Chapter 1

Literature Review

Overview of plant defence response mechanisms

1.1 INTRODUCTION

Pathogens and insect pests cause widespread losses to agriculture throughout the world on an annual basis. In developed countries, losses are typically around 20% of potential yield, while in developing countries losses are normally significantly greater (Anderson et al., 2005). Plant disease resistance is a prerequisite for the successful utilisation of crop species in modern agriculture. One of the major challenges facing modern agriculture is to achieve a satisfactory, but environmentally friendly control of plant diseases. Although the extensive use of pesticides and fungicides remains the main strategy of disease control, conventional breeding approaches have been very successful in introducing resistance (R) genes from wild populations into commercial crop cultivars. Although some R genes have provided excellent disease control in large scale commercial production for more than 15 years, this resistance is often not durable as pathogens are able to evolve quickly and overcome it (Hammond-Kosack and Jones, 2000).

Recent advances in molecular biology have improved our understanding of plant-pathogen interactions through the isolation of a number of R genes, and analysis of signalling pathways leading to the hypersensitive response (HR) and systemic acquired resistance (SAR) (Ryals et al., 1996; Dempsey et al., 1999). This knowledge has enabled more sophisticated breeding strategies to be employed using marker-assisted selection (Ayliffe and Lagudah, 2004). The completion of Arabidopsis and rice genome sequences (The Arabidopsis Genome Initiative, 2000; Yu et al., 2002; Goff et al., 2002), and the current sequencing of crop plant genomes, together with improved knowledge of plant defence response mechanisms through functional analyses, will pave the way for the development of transgenic crops with increased disease resistance, or the development of novel pesticides capable of activating plant defence responses.

The presented review introduces pearl millet, an indigenous African crop, and the diseases associated with growing this subsistence crop. In addition, an overview of plant defence response mechanisms is presented, and wherever possible, advances in understanding cereal specific disease resistance and defence responses are presented. The application of DNA microarrays as a tool to study global gene expression changes during plant defence response is also considered. Finally, the literature review is put into context through discussion of the aims of the project and experimental approaches adopted in this study to elucidate defence response mechanisms in pearl millet.

1.2 PEARL MILLET

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] was domesticated from wild grasses of the southern Sahara approximately 4000 years ago. It has since become widely distributed across the semiarid tropics of Africa and Asia, and is also extensively grown as a summer annual grazing crop in the southern United States and tropical and subtropical regions of the world (Goldman et al., 2003). In 2003 pearl millet was the world's sixth largest cereal crop with 29 million metric tons produced on 36 million hectares of cultivated land (http://apps.fao.org/). Approximately half of the world's pearl millet is grown in Africa, with the continent producing 14 million metric tons in 2003 (http://apps.fao.org/). In Southern Africa, the commercialisation of agriculture has resulted in maize partially or completely displacing pearl millet as a traditional food crop. In South Africa, only 12 000 tons of pearl millet was produced in 2003, and this was mainly for subsistence purposes. Almost all millet is produced by small scale farmers for household consumption and localised trade.

Pearl millet is a crop of vital importance to millions of African families living in semi-arid regions of the continent. Millet is one of the world's most resilient crops. In many areas where millet is the staple food, nothing else will grow. Pearl millet is supremely adapted to heat and aridity, and production is likely to increase as the world gets hotter and drier. Of all the major cereals, it is the one most able to tolerate extremes of heat and drought. It yields reliably in

regions too hot and too dry to consistently support good yields of maize or even sorghum. Pearl millet is easy to grow and suffers less from disease and insect pests than sorghum, maize or other grains (National Research Council, 1996).

Pearl millet grain is nutritious, and has higher protein and energy levels than maize or sorghum (National Research Council, 1996). Carbohydrates usually make up about 70 percent of the dry grain, and they consist almost exclusively of starch. The grain contains at least nine percent protein and a good balance of amino acids. It has roughly twice the fat content (5-7%) of most standard cereals, and is particularly high in calcium and iron. The vitamin values of pearl millet grain are generally somewhat lower than those of maize, although the levels of vitamin A and carotene are good, particularly for a cereal. Importantly, it has neither the tannins nor other compounds that reduce digestibility in sorghum. Pearl millet is a versatile foodstuff, and is mainly used as whole seed, cracked seed, ground flour, dough, or a grain like rice. These are made into unfermented breads (roti), fermented foods (kisra and gallettes), thin and thick porridges (toh), steam cooked dishes (couscous), non alcoholic beverages and traditional beers. Grain from certain cultivars is roasted whole and consumed directly (http://africancrops.net).

In recent years, considerable advances have been made in the understanding of the genetics of the crop. *P. glaucum* is diploid (2n=2x=14) with a haploid DNA content of 2.4 pg. Detailed genetic maps of some 300 loci spread over 7 linkage groups are available (Liu et al., 1994). Despite these advances, pearl millet is poorly supported by science and politics. Over the past two decades, production in West Africa has only increased by 0.7 percent per year, the lowest growth rate of any food crop in the region (National Research Council, 1996). Over the decades, more and more farmers, especially in southern Africa, have abandoned pearl millet farming and switched to maize. This is due to a number of reasons. First, research efforts have made maize more productive than pearl millet; second, government incentives have given maize an added financial advantage; and finally, easier processing has made maize

more convenient to use. However, with water steadily becoming a limiting resource to numerous economies, pearl millet could become a vital resource.

1.3 PEARL MILLET DISEASES

Due to the subsistence nature of farming pearl millet in developing countries, very few statistics are available for crop losses resulting from disease. However, pearl millet suffers less from disease and insect pests than sorghum, maize or other grains (National Research Council, 1996), but this could be due to the planting of landraces as opposed to hybrids in many developing areas.

Downy mildew is the major biotic cause of yield loss in pearl millet, and is caused by the biotrophic oomycete fungus *Sclerospora graminicola* (Sacc.) Schroet. The disease is widespread in India and West Africa, but does not occur on pearl millet in the Americas. Downy mildew is not found in South Africa as the climate is too dry to support *S. graminicola* infections (de Miliano, personal communication). The disease is economically important, since it causes more than 60-70% loss of yield in susceptible hybrids (Singh et al., 1990; Singh, 1995). The life cycle of *S. graminicola* comprises both sexual and asexual phases. The sexual stage produces oospores that become soil- or seedborne, thus providing the primary source of inoculum each season. Seedlings growing in soils infested with oospores become systemically infected and show chlorosis. Under humid conditions, systemically infected leaves produce sporangia, and release zoospores that encyst, germinate and invade the foliage to produce secondary infections (Jones et al., 2001). Disease symptoms include leaf chlorosis, stunted growth with no production of panicles, and green ear symptoms which result from transformation of floral parts into leafy structures. Breeding for resistance to downy mildew is a high priority for pearl millet breeders.

Smut [*Moesziomyces penicillariae* (Bref.) Vanky] and ergot (*Claviceps* sp.) are inflorescence diseases. These fungal pathogens are widely distributed across the pearl millet growing areas of the world; however *Claviceps fusiformis* Loveless has not been reported on pearl millet in the United States

and strict quarantine procedures are in place to restrict its entry (http://www.cpes.peachnet.edu). Although losses are considerably less than downy mildew, these inflorescence diseases still result in reduced seed yield. The introduction of hybrid and exotic breeding lines has greatly increased the severity of these diseases in India and Africa respectively (King, 1992; Panwar and Rathi, 1997). Field screening techniques for smut and ergot are available and stable resistances have been identified (King, 1992; de Milliano, 1992).

The two most destructive diseases to pearl millet in the United States are rust (caused by *Puccinia substriata* Ell. & Barth. var. *indica* Ramachar & Cumm.) and pyricularia leaf spot [*Pyricularia grisea* (Cke.) Sacc] (Morgan et al., 1998). Although rust is fairly widespread and is distributed throughout the Americas, Asia and Africa, pyricularia leaf spot appears to be limited to India, Singapore and the United States (http://www.cpes.peachnet.edu). General disease resistance to both pathogens has been transferred into agronomically acceptable forage and grain cultivars. However, the diverse nature of *P. substriata* var. *indica* has hampered efforts to breed for stable resistance and biomass production (Wilson and Gates, 1999). Even low levels of rust infection result in significant losses of digestible dry matter, and as a result, this disease has become an important limiting factor for grain and forage production in the United States. *P. substriata* var. *indica* is a macrocyclic rust that causes small reddish orange round uredinia to develop mainly on pearl millet foliage. As the severity of the infection increases, leaf tissue wilts and becomes necrotic from the leaf apex to the base. Pearl millet rust is fairly widespread throughout growing regions of South Africa (de Miliano, personal communication).

Bacterial and viral diseases are of minor importance to the extent that disease causal agents are not always identified (King, 1992). Nematodes are likewise probably widespread, but their importance in pearl millet production is virtually unknown. A recent study indicated that pearl millet hybrids exhibited differences in resistance to the nematode species *Meloidogyne incognita* (Kofoid and White) Chitwood and *Paratrichodorus minor* (Colbran) Siddiqi

(Johnson et al., 1999). Johnson and coworkers hypothesised that the fibrous rooting system and root branching of mature pearl millet plants probably allows this crop to flourish under certain populations of nematodes.

1.4 PATHOGEN RECOGNITION BY PLANT CELLS

Plants are sessile organisms, anchored to the ground through the root system for acquisition of nutrients and water, and can therefore not move to escape environmental challenges. Biotic stresses result from constant attack by fungi, bacteria, nematodes, feeding insects and viruses. In addition to passive protection provided by the waxy cuticle and preformed antimicrobial compounds such as saponins (Osbourn, 1996), plants have evolved sophisticated defence mechanisms to perceive pathogen attacks, and to translate that perception into an adaptive response.

As a first step, plants recognise the presence of a pathogen when pathogen derived molecules (i.e. elicitors) bind to receptors. Recognition is accomplished by the detection of elicitors (i.e. peptide, oligosaccharide or lipid based signalling molecules) that originate from the pathogen or cell wall degradation products (Romeis, 2001). In gene-for-gene plant-pathogen interactions, these race-specific elicitors are encoded by pathogen avirulence (*avr*) genes and their specific recognition is conferred by corresponding plant disease resistance (*R*) genes. R gene products function extracellularly or intracellularly. Plants also possess a broader basal surveillance involving perception systems for non-race specific microbe derived molecules termed pathogen associated molecular patterns (PAMPs) (Gomez-Gomez, 2004). In the case of non-race-specific elicitors, high affinity binding receptors located in the plasma membrane are responsible for pathogen perception. Upon pathogen recognition, signalling events become initiated that trigger early cellular responses such as changes in ion fluxes, synthesis of reactive oxygen species leading to the development of the hypersensitive cell death response (HR) and changes in gene transcription. Delayed defence responses include the production of antimicrobial compounds, cell wall fortification and the activation of systemic acquired resistance (SAR), which reflects a long lasting resistance that is established in non-infected areas of the plant.

The speed with which the plant cell can mobilise its defences often determines whether it survives or succumbs to the attack. When the induced responses are triggered rapidly and coordinately during a given plant-pathogen interaction, the plant is resistant to disease. A susceptible plant responds more slowly with an onset of defence mechanisms after infection. Thus, the timely recognition of an invading microorganism and the rapid and effective induction of defence responses appear to be a key difference between resistant and susceptible plants (Hammond-Kosack and Parker, 2003).

Pathogens can be divided into two basic categories depending on the infection strategy deployed to utilise the host plant as a substrate. Biotrophic pathogens use living plant cells as a nutrient source, and have developed specialised feeding structures, or haustoria (Hammond-Kosack and Jones, 2000). Biotrophs require a living host to complete their lifecycle, and therefore aim to evade recognition by the plant. On the other hand, necrotrophic pathogens kill the host and absorb nutrients from the dead plant tissue. These pathogens secrete toxins or cell wall degrading enzymes to kill and macerate plant tissue preceding recognition and the subsequent activation of defence responses (Hammond-Kosack and Jones, 2000). Hemibiotrophic fungi sequentially deploy a biotrophic and then a necrotrophic mode of nutrition. The switch is usually triggered by increasing nutritional demands as the fungal biomass increases (Hammond-Kosack and Jones, 2000).

Gene-for-gene resistance

A compatible plant-pathogen interaction occurs when a virulent pathogen penetrates a susceptible plant and causes disease. Alternatively, an incompatible interaction takes place when an avirulent pathogen attacks a resistant plant, which is able to rapidly activate defence responses, thus preventing the development of disease. The gene-for-gene model for plant disease resistance proposed that an incompatible reaction results from the interaction of the product of a plant resistance (*R*) gene with the product of the corresponding avirulence (*avr*) gene (Flor, 1971). Subsequent research has

shown that there are many exceptions to this model and that most R-Avr protein interactions are not direct, but instead involve perception of pathogen derived proteins within a complex (Hammond-Kosack and Parker, 2003).

R genes

In the past decade, many R genes have been isolated that confer pathogen resistance to various plant species against a wide range of pathogens. An ever increasing number of R genes have been isolated from wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*) (see Table 1.1), the three crop species that account for over 85% of cereal production (FAO Statistical Databases; FAOSTAT 2004). Significant advances have also been made in understanding the molecular basis of pathogen recognition in barley (*Hordeum vulgare*). These four species, however, are the only cereals from which functional disease resistance genes have been isolated and characterised (Ramalingam et al., 2003; Ayliffe and Lagudah, 2004).

R genes can be classified into six classes based on their predicted protein structures (Hammond-Kosack and Jones, 2000; Hammond-Kosack and Parker, 2003). These six classes of R genes, with examples of each, are outlined in Table 1.1. Structures of the different R gene proteins are illustrated in Figure 1.1.

The largest class of R genes encode proteins containing a central domain with a nucleotide binding site (NBS), which binds either ATP or GTP (Saraste et al., 1990), and a carboxy terminal domain consisting of a series of degenerate leucine rich repeat residues (LRR). Amongst plant species, NBS-LRR proteins can be further divided into two subgroups; those containing an amino terminus with homology to the Drosophila Toll protein and mammalian interleukin-1-receptor (TIR-NBS-LRR); and those which do not contain this domain, but often have it substituted with a leucine zipper or coiled-coil domain (CC-NBS-LRR). A major distinction between monocotyledonous and dicotyledonous species is that only CC-NBS-LRR genes have been identified in monocots, whereas both subgroups are found in dicots, with TIR-NBS-LRR genes being the more abundant class (Dangl and Jones, 2001; Ayliffe and

Lagudah, 2004). The rice genome sequence has enabled scientists to estimate the number of NBS-LRR encoding genes existing in a cereal genome. To date about 600 NBS-LRR genes have been identified in the rice genome (Goff et al., 2002). This compares with the 149 NBS-LRR genes present in the Arabidopsis genome, 60% of which encode TIR-NBS-LRR proteins, while 40% encode CC-NBS-LRR proteins (Dangl and Jones, 2001).

Several other cereal resistance genes have been identified that do not encode NBS-LRR proteins. The rice *Xa21* and *Xa26* genes, which confer resistance to the bacterial pathogen *Xanthomonas oryzae*, each encode a protein comprised of an amino terminal extracellular LRR joined by a transmembrane domain to a cytoplasmic C-terminal serine/threonine protein kinase domain (Song et al., 1995; Sun et al., 2004).

In contrast, the non-race specific barley *Rpg1* stem rust resistance gene only encodes an intracellular protein kinase with two tandem kinase domains (Horvath et al., 2003). Possible parallels exist between this cereal gene and the tomato *Pto* gene which encodes a protein kinase that confers race specific resistance to *Pseudomonas syringe*. However, to confer disease resistance, the *Pto* gene product also requires the LRR containing protein Prf. It will be of interest to determine whether the *Rpg1* gene additionally requires an NBS-LRR gene for defence gene activation.

Maize *Hm1* encodes a unique R protein that confers resistance to the leaf spot fungus *Helminthosporium maydis*. This nectrotrophic fungus produces a race specific toxin, HC toxin that inhibits the activity of histone deacetylase, an enzyme that is required for activation of plant defence responses. The maize *Hm1* resistance gene encodes a reductase enzyme that specifically detoxifies the HC toxin (Johal and Briggs, 1992).

Table 1.1. The six classes of plant resistance genes [adapted from Hammond-Kosack and Jones (2000); Hammond-Kosack and Parker (2003); Ayliffe and Lagudah (2004)].

Class	R protein predicted features	Gene	Plant	Pathogen	Pathogen type
1	Detoxifying enzyme	*Hm1*	Maize	*Helminthosporium maydis* (race 1)	Necrotrophic fungus
2a	Intracellular protein kinase	*Pto*	Tomato	*P. syringae* pv *tomoato (avrPto)*	Extracellular bacteria
2b	Intracellular protein kinase with 2 tandem kinase domains	*Rpg1*	Barley	*Puccinia graminis* f.sp. *tritici*	Biotrophic intracellular fungus
3a	TIR-NBS-LRR	*N*	Tobacco	Tobacco mosaic virus	Intracellular virus
		RPP1, RPP4, RPP5	Arabidopsis	*Peronospora parasitica (avrRPP1, avrRPP4, avrRPP5)*	Biotrophic intracellular Oomycete
3b	TIR-NBS-LRR-NLS-WRKY	*RRS-1*	Arabidopis	*Ralstonia solanacearum*	Extracellular bacteria
3c	CC-NBS-LRR	*RPS2*	Arabidopsis	*P. syringae pv maculicola (avrRpt2)*	Extracellular bacteria
		Mla1/Mla6	Barley	*Blumeria graminis* f.sp. *hordei* (race 1, race 6)	Biotrophic fungus
		Rp1-D	Maize	*Puccinia sorghi*	Biotrophic intracellular fungus
		Rp3	Maize	*Puccinia sorghi*	Biotrophic intracellular fungus
		Lr10, Lr21	Wheat	*Puccinia triticina*	Biotrophic intracellular fungus
		Pm3	Wheat	*Blumeria graminis*	Biotrophic fungus
3d	NBS-LRD	*Pi-ta*	Rice	*Magnaporthe grisea (avrPita)*	Hemibiotrophic intracellular fungus
4a	Extracellular LRR with single membrane spanning region and short cytoplasmic carboxyl terminus (eLRR-TM)	*Cf-9, Cf-2, Cf-4, Cf-5*	Tomato	*Cladosporium fulvum (Avr 9, Avr2, Avr4, Avr5)*	Biotrophic extracellular fungus
4b	CC-eLRR-TM-ECS	*Ve1*	Tomato	*Verticillium albo-atrum*	Extracellular fungus
	eLRR-TM-PEST-ECS	*Ve2*		*Verticillium albo-atrum*	Extracellular fungus
5	Extracellular LRR with single membrane spanning region and cytoplasmic kinase domain (eLRR-TM-kinase)	*Xa-21*	Rice	*Xanthomonas oryzae* pv *oryzae* (all races)	Extracellular bacteria
6	G protein coupled receptor	*mlo*	Barley	*Blumeria graminis* f.sp. *hordei*	Biotrophic fungus

TIR, Toll interleukin1 resistance domain; NBS, nucleotide binding site; LRR, leucine rich repeat; NLS, nuclear localisation sequences; WRKY, WRKY transcription factor; CC, coiled coil domain; LRD, leucine rich domain, eLRR, extracellular leucine rich repeat; TM, transmembrane; ECS, endocytosis signal; PEST, Pro-Glu-Ser-Thr; G, GTP binding domain.

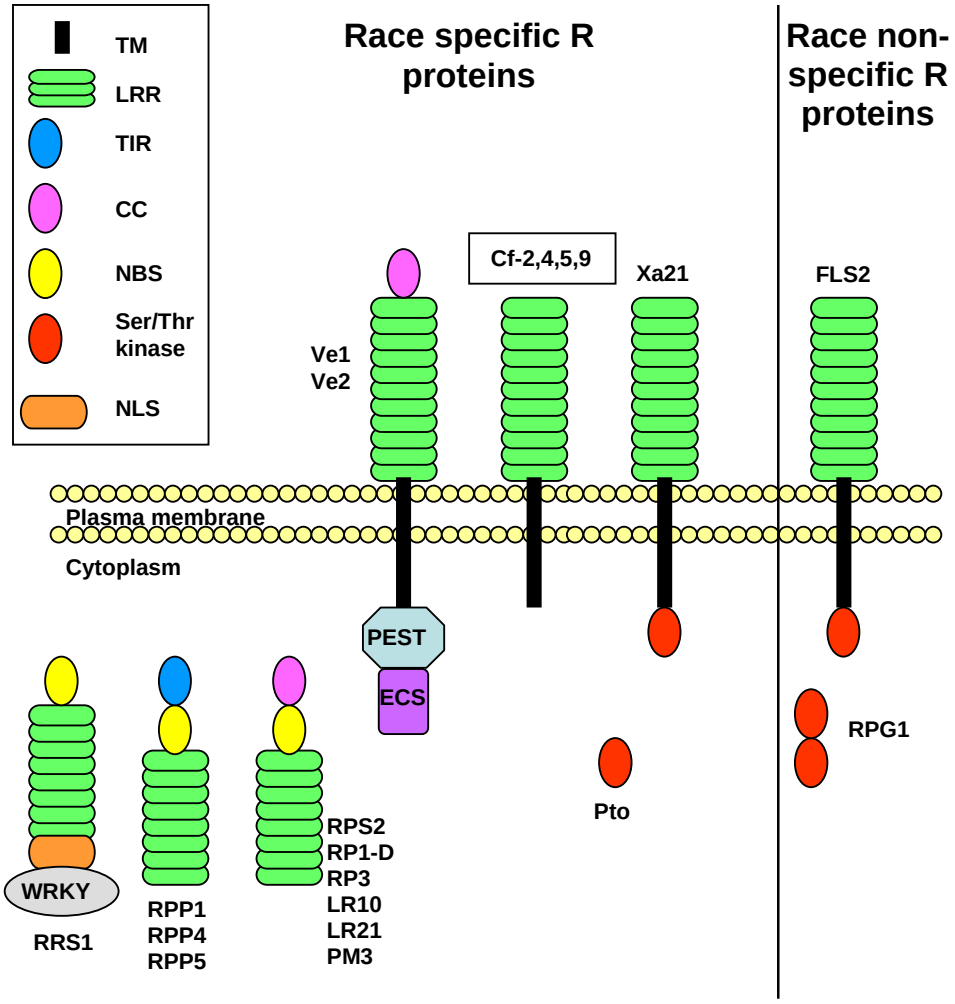

Figure 1.1. Schematic representation of the predicted domains of R proteins which confer either race specific or race non-specific resistance (adapted from Hammond-Kosack and Parker 2003). TM, membrane spanning domain; LRR, leucine rich repeat; TIR, Toll/interleukin-1. resistance domain; CC, coiled coil domain; NBS, nucleotide binding site; NLS, nuclear localisation sequences; PEST, Pro-Glu-Ser-Thr-like sequence for protein degradation; ECS, endocytosis signal.

The barley *mlo* resistance gene is distinct from other classified R genes in that it is recessive and confers resistance against all known isolates of the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei (Bgh)*. The *Mlo* gene was recently isolated and shown to encode a novel 533 amino acid protein predicted to form seven transmembrane helical bundles and may well be a G protein linked receptor (Figure 1.2) (Büschges et al., 1997; Elliott et al., 2005).

A number of mutation induced *mlo* resistance alleles have been molecularly characterised, each resulting either in single amino acid substitutions, deletions or frame shifts of the wild type gene (Shirasu et al., 1999). Requirement of the wild type protein for successful *Bgh* pathogenesis may indicate a role of MLO as endogenous plant defence modulator. Alternatively, MLO might be targeted by the fungal pathogen for suppression of host defence pathways (Elliott et al., 2005).

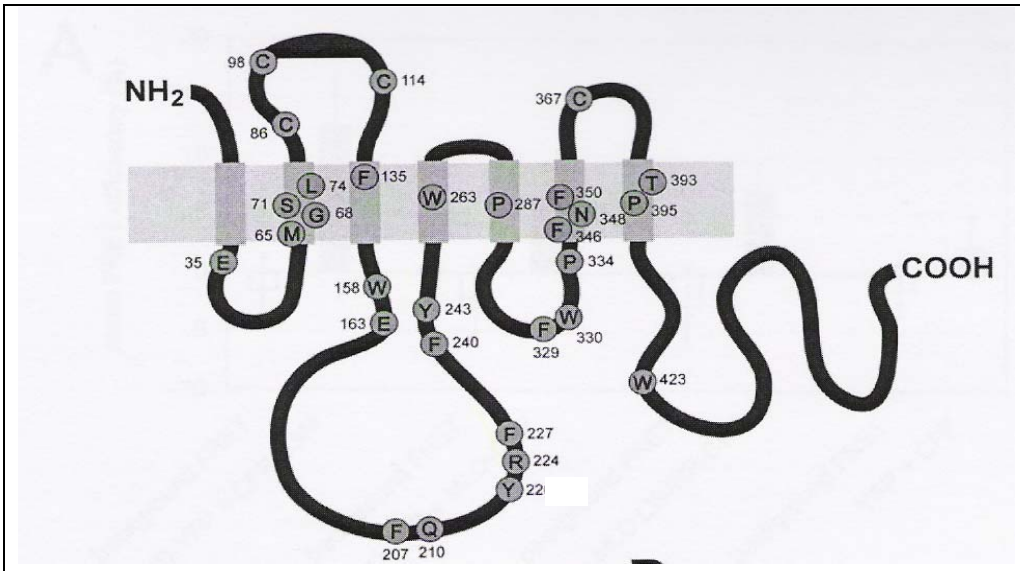

Figure 1.2. Schematic representation of MLO depicting strictly conserved residues within the MLO protein family. The light grey box represents the lipid bilayer, whereas smaller dark grey boxes symbolise the seven transmembrane domains. Invariant amino acids of a sample of 38 full length MLO sequences from different plant species are shown as circles labelled using the one letter amino acid code. Numbering of amino acids corresponds to barley MLO (Elliott et al., 2005).

The guard hypothesis

A number of recent studies have indicated that most R-Avr protein interactions are not direct, but instead involve perception of pathogen derived proteins within a complex (Mackey et al., 2002; Van der Hoorn et al., 2002). Rather, it is likely that R proteins may recognize the activities of multiple avirulence factors that similarly manipulate the same host target in order to enhance pathogen virulence. The “guard hypothesis”, proposed by Dangl and Jones

(2001), postulates that the corresponding R protein monitors the integrity of that particular host target in order to detect manipulation of it by the avirulence factors, and subsequently induce defence responses. Therefore, R proteins might "guard" a limited set of key cellular targets of pathogen virulence effectors. In the absence of the host R protein, the pathogen avirulence protein could interfere with a positive plant defence regulator or promote a plant defence suppressor. This would be crucial to successful pathogen proliferation (Hammond-Kosack and Parker, 2003).

The guard model provides explanation for previously unexpected findings, where specific Avr proteins associate with a seemingly inappropriate R protein (Leister and Katagiri, 2000), that unrelated Avr proteins target the same molecule in the plant cell (Kim et al., 2002), that R proteins can functionally interfere with one another (Ritter and Dangl, 1996), and explains why direct interactions between R and Avr products have been difficult to detect (Gómez-Gómez, 2004). Direct interaction between R and Avr proteins has only been detected during the incompatible reaction between rice and the rice blast fungus *Magnoporthe grisea*. The rice *Pi-ta* gene encodes an NBS-LRR protein that recognises *Magnaporthe* isolates in a race specific fashion. The corresponding pathogen avirulence gene product (AVR-Pita) is predicted to be a small, secreted metalloprotease protein. Direct physical interaction occurs between the plant resistance protein and the pathogen avirulence protein both *in vitro* and *in vivo* (Jia et al., 2000). It is though that the N terminal region of Pi-ta is responsible for the interaction with AVR-Pita. In a refinement of the guard hypothesis, it has been suggested that during a compatible reaction this domain might interact with yet another pathogen protein that then would preclude the interaction with the AVR-Pita leading to pathogen virulence (Gómez-Gómez, 2004).

Given the large number of possible effectors from just a single strain of one pathogen (Collmer et al., 2002), it is unlikely that the repertoire of R proteins in the plant could be sufficient to mediate direct recognition of all the possible virulence factors from a pathogen (Mackey et al., 2003). Therefore, in order for the plant to effectively protect itself against a variety of pathogens, from

viruses to bacteria, from fungus to aphids, it is thought that pathogen virulence factors only target a limited set of host proteins that are important in plant defence responses (Gómez-Gómez, 2004).

Pathogen associated molecular patterns

In addition to *Avr-R* gene product interactions, plants possess a broader, more basal surveillance involving sensitive perception systems for numerous microbe derived molecules. These molecules mediate activation of plant defence responses in a non-cultivar specific manner, and have been described as general elicitors (Boller, 1995). These non-specific elicitors are constitutively present in the pathogen, and are essential for the functioning of the microorganism. As a result, they are conserved within a class of microbes, and have recently been termed pathogen associated molecular patterns (PAMPs) (Gómez-Gómez, 2004).

General elicitors involved in the activation of plant defence responses have been isolated from viruses, bacteria, fungal and oomycete pathogens. They act as signalling molecules at low concentrations, have diverse structures and include polygalacturonides, β-glucans, chitosan, lipids and proteins (Boller, 1995). Other examples of PAMPs include the elicitor PaNie from the phytopathogenic oomycete *Pythium aphanidermatum* and other fungi (Veit et al., 2001); the elicitor Pep-13 conserved among different oomycete transglutaminases (Brunner et al., 2002); fungal chitin (Stacey and Shibuya, 1997; Ramonell et al., 2002); arachidonic acid, a fatty acid component of oomycete mycelia (Geetha et al., 1996); and lipopolysaccharides that form an integral component of the cell surface of Gram negative bacteria that invades plants (Erbs and Newman, 2003). Two other bacterial PAMP elicitors include bacterial cold shock protein (CSP) (Felix and Boller, 2003) and bacterial flagellin (Felix et al., 1999). All these molecules are produced by bacteria or fungi, but not by plant cells, and their recognition by plant receptors signals the presence of potential phytopathogens.

Plant recognition of bacterial flagellin provides an excellent example of the plant perception systems for PAMP molecules. Flagella are essential for

bacterial mobility in response to a changing environment. Flagellin represents the building block of the flagellar filament of eubacteria, and is comprised of conserved domains in the N and C termini of the protein, while the central domain is hypervariable (Felix et al., 1999). Like other PAMPs, flagellae are essential for bacterial viablility, and mutations in the flagellin protein that compromise flagella function would have deleterious consequences for the bacteria. Recent experiments have shown that addition of crude bacterial extracts containing extracellular flagellin monomers caused medium alkalinisation and ethylene production in Arabidopsis, tomato, tobacco and rice cell cultures (Felix et al., 1999; Che et al., 2000) as well as callose deposition and defence gene activation in Arabidopsis (Gómez-Gómez, 2004), and transcriptional reprogramming in rice (Fujiwara et al., 2004).

A breakthrough in the understanding of flagellin recognition came from the map based cloning of Arabidopsis *FL*agelling *S*ensing (*FLS2*) protein (Gómez-Gómez and Boller, 2002). FLS2 is a receptor-like kinase with structural similarities to R proteins. It contains a predicted signal peptide, and extracellular LRR domain, a transmembrane domain and an intracellular serine/threonine kinase domain (Figure 1.1). The overall structure suggests a role for FLS2 in the perception of an extracellular signal and transduction of the signalling event through the intracellular kinase domain (Gómez-Gómez and Boller, 2002). The search for further PAMP receptors in plants is likely to provide good targets for engineering durable resistance control (Hammond-Kosack and Parker, 2003).

1.5 PLANT DEFENCE SIGNALLING NETWORKS

Plant-pathogen recognition causes the rapid activation of appropriate defences. Upon elicitor binding to receptors, defence signalling pathways are activated that eventually lead to a defence response. Intracellular signalling is instigated when the occupied receptor directly or indirectly activates a downstream effector enzyme to produce a specific second messenger. In its turn, the second messenger binds to and activates, for example, a protein kinase that is at the start of a kinase cascade. This leads to different responses, such as transcriptional activation of defence related genes (Laxalt

and Munnik, 2002). Experimental evidence suggests that defence signalling is complex and involves an interplay between protein kinases, phospholipids, and defence signalling molecules such as nitric oxide (NO), reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (McDowell and Dangl, 2000; Hammond-Kosack and Parker, 2003).

Mitogen activated protein kinases

Mitogen activated protein kinase (MAPK) cascades are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses, and are found in all eukaryotes analysed to date (Innes, 2001). The basic assembly of a MAPK cascade is a three kinase module conserved in all eukaryotes. MAPK, the last kinase in the cascade, is activated by a kinase relay consisting of a MAPK kinase (MAPKK or MEK), which, in turn, is activated by a MAPKK kinase (MAPKKK or MEKK).

MAPKs are activated by a variety of stress stimuli including wounding, temperature drought, salinity, osmolarity, UV irradiation, ozone and reactive oxygen species. MAPKs from several plant species were also shown to be activated during plant responses to elicitors or pathogens (Romeis, 2001; Zhang and Klessig, 2001). A significant advance in our understanding of plant MAPKs was recently made by Asai and coworkers (Asai et al., 2002) in which a complete plant MAPK cascade was identified that functions downstream of the Arabidopis flagellin receptor FLS2 (Figure 1.3). In the Arabidopsis FLS2 pathway, the flagellin peptide present in the extracellular media interacts with the extracellular LRR FLS2 domain. This interaction leads to heterodimerisation or dimerisation of the receptor complex and activation of the FLS2 kinase domain. A kinase associated protein phosphatase (KAPP) is a negative regulator in this pathway (Gómez-Gómez, 2004). The FLS2 kinase activity is responsible for the phosphorylation and activation of the Arabidopsis mitogen kinase kinase 1 (AtMEKK1) which then phosphorylates Arabidopsis mitogen kinase 4 and 5 (AtMAKK4/5). These kinases in turn phosphorylate and activate Arabidopsis mitogen kinase 6 and 3 (AtMK6/3) and lead to the activation of the WRKY transcription factors WRKY22 and WRKY29 that activate the transcription of defence genes. Infection of Arabidopsis plants

constitutively expressing components of the flagellin responsive MAPK cascade led to enhanced resistance to the virulent pathogens *Pseudomonas syringae* and *Botrytis cinerea* (Asai et al., 2002). These results suggest that defence responses activated by the flagellin MAPK cascade are effective against both fungal and bacterial pathogens, and that signalling events initiated by diverse pathogens converge into a conserved MAPK cascade.

In tobacco, activation by fungal elicitors of AtMK3 and AtMK6 orthologues, WIPK and SIPK respectively, is similar to activation of the flagellin MAPK cascade in Arabidopsis (Asai et al., 2002). Furthermore, Yang and coworkers (2001) identified a tobacco MAPKK, NtMEK2, which can activate WIPK and SIPK. Constitutive expression of NtMEK2 in tobacco leaves lead to the induction of hypersensitive cell death and the expression of defence genes in the absence of pathogens. These results suggest that the MAPK cascade containing NtMEK2, WIPK and SIPK is involved in the expression of fungal pathogen defence responses in tobacco (Yang et al., 2001). The Arabidopsis orthologues of NtMEK2 are MK4 and MK5, indicating the importance of the flagellin MAPK cascade in pathogen defence, and further suggesting that signalling events initiated by diverse pathogens converge into a conserved MAPK cascade (Asai et al., 2002).

Recently, a number of rice MAPKs have been identified that play a role in signalling following pathogen attack and wounding (Rakwal and Agrwal, 2003; Agrwal et al., 2003; Kim et al., 2003). Rice MAPKs OsBWMK1, OsWJUMK1, OsMSRMK2, OsBIMK1 and OsMSRMK3 were all shown to be upregulated by SA, JA and ethylene (Agrwal et al., 2003). In addition, OsBIMK1 was rapidly induced during an incompatible reaction between a blast resistant rice genotype and *Magnoporthe grisea* (Song and Goodman, 2002).

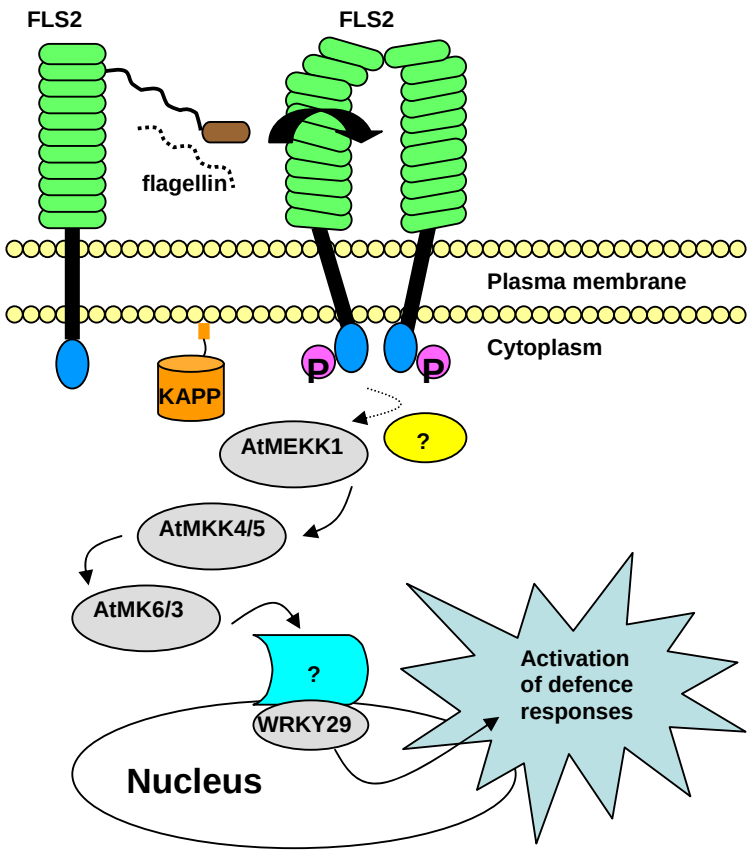

Figure 1.3. Main components of the signal transduction pathway involved in flagellin signalling in Arabidopsis (adapted from Gomez-Gomez, 2004). In the Arabidopsis FLS2 pathway, the flagellin peptide present in the extracellular media interacts with the extracellular LRR FLS2 domain. This interaction, which may also involve additional components, leads to heterodimerisation or dimerisation of the receptor complex and activation of the FLS2 kinase domain. A kinase associated protein phosphatase (KAPP) is a negative regulator of this pathway. The FLS2 kinase activity is responsible for the phosphorylation and activation of Arabidopsis mitogen kinase kinase 1 (AtMEKK1), which then phosphorylates Arabidopsis mitogen kinase kinase 4 and 5 (AtMAKK4/5). These kinases in turn phosphorylate and activate Arabidopsis mitogen kinase 6 and 3 (AtMK6/3) and leads to the activation of the transcription factor WRKY29, which activates the transcription of defence genes.

Calcium dependent protein kinases

Numerous stimuli can alter the Ca^{2+} concentration in the cytoplasm, a factor common to many physiological responses in plant and animal cells. In general, in the absence of a stimulus, cytosolic Ca^{2+} concentration in plant cells is maintained at a concentration of approximately 100 nM. However, Ca^{2+} concentration in the cell wall and organelles is in the millimolar range (Yang and Poovaiah, 2003). In response to a variety of stimuli, the cytosolic Ca^{2+} concentration in plants is rapidly elevated via an increased Ca^{2+} influx, and then quickly returns to the basal level by Ca^{2+} efflux – this produces a Ca^{2+} spike. Specific responses to different stimuli could be achieved through variations in the amplitude, duration, location, and frequency of these Ca^{2+} spikes (Ludwig et al., 2004).

Calcium binding proteins decode information contained in the temporal and spatial patterns of these Ca^{2+} signals and bring about changes in metabolism and gene expression. In addition to calmodulin, a calcium binding protein found in all eukaryotes, plants contain a large family of calcium dependent protein kinases (CDPKs). CDPKs, one of the largest subfamilies of plant protein kinases, possess a characteristic structure in which an N terminal serine/threonine protein kinase domain is fused to a carboxyl terminal calmodulin like domain containing four EF hand calcium binding sites (Harmon et al., 2000). A junction domain between the kinase and calmodulin like domain functions as a pseudo substrate autoinhibitor that inhibits phosphorylation in the absence of Ca^{2+} and keeps the CDPK in a state of low activity (Harmon et al., 2000).

Elicitor induced calcium influx and protein kinase activity have been reported in many pathosystems as one of the earliest responses required for further downstream signalling. CDPKs are therefore ideally structured for sensing changes in intracellular calcium concentration and translating them into kinase activity. Treatment of tobacco with non specific elicitors and wounding caused an accumulation of NtCDPK1 transcripts (Yoon et al., 1999). A maize CDPK (ZmCPK10) is also transcriptionally activated in response to fungal infection and treatment of fungal elicitors (Murillo et al., 2001). Activation of ZmCPK10

was accompanied by an increase in the level of maize PR proteins. One of the best biologically characterised CDPKs is NtCDPK2 from tobacco. This enzyme was found to be upregulated in response to treatment with the *Cladosporium fulvum* Avr9 peptide in transgenic tobacco plants expressing the Cf-9 resistance (Romeis et al., 2001). Furthermore, Romeis and coworkers (2001) also reported that CDPK silenced Cf-9 *Nicotiana benthamiana* plants showed a reduced and delayed hypersensitive response after race specific elicitation in the Cf-9:Avr9 gene for gene interaction.

Phospholipid signalling

Phospholipid derived molecules are emerging as novel secondary messengers in plant defence signalling. A downstream product of both the phospholipase C (PLC) and phospholipase D (PLD) pathways is phosphatidic acid (PA). Recent research suggests that PA plays a role in defence response signalling. Evidence for PLC involvement in plant defence response is through the application of PLC inhibitors. These inhibitors were shown to block a race specific defence response reaction, as well as inhibit reactive oxygen species formation and MAPK cascade activation (Laxalt and Munnik, 2002). One of the first reports that implicated PLD in plant-pathogen interactions described the induction of PLD gene expression in rice upon infection by *Xanthomonas oryzae* (Young et al., 1996). In soybean, PA activates a MAPK cascade via a protein kinase that has not yet been identified, and when PA production is suppressed, wound activation of the MAPK is also inhibited (Lee et al., 2001). Furthermore, Farmer and Choi (Farmer and Choi, 1999) showed that a carrot CDPK was activated by PA and Ca^{2+} *in vitro*, and further studies indicated that phosphoinositide dependent protein kinase 1 (PDK1) specifically binds PA (Deak et al., 1999). PA signalling is thought to be located upstream of the oxidative burst, as treatment of tobacco cells with PA induced an oxidative burst (Laxalt and Munnik, 2002). Future research will involve the identification of downstream target proteins for PA, beginning with proteins that bind PA so that PA binding domains can be characterised.

Reactive oxygen species and nitric oxide

Reactive oxygen species (ROS) have been proposed to serve as diffusible intercellular signals and/or second messengers for the activation of various defence genes in animals, plants and bacteria (Mehdy et al., 1996). Upon pathogen attack, O_2- accumulates in a process known as the oxidative burst, and is rapidly dismutated to H_20_2 non-enzymatically, or by the action of superoxide dismutase (Wojtaszek, 1997; Grant and Loake, 2000). Activation of the oxidative burst in the plant is part of an integrated signal system that involves salicylic acid and perturbations of the cytosolic Ca^{2+} (Alvarez et al., 1998). Several sources are known to exist for the generation of ROS including a plasmamembrane located NADPH oxidase, a cell wall peroxidase and amine, diamine and polyamine oxidase type enzymes (Grant and Loake, 2000).

ROS play a role in activating a number of plant defence responses to pathogen attack. These include induction of phytoalexin production, oxidative cross linking of cell wall hydroxyproline rich proteins to reinforce the cell wall against pathogen degrading enzymes, and a role in initiation of programmed cell death leading to the formation of the hypersensitive response (Mehdy et al., 1996; Wojtaszek, 1997). This hypersensitive cell death results in a restricted lesion delimited from surrounding healthy tissue and is thought to contribute to pathogen restriction. However, the oxidative burst is necessary but not sufficient to trigger host cell death, and experimental evidence indicates that nitric oxide (NO) cooperates with ROS in the activation of hypersensitive cell death (Delledonne et al., 1998).

NO is a diffusible molecular messenger in animals and plants, and exerts a number of diverse signal functions in plants. It is a free radical that can either gain or lose an electron to energetically more favourable structures such as the nitrosonium cation (NO^+) and the nitroxyl radical (NO^-). NO has recently been identified as an essential molecule that mediates hypersensitive cell death and defence gene activation in plants (Delledonne et al., 1998; Durner et al., 1998; Tada et al., 2004) (Figure 1.4).

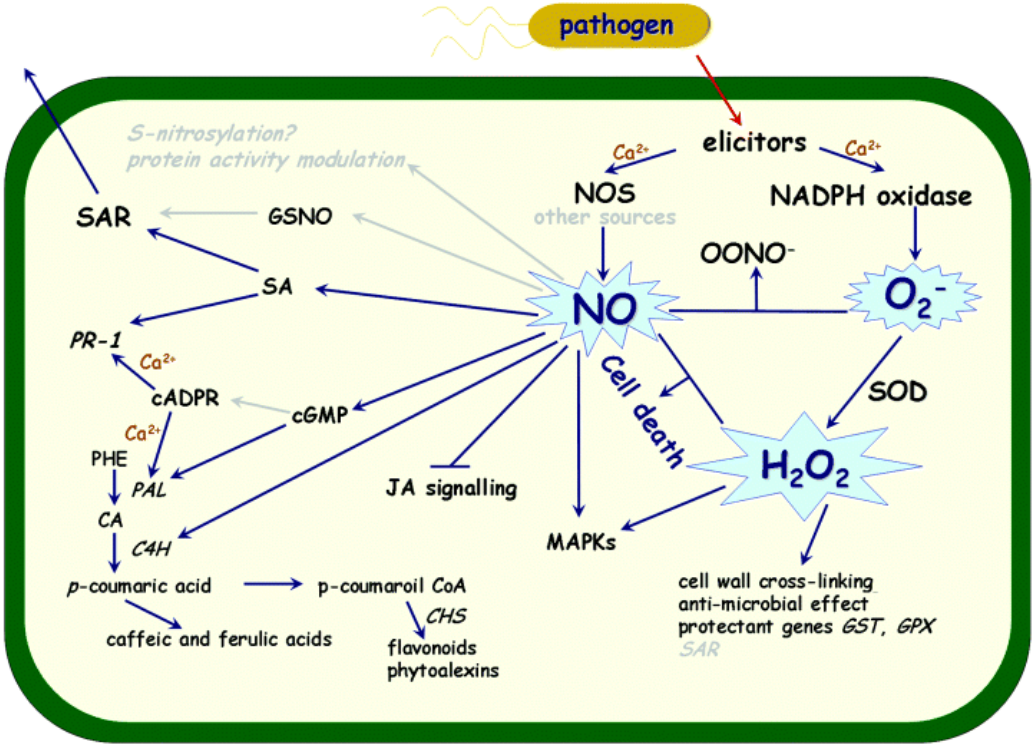

Figure 1.4. Representation of NO signalling functions during HR (adapted from Romero-Puertas et al. 2004). Grey arrows represent potential NO functions and synthesis, blue arrows represent experimental supported results. CHS, chalcone synthase; C4H, cinnamic acid-4-hydroxylase; CA, cinnamic acid, Ca^{2+}, calcium influx; cADPR, cyclic ADP ribose; cGMP, cyclic GMP, GPX, glutathione peroxidase; GSNO, S-nitroso-L-glutathione; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; HR, hypersensitive response; JA, jasmonic acid; MAPK, mitogen activated protein kinase; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PAL, phenylalanine ammonia lyase; PHE, phenylalanine; PR, pathogenesis related protein; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase.

In plants, NO can be synthesised either by an inorganic nitrogen pathway or by enzymatic catalysis. Slow spontaneous liberation of NO occurs from nitrite at neutral pH, and rate of release can be accelerated at acidic pH and presence of reducing agents (Romero-Puertas et al., 2004). In plants, the first enzyme found to be implicated in NO synthesis was nitrate reductase (NR). This protein has a fundamental role in nitrogen assimilation, and catalyses the NAD(P)H dependent reduction of nitrite to NO (Wendehenne et al., 2004). Recently, a pathogen inducible nitric oxide synthase (iNOS) was identified in tobacco and Arabidopsis (Chandok et al., 2003). iNOS is a variant of the P

protein of the glycine decarboxylase complex, displays typical NOS activity and requires the same cofactors as its mammalian counterpart (Chandok et al., 2003). However, with the exception of a few conserved domains, very little homology exists between plant and mammalian NOS proteins, implying that plant iNOS probably uses distinct chemistry to generate NO (Wendehenne et al., 2004).

NO signalling in plants (illustrated in Figure 1.4) is exerted through the secondary messengers cyclic GMP (cGMP), cyclic ADP-ribose (cADPR) and Ca^{2+}. NO binds to soluble guanylate cyclase, thereby activating the enzyme and increasing the level of cGMP. cGMP has been shown to induce the levels of a number of defence related proteins including pathogenesis related 1 protein (PR1), phenylalanine ammonia lyase (PAL) and cinnamate-4-hydroxylase (C4H) as well as the level of antimicrobial flavonoids and phytoalexins (Durner et al., 1998; Modolo et al., 2002; Polverari et al., 2003). One mode of action of cGMP is to stimulate synthesis of cADPR, a second messenger that stimulates Ca^{2+} release through intracellular Ca^{2+} permeable ryanodine receptor channels (RYR). Like cGMP, cADPR application in tobacco has also been shown to increase PAL and PR1 levels, a phenomenon that is amplified when cGMP and cADPR were added simultaneously (Durner et al., 1998). Thus, cGMP and cADPR appear to act synergistically to increase defence gene expression.

In animal cells, programmed cell death (PCD) is mainly mediated by peroxynitrite ($ONOO^-$) that is formed from NO and superoxide ($O_2^{\bullet -}$). In contrast, evidence from soybean cells indicates that HR associated cell death appears to be mediated by the relative levels of NO and H_2O_2 that is formed by dismutation of $O_2^{\bullet -}$ (Delledonne et al., 2001). Consistent with this conclusion, only the simultaneous increase of NO and H_2O_2 in tobacco cells induced cell death that had typical cytological and biochemical features of PCD (de Pinto et al., 2002). However, evidence from a study by Zhang and coworkers (2003) suggests that NO regulates HR cell death, but NO synthesis may not be a prerequisite for initiating the PCD signalling pathway. These authors showed that NO production in *P. syringae*-inoculated Arabidopsis did

not precede the HR, but rather occurred concurrently with HR. Because NO was first detected in the extracellular spaces, and then in the cytoplasm of nearby cells that died soon afterwards, it was proposed that NO facilitates the cell to cell spread of the HR.

Salicylic acid and systemic acquired resistance

In addition to the hypersensitive response that blocks the local growth of an infecting pathogen, a secondary defence response can be triggered that renders uninfected parts of the plant resistant to a variety of normally virulent pathogens (Ryals et al., 1996). This broad spectrum disease resistance is known as systemic acquired resistance (SAR). Salicylic acid accumulates after pathogen infection in a wide range of plants, and has been found to accumulate in systemic tissue following pathogen infection and is closely associated with the development of SAR (Ryals et al., 1996; Dempsey et al., 1999). In addition, exogenous application of SA or its analogues, such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) has been shown to induce SAR (Kessmann et al., 1994; Lawton et al., 1996). Associated with the SA accumulation and the onset of SAR is the induction of a group of pathogenesis related (PR) genes, which encode small secreted or vacuole targeted proteins with antimicrobial properties (Ryals et al., 1996; Dong, 2004).

SA is synthesised in plants either via the PAL pathway, or via isochorismate synthase (ICS) (Wildermuth et al., 2001). Evidence suggests that SA synthesised through ICS has an important role in plant defence against pathogens, and that it is required for PR1 gene expression and SAR defence responses. However, SA also potentiates cell death in response to particular pathogens or fungal elicitors (Dempsey et al., 1999). As plants that are defective in ICS gene expression still exhibit cell death when infected with necrotising pathogens, SA that potentiates plant cell death is probably synthesised through PAL (Wildermuth et al., 2001).

Evidence for the key role of salicylic acid in defence response came from the analysis of transgenic plants expressing the bacterial *nahG* gene, which

encodes the enzyme salicylate hydroxylase that inactivates salicylic acid by converting it to catechol. Transgenic NahG plants are unable to accumulate salicylic acid, and are also incapable of developing SAR, indicating that salicylic acid is required for the expression of SAR (Gaffney et al., 1993).

Studies on Arabidopsis mutants aimed at identifying components of the SA dependent signalling pathway led to the isolation of the *NPR1* gene which is a key regulator in transducing the SA signal leading to PR gene expression and SAR (Cao et al., 1998). NPR1 senses the SA signal through a change in redox potential in the cell (Mou et al., 2003). A low redox potential leads to the dissociation of the NPR1 oligomer in the cytoplasm through reduction of the disulphide bonds that hold the oligomer together. The dissociated NPR1 monomer translocates into the nucleus where it activates SA inducible genes through interaction with a subclass of the TGA transcription factors (Zhang et al., 1999; Zhou et al., 2000; Després et al., 2003). *NPR1* encodes a novel protein with ankyrin repeats, which are necessary and sufficient for the interaction with TGA transcription factors, although high affinity interactions also require the N terminal one third of NPR1 (Zhang et al., 1999). Despres and coworkers reported that TGA1 and TGA4 interact with NPR1 following SA treatment. Both TGA1 and TGA4 have unique cysteine residues that are oxidised in the cell's unreduced state, forming an intramolecular disulphide bond. However, SA induction leads to the establishment of a reduced state, which breaks the disulphide bonds, allowing interaction between NPR1 and TGA1 and TGA4, and subsequent PR gene expression. Besides TGAs, WRKY transcription factors have been suggested to play an important role in SAR related gene expression (Maleck et al., 2000; Yu et al., 2001). Significantly, WRKY transcription factors have recently been shown to bind the *NPR1* promoter, indicating that they positively regulate NPR1 expression during SAR (Yu et al., 2001).

Evidence that monocot and dicot plants share a conserved signal transduction pathway controlling *NPR1* mediated resistance came from a study in which Arabidopis *NPR1* was over expressed in rice plants (Chern et al., 2001). Transgenic rice plants challenged with the rice bacterial blight pathogen,

Xanthomonas oryze pv. *oryzae* (*Xoo*), displayed enhanced resistance to *Xoo.* Four rice bZIP proteins (rTGA2.1, rTGA2.2, rTGA2.3 and rLG2) were found to directly interact with NPR1 (Chern et al., 2001). Chern and coworkers (Chern et al., 2005) have recently reported the isolation of a rice NPR1 homologue (*NH1*). Transgenic rice plants overexpressing NH1 acquired high levels of resistance to *Xoo*, and yeast two hybrid studies indicated that NH1 interacts with the rice transcription factor rTGA2.2.

Nitric oxide is thought to play an important role in signalling pathways leading to SAR. NO treatment induces SA accumulation and its conjugates in tobacco (Durner et al., 1998). Furthermore, activation of PR1 by NO is mediated through SA, because it is blocked in transgenic *NahG* plants which are unable to accumulate SA (Durner et al., 1998). Although SA is an important molecule required for defence gene induction in uninfected distal tissue, it is not the key signal that activates SAR (Mauch-Mani and Metraux, 1998). Durner and colleagues (1998) have proposed that nitroso glutathione (GSNO) is a potential candidate for long distance signalling involved in SAR. These authors showed that GSNO is a powerful inducer of plant defence genes. GSNO has also been shown to induce systemic resistance against TMV infection in tobacco (Song and Goodman, 2001). Furthermore, glutathione is a major metabolite in phloem, where the SAR signal is most probably transmitted (Romero-Puertas et al., 2004).

A recent study illustrated the importance of S-nitrosoglutathione reductase (GSNOR) in Arabidopsis defence responses (Feechan et al., 2005). S-nitrosylation of the antioxidant tripeptide glutathione forms GSNO, which is thought to function as a mobile reservoir of NO bioactivity. GSNOR is able to metabolise GSNO as well as other protein S-nitrosothiols (SNOs). Feechan and coworkers (2005) showed that an increase in GSNOR activity led to decreased formation of SNOs, enhancing protection against ordinarily virulent microbial pathogens. Conversely, loss of GSNOR activity led to increased SNO levels, and both basal and nonhost disease resistance were also compromised (Feechan et al., 2005). Importantly, GSNOR was shown to positively regulate the signalling network controlled by SA in Arabidopsis.

Jasmonic acid and ethylene

NahG plants have also been valuable tools in the discovery of novel, salicylic acid independent defence pathways that, like SAR, convey broad spectrum systemic resistance. Several research groups demonstrated that specific defence responses are unaffected by the absence of salicylic acid in the NahG plants, which indicates that these defence reactions operate independently of salicylic acid (Pieterse and Van Loon, 1999). This is supported by the discovery that several defence responses can be activated without an increase in the level of salicylic acid or salicylic acid marker gene expression. In this light, the plant growth regulators jasmonic acid and ethylene have emerged as important signalling molecules in salicylic acid independent signalling.

Jasmonic acid (JA) is a fatty acid hormone derived from linolenic acid via the octadecanoid pathway (Turner et al., 2002). JA and its methyl ester methyl jasmonate (MeJA – collectively referred to as jasmonates) act as signalling molecules in many processes in plants including pollen and seed development, and defence against wounding, ozone, insect pests and microbial pathogens (Kunkel and Brooks, 2002; Voelckel and Baldwin, 2004).

Evidence indicates that jasmonates and ethylene act synergistically to induce defence responses in plants. Both are rapidly produced when the plant is attacked by a pathogen, particularly during necrotising infections where the rise in jasmonic acid levels even extends to systemic tissues (Penninckx et al., 1996). Moreover, exogenous application of these signalling molecules induces a set of defence genes that are also activated upon pathogen infection, among which are genes encoding plant defensins or thionins which exhibit antimicrobial activity (Terras et al., 1995; Epple et al., 1997). In Arabidopsis NahG plants, pathogen induced systemic activation of the plant defensin PDF1.2 is unaffected, indicating that this regulatory pathway is salicylic acid independent. Interestingly, *PDF1.2* gene expression is blocked in the ethylene insensitive mutant *ein2* and the jasmonic acid insensitive mutant *coi1* demonstrating that the signalling pathway involved in *PDF1.2*

induction requires components of the ethylene and jasmonic acid response (Penninckx et al., 1996; Pieterse and Van Loon, 1999).

Jasmonic acid has emerged as an important signal in a plant's wound response against insect and herbivore feeding (Wasternack and Parthier, 1997; Leon et al., 2001). Wounding not only causes a rapid production of jasmonic acid, but levels of ethylene increase as well. O'Donnell and coworkers (1996) demonstrated that neither wounding, nor jasmonic acid was able to induce the expression of the tomato *pin* (proteinase inhibitor) gene, a marker of the wound response, in the presence of ethylene inhibitors. Similarly, ethylene is unable to activate *pin* gene expression by itself, an indication that ethylene must sensitize the tissue to the inducing action of jasmonic acid (O'Donnell et al., 1996).

Although both wounding and pathogen attack involve the production of jasmonic acid and ethylene, several lines of evidence indicate that their respective response pathways are distinct. For example, in tobacco, wounding and pathogen attack show differential activation of different members of the *PR* gene families (Bol et al., 1996). Wounding appears to inactivate basic *PR* genes, whereas pathogen attack predominantly leads to acidic *PR* gene expression. A possible cause might be that upon wounding only jasmonic acid and ethylene appear to play a role, whereas after pathogen infection salicylic acid is produced as well. Salicylic acid and its functional analogues inhibit jasmonic acid induced defence gene expression (Kunkel and Brooks, 2002).

The study of Arabidopsis mutants have helped to elucidate and highlight the complexity of jasmonate signalling during defence response. The *coi1* mutation defines an Arabidopsis gene that functions in the jasmonate signalling pathway required for pollen development and defence against pathogens and insects. Characterisation of the COI1 gene indicated that it encodes a protein containing leucine rich repeats and an F box motif (Xie et al., 1998). F box proteins are known to function as receptors that selectively recruit regulatory proteins as substrates for ubiquitination. Thus, it appears

that COI1 is required to degrade a repressor of the jasmonate signalling pathway. Experiments using coimmunoprecipitation suggest that COI1 forms part of a functional E3 type ubiquitin ligase complex, and mediates ubiquitination of histone deacetylase, leading to the activation of the jasmonate responsive genes (Devoto et al., 2002).

Cross talk between signalling pathways

The interactions between SA and JA signalling appear to be complex, and there are a number of examples of antagonistic action between the two pathways. Expression of the JA/ET independent gene *PDF1.2* is strongly inhibited by SA, as demonstrated by increased *PDF1.2* expression in *nahG* plants following infection with *Alternaria brasicola* (Penninckx et al., 1996). Furthermore, Arabidopsis mutants *eds4* and *pad4*, which are impaired in SA accumulation, displayed increased PDF1.2 expression after treatment with MeJA (Gupta et al., 2000). Characterisation of the Arabidopsis JA signalling mutant *mpk4* provided genetic evidence that JA signalling negatively regulates the expression of SA mediated defences (Petersen et al., 2000). In addition to exhibiting impaired JA signalling, *mpk4* plants constitutively express SA mediated defences (Petersen et al., 2000).

Evidence from Arabidopis mutants seems to indicate that SA dependent defence responses are effective against biotrophic pathogens, whereas JA/ethylene dependent defence responses are effective against necrotrophic pathogens (Murray et al., 2002; Kunkel and Brooks, 2002). The existence of multiple defence mechanisms might be the evolutionary answer of the plant to challenges from different groups of pathogens. Although the HR, which is strongly associated with the accumulation of SA, restricts the growth of biotrophs, it may strengthen the virulence of necrotrophic pathogens and promote infection. This hypothesis was supported by the Arabidopsis mutant *dnd1*. *dnd1* fails to produce a normal HR, and was shown to suppress growth of the necrotrophic pathogen *Botrytis cinerea* (Govrin and Levine, 2000). It is therefore possible that plants have evolved a JA/ET signalling pathway in order to combat necrotrophic pathogens. In this manner, downregulation of

SA dependent defence responses by JA would be a logical evolutionary adaption.

Evidence from DNA microarray studies (reviewed in section 1.7) indicates that positive interactions do exist between SA and JA/ET pathways. Microarray analysis of Arabidopsis plants that had been exposed to a variety of defence inducing treatments revealed that more than 50 defence related genes were coinduced by SA and JA, suggesting that the two signals coordinately regulate these genes (Schenk et al., 2000). Similarly, Salzman and colleagues (Salzman et al., 2005) performed a microarray study in sorghum, which showed that genes from the octadecanoic acid pathway responsible for JA synthesis were induced by SA as well as JA, and that mutual antagonisms, as well as synergistic effects, existed between SA and JA/ET pathways.

1.6 BIOCHEMISTRY OF PLANT DEFENCE RESPONSES

A major target of signal transduction is the cell nucleus where the terminal signals lead to the transcriptional activation of numerous genes, and consequently to the *de novo* synthesis of a variety of proteins and antimicrobial compounds. Studies with different plant pathology systems have revealed that the active response of plants to attempted pathogen infection is associated with dramatic reprogramming of cellular metabolism (Rushton and Somssich, 1998). Expression of a large array of genes whose products are involved both in diverse primary and secondary metabolic pathways are rapidly induced or strongly upregulated. These include genes encoding enzymes of the shikimate and the general phenylpropanoid pathways along with enzymes from subsequent branch pathways (Rushton and Somssich, 1998). Another group of genes, the PR genes, are closely associated with the defence response (Van Loon and van Strien, 1999). In addition, a range of secondary signalling molecules are generated to ensure coordination of the defence response both temporally and spatially, resulting in rapid containment of the pathogen.

Pathogenesis related proteins

A major response to pathogen attack is the expression of a number of plant genes which encode PR proteins. The PRs have typical physiochemical properties that enable them to resist acidic pH and proteolytic cleavage and survive the harsh environments where they occur, which include vacuoles, cell wall or intercellular spaces. PR proteins were first discovered and classified in tobacco, but have subsequently been found to occur in other plant species including monocots (Stintzi et al., 1993). Currently, fourteen families of PR proteins have been classified (Table 1.2). Within each PR family, a type member has been defined, the nucleotide sequence of the mRNA of which may be used in the search for homologues in the same or different plant species (Van Loon and van Strien, 1999).

For the majority of the PR families, activities are known or can be inferred. The PR2 family consists of endo-β-1,3-glucanases, and PR3, PR4, PR8 and PR11 are all classified as endochitinases. A way of distinguishing these types of chitinases is by class, based on their different specific activities on a range of substrates, with class III (PR8) basic isoforms possessing substantial lysozyme activity (Van Loon and van Strien, 1999). The PR5 family belongs to the thaumatin like proteins with homology to permatins that permeabilise fungal membranes (Vigers et al., 1991). PR6 are proteinase inhibitors implicated in defence against insects and other herbivores, microorganisms and nematodes. PR7 has so far been characterised only in tomato, where it is a major PR and acts as an endoproteinase. The PR9 family of peroxidases is likely to function in strengthening plant cell walls by catalysing lignin deposition in reaction to microbial attack. The PR10 family is structurally related to ribonucleases, however their capability to cleave viral mRNA remains to be demonstrated. The PR12 defensins, PR13 type thionins and PR14 type lipid transfer proteins all exhibit antifungal and antibacterial activity, exerting their effect at the level of the plasma membrane of the target microorganism (Bohlmann, 1994; Garcia-Olmedo et al., 1995; Broekaert et al., 1997). The only PR family for which no fuction or relationship is known is the PR1 protein family (Van Loon and van Strien, 1999).

In Arabidopsis PR proteins dependent on the accumulation of SA comprise PR1, PR2 and PR5, with PR1 being the predominant proteins (Uknes et al., 1992). However, SA independent but jasmonate dependent induction of the plant defensin gene *pdf1.2* as well as PR3 and PR4 is associated with the induced resistance against necrotrophic fungi (Van Loon and van Strien, 1999). In maize, treatment with SA, BTH and INA lead to the rapid induction of maize PR1 and PR5 genes, as well as increased resistance to the downy mildew pathogen *Peronsclerospora sorghi* (Morris et al., 1998). Furthermore, infection with *Puccinia sorghi* (causal agent of rust) and *Bipolaris maydis* (causal agent of Southern corn leaf blight) caused an increase in *PR1* and *PR5* gene expression. The existence of a chemically inducible disease resistance and *PR1* and *PR5* gene expression in maize indicates that maize is similar to dicots in many aspects of induced resistance, and supports the notion of an ancient plant inducible defence pathway against pathogen attack that is shared between monocots and dicots.

Table 1.2. The families of pathogenesis related proteins (adapted from van Loon and van Strien 1999)

Family	Type Member	Properties
PR1	Tobacco PR1-a	Unknown
PR2	Tobacco PR2	B-1,3-glucanase
PR3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR4	Tobacco R	Chitinase type I, II
PR5	Tobacco S	Thaumatin like
PR6	Tomato Inhibitor I	Proteinase inhibitor
PR7	Tomato P69	Endoproteinase
PR 8	Cucumber chitinase	Chitinase type III
PR9	Tobacco lignin forming peroxidase	Peroxidase
PR10	Parsley PR1	Ribonuclease like
PR11	Tobacco class V chitinase	Chitinase type I
PR12	Radish Rs-AFP3	Defensin
PR13	Arabidopsis THI2.1	Thionin
PR14	Barley LTP4	Lipid transfer protein

Cell wall fortification

Microbes must negotiate the plant cuticle and the plant cell wall to reach the cell and cause disease. Plant cell walls are complex consisting of proteins carbohydrates, lignin and water with encrusting molecules including cutin, suberin and certain inorganic compounds (Shailasree et al., 2004). Exposure to pathogens brings about further change in composition and structure.

One type of cell wall fortification that occurs rapidly in response to biotrophic fungal invasion is the formation of papillae. These papillae, which are primarily composed of callose (a β-1,3-glucan polymer) and lignin, are thought to act as a physical barrier blocking fungal penetration into plant cells (Hammond-Kosack and Jones, 2000). Callose plays a further role in plant defence through the blockage of plasmodesmata which impedes cell to cell movement of viruses (Beffa et al., 1996).

Extracellular basic hydroxyproline rich glycoproteins (HRGPs) contribute to cell wall fortification in two ways. Firstly, preformed HRGPs rapidly crosslink to the cell wall matrix by way of tyrosine through reaction with induced H_2O_2. This may constitute one of the earliest defence responses accompanying the oxidative burst (Bradley et al., 1992). Later, *de novo* HRGP synthesis initiates additional lignin polymerisation to further reinforce cell walls (Hammond-Kosack and Jones, 2000). Shailasree and colleagues (2004) demonstrated that pearl millet cultivars rapidly accumulated HRGPs in response to infection with *S. graminicola*. These authors also showed that HRGPs accumulated to a higher level in resistant cultivars compared to susceptible ones, and crosslinking of HRGPs to the cell wall only occurred in downy mildew resistant pearl millet cultivars.

Plant pathogens produce a number of cutinases and cell wall hydrolysing enzymes, such as pectinases, cellulases, xylanases and polygalacturonases (PG), which attack the various cell wall polymers. Another class of plant defence related extracellular proteins are polygalacturonase inhibiting proteins (PGIPs), which have been shown to inhibit PG activity (De Lorenzo and

Ferrari, 2002). It has been hypothesised that PGIPs may retard PG function, which would lead to elevated abundance of oligogalacturonides with a chain length of >8 units. These, in turn, may trigger additional defence responses (De Lorenzo and Ferrari, 2002). Alternatively, PGIPs may slow the rate of hyphal extension so that other components of the defence response can be more effectively deployed (Hammond-Kosack and Jones, 1996). Interestingly, PGIPs possess a LRR domain similar to that predicted for several of the cloned R gene products (Bent, 1996). Recently, Kemp and coworkers (Kemp et al., 2003) provided the first evidence for the presence of a PGIP in a monocotyledonous cereal. These authors isolated a PGIP from wheat that was closely associated with the cell wall, and exhibited a highly selective inhibitory activity against PGs from various fungi. N-terminal sequencing of the wheat PGIP showed that the protein displayed no similarity to any other characterised PGIP.

Lipoxygenases

Lipoxygenases (LOXs) are a class of non-heme, iron containing dioxygenases that catalyse the oxygenation of polyunsaturated fatty acids with a 1,4-*cis,cis*-penatadiene structure to form conjugated diene hydroperoxide. Products of LOX action include plant defence compounds such as jasmonates, lipid peroxides, and antimicrobial and antifungal compounds such as leaf aldehydes or divinyl ethers (Babitha et al., 2004). Rapid increases in LOX enzyme activity and/or mRNA and protein levels are frequently found to be associated with *R-Avr* gene mediated incompatibility (Hammond-Kosack and Jones, 1996). Increased LOX activity may contribute to resistance in a number of ways. For example, the primary products of LOX, the fatty acid hydroperoxides, are very reactive and may cause oxidative damage to membranes, leading to leakage of cellular contents, and ultimately plant cell death (Hildebrand, 1989). Babitha and coworkers (2004) recently reported the induction of LOX in downy mildew resistant pearl millet seedlings upon inoculation with *S. graminicola*, and demonstrated that *de novo* synthesis of the LOX6 isozyme was linked to resistance to downy mildew.

Phytoalexins

Phytoalexins are low molecular mass, lipophilic compounds that accumulate rapidly at sites of incompatible pathogen infection. Phytoalexin biosynthesis occurs after primary metabolic precursors are diverted into secondary metabolic pathways. For example, phenylalanine is diverted into the synthesis of various flavonoid phytoalexins by the *de novo* synthesis of PAL, an enzyme that controls a key branchpoint in the phenylpropanoid biosynthetic pathway (Hammond-Kosack and Jones, 2000). Well characterised phytoalexins include camalexin from wild type Arabidopsis plants (Browne et al., 1991) and the grapevine phytoalexin resveratrol (Hain et al., 1993). The biosynthesis of resveratol was engineered in tobacco by constitutively expressing the terminal biosynthetic enzyme stilbene synthase (Hain et al., 1993). Transgenic tobacco plants exhibited enhanced resistance to the necrotrophic fungus *B. cinerea*. Grayer and Kokubun (Grayer and Kokubun, 2001) showed that the rice leaf phytoalexins sakuranetin and momilactone A were produced within three days of inoculation with the rice blast fungus *M. grisea*. Further studies suggested that blast resistant cultivars produced much higher quantities of phytoalexins than blast sensitive cultivars.

Transcription factors

Transcriptional activation of genes is a vital part of the plants defence system against pathogens. Differences in the expression patterns of pathogen responsive genes are a result of the architecture of the promoters. Research carried out over the past few years has been productive in identifying promoter elements and transcription factors that bind to these elements that are important for regulating plant responses to pathogens. Transcription factors that play an important role in defence response belong to WRKY, bZIP, ethylene responsive element binding proteins (EREBP), Whirly and Myb protein families. EREBPs, WRKY and Whirly proteins appear to be unique to plants, whereas other transcription factors such as bZIP and Myb proteins also have counterparts in animals (Rushton and Somssich, 1998).

WRKY proteins comprise a large family of transcription factors with 74 members in Arabidopsis and more than 90 members in rice (Ülker and

Somssich, 2004). Common to these proteins is a DNA binding region of approximately 60 amino acids in length (the WRKY domain) that contains the conserved amino acid motif WRKYGQK, adjacent to a zinc finger like motif. WRKY factors show high binding affinity to a DNA sequence designated the W box that displays the characteristic DNA sequence (C/T)TGAC(T/C). W box dependent binding activity requires both the invariable WRKY amino acid signature and the cysteine and histidine residues of the WRKY domain that tetrahedrally coordinate a zinc atom (Ülker and Somssich, 2004). Transcriptome analyses have revealed that W boxes and related sequence motifs are ubiquitously conserved in upstream regions of genes that are upregulated during SAR, R gene mediated resistance or basal resistance (Maleck et al., 2000; Ramonell et al., 2002).

Specific WRKY family members show enhanced expression and/or DNA binding activity following induction by a range of pathogens, defence signals and wounding (Euglem et al., 2000). Several lines of evidence also exist which show a role for WRKY transcription factors in MAPK kinase signalling cascades. Two Arabidopsis WRKY factors (*At*WRKY22 and *At*WRKY29) have been identified as important downstream components of a MAPK pathway that confers resistance to both bacterial and fungal pathogens (Asai et al., 2002); see section 1.5). Similarly chitin also upregulates a group of WRKY genes including *At*WRKY22 and *At*WRKY29 (Wan et al., 2004). Furthermore, Arabidopsis WRKY70 was recently identified as a common regulatory component of SA and JA dependent defence signalling (Li et al., 2004). WRKY70 acts as an activator of SA signalling, but as a repressor of the JA signalling pathway, thus mediating crosstalk between these antagonistic pathways.

bZIPS represent a large family of transcription factors that possess a basic region that binds DNA and a leucine zipper dimerisation motif. One class of bZIP proteins that is linked to plant defence responses comprises the TGA element binding factor proteins. TGA transcription factors were originally identified by their ability to bind to the *as*1 like elements (CTGACGTAAGGGATGACGCAC), a class of general stress responsive

elements (Rushton and Somssich, 1998). Some stress responsive genes that contain as1 like elements and are regulated by TGA factors include PR1 and glutathione *S*-transferase 6 (*GST6*) genes (Singh et al., 2002). A major advance was the discovery that TGA family members bind to the NPR1 in Arabidopsis as well as its homologue NH1 in rice (Després et al., 2003; Chern et al., 2005). NPR1 is a key component in SA defence signalling, and treatment of plants with SA increases the interaction between TGA proteins and NPR1, and enhances the DNA binding activity of specific TGA proteins (Fan and Dong, 2002).

EREBPs share a conserved 58-59 amino acid domain (the ERFdomain) that can bind to a GCC box *cis* element. GCC boxes (AGCCGCC) and related cis-elements mediate gene expression in response to various pathogens and defence elicitors (Euglem, 2005). Further evidence for the role of EREBPs in defence has come from yeast two hybrid experiments in which PTO kinase, a product of the tomato *Pto* R gene, was shown to interact with and phosphorylate an EREBP called PTO-INTERACTING4 (PTI4) (Zhou et al., 1997). As a result, the DNA binding capacity of PTI4 to the GCC box is increased. Thus, PTO appears to confer resistance to *Pseudomonas syringae* strains carrying the corresponding *avrPto* avirulence genes by activating a signalling pathway that leads through EREBPs to activate PR genes containing GCC boxes.

Whirly transcription factors are single stranded DNA binding proteins that were first identified in potato were they were found to activate or repress the expression of the potato *PR10a* gene (Boyle and Brisson, 2001). These transcription factors obtained their name from the 'Whirly-gig' like structures they form through the association of four protomers in a cyclic C4 symmetry (Desveaux et al., 2005). Studies on Arabidopsis mutants defective in Whirly transcription factor function indicated that the Whirly transcription factor AtWhy1 is required for SA dependent R mediated resistance, basal resistance and SAR (Desveaux et al., 2005). In response to SA treatment, AtWhy1 tetramers bind to single stranded GTCAAAA/T containing DNA. However, AtWhy1 SSB activity is independent of NPR1, which suggests that AtWhy1 is

likely to play a role in NPR1-independent salicylic signalling pathways (Desveaux et al., 2005). Although not yet studied in a lot of detail, Whirly family members have also been identified in the cereals wheat (2), rice (2) and maize (2), and partial sequences have also been identified in sugar cane (*Saccharum officinarum*) and rye (*Secale cereale*).

Certain Myb transcription factors have also been found to be upregulated during pathogen attack and other defence related stimuli (Rushton and Somssich, 1998; Euglem, 2005). A combined metabolomic and transcriptomic analysis of the Arabidopsis mutant *pap1-D,* which overexpresses the Myb transcription factor PAP1, indicated that genes and subsequent metabolites involved in the phenylpropanoid and flavonoid biosynthetic pathways were significantly upregulated (Tohge et al., 2005). Genes and metabolites from these pathways are known to play an important role in plant defence response (Hammond-Kosack and Jones, 2000). One Myb binding motif (type 1, GG/TTA/TGG/TT) is generally conserved in promoters of WRKY genes, which further supports the suggestion that Myb factors have roles in defence regulation (Euglem, 2005).

1.7 DNA MICROARRAYS: TOOLS FOR STUDYING GLOBAL GENE EXPRESSION CHANGES DURING PLANT DEFENCE RESPONSE

The analysis of plant-pathogen interactions and defence signalling processes in plants have traditionally been reductionist in approach, and have focussed on only one or a few defence response genes at any one time. From such studies, it has not been possible to assess the extent of overlap of gene activation by different signals and pathogens during defence response. The advent of DNA microarray technology has revolutionised the study of plant-pathogen interactions, and can provide information on the expression patterns of thousands of genes in parallel.

DNA microarray technology

The key principle behind microarray technology is the large scale hybridisation of fluorescently labelled nucleic acid molecules from biological samples to be

analysed to an array of complementary single stranded DNA sequences immobilised on a solid surface. Two types of microarrays are commonly used in transcriptome analysis; spotted or deposition microarrays and Affymetrix microarrays (www.affymetrix.com). In spotted microarrays, collections of DNA samples are deposited onto a glass slide using robotics. These microarrays are highly flexible as they may be constructed from anonymous clones found in genomic, subtractive, differentially displayed or normalised libraries or from commercially synthesised long (n = 50-70) oligonucleotides (Ramonell and Somerville, 2002). Alternatively, Affymetrix chips consist of an array of oligonucleotides (n = 20-25) that have been synthesised *in situ* on a solid substrate using photolithography. Each gene to be analysed is typically represented by twenty specific probes on the chip. In contrast to spotted microarrays, Affymetrix chips require prior knowledge of DNA sequence information but permit single base change analysis. However, Affymetrix arrays are only available commercially, and their expense often limits the number and scale of experiments that can be performed in a typical laboratory. An advantage of using spotted oligonucleotide and Affymetrix microarrays over cDNA microarrays is that they offer sequence specific detection of gene expression, which is especially important when studying the expression of different gene family members.

In spotted microarrays, relative mRNA abundances are compared in different samples by extraction of RNA from samples, conversion to cDNA, labelling with different fluorescent dyes, and simultaneous hybridisation of samples to the array. The amount of each labelled target bound to each spot on the array is then quantified. The ratio of signal intensities between control and test cDNA targets reflects the induced/repressed or unchanged expression of mRNA species under study.

Described below are a number of examples of how DNA microarrays have been employed to examine plant-pathogen interactions, the plant's response to mechanical wounding and insect damage, and to study coordinated responses between different signalling pathways. The chosen examples by no means represent all microarray defence profiling studies, but have rather

been chosen to illustrate studies in non model plants and cereal crops, defence response to elicitor treatment, and detailed analysis of coordinated defence responses.

Profiling plant-pathogen interactions

A number of microarray experiments have been performed to examine the effect of pathogens on plant gene expression. Baldwin and coworkers. (Baldwin et al., 1999) undertook one of the first plant-pathogen interaction studies using DNA microarray technology. These authors used an Affymetrix chip representing 1500 maize ESTs, and observed that 117 genes were either induced or repressed six hours after challenge with the fungal pathogen *Helminthosporium maydis*. The same maize gene chip was subsequently used to investigate the differential gene expression in the *Les9* maize mutant, which forms spontaneous lesions and exhibits an upregulation of defence related genes and enhanced resistance to *Bipolaris maydis* (Nadimpalli et al., 2000). One third of the genes on this array were defence related genes, and nearly 70 genes showed changes in mRNA abundance of twofold or higher. Most of these genes were involved in defence, or were unknown or not previously implicated in plant defence. Unfortunately, neither study revealed the identities of induced or repressed genes except for the *Zm-hir3* gene which is implicated to be involved in cell death through ion channel regulation (Kazan et al., 2001).

Dowd and coworkers (Dowd et al., 2004) recently performed a gene expression profiling study to examine the changes in cotton root and hypocotyls tissues in response to infection with *Fusarium oxysporum* f. sp. *vasinfectum*. As cotton is a non model plant system, the authors prepared microarrays using clones from two cDNA libraries. One library was prepared from infected tissues from several time points after infection, and a second library was prepared from uninfected cotton tissues. Following microarray hybridisations, differentially expressed genes were sequenced and analysed for sequence homology to known genes. Microarray analysis of this susceptible plant pathogen interaction revealed different gene expression profile changes in cotton root and hypocotyls tissues. In hypocotyls tissues

infected with *F. oxysporum* f. sp. *vasinfectum*, increased expression of defence related genes was observed, whereas few changes in expression levels of defence related genes were found in infected root tissues. In infected roots, more plant genes were repressed than induced, especially at earlier stages of infection. Although many known cotton defence responses were identified including induction of PR genes, gossypol biosynthesis genes, potential new defence responses were also identified such as the biosynthesis of lignans.

In addition to plant-pathogen gene profiling, microarray profiling has been applied to study the effect of the addition of elicitors to plant cultures (Ramonell et al., 2002; Akimoto-Tomiyama et al., 2003; Fujiwara et al., 2004). Ramonell et al. (2002) characterised gene expression patterns in Arabidopsis in response to chitin treatment using an Arabidopsis microarray consisting of 2375 EST clones representing putative defence related and regulatory genes. These authors identified 71 genes whose expression was altered more than three fold in response to chitin treatment. Many of these genes were reported to be elicited by various pathogen related stimuli in other plants. Interestingly, the chitin gene expression profile was different to those obtained previously for SA, methyl jasmonate and ethylene treatment in Arabidopisis (Schenk et al., 2000) suggesting that perhaps chitin acts in parallel with these defence signalling molecules to influence the outcome of encounters between fungal and/or insect pathogens and plants (Ramonell et al., 2002). In a similar experiment Akimoto-Tomiyama et al. (2003) found that N-acetylchitooligosaccharides significantly induced 166 and repressed 93 out of 8987 randomly chosen rice ESTs in suspension cultured rice cells. Of the 259 ESTs identified as responsive to N-acetylchitooctaose, 18 genes were found to be involved in signal transduction, including 5 CDPKs.

Fujiwara et al. (2004) employed rice cDNA microarray analysis to examine gene expression changes in suspension cultured rice cells in response to treatment with compatible and incompatible strains of *Acidovorax avenae*. In all, 131 genes were differentially expressed between compatible and incompatible interactions. Ninety four genes were up regulated and 32 genes

were down regulated during incompatible interactions, whereas only 5 genes were up regulated during compatible reactions. Among the 126 genes that were up- or down regulated during incompatible interactions, expression of 46 genes was decreased when cultured rice cells were inoculated with a flagellin deficient incompatible strain, indicating that approximately 37% of the 126 genes were directly controlled by flagellin perception. Interestingly, OsCDPK7 was found to play a role in flagellin perception. Akimoto-Tomiyana et al. (2003) also demonstrated that this CDPK is also involved in N-actelychitooctaose and jasmonic acid perception.

Profiling plant responses to insect attack

Herbivorous insects, unlike most plant pathogens, are physiologically independent of their host plant. They force their way through a plant's outer protective barriers with mandibles and mouthparts that cause mechanical wounds into which herbivore specific elicitors are likely to be introduced during the interaction. Reymond and coworkers (Reymond et al., 2000) used Arabidpsis microarrays to analyse and compare the expression of 150 defence related genes after mechanical wounding and insect feeding by larvae of the cabbage butterfly (*Pieris rapae*). The interaction between jasmonate and wound response signal transduction pathways was also examined in this study. Although PR proteins, genes involved in the biosynthesis or metabolism of jasmonic acid, genes involved in the tryptophan pathway, and genes encoding PAL and chalcone synthase showed coordinated expression, expression profiles of genes after mechanical wounding were more similar to those of water stress than insect feeding. This suggested that this insect has developed feeding strategies to minimise the activation of stress inducible defence related genes.

In a similar study, Zhu-Salzman et al. (Zhu-Salzman et al., 2004) performed sorghum expression profiling in response to greenbug aphid (*Schizaphis graminum*) infestation, as well as MeJA and SA treatments. It has been proposed that phloem feeding insects are perceived as pathogens due to similarities in the manner of penetration of plant tissues by fungal hyphae and aphid stylets, and to some extent, by similar hydrolytic enzymes released

during fungal growth and insect feeding (Walling, 2000). In accordance with this view, Zhu-Salzman et al. (2004) observed strong induction of SA regulated PR genes by green bug feeding, but weak induction of wounding and JA related genes. However, infestation tests on control and MeJA treated plants indicated that MeJA treatment deters greenbug infestation, and confirmed that JA regulated pathways were effective in plant defence against greenbugs. Furthermore certain genes were activated exclusively by greenbugs, and may represent unique signal transduction events independent of JA and SA regulated pathways. Taken together, these results suggest that plants co-ordinately regulate defence gene expression when attacked by phloem feeding aphids, but also suggest that aphids are able to avoid triggering activation of some otherwise potentially effective plant defensive machinery, possibly through their particular mode of feeding (Zhu-Salzman et al., 2004).

In an elegant experiment, Voelckel and Baldwin (Voelckel and Baldwin, 2004) profiled wild tobacco's (*Nicotiana attenuata*) response to attack by sap feeding mirids (*Tupiocoris notatus*) and chewing hornworms (*Manduca sexta*). Microarrays enriched in herbivore elicited genes (cloned by differential display reverse transcription PCR and subtractive hybridisation) were used to characterise single, sequential, or simultaneous attacks by these two main predators of *N. attenuata.* Principle component analysis (PCA) identified distinctly different imprints left by individual attack from the two species after 24 h, but not after 5 days. Moreover, imprints of sequential or simultaneous attacks differed significantly from those of a single attack. On the individual gene level this means that when a plant experiences different biological stressors sequentially or simultaneously, a different suite of genes is induced. Attack from both herbivores elicited a switch from growth to defence related transcriptional processes, and herbivore specific changes occurred mainly in primary metabolism and signalling cascades.

Analysis of coordinated defence responses

In the year 2000, two milestone microarray profiling papers appeared that represented the first large scale analyses of complex signalling pathways and

coordinated gene expression between signalling pathways in Arabidopsis. Maleck et al. (2000) applied microarray technology to provide a comprehensive description of SAR in Arabidopsis. These researchers employed an Arabidopsis microarray to profile expression changes in 7000 genes under 14 different conditions related to SAR. About 300 genes were identified whose expression level changed significantly in response to SAR treatment. Many of these genes were novel, and had not previously been shown to play a role in SAR. Clustering of differentially expressed genes, and subsequent analysis of a gene cluster containing PR1 (a common marker of SAR) revealed that all 26 genes in this cluster contained core binding sites for WRKY transcription factors (W boxes) in their promoter region. This suggests a role for PR1 WRKY transcription factors in PR1 regulation and SAR. Using a pathology related microarray comprising 2375 elements, Schenk and coworkers (2000) examined gene expression patterns in Arabidopsis infected with the fungal pathogen *Alternaria brassicola* or treated with the defence signalling molecules SA, MeJA or ethylene. Comparison of plants subjected to each of the four treatments showed that 126 genes were coordinately regulated by overlapping defence pathways. The most significant overlap was observed in the 55 genes that were induced by both SA and MeJA treatment. This suggests that the extent of overlap between the two pathways was much greater than previously anticipated. In addition, 50% of the genes induced by MeJA treatment were also induced by ethylene treatment, suggesting coordination between these defence signalling pathways.

In a recent study, Salzman et al. (2005) undertook a similar study to Schenk and coworkers, and examined gene expression in sorghum in response to the defence signalling compounds SA, MeJA and the ethylene precursor aminocyclopropane carboxylic acid (ACC). Expression profiles were generated using a microarray containing 12982 non redundant sorghum ESTs. Comparison of plant expression profiles in response to the three treatments yielded similar results to Schenk et al. (2000). In total 727 genes were co-ordinately regulated by overlapping defence pathways, with the largest group of coregulated genes occurring between SA and MeJA. Interestingly, synergistic as well as antagonistic effects on regulation of some

genes were observed between SA and JA pathways. For example, genes of the octadecanoic acid pathway leading to JA synthesis were induced by SA as well as by MeJA. In contrast, many of the genes induced by either MeJA or SA were induced at lower levels when plants were treated with a combination of MeJA+SA. This suggests that two or more independent molecular switches control flux through the SA and JA pathways. Apparently both pathways can be switched off (coantagonism) or on (synergism) simultaneously (Salzman et al., 2005).

The large scale analysis of defence associated genes has accelerated our understanding of how complex defence networks operate during plant-pathogen interaction. As the genomes of more crop species are fully sequenced and annotated, microarrays will be used to study signalling networks governing plant defence in these plants. Ultimately, as our understanding of basic processes involved in host-pathogen interactions improves, the development of better disease protection strategies in agriculturally important plants will follow.

1.8 AIMS OF THE PROJECT

Pearl millet is the world's sixth most important cereal crop, but very little funding has been diverted towards pearl millet research. Of all the major cereals, pearl millet is the most tolerant of heat and drought. Thus, as the world becomes hotter and drier, this crop will have the power to yield reliably in regions too arid and too hot to consistently support other grains. Despite its thermotolerant properties, pearl millet is still affected by a number of diseases which can result in significant yield reductions. The aim of this project was therefore to elucidate and understand defence response mechanisms and signalling networks in pearl millet. We hypothesised that treatment of monocots with pathogen elicitors and defence signalling molecules would result in differential expression of defence related genes, and chose to evaluate this hypothesis in the non-model monocot, pearl millet. This was accomplished through the construction of a pearl millet cDNA library enriched for defence response genes, which was subsequently used to profile pearl millet's response to the defence signalling molecules NO, SA and MeJA, and

to infection with the compatible biotrophic rust fungus *P. substriata* var. *indica*. Selected genes will be used in future cereal transformation projects in which key regulators of defence signalling networks will be introduced into pearl millet lines or other cereal crops to convey resistance to chosen pathogens.

Chapter 2 outlines the construction of a pearl millet cDNA library that was enriched in defence response genes. This was accomplished by treating pearl millet seedlings with the pathogen elicitors chitin and flagellin as well as subjecting the leaves to mechanical wounding. Suppression Subtractive Hybridisation (SSH) was performed to isolate transcripts that were either up- or downregulated in response to treatment. A quantitative measure for screening pearl millet SSH cDNA libraries using cDNA microarray analysis is presented. This method was employed to identify true positive and false positive clones from the SSH cDNA libraries. The technique also enabled differentiation between abundantly expressed and rare transcripts following elicitor treatment. Based on screening results, a selection of genes was sequenced, and analysed for sequence homology to known genes. Sequence analysis revealed similarities to many genes known to be involved in defence response processes.

Chapter 3 examines transcriptional changes in pearl millet related to NO action following treatment with the NO donor sodium nitroprusside (SNP). Comparisons are made with NO mediated transcriptional changes known to occur in Arabidopsis following SNP treatment.

In Chapter 4, a pathogen infection trial was performed to assess whether treatment of pearl millet with the defence signalling molecules MeJA and SA conferred resistance to the biotrophic rust pathogen *P. substriata* var *indica*. Furthermore, a microarray profiling study is presented which outlines pearl millet's response to MeJA and SA treatment, as well as infection with *P. substriata* var. *indica*. Comparisons were made between treatments to determine whether there was any overlap between jasmonate and salicylate pathways in pearl millet, and to assess pearl millet's transcriptional response to pathogen attack.

Finally, Chapter 5 discusses the results with reference to current ideas around defence response and signalling networks, and the potential for its application in the development of higher yielding cereal crops.

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Chapter 2

Construction and characterisation of a pearl millet defence response cDNA library

2.1 ABSTRACT

Efficient construction of cDNA libraries enriched for differentially expressed transcripts is an important first step in many biological investigations. In order to construct a pearl millet cDNA library enriched for defence response genes, suppression subtractive hybridisation (SSH) was employed following wounding and treatment of pearl millet plants with the pathogen elicitors chitin and flagellin. A forward and reverse library was constructed to identify genes that are up and down regulated during the defence response, respectively. Furthermore, a quantitative procedure for screening cDNA libraries constructed by SSH is presented. Following two colour Cy dye labelling and hybridisation of subtracted tester with either unsubtracted driver or unsubtracted tester cDNAs to the SSH libraries arrayed on glass slides, two values were calculated for each clone, an enrichment ratio 1 (ER1) and an enrichment ratio 2 (ER2). A third enrichment ratio 3 (ER3), was also calculated following hybridisation of unsubtracted tester and unsubtracted driver cDNAs. Graphical representation of ER1 and ER2, or ER3 plotted against inverse ER2 enabled identification of clones that were likely to represent up regulated transcripts. Normalisation of each clone by the SSH process was determined from the ER2 values, thereby indicating whether clones represented rare or abundant transcripts. Differential expression of pearl millet clones identified by this quantitative approach was verified by inverse Northern blots. Sequence analysis was performed on clones shown to be up regulated during the defence response identified from plots of ER1 versus ER2, and ER3 versus inverse ER2. This pearl millet cDNA library serves as a basis for further microarray studies to examine the effect of defence signalling molecules and pathogen infection on pearl millet gene expression.

2.2 INTRODUCTION

Pearl millet [*Pennisetum glaucum* (L.) R. Br] is a member of the Gramineae family that includes many major monocotyledonous agricultural crop species such as maize, rice, wheat, sorghum, barley and oats. Many of these species have large, complex genomes that present a substantial challenge to molecular studies (Carson et al., 2002). Much of what is known at the genetic level for crop plants has been obtained through genetic mapping and synteny comparisons with species with relatively small genomes such as sorghum and rice (Devos and Gale, 2000; Gale and Devos, 1998; Keller and Feuillet, 2000). In recent years, completion of the rice genome sequence has added to our knowledge of cereal genome structure and complexity (Goff et al., 2002; Yu et al., 2002). However, information at the level of gene sequence and function is still very limited for most non-model crop species. As a consequence, research groups have employed Expressed Sequence Tags (ESTs) as a method for gene discovery in crop species with complex genomes (Akimoto-Tomiyama et al., 2003; Carson et al., 2002; Hein et al., 2004).

ESTs provide a rapid method to establish an inventory of expressed genes through determination of single pass sequences of 200 to 900 bp from one or both ends of randomly isolated gene transcripts that have been converted to cDNA. The sequences are sloppy and have a relatively high error rate, but, in most cases, they are sufficiently accurate to unambiguously identify the corresponding gene through homology comparisons with known genes. In addition, high throughput technology and EST sequencing projects can result in identification of significant portions of an organism's gene content and thus can serve as a foundation for initiating genome sequencing projects (Alba et al., 2004). Most importantly, thousands of sequences can be determined with limited investment.

In order to add value to EST data, several techniques exist to isolate and characterise cDNA fragments that are differentially expressed under specific conditions. These include differential display reverse transcriptase PCR (DD RT-PCR), cDNA amplified fragment length polymorphism (cDNA-AFLP), serial analysis of gene expression (SAGE) and suppression subtractive

hybridisation (SSH) (Bachem et al., 1996; Diatchenko et al., 1996; Liang and Pardee, 1992; Velculescu et al., 1995). In DD RT-PCR cDNA is synthesised from RNA using reverse transcriptase and an oligo dT primer that anneals to the 3' polyA tail of mRNA. Thereafter, subsets of cDNA populations for comparison are amplified with short, non-specific oligonucleotide primers, in combination with oligo dT primers, and visualised on polyacrylamide gels. Differentially expressed cDNA fragments are isolated from the gel and sequenced. Like DD RT-PCR, cDNA-AFLP is derived from a DNA fingerprinting method and also involves the selective PCR amplification of subsets of cDNA populations for comparison on polyacrylamide gels. However, cDNA-AFLP is an improvement on DD RT-PCR in that amplification is specific, using primers with higher annealing temperatures that bind to adaptors ligated to the ends of double stranded cDNA molecules following restriction digestion. SAGE is an elegant technique that combines differential display and cDNA sequencing approaches, and it has the advantage of being quantitative. Unfortunately, SAGE is laborious and requires an extensive foundation of sequence information. All three techniques described above are often limited by their ability to capture low abundance transcripts (Alba et al., 2004).

Suppression subtractive hybridisation (SSH) is a powerful technique to enrich libraries with differentially expressed cDNAs, and can be combined with large scale sequencing approaches (Birch and Kamoun, 2000). The SSH technique utilises subtractive hybridisation to selectively remove cDNA from genes that are expressed in both control and experimental samples, and a post hybridisation PCR step to preferentially amplify cDNA unique to the experimental sample (Figure 2.1). One of its main advantages is that it includes a normalisation step that enables the detection of low abundance differentially expressed transcripts such as many of those likely to be involved in signalling and signal transduction, and might thus identify essential regulatory components in several biological processes (Birch and Kamoun, 2000). A further advantage of SSH is that it yields cDNA fragments that can be used directly for the construction of DNA microarrays.

In this study, we aimed to isolated pearl millet genes that are involved in defence response. In order to achieve this, we treated pearl millet seedlings with the pathogen elicitors chitin and flagellin and mechanically wounded the leaves. Differential gene isolation was accomplished through application of SSH to enrich cDNA libraries for genes up- or down regulated in response to elictor treatment. The SSH procedure was chosen for several reasons: it includes a normalisation step, it enriches for differentially expressed transcripts, and it yields cDNA fragments that can be used directly for the construction of DNA microarrays. The normalisation step is particularly important because a few defence genes, such as those encoding the pathogenesis related (PR) proteins, are abundantly induced during defence response, potentially obscuring important defence specific transcripts expressed at much lower levels (Mahalingam et al., 2003).

In previous studies, SSH libraries were screened to identify cloned differentially expressed genes by colony blot hybridisation, inverse Northern analysis or cDNA AFLP (Birch et al., 1999; Hein et al., 2004; Mahalingam et al., 2003). However, these methods are time consuming, and do not allow the level of enrichment of a transcript to be quantified. SSH has also been used as a method to generate a cDNA library to use in subsequent cDNA microarray expression profiling (Yang et al., 1999). In this study, cDNA microarrays were used to screen PCR amplified clones from forward and reverse subtracted SSH libraries to identify genes from pearl millet that are up- or down regulated during defence responses, respectively. This quantitative approach of determining the extent to which transcripts were enriched by the SSH process allowed us to identify and exclude clones that were not derived from differentially expressed transcripts and to determine whether transcripts were rare or abundant. Based on cDNA microarray analysis of forward and reverse subtracted pearl millet SSH libraries, SSH clones were selected for sequence analysis. A number of genes exhibited significant similarities to genes associated with plant defence and stress responses.

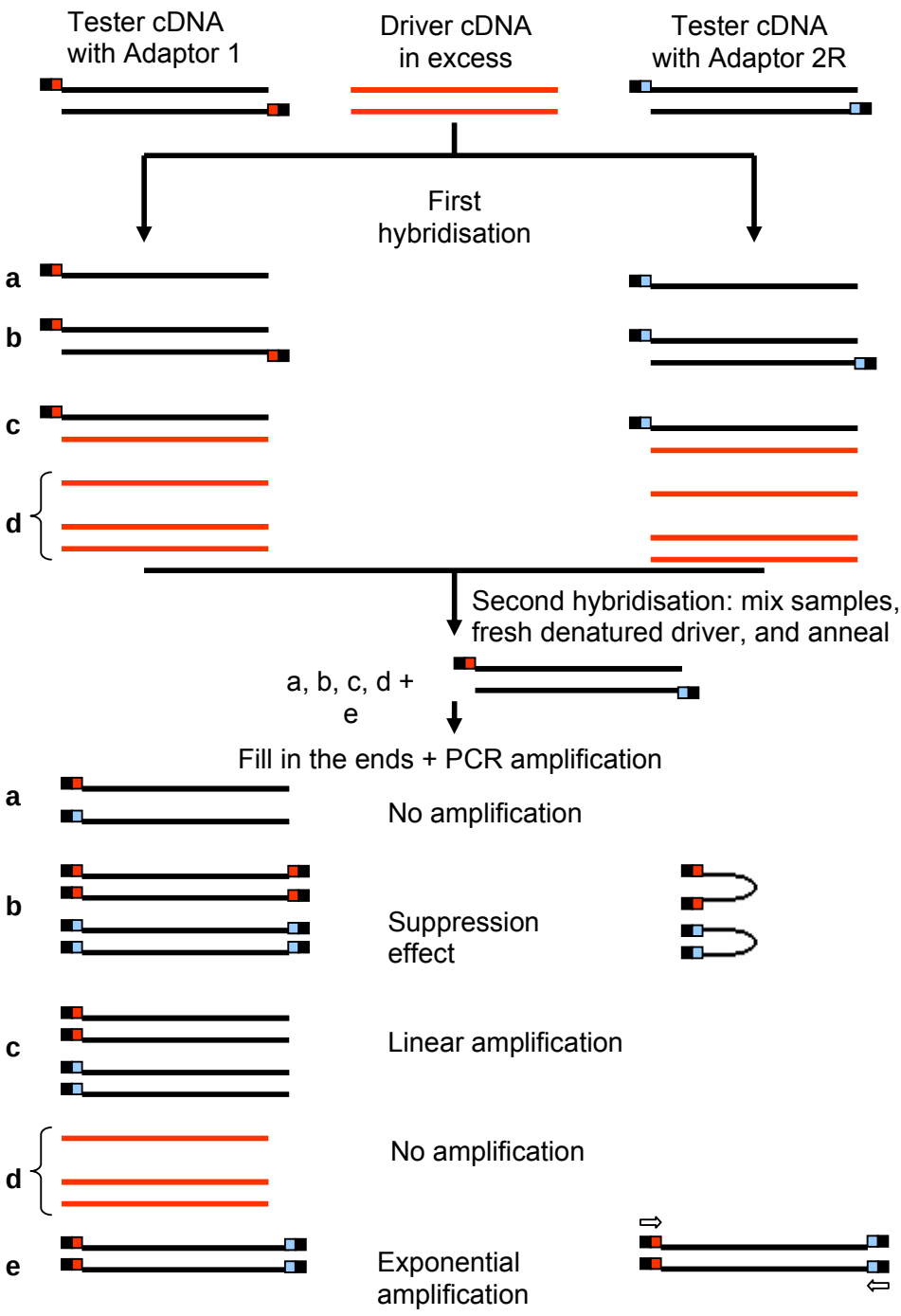

Figure 2.1. Schematic representation of SSH (Diatchenko et al. 1996). Solid lines represent the *Rsa*I digested tester (black) or driver cDNA (red). Solid boxes represent the outer part of adaptor 1 and 2 longer strand and corresponding SSH1 primer sequence. Red and blue boxes represent the inner part of the adaptors and correspond to nested PCR primers 1 and 2R respectively.

2.3 MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma (Aston Manor, South Africa) unless otherwise stated. Sequences of adaptors and primers used in PCR, SSH and sequencing reactions are shown in Table 2.1.

Plant material and growth

Pearl millet breeding lines ICML12=P7 and 842B were obtained from ICRISAT India and ICRISAT Zimbabwe respectively. ICML12=P7 is resistant to downy mildew caused by the oomycetous fungus *Sclerospora graminicola*, and rust (causal agent: *Puccinia substriata* var. *indica*) (Singh et al., 1990), whereas 842B is moderately susceptible to *S. graminicola* infections (M. O'Kennedy, personal communication).

Pearl millet seed was sterilised by briefly rinsing with 70% ethanol, followed by 20 min incubation in 0.7% sodium hypochlorite. Following three washes with sterile distilled water, seeds were plated on half strength MS medium (Murashige and Skoog, 1962), and incubated at 25ºC with a 16 hour light/8 hour dark photoperiod.

Elicitor preparation

The fungal elicitor chitin was purchased from Sigma Aldrich (Sigma catalogue number C-3641). The bacterial elicitor flagellin (Felix et al., 1999) was prepared from *Bacillus* sp. alk 36 (E. Berger, personal communication). Briefly, Luria broth pH 8.5 (Sambrook et al., 1989), was inoculated with a colony of *Bacillus* sp. alk 36 and grown with shaking at 42ºC for 48 hours. An equal volume of 0.1 N NaOH was added to the culture, which was then left at room temperature for 30 min to strip the flagellin from the cell wall (cell bound fraction). Samples were centrifuged at 6000 rpm for 10 min to pellet bacteria, and the supernatant containing the flagellin was transferred to a new tube. Alternatively, ten millilitres of bacterial culture was pelleted at 6000 rpm for 10 min, and proteins remaining in the supernatant were precipitated (extracellular fraction). An equal volume of 10% trichloroacetic acid was added to the cell

bound and extracellular supernatants, which were incubated at -20ºC for one hour to precipitate the proteins. Extracellular and cell bound proteins were collected by centrifugation at 11 000 rpm for 20 minutes, the protein pellet was dried in a laminar flow bench, and resuspended in a total volume of 1 ml phosphate buffered saline (PBS) (Sambrook et al., 1989). Five micrograms of protein was analysed on a 10% SDS polyacrylamide gel (Ausubel et al., 2005) to assess the presence and quality of flagellin in the crude extracellular protein extract. Proteins were detected by staining the polyacrylamide gel in 0.1% Coomassie Brilliant R, 50% methanol, 10% acetic acid.

Presence of the flagellin in the protein extract was confirmed by Western blot analysis according to the method of (Ausubel et al., 2005). Briefly, 2.5 µg protein was run on a 10% SDS polyacrylamide gel, and transferred to polyvinyldifluoride (PVDF) membrane at 15V overnight in CAPS (3-[cyclohexylamino]-1-propane-suphonic acid) buffer (10 mM CAPS 3% methanol, pH 10.5). Following protein transfer, the membrane was incubated in blocking solution (10 mM Tris, pH 7.5, 150 mM NaCl, 3% milk powder and 0.1% Tween 20) for 2 hours at room temperature. Rabbit antibodies raised against the *Bacillus* sp. alk36 flagellin protein were diluted 1:2000 in blocking solution and added to the membrane for 2 hour at 37ºC. The membrane was subjected to three washes of five minutes each with washing buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) to remove unbound antibodies. Following an hour incubation at 37ºC with Anti-Rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) (diluted 1:1000 in blocking buffer), the membrane was once again subjected to three washes of five minutes each in washing buffer. The membrane was equilibrated in detection buffer (100 mM Tris, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) subsequent to antibody detection with NBT/BCIP solution (Roche Diagnostics, Mannheim, Germany) (200 µl in 10 ml detection buffer). The membrane was incubated in the dark until bands appeared. The reaction was stopped by the addition of TE, pH 8.0 (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0).

Treatment of pearl millet seedlings with elicitors

Leaves of ten day old ICML12=P7 and 842B pearl millet seedlings were wounded by pricking leaves at one centimetre intervals with a sterile needle. The abaxial surface of ICML12=P7 leaves was inoculated with a total of 100 µl of either 100 mg/ml chitin, or a crude boiled extract of flagellin. Control 842B plants were treated with sterile deionised water and not wounded. Each elicitor and control inoculation was repeated in triplicate. Plates containing pearl millet seedlings were sealed with Micropore™ tape (3M, Isando, South Africa), and were incubated at 25ºC with a 16 hour light/8 hour dark photoperiod. Necrotic lesion formation was observed under a dissecting microscope at 24, 48 and 96 hours post inoculation.

RNA isolation

Pearl millet leaves were harvested 5, 14 and 24 h post elicitor treatment (hpe), and immediately placed in liquid nitrogen. Total RNA was prepared from ten day old chitin or flagellin inoculated ICML12=P7 leaves, or untreated 842B leaves using a Plant RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA yield was determined by measuring absorbency at 260 nm, and RNA integrity was determined by electrophoresing two micrograms total RNA through a 1.2% agarose gel. Poly (A)+ RNA was purified from total RNA using an Oligotex® mRNA Mini Kit (Qiagen).

RT-PCR amplification of the actin gene

A two step reverse transcriptase PCR (RT-PCR) was performed to amplify the actin gene from pearl millet mRNA samples. First strand cDNA synthesis reactions were performed using a *C. therm* RT-PCR kit (Roche Diagnostics) according to the manufacturer's instructions. Each 20 µl reaction contained 1 µM actin forward primer (Table 2.1), 200 µM dNTPs, 3% (v/v) DMSO, DTT, 1 X RT buffer (Roche Diagnostics), 3 units *C. therm* polymerase (Roche Diagnostics) and 50 ng mRNA. The reaction was incubated at 60ºC for 30 min, followed by 94ºC for 2 min to inactivate the reaction.

PCR amplification of the pearl millet actin gene was performed on genomic DNA, or first strand cDNA synthesized during the RT reaction. Each twenty

five microlitre reaction consisted of 1 μM of each of actin forward and reverse primers (Table 2.1), 100 μM dNTPs, 1.5 mM $MgCl_2$, 1 X ammonium acetate reaction buffer (Biolines, London, UK), 1 unit of Taq DNA polymerase (Biolines BioTaq) and 5 μl RT product. Reactions were incubated at 94ºC for two minutes to denature the DNA template, followed by 30 cycles each with a 94ºC denaturation step, a 55ºC annealing step and a 72ºC. A final elongation cycle of 72ºC for 3 minutes was included. RT-PCR products were electrophoresed through a 2% agarose gel to assess actin cDNA and genomic DNA product size.

cDNA synthesis from pearl millet mRNA samples

Double stranded cDNA was synthesized from 2 μg pearl millet mRNA using a cDNA Synthesis System (Roche Diagnostics). cDNA synthesis reactions were purified using a MinElute Reaction Cleanup Kit (Qiagen). The cDNA preparations were resuspended in a final volume of 20 μl of nuclease free water. Integrity of the synthesized cDNA was assessed by performing PCR amplification of the actin gene using 1 μl of the cDNA product.

Suppression subtractive hybridisation

Subtractive hybridisation was performed as described by Diatchenko et al. (1996) using a PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, CA) with modifications. cDNA preparations from chitin treated leaves and flagellin treated leaves isolated 5, 14 and 20 hpe were pooled in equal proportions. For the forward subtractive cDNA library, pooled cDNA obtained from treated ICML12=P7 leaves was used as the 'tester' and that from the control 842B leaves as 'driver' to isolate fragments corresponding to genes whose expression level was increased following elicitor treatment. The reverse subtraction was performed with the control sample as tester and the treated sample as driver to isolate fragments corresponding to genes whose expression level decreased following elicitor treatment. Approximately 400 ng of tester and driver cDNA were digested with *Rsa*I in a 40 μl reaction mixture containing 30 units of enzyme (Roche Diagnostics) for 4 hours at 37ºC. The restricted cDNA fragments were purified using a MinElute Reaction Cleanup Kit (Qiagen), and eluted from MinElute columns in 10 μl sterile water.

Digested tester cDNA (1µl) was diluted in 5 µl of water. A 2 µl aliquot of the diluted tester cDNA was then either ligated to 2 µl of adaptor 1 (10 µM) or 2 µl of adaptor 2R (10 µM) (Table 2.1) in separate ligation reactions in a total volume of 10 µl at 14ºC overnight, using 2 units of T4 DNA ligase (BD Biosciences Clontech) in the buffer supplied by the manufacturer. After ligation, reactions were heated to 72ºC for 5 minutes to inactivate the ligase.

Thereafter, 1.5 µl driver ds cDNA, together with 1 µl hybridisation buffer (BD Biosciences Clontech), was added to each of two tubes containing 1.5 µl of adapter 1 and adapter 2R ligated tester cDNA (1:10 diluted) respectively. The total reaction volume of each of the samples was 4 µl. The solution was overlaid with mineral oil, the DNA was denatured (1.5 min, 98ºC), and then allowed to anneal for 12 hours at 68ºC. After this first hybridisation, the two samples were combined and a fresh portion of heat denatured driver (100 ng) in 1 µl hybridisation buffer was added. The sample was left to hybridise for an additional 16 hours at 68ºC. The final hybridisation reaction was diluted in 100 µl of dilution buffer (BD Biosciences Clontech), heated at 68ºC for 7 min, and stored at -20ºC until use. The final ratio of tester to driver in both the forward and reverse subtraction experiments was 300:1.

Six separate suppressive PCR amplification reactions were performed for the forward and reverse subtracted cDNA samples. Primary PCR was conducted in a 25 µl volume that contained 1 µl of subtracted cDNA, 1 µl primer SSH1 (10 uM) (Table 2.1), and 23 µl of PCR master mixture prepared using the Advantage cDNA PCR Core Kit (BD Biosciences Clontech). PCR was performed with the following parameters: 75ºC for 5 min; 30 cycles at (94ºC for 30 sec, 66ºC for 30 sec, 72ºC for 90 sec); and a final extension at 72ºC for 5 min. The amplified products were diluted ten fold in sterile deionised water. One microlitre of the product was then used as a template in secondary PCR for 25 cycles under the same conditions, except PCR primer SSH1 was replaced with nested PCR primers 1 and 2R (Table 2.1), and the primer annealing temperature was 68ºC. The PCR products were analysed by 2% agarose gel electrophoresis.

cDNA enriched for differentially expressed transcripts was termed subtracted tester (ST), whereas unsubtracted tester (UT) cDNA was prepared from treated pearl millet plants, and unsubtracted driver (UD) cDNA was prepared from control pearl millet plants. For preparation of the reverse subtracted cDNA library, the tester cDNA was prepared from control plants, and driver cDNA from treated pearl millet plants.

Southern blot analysis

The efficiency of the cDNA subtraction was tested by Southern hybridization (Southern 1975). Non-radioactive DNA probes were prepared by digesting 10 µl of each of forward subtracted tester cDNA, reverse subtracted tester cDNA, unsubtracted tester cDNA and unsubtracted driver cDNA with *Rsa*I to remove adapter sequences. Restriction digests were electrophoresed through a 1.5% low melting point agarose gel, PCR products minus adaptors were excised from the gel, and gel purified using a Qiaquick Gel Extraction Kit (Qiagen). Products were labelled with digoxygenin (DIG)-dUTP molecules using a DIG Random Prime Labelling Kit (Roche Diagnostics).

DNA blots were prepared by electrophoresing forward subtracted tester cDNA, reverse subtracted tester cDNA, unsubtracted tester cDNA and unsubtracted driver cDNA through a 2% agarose gel, followed by capillary transfer of SSH products to a positively charged nylon membrane (Roche Diagnostics) using 1.5 M NaCl/0.5 N NaOH as a transfer buffer. DNA was fixed to the membrane by exposure to UV light (312 nm) for three minutes. Nylon bound cDNA was hybridised to either 5 ng/ul DIG-labelled forward subtracted tester cDNA, reverse subtracted tester cDNA, unsubtracted tester cDNA or unsubtracted driver DNA. Hybridisation signals were detected using CPD star (Roche Diagnosticss).

Cloning and analysis of the subtracted cDNA

Secondary PCR products were purified using a MinElute PCR Purification Kit (Qiagen). Subtracted cDNA fragments were ligated into pGEMT-easy using a pGEMT-easy cloning kit (Promega, Madison, WI, USA). Recombinant plasmids were transformed into electrocompetant *Escherichia coli* DH10B

cells (Ausubel et al., 2005). Individual colonies containing recombinant plasmids were inoculated into 150 µl Luria broth in 96 well microtitre plates. Cultures were grown overnight at 37ºC with gentle shaking (100 rpm), after which 150 µl 50% glycerol was added to each of the wells. Microtitre plates were stored at -80ºC until use.

Nucleotide sequencing of selected cDNA clones was performed by Inqaba Biotechnological Industries (Pty) Ltd. and Scottish Crop Research Institute, Dundee, Scotland, UK, using T7 or Sp6 primer sequences (Table 2.1). Each sequence was edited to correct sequencing ambiguities and remove the plasmid and SSH adaptor sequences. The edited sequences were used to query the NCBI (National Center for Biotechnology Information, USA) database using the BlastX, BlastN and dBEST algorithms (Altschul et al., 1990). The cDNAs were classified according to the E-values generated in the BLAST searches. E-values <1e-05 were deemed to indicate significant homology, whereas cDNAs with E-values >1e-05 were deemed to have no significant homology to any known protein and assumed to be novel (Shim et al., 2004). Sequences were checked for stop codons to ensure that cDNA fragments represented a portion of an open reading frame.

cDNA microarray analysis

Libraries containing 960 forward subtracted and 960 reverse subtracted pearl millet SSH clones were arrayed onto silanised microarray slides (Amersham Biosciences, Little Chalfont, UK) using an Array Spotter Generation III (Molecular Dynamics Inc., Sunnyvale, CA, USA). Cloned inserts were PCR amplified using SP6 and T7 primers and visualized on a 1% agarose Electro-FastSTRETCH gel (ABgene®, Epsom, UK). PCR products were purified using Multiscreen® PCR Purification Plates (Millipore, Molsheim, France) and eluted in 50 µl sterile distilled water. The probe was dried in a vacuum centrifuge and resuspended to a final volume of 20 µl in 50% DMSO prior to being robotically printed onto glass slides. On average, 200 pg of each pearl millet SSH fragment was spotted on each slide. The *uidA, luc* and *bar* genes and a fungal ITS fragment were also printed to serve as controls for global normalisation. Following removal of adaptor sequences, 200 ng ST, UT or

UD cDNA probes from forward and reverse subtracted libraries were labelled by incorporation of Cy™5 or Cy™3 dUTP (Amersham Biosciences) using Klenow enzyme (USB, Cleveland, Ohio, USA) as previously described (Ramonell et al., 2002). Each hybridisation was performed in duplicate with the reverse Cy dye labelling of the probes. Reactions were spiked with Cy labeled *uidA* (0.3 ng), *luc* (0.03 ng) and *bar* (3 ng) genes and a fungal ITS fragment (3 ng). After incubation at 37ºC for 20 hours, probes were purified using a Multiscreen® PCR Purification Plate (Millipore) and eluted in 45 µl sterile distilled water. The probe was dried in a vacuum centrifuge and resuspended in hybridisation buffer (Amersham Biosciences). The glass slide was initially incubated in pretreatment solution (3.5X SSC; 0.2% SDS; 1% bovine serum albumin) without probe at 60ºC for 20 min. The glass slide was placed in a HybUP hybridisation chamber (NB Engineering, Pretoria, South Africa) with the probe at 42ºC for 16 h. After hybridisation, slides were washed for 4 min at 42ºC with 1 X SSC/0.2% SDS, 0.1 X SSC/0.2% SDS (twice) followed by three washes in 0.1 X SSC for 1 min at room temperature. Slides were rinsed with distilled water, dried with high pressure nitrogen, and scanned with a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA, USA). The computer program ArrayVision™ (Molecular Dynamics Inc.) was used to localise and integrate every spot on the array.

Enrichment ratios of ST:UD (ER1), ST:UT (ER2) and UT:UD (ER3) were calculated for both forward and reverse subtracted pearl millet SSH libraries from experiments performed in duplicate. For each clone, background signal intensities were subtracted, signal intensities of duplicate spots on glass slides were averaged, and spots with a signal/noise ratio of less than 3 were rejected. Global normalisation of data for the Cy dye effect was performed using a control gene set to calculate normalisation functions c and c' for each pair of dye swap slides (Yang et al., 2002). This was accomplished by plotting the Cy3 versus the Cy5 value for each of the control spots and fitting a linear regression line through the data points. The $\log_2$ value of the gradient of the regression line was used to calculate c (or c' in the dye swap slide). To determine ER1, slides were hybridised with ST and UD. ER1 was calculated using the following formula: ½[$\log_2$ Cy3 ST/Cy5 UD – c – ($\log_2$Cy3UD/Cy5ST

– c')](Yang et al., 2002). ER2 was calculated in the same way following hybridisations with ST and UT. UT/UD values (Table 2.2) were calculated from the ER1 and ER2 values as follows: since ER1 – ER2 ~ $\log_2$ ST/UD – $\log_2$ST/UT = $\log_2$ UT/UD, therefore UT/UD = antilog of (ER1-ER2) in the base 2. Alternatively, a simple data analysis pipeline named `SSHscreen` using "linear models for microarray data" (`limma`) functions in the R computing environment was employed to calculate and plot ER1, ER2 and ER3 values (Berger et al., 2006) (http://www.stats.ox.ac.uk/~vos/SSHscreen/). ER1, ER2 and ER3 calculations differ from the above described method in that `SSHscreen` software implements both within- and between-array normalisations. A global loess (lowess) normalisation is used to perform within array normalization. Quantile normalisation is used to perform between array normalisation. This ensures that the distribution of red and green channels for each array becomes essentially the same, as well as the distribution across arrays. Following normalisation of fluorescence values, enrichment ratios are calculated as follows in `SSHscreen`: ER1 = $\log_2$ ST/UD; ER2 = $\log_2$ ST/UT; and ER3 = $\log_2$ UT/UD.

rRNA redundancy for each pearl millet libraries was determined by hybridising glass microarray slides with pearl millet rDNA probes respectively. A clone was considered to have hybridised to a rDNA probe if its fluorescence was more than two standard deviations above local background fluorescence (Leung and Cavalieri, 2003).

Inverse Northern dot blots

Twenty clones which showed differential expression by microarray analysis were selected Northern analysis. Amplified, denatured inserts of selected clones from pearl millet SSH libraries were applied to a positively charged Hybond nylon membrane (Amersham Biosciences) as described previously (Hein et al., 2004). Poly (A)+ mRNA, used for pearl millet probe generation, was isolated from 50 µg freshly prepared total RNA (tester and driver). cDNA was labeled with DIG-dUTP using a DIG DNA Labeling and Detection Kit (Roche Diagnostics). Hybridisations were performed as described previously

(Southern, 1975), using 20 ng/µl cDNA. Hybridisation signals were detected using CDP Star (Roche Diagnostics).

ArrayVision™ (Molecular Dynamics Inc.) was used to calculate signal density, following normalisation by comparing values of rDNA dots. Inverse Northern expression ratios were calculated by dividing normalised density measurements for each clone hybridised with the tester probes, with values for the same clones hybridised with the driver probe.

Table 2.1. Sequences of adaptors and primers used in PCR, SSH and sequencing reactions (adapted from PCR Select cDNA Subtraction Kit Manual, BD Biosciences Clontech).

SSH Adaptor 1:
5'CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
3'- GGCCCGTCCA-5'

SSH Adaptor 2R
5'CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3'
3'-GCCGGCTCCA-5'

Primer SSH1
5'CTAATACGACTCACTATAGGGC-3'

Nested PCR primer 1
5'TCGAGCGGCCGCCCGGGCAGGT-3'

Nested PCR primer 2R
5'AGCGTGGTCGCGGCCGAGGT-3'

Actin forward primer
5'ACCGAAGCCCCTCTTAACCC-3'

Actin reverse primer
5'GTATGGCTGACACCATCACC-3'

Sp6 primer
5'TATTTAGGTGACACTATAG-3'

T7 primer
5'TAATACGACTCACTATAGGG-3'

2.4 RESULTS AND DISCUSSION

Purification of flagellin from *Bacillus* sp. alk 36

Felix and coworkers (1999) identified eubacterial flagellin, the protein that builds up the filament of eubacterial flagella, as a potent elicitor of plant cells from different species. The elicitor active epitope could be localised to the most conserved domain in the N-terminal part of flagellin, and synthetic peptides spanning 15-22 amino acid residues of this domain showed full biological activity as elicitors. In this study, we had available to us a *Bacillus sp.* alk 36 strain that overproduced flagellin (E Berger, personal communication). Comparison of the *Bacillus sp.* alk 36 strain N-terminal amino acid sequence (E. Berger, personal communication) with the conserved flagellin domain identified by Felix and coworkers (Felix et al., 1999), indicated that *Bacillus sp.* alk36 shared a high degree of homology with the eubacterial consensus sequence, and more importantly with the 22 amino acid conserved domain (Figure 2.2). We thus decided to test the crude *Bacillus* sp. *alk 36* flagellin extracts for their ability to induce defence responses in pearl millet seedlings.

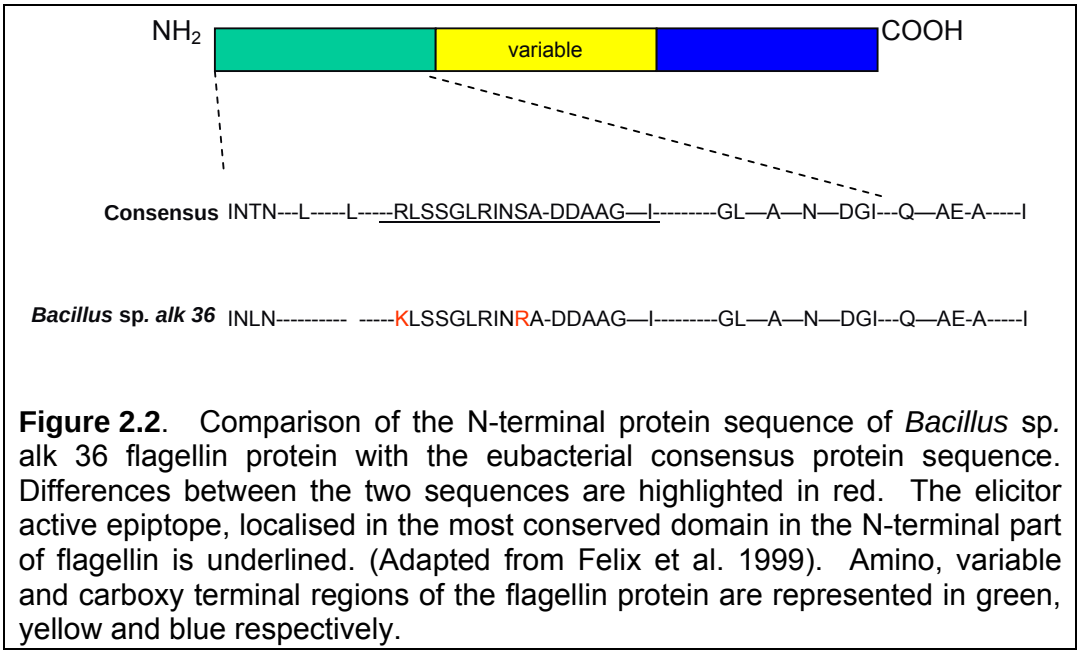

Figure 2.2. Comparison of the N-terminal protein sequence of *Bacillus* sp. alk 36 flagellin protein with the eubacterial consensus protein sequence. Differences between the two sequences are highlighted in red. The elicitor active epiptope, localised in the most conserved domain in the N-terminal part of flagellin is underlined. (Adapted from Felix et al. 1999). Amino, variable and carboxy terminal regions of the flagellin protein are represented in green, yellow and blue respectively.

SDS PAGE of cell bound and extracellular *Bacillus* sp. alk36 extracts indicated that extract was primarily enriched in a 34 kDa protein representative of the flagellin protein (Figure 2.3A). This was confirmed with Western blot analysis using a polyclonal antibody raised against the *Bacillus* sp. alk 36 flagellin protein (Figure 2.3B). The Western blot indicated that the cell bound protein fraction contained more flagellin than the extracellular protein fraction. This suggests that more flagellin is found in the cell wall than is excreted extracellularly by the bacteria during cell growth. This result was expected as the bacterial flagella are anchored to the cell wall.

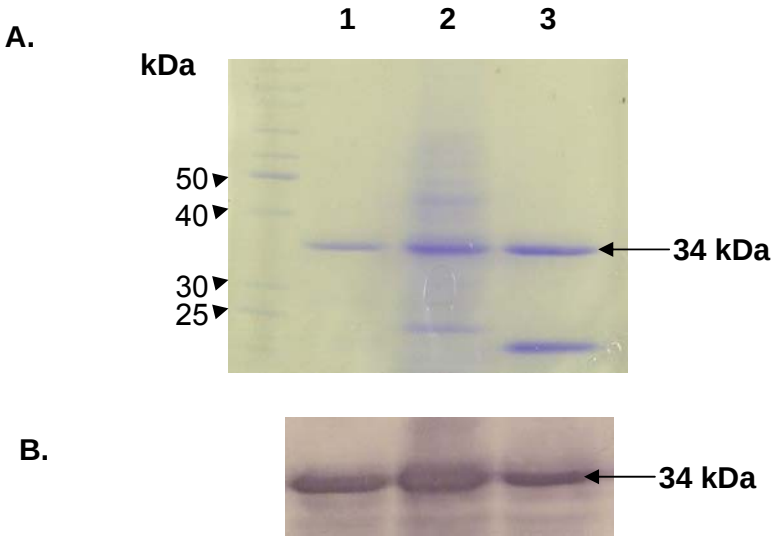

Figure 2.3. SDS-PAGE analysis of extracellular and cell bound protein extracts from *Bacillus* sp. alk 36. (A) Comassie blue stained extracellular (lanes 1 & 3) and cell bound (lane 2) protein extracts. (B) Western analysis of extracellular (lanes 1 & 3) and cell bound (lane 2) protein extracts indicating the presence of the 34 kDa flagellin protein in the extracts.

Chitin and flagellin induce defence responses in pearl millet

Assessment of the response of pearl millet lines to microbial elicitor application was performed using an assay that assessed hypersensitive response (Geetha et al., 1996). Geetha and coworkers (1996) developed the assay to determine the effect of the oomycete elicitors, arachidonic acid and eicosapentaenoic acid on induction of the hypersensitive response in pearl millet. These authors pricked pearl millet leaves with a fine needle, applied the elicitor to the surface of two day old pearl millet seedlings, and assessed HR lesion development. Treatment of pearl millet line ICML12=P7 with chitin and flagellin indicated that necrotic lesions formed around leaf wound point 24 hours after microbial elicitor treatment (Figure 2.4). No lesions formed around the wound point of control plants that had been treated with water. Interestingly, the types of lesions formed following chitin and flagellin treatment were quite different. Chitin treated leaves formed dark brown lesions that extended up to 10 mm from the wound point. On the other hand, cells around the wound point of leaves treated with the flagellin enriched protein extract, became bleached and translucent (Figure 2.4). This phenomenon is known as water soaking, and is a typical symptom of bacterial leaf infections (Esnault et al., 1993; Hauck et al., 2003).

Higher plants initiate various defence reactions when invaded by pathogens. Molecules released or generated during microbial entry, so called elicitors, are thought to act as the chemical cues that are perceived by the plant and activate defence, including early responses such as the oxidative burst (Felix et al., 1999). Specific elicitors produced by pathogenic microbes include oligosaccharides, polysaccharides, proteins, and glycoproteins.

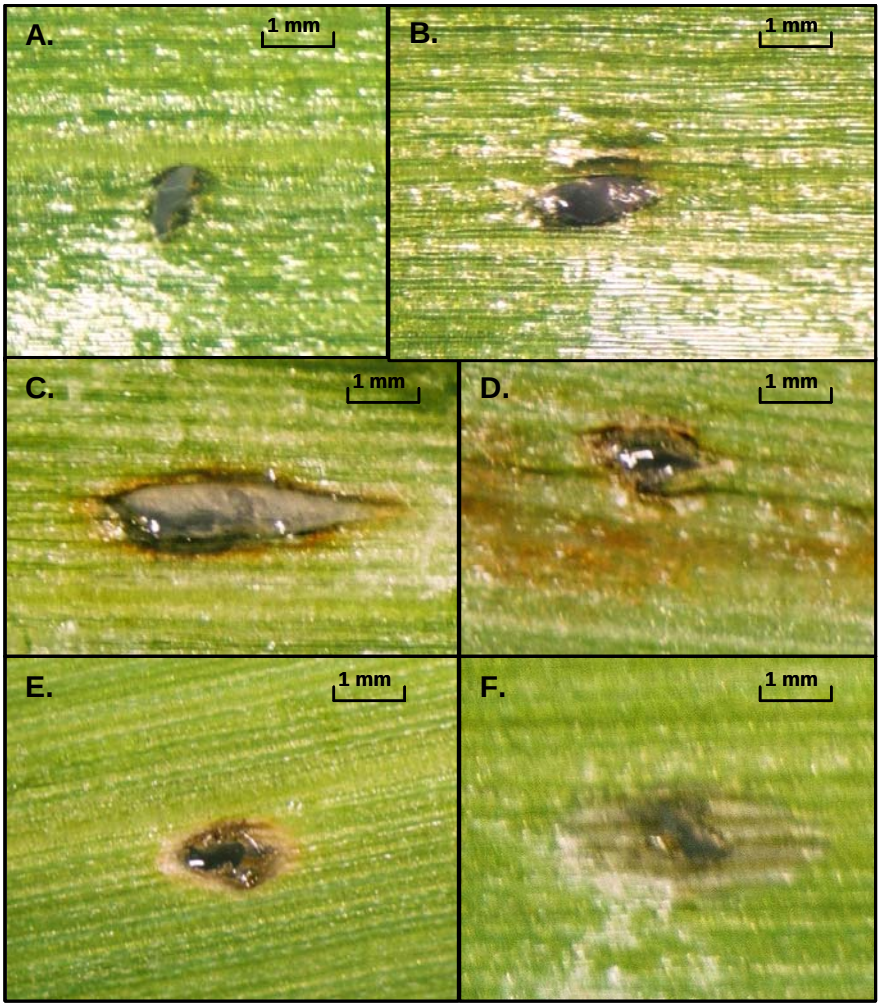

Figure 2.4. Treatment of pearl millet leaves with elicitors. Leaves were pricked with a fine needle, and either water (A & B), chitin (C & D), or an enriched flagellin extract (E & F) was applied to the abaxial surface of the leaf. Necrotic lesions were observed under a dissecting microscope (25X magnification) 24 hours after treatment.

Chitin oligomers, which can be generated from fungal cell walls by endochitinase, have been shown to induce defence related cellular responses in many plants. Such responses include medium alkalinization, cytoplasmic acidification, ion flux, membrane depolarization, protein phosphorylation and phytoalexin production (Ramonell et al., 2002). Microarray analysis of chitin elicitation in Arabidopsis indicated that a number of transcripts exhibited

altered accumulation as early as 10 minutes after seedling exposure to chitin (Ramonell et al., 2002). Transcript levels of 61 genes were altered three fold or more in chitin treated seedlings relative to control seedlings. Similarly, treatment of rice suspension cultures with N-acetylchitooligosaccharides (COS) strongly induced defence gene expression and cell death, with rapid production of H_20_2 preceeding cell death. COS treatment could also induce visible cell death on rice leaves (Ning et al., 2004). Microarray analysis of COS treated rice suspension cultures also showed that there was significant transcriptional reprogramming in response to elicitor treatment (Akimoto-Tomiyama et al., 2003).

Flagellin, and peptides corresponding to the most conserved domain of eubacterial flagellin (flg15 and flg22) have been shown to act as potent elicitors in cells of different species (Felix et al., 1999; Gómez-Gómez et al., 1999). Flagellin and the flg22 peptide caused medium alkalinisation, indicative of necrosis and induction of HR, in a number of dicotyledonous cell suspension cultures, including those of tomato, potato, tobacco and Arabidopsis. In Arabidopsis seedlings, flg22 treatment caused callose deposition, induction of genes coding for pathogenesis related proteins, and strong inhibition of growth (Gómez-Gómez et al., 1999). However, rice suspension cultures did not show a detectable response to flagellin, flg22 or flg15, although they reacted with an alkalinisation response to chitin oligomers (Felix et al., 1999). Subsequent studies by Che and coworkers (Che et al., 2000) demonstrated that flagellin isolated from an incompatible strain of *Acidovorax avenae* induced a hypersensitive response in cultured rice cells, but no response was observed following treatment with a compatible strain of *A. avenae*. However, Pfund and coworkers (Pfund et al., 2004) showed that flagellin from *Ralstonia solanacearum* cells was not the major elicitor of defence responses in Arabidopsis. Instead, these authors concluded that the primary eliciting activity in boiled *R. solanacearum* extracts applied to Arabidopsis was attributable to one or more proteins other than flagellin. Interestingly, the flagellin receptor *FLS2* was found to be highly expressed in the plant vasculature (Gómez-Gómez and Boller, 2002). As *R. solanacearum*

is a vascular pathogen, it is possible that it has evolved due to strong selection pressure to elude detection via FLS2.

It is thus possible that the hypersensitive response observed in pearl millet following treatment with crude flagellin extract could be due to a protein in the extract other than flagellin. In order to test this hypothesis, it will be necessary to treat pearl millet with purified flagellin. This will be the subject of a future study in the laboratory.

RNA isolation and cDNA synthesis

Pearl millet ICML12 plants were wounded and inoculated with chitin or flagellin. Total RNA was extracted at 5, 14 and 24 hours post inoculation. RNA extractions at each time point were performed in triplicate. RNA was also extracted from untreated pearl millet 842B plants. Total RNA samples were electrophoresed through agarose gels in order to assess the integrity of the RNA. Visualisation of ribosomal RNA bands indicated that the RNA had not degraded. An example of pearl millet total RNA separated by agarose gel electrophoresis is indicated in Figure 2.5. mRNA was purified from total RNA in order to minimise the number rRNA clones coming through the SSH procedure.

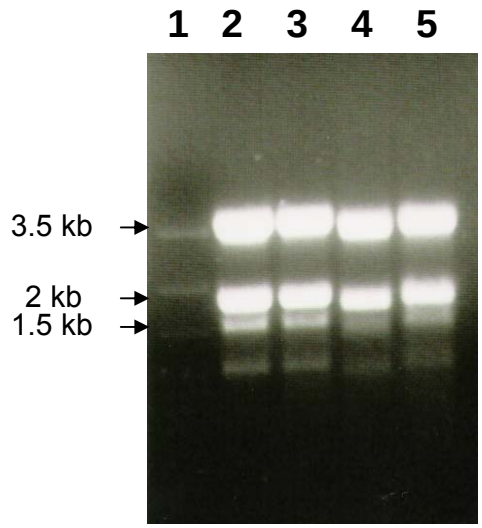

Figure 2.5. Formaldehyde agarose gel electrophoresis of total RNA isolated from pearl millet leaves. Lane 1, molecular weight marker; lane 2, RNA isolated from untreated 842 leaves; lanes 3-5, RNA isolated from ICML12=P7 leaves that had been wounded with a fine needle and inoculated with a crude flagellin extract. Total RNA was isolated from leaves 5 h (lane 3), 14 h (lane 4) and 24 h (lane 5) after treatment with flagellin. Ribosomal RNA (rRNA) subunits are visible as bands on the agarose gel.

RT-PCR of the pearl millet actin gene was performed on purified mRNA to assess for genomic DNA contamination. As little genomic sequence information is available for pearl millet, primers designed to a conserved region of the banana actin gene were used to amplify a fragment of the pearl millet actin gene. Preliminary PCR studies indicated that these primers spanned an intron in the pearl millet actin gene (results not shown). Therefore, amplification of the actin gene fragment from genomic DNA yields a 280 bp fragment, whereas RT-PCR from pearl millet mRNA, in which the intron has been excised, yields a 170 bp fragment. Sequence analysis of the pearl millet genomic DNA actin gene product showed that it exhibited 87% homology to the maize actin 1 gene (GenBank accession number J01238) over 140 bp (results not shown). RT-PCR of ICML12=P7 and 842B mRNA samples yielded a 170 bp fragment, but not a 280 bp fragment (Figure 2.6). This indicated that the sample did not harbour any contaminating genomic DNA that would negatively influence cDNA subtraction experiments.

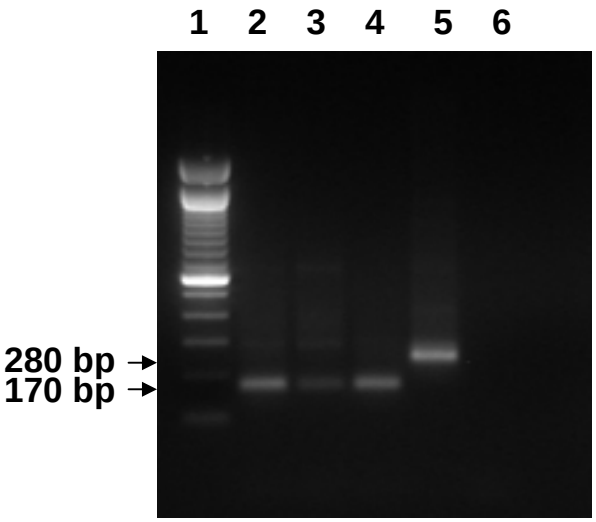

Figure 2.6. RT-PCR of an actin gene fragment from mRNA extracted from pearl millet leaves. mRNA samples were subjected to RT-PCR to amplify an actin gene fragment, and amplification products were separated by agarose gel electrophoresis. Lane 1, 100 bp ladder (Invitrogen); lane 2, untreated 842B actin RT-PCR products; lane 3, chitin treated ICML12=P7 actin RT-PCR products; lane 4, flagellin treated ICML12=P7 actin RT-PCR product; lane 5, actin gene fragment amplified from 842B genomic DNA; lane 6, water control. An intron has been spliced from actin mRNA yielding a 170 bp product, as compared the actin PCR product from genomic DNA, which possesses the intron sequence, and is 280 bp.

In preparation for SSH experiments, which require DNA as a starting material, chitin and flagellin treated ICML12=P7 mRNA samples and 842B mRNA samples were converted to cDNA. Conversion of mRNA to cDNA was tested by PCR amplification of the pearl millet actin gene fragment from cDNA. If mRNA was not converted to cDNA, no amplification of this gene would occur, as Taq polymerase is unable to use mRNA as a template.

PCR amplification of the actin gene fragment from ICML12=P7 and 842B cDNA samples yielded 170 bp fragments (Figure 2.7). As the actin cDNA gene was synthesized from an mRNA template, it lacked all introns that are present in genomic DNA. Therefore, the actin cDNA amplification product is the same size as the mRNA RT-PCR product (170 bp).

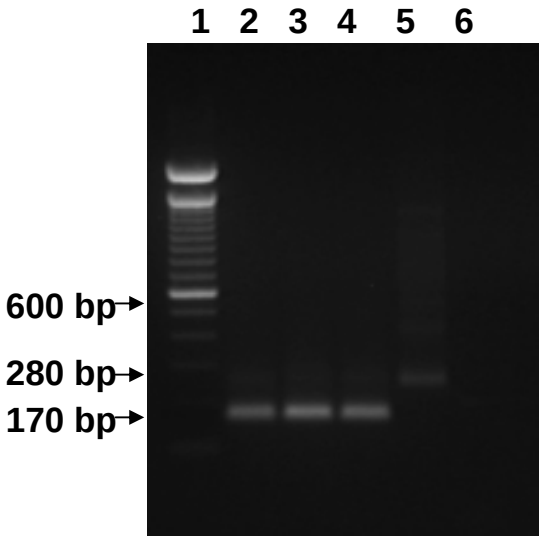

Figure 2.7. Agarose gel electrophoresis of actin fragments PCR amplified from pearl millet cDNA. The actin gene fragment was amplified from 842B cDNA from untreated leaves (lane 2), ICML12=P7 cDNA from chitin treated cDNA (lane 3), ICML12=P7 cDNA from flagellin treated cDNA (lane 4), 842B genomic DNA (lane 5), and water control (lane 6). The 170 bp actin cDNA fragments lacked the intron present in the 280 bp genomic DNA actin PCR product.

Isolation of defence related pearl millet genes by SSH

When performing SSH, experimental design is important so that cDNA libraries are constructed that capture the maximum number of differentially expressed genes between control untreated samples (named “Driver”) and treated samples (named “Tester”). An important issue to consider is whether the experimental aim requires a narrow or a wide subtraction. Hein and coworkers (Hein et al., 2004) performed a narrow subtraction in an investigation of early responses (within 1-5 h) of barley plants to avirulent and virulent races of a fungal pathogen, and identified 21 differentially expressed transcripts. In contrast, a wide subtraction was applied in this study in order to identify as many transcripts as possible that are differentially expressed in the general defence response of the pearl millet plant to a range of pathogens. This was accomplished by subtracting control cDNA from untreated leaves of a downy mildew susceptible pearl millet line (842B) (Driver) from cDNA from leaves of a disease resistant pearl millet line (ICML12=P7) that had been wounded and treated separately with elicitors of bacterial and fungal origin (Tester). Furthermore, cDNA from a range of different time points after

treatment (5, 14, and 24h) was pooled for the Tester, and at the corresponding times for the Driver. A reverse subtraction cDNA library was also constructed in which cDNA from treated ICML12=P7 leaves was subtracted from that of control 842B leaves. In this manner, genes that are down regulated during pearl millet defence response were also captured, which are likely to be as important as up regulated genes (Cao et al., 2004).

Efficiency of the SSH reaction was assessed by PCR amplification of the subtracted products, and Southern hybridisation of forward and reverse subtracted products to tester and driver probes. PCR amplification of subtracted tester samples, compared to amplification of unsubtracted tester and driver samples, indicated that the subtracted tester had been enriched for a number of differentially expressed products as shown by distinct bands in the subtracted tester sample when compared to the product "smear" in unsubtracted tester and driver samples (Figure 2.8).

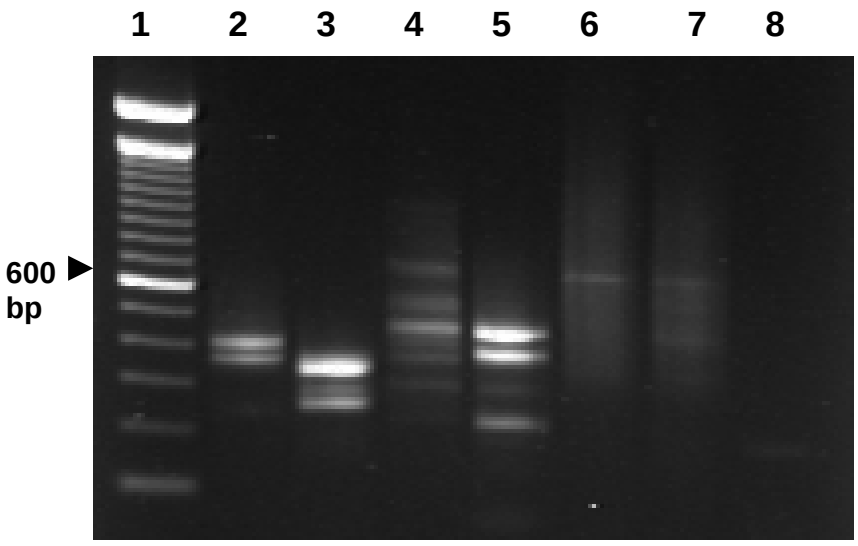

Figure 2.8. Agarose gel electrophoresis of amplified SSH products from forward subtracted pearl millet SSH products. Four individual secondary PCR reactions were set up in order to bulk up primary PCR SSH products. Lane 1, 100 bp marker (Invitrogen); lanes 2-5, secondary amplification of subtracted tester cDNA; lane 6, amplification of unsubtracted tester cDNA; lane 7, amplification of unsubtracted driver cDNA; and lane 8, water control.

In addition to PCR amplification, the efficiency of subtraction was further evaluated by Southern analysis. Forward subtracted, unsubtracted tester, reverse subtracted and unsubtracted driver PCR products were electrophoresed through an agarose gel, blotted onto a nylon membrane and independently hybridised with complex probes derived from forward subtracted cDNAs, unsubtracted tester cDNAs (UT), reverse subtracted cDNAs and unsubtracted driver cDNAs (UD). Results showed that forward subtracted cDNA probes bound mainly to the forward subtracted tester PCR product, with far less hybridisation to reverse subtracted cDNAs, unsubtracted tester and driver PCR products (Figure 2.9, filter A). Furthermore, very little UT and UD probe hybridised to the forward subtracted material (Figure 2.9, Filter B and Filter D), demonstrating gene transcripts common to both UD and UT had been removed by the subtraction, and thus implying enrichment for tester specific transcripts.

Forward and reverse subtracted tester PCR products were cloned into the pGEMT-Easy and transformed into *E.coli* DH10B cells. The SSH procedure yielded a forward subtracted cDNA library of 4 X 10^5 clones, and a reverse subtracted cDNA library of 2.8 X 10^5 clones. Nine hundred and sixty clones were picked from each library for further analysis.

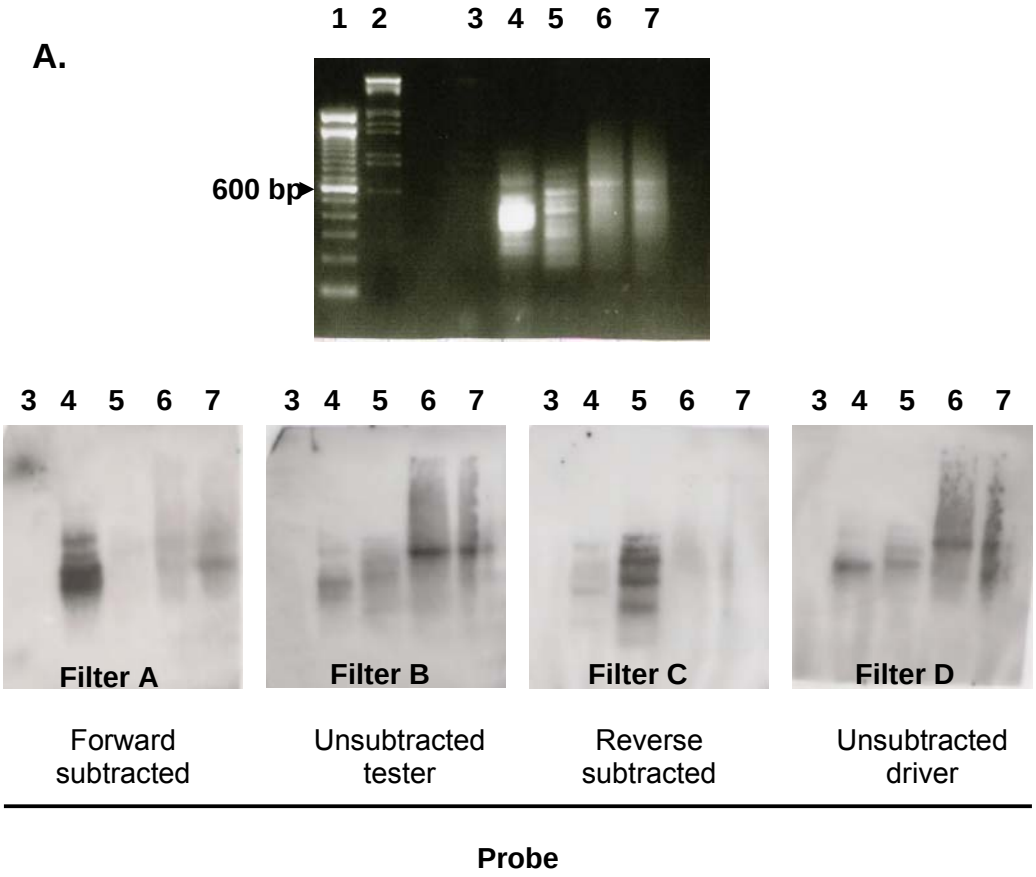

Figure 2.9. Southern analysis of forward subtracted, unsubtracted tester, reverse subtracted unsubtracted driver cDNA amplfication products. (A) Agarose gel electrophoresis of amplification products. Amplification products in (A) were transferred to a positively charged nylon membrane and independently hybridised with complex probes derived from forward subtracted cDNAs (Filter A), unsubtracted tester cDNAs (Filter B), reverse subtracted cDNAs (Filter C) and unsubtracted driver cDNAs (Filter D). Lane 1, 100 bp marker; lane 2, λDNA marker III (Roche Diagnostics); lane 3, DIG-labelled λDNA marker III (Roche Diagnostics); lane 4, forward subtracted cDNA; lane 5, reverse subtracted cDNA; lane 6, unsubtracted tester; lane 7, unsubtracted driver.

Screening of the SSH libraries using glass slide microarrays.

cDNA microarray technology was used to perform a high throughput screen of the pearl millet SSH cDNA libraries to identify genes expressed in response to pathogen elicitor treatment, and obtain information about the relative abundance of these gene transcripts upon induction of plant defense responses. The aim was to identify and discard “housekeeping” and rRNA genes that had escaped subtraction, and to select defence response associated genes for sequencing.

The SSH libraries arrayed on the glass slides were screened with rDNA clones from pearl millet. The rDNA probes hybridised to 5% of the forward subtracted pearl millet clones and less than 1% of the reverse subtracted clones (data not shown).

After hybridisation with combinations of the SSH cDNAs, enrichment ratios were calculated for the forward subtracted library for ST:UD (ER1) and ST:UT (ER2) (Van den Berg et al., 2004). A positive ER1 or ER2 value indicates transcripts that have been enriched during subtraction relative to their levels in UD or UT, respectively. Conversely, negative ER1 or ER2 values indicate transcripts that have been reduced in abundance during SSH relative to their levels in UD or UT, respectively, due to normalisation. Normalisation equalises the concentration of individual transcripts, which may be present at very different concentrations prior to normalisation (Diatchenko et al., 1996). This has the advantage of enriching for rare transcripts in the subtracted tester samples and reducing the levels of abundant transcripts.

Relative abundance of cDNAs in UD and UT for the forward subtracted library was visualized by plotting ER1 versus ER2 for individual forward subtracted pearl millet clones (Figure 2.10). The diagonal line on each graph (ER1=ER2) in Figure 2.10 represents similar levels of enrichment / normalisation during SSH relative to UD and UT. Clones lying on this line are derived from transcripts of equal abundance in UD and UT. The majority of clones (77 % of forward subtracted pearl millet SSH clones) lie above this line (ER1>ER2), indicating a greater abundance of these transcripts in UT than in UD, and confirming that each SSH has enriched for transcripts that are up regulated in the tester. Clones above the diagonal line with positive ER2 values are likely to be derived from low abundance transcripts; expression of some of these may be difficult to detect in Northern blots and accurate comparisons of gene expression between tester and driver may require real time RT-PCR.

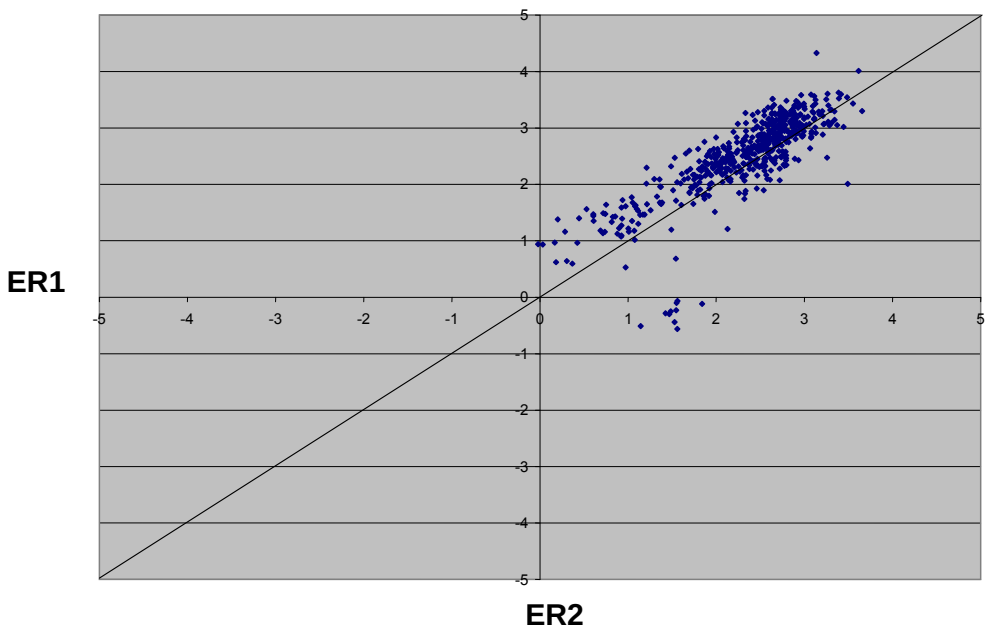

Figure 2.10. Screening the pearl millet forward subtracted SSH library on glass slide microarrays. SSH enrichment ratio 1 (ER1) is plotted against SSH enrichment ratio 2 (ER2). ER1 was calculated for each clone by $\log_2$ transforming the value of the subtracted tester fluorescence divided by the unsubtracted driver fluorescence. ER2 for each clone was calculated by $\log_2$ transforming the value of the ST fluorescence divided by the unsubtracted tester (UT) fluorescence. The diagonal line indicates clones derived from equal abundance in UD and UT (i.e. ER1=ER2). Clones that lie above the diagonal line represent transcripts that are induced upon treatment (ER1>ER2), while those below the line indicate clones that have escaped the subtraction (ER2<ER1). Clones above the line with a positive ER2 value represent rare transcripts, whereas clones above the line with a negative ER2 value are regarded as abundant and have been reduced in relative concentration during normalisation.

Data represented in Figure 2.10 was analysed in Microsoft Excel, and global normalisation using spiked controls was applied. In the spiked controls method, DNA sequences from an organism different from the one being studied are spotted on the array (with replication), and included in the two different samples to be labelled at an equal amount. These spotted control sequences should thus have equal red and green intensities and can be used for normalisation. In this experiment, the control genes *bar*, *luc*, *uid*A and ITS were added in equal quantities into the Cy3 and Cy5 labelling mixes. The first

transformation applied to expression data, referred to as normalisation, adjusts the individual hybridisation intensities to balance them appropriately so that meaningful biological comparisons can be made. There are a number of reasons why data must be normalised, including unequal quantities of starting RNA, differences in labelling or detection efficiencies between fluorescent dyes used, and systematic biases in the measured expression levels (Quackenbush, 2001). However, global normalisation using spiked controls is not an ideal method to normalise differences in Cy dye labelling because of evidence of spatial or intensity dependent dye biases in numerous experiments (Yang et al., 2002). For this reason, a simple data analysis pipeline named `SSHscreen` using "linear models for microarray data" (limma) functions in the R computing environment (Smyth, 2004) was developed to analyse spot intensity data, thereby screening clones in the forward and reverse subtracted SSH cDNA libraries to identify those that are significantly differentially expressed (Berger et al., 2006) (http://www.stats.ox.ac.uk/~vos/SSHscreen/). `SSHscreen` applies locally weighted linear regression (lowess) analysis (Yang et al., 2002) to microarray data to account for differences in dye intensity between two samples, and also employs statistical tests [T-test with an output of p-values that are corrected for multiple testing by controlling the false discovery rate (FDR) correction] to provide confidence in the choice of genes for further study.

`SSHscreen` produces MA-plots (Yang et al., 2002) for the raw and normalised data for each slide. A MA–plot is a scatter plot of log intensity ratios $M = log_2(R/G)$ versus average log intensities $A = log_2(R*G)/2$, where R and G represent the fluorescence intensities in the red and green channels respectively. After the within-array global (lowess) normalisation and between-array quantile normalisation the distribution of red and green channels for each array should become essentially the same, as well as the distribution across arrays. Figure 2.11 shows slide pairs used to perform ER3 versus inverse ER2 plots before and after with-in array normalisation for the reverse subtracted library. After normalisation, the distribution of M values of spots on a given slide is not biased towards one dye, and is centred around 0.

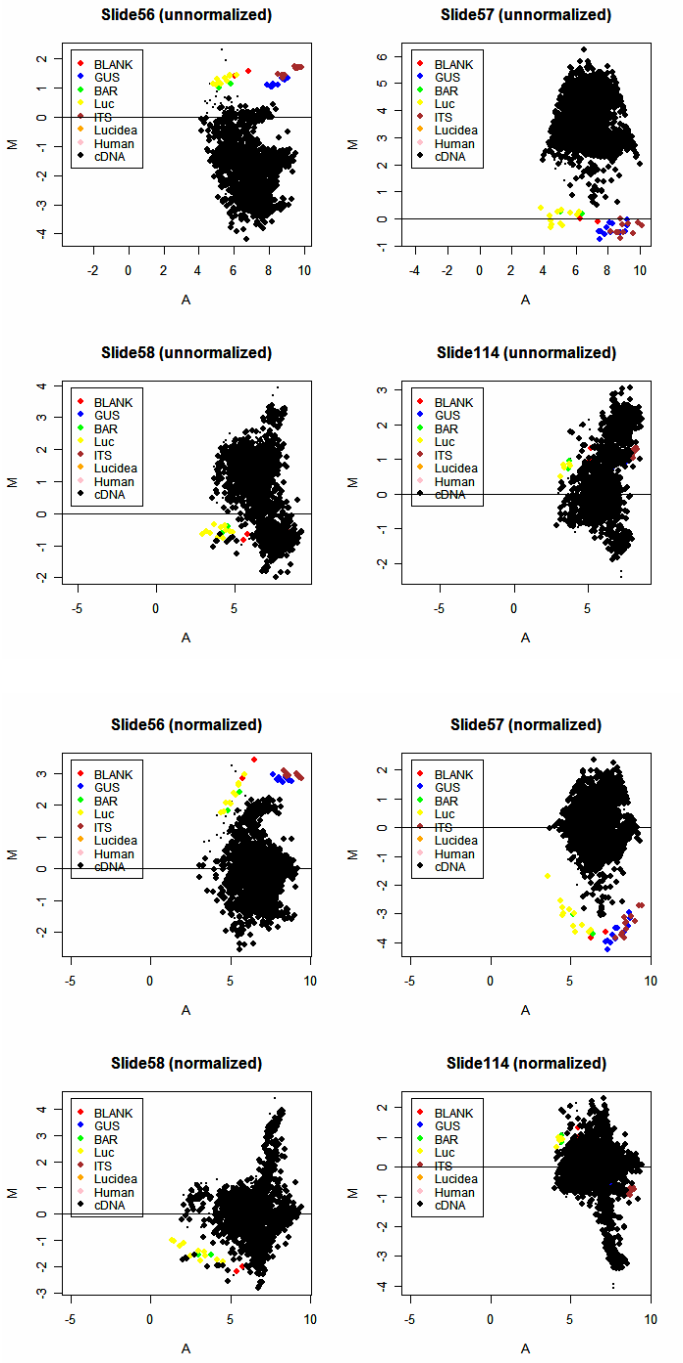

Figure 2.11. M vs A plots for microarray slides before and after global lowess normalisation using SSHscreen. Data from slides 56 and 57 were used to calculate inverse ER2, and data from slides 58 and 114 were used to calculate ER3 for the reverse subtracted library. Slide 56 was probed with subtracted untreated sample (Cy3) and unsubtracted untreated sample (Cy5). Slide 58 was probed with unsubtracted treated sample (Cy5) and unsubtracted untreated sample (Cy 3). Slide 57 and slide 114 represent dye swap comparisons of slides 56 and 114 respectively.

Furthermore, comparison between normalised dye swap slide pairs shows that MA-plot distributions are mirror images of each other, suggesting that clones that are shown to be up- or down regulated on one slide, show similar expression patterns on the reverse slide.

Figure 2.12 shows the plot of ER3 versus inverse ER2 for the forward subtracted pearl millet library as analysed with `SSHscreen`. Data points that are above the x-axis of the graph (ER3>0 ~ UT>UD) represent genes up regulated by the treatment. These are the expected clones in the SSH library that can be chosen for further study. Data points below the x-axis of the graph (ER3<0 ~ UT<UD) represent genes that are down regulated by the treatment, which should not be represented in the SSH library and have most likely escaped subtraction. The x-axis of the graph (ER3=0 ~ UT=UD) represents housekeeping genes that have similar levels in the untreated (Driver) and treated (Tester) samples. The inverse ER2 values reflect the level of normalisation for each gene, from which one can infer whether it is rare or abundant in the treated sample (UT). Data points in quadrant 1 (top left) of Figure 2.12 represent up regulated genes that are rare in the UT sample (negative 1/ER2 and positive ER3). This information is useful since these will have a low level of mRNA transcripts in the treated sample, which may need a sensitive verification method such as real time RT PCR. In contrast, data points in quadrant 2 (top right) represent up regulated genes that are abundant in the UT sample that should require a less sensitive verification technique such as Northern blot analysis. The clones that are statistically significantly up- or down regulated, as determined by the linear model, are also indicated in Figure 2.12. The advantage of the plot in Figure 2.12 over that in Figure 2.10 is that it places the data for genes with different behaviours in each of the four quadrants which allows for easy interpretation.

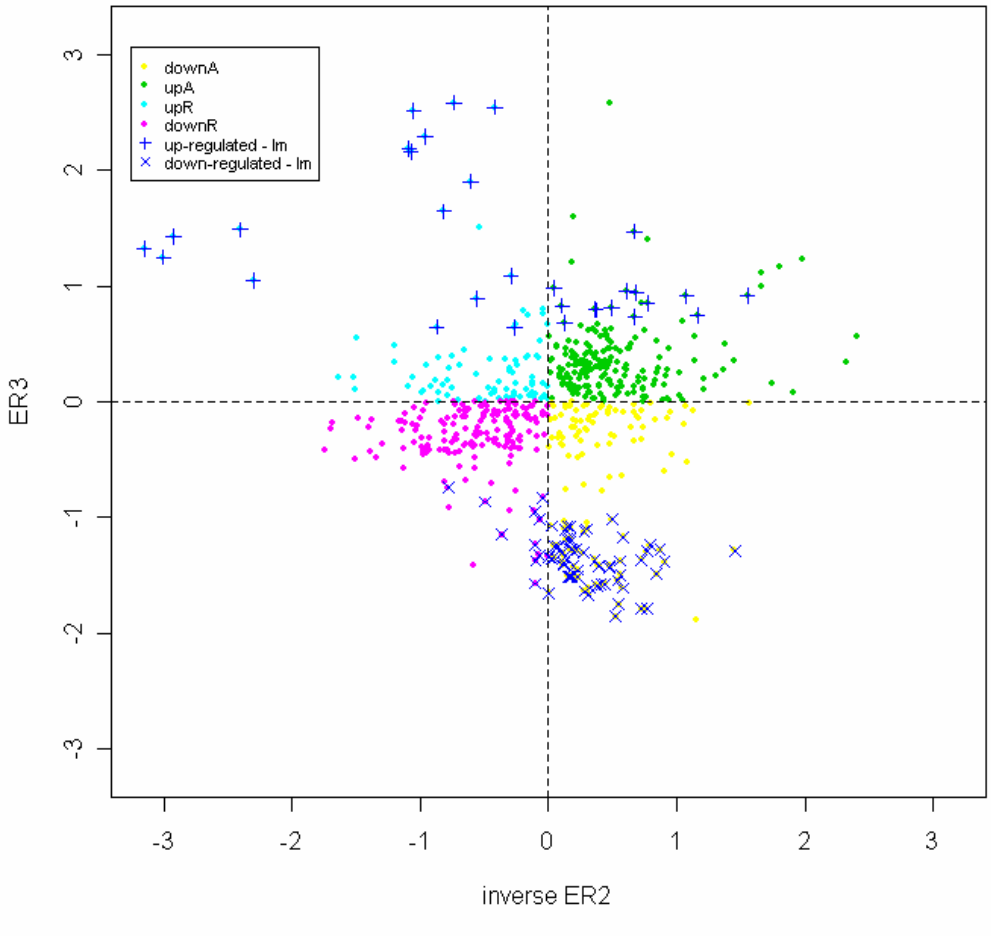

Figure 2.12. ER3 vs. Inverse ER2 plot produced by SSHscreen for data for the forward subtracted pearl millet SSH cDNA library.

Slight discrepancies exist between data analysed using global normalisation using spiked controls (Figure 2.10) and lowess global normalisation (Figure 2.12). On the whole, global normalisation using spiked controls indicated that most of the isolated genes were up regulated in the treated tissue, whereas application of lowess normalisation suggested that approximately half of the isolated genes are truly up regulated following elicitor treatment. However, because of problems with using spiked controls in global normalisation, and the advantage of employing statistical tests to provide confidence in the choice of genes for further study, we have chosen to apply `SSHscreen` to analyse all SSH data produced in our laboratory.

Figure 2.13 shows a similar plot for genes represented by the reverse subtracted library. Clones in the reverse library representing genes that are down regulated during pathogen attack are represented by blue (down regulated rare) and green (down regulated abundant) spots. Pink and yellow spots show clones that have escaped the subtraction procedure and actually represent genes that are up regulated during defence response. Genes exhibiting the highest statistical significance are shown by crosses (down regulated, significant) and plusses (up regulated significant).

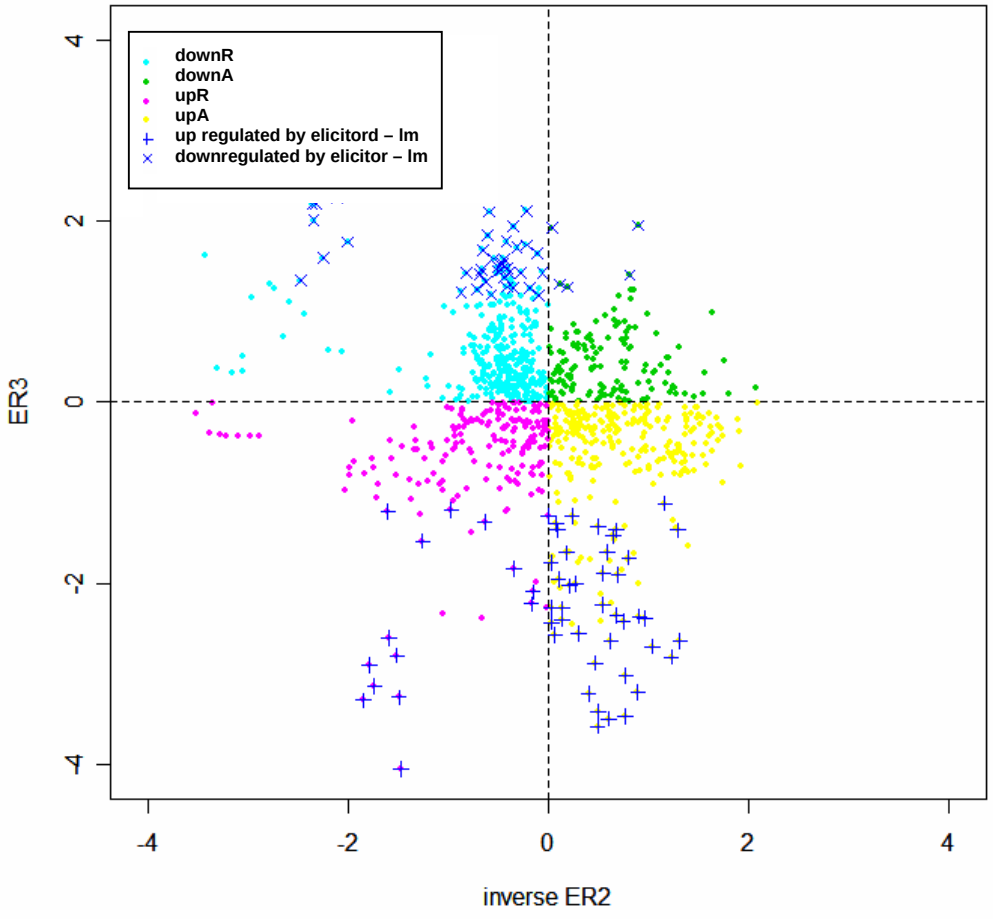

Figure 2.13. ER3 vs. Inverse ER2 plot produced by SSHscreen for data for the reverse subtracted pearl millet SSH cDNA library.

Inverse Northern analysis of selected transcripts

To validate conclusions drawn from comparisons of ER1 and ER2 and to confirm that clones are derived from differentially expressed transcripts, twenty clones from forward subtracted SSH library were selected for inverse Northern analyses. These clones were arrayed in duplicate on dot blots and hybridised to freshly prepared non amplified tester and driver cDNAs.

ER1 and ER2 ratios from the microarray screening (Figure 2.10) together with the inverse Northern expression ratios of the selected pearl millet clones are shown in Table 2.2. Clones with ER1>ER2 had inverse Northern expression ratios greater than one, confirming that these clones represented transcripts that were up regulated in the tester compared to the driver (data for 19 pearl millet clones from the forward subtracted SSH library shown in Table 2.2). In the case of clone 10-C6, ER1<ER2, and this clone exhibited an inverse Northern expression ratio of less than one confirming that it represented a transcript that was more abundant in the driver than the tester, and had escaped the subtraction process. In order to relate the inverse Northern and microarray data, a ratio of UT/UD was calculated from the ER1 and ER2 ratios (Table 2.2). As expected, inverse Northern ratios correlated with UT/UD ratios, ie clones with inverse Northern ratios >1 had UT/UD values > 1, whereas clones with inverse Northern ratios <1 gave UT/UD < 1 (Table 2.2). Some of the clones selected for Northern analyses were subjected to sequence analysis. These clones are represented in bold in Table 2.3.

Table 2.2. Validation of microarray screening of selected pearl millet SSH clones by inverse Northern data.

Clone number	ER1 [1]	ER2 [2]	UT/UD ratio [3]	Inverse Northern expression ratio [4]
3-D5	1.4	0.2	2.3	34.3
4-H11	1.2	0.3	1.9	21.1
10-B7	1.0	0.2	1.7	11.1
4-H9	0.6	0.3	1.2	9.3
5-C3	1.5	0.7	1.7	7.9
1-B7	1.6	0.5	2.1	7.2
6-A2	1.2	0.7	1.4	6.3
8-D7	1.0	0.4	1.5	6.2
4-A2	0.9	0.03	1.8	5.8
4-E12	0.9	-0.02	1.9	5.4
7-D7	1.6	0.8	1.7	5.3
2-A12	3.6	3.4	1.1	3.6
6-F1	2.6	2.0	1.5	3.3
2-A8	3.6	3.3	1.2	2.6
6-H1	2.2	1.9	1.2	2.5
6-C2	3.0	2.7	1.2	2.3
6-D1	2.6	2.5	1.1	1.5
3-H3	4.3	3.1	2.3	1.3
6-G2	2.0	1.7	1.2	1.1
10-C6	-0.3	1.5	0.3	0.9

[1 and 2] ER1 and ER2 were calculated from the microarray screening as $\log_2$ (ST/UD) and $\log_2$ (ST/UT), respectively.

[3] UT/UD = antilog of (ER1 – ER2) in the base 2

[4] Inverse Northern expression ratio was calculated as follows: Density of tester/Density of driver samples after normalization of the data using a rDNA clone.

The above results effectively demonstrated the use of cDNA microarrays to screen forward and reverse subtracted pearl millet SSH libraries in a rapid, high throughput manner. The major advantages of this screening method are that it provides an objective and quantitative way to identify differentially expressed genes as well as to determine the relative abundance of transcripts in the original UT samples. Furthermore, application of `SSHscreen` allows for statistical tests of microarray data to provide confidence in choice of genes for further studies.

Previous studies have used inverse Northern blots to screen SSH libraries in which PCR products or colonies are dotted onto nylon membranes and the

driver and tester cDNAs are labelled with radioactivity (Diatchenko et al., 1996; Mahalingam et al., 2003). This method has disadvantages, namely that comparisons are made between two separate membrane hybridisations which introduces error and the interpretation is qualitative (Mahalingam et al., 2003) unless a laboratory has access to a phosphorimager. In contrast to membrane based methods, hybridisation to glass slide cDNA microarrays can be performed with different fluorescent tags, which allows a direct comparison of the relative abundance of transcripts in ST, UT and UD. Furthermore, hybridisations are performed on a small surface area, which reduces the amount of labelled probe needed. Finally, the computerised scanning of the array provides a high throughput quantitative method to choose which genes to sequence and study further using Northern Blot analysis, real time RT-PCR or a custom microarray.

Sequence analysis of selected clones

A random selection of clones from the forward and reverse subtracted SSH libraries with either ER1>ER2, or positive ER3 values, were chosen for sequence analysis (Table 2.3). In total, 120 clones were sequenced, of which 117 reactions produced results of good quality. In many cases, the same sequences were represented by more than one clone, indicative of enrichment in the SSH. Homology comparisons indicated that 55 of the 117 sequences represented clones with unique identity. A number of clones showed significant similarities to genes associated with plant defence and stress responses.

Pathogenesis related protens (PRs) (4-B7) are plant proteins induced in response to infection by pathogens including viruses, fungi and bacteria. PR1 expression is mediated via the SA signalling pathway, and is a typical marker protein of systemic acquired resistance.

Glutaredoxins (1-G9) are small ubiquitous proteins of the thioredoxin family, which protect the plant from oxidative stress. These proteins catalyse dithiol-disulphide exchange reactions or reduce protein-mixed glutathione disulphides (Rouhier et al., 2005). Glutathione S-transferases (6-E3) are also

involved in protecting the plant from oxidative stress, and catalyse the conjugation of hydrophobic, electrophilic compounds with the tripeptide glutathione to form polar, non-toxic peptide conjugates (Wilce and Parker, 1994).

Other induced defence response genes include farnesyl pyrophosphate synthetase (2-D2), which is involved in lesion formation in diseased leaves (Manzano et al., 2004), UDP-glucose:salicylic acid glucosyl transferase (1-B9), HSP70 (2-F11), ubiquitin associated protein (6-B5), alanine aminotransferase (6-B6). HSP70 has been found to be up regulated during the hypersensitive response (Birch et al., 1999), and recently Kanzaki and coworkers (Kanzaki et al., 2003) showed through virus induced gene silencing (VIGS) that HSP70 is an essential component of the plant defence signal transduction pathway. Glucosyl transferases catalyse the transfer of glucose residues to numerous substrates and regulate the activity of compounds that play important roles in plant defence against pathogens, such as salicylic acid (Chong et al., 2002). Aminotransferases have recently been shown to play a role in "enzymatic disease resistance" to downy mildew (cased by *Pseudoperonospora cubensis*) in *Cucumis melo* (wild melon) (Taler et al., 2004). S-adenosylmethionine decarboxylase (6-H1) is an important enzyme in polyamine biosynthesis, and catalyses the decarboxylation of S-adenosyl methionine (SAM) into decarboxylated SAM which provides the aminopropyl moiety required for spermidine and spermine biosynthesis from putresine. Recently, a preliminary link was made between polyamines and plant defence response where the polyamine spermine was hypothesised to act as an inducer of PR proteins, and as a trigger for caspase-like activity and hence HR (Walters, 2003).

Genes involved in signal transduction are represented in the pearl millet SSH library. Receptor kinases (4-E8) are involved in perception of the pathogen signal, and initiation of subsequent intracellular transduction to elicit plant defence responses (Hammond-Kosack and Parker, 2003). The calcium binding EF hand protein (5-B12) is one of four similar domains which form a calcium dependent protein kinase (CDPK). Pathogen attack cause

perturbations in cellular calcium (Ca^{2+}) levels. CDPKs decode information contained in the temporal and spatial signals of these Ca^{2+} signals and bring about changes in metabolism and gene expression (Harmon et al., 2000).

Induction of genes encoding chlorophyll A/B binding (CAB) (5-H9) proteins by the pathogen elicitor treatment suggests the existence of crosstalk between defence and other signalling pathways. Schenk and coworkers (Schenk et al., 2000) obtained similar results when they treated Arabidopsis plants with SA and observed the up regulation of *cab* genes. Results presented here further support the hypothesis that defence mediated signalling pathways crosstalk with the pathway regulated with the phytochromeA/red light, leading to induction of *cab* genes. Availability of an Arabidopsis phytochrome A and B signalling mutant (*psi2*) showing elevated levels of PR gene expression also suggests that light signal transduction and pathogenesis related gene signalling pathways are connected (Genoud et al., 1998).

SSH revealed a number of genes involved in transcription and translation that are up regulated in response to pathogen elicitor treatment. These include transcription factor EREBP1 (10-C3), transcription factor BTF3 (4-G8), translation initiation factor 5A (10-A9), protein translation factor Sui1 (10-F7) and elongation factor 1 alpha (3-C8). Of particular interest to plant defence response interactions is transcription factor EREBP1 which is known to mediate gene expression in response to various pathogens and defence elicitors (Euglem, 2005).

The pearl millet SSH defence response library was found to contain a number of unknown proteins (10-A1, 10-A3, 10-A4, 10-B7,1-C12, 1-H6, 2-C10, 2-C8, 4-A2, 5-A8, 5-B1, 6-A2, 6-B1, 6-G9, 7-A11, 7-F2, 9-D10), which potentially code for novel, uncharacterised genes involved in plant defence response. These genes are therefore interesting candidates for further studies involving either RNA silencing (Vance and Vaucheret, 2001) or virus induced gene silencing (VIGS) (Burch-Smith et al., 2004) to determine their role in plant defence response.

Several research groups have employed SSH to study plant gene expression in response to pathogen infection or insect feeding. Birch and coworkers (Birch et al., 1999) used SSH to isolate and study potato genes that are induced during an early stage of the hypersensitive response to *Phytophthora infestans.* Mahalingam and coworkers (2003) applied SSH to identify a total of 1058 Arabidopsis genes that are differentially expressed in response to ozone, bacterial and oomycete pathogens and the defence signalling molecules salicylic acid and jasmonic acid. SSH was also employed to study gene expression changes in rice in response to treatment with the blast fungus (*Pyricularia grisea*) (Xiong et al., 2001), and more recently to characterise the early transcriptional changes involving multiple signalling pathways in the *Mla*13 barley interaction with powdery mildew (*Blumeria graminis* f. sp. hordei) (Hein et al., 2004). A number of studies have also utilised SSH to create defence response cDNA libraries of non-model organisms, and combined these experiments with cDNA microarray analyses to examine gene expression in response to pathogen/pest treatment. Combined SSH/microarray studies have been performed in wild rice (*Oryza minuta*) to identify fungal (*Magnaporthe grisea*) stress induced genes and planthopper induced genes respectively (Cho et al., 2005; Shim et al., 2004). Zhu-Salzman and coworkers (Zhu-Salzman et al., 2004) constructed a cDNA library by differential subtraction with cDNAs prepared from sorghum seedlings infested by greenbug aphids and those from uninfested seedlings. Subsequent expression profiling using DNA microarray identified transcripts from this cDNA collection responsive to greenbug feeding, methyl jasmonate, or salicylic acid application. The forward and reverse subtracted pearl millet SSH cDNA libraries therefore present a resource for cDNA microarray studies to examine gene expression changes in pearl millet in response to various pathogen related stresses.

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Table 2.3. Selected genes from forward and reverse SSH libraries chosen for sequence analysis. Clones shown in bold represent sequences that were subjected to inverse Northern analysis (Table 2.2). Clone 10-A1 is identical to clones 3-D5, 4-H11, 5-C3. Clone 3-C8 is identical to 7-D7, and 8-F3 identical to 6-F1. Clones with “no matches” exhibit no homology to sequences in BlastX, BlastN and dBEST databases. Clones with “no identity” have homology to sequences in BlastN and dBEST databases, but the sequence is not characterised. Unknown proteins represent clones with homology to annotated but uncharacterised sequences in the BlastX database.

Clone ID	Size bp	Origin of similar sequence	Protein/DNA similarity	BLAST X		BLAST N		D BEST		Redund-ancy
				Accession no.	Similarity	Accession no.	Similarity	Accession no.	Similarity	
1-B7	219	*Oryza sativa*	Small nuclear ribonucleoprotein polypeptide E	XP_463967	1e-12 (87%, 40 aa)	AK059440	2e-31 (89%, 124 bp)	CF761402	7e-39 (91%, 124 bp)	4
1-B9	474	*Oryza sativa*	UDP-glucose:salicylic acid glucosyl transferase	BAD34358	5e-08 (52%, 50 aa)			BM269408	7e-13 (82%, 137 bp)	
1-C12	278	*Pennisetum glaucum*	No identity					CD725272	e-120 (97%, 261 bp)	
1-E11	338	*Oryza sativa*	Fructose-bisphosphate aldolase	T03679	1e-04 (91%, 23 aa)	AY103557	1e-24 (4%, 77 bp)	CK802616	4e-32 (94%, 93 bp)	3
1-G11	614	*Populus nigra*	Cytosolic phosphoglycerate kinase 1	BAA33801	8e-50 (83%, 123 aa)	AY103553	1e-99 (87%, 371 bp)	CD726515	0 (95%, 581 bp)	
1-G9	516	*Oryza sativa*	Glutaredoxin	CAA54397	3e-46 (87%, 105 aa)	AY104653	e-107 (89%, 336 bp)	CD227215	e-124 (91%, 340 bp)	
1-H6	300	*Pennisetum ciliare*	No identity					BM084147	8e-24 (88%, 127 bp)	4
2-C10	409	*Oryza sativa*	Unknown protein	NP_915800	1e-11 (94%, 37 aa)	AY107683	5e-27 (88%, 117 bp)	AI637116	7e-19 (88%, 95 bp)	2
2-C8	93		No matches							
2-D2	394	*Oryza sativa*	Farnesyl-pyrophosphate synthetase	NP_917118	4e-63 (98%, 117 bp)	AF330037	1e-64 (92%, 345 bp)			
2-E10	421	*Glycine max*	Aspartic proteinase 1	BAB62890	8e-26 (86%, 61 aa)	AY112455	7e-54 (91%, 162 bp)	CN145287	2e-78 (88%, 301)	
2-F11	290	*Oryza sativa*	HSP70	NP_915417	1e-39 (93%, 87 aa)	AF005993	2e-68 (92%, 250 bp)	CD726670	e-130 (98%, 250 bp)	
2-G12	409	*Oryza sativa*	Triose phosphate/phosphate translocator			Z26595	4e-09 (92%, 51 bp)	BM084392	e-113 (93%, 300 bp)	2
2-G6	430	*Oryza sativa*	Tryptophan synthase alpha chain	XP_476874	1e-22 (79%, 64 aa)	AF271384	3e-53 (85%, 253 bp)	CF761257	6e-78 (90%, 242 bp)	
3-C12	498	*Oryza sativa*	Immunophilin	XP_467909	8e-55 (89%, 112 aa)	AY081564	e-144 (93%, 349 bp)	CN844676	e-144 (93%, 3449 bp)	3
3-C8	363	*Zea mays*	Alpha subunit of translation elongation factor 1	BAA08249	2e-10 (96%, 32 aa)	D63581	3e-40 (91%, 130 bp)	CD726323	e-130 (94%, 310 bp)	3
3-D2	215	*Oryza sativa*	Small nuclear ribonucleoprotein polypeptide E	XP_463967	4e-13 (90%, 40 aa)	AK059440	7e-34 (90%, 124 bp)	CN487001	7e-39 (91%, 124 bp)	3
4-A2	234		No matches							
4-A8	424	*Oryza sativa*	Pyrrolidone carboxyl peptidase-like protein	XP_479284	3e-16 (89%, 46 aa)	AP005577	2e-32 (91%, 114 bp)	CK985460	4e-42 (94%, 114 bp)	
4-B7	287	*Zea mays*	Pathogenesis-related protein 1	A33155	1e-23 (77%, 61 aa)	U82200	5e-17 (81%, 183 bp)	CD998687	3e-17 (81%, 182 bp)	2
4-D8	82	*Rickettsia akari*	hypothetical protein RakaH01001082		5e-18 (59%, 49 aa)					5
4-E8	508	*Arabidopsis thaliana*	Receptor kinase	AAM20287	4e-42 (56%, 132 aa)	AL606691	3e-10 (91%, 57 bp)	CD220911	e-125 (89%, 386 bp)	2
4-G8	429	*Oryza sativa*	Transcription factor BTF3	AAO72645	7e-40 (75%, 114 aa)	AY103817	e-103 (87%, 393 bp)	CD724954	0 (98%, 412 bp)	
4-H9	419	*Zea mays*	Cytosolic glyceraldehyde 3-phosphate dehydrogenase			X07156	1e-18 (91%, 125 bp)	CD725014	e-154 (99%, 290 bp)	5
5-A8	367	*Zea mays*	No identity			AY104556	3e-37 (91%, 125 bp)	CA299434	8e-43 (89%, 161 bp)	
5-B1	389	*Zea mays*	No identity			AY109519	6e-08 (93%, 49 bp)	CD724823	6e-50 (99%, 107 bp)	
5-B12	553	*Hordeum vulgare*	Calcium binding EF-hand protein	CAB71337	1e-53 (58%, 174 aa)	AK101337	8e-39 (82%, 281 bp)	CF059716	3e-43 (86%, 173 bp)	
5-D6	563	*Oryza sativa*	Histone H2B.2	XP_483094	3e-28 (98%, 66 aa)	D37943	1e-96 (97%, 202 bp)	CA147135	e-107 (93%, 278 bp)	
5-G4	449	*Oryza sativa*	Phosphatidylserine decarboxylase	NP_914239	7e-54 (85%, 127 aa)	AY110670	e-122 (90%, 351 bp)	CD226354	e-140 (92%, 367 bp)	3
5-H5	576	*Oryza sativa*	AcinusL protein-like	XP_479211	5e-31 (48%, 175 aa)	AY105343	e-153 (89%, 477 bp)	CA068506	e-156 (90%, 457 bp)	2
5-H9	234	*Zea mays*	Chlorophyll a/b binding protein			X63205	2e-06 (100%, 30 bp)			7
6-A2	363		No matches							
6-B1	534		No matches							
6-B5	525	*Oryza sativa*	Ubiquitin-associated (UBA) protein	XP_466502	4e-25 (43%, 107 aa)	AY596599	1e-28 (83%, 200 bp)	BF657757	7e-38 (81%, 312 bp)	
6-B6	469	*Panicum miliaceum*	Alanine aminotransferase	CAA49199	6e-80 (94%, 151 aa)	AB007405	e-151 (93%, 450 bp)	CN136122	0 (94%, 450 bp)	
6-D12	327	*Oryza sativa*	Inorganic pyrophosphatase	XP_476313	3e-47 (95%, 100 aa)	D13472	e-111 (93%, 282 bp)	CN146327	e-118 (93%, 290 bp_	
6-E3	750	*Zea mays*	Glutathione S-transferase (GST40)	AAG34848	3e-60 (72%, 162 aa)					
6-E11	341	*Oryza sativa*	Plastocyanin, chloroplast precursor	P20423	2e-15 (97%, 38 aa)	AF009412	3e-28 (88%, 118 bp)	CD724564	e-145 (100%, 262 bp)	6
6-G9	368	*Pennisetum glaucum*	No identity					CD725231	e-168 (98%, 328 bp)	3
6-H1	1400	*Zea mays*	S-adenosylmethionine decarboxylase	CAA69075	6e-58 (96%, 120 aa)					
7-A11	198		No matches							
7-F2	366	*Sorghum bicolor*	No identity			AY104556	1e-39 (92%, 125 bp)	CN133956	8e-40 (92%, 125 bp)	
7-H11	462	*Sorghum bicolor*	Mitochondrial aldehyde dehydrogenase	BAB92019	8e-72 (95%, 141 aa)	AB084898	e-153 (91%, 416 bp)	BM326464	e-153 (91%, 416 bp)	
8-D10	581	*Arabidopsis thaliana*	Membrane-associated salt-inducible protein-like	BAB08985	5e-72 (67%, 186 aa)	XM_474412	e-151 (87%, 561 bp)	CD980699	e-152 (90%, 453 bp)	
8-D12	537	*Arabidopsis thaliana*	Hydrolase, alpha/beta fold family protein	NP_974605	2e-53 (79%, 123 aa)	AP006175	1e-25 (90%, 99 bp)	CA484537	e-127 (89%, 382 bp)	
8-D7	622	*Oryza sativa*	MATE efflux protein family protein	XP_478265	2e-37 (59%, 122 aa)	XM_478265	2e-36 (83%, 233 bp)	CB925276	5e-61 (82%, 398 bp)	
8-F3	470	*Zea mays*	Enolase	CAA39454	1e-76 (96%, 150 aa)	X55981	e-150 (90%, 431 bp)	CN146083	0 (93%, 450 bp)	2
9-D10	22		No matches							
10-A1	216	*Zea mays*	No identity			AY104344	1e-10 (93%, 49 bp)	BF586083	2e-23 (85%, 162 bp)	
10-A3	52		No matches							13
10-A4	255		No matches							
10-A9	542	*Zea mays*	Translation initiation factor 5A	CAA69225	4e-70 (94%, 135 aa)	AF034943	e-145 (94%, 408 bp)	CD445022	e-148 (91%, 408 bp)	3
10-B7	536	*Arabidopsis thaliana*	Unknown protein	AAP37829	8e-20 (78%, 57 aa)	AY106780	6e-18 (85%, 122 bp)	AW120334	9e-19 (85%, 123 bp)	
10-C3	641	*Oryza sativa*	Transcription factor EREBP1	XP_468125	2e-66 (69%, 196 aa)	AY108380	2e-98 (86%, 409 bp)	CN130950	e-117 (87%, 441 bp)	
10-F7	383	*Oryza sativa*	Protein translation factor Sui1	XP_475493	1e-16 (97%, 43 aa)	AY104121	3e-65 (93%, 189 bp)	CD724989	0 (100%, 342 bp)	8

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