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PREFACE

This thesis is a compilation of three microarray studies conducted over five years pertaining to the investigation of defence responses against *Ralstonia solanacearum* in the model plant *Arabidopsis thaliana*. Each research Chapter has been written in the format of publishable units.

At the outset, it should be mentioned that the transcript profiling and bioinformatics approach to find genes involved in a biological process is limited. Further characterisation of these genes, such as gene function studies, is necessary to determine whether the gene does have a role in resistance or susceptibility.

Chapter 1 is a literature review, which discusses the pathogen *R. solanacearum* and the model plant *Arabidopsis thaliana*, the defence responses within plants and the use of microarray technology to study plant-pathogen interactions.

Chapter 2 is an extension of the literature review focusing on considerations for the design of microarray experiments aimed at an audience that wishes to embark on microarray experiments for the first time. This Chapter has been published in the South African Journal of Science in 2005 (Naidoo S., Denby K.J. and Berger D.K. (2005) Microarray experiments: considerations for experimental design. South African Journal of Science 101, 347-354).

Chapter 3 represents the first microarray expression profiling experiment conducted towards optimising the technology. The results of this experiment provided interesting candidate genes that could be involved in defence against *Pst* in the Arabidopsis mutant *cir1*. This Chapter was published in the South African Journal of Botany in 2007 (Naidoo S., Murray S.L., Denby K.J. and Berger D.K. (2007). Microarray analysis of the Arabidopsis thaliana *cir1* (*c*onstitutively *i*nduced *r*esistance 1) mutant reveals candidate defence response genes against *Pseudomonas syringae* pv *tomato* DC3000. South African Journal of Botany 73, 412-421).

Chapter 4 investigates the differential expression pattern in Arabidopsis ecotype Col-5, which is susceptible to *R. solanacearum*. This Chapter has been written in the format of an article aimed at the Journal of Functional Plant Biology and will be submitted for review shortly.

Chapter 5 deals with a resistant interaction between Arabidopsis ecotype Kil-0 and a Eucalyptus isolate of *R. solanacearum*. The basis of this resistance is explored at the transcript level using whole genome micoarrays.

Finally, Chapter 6 provides a summary of the results obtained in this study and provides a comparison of susceptible and resistant interactions against *R. solanacearum*. The impact of this research in understanding the plant defence response against *R. solanacearum* is discussed and future research is considered.

SUMMARY

Ralstonia solanaearum, a soil borne pathogen infects several important crops causing wilting. In 2000-2001, two eucalyptus isolates, BCCF 401 and BCCF 402 were isolated from plantations in Kwa-Zulu Natal and the Democratic Republic of Congo, respectively. *Arabidopsis* has been recognised as a host for *R. solanacearum* and as such has been adopted as a model to understand the plant defence response against this pathogen. The aim of this study was to use microarray expression profiling techniques to elucidate the plant defence response and to identify candidate genes possibly contributing towards resistance against the pathogen. As a means to optimise microarray expression profiling, the differential expression in an *Arabidopsis* mutant, *cir1* (constitutively induced resistance 1) and wild-type plants was investigated using a custom 500-probe microarray. Several genes were found to be induced in *cir1* at a significance threshold of $-\log_{10}(p)$ equal to 3 ($p< 0.001$) using a mixed model ANOVA approach. The genes 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), AtEREBP-4 (ethylene response element binding protein 4) and HFR1 (long hypocotyl in far-red 1) were further identified as possible candidate genes which may contribute to disease resistance in *cir1* against *Pseudomonas syringae* pv. *tomato.*

A similar transcript profiling approach, using the optimised protocols, was adopted to investigate the compatible interaction between *Arabidopsis* ecotype Col-5 and the *R. solanacearum isolate* BCCF 401. A screen of 5000 *Arabidopsis* ESTs revealed approximately 120 genes differentially regulated by *R. solanacearum* infection at a significance threshold of $p<0.03$ (Bonferroni corrected). Subsequent bioinformatic comparisons revealed that abscisic acid responses appear to be induced in Col-5 in response to the pathogen and that *R. solanacearum* induces an expression profile consistent with a necrotroph. The basal defence responses in Col-5 against *R. solanacearum* infection were investigated by comparing the expression data to that during treatment with the pathogen associated molecular patterns (PAMPs) flg22 and lipopolysaccharide, and the Type Three Secretion System deficient *Pst hrp*$^-$ mutant. Expression patterns for a subset of these genes were suggestive of host basal defences manipulated by the pathogen. It is hypothesised that genetic engineering to alter the expression of these “pathogen-manipulated” genes could contribute to resistance against *R. solanacearum* in the host.

In order to further elucidate the defence response to *R. solanacearum*, expression profiling was performed in the resistant ecotype Kil-0 challenged with isolate BCCF 402 using whole-genome *Arabidopsis* 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. Using a quantitative RT-PCR approach, it was shown that the expression of 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 role of these genes in defence against the pathogen remains to be elucidated by gene function studies. The current study has, however allowed the identification of important candidate genes that could be targeted in future to improve resistance against *R. solanacearum* in *Eucalyptus*.

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ABBREVIATIONS

ABA	abscisic acid
Avr	avirulence
BCCF	Bacterial Culture Collection FABI
BGT	Bacto-agar Glucose Triphenyltetrazolium chloride
bp	base pairs
cDNA	complementary DNA
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpi	days post inoculation
EDTA	ethylenediamine tetraacetic acid
EST	expressed sequence tag
ET	ethylene
hr	hour
HR	hypersensitive response
ISR	induced systemic resistance
JA	jasmonic acid
kb	kilobase
min	minute
mRNA	messenger ribonucleic acid
MeJA	methyljasmonate
MS	Murashige and Skoog media
ng	nanogram
NO	nitric oxide
PCR	polymerase chain reaction
pmol	picomole
PR	pathogenesis related
qRT-PCR	quantitative reverse transcriptase PCR
Rif^r	rifampicin resistant
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute

RT	reverse transcriptase
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SSC	sodium chloride / sodium citrate
UV	ultraviolet
µg	microgram

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List of research project topics and materials

CHAPTER 1
LITERATURE REVIEW

1.1. Introduction

Plant diseases represent a major threat to the human food source globally. Efforts to curb such diseases have involved control measures such as using suppressive soils, alteration of farming practices, treating plants with chemical sprays and classical breeding to produce varieties with enhanced tolerance or resistance against the disease (Agrios, 1997). An integrative approach, which incorporates several methods of control, has been recognised as most successful in curbing disease incidence. Progress towards producing resistant or tolerant plant varieties has been accelerated by the availability of genomic tools; in particular, the adoption of *Arabidopsis thaliana* as a model plant. Various plant pathogens are also virulent on Arabidopsis, providing a model to conduct pathogenicity tests. Such studies in Arabidopsis and other plant species have shown that plants have a sophisticated and complex immune system reminiscent of the animal immune system involving receptors, signalling pathways and the activation of antimicrobial and antifungal proteins for protection (reviewed in Jones and Dangl, 2006). It is expected that by elucidating these defences, genes involved in resistance or susceptibility can be identified. The long-term goal is that orthologues of these genes can be identified in the more important crop plants and targeted for genetic modification or in breeding programs for crop improvement.

The particular pathogen discussed in this study is the bacterial wilt bacterium *Ralstonia solanacearum.* The pathogen has a wide host range and as such presents a problem worldwide. This review focuses on the epidemiology, molecular and genetic characteristics of the pathogen and efforts towards determining resistance against the pathogen. The current knowledge regarding the plant defence response deemed most pertinent to this study is presented. Information on the application of microarray expression profiling and tools for data mining are also provided in this review.

1.2. *Ralstonia solanacearum*

Phytopathogenic bacteria multiply in the apoplast of plant cells and remain extracellular (Staskawicz et al., 2001). Some of the most common plant pathogenic genera of bacteria include *Agrobacterium*, *Clavibacter, Pectobacteria, Pseudomonas, Xanthomona*s, and *Streptomyces* (Agrios, 1997). Infected plants show a variety of symptoms, such as leaf spots and blights, soft rots, wilts, and cancers. One example of a pathogen that causes wilting disease is *Ralstonia solanacearum.*

The genus *Ralstonia* belongs to the β-proteobacteria (Palleroni et al., 1973). *R. solanacearum* is a gram-negative aerobic bacterium, which is rod-shaped and has polar flagella (Holt et al., 1994). *Ralstonia solanacearum* has a wide host range. Orignially known as *Pseudomonas solanacearum* (Yabuuchi et al., 1995), the pathogen is considered one of the most important plant pathogenic bacteria due to the economic losses that occur globally resulting from bacterial wilt disease caused by *R. solanacearum* (Hayward, 1991).

1.2.1 Host Range and Epidemiology

R. solanacearum infects over 200 plant species representing more than 50 plant families. Hosts include solanaceous crops such as tobacco, tomato, potato and eggplant (Agrios, 1997), leguminous plants such as groundnut and French bean (Genin and Boucher, 2002), and in monocotyledonous plants, such as banana, the pathogen causes Moko disease. *R. solanacearum* also causes bacterial wilt disease on several shrub and tree species such as cashew, mulberry, olive (He et al., 1983; Shiomi et al., 1989) and *Eucalyptus*. *Eucalyptus* was initially reported as a host in Brazil and China but is currently also a host of the pathogen in Australia and Africa i.e. South African and Uganda (Hayward, 1991; Hayward et al., 1994; Coutinho et al., 2000; Roux et al., 2001). The severity of the disease in Africa may be underestimated as a limited number of *Eucalyptus* plantations have been surveyed. There is a discrepancy in the distribution of bacterial wilt on specific hosts i.e. bacterial wilt may pose a problem on a certain host in one geographic location, and be absent from the same host in another location. This suggests that a combination of environmental factors conducive to disease incidence is necessary for *R. solanacearum* prevalence on a particular host (Hayward, 1991).

R. solanacearum has the ability to survive in the soil in the absence of a host for extended periods as well as in the protected niche of a weed's rhizosphere (Hayward, 1991). High soil moisture in well-drained soils is conducive to *R. solanacearum* survival, however, its survival in the soil is temperature dependent. A high day temperature of 40°C maintained for more than four hours has been shown to reduce bacterial populations (van Elsas et al., 2000) although an increase in ambient temperature between 30-35°C has been correlated with an increase in disease incidence and rate of onset of bacterial wilt on hosts such as tomato (Hayward, 1991). Some soil types suppress the pathogen as the soil moisture determines the antagonistic population levels, which compete with *R. solanacearum*. Nematode infestation (*Meloidogyne* species) also contributes to spread of the disease. This is thought to be

primarily a result of the increase in wounding of plants by the nematodes, which promotes bacterial infection, however, the nematode may also modify plant tissue making it suitable for bacterial invasion (Hayward, 1991).

R. solanacearum is also able to survive in aquatic habitats and contaminated irrigation water and municipal wastewater, used in the processing of diseased plant tissue, have been recognised as sources of inoculum (Elphinstone et al., 1998; Janse et al., 1998).

A host may often be regarded as healthy since disease symptoms are not visible however the pathogen can be present in the plant at high inoculum levels. The pathogen over-winters in diseased plants or plant debris, in vegetative propagative organs such as potato tubers or banana rhizomes, on the seeds of some crops like capsicum and tomato, and in the rhizosphere of weed hosts e.g. *Solanum dulcamara*, *Solanum carolinense* and *Solanum cinereum* (Hayward, 1991; van Elsas et al., 2000). This results in latent infection as the host is sometimes further cultivated (Denny et al., 2001).

The pathogen enters the host via root wounds, which may be caused by insects, nematodes, cultural practices or sites of secondary root emergence (Kelman and Sequeira, 1965). The bacteria move towards the xylem vessels where they multiply and spread (Salanoubat et al., 2002). The root cortex and vascular parenchyma are colonised and cell walls are disrupted. This facilitates the spread of the pathogen through the vascular system. The bacteria accumulate in pockets filled with slimy masses and cellular debris (Hayward, 1991; Vasse et al., 1995; Genin and Boucher, 2002). The colonising *R. solanacearum* bacteria cause rot and tissue disintegration as a result of secreted extracellular products. These include an acidic, high molecular mass, extracellular polysaccharide (EPS1) and several plant cell wall-degrading enzymes: endo-polygalacturonase (PehA), two exo-polygalacturonases (PehB and PehC), endoglucanase (Egl) and a pectinmethylesterase (Pme). Recently a new cell wall degrading enzyme has been identified following sequencing of *R. solanacearum*: an exoglucanase 1,4 β-cellobiosidase (Salanoubat et al., 2002). Together the endo-polygalacturonases and exo-polygalacturonases are thought to contribute substantially to the virulence of *R. solanacearum* (Genin and Boucher, 2002).

The accumulation of the bacteria in pockets in the vascular bundle, pith and the cortex, effectively destroys the plant's vascular system. Stems, roots and tubers discolour through

necrosis. These tissues will also exude whitish-coloured exudates under conditions of severe infection. The plants wilt completely, with younger plants wilting more rapidly than the older plants, followed by rotting and disintegration of the roots (Agrios, 1997).

Experiments using *R. solanacearum* constitutively expressing green fluorescent protein (gfp38) have demonstrated the progress of infection and timing of disease symptoms (Denny and Lui, 2002). The colonisation of the epidermal cells of lateral roots of tomato plants was observed within one day of soil drenching with the *R. solanacearum* strain AW1-gfp38. By four days, one or more xylem vessels were colonised throughout the plant. At this stage wilt symptoms were not present on the plant. The bacteria eventually entered the stem pith and the cortex spreading to all plant tissues coinciding with visible wilt symptoms.

1.2.2 Classification and Control

The broad host range and diversity within the *R. solanacearum* species has complicated classification over the years. The accepted convention is to employ a two-fold classification system, which is based on the host range of the strains to classify them into races (Buddenhagen et al., 1962), and the ability of the strains to oxidise various disaccharides and hexose alcohols to classify them into biovars (Hayward, 1964). RFLPs on the *hrp* gene region and 16S rRNA sequence analysis have also been used as the basis of a classification system for *R. solanacearum* (Cook et al., 1989; Cook et al., 1994; Poussier and Luisetti, 2000). Recently, Prior and Fegan (2005) described a classification system based upon phylogenetic analysis of sequence data generated from the 16S-23S internal transcribed spacer (ITS) region, the endoglucanase gene and the *mutS* gene of *R. solanacearum*. The *R. solanacearum* species complex subdivided into four monophyletic clusters of strains called phylotypes. The phylogenetic analysis revealed that each phylotype broadly originated from the same location. Within each phylotype, there were a number of strains containing highly conserved sequences, which were grouped as sequevars. Some of these sequevars are pathogenic on the same hosts or strains of common geographic origin (Prior and Fegan, 2005). The robustness of this phylogeny was demonstrated by Guidot *et al*. (2007) who showed, based on *R. solanacearum* microarray genomic data, that the organismal phylogenetic relationships of a set of strains chosen as representative of the four phylotypes matched the classification scheme of Prior and Fegan (2005). The relationship between races, biovars, RFLP and phylotype division for the classification of *R. solanacearum* is summarised in Table 1.1.

Table 1.1 Characteristics of races and their relationship to Biovars, RFLP and phylotype subdivisions of *R. solanacearum*.

Race[a]	Host Range[a]	Geographical Distribution	Biovar[b]	RFLP Division[c, d]	Phylotype[f]
1	wide	Asia, Australia,	3, 4	I	I
		Americas	1	II & III[e]	II
		Africa			III
2	Banana and other Musa spp.	Caribbean, Brazil,	1	II	II
		Philippines			IV
		Indonesia			
3	Potato	Worldwide	2	II	II
4	Ginger	Asia	3, 4	I	I
5	Mulberry	China	5	I	I

[a]Buddenhagen (1962), [b]Hayward (1964), [c]Poussier et al. (2000); [d]Cook and Sequierra, (1994); [e]Fouchè-Weich et al. (2006); [f]Prior and Fegan (2005).

The PCR-RFLP approach was adopted by Fouchè-Weich et al. (2006) in determining the causal agent of bacterial wilt from eucalypt plantations in the Democratic Republic of Congo (DRC), South Africa, and Uganda and from potato fields in South Africa. The eucalypt isolates were identified as *R. solanacearum* biovar 3 while the potato isolates, except for one, were classified as biovar 2. This study further qualifies the PCR-RFLP approach as a useful tool for classification of *R. solanacearum*.

The importance of classification is that it is useful to identify suitable control measures against the disease outbreak. Various strategies have been employed to control the pathogen and are discussed below.

A certification scheme on seed potato crops is employed in the South African potato industry, preventing disease spread. Routine field inspections and compulsory laboratory tests of all registered seed plantings are performed (Swanepoel and Theron, 1999). If bacterial wilt is detected in a registered seed tuber planting, a quarantine control measure is implemented. Adjacent fields in a 50 m radius of the planting site are also considered infected and planting is terminated. The cultivation of seed tubers in infected fields is not allowed for 8 years if *R. solanacearum* biovar 2 is found to be the causal agent of bacterial wilt in that field, however;

if biovar 3 is found to be the causal agent, no planting will resume on the field (Swanepoel and Theron, 1999). The potato industry has a zero tolerance for *R. solanacearum* in potato seed tubers and fields testing positive for *R. solanacearum* infection are no longer registered for seed tuber production.

Intercropping is a method to reduce soil populations of the pathogen and limits root-to-root transmission (Hayward, 1991). Pegg and Moffet (1971) found long-term crop rotations with either rye or winter oats, together with fallowing in infested ginger fields reduced bacterial populations. Amendment of soil is also potentially a good control strategy as a soil mixture known as S-H developed in Taiwan (Hayward, 1991) showed broad-spectrum activity against soil-borne disease including bacterial wilt. Based on the observation that bacterial wilt never occurs on the seashell ridges of coastal plains, Power (1983) reported on the use of sea-shell grit (42% CaO) as a soil additive at a rate of approximately 1 m^3 per 15 m^3 sandy or clay bacterial wilt-infested soil mixed into the tilled layer. This amendment resulted in a decrease in bacterial populations. Similarly, silicon application to the soil of tomato plants reduced bacterial wilt symptom development (Schacht and Wydra, 2006; Wydra and Beri, 2006). These experiments resulted in an increase in cell wall derived polygalacturonase inhibiting proteins (PGIPs) and structural changes to the xylem cell wall contributing to inhibition of bacterial wilt. Another approach is to change the planting season to a season unfavourable for bacterial wilt development. This process, termed disease avoidance, can decrease crop losses by the disease but is limited to those crops which are not propagated further as the plant material will harbour a latent infection (Hayward, 1991).

Biological control can be achieved by using antagonistic rhizobacteria and avirulent mutants of *R. solanacearum*. This biological control may be attributed to either induced resistance, protection by competitive exclusion, active colonisation of the rhizosphere with antagonistic soil bacteria or bacteriocin- and bacteriophage-producing strains of *R. solanacearum* (reviewed in Hayward, 1991). The use of specific bacteriophage: *R. solanacearum* mixtures in irrigation water reduced the disease incidence to 0-5% in tomato plants under glasshouse trials (Álvarez et al., 2006). *Pseudomonas putida* is another biocontrol agent, which has shown some promise under laboratory conditions. Moderately resistant and susceptible potato seed tubers were coated with the endophytic antagonistic *P. putida* strain BA28 and planted in infected soil. A decrease in symptom and latent infection was observed up to a level of 95% in moderately resistant cultivars (Priou et al., 2006b). In China, a biocontrol pesticide has

been developed called KangDiLeiDe comprising a $1x10^7$ cfu/g granular formula of the rhizobacteria *Paenibacillus polymyxa,* strain HY96-2 (Li et al., 2006). This formulation has been shown to be effective against bacterial wilt in the field, inhibiting bacterial wilt of tomato, eggplant, green pepper and tobacco. This commercial product is also effective against other plant diseases such as Fusarium wilt of tomato and watermelon, seedling *Rhizoctonia*, damping off (*Pythium aphanidermatum),* tobacco brown leaf spot (*Alternaria altelnata*) and soybean Fusarium root rot (*Fusarium orthocreras*).

Avirulent mutants of *R. solanacearum* produced by Tn5 mutagenesis were able to prevent subsequent colonisation by wild-type strains to a limited degree (Hayward, 1991). Vesicular-arbuscular mycorrhizae (VAM) have been used in the Phillipines for the protection of plants from bacterial wilt. The mechanism of protection may be a competition effect between the mycorrhizae and pathogen or due to the mechanical barrier in the form of VAM vesicles and hyphae, which inhibit the bacterial pathogen from deeper penetration into the host tissues (Halos and Zorilla, 1979).

It has been recognised that there is no universal means of control. However an integrated approach which involves preventative measures combined with the use of resistant cultivars is one of the most successful ways to reduce disease incidence (Poussier et al., 2002). In Japan, for example, bacterial wilt on potato was addressed by a combination of soil fumigation with chloropicrin, using a tolerant cultivar and delayed planting during cooler temperatures (Hayward, 1991).

Host-plant resistance has been successful in tobacco and peanut and to some extent in tomato, but immunity has not been identified in potato (Thoquet et al., 1996; Thouquet et al., 1996b). A wide screen for potato genotypes resistant against *R. solanacearum* biovar 2A was recently performed at the International Potato Center in wild species of potato (Priou et al., 2006a). Three genotypes of *Solanum acaule* and one of *S. tuberosum* subsp. *andigena* (primitive weed) showed no latent infection in tubers and stems or disease symptoms indicating high levels of resistance. Plans are underway to transfer this genetic resistance to commercial potato varieties. In Brazil, a similar strategy is underway following the identification of two wide-spectrum (*R. solanacearum* biovars 1 and 2) resistant potato clones (Lopez et al., 2006).

Genetic engineering techniques are being employed to engineer resistance in potato by introducing lysozyme, cecropins and insect-derived antibacterial proteins (Montanelli and Nascari, 1998). The observed interaction between PGIPs from tomato against polgygalacturonases from *R. solanacearum* (Schadt and Wydra, 2006) provides another potential target for improving resistance against bacterial wilt through genetic engineering.

1.2.3 Molecular Studies of *R. solanacearum*

R. solanacearum strains, e.g. K60, GMI1000 (Boucher et al., 1985) and AW (Schell, 1987), have been intensely studied at the molecular level in order to characterise the pathogenicity factors used by the bacterium. Some of the most interesting findings unveiled by genome sequencing of the pathogen include clues as to what contributes to the bacteria's pathogenicity, complexity, potential plasticity and ability to adapt to diverse ecological niches.

The genome sequence of a French Guyana isolate of *R. solanacearum,* GMI1000, pathogenic on tomato, was completed in 2002 (Salanoubat et al., 2002). *R. solanacearum* has a bipartitie genome structure organised into two replicons: a 3.7 Mb chromosome, which houses the mechanisms required for survival, and a 2.1 Mb megaplasmid, which carries duplicates of metabolic genes as well as the *hrp* genes necessary for virulence (Genin and Boucher, 2002; Salanoubat et al., 2002). The genome has a high G+C content of 67% and contains genes which potentially encode approximately 5120 proteins. The megaplasmid encodes genes for flagellin biosynthesis as well as essential pathogenicity functions, catabolism of aromatic compounds, copper- and cobalt/zinc/cadmium-resistance gene clusters. The megaplasmid also contains duplications of several important genes such as three tRNAs and a second subunit α of DNA polymerase III. The presence of genes coding for several enzymes involved in the metabolism of small molecules on the megaplasmid and absence of these gene counterparts on the chromosome in comparison to other bacteria, suggests that the megaplasmid is in the process of acquiring new functions via duplication or translocation of essential genes from the chromosome (Genin and Boucher, 2002). This characteristic of the megaplasmid is thought to contribute to the overall fitness of the bacterium as well as the potential plasticity of the genome. Other factors, which contribute to the latter phenomenon in *R. solanacearum* are the high number of transposable elements and that 7% of the genome corresponds to Alternative Coding Usage Regions (ACURs). The presence of ACURs and transposable elements suggests that the pathogen is able to acquire and recombine exogenous DNA through natural

transfer (Salanoubat et al., 2002). These ACURs may be pathogenicity islands acquired by horizontal gene transfer which may be involved in a duplication or evolution process, thus allowing the acquisition, loss and rearrangement of genetic material (Genin and Boucher, 2002). Evidence of a tandem repeat of a 31 kb region flanked by insertion sequences in the megaplasmid is consistent with the suggested genetic rearrangement in *R. solanacearum*. Such genomic instability is probably responsible for the genomic diversity of the species (Genin and Boucher, 2002).

The 8x draft sequence of a Geranium strain of *R. solanacearum* UW551 was completed in 2006 (Gabriel et al., 2006). This isolate was considered a United States Department of Agriculture Select Agent and was shown to be pathogenic on geranium, tomato, and potato. The genomes of UW551 and GMI1000 were compared and 71% syntenic gene organisation was observed between the two genomes however the largest physical difference between the genomes was the presence of a cluster of 38 probable prophage genes in UW551. These prophage genes may contribute to pathogenicity as suggested in *R. solanacearum* strain K60 (Brown and Allen, 2004). UW551 belongs to race 3, biovar 2 while GMI1000 belongs to race 1, biovar 3. Comparative genomics allowed the identification of a 22kb region present in GMI1000 that is absent from UW551, which encodes for genes required for the utilisation of the 3 sugar alcohols that distinguish biovars 3 and 4 from biovars 1 and 2 (Gabriel et al., 2006). A PCR-based diagnostic marker was developed for race 3 biovar 2 strains resulting from unique genes in UW551 which was found to be race 3, biovar 2-specific after PCR across 58 strains from different races and biovars.

Brown and Allen (2004) used an *in planta* expression technology to identify which *R. solanacearum* strain K60 genes are expressed during growth in tomato plants. The expression of genes in *R. solanacearum* in the xylem is suggestive of a pathogen, which adapts to the host environment. A small percentage of the genes identified may play a role in bacterial stress response pathways by neutralising plant derived reactive oxygen species or toxins. There was also evidence for possible DNA rearrangement and the involvement of phages during pathogenicity and development within the host. Regulators specifically expressed within the plant may be required for *R. solanacearum* pathogenesis (Brown and Allen, 2004). Genome sequencing of the two *R. solanacearum* strains GMI1000 and UW551 (Salanoubat et al., 2002; Gabriel et al., 2006) also revealed the presence of several proteins secreted by the Type Two and Type Three Secretion Systems and *in planta* expression technology (Brown

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and Allen, 2004) showed expression of several of these genes which are important for disease development in hosts.

R. solanacearum Type Two Secretion System

The *R. solanacearum* Type Two Secretion System (T2SS) secrete factors such as plant cell wall degrading pectinases (PehA, PehB, PehC and Pme) an endoglucanase (Egl), polygalacturonases (PG) and extracellular polysaccharide (EPS) (Allen et al., 1991; Denny and Baek, 1991; Gonzalez and Allen, 2003). Each of these factors contributes to successful pathogen colonisation and disease development. Before proteins can be secreted by the T2SS, they have to first be secreted through the cytoplasmic membrane into the periplasm. The twin arginine protein translocation (Tat) system is one way in which proteins can be translocated into the periplasm. González et al. (2007) showed that mutation of a key component of this system (TatC) resulted in reduced virulence of GMI1000. Bioinformatic analysis suggests that over 70 proteins are translocated by the Tat system. Mutation of two of these proteins which were previously shown to be induced in a host by *in vivo* expression technology (RSp1521 and RSp1575) were significantly reduced in virulence (Brown and Allen, 2004; Gonzalez et al., 2007). RSp1521 is suspected to be involved in acid tolerance and could contribute to tolerance of the acidic pH within the host environment while RSp1575 is thought to play a role in *R. solanacearum* taxis which is the ability of the pathogen to move to more favourable environments within the host. This example demonstrates the importance of the *R. solanacearum* T2SS in contributing to virulence.

R. solanacearum Type Three Secretion System

R. solanacearum employs the Type Three Secretion System (TTSS), which is one of three distinct pathways via which gram-negative bacteria secrete proteins across their inner and outer membranes (Salmond and Reeves, 1993). The *hrp* cluster of genes is required for the production of the TTSS (Genin et al., 1992). By inactivating one of the *hrp* genes in *R solanacearum*, Arlat *et al*. (1992) found that the pathogen was unable to cause disease and multiply in susceptible plants and lost the ability to cause a hypersensitive response in resistant plants. The TTSS allows the delivery of virulence proteins (effector and accessory proteins) directly into host cells and requires the production of a *Hrp* pilus, coded for by the *hrpY* gene, which is thought to direct protein translocation across the cell wall (Van Gijsegem et al., 2000). The Hrp TTSS is regulated by a complex signal transduction cascade, which responds to a specific inducing signal (reviewed in Schell, 2000). The *hrpB* gene codes for the

regulator of this system (Van Gijsegem et al., 1995). Maximal expression of the *hrpB* gene was attained in response to physical contact of the bacteria with plant cells or cell wall fragments (Aldon et al., 2000). This contact-dependent activation is thought to ensure the translocation of effector proteins into the plant cells at the appropriate time and place. The nature of the *hrp*-inducing compound from the plant cell wall is not known, however, the bacterial receptor involved is suggested to be the outer membrane protein PrhA (Aldon et al., 2000). PrhA in turn transfers the signal to HrpB via the following cascade: PrhA-PrhR/PrhI-PrhJ-HrpG (Brito et al., 1999; Aldon et al., 2000; Brito et al., 2002; Cunnac et al., 2004b). The research contributing to the discovery of *R. solanacearum* effector proteins is discussed further.

Cunnac et al. (2004b) compared the structures of two *HrpB*- dependent promoters *hrpY* and *popABC* and found a conserved DNA motif, referred to as the hrp_{II} box, which was thought to confer HrpB-dependent activation. Based on this conserved sequence, 114 candidate genes encoding TTSS effectors were identified. A subsequent study by Cunnac et al. (2004a), using insertion mutagenesis of 56 of these candidate HrpB TTSS dependent genes, resulted in the identification of 48 novel *HrpB*-regulated genes. The authors proved biochemically that 5 of these proteins were translocated into plant host cells via the TTSS. These include PopP2, RipA (Ralstonia effector injected into plant cells), RipB, RipG and RipT. The type III secretome appears highly conserved in *Ralstonia spp*. In UW551, only 6 or 7 effectors appear to be missing compared to GMI1000 and three effectors: RRSL00326, RRSL01019, and RRSL03923, were found to be unique to UW551 (Mukaihara et al., 2004; Gabriel et al., 2006). The number and type of effectors contained within the different strains may influence the hosts on which each strain can be a successful pathogen. A study by Mukaihara et al. (2004) identified 30 novel *HrpB* activated genes outside the *hrp* gene cluster using a transposon/promoter trap system. Most of these genes contained a plant-inducible promoter box motif in their promoter regions, which is thought to be the recognition motif for HrpB. However, interaction of HrpB with this motif has not yet been demonstrated (Mukaihara et al., 2004). The specific role of HrpB was revealed in a whole genome microarray screen of wildtype, *hrpB* deficient and high-expressing *hrpB R. solanacearum* strains (Occhialini et al., 2005). The *hrpB* gene is thought to function as a master switch controlling a physiological change during the shift from saprophytic to parasitic life. The *hrpB* gene positively regulates seventy-nine effectors or TTSS accessory proteins. Only 50 are negatively controlled by the

gene. The lack of a conserved *hrp*$_{II}$ box sequence in several of the *HrpB*-regulated genes suggests that these genes may be regulated indirectly by *hrpB* (Occhialini et al., 2005).

There are other regulatory pathways controlling the TTSS. Recently, Genin et al. (2005) investigated the relationship between the two regulatory pathways controlled by PhcA and HrpB respectively. Their results indicated that inactivation of *phcA* strongly activated *hrp* gene transcription in complete medium i.e. conditions under which *hrp* genes are normally expressed at background levels. The specific activation of *HrpB* by the inactivation of *phcA* required the *hrpG* gene. The *hrpG* gene is the regulator acting upstream of *hrpB* in the pathway induced by the bacterial-plant cell contact that leads to the induction of effector proteins. Over-expression of *phcA* reduced the ability of *R. solanacearum* to elicit a hypersensitive response on tobacco leaves. PhcA therefore appears to negatively regulate *hrp* gene expression, possibly contributing to reduced virulence. Studies using a *lacZ* operon fusion to PrhIR in *R. solanacearum* strain OE1-1 and OE1-1phcA suggest that PhcA negatively regulates the expression of PrhIR possibly attenuating the signal cascade leading to *hrpB* activation (Hikichi et al., 2007). PhcA also negatively regulates PehSR, which is in turn responsible for the production of polygalacturonases PehA, PehB and PehC via the T2SS (Allen et al., 1997). Further studies in OE1-1 suggest cooperation between the T2SS and the TTSS in that *pehC* was positively regulated by HrpB (Hikichi et al., 2007). Figure 1.1. summarises the hrpB and PhcA regulatory pathways which contribute to the virulence of *R. solanacearum* and the secretion of cell wall degrading enzymes via the T2SS and the secretion of effector proteins via the TTSS pathway into the host cell.

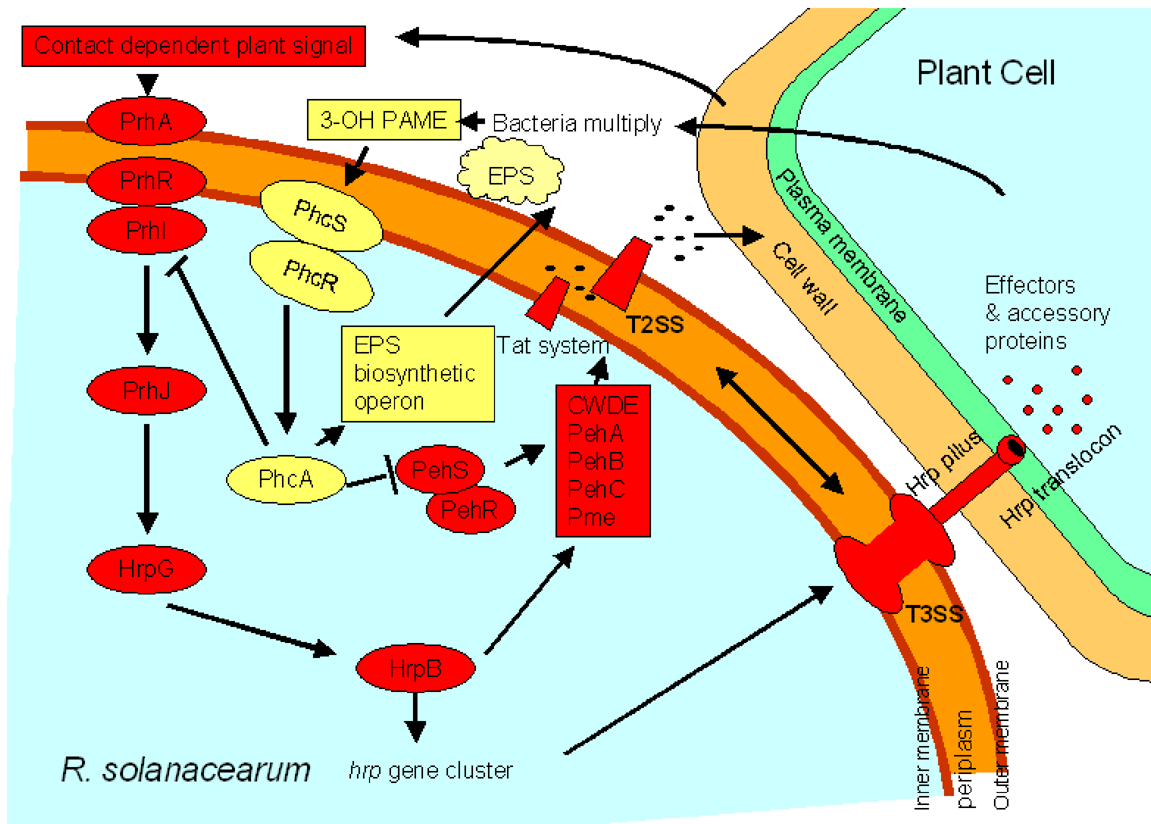

Figure 1.1 Schematic diagram representing the regulation of virulence in *R. solanacearum* adapted from Hikichi et al., 2007; Buttner and Bonas, 2006. Pathways have been studied in *R. solanacearum* strains GMI1000, K60 or OE1-1. Abbreviations are as described in the text. Symbols are: positive regulation →, negative regulation ⊥ and cooperative interactions ↔.

There is evidence that TTSS effectors can 1) interfere with the host's transcriptional machinery, 2) cleave plant proteins as cysteine proteases, and 3) interfere with the host ubiquitin/ proteasome pathway (Szurek et al., 2001; Axtell et al., 2003; Kim et al., 2005). Angot et al. (2006) recently demonstrated the latter phenomenon in *R. solanacearum*. Seven TTSS effectors were identified with plant-specific leucine-rich repeats (LRR) and were termed GALA as they contain a conserved GAxALA domain. Sequence analysis revealed that each of these GALA proteins contain an F-box domain. In eukaryotes, the F-box domain interacts with the SKP-1 protein which in turn interacts with Cullin1 forming the SCF-type E3 ubiquitin ligase complex. This leads to the ubiquitination of specific proteins and the ubiquitin tagged proteins are either modified or are degraded by the 26S proteasome. Using a yeast two-hybrid system, the authors showed that the GALA proteins were able to interact with 19 different SKP-1-like proteins from Arabidopsis in the same manner as true Arabidopsis F-box proteins. It is possible that the GALA F-box proteins were once acquired from plant DNA by

R. solanacearum via lateral gene transfer. A mutant strain of GMI1000, which has all seven GALA genes deleted, showed reduced pathogenesis on Arabidopsis and tomato. Single GALA gene mutations failed to produce a phenotype change on Arabidopsis and tomato however, infections with a mutant of GALA7 on *Medicago truncatula* showed a drastic reduction in wilting symptoms suggesting that GALA7 is a host-specific factor required for disease on *M. truncatula*.

Meyer et al. (2006) identified two novel Hrp-secreted proteins PopF1 and PopF2 in GMI1000 showing similarity to the TTSS accessory proteins of the YopB family from bacteria (*Yersinia pestis*), which are pathogenic on animals and humans. YopB, together with other accessory proteins, is thought to associate into a translocon, which in turn is required for the translocation of effector proteins across the plasma membrane into mammalian host cells (Sarker et al., 1998). Thus these specific TTSS accessory proteins are referred to as translocators. The strain UW551, which belongs to a different phylotype than GMI1000, also contained two translocators, one of which was different to that identified in GMI1000. This suggests that the *R. solanacearum* translocators may be variable in different strains (Meyer et al., 2006).

Quorum sensing

Quorum sensing enables bacteria to determine their local population density by the secretion and detection of small, diffusible signal molecules. The Phc regulatory system is responsible for the regulation of the traits required for virulence in a population density–dependent manner (Clough et al., 1997). At the centre of this regulation is PhcA whose activity is modulated by an endogenous volatile signal molecule 3-hydroxypalmitic acid methyl ester (3-OH PAME). In *R. solanacearum*, quorum sensing may be important as the bacteria make the transition from a saprophytic lifestyle to a parasitic one. Low levels of 3-OH PAME lead to a decrease in PhcA activity which in turn results in reduced extrapolysaccharide and exoenzyme synthesis, but enhanced motility and siderophore production, while high levels of 3-OH PAME (>5 nM at a high cell density in the vascular system) promotes PhcA activity leading to enhanced expression of EPS and exoenzymes and decreased motility and siderophore synthesis (Clough et al., 1997; represented in Figure 1). In this manner, the Phc regulatory system serves as a master control switch, which is able to turn on behaviours suited to free-living survival and via its negative interaction with *hrpB*, is able to turn off pathogenesis (reviewed in Hikichi et al., 2007; Figure 1.1).

PhcA is also known to positively regulate the production of a second quorum sensing molecule acylhomoserine lactone (Flavier et al., 1997). This secondary regulatory system is mediated by the SolI-SolR regulators, which are suggested to operate after the virulence factors have performed their function, activating genes towards the terminal stages of the disease. PhcA may thus play an important regulatory role in quorum sensing by decreasing virulence via negatively regulating *hrp* gene expression and positively regulating acylhomoserine lactone. Another quorum sensing system may be present in *R. solanacearum* exemplified by a pair of ORFs showing homology to SolI-SolR on the megaplasmid of GMI1000 (Genin and Boucher, 2002).

Phenotypic conversion

R. solanacearum is able to convert from a mucoid colony morphology to a non-mucoid morphology in a process called 'Phenotypic conversion', which is effected by spontaneous or induced mutations in *phcA*. Although this conversion results in reduced virulence of the pathogen, some PC-type mutants are able to revert to a virulent state in a susceptible host (Poussier et al., 2003). One possible mechanism of phenotypic conversion was demonstrated by Poussier et al. (2003) who showed that an inversion caused by a 64bp perfect tandem repeat in *phcA* was reversed *in vitro* in the presence of tomato root exudates. By entering this dormant-like 'viable but not culturable' state via mutational conversion, *R. solanacearum* is able to adapt to a saprophytic lifestyle and is able to survive for long periods in the soil (Denny et al., 1994).

Hormone synthesis

The biosynthesis of "plant-like" hormones such as ethylene gas, auxin, and the cytokinin trans-zeatin occurs in *R. solanacearum* (Freebain and Buddenhagen, 1964; Phelps et al., 1968; Akiyoshi et al., 1987). Genes potentially involved in auxin and trans-zeatin synthesis exist in GMI1000 and genes encoding ethylene forming enzyme and a 1-aminocyclopropane-1-carboxylate deaminase that is involved in ethylene degradation, were identified on the megaplasmid. These signalling molecules are likely to play a role in disease development (Genin and Boucher, 2002).

The production of plant-like hormomes may be a virulence strategy by the pathogen to manipulate host defences. For example, several strains of the bacterial pathogen *Pseudomonas*

syringae produce coronatine, a bacterial toxin which is most similar to jasmonate-isoleucine which is the active form of Methyl jasmonate (MeJA), the endogenous plant hormone involved in defense signaling (Bender et al., 1999; Staswick and Tiryaki, 2004). A mutant of *P. syringae* pv. *tomato* (*Pst*) unable to produce coronatine was less virulent on Arabidopsis and this reduction in virulence was associated with high activation of host defence response genes (Mittal and Davis, 1995). Arabidopsis *coi1* mutants insensitive to coronatine also show an enhanced resistant phenotype to *P. syringae* associated with an increase in expression of *PR-1* and SA levels (Kloek et al., 2001). Together, these studies provided evidence that coronatine is involved in inhibiting host defences in order to colonise the plant tissue. It is suggested that this occurs in a COI1-dependent manner to interfere with SA signaling which is required for defence against the pathogen (Kloek et al., 2001). A more recent role for coronatine in defence is described by Melotto et al. (2006) who showed that coronatine was able to interfere with PAMP-induced stomatal closure and inhibited ABA-induced stomatal closure suggesting that coronatine suppresses stomatal defenses allowing the pathogen entry into the host via the stomata.

Molecular experiments have provided interesting insights into the virulence mechanisms employed by *R. solanacearum*. For example, genes expressed during its pathogenic lifestyle, its TTSS and regulation thereof, quorum sensing, and hormone synthesis. However, many more questions remain. It is hoped that further molecular evidence will provide answers to questions such as: 1) what determines host-specificity in *R. solanacearum*, 2) how do plant-like hormones contribute to bacterial virulence in the host, 3) what are the cues involved in quorum sensing? This information will be valuable in manipulating the pathogen to reduce its virulence against important crop plants.

1.3. *Arabidopsis thaliana*

Arabdiopsis thaliana (L.) Heynh, commonly referred to as thale cress or mouse-eared cress, is a small plant in the mustard family (Anderson et al., 2000). This plant has become widely established as the model plant system owing to its quick regeneration time (approximately 6 weeks from seed to seed), the ability to produce thousands of seed, its ability to be transformed by *Agrobacterium tumefaciens* and in particular its relatively small genome size (The Arabidopsis Genome Iniative, 2000). The entire genome sequence of the plant was completed in 2000 (approximately 118 998Mbp in size) and since then 32,041 genes have been annotated by The Arabidopsis Genome Initiative (TIGR). The annotated set of proteins

have been classified using a set of controlled vocabularies termed Gene Ontologies (GO). GO provide classifications for proteins under the following categories: molecular function, biological process and cellular component. Figure 1.2 indicates the percentage of annotated Arabidopsis genes for each GO category represented as pie charts. The pie charts indicate that there are a large proportion of genes with GO classifications that are not defined. The challenge for the Arabidopsis community is to uncover the biochemical, molecular and biological roles of these genes.

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A

Functional Categorization by annotation for : GO Cellular Component

- unknown cellular components: 29.571% (raw value = 9193)
- other membranes: 22.655% (raw value = 7043)
- other intracellular components: 10.728% (raw value = 3335)
- chloroplast: 7.984% (raw value = 2482)
- nucleus: 7.964% (raw value = 2476)
- other cytoplasmic components: 7.08% (raw value = 2201)
- mitochondria: 3.718% (raw value = 1156)
- plastid: 2.705% (raw value = 841)
- ribosome: 1.686% (raw value = 524)
- plasma membrane: 1.216% (raw value = 378)
- cytosol: 1.081% (raw value = 336)
- ER: 0.994% (raw value = 309)
- cell wall: 0.785% (raw value = 244)
- extracellular: 0.737% (raw value = 229)
- Golgi apparatus: 0.692% (raw value = 215)
- other cellular components: 0.405% (raw value = 126)

B

Functional Categorization by annotation for : GO Biological Process

- other metabolic processes: 21.004% (raw value = 10602)
- other cellular processes: 20.534% (raw value = 10365)
- unknown biological processes: 20.34% (raw value = 10267)
- protein metabolism: 8.356% (raw value = 4218)
- cell organization and biogenesis: 4.016% (raw value = 2027)
- transport: 3.99% (raw value = 2014)
- developmental processes: 3.798% (raw value = 1917)
- other biological processes: 3.621% (raw value = 1828)
- response to abiotic or biotic stimulus: 3.564% (raw value = 1799)
- transcription: 3.53% (raw value = 1782)
- response to stress: 2.571% (raw value = 1298)
- signal transduction: 1.967% (raw value = 993)
- electron transport or energy pathways: 1.698% (raw value = 857)
- DNA or RNA metabolism: 1.01% (raw value = 510)

C

Functional Categorization by annotation for : GO Molecular Function

- unknown molecular functions: 28.065% (raw value = 9501)
- other binding: 10.98% (raw value = 3717)
- hydrolase activity: 8.566% (raw value = 2900)
- other enzyme activity: 8.554% (raw value = 2896)
- transferase activity: 7.21% (raw value = 2441)
- protein binding: 6.091% (raw value = 2062)
- DNA or RNA binding: 5.429% (raw value = 1838)
- kinase activity: 5.03% (raw value = 1703)
- transcription factor activity: 4.986% (raw value = 1688)
- transporter activity: 4.939% (raw value = 1672)
- nucleotide binding: 3.748% (raw value = 1269)
- other molecular functions: 2.469% (raw value = 836)
- nucleic acid binding: 1.684% (raw value = 570)
- structural molecule activity: 1.533% (raw value = 519)
- receptor binding or activity: 0.715% (raw value = 242)

Figure 1.2. The functional categorisation of all annotated Arabidopsis genes under gene ontologies for A) Biological Process, B) Cellular Component and C) Molecular Function. The categories were derived from TAIR (www.arabidopsis.org) version 7 which contains 32 041 genes.

Several ecotypes of Arabidopsis have been collected from various geographic locations around the world. Approximately 142 ecotypes exist in the native population that are available for research (Mitchell-Olds, 2001). This natural genetic variation has been exploited for molecular studies to identify genes associated with traits of interest. An example of this is the genetic variation in the ecotypes Landsberg (Ler) and Columbia (Col-0), which showed late and early flowering times respectively (Lee et al., 1993). The late-flowering trait segregated as a single dominant gene in genetic crosses of these ecotypes and the *FLA* gene was identified on Arabidopsis chromosome 4 (Lee et al., 1993). The recent work by Clark et al. (2007) further exemplifies the genetic diversity of Arabidopsis ecotypes. The genomes of twenty diverse *Arabidopsis thaliana* ecotypes (Bay-0, Bor-4, Br-0, Bur-0, C24, Cvi-0, Est-1, Fei-0, Got-7, Ler-1, Lov-5, Nfa-8, Rrs-7, Rrs-10, Sha, Tamm-2, Ts-1, Tsu-1, Van-0) were examined and compared to the reference ecotype Col-0. A large number of non-redundant single nucleotide polymorphisms were identified (>1 million) and approximately 4 percent of each ecotypes' genome was different to the reference genome. Exceptionally high polymorphism levels between ecotypes were noted in genes mediating interaction with the biotic environment.

Arabidopsis exhibits all of the major kinds of defence responses described in other plants and a large number of virulent and avirulent bacterial, fungal and viral pathosystems have been established. Various mutants defective in almost every aspect of plant growth and development have been identified and most of our understanding of the plant defence response comes from studies on Arabidopsis mutants and interactions with various pathogens (Glazebrook et al., 1997). These mutants are discussed briefly in the proceeding section on plant defence responses. Together, these attributes make Arabidopsis an attractive model plant for molecular experiments pertaining to plant defences.

1.4. Plant Defence Response

Apart from abiotic stresses, plants encounter various biotic stresses daily and have both preformed and inducible defence systems to protect themselves from such onslaughts (reviewed in Thatcher et al., 2005; Ingle et al., 2006; Jones and Dangl, 2006).

Preformed defences include the dense epidermal layers and waxy cuticle of leaves and the presence of hairs and trichomes on surfaces providing plants with protection against insect feeders. The pectin and lignin component of plant cell walls also provide a barrier against

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pathogen invasion (Agrios, 1997). Preformed chemical defences such as antimicrobial peptides and toxic secondary compounds can be released upon insect or pathogen attack (Zhao et al., 2005). Such a chemical defence mechanism is the glucosinolate-myrosinase system in the *Brassicaceae* species (Halkier and Gershenson, 2006). Glucosinolates and myrosinase are stored in separate compartments in plant cells and myrosinase cleaves non-toxic glucosinolates upon wounding and pathogen attack (i.e. insects). This results in the production of isothiocyanates, which are harmful to a wide range of plant pathogens. Such preformed defences, which provide general resistance of an entire plant species to all strains of a particular pathogen, is a form of non-host resistance (Heath, 2000).

A pathogen that overcomes preformed defences encounters inducible defence responses. Induced responses result from the plant's ability to recognise non-self. This ability to recognise non-self is likened to innate immunity in animals (Jones and Dangl, 2006). Plants lack an adaptive immune system involving somatic recombination of genes, and have no circulating immune cells, thus, they rely on the innate defences of each cell to respond to microbial attack. Plant innate immunity can be divided into two branches: the basal defence system and gene-for-gene mediated defences.

1.4.1 Basal Defence

Successful basal defences provide resistance against heterologous pathogens and may be described as an inducible form of non-host resistance. Pathogen recognition is brought about by general elicitors called pathogen associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs) located either at the cell surface or inside the cell (Dardick and Ronald, 2006). These PAMPs are usually molecules that are essential for the pathogen's lifecycle. PAMPs include bacterial lipopolysaccharides (LPS), flagellin, cold-shock protein , elongation factor Tu, and fungal glucan, chitin and ergosterol, which trigger basal defence responses independently of the genotype of the particular pathogen (reviewed in Nurnberger and Lipka, 2005).

Flagellin, the protein subunit of the bacterial surface structure flagellum, is one of the most well studied bacterial PAMPs and induces a defence response in both animals and plants (Felix et al., 1999). A highly conserved stretch of 22 amino acids from the N-terminus of flagellin, flg22, is able to induce the defence response to a higher level than flagellin itself; suggesting that plants have evolved PRRs that recognize short highly conserved amino acid

sequences on microbial proteins (Felix et al., 1999). It is also possible that microbes are able to avoid detection by specific PRRs; although the *R. solanacearum* pathogen possesses functional flagellin, it is not responsible for the activation of a defence response in Arabidopsis (Pfund et al., 2004).

To date, few receptors for PAMPs have been identified in plants. The best characterised is the flagellin receptor FLS2 (flagellin sensitive 2). FLS2, a 120 kDa receptor-like kinase (RLK), was identified by screening Arabidopsis mutants, which did not respond to flg22 (Gomez-Gomez and Boller, 2002). FLS2 contains a predicted signal peptide, an extracellular LRR domain, a transmembrane domain, and an intracellular Ser/Thr protein kinase domain, typical of a receptor kinase. FLS2 was recently shown to bind to flg22 via interaction with the extracellular LRR domain of the FLS2 receptor by chemical cross-linking and immunoprecipitation (Chinchilla et al., 2006). This leads to the activation of a MAPK signaling cascade resulting in defence gene activation (Asai et al., 2002).

Successful basal defence, resulting in signaling events that are able to overcome the pathogen is collectively known as PAMP-triggered immunity or PTI (Jones and Dangl, 2006). However, some pathogens are able to suppress basal defences by delivering specific effector proteins to the plant cells suppressing plant defence. This is known as effector triggered susceptibility (Jones and Dangl, 2006). Evidence for this comes from recent expression profile studies which show that PAMPs from *E. coli* and TTSS-deficient *P. syringae* mutants induce genes in Arabidopsis which are either repressed or not induced by virulent *P. syringae* (Thilmony et al., 2006). Truman et al. (2006) also showed 888 genes modulated by effectors in Arabidopsis. These effectors are capable of suppressing extracellular receptors (e.g. FLS2) and attenuate kinase signalling (Thilmony et al., 2006). Effectors were also largely responsible for the suppression of PAMP-induced cell wall modifications, such as the phenylpropanoid pathway required for lignin deposition, which would be required to restrict bacterial growth (Truman et al., 2006). He et al. (2006) demonstrated the specific suppression of PAMP-induced responses by the effectors AvrPto and AvrPtoB from *P. syringae* in Arabidopsis protoplasts. This suppression occurs upstream of the MAPK signalling cascade at the plasma membrane. AvrRpt2 or AvrRpm1, effectors with known virulence effects, did not suppress early PAMP-specific gene activation or MAPK signalling, suggesting that effector proteins may block the PAMP-induced defence response in different ways (He et al., 2006).

1.4.2 Gene-for-gene defence

Gene-for-gene resistance (also known as cultivar-specific resistance) occurs when specific members of a plant species have acquired resistance to a particular race of a pathogen (Hammond-Kosack and Parker, 2003).

Flor (1971) proposed the gene-for-gene model, which states that for every gene of resistance in the host plant, there was a corresponding gene for avirulence in the pathogen and for every gene of virulence in the pathogen; there is a gene for susceptibility in the host plant. This resistance is suggested to be controlled by a receptor-ligand model implying that effector proteins act as ligands to bind and activate a matching *R* gene-encoded receptor (Hammond-Kosack and Parker, 2003). An example of a direct interaction between *R* and *Avr* gene products comes from the work of Jia et al. (2000) who showed, using the yeast-two hybrid system, the physical interaction between the rice Pi-ta protein and Avr-Pita from the rice blast fungus, *Magnaporthe grisea,* at the site of the leucine rich domain on the R protein. This direct interaction was further confirmed using *in vitro* binding experiments involving bacterially produced recombinant proteins. A single amino acid substitution in the Pi-ta leucine rich domain or in Avr-Pita resulted in the loss of resistance and the interaction observed between the two proteins in the yeast-two hybrid study and the *in vitro* assay was disrupted. Experimental data often does not support the direct interaction of *R* and *Avr* genes thus the guard hypothesis was proposed as an alternative (Dangl and Jones, 2001). This hypothesis proposes that the R protein interacts directly with another plant protein (the guardee) and not the pathogen effector directly. Any attempt by the pathogen to modify the guardee activates the R protein, triggering resistance (Dangl and Jones, 2001). Evidence of a guarded protein was obtained from investigations into Arabidopsis RIN4, a regulator of PAMP signaling (Mackey et al., 2002). Two *P. syringae* effector proteins, AvrRpm1 and AvrRpt2, manipulate RIN4, interfering with the activation of basal defences. Perturbations in RIN4 are sensed by the R proteins RPM1 and RPS2, resulting in the activation of defense responses (Mackey et al., 2002; Kim et al., 2005).

R genes, although functionally diverse, share some structural similarity and have been divided into six classes depending on their predicted protein structure and function (Hammond-Kosack et al., 2000). Many R proteins contain a series of LRRs, a nucleotide-binding site (NBS), and an amino-terminal TIR (Toll and Interleukin-1 receptor) or CC (coiled-coil) structure (Feys and Parker, 2000; Ellis et al., 2000; Holt et al., 2003). Only CC-NBS-LRR

genes have been identified in monocotyledonous plants, while both CC-NBS-LRR and TIR-NBS-LRR genes have been identified in dicotyledonous plants (Dangl and Jones, 2001). For example, RPP5 and RPS4 belonging to the TIR-NBS-LRR class of R proteins confer resistance to the oomycete *H. parasitica* and bacterium *P. syringae*, respectively, in Arabidopsis (Gassmann et al., 1999; Noel et al., 1999). The CC-NBS-LRR-type R proteins RPM1 and RPS2, afford resistance to different *P. syringae* strains expressing the corresponding effector genes (Holub, 2001). Different *R* genes may utilise different signalling components. Experiments on Arabidopsis mutants *ndr1* (nonrace-specific disease resistance 1) and *eds1* (enhanced disease susceptibility 1) revealed two possible disease resistant pathways required by *R* genes (Aarts et al., 1998). The *R* genes *RPP2, RPP4, RPP5, RPP21* require EDS1 to confer resistance to *H. parasitica* carrying the corresponding *Avr* genes and similarly *RPS4* requires EDS1 to confer resistance to *P. syringae* carrying *AvrRps4* with little or no requirement for NDR1, while the *R* genes *RPS2*, *RPM1*, and *RPS5*, operate independently of EDS1 and are NDR1-dependent. *RPP8*, which like *RPS5* has a LZ-NBS-LRR motif, has no requirement for either NDR1 or EDS1 suggesting that another signaling pathway may be required by this R-gene to confer resistance and that the structural motifs can not be used as markers for NDR1 or EDS1 dependency (Aarts et al., 1998).

NBS-LRR proteins are effective in mediating resistance to biotrophs (pathogens that require live host tissue to grow) but not against necrotrophs (pathogens that kill host tissue during colonisation) (Glazebrook, 2005). Jones and Dangl (2006) describe the responses following recognition of a specific pathogen effector by the NBS-LRR protein, as effector-triggered immunity (ETI). ETI produces an amplified defence response (in comparison to PTI) inducing the hypersensitive response (HR), which is localised cell death at the point of infection to restrict pathogen spread (Greenberg, 1997). A pathogen can evolve to gain new effectors to suppress ETI and in turn, the plant can acquire a new NBS-LRR protein, which can recognise the new effector, to induce ETI again (Jones and Dangl, 2006).

It should be noted that not all *R* genes contain the NBS-LRR domain; rice *Xa21* and *Xa26* encode a protein comprised of an amino terminal extracellular LRR joined by a transmembrane domain to a cytoplasmic C-terminal serine/threonine kinase domain (Song et al., 1995; Sun et al., 2004), while the barley *Rpg1* gene encodes an intracellular protein kinase with two tandem kinase domains (Horvath et al., 2003). Another example of a resistance gene with a distinct protein structure is the recessive barley *mlo* resistance gene. This gene confers

resistance against all known isolates of the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) in barley. The *Mlo* gene encodes a novel 533 amino acid protein predicted to form seven transmembrane helical bundles and is thought to be a G protein coupled receptor. MLO is thought to be either an endogenous plant defence modulator or a target by the fungal pathogen for suppression of host defence pathways (Elliott et al., 2005).

This paragraph defines the terms that will be used in the following sections on plant defence. The interaction between R and avr gene products resulting in no disease is referred to as an incompatible interaction and the pathogen is described as avirulent whereas a plant-pathogen interaction that results in susceptibility that is either effector-triggered or due to unsuccessful basal defence responses (e.g. unsuccessful PTI) is termed a compatible interaction and the pathogen is said to be virulent (Dangl and Jones, 2001; Jones and Dangl, 2006).

1.4.3 Systemic defences

The earlier resistance responses discussed are local responses against pathogens. Broader resistance responses can be induced via perception of a systemic signal originating from the point of infection, e.g. Systemic Acquired Resistance (SAR). SAR is produced as a result of a pathogen-triggered localised cell death (e.g. the HR) which in turn results in a systemic signal being transmitted to various parts of the plant protecting it from further pathogen attack (Uknes et al., 1993). SAR is known to provide long-lasting (a few weeks to a few months) resistance against various viral, bacterial, fungal and oomycete pathogens, which are usually virulent (Thomma et al., 2001; Durrant and Dong, 2004).

A second type of systemic induced response is Induced Systemic Resistance (ISR), which is mediated by certain rhizobacteria (Pieterse et al., 1998). ISR has been demonstrated against fungal, bacterial, and viral pathogens in various plants including *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato (reviewed in van Loon et al., 1998). The bacterial determinants required for the induction of ISR include lipopolysaccharide, siderophores and the production of SA by the rhizobacteria. Col-0 plants grown in soil containing the ISR-inducing rhizobacterium *P. fluorescens*, and subsequently treated with SA or exposed to avirulent *Pst,* showed increased resistance to virulent *Pst* (van Wees et al., 2000). Similarly, growth of the *cpr1* (constitutive expressor of PR-1) mutant, which constitutively expresses SAR, in soil containing *P. fluorescens* improved resistance to virulent *Pst*. These results

indicate that the simultaneous activation of ISR and SAR results in an additive resistance effect.

1.4.4 Defence signalling events

General elicitors (PAMPs) and specific effectors (avr gene products) elicit overlapping signal responses in the plant when an R protein is present (Kim et al., 2005) however, the induction of defence genes is more rapid and enhanced in response to specific effectors (Tao et al., 2003). de Torres et al. (2003) showed that within the first 2 hrs of infection, virulent and avirulent pathogens induce similar host transcriptional changes. Upon pathogen recognition, phosphorylation and dephosphorylation events take place, increase of cytosolic Ca^{2+} concentration, other ion fluxes and alkanisation of the apoplast occurs (reviewed in Thatcher et al., 2005). Callose in the form of papillae is deposited at the site of pathogen invasion. Mitogen-activated protein kinases (MAPK) and NADPH oxidase are activated and reactive oxygen species (ROS) are produced (Zhao et al., 2005). Early expression of defence genes may occur and the kinase cascades and ROS amplify the defence signal and downstream reactions are activated which involve the signalling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene. The signal is transmitted to adjacent cells as well as intracellularly, resulting in the production of phytoalexins, toxic antimicrobial substances. Defence related proteins such as pathogenesis-related (PR) proteins, which have antimicrobial activity serve to contain the infection (reviewed in Thatcher et al., 2005). Figure 1.3 illustrates these defence signalling events.

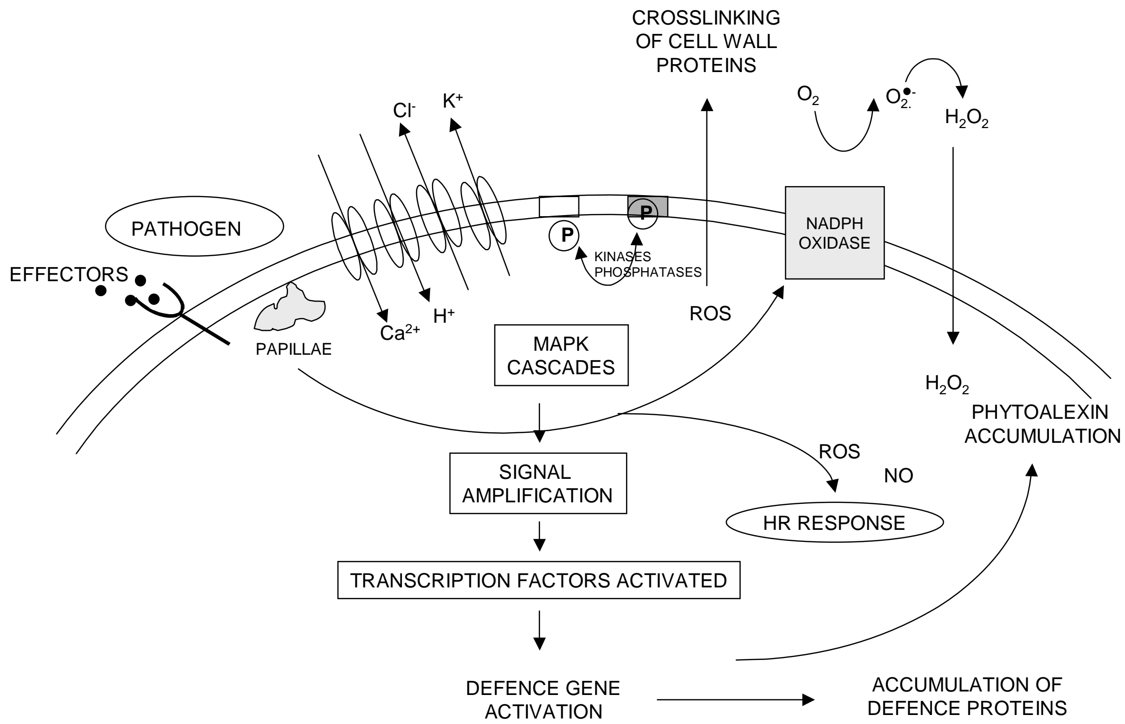

Figure 1.3. A simple model of plant responses induced by specific effectors or non-specifc pathogen-derived elicitors (adapted from Buchanan et al., 2002; Thatcher et al., 2005).

The signaling events described further are discussed in the order in which they appear on the diagram (figure 1.3).

Calcium signalling

A calcium spike characterised by a rapid elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]$ and a rapid return to basal levels often occurs in response to a variety of stimuli including pathogen attack (Yang and Poovaiah, 2003). Calcium binding proteins such as calmodulin and Calcium Dependent Protein Kinases (CDPKs) are responsible for decoding these patterns of Ca^{2+} signals. The characteristic structure of CDPKs is an N terminal serine/threonine protein kinase domain fused to a carboxyl terminal calmodulin-like domain containing four EF hand calcium binding sites (Harmon et al., 2000). Under non-stress conditions, CDPK remains in a state of low activity due to a junction domain between the kinase and calmodulin like domain that inhibits phosphorylation in the absence of Ca^{2+} (Harmon et al., 2000). CDPK was shown to be transcriptionally activated in response to wounding in tobacco (Yoon et al., 1999), in response to fungal elicitors in maize (Murillo et al., 2001) and in response to treatment with *Cladosporium fulvum* Avr9 peptide in transgenic tobacco plants expressing Cf-9 resistance (Romeis et al., 2001).

Elicitors of plant defence (cryptogein and oligogalacturonides) induce changes in cytosolic free Ca^{2+} concentrations (Lecourieux et al., 2002). Lecourieux et al. (2002) showed that the increase in cytosolic free calcium in plant cell suspension cultures was mediated by cryptogein-receptor interaction and this long-sustained increase was thought to be responsible for sustained mitogen activated protein kinase activation. The increase in cytosolic free Ca^{2+} originates from a calcium influx, which in turn leads to calcium release from internal stores and additional Ca^{2+} influx. H_20_2 also brings about cytosolic Ca^{2+} increases and is thought to activate calcium channels in the plasma membrane.

Different calcium responses have been reported during virulent and avirulent infection in Arabidopsis (de Torres et al., 2003). Levels of cytosolic $[Ca^{2+}]$ in the incompatible interaction (*Pst* DC3000 avrRpm1 and RPM1) interaction began to rise 1 hr after infiltration, reached a maximum 2 hrs post infection and began to decrease over the next two hours, whereas in a compatible interaction (Pst DC3000 and RPM1), cytosolic $[Ca^{2+}]$ levels remained low during this period (de Torres et al., 2003). The rise in cytosolic $[Ca^{2+}]$ an hour after infiltration with *Pst* DC3000 (*avrRpm1*) corresponded with an induction of *avrRpm1 in planta*. This suggests

that the delivery of specific effectors is necessary for the induction of high levels of cytosolic calcium (de Torres et al., 2003).

Reactive Oxygen Species

During non-stress conditions, the formation and scavenging of ROS in the cell are in balance. However during several forms of abiotic and biotic stress, the production of ROS increases. These include the superoxide anion $O_2^{\cdot-}$, hydroxyl radical ($OH\cdot$) and H_2O_2. Such increases could potentially result in cellular damage, inactivation of enzymes or cell death if the amount of ROS generated exceeds the capacity of the scavenging enzymes (Foyer et al., 1994). ROS is produced by plasma membrane-bound NADPH oxidases and cell wall-bound peroxidases and amine oxidases in the apoplast during defence responses (Mahalingam and Fedoroff, 2003; Laloi et al., 2004). The oxidative burst is one of the most immediate pathogen-induced defence responses and is characterized by a rapid and transient production of large amounts of ROS at the site of attempted pathogen invasion (Wojtaszek, 1997). It is thought that a NADPH oxidase homologous to that of activated mammalian phagocytes and neutrophils is responsible for the generation of apoplastic O^{2-} at the site of attempted pathogen invasion (Keller et al., 1998; Overmyer et al., 2003; Laloi et al., 2004). The NADPH oxidase encoding genes *AtRBOHD* and *AtRBOHF* in Arabidopsis are required for full ROS generation during bacterial and fungal attack (Torres et al., 2002). After pathogen attack, the accumulation of extracellular hydrogen peroxide is proposed to crosslink the cell wall proteins, strengthening the wall (Neill et al., 2001). Peroxidases have been suggested to contribute to the oxidative burst (Wojtaszek, 1997).

Not only is the oxidative burst directly harmful to invading pathogens but it also contributes to cell death as ROS generated via the oxidative burst play a central role in the development of the HR (Lamb and Dixon, 1997; Grant and Loake, 2002). ROS is also potentially a signal for plant defence responses and has the ability to diffuse across membranes and reach locations far from the site of its original generation (Wojtaszek, 1997). It is also evident that increased ROS generation enhances the accumulation of SA and *PR* gene transcripts (Chen et al., 1995; Maleck and Dietrich, 1999). Excess light also induces an increase in ROS generation and mechanisms for plant defence against pathogens were linked to the light-sensing network (Karpinski et al., 2003). Genoud et al. (2002) demonstrated that phytochrome signaling controlled by PHYA and PHYB photoreceptors modulated induction of *PR-1* by SA

and its functional analogs. In addition, the growth of avirulent *Pst* was enhanced in Arabidopsis *phyA* and *phyB* mutants.

Nitric Oxide

Nitric oxide was initially identified as an important messenger in animal cells and the NO burst is a hallmark of the innate defence response (Mayer and Hemmes, 1997). In plants NO is involved in developmental regulation and promotion of germination and importantly is a mediator in plant defence signaling (Wendehenne et al., 2004; Delledonne, 2005). Zeidler et al. (2004) reported a rapid burst of NO in Arabidopsis cells in recognition of bacterial LPS. LPS from animal and plant pathogens were shown to induce NO synthase *AtNOS1* as well as activate several defence genes (Zeidler et al., 2004). Zeidler et al. (2004) also demonstrated the essential role of NO as *AtNOS1* mutants were more susceptible to virulent *Pst* than wild-type plants demonstrating the role of NO in basal defence. NO is also considered an important intercellular signal activating the HR as it is also implicated in triggering cell death together with ROS (Romero-Puertas et al., 2004; Tada et al., 2004; Zeidler et al., 2004). In addition, NO is induced concurrently with the HR and is proposed to facilitate the cell-to-cell spread of the HR (Zhang et al., 2003).

By analogy to mammalian systems, NO signaling in plants is thought to occur in the following way: NO binds to soluble guanylate cyclase activating the enzyme and increasing the level of cyclic GMP (cGMP). cGMP is able to stimulate synthesis of cyclic ADP-ribose (cADPR), a second messenger that stimulates Ca^{2+} release through intracellular Ca^{2+} permeable ryanodine receptor channels. Both messengers cGMP and cADPR have been shown to induce the levels of a number of defence related proteins including pathogenesis related protein 1 (PR-1) and phenylalanine ammonia lyase (PAL). Simultaneous addition of cGMP and cADR amplified the levels of PR-1 and PAL in tobacco indicating that these two messengers may act synergistically to increase defence gene expression (Durner et al., 1998). A soluble guanylate cyclase identified in Arabidopsis (AtGC1) lacks a NO domain (Ludidi and Gehring, 2003), thus the soluble guanylate cyclase required for NO signaling remains to be identified.

Mitogen Activated Kinases

Downstream of elicitor-receptor interactions, Mitogen Activated Kinase (MAPK) cascades are induced. This cascade involves a three-kinase relay: MAPKK kinase activates MAPK

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kinase, which in turn activates MAPK. MAPKs are activated by a variety of abiotic stresses including wounding, temperature, drought, and salinity but are also induced during plant responses to elicitors or pathogens (Romeis, 2001).

A complete plant MAPK cascade was recently described which functions downstream of the receptor kinase FLS2 receptor in Arabidopsis (Asai et al., 2002). Flg22 was shown to interact with FLS2 in Arabidopsis and in tomato plants expressing Arabidopsis *FLS2* (Chinchilla et al., 2006). It is thought that the interaction between flg22 and FLS2 occurs at the LRR domain of the receptor as has been demonstrated in mammalian systems (Mizel et al., 2003; Chinchilla et al., 2006) This interaction leads to the heterodimerisation or dimerisation of the receptor complex and activation of the FLS2 kinase domain. The FLS2 kinase is responsible for the phosphorylation and activation of the Arabidopsis MAPK kinase kinase 1 (AtMEKK1) which in turn phosphorylates Arabidopsis MAPK kinase 4 and 5. These kinases then phosphorylate and activate Arabidopsis MAPK 6 and 3, leading to the activation of the WRKY transcription factors WRKY22 and WRKY29 that activate the transcription of defence genes. Arabidopsis plants which constitutively expressed components of the flagellin responsive MAPK cascade showed enhanced resistance to the usually virulent bacterial and fungal pathogens *P. syringae* and *Botrytis cinerea* (Asai et al., 2002). Botrytis does not have flagellin; therefore these results suggest that signalling events initiated by diverse pathogens converge into a conserved MAPK cascade.

1.4.5 The role of phytohormone signalling in plant defence

Jasmonic Acid

The jasmonates, especially the phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are produced by the octadecanoid pathway from the major plant membrane lipid linolenic acid, and are known to regulate developmental processes such as embryogenesis, pollen and seed development, and root growth (Farmer et al., 2003; Liechti et al., 2006). JAs also mediate resistance to insects, microbial pathogens, and abiotic stress responses to wounding and ozone. A cyclopentenone precursor of JA, 12-oxo-phytodienoic acid (OPDA) is also able to induce defence gene expression (Farmer et al., 2003).

Arabidopsis mutants impaired in the perception of JA (e.g. *coi1*) exhibit enhanced susceptibility to a variety of necrotrophic pathogens, including the fungi *Alternaria brassicicola*, *B. cinerea*, and *Pythium* sp., and the bacterium *Erwinia carotovora* (Thomma et

al., 1998; Norman-Setterblad et al., 2000; Thomma et al., 2001). In some cases, such as the Arabidopsis *constitutive expression of vsp1 (cev*1) mutant, which exhibits constitutive JA signaling, JA plays a role in resistance against biotrophic pathogens: *E. cichoracearum* and *P. syringae* pv. *maculicola* possibly through suppression of SA responses (Ellis et al., 2002).

The metabolism of JA can occur via methylation to MeJA or conjugation to amino acids (Liechti et al., 2006). JAR1 (Jasmonic acid resistance 1) has been demonstrated to be a JA-amino acid synthetase conjugating JA to isoleucine (Staswick and Tiryaki, 2004). JA isoleucine has been described as the active form of JA and was able to complement the root growth inhibition seen in *jar1-1*, fully complementing the defect in the *jar1-1* mutant (Staswick and Tiryaki, 2004). Arabidopsis *jar1* plants are less sensitive to the exogenous application of JA and are susceptible to certain pathogens and unable to induce ISR (Staswick et al., 1998).

Although no receptor for JA has been characterized, it has been suggested that the receptor may be COI1 (coronatine insensitive 1), which plays a central role in JA signaling (Xie et al., 1998; Liechti et al., 2006). COI1 has been suggested as the JA receptor due to the analogy to the auxin system wherein TIR, the F-box component of the SCF^{TIR} complex, was found to be the receptor for auxin (Kepinski and Leyser, 2005). COI1 is the F-box component of the SCF^{COI1} complex, which was shown to target the repressors Jasmonate insensitive 3 (JAI3) and Jasmonate Zim-domain (JAZ) proteins for degradation upon jasmonate perception (Chini et al., 2007). These repressors are analogous to the auxin repressors identified previously. AtMYC2 interacts with JAI3 and JAZ proteins and it is suggested that JAI3 and JAZ are repressors of MYC2 (Chini et al., 2007). The production of JA leads to the production of defence related genes such as *plant defensin 1.2 (PDF1.*2), *hevein-like protein (HEL*), and *basic chitinase (CHI*B), which are induced cooperatively by JA and ET in Arabidopsis (Penninckx et al., 1998; Norman-Setterblad et al., 2000). In addition, the production of JA leads to the induction of *vegetative storage protein (VSP*), i.e. proteins that play important nutritional roles during plant development and *thionin 2.1 (THI2.*1). These genes are often used as markers for JA-dependent defence responses (Berger et al., 1995; Epple et al., 1995; Penninckx et al., 1998).

The MYC transcription factor AtMYC2 is involved in JA signaling. The Arabidopsis mutant jasmonate insensitive 1 (*jin1*) encodes AtMYC2, which is a nuclear-localised basic helix-

loop-helix-leucine zipper transcription factor (Lorenzo et al., 2004). The expression of this transcription factor is rapidly induced by JA in a COI-1 dependent manner. Mutations in AtMYC2 prevent the activation of *VSP*, which is required for defence against herbivores and wounding; however the expression of JA-induced genes involved in pathogen defence is enhanced. In this way, AtMYC2 mutant plants show enhanced resistance to the necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* (Lorenzo et al., 2004).

JA has been implicated in systemic signaling. JA, MeJA and the oligopeptide systemin (derived from pro-systemin), are considered central players in mediating the long-distance systemic wound signal (Ryan and Moura, 2002; Bostock, 2005; Schilmiller and Howe, 2005). The production of systemin is induced by wounding which in turn regulates the activation of over 20 defensive genes in response to herbivore and pathogen attack (Pearce et al., 1991; Ryan, 2000). The release of systemin from primary wound sites promote proteinase inhibitor gene expression and contributes to the long-distance defence response by activating and amplifying JA production in vascular tissues (Schilmiller and Howe, 2005). JA has also been recently described to play a role in the establishment of SAR (Truman et al., 2007) and ISR (Glazebrook et al., 1996). These systemic resistance responses are discussed in section 4.5.3. under salicylic acid.

Ethylene

Ethylene is produced during early responses to pathogen attack and leads to the induction of defence genes such as *PR-1*, basic *β-1,3-GLUCANASE*, and *CHIB* (Deikman, 1997; Thomma et al., 1998). Although ethylene is known to contribute to resistance in some interactions, it is also a promoter of disease development in others (Thomma et al., 1998; Hoffman et al., 1999; Thomma et al., 1999; Norman-Setterblad et al., 2000). For example, the Arabidopsis *ethylene-insensitive 2 (ein2)* plants displayed enhanced susceptibility to *B. cinerea* and *P. carotovora* (Thomma et al., 1999; Norman-Setterblad et al., 2000) while infection of *ein2* with virulent *P. syringae* and *Xanthomonas campestris* resulted in reduced disease symptoms (Bent et al., 1992). This is due to antagonism between the signaling pathways SA and ET/JA.

Some of the mutations affecting ET signal transduction have identified transcription factors such as the ERF1 protein, which belongs to a family of ET response element binding factor (ERF) proteins. These proteins are also referred to as ethylene response element binding proteins (EREBPs) and are transcription factors unique to plants (Fujimoto et al., 2000).

These EREBPs bind to the GCC box of promoters of *β-1,3-glucanase*, *CHIB*, and *PDF1.2,* known pathogenesis-related genes (Ohme-Takagi and Shinshi, 1995; Solano et al., 1998; Wang et al., 2002). Over expression of *ERF1* in Arabidopsis confers resistance to the necrotrophs *B. cinerea* and *P. cucumerina* but is ineffective at providing resistance against the biotroph *P. syringae* (Berrocal-Lobo et al., 2002). In contrast, over expression of a tomato *ERF* gene, *PTI5* in tomato provided enhanced resistance against the biotrophic pathogen *Pst* (He et al., 2001). This supports a diverse role for plant ERF transcription factors in plant defence.

Salicylic Acid

Salicylic Acid (SA) levels have been shown to increase in response to pathogen attack at the site of infection, and the exogenous application of SA protects plants against pathogens and induces the expression of defence-related genes (van Loon, 1997; Glazebrook, 2005). SA is also involved in the establishment of Systemic Acquired Resistance (SAR). Virulent pathogens do not usually trigger HR, however, they can induce SA signaling as part of the basal defence response by the plant in an attempt to contain their growth (Glazebrook et al., 1997). The PR proteins beta-1, 3-glucanases, thaumatin-like proteins, chitinases, and PR-1 are induced during SA accumulation and SAR and serve as molecular markers for the onset of the defence response (van Loon, 1997; Durrant and Dong, 2004).

The first studies highlighting the importance of SA in defence signaling employed transgenic Arabidopsis plants, which express the bacterial SA-degrading enzyme salicylate hydroxylase *(NahG*). This enzyme converts SA to inactive catechol and *NahG* plants display enhanced susceptibility to several fungal, bacterial, oomycete and viral pathogens (Gaffney et al., 1993; Delaney et al., 1994). It has been recently suggested that the observed disease susceptibility phenotype might partly arise from the SA degradation product catechol rather than the lack of SA itself (Heck et al., 2003). The accumulation of catechol might trigger increased production of hydrogen peroxide which may be toxic to the cell, masking the true phenotype of the lack of SA. Evidence for this was obtained from experiments wherein *NahG* plants, treated with catalase, showed increased resistance to *P. syringae* pv. *phaseolicol*a (van Wees and Glazebrook, 2003).

True SA mutants such as *sid2* (SA induction deficient) show high levels of susceptibility to both virulent and avirulent forms of *P. syringae* and *H. parasitica* compared to the wild-type

(Nawrath and Metraux, 1999). The *sid2* mutant is deficient in isochorismate synthase (ICS1) and shows a drastic reduction in the accumulation of SA. This phenotype suggested that most of the SA hormone is produced from isochorismate (Wildermuth et al., 2001). The alternative pathway responsible for SA accumulation is the shikimate-phenylalanine pathway.

The nature of the signal for the establishment of SAR was initially suggested to be SA itself (Shulaev et al., 1995) but this theory has been contested in light of evidence that the detachment of leaves from *P. syringa*e-infected plants before SA levels rose did not block SAR development (Rasmussen et al., 1991). Current evidence suggests that the transmission of the signal may be by a lipid based molecule either *AtDIR1,* which encodes a putative apoplastic lipid transfer protein (Maldonado et al., 2002) or Tobacco SA-BINDING PROTEIN 2 (NtSABP2), which when silenced resulted in diminished local and systemic resistance (Kumar and Klessig, 2003).

Recently, the role of jasmonates in systemic immunity has been described (Truman et al., 2007). JA, and not SA, rapidly accumulates in the phloem exudates of leaves, which have been challenged with avirulent *P. syringae,* implicating JA in the early initiating phase of SAR. The induction of JA biosynthetic genes and JA responsive genes such as: *VSP2*, *CORI1*, *CORI3* (coronatine induced) and *AtMYC2* in systemic leaves occurred within 4 hours of avirulent pathogen challenge while the JA marker genes associated with local pathogen responses *Thi2.1* and *PDF1.2* were not induced systemically. Foliar application of JA resulted in responses characteristic of SAR. These responses were not observed in mutants defective in JA responses. Together the data provides evidence that jasmonate signaling acts in tandem with SA to mediate SAR and that JA signaling mediates early long-distance information transfer (Truman et al., 2007). ISR, induced by non-pathogenic rhizobacteria, also requires JA as well as ET but is SA independent (Glazebrook et al., 1996). The requirement for ET was demonstrated by Pieterse et al. (1998) in studies showing that the ET response mutant *etr1 (ethylene-resistant 1)* failed to develop pathogen resistance in response to nonpathogenic rhizobacteria. ISR also requires NPR1, a protein also required for the establishment of SAR, suggesting that SA-mediated SAR works in parallel with JA/ET-mediated ISR or that NPR1 acts independently of SA.

SA treatment also induces the expression of WRKY proteins, which are a family of transcription factors unique to plants, that contain either one or two WRKY domains, a 60-

amino-acid region that contains the amino-acid sequence WRKYGQK and a zinc-finger-like-motif (reviewed in Eulgem et al., 2000; reviewed in Singh et al., 2002). Microarray expression profiling indicated 49 of the 72 Arabidopsis WRKY genes showed enhanced expression in response to SA treatment or infection by a bacterial pathogen (Dong et al., 2003). WRKY proteins bind to the W-box, a motif found in the promoters of several plant defence genes (Chen et al., 2002). The promoters of these *AtWRKY* genes are also rich in W-boxes suggesting WRKY factors may function in transcriptional cascades. WRKY proteins also regulate the expression of the regulatory genes NPR1 and receptor protein kinases (Robatzek and Somssich, 2002). As described earlier, Asai et al. (2002) showed that Arabidopsis AtWRKY22 and AtWRKY29 functioned down-stream of the flagellin receptor to contribute to conferring resistance against *P. syringae* and *B. cinerea*. Over expression of AtWRKY29 was sufficient to provide enhanced resistance against *P. syringae* and *B. cinerea* (Asai et al., 2002). In contrast, over expression of WRKY25 resulted in enhanced susceptibility to *P. syringae* compared to wild-type plants with reduced expression of PR-1 (Zheng et al., 2007). These results suggest that WRKY25 is a negative regulator of SA-mediated defence responses.

Abscisic Acid

The role of Abscisic Acid (ABA) is being recognised as important in biotic stress responses as increasing evidence suggests 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). The role of ABA is somewhat controversial however, as exogenous application of ABA prior to inoculation with the pathogen increases susceptibility of barley *(Hordeum* sp.), tomato, soybean *(Glycine ma*x), potato, and Arabidopsis (Edwards, 1983; Ward et al., 1989; Audenaert et al., 2002; Mohr and Cahill, 2003) and ABA deficiency results in improved plant resistance (Kettner and Dorffling, 1995; Audenaert et al., 2002; Mohr and Cahill, 2003; Anderson et al., 2004). The ABA biosynthetic mutant, *aba2-1* for example, showed enhanced resistance to the necrotrophic fungal pathogen *Fusarium oxysporum* (Anderson et al., 2004). In contrast, Adie et al. (2007) showed, through transcriptome analysis, that ABA up-regulated approximately a third of the genes induced by another necrotroph, *Pythium irregulare* in Arabidopsis. ABA-deficient mutants were more susceptible to *P. irregulare* and *A. brassicicola* than wild-type plants, suggesting a positive role for ABA in plant defence against these pathogens. Together, this work suggests that ABA is not a positive signal for plant defence against all necrotrophs. Pathogens are also capable of

producing ABA and are thought to enhance host susceptibility by manipulating host defences, e.g. *Botrytis* (Marumo et al., 1982). This suggests that the up-regulation of ABA responsive genes in the host may not necessarily be due to the plant. Microarray expression profiling of *Pst* infected Arabidopsis plants indicate that pathogen effectors target the ABA signaling pathway within the plant, leading to enhanced susceptibility. Disease was reduced in an ABA biosynthetic mutant and in Arabidopsis plants expressing the bacterial effector AvrPtoB (de Torres-Zabala et al., 2007). Thus, the biotrophic pathogen *Pst* is able to control the plant's ABA signaling pathway to cause disease.

Ton and Mauch-Mani (2005) proposed that ABA can enhance plant resistance towards pathogens via its positive effect on callose biosynthesis after pathogen recognition. Callose-deficient mutants (*pmr4*) showed enhanced susceptibility to *P. irregulare* infection compared to wild-type plants however, ABA-deficient mutants did not show a significant defect in callose production compared to wild-type plants in response to pathogen infection (Adie et al., 2007). This implies that the production of callose is not only regulated by ABA.

ABA seems to influence biotic stress responses by interfering with defence signaling regulated by SA, JA, and ET, but also through shared components of stress signaling (Mauch-Mani and Mauch, 2005). Recent evidence also implicates ABA signaling in effecting stomatal closure in response to bacterial PAMPs (Melotto et al., 2006). The bacterial toxin coronatine (COR) was able to inhibit the PAMP-induced ABA signaling in the guard cell, effecting stomatal opening. Stomata serve as sites of entry for pathogenic bacteria and thus, the closure of the stomata mediated in part via ABA signaling, supports a positive role for ABA signaling in plant defence (Melotto et al., 2006).

The production of plant-like hormones such as ABA (Marumo et al., 1982) and coronatine by pathogens complicates the study of plant defence, as it is difficult to dissect which defence signalling events are induced by plant hormones or by the pathogen. A future strategy to study ABA signalling, would be to use pathogens deficient in ABA production to address this.

Cross-talk

Crosstalk can be described as a network of signal interactions in which functional outcomes can be positive, negative, or neutral (Bostock, 2005). Most of the interaction between SA and JA appears to be mutually antagonistic. For example, expression of the JA/ET dependent gene

PDF1.2 was increased in *nahG* plants infected with *Alternaria brassicola* (Penninckx et al., 1996). The Arabidopsis mutants *eds4* and *pad4* that are impaired in SA accumulation, displayed increased PDF1.2 expression upon MeJA treatment (Gupta et al., 2000). In addition, the JA signalling mutant *mpk4* constitutively expresses SA mediated defences (Petersen et al., 2000).

The plant specific transcription factor WRKY70 appears to be a node of convergence between SA and JA signaling indicating that WRKY70 integrates defence signals (Li et al., 2004). Plants overexpressing WRKY70 showed decreased JA- but enhanced SA-dependent defence activation, resulting in improved resistance to *Pectobacterium carotovora* and *P. syringae* (Li et al., 2004). Experiments using the latter pathogen, revealed the respective up and down-regulation of JA and SA-specific clusters of genes in Arabidopsis following *Pst* DC3000 infection; further suggesting that these pathways act antagonistically during defence against this pathogen. In-depth microarray expression profiling experiments on *P. syringae* challenged Arabidopsis signaling mutants (*eds3, eds4, eds5, eds8, pad1, pad2, pad4, NahG, npr1, sid2, ein2, coi1*) and wild-type plants also revealed distinct clusters of JA/ET and SA genes suggesting antagonism between the JA/ET and SA pathways during plant defence against the pathogen (Glazebrook et al., 2003).

Positive interactions also exist between SA and JA/ET pathways as microarray analysis of Arabidopsis plants treated with various defence inducing treatments showed co-ordinated regulation of several genes by SA and JA (Schenk et al., 2000). Synergism between the SA and JA pathways was also revealed by a microarray study in sorghum, which showed that genes from the octadecanoic pathway, responsible for JA synthesis, were induced by SA as well as JA (Salzman et al., 2005). As discussed earlier, Truman et al. (2007) also provides evidence for a positive interaction between SA and JA in the establishment of SAR.

Crosstalk between JA and ethylene signaling is mostly positive. An example of this is the activation of ERF1, which is synergistically activated by ET and JA, and ERF1 integrates these signals for the activation of plant defences (Lorenzo et al., 2003). SA-dependent defence responses are considered effective mainly against biotrophic pathogens, such as the oomycete *H. parasitica*, the fungus *Erysiphe orontii*, and the bacterium *P. syringae* (Glazebrook, 2005). It is possible that plants have evolved a JA/ET signalling pathway in order to combat necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Thomma et al., 1998). Figure

1.4 illustrates the crosstalk between the SA, JA, ET and ABA signaling pathways and the production of the PR-proteins required for defence against pathogens. These examples demonstrate the ability of plants to fine-tune their defence responses to different pathogens via crosstalk.

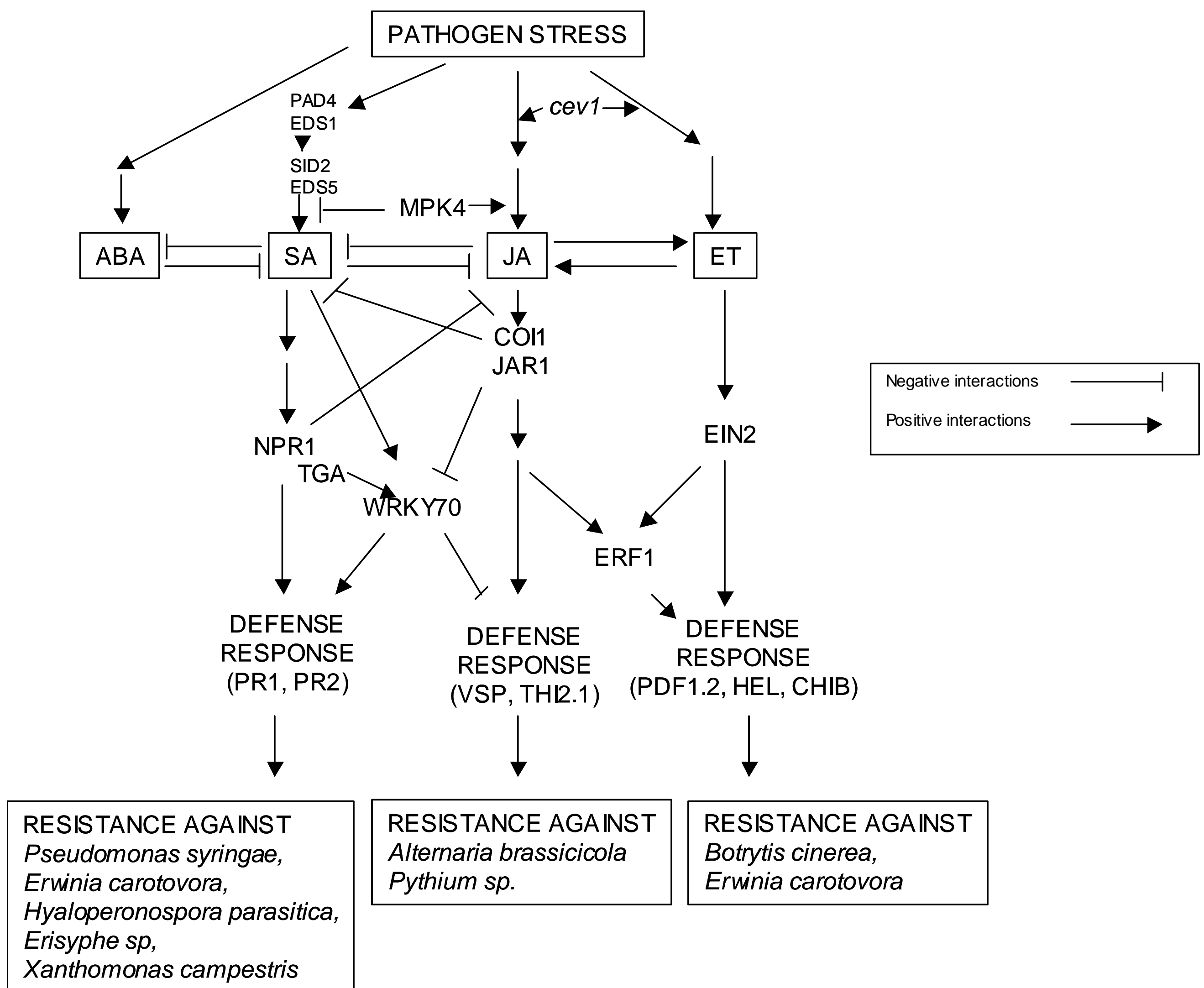

Figure 1.4. Signalling pathways mediated by ABA, SA, JA and ET in response to pathogen stress. Not all identified defence mutants are shown (Kunkel and Brooks, 2002; Durrant and Dong, 2004).

1.5. The Arabidopsis- Ralstonia Plant-Pathogen interaction

Several studies have investigated the interaction between strains of *R. solanacearum* and various ecotypes of Arabidopsis (Ho and Yang, 1999; Deslandes et al., 2003; Godiard et al., 2003). In Arabidopsis, multigenic (Godiard et al., 2003) and single-gene resistance (Deslandes et al., 2002) have been described against *R. solanacearum.*

R. solanacearum strain Ps95 induced a hypersensitive response, typically observed by other pathogens such as *Pst (avrB)* when infiltrated onto leaves of Arabidopsis ecotype S96 (Ho and Yang, 1999). The hypersensitive response was accompanied by the enhanced expression of the defence response genes *PR-1, GST1* and Cu/Zn superoxide dismutase. The induction of these genes was delayed in susceptible Arabidopsis ecotypes compared to resistant ecotypes. Genetic crosses between the resistant and the susceptible Arabidopsis ecotype N913 indicated that resistance to *R. solanacearum* Ps95 was due to a single dominant locus.

Godiard et al. (2003) showed that *R. solanacearum* tomato isolate 14.25 wilted Arabidopsis ecotype Landsberg erecta (L*er*) but did not cause wilt symptoms on Col-0. Genetic analysis revealed that resistance in Col-0 was governed by three quantitative trait loci: QRS1 (Quantitative Resistance to *Ralstonia solanacearum* 1), QRS2, and QRS3 on chromosomes 2 and 3. Polygenic resistance to *R. solanacearum* has also been described in tomato (Thouquet et al., 1996a, b). The ERECTA gene, which is a developmental regulator affecting the development of aerial organs, encodes for a leucine rich repeat receptor like kinase (LRR-RLK) and maps closely to QRS1 (Godiard et al., 2003). Transformation of susceptible L*er* plant with the wild-type ERECTA gene resulted in enhanced resistance to Ralstonia infection, showing that part of the resistance in Col-0 is controlled by ERECTA (Godiard et al., 2003). ERECTA may function in signal perception or transduction and the LRR domain is suggested to perceive signals from developmental cues as well as biotic stimulus such as *R. solanacearum* infection thus indicating a cross-talk between developmental signals and pathogen signals (Godiard et al., 2003).

Experiments, which paved the way towards the discovery of the first R-gene against *R. solanacearum,* were performed by Deslandes et al. (1998). Various Arabidopsis ecotypes were infected with GMI1000 and a GMI1000 *hrp*⁻ mutant. Results indicated that Col-5 was susceptible to the pathogen in a hrp-dependent manner while Nd-1 was resistant. Further work

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by Deslandes et al. (2002) identified two RRS1 (Resistance to *Ralstonia solanacearum* 1) alleles implicated in resistance (RRS1-R) and susceptibility (RRS1-S) in Arabidopsis ecotypes Nd-1 and Col-5 respectively. The RRS1-R gene conferred resistance to GMI1000. RRS1-R and RRS1-S contained the structural motif TIR-NBS-LRR that is characteristic of R gene motifs however, RRS1-S contains a stop codon resulting in the formation of a protein truncated by 90 amino acids.

The corresponding avr protein termed PopP2 encoded by *R. solanacearum* GMI1000 was described by Deslandes et al.(2003). PopP2 mutants of GMI1000 failed to produce an incompatible interaction with Nd-1 suggesting that the interaction between Arabidopsis RRS1-R and *R. solanacearum* PopP2 is necessary to confer resistance. It was further established that the two proteins directly interact with each other in a yeast two-hybrid screen, providing evidence for a direct interaction between R-Avr proteins in contrast to the guard model (Deslandes et al., 2003). Localisation experiments using PopP2::GFP and RRS1::GFP fusions in protoplasts indicated that the PopP2 effector is specifically targeted to the plant nucleus and that the nuclear localisation of the RRS1 proteins is dependent on the presence of PopP2.

Possible roles were proposed for the RRS protein structure in conferring resistance: the NH_2 terminus may bind to a pathogen-derived signal e.g. PopP2 at the LRR motif, which is known to facilitate protein-protein binding. This recognition event could then lead to the activation of the WRKY transcription factor domain at its C-terminal end activating particular defence genes in response to the pathogen. RRS1-R is identical to SLH1 (sensitivity to low humidity 1) described by Noutoshi et al. (2005) in the Arabidopsis ecotype No-0. SLH1 also functions as an *R* gene against *R. solanacearum* GMI1000. A 3bp insertion in the WRKY domain of the *slh1* added a single amino acid to the WRKY domain reducing its DNA binding ability. *Slh1* is a "gain of function" mutant that showed constitutive defence gene activation compared to wildtype plants. These results suggest that the WRKY domain of SLH1 (RRS1-R) is a negative regulator of defence. A model proposed by Noutoshi et al. (2005) suggests that SLH1 is a transcriptional repressor of plant defence genes (Figure 1.5, a). The WRKY domain is thought to bind to the W-boxes of the promoters of plant defence genes and repress their expression. During pathogen attack, the Avr protein binds to the WRKY domain causing dissociation of SLH1 from the promoters, resulting in defence gene activation. In *slh1*, the perturbation in the WRKY domain results in permanent dissociation from these promoters

leading to resistance. In theory, based on this model, it would be expected that knocking-out SLH1 would result in defence gene activation however, this was not the case. The authors suggest that the TIR-NB-LRR portion of SLH1 may be necessary for the activation of defence genes. A second model that was proposed was that the WRKY domain of SLH1 acts as a "guardee" in a typical guard model (Figure 1.5 b). It is thought that the TIR–NB–LRR portion of the protein may interact via intramolecular associations with the WRKY domain. The Avr protein PopP2 may target the WRKY domain, either modifying it or causing its disassociation from the TIR–NB–LRR domain. Such modification is perceived by the R-gene SLH1 or RRS1-R, leading to the activation of down-stream defences (Figure 1.5 b).

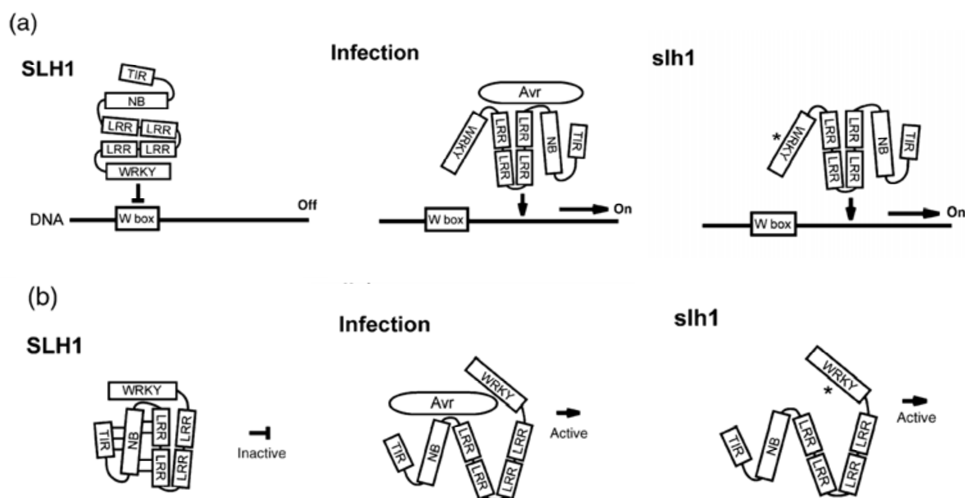

Figure 1.5. Two models proposed by Noutoshi et al. (2005) describing the interaction between SLH1 (RRS1-R) and the Avr protein (PopP2) in producing resistance against *R. solanacearum*. Model a) shows SLH1 as a transcriptional repressor in wild-type plants, negatively interacting with the W-box. Upon pathogen attack, the avr gene alleviates this repression resulting in transcriptional activation of SLH1 and subsequent resistance. In slh1, WRKY DNA binding activity is impaired (prevents binding to the W-box) leading to transcriptional activation. Model b) suggests that the WRKY domain of SLH1 functions as a "guardee" monitoring changes in SLH1. Interaction between Avr and SLH1 results in the detachment of the WRKY domain from the amino-terminal regions. The intermolecular interactions within the protein dissociate leading to the activation of SLH1. The mutation in slh1 disrupts protein-protein interactions between the WRKY domain and the amino-terminal domains of SLH1 leading to activation and resistance against *R. solanacearum*.

The resistance mediated by RRS1-R is SA and NDR1 dependent (Deslandes et al., 2002). However, the resistance is apparently independent of ET signalling. The role of ET in Arabidopsis against *R. solanacearum* was described by Hirsch et al. (2002) who suggested that ET plays a role in wilt symptom development and not in resistance. Evidence for this

conclusion was based on the delayed wilt-symptom development in ethylene insensitive mutant *ein2-1* challenged with GMI1000 and the accumulation of *PR-3* and *PR-4* ethylene-responsive transcripts in susceptible Col-0 plants which was not observed in *ein2-1* and resistant Nd-1 plants. Homozygous *ein2-1* plants in homozygous *RRS1-R* background remain resistant to strain GMI1000. Recent evidence also suggests that 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 the necrotrophic pathogen *P. cucumerina* and to *R. solanacearum* independently of SA, JA and ethylene (Hernandez-Blanco et al., 2007). Interestingly, primary cell wall mutants did not have the same effect on resistance against these pathogens. 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*. This observation was supported by experiments on ABA mutants (*abi1-1*, *abi2-1* and *aba1-6*), compromised in ABA-signalling, which showed enhanced susceptibility to *R. solanacearum*.

An Arabidopsis putative receptor-like kinase (*At-RLK3)*, proposed as a new class of receptor-like protein kinases, is activated preferentially during the incompatible interaction with *R. solanacearum* GMI1000 in ecotype Nd1 (Czernic et al., 1999). No induction of the gene was observed during the compatible interaction with ecotype Col-5 or with the control *hrp*$^-$ bacteria. The functional role of *At-RLK3* has not been elucidated, however, the rapid induction of the gene in suspension cells and in root, shoot and leaves is in accordance with a function in rapid signaling through dephosphorylation events leading to the activation of target genes (Czernic et al., 1999).

Work by Pfund et al. (2004) demonstrated that flagellin, derived from *R. solanacearum* isolate K60, was not a major elicitor of plant defence responses. Mutants defective in *fliC* (gene encoding flagellin) or *flhDC* (encodes the master regulator of flagellin biosynthesis) exhibited the same responses as wild-type bacterial extracts on tomato plants challenged with the bacteria. Arabidopsis plants either containing the FLS2 or lacking the receptor were also challenged with the wild-type and aflagellate *R. solanacearum* strains by wounding the plant roots and applying the bacteria onto the soil surface. Similar disease levels were observed in both types of interactions, suggesting that *R. solanacearum* flagellin may not be recognized by the Arabidopsis FLS2 flagellin-recognition system. FLS2 is highly expressed in the plant

vasculature (Gomez-Gomez and Boller, 2002) and a vascular pathogen such as *R. solanacearum* may have developed a type of flagellin to evade recognition by the host.

Recently, a pathosystem between *R. solanacearum* and a leguminous host, *Medicago truncatula* has been described (Vailleau et al., 2007). An *in vitro* root inoculation method using strain GMI1000, revealed a resistant and susceptible line of *Medicago truncatula*. Recombinant inbred lines (RILs) generated from this cross were used to identify a major QTL for resistance on chromosome 5.

1.6. Microarrays

Microarray technology developed concurrently with the completion of the whole-genome sequencing of Arabidopsis ecotype Col-0 (Schena et al., 1995; The Arabidopsis Genome Iniative, 2000). Microarrays might be regarded as a large-scale reverse northern-dot blot, which allow researchers to screen thousands of genes simultaneously. Several types of microarrays exist, which investigate organisms at molecular and cellular levels i.e. DNA microarrays, protein microarrays and tissue microarrays. DNA microarrays may be spotted such as cDNA microarrays and oligonucleotide microarrays, or synthesised directly onto the microarray support i.e. the Affymetrix GeneChip® system. The technology has wide applications. They may be used for genome analysis (detection of copy number, mutation detection and SNP genotyping), expression profiling, gene discovery, diagnostics and re-sequencing of organisms' genomes (Bowtell and Sambrook, 2003; Schena et al., 1998).

cDNA microarrays, used for expression profiling, are discussed further as an example of microarray technology. This type of microarray platform is prepared from cDNA libraries with known expressed sequence tags (ESTs) representing individual genes. These ESTs are amplified, purified and spotted at a high density onto microscope glass slides using a robotic printer. The microscope slide has a specific surface chemistry such as a positive poly-lysine or aminosilane substrate, which allows for the binding of the negatively charged DNA (Harrington et al., 2002). Two different RNA populations derived from differentially treated material (e.g. control and experiment) are each labelled with a different coloured dye (Cy3 or Cy5) and hybridised to the microscope slide. The accepted terminology applied in the microarray community is that the "probe" is a tethered, unlabeled molecule of known sequence and the "target" being interrogated is labelled, in solution and undefined (Bowtell and Sambrook, 2003). After washing the slide to remove unbound target, the slide is scanned

using lasers, which excite the dyes. The resulting fluorescence is then computed for each spot providing a measure of the transcript abundance for each spot in the samples investigated (i.e. control and experiment) (Dolan et al., 2001).

1.6.1 Experimental Design

In its short history, microarray technology has made valuable contributions to plant science research and the technology has become more sophisticated over the years. However, several considerations remain (Hoheisel, 2006). The process of normalisation is important and each experiment requires careful planning in order to ensure that the correct number of replicates is included and that appropriate controls are in place to allow for the elimination of dye-bias, spatial bias and artefacts. These issues are reviewed in Chapter 2.

1.6.2 Microarray data normalisation and analysis

Microarray data analysis is a challenge to researchers due to the large amount of data generated by the experiments. This data has to be captured, normalised and then analysed for differential expression. Many open-source software packages are available for microarray data normalisation and analysis. These include TM4 microarray software suite (http://www.tigr.org/software/tm4), Gene Expression Pattern Analysis Suite GEPAS (http://gepas.bioinfo.cnio.es) and the Bioconductor libraries in the statistical language R (www.bioconductor.org Gentleman et al., 2004).

Data normalisation methods

Normalisation is necessary to remove systematic errors and bias introduced by the microarray experimental platform. Data normalisation involves 1) extraction of the data and removing artefacts, 2) within-array normalisation, which allows for the comparison of the Cy3 and Cy5 signals of a two-colour microarray and 3) between-slide normalisation, which allows for comparison of signals on different arrays (reviewed in Stekel, 2003). The software, which allows extraction of the measurement of Cy3 and Cy5 intensities for each spot, allows spots with poor spot morphology to be flagged. This flagged data can either be excluded completely from the subsequent analysis or in the case of a small microarray, which would not be too time-consuming, each flagged spot can be used in the dataset but cross-checked on the image to ensure that the flagging is appropriate. The background for each spot, which is thought to represent non-specific hybridisation of labelled target or the fluorescence from the slide surface itself, is calculated either locally around each spot or globally across the slide. This is

subtracted from the Cy3 and Cy5 feature intensity. In cases where the slide background is quite low and is evenly distributed across the slide, background subtraction may not be necessary (Quakenbush, 2002). The data is then transformed into log_2 values for the Cy3 and Cy5 signals for each spot. This data is usually entered into microarray analysis software programs. The log_2 transformation results in a normalised distribution of intensities for an array with numerous spots.

Systematic bias resulting from 1) the differential incorporation of the Cy3 and Cy5 dyes into DNA, 2) the different emission responses of the Cy3 and Cy5 dyes to the excitation laser and 3) spatial biases e.g. the deposition of different amounts of DNA by the robotic print-tip, have to be removed prior to data analysis (Quakenbush, 2002; Stekel, 2003). If the microarray contains a large number of spots representing a large portion of an organism's genome, the assumption can be made that most of the genes on the array should not be differentially expressed. Then, total intensity dependent normalisation can be applied in a linear or non-linear manner to address this systematic variation between dyes (Quakenbush, 2002; Yang et al., 2002). The behaviour of the dyes on a slide can be tested by producing a scatterplot of the Cy3 versus the Cy5 intensity values for each gene. An ideal result would be a linear regression through the points with a slope of 1 and an intercept of 0. Variations in these values indicate a dye bias. In some cases, the assumption that the expression of most genes would not change, may not hold: e.g. if samples were treated with a transcriptional inhibitor, then most genes on the array would be expected to change. For these experiments, other types of normalisation, based on spike-in controls may be more appropriate (reviewed in Chapter 2).

Non-linear normalisation involves a lowess (locally weighted scatterplot smoothing) regression, which performs a large number of local regressions in overlapping windows across the whole range of the data set (Cleveland, 1979). Spatial effects generated by uneven hybridisation of the targets or through uneven scanning of the slide surface can be corrected by using a two-dimensional lowess regression, which fits a two-dimensional polynomial surface to the data. Spatial bias generated by print-tip variation is best corrected using a print-tip or block-by-block lowess regression. This procedure completes a one-dimensional lowess regression on each block (printed by a different print-tip) of the microarray slide separately (Smyth et al., 2003). An alternative to these normalisation methods is the "robust spline" normalisation, which may be regarded as a compromise between global normalisation and

lowess normalisation. This type of normalisation may be applied when most of the spots printed by each robotic pin appear yellow i.e. are not differentially expressed (Schadt et al., 2001).

Following within-slide normalisation, slides have to be normalised between each other in order to make comparisons between samples hybridised to different arrays as each hybridisation reaction may be different on each slide, resulting in the intensities across the slides being different (Quakenbush, 2002). A box plot generated for each slide helps one visualise the distributions of log intensities on several arrays. The box itself represents the standard deviation of the distribution while the line through the center of the box represents the mean of the distribution. Horizontal lines termed "whiskers", that represent the extreme values of the distribution, bracket this plot. The central assumption made when normalising between arrays is that the variations in the distributions between arrays are a result of experimental conditions and do not represent biological variability. This needs to be checked for particular experiments and if the distributions are different for a particular treatment, then this approach is not valid. The data can be scaled to ensure that the means of the distribution are equal or alternatively, the median can be used which provides a more robust measure of the average intensity on an array in situations where there are outliers or the intensities are not normally distributed (Yang et al., 2002).

A similar method to scaling is centering of the data. This involves subtracting the mean measurement of the array for each element on the array and dividing by the standard deviation (Stekel, 2003). A more complicated alternative to centering is distribution normalisation (e.g. quantile normalisation), which ensure that the distributions of the data on each of the arrays are identical. This is achieved by centering the data, ordering the centered measurements from lowest to highest, computing a new distribution based on the average value for the gene from each of the arrays and replacing each measurement on each array with the corresponding average in the new distribution so that each array will have a mean of 0, a standard deviation of 1 and identical distributions to all the other arrays (Bolstad et al., 2003).

Data Analysis and Hypothesis Testing

The data analysis process is quite distinct from the normalisation steps described above in that the former process is used to answer the scientific question for which the microarray experiment has been designed. The statistical analysis may involve hypothesis testing to

determine whether a gene is differentially expressed or not. Statistical analysis becomes more sophisticated as the complexity of the design of a microarray experiment increases. Several authors have reviewed data analysis methods (Cui and Churchill, 2003; Parmigiani et al., 2003; Smyth et al., 2003; Speed, 2003; Smyth, 2004). Two simple methods of analysis pertaining to differential expression of genes in a direct comparison and used in this study are discussed viz: the Analysis of Variance (ANOVA), implemented in the software package Statistical Analysis Software (SAS), and linear models for microarrays (`limma`), implemented in R.

T-tests are commonly used in statistics and requires that the distribution of the data being tested is normal. This test can be used to compare two conditions when there is replication of samples. The t-test statistic for paired data is calculated as follows:

$$t = \left(\frac{\overline{Y_{g1..}} - \overline{Y_{g2..}}}{\sqrt{\frac{\sigma^2_{g1}}{n_{g1}} + \frac{\sigma^2_{g2}}{n_{g2}}}} \right)$$

where σ^2_{g1} is the standard deviation of observations for gene g, under treatment one, and n_{g1} is the number of spots under treatment one, for the particular gene. σ^2_{g2} is the standard deviation of observations for gene g under treatment two, and n_{g2} is the number of spots under treatment two, for the particular gene. The null hypothesis for every gene is that there is no difference in gene expression due to the treatment (i.e. Ho: $\mu 1=\mu 2$, where μ represents the mean expression for a gene).

The ANOVA model is a powerful approach for microarray experiments with multiple factors and/ or several sources of variation. The mixed model ANOVA essentially performs a global normalisation and is referred to as a "mixed" model as some effects are random while other effects are fixed (Wolfinger et al., 2001). Originally, the ANOVA applied by Wolfinger et al. (2001) did not account for dye effects and it was proposed by Kerr and Churchill (2001) that such an effect should be included into the model for flip-dye comparisons. The assumptions of this ANOVA and the formula is indicated below:

$$y_{gijks} = \mu + D_k + T_i + A_j + (TA)_{ij} + \varepsilon_{gijks}$$

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where μ represents the overall mean value, D is the dye effect, T is the main effect for treatments, A is the main effect for arrays and TA is the interaction between arrays and treatments and ε is the random error. This model (*y*) calculates for the *g*th gene, the effect of the *k*th dye, the *i*th treatment, the *j*th array and the *s*th replicate spot on the slide. The assumptions that were made were that the effects $A_j, (TA)_{ij}, \varepsilon_{gij}$ are normally distributed with zero means and variance components σ^2_A, σ^2_{TA} *and* σ^2_ε respectively, and the latter named effects are independent both across their indices and with each other, and μ is a fixed effect.

The resulting p-value from a statistical analysis is the probability of observing a statistic that is at least as extreme as the observed statistic in the data. The smaller the p-value, the less likely it is that the observed data have occurred by chance and the more significant the result (Dudoit et al., 2003). Calculated p-values are adjusted to control for the discovery of false positives. The multiple testing of microarray data generates this error. The family-wise error rate (FWER) or false discovery rate (FDR) may be controlled during microarray data analysis. The Bonferroni adjustment is an example of a method, which controls the FWER but is regarded as a strict form of adjustment (Stekel, 2003). This adjustment is computed for each gene by multiplying the calculated p-values by the number of genes in the analysis (Stekel, 2003). False discovery rate (FDR) adjustment however, is a more favourable form of adjustment to reduce the number of false positives obtained from the analysis e.g. Benjamini and Hochberg (1995).

R is an open-source statistical package, which is available for Unix, Windows and Macintosh and has a wide range of statistics and graphing functions (Gentleman et al., 2004). R is command line driven and a number of groups have written packages for microarray normalisation and analysis in R. The Bioconductor package `marray`, run in R, provides functions for reading, producing diagnostic plots and normalising spotted microarray data. `Limma` is a package for the analysis of gene expression microarray data also found within R, especially the use of linear models for analysing designed experiments and the assessment of differential expression (Smyth, 2004). The package allows the user to analyse comparisons between many RNA targets simultaneously. Although the `Limma` package overlaps with `marray` in functionality, it is based on a more general separation between within array and between array normalization (Gentleman et al., 2004).

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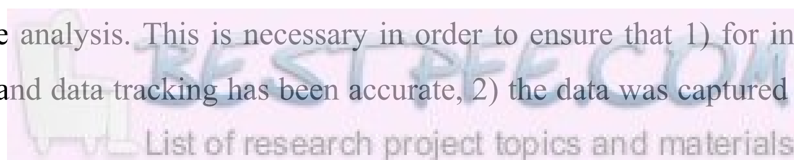
The core of the `Limma` package is an implementation of the empirical Bayes linear modeling approach of Smyth (2004). The empirical Bayes approach, which is essentially based on the t-test described on page 48, results in a more stable inferences when the number of arrays is small and allows for incomplete data arising from data flagging. Information is borrowed across the range of genes, which assists in inference about each gene individually. The approach according to Smyth (2004) is equivalent to shrinkage of the estimated sample variances towards a pooled estimate. The posterior variance is substituted into the classical t-statistic in place of sample variance. In this way, the number of hyperparameters in the model (e.g. dye, slide, etc.) which need to be estimated is reduced and prior knowledge of the proportion of differentially expressed genes is not needed. The moderated t-statistic follows a t-distribution with augmented degrees of freedom and the approach also uses moderated F-statistics in which the posterior variance is substituted for the sample variance in the denominator in order to accommodate tests of composite null hypotheses. In the `limma` model, a B-statistic is also calculated as further evidence for differential expression (Smyth, 2004). The B-statistic is in simple terms the logarithm of a ratio of probabilities. The numerator is the probability that a gene is differentially expressed while the denominator is the probability that it is not. These probabilities are referred to as posterior probabilities as they are calculated in light of the entire data set. Therefore, the B statistic is a logarithm of the posterior odds of differential expression (Lonnstedt and Speed, 2002). For non-statisticians, Wettenhall and Smyth (2004) have designed a graphical user interface for the linear modeling of microarrays called *`LimmaGUI`*, which reduces the difficulty of specifying appropriate design and contrast matrices using a command-line interface.

The analysed microarray data is often graphically represented in the form of a “volcano plot” which is a scatterplot of the negative $\log_{10}$-transformed p-values from the specified test against the $\log_2$ fold change that is calculated from the ratio of one condition compared to another (Stekel, 2003).

1.6.3 Verification of Microarray Data

After data analysis, microarray expression data is also subject to verification experiments such as Northern blot analysis, semi-quantitative RT-PCR, quantitative RT-PCR or reverse dot-blots analysis (Canales et al., 2006). This process is usually performed on a subset of genes resulting from the analysis. This is necessary in order to ensure that 1) for in-house spotted arrays, the clone and data tracking has been accurate, 2) the data was captured accurately and



3) the assumptions made during normalisation and statistical data analysis were correct. Following verification, the data can be trusted to derive biological meaning (Canales et al., 2006). This is especially important, for example, for gene discovery experiments wherein a large amount of time and money will be subsequently invested to investigate candidate genes in gene knockdown or over-expression studies. Until microarray methods become standardised, verification of the data will remain necessary (Ruan et al., 1998) however, experiments by Canales et al. (2006) support the use of microarray platforms for the quantification of gene expression. A comparison of microarray platform data and quantitative RT-PCR data showed correlation between the two methods of quantification. This suggests that in the future, microarray expression data may not necessarily be subjected to verification using a different platform.

The Minimum Information About a Microarray Experiment (MIAME), is an effort by The Microarray Gene Expression Data Society (MGED) to standardise microarray data in that researchers provide a set of associated information for each microarray experiment conducted (Brazma et al., 2001). This is necessary to ensure that microarray data can be easily interpreted, repeated if necessary and the results can be verified independently. By standardising the recording and reporting of microarray data, the establishment of databases and public repositories has been facilitated over the years (e.g. MicroGen, ArrayExpress and MAGE-TAB) and new data analysis tools have also been developed (e.g. MARS) (Brazma et al., 2003; Burgarella et al., 2005; Maurer et al., 2005; Rayner et al., 2006). However, efforts in this area are still underway as it has been recognised that a lack of standardisation in terms of format and comparatibility confounds integrative microarray research (Larsson and Sandberg, 2006). Researchers in the plant community are encouraged to use the MIAME/Plant standard to facilitate data mining (Zimmermann et al., 2006). MIAME/Plant, an extension of MIAME, include standards for important parameters and ontologies, which extend the basic experiment and sample annotations of MIAME. These standards describe the biological details that should be captured in a plant microarray experiment e.g. growth conditions, age of plants, harvesting time, harvested organs, etc. MIAME/Plant guidelines are accessible on the Microarray Gene Expression Data Society [http://www.mged.org], the Nottingham Arabidopsis Stock Center [http://www.arabidopsis.info] and The Arabidopsis Information Resource [http://arabidopsis.org/info/expression] websites.

1.7. Arabidopsis microarrays to study plant-pathogen interactions.

Custom-designed as well as partial or whole-genome Arabidopsis microarrays have been used extensively for the study of plant-pathogen interactions. Five examples, which have used different Arabidopsis microarray platforms containing various numbers of genes, are outlined below to demonstrate the use of different Microarray platforms to answer questions pertaining to the plant defence response.

Ramonell et al. (2002) investigated gene expression patterns in Arabidopsis in response to chitin treatment. The microarray contained 2 375 EST clones representing putative defence related and regulatory genes. Seventy-one genes whose gene expression was altered more than three fold in response to chitin (a fungal PAMP) treatment were identified. Ziedler *et al*., (2004) used a custom set of defence response genes from *Arabidopsis* to investigate the response to the bacterial PAMP LPS. Together, such studies contributed to our understanding of the basal defence response mediated by PAMPs. A customised cDNA microarray consisting 150 ESTs was used to analyse the plant response to mechanical wounding, insect feeding and water-stress (Reymond et al., 2000). This study demonstrated that multiple stress responses induce similar expression profiles and there is significant overlap between abiotic and biotic stress responses.

Tao et al., 2003 investigated the difference in compatible and incompatible interactions using the Affymetrix GeneChip® microarrays containing 8000 genes. The results suggested that the difference between incompatible and compatible interactions is largely quantitative with the amplitude of induction of genes reaching higher levels earlier in incompatible interactions than in compatible interactions i.e. PTI and ETI largely overlap but it is the timing and amplitude of responses which differ (Tao et al., 2003).

Truman et al. (2006) also showed some overlap between basal and gene-for-gene defence responses and described a set of 96 core genes involved in basal defence based on an overlap of several microarray data-sets investigating basal defence responses to the *P. syrinage hrpA* mutant at early and late time points (2, 4 and 12 hours post inoculation (hpi)). Based on microarray expression profiles of Arabidopsis plants challenged with *P. syringae hrpA* mutants (deficient in TTSS effector delivery), avirulent *P. syringae* DC3000 AvrRpm1 (AvrRpm1 specific TTSS effector) and virulent *P. syringae* DC3000, using the Affymetrix GeneChip® 8000 microarrays, a set of 880 genes were found to be modified by the TTSS

effector proteins of the bacteria 12 hpi. The apparent suppression of leucine rich receptor proteins and the induction of protein phosphatases by the pathogen suggests that the TTSS effector proteins contribute to avoiding the host recognition by depressing host extracellular receptors and enhancing kinase signaling pathways. Genes involved in phenylpropanoid biosynthesis, implicated in lignin deposition, cell wall modification and subsequent restriction of the passage of water and nutrients to the invading bacteria, are also modified by the pathogen Type III effector system. These experiments not only provided further evidence of the suppression of basal defences by virulent pathogens but also allowed for the length and breadth of this suppression to be seen.

Thilmony et al. (2006) used full-genome Affymetrix GeneChip® microarrays to investigate the basal response of plants to PAMPs, TTSS and COR (coronatine, a bacterial toxin) during infection using the human pathogen *Escherichia coli* O157:H7, *hrp*$^-$, COR$^-$ and TTSS$^-$ mutants of *Pst* DC3000. Using a *flic*$^-$ mutant of the human pathogen revealed that flagellin does not contribute uniquely to PAMP-induced transcriptional changes after bacterial inoculation. Plant and human bacterial PAMPs induced similar transcriptional responses while the TTSS and COR virulence factors induced some distinct expression profiles. There was also evidence for TTSS effector-mediated suppression of basal defence associated genes (Thilmony et al., 2006).

Mahalingham et al. (2006) used a 70-mer whole genome spotted Arabidopsis microarray to investigate the role of oxidative signalling in plants. Transcript profiling of ozone (an elicitor of endogenous reactive oxygen species) treated Wassileskija plants revealed 371 genes differentially expressed by the treatment. Genes involved in proteolysis and hormone responsive genes were induced early during treatment while genes involved in carbon utilisation, energy pathways and signalling were down regulated (Mahalingham et al., 2006). Comparison to other microarray data revealed that 60% of the ozone-repressed genes were also strongly repressed by methyl jasmonate treatment in accordance with previous studies which showed the interaction between ozone and signalling hormones jasmonate, ethylene and SA (Kangasjärvi et al., 1994; Kangasjarvi et al., 2005).

The examples described here also illustrate the applicability of microarrays to study various aspects of plant defence and include, but are not limited to: the discovery of defence genes (Ramonell et al., 2002); the identification of the role of effectors (Thilmony et al., 2006;

Truman et al., 2006), and the study of the interaction between biotic and abiotic pathways (Mahalingam et al., 2006).

1.7.1 Arabidopsis Databases and bioinformatics tools

Various Arabidopsis microarray databases allow public access to microarray data generated by Affymetrix or GeneChip® arrays or EST microarrays e.g. The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org/), Nottingham Arabidopsis Stock Centre's microarray database (NASCArrays, http://arabidopsis.info/), the Stanford Microarray Database (SMD, http://genome-www5.stanford.edu/) and the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo). The challenge of a microarray experiment is to make biological inferences from microarray data. Web-based tools available in databases such as GENEVESTIGATOR (Zimmermann et al., 2004; Zimmermann et al., 2005) and DRASTIC--INSIGHTS (Database Resource for Analysis of Signal Transduction In Cells, Button et al., 2006) provide researchers with tools which facilitate gene mining by mapping and categorising data in relation to known information. In GENEVESTIGATOR, the following queries can be made using the available tools: 1) How is the gene of interest expressed under a certain treatment or tissue, 2) How do the expression profiles of genes compare to each other, 3) what are the GO annotations of genes, 4) how do expression profiles map to metabolic and regulatory pathways, 5) which genes are expressed under certain conditions or tissue type (Zimmerman et al., 2005). The DRASTIC__INSIGHTS database is focused on signal transduction pathways in plants and allows potential response pathways to be inferred. The data within this database is derived from microarray experiments as well as manually curated records including data from plant northern blots, ESTs, cDNA-AFLPs, quantitative RT–PCR and massively parallel signature sequencing. The pathway and roadmap tools found in DRASTIC—INSIGHTS allow the identification of co-regulated genes, which may be involved in the same signal transduction pathway (Button et al., 2006). Not only do these tools allow for the validation of existing microarray results and the identification of specific marker genes, but also allow for the generation of new testable hypotheses.

In summary, several experiments have demonstrated the success of using Arabidopsis microarrays as a tool in understanding plant defence responses. Open-source or commercial statistical packages can be employed to determine which genes are responding significantly to the pathogen. Thereafter, various data mining tools are available to derive biological meaning

from these gene clusters. Together, this evidence creates a strong argument for the use of Arabidopsis microarray expression profiling to understand plant defence responses.

1.8. Aims

This study focuses on the plant defence response against *R. solanacearum* in the model plant *Arabidopsis thaliana*. The aims of this study were to:

1) Qualify the use of microarray expression profiling technology to study plant defence responses in our laboratory. This was achieved in the study described in Chapter 3, which utilised optimised microarray protocols to investigate the plant defence response in the Arabidopsis *cir1* (*c*onstitutively *i*nduced *r*esistance 1*)* mutant compared to the wild-type plant.
2) Investigate a susceptible interaction between *Arabidopsis* and *R. solanacearum* using microarray expression profiling of the host and subsequent bioinformatic analysis to determine host transcriptional responses to the pathogen. This was accomplished by performing microarray analysis on Arabidopsis ecotype Col-5 infected with a *Eucalyptus* isolate of *R. solanacearum* isolate BCCF 401 (Chapter 4).
3) Determine which plant defence response genes respond to *R. solanacearum* challenge in a resistant interaction between Arabidopsis ecotype Kil-0 and isolate BCCF 402. Whole-genome microarrays and qRT-PCR expression profiling were performed for this investigation, which is detailed in Chapter 5.

1.9. References

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CHAPTER 2
MICROARRAY EXPERIMENTS: CONSIDERATIONS FOR EXPERIMENTAL DESIGN

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2.1. Abstract

Microarrays are useful tools to investigate the expression of thousands of genes rapidly. However researchers remain reluctant to delve into the technology largely due to the expense. Careful design of a microarray experiment is key to generating cost-effective results. This review explores issues that researchers are faced with when embarking on a microarray experiment for the first time. This includes decisions about which microarray platform is available for the organism of interest, the degree of replication (biological and technical) needed and which design (direct or indirect, loop or balanced block) is suitable.

2.2. Introduction

Initially conceived and implemented ten years ago (Schena et al., 1995), microarray technology has become an attractive choice for researchers to screen the expression of thousands of genes simultaneously. During its short history, the technology has made invaluable contributions to various scientific fields. An example of such an achievement is evident in human cancer research and the development of a prognostic tool based on gene expression profiles in early breast tumours (van't Veer et al., 2002). This assists doctors in predicting whether severe cancer will develop which warrants aggressive therapy such as chemotherapy and hormone treatments, and prevents low-risk patients from receiving harsh treatments unnecessarily, since surgery and radiotherapy are sufficient in these cases. Concurrent with the sequencing of whole genomes, microarray technology has become more sophisticated, allowing high-density arrays and consequently high-throughput of data. Despite the recent advancements of the technology, several questions remain, especially to those researchers embarking on microarray experiments for the first time. The design of the experiment depends firstly on the biological question being asked, as well as the organism being studied. Different microarray platforms exist and selection of the correct design influences the analysis of the data to obtain biologically significant results. This review serves to aid those researchers wishing to employ microarrays for their biological organism of interest by outlining the principles of experimental design.

2.3. Microarray platforms

Microarrays are conceptually quite simple and may be regarded as a large-scale reverse Northern blot. Several types of microarray platforms exist: spotted microarrays, such as cDNA microarrays and oligonucleotide arrays, and the Affymetrix GeneChip® system, which involves synthesis of oligonucleotides directly onto the microarray support. In South Africa, two microarray facilities are available: the African Centre for Gene Technologies (ACGT) Microarray facility at the University of Pretoria (http://microarray.up.ac.za) and capar at the University of Cape Town's Molecular and Cell Biology Department (http://www.capar.uct.ac.za). Both facilities are capable of producing cDNA and oligonucleotide microarrays at high densities.

The diagram in Figure 2.1 describes a typical microarray experiment that uses a cDNA microarray platform. cDNA fragments, representing different genes, are amplified and spotted at high density onto microscope glass slides with special surface chemistry that allows

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binding of the spotted DNA. Two different cDNA populations derived from independent RNA samples are labelled with red (Cy5) and green (Cy3) fluorescent dyes respectively and hybridised to the slide. The array is subsequently washed and scanned by lasers that excite the different dyes. A fluorescent signal is computed for each spot on the array and the ratio of Cy3:Cy5 induced fluorescence for each spot corresponds to the relative amount of transcript in the samples. In microarray experiments, the selection of candidate genes will depend on the criteria set by the researcher to describe differential expression. Previously, those genes that satisfied the criteria of having a fold change greater than two were considered differentially expressed. However, the role of statistics in determining significance of results has become increasingly important and only those genes that are shown to be differentially expressed with statistical support across replicates are selected (Stekel, 2003). It is for the latter reason that any microarray experiment would benefit from the expertise of a statistician who would be able to advise on the experimental design and subsequent analysis for a particular biological question.

For those organisms with little or no genome sequence available, arrays can be constructed by picking clones from a cDNA library and amplifying the insert cDNAs prior to spotting. The identity of selected clones can be determined after microarray analysis (Gibson and Muse, 2002). It is important to normalise the cDNA library prior to preparation of the microarray in order to reduce the redundancy of clones. Redundant clones only contribute to increased expense during amplification of the library. The preparation of microarrays from a normalised cDNA library is a viable strategy especially for uniquely South African organisms demonstrated in the desiccation tolerant plant *Xerophyta humilis* (Collet et al.,2004). Another method to generate a normalised cDNA library is by using a subtractive hybridisation technique such as Suppressive Subtractive Hybridisation (SSH). An SSH library is created by subtracting the transcripts common to both samples so the resulting cDNA clones are derived from transcripts present in one sample (tester), e.g. disease tissue, but not in the other (driver), e.g. healthy tissue. The SSH technique allows the detection of low-abundance differentially expressed transcripts and may identify essential regulatory components in a number of biological processes (Diatchenko et al., 1999). Yang *et al*. (1999) successfully combined SSH and microarrays to identify genes differentially expressed in breast cancer cell lines and microarrays have also been used to screen clones derived from SSH libraries to identify up-regulated genes in banana and pearl-millet during defence responses (van den Berg et al, 2004).

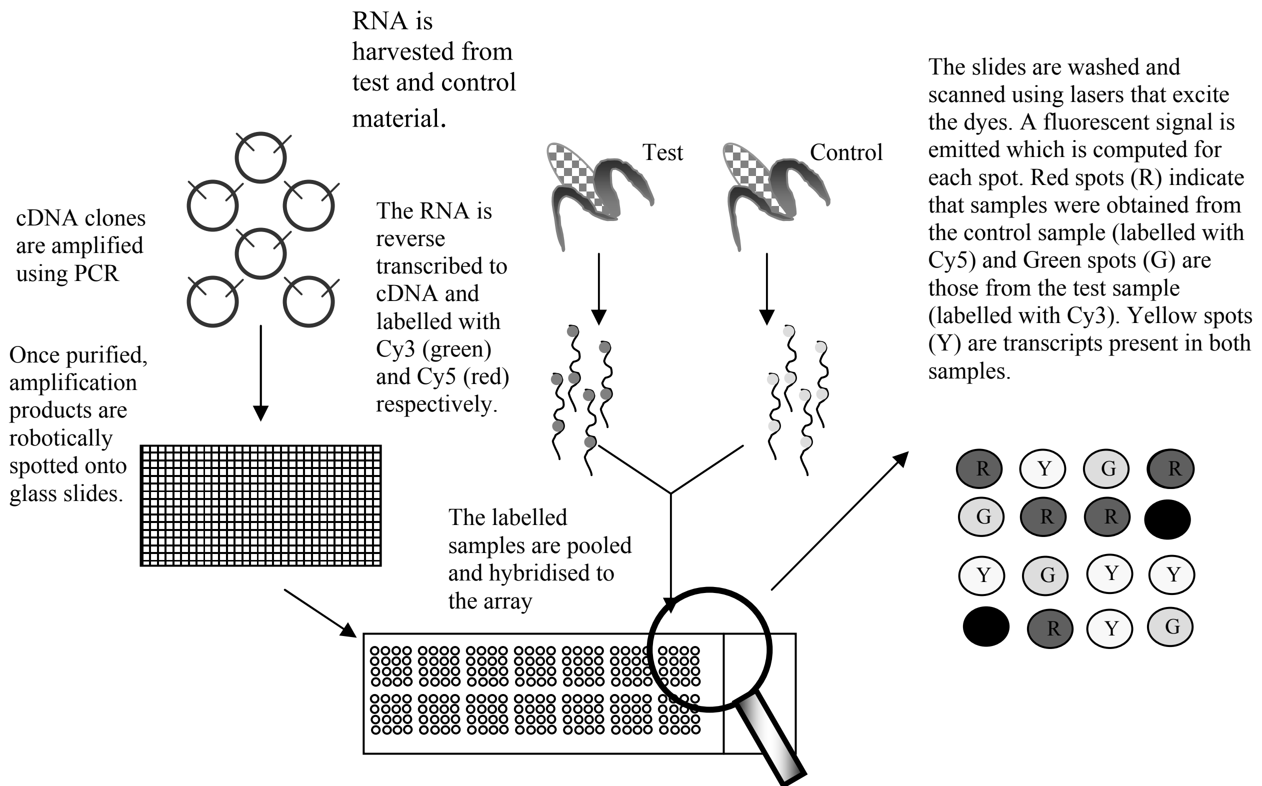

Figure 2.1 An example of a typical microarray experiment using dual colour labelled cDNA samples hybridised to glass slides containing amplified cDNA fragments.

As an alternative to preparing your own cDNA libraries, arrays can be prepared from amplification of sequenced cDNA clones called Expressed Sequence Tags (ESTs). Currently, there are several million ESTs from various organisms in the NCBI public collection (http://www.ncbi.nlm.nih.gov/dbEST/). Ideally each EST should represent a unique gene, referred to as a unigene set. Unigene sets for most genomes were initially assembled using software that identifies unique clones in EST databases. With the availability of whole genome sequences, new unigene sets are becoming available. Some clones are genomic clones representing predicted genes for which no EST has been identified (Gibson and Muse, 2002). The advent of whole genome sequences also allows one to custom-design arrays with genes predicted or known to be involved in a particular biological process. Kidson *et al.* (personal communication[c]) customised an array consisting of 384 amplified ESTs involved in eye-development. Other EST collections, like that consisting of 6000 *Arabidopsis* ESTs, has a wider application (Naidoo, unpublished[d]). However, an expensive step in cDNA microarray

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[d]Sanushka Naidoo, Chapter 4.

analysis is the amplification of the EST set. This promotes the case for preparing smaller custom arrays rather than using large collections.

Affymetrix GeneChip® technology uses a series of 25mer oligonucleotides (Lipshutz et al., 1999). These oligonucleotides are designed using a computer algorithm to represent known or predicted open reading frames. This technology is limited to organisms with a significant amount of genome information. There are between 10-20 different oligonucleotides representing each gene to control for variation in hybridisation efficiency due to factors such as GC content. A control for cross-hybridisation with similar short sequences in transcripts other than the one being probed for is a mismatch oligonucleotide next to each oligonucleotide with a single base pair change at the centre of the oligonucleotide. Under stringent hybridisation conditions, this control should not hybridise to the exact match cDNA. The level of expression of each gene is calculated using a procedure provided by the Affymetrix software, which calculates the weighted average of the difference between the perfect match and mismatch. The high-density arrays are constructed on silicon wafers using a technique called photolithography and combinatorial chemistry. The process used to prepare the arrays is expensive and processing requires a proprietary hybridisation station, scanner and software, putting a constraint on the number of slides that can be purchased for replication and availability to South African researchers. The target cDNA is labelled using amplified RNA and only a single sample is hybridised to each chip. Although Affymetrix GeneChip®arrays can accommodate a higher density of genes and are probably considered the "gold standard" of microarray technology, they are limited to model organisms while cDNA methods can be applied to any organism, are cheaper and more repetitions can be achieved. This enhances statistical analysis and can be more flexible in design (Gibson and Muse, 2002).

Spotted oligonucleotides have grown in popularity and are considered a hybrid technology, combining the uniformity of Affymetrix GeneChips®and the versatility of cDNA microarrays. This method also removes the variability inherent in amplification of cDNA clones. This technology involves spotting 50-70mer oligonucleotides onto glass slides. Subsequent probe preparation and hybridisation is similar to that of cDNA microarrays. Hughes *et al.* (2001) found 60mer oligonucleotides were able to reliably detect transcript ratios at one copy per cell

in complex biological samples. These results are in accordance to data obtained with robotically printed cDNA arrays.

Recently, Yauk *et al*. (2004) compared six microarray platforms, two cDNA and four oligonucleotide (including 25mer Affymetrix microarrays, 30mer spotted microarrays and 60mer oligonucleotides synthesised *in situ*). The objective of this exercise was to determine whether gene expression profiles are influenced more by biology or by artefacts of the technology. There was significant difference in the ability of the different platform types to detect differential expression in the two very different cell types that were used for the study. More differentially expressed genes were identified using the oligonucleotide rather than the cDNA based platforms. The validation exercises using Northern hybridisations and RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) supported the suggestion that cDNAs are less sensitive than the oligonucleotide platform. The authors conclude that with high-quality microarrays and the appropriate normalisation methods, the primary factor determining variance is biological rather than technical. This provides reassurance that if one cannot afford the Affymetrix platform, biologically meaningful data can still be obtained using cDNA microarrays or spotted oligonucleotide arrays.

The Affymetrix system may not be the first choice for South African researchers primarily due to the cost and their limitation to model organisms. Spotted oligonucleotides provide a good alternative and are commercially available for organisms with a large amount of sequence information. The cDNA microarray platform remains the only alternative for organisms with limited sequence information. Given the cost of microarray experiments, it is important that attention be given to the design of the experiment. Typically one would wish to obtain the best possible results with the particular resources available to answer the question of interest. The issues on design discussed below pertain to two-colour dye experiments such as those using the cDNA and spotted oligonucleotide platforms. The Affymetrix system, which uses a single labelled sample during hybridisation, is not discussed further.

2.4. Questions on design

When one embarks on a microarray experiment, several questions should be considered. Logically the first being, what exactly is the researcher investigating i.e. what is the biological question or hypothesis being tested? Will the microarray experiments be able to address the question and how will the results of the microarray experiments contribute to the research as a

whole? Would an alternative method be better, such as quantitative RT-PCR, SAGE (Serial Analysis of Gene Expression), cDNA-AFLP (Amplified Fragment Length Polymorphism) or ddRTPCR (differential display Reverse Transcriptase PCR)? In order to determine precisely what comparisons are being made, Yang and Speed (2003) advise that the priority of the different scientific questions being asked should be identified along with the types and number of samples available.

On the technical side, another important consideration is whether the RNA sample is limiting and whether the process prior to hybridisation i.e. RNA isolation, RNA extraction and labelling are optimised for the organism of interest. If one wishes to identify a few genes to work on further, one should determine prior to the microarray experiments which method will be appropriate to verify the data obtained from the experiments as a considerable amount of RNA is required for Northern hybridisation while quantitative RT-PCR remains the method of choice for several researchers due to the low amount of starting material required. Other experiments, such as those which compare expression profiles, rely on the strength of the statistical analysis to make conclusions and do not require verification (Rockett and Hellmann, 2004).

The data from spotted microarray experiments often has to be normalised prior to analysis due to variability in labelling efficiency contributed by the two different dyes. For this purpose control spots are often necessary. A researcher has to determine what types of controls would be most appropriate for the tissue type being used. External or spike-in controls aid the researcher in determining whether labelling and hybridisation has worked well (Yang and Speed, 2003). This method utilises genes from an organism different to the one being studied or synthetic genes with no significant regions of homology to genes on the microarray to prevent cross-hybridisation. These genes are spotted onto the microarray and their corresponding RNA transcript is included in both the target samples, which are subsequently labelled with the red and green dyes and hybridised to the slide. Spike artificial RNA controls and corresponding DNA targets to be spotted are commercially available e.g. Lucidea™ Universal ScoreCard™ (Amersham Biosciences). Hybridisation results in predictable red and green fluorescence intensities at the target spots relative to the different concentrations of spiked RNA added to the samples. This controls for labelling efficiency but does not control for the difference in the amount of RNA in the two samples being hybridised. Negative controls (no DNA or DNA that is unlikely to cross-hybridise e.g. from an unrelated organism)

are often included on spotted microarrays to determine the background fluorescence and whether the hybridisation conditions are stringent enough.

Internal controls may be housekeeping genes or genes known to be constitutively expressed between the test and control sample. Housekeeping genes are required for fundamental cellular processes in different cell types and tissues. The expression of housekeeping genes does not depend on the physiological, developmental or pathological state of the tissue e.g. actin and GAPDH in some cell types (Yang and Speed, 2003). One problem with housekeeping genes is that they tend to be highly expressed and may not be representative of genes of interest which tend to be expressed less and are more likely to be subjected to intensity dependent bias. The selection of an appropriate housekeeping gene for a particular condition is also a contentious issue, since so-called "housekeeping" genes do not remain constitutively expressed under some conditions (Wu et al., 2001). One way to identify internal control (housekeeping) genes is to data-mine previous microarray experiments for genes whose expression levels do not vary under various treatments. This approach is useful for model organisms for which there is a large amount of publicly available microarray data (e.g. *Arabidopsis*, human, etc.).

It is advisable to first test candidate internal control housekeeping genes for stable expression in the tissues of interest using sensitive methods such as quantitative RT-PCR. Vandesompele *et al*. (2002) developed a procedure in Microsoft Excel to analyse real-time quantitative RT-PCR data of putative housekeeping genes. They tested 10 commonly used housekeeping genes and confirmed that normalisation using a single housekeeping gene was unreliable. This procedure, which is also applicable to microarray data, uses the geometric mean of relative expression levels from carefully selected housekeeping genes to calculate a normalisation factor.

Normalisation removes unwanted systematic bias from microarray data. This includes within-slide normalisation to remove effects of dye bias and spatial bias (e.g. spotter print tip variation). Arrays with a large number of spots representing a large portion of an organism's genome can be normalised based on the assumption that most of the genes on the array should not be differentially expressed and should thus remain yellow (Stekel., 2003). If this assumption holds, then a linear or non-linear regression can be applied. The linear regression

method, referred to as total intensity normalisation, assumes that the relationship between the Cy3 and Cy5 channels is linear. However, this is not true for most microarray experiments. ANOVA models have also been applied for normalisation (Kerr *et al*., 2000, Wolfinger *et al*., 2001). Normalisation of spatial biases can also be incorporated into the latter ANOVA models. LOWESS (LOcally WEighted Scatterplot Smoothing; also known as loess) is a commonly used non-linear regression method for microarray data, and performs a series of local regressions in overlapping windows through the range of the data (Cleveland, 1979). The regression is then joined to form a smooth curve. Spatial biases can also be corrected separately using the LOWESS regression. In customised arrays containing a small number of genes biased towards a certain condition e.g. disease or salt-stress, control spots are required for normalisation (Yang and Speed, 2003). These could be a set of validated housekeeping genes, however several that are expressed at a range of intensity levels should be used in order to perform a non-linear normalisation. Additionally, prior to analysis, slides are also subject to between-slide normalisation, which allows comparison of multiple arrays on an equal footing. Basic normalisation issues in the context of experimental design have been covered in this review; the reader is referred to Stekel (2003), Yang *et al*. (2002), Quackenbush (2002), Futschik and Crompton (2004) for normalisation in the context of microarray data analysis.

2.5. Replication

In order to apply a statistical test and reduce the variability inherent in microarray experiments, replication is necessary. There are different levels of replication: technical and biological (Yang and Speed, 2002). One type of technical replication is spot duplication. If space permits, cDNAs can be spotted in duplicate on every slide and the degree of conformity between duplicate spot intensities is a good indicator of the quality of the slide and hybridisation. It is advisable, however, that duplicate spots be well spaced rather than spotted adjacently as this allows a better inspection of the degree of variability across the slide. Replicate slides hybridised with target RNA from the same preparation is also considered technical replication. Statisticians prefer the latter type of technical replication, as replicate spots on the same slides are not independent of each other (Yang and Speed, 2002). Biological replicates could be hybridisations performed using RNA from independent preparations from the same source or preparations from a different source e.g. different organisms or different versions of a cell line. The latter type of biological replication encompasses a greater degree of variation in measurements. For example, an experiment

investigating drug treatment in mice is subject to the variation within the mice population such as difference in immune system, sex, age etc. The greater variability inherent in this form of replication contributes to a greater generalisation of the experimental results (Vandesompele et al., 2002). Typically, a researcher should use biological replicates to validate generalisations of conclusions and technical replicates to reduce the variability of these conclusions (Yang and Speed, 2002).

Often, pooling RNA from a number of similar sources is unavoidable in order to have sufficient amounts for a single hybridisation. One way of overcoming the problem of limited amounts of starting material is RNA amplification (Livesey, 2003). Pooling may also be used by researchers to reduce the number of arrays in order to save on cost (Dobbin and Simon, 2003). However, a single pool of many samples does not allow for the estimation of technical and biological variability. Shih *et al*. (2004) show statistically that there is a loss of degrees of freedom and a decrease in power when pooling and suggest that if pooling is used, the number of different pools should not be too small and the number of individuals should be appropriately increased in order to compensate for this (Shih et al., 2004). The decision to pool is at the discretion of the researcher as it is sometimes not appropriate to pool samples. For example, when studying the effect of a drug on cancer patients, the gene expression in different patients is of interest. In this case, hybridisations with individual samples should be carried out. On the other hand, in an investigation of two inbred homozygous ecotypes of *Arabidopsis*, differences between the individual plants are not of interest, so pooling may be better justified.

Another form of replication, dye swap replications are hybridisations that are repeated with the dye assignments reversed in the second hybridisation. This method is useful to reduce the systematic differences in the red and green intensities, which have to be corrected during normalisation (Dobbin et al., 2003). A dye swap replicate can be performed for both a technical and biological replicate. Dye-swap pairs are not routinely warranted and may be excluded when indirect comparisons, such as those involving a common reference sample, are performed since this design is based on differences between slides and the repeatable colour bias is removed during the analysis (Dobbin et al., 2003). Similarly, a balanced block design negates the use of dye swap replication as the design inherently compensates for the dye effect (Dobbin and Simon, 2003). Balancing the dyes using the latter method is favoured over

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repeating each comparison with a dye swap, as this would require the use of more slides and thus increase the expense of the experiment (Dobbin et al., 2003).

2.6. Design types

In cancer studies, Golub *et al*. (1999) identified three categories into which a microarray experiment can fall, depending on the objective. These categories (class comparison, class discovery and class prediction) are applicable to most microarray experiments regardless of the organism being studied. In class comparisons, researchers are interested in comparing samples with each other (Yang et al., 2002). An example of this would be comparing gene expression profiles in wild-type mice with a mutant strain. Class predictions involve using the expression profiles generated by class comparisons and applying a multigene statistical model to determine in which class a new sample belongs. A strategy is to first make a class comparison to identify genes differentially expressed between cancer patients who respond to a particular treatment and those who don't. Subsequently, a commonly used class prediction approach would involve developing a univariate statistical model to identify a subset of genes that would help predict whether a new patient will respond to that therapy on the basis of their tumour expression profile (Dobbin and Simon, 2003). Class discovery involves those studies in which the samples are not predefined into different classes before the microarray experiment. The objective is to discover clusters of the samples based on gene expression profiles. Once the classification is made, the next step would be to characterise the cluster. An example of this would be a set of tumour samples that one wishes to divide into sub-classes based on gene expression profiles (Bittner et al., 2000). Other studies that investigate which classes of genes are co-regulated, for example in a time-course experiment, are also considered as class discovery. When samples have to be co-hybridised as in the case of spotted microarrays, careful design for pairing and labelling samples is required. Designs may involve direct or indirect comparisons and more than one option may exist to answer the same question.

Direct Comparison

Yang and Speed (Yang and Speed, 2003), stress the importance of deciding whether to use direct (within slides) or indirect comparisons (between slides). In our laboratory, investigation of differentially expressed genes in a mutant *Arabidopsis* plant involved a direct comparison design. Figure 2.2A illustrates the comparisons made. The mutant RNA sample was co-hybridised with the wild-type RNA sample on the same slide. For the repeat slide, the same

comparison was made with the dye assignments reversed. The platform used for this experiment was a cDNA microarray containing 500 *Arabidopsis* ESTs and several controls including a commercially available spike-in control (Lucidea™ Universal ScoreCard™, Amersham Biosciences) and several housekeeping genes e.g. actin and ß-tubulin. Negative controls of mouse genes, with no known homology to *Arabidopsis*, were also included. Spots were duplicated on the slide. Each sample contained leaf material pooled from 6-8 plants and two types of biological replicates were performed: one using independent RNA preparations of the leaf material from trial 1 and the other using leaf material harvested from a completely different trial (trial 2). Two technical replicates were performed per biological replicate. In total, twelve slides were used for this study. The correlation between all the mutant:wildtype gene expression ratios in each of the replicates was calculated. Table 2.1 lists the correlation between the two types of technical replicates and the biological replicates. It is evident that the correlation between biological replicates derived from independent trials is less than that for biological replicates derived within the same trial. Thus, it is advisable that when making generalisations, a biological replicate be included which is completely independent of the first. The data was analysed using a mixed model ANOVA (Wolfinger et al., 2001) and approximately 2% of the genes arrayed were regarded as differentially expressed at a significance threshold of $-\log_{10}(p)$ equals 5 (that is, $p < 0.00001$, Bonferroni adjusted to correct for multiple testing) (data not shown).

Table 2.1. Correlation between technical and biological replicates in a direct class comparison between a mutant and a wild-type *Arabidopsis* plant.

Comparison	**Correlation**
Duplicate spots on 1 slide	0.93
Between two technical replicates (slides)	0.92
Between biological replicates from within a trial	0.84
Between biological replicates from independent trials	0.72

Reference Design

A commonly used method of indirect comparison for microarray experiments is a reference design (Churchill, 2002). This design uses an aliquot of a common reference RNA and the intensity of hybridisation of a test RNA sample is always compared to hybridisation of the reference RNA to the same spot. A reference sample should be in large supply and is sometimes prepared by constructing complex mixtures of RNA in order to achieve maximum hybridisation to the array. Such reference samples are commercially available e.g. Stratagene® Universal Mouse reference RNA set. Another method of preparing reference samples

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involves pooling aliquots of test samples that are to be investigated in the experiment. Thus, every sample present in the test sample is present in the reference sample and the relative amounts of each RNA species will be the same. This implies that in any test versus reference comparison, the RNA concentrations will not be vastly different as each test sample is represented in the reference sample; a strategy which facilitates normalisation (Churchill, 2002). Figure 2.2B illustrates a reference design. For example, suppose one wishes to identify genes that are differentially expressed in two transgenic plant lines, then samples from the untransformed line, transgenic plant line 1 and transgenic plant line 2 can be individually compared to a reference sample in this case made up of a pool of equal amounts of RNA from each sample. In a reference design, the reference sample is labelled with the same dye each time. It is generally assumed that any remaining dye bias not removed by normalisation affects all the arrays similarly and does not bias comparison between the samples (Dobbin and Simon, 2002). However, recently Dombowski *et al*. (2004) suggest that gene-specific dye bias exists in microarray reference designs. If this is a significant variable, then microarray data will have to be validated before conclusions can be made or a reverse dye comparison could be incorporated in a biological replicate to account for the dye effect on specific genes (Tempelmen, 2005). There are two steps connecting two samples in a reference design, so each comparison can be made equally efficiently. An advantage of this method is that as long as the amount of reference sample is not limiting, the design can be extended to handle large numbers of samples and in class discovery experiments samples from a new class can be added at a later stage (Churchill, 2002).

Balanced Block Design

A drawback of the reference design is that half of the hybridisations are used for the least interesting sample, the reference (Dobbin and Simon, 2003). An alternative is a balanced block design. In a simple situation, suppose one wishes to compare 4 mutant mice with 4 wild-type mice. One could hybridise on each array one mutant sample with a wild-type sample. Half the arrays should have the mutant samples labelled with the red dye and the wild-type samples with the green dye while the other half of the slides should be hybridised with the samples labelled inversely. One disadvantage of the balanced block design is that cluster analysis of the expression profiles cannot be performed effectively. The common reference design is more amenable for the latter purpose as the relative expression measurements are consistent with regard to the same reference. Without a common reference, as in the balanced block design and direct comparison, comparison of samples on different

arrays can be skewed by variation in size and shape of corresponding spots on different arrays (Dobbin and Simon, 2003). The balanced block design is most effective when comparing two classes and can accommodate *n* samples of each type using *n* arrays. The advantage of the balanced block design is that half the amount of slides can be used compared to a reference design or direct comparison. However, the balanced block design loses to the reference design when there is large variability between samples and when the number of samples and not the number of arrays is in limited supply (Dobbin and Simon, 2003).

Loop Design

A loop design involves array hybridisations that link the samples together in a loop. The comparisons being made control for variation in spot size and sample distribution patterns using a statistical model (Kerr and Churchill, 2001). The example illustrated in Figure 2.2B could be designed in a loop-wise fashion. This is illustrated in Figure 2.2C. This design uses two aliquots of each sample and *n* arrays are used to study *n* samples. It is advisable to repeat the loop with the dye assignments reversed using the same sample (technical replicates) or employ a balanced block design by performing the loop with the biological replicates labelled with the reverse dyes to account for the general dye bias. Comparison of two samples far apart in the loop is inherently more variable in a loop design and is more susceptible to fail if there are two or more bad quality arrays. This can result in collapse of the loop, which would have to be solved by repeating the bad quality arrays (Dobbin and Simon, 2002).

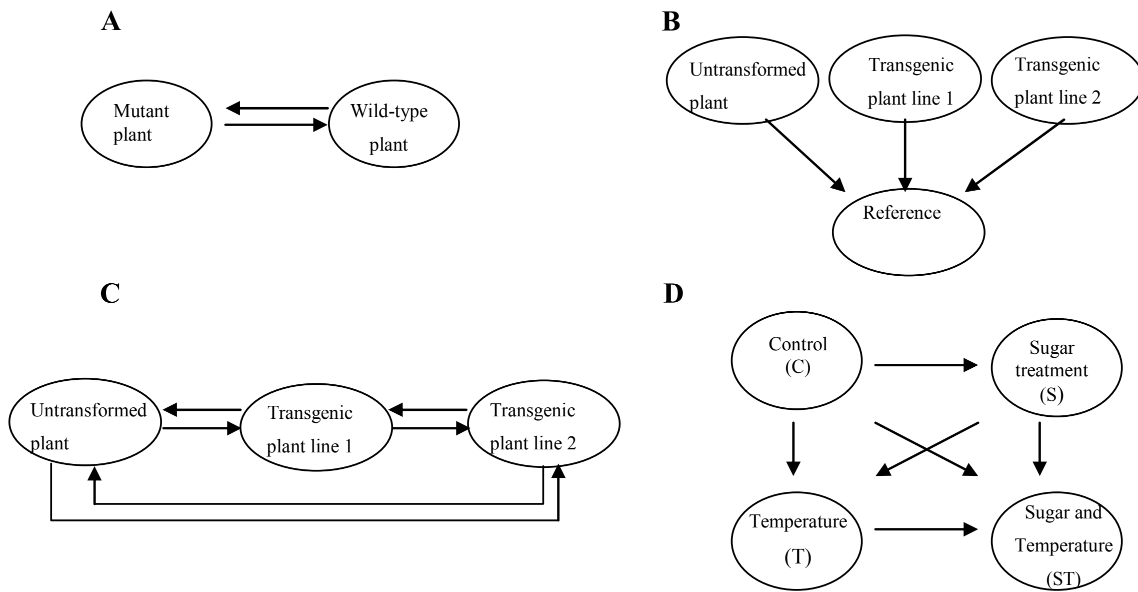

Figure 2.2. Diagrammatic representations of microarray experiment designs. The head of the arrow indicates that the sample was labelled with Cy5, while the tail represents a sample that is labelled with Cy3. A: direct comparison between a mutant and wild-type *Arabidopsis* plant. B: An indirect comparison using a reference design. C: A loop design investigating differentially expressed genes in transgenic plant lines. D: A factorial experiment investigating the interaction between two factors: temperature and sugar.

2.7. Factorial Experiments

The previous types of designs have been single factor experiments (for example, time, genotype, tissue type or treatment) but experiments investigating two or more factors require a more complex design. Factorial experiments can be used to study the expression profiles resulting from single factors or those resulting from the combined effect of two or more factors (Yang and Speed, 2003). For example, one may wish to investigate the growth of bacterial cells under two conditions: high sugar content and high temperature. Figure 2.2D illustrates the comparisons that can be made. Let C denote expression of the untreated control sample and S, the expression of samples grown in media containing high sugar and T, the expression of those samples grown at high temperature and ST, expression of bacteria treated with both factors simultaneously. Then, the impact on gene expression of sugar treatment (S) in the absence of the high temperature (T) can be assessed by log (S/C) and similarly the effect of high temperature can be estimated by log (T/C) in the absence of the effect of sugar treatment. The effect of the factor S in the presence of T is measured by log (ST/T) and a similar calculation can be made for factor T. The interaction of the two treatments, which is in

effect measuring the extent to which the differential expression of a gene induced by sugar is dependent on whether the high temperature (T) is present, is indicated by: log (ST/T)-log (S/C)= log (ST x C/T x S). The same experiment can be repeated with the samples labelled with reverse dyes, after which the data for the two experiments can be combined to normalise the dye bias. Subsequently, the same calculation can be performed to determine the treatment effect (Yang and Speed, 2003).

2.8. Time-course experiments

Several designs are possible for time-course experiments, but these depend on the comparisons of interest and the number of time points (Yang and Speed, 2002). Most studies are aimed at identifying co-regulated genes, which falls under gene class discovery (Dobbin and Simon, 2003). For example, in an experiment investigating the effect of ozone treatment on cells over time, the designs represented in Figure 2.3 could be used. The design in Figure 2.3A would be suitable if one were interested in the relative changes between time points two, three and four and the initial time point. However, if comparisons between consecutive time points are of interest, then a sequential comparison (Figure 2.3B) or a loop design (Figure 2.3C) may be more appropriate. A reference design could also be used (Figure 2.3D) but, like the loop design, would require four slides while designs A and B use three slides. However, the dye bias would have to be removed in a loop and sequential design, necessitating the use of more slides, with dye assignments reversed. Deciding between a reference or loop design is influenced by several factors, however, Kerr and Churchill (2001) provide ANOVA models to evaluate the micorarray design and assist in selecting a loop or reference design for particular experimental objectives. Vinciotti *et al*. (2004) evaluated a loop versus a reference design in two sets of microarray experiments and concluded that the loop design attained a higher precision than the reference. The authors advise how simple loop designs can be extended to more realistic experimental designs.

Table 2.2 lists examples of different types of microarray experimental design employed by different researchers. The degree of replication, number of slides and aim of the experiment are included. As the examples suggest, specific designs are more appropriate for different studies and a valuable practice is to formulate the specific question one wishes to answer at the outset of the experiment.

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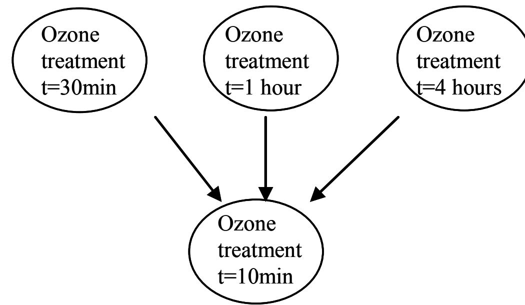
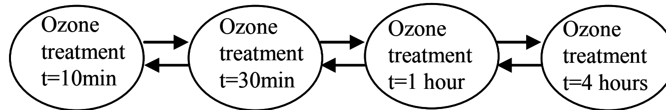
A lides required



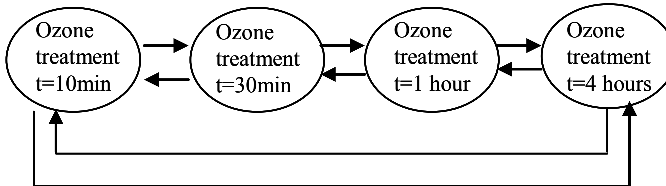
3 slides (no dye reversal required)
6 slides for 1 biological replicate

B



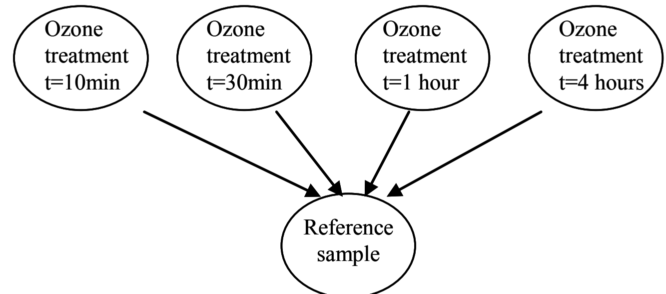
6 slides (direct comparison with a dye swap)
6 slides (balanced block design with 1 biological replicate)

C



8 slides (direct comparison with a dye swap)
8 slides (balanced block design with 1 biological replicate)
16 slides (direct comparison, dye swap and 1 biological replicate)

D



4 slides (no dye reversal required)
8 slides for 1 biological replicate

Figure 2.3. Possible designs and the minimum number of slides required for a time course experiment. Design (A) uses the first time-point as a reference while design (B) is a comparison between consecutive time points. A Loop design is indicated in (C) and a reference design in (D). Each box represents a sample while the arrow represents a slide. The head and tail of the arrows correspond to samples labelled with Cy5 and Cy3 dyes respectively.

Table 2.2. Some published examples of microarray designs.

Design	*Type of study*	Question	Replication	Number of Slides	Reference
Direct comparison	Single-factor experiment Class comparison	Which genes are differentially expressed genes in *Phythophthora infestans* infected leaves of *Arabidopsis*?	2 spots/gene/slide 2 technical replicates Individual plants pooled (no biological replication) Dye swap: yes	4	Huitema *et al*., 2003
Loop	Two-factor experiment Class comparison	How many genes are differentially expressed within and between natural populations of teleost fish?	2 spots/gene/slide 1 technical replicate Individual fish compared (15 biological replicates) Dye swap: yes	60	Oleksiak *et al*., 2002
Balanced Block in a loop design	Single-factor experiment Class comparison	Which genes are differentially expressed in parasitic and infectious larval stages of the common canine parasite *Ancylostoma caninum*?	2 spots/gene/slide 1 technical replicate 2 strains assessed (1 biological replicate) Dye swap: no	24	Moser *et al*., 2005
Reference	Single-factor experiment Class comparison	Which genes are preferentially expressed in the retina?	2 technical replicates (Minimum of 1 biological replicate) Dye swap: no	18	Chowers *et al*., 2003

2.9. Sample size

A question facing researchers during design consideration is the number of slides to use for a particular experiment. In microarray experiments, the variance of the relative expression levels across hybridisations varies greatly across genes, so sample size is a difficult question to address (Yang and Speed, 2003). Power analysis can be used to determine the number of replicates required in an experiment given that an estimate of the technical variability is known (Stekel., 2003).

A common approach is to consider a null hypothesis for every gene in a microarray experiment. For example, in the experiment in Figure 2.2A, the null hypothesis could be that a given gene is not differentially expressed between the mutant and wild-type plant. In this type of class comparison experiment, one would be interested in identifying those genes that do not adhere to the null hypothesis. False positives would be genes identified as being differentially expressed when they are not, whereas false negatives would be genes that are identified as not differentially expressed when they actually are. False positive results, where the null hypothesis is rejected when it is true, may be referred to as type I errors. The confidence of a statistical test is the probability of not getting a false positive result (i.e. the probability of accepting the null hypothesis when it is true). False negative results, where the null hypothesis is accepted when it is false, are called type II errors. The power of a statistical test is the probability of not getting a false negative result (i.e. the probability of not accepting the null hypothesis when it is false). While type I errors can be controlled explicitly when a significance level for the statistical test is selected (e.g. 1% significance threshold), type II errors are controlled implicitly via the experimental design. The power of an experiment relies on the number of replicates used. Thus, the number of replicates one chooses is determined by the power one wishes to attain in the analysis (Stekel., 2003). When a more stringent significance threshold is set, greater confidence but less power is achieved, and, conversely, a lower significance threshold means less confidence and greater power. Depending on the experiment in question, one can judge as to whether a type I or a type II error is more acceptable. For example, if the purpose of the experiment is to identify possible genes involved in disease resistance to a certain plant pathogen and much time and money will be subsequently spent researching each chosen candidate gene, then it is more important that type I errors (false positives) are avoided. However if the microarray is being used as a diagnostic tool for cancer, then type II errors (false negatives) are less desirable as a patient falsely diagnosed as being cancer-free could develop a fatal tumour, which would have been

otherwise treated (Stekel., 2003). The reader is directed to Stekel (2003), Tempelman (2005) and Zien *et al*. (2002); useful sources that help one determine the number of microarrays needed to ascertain differential gene expression.

2.10. Analysis

The particulars of analysis have not been discussed in this review, however more software is emerging which is open-source, user-friendly and can be applied to various methods of microarray design. These include TM4 microarray software suite (http://www.tigr.org/software/tm4), Gene Expression Pattern Analysis Suite GEPAS (http://gepas.bioinfo.cnio.es) and Bioconductor (www.bioconductor.org) and R (www.r-project.org).

Whilst this review aims to give non-statisticians an overview of how to approach microarray experimental design and suitable design parameters for particular types of experiment, it is advisable to enlist the assistance of a statistician at the very beginning of your microarray experiment. Expertise in this area is growing as microarray technology generates more interest among statisticians. It is encouraging to note that the capacity for successful completion of microarray experiments exists in South Africa. The quality of publications generated from both local microarray facilities attest to this.

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

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3.1. Abstract

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

3.2. Introduction

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

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

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

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

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

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

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

3.3. Materials and Methods

Plant Growth

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

Microarray experiments

Experimental Design

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

Array Design

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

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

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

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

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

Samples

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

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

Hybridisations

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

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

Data Analysis

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

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

Northern blot analysis

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

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

Quantitative reverse-transcription PCR (qRT-PCR)

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

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

Data comparison and Hypothesis testing

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

3.4. Results

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

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

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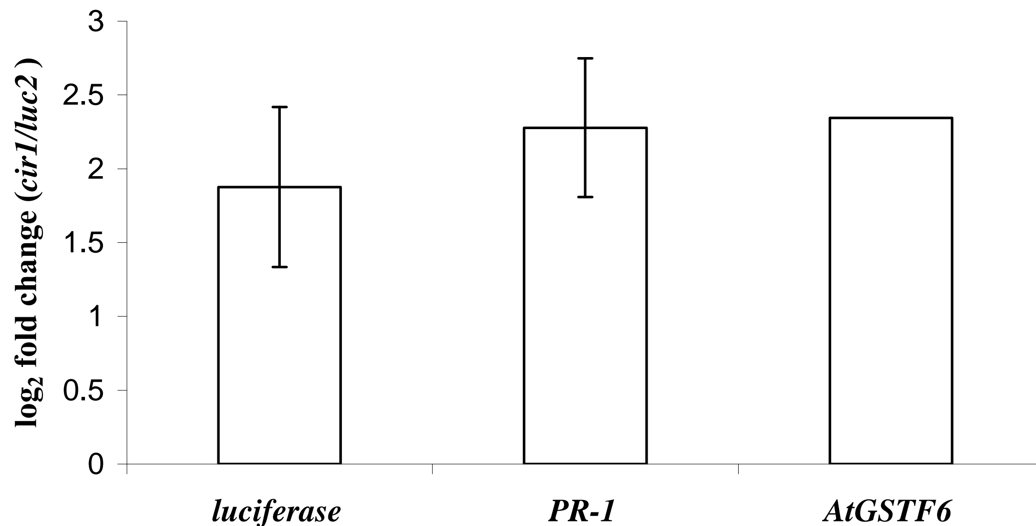

Figure 3.1. Expression of defence marker genes in *cir1* and *luc2* plants. Expression is represented by qRT-PCR data for *PR-1* and the luciferase reporter gene, while Northern blot analysis data from a single experiment, quantified by densitometry, is represented for *AtGSTF6*. Error bars represent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

Expression profiling of cir1 and luc2

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

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

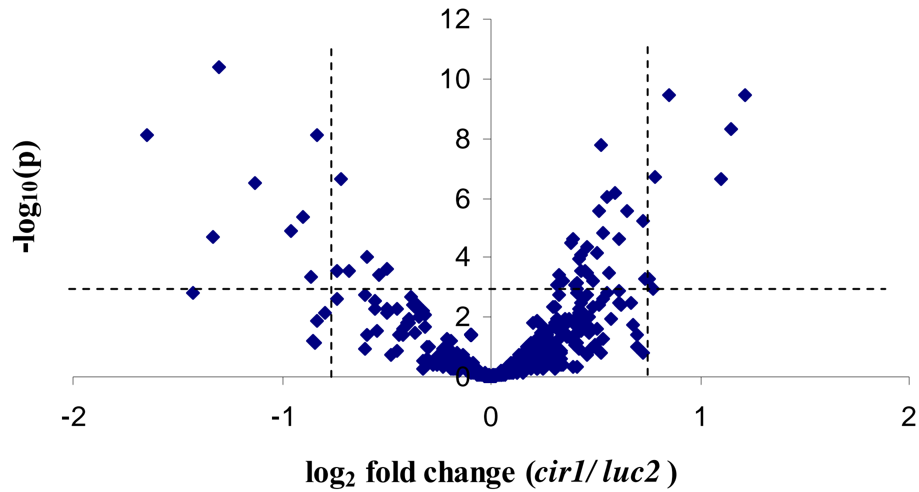

Figure 3.2. A volcano plot generated in SAS v8.2 of microarray data comparing expression in *cir1* and the *luc2*. Those ESTs with a p-value greater than $-\log_{10}(p)=3$ (p=0.001) and a $\log_2$ fold change greater than 0.75 or less than –0.75 were selected as differentially expressed.

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

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

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

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

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

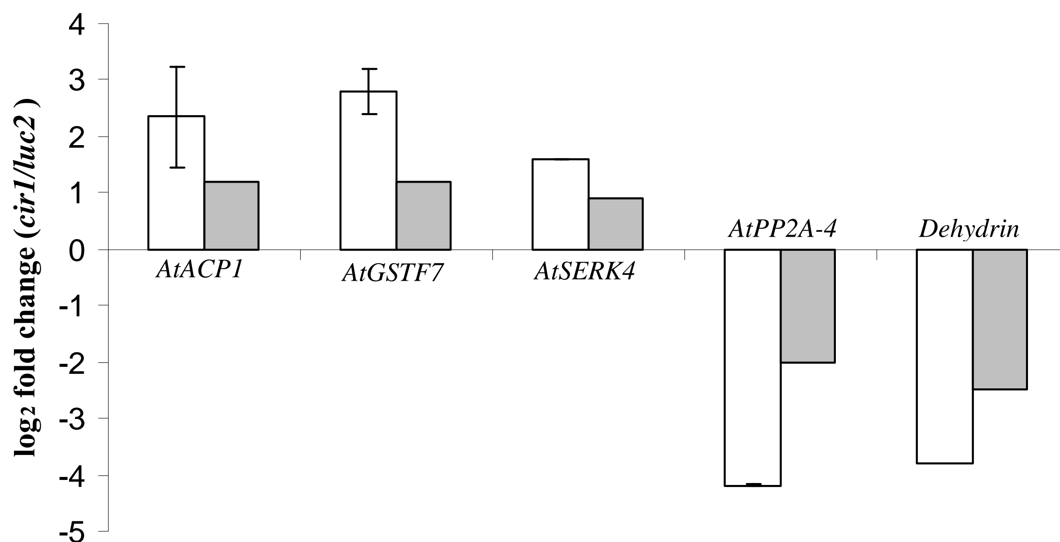

Figure 3.3. Confirmation of differential expression in *cir1* versus *luc2* plants observed in microarray studies. qRT-PCR results are shown for *AtACP1, AtGSTF7*, *AtSERK4* and *AtPP2A-4* (white bars). Northern blot analysis was performed for the dehydrin gene and quantified by densitometry (the result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

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

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

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

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

Gene Name	GO Biological Process	*cir1**	COMPATIBLE		INCOMPATIBLE		
			Pst 6hpi[1]	*Pst* 24hpi[1]	*Pst*(*avrB*) 6hpi[1]	*Pst*(*avrB*) 24hpi[1]	*Pst* (*avrRpt2*)[2]
PR-1	SAR, defence response	Induced	Induced	Induced	Induced	Induced	Induced
PR-2	SAR, response to cold	Induced	No change	Induced	Induced	Induced	Induced
PR-5	Regulation of anthocynin biosynthesis, SAR, response to UV-B.	Induced	Induced	Repressed	No change	Repressed	Induced
PDF1.2	Jasmonic acid and ethylene, insect & wound response	Induced	Induced	Repressed	Repressed	Repressed	X
AtGSTF6	Response to water deprivation andoxidative stress.	Induced	X	X	X	X	Induced
AtGSTF7	Toxin catabolism	Induced	X	X	X	X	Induced
AtP2C-HA	Protein amino acid dephosphorylation	Induced	No change	Induced	Induced	Induced	X
AtACP1	Hyperosmotic salinity response	Induced	Repressed	Induced	Induced	Induced	X
AtPAL1	Phenylpropanoid biosynthesis, wounding & oxidative stress	Induced	Repressed	Induced	Induced	Induced	Induced
tryptophan synthase -like	Tryptophan biosynthesis	Induced	No change	Induced	Induced	Induced	X
AtSERK4	Protein amino acid phosphorylation	Induced	No change	No change	No change	No change	Repressed
thioesterase	Unknown	Induced	No change	No change	No change	No change	X
AtEREBP-4	Regulation of transcription	Repressed	No change	No change	Repressed	Repressed	Repressed
HFR1	Signal transduction	Repressed	Repressed	No change	No change	Repressed	X
jacalin lectin	Response to cold	Repressed	Repressed	Induced	Repressed	Induced	Induced
AtDREB2A	Response to water deprivation	Repressed	No change	Induced	Induced	Induced	Induced
dehydrin	Response to ABA stimulus, cold acclimation, water deprivation.	Repressed	No change	Induced	Induced	Induced	X
AtMYB47	Jasmonic acid stimulus, response to salt stress	Repressed	Induced	Repressed	Induced	Repressed	X
AtPP2A-4	Protein amino acid dephosphorylation	Repressed	X	X	X	X	X
EF1-α	Translational elongation	Repressed	X	X	X	X	X

*expression compared to *luc2*

X No Data | Repressed | No Change | Induced

3.5. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4.1 Abstract

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

4.2 Introduction

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

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

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

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

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

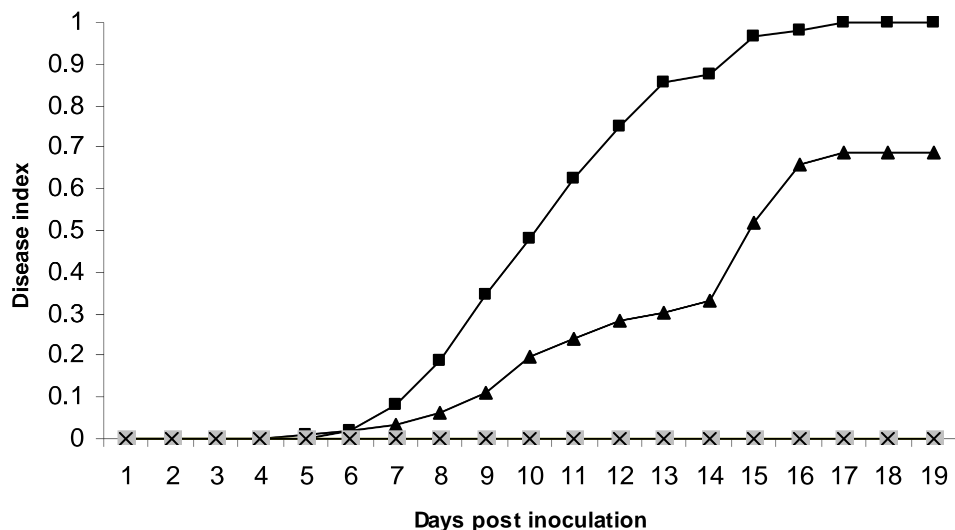

Figure 4.1. Disease index for Arabidopsis ecotypes infected with strains of *R. solanacearum.* Ecotype Col-5 infected with *R. solanacearum* ecotype GMI1000 (■), ecotype Col-5 infected with isolate BCCF 401 (▲), ecotype Nd-1 after challenge with GMI1000 (■) and ecotype Nd-1 after challenge with BCCF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

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

4.3 Materials and Methods

4.3.1 Plant material

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

4.3.2 Inoculations

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

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

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

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

4.3.3 RNA isolation

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

4.3.4 Microarray experiments

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

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

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

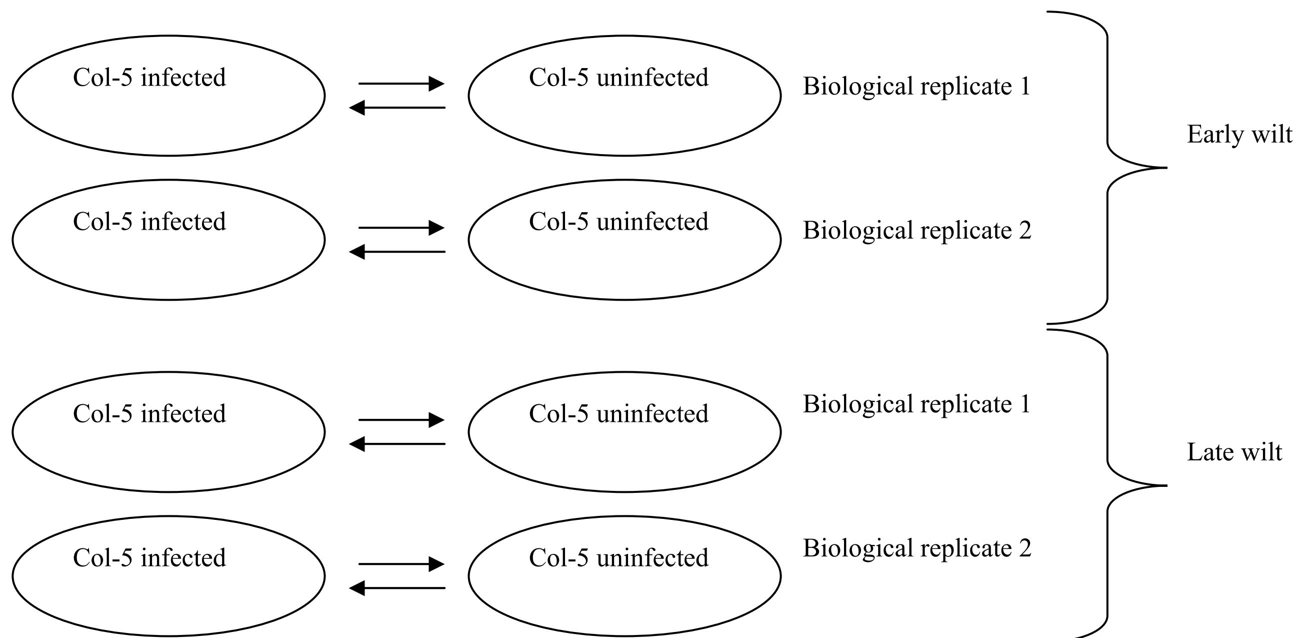

Figure 4.2 Experimental Design employed in microarray expression profiling between Col-5 plants infected with *R. solanacearum* BCCF 401 and Col-5 plants that were uninfected at two time-points: early wilt and late wilt. Each oval represents a sample. The arrows represent a slide and the head of the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

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

4.3.5 Quantitative reverse-transcription PCR (qRT-PCR)

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

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

4.3.6 Gene ontologies

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

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

4.3.7 Data comparison and hypothesis testing

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

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

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

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

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

4.4 Results

4.4.1 R. solanacearum infections

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

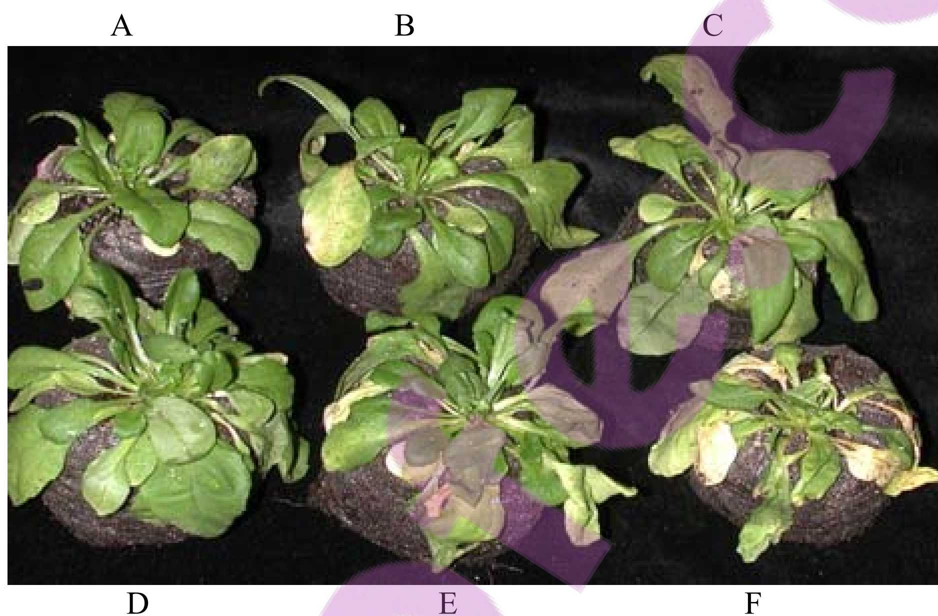

Figure 4.3 Wilt symptoms on Col-5 inoculated with $1X10^8$ cfu/ml of *R. solanacearum* isolate K using a root-inoculation method. The control plants were inoculated with a suspension of media and water. A: healthy control plant, no wilt symptom; plant showing wilt symptom B: 0.5; C: 1; D: 2; E: 3 and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

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

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

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

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

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

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

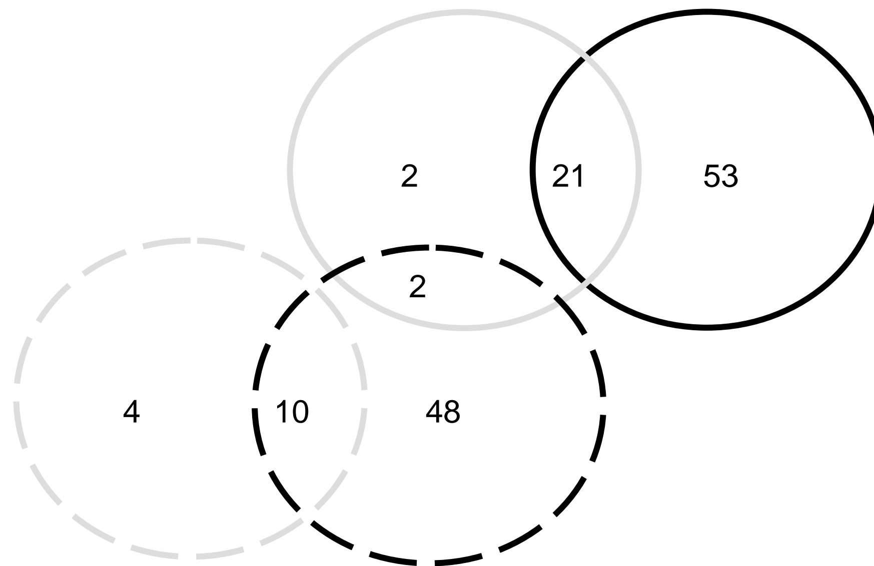


Figure 4.4. Venn diagram representing up-regulated (solid lines) and down-regulated (dashed lines) gene sets in early (grey circles) and late wilt stages (black circles) in response to *R. solanacearum* BCCF 401 infection. Genes were selected following mixed model ANOVA analysis and only those genes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

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

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

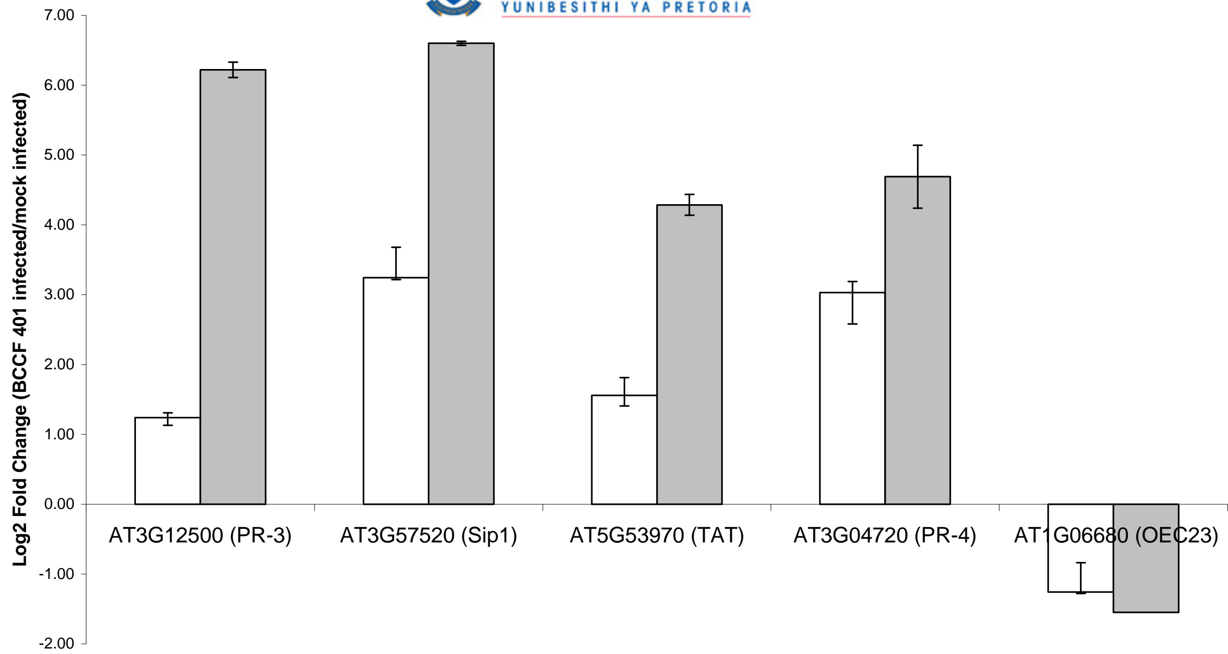
4.4.3 ***Confirmation of Microarray Data***

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

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

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

A



B

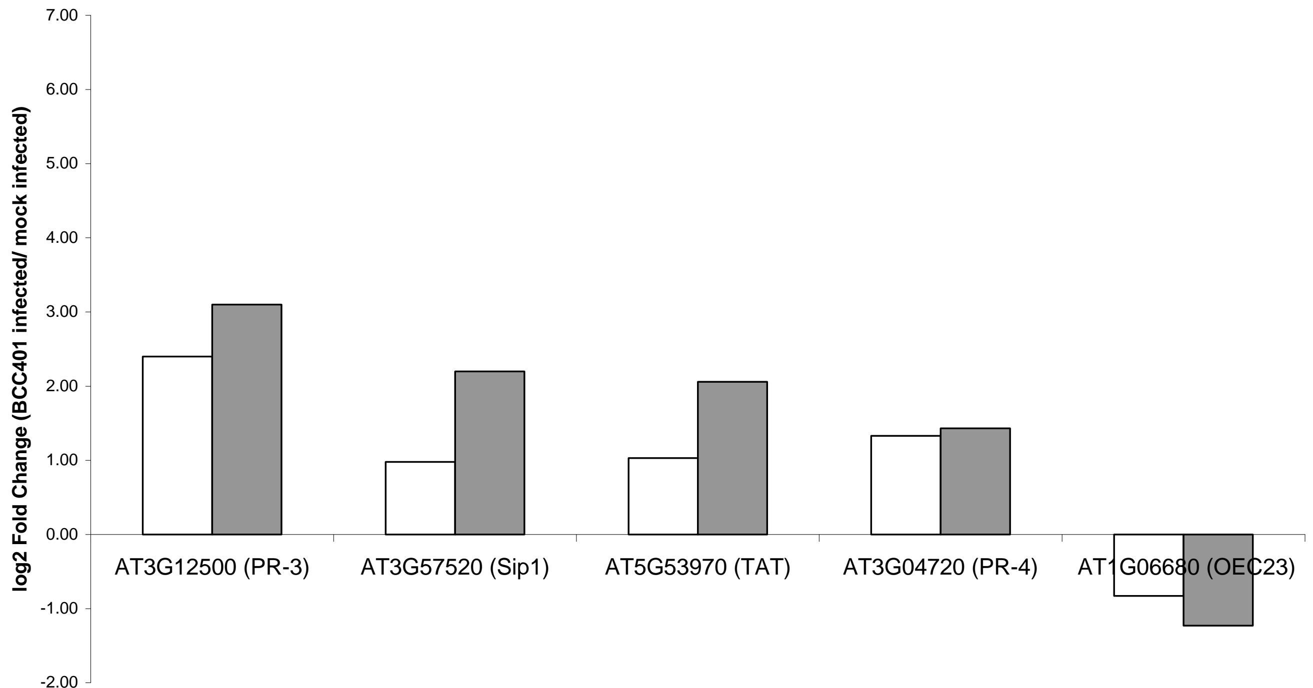


Figure 4.5 Expression data for selected Arabidopsis genes during *R. solanacearum* BCCF 401 infection relative to mock-inoculations at the same time-points. A) qRT-PCR results and B) microarray results. The gene expression for the up-regulated genes basic endochitinase (PR-3), Seed imbibition protein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

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

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

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

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

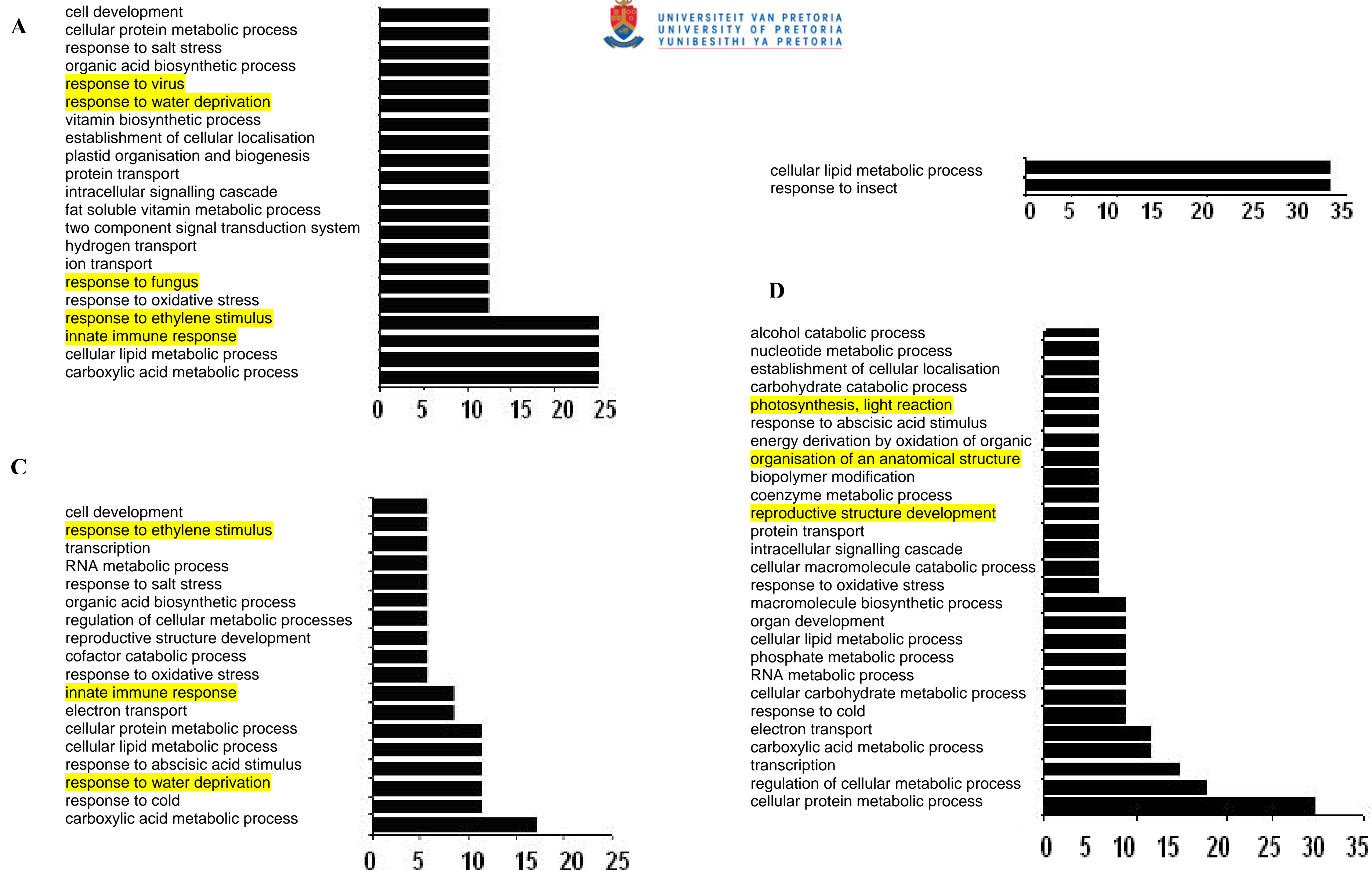


Figure 4.6 GO, biological process, categorization of Arabidopsis genes differentially regulated after inoculation with *R. solanacearum* BCCF 401 using FatiGo at level 5. A) up-regulated at early wilt (27 genes), B) down regulated at early wilt (14 genes), C) up-regulated at late wilt (69 genes), D)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

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

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

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

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

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

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

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

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

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

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

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

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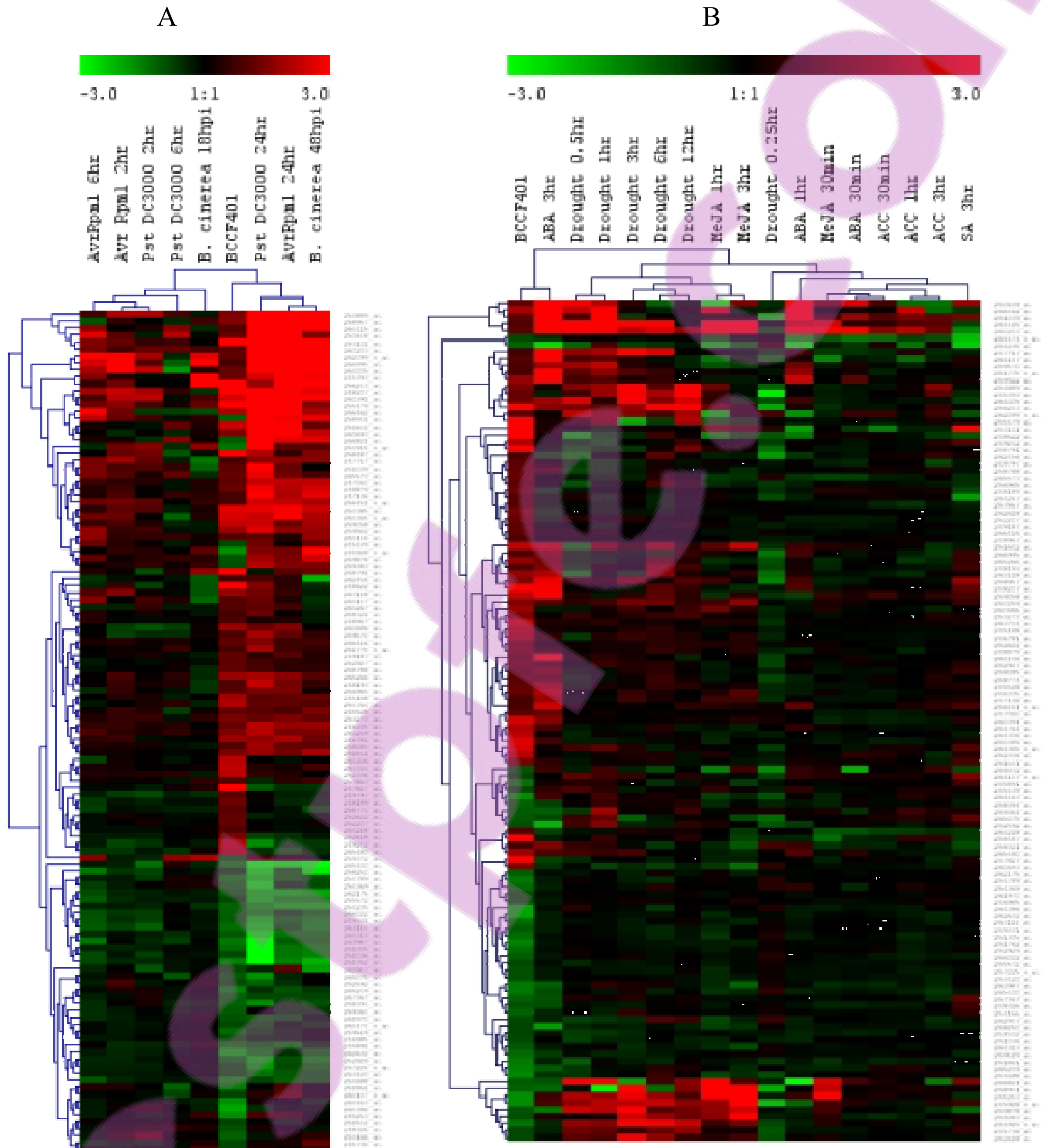

Figure 4.7 Hierarchical clustering (complete linkage, Manhattan distance) of the 120 Arabidopsis genes that are differentially regulated during *R. solanacearum* infection 7 days after infection across various different conditions: (A) *Pst and B. cinerea* infection (B), drought stress and hormone treatment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

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

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

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

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

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

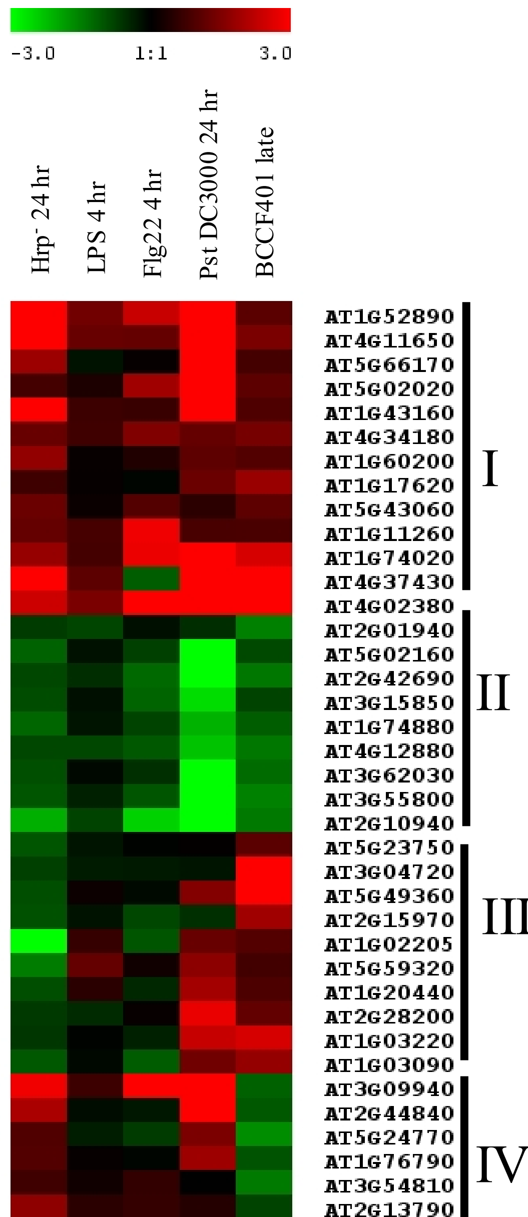

Figure 4.8 Arabidopsis genes showing basal defence response against *R. solanaceearum* in comparison to *Pst* DC3000 and PAMP-induced genes. Cluster I are genes induced by PAMPs and effectors, cluster II are genes which are repressed by PAMPs and effectors, cluster III are genes which are repressed by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

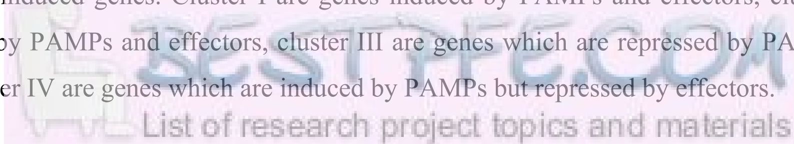
List of research project topics and materials

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

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

4.4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

4.5. References

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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*.

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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.

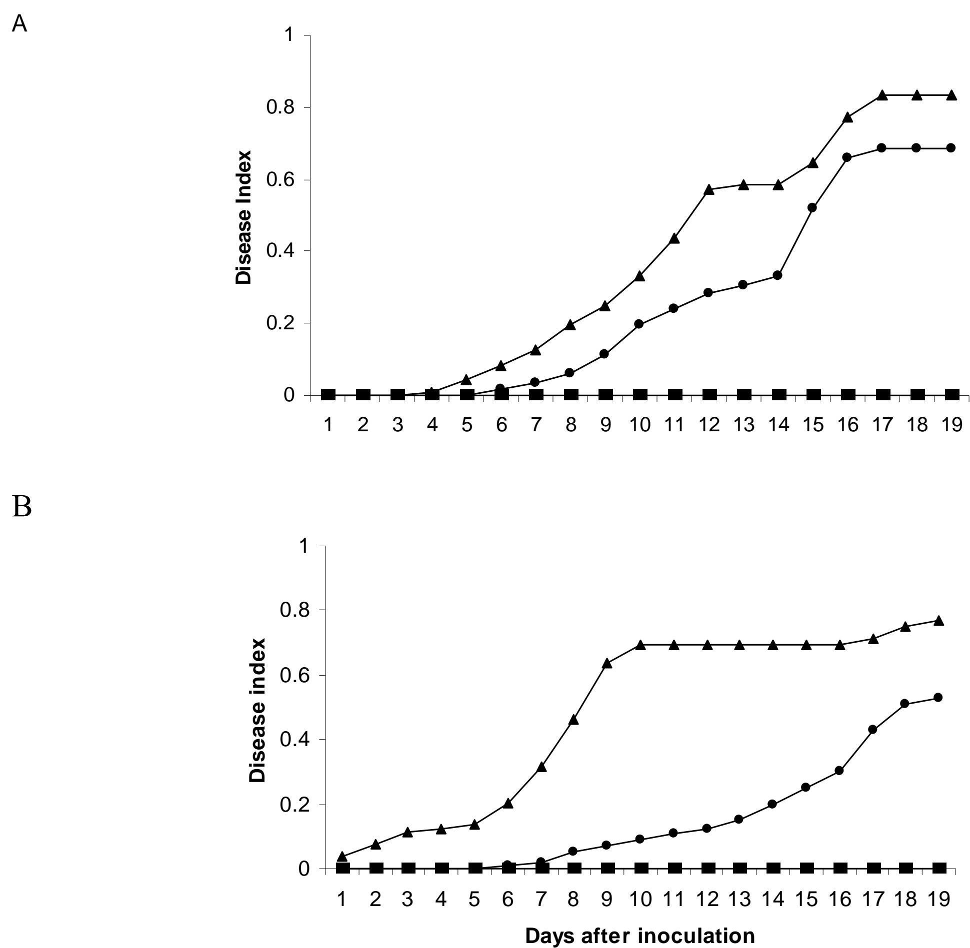

Figure 5.1 Disease index of ecotypes Col-5 (●), Kil-0 (■) and Be-0 (▲) for 19 days after infection with *R. solanacearum* isolates A) BCCF 401 and B) BCCF 402. The data shown has been derived from a single infection trial in which seven independent plants per ecotype were infected with each bacterial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

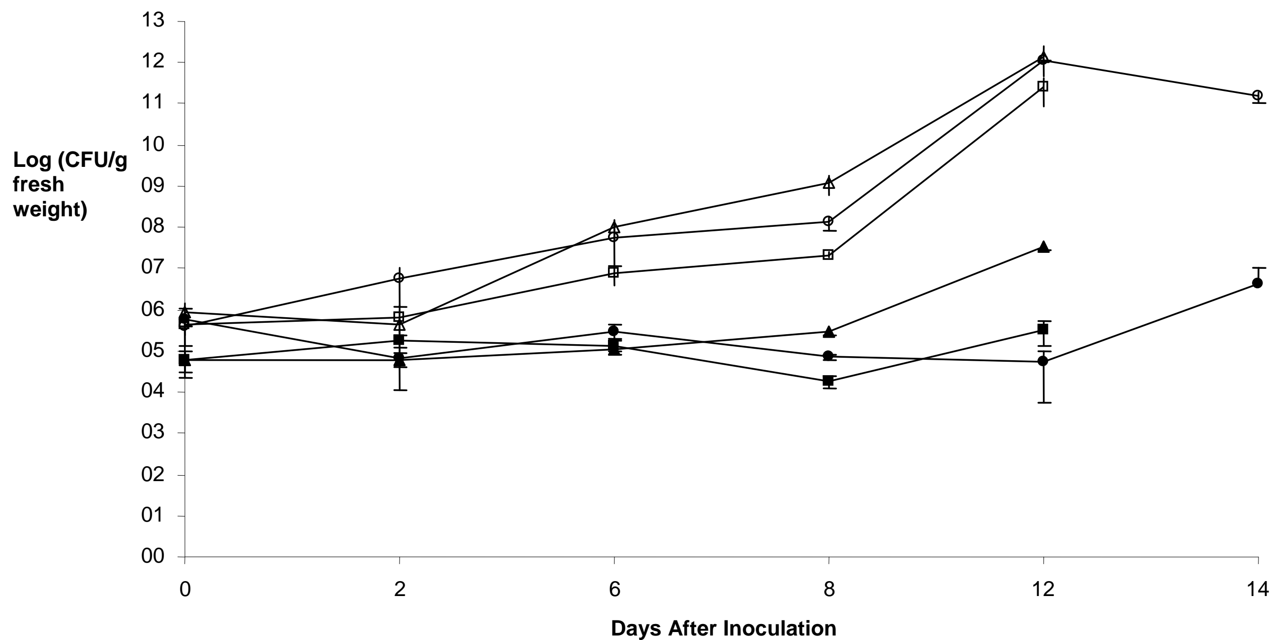

Figure 5.2 Internal bacterial growth curves for BCCF 402 strains in Arabidopsis leaves following root inoculation. Ecotypes Col-5 (○), Kil-0 (□) and Be-0 (Δ) were infected with the rifampicin mutant of BCCF 402 and with the *hrp*⁻ mutant of BCCF 402, indicated in filled symbols: Col-5 (●), Kil-0 (■) 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°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).

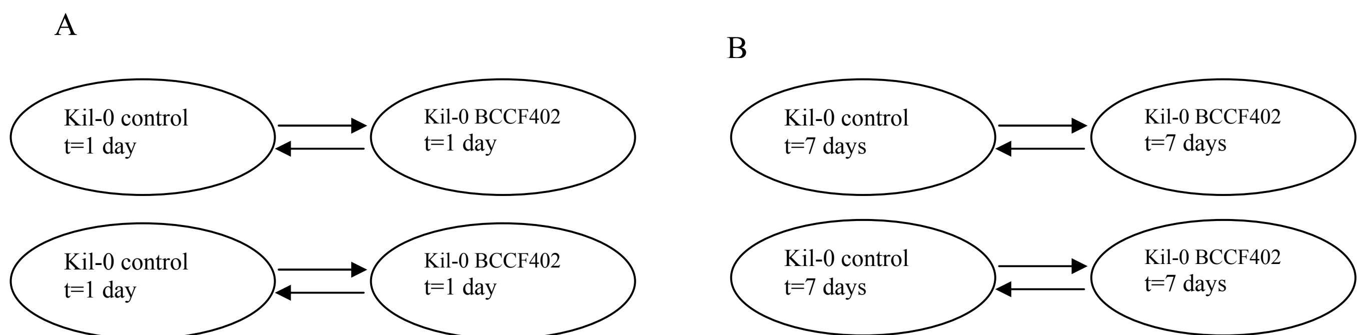

Figure 5.3. Oval and arrow representation of the experimental design employed in microarray comparisons between Arabidopsis ecotype Kil-0 infected with *R. solanacearum* isolate BCCF 401 and Kil-0 plants which were uninfected at one (A) and seven days (B) after infection respectively. Ovals represent 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 50^0C for 10 sec over the water vapour. The slides are snap-dried on a heating block at 65^oC 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°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°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.

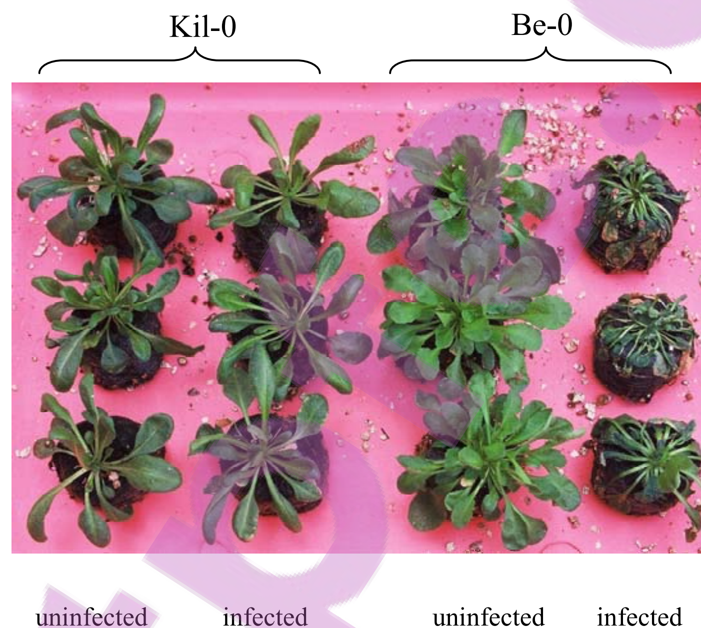

Figure 5.4. The result of BCCF 402 infection on ecotypes Kil-0 and Be-0 after one week compared to uninfected plants. Infected Be-0 plants (right) become wilted while infected Kil-0 plants show no wilt symptoms.

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.

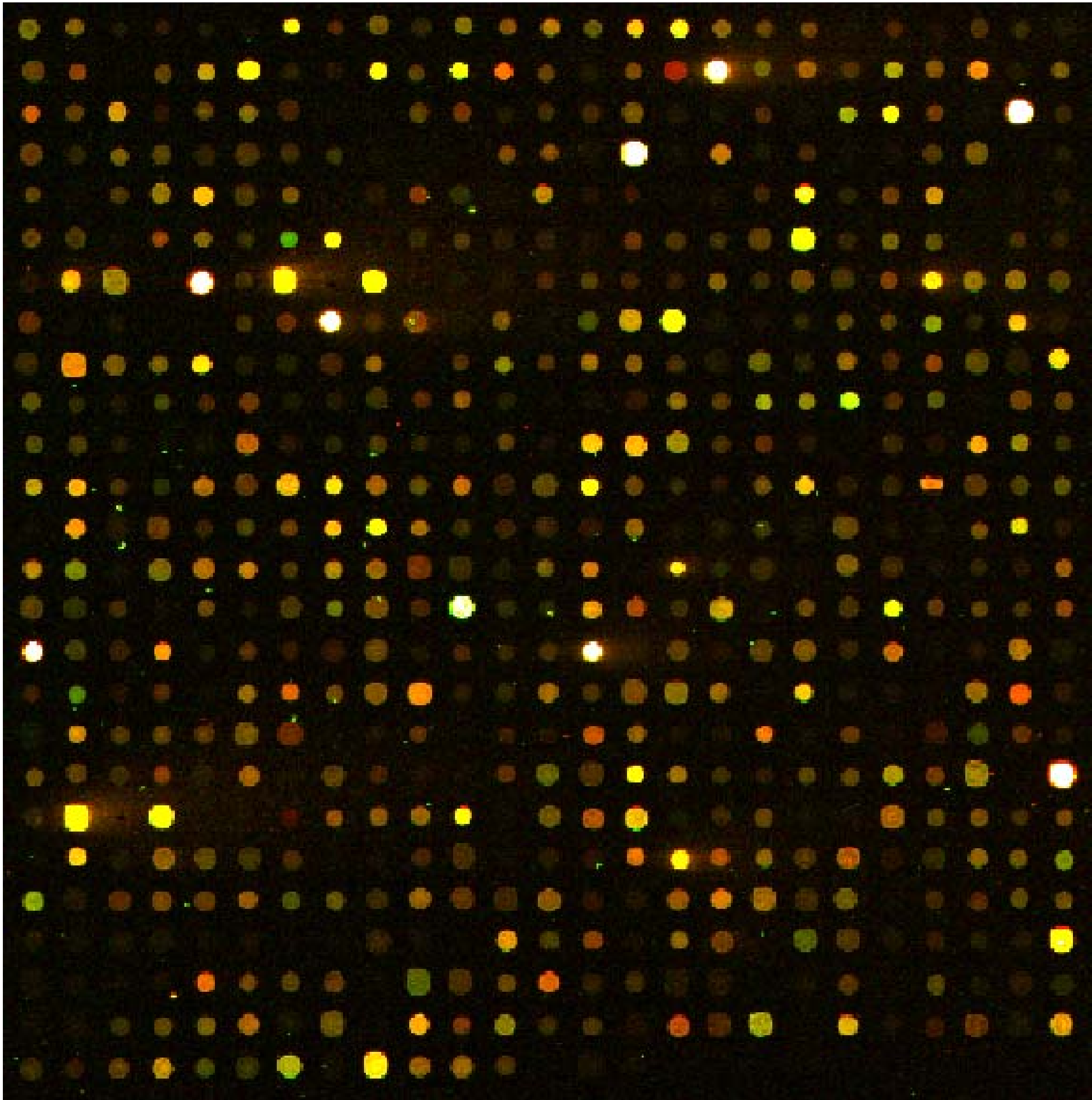

Figure 5.5 Image of section of Arabidopsis thaliana 29K microarray slide after hybridisation to Kil-0 infected material labelled with Cy5 (red) and Kil-0 uninfected material labelled with Cy3 (green) at t=7 days after inoculation Bright orange-to red spots are genes which are possibly induced by *R. 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.

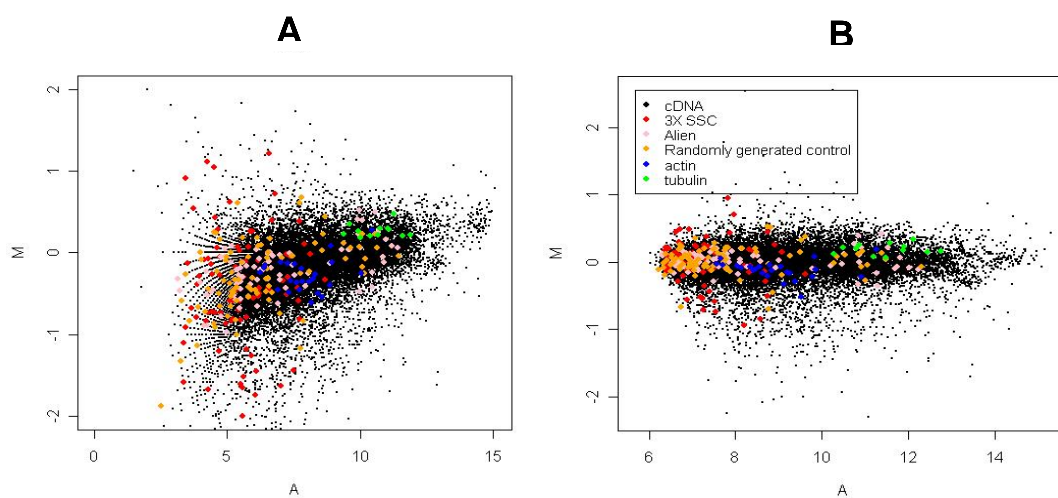

Figure 5.6 MA-plots for a microarray slide before (A) and after (B) print –tip loess normalisation. Before normalisation, there appears to be a bias towards the green dye however, after normalisation, the data becomes centred around zero.

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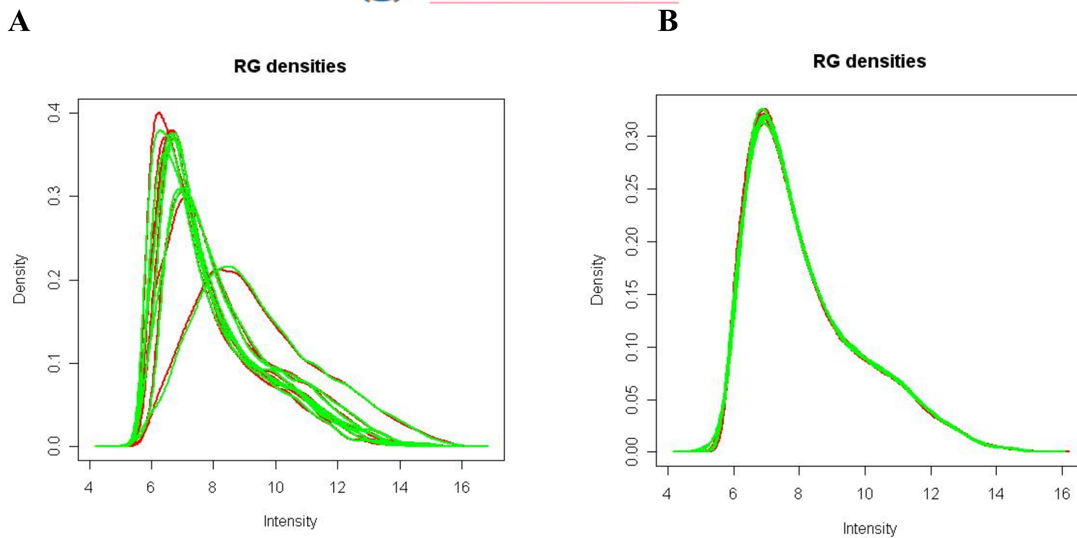

Figure 5.7 RG densities before (A) and after (B) between slide normalisation using A-quantile normalisation in the limma package in R version 2.1.1 (Bolstad et al., 2003; Smyth, 2004).

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 • serine-type endopeptidase inhibitor activity • translation initiation factor activity • peroxidase activity • oxidoreductase activity	• Oxygen and reactive oxygen species metabolic process • Response to bacterium • Response to fungus • Unidimensional cell growth • Translational initiation • 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.

LTP3

log2 ratio (infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

*

LTP4

log2 ratio (infected/ control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

LTP pseudogene

log2 ratio (infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

PRX34

log2 ratio (infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

SAG13

log2 ratio (infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

PPR

log2 ratio (infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

AIG

log2 ratio (infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

SUI1

log2 ratio(infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

SKP5

log fold change (infected/ control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

Expressed Protein

log2 ratio(infected/control)

Be-0 1dpi Be-0 4dpi Be-0 7dpi Kil-0 1dpi Kil-0 4dpi Kil-0 7dpi

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.

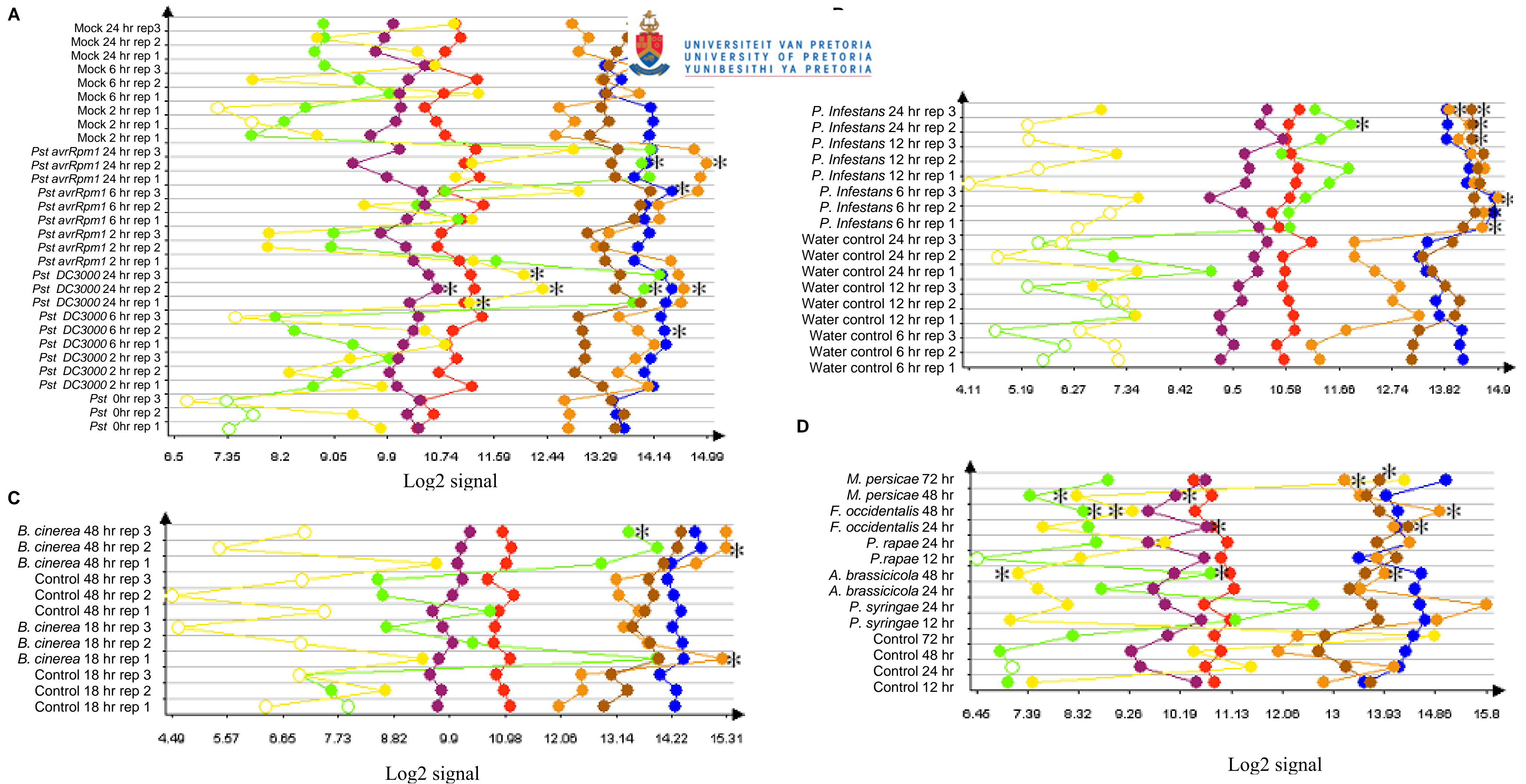

Figure 5.9 Expression profiles of 7 selected Arabidopsis genes during infection with A) *P. syringae,* B) *P. infestans* C) *B. cinerea* and D) during fungal (*A. brassicicola*; *F. occidentalis*), bacterial pathogen (*Pst DC3000*) and insect attack (*M. persicae*). Closed circles represent genes with 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

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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

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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

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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.

6.6 References

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APPENDIX A

Arabidopsis 5000 cDNA microarray elements

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AT5G16880 AT5G18430 AT5G19820 AT5G21060 AT5G22780 AT5G24300 AT5G26240 AT5G28150 AT5G37370 AT5G39670 AT5G41520 AT5G43280 AT5G44340 AT5G46190 AT5G47450 AT5G48570
AT5G17020 AT5G18600 AT5G19860 AT5G21090 AT5G22790 AT5G24320 AT5G26260 AT5G28300 AT5G37370 AT5G39950 AT5G41680 AT5G43320 AT5G44380 AT5G46210 AT5G47560 AT5G48580
AT5G17170 AT5G18620 AT5G19890 AT5G21105 AT5G22830 AT5G24460 AT5G26260 AT5G28500 AT5G37475 AT5G39990 AT5G41700 AT5G43330 AT5G44380 AT5G46210 AT5G47560 AT5G48630
AT5G17190 AT5G18630 AT5G19940 AT5G21170 AT5G22850 AT5G24490 AT5G26340 AT5G28500 AT5G37510 AT5G40020 AT5G41810 AT5G43370 AT5G44380 AT5G46230 AT5G47570 AT5G48800
AT5G17310 AT5G18650 AT5G19950 AT5G21222 AT5G23010 AT5G24490 AT5G26360 AT5G28500 AT5G37510 AT5G40150 AT5G41940 AT5G43450 AT5G44410 AT5G46250 AT5G47640 AT5G48810
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AT5G17380 AT5G18860 AT5G20030 AT5G21940 AT5G23060 AT5G24610 AT5G26742 AT5G28750 AT5G37690 AT5G40170 AT5G41990 AT5G43520 AT5G44680 AT5G46290 AT5G47680 AT5G48920
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AT5G17380 AT5G19000 AT5G20090 AT5G21990 AT5G23190 AT5G24760 AT5G26830 AT5G29000 AT5G37740 AT5G40230 AT5G42000 AT5G43700 AT5G44790 AT5G46440 AT5G47710 AT5G49100
AT5G17420 AT5G19030 AT5G20150 AT5G22000 AT5G23210 AT5G24770 AT5G26860 AT5G30495 AT5G37770 AT5G40270 AT5G42020 AT5G43710 AT5G44820 AT5G46500 AT5G47720 AT5G49160
AT5G17440 AT5G19120 AT5G20160 AT5G22030 AT5G23210 AT5G24800 AT5G26980 AT5G30510 AT5G38030 AT5G40390 AT5G42020 AT5G43750 AT5G44860 AT5G46630 AT5G47730 AT5G49210
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AT5G49570	AT5G51400	AT5G52920	AT5G54590	AT5G56010	AT5G57580	AT5G58950	AT5G60530	AT5G61670	AT5G63590	AT5G64470	AT5G66320
AT5G49650	AT5G51430	AT5G52960	AT5G54600	AT5G56020	AT5G57625	AT5G58990	AT5G60570	AT5G61730	AT5G63590	AT5G64570	AT5G66390
AT5G49660	AT5G51490	AT5G53000	AT5G54660	AT5G56030	AT5G57655	AT5G59010	AT5G60580	AT5G61780	AT5G63600	AT5G64740	AT5G66400
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AT5G49665	AT5G51570	AT5G53140	AT5G54730	AT5G56130	AT5G57710	AT5G59090	AT5G60600	AT5G61970	AT5G63620	AT5G65110	AT5G66420
AT5G49770	AT5G51680	AT5G53160	AT5G54740	AT5G56170	AT5G57710	AT5G59150	AT5G60640	AT5G62050	AT5G63790	AT5G65170	AT5G66450
AT5G49810	AT5G51830	AT5G53180	AT5G54770	AT5G56280	AT5G57800	AT5G59160	AT5G60640	AT5G62090	AT5G63840	AT5G65180	AT5G66580
AT5G49820	AT5G51840	AT5G53180	AT5G54810	AT5G56350	AT5G57900	AT5G59160	AT5G60660	AT5G62150	AT5G63850	AT5G65210	AT5G66675
AT5G49940	AT5G51890	AT5G53310	AT5G54960	AT5G56360	AT5G57900	AT5G59210	AT5G60660	AT5G62190	AT5G63890	AT5G65380	AT5G66690
AT5G49945	AT5G52040	AT5G53340	AT5G55070	AT5G56500	AT5G58030	AT5G59290	AT5G60680	AT5G62330	AT5G63890	AT5G65480	AT5G66760
AT5G49980	AT5G52180	AT5G53350	AT5G55120	AT5G56550	AT5G58070	AT5G59300	AT5G60700	AT5G62390	AT5G63910	AT5G65490	AT5G66810
AT5G50150	AT5G52200	AT5G53370	AT5G55130	AT5G56590	AT5G58130	AT5G59320	AT5G60790	AT5G62460	AT5G63910	AT5G65520	AT5G66850
AT5G50200	AT5G52240	AT5G53370	AT5G55210	AT5G56650	AT5G58160	AT5G59440	AT5G60790	AT5G62570	AT5G64040	AT5G65590	AT5G66860
AT5G50250	AT5G52280	AT5G53420	AT5G55220	AT5G56670	AT5G58240	AT5G59450	AT5G60800	AT5G62610	AT5G64050	AT5G65620	AT5G66900
AT5G50320	AT5G52310	AT5G53420	AT5G55280	AT5G56750	AT5G58250	AT5G59450	AT5G60950	AT5G62650	AT5G64080	AT5G65710	AT5G66910
AT5G50450	AT5G52420	AT5G53460	AT5G55290	AT5G56840	AT5G58260	AT5G59550	AT5G61020	AT5G62680	AT5G64080	AT5G65750	AT5G66910
AT5G50560	AT5G52430	AT5G53460	AT5G55390	AT5G56890	AT5G58290	AT5G59710	AT5G61020	AT5G62720	AT5G64090	AT5G65760	AT5G66950
AT5G50600	AT5G52440	AT5G53490	AT5G55480	AT5G56940	AT5G58290	AT5G59780	AT5G61190	AT5G62790	AT5G64100	AT5G65840	AT5G67030
AT5G50740	AT5G52470	AT5G53530	AT5G55500	AT5G56950	AT5G58290	AT5G59780	AT5G61230	AT5G62890	AT5G64100	AT5G65960	AT5G67160
AT5G50760	AT5G52510	AT5G53900	AT5G55540	AT5G56980	AT5G58330	AT5G59790	AT5G61270	AT5G63110	AT5G64120	AT5G66010	AT5G67250
AT5G50780	AT5G52510	AT5G53900	AT5G55610	AT5G57020	AT5G58375	AT5G59850	AT5G61410	AT5G63160	AT5G64130	AT5G66040	AT5G67290
AT5G50850	AT5G52510	AT5G53970	AT5G55630	AT5G57040	AT5G58420	AT5G59870	AT5G61410	AT5G63190	AT5G64200	AT5G66052	AT5G67290
AT5G50920	AT5G52530	AT5G54080	AT5G55710	AT5G57050	AT5G58500	AT5G59960	AT5G61500	AT5G63190	AT5G64260	AT5G66090	AT5G67300
AT5G51010	AT5G52540	AT5G54145	AT5G55730	AT5G57120	AT5G58530	AT5G59960	AT5G61500	AT5G63190	AT5G64260	AT5G66120	AT5G67380
AT5G51040	AT5G52820	AT5G54290	AT5G55730	AT5G57120	AT5G58575	AT5G59970	AT5G61530	AT5G63200	AT5G64310	AT5G66140	AT5G67420
AT5G51070	AT5G52840	AT5G54370	AT5G55850	AT5G57150	AT5G58620	AT5G59980	AT5G61590	AT5G63260	AT5G64350	AT5G66160	AT5G67440
AT5G51110	AT5G52840	AT5G54370	AT5G55930	AT5G57340	AT5G58640	AT5G60040	AT5G61600	AT5G63330	AT5G64380	AT5G66170	AT5G67440
AT5G51120	AT5G52880	AT5G54540	AT5G55940	AT5G57345	AT5G58730	AT5G60300	AT5G61600	AT5G63400	AT5G64430	AT5G66230	AT5G67480
AT5G51130	AT5G52900	AT5G54570	AT5G56000	AT5G57370	AT5G58787	AT5G60390	AT5G61650	AT5G63460	AT5G64440	AT5G66240	AT5G67630

APPENDIX B

Differentially regulated Col-5 genes

Table of Arabidopsis Col-5 genes differentially regulated (fold change > 0.75; p<0.03, Bonferroni adjusted) after infection with BCCF 401 compared to uninfected plants.

	Up regulated Early wilt	Up regulated Late wilt	Up regulated Early & late wilt	Down regulated Early wilt	Down regulated Late wilt	Down regulated Early & late wilt
1	AT1G47830.1	AT1G03090.1	AT1G06570.1	AT1G75040.1	AT1G03130.1	AT1G06680.1
2	AT1G51670.1	AT1G03220.1	AT1G31130.1	AT1G04250.1	AT1G10150.1	AT1G70410.2
3	AT3G10630.1	AT1G11260.1	AT1G32450.1	AT3G28300.1	AT1G12000.1	AT1G73330.1
4	AT3G28710.1	AT1G17620.1	AT1G78890.1	AT5G05690.1	AT1G12270.1	AT2G44840.1
5		AT1G20440.1	AT2G33150.1		AT1G13260.1	AT3G14210.1
6		AT1G43160.1	AT2G42890.2		AT1G47830.1	AT3G22231.1
7		AT1G52890.1	AT3G01420.1		AT1G51670.1	AT3G28300.1
8		AT1G60200.1	AT3G04720.1		AT1G55450.1	AT4G15440.1
9		AT1G72770.1	AT3G12500.1		AT1G65960.1	AT5G24770.1
10		AT1G74020.1	AT3G57520.2		AT1G69120.1	AT5G61650.1
11		AT1G75170.3	AT4G11650.1		AT1G74880.1	
12		AT2G01340.1	AT4G13250.1		AT1G75750.1	
13		AT2G15970.1	AT4G16260.1		AT1G76790.1	
14		AT2G22470.1	AT4G37430.1		AT2G01940.2	
15		AT2G28200.1	AT4G39090.1		AT2G05920.1	
16		AT2G34500.1	AT5G11520.1		AT2G10940.2	
17		AT2G38710.1	AT5G49360.1		AT2G13790.1	
18		AT2G47770.1	AT5G52310.1		AT2G42690.1	
19		AT3G02550.1	AT5G53970.1		AT2G44210.1	
20		AT3G03470.1	AT5G58500.1		AT3G09940.1	
21		AT3G10740.1	AT5G59320.1		AT3G13140.1	
22		AT3G11780.1			AT3G15530.1	
23		AT3G13450.1			AT3G15850.1	
24		AT3G17780.1			AT3G16470.2	
25		AT3G22840.1			AT3G27830.1	
26		AT3G26100.1			AT3G45140.1	
27		AT3G28550.1			AT3G45640.1	
28		AT3G44880.1			AT3G54810.2	
29		AT3G45310.2			AT3G55800.1	
30		AT3G48880.2			AT3G58760.1	
31		AT3G50370.1			AT3G62030.1	
32		AT3G55610.1			AT4G01050.1	
33		AT3G58750.1			AT4G11320.1	
34		AT4G02380.1			AT4G12880.1	
35		AT4G15530.3			AT4G13830.2	
36		AT4G19920.1			AT4G16670.1	
37		AT4G34180.1			AT4G21720.1	
38		AT4G37390.1			AT4G23750.2	
39		AT5G02020.2			AT4G24190.2	
40		AT5G06760.1			AT4G32260.1	
41		AT5G13800.1			AT4G38970.1	
42		AT5G13800.1			AT5G02160.1	
43		AT5G21990.1			AT5G09220.1	
44		AT5G23750.2			AT5G38410.1	
45		AT5G27350.1			AT5G38420.1	
46		AT5G42250.1			AT5G40950.1	
47		AT5G43060.1			AT5G52820.1	
48		AT5G45350.1			AT5G67290.1	
49		AT5G46180.1				
50		AT5G54080.2				
51		AT5G60580.3				
52		AT5G66170.2				
53		AT5G66760.1				

APPENDIX C

flg22 region of several species of bacteria

(adapted from Felix et al., 1999)

APPENDIX D

Alignment of the lipopolysaccharide heptosyltransferase gene

Amino acid alignment of the lipopolysaccharide heptosyltransferase gene from *Pst DC3000* (top) and *R. solanacearum* isolate GMI1000 (bottom). (*) represent identical residues, (:) represent conserved substitutions and (.) represents semi-conserved substitutions.

```
MRVLIVKVSSLGDVVHCTPVVADILRAHPGAEIDWVVEEGFAGIVRIVRG 50
MRVLLIKTSSLGDVIHALPALTDAARALPGIRFDWVVEEGFAEIPAWHPA 50
****::*.******:*. *.::*  ** ** .:********* *     .

VQDVIPFALRRWRKSLASGATWGEMAAFRRALRAKPYDVVLDTQGLIKTA 100
VDAVIPVAIRRWRKNLWQTFRSGEWRRFKARVREQRYDLVIDAQGLFKSA 100
*: ***.*:*****.* .    **   *:  :* : **:*:*:***:*:*

LVAAQARLAPNGFVAGLGNRTDGAGYEPLARLFYQREVHMEPRVHVVERS 150
WLTRYIDAP----VAGLDR---DSAREPVASRFYDRALPVARGQHAVERL 143
 ::     .    ****..   .:. **:*  **:* : :    *.***

RRMVAEALGYAVPETI-DFGLQPPASLPFALPRPYVALVHATSRADKGWP 199
RQLFAQALGYPLPSGMGDYGLKPLAALDDTLQAPFVLFLHGTTWDTKHWP 193
*::.*:****.:*. : *:**:* *:*  :*  *:* ::*.*:   * **

QDAWVDVARALLARDYALALPWGSETERRTSEAIREAIVAAVPGTLGRIV 249
ELYWRQLAELMVARGLHVQLPWGNPTEKARAERIAEGLESAH-------- 235
:  * ::*. ::**.  : ****. **:  :* * *.: :*

IPPRMSLPDVTAFLDQSTAVVGVDTGLVHIAAAMCKPTVALYNFSTSWRT 299
VLPKLNLAGVARVLASAQACVAVDTGIGHLAAALDVPTVSLFGPTNPGLT 285
: *::.*..*: .* .: * *.****: *:***:  ***:*:. :..  *

GGYWTPKVHDLG-------CAEAHPTSAQALDALR--------------- 327
GAYGKSQVHLASDYPGCTPCLQKKCTYQPSADDQRRFDLKREWPLCFTRL 335
*.* ..:**  .       * : : *   : *  *

-------ALGVL------- 332
NPERVASQLGALLLAKEPG 354
        **.*
```

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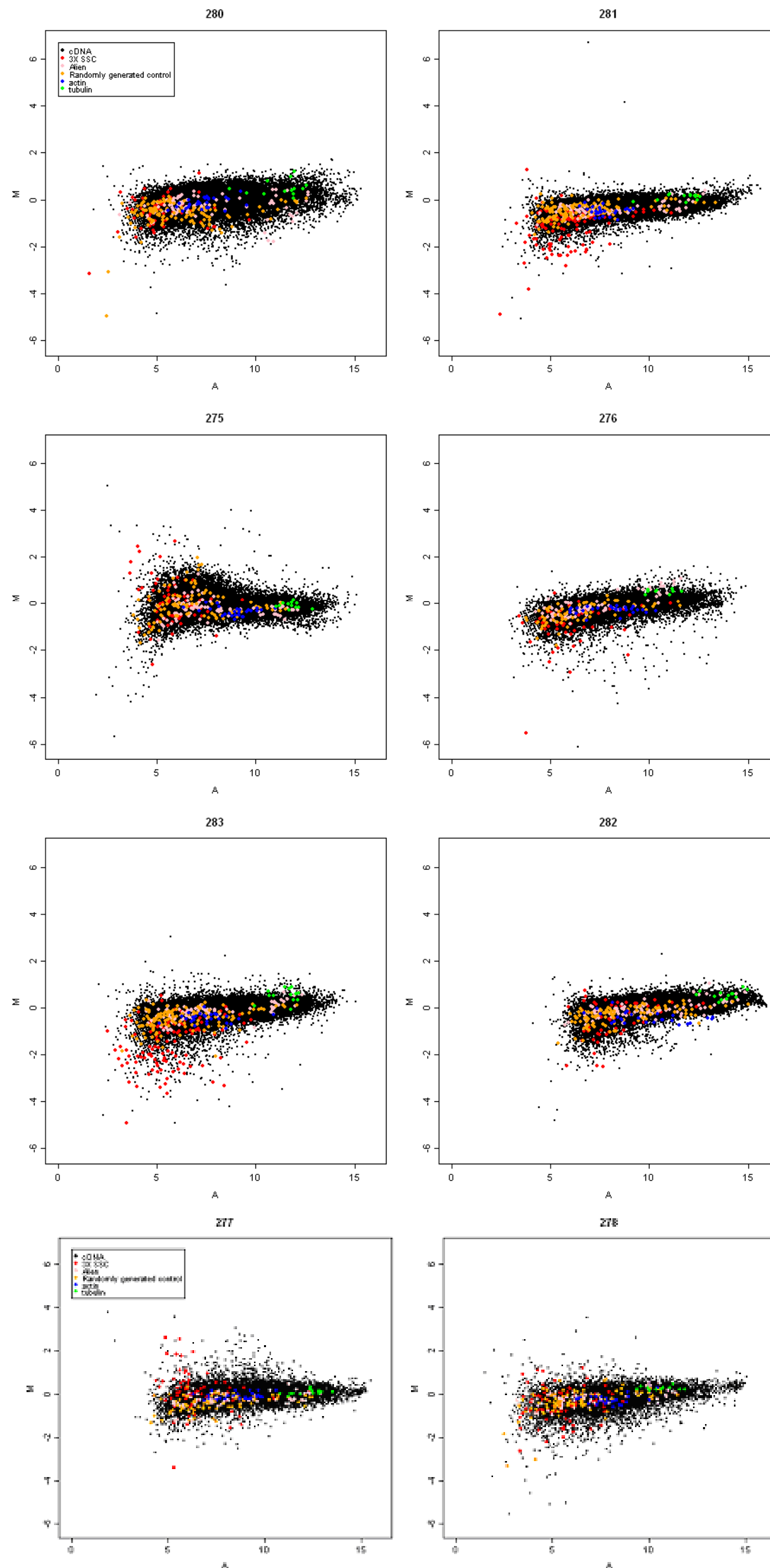
APPENDIX E

MA Plots before within slide normalisation.



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APPENDIX E

MA plots after within slide normalisation.

280

cDNA
3X SSC
Alien
Randomly generated control
actin
tubulin

281

275

276

283

282

277

cDNA
3X SSC
Alien
Randomly generated control
actin
tubulin

278

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APPENDIX F

Constitutive expression of 10 genes in Kil-O and Be-O

relative to the control (At1g18070.1) for two biological replicates.

LTP3

Expression relative to control gene
10 8 6 4 2 0
1 2
Replicate
Be-0
Kil-0

LTP4

Expression relative to control gene
3 2.5 2 1.5 1 0.5 0
1 2
Replicate
Be-0
Kil-0

LTP pseudogene

Expression relative to control gene
0.025 0.02 0.015 0.01 0.005 0
1 2
Replicate
Be-0
Kil-0

PRX34

Expression relative to control gene
2.5 2 1.5 1 0.5 0
1 2
Replicate
Be-0
Kil-0

AIG

Expression relative to control gene
15 10 5 0
1 2
Replicate
Be-0
Kil-0

SAG13

Expression relative to control gene
2 1.5 1 0.5 0
1 2
Replicate
Be-0
Kil-0

PPR

Expression relative to control gene
0.04 0.03 0.02 0.01 0
1 2
Replicate
Be-0
Kil-0

SUI1

Expression relative to control gene
0.06 0.05 0.04 0.03 0.02 0.01 0
1 2
Replicate
Be-0
Kil-0

SKP5

expression relative to control gene
15 10 5 0
1 2
Replicate
Be-0
Kil-0

Expressed protein

Expression relative to control gene
24.5 24 23.5 23 22.5 22 21.5
1 2
Replicate
Be-0
Kil-0