# CHAPTER 5
# TRANSCRIPTOME ANALYSIS OF AN *ARABIDOPSIS THALIANA* ECOTYPE SHOWING RESISTANCE TO AN AFRICAN ISOLATE OF *RALSTONIA SOLANACEARUM*.

## 5.1 Abstract

The soil-borne vascular pathogen *Ralstonia solanacearum*, causes wilting on several plant species, including tree species such as *Eucalyptus*, and results in devastating crop losses worldwide. Resistant plant varieties are desirable as part of an integrated approach of disease control. Resistance against *R. solanacearum* has previously been identified in the model plant *Arabidopsis thaliana.* Previous work has revealed a novel pathosystem between a *Eucalyptus* isolate of *R. solanacearum* (BCC 402, CK) and the *Arabidopsis* ecotypes Be-O and Kil-O. Isolate BCCF 402 caused disease symptoms on Be-O three to five days after infection while Kil-O remained healthy two weeks after infection, at which time Be-O was completely dead. The resistant interaction between BCC 402 and Kil-O was investigated using whole-genome microarrays. Thirteen genes were found to be differentially expressed in Kil-0 at a p-value <0.01 and fold change greater than 1.65. A comparison of the expression of several of these genes in the susceptible ecotype Be-O indicated that transcripts of lipid transfer protein 3 (LTP3), peroxidase (PRX34), tropinone reductase (SAG13), avirulence-induced gene (AIG), translation initiation factor (SUI1), SKP1 interacting partner 5 (SKP5) and an "expressed protein" are preferentially expressed to a higher level earlier in the resistant interaction than in the susceptible one. The latter genes are worthy of further investigation in gene functional studies to clarify their role in resistance against *R. solanacearum*.

## 5.2. Introduction

The *Ralstonia solanacearum* and *Arabidopsis thaliana* pathosystem has proved a useful tool to understand the plant defence response. Deslandes et al. (1998) showed that the ecotype Col-0 was susceptible to the French Guyana tomato isolate of *R. solanacearum*, GMI1000. Nd-1 was completely resistant. These phenotypes were accompanied by a high bacterial load in the susceptible ecotype and a low bacterial load in the resistant ecotype. This pathosystem formed the basis of studies, which identified the first R-gene against *R. solanacearum* (Deslandes et al., 2003). Genetic crosses between Col-0 and Nd-1 and subsequent pathogen challenges revealed a 1:3 segregation of resistance: susceptibility in the F2 progeny, suggesting that resistance was governed by a single recessive gene. The R gene in Nd-1 was termed RRS1-R (Resistance to *Ralstonia solanacearum* 1) and the susceptible allele was termed RRS1-S. The bacterial *avr* gene referred to as *popP2* determines resistance against GMI1000 in Nd-1 (Deslandes et al., 2003). It was initially suggested that the RRS1-R and PopP2 gene products interact with each other directly, however a recent proposed model for SLH1 (sensitivity to low humidity 1) in *A. thaliana* ecotype No-1, which is identical to RRS1-R in *A. thaliana* ecotype Nd-1, supports the guard hypothesis (Noutoshi et al., 2005).

A previous screen, conducted using African isolates of *R. solanacearum* from the Congo, Uganda and South Africa against Arabidopsis ecotypes Col-5, Be-0, Kil-0, Sf-2, La*er* and Cvi indicated varying degrees of susceptibility or resistance to the *Eucalyptus* isolates K (BCCF 401), CK (BCCF 402), CC (BCCF 403) and 27B (BCCF 427) (Weich, 2004). The susceptible interaction between Col-5 and the Eucalyptus isolate BCCF 401 has been described in Chapter 4. The Eucalyptus isolate BCCF 402 was found to be more virulent than BCCF 401, and caused wilting symptoms earlier in susceptible interactions with Be-0 (Weich, 2004). Figure 5.1 shows the disease index for the ecotypes Col-5, Be-0 and Kil-0 infected with BCCF 401 and BCCF 402. Ecotypes Col-5, Be-0 and were consistently susceptible to isolates BCCF 401 and BCCF 402 while Kil-0 showed a degree of tolerance or resistance to BCCF 401 and BCCF 402 (Weich, 2004). A spontaneous rifampicin resistant mutant of BCCF 402 was selected, and tested to confirm that it showed the same level of symptoms on Arabidopsis plants as the wild-type BCCF 402. It was used to determine the internal bacterial growth curves for Col-5, Be-0 and Kil-0 by counting bacterial colonies from leaf extracts on agar plates containing rifampicin, to avoid confounding the data with other (rifampicin sensitive) bacterial species. Figure 5.2 represents the titre of bacteria in the ecotypes after infection with BCCF 402. A BCCF 402 *hrp*$^{-}$ mutant, which has an insertion of a kanamycin

resistance cassette in the *hrpB* gene, disrupting the bacterial TTSS and subsequently unable to cause disease, was used as a control. Both Kil-O and Be-0 appear to support a high bacterial load with the resistant ecotype being able to support one order of magnitude lower bacterial numbers than the susceptible ecotype. Based on this data, the interaction between Kil-0 and the more virulent Eucalyptus isolate BCCF 402 was selected for a study investigating resistance against *R. solanacearum*. Early time-points after infection were of interest and thus, the susceptible interaction between Be-0 and BCCF 402, which shows a higher degree of wilting earlier-on compared to the Col-5 and BCCF 401 or BCCF 402 interaction, was selected as the susceptible interaction in this study.

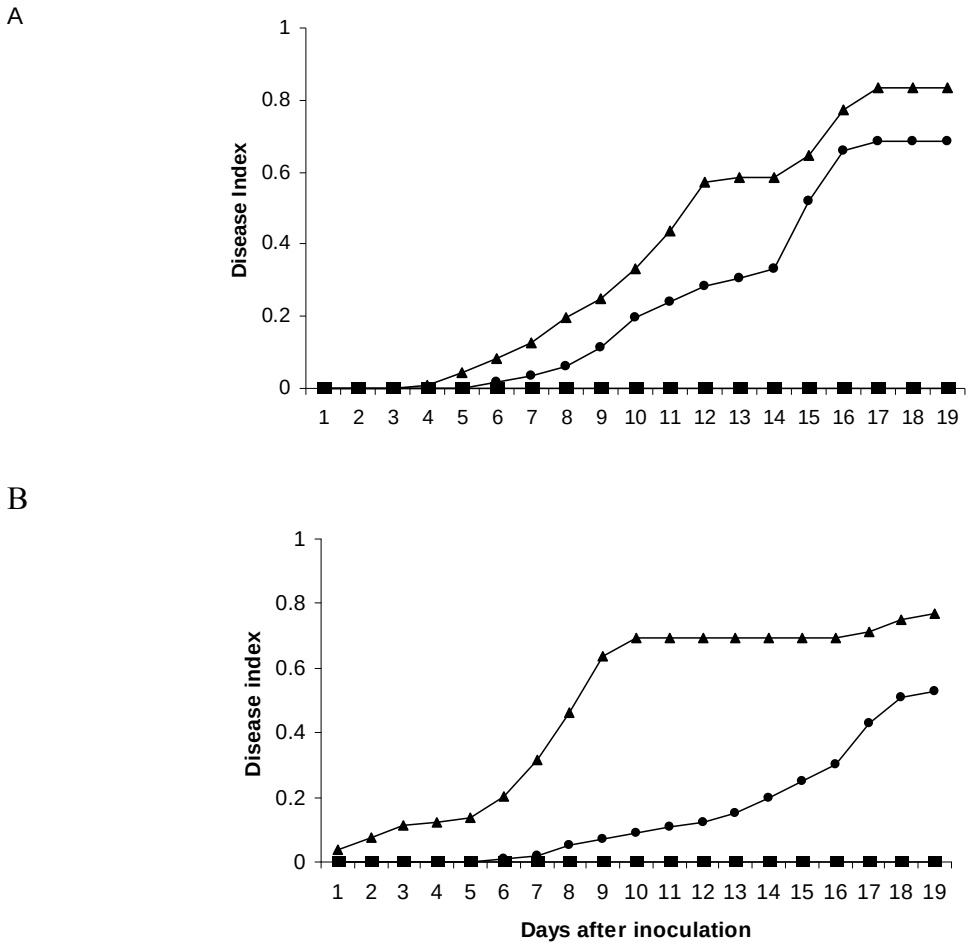


Figure 5.1 Disease index of ecotypes Col-5 (•), Kil-0 (■) and Be-0 (▲) for 19 days after infection with *R. solanacearum* isolates A) BCCF 401 and B) BCCF 402. The data shown has been derived from a single infection trial in which seven independent plants per ecotype were infected with each bacterial isolate. Replicate trials produced similar results (data not shown). Data from Weich (2004).

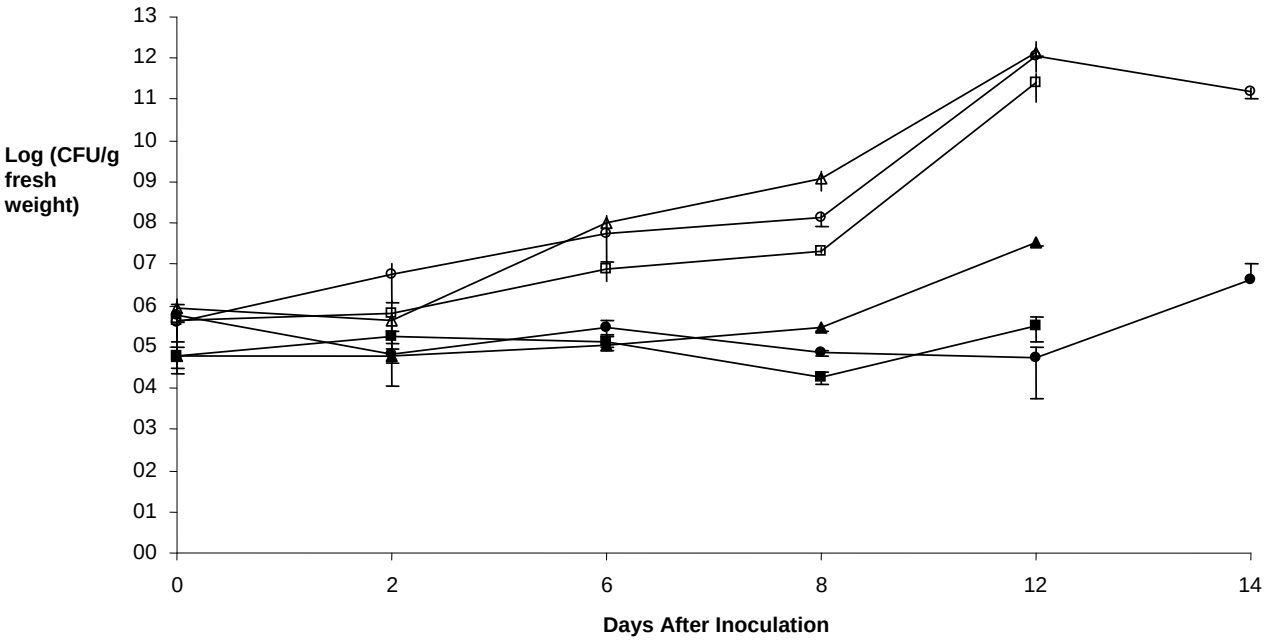


Figure 5.2 Internal bacterial growth curves for BCCF 402 strains in Arabidopsis leaves following root inoculation. Ecotypes Col-5 (○), Kil-0 (□) and Be-0 (Δ) were infected with the rifampicin mutant of BCCF 402 and with the *hrp*⁻ mutant of BCCF 402, indicated in filled symbols: Col-5 (●), Kil-0 (■) and Be-0 (▲). The data was derived from triplicate assays on three plants per time-point and bacterial strain-ecotype combination. Data from Weich (2004).

The scientific question, which was posed, was, "which Arabidopsis genes are responding differently in the resistant interaction (Kil-0) against BCCF 402 compared to the susceptible interaction (Be-0) against BCCF 402?" The step-wise strategy which was followed involved 1) the investigation of transcripts differentially regulated in Kil-0 infected plants compared to Kil-0 uninfected plants using whole-genome microarrays, 2) qRT-PCR comparison of the expression levels of these genes in the susceptible interaction with Be-0. It is hypothesised that genes induced in Kil-0 and not in Be-0, or genes that are induced earlier in Kil-0 compared to Be-0 are potential candidate genes conferring resistance against the pathogen. Thirteen genes, induced at $p<0.01$ and fold change > 1.7, were obtained using linear models for microarrays analysis in the R computing environment and explore their role in defence against *R. solanacearum* using bioinformatics comparisons.

## 5.3. Materials and Methods

### 5.3.1 *Plant material*

Seeds of Arabidopsis ecotype Killean (Kil-O) and Bensheim (Be-O) were obtained from The Nottingham Arabidopsis Stock Centre (NASC, www.arabidopsis.info) and sterilized using washing steps with 70% ethanol, 1.5% sodium hypochlorite and 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.

### 5.3.2 Inoculations

*R. solanacearum* isolate BCCF 402 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 according to Deslandes et al. (1998).

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 aerial tissue of between 6-8 individual Be-O and Kil-O plants was harvested 1, 4 and 7 days after inoculation. Similarly, control plants showing no wilt symptoms at the respective time-points were harvested. The experiment was performed in triplicate.

### 5.3.3 RNA isolation

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

### 5.3.4 Microarray experiments

*Experimental Design*

The experimental design was a direct comparison (reviewed in Naidoo et al., 2005) between Kil-O infected and Kil-O uninfected tissue at two time points: 1 day post inoculation and 7 days post inoculation. Two biological replicates were performed i.e. the experiment was repeated on two different occasions with plants grown under the same conditions. A technical replicate which was a dye-swap was included within each biological replicate. In total, 8 slides were used. Figure 5.3 indicates the experimental design followed, represented by ovals (treatment) and arrows (slides).

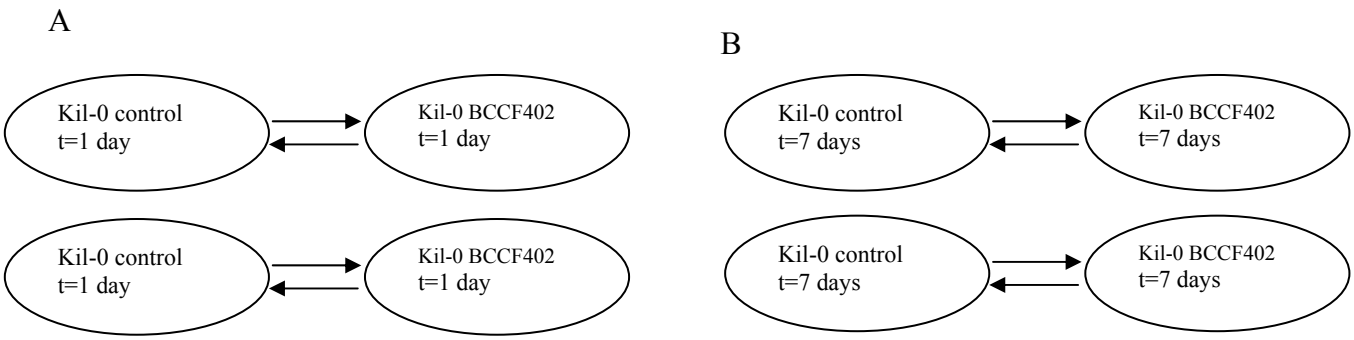


Figure 5.3. Oval and arrow representation of the experimental design employed in microarray comparisons between Arabidopsis ecotype Kil-0 infected with *R. solanacearum* isolate BCCF 401 and Kil-0 plants which were uninfected at one (A) and seven days (B) after infection respectively. Ovals represent the samples that were compared. The head of the arrow indicates a sample labelled with the Cy5 dye and the tail of the arrow indicates a sample that was labelled with the Cy3 dye. Opposite arrows indicate reversal of dye assignments in replicate experiments. Each arrow indicates a slide.

*Whole-genome Microarrays*

Microarray slides containing 70mer oligonucleotides representing approximately 29000 Arabidopsis genes were purchased from the University of Arizona, USA. Prior to hybridisation, slides were rehydrated by holding the slides over a water bath at $50^0$C for 10 sec over the water vapour. The slides are snap-dried on a heating block at $65^o$C for 5 sec and allowed to cool for a minute. The steaming, drying and cooling steps were repeated a total of four times. The slides were then cross-linked using a UV Stratalinker at 180 mJ. Slides were washed in 1% SDS for 5 min at room temperature, dipped ten times in sterile ddH20, dipped five times in 100% ethanol and centrifuged to dryness at 200g for 4 min.

*Target preparation and hybridisations*

Targets were labelled using 15 µg total RNA per labelling reaction with the indirect labelling method according to The Institute for Genome Research (TIGR) protocol SOP #M004 (http://pga.tigr.org/sop/M004_1a.pdf). cDNA was purified prior to dye-coupling using the Qiagen PCR purification kit (Qiagen) and again after labelling. The labelled targets were combined with 3xSSC, 1.5% BSA, 0.1% SDS in a total volume of 40 µl and added to the microarray slide under a clean coverslip. The hybridisation was allowed to proceed overnight at 55$^{o}$C in a Telechem hybridisation chamber (Telechem International Inc., California, USA). Slides were washed in a solution of 2xSSC, 0.5% SDS for 5 min at 55$^{o}$C, followed by a wash in 0.5% SDS for 5 min at room temperature, and a final wash of 0.05% SDS for 5 min at room temperature. Slides were dried by centrifuging at 1000 rpm for 4 min. The slides were scanned using the Axon GenePix 400B Scanner (Axon Instruments, Foster City, CA, USA).

*Data Analysis*

Data was captured using GenePix Pro v 5.0 (Axon Instruments) and spots with poor morphology were flagged. The gene pix results (gpr) files were inputted into the `marray` package in R version 2.1.1. for quality control and subsequently, the linear models for microarrays (`limma`) package was used for data analysis. In `marray`, Minus versus Addition (MA) plots for foreground and background data values were generated for each slide. Using the `marray` package, local background subtraction was performed for each feature on each slide using the adaptive foreground and offset=50. In `limma`, print-tip loess normalization was performed within each slide and A-quantile normalization was performed between slides. A top-table of differentially expressed genes was obtained for each time-point i.e. 1 day post inoculation and 7 days post inoculation.

### 5.3.5 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 5 (Scientific & Educational Software, Cary, North Carolina, USA). Primer sequences are listed in the table below. 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 10 µl reaction. All PCR reactions were performed in duplicate and a biological replicate was also 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 primer library for Arabidopsis Pathogen-inducible genes (Sigma-Aldrich, catalogue number P5621) 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'), which appeared to be expressed constitutively in microarray experiments (fold change = 1, and p value= 0.000315) and cross-checked with Affymetrix data under various biotic stress conditions, 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. Data acquisition was performed between 72°C and 80°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.

**Table 5.1 qRT-PCR primer pairs for 13 genes induced by *R. solanacearum* infection in Kil-0.**

| AGI number | Gene Description | Sequence forward 5'-3' | Sequence reverse 5'-3' |
|---|---|---|---|
| At5g59320 | lipid transfer protein 3 (LTP3) | AGGTAGCTTGGCTCCATGTG | ATGCTAACACCGCACTTTCC |
| At5g59310 | lipid transfer protein 4 (LTP4) | AGTTGGTGCTCGTGGAGATG | TGTGGCACAGTGGCAAGTAG |
| At5g59330 | LTP family protein psuedogene | GGCTTTGGCTCTCAGGTTCT | GAGACCAGAAATGCCCTTTG |
| At2g12945 | hypothetical protein A | TGATTTTGCAGCCATGATTC | CATGATCTTTCCCCATGATT |
| At3g49120 | peroxidase | TATCCAGAGCGACCAAGAGT | ACCACATCATGGAGCAGAGA |
| At2g29350 | tropinone reductase | TGGGCGAGCGACAACATAAG | GAAATGCCCACAAGCGGTGA |
| At1g07590 | pentatricopeptide (PPR) repeat-containing protein | GGGTGATGGGTTTCCAGTTC | GAGGGACACGGGTAAATAGC |
| At1g56555 | hypothetical protein B | TGACAGATACGCTCGTGGTC | CTGTGGTTGGCCAAGTGTTA |
| At5g43580 | protease inhibitor | TGCAGGAGAAGGGATGAAGA | TTGGCCGTCACTTTCGTGTT |
| At3g28940 | avirulence-responsive protein (AIG) | TGGTTCCCGCTCAACTCCAC | TGAAGCCGTCTCCATTCCTC |
| At5g54940 | eukaryotic translation initiation factor SUI1 | TCAGATGCACCAGGAGCTAA | ACCGTTGCAGCAGAAATCTT |
| At3g54480 | SKP1 interacting partner 5 (SKIP5) | CCAAGTCTCCCCTTGTTGAA | GTGAGCACTGCTGGAGATGA |
| At3g11770 | expressed protein | AAGTCCGAATGGCGTCTATG | GCGAGGTCTTCTTGAATCTG |

### 5.3.6 Bioinformatic analysis

Bioinformatic comparisons were performed for seven selected genes (LTP3, PRX34, SAG13, AIG, SUI1, SKP5 and expressed protein) using the GeneVestigator v3 tool (Zimmermann et al., 2004; www.genevestigator.ethz.ch/). Microarray data was selected from the following experiments: AT-106 (*Pseudomonas syringae*), AT-108 (*Phytophthora infestans*), AT-161 (*Pathogen, insect attack*) and AT-147 (*Botrytis cinerea*). This Affymetrix data was available as log2 signal values having been analysed using the MAS v5.0 scaling protocol. Electronic

Northern images were generated in GeneVestigator for each of the experiments. Student T-tests were conducted in MS Excel to determine which genes were significantly differentially regulated by a treatment compared to the control. In the case where no replicate data was available for an experiment (AT-161), a log2 fold change > 1.5 was considered significant.

### 5.4 Results

*R. solanacearum* isolate BCCF 402 is virulent on Arabidopsis ecotype Be-0 but does not induce symptoms on ecotype Kil-0. Three consecutive pathogenicity trials consistently produced the same results. The severe wilting symptoms sustained by Be-0 compared to ecotype Kil-0 are apparent in Figure 5.4.

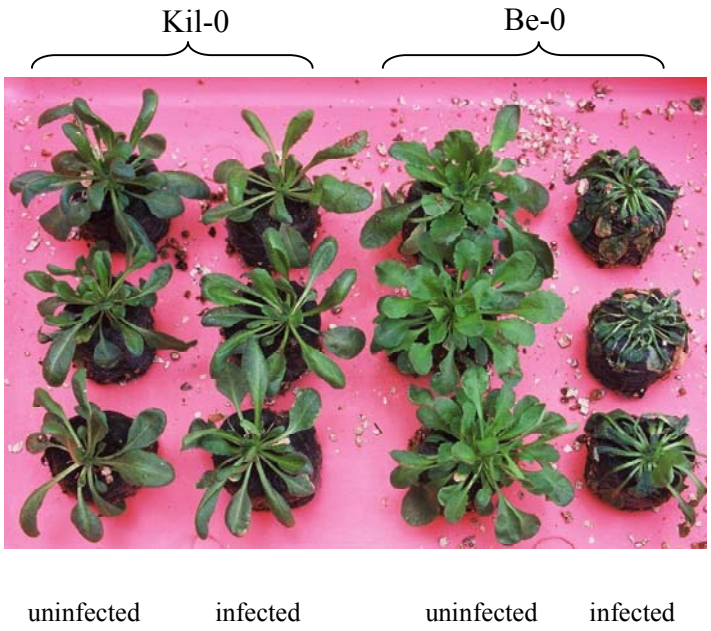


Figure 5.4. The result of BCCF 402 infection on ecotypes Kil-0 and Be-0 after one week compared to uninfected plants. Infected Be-0 plants (right) become wilted while infected Kil-0 plants show no wilt symptoms.

Differential gene expression in Kil-0 was investigated by performing microarray hybridisations at two time-points: one day and one week after inoculation with BCCF 402. Figure 5.5 shows the result of a typical hybridisation on the Arabidopsis whole genome microarray.

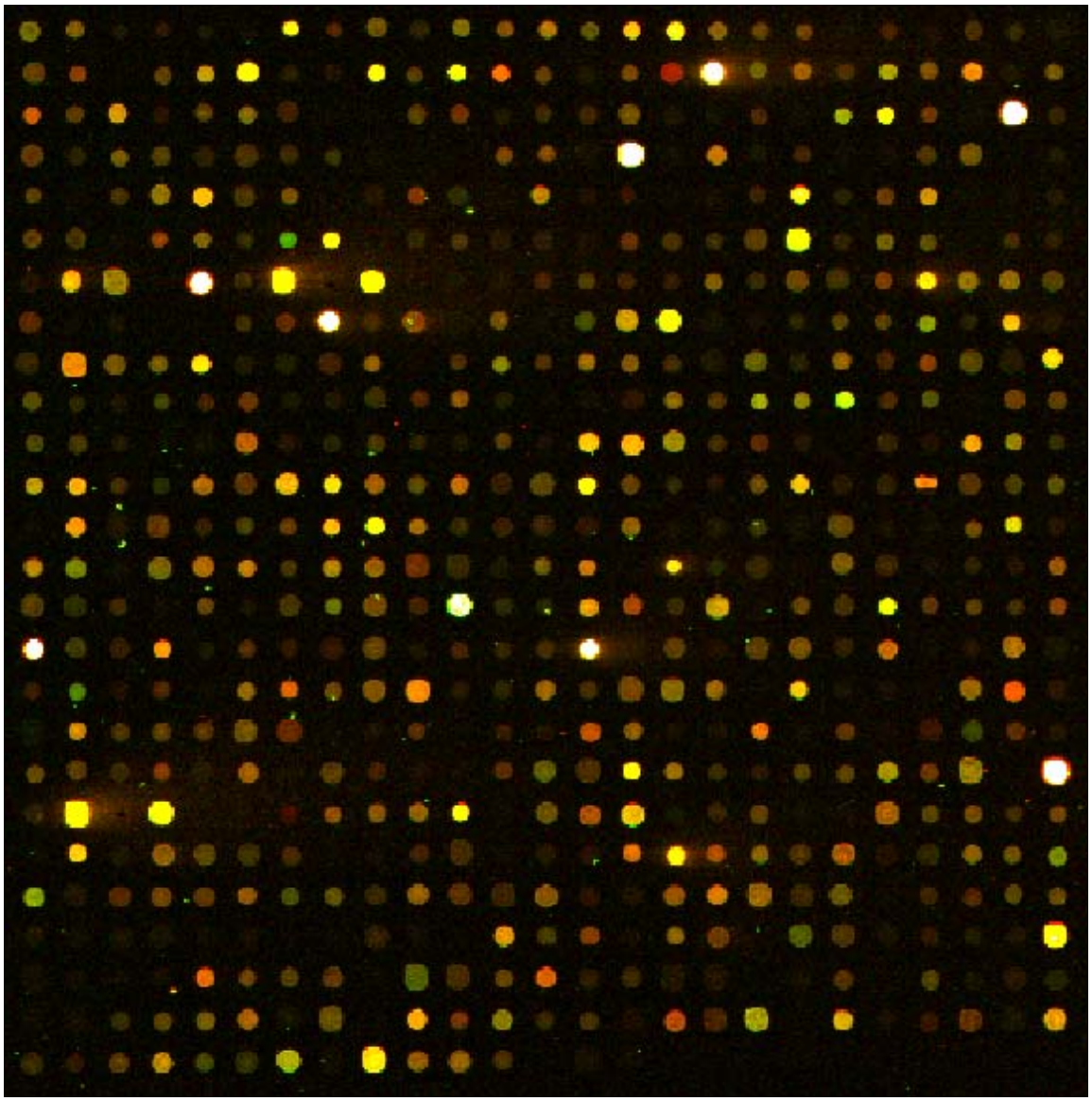

Figure 5.5 Image of section of Arabidopsis thaliana 29K microarray slide after hybridisation to Kil-0 infected material labelled with Cy5 (red) and Kil-0 uninfected material labelled with Cy3 (green) at t=7 days after inoculation Bright orange-to red spots are genes which are possibly induced by *R. solanacearum* BCCF 402 infection while green spots are indicative of genes which may be down-regulated by the pathogen. Most of the genes on the array are yellow, indicating that they are not differentially expressed by the treatment.

The captured microarray data was normalised using a print-tip loess normalisation. Figure 5.6 shows the MA-plots for the raw and normalised data for a single slide. M refers to Minus and is the $\log_2$R-$\log_2$G, while A refers to Addition and is the average intensity calculated as ($\log_2$R+$\log_2$G)/2, where R is the fluorescence intensity in the red channel and G, the green (Yang et al., 2002). After normalisation, the distribution of M values for the spots on the slide is closely centered around 0. Over 50% of the spots on each slide showed a detectable expression level i.e. greater than 2x the standard deviation of the background. This control measure indicated that the microarray slides were of acceptable quality for analysis. The pre and post normalisation MA-plots for all of the microarray slides are available in Appendix E. The assumption that is made for this type of normalisation is that most of the spots on the whole-genome array would not be differentially expressed and thus their M-values would be close to 0. Following normalisation within a slide, A-quantile normalisation was performed between slides. Figure 5.7 shows the R and G fluorescence densities for all of the slides after A-quantile normalisation. The possibility that the odd distribution seen for one of the slides in Figure 5.7 (A) was due to technical variation rather than biological variation was addressed by repeating the slide. Similar results were obtained which suggested that the distribution was due to biological variation.

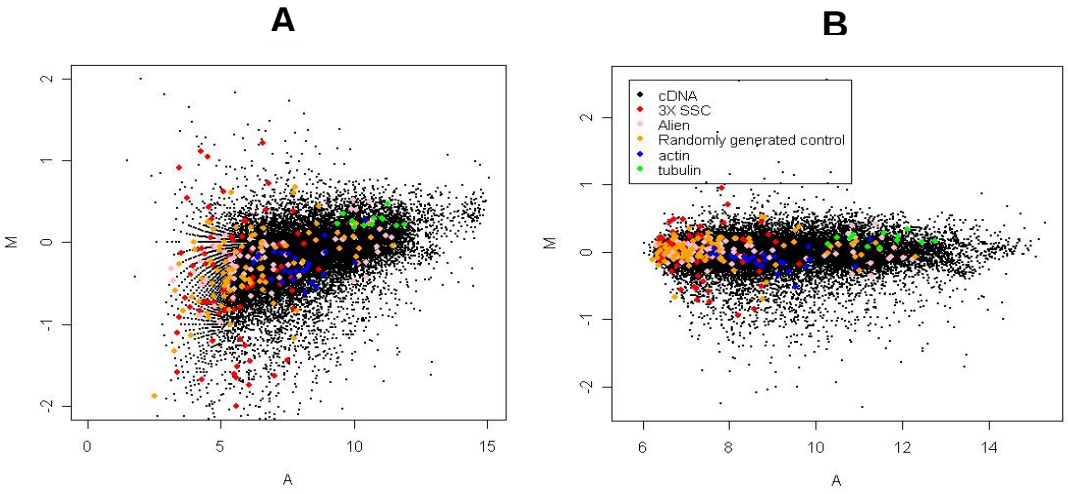


Figure 5.6 MA-plots for a microarray slide before (A) and after (B) print –tip loess normalisation. Before normalisation, there appears to be a bias towards the green dye however, after normalisation, the data becomes centred around zero.

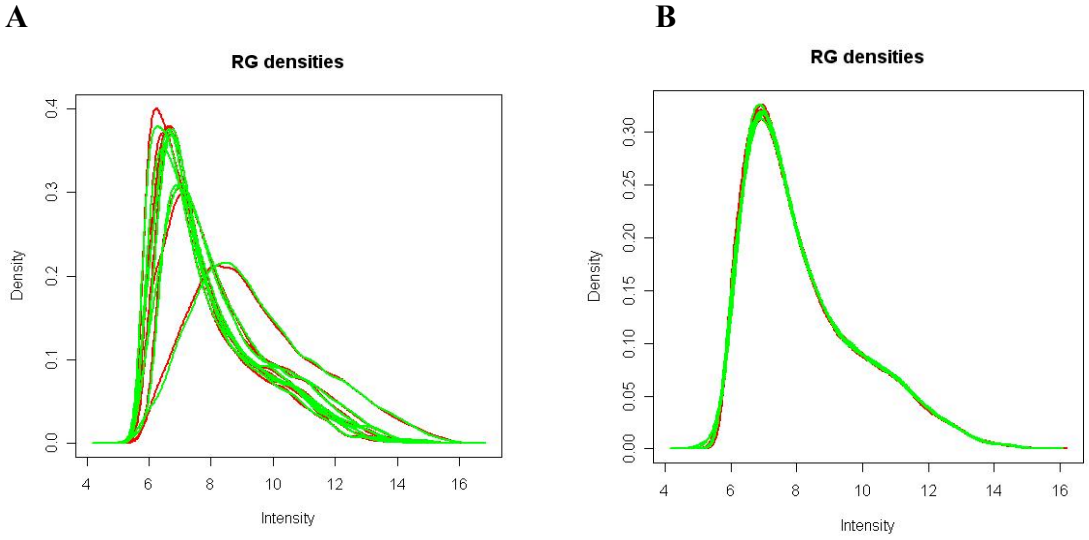


Figure 5.7 RG densities before (A) and after (B) between slide normalisation using A-quantile normalisation in the limma package in R version 2.1.1 (Bolstad et al., 2003; Smyth, 2004).

A Bayesian method of analysis (Smyth, 2004) was employed on the normalised data to determine differentially expressed genes. In this approach, information is borrowed across the range of genes, which assists in inference about each gene individually. Correction for multiple testing was performed using FDR. Table 5.2 shows the results that were obtained.

**Table 5.2. Genes up regulated in response to *R. solanacearum* BCCF 402 infection in Arabidopsis ecotype Kil-0, one[a] and seven days after inoculation. The expression data is ordered from most induced to least induced at a significance threshold of p<0.01.**

| AGI number | Description | log2 fold change | p-value | Fold change |
|---|---|---|---|---|
| At5g59320 | lipid transfer protein 3 (LTP3) | 2.08 | 4.31E-03 | 4.23 |
| At5g59310 | lipid transfer protein 4 (LTP4) | 1.90 | 4.86E-03 | 3.72 |
| At5g59330 | LTP family protein pseudogene | 1.51 | 2.09E-02 | 2.85 |
| At2g12945 | hypothetical protein A | 1.24 | 4.86E-03 | 2.36 |
| At3g49120 | peroxidase (PRX34) | 1.15 | 4.31E-03 | 2.21 |
| At2g29350 | tropinone reductase (SAG13) | 1.02 | 2.09E-02 | 2.03 |
| At1g07590 | pentatricopeptide repeat-containing protein (PPR) | 0.98 | 4.86E-03 | 1.97 |
| At1g56555 | hypothetical protein B | 0.88 | 2.09E-02 | 1.84 |
| At5g43580 | serine-type endopeptidase inhibitor | 0.85 | 1.80E-02 | 1.81 |
| At3g28940 | avirulence-responsive protein (AIG) | 0.82 | 4.86E-03 | 1.77 |
| At5g54940 | eukaryotic translation initiation factor SUI1 | 0.79 | 2.09E-02 | 1.72 |
| At3g54480 | SKP1 interacting partner 5 (SKIP5) | 0.75 | 2.09E-02 | 1.68 |
| At3g11770[a] | expressed protein | 0.72 | 5.34E-03 | 1.65 |

One gene, expressed protein (At3g11770), was found to be induced in Kil-0 infected plants one-day post inoculation following data analysis, while 12 genes were selected as differentially expressed at seven days post inoculation at a fold change greater than 1.65. Only up-regulated genes were observed after data analysis at the two time points investigated. Genes, which were marginally down regulated, were the expressed proteins At4g04985, At5g59020, and At1g54095 which had $\log_2$ fold-changes of –0.4 (fold change = 0.74; 1.3X down-regulated), -0.52 (fold change = 0.70; 1.4X down-regulated) and -0.60 (fold change = 0.66; 1.5X down-regulated).

The number of genes found to be differentially expressed in Kil-0 after infection compared to control plants at the two time points were lower than expected. The possibility that the microarray data had been over-normalised was explored by performing the analyses using global loess normalisation and similar results were obtained (results not shown). In addition, the data was independently analysed using another software package (MAANOVA in `limma`) and similar results were obtained. The variation between the 4 replicates of each data point (i.e. dye swap and biological replicate) investigated using MAANOVA indicated that the treatment accounts for most of the variation observed after normalisation and variation due to dye and slide is minimal. This suggests that the slides were not so variable as to result in a large number of false negatives (i.e. differentially expressed genes that were scored as not significantly differentially expressed)(data not shown).

The gene ontologies of the selected genes were investigated using MADIBA (www.bi.up.ac.za/MADIBA/). Table 5.3 shows the gene ontologies for the three categories: cellular component, biological process and molecular function. Two ontologies: response to bacterium and response to fungus, in the category molecular function (Table 5.3) suggest that microarray expression profiling has revealed possible defence response genes in Kil-0 infected with BCCF 402 compared to uninfected plants.

**Table 5.3 Gene Ontology of 13 genes induced in Kil-0 infected plants showing a fold change of >1.65. The ontology that is over-represented in the cluster compared to the gene ontology annotations in the whole *A. thaliana* genome is shown for each category using a hypergeometric test and FDR corrected using the Holm correction, at a significance threshold of p<0.05 (www.bi.up.ac.za/MADIBA/).**

| Cellular Component | Biological process | Molecular function |
|---|---|---|
| • cellulose and pectin containing cell wall | • lipid binding<br>• serine-type endopeptidase inhibitor activity<br>• translation initiation factor activity<br>• peroxidase activity<br>• oxidoreductase activity | • Oxygen and reactive oxygen species metabolic process<br>• Response to bacterium<br>• Response to fungus<br>• Unidimensional cell growth<br>• Translational initiation<br>• Response to light stimulus |

Following the data analysis, quantitative RT-PCR was performed on several of the selected genes to confirm the expression ratios from microarray analysis as well as to determine their expression during the susceptible interaction with BCCF 402. It was hypothesised that genes required for successful resistance would be induced in Kil-0 specifically in response to the pathogen and induced either only later or not at all in Be-0. This trend has been observed in compatible and incompatible interactions with avirulent and virulent *Pst* infections in Arabidopsis ecotype Col-0 (Tao et al., 2003). To test this hypothesis, a 4-day time-point (4 dpi) in Kil-0 and Be-0 was included for expression profiling using qRT-PCR. All the expression values were standardised against the expression of the control gene (At1g18070.1). Figure 5.8 shows the expression ratios of the individual genes in the resistant and susceptible interactions with BCCF 402 relative to the respective uninfected plants.

In most cases tested, the expression pattern of the genes in the qRT-PCR expression profiling matched the expression pattern obtained in the microarry experiments with the amplitude of expression being higher in the qRT-PCR experiments. Fold change in gene expression has been shown to differ between microarray and qRT-PCR quantification (Czechowski et al., 2004) especially for genes expressed at low levels, however it is important that the pattern of gene expression is similar. Furthermore, PRX34, AIG, SUI (Figure 5.8) appear to be expressed earlier in Kil-0 infections than in Be-0 i.e. 4 days after inoculation. LTP3 is induced to a higher level in Kil-0 4 days after inoculation than in Be-0 at the same time-point. A t-test suggests that the difference in LTP3 expression between Be-0 and Kil-0 4 days after inoculation is significant (approximately 2x greater in Kil-0). Genes induced more in Kil-0 than Be-0 7 days after inoculation include: PRX34, SAG13, AIG, SKIP5 and expressed

protein (Figure 5.8). LTP4, LTP psuedogene and PPR are induced in both Be-0 and Kil-0 7 days after inoculation and could arguably be indicative of PAMP-triggered immunity.

The expressed protein (At3g11770), induced in Kil-0 challenged with BCCF 402 one day after inoculation, was induced to a high level in Kil-0 but remained uninduced in Be-0 at the time-points tested (figure 5.8). The qRT-PCR experiments for hypothetical proteins A and B and serine-type endopeptidase inhibitor were not successful and were not included.

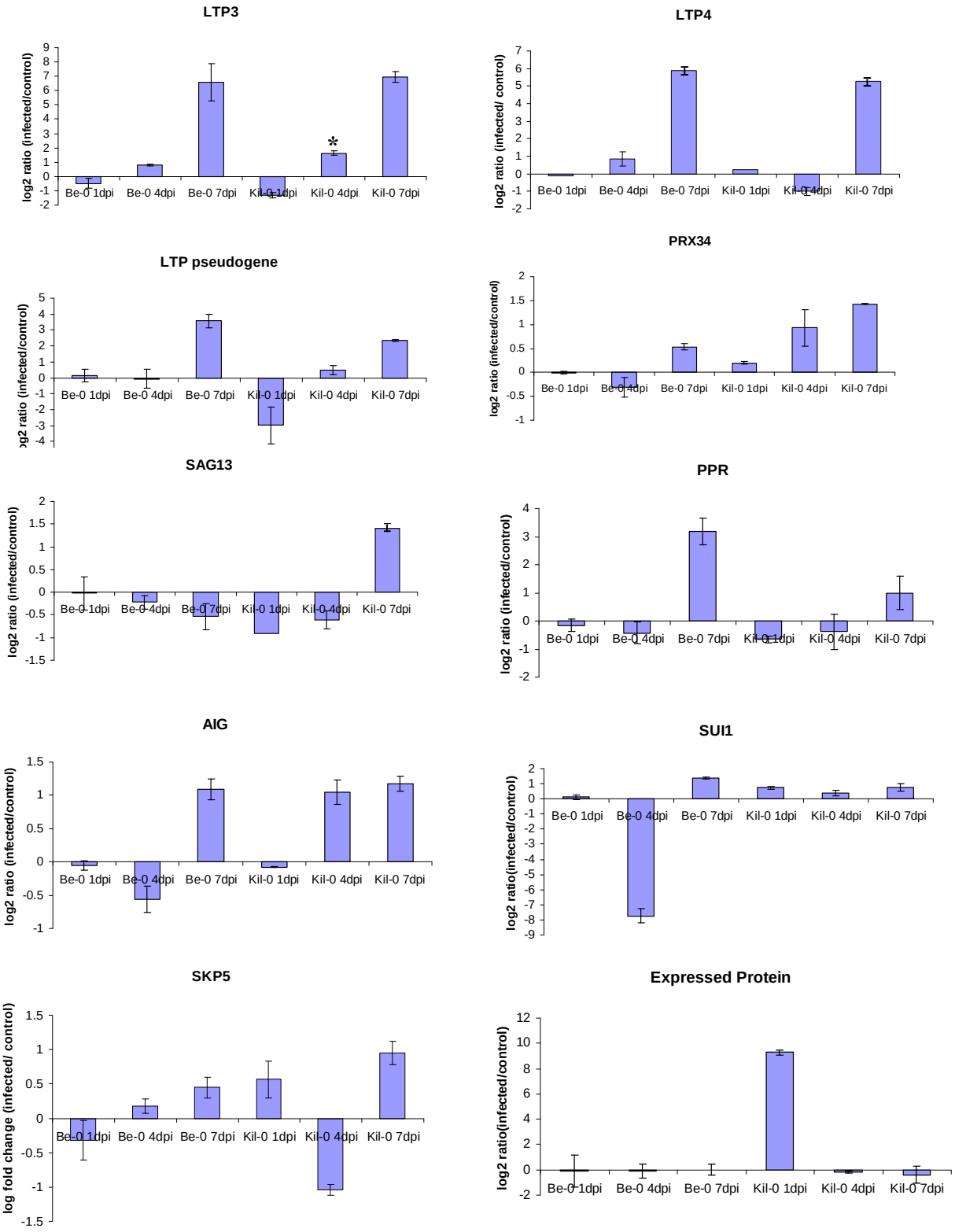


Figure 5.8 Expression ratios for genes differentially expressed in Be-0 and Kil-0 after infection with *R. solanacearum* isolate BCC402 as determined by qRT-PCR. Samples were harvested 1, 4 and 7 days after inoculation. Error bars represent the standard deviation between replicate experiments. A * denotes that the expression of LTP3 in Kil-0 was significantly different to that in Be-0 during infection ($p<0.05$, T-test).

Following qRT-PCR, 7 genes were selected for further investigation using the bioinformatics tool GeneVestigator (v3) (Zimmermann et al., 2006). Expression profiles of LTP3, PRX34, SAG13, AIG, SUI1, SKP5 and expressed protein were compared under challenge with *P. syringae*, *P. infestans*, *B. cinerea* and under insect and pathogen attack (*M. persicae, F. occidentalis, A. brassicicola and P. syringae*). These results are shown in Figure 5.9.

Some genes such as PRX34 (orange) and SAG13 (green) are induced during most pathogen treatments (Figure 5.9), including compatible and incompatible interactions with *Pst* (Figure 5.9 A), *B. cinerea* infection (Figure 5.9 C), *A. brassicicola, F. occidentalis and M. persicae* infection (Figure 5.9 D). SAG13 is also induced during *P. infestans* infection (Figure 5.9 B).

LTP3 (yellow dots) appears to be induced during *Pst DC3000* infection 24 hrs after infection compared to the mock inoculated plants at the same time-points (Figure 5.9 A) however, LTP3 is down-regulated by *F. occidentalis*, *A. brassicicola* and *M. persicae* challenge in Col-0 plants in comparison to uninfected plants (Figure 5.9 D).

AIG (brown dots) is induced after infection with *F. occidentalis* infection and *M. persicae* challenge (Figure 5.9 D). SUI1 (blue dots) is marginally induced after 6 hrs of *Pst* challenge in both incompatible and compatible interactions (1.5 fold and 1.7 fold respectively) (Figure 5.9 A) and is induced during *P. infestans* infection (Figure 5.9 B).

The expressed protein (purple dots) is marginally induced by *Pst DC3000 infection* (1.6 fold) (Figure 5.9 A) and is induced by *M. persicae* and *A. brassicicola* infection (Figure 5.9D). SKP5 does not appear to be induced during infection with any of the pathogens investigated in Figure 5.9. Together, this data supports a role for LTP3, PRX34, AIG, SAG13, SUI1 and expressed protein in plant defence and qualifies them as candidates for the resistance response in Kil-0.

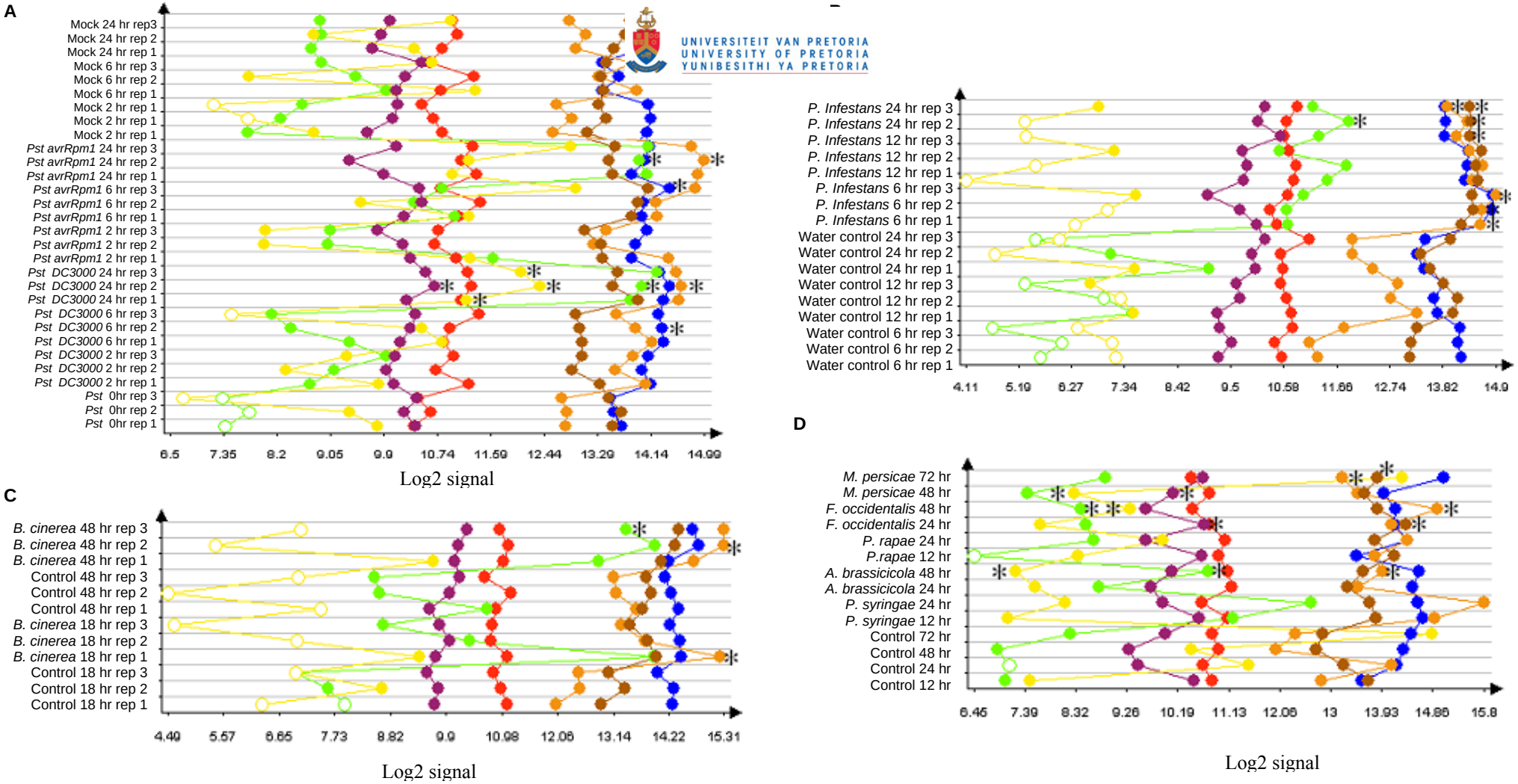


Figure 5.9 Expression profiles of 7 selected Arabidopsis genes during infection with A) *P. syringae,* B) *P. infestans* C) *B. cinerea* and D) during fungal (*A. brassicicola*; *F. occidentalis*), bacterial pathogen (*Pst DC3000*) and insect attack (*M. persicae*). Closed circles represent genes with significant signal values ($p<0.06$) while open circles represent genes with a p value > 0.06 on the Affymetrix microarrays. Genes are represented by the following colours: LTP3 – yellow, PRX34- orange, SAG13 – green, AIG – brown, SUI1 – blue, SKP5 – red, expressed protein – purple. A * indicates expression values, which are significantly different from the control at the respective time-point ($p<0.05$, student's T-test).

## 5.5 Discussion

A pathosystem wherein Arabidopsis ecotype Kil-0 was resistant to *R. solancearum* isolate BCCF 402 and ecotype Be-0 was susceptible, was exploited for gene expression profiling. The genes that are differentially expressed in Kil-0 during infection with BCCF 402 compared to Kil-0 uninfected plants were investigated using Arabidopsis whole-genome microarrays. One day post infection was selected as a time-point for investigation in order to capture early defence response events close to the recognition event. Figure 5.1 A shows that at this time-point, no wilt symptoms are apparent on either Kil-0 or Be-0 however, both ecotypes contain the same bacterial numbers (Figure 5.2). Seven days post infection was also selected as a time-point for investigation based on the wilt symptoms observed in Be-0 at this time-point and no apparent symptoms in Kil-0 however, there is one order of magnitude higher bacterial numbers in Be-0 compared to Kil-0 at this time point (Figure 5.2). One gene was induced one day after infection while 13 genes were induced 7 days after infection in Kil-0 (Table 5.2). Technical reasons were explored for the few genes found to be significantly differentially expressed under BCCF 402 infection in Kil-0 and conclude that the results obtained are robust and that the microarray data were of good quality. Evidence for this comes from the good correlation of the qRT-PCR data 7 days after infection in Kil-0 for the 10 genes investigated (Figure 5.8). Thus, the reason for the few number of significant genes is probably biological.

In an experiment investigating the effect of clubroot disease on Arabidopsis, using the ATH1 array (a 22K Affymetrix microarray), more than 1000 genes were differentially expressed ($p<0.04$) at each time point (Siemens et al., 2006). This trend would be expected when investigating 29 000 genes in a single experiment and it is evident that one day post inoculation, approximately $10^6$ cfu/ml of bacteria per gram of tissue is present in aerial tissue of Kil-0 (Figure 5.1; Weich, 2004) which would suggest that a large proportion of the plants transcriptome would respond to the bacterial infection. The small numbers of genes induced after inoculation with BCCF 402 in Kil-0 may be attributed to:

1) The time points being investigated. Perhaps *R. solanacearum* had not reached the leaves during these time-points, so little difference between uninfected and infected tissues were observed. An *in vitro* method of inoculation was employed to infect the model legume *Medicago truncatula* with GFP labeled GMI1000. These results indicate that bacteria reach the stems and leaves only 2-3 days after infection at the root (Vailleau et al., 2007). Figure 5.2 indicates a high amount of bacteria ($10^6$ cfu/ml) in aerial tissue of Kil-0 and Be-0 one day after inoculation with the bacteria. This

amount of bacteria is also observed in the two ecotypes immediately after infection. It is possible that the amount of bacteria observed in the aerial part of the plant at this early time-point is as a result of capillary action via transpiration. If so, the plant may not have had adequate time to recognize or to respond to the pathogen itself. One way to determine whether bacteria have entered the leaves in Kil-0 at the indicated time-points would be to develop a bacterial specific quantitative PCR assay.

2) There is constitutively high expression of some of the genes in Kil-0, which renders the plant resistant to the pathogen and as such a comparison of Kil-0 infected versus Kil-0 uninfected would not reveal these genes. This possibility was investigated by comparing the expression levels of each of the 10 candidate genes in Be-0 and Kil-0 without pathogen infection by qRT-PCR and it was found that the expression levels in Kil-0 were constitutively higher than in Be-0 for seven of the 10 genes 1 day after inoculation (appendix F).
3) There are some unique genes in Kil-0, which are not present in Col-0, which was used to derive the microarray. To address this possibility, an SSH library has been constructed from a subtraction of Kil-0 infected plants and Kil-0 uninfected plants at various time-points post infection (2, 8, 12, 24, 48, 96 and 168 hrs after infection)(data not included in this thesis).
4) The response in Kil-0 is mostly in the roots and not the leaves. It has been demonstrated that in a resistant line of *Medicago truncatula*, GMI1000 is limited in the root system of the plants (Vailleau et al., 2007). This suggests that in a resistant interaction, there may be a mechanism whereby the pathogen is contained within the root system. This theory is negated by data (Figure 5.2) indicating high levels of the bacteria in aerial parts of the plant in Kil-0.
5) Another likely scenario is that changes in transcription are only seen in the cells encountering bacteria and in the resistant interaction the bacteria spreads less and as such less cells respond to the pathogen. By taking the aerial parts of the plant, fewer cells have induction of defence genes.

Despite the concern over the number of genes found to be differentially expressed in this study, transcript profiling did reveal several interesting genes induced in Kil-0 after infection with BCCF 402. This strategy was employed to identify candidate genes involved in resistance against BCCF 402. It was hypothesized that genes required for defence in a resistant interaction would be induced earlier and/ or higher than in a susceptible interaction.

The results identified 7 genes, which show higher expression earlier in Kil-0 infections relative to the susceptible interaction with Be-0 in qRT-PCR experiments (Figure 5.8). These genes were: LTP3, PRX34, SAG13, AIG, SUI1, SKP5 and an "expressed protein". GeneVestigator data suggests that several of these genes have previously been implicated in the plant defence response against various pathogens (Figure 5.9). The discussion that follows speculates on the possible role of these genes in defence against *R. solanacearum* in Kil-0.

LTPs are important antimicrobial peptides involved in plant defence against pathogens (García-Olmedo et al., 1995). Barley LTP2 expression in tobacco and Arabidopsis transgenic plants reduced necrotic effects of Pseudomonas (Molina and García-Olmedo, 1997). Ge et al. (2003) showed that LTP110, a lipid transfer protein from rice had antifungal activity against *P. oryzae* and antibacterial activity against *Xanthomonas in vitro* to a limited extent. Early studies on resistance against *R. solanacearum* suggest that the exogenous application of LTP was able to reduce growth of the pathogen *in vitro* (Segura et al., 1993). LTP3 and LTP4 were among the genes shown to be constitutively induced in Arabidopsis irregular xylem (*irx)* mutants (*irx1, irx 3 and irx5*), which were resistant against *R. solanacearum* GMI1000 (Hernandez-Blanco et al., 2007). The up-regulation of specific LTP genes in a resistant interaction between *R. solanacearum* and Arabidopsis mutants supports a role for these genes in host defence against the pathogen. In addition, the slightly higher induction of LTP3 in Kil-0 compared to Be-0 four days after inoculation with the pathogen may reflect the importance of LTP3 in defence against *R. solanacearum* (Figure 5.8).

Peroxidase gene (PRX34) expression was found to be mainly in root tissue of Arabidopsis plants compared to stem and leaf tissue (Passardi et al., 2005). This cell-wall bound class III peroxidase is thought to be responsible for the production of reactive oxygen species ($H_2O_2$) during plant defence and pathogen attack (Mahalingam and Federoff, 2003). Bindschedler et al. (2006) introduced French bean peroxidase (FBP1) into Col-0 plants in an antisense orientation. FBP1 has high amino acid identity (53%) to PRX34 (At3g49120) from Arabidopsis. The transgenic knock-down Arabidopsis plants displayed an impaired oxidative burst, had low transcript levels of PRX34 and displayed higher susceptibility to the fungi *Golovinomyces orontii*, *B. cinerea* and bacteria *Pst DC 3000* and *P. syringae* pv. *maculicola*. FBP1 plants had a reduction of wall-bound cationic peroxidase activity suggesting that PRX34, which has predicted amino-terminal secretion sequence, is localised to the cell wall. This result implicates PRX34 in generating $H_2O_2$ during defence and indicates its importance

for resistance against different pathogens. The higher induction of PRX34 in Kil-0 and not in Be-0 earlier on during *R. solanacearum* challenge is consistent with a role for PRX34 in defence against *R. solanacearum* (Figure 5.9). The activation of PRX34 in Kil-0 may be indicative of the oxidative burst associated with the formation of the HR (Wojtaszek, 1997). BCCF 402 induces a HR on tobacco leaves after infiltration, but this response has not been confirmed to occur in Arabidopsis leaves (Weich, 2004). It is possible that the HR is induced in roots of Kil-0. If so, the production of the HR in Kil-0 and not in Be-0 would be consistent with an incompatible and compatible interaction respectively.

AIG (*avrRpt2*-induced gene) is induced early on in response to *avrRpt2* in a *RPS2* dependent manner (Reuber and Ausubel, 1996). However, AIG is not induced by *avrRpm1* and *avrB*. Thus, AIG is used as a marker of RPS2 mediated responses in Col-0. One could speculate that the induction of AIG in Kil-0 and not Be-0 suggests that a similar *avrRpt2* effector in *R. solanacearum* could be inducing AIG in Kil-0 or that AIG guards the same host protein as RPS2 that is modified by *avrRpt2* from *Pst* and a second effector from *R. solanacearum*. A PCR specifically targeting RPS2 would be useful to determine whether this R-gene is present in Kil-0. To compliment this exercise, one could also determine whether BCCF 402 has *avrRpt2* (by PCR or Southern blotting) which contributes to an R-avr interaction in Kil-0. It is important to note that RPS2 (located on chromosome 4) is not closely linked to RRS1-R (chromosome 5).

Tropinone reductase is a short-chain dehyrogenase involved in the synthesis of tropane alkaloids, important defence compounds in plants (De Luca and St Pierre, 2000). Tropinone reductase is similar to senescence associated gene 13 (SAG13, 90% nt identity) which, is induced by most types of pathogen challenge (Figure 5.9) and is used as a marker gene of programmed cell death (Lohman et al., 1994). The gene is expressed in mature leaves even when senescence is not apparent and its expression is observed to increase in senescing leaves (Swartzberg et al., 2006). SAG13 has also been shown to be induced by ozone treatment in Arabidopsis leaves, SA, ABA and ethylene treatment (Miller et al., 1999; Morris et al., 2000; Barth et al., 2004) and is induced to high levels in the Arabidopsis gain-of-function mutant *slh1* that has an amino acid change in the WRKY domain of the RRS1-R gene (Notoushi et al., 2005). The high expression levels of SAG13 in Kil-0 could be a marker of a SA defence response as observed for *slh1*. The role of SA in defence in Kil-0 could be investigated by creating crosses between Kil-0 and SA mutants. A susceptible phenotype after *R.*

*solanacearum* challenge on the progeny would indicate a SA-dependent type of defence response.

The expressed protein shown to be induced 1-day post inoculation in Kil-0 is consistent with a defence response gene to some extent as its expression is induced during *Pst DC3000, A. brassicicola* and *M. persicae* challenge in Col-0 (Figure 5.9). This gene may represent a resistance response in the ecotype Kil-0 to *R. solanacearum* and is thus worthy of further investigation. According to TAIR, the expressed protein was obtained from an EST library derived from mixed floral buds and roots (9:1 ratio) from Arabidopsis ecotypes Ws and Ler. It would be necessary to perform regular bioinformatics analysis on this gene to determine which annotated orthologue it has homology to or to perform a yeast-two hybrid screen of the expressed protein cDNA and a library of *A. thaliana* genes to determine which protein(s) this "expressed protein" interacts with.

The sequence of the eukaryotic SUI1 is similar to bacterial SUI1 and is involved in stabilising mRNA and initiator tRNA binding to the 40S ribosomal subunit (Kyrpides and Woese, 1997). Given its role, it would be expected that the expression of SUI1 would be similar to that of a housekeeping gene however, SUI1 is induced during compatible and incompatible *Pst* infection as well as during *P. infestans* infection (Figure 5.9). It is plausible that the initiation of translation in Kil-0 is an important factor contributing to its enhanced resistance against the pathogen compared to the late induction of the gene in Be-0. This SUI1 gene is not uniquely pathogen-induced. Arabidopsis has several SUI1-like genes (AT1G09150, AT1G54290, AT1G71350, AT4G27130, AT5G11900, AT5G54760), one of which (At4g27130) is also induced by pathogen treatments (as determined by GeneVestigator, results not shown).

SKIP5 is induced by Kil-0 7 days after infection but appears to be uninduced in Be-0 or in Col-0 under various pathogen challenge (Figure 5.9). The SCF-type E3 ubiquitin ligase complex is responsible for post-translational modification of proteins in plants (reviewed in Angot et al., 2006). The ubiquitin tagged proteins are either modified or are degraded by the 26S proteasome during plant development. Specific effectors of *R. solanacearum*, referred to as GALA proteins, are able to mimic components of the SCF-type E3 ubiquitin ligase complex. For example, they mimic F-box proteins which are capable of interacting with various Arabidopsis SKP1-like proteins (Angot et al., 2006). This "high-jacking" of the plants machinery is thought be a virulence strategy by the pathogen to promote disease. The higher

induction of Arabidopsis SKIP5 in Kil-0 7 days after inoculation may reflect a strategy by the plant to counter this process. SKIP5 is among the 7 SKIP cDNAs shown to interact with SKP1 in a two-hybrid screen conducted by Farrás et al. (2001) and also encodes an F-box protein. One may therefore hypothesise that the role of SKIP5 in *R. solanacearum* defence is perhaps to compete with the GALA F-box proteins to interact with SKP1 and thus minimise manipulation by these pathogen effectors.

This study investigated transcript levels and it remains to be investigated whether this translates into enhanced levels of the protein. Western blot analysis or large scale proteomic analysis would be necessary to detect enhanced protein levels possibly required for defence (Baginsky and Gruissem, 2006). The next step towards determining whether these genes are necessary for resistance in Kil-0 is to perform gene function studies. Although T-DNA insertion lines are available for each of these candidate genes in Col-0 no phenotype has been reported as yet (data not shown). *R. solanacearum* BCCF 402 pathogen challenges of these lines would indicate whether the knock-out of the gene results in an enhanced susceptibility phenotype such as the early wilt symptoms observed for Be-0. Over-expression of the promising candidates in Be-0 or knockouts in Kil-0 would then be used to ascertain the role of the gene in plant defence against *R. solanacearum*. Once a gene has been identified as important for resistance in Kil-0 its orthologue can be identified in the host (Eucalyptus) and potentially manipulated therein to afford resistance against *R. solanacearum.*

The Be-0/ Kil-0 *R. solanacearum* BCCF 402 pathosystem has provided a useful tool to identify candidate genes involved in resistance against *R. solanacearum* however the high amount of bacteria in Kil-0 (an order of magnitude less than found in Be-O) is not consistent with other resistant interactions (e.g. Nd1 and Col-0; Deslandes et al., 1998) and more recently, bacterial numbers in a resistant *M. trunculata* line was $1x10^5$ times less than the susceptible line (Vallieau et al., 2007). The high bacterial numbers in Kil-0 measured by colony counting, suggests that Kil-0 may show tolerance and not resistance (Weich, 2004). Tolerant plants are able to survive pathogen infection, may remain symptom free but are able to accommodate high amounts of the pathogen (Agrios, 1997). This is being addressed by creating an accurate quantitative PCR assay designed to specifically amplify the *flic* gene from *R. solanacearum* (Schonfeld et al., 2003) to determine whether bacterial numbers in Kil-0 are limited in this ecotype. If so, then Kil-0 could be regarded as resistant and not tolerant. The question that would then remain would be whether the resistance in Kil-0 is a single gene

resistance governed by an R-gene or whether resistance is governed by multiple loci. Efforts are underway to perform the genetic crosses with Kil-0 and Be-0 and subsequent pathogenicity trials to address this question. If resistance is due to an R-gene, this would provide another target for manipulation via genetic engineering to afford resistance against *R. solanacearum* in hosts. However, the advantage of manipulating multiple genes down-stream of the recognition event (R-Avr interaction) such as those candidate genes identified in the current study would be that resistance against *R. solanacearum* would not be easily overcome.

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# CHAPTER 6
# SUMMARY & CONCLUDING DISCUSSION

## 6.1 Summary

*R. solanacearum* is a devastating disease affecting various crop species world-wide (Hayward, 1991). Importantly, the pathogen has been identified in *Eucalyptus* plantations in Africa (Coutinho et al., 2000). It is predicted that global warming will result in further disease incidence as changing temperatures may alter the geographical range of pathogens resulting in infection on new hosts (P. Birch[1], personal communication). *Eucalyptus* is exploited for wood and fibre production and is increasingly clonally propagated. The prevalence of bacterial wilt on *Eucalyptus* plantations could thus have devastating consequences for the forestry industry. One way to reduce disease incidence is to develop varieties with improved resistance against *R. solanacearum*. To this end, the current study exploited the model plant Arabidopsis to investigate the *R. solanacearum*/plant interaction. The aim of this study was to investigate the defence response against *R. solanacearum* in Arabidopsis and to identify candidate genes involved in resistance or susceptibility against the pathogen. A previous screen of several Arabidopsis ecotypes and a panel of African *R. solanacearum* isolates revealed that Arabidopsis ecotypes Col-5 and Be-0 were susceptible to *Eucalyptus* pathogens of *R. solanacearum* (BCCF 401 and BCCF 402) while ecotype Kil-0 was resistant (Weich, 2004). These interactions were used to investigate the plant defence response against *R. solanacearum.*

Initially, a microarray expression profiling system was developed and optimised using a custom microarray consisting of 500-defence response related cDNA probes (Chapter 3). The experimental question that was addressed was which genes are differentially expressed in a mutant (*cir1* (constitutively induced resistance 1), which previously showed enhanced resistance to the pathogenic biotrophic bacterium *Pst DC3000*, compared to wild-type (*luc2*) Arabidopsis plants without pathogen attack. The cDNA microarray expression profiling methodology was optimised to include the Trizol RNA isolation method, indirect labelling, and a mixed model ANOVA approach for data analysis. Several genes were found to be induced in *cir1* compared to *luc2* at a significance threshold of $p<0.01$ and fold change > 1.7 expression. These included 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

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hypocotyl in far-red 1) in *cir1*. Publicly available microarray data showed similar expression profiles for these genes in Arabidopsis plants infected with *Pst*, suggesting that these genes contribute to disease resistance in *cir1*. qRT-PCR confirmed the expression patterns of a subset of these genes providing evidence that the microarray expression profiling procedure was robust. An important conclusion from this study was that microarray expression profiling in our hands was successful in identifying genes involved in the plant defence response. The methodologies optimised in these experiments were employed in the subsequent microarray study (Chapter 4). It is logical that those candidate genes identified as possibly playing a role in *Pst* resistance should be subjected to gene function studies either by overexpression in a wild-type background or be knocked-out in *cir1* and subsequently challenged with *Pst* to determine their role in resistance against the pathogen. The focus of this PhD study, however was to investigate defence responses against a different bacterial pathogen: *R. solanacearum,* thus the latter work was not continued. Interestingly, preliminary infection trials showed that *cir1* was not resistant to *R. solanacearum* isolate GMI1000 or BCCF 402.

We investigated the susceptible interaction between *R. solanacearum* isolate BCCF 401 and Arabidopsis ecotype Col-5 using a cDNA microarray consisting of 5000 unigenes. Two time-points were investigated: early wilt and late wilt induced by *R. solanacearum* infection compared to the uninfected plants at the respective time-points. Three themes emerged from the results of expression profiling and bioinformatics comparison to publicly available data: 1) *R. solanacearum*-induced expression profiles are similar to that induced by *B. cinerea and P. syringae* during necrotrophic phases suggesting that *R. solanacearum* is a necrotroph (Glazebrook, 2005; Toth and Birch, 2005), 2) *R. solanacearum*-induced expression profiles are similar to that induced by ABA treatment suggesting a role for ABA signalling in response to the pathogen in Col-5 and 3) There are basal defence responses active in Col-5 in response to *R. solanacearum;* some of which may be manipulated by the pathogen.

The pathosystem developed by Weich (2004) was exploited to investigate the defence response against *R. solanacearum*. Arabidopsis ecotype Be-0 was more susceptible than ecotype Col-5 to *R. solanacearum* isolate BCCF 401 or BCCF 402 showing wilt symptoms as early as 4 dpi in most trials. Kil-0 was consistently resistant and showed little to no wilt symptoms even two weeks after inoculation with either strain. The resistant interaction between *R. solanacearum* BCCF 402 and Kil-0 was subjected to microarray expression profiling to determine which genes were differentially regulated in Kil-0 in response to the

pathogen. Thirteen genes were shown to be differentially regulated in challenged Kil-0 plants compared to mock inoculated plants. Subsequent qRT-PCR experiments investigated the expression profiles of a subset of these genes during the susceptible interaction. Seven of these genes: LTP3, PRX34, AIG, SAG13, SUI1, SKP5 and an "expressed protein" were further qualified as candidate genes conferring defence against *R. solanacearum* in Kil-0 based on the criteria that they were expressed earlier and/ or to a higher level in a resistant interaction compared to a susceptible interaction. Furthermore, bioinformatics comparison of these genes in microarray studies investigating other pathogen challenges provides evidence for several of these genes as good candidates for defence against *R. solanacearum* in Kil-0.

In this chapter, a comparison is made between the susceptible and resistant interactions between Arabidopsis ecotypes and *R. solanacearum* at the transcript level. The selection of candidate defence response genes for improving resistance against *R. solanacearum* initially in Arabidopsis, and with time, *Eucalyptus* are discussed.

## 6.2 Comparison between susceptible and resistant *Arabidopsis-R. solanacearum* interactions.

Table 6.1 draws a comparison between the resistant interaction Kil-0 challenged with *R. solanacearum* BCCF 402 with the susceptible interaction Col-5 challenged with BCCF 401. Several of the genes identified as significantly differentially expressed in the resistant interaction with Kil-0 and BCCF 402 are absent from the 5000 unigene cDNA microarray used for expression profiling of Col-5 infected with BCCF 401 (Table 6.1).

Two genes, which are induced in the susceptible interaction with BCCF 401 in ecotype Col-5, are marginally induced in Kil-0 challenged with BCCF 402 compared to the respective mock-inoculated plants i.e. glycosyl hydrolase family protein and PR-3 (Table 6.1). This suggests that high expression of these PR-genes may not be required for defence against *R. solanacearum*. This is in accordance with Hirsch et al. (2002) who suggested that the induction of PR-3 was consistent with the disease symptom rather than defence against *R. solanacearum.* PR-5 is down regulated in Col-5 but is not shown to be up-regulated in Kil-0 during pathogen challenge at the time-points investigated in this microarray expression profiling study. Northern blot analysis confirms that PR-5 is also down-regulated in ecotype Be-0 (results not shown). PR-5 is a marker of the SA signalling pathway. It may be predicted that suppression of this pathway could be a strategy by the pathogen to elicit disease and

results in Chapter 4 (Table 4.3) suggest that PR-5 may be a potential target or down-stream effect of possible effector manipulation. The results of Hirsch et al. (2002) showed that Arabidopsis *cpr1* and *cpr5* mutants, which have constitutively high levels of SA and PR-1 and PR-5 gene expression respectively, remained susceptible to isolate GMI1000.

**Table 6.1. Arabidopsis genes significantly differentially regulated in Kil-0 infected with *R. solanacearum* BCCF 401 and their expression in Col-5 infected with *R. solanacearum* BCCF 401 relative to mock inoculated plants.**

| TAIR ID | DESCRIPTION | log2 fold change (Kil-0 infected with BCCF 402/ Kil-0 uninfected) | Probe present on 5000 cDNA microarray? | log2 fold change (Col-5 infected with BCCF 401/ Col-5 uninfected) |
|---|---|---|---|---|
| At5g59320 | lipid transfer protein 3 (LTP3) | 2.08 | YES | 3.20 |
| At1g64360 | expressed protein | 0.67 | YES | NS |
| At1g54095 | expressed protein | -0.60 | NO | - |
| At3g49120 | peroxidase (PRX34) | 1.15 | NO | - |
| At1g07590 | pentatricopeptide (PPR) repeat-containing protein | 0.98 | NO | - |
| At5g59310 | lipid transfer protein 4 (LTP4) | 1.90 | NO | - |
| At2g12945 | hypothetical protein | 1.24 | NO | - |
| At3g28940 | avirulence induced gene (AIG) | 0.82 | NO | - |
| At4g16260 | glycosyl hydrolase family 17 protein | 0.47 | YES | 2.42 |
| At5g43580 | putative protease inhibitor | 0.85 | NO | - |
| At3g12500 | basic endochitinase (PR-3) | 0.61 | YES | 2.70 |
| At3g04720 | hevein-like protein precursor (PR-4) | NS | YES | 1.40 |
| At1g75040 | thaumatin (PR-5) | NS | YES | -1.08 |
| At2g29350 | tropinone reductase (SAG13) | 1.02 | NO | - |
| At5g54940 | eukaryotic translation initiation factor (SUI1) | 0.79 | NO | - |
| At5g20160 | ribosomal protein L7Ae family protein | 0.61 | YES | NS |
| At3g54480 | SKP1 interacting partner 5 (SKIP5) | 0.75 | YES | NS |
| At1g56555 | hypothetical protein | 0.88 | NO | - |
| At5g59330 | hypothetical protein | 1.51 | NO | - |
| At3g11770 | expressed protein | 0.72 | NO | - |

NS: Not significant

## 6.3 Comparison to the expression profiles of *At irx* mutants resistant to *R. solanacearum* GMI1000.

At the time of compiling this thesis, the only other known transcript profiling experiment conducted on plants showing resistance to *R. solanacearum* was performed by Hernāndez-Blanco et al. (2007). The secondary cell wall mutants of At *irx1* and *irx5* were resistant to *R. solanacearum* isolate GMI1000. Microarray expression profiling was performed on the *irx* mutants compared to the wild-type plants and a common set of constitutively expressed genes in the *irx* mutants was identified.

LTP3 and LTP4 are the only two genes which are induced in Kil-0 plants challenged with *R. solanacearum* and constitutively expressed in *irx* mutants which are resistant against GMI1000 (Hernandez-Blanco et al., 2007). The observation that only two genes are commonly induced in the resistant Kil-0 ecotype (LTP3 and LTP4) and constitutively induced in the *irx* mutants, which are resistant to GMI1000, suggests that different resistant mechanisms are involved in Kil-0 and in the secondary cell wall mutants (*irx1 and irx5*) against *R. solanacearum*. The authors Hernāndez-Blanco et al. (2007) suggest that the antimicrobial proteins constitutively expressed by the mutants create a hostile environment for the pathogen. LTP3 is induced to a higher level earlier in Kil-0 compared to Be-0 upon pathogen challenge with BCCF 402 (Chapter 5) and LTP3 is also induced during the susceptible interaction in Col-5 after infection with BCCF 401 (Chapter 4). This suggests that LTP3 is a key gene involved in defence against *R. solanacearum* in plants. LTPs are important antimicrobial peptides involved in plant defence against pathogens (García-Olmedo et al., 1995). Earlier experiments by Molina et al. (1993) showed that LTPs isolated from barley and maize leaves were able to inhibit the growth of *R. solanacearum in vitro* and the over expression of barley LTP2 in Arabidopsis and tobacco plants were able to reduce disease incidence caused by *P. syringae* (Molina and García-Olmedo, 1997). A similar transgenic approach, over-expressing LTP3 in susceptible Arabidopsis would be necessary to determine the role of LTP3 in defence against *R. solanacearum.*

Four genes, which are repressed in Col-5 during *R. solanacearum* infection with isolate BCCF 401 compared to mock inoculated plants, are constitutively induced in the *irx* mutants compared to wild-type plants. These are the integrin-related protein 14a, vegetative storage protein 2 (VSP2), O-methyltransferase family 2 protein and a jacalin lectin family protein (Table 6.1). Although the role of integrin-related protein 14a and O-methyltransferase family 2 protein in plant defence is unknown, vegetative storage protein 2 and jacalin lectin family protein are both jasmonate-responsive (Leon et al., 1998; Liu et al., 2005). VSP2 is induced during oxidative stress, wounding and has been indirectly shown to be involved in defence against insects (Liu et al., 2005). VSP2 has also been identified in Chapter 4 as a potential *R. solanacearum* effector target gene. It is possible that the up regulation of these genes in Col-5 would provide further protection against *R. solanacearum*. Other genes indicated in Table 6.2 which are similarly expressed in Col-5 in response to *R. solanacearum* and in *irx mutants* compared to the control plants, have also been identified in Chapter 4 (Figure 4.8) as possible genes involved in PTI and in this way, may play a role in defence against *R. solanacearum*.

**Table 6.2 Comparison of expression profiles for selected Arabidopsis genes differentially expressed in Col-5 plants infected with *R. solanacearum* BCCF 401 compared to uninfected plants (p<0.03, Bonferroni adjusted) and genes constitutively induced in *irx* mutants, *irx1* and *irx5* compared to wild type plants (Hernāndez-Blanco et al., 2007). Red boxes represent up-regulated genes while green boxes represent down-regulated genes. ABA responsive genes are indicated in bold type.**

| TAIR ID | DESCRIPTION | Col-5 | irx mutants |
|---|---|---|---|
| AT3G28290 | Integrin-related protein 14a | | |
| **AT5G24770** | **vegetative storage protein 2 (VSP2)** | | |
| **AT1G76790** | **O-methyltransferase family 2 protein** | | |
| AT3G16470 | jacalin lectin family protein | | |
| **AT1G02205** | **CER1 protein, identical to maize gl1 homolog (glossy1 locus)** | | |
| **AT5G59320** | **lipid transfer protein 3 (LTP3)** | | |
| **AT5G13800** | **hydrolase, alpha/beta fold family protein** | | |
| **AT5G06760** | **late embryogenesis abundant group 1 domain-containing protein** | | |
| **AT1G43160** | **AP2 transcription factor family (RAP2.6)** | | |
| **AT2G47770** | **Disease resistance protein (TIR class)** | | |
| **AT2G39800** | **delta 1-pyrroline-5-carboxylate synthetase A (P5CS1)** | | |
| **AT1G72770** | **protein phosphatase 2C P2C-HA (AtP2C-HA)** | | |
| **AT1G52890** | **no apical meristem (NAM) family protein** | | |
| **AT1G13260** | **DNA-binding protein RAV1** | | |
| AT3G54810 | zinc finger family protein, GATA transcription factor 3 | | |
| **AT2G44210** | **expressed protein** | | |

## 6.4 The role of ABA in resistance against *R. solanacearum*

There is increasing evidence to suggest that ABA is significantly involved in the interactions between plants and pathogens (Audenaert et al., 2002; Anderson et al., 2004; Thaler and Bostock, 2004; Ton and Mauch-Mani, 2004). Adie et al. (2007) indicated that ABA is required for defence against the necrotroph *P. irregulare* in Col-0 while Hernāndez-Blanco et al. (2007) showed that ABA mutants were more susceptible to *R. solanacearum* isolate GMI1000 compared to wild-type plants (Col-0). The *irx* mutants also show an induction of ABA-responsive genes compared to wild-type plants (Hernāndez-Blanco et al., 2007), some of which are genes induced in response to BCCF 401 in Col-5 (Table 6.2; bold type font).

If ABA is required for resistance, why is the expression of ABA-responsive genes in Col-5 not sufficient to confer resistance against the pathogen? This may be due to two factors: 1) the induction of ABA signaling required for resistance occurs later and / or to a lower level in

Col-5 than in a resistant interaction, 2) the induction of ABA-responsive genes in Col-5 is a result of wilting caused by *R. solanacearum* infection and not a reflection of the defence signaling pathway. The role of ABA in defence against *R. solanacearum* is further questionable if the resistant interaction between Kil-0 and *R. solanacearum* is considered. Expression profiling of this interaction suggests that there is induction of few ABA-responsive genes (LTP3, LTP4, glycosyl hydrolase family protein, PR-3, putative protease inhibitor, SAG13 and ribosomal protein L7Ae; as determined from NASCARRAYS-176 ABA treatment data). To test the role of ABA signaling in resistance in Kil-0, ABA mutants in a Kil-0 background would have to be challenged by *R. solanacearum.*

### 6.5 Further Work

The approach that has been undertaken in this study is one of gene discovery. During the course of this study an SSH library was prepared from a subtraction of cDNA from Kil-0 infected plants and cDNA from Kil-0 uninfected plants at various time-points (Mcleod and Naidoo, unpublished). This library provides another tool, which will be exploited in the future to identify candidate genes involved in defence against *R. solanacearum*. Together the microarray transcription profiling and bioinformatics approach used in the current study has identified candidate defence response genes against *R. solanacearum* in Arabidopsis. Gene function studies involving over-expression of candidate genes in Arabidopsis under the control of a constitutive or inducible promoter; knock-down using RNAi or VIGs (Burch-Smith et al., 2004), or knock-outs using T-DNA insertion mutagenesis (Woody et al., 2007), and subsequent challenge of the mutant plants with *R. solanacearum* would be used to determine the role of these genes in defence against the pathogen. One consideration is that this study has investigated transcript levels in resistant and susceptible interactions however these levels are not automatically representative of protein levels. Western blots or proteomic profiling approaches would be useful to determine whether high levels of the proteins are also produced. A yeast-two hybrid system could also be employed to determine whether any of the suites of genes induced in the resistant and susceptible interactions actually interact with each other or with other proteins using an Arabidopsis cDNA library of the prey. An important consideration is that multiple genes may act in concert to provide resistance against *R. solanacearum* in Kil-0, thus gene function studies of single genes may be uninformative and gene pyramiding may be necessary.

Once the role of a gene is characterised in Arabidopsis, its orthologue can be identified in Eucalyptus. The US Department of Energy -Joint Genomes Institute (JGI) has approved the sequencing of the *Eucalyptus grandis* genome (IUFRO 2007 Tree Biotechnology Congress, Azores, 8th June, 2007). The availability of this genome sequence would expedite the search for *Eucalyptus* orthologues. In addition, the *Eucalyptus* genome would provide another genomic tool to elucidate host defence responses against *R. solanacearum* using the transcriptome profiling approach employed in the current study. Recent progress has been made towards genetically engineering *Eucalyptus* (Van Beveren et al., 2006) and as such, candidate defence genes could be tested within the natural host against strains of *R. solanacearum*. If the desired phenotype is obtained, one could then infer that the gene is important for defence against the pathogen and one could subsequently target the gene to provide crop protection. Existing varieties which show enhanced expression levels of the target gene could be identified and intoduced into a breeding program or genetically modified trees would have to be produced in order to improve resistance against *R. solanacearum*.

The question that remains from this study is whether resistance in Kil-0 is multigenic or governed by a single recessive gene. *R. solanacearum* infections of the F2 progeny generated from a cross of Kil-0 and Be-0 are underway to address this question. If resistance against *R. solanacearum* were due to a single *R* gene such as the case in Nd1 (Deslandes et al., 1998), further fine mapping would have to be implemented to identify the *R* gene. The implication of a single *R gene* in Kil-0 is that it would provide an attractive biotechnology target for manipulation in *Eucalyptus*, to enhance resistance against *R. solanacearum* isolates carrying the corresponding *Avr* gene.

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# APPENDIX A

## Arabidopsis 5000 cDNA microarray elements

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT1G01040 | AT1G02870 | AT1G04140 | AT1G05850 | AT1G07040 | AT1G08410 | AT1G09830 | AT1G11310 | AT1G12440 | AT1G13600 | AT1G15340 | AT1G16870 | AT1G18570 | AT1G20370 | AT1G21730 | AT1G23440 |
| AT1G01080 | AT1G02880 | AT1G04250 | AT1G05850 | AT1G07080 | AT1G08510 | AT1G09870 | AT1G11310 | AT1G12520 | AT1G13640 | AT1G15340 | AT1G17010 | AT1G18580 | AT1G20380 | AT1G21760 | AT1G23480 |
| AT1G01090 | AT1G02890 | AT1G04250 | AT1G05890 | AT1G07140 | AT1G08570 | AT1G09970 | AT1G11360 | AT1G12550 | AT1G13710 | AT1G15350 | AT1G17050 | AT1G18620 | AT1G20440 | AT1G21780 | AT1G23740 |
| AT1G01100 | AT1G02920 | AT1G04260 | AT1G05960 | AT1G07170 | AT1G08630 | AT1G10020 | AT1G11380 | AT1G12570 | AT1G13960 | AT1G15350 | AT1G17080 | AT1G18700 | AT1G20460 | AT1G21830 | AT1G23800 |
| AT1G01230 | AT1G02950 | AT1G04290 | AT1G05990 | AT1G07230 | AT1G08680 | AT1G10030 | AT1G11410 | AT1G12680 | AT1G14000 | AT1G15370 | AT1G17100 | AT1G18710 | AT1G20500 | AT1G21880 | AT1G23820 |
| AT1G01240 | AT1G03030 | AT1G04300 | AT1G06040 | AT1G07230 | AT1G08710 | AT1G10040 | AT1G11450 | AT1G12740 | AT1G14010 | AT1G15400 | AT1G17200 | AT1G18730 | AT1G20620 | AT1G21980 | AT1G23850 |
| AT1G01300 | AT1G03060 | AT1G04330 | AT1G06050 | AT1G07310 | AT1G08730 | AT1G10060 | AT1G11480 | AT1G12760 | AT1G14150 | AT1G15500 | AT1G17220 | AT1G18800 | AT1G20670 | AT1G22360 | AT1G23860 |
| AT1G01430 | AT1G03080 | AT1G04410 | AT1G06060 | AT1G07320 | AT1G08770 | AT1G10070 | AT1G11530 | AT1G12780 | AT1G14170 | AT1G15670 | AT1G17330 | AT1G18880 | AT1G20693 | AT1G22530 | AT1G23860 |
| AT1G01470 | AT1G03090 | AT1G04410 | AT1G06110 | AT1G07350 | AT1G08830 | AT1G10130 | AT1G11545 | AT1G12810 | AT1G14320 | AT1G15690 | AT1G17340 | AT1G18970 | AT1G20696 | AT1G22530 | AT1G23900 |
| AT1G01490 | AT1G03106 | AT1G04440 | AT1G06260 | AT1G07350 | AT1G08880 | AT1G10150 | AT1G11580 | AT1G12830 | AT1G14380 | AT1G15740 | AT1G17420 | AT1G19100 | AT1G20760 | AT1G22700 | AT1G23950 |
| AT1G01620 | AT1G03130 | AT1G04490 | AT1G06270 | AT1G07360 | AT1G08940 | AT1G10170 | AT1G11650 | AT1G12840 | AT1G14400 | AT1G15740 | AT1G17470 | AT1G19330 | AT1G20850 | AT1G22710 | AT1G23950 |
| AT1G01770 | AT1G03210 | AT1G04680 | AT1G06410 | AT1G07370 | AT1G08980 | AT1G10200 | AT1G11650 | AT1G12840 | AT1G14410 | AT1G15740 | AT1G17530 | AT1G19350 | AT1G20850 | AT1G22800 | AT1G24020 |
| AT1G01820 | AT1G03220 | AT1G04690 | AT1G06410 | AT1G07470 | AT1G08990 | AT1G10270 | AT1G11680 | AT1G12850 | AT1G14450 | AT1G15750 | AT1G17580 | AT1G19370 | AT1G20900 | AT1G22840 | AT1G24050 |
| AT1G01830 | AT1G03220 | AT1G04750 | AT1G06450 | AT1G07600 | AT1G09070 | AT1G10290 | AT1G11700 | AT1G12930 | AT1G14580 | AT1G15820 | AT1G17620 | AT1G19400 | AT1G20920 | AT1G22850 | AT1G24090 |
| AT1G01910 | AT1G03290 | AT1G04790 | AT1G06460 | AT1G07640 | AT1G09130 | AT1G10390 | AT1G11820 | AT1G13020 | AT1G14610 | AT1G15880 | AT1G17620 | AT1G19540 | AT1G20960 | AT1G22920 | AT1G24260 |
| AT1G02080 | AT1G03310 | AT1G04850 | AT1G06570 | AT1G07790 | AT1G09140 | AT1G10460 | AT1G11840 | AT1G13060 | AT1G14720 | AT1G15950 | AT1G17730 | AT1G19570 | AT1G20980 | AT1G22930 | AT1G24460 |
| AT1G02140 | AT1G03350 | AT1G04850 | AT1G06630 | AT1G07890 | AT1G09210 | AT1G10470 | AT1G11860 | AT1G13090 | AT1G14740 | AT1G16010 | AT1G17745 | AT1G19570 | AT1G21010 | AT1G22940 | AT1G24510 |
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| AT2G23820 | AT2G25800 | AT2G27285 | AT2G28960 | AT2G30570 | AT2G32520 | AT2G34040 | AT2G35790 | AT2G37500 | AT2G39100 | AT2G40300 | AT2G41770 | AT2G43330 | AT2G44790 | AT2G46090 | AT2G47510 |
| AT2G23910 | AT2G25810 | AT2G27310 | AT2G29080 | AT2G30590 | AT2G32520 | AT2G34170 | AT2G35840 | AT2G37520 | AT2G39260 | AT2G40360 | AT2G41900 | AT2G43340 | AT2G44840 | AT2G46100 | AT2G47620 |
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| AT4G00752 | AT4G02270 | AT4G05070 | AT4G09580 | AT4G12230 | AT4G14020 | AT4G15550 | AT4G16830 | AT4G18260 | AT4G20150 | AT4G22330 | AT4G24050 | AT4G25520 | AT4G27040 | AT4G28680 | AT4G30090 |
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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
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| AT4G32010 | AT4G33360 | AT4G35060 | AT4G36640 | AT4G37870 | AT4G38980 | AT5G01090 | AT5G02540 | AT5G04600 | AT5G06160 | AT5G07590 | AT5G09450 | AT5G10960 | AT5G12950 | AT5G13850 | AT5G15230 |
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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
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| AT5G17440 | AT5G19120 | AT5G20160 | AT5G22030 | AT5G23210 | AT5G24800 | AT5G26980 | AT5G30510 | AT5G38030 | AT5G40390 | AT5G42020 | AT5G43750 | AT5G44860 | AT5G46630 | AT5G47730 | AT5G49210 |
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| AT5G17560 | AT5G19180 | AT5G20250 | AT5G22060 | AT5G23240 | AT5G24930 | AT5G27150 | AT5G33320 | AT5G38410 | AT5G40520 | AT5G42080 | AT5G43780 | AT5G45050 | AT5G46700 | AT5G47820 | AT5G49360 |
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