is susceptible to isolate BCCF401 from Eucalyptus

We investigated the susceptibility of *Arabidopsis thaliana* to a Eucalyptus isolate of *R. solanacearum* and demonstrated that the model plant acted as a host for the forest pathogen. Arabidopsis has been adopted as a model plant for plant pathogen studies as pathogens from several plant species have been shown to be pathogenic on Arabidopsis (Glazebrook 2005); however, to our knowledge this is the first report of a forest tree pathogen which has been used to infect Arabidopsis. This pathosystem provides an opportunity to exploit the genetic resourses available for Arabidopsis to understand the response of the host *Eucalyptus* to *R. solanacearum*. Based on this pathosystem, the plant defence responses against *R. solanacearum* were investigated using microarray expression profiling of 5000 unigenes. We identified 141 genes that were significantly differentially regulated at early and/or late wilt, with 33 genes differentially regulated at both time-points (Fig. 2). Six genes were differentially regulated only during early wilt, whereas 102 genes were differentially regulated at late wilt, indicating that the bulk of gene expression changes occurred during late wilt (Fig. 2).

4.2 Signalling pathways in response to *R. solanacearum* BCCF401 infection

The induction of the marker genes for the JA/ET signalling pathway, *PR-3* and *PR-4* by *R. solanacearum* was detected by microarray analysis and RT-qPCR (Fig. 3). 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 GMI1000 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). *Ralstonia 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' jasmonate-isoleucine conjugate involved in defence signalling (Grant and Jones 2009). The JA signalling pathway antagonises the SA pathway, which is important for defence against *P. syringae*. The fact that there is coordinated expression of type three effectors and ethylene biosynthesis and that the levels of bacterial ethylene production causes the induction of plant ethylene marker genes increases the possibility that *R. solanacearum* may manipulate the plant ethylene signalling response to enhance susceptibility (Valls et al. 2006). Cluster V of Fig. 4 also indicates that *PR-4* (At3g04720) is induced by *R. solanacearum* effectors but is not induced by PAMPs, suggesting pathogen manipulation of signalling leads to induction of PR-4.

The SA marker gene PR-5 was repressed at late-wilt time points in Col-5 suggesting that this pathway may be repressed during a compatible interaction; however, Hirsch et al. (2002) demonstrated that the SA mutants *cpr1* and *cpr5*, which show constitutively elevated levels of SA and PR gene expression, and *NahG* transgenic plants, which had depleted endogenous SA, were as susceptible to *R. solanacearum* GMI1000 as wild-type plants. The hypothesis that SA does play a limited role in plant defence against *R. solanacearum* is supported by Deslandes et al. (2002) who observed susceptibility to the pathogen in Nd1 plants homozygous for *NahG*. In *Eucalyptus urophylla* seedlings, the application of exogenous SA as a soil drench induced resistance against *R. solanacearum*, supporting a role for this signalling hormone in its natural host (Ran et al. 2005a).

Based on the RCC results in Table 1, it can be predicted that the ABA signalling pathway is operating in response to *R. solanacearum* infection. This observation is supported by Hu et al. (2008) who showed that approximately 40% of the genes up-regulated during wilting caused by GMI1000 infection in susceptible Arabidopsis plants were involved in ABA biosynthesis and signalling. These results lead to the conclusion that ABA may enhance susceptibility to *R. solanacearum*, particularly during wilting at the later stages of infection. However, this is in contrast to reports that ABA may also play a role in resistant interactions. 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. We hypothesize that this conundrum may be explained by differences in timing, i.e. ABA signalling at early stages of infection leads to resistance, whereas an enhanced ABA response at late stages of disease serves to enhance the wilting response, as is proposed for ethylene. Measurement of ABA levels during the time course of infection in different interactions may shed light on this question.

4.3 Manipulation of defence responses by *R. solanacearum* BCCF401

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). We identified several genes with an increased expression during *R. solanacearum* infection or *Pst* DC3000 infection compared with 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 Fig. 4 are potential biotechnology targets, which if uncoupled from pathogen manipulation (i.e. by overexpression of genes in cluster IV; or repression of genes in cluster III/V), may enhance resistance against *R. solanacearum*. GMI1000 infection of Arabidopsis plants also revealed a high percentage of transcripts which were induced by flg22 (Hu et al. 2008), suggesting that basal defence responses are generally active against *R. solanacearum*.

In cluster III (Fig. 4), the gene At5g49360 (*beta-xylosidase 1*) is up-regulated by bacterial effectors from *Pst* and *R. solanacearum* but is down-regulated by PAMPs. This may reflect efforts by the pathogen to promote host cell wall degradation and facilitate spread of the pathogen. The cell wall polysaccharide substrates of beta-xylosidase 1 are xylan, arabinan and arabinoxylan (Minic 2008).

Another noteworthy gene which appears to be manipulated by *R. solanacearum* effectors is At2g13790 (*AtSERK4*, Fig. 4 Cluster IV). AtSERK4 is the closest paralog of the Arabidopsis receptor like kinase BAK1 (He et al. 2007; Hernandez-Blanco et al. 2007). BAK1 is a signalling partner of the flagellin receptor FLS2 and is thought to be a shared signalling partner for other PAMPs besides flagellin (Shan et al. 2008). Effectors from *Pst*, AvrPto and AvrPtoB bind BAK1 and interfere with the association of FLS2 with BAK1 during infection and impede BAK1-dependent host immune responses (Shan et al. 2008). AvrPto and AvrPtoB are also able to interact with AtSERK4 (Shan et al. 2008). The down-regulation of *AtSERK4* by *R. solanacearum* effectors may reflect attempts by the pathogen to suppress AtSERK4-dependent defences. Interestingly, in a resistant interaction between *R. solanacearum* GMI1000 and Nd-1, the expression of *AtSERK4* does not change (data not shown) suggesting that in a resistant interaction, the suppression by *R. solanacearum* effectors may be relieved.

In cluster V of Fig. 4, up-regulation of a marker gene of the ethylene pathway (*PR-4*; At3g04720) could represent pathogen manipulation at the level of phytohormones, because *R. solanacearum* is known to produce ethylene (Aldon et al. 2000).

Ralstonia. solanacearum contains over 70 putative effectors (Cunnac et al. 2004a,b; Occhialini et al. 2005; Angot et al. 2006; Meyer et al. 2006). *Ralstonia solanacearum* Petunia strain UW551, which belongs to race 3, biovar 2 has only 6 or 7 effectors 'missing' compared to GMI1000, and three effectors: RRSL00326, RRSL01019, and RRSL03923, are unique to UW551 (Mukaihara et al. 2004; Gabriel et al. 2006; Adie et al. 2007). BCCF401 belongs to the same race and biovar as GMI1000 (race 1, biovar 3), thus the two pathogens may share common effectors. Thus, it is intriguing as to why the two *R. solanacearum* strains GMI1000 and BCCF401 induce different expression profiles of some genes in Col-5, e.g. in cluster VI of Fig. 4, At2g01940 (*shoot gravitropism 5*) appears to be manipulated specifically by *R. solanacearum* GMI1000 effectors. The gene is also moderately induced in a resistant interaction between *R. solanacearum* GMI1000 and Arabidopsis ecotype Nd1 (data not shown). *Shoot gravitropism 5* has transcription factor activity and mutant plants showed abnormal gravitropic response in inflorescence stems, while gravitropism in hypocotyls and roots remained unaltered (Morita et al. 2006). The role of this gene in defence against *R. solanacearum* is unknown and due to the fact that it is uniquely regulated by *R. solanacearum* GMI1000 makes it an attractive target for gene function studies.

Experiments are underway to determine the significance of some of the putative effector target genes using T-DNA knockout lines in Col-0. However, a single knock-out in the host may not reveal the desired phenotype as demonstrated by Hu et al. (2008) who screened 45 null mutants and found only two which showed a delay in wilt symptom development after challenge by *R. solanacearum* GMI1000 suggesting that effectors have multiple host targets to effect disease.

The availability of the genome sequence of *Eucalyptus grandis* in 2010 (Myburg et al. 2008) will provide a resource to design Eucalyptus-specific primers to target the Eucalyptus orthologs of host genes identified in this study. It would be of interest to determine whether the same signalling pathways are induced by the pathogen and whether the same targets are being manipulated in Eucalyptus. These results will strengthen the motivation for using Arabidopsis as a model for tree pathogens and allow for the identification of candidate genes which may be targeted to improve defence against *R. solanacearum*.

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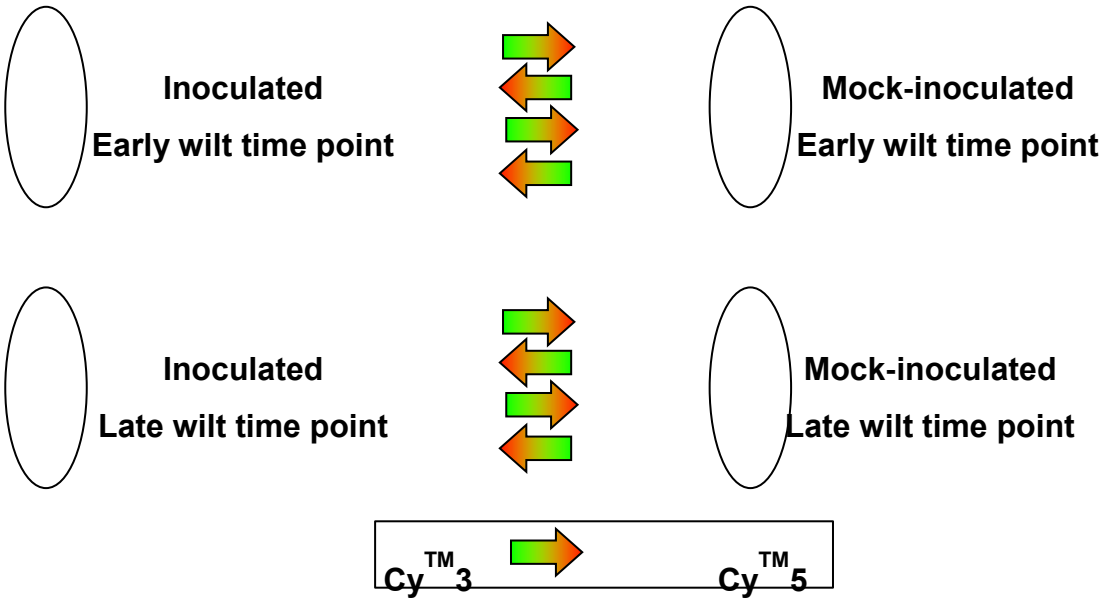
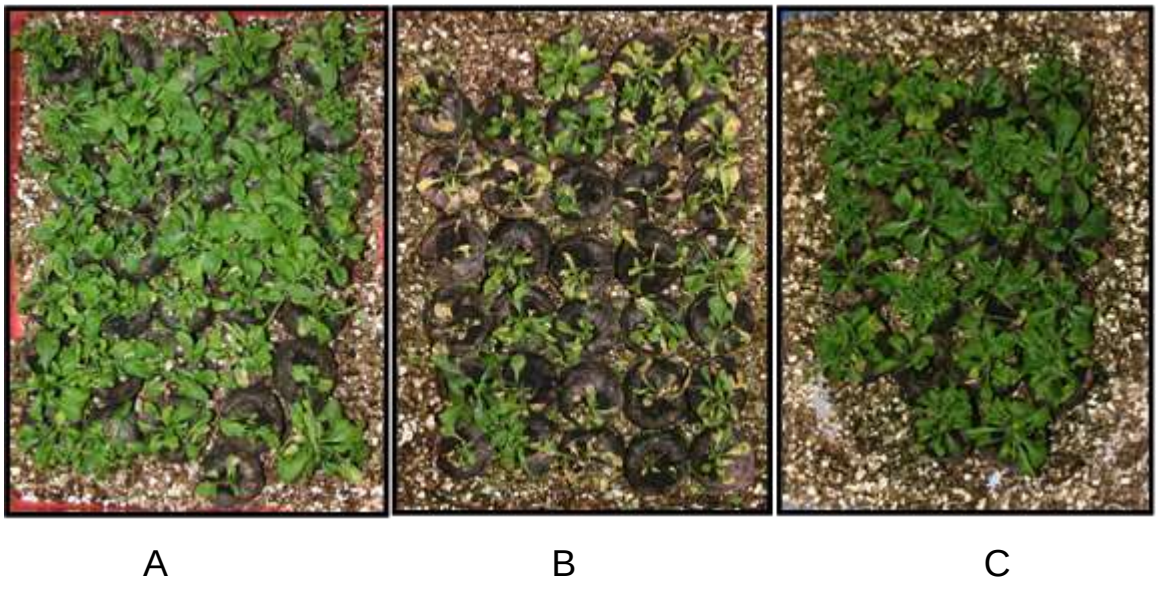
Supporting Information



Supplementary Figure S1. Microarray experimental design used to compare expression patterns in *Arabidopsis thaliana* Col-5 plants inoculated with *Ralstonia solanacearum* BCCF401 and mock-inoculated plants during early wilt and late wilt stages. Ovals represent samples and arrows represent slides. The head of the arrow represents samples labelled with the Cy™5 dye and the tail of the arrow represents samples labelled with the Cy™3 dye. Arrows in opposite directions indicate a dye swap. The design includes biological replicates of each treatment.



Supplementary Figure S2.*Arabidopsis thaliana* ecotypes inoculated with *Ralstonia solanacearum* BCCF401 at 21 days after inoculation. Ecotype Col-5 inoculated with the *hrp*⁻ mutant of isolate BCCF401 (A), ecotype Col-5 inoculated with BCCF401* (B) and ecotype Nd-1 inoculated with BCCF401* (C).

Supplementary Table 1: Microarray expression ratios of genes that are differentially expressed at the early and late wilt stage in *Arabidopsis thaliana* Col 5 plants infected with *Ralstonia solanacearum* BCCF401.

		EARLY WILT	
TAIR Accession	**Description**	**log_2 Fold Change**	**Negative log_{10}(p value)**
AT4G11650	ATOSM34 (OSMOTIN 34)	3.64	2.72
AT5G59320	LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding	2.67	1.75
AT3G01420	ALPHA-DOX1 (ALPHA-DIOXYGENASE 1)	2.45	5.55
AT3G12500	ATHCHIB (BASIC CHITINASE); chitinase	2.40	2.47
AT1G47830	clathrin coat assembly protein, putative	2.33	2.04
AT4G16260	glycosyl hydrolase family 17 protein	2.19	6.59
AT4G37430	CYP91A2 (CYTOCHROME P450 MONOOXYGENASE 91A2); oxygen binding	1.86	1.92
AT3G28710	H+-transporting two-sector ATPase, putative	1.78	2.05
AT1G78890	similar to Os08g0230000 [Oryza sativa (japonica cultivar-group)]	1.41	1.75
AT1G32450	proton-dependent oligopeptide transport (POT) family protein	1.34	1.80
AT3G04720	PR4 (PATHOGENESIS-RELATED 4)	1.33	7.26
AT1G31130	similar to conserved hypothetical protein [Medicago truncatula] (GB:ABE90086.1)	1.22	3.75
AT5G52310	COR78 (COLD REGULATED 78)	1.14	1.58
AT4G13250	short-chain dehydrogenase/reductase (SDR) family protein	1.07	1.72
AT5G53970	aminotransferase, putative	1.03	2.52
AT3G57520	ATSIP2 (ARABIDOPSIS THALIANA SEED IMBIBITION 2)	0.98	2.12
AT1G51670	contains domain Cysteine proteinases (SSF54001)	0.96	2.31
AT5G11520	ASP3 (ASPARTATE AMINOTRANSFERASE 3)	0.95	1.67
AT3G10630	glycosyl transferase family 1 protein	0.91	2.46
AT1G06570	PDS1 (PHYTOENE DESATURATION 1)	0.91	2.66
AT5G58500	similar to hypothetical protein [Oryza sativa (japonica cultivar-group)]	0.87	4.39
AT2G42890	AML2; RNA binding	0.86	2.20
AT5G49360	BXL1 (BETA-XYLOSIDASE 1); hydrolase, hydrolyzing O-glycosyl compounds	0.85	5.05
AT4G39090	RD19 (RESPONSIVE TO DEHYDRATION 19); cysteine-type peptidase	0.83	10.43
AT2G33150	PED1 (PEROXISOME DEFECTIVE 1)	0.82	2.24

		EARLY WILT	
TAIR Accession	**Description**	**log_2 Fold Change**	**Negative log_{10}(p value)**
AT3G22231	PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)	-1.81	1.58
AT5G24770	VSP2 (VEGETATIVE STORAGE PROTEIN 2); acid phosphatase	-1.30	2.78
AT1G73330	ATDR4 (Arabidopsis thaliana drought-repressed 4)	-1.17	2.29
AT2G44840	ATERF13/EREBP (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 13)	-1.13	1.55
AT4G15440	HPL1 (HYDROPEROXIDE LYASE 1)	-1.11	2.76
AT1G75040	PR5 (PATHOGENESIS-RELATED GENE 5)	-1.09	3.06
AT1G14030	RIBUSCO large subunit N-methyltransferase, putative	-1.02	1.70
AT5G09810	ACTIN 2	-0.98	4.88

Supplementary Table 1: Microarray expression ratios of genes that are differentially expressed at the early and late wilt stage in *Arabidopsis thaliana* Col 5 plants infected with *Ralstonia solanacearum* BCCF401.

		EARLY WILT	
TAIR Accession	**Description**	**log_2 Fold Change**	**Negative log_{10}(p value)**
AT1G70410	carbonic anhydrase, putative / carbonate dehydratase, putative	-0.89	1.80
AT1G04250	AXR3 (AUXIN RESISTANT 3); transcription factor	-0.88	2.19
AT3G28300	AT14A	-0.85	2.25
AT3G14210	ESM1 (EPITHIOSPECIFIER MODIFIER 1)	-0.83	2.93
AT1G06680	PSBP-1 (OXYGEN-EVOLVING ENHANCER PROTEIN 2)	-0.83	2.53
AT5G61650	CYCP4;2 (CYCLIN P4;2); cyclin-dependent protein kinase	-0.80	1.85

Supplementary Table 1: Microarray expression ratios of genes that are differentially expressed at the early and late wilt stage in *Arabidopsis thaliana* Col 5 plants infected with *Ralstonia solanacearum* BCCF401.

		LATE WILT	
TAIR Accession	**Description**	**log2 Fold Change**	**Negative log_{10}(p value)**
AT3G01420	pathogen-inducible alpha-dioxygenase (Nicotiana attenuata)	4.49	7.91
AT4G11650	Osmotin-like protein (OSM34)	4.27	2.22
AT5G59320	Lipid transfer protein 3 (LTP3)	3.84	3.36
AT4G37430	Cytochrome P450 81F1 (CYP81F1)	3.60	3.08
AT2G47770	Benzodiazepine receptor-related, contains weak similarity to Peripheral-type benzodiazepine receptor (PBR)	3.57	2.35
AT3G12500	Basic endochitinase, identical to basic endochitinase precursor SP:P19171 from (Arabidopsis thaliana)	3.11	2.61
AT2G22470	Arabinogalactan-protein (AGP2)	3.08	2.69
AT4G16260	Glycosyl hydrolase family 17 protein	2.65	5.51
AT1G03220	Extracellular dermal glycoprotein, putative / EDGP	2.55	3.05
AT5G11520	Aspartate aminotransferase, chloroplast (YLS4)	2.35	3.19
AT2G34500	Cytochrome P450 family protein	2.33	2.43
AT3G57520	Alkaline alpha galactosidase, putative, similar to alkaline alpha galactosidase II (Cucumis melo)	2.28	2.67
AT4G13250	Short-chain dehydrogenase/reductase (SDR) family protein	2.18	2.67
AT5G53970	Encodes tyrosine aminotransferase which is strongly induced upon aging and coronatine treatment	2.06	5.67
AT1G74020	Strictosidine synthase family protein	2.05	2.87
AT4G19920	Disease resistance protein (TIR class), putative	2.01	2.55
AT1G32450	Proton-dependent oligopeptide transport (POT) family protein	1.91	4.31
AT1G52890	No apical meristem (NAM) family protein	1.84	2.46
AT3G44880	Rieske (2Fe-2S) domain-containing protein	1.83	2.34
AT3G03470	Cytochrome P450, putative	1.81	9.42
AT2G33150	Encodes a peroxisomal 3-ketoacyl-CoA thiolase	1.79	3.20
AT1G11260	Glucose transporter (STP1)	1.77	2.27
AT1G43160	Encodes a member of the ERF subfamily B-4 of ERF/AP2 transcription factor family (RAP2.6).	1.69	3.35
AT5G66760	Succinate dehydrogenase (ubiquinone) flavoprotein subunit	1.67	2.75

Supplementary Table 1: Microarray expression ratios of genes that are differentially expressed at the early and late wilt stage in *Arabidopsis thaliana* Col 5 plants infected with *Ralstonia solanacearum* BCCF401.

		LATE WILT	
TAIR Accession	**Description**	**log2 Fold Change**	**Negative log_{10}(p value)**
AT1G31130	Expressed protein	1.56	3.28
AT5G66170	Senescence-associated family protein	1.53	2.33
AT5G52310	Low-temperature-responsive protein 78 (LTI78) / desiccation-responsive protein 29A (RD29A)	1.51	1.56
AT1G78890	Expressed protein	1.50	2.39
AT5G46180	Ornithine aminotransferase, putative	1.50	2.71
AT5G06760	Late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein	1.50	3.70
AT4G39090	Cysteine proteinase RD19a (RD19A) / thiol protease	1.48	17.86
AT5G49360	Glycosyl hydrolase family 3 protein	1.47	4.46
AT2G42890	RNA recognition motif (RRM)-containing protein	1.47	9.13
AT3G45310	Cysteine proteinase, putative	1.43	2.47
AT3G04720	Hevein-like protein (HEL)	1.43	5.58
AT3G10740	Glycosyl hydrolase family protein 51	1.42	3.79
AT5G49360	Glycosyl hydrolase family 3 protein	1.36	4.74
AT3G02550	LOB domain protein 41 / lateral organ boundaries domain protein 41 (LBD41)	1.31	2.68
AT5G54080	Homogentisate 1,2-dioxygenase / homogentisicase/homogentisate oxygenase / homogentisic acid oxidase (HGO	1.28	2.89
AT4G34180	Cyclase family protein, contains Pfam profile: PF04199 putative cyclase	1.24	4.26
AT5G23750	Remorin family protein	1.23	2.19
AT1G03090	Methylcrotonyl-CoA carboxylase alpha chain, mitochondrial / 3-methylcrotonyl-CoA carboxylase 1 (MCCA)	1.21	2.02
AT4G02380	Late embryogenesis abundant 3 family protein / LEA3 family protein	1.20	2.95
AT1G06570	4-hydroxyphenylpyruvate dioxygenase (HPD),	1.19	3.24
AT3G13450	2-oxoisovalerate dehydrogenase / 3-methyl-2-oxobutanoate dehydrogenase	1.18	4.02
AT5G13800	Hydrolase, alpha/beta fold family protein, low similarity to hydrolase	1.18	2.71
AT1G20440	Dehydrin (COR47), identical to dehydrin COR47	1.16	2.04
AT3G22840	Chlorophyll A-B binding family protein / early light-induced protein (ELIP)	1.16	2.13
AT2G15970	Cold-acclimation protein, putative (FL3-5A3)	1.15	2.04
AT5G13800	Hydrolase, alpha/beta fold family protein, low similarity to hydrolase (Terrabacter sp. DBF63) GI:14196240	1.15	2.38
AT4G37390	Encodes an IAA-amido synthase that conjugates Asp and other amino acids to auxin in vitro	1.14	6.08
AT3G11780	MD-2-related lipid recognition domain-containing protein	1.14	2.89
AT5G43060	similar to cysteine proteinase RD21A precursor (thiol protease) GI:435619, SP:P43297 from (Arabidopsis thaliana)	1.14	4.75
AT4G15530	Similar to pyruvate,orthophosphate dikinase [Flaveria brownii] (GB:CAA55784.1);	1.13	7.38
AT5G58500	Expressed protein, contains Pfam profile PF04852	1.12	2.47
AT5G42250	Alcohol dehydrogenase, putative, similar to alcohol dehydrogenase ADH GI:7705214 from (Lycopersicon esculentum);	1.10	2.73
AT2G01340	Expressed protein	1.10	2.46
AT2G28200	Similar to zinc finger (C2H2 type) family protein [Arabidopsis thaliana] (TAIR:At5g04390.1)	1.09	2.23
AT1G75170	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein, similar to polyphosphoinositide binding protein Ssh1p (GI:2739044) {Glycine max}	1.09	2.10

Supplementary Table 1: Microarray expression ratios of genes that are differentially expressed at the early and late wilt stage in *Arabidopsis thaliana* Col 5 plants infected with *Ralstonia solanacearum* BCCF401.

		LATE WILT	
TAIR Accession	**Description**	**log2 Fold Change**	**Negative log_{10}(p value)**
AT5G02020	Expressed protein	1.08	4.35
AT3G50370	Expressed protein	1.03	2.94
AT1G72770	Protein phosphatase 2C P2C-HA	1.01	3.73
AT3G48880	F-box family protein, N7 protein - Medicago truncatula, EMBL:CAA768	1.00	2.01
AT1G02205	CER1 protein, identical to maize gl1 homolog (glossy1 locus)	1.00	11.05
AT5G45350	Proline-rich family protein	0.99	3.13
AT3G17780	Expressed protein	0.98	2.42
AT3G28550	Proline-rich extensin-like family protein	0.95	2.17
AT5G60580	Zinc finger (C3HC4-type RING finger) family protein	0.95	2.68
AT5G27350	Sugar-porter family protein 1 (SFP1)	0.92	4.26
AT3G26100	Regulator of chromosome condensation (RCC1) family protein	0.91	2.17
AT3G58750	Encodes a peroxisomal citrate synthase that is expressed throughout seedling and shoot development.	0.89	2.79
AT3G55610	Delta 1-pyrroline-5-carboxylate synthetase B / P5CS B (P5CS2), identical to SP|P54888	0.85	2.37
AT5G21990	Tetratricopeptide repeat (TPR)-containing protein, contains Pfam profile PF00515: TPR Domain	0.84	2.39
AT1G60200	Splicing factor PWI domain-containing protein	0.84	3.12
AT1G17620	Expressed protein	0.83	2.79
AT2G38710	AMMECR1 family, similar to AMMECR1 (GI:6063688)	0.81	2.70

		LATE WILT	
TAIR Accession	**Description**	**log2 Fold Change**	**Negative log_{10}(p value)**
AT3G22231	Encodes a member of a novel 6 member Arabidopsis gene family (PPC1)	-2.79	1.70
AT3G54810	Encodes a protein containing a GATA type zinc finger domain	-2.41	2.41
AT2G44840	Encodes a member of the ERF subfamily B-3 of ERF/AP2 transcription factor family.	-1.96	2.31
AT5G52820	WD-40 repeat family protein / notchless protein	-1.68	2.29
AT5G24770	Vegetative storage protein 2 (VSP2)	-1.67	2.23
AT3G13140	Hydroxyproline-rich glycoprotein family protein	-1.60	4.63
AT3G45640	Mitogen-activated protein kinase, putative / MAPK, putative (MPK3)	-1.54	2.55
AT3G15530	Expressed protein	-1.51	2.40
AT4G16670	Expressed protein	-1.45	2.79
AT1G14030	RIBUSCO large subunit N-methyltransferase, putative	-1.43	3.53
AT5G38410	RuBisCO small subunit 3B (RBCS-3B) (ATS3B),	-1.42	2.04
AT5G02160	Expressed protein	-1.42	3.28
AT4G12880	Plastocyanin-like domain-containing protein	-1.42	2.19
AT4G11320	Cysteine proteinase, putative, contains similarity to cysteine proteinase RD21A	-1.37	2.04
AT1G73330	Protease inhibitor, putative (DR4), identical to Dr4 GI:469114 from (Arabidopsis thaliana);	-1.32	3.10
AT4G15440	member of the CYP74B cytochrome p450 family	-1.28	2.79

AT1G75750	Gibberellin-regulated protein 1 (GASA1)	-1.28	3.28
AT1G10150	Expressed protein	-1.27	2.16
AT3G55800	Encodes the chloroplast enzyme sedoheptulose-1,7-bisphosphatase (SBPase)	-1.25	2.12
AT1G12270	Stress-inducible protein, putative, similar to sti (stress inducible protein) (Glycine max)	-1.25	4.84
AT3G45140	Lipoxygenase (LOX2), identical to SP|P38418	-1.24	2.91
AT4G13830	DNAJ heat shock N-terminal domain-containing protein (J20)	-1.23	2.36
AT1G06680	Photosystem II oxygen-evolving complex 23 (OEC23)	-1.23	3.23
AT4G38970	Fructose-bisphosphate aldolase, putative	-1.22	2.13
AT3G58760	Ankyrin protein kinase	-1.17	2.14
AT3G09940	Monodehydroascorbate reductase, putative,	-1.15	2.19
AT1G76790	O-methyltransferase family 2 protein, similar to caffeic acid O-methyltransferase	-1.15	2.11
AT3G15850	similar to delta 9 acyl-lipid desaturase (ADS1)	-1.15	3.08
AT2G44210	Expressed protein, Pfam profile PF03080: Arabidopsis proteins of unknown function	-1.15	2.10
AT1G69120	Floral homeotic protein APETALA1 (AP1) / agamous-like MADS box protein (AGL7),	-1.10	2.19
AT5G44340	beta tubulin	-1.10	2.32
AT1G55450	embryo-abundant protein-related, similar to embryo-abundant protein	-1.08	3.50
AT5G40950	50S ribosomal protein L27	-1.07	3.16
AT2G01940	Similar to zinc finger (C2H2 type) family protein [Arabidopsis thaliana]	-1.06	2.54
AT5G61650	Cyclin family protein, similar to cyclin 2 (Trypanosoma brucei)	-1.05	1.64
AT1G75040	PR5 (PATHOGENESIS-RELATED GENE 5)	-1.05	4.25
AT4G21720	Expressed protein, (Arabidopsis thaliana)	-1.05	3.53
AT1G74880	Encodes subunit NDH-O of NAD(P)H:plastoquinone dehydrogenase complex (Ndh complex)	-1.04	2.29
AT3G28300	Integrin-related protein 14a	-1.00	1.67
AT2G05920	Subtilase family protein, contains similarity to cucumisin-like serine protease	-0.99	3.84
AT3G62030	Peptidyl-prolyl cis-trans isomerase, chloroplast A-binding protein (ROC4)	-0.98	2.57
AT4G23750	Encodes a member of the ERF subfamily B-5 of ERF/AP2 transcription factor family	-0.98	2.08
AT2G42690	Lipase, putative, similar to lipase (Dianthus caryophyllus)	-0.97	2.09
AT1G65960	Similar to glutamate decarboxylase 1 (GAD 1) [Arabidopsis thaliana]	-0.97	2.49
AT1G13260	DNA-binding protein RAV1 (RAV1)	-0.95	5.68
AT3G27830	50S ribosomal protein L12-1, chloroplast (CL12-A)	-0.95	2.49
AT3G14210	Myrosinase-associated protein	-0.94	1.70
AT3G16470	Jacalin lectin family protein	-0.94	2.28
AT2G10940	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	-0.93	2.62
AT1G03130	Photosystem I reaction center subunit II, chloroplast precursor	-0.92	2.41
AT4G32260	identical to cDNA chloroplast ATP synthase beta chain precursor (atpG) GI:5730140	-0.92	2.62
AT4G24190	Shepherd protein (SHD) / clavata formation protein, putative, nearly identical to SHEPHERD	-0.91	2.35
AT5G67290	FAD-dependent oxidoreductase family protein	-0.86	2.12
AT4G01050	Hydroxyproline-rich glycoprotein family protein, contains a rhodanese homology domain	-0.86	3.66
AT1G70410	Carbonic anhydrase, putative / carbonate dehydratase, putative,	-0.85	2.33
AT1G12000	Pyrophosphate--fructose-6-phosphate 1-phosphotransferase beta subunit, putative	-0.84	2.39
AT5G38420	RuBisCO small subunit 2B (RBCS-2B)	-0.84	2.74

AT5G09220	Amino acid permease 2 (AAP2),	-0.81	2.71
AT2G13790	Leucine-rich repeat family protein / protein kinase family protein	-0.80	4.12

Supplementary Table 2. Gene Ontologies for 5000 unigenes represented on the Arabidopsis cDNA microarray used for expression profiling of the susceptible interaction between *Ralstonia solanacearum* BCCF401 and *Arabidopsis thaliana* 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