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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*, *Xanthomonas*, 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. Originally 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

^aBuddenhagen (1962), ^bHayward (1964), ^cPoussier et al. (2000); ^dCook and Sequiera, (1994); ^eFouchè-Weich et al. (2006); ^fPrior 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 m³ per 15 m³ 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 1×10^7 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 orthoceras*).

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 polygalacturonases 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 bipartite 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

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 OE1-1*phcA* 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.

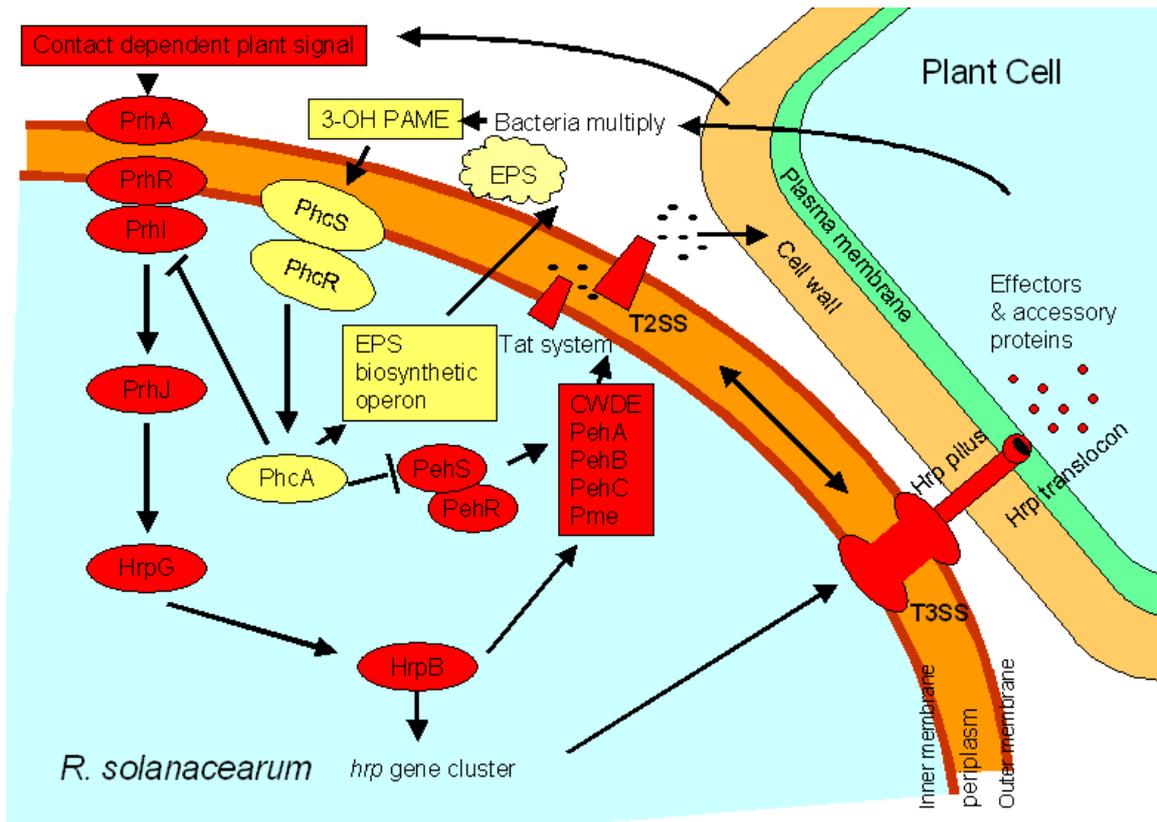


Figure 1.1 Schematic diagram representing the regulation of virulence in *R. solanacearum* adapted from Hikichi et al., 2007; Buttner and Bonas, 2006. Pathways have been studied in *R. solanacearum* strains GMI1000, K60 or OE1-1. Abbreviations are as described in the text. Symbols are: positive regulation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

Arabidopsis 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 Initiative, 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.

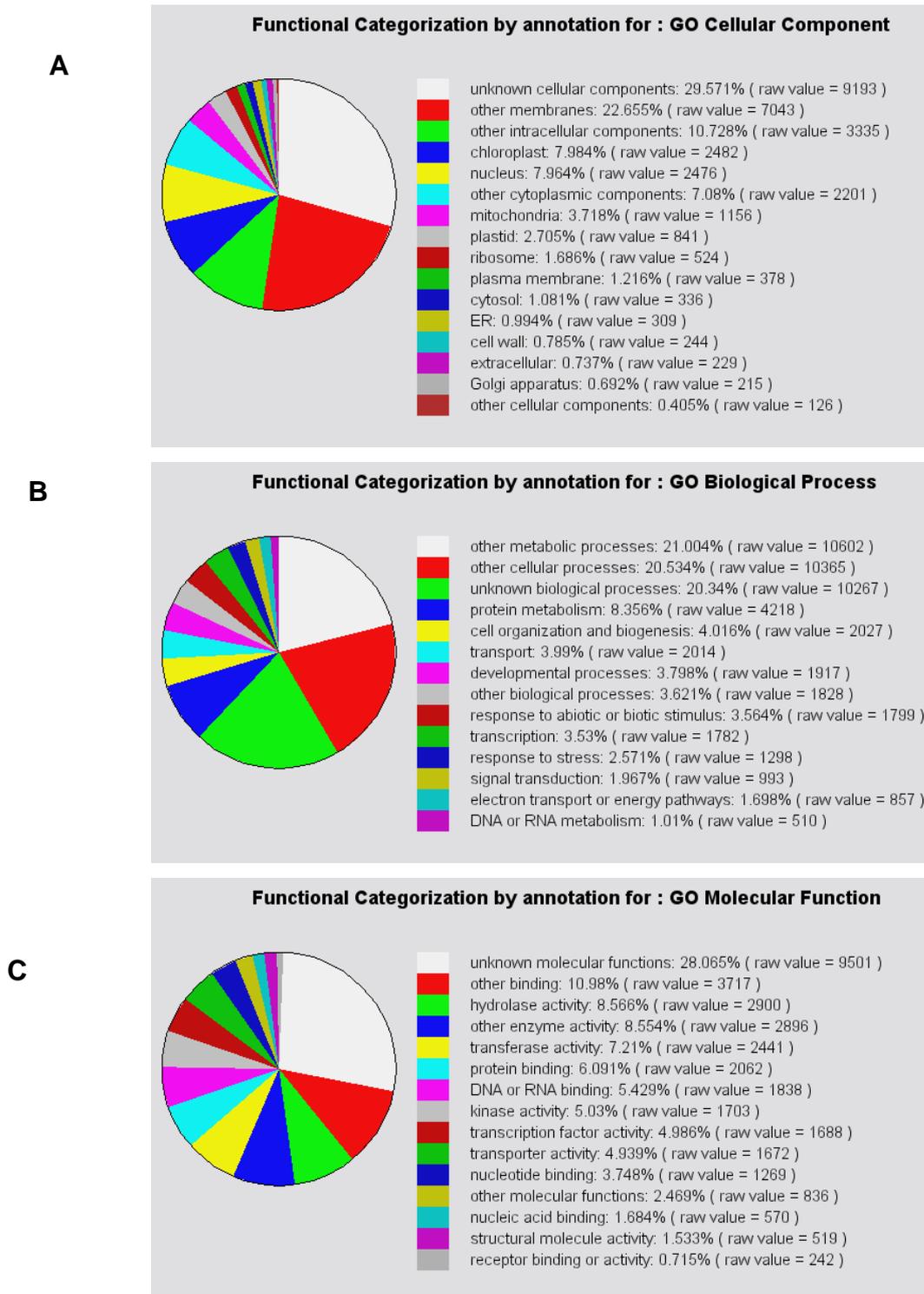


Figure 1.2. The functional categorisation of all annotated Arabidopsis genes under gene ontologies for A) Biological Process, B) Cellular Component and C) Molecular Function. The categories were derived from TAIR (www.arabidopsis.org) version 7 which contains 32 041 genes.

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

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 alkalinisation 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.

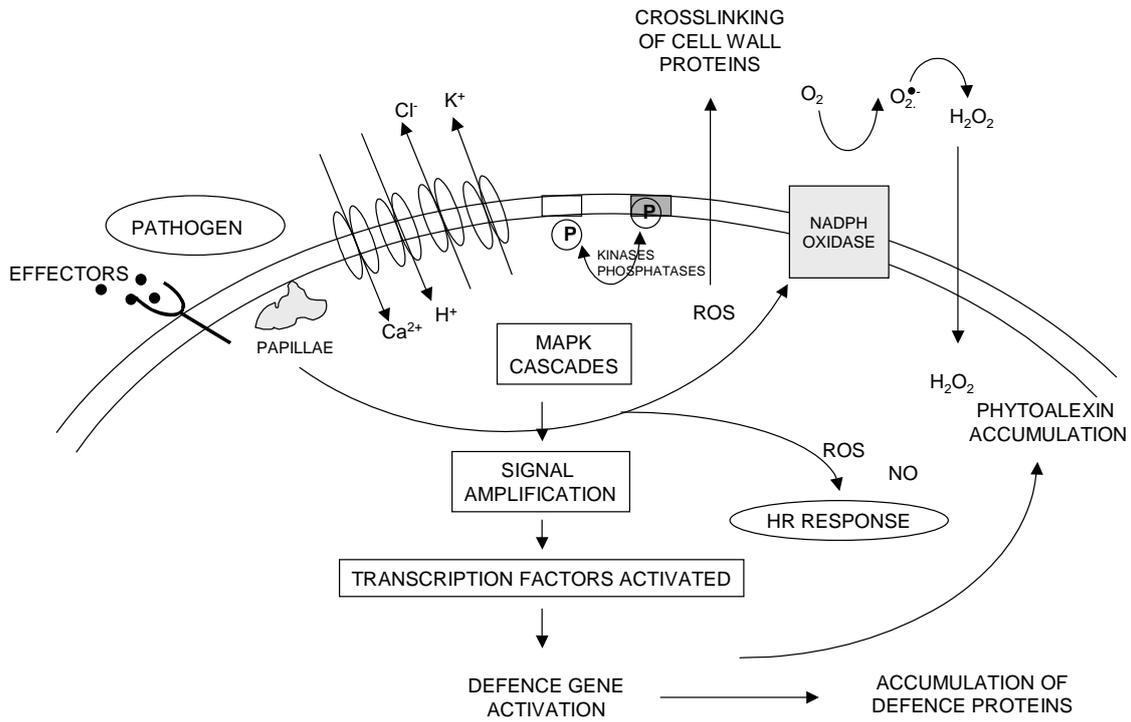


Figure 1.3. A simple model of plant responses induced by specific effectors or non-specific pathogen-derived elicitors (adapted from Buchanan et al., 2002; Thatcher et al., 2005).

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 ($[\text{Ca}^{2+}]$) 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_2O_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 $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]$ levels remained low during this period (de Torres et al., 2003). The rise in cytosolic $[\text{Ca}^{2+}]$ 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^- , 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

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 (cev1)* 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 (CHIB)*, 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. *phaseolicola* (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. syringae*-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*, *COR11*, *COR13* (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 max*), 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 brassicicola* (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.

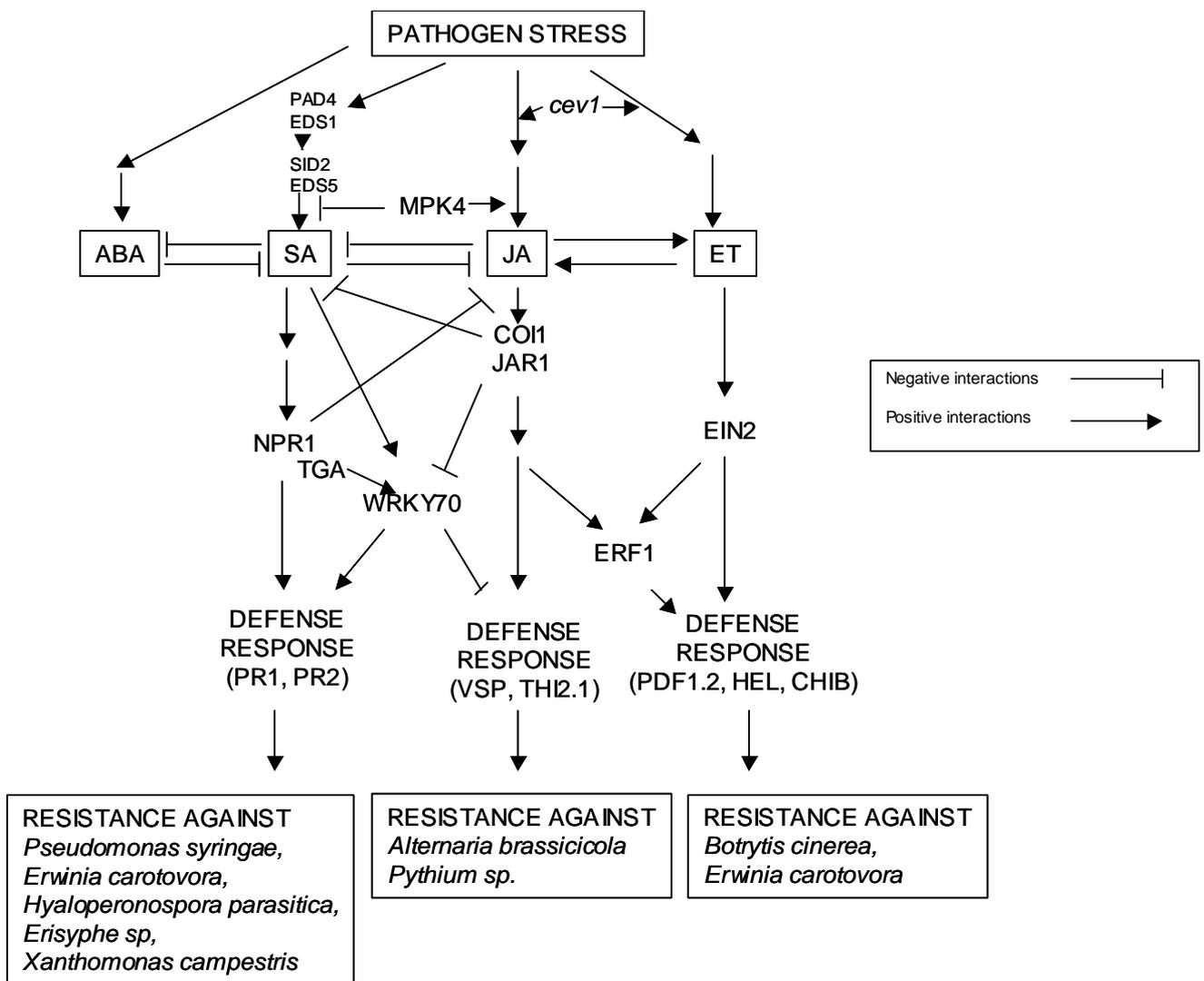


Figure 1.4. Signalling pathways mediated by ABA, SA, JA and ET in response to pathogen stress. Not all identified defence mutants are shown (Kunkel and Brooks, 2002; Durrant and Dong, 2004).

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 (*Ler*) 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 *Ler* 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

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 NH₂ 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).

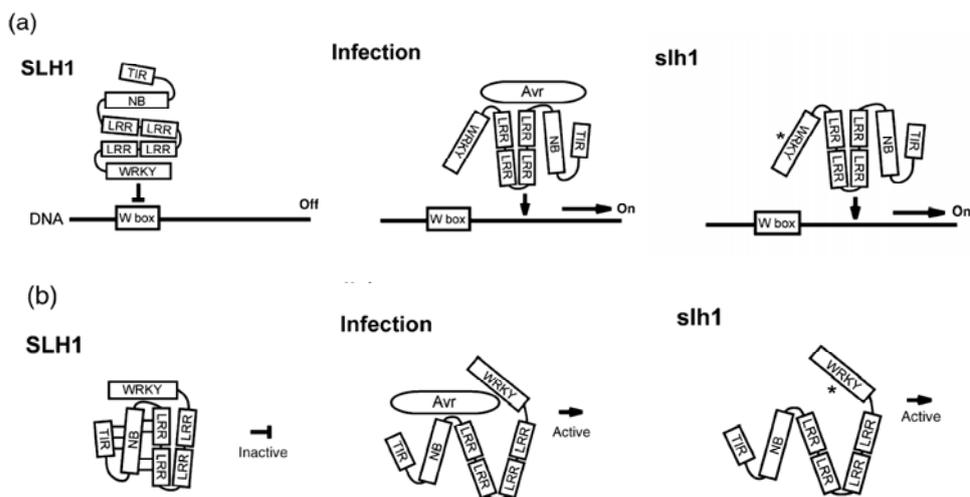


Figure 1.5. Two models proposed by Noutoshi et al. (2005) describing the interaction between SLH1 (RRS1-R) and the Avr protein (PopP2) in producing resistance against *R. solanacearum*. Model a) shows SLH1 as a transcriptional repressor in wild-type plants, negatively interacting with the W-box. Upon 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* (irregular xylem 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 (Vaillau 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 Initiative, 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

lowest 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:

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

where σ_{g1}^2 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_{g2}^2 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. $H_0: \mu_1 = \mu_2$, 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:

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

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 $\sigma^2_A, \sigma^2_{TA}$ 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).

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 comparability 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. syringae 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 *Wassilekija* 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* (constitutively induced resistance 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 (Skena 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

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).

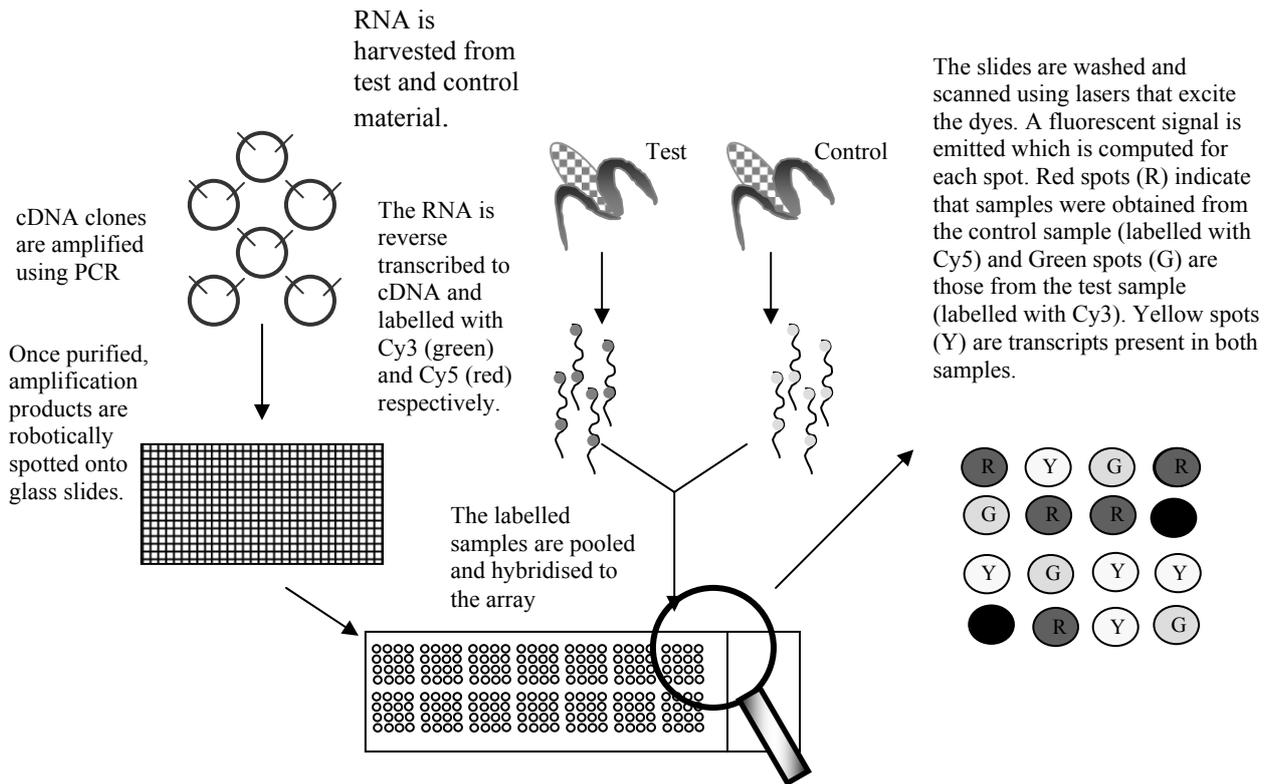


Figure 2.1 An example of a typical microarray experiment using dual colour labelled cDNA samples hybridised to glass slides containing amplified cDNA fragments.

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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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 ddRT-PCR (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

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 β -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

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).

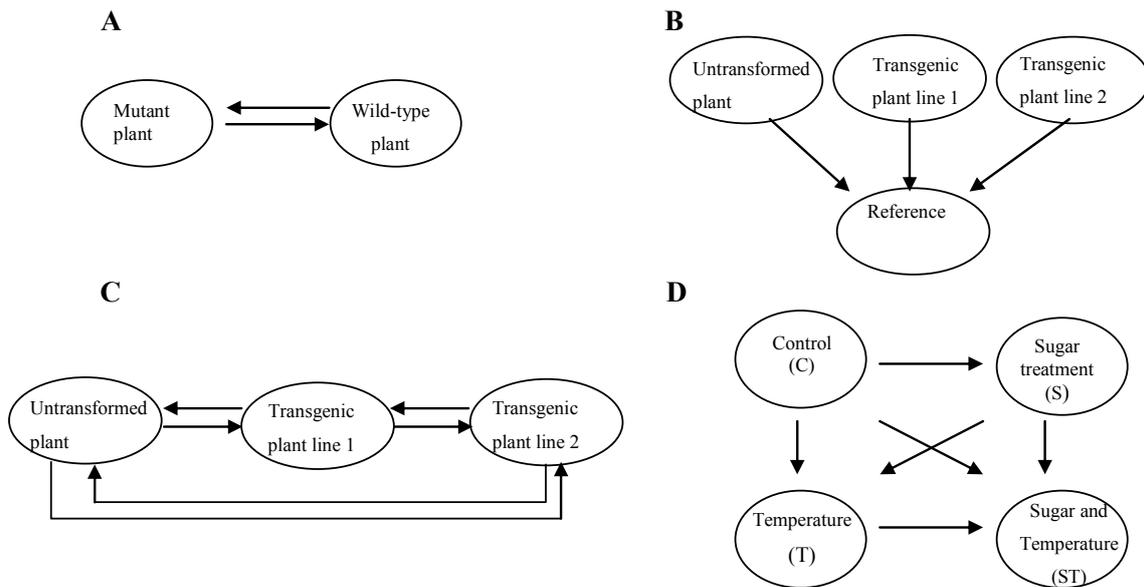


Figure 2.2. Diagrammatic representations of microarray experiment designs. The head of the arrow indicates that the sample was labelled with Cy5, while the tail represents a sample that is labelled with Cy3. A: direct comparison between a mutant and wild-type *Arabidopsis* plant. B: An indirect comparison 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: $\log (ST/T) - \log (S/C) = \log (ST \times C/T \times S)$. 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).

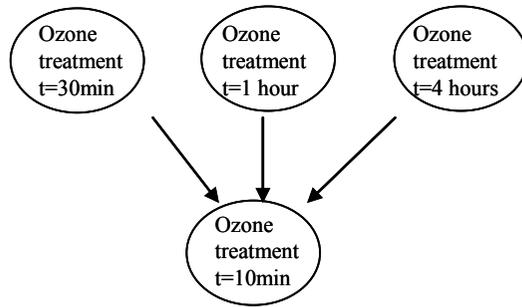
2.8. Time-course experiments

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 microarray 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.

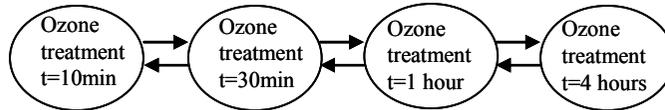


A



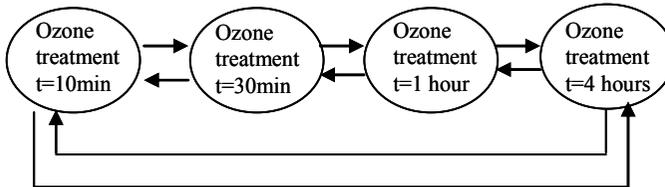
3 slides (no dye reversal required)
6 slides for 1 biological replicate

B



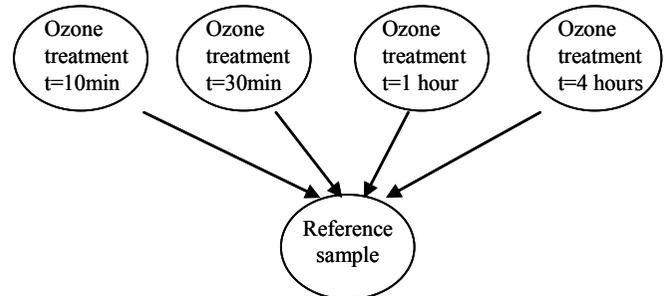
6 slides (direct comparison with a dye swap)
6 slides (balanced block design with 1 biological replicate)

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.

<i>Design</i>	<i>Type of study</i>	<i>Question</i>	<i>Replication</i>	<i>Number of Slides</i>	<i>Reference</i>
Direct comparison	Single-factor experiment Class comparison	Which genes are differentially expressed genes in <i>Phytophthora infestans</i> infected leaves of <i>Arabidopsis</i> ?	2 spots/gene/slide 2 technical replicates Individual plants pooled (no biological replication) Dye swap: yes	4	Huitema <i>et al.</i> , 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 <i>et al.</i> , 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 <i>Ancylostoma caninum</i> ?	2 spots/gene/slide 1 technical replicate 2 strains assessed (1 biological replicate) Dye swap: no	24	Moser <i>et al.</i> , 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 <i>et al.</i> , 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.

Acknowledgements.

We acknowledge the support of the Mellon Foundation and the National Research Foundation, South Africa.

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