Chapter 3

Nitric oxide mediated transcriptional changes in pearl millet

3.1 ABSTRACT

Nitric oxide (NO) is an important signalling molecule that plays a key role in the activation of defence response mechanisms in plants. In the current study, we investigated the transcriptional changes in pearl millet plants over time following application of 1 mM sodium nitroprusside (SNP), a donor of NO. Gene expression changes were examined using a pearl millet cDNA microarray that contained a biased representation of defence response genes. Altered expression patterns were detected for 45 of the 1920 cDNAs examined, of which 24 cDNAs were up regulated and 21 cDNAs were down regulated in response to NO treatment. Comparison of pearl millet expression profiles with those of Arabidopsis plants treated with a NO donor, suggested that there was very little overlap in gene expression profiles. Most of the transcripts exhibiting differential expression in pearl millet have not been previously implicated in NO signalling in plants

3.2 INTRODUCTION

Attempted infection of plants by an avirulent pathogen elicits a number of defence responses, many of which lead to cell death at the site of pathogen infection. This hypersensitive cell death results in a restricted lesion delimited from surrounding healthy tissue, and is thought to play a role in containment of pathogen spread throughout the plant. Closely associated with the hypersensitive response is the oxidative burst, which is characterised by the rapid production of the reactive oxygen intermediates (ROIs) superoxide (O_2^-) and hydrogen peroxide (H_20_2). This oxidative burst drives cross linking of the cell wall, induces several plant genes involved in cellular protection and defence, and is necessary for the initiation of HR. However, the oxidative burst has been shown to be necessary but not sufficient to trigger host cell death, and experimental evidence indicates that nitric oxide (NO) cooperates with ROIs in the activation of HR (Delledonne et al., 1998).

The role of NO as a biological messenger in animals has been well documented, and has been shown to control blood pressure homeostasis, platelet aggregation, and transmission of signals by the nervous system. NO is also known to play a key role in the activation of macrophages and cellular defences against microbial pathogens (Mayer and Hemmens, 1997). Recent studies have also suggested that NO is involved in several plant functions including stress response (Garcia-Mata and Lamattina, 2001), growth and development (Beligni and Lamattina, 2000), senescence (Leshem and Pinchasov, 2000) and iron homeostasis (Murgia et al., 2002). NO has also been shown to interact with plant hormone signalling pathways such as indole acetic acid (IAA) and abscisic acid (ABA) (Garcia-Mata and Lamattina, 2002; Pagnussat et al., 2003). Furthermore, NO has 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).

In plants, NO can by synthesised enzymatically, or by non-enzymatic reduction of apoplastic nitrite under acidic conditions (Parani et al., 2004). Recent evidence suggests that plants, like animals, use multiple enzymes for the synthesis of this critical signalling molecule (Chandok et al., 2003). Nitrate reductase is believed to be responsible for NO production in uninfected or non-elicited plants, whereas nitric oxide synthase, a variant of the P protein of the glycine decarboxylase complex, is induced in response to pathogen attack (Chandok et al., 2003). NO production leads to increases in the gene expression levels of the defence related proteins phenylalanine lyase (PAL) and pathogenesis related protein 1 (PR1) (Durner et al., 1998), and experimental evidence suggests that NO is required for the full function of salicylic acid (SA) as an inducer of systemic acquired resistance (Song and Goodman, 2001). Like mammals, NO signalling in plants has been shown to operate through cGMP- and cADP ribose-dependent pathways (Durner et al., 1998; Klessig et al., 2000). However, NO can also act on many other potential cell targets, such as metal- and thiol-containing proteins and enzymes, such as catalases and peroxidases, guanylate cyclase, receptors and transcription factors (Polverari et al., 2003).

The existence of multiple mechanisms of NO action makes dissection of specific pathways difficult. However, three recent studies have helped to understand the role of NO in regulating gene transcription in Arabidopsis. Huang and coworkers (Huang et al., 2002) applied DNA microarray analysis to examine the affects of NO on transcriptional activation in Arabidopsis cell suspension cells using microarrays containing 200 cDNAs involved in or associated with plant defence, and 50 cDNAs associated with primary metabolism. Parani and associates (Parani et al., 2004) improved on this study by employing a whole genome ATH1 microarray, representing over 24000 genes, to study changes in gene expression in whole Arabidopsis plants in response to treatment with a NO donor. Polverari and coworkers (Polverari et al., 2003) complemented microarray studies by performing cDNA-AFLP to examine nitric oxide mediated transcriptional changes in *Arabidopsis thaliana*. Application of this technique enabled the authors to detect gene expression patterns of approximately 2500 cDNAs. In the current study, cDNA microarray analysis was applied to examine the effects of NO on a non-model cereal plant, pearl millet [*Pennisetum glaucum* (L.) R. Br], and to identify genes that are differentially expressed following exogenous application of a NO donor. Furthermore, comparisons were made with reports that analysed transcriptional changes in NO treated Arabidopsis to determine if there were similarities in responses between this model plant and pearl millet.

3.3 MATERIALS AND METHODS

NO treatment of pearl millet

Pearl millet (ICML12=P7) 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. After one week (once the seeds had germinated) seedlings were transferred to seedling trays containing sterilised vermiculite that had been fertilised with Hoagland's solution (Hoagland and Arnon, 1950). Plants were grown for a further six weeks under 16 h light (140 umol/m^2/s) and 8 h dark cycles at a constant temperature of 25ºC and 85% relative humidity. For NO treatment, seven week old plants were irrigated with 1 mM sodium nitroprusside (SNP) (Sigma, Aston Manor, South Africa) in water, and leaf tissue was harvested 0, 1, 3 and 6 hours post treatment, and the plants were immediately frozen in liquid nitrogen for storage until RNA purification. Plants were treated during the light period, and two replications with 9 plants in each were included. Plants within replicates were pooled to reduce variation prior to RNA extraction.

RNA isolation and purification

Total RNA was isolated from the frozen seedlings using Qiazol™ Lysis Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol with modifications. Briefly, one gram of plant material was placed in liquid nitrogen and ground to a fine powder with the aid of a mortar and pestle. Frozen samples were added to 15 ml Qiazol™ reagent (Qiagen), incubated at 60ºC for 5 minutes, and then vortexed for 15 seconds. Plant debris was pelleted by centrifugation at 9000 rpm for 10 minutes. Three millilitres of chloroform was added to the supernatant, the samples were vortexed vigorously for 15 seconds, and then centrifuged at 9000 rpm for 15 minutes. The upper aqueous phase was carefully removed and total RNA was precipitated by the addition of a half volume of each of isopropanol and 0.8 M sodium citrate/1.2 M NaCl solution. RNA was pelleted by centrifugation at 9000 rpm for 10 minutes, and washed with 75% ethanol. The dried RNA

pellets were resuspended in 200 µl nuclease free water (Ambion, Huntingdon, Cambridgeshire, UK). Total RNA was treated with RNAse free DNAse1 (Qiagen) and further purified using an RNeasy® Minelute™ Kit (Qiagen) to remove contaminating genomic DNA, carbohydrates and polyphenols.

RNA yield and purity was determined by measuring absorbency at 260 nm, 280 nm and 230 nm using a Nanodrop ND-1000 Spectrophotometer. RNA integrity was assessed by electrophoresing five micrograms total RNA through a 1.2% formaldehyde denaturing agarose gel (results not shown).

Microarray preparation

cDNA inserts from subtracted pearl millet collections (see Chapter 2 for details) (Van den Berg et al., 2004), which are biased in representation of defence response genes, were PCR amplified in 100 µl reactions in a 96 well microplate format, using Sp6 (5'TATTTAGGTGACACTATAG-3') and T7 primers (5'TAATACGACTCACTATAGGG-3'). PCR products confirmed by gel electrophoresis were purified using Multiscreen® PCR Purification Plates (Millipore, Molsheim, France), and resuspended in 50 µl sterile water. Purified cDNA inserts were vacuum dried, and resuspended in a final volume of 20 µl 50% DMSO in preparation for array spotting. cDNA inserts (200 pg) were printed in duplicate onto Corning® Gaps II (Corning, NY, USA) aminosilane coated microscope slides using an Array Spotter Generation III (Molecular Dynamics Inc., Sunnyvale, CA, USA) at the ACGT Microarray facility (http://www.microarray.up.ac.za). The lucidea spiked control set (Amersham Biosciences) was also arrayed to serve as controls for labelling and hybridisation reactions.

Fluorescent probe preparation, hybridisation and scanning

Indirect aminoallyl labelling reactions using total RNA were performed using slight modifications of published protocols (http://www.tigr.org/tdb/microarray/protocols.html). Labelled cDNA from each time point was cohybridised with that from a reference sample to profile expression changes following exposure to SNP. RNA samples representative of each time point were labelled with Cy3, and the reference sample was

labelled with Cy5 dye (Figure 3.1). A reference RNA sample was prepared by pooling 50 µg RNA from each time point from each biological replicate. In brief, 15 µg total RNA was reverse transcribed using SuperScript™II (Invitrogen, Carlsbad, CA, USA) in the presence of 6 µg random hexamers (Invitrogen), 1 ug oligo(dT)$_{20}$ (Invitrogen), and 1 X aminoallyl-dNTP labelling mix (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.3 mM dTTP, 0.2 mM aminoallyl-dUTP). Reactions were incubated at 42ºC overnight. Thereafter, RNA was hydrolysed at 65ºC for 15 min by the addition of 0.2 N NaOH and 0.1 mM EDTA. Tris, pH7.4 (0.3 M) was added to neutralise the reaction before cDNA was purified using a modified Qiaquick PCR Purification kit (Qiagen) protocol. Manufacturer's instructions were followed except that columns were washed with phosphate wash buffer (5 mM KPO_4, pH 8.5 in 80% ethanol), and cDNA was eluted in 4 mM KPO_4, pH 8.5. cDNA samples were vacuum dried and resuspended in 4.5 µl 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0, to which an equal volume of NHS-Cy dye (Amersham BioSciences, Little Chalfont, UK) (prepared in DMSO) was added. The dye coupling reaction was incubated for 1 h in the dark at room temperature. Thirty five microlitres 100 mM sodium acetate, pH 5.2 was added to neutralise the reaction, and uncoupled dye was removed by purifying the sample using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Labelling efficiency was determined by measuring absorbency with a Nanodrop ND-1000 Spectrophotometer at 260 nm to determine cDNA concentration, and either 550 nm or 650 nm to determine Cy3 and Cy5 concentrations, respectively. The following calculations were performed for each sample to assess the quality of the labelled product: pmol nucleotides = [(OD260*volume*37 ng/µl*1000 pg/ng)/324.5 pg/pmol], pmol Cy3 = [OD_{550}*volume)/0.15] or pmol Cy5 = [(OD_{650}*volume)/0.25], and nucleotides/dye ratio = [pmol DNA/pmol Cy dye]. Only cDNA samples with dye incorporation of greater than 100 pmol and a nucleotides/dye molecule ratio of less than 50 were considered for hybridisation reactions. Probes were prepared by combining selected test and reference samples, which were then dried in a vacuum desiccator, and resuspended in 45 µl hybridisation buffer [50% formamide, 25% hybridisation buffer (Amersham Biosciences), 25% deionised water]. Probes were denatured at 92ºC for 5 min.

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. Thereafter, the slide was placed in a HybUP hybridisation chamber (NB Engineering, Pretoria, South Africa) with the denatured probe solution 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 by centrifugation (2000Xg for 2 min), and scanned with a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA, USA). Separate images were acquired for each fluorophore at a resolution of 10 µm per pixel.

Microarray data analysis

Scanned images (tiff images) were imported into GenePix Pro 5.0 (Axon Instruments), and spot intensities from scanned slides were quantified. Grids were predefined and manually adjusted to ensure optimal spot recognition, and spots with dust or locally high background were flagged as bad. Intensity data for individual slides were imported into `limmaGUI` (linear models for microarray data Graphical User Interface) (Smyth, 2004) in the R computing environment. Data from each microarray slide was normalised using the global lowess algorithm. Results from biological and slide replicates within each of the time points was collated, and linear models were computed to contrast gene expression between time points. Expression data values for each time point were then imported into Microsoft Excel and filtered by expression values to eliminate genes with mean fold changes of less than twofold up or down, and further filtered by confidence (one sample Student's t test P-value, using FDR multiple testing correction), to retain only genes in which expression changes of SNP treated versus untreated control (time = 0 h) were significant at $P \leq 0.05$. The resulting data were visualised and further explored using TIGR MeV (Saeed et al., 2003). Hierachical clustering was performed using average linkage clustering and Euclidean distance measures. For K-means clustering, 50 iterations were performed, and the smallest number of meaningful clusters was determined empirically viz. K-

means was repeated a number of times, starting with a large number clusters, until the profiles were stable.

DNA sequencing and data analysis

Nucleotide sequencing of selected cDNA clones was performed by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) using T7 or Sp6 primers. Each sequence was edited to correct sequencing ambiguities and remove the plasmid and SSH adaptor sequences. cDNA fragments were analysed to ensure they contained no stop codons and represented fragments of open reading frames. cDNA identities were determined by sequence comparison with the GenBank database using the BlastX, BlastN and dbEST algorithms (Altschul et al., 1990).

Reverse transcriptase quantitative PCR (qRT-PCR)

Gene specific primers were synthesised for an endogenous control (18S rRNA) and 4 selected probe sets [elongation factor 1 alpha (EF1α), manganese superoxide dismutase (Mn SOD), β-glucosidase (β-gluc), chlorophyll a/b binding protein (CAB)], and qPCR was performed to verify the microarray results. Optimal primer design was performed using Primer3 software (http://frodo.wi.mit.edu), and internal primer secondary structure (hairpins, dimers, palindromes, repeats) was assessed using Net Primer software (www.PremierBioSoft.com). Primers used in qRT-PCR confirmation of microarray data are shown in Table 3.1.

Table 3.1. Primers used in qRT-PCR confirmation of microarray data

Target gene	Forward primer	Reverse primer	Product size(bp)
18S rRNA	GCCATCGCTCTGGATACATT	TCATTACTCCGATCCCGAAG	83
EF1α	CTCTTGGTCGCTTTGCTGTT	ACCAGTGGGTCCTTCTTCT	86
Mn SOD	TGTTCTGGTGCAACTCTGCT	ATTGCGGAGGACTGAATCAC	71
β-gluc	AGCTGCAAGGATGAACGACT	ATCGGTGAAGGATGGTAGCC	112
CAB	CACACACTCTCTCTCGCCTCT	CAAAGGAGCCCACCTTGAT	94

DNA free total RNA from each of the SNP treated samples (0, 1, 3, 6 hpt) was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit

(Roche Diagnostics). Each 20 µl reaction contained 1 µg total RNA, 60 µM random hexamer primer, 1 X Transcriptor RT reaction buffer, 20 U Protector RNase Inhibitor, 1 mM dNTP mix, 10 U Transcriptor reverse transcriptase. Reactions were incubated at 55ºC for 30 min, after which the Transcriptor reverse transcriptase was inactivated by heating to 85ºC for 5 min. Reactions were stored at -20ºC until use in real time PCR reactions.

Conditions for all PCR reactions were optimised in a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA, USA) with regard to forward and reverse primer concentrations, $MgCl_2$ and dNTP concentration (Roche Diagnostics), and annealing temperature. Optimised results were applied to the LightCycler PCR protocol. Real time PCR reactions were prepared using a LightCycler® FastStart DNA Master^{PLUS} SYBR Green 1 kit (Roche Diagnostics). Each 20 µl reaction contained the following components prepared to the indicated final concentration: 10 µl water, 2 µl forward primer (0.5 µM), 2 µl reverse primer (0.5 µM), 4 µl 5 X LightCycler Master Mix and 2 µl cDNA (1:20 dilution). Reactions were added to glass capillaries and placed into the LightCycler rotor (Roche Diagnostics). The following LightCycler experimental run protocol was used: denaturation cycle (95ºC for 10 min), amplification and quantification cycle repeated 45 times (95ºC for 10 s, 58ºC for 10s, 72ºC for 6 s with a single fluorescence measurement), melting curve cycle (65-95ºC with a heating rate of 0.1ºC per second and continuous fluorescence measurement), and finally a cooling step to 40ºC. LightCycler software 3.3 (Roche Diagnostics) was employed to calculate crossing points (C_T) for each transcript. C_T is defined as the point at which the fluorescence rises appreciably above the background fluorescence (Pfaffl, 2001).

3.4 RESULTS AND DISCUSSION

Experimental design

A common reference design was employed to examine changes in transcription over time in response to treatment with a NO donor (Figure 3.1). This design uses an aliquot of a common reference RNA, and the intensity of hybridisation of a test RNA is compared to hybridisation of the reference RNA at the same spot (Naidoo et al., 2005). The reference sample was prepared by pooling equal amounts of RNA from test samples. In this manner, every sample present in the test sample is present in the reference sample, and so the relative amounts of each RNA species will be comparable. In order to minimise experimental variability and ensure accurate representation of changes in mRNA abundance, both biological and technical replicates were employed for all time points (Figure 3.1). Two biological replicates were collected from each of two independent SNP treatment experiments. At each time point, nine seedlings were harvested from each biological replicate for RNA extraction and production of cDNA, microarray probes, and quantitative reverse transcription (RT)-PCR (qRT-PCR). Technical replication was twofold – firstly, cDNAs were spotted in duplicate on every slide, and secondly, RNA preparations from each of the time point comparisons were hybridised in duplicate against the common reference control RNA sample in at least one of the biological replicates. Test samples were labelled with Cy3 and the reference sample with Cy5 in each hybridisation reaction. Dye swap experiments were not performed, as in a reference design, it is assumed that any remaining dye bias not removed by normalisation affects all the arrays similarly, and does not bias comparison between samples (Naidoo et al., 2005). Three microarray slides were hybridised for each of the time point comparisons following SNP treatment, and a total of 12 slides were included in `limma` data analyses to identify significantly regulated genes. Data was further filtered for significance in Microsoft Excel to retain genes passing the twofold response cut off at $P \leq 0.05$.

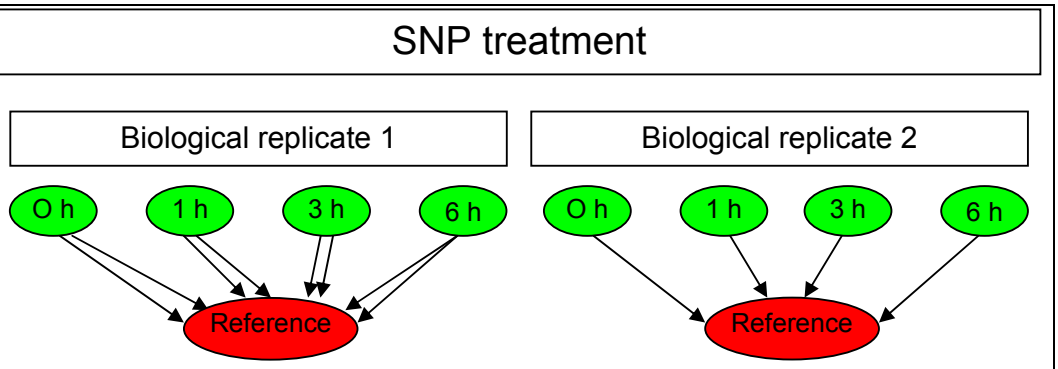

Figure 3.1. Diagrammatic representation of experimental design and replication of pearl millet SNP treatment microarray experiment. A reference RNA sample was prepared by pooling 50 µg RNA from each time point from each biological replicate. The head of the arrow indicates that the sample was labelled with Cy5 (shown in red), while the tail represents a sample that was labelled with Cy3 (shown in green). Each arrow represents a single hybridisation experiment.

Microarray analysis of NO-elicited gene expression

In order to understand the diverse nature of NO signalling, recent studies have focussed on large scale gene expression profiling using cDNA-AFLP (Polverari et al., 2003), oligonucleotide microarrays (Parani et al., 2004) or cDNA microarrays (Huang et al., 2002). Although these studies have identified numerous genes which had previously not been implicated in NO signalling, they all examined transcriptional changes in Arabidopsis in response to NO treatment. The presented study therefore focussed on identifying NO responsive genes in the non-model crop pearl millet. We examined changes that occur in transcript abundance corresponding to 1960 pearl millet cDNAs with a biased representation of defence responsive genes. Following treatment with 1 mM SNP, 45 cDNAs showed a significant change in gene expression (two-fold change, $p \leq 0.05$) at at least one time point, when compared to the untreated sample (time 0 h). Hierarchical clustering was performed to identify groups of cDNAs with similar expression patterns in SNP treated pearl millet plants (Figure 3.2). This revealed five biologically meaningful expression profiles, which were further confirmed by K-means clustering (Figure 3.3). In total, 24 cDNAs were up regulated in response to SNP treatment, and 21 cDNAs were down regulated. Expression profiles (Figure 3.3) revealed that 21 cDNAs were up regulated over a 6 hour period (Profile 1), and three cDNAs displayed a sharp increase in expression one

hour after treatment, but expression levels returned to basal levels 3 h post treatment (Profile 2). Eight cDNAs showed similar expression to the untreated sample at 1 h and 3 h post treatment, but exhibited significant down regulation 6 h post treatment (Profile 3), while Profile 4 contains cDNAs which show a significant decrease in expression 3 h and 6 h post treatment. Profile 5 represents transcripts which steadily decline in levels 1h and 3 h post treatment, but start to recover at 6 h post treatment.

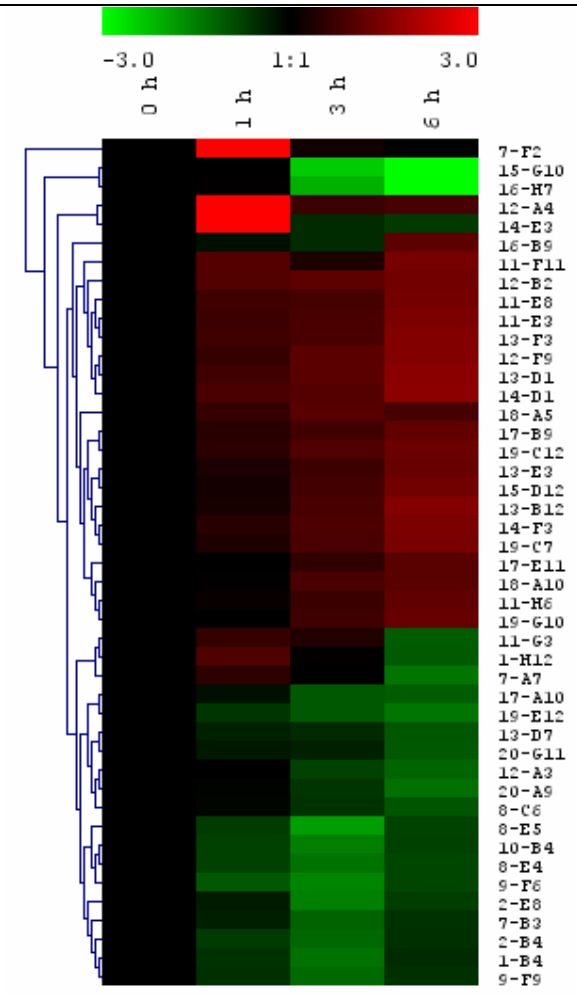

Figure 3.2. Hierarchical cluster of 45 cDNAs with two-fold or more changes in transcript abundance for at least one time point in response to SNP treatment. Each cDNA is represented by a single row of coloured boxes, and each column represents a time point. Induction (or repression) ranges from pale to saturated red (or green) with a $\log_2$ fold change scale bar shown above the cluster.

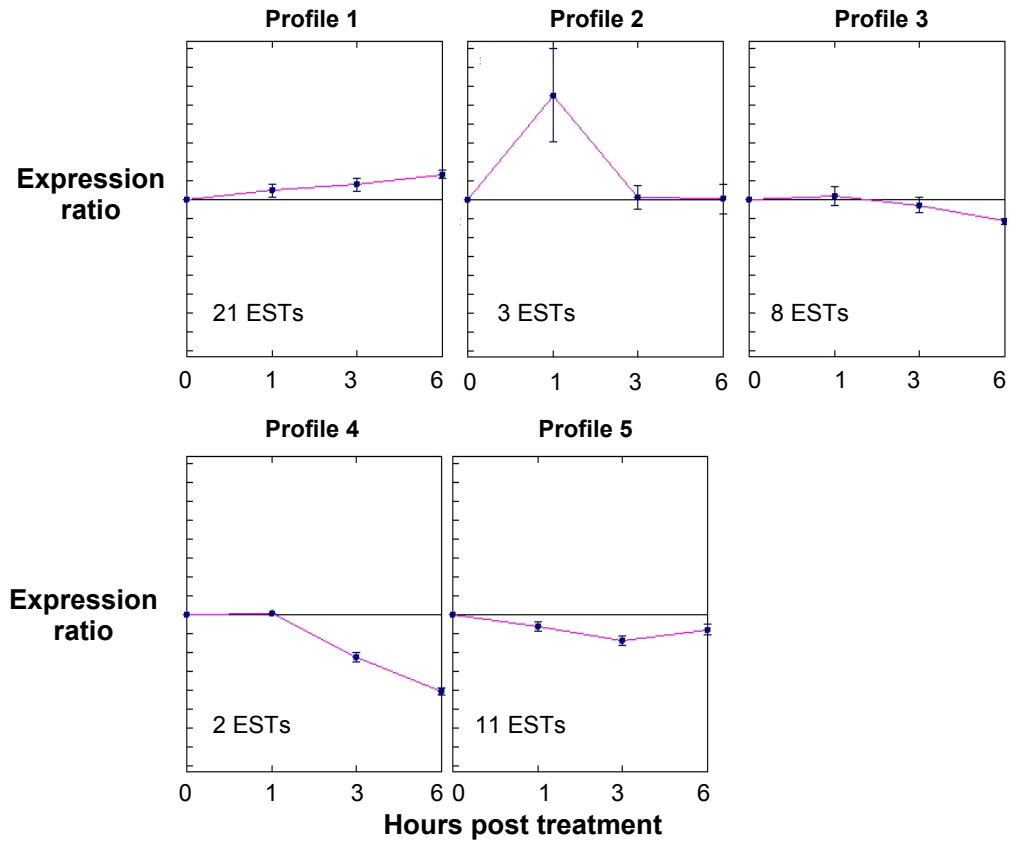

Figure 3.3. Expression profiling of pearl millet cDNAs differentially expressed in response to SNP treatment. Expression ratios were calculated for each time point after treatment relative to time 0 (untreated sample). Error bars indicate standard deviations between transcript expression levels within each profile.

A selection of cDNAs in each cluster was analysed by DNA sequencing to reveal function, and to give an indication as to the functional categories of cDNAs represented by each profile (Table 3.2). Sequence analysis revealed a number of redundant cDNAs within each profile. This serves as additional confirmation that genes represented in a profile truly exhibit expression patterns deduced from cDNA microarray analysis. However, none of the clones exhibiting no significant homology to sequences in the Genbank were redundant. In total, sequence analysis identified 14 unique gene clusters that were NO responsive.

Table 3.2. Selected genes from profiles 1 to 5 that showed differential expression in SNP treated pearl millet seedlings. "No significant similarity" indicates that there was no homology to sequences in the BlastX database.

Profile clone ID	Expression profile	Putative protein name	Blast accession number	Putative function
Profile 1	Up regulated			
11-F11		No significant similarity		
12-F9		Chlorophyll a/b binding protein	P12329	Photosynthesis
13-B12		No significant similarity		
13-F3		Chlorophyll a/b binding protein	NP917525	Photosynthesis
14-F3		β-glucosidase	AAK07429	Defence
18-A5		Chlorophyll a/b binding protein	BAD61582	Photosynthesis
19-C7		Chlorophyll a/b binding protein	BAD61582	Photosynthesis
Profile 2	Up regulated			
7-F2		No significant similarity		
12-A4		No significant similarity		
Profile 3	Down regulated			
1-H12		Phosphoenylpyruvate carboxylase	AAM15963	Photosynthesis
7-A7		No significant similarity		
12-A3		Pyruvate dehydrogenase kinase	NP909820	Respiration
Profile 4	Down regulated			
15-G10		Manganese superoxide dismutase	CAD42944	ROS scavenger
16-H7		Manganese superoxide dismutase	CAD42944	ROS scavenger
Profile 5	Down regulated			
8-E5		Elongation factor 1α	AAF42980	Protein synthesis
10-B4		Elongation factor 1α	AAF42980	Protein synthesis
17-A10		Actin	AAW34192	Cell structure
19-E12		Actin	AAX09593	Cell structure

Profile 1 is typified by mainly chlorophyll a/b binding protein genes, which suggest crosstalk between defence and phytochrome signalling pathways. Chlorophyll a/b binding proteins have been previously shown to be up regulated in defence response signalling (Schenk et al., 2000). The Arabidopsis phytochrome signalling mutant (*psi2*) (which is characterised by a hyperactive phytochrome signalling pathway) exhibits elevated levels of PR1 gene expression (Genoud et al., 2002). Furthermore, pathogen (*Pseudomonas syringae* pv. *tomato*) growth was found to be elevated in Arabidopsis mutants (*phyA* and *phyB*) affected in light perception, but was

clearly reduced in the *psi2* mutant hypersensitive to light (Genoud et al., 2002). These authors also showed that the formation of HR is strongly reduced in the absence of phytochrome signalling and is amplified in the *psi2* mutant. These results clearly suggest that light signal transduction and pathogenesis related gene signalling pathways are connected. Three different genes coding for chlorophyll a/b binding proteins are present in this profile (homology to BlastX accession numbers P12329, NP917525 and BAD61582). Profile 1 also contains a β-glucosidase gene, which is up regulated over a six hour period in response to SNP treatment. The cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) are secondary metabolites that occur in high abundance as glucosides in the cereals maize, wheat and rye. Upon pathogen attack, the hydroxamic acid glucoside is cleaved by β-glucosidase to release a toxic aglucone that is unstable and decomposes to the reactive benzoxaxolinones, which are toxic to invading pathogens (Nikus et al., 2001).

Two cDNAs (7-F2 and 12-A4) were sequenced from Profile 2, both of which were found to have no significant homology to genes in the GenBank. However, sequence alignments indicated that the two sequences did not display any homology, and represented two different pearl millet transcripts.

Eight cDNAs are represented by Profile 3. Sequence analysis of three cDNAs revealed a phosphoenolpyruvate carboxylase, a pyruvate dehydrogenase kinase, and a cDNA with no significant homology to sequences in the GenBank. The identity of these cDNAs suggests that this profile is representative of genes coding for enzymes involved in basic cell metabolism. Phosphoenolpyruvate carboxylase plays an important role in photosynthesis, and catalyses the fixation of CO_2 to yield oxaloacetate. In the leaves of Crassulacean Acid Metabolism (CAM) and C_4 plants (pearl millet is a C_4 plant), it catalyses the primary fixation step in atmospheric CO_2 assimilation, while in C_3 leaves and non-photosynthetic tissue it replenishes TCA cycle intermediates and allows respiration to continue (Hartwell et al., 1999). In response to pathogen attack, as mimicked by the application of SNP in these

experiments, the cell obviously down regulates its basic functions such as photosynthesis (and thus, phosphoenolpyruvate carboxylase), and puts more energy into defence responses to fight off invading pathogens. A gene coding for an enzyme involved in regulating respiration, pyruvate dehydrogenase kinase (PDK), was also down regulated in response to SNP treatment. The pyruvate dehydrogenase complex (PDC) is a multienzyme structure that catalyses the oxidative decarboxylation of pyruvate, yielding CO_2, acetyl-CoA and NADH (Thelen et al., 2000). PDK inactivates mitochondrial PDC by phosphorylating specific Ser residues, and is the primary regulator of flux through the mitochondrial PDC. Down regulation of PDK by plant cells would lead to activation of the PDC and thus an increase in respiration, which may provide the necessary energy for activation of plant defence responses (as elicited by SNP application) to counteract pathogen attack.

Two transcripts are representative of Profile 4, both of which display homology to a manganese superoxide dismutase gene. During the HR, superoxide dismutase (SOD) accelerates O_2^- dismutation to H_2O_2 to minimise the loss of NO by reaction with O_2^-, and to trigger hypersensitive cell death through NO/ H_2O_2 cooperation (Delledonne et al., 2001). However, when the NO/O_2^- balance is in favour of NO, there is no O_2^- left for SOD mediated dismutation to H_2O_2. Thus, treatment of pearl millet plants with the NO donor, SNP, should have yielded high levels of NO that would have reacted with O_2^- to produce ONOO-, and limited SOD dismutation to H_2O_2. This presumably resulted in decreased expression of SOD genes as observed in profile 4. Similar results were obtained in oat plants, which exhibited a significant decrease in H_2O_2 accumulation when treated with the NO donor S-nitroso-*N*-acetylpenicillamine (SNAP). Conversely, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) markedly enhanced H_2O_2 accumulation (Tada et al., 2004).

Profile 5 contains transcripts with homology to genes involved in cell housekeeping type functions. Clones 8-E5 and 10-B4, which are redundant, exhibit homology to elongation factor 1α. The alpha subunit of translation elongation factor 1, EF1α, is the most abundant component in the

translational machinery and plays a central role in polypeptide chain elongation in eukaryotes. It catalyses the binding reaction of aminoacyl t-RNAs to the acceptor site (A site) on the ribosome. However, it has been shown that EF1α is a multifunctional protein and is involved in other cellular processes such as binding actin, acting as a microtubule severing protein, binding calmodulin, and participating in the degradation of certain proteins via the ubiquitin pathway (Cao et al., 1997). Many of these basic cellular processes would be down regulated during pathogen attack, which would lead to a decrease in EF1α gene expression. Profile 5 also contains two down regulated cDNAs with homology to actin. These two clones are not redundant and exhibit homology to different actin genes in the Genbank. Actin filaments play an important role in cell elongation, and growing evidence shows that the actin cytoskeleton is a key effector of signal transduction, which controls and maintains the shape of plant cells, as well as playing roles in plant morphogenesis (Vantard and Blanchoin, 2002). Studies in rat cells have shown that disruption of the actin cytoskeleton lead to an increase in NOS protein expression, and thus NO formation (Zeng and Morrison, 2001). Perhaps a similar mechanism exists in plants, and increased levels of endogenous NO would result in decreased actin synthesis, and disruption of the actin cytoskeleton.

Verification of gene expression changes

For verification of the differential expression of genes obtained from cDNA microarray analysis, qRT-PCR was performed for selected genes. These included two up regulated (chlorophyll a/b binding protein and β-glucosidase) and two down regulated (manganese superoxide dismutase and elongation factor 1 alpha) genes, and 18S rRNA served as an endogenous control gene. The purpose of the endogenous control gene is to normalise qRT-PCR data for the amount of RNA added to each of the reverse transcription reactions (Pfaffl, 2001).

Standard curves were calculated for each of the five genes subjected to qRT-PCR (Figure 3.4). Standard curves were determined by making dilutions of cDNA prepared from plants 3 h after SNP treatment, and subjecting these

dilutions to real time PCR using the primer sets for the different genes. Crossing points (C_T) were plotted against log ng, and a linear regression curve was fitted. Slope of the regression curve (m value) and the y-intercept value (c) were used to calculate the amount of ng of a particular transcript present in test samples. m values for each of the regression curves representative of the different genes tested were similar, and ranged between 3.2 and 3.7 (Figure 3.4). This is indicative that the efficiency of the qRT-PCR reactions for each of the genes tested was similar. However, y-intercept (c) values differed widely, and varied from approximately 5.2 for the 18S rRNA gene product to 22.2 for the manganese superoxide dismutase gene product. c values give an indication as to how abundant a transcript is in a sample. High c values, such as that obtained for 18S rRNA, suggest that 18S rRNA is abundant in the sample. qRT-PCR products for each of the five genes tested were visualised on an agarose gel to ensure that single transcript products were obtained, and to verify LightCycler melting curve analyses that indicated that qRT-PCR reactions were free of primer dimers (Figure 3.5).

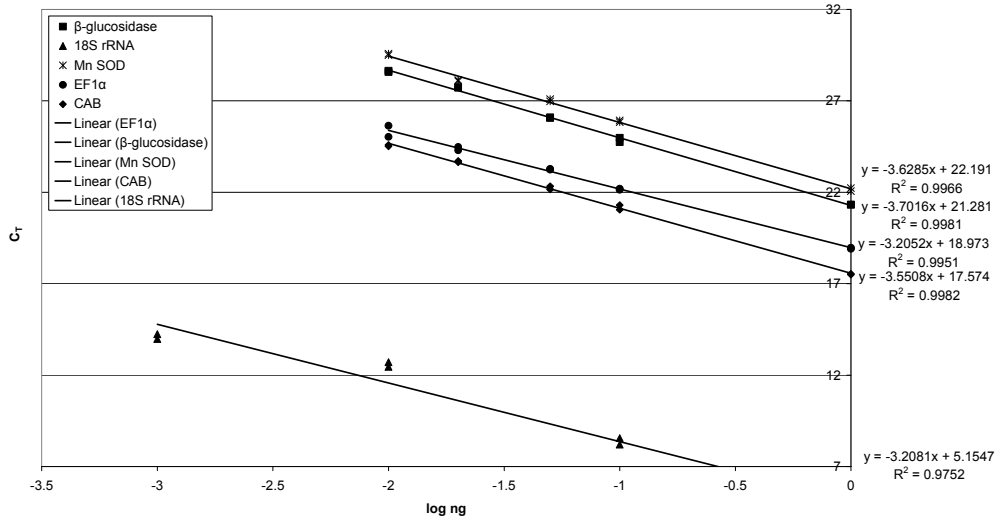

Figure 3.4. Standard concentration curves for each of the five transcripts subjected to real time PCR. Genes subjected to transcript profiling using qRT-PCR include β-glucosidase, manganese superoxide dismutase (Mn SOD), elongation factor 1 α (EF1α) and chlorophyll a/b binding protein (CAB). 18S rRNA served as an endogenous control.

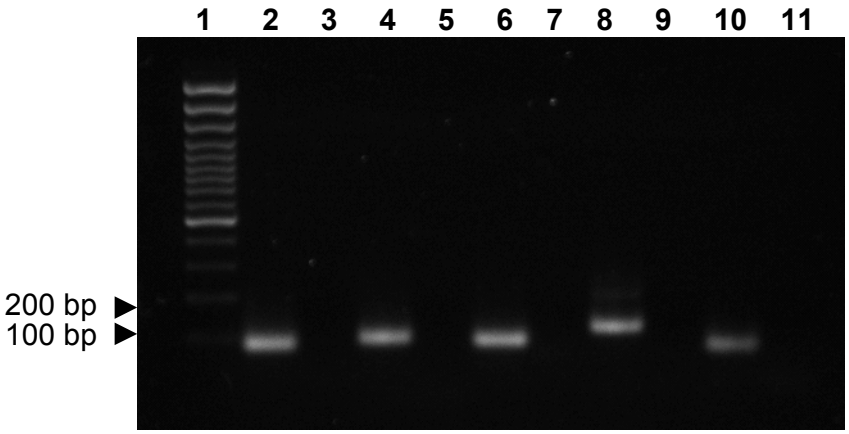

Figure 3.5. Agarose gel electrophoresis of qRT-PCR products responsive to SNP treatment. Lane 1, 100 bp ladder (Fermentas); lane 2-11, 18S rRNA transcript (lane 2); 18S rRNA water control (no cDNA added to the reaction) (lane 3); chlorophyll a/b binding protein transcript (lane 4); chlorophyll a/b binding protein water control (lane 5); EF1α transcript (lane 6); EF1α water control (lane 7); β-glucosidase transcript (lane 8); β-glucosidase water control (lane 9); Mn superoxide dismutase transcript (lane 10); Mn superoxide dismutase water control (lane 11).

qRT-PCR was applied to confirm microarray data for four genes exhibiting differential gene expression over time following treatment of pearl millet with SNP. Changes in gene expression at the different time points post SNP treatment were calculated relative to the 0 h timepoint (no treatment). The changes in expression levels observed using qRT-PCR were similar to or greater than the levels obtained by microarray analysis (Figure 3.6). Discrepancies in gene expression levels between the two methods have been well documented (Dowd et al., 2004; Parani et al., 2004; Salzman et al., 2005), and are often attributed to cross hybridisation of gene family members on cDNA microarrays, differences in hybridisation on surfaces versus solution hybridisation, and/or better quantification of low abundance transcripts by qRT-PCR. However, expression pattern trends observed over time with qRT-PCR data were similar to those obtained from microarray data (Figure 3.6). For example, although chlorophyll a/b binding protein transcript levels were much greater in qRT-PCR experiments, both qRT-PCR and microarray data indicated that this transcript steadily increased over a six hour period in

response to SNP treatment. Only the EF1α transcript exhibited discrepancies between the two qRT-PCR biological replicates. Although qRT-PCR1 showed similar gene expression pattern over time to the microarray expression data, qRT-PCR2 data suggested that this gene was up regulated after 6 hours (Figure 3.6). We can therefore not conclusively state that this gene is down regulated in response to NO treatment.

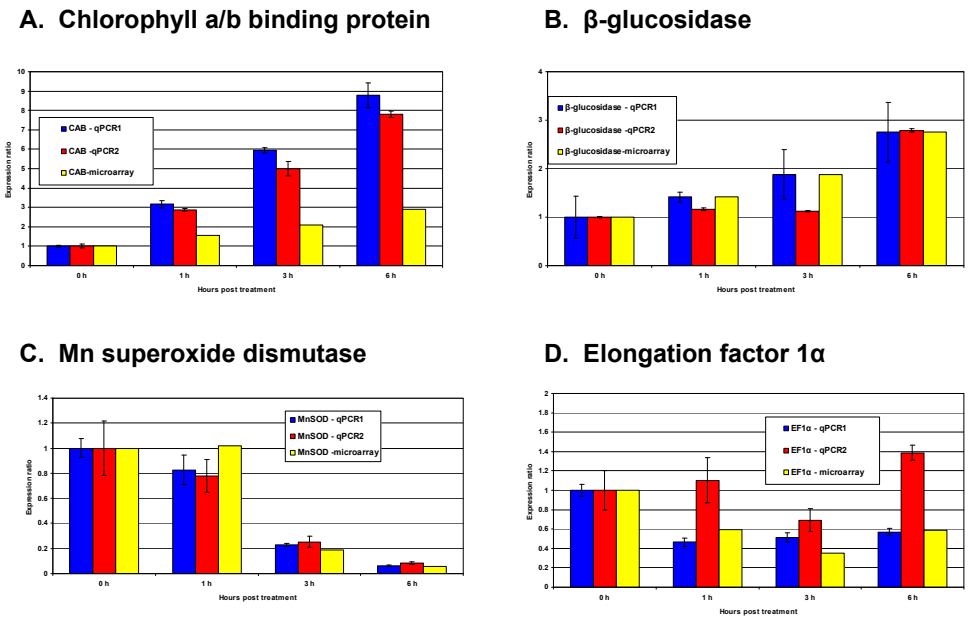

Figure 3.6. Comparison of gene expression data from qRT-PCR and microarray hybridisation experiments. Changes in gene expression at the different time points post SNP treatment were calculated as the fold change relative to the untreated control (0 h treatment).

Comparison of NO mediated transcriptional changes in pearl millet and Arabidopsis

Three recent studies examined NO induced transcriptional changes in the model plant Arabidopsis (Huang et al., 2002; Polverari et al., 2003; Parani et al., 2004). Two studies undertook microarray profiling experiments, and one performed cDNA-AFLP to examine gene expression changes in response to NO donor treatment. All three reports noted that there was a distinct increase in transcripts coding for proteins involved in plant defence response, cellular detoxification and transcription. Huang and coworkers (2002) observed

transcriptional changes in 39 genes out of a total of 250 cDNA fragments (sequenced and non-redundant) tested, Parani et al. (2004) detected changes in a total of 422 genes on a whole genome ATH1 microarray (24 000 genes), and Polverari et al. (2003) noted changes in 120 out of 2500 cDNA transcripts examined. Comparison of NO responsive genes in pearl millet and Arabidopsis showed that there was very little overlap in gene expression profiles. In fact, genes such as β-glucosidase, phosphoenylpyruvate carboxylase, pyruvate dehydrogenase kinase, manganese superoxide dismutase, elongation factor 1α and actin, identified in pearl millet as being NO responsive, have not previously been shown to be involved in NO signalling. In total, 45 out of 1920 pearl millet defence related transcripts exhibited a significant change in gene expression in response to NO treatment. Eighteen of the 45 transcripts have been sequenced, but further sequence analysis could reveal transcripts with homology to those represented in the Arabidopsis NO responsive transcript studies. However, sequence analysis of pearl millet transcripts within the profiles represented in Figure 3.3 suggests that there is a fair amount of redundancy (Table 3.2). Alternatively, observed differences in pearl millet's and Arabidopsis' response to NO could be due to the fact that the pearl millet cDNA library was constructed from time points post elicitor treatment (5, 14, 20 h) that were later than time points examined following SNP treatment (1, 3, 6 h post treatment). Therefore the pearl millet cDNA library does not potentially contain a wide array of early responsive defence transcripts. On the other hand, pearl millet plants, and perhaps cereal plants in general, do not respond in the same manner to NO donor treatment as Arabidopsis plants do.

In conclusion, NO plays an important role in plant defence response to invading pathogens. The current study clearly shows that pearl millet responds to treatment with a nitric oxide donor, and alters the expression profiles of a number of transcripts. Comparison of pearl millet NO responsive genes with *Arabidopsis thaliana* NO responsive genes revealed very little overlap. Most of the genes exhibiting significant differential expression in pearl millet have not been previously implicated in NO signalling in plants.

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Chapter 4

Evaluation of pearl millet defence signalling pathways involved in leaf rust (*Puccinia substriata)* resistance and perception

4.1 ABSTRACT

Studies were undertaken to assess induction of defence response pathways in pearl millet in response to infection with the leaf rust fungus *Puccinia substriata*. Pathology studies indicated that pretreatment of pearl millet with salicylic acid (SA) conferred resistance to a virulent isolate of the rust fungus, whereas methyl jasmonate (MeJA) did not significantly reduce infection levels. These results imply that the salicylate defence pathway is induced in response to an incompatible rust infection. However, large scale gene expression profiling of pearl millet in response to treatment with MeJA, SA and a virulent rust isolate showed that the compatible rust infection increased transcript abundance of a number of genes that were common to jasmonate and salicylate defence signalling, as well as transcripts that were unique to rust infection. These results suggest that the plant adopts elements from a number of defence signalling pathways in an attempt to ward off infection by the compatible biotrophic rust fungus. However, in view of results obtained from pearl millet defence chemical treatments, it is probably genes that are significantly induced in response to SA, but to a lesser extent by MeJA that actually confer resistance to an avirulent rust isolate. Gene expression analysis also revealed substantial overlap in gene expression responses between the treatments, with MeJA and SA treatments exhibiting the largest number of coinduced transcripts (67). DNA sequence analyses of 135 cDNAs displaying two-fold or more changes in gene expression in at least one of the treatments yielded 66 unigenes (51% redundancy) that encoded proteins functioning in direct defence, oxidative burst, abiotic stress, basic/secondary metabolism, protein synthesis and cell signalling, and photosynthesis, as well as proteins of unknown function.

4.2 INTRODUCTION

Plants respond to invasion by pathogens by activating a complex set of transcriptional and biochemical changes, which induce the production of reactive oxygen species and the development of the hypersensitive response (HR), fortification of cell walls by the cross-linking of cell wall proteins, biosynthesis of phytoalexins, and the accumulation of anti-microbial proteins. The signal transduction network controlling defence activation is comprised of several interacting pathways. Three signalling molecules, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are known to play key roles in various aspects of plant defence signal transduction. These signalling molecules are involved in what appears to be two major defence signalling pathways: a SA dependent pathway and a SA-independent pathway that involves JA and ET (Kunkel and Brooks, 2002).

Evidence suggests that defence signalling pathways do not function independently, but rather influence each other through a complex network of regulatory mechanisms (Kunkel and Brooks, 2002). However, the analysis of signalling processes and their interactions in plants have traditionally been reductionist in approach and have focussed on only one or a few genes at any one time (Kunkel and Brooks, 2002). From such studies it has not been possible to assess the extent of overlap of gene activation by different signals and pathogens in defence response. However, a number of recent studies have applied DNA microarray global gene expression profiling, which enables expression analysis of thousands of genes in parallel, to improve our understanding of the molecular basis of plant defence response mechanisms (Schenk et al., 2000; Maleck et al., 2000; Glazebrook et al., 2003; Zhu-Salzman et al., 2004; Salzman et al., 2005). Results from these studies indicate the existence of a substantial network of regulatory interactions and coordination during plant defence signalling pathways, notably between the salicylate and jasmonate pathways that were previously thought to act in an antagonistic fashion.

To date, most genome scale studies of gene expression in response signalling molecules have been performed in the model plant *Arabidopis*

thaliana. Recently two studies were published on gene expression profiling in response to defence signalling molecules in the C4 monocot sorghum (*Sorghum bicolor*) (Zhu-Salzman et al., 2004; Salzman et al., 2005). In the present study, we further improved our understanding of defence signalling pathways in C4 monocotyledonous crop plants by examining the transcriptional response of pearl millet [*Pennisetum glaucum* (L.) R. Br] at different time points after inoculation with the compatible rust fungus *Puccinia substriata* Ellis & Barth. var *indica* Ramachar & Cummins, and contrasting this regulation with JA- and SA-regulated gene expression. Investigations were made to determine whether MeJA and SA signalling is antagonistic as has previously been reported for Arabidopsis (Kunkel and Brooks, 2002), and whether infection with a biotrophic rust fungus elicits gene expression more similar to the SA than JA signalling pathway.

4.3 MATERIALS AND METHODS

Treatment of pearl millet plants

Pearl millet seed (line ICML12=P7) 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. After one week (once the seeds had germinated) seedlings were transferred to seedling trays containing sterilised vermiculite that had been fertilised with Hoagland's solution (Hoagland and Arnon, 1950). Plants were grown for a further six weeks under 16 h light (140 umol/m^2/s) and 8 h dark cycles at a constant temperature of 25ºC and 85% relative humidity.

P. substriata var. *indica* cultures, isolated from infected pearl millet plants grown in KwaZulu-Natal, South Africa, were maintained on pearl millet ICML12=P7 plants. For inoculations, 5 mm leaf segments containing uredospores were gently pressed onto the adaxial surface of 2 week old pearl millet (ICML12=P7) seedlings that had been misted by spraying sterile double

distilled water from an "atomiser" spray bottle. Seedlings were incubated in the dark for two days, and were thereafter maintained under 16 h light (140 umol/m^2/s) and 8 h dark cycles at a constant temperature of 25ºC and 85% relative humidity. Rust infected pearl millet leaf material was harvested 0, 20, 120 and 192 h post inoculation. For SA and methyl jasmonate (MeJA) treatments, seven week old plants were sprayed until runoff with either 5 mM sodium salicylate (Sigma, Aston Manor, South Africa), prepared in 0.1% Tween20 (Sigma), or 500 µM MeJA (Sigma) in 0.1% ethanol, 0.1% Tween20, respectively. Both SA and MeJA treated leaf tissue was harvested 0, 12, 24 and 48 hours post treatment (Schenk et al., 2000). Plants were immediately frozen in liquid nitrogen and stored at -80ºC until RNA purification. All treatments (rust, SA and MeJA) were applied during the light period, and two replications with 9 plants in each were included. Plants within replicates were pooled to reduce variation prior to RNA extraction.

Chemical induction/pathogenicity trials

For chemical induction experiments, three week old ICML12=P7 plants were treated with either water, 5 mM sodium salicylate (Sigma) prepared in 0.1% Tween20, or 500 µM MeJA (Sigma) in 0.1% ethanol containing 0.1% Tween20 until run off. Plants were incubated for 24 h, after which 50 µl of freshly collected *P. substriata* urediniospores were applied to the fourth leaf of each plant. Seedlings were incubated in the dark for two days, and were thereafter maintained under 16 h light (140 umol/m^2/s) and 8 h dark cycles at a constant temperature of 25ºC and 85% relative humidity until rust pustules developed on the leaf surface. Each treatment consisted of two biological replicates each containing 7 plants. Results were analysed in Microsoft Excel using a Student's *t*-test assuming unequal variances.

RNA isolation and purification, Microarray preparation, Fluorescent probe preparation, hybridisation and scanning, Microarray data analysis, and **DNA sequencing and data analysis** were performed as outlined in Chapter 3 (Materials and methods).

Quantitative PCR

Gene specific primers were synthesised for an endogenous control (18S rRNA) and five selected probe sets [ASR2, β-glucosidase (β-gluc), Ca EF hand protein, manganese superoxide dismutase (Mn SOD) and thionin], and used in reverse transcriptase quantitative PCR (qRT-PCR) reactions to verify expression ratios obtained from microarray analysis. Optimal primer design was performed using Primer3 software (http://frodo.wi.mit.edu), and internal primer secondary structure (hairpins, dimers, palindromes, repeats) was assessed using Net Primer software (www.PremierBioSoft.com). Primers used in qRT-PCR confirmation of microarray data are shown in Table 4.1.

Table 4.1. Primers used in qRT-PCR confirmation of microarray data

Target gene	Forward primer	Reverse primer	Product size(bp)
18S rRNA	GCCATCGCTCTGGATACATT	TCATTACTCCGATCCCGAAG	83
ASR2	GCCACAACTGAAGAGACACC	ACGCACACACAAATCGAGAG	111
β-gluc	AGCTGCAAGGATGAACGACT	ATCGGTGAAGGATGGTAGCC	112
Ca EF hand	ATTAGTCCCCATTCCCCTTC	TAACATCCGCAGAGATCGAG	94
Mn SOD	TGTTCTGGTGCAACTCTGCT	ATTGCGGAGGACTGAATCAC	71
thionin	AGGGGTGTCAAGATCAGCAG	GCAGCAACTCTTGCCTTTCT	99

DNA free total RNA from each of the four MeJA, SA and rust post treatment timepoints was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). qRT-PCR reactions were set up and performed as outlined in Chapter 3 (Materials and methods).

4.4 RESULTS AND DISCUSSION

Experimental system: rust infection of pearl millet seedlings

In pearl millet, common rust, caused by *Puccinia substriata*, is a disease that is characterised by the formation of rust pustules on leaves in productively infected plants. 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 the growing regions of South Africa, and is a major constraint to biomass production (de Miliano, personal communication).

A number of publications in the literature detail methods of rust infection of plant seedlings. These include suspending urediniospores in a kerosene solution and spraying seedlings, dewaxing leaves prior to inoculation with uredospores (Beckett et al., 1990), mixing uredospores with talc powder and applying to the leaf surface (Freytag and Mendgen, 1991; Larous and Losel, 1993), and particle bombardment of leaf segments with ureodiniospores (Girgi et al., 2006). However, we sought an application method that would result in minimal damage to the pearl leaf surface, and that would only initiate transcriptional changes in pearl millet in response to rust treatment. For this reason, we applied a rust infection method that was developed in kikuyu grass (Adendorff and Rijkenberg, 2000), in which infected leaf segments containing *P. substriata* urediniospores were gently pressed against the adaxial surface of two week old pearl millet seedlings. The symptoms that are characteristic of rust infection in the field were obtained over an 8 day period in our experimental system (Figure 4.1). Chlorotic lesions developed on leaf surfaces within 5 days or 120 hours postinfection (120 hpi), and fully developed rust symptoms were visible within 8 dpi (192 hpi) (Figure 4.1).

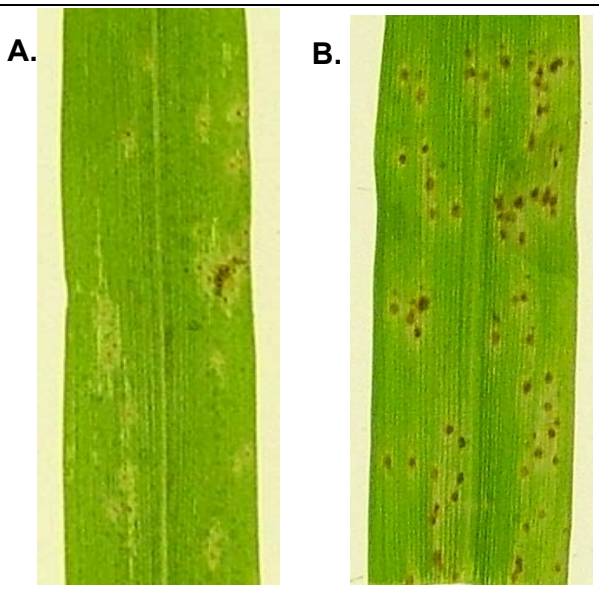

Figure 4.1. Development of rust symptoms on pearl millet leaves. (A) 120 hpi and (B) 192 hpi with *P. substriata.*

Pearl millet breeding line ICML12=P7 was chosen for defence response mechanism studies because of its documented resistance to two major pearl millet diseases, viz. downy mildew caused by the oomycetous fungus *Sclerospora graminicola*, and rust (causal agent: *Puccinia substriata*) (Singh et al., 1990). However, infection assays presented in this study show that line ICML12=P7 exhibits a compatible interaction with common rust, as evidenced by the formation of rust pustules on infected leaves (Figure 4.1). The rust isolate used in this study was collected from infected pearl millet leaves from KwaZulu-Natal, South Africa, so it is possible that ICML12=P7 is not resistant to this isolate of rust, but contains R-gene resistance to rust isolates from India, where it was originally tested for resistance.

Chemically induced disease resistance in pearl millet

Experiments were performed to assess whether treatment of pearl millet with the defence signalling molecules MeJA and SA elicited a defence response that would render pearl millet less susceptible to rust infection. Three week old pearl millet plants were treated with water, MeJA or SA, and the fourth leaf of each pearl millet plant was inoculated 24 h later with freshly collected *P. substriata* urediniospores. Rust pustules began to develop on water and

MeJA treated leaves within 7 days of infection, whereas rust symptoms only became evident on SA treated leaves after nine days. Some of the SA treated leaves only developed chlorotic lesions and did not develop full rust like symptoms. Although SA treated leaves did develop rust pustules, the number of pustules per leaf was far less than those on water and MeJA treated plants (Figures 4.2 and 4.3). It thus appears that application of SA to pearl millet leaves is able to protect the plant against subsequent attack by a compatible rust fungus. Similar results were obtained in maize plants in which application of the SA analogue, benzo(1,2,3)thiadiazole-7-carbothioc acid S-methyl ester (BTH), increased resistance to downy mildew caused by *Peronsclerospora sorghi* (Morris et al., 1998).

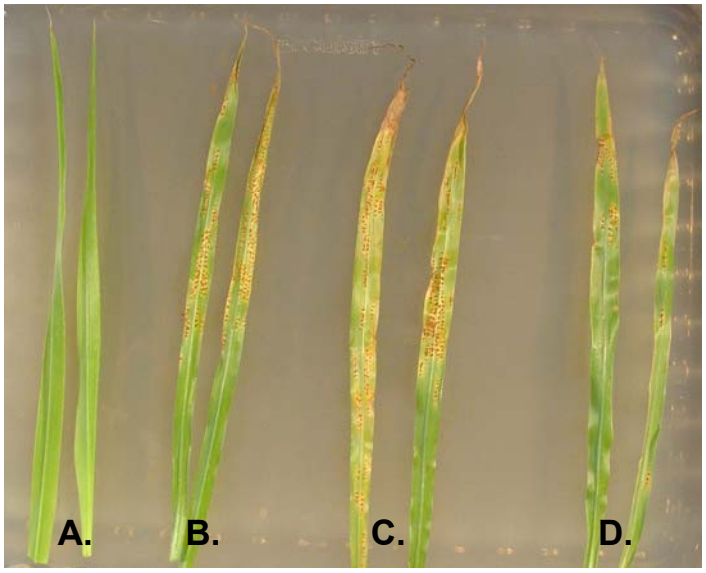

Figure 4.2. Comparison of defence signalling molecule treatments on reducing rust susceptibility in pearl millet. Three week old ICML12=P7 pearl millet plants were sprayed with either water (A&B), MeJA (C) or SA (D), and the fourth leaf of each plant was inoculated with freshly collected *P. substriata* urediniospores 24 h later (B, C, D). Rust pustules developed within 7 days of inoculation on water (B) and MeJA (C) treated plants, and after nine days on SA (D) treated plants.

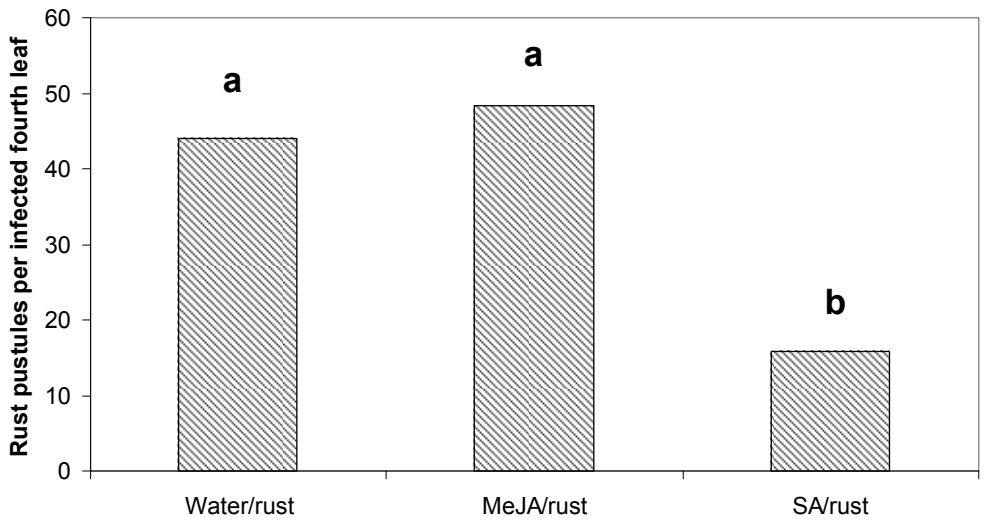

Figure 4.3. Mean number of rust pustules per ICML12=P7 pearl millet leaf 10 days post rust infection. Bars with identical letters are not significantly different from each other (Students *t* test at $p<0.05$).

Microarray experimental design

Gene expression changes over time following rust inoculation or treatment with either SA or MeJA were assessed using a direct-sequential loop design (Kerr and Churchill, 2001; Yang and Speed, 2002) (Figure 4.4). Expression profiles obtained with these designs derive from pair-wise comparisons of adjacent time points, allowing direct comparison of expression differences between time points. Such comparisons can only be made indirectly when designs utilising a common reference are employed, which may make subtle differences from one time point to another difficult to detect (Alba et al., 2004). Equally important, the direct-sequential loop design increases precision for some pair-wise comparisons in the time course, which reduces mean variance for data collected in this way (Alba et al., 2004).

Two sample replicates (each consisting of nine plants) were harvested for each time point (biological replicates), and one of these samples was further analysed with a dye swap (technical replicate) (Figure 4.4). Samples were collected from rust infected leaves at 0, 20, 120 and 192 hpi, whilst samples

were harvested from MeJA and SA treated plants at 0, 12, 24 and 48 h post treatment (Schenk et al., 2000).Further technical replication within microarray slides was employed through cDNA spot duplication. For each spot, the fluorescence intensity of red and green channels was measured. A typical microarray image obtained after hybridisation and scanning is represented in Figure 4.5. Fluorescence data from a total of 12 slides were imported into `limmaGUI` in the R computing environment where data was normalised (within array global lowess normalisation and between array quantile normalisation), and linear models were fitted in order to contrast post treatment expression values with the non-treated sample (time = 0 h). Data was further filtered for significance in Microsoft Excel to retain genes passing the twofold response cut off at P≤0.05.

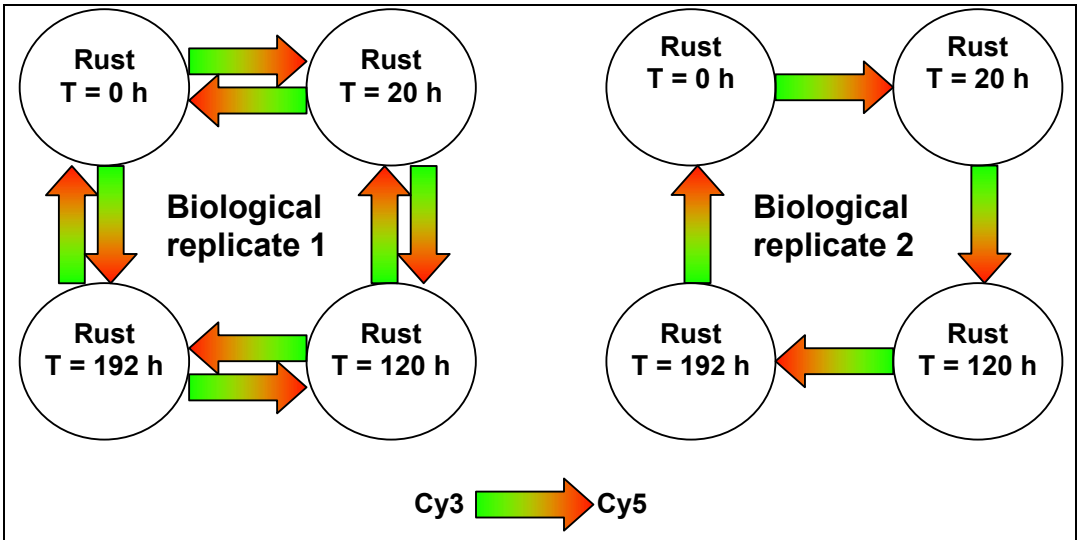

Figure 4.4. Diagrammatic representation of a direct sequential loop design applied to analyse gene expression changes in rust infected pearl millet plants over time. Each circle represents an RNA sample extracted from pearl millet leaves at a specified time post rust infection. The head of the arrow indicates that the sample was labelled with Cy5 (shown in red), while the tail represents a sample that was labelled with Cy3 (shown in green). Each arrow represents a single hybridisation experiment. Similar experimental designs were applied to analyse pearl millet plants that had been treated with either SA or MeJA.

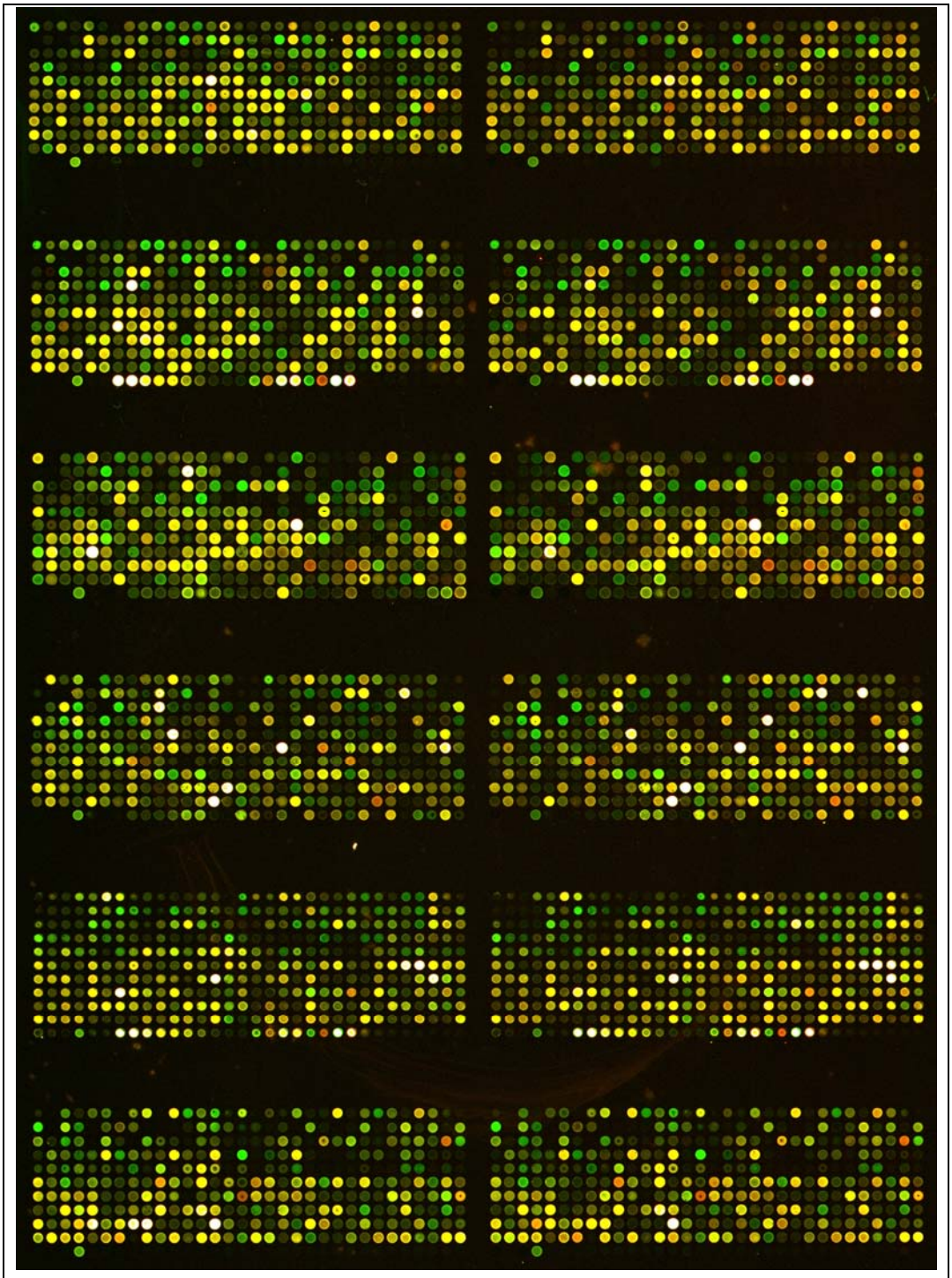

Figure 4.5. Example of a pearl millet microarray image following hybridisation with differentially labelled RNA samples, and scanning with a Genepix™ 4000B scanner (Axon instruments). In this particular example, RNA extracted from pearl millet plants 0 h post MeJA treatment was labelled with Cy5 dye, and RNA isolated from plants 48 h post MeJA treatment was labelled with Cy3 dye.

Overall gene regulation in response to MeJA, SA and rust

Of the 1920 pearl millet cDNAs present on the microarray, a total of 471 were significantly regulated (more than 2 fold induced or repressed; $P<0.05$) in at least one of the twelve conditions (3 treatments each with 4 timepoints) analysed. The largest group of differentially expressed genes were identified in the MeJA treatment, whilst the rust treatment resulted in the least genes being differentially expressed (Figure 4.6). After fungal inoculation, transcript levels of only 5 cDNAs were uniquely increased, whereas those of 26 cDNAs were decreased (Figure 4.6). The transcript abundance of 165 cDNAs for MeJA, and 93 cDNAs for SA was increased as a result of treatment with these signal molecules, respectively. Transcript abundance of 7 and 42 cDNAs were reduced after treatment with MeJA and SA respectively.

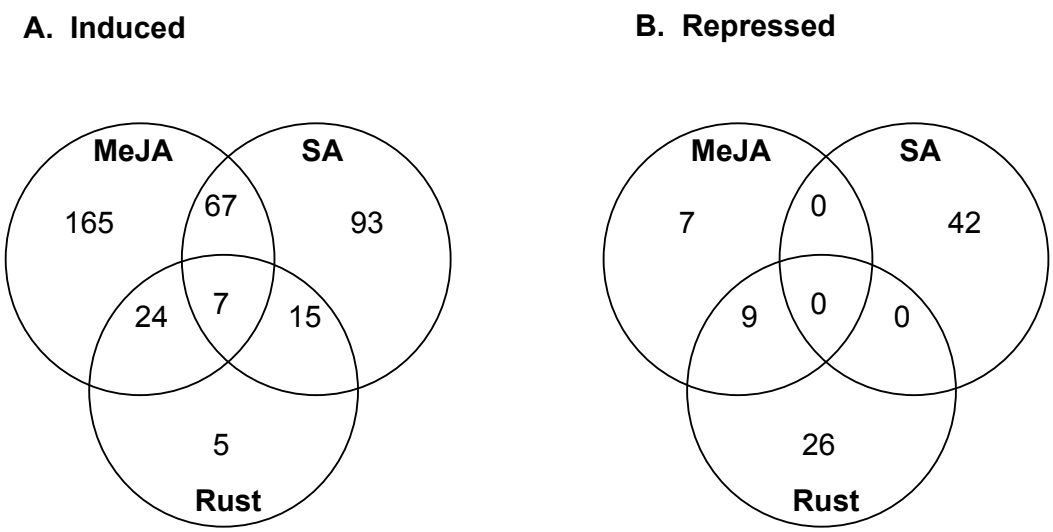

Figure 4.6. Venn diagrams depicting regulatory relationships of pearl millet transcripts significantly induced (A) or repressed (B) more than two fold ($P<0.05$) relative to untreated controls by MeJA, SA and rust treatments.

Functional classification of genes with altered expression patterns

Of the 471 cDNAs exhibiting differential expression in response to MeJA, SA and/or rust treatment, 135 cDNAs were selected for sequence analysis. Putative functions of each of the cDNAs were determined by comparing them to previously reported databases using the BLASTX programme (Altschul et al., 1990) with an E-value cut off of 10^{-5}. Of these 135 cDNAs sequenced, 85 (63%) were found to have significant homology to previously known genes,

while 50 (37%) represented unique genes. In total, the selected cDNAs were found to represent 66 unigenes (51% redundancy). Of the 66 unigenes, 43 exhibited similarity to annotated genes, whilst 23 showed no similarity to sequences in the database. The five most redundant clones in the subtracted library are summarised in Table 4.2.

Table 4.2. The five most redundant clones in the pearl millet defence library as revealed by sequencing selected clones exhibiting differential expression in response to MeJA, SA and/or rust treatment.

Clone ID	Putative ID	Number of hits	Percentage of total sequenced clones
6-C3	No significant similarity	22	16.3
7-E6	Protein translation factor Sui1	9	6.7
10-C2	No significant similarity	7	5.2
4-B11	MtN3-like protein	6	4.4
16-B8	ASR2	5	3.7
Sum of top ten redundant clones		49	36.3

cDNAs with homology to plant genes were classified according to function (Table 4.3). Functional groups include genes that are known to be involved in plant defence, the oxidative burst, abiotic stress, basic or secondary metabolism, protein synthesis, cell signalling and photosynthesis.

The first group contained genes that have previously been implicated in the plant defence response. This group includes a few well documented defence response gene products such as pathogenesis related 1 protein, a disease resistance protein (homologous to an Arabidopsis disease resistance protein that has been annotated but not characterised) and thionin, as well as lesser characterised defence related genes such as an aspartic proteinase, pore forming like toxin *Hfr*-2, and a brown leafhopper susceptibility protein. Interestingly though, microarray studies indicated that PR1 mRNA, which is considered a marker gene of SA induced signal transduction (Ward et al., 1991), was not significantly up regulated in pearl millet in response to SA treatment. On the other hand, PR1 mRNA was highly up regulated in response to rust infection, and also surprisingly up regulated in response to MeJA. This is in contrast to Arabidopsis studies which show that PR1 is down

Table 4.3. Expression profiles of sequenced cDNAs that are more than twofold induced (ratio>2, P<0.05,shaded in red) or repressed (ratio<0.5, P<0.05, shaded in green) by MeJA, SA and rust treatments.

CLONE	BLAST ID	PUTATIVE FUNCTION	e-value	MeJA 12 h Ratio	MeJA 24 h Ratio	MeJA 48 h Ratio	SA 12 h Ratio	SA 24 h Ratio	SA 48 h Ratio	Rust 20 h Ratio	Rust 120 h Ratio	Rust 192 h Ratio
		Defence										
2-E10	AY112455	Aspartic proteinase 1	8.00E-26	1.6	1.6	1.3	1.8	1.6	1.2	2.1	1.2	1.5
4-A1	AAC25629	Pathogenesis related protein 1	2.00E-20	1.1	2.9	2.3	1.1	2.2	2.2	16.9	67.4	49.5
8-B2	BAD34358	Putative UDP-salicylic acid glucosyltransferase	5.00E-08	1.3	1.8	2.7	6.1	2.8	3.6	72.5	49.5	7.8
11-A10	AAQ54304	Putative brown plant hopper susceptibility protein	2.00E-05	0.5	1.4	1.1	0.6	1.4	0.7	2.1	1.7	1.8
11-F3	AAK07429	Beta glucosidase	3.00E-47	1.5	2.7	1.5	2.4	2.1	1.5	2.4	3.4	3.8
15-G7	AAA91048	Thionin	5.00E-15	5.3	3.3	3.2	2.4	2.6	6.0	1.1	1.3	1.4
16-E11	AAW48295	Pore-forming toxin-like protein Hfr-2	1.00E-13	1.8	5.7	5.2	1.1	0.6	1.0	0.8	0.7	0.9
19-H3	AAM45000	Putative disease resistance protein	6.00E-37	1.7	1.9	2.1	1.4	1.0	0.9	3.2	2.6	1.7
		Oxidative Burst										
1-G9	AY104653	Glutaredoxin	3.00E-46	1.3	1.1	1.3	2.5	1.7	1.0	1.1	2.0	3.4
12-C6	NP_919535	Putative peroxidase	3.00E-53	1.1	1.9	0.9	0.5	0.4	0.5	1.2	1.5	0.5
15-G10	CAD42944	Manganese superoxide dismutase	1.00E-06	3.8	0.4	0.7	8.1	1.0	1.6	0.6	0.3	0.2
18-E3	P18123	Catalase isoenzyme 3	2.00E-20	0.3	2.9	2.5	0.3	2.6	1.2	1.0	3.3	3.6
		Stress										
2-F11	CAA05547	Putative HSP70	1.00E-39	1.1	0.9	1.0	3.3	1.1	0.9	1.2	0.5	0.2
8-D7	XM_478265	Putative MATE efflux protein family protein	2.00E-37	1.8	0.7	1.6	3.4	1.8	1.5	0.8	0.7	0.6
13-G1	XP_483156	Putative dehydration-responsive protein RD22	3.00E-17	1.4	9.5	4.9	1.9	4.0	1.4	1.9	1.6	1.0
16-B8	BAD28236	Putative ASR2	2.00E-11	0.9	1.7	2.4	0.6	2.4	2.3	1.0	1.4	3.3
		Basic/secondary metabolism										
1-D3	NP_917118	Putative farnesyl-pyrophosphate synthetase	3.00E-62	2.2	2.5	2.6	1.2	2.3	1.6	1.1	2.4	0.4
3-B6	AAP51748	Serine carboxypeptidase	2.00E-33	1.2	0.8	3.2	6.4	4.6	3.5	8.3	17.7	91.8
5-B6	P12783	Phosphoglycerate kinase, cytosolic	1.00E-49	1.1	1.0	1.1	2.2	1.6	1.2	0.8	1.3	1.5
6-F1	X55981	2-phosophoglycerate dehydrogenase (enolase)	2.70E-54	3.1	2.7	2.5	2.4	2.1	1.3	0.9	0.7	0.7
6-H2	CAA69075	S-adenosylmethionine decarboxylase	8.00E-37	1.3	1.3	1.3	2.1	1.4	1.1	1.1	1.3	1.4
7-E2	XP_476313	Putative inorganic pyrophosphatase	3.00E-46	5.7	5.2	4.5	1.1	1.1	1.1	7.8	11.9	8.9
7-G5	AAA33466	Glyceraldehyde 3-phosphate dehydrogenase, phosphorylating	4.00E-67	14.8	15.1	11.7	4.2	2.2	0.7	1.8	0.8	0.5
10-H1	AF271384	Putative tryptophan synthase alpha chain	1.00E-21	1.5	2.4	1.2	1.4	1.2	1.0	3.0	2.1	1.0
13-D2	XP466501	Rhodanese-like domain-containing protein	3.00E-21	0.9	1.1	0.7	0.5	0.5	0.7	1.1	0.8	0.7
14-B12	P49105	Glucose-6-phosphate isomerase	3.00E-60	0.7	1.2	0.6	0.5	0.8	0.9	0.6	0.9	1.6
		Protein synthesis										
7-A8	D63581	Elongation factor 1 alpha	2.00E-10	1.1	1.2	1.1	2.0	1.8	1.3	1.4	0.9	0.8
7-E6	XP_475493	Putative protein translation factor Sui 1	4.00E-15	7.9	9.4	7.5	3.4	2.1	1.2	1.7	1.1	1.0
10-C3	AY108380	Putative transcription factor EREBP1	2.00E-66	1.6	2.5	1.6	1.2	1.0	0.9	1.8	1.6	1.4
20-G6	ABA99797	DNA binding protein	1.00E-05	0.4	0.8	0.7	0.2	0.8	0.7	0.8	1.1	1.6

CLONE	BLAST ID	PUTATIVE FUNCTION	e-value	MeJA 12 h Ratio	MeJA 24 h Ratio	MeJA 48 h Ratio	SA 12 h Ratio	SA 24 h Ratio	SA 48 h Ratio	Rust 20 h Ratio	Rust 120 h Ratio	Rust 192 h Ratio
		Signalling										
1-E5	XP_466502	Putative ubiquitin-associated (UBA) protein	3.00E-14	7.0	6.7	5.0	1.4	2.0	3.3	1.3	0.8	1.0
14-C1	NP_909820	Putative pyruvate dehydrogenase kinase 1	3.00E-67	1.8	5.0	5.7	1.1	0.8	1.3	0.4	0.6	0.9
5-B12	AK101337	Putative calcium binding EF-hand protein	1.00E-53	2.2	0.7	1.7	4.9	3.0	3.2	0.5	0.5	0.5
		Photosynthesis										
1-G12	Z26595	Triose phosphate/phosphate translocator	7.00E-08	3.8	4.5	3.7	1.5	1.1	0.9	1.3	0.8	0.8
1-H12	AAM15963	Putative phosphoenolpyruvate carboxylase	5.00E-16	1.1	0.9	1.6	1.8	2.3	2.9	0.4	0.9	0.7
6-B6	AB007405	Alanine aminotransferase		0.9	1.0	1.4	2.7	1.8	1.3	1.4	1.1	1.1
12-F9	P12329	Chlorophyll a/b binding protein 1, chloroplast precursor (LHCII type I CAB-1)	4.00E-10	0.3	1.0	1.1	0.5	1.0	1.1	0.6	1.4	1.6
14-H11	CAA44881	Type III LHCII CAB precursor protein	1.00E-34	0.4	2.8	2.6	0.4	2.9	1.4	1.1	2.8	3.1
16-B10	AAQ55066	Photosystem II subunit PsbS precursor	2.00E-47	1.7	0.6	0.9	1.9	1.0	1.2	0.3	0.5	0.5
18-D6	XP_483783	Putative photosystem I reaction centre subunit II, chloroplast precursor	2.00E-24	0.6	0.6	0.4	0.8	0.6	1.0	0.4	0.9	1.6
		Other										
3-F10	BAA04615	Rice homologue of Tat binding protein	6.00E-41	3.1	3.7	3.5	1.5	1.4	1.0	2.6	1.1	1.5
4-B11	BAD82209	MtN3-like	3.00E-42	3.2	1.1	1.9	4.1	1.6	1.4	0.5	0.5	0.2
13-C4	ABA95153	Putative transposon protein, En/SPM subclass	2.00E-11	1.1	0.9	0.9	0.9	0.6	1.0	2.0	1.0	0.8
6-F3		No significant similarity		5.5	6.2	5.4	1.9	1.5	1.0	2.0	1.2	0.4
2-D3		No significant similarity		2.5	1.6	1.4	1.9	1.3	1.1	1.2	1.3	0.5
3-C6		No significant similarity		17.3	21.0	16.2	4.1	2.6	1.1	2.0	0.8	1.0
4-A9		No significant similarity		2.0	2.2	1.9	1.2	1.3	0.8	1.6	0.9	1.5
5-B1		No significant similarity		0.8	1.5	1.4	1.8	1.7	0.8	3.0	1.7	1.5
5-B9		No significant similarity		2.0	2.0	1.8	0.9	1.0	0.8	1.0	1.2	1.4
5-F7		No significant similarity		2.6	2.4	2.8	1.0	3.0	2.1	1.1	0.9	1.1
5-H11		No significant similarity		0.6	0.3	0.4	0.9	1.3	0.9	0.1	0.2	0.5
6-A1		No significant similarity		2.2	2.1	1.8	1.1	1.0	1.1	0.9	0.9	0.8
6-A4		No significant similarity		0.4	0.3	0.3	0.7	0.5	0.9	0.4	0.7	1.3
6-C3		No significant similarity		7.8	8.7	8.2	2.9	1.7	1.4	2.1	1.2	0.9
6-E1		No significant similarity		5.3	6.0	4.6	1.9	1.5	1.3	2.1	1.0	0.5
6-F5		No significant similarity		2.0	0.7	1.4	1.9	3.8	6.4	1.0	4.2	14.1
6-G9		No significant similarity		4.0	4.5	3.8	1.4	0.9	0.9	2.0	1.7	0.7
6-H12		No significant similarity		4.7	5.4	3.6	1.6	1.3	0.8	2.0	1.2	0.9
7-A7		No significant similarity		1.0	0.7	1.5	1.9	2.5	3.2	0.3	0.9	0.8
7-A10		No significant similarity		2.6	2.7	2.1	1.0	0.9	0.8	1.4	1.6	1.2
8-F10		No significant similarity		1.0	1.3	1.2	1.2	1.2	0.8	2.4	2.0	1.7
10-C4		No significant similarity		1.4	2.5	1.1	1.0	0.6	0.9	2.2	2.0	1.3
11-F11		No significant similarity		0.6	0.8	1.0	0.5	1.7	1.4	1.0	1.8	2.1
14-B2		No significant similarity		1.8	0.8	1.9	0.6	0.7	1.1	1.1	0.4	0.5
15-B6		No significant similarity		0.9	1.6	1.3	0.7	2.0	1.1	1.9	2.5	2.8
16-B9		No significant similarity		0.6	1.5	1.3	0.6	2.1	1.1	1.5	2.2	2.7

regulated in response to MeJA treatment (Schenk et al., 2000). A thionin gene transcript was induced in response to MeJA and SA treatments, but not rust infection. Thionin has been shown to be up regulated in response to MeJA treatment in both barley (Andresen et al., 1992) and Arabidopsis (Vignutelli et al., 1998), and was also induced in Arabidopsis in response to the nectrotrophic fungal pathogen *Fusarium oxysporum* f sp. *matthiolae* (Vignutelli et al., 1998). A β-glucosidase was also induced in response to all three treatments. The cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) are secondary metabolites that occur in high abundance as glucosides in the cereals maize, wheat and rye. Upon pathogen attack, the hydroxamic acid glucoside is cleaved by β-glucosidase to release a toxic aglucone that is unstable and decomposes to the reactive benzoxaxolinones, which are toxic to invading pathogens (Nikus et al., 2001).

Plant aspartic proteinases exhibit antimicrobial activity (Guevara et al., 2001) and have thus been implicated in plant defence. Recently, an aspartic proteinase was shown to be induced in potato cultivars in response to *Phytophthora infestans* infection (Guevara et al., 2005). The exact role of aspartic proteinases in defence response is still speculative, and Rodrigo and coworkers (Rodrigo et al., 1991) reported that the constitutive expression of aspartic proteinases degrades PR proteins and suggest that these proteinases might be involved in the turnover of PR proteins as well as the pathogenesis process itself. Although a UDP-glucose:salicylic acid glucosyltransferase was up regulated in pearl millet in response to MeJA, SA and rust, it was only significantly induced in response to SA treatment (more than two fold induced; $P<0.05$). This enzyme is capable of forming SA 2-O-β-D-glucoside (SAG) and glucosyl salicylate (GS) following inoculation of tobacco leaves with incompatible pathogens (Lee and Raskin, 2005). The Hessian fly responsive-2 (*Hfr*-2) gene, which codes for a pore forming toxin-like protein, was recently identified in wheat (Puthoff et al., 2005), and its involvement in interactions with insects is supported by experiments demonstrating its up regulation by both fall armyworm and bird cherry-oat aphid infestations, but not virus infection. Examination of wheat defence

response pathways showed *Hfr*-2 up regulation following MeJA treatment, and only slight up regulation in response to SA, abscisic acid and wounding treatments (Puthoff et al., 2005). Significantly, in this pearl millet microarray study, *Hfr*-2 was only induced in response to MeJA sprays, and not SA or rust treatments. Disease resistance proteins are involved in plant pathogen recognition and mediating race specific or non-specific race resistance (Hammond-Kosack and Parker, 2003).

The second group of genes contains genes that are implicated in the oxidative burst and programmed cell death (Table 4.3). Following the oxidative burst, a number of antioxidant genes are activated in areas around the site of infection in order to minimise damage of healthy tissue. These include peroxidases, catalases, superoxide dismutases and thioredoxins (Table 4.3). Superoxide dismutase catalyses the first step in the scavenging system of active oxygen species by disproprionation of the superoxide anion radical to hydrogen peroxide and molecular oxygen (Kaminaka et al., 1999). Catalases are hydrogen peroxide scavengers, with peroxidases showing a higher affinity for hydrogen peroxide (Palatnik et al., 2002). Glutaredoxins 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). Interestingly, some glutaredoxin targets identified include catalases, and peroxidases, as well as alanine aminotransferase, and heat shock protein (Rouhier et al., 2005). With the exception of the putative peroxidase, all of these glutaredoxin targets were induced in response to SA treatment in pearl millet (Table 4.3).

A number of abiotic stress related genes were differentially regulated in response to MeJA, SA and/or rust treatment in pearl millet, highlighting the similarities in response to biotic and abiotic stresses (Table 4.3). Both the HSP70 gene and a multidrug and toxic compound extrusion (MATE) efflux protein gene were up regulated in response to SA treatment. A recent study that employed virus induced gene silencing of the HSP70 gene showed that this protein is an essential component of the plant defence signal transduction

pathway (Kanzaki et al., 2003). Members of the MATE protein family are putative secondary transporters, unique to plants and microbes, that remove both toxins and secondary metabolites from the plant cell cytoplasm for storage in the vacuole (Diener et al., 2001). A dehydration responsive protein gene, *rd22*, was induced in pearl millet in response to MeJA and SA treatment. In Arabidopsis, *RD22* mRNA was induced by salt stress, water deficit and abscisic acid treatment (Yamaguchi-Shinozaki and Shinozaki, 1993). An abscisic acid-, stress-, ripening-induced (ASR2) (Cakir et al., 2003) protein gene was up regulated in response to all three treatments in pearl millet.

Three genes involved in secondary metabolism were up regulated in pearl millet in response to defence signalling molecule or fungal treatment. These include a farnesyl-pyrophosphate synthetase gene, a serine carboxypeptidase gene and an S-adenosylmethionine decarboxylase gene. All three of these genes have previously been implicated to play a role in plant defence response mechanisms. Farnesyl pyrophosphate synthetase has been shown to be involved in lesion formation in diseased leaves (Manzano et al., 2004) and serine carboxypeptidase is a wound inducible gene product (Moura et al., 2000) that functions in signal transduction components via the brassionsteroid pathway (Li et al., 2001). S-adenosylmethionine decarboxylase 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).

Pearl millet defence signalling molecule inducible genes involved in protein synthesis and signalling that have previously been shown to play roles in plant defence responses include the transcription factor EREBP1, and a ubiquitin associated (UBA) protein gene and a calcium EF hand protein gene. Transcription factor EREBP1 mediates gene expression in response to

various pathogens and defence elicitors (Euglem, 2005). The calcium binding EF hand protein is one of four similar monomers which form a multiprotein complex 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).

Verification of gene expression changes

In order to verify differential gene expression levels of genes observed in cDNA microarray analysis, qRT-PCR was performed for selected genes. Genes were selected on the basis of their documented involvement in plant defence response. In this regard, the following genes were chosen for qRT-PCR analysis in pearl millet: the abiotic stress protein gene *ASR2*; the defence related gene encoding β-glucosidase; the Ca EF hand signalling protein gene; a manganese superoxide dismutase gene whose product is involved in scavenging radical oxygen species; and a thionin gene, which is well documented defence response gene. The changes in expression levels observed using qRT-PCR were similar to or greater than levels obtained by microarray analysis (Figure 4.7). On the whole, expression trends observed over time for each of the treatments using microarray analysis were similar to qRT-PCR expression trends.

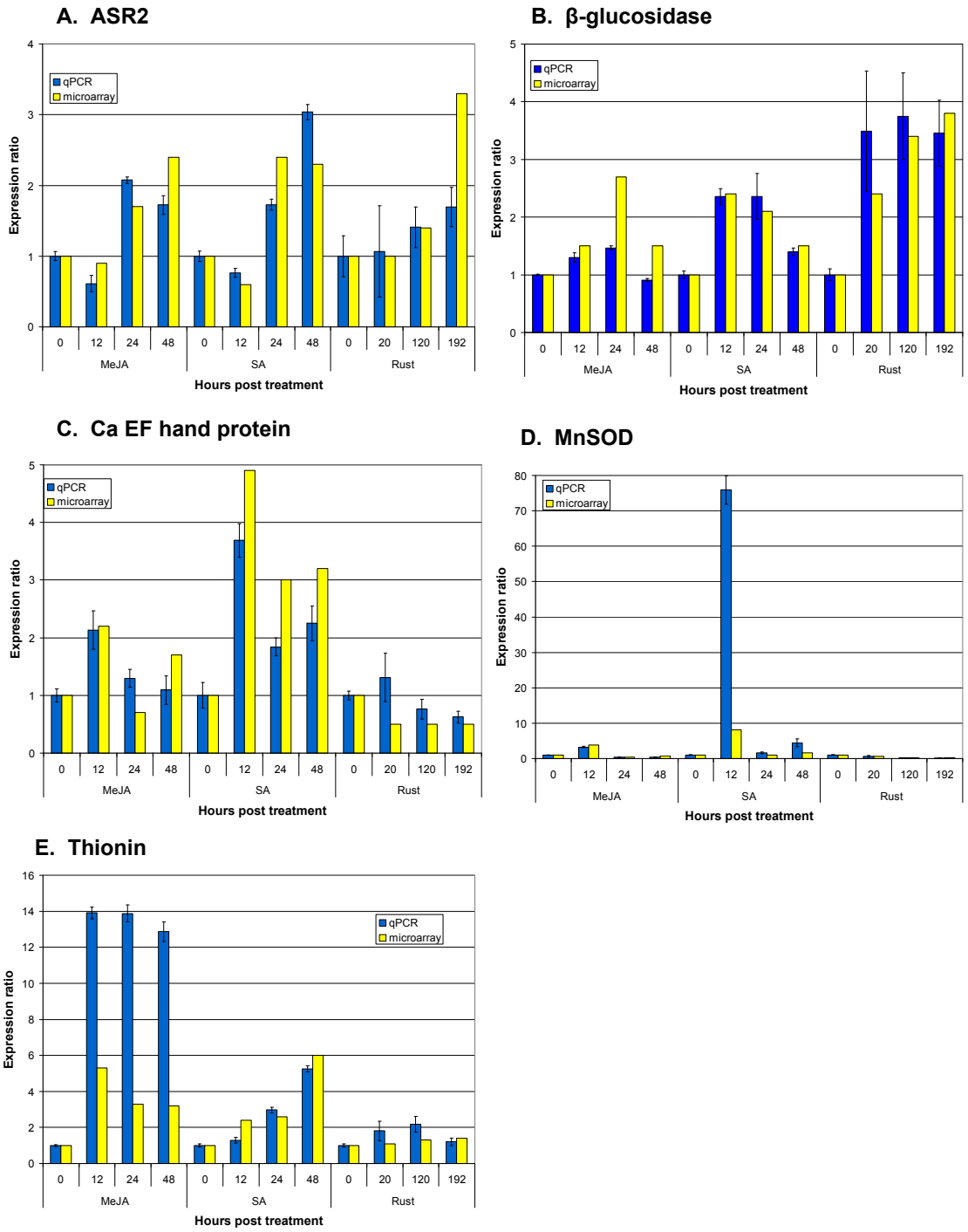

Figure 4.7. Expression ratios for selected genes determined by microarray analysis and qRT-PCR. Changes in gene expression at different time points post MeJA, SA or rust treatment were calculated as the fold change relative to the untreated control (0 h treatment).

Characterisation of pearl millet's response to rust infection

Evidence from Arabidopsis studies suggests that defence against biotrophic pathogens is mounted via the SA defence signalling pathway, whereas resistance to nectrotrophic pathogens and insect attack is elicited through the jasmonic acid/ethylene signalling pathway (Murray et al., 2002). Infection of pearl millet plants with a compatible biotrophic rust pathogen, *P. substriata*, and subsequent microarray analysis suggested that pearl millet responds to this biotrophic pathogen by inducing genes from both the SA and jasmonic acid signalling pathways (Figure 4.6). In order to ascertain if pearl millet's response to a compatible rust infection was more similar to one of the defence signalling pathways, cDNA expression ratios in response to MeJA, SA and rust treatment were clustered, and a tree was built using an average-linkage algorithm (Saeed et al., 2003). Figure 4.8 shows that over time, pearl millet gene expression changes in response to MeJA and SA treatments are more similar to each other, than either treatment is to gene expression changes following the compatible rust infection. These results suggest that the plant adopts elements from a number of defence signalling pathways in an attempt to ward off attack by a virulent *P. substriata* isolate. Zhu-Salzman and colleagues (2004) also showed that when sorghum is attacked by a phloem-feeding greenbug aphid, the plant activates both JA and SA regulated genes, as well as genes outside known wounding and SA signalling pathways. These results all suggest that defence signalling in monocotyledonous plants is complex. Whilst pathogen attack may result in the induction of a number of defence signalling pathways, a single defence signalling pathway may be responsible for resistance to an avirulent pathogen. It is possible that virulent pathogens have evolved mechanisms to avoid induction of all the elements of the signalling pathway conferring resistance (Zhu-Salzman et al., 2004).

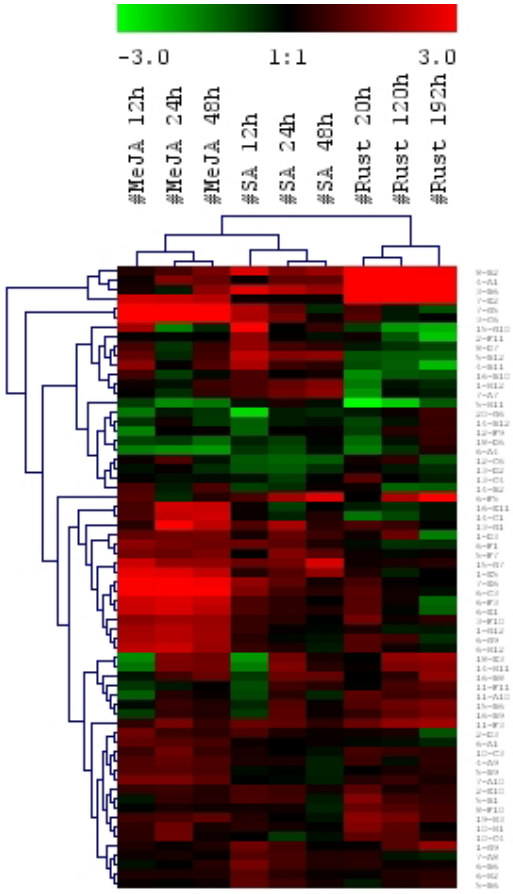

Figure 4.8. Hierachical cluster of 66 sequenced pearl millet cDNAs with two-fold (P<0.05) or more changes in transcript abundance in response to MeJA, SA and rust treatment. Each gene is represented by a single row of coloured boxes, and a single column represents a time point following a particular treatment. Induction (or repression) ranges from pale to saturated red (or green) with a fold change scale bar shown above the cluster.

Although gene expression studies indicate that different defence signalling pathways are activated in response to infection with a compatible rust fungus, chemical induction studies suggest that induction of the salicylate pathway is able to render resistance to pearl millet plants infected with virulent *P. substriata*. Thus, although *P. substriata* infection elicits genes from different defence signalling pathways, it is probably genes uniquely up regulated in response to SA that confer resistance to virulent rust isolates. In this study, a number of candidate genes were significantly induced by SA but not up regulated to the same extent by MeJA (Table 4.4). Some highly SA responsive genes include well characterised defence response genes

Table 4.4. Expression profiles of sequenced cDNAs that are more than twofold induced (ratio>2, P<0.05, shaded in red) or repressed (ratio<0.5, P<0.05, shaded in green) by SA treatment but not by MeJA application.

CLONE	BLAST ID	PUTATIVE FUNCTION	e-value	MeJA 12 h Ratio	MeJA 24 h Ratio	MeJA 48 h Ratio	SA 12 h Ratio	SA 24 h Ratio	SA 48 h Ratio
		Defence							
8-B2	BAD34358	Putative UDP-salicylic acid glucosyltransferase	5.00E-08	1.3	1.8	2.7	6.1	2.8	3.6
		Oxidative Burst							
1-G9	AY104653	Glutaredoxin	3.00E-46	1.3	1.1	1.3	2.5	1.7	1.0
12-C6	NP_919535	Putative peroxidase	3.00E-53	1.1	1.9	0.9	0.5	0.4	0.5
		Stress							
2-F11	CAA05547	Putative HSP70	1.00E-39	1.1	0.9	1.0	3.3	1.1	0.9
8-D7	XM_478265	Putative MATE efflux protein family protein	2.00E-37	1.8	0.7	1.6	3.4	1.8	1.5
		Basic/secondary metabolism							
3-B6	AAP51748	Serine carboxypeptidase	2.00E-33	1.2	0.8	3.2	6.4	4.6	3.5
5-B6	P12783	Phosphoglycerate kinase, cytosolic	1.00E-49	1.1	1.0	1.1	2.2	1.6	1.2
6-H2	CAA69075	S-adenosylmethionine decarboxylase	8.00E-37	1.3	1.3	1.3	2.1	1.4	1.1
13-D2	XP466501	Rhodanese-like domain-containing protein	3.00E-21	0.9	1.1	0.7	0.5	0.5	0.7
14-B12	P49105	Glucose-6-phosphate isomerase	3.00E-60	0.7	1.2	0.6	0.5	0.8	0.9
		Protein synthesis							
7-A8	D63581	Elongation factor 1 alpha	2.00E-10	1.1	1.2	1.1	2.0	1.8	1.3
		Photosynthesis							
1-H12	AAM15963	Putative phosphoenolpyruvate carboxylase	5.00E-16	1.1	0.9	1.6	1.8	2.3	2.9
6-B6	AB007405	Alanine aminotransferase		0.9	1.0	1.4	2.7	1.8	1.3
12-F9	P12329	Chlorophyll a/b binding protein 1, chloroplast precursor (LHCII type I CAB-1)	4.00E-10	0.3	1.0	1.1	0.5	1.0	1.1
		Other							
6-A4		No significant similarity		0.4	0.3	0.3	0.7	0.5	0.9
16-B9		No significant similarity		0.6	1.5	1.3	0.6	2.1	1.1

encoding glutaredoxin, Mn SOD, HSP70, MATE efflux protein, Ca EF hand protein (Table 4.3 and Table 4.4). Interestingly, application of SA or its synthetic mimics protected barley plants systemically against *Bgh* infection (Bessert et al., 2000). Chemically induced genes were found to encode a lipoxygenase, a thionin, and acid phosphatase, a Ca EF hand protein, a serine proteinase inhibitor, a fatty acid desaturase and several proteins of unknown function. Except for the Ca EF hand protein and a gene of unknown function, the genes were also induced by exogenous application of jasmonates. However, like pearl millet, treatments that raised endogenous jasmonastes, as well as wounding, were less effective in conferring resistance to *Bgh* (Bessert et al., 2000). Treatment of pearl millet plants with an avirulent *P. substriata* strain and subsequent microarray analysis would reveal whether an incompatible reaction elicits more elements of the salicylate defence response pathway.

Coordination between plant defence response signalling pathways

Many genes were coregulated by MeJA, SA and rust, and the largest number of coinduced cDNAs in pearl millet were between MeJA and SA (67 cDNAs) (Figure 4.5). Similar results were obtained in Arabidopsis (Schenk et al., 2000) and sorghum (Salzman et al., 2005), where coregulation was observed between MeJA, SA and ethylene signalling pathways, with the highest amount of coregulation between jasmonate and salicylate pathways. This contradicts data obtained from Arabidopsis mutants defective in salicylate and jasmonate signalling pathways in which studies have indicated that the primary mode of interaction between these signalling pathways is mutual antagonism (Kunkel and Brooks, 2002). For example, two Arabidopsis mutants (*eds4* and *pad4*) with reduced levels of SA displayed increased expression of the JA/ethylene dependent gene *PDF1.2* after treatment with MeJA (Gupta et al., 2000). Conversely, the Arabidopsis mutant *mpk4*, which is defective in JA dependent PDF1.2 expression, displayed constitutive activation of SA dependent signalling (Petersen et al., 2000). Such antagonism has probably evolved in plants in order to conserve resources by limiting defence responses to genes effective against biotrophs versus nectrotrophs or insects (Kunkel and Brooks, 2002; Salzman et al., 2005).

However, defence signalling pathway studies in mutants tends to be reductionist in approach with only one or a few genes examined in isolation. The emergence of DNA microarrays have helped improve our understanding of cross-talk between defence signalling pathways as they have created an opportunity to study the expression of thousands of genes in parallel. Such studies in Arabidopsis (Schenk et al., 2000), sorghum (Salzman et al., 2005) and pearl millet suggest substantial coregulation among different plant defence pathways. Although some antagonism exists, this appears to be specific to particular genes. Synergism between defence pathways enables the plant to mount a response that targets several of the pathogen's virulence factors or invasion strategies at once.

In conclusion, cDNA microarray analysis of pearl millet exposed to MeJA and SA enhanced our understanding of transcriptional changes and mechanisms of action of defence signalling pathways in a non-model cereal crop. Even though the number of cDNAs analysed represent a subset of the entire pearl millet defence transcriptome, the results demonstrated that a substantial network of regulatory interactions exists between the salicylate and jasmonate pathways, which were previously believed to act in an antagonistic manner. Infection with the compatible biotrophic rust pathogen, *P. substriata*, induced genes common to both the jasmonate and salicylate signalling pathway, suggesting that pearl millet activates genes from a number of defence signalling pathways in an attempt to prevent infection by the virulent pathogen. However, treatment with SA prior to rust infection rendered the pearl millet plants more resistant to the development of rust symptoms. These results indicate that it is elements of the salicylate defence pathway that actually render pearl millet resistant to rust infection.

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Chapter 5

Concluding remarks and future prospects

Pearl millet is the world's sixth most important cereal crop (National Research Council, 1996), and is a staple food source for millions of African families living in semi-arid regions of the continent. Yet, despite its importance and ability to yield consistently, especially in extreme heat and drought conditions, very little research and resources have been directed towards understanding mechanisms governing this crop's resilience to biotic and abiotic stresses. The research outlined in this thesis therefore aimed to elucidate defence response mechanisms in pearl millet, and to identify broad spectrum defence response genes that could be used in future genetic engineering experiments to improve pearl millet and other cereal crops against pathogen attack. This was accomplished through the construction and characterisation of a pearl millet defence response cDNA library, which was subsequently utilised in large scale gene expression studies to profile pearl millet's response to the defence signalling compounds nitric oxide, methyl jasmonate and salicylic acid, and to the biotrophic rust fungus *Puccinia substriata* var. *indica*.

At the onset of this study, very little gene sequence information was available for pearl millet. As a result, a pearl millet defence response cDNA library was constructed and characterised. This was accomplished by treating pearl millet plants with the fungal and bacterial defence elicitors chitin and flagellin, respectively, and wounding the plants. Following RNA extraction and cDNA synthesis, the cDNA was enriched for defence response transcripts by employing suppression subtractive hybridisation (SSH) (Diatchenko et al., 1996). A forward and reverse library was constructed to identify genes which are up- or down regulated during the defence response, respectively. In an effort to characterise the cDNA libraries, a quantitative cDNA microarray-based screening method was developed that enabled identification of false positive transcripts, as well as clones which represented rare or abundant transcripts. Based on this screening method, a number of clones were selected for sequence analysis, and their identity ascertained through

homology searches with previously sequenced genes. This revealed a number of genes known to play important roles during pathogen attack.

The pearl millet defence response library, consisting of 1920 cDNAs either up- or down regulated in defence response, was spotted onto a glass slide microarray and used in transcript profiling studies to examine pearl millet's response to the defence signalling molecules NO, MeJA and SA. Whilst only 45 cDNAs responded significantly to NO treatment, 279 and 224 cDNAs responded to MeJA and SA sprays, respectively. Table 5.1 summarises all sequenced genes which exhibited differential expression in response to pathogen elicitors, NO, MeJA, SA and rust infection. Closer examination of MeJA and SA responsive genes revealed that many of the induced transcripts were common to both signalling pathways, demonstrating that a substantial network of regulatory interactions exists between the salicylate and jasmonate pathways, which were previously believed to act in an antagonistic manner (Kunkel and Brooks, 2002).

Pathology studies indicated that pretreatment of pearl millet with SA conferred resistance to a virulent isolate of the biotrophic rust, *P. substriata* var. *indica*, whereas MeJA did not significantly reduce infection levels. Transcript profiling of a susceptible pearl millet cultivar in response to infection with rust revealed that genes common to both the jasmonate and salicylate pathways were induced, suggesting that pearl millet has evolved its defence responses by adopting elements from both pathways in an attempt to prevent infection by the virulent pathogen. However, as implied from chemical induction and subsequent pathology studies, it is probably the salicylate defence signalling pathway that confers resistance to avirulent rust isolates. Treatment of pearl millet plants with an avirulent *P. substriata* strain and subsequent microarray analysis would reveal whether an incompatible reaction elicits more elements of the salicylate defence response pathway.

Sequence analysis of pearl millet cDNAs responsive to chitin, flagellin, wounding, NO, MeJA, SA and rust infection revealed genes with homology to genes coding for previously characterised defence-related proteins such as

aspartic proteinase, pathogenesis related protein 1, β-glucosidase, thionin, a receptor-like kinase, a putative disease resistance protein, glutaredoxin, peroxidase, catalase, manganese superoxide dismutase, HSP70, transcription factor EREBP1, and a calcium binding EF hand protein (See Table 5.1). However, a number of cDNAs exhibited no similarity to genes in the GenBank database. These cDNAs could encode novel defence response genes that have not been previously characterised. Studies are presently underway to characterise the role of some of these genes in defence in pearl millet through virus induced gene silencing (VIGS). In VIGS, viruses engineered to carry sequences derived from plant gene transcripts activate the host's sequence-specific RNA degradation system. This mechanism targets the RNAs of the viral genome for degradation, and as the virus contains transcribed plant sequence, homologous host mRNAs are also targeted for destruction (Scofield et al., 2005). VIGS has been successfully applied to examine components of the defence response system in both barley (Hein et al., 2005) and wheat (Scofield et al., 2005).

Broad spectrum disease resistance genes, exhibiting up regulation under a number of defence conditions tested, could also be introduced into pearl millet to convey resistance to a number of pathogens (Gurr and Rushton, 2005). Good examples of such genes identified in this study which are up regulated in both jasmonate and salicylate defence signalling include genes coding for thionin, catalase, putative dehydration-responsive protein RD22, ASR2, calcium binding EF hand protein. Receptor-like kinases, such as the Arabidopsis FLS2, which controls perception of a portion of the highly conserved bacterial flagellin protein, are excellent candidates for genetic engineering resistance to a broad range of pathogens (Hammond-Kosack and Parker, 2003). This study identified a pearl millet receptor-like kinase gene (clone 4-E8), which was induced in response to chitin and/or flagellin. The *Arabidopsis thaliana* homologue of this gene (AAM20287) has been annotated, but not characterised. In pearl millet, this receptor-like kinase appears to play a role in chitin or flagellin perception as it is not up regulated under other defence inducing conditions such as NO, MeJA, SA or rust treatment (See Table 5.1). Further characterisation of this gene through

studies such as VIGS might reveal a candidate gene for improved resistance to a particular pathogen group (e.g. fungi or bacteria) in pearl millet. Successful transformation of pearl millet has recently been achieved (Girgi et al., 2002), providing opportunities to enhance the gene pool of this crop regarded as an African jewel (National Research Council, 1996). However, to minimise the cost of defence activation on plant yield a new repertoire of pathogen promoters is required (Gurr and Rushton, 2005). Defence inducible genes identified in this investigation will provide a novel source of material for promoter mining studies.

The main constraint in the study was that the pearl millet SSH cDNA library does not represent all pearl millet defence response genes. The cDNA library contains only copies of transcripts of genes expressed in the material from which the mRNA was extracted. Therefore, if the defence signalling molecules NO, MeJA and SA, or the rust fungus induce or repress the expression of genes whose expression is unaffected in the basal defence responses against wounding or elicitor treatment, then this will not be discovered, as these genes are not present in the SSH library and therefore the microarray.

In conclusion, this study has yielded significant insight into defence response mechanisms in pearl millet. At the onset of the project we hypothesised that treatment of monocots with pathogen elicitors and defence signalling molecules would result in differential expression of defence related genes. Results from this study suggest that many previously characterised defence genes are up regulated in response to elicitor treatment, defence signalling molecules and pathogen infection. In addition, a number of genes previously shown to be involved in the oxidative burst, stress, basic and secondary metabolism, protein synthesis, photosynthesis and signalling exhibit differential expression under defence inducing conditions. Furthermore, a number of genes with no homology to sequences in the Genbank were up regulated during defence inducing conditions

Table 5.1. Summary of response of sequenced pearl millet genes to treatment with chitin/flagellin/wounding, NO, MeJA, SA and rust. Up indicates that the gene was up regulated in response to a treatment, down indicates that the gene was down regulated, and no change shows that there was no change in gene expression in response to the treatment.

Cluster ID	Gene annotation	Origin of similar sequence	Accession number	e-value	Library	chi/flg/wound	NO	MeJA	SA	Rust
Defence										
1	Thionin BTH7 precursor	*Hordeum vulgare*	AAA91048	5E-15	reverse	up	no change	up	up	no change
2	Putative UDP-salicylic acid glucosyltransferase	*Oryza sativa*	BAD34358	5E-08	forward	up	no change	no change	up	no change
3	Putative disease resistance protein	*Arabidopsis thaliana*	AAM45000	6E-37	reverse	up	no change	up	no change	up
4	Putative brown plant hopper susceptibility protein		AAQ54304	2E-05	reverse	up	no change	no change	no change	up
5	Pore-forming toxin-like protein Hfr-2	*Triticum aestivum*	AAW48295	1E-13	reverse	up	no change	up	no change	no change
6	Aspartic proteinase 1	*Glycine max*	BAB62890	8E-26	forward	up	no change	no change	no change	up
7	Pathogenesis related protein 1	*Zea mays*	AAC25629	2E-20	forward	up	no change	up	no change	up
8	Beta glucosidase	*Musa acuminata*	AAK07429	3E-47	reverse	up	up	up	up	up
9	Hydrolase, alpha/beta fold family protein	*Arabidopsis thaliana*	NP_974605	2E-53	forward	up	no change	no change	no change	no change
10	Pyrrolidone carboxyl peptidase-like protein	*Oryza sativa*	XP_479284	3E-16	forward	up	no change	no change	no change	no change
Oxidative burst										
11	Putative peroxidase	*Oryza sativa*	NP919535	3E-53	reverse	up	no change	no change	down	no change
12	Manganese superoxide dismutase	*Taiwanofungus camphora*	CAD42944	1E-06	reverse	up	down	up	up	no change
13	Glutaredoxin	*Oryza sativa*	CAA54397	3E-46	forward	up	no change	no change	up	no change
14	Catalase isoenzyme 3	*Zea mays*	P18123	2E-20	reverse	up	no change	up	up	up
15	Glutathione S-transferase (GST40)	*Zea mays*	AAG34848	3E-60	forward	up	no change	no change	no change	no change
16	Fructose-bisphosphate aldolase	*Oryza sativa*	AY103557	1E-24	forward	up	no change	no change	no change	no change
Stress										
17	Putative MATE efflux protein family protein	*Oryza sativa*	XP478265	2E-37	forward	up	no change	no change	up	no change
18	Putative dehydration-responsive protein RD22	*Oryza sativa*	XP483156	3E-17	reverse	up	no change	up	up	no change
19	Membrane-associated salt-inducible protein-like	*Arabidopsis thaliana*	BAB08985	5E-72	forward	up	no change	no change	no change	no change
20	Putative ASR2	*Oryza sativa*	BAD28237	2E-11	reverse	up	no change	up	up	up
21a	Heat shock protein 70	*Oryza sativa*	CAA47948	9E-37	forward	up	no change	no change	up	no change
21b	Heat shock protein 70	*Arabidopsis thaliana*	CAA05547	2E-37	forward	up	no change	no change	up	no change
Basic/secondary metabolism										
22	Serine carboxypeptidase	*Oryza sativa*	NP919461	2E-33	forward	up	no change	up	up	up
23	S-adenosylmethionine decarboxylase	*Zea mays*	CAA69075	8E-37	forward	up	no change	no change	up	no change
24	Rhodanese-like domain-containing protein	*Oryza sativa*	XP466531	3E-35	reverse	up	no change	no change	down	no change
25a	Putative tryptophan synthase alpha chain	*Oryza sativa*	XP476874	1E-21	forward	up	no change	up	no change	up
25b	Putative tryptophan synthase alpha chain	*Oryza sativa*	Z26595	4E-09	forward	up	no change	no change	no change	down
26	Putative inorganic pyrophosphatase	*Oryza sativa*	XP476313	3E-46	forward	up	no change	up	no change	no change
27	Putative farnesyl-pyrophosphate synthetase	*Oryza sativa*	NP917118	3E-62	forward	up	no change	up	no change	no change
28a	Glyceraldehyde 3-phosphate dehydrogenase, phosphoryl	*Zea mays*	AAA33466	4E-67	forward	down	no change	up	up	no change
28b	glyceraldehyde 3-phosphate dehydrogenase	*Hordeum vulgare*	CAA42901	2E-03	forward	up	no change	up	up	no change
28c	Cytosolic glyceraldehyde 3-phosphate dehydrogenase	*Zea mays*	X07156	1E-18	forward	up	no change	no change	no change	no change
29	Glucose-6-phosphate isomerase	*Zea mays*	P49105	3E-60	reverse	up	no change	no change	down	no change
30	Tryptophan synthase alpha chain	*Oryza sativa*	XP_476874	1E-22	forward	up	no change	no change	no change	no change
31	2-phosophoglycerate dehydrogenase (enolase)			3E-54	forward	up	no change	up	up	no change
32	Phosphatidylserine decarboxylase	*Oryza sativa*	NP_914239	7E-54	forward	up	no change	no change	no change	no change
33	Inorganic pyrophosphatase	*Oryza sativa*	XP_476313	3E-47	forward	up	no change	no change	no change	no change
34	Mitochondrial aldehyde dehydrogenase	*Sorghum bicolor*	BAB92019	8E-72	forward	up	no change	no change	no change	no change

Table 5.1. cont.

Cluster ID	Gene annotation	Origin of similar sequence	Accession number	e-value	Library	chi/flg/wound	NO	MeJA	SA	Rust
Protein synthesis										
35	Putative transcription factor EREBP1	*Oryza sativa*	XP_468125	2E-66	forward	up	no change	up	no change	no change
36	Putative protein translation factor Sui 1	*Oryza sativa*	XP475493	4E-15	forward	up	no change	no change	up	no change
37a	Elongation factor 1 alpha	*Zea mays*	BAA08249	2E-10	forward	up	no change	no change	up	no change
37b	Elongation factor 1 alpha	*Zea mays*	AAF42980			up	down	no change	no change	no change
38	DNA binding protein	*Oryza sativa*	ABA99799	1E-05	reverse	up	no change	down	down	no change
39	Transcription factor BTF3	*Oryza sativa*	AAO72645	7E-40	forward	up	no change	no change	no change	no change
40	Histone H2B.2	*Oryza sativa*	XP_483094	3E-28	forward	up	no change	no change	no change	no change
41	Translation initiation factor 5A	*Zea mays*	CAA69225	4E-70	forward	up	no change	no change	no change	no change
Signalling										
42	Putative ubiquitin-associated (UBA) protein	*Oryza sativa*	XP466502	3E-14	forward	up	no change	up	no change	no change
43	Putative calcium binding EF-hand protein	*Hordeum vulare*	CAB71337	1E-53	forward	up	no change	up	up	no change
44	Receptor kinase	*Arabidopsis thaliana*	AAM20287	4E-42	forward	up	no change	no change	no change	no change
45a	Cytosolic phosphoglycerate kinase	*Populus nigra*	BAA33801	8E-50	forward	up	no change	no change	up	no change
45b	Phosphoglycerate kinase, cytosolic	*Triticum aestivum*	P12783	1E-49	forward	up	no change	no change	up	no change
46a	Putative pyruvate dehydrogenase kinase 1	*Oryza sativa*	XP479264	3E-67	reverse	down	no change	no change	up	no change
46b	Putative pyruvate dehydrogenase kinase	*Oryza sativa*	NP909820	1E-27	reverse	down	down	up	no change	no change
Photosynthesis										
47a	Putative chorophyll a/b binding protein	*Oryza sativa*	NP917525	4E-06	reverse	up	up	no change	down	no change
47b	Light harvesting chlorophyll a/b binding protein	*Zea mays*	CAA39376	2E-06	reverse	up	no change	down	no change	down
47c	Chlorophyll a/b binding protein 1 (LHCII type I CAB-1)	*Oryza sativa*	P12329	4E-10	reverse	up	up	no change	down	no change
47d	Chlorophyll a/b binding protein	*Zea mays*	X63205	2E-06	forward	up	no change	no change	no change	no change
47f	Chlorophyll a/b binding protein	*Oryza sativa*	BAD61582			up	up	no change	no change	no change
47g	Type III LHCII CAB precursor protein	*Hordeum vulgare*	CAA44881	1E-34	reverse	up	no change	no change	up/down	up
48	Chlorplast PSI reaction centre			9E-26	forward	up	no change	no change	up	no change
49	Putative photosystem I reaction centre subunit II		XP483783	2E-24	reverse	up	no change	down	no change	down
50	Photosystem II subunit PsbS precursor	*Zea mays*	AAQ55066	2E-47	reverse	up	no change	no change	no change	down
51	Plastocyanin, chloroplast precursor	*Oryza sativa*	P20423	2E-15	forward	up		no change	no change	no change
52	Putative phosphoenolpyruvate carboxylase	*Setaria italica*		5E-16	forward	up	down	no change	up	down
53	Alanine aminotransferase	*Panicum milaceum*	CAA49199		forward	up		no change	up	no change
54	Triose phosphate/phosphate translocator	*Zea mays*	Z26595	7E-08	forward	down	no change	up	no change	no change
Cell structure										
55	Actin	*Linum usitatissimum*	AAW34192	5E-40		up	down	no change	no change	no change

Table 5.1 cont

Cluster ID	Gene annotation	Origin of similar sequence	Accession number	e-value	Library	chi/flg/wound	NO	MeJA	SA	Rust
Other										
56	Rice homologue of Tat binding protein	*Oryza sativa*	BAA04615	6E-41	forward	up	no change	up	no change	no change
57	Immunophilin	*Oryza sativa*	XP_467909	8E-55	forward	up		no change	no change	no change
58	Small nuclear ribonucleoprotein polypeptide E	*Oryza sativa*	XP_463967	1E-12	forward	up		no change	no change	no change
59	Unknown protein	*Oryza sativa*	NP_915800	1E-11	forward	up		no change	no change	no change
60	Unknown protein	*Arabidopsis thaliana*	AAP37829	8E-20	forward	up		no change	no change	no change
61	AcinusL protein-like	*Oryza sativa*	XP_479211	5E-31	forward	up		no change	no change	no change
62	Pistil specific extensin like protein				forward	up	no change	up	no change	no change
63	Putative transposon protein, CACTA, En/SPM subclass	*Oryza sativa*	ABA95153	2E-11	reverse	up	no change	no change	no change	up
64	MtN3-like	*Oryza sativa*	BAD82209	8E-41	forward	up	no change	up	up	no change
65	hypothetical protein RakaH01001082	*Rickettsia akari*		5E-18	forward	down		no change	no change	no change
66	Putative Bowman Birk trypsin inhibitor	*Oryza sativa*	CAB88391	4E+01	forward	up	no change	up	up	no change
67	No significant similarity				reverse	up	up	no change	up	up
68	No significant similarity				forward	down	no change	up	no change	up
69	No significant similarity				reverse	up	up	no change	no change	up
70	No significant similarity				reverse	up	no change	no change	no change	up
71	No significant similarity				forward	up	no change	up	no change	no change
72	No significant similarity				forward	down	no change	up	up	no change
73	No significant similarity				forward	up	no change	up	no change	no change
74	No significant similarity				forward	up	no change	no change	no change	up
75	No significant similarity				forward	reject	no change	up	no change	no change
76	No significant similarity				forward	up	no change	down	no change	no change
77	No significant similarity				forward	down	no change	up	no change	no change
78	No significant similarity				forward	down	no change	no change	no change	down
79	No significant similarity				forward	up	no change	up	no change	down
80	No significant similarity				forward	up	no change	no change	no change	up
81	No significant similarity				forward	up	no change	up	no change	no change
82	No significant similarity				forward	up		no change	no change	no change
83	No significant similarity				forward	up		no change	no change	no change
84	No significant similarity				forward	down		no change	no change	no change
85	No significant similarity				forward	up		no change	no change	no change
86	No significant similarity				forward	up		no change	no change	no change
87	No significant similarity				forward	up		no change	no change	no change
88	No significant similarity				reverse	up	up	no change	no change	no change
89	No significant similarity				forward	up		no change	no change	no change
90	No significant similarity				forward	up	up	no change	no change	no change
91	No significant similarity				forward	up		no change	no change	no change
92	No significant similarity				forward	up		no change	no change	no change
93	No significant similarity				forward	up		no change	up	no change
94	No significant similarity				forward	up		no change	no change	no change
95	No significant similarity				forward	up		no change	no change	no change
96	No significant similarity				forward	up	down	no change	up	down
97	No significant similarity				forward	up	no change	no change	no change	up
98	No significant similarity				forward	up	no change	up	no change	no change
99	No significant similarity				reverse	up		no change	no change	down
100	No significant similarity				forward	up	no change	up	no change	no change

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