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Specific changes in the *Arabidopsis* proteome in response to bacterial challenge: differentiating basal and *R*-gene mediated resistance

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Abstract

Alterations in the proteome of *Arabidopsis thaliana* leaves during early responses to challenge by *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) were analysed using two-dimensional (2D) gel electrophoresis. Protein changes characteristic of the establishment of basal resistance and *R*-gene mediated resistance were examined by comparing responses to DC3000, a *hrp* mutant and DC3000 expressing *avrRpm1* respectively. The abundance of selected transcripts was also analysed in GeneChip experiments. Here we present data from the soluble fraction of leaf protein, highlighting changes in two antioxidant enzyme groups; the glutathione S-transferases (GSTs F2, F6, F7 and F8) and peroxiredoxins (PrxA, B and IIE). Members of both enzyme groups showed signs of specific post-translational modifications, represented by multiple spots on gels. We suggest that oxidation of specific residues is responsible for some of the spot shifts. All forms of the GST proteins identified here increased following inoculation with bacteria. GSTF8 showed particularly dynamic responses to pathogen challenge, the corresponding transcript was significantly up-regulated by 2 h after inoculation, and the protein showed post-translational modifications specific to an incompatible interaction. Differential changes were observed with the peroxiredoxin proteins; PrxIIE and to a lesser extent PrxB, no change was observed with PrxA, but a truncated form PrxA-L was greatly reduced in abundance following bacterial challenges. Our data suggest that bacterial challenge generally induces Prxs and the antioxidants GSTs, however individual members of these families may be specifically modified dependent upon the virulence of the DC3000 strain and outcome of the interaction. Finally, proteomic and transcriptomic data derived from the same inoculation system are compared and the advantages offered by 2D gel analysis discussed in light of our results.
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1. Introduction

The bacteria and fungi that colonise living plants and cause disease have evolved the ability to overcome the basal resistance of their hosts to infection. Superimposed on the establishment of basic parasitism (or pathogenicity) is the phenomenon of varietal resistance. Failure to colonise resistant host cultivars is very often associated with activation of the hypersensitive reaction (HR). The HR involves various biochemical perturbations including changes in ion fluxes, lipid peroxidation, protein phosphorylation, a burst of reactive oxygen species which includes de novo nitric oxide (NO) generation, and leads ultimately to a form of rapid programmed cell death. The current paradigm is that plant pathogens may

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produce numerous proteinaceous virulence factors (effectors), which act synergistically to suppress basal resistance (Collmer et al., 2002). Some of these effectors may become recognised by the product of plant resistance (*R*) genes and, therefore, be identified as determinants of avirulence (*avr*); creating the patterns of gene-for-gene interaction observed in many diseases (Flor and Comstock, 1972; Jackson et al., 1999; Tsiamis et al., 2000).

The ability of plant pathogenic bacteria, such as pathogens of *Pseudomonas syringae*, to deliver effector proteins to plant cells is dependent on their possession of a functional type III secretion system (TTSS, Cornelis and Van Gijsegem, 2000). Mutation in the TTSS leads to failure to elicit the HR in in resistant varieties of their host and certain non-host plants, and also loss of pathogenicity. The *hrp* genes encoding the TTSS in *P. syringae* include *hrpA* which encodes an extracellular protein that forms a pilus conduit which delivers effectors across the plant cell wall and into the plant cytoplasm (Li et al., 2002; Jin and He, 2001; Jin et al., 2003).

Although *hrp* mutants and saprophytic bacteria fail to cause macroscopic symptoms such as the HR in plants they do activate some typical defence responses (Jakobek and Lindgren, 1993). Comparison of early transcripts induced in *Arabidopsis* by wild type *P.s.* pv. *tomato* DC3000 (DC3000) and a DC3000 *hrpA* mutant, revealed the rapid induction of the same genes following inoculation. These results indicated the existence of a basal recognition mechanism allowing detection of any invading microbe (de Torres et al., 2003). Microscopical studies of interactions between *hrp* mutants of *P.s.* pv. *phaseolicola* and *Xanthomonas campestris* pv. *vesicatoria* and a range of plants, including *Arabidopsis*, have revealed highly localised alterations occurring in plant cells adjacent to the compromised bacteria (Bestwick et al., 1995, 1998; Brown et al., 1995, 1998). Changes to the plant cell wall included incorporation of phenolics and hydroxyproline and proline rich glycoproteins and were associated with the accumulation of H_2O_2 and peroxidase activity at reaction sites (Bestwick et al., 1998; Hauck et al., 2003). The oxidative burst associated with basal resistance in *Arabidopsis* and other plants is much less intense than that observed during the HR (Grant et al., 2000; Bestwick et al., 1995; Brown et al., 1998). Clearly, regulation of the intensity and localisation of redox reactions has an important role in co-ordination of the plant's response to both pathogenic and non-pathogenic bacteria.

Whereas gene-for-gene based resistance involves recognition of the proteins encoded by *avr* genes, basal defences are thought to be activated by surface derived molecules referred to as pathogen associated molecular patterns (PAMPS). Recognition of PAMPS such as bacterial lipopolysaccharides (LPS) is considered an example of the expression of innate immunity in vertebrate and invertebrate organisms. The perception of LPS in mammals is mediated through Toll-like receptors, such as TLR4, that have pattern recognition capabilities (Anderson, 2000; Nurnberger and Brunner, 2002). Recognition of PAMPS by plant cells may involve similar receptor proteins for example; TLRs are known to be involved in the responses of plants to flagellin (Dangl and Jones, 2001; Gomez-Gomez and Boller, 2000). As all forms of immunity in plants may be classed as innate, the response to LPS and other PAMPS should be considered an expression of a basal resistance which must be overcome by any microbe attempting to parasitise plants. Superimposed on this PAMP-mediated basal resistance are the more specific gene-for-gene interactions that control varietal resistance and the HR.

The proteins which control the co-ordination of the plant's response and which may be targets of effectors delivered into plant cells by bacterial pathogens are poorly defined. Recent landmark studies have highlighted the importance of post-transcriptional modification (PTM) and protein relocation as components of the plant's response (Boyes et al., 1998; Romeis et al., 1999; Nimchuk et al., 2000). Comparative proteomics offers the opportunity to understand signalling hierarchies activated during the plant's perception of an invading microbe, inform which post-translational modifications occur and measure relative protein abundance not possible through the correlative examination of transcript abundance.

Using an integrated comparative proteomics and transcriptomics approach we are dissecting the signalling and response pathways operating during basal and gene-for-gene mediated resistance in the model pathosystem *P.s.* pv. *tomato* strain DC3000 and *Arabidopsis thaliana*. The compatible interaction between DC3000 and *Arabidopsis* accession Columbia (Col-5) allows us to examine the establishment of successful parasitism. By analysis of a transconjugant of DC3000 expressing *avrRpm1* we investigate the gene-for-gene interaction through the cognate *R*-gene *RPM1* in Col-5 (Grant et al., 1995). The HR induced by the *avrRpm1*/*RPM1* interaction is compared with basal resistance through examination of responses to the *hrpA* mutant of DC3000. We recently reported results of experiments addressing transcript profiles determined by differential cDNA AFLP display (de Torres et al., 2003). Key findings were (1) that certain transcripts were upregulated in response to bacterial challenge irrespective of virulence and, (2) transcripts specific to the HR were induced relatively late in the infection process, subsequent to increases in cytosolic calcium and reactive oxygen species. The delayed induction of transcripts suggested preformed components and possibly post-translational modifications contributed to the early events associated with induced resistance. In this article

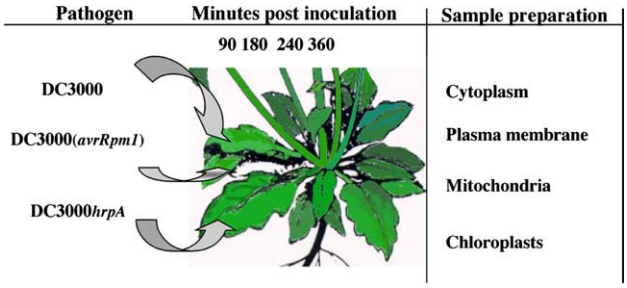

Fig. 1. Summary of experimental method. Five week old leaves of *A. thaliana* were challenged with near isogenic lines of *P. syringae* pv. *tomato* DC3000 (DC3000), designed to elicit different defence responses.

we focus on early changes in the proteome by the analysis of challenged tissues 1.5, 3, 4 and 6 h after inoculation as summarised in Fig. 1, and relate these to changes in the transcriptome identified by Affymetrix GeneChip analysis 2 and 4 h after bacterial challenges. The first proteomics profile completed is described here, involving comparison of total soluble proteins. We specifically focus upon two subsets of proteins involved in redox responses, glutathione S transferases (E.C. 2.5.1.18) and peroxiredoxins (Prxs) (E.C. 1.11.1), and use these examples to highlight complexities of comparative proteomics and transcriptomics.

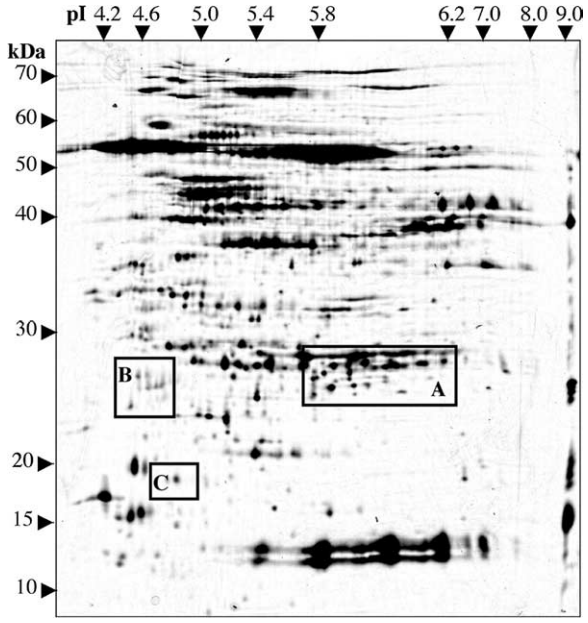

Fig. 2. Representative 2D SDS gel resolving total soluble proteins of *Arabidopsis* leaf tissue 4 hpi after challenge with DC3000*hrpA*. The first dimension was focussed across a 3–9 pH unit range and the second dimension resolved proteins between 10 and 70 kDa. Boxed regions represent; A – GSTs proteins, B – 2-cys Prxs, C – Prx IIE. Proteins >50 kDa were excluded from this analysis due to contamination by rubisco large subunit and/or poor resolution.

2. Results

2.1. Proteomics

2.1.1. System development

Methods were developed to allow reproducible production of 2D IEF/SDS PAGE gels for each extract of total soluble proteins. An essential feature of the design of our experiment was the preparation of gels from three replicate samples of challenged tissue. The triplicate analyses allowed the differences between protein abundance determined to be assessed statistically. In this way critical differences between treatments were resolved.

A typical 2D gel illustrating the resolution of protein spots is presented in Fig. 2. Regions of gels containing proteins greater than 50 kDa were poorly resolved mainly due to the presence of high concentrations of the large subunit of Rubisco. Following removal of the >50 kDa region and additional saturated spots, approximately 800 proteins were matched on each of the 45 gels and were included in the analysis. In total, 120 spots (~5%) showed some statistically significant changes in density between treatments and time points ($P < 0.05$). Two subsets of proteins, which consistently showed clear differences in abundance after various challenges and time intervals, were glutathione S-transferases (GSTs) and peroxiredoxins (Prxs). Both of these groups of enzymes were considered likely to have significant roles in the regulation of redox conditions within infected tissue. The features of the proteins identified and the designations of their encoding genes are summarised in Table 1.

2.1.2. Changes in glutathione S-transferases

The positions of gel spots identified as isoforms of GSTs from peptide MS fingerprints and MS/MS sequence data are shown in Fig. 3. Proteins encoded by GSTF2, GSTF6, GSTF7, and GSTF8 all changed in abundance after bacterial challenge, irrespective of the type of interaction with *Arabidopsis*. Significantly, charge isomers of GSTF6, 7 and 8 were resolved in samples from all tissues including those receiving mock inoculation. The acidic and basic forms of the proteins are designated a and b. The shifts in pI observed in our gels, between acidic and more basic forms of GSTs F6, F7 and F8 are likely to be due to oxidation of methionine residues. This modification was observed in the mass spectra of GSTF8 at residues Met64, Met84, Met173 and Met176 and less frequently in GSTF6 (Met126) and GSTF7 (Met99). No oxidation of methionine was observed in GSTF2 mass spectra. The patterns of oxidation are in agreement with the observed shifts in pI with more oxidation leading to increasingly acidic spots.

Differences found in protein abundance, as quantified from spot densities, are reported in Figs. 4–7. Note that

Table 1
Summary of GSTs and Prx identified in this study

Protein		Gene MIPS code	Observed Mr	Predicted Mr	Observed p*I*	Predicted p*I*	Subcellular location
GSTF2		At4g02520	28.4	24.1	5.95	5.92	Membrane associated (Zettl et al., 1994)
GSTF6	a	At1g02930	27.4	23.4	5.85	6.17	Assumed to be cytosolic
	b		27.5		5.79		
GSTF7	a	At1g02920	27.7	23.5	5.90	6.14	Assumed to be cytosolic
	b		28.0		5.98		
GSTF8	a	At2g47730	26.9	29.2	5.70	8.90	Chloroplast (Wagner et al., 2002)
	b		26.8	(24.1)	5.85	(6.09)	
PrxA		At3g11630	24.8	29.1	4.7	4.91	Chloroplast (Horling et al., 2003)
(main spot)			25.9	(22.8)	4.8	(5.01)	
PrxB		At5g06290	26.5	29.6	4.5	4.71	Cloroplast (Horling et al., 2003)
				(24.6)		(5.07)	
Prx II E		At3g52960	19.3	24.2	4.9	5.03	Chloroplast (Horling et al., 2003)
				(17.3)		(5.02)	

Mass and p*I* predictions in brackets are for the mature chloroplast proteins. Some of the discrepancies in mass observed could be due to removal of the transit peptides for the chloroplast localised proteins. Post-translational modifications are probably responsible for gain of mass and alterations to p*I*.

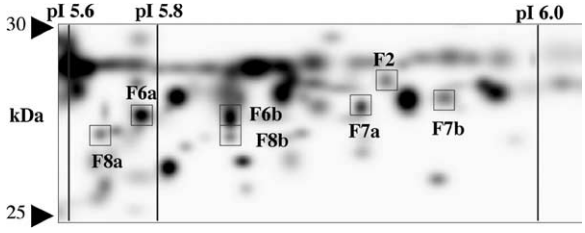

Fig. 3. Location of the forms of GSTs from the soluble protein fraction of *Arabidopsis* leaves identified in this study. The image is a composite of gels. Where two spots were identified as the same protein they are labelled as follows; 'a' (acidic) and 'b' (basic). F2; GSTF2, F6a and F6b; GSTF6, F7a and F7b; GSTF7, F8a and F8b; GSTF8.

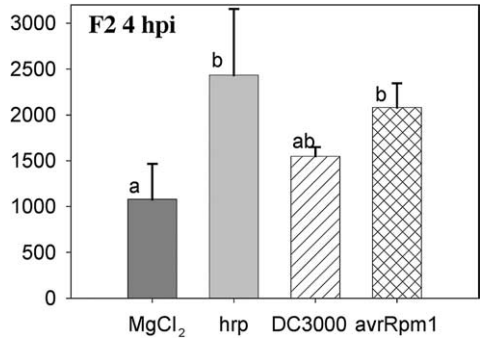

Fig. 4. Changes in spot density for GSTF2 determined at 4 hpi with $MgCl_2$ (10 mM, mock inoculation); DC3000*hrpA* mutant; DC3000 and DC3000(*avrRpm1*). Data record the mean values from three gels per treatment, bars = SD, different letters represents treatments that are significantly different ($P < 0.05$, Student's *t*-test).

data from only those time points at which statistically significant differences between inocula were found are presented. Increases in GSTF2 were found after all bacterial challenges by 4 h after inoculation, being greatest in response to the *hrp* mutant (Fig. 4). There were no significant differences between GSTF2 levels at earlier time points or after 6 h (data not shown). In contrast, two charge isomers of GSTF6 were identified and both the acidic (a) and basic (b) forms of GSTF6 increased more rapidly than GSTF2 in all bacterial challenges (Fig. 5). A significant increase in GSTF6a protein in leaves challenged with bacteria rather than the mock inoculation was detected at 3 hpi, and abundance slowly increased over the subsequent 3 h (Fig. 5). The more basic form of GSTF6 (b) showed significant increase from mock inoculation only at 4 hpi. The accumulation of GSTF6b was transient and indistinguishable from mock challenge at 6 hpi. Two charge isomers of GSTF7 were also detected, but the kinetics of accumulation differed from GSTF6. GSTF7 was significantly elevated in all bacterial challenges 3 hpi, and further accumulated to 6 hpi (Fig. 6).

In contrast to the other GSTs, increases in abundance of GSTF8a (compared with the $MgCl_2$ mock inoculation), were only recorded during the HR activated by the *avrRpm1*/*RPM1* interaction. Data for GSTF8a and GSTF8b 4 hpi are compared in Fig. 7, which illustrates that no changes in abundance were found with the basic form at this time. Our data suggest the acidic form of GSTF8 appears to be specifically associated with tissue undergoing the hypersensitive response.

2.1.3. Changes in peroxiredoxins

The second family of enzymes to be highlighted for statistically significant differences between protein abundance in mock and bacterial suspension inoculations were the Prxs. The regions of gels found to contain the Prxs are shown in Fig. 2 and in more detail in Figs. 8 and 9. The lower MW Prx was found to be Prx IIE and this increased after all bacterial challenges by 4 hpi (Fig. 8).

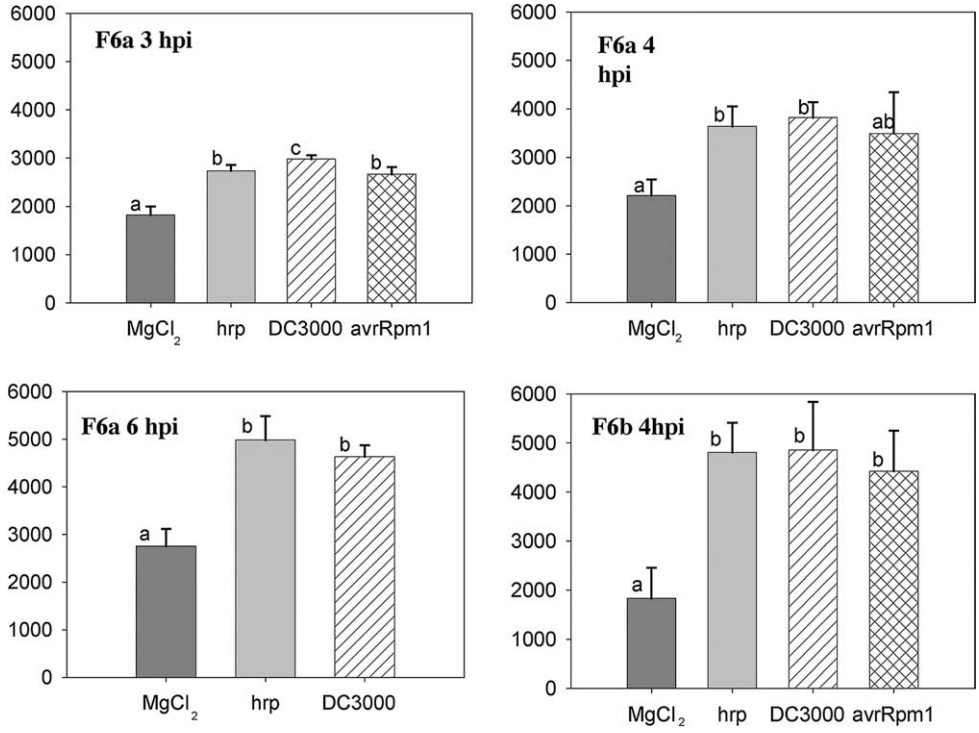

Fig. 5. Changes in spot density for GSTF6 acidic (a) and basic (b) forms, determined at 3, 4 and 6 hpi with $MgCl_2$ (10 mM, mock inoculation); DC3000*hrpA* mutant; DC3000 and DC3000(*avrRpm1*). Data record the mean values from three gels per treatment, bars = SD, different letters identify treatments that are significantly different ($P < 0.05$, Student's *t*-test).

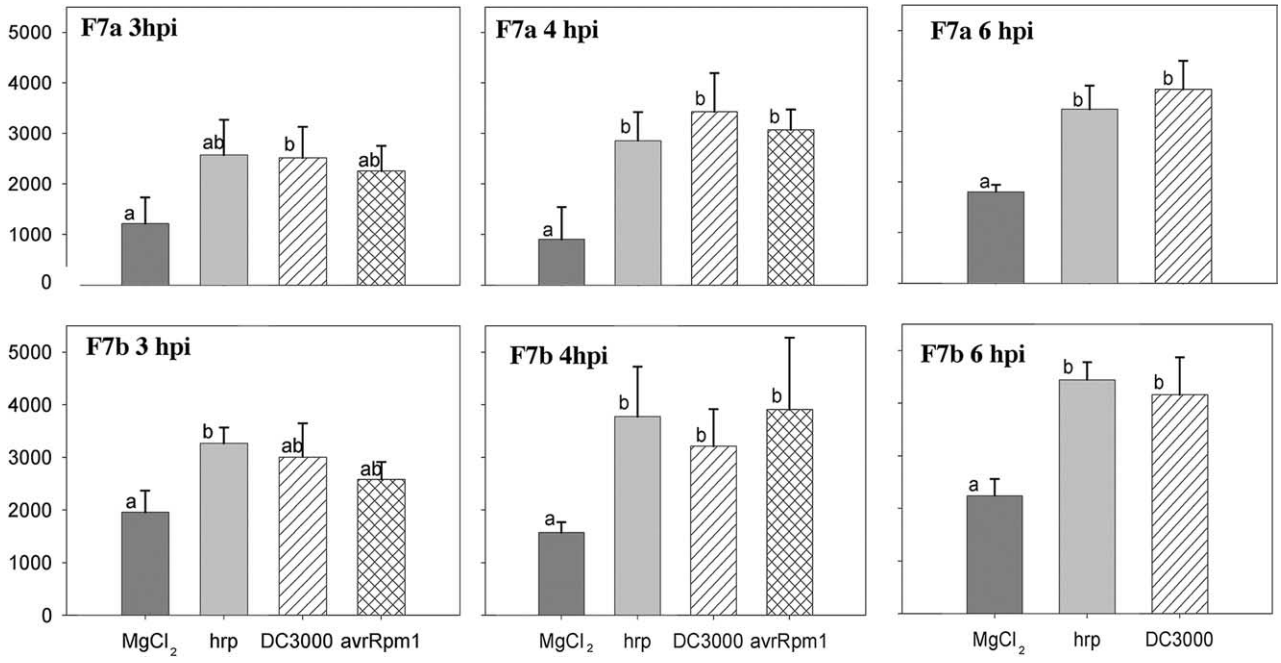

Fig. 6. Changes in spot density for GSTF7 acidic (a) and basic (b) forms, determined at 3, 4 and 6 hpi with $MgCl_2$ (10 mM, mock inoculation); DC3000*hrpA* mutant; DC3000 and DC3000(*avrRpm1*). Data record the mean values from three gels per treatment, bars = SD, different letters represent challenges that are significantly different for that spot at each timepoint ($P < 0.05$, Student's *t*-test).

A more complex pattern was resolved for the two closely related 2-cys Prx isoforms, PrxA and PrxB (mature proteins differing by only seven amino acids) encoded by *At3g11630* and *At5g06290*, respectively. Forms of the 2-cys Prxs were identified as separate species and also as a mixed spot in all leaf samples

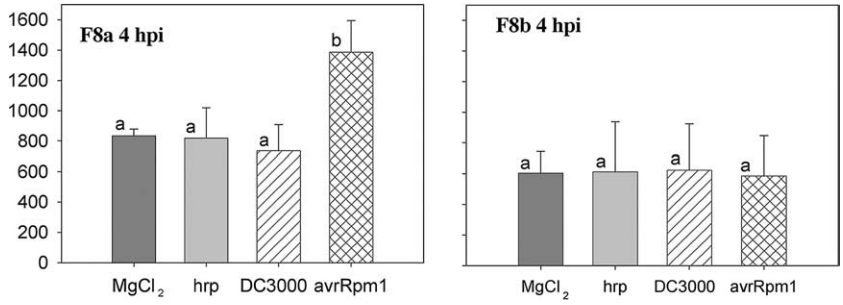

Fig. 7. Changes in spot density for GSTF8 acidic (a) and basic (b) forms, determined at 4 hpi with $MgCl_2$ (10 mM, mock inoculation); DC3000*hrpA* mutant; DC3000 and DC3000(*avrRpm1*). Data record the mean values from three gels per treatment, bars = SD, different letters correspond to spots that differ significantly at the time of that bacterial challenge ($P < 0.05$, Student's *t*-test).

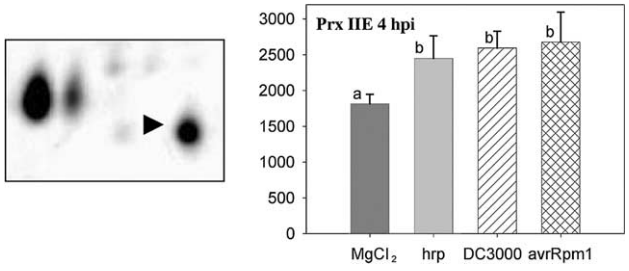

Fig. 8. Representative gel showing peroxiredoxin IIE (PrxIIE, marked with arrow). The histogram shows changes in spot density for PrxIIE, determined at 4 hpi with $MgCl_2$ (10 mM, mock inoculation); DC3000*hrpA* mutant; DC3000 and DC3000(*avrRpm1*). Data record the mean values from three gels per treatment, bars = SD, different letters represent significantly different responses ($P < 0.05$, Student's *t*-test).

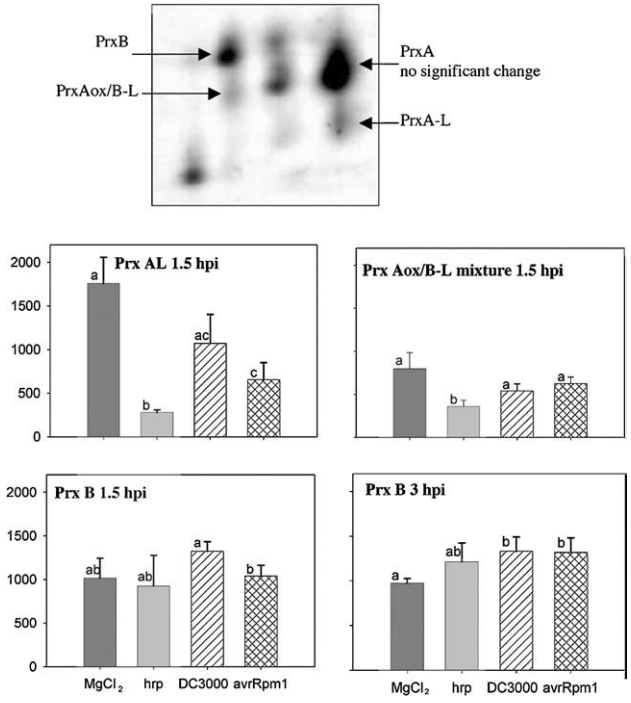

Fig. 9. A representative gel showing peroxiredoxins A and B (Prx A, Prx B). Data record the average of three gels per treatment with the error bar marking the standard deviation. Letters summarise Student's *t*-test, *P*-values less than 0.05 were considered significant.

(Fig. 9). The mixed spot comprised a low MW form of PrxB (designated PrxB-L) and a more acidic form of PrxA (termed PrxA-ox). The likely cause of the spot-shifts to varying pIs observed for 2-cys PrxA is over-oxidation of the cysteine residue within the active site (hence the PrxA-ox designation, reviewed by Wood et al. (2003)). Overoxidised, inactive, forms of Prxs are visible in 2D gels through a shift to a more acidic pI as demonstrated by Woo et al. (2003). The tryptic peptide containing the peroxidatic cysteine can be predicted, and has been observed in our mass spectra (data not shown). A possible cause of the loss of mass between the main PrxA and B spots and the lower, differential spots (designated PrxA-L and PrxB-L), is a C-terminal truncation of ~13 amino acids as previously observed in related Prx from erythrocytes and yeast (Koo et al., 2002; Cha et al., 2000). Changes in abundance of PrxB followed a pattern similar to that observed with PrxIIE, (and also in general the GSTs). Increases over mock inoculation were observed after all bacterial treatments (Fig. 8). Intriguingly, although a strong spot corresponding to PrxA showed no significant change after inoculation, a lower MW form PrxA-L was significantly reduced following bacterial challenge and in particular after inoculation with the *hrp* mutant (Fig. 8). A similar reduction in abundance of the protein mixture (PrxA-ox and PrxB-L) was recorded in tissues challenged with the non-pathogenic *hrp* mutant.

2.2. Transcriptomics

Expression profiles recorded for the genes encoding the GSTs and Prx proteins highlighted by our proteomic analysis are summarised in Table 2. Previous work had shown that the engagement of transcripts was very similar after challenge with DC3000, DC3000(*avrRpm1*) and the *hrp* mutant within 2 hpi (de Torres et al., 2003). Therefore, it was not considered necessary to include DC3000 or DC3000(*avrRpm1*) in the Affymetrix Gene-

Table 2
Summary of signal values (mean ± SD) recorded following global scaling using MAS 5.0 for the Affymetrix transcription data corresponding to the genes used in this study

Gene	$MgCl_2$ 2 hpi	*hrpA* 2 hpi	$MgCl_2$ 4 hpi	*hrpA* 4 hpi	DC3000 4 hpi	*avrRpm1* 4 hpi
Signal values after mock challenge or inoculation with bacterial suspensions						
GSTF2	2302 ± 147 a	2630 ± 172 a	2495 ± 150 a	2747 ± 844 a	2923 ± 802 a	3114 ± 704 a
GSTF6/7	3360 ± 255 a	3702 ± 185 a	2993 ± 157 a	4045 ± 1826 a	4316 ± 1487 a	4044 ± 1121 a
GSTF8	**2381 ± 91 a**	**3513 ± 140 b**	2500 ± 246 a	3157 ± 780 a	3297 ± 828 a	3743 ± 538 a
Prx A	1329 ± 243 a	1368 ± 148 a	1076 ± 225 a	980 ± 158 a	975 ± 123 a	884 ± 247 a
Prx B	394 ± 102 a	357 ± 96 a	**289 ± 28 a**	**194 ± 68 b**	**191 ± 28 b**	**169 ± 22 b**
PrxIIE	235 ± 31 a	303 ± 81 a	**257 ± 3 a**	**238 ± 30 ab**	**231 ± 11 b**	**187 ± 23 c**

Experiments were undertaken using the same inocula and growth conditions as used in the proteomics profiling. Significant differences in transcript accumulation between different treatments are shown in bold (Student's *t*-test $P < 0.05$). The probe set (262119-S-at) cross hybridises to both *GST6* and *GST7*, therefore these genes cannot be distinguished. All raw data are available at http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid = 59.

Chip experiment at 2 h. The complete Affymetrix data based upon triplicate GeneChip experiments from the leaves sampled at 2 and 4 hpi, using exactly the same inocula and growth conditions as for the proteomic profiling are available from the Nottingham *Arabidopsis* Stock Centre (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid = 59).

Increases in GSTF2, GSTF6 and GSTF7 proteins were not correlated with any significant changes in transcription of their corresponding genes at 2 or 4 hpi with any pathogen strain. It should be noted that transcripts of the tandemly localised *GSTF6* and *GSTF7* cannot be distinguished in the transcript profile as they cross hybridise to the same probe-set. The only difference from mock challenged tissue was a significant accumulation of *GSTF8* at 2 hpi with bacterial inoculation. Despite the lack of statistically significant differences ($P < 0.05$), we observed a clear trend towards increased *GST* transcript abundance as has been previously noted on RNA blots (Lieberherr et al., 2003). For example, the percentage increases recorded 4 hpi with DC3000(*avrRpm1*) compared with the mock control for *GSTs F*2, *F*6 and *F*8 were 25%, 35% and 49%, respectively. Note that previous studies have shown by RNA blots that *GSTs* are not expressed to high levels in uninoculated tissue (Lieberherr et al., 2003). The abundance of transcripts we observed following inoculation with $MgCl_2$ alone indicates therefore that these *GST*s also respond to the abiotic stress of infiltration.

The absolute levels of transcripts corresponding to *PrxB* and *PrxIIE* were ~4 times lower than *PrxA*. These levels are consistent with the observed differences in spot intensities of the resultant gene products, PrxB and PrxIIE relative to the more abundant PrxA (Fig. 8). Although transcript levels of *PrxA* and *PrxIIE* show no significant change, *PrxB* expression was significantly repressed at 4 hpi with all bacterial strains, suggesting that the observed reduction in PrxB following pathogen challenge (Fig. 8) is in part related to transcriptional suppression.

Table 3 provides a summary comparing the protein and transcript abundance data recorded for the different members of the GST and Prx families. In general, a good correlation was found between the protein and mRNA levels recorded for Prxs. However, in the GST family there were few parallels between changes observed at the transcript and protein levels, particularly when PTMs are considered.

Table 3
Summary of differences in abundance of proteins and transcripts recorded after bacterial challenge compared with mock inoculation

Gene and protein	Difference in transcript abundance at 2 hpi	Difference in transcript abundance at 4 hpi	Difference in spot density at 4 hpi
GSTF2	None	Increase (T)	Increase*
GSTF6/7	None	Increase (T)	–
F6a	–	–	Increase*
F6b	–	–	Increase*
F7a	–	–	Increase*
F7b	–	–	Increase*
GSTF8	Increase*	Increase (T, *avrRpm1*)	-
F8a	–	–	Increase* (*avrRpm1*)
F8b	–	–	None
PrxA	None	None	None
A-L	–	–	Decrease* (*hrp* mutant)
PrxB	None	Decrease*	None
PrxIIE	Increase*	Decrease* (*avrRpm1*)	Increase*

Where increases or decreases were observed they are classified as being significantly different (*) or as trends (T), specific treatments are indicated in brackets.

3. Discussion

3.1. Proteomics of the defence response

Two clear advantages of proteomic analyses over transcriptional profiling are (i) differential abundance of proteins actually present at the time of sampling are distinguished and (ii) different forms of the same protein can be resolved. Analysis of the spot behaviour from this study underlines the dynamic nature of the defence proteome. Over 120 differential spots were identified over the time course, representing changes in ~5% of the resolved proteome. Of the 53 proteins identified, 24% were found to map to two or more gel spots. For the two protein families analysed in detail here, we hypothesise that oxidation of methionine residues in the GST family, and proteolysis and oxidation of cysteine residues in the 2-cys Prxs, could be responsible for the spot shifts observed. We cannot, however, rule out the possibility that other PTMs contribute to the spot shifts recorded.

3.2. Function of GSTs

GSTs are a large family of enzymes that catalyze the addition of the tripeptide glutathione (GSH) to a number of electrophilic compounds and constitute one of the primary defence mechanisms against cellular damage by these reactive species (reviewed in Marrs, 1996; McGonigle et al., 2000; Rinaldi et al., 2002). Members of the GST family have long been used as markers of stress; their best characterised function is the detoxification of endo- and xenobiotics. Little is currently known about the role of various GSTs in normal physiological and developmental processes (Smith et al., 2003). Some GSTs have been shown to have glutathione peroxidase activity but plant GPX are slow to catalyse H_2O_2 in contrast to lipid and alkyl peroxides, which are rapidly catalysed (Eshdat et al., 1997; Noctor et al., 2002). GSTs appear to be ubiquitous in plants and *Arabidopsis*, like other organisms, encodes a large GST gene family, comprising 53 GST genes distributed across the six sequence related classes, Phi (F), Zeta (Z), Tau (U), Theta (T), Lambda (L) and glutathione-dependent dehydroascorbate reductases (Edwards et al., 2000; Dixon et al., 2002; Wagner et al., 2002).

All four of the GSTs discussed here are members of the Phi class and are reported to possess both transferase and peroxidase activities. Heterogeneously expressed *At* GSTF2 and *At* GSTF8 catalyze the glutathione-dependent detoxification of the cytotoxic linoleic acid hydroperoxide, 13-hydroperoxy-*cis*-9, *trans*-11-octadienoic acid, to the respective alcohol (Wagner et al., 2002). Interestingly, the 4 GSTs described here have different cellular addresses. GSTF6 and GSTF7 are assumed to be cytosolic enzymes and GSTF2 was originally isolated as an auxin binding protein localised to the membrane fraction of *Arabidopsis* (Zettl et al., 1994; Smith et al., 2003). Uniquely, GSTF8 has been putatively localised to the chloroplast through recognition of a signal peptide (Wagner et al., 2002). GSTF8 was the only GST to show specificity for the *R*-gene response. Other transcripts specific to *RPM1* elicitation encode products predicted to be localised to the chloroplast (de Torres et al., 2003). Our ongoing proteomics profiling includes analysis of fractionated organelles and these experiments should highlight the significance of protein localisation. It is also notable that of these four GSTs only GSTF8 possesses a cysteine residue (cys139), which could be subject to oxidation, perhaps making these enzymes less susceptible to oxidative damage. The oxidation of methionine residues by ROS has been proposed as an antioxidant scavenging mechanism, or to be involved in cell signalling and to regulate enzyme activities (Stadtman et al., 2003).

Most GSTs have been characterized through their rapid transcriptional regulation, particularly in response to stress treatments (Marrs, 1996; Wagner et al., 2002). Following pathogen challenge a number of GST transcripts are rapidly up-regulated, characteristically preceding the induction of pathogenesis related (PR) transcripts (Mauch and Dudler, 1993; Greenberg et al., 1994; Alvarez et al., 1998; Maleck et al., 2000). These studies have led to the conclusion that certain GSTs may play a role in redox control under conditions of oxidative stress, limiting the extent of cell death during the HR. Predominant amongst these is *GSTF8*, which is induced by H_2O_2 through the activation of MPK3/MPK6 (Kovtun et al., 2000) and has a promoter containing an *as*-1 motif which is implicated in response to oxidative stress (Garreton et al., 2002). Previous studies on *GST* regulation in defence responses have failed to account for basal responses to bacterial challenge and have correlated *GST* changes with avirulent or virulent pathogens relative to control. Our transcriptome data suggest that most *GSTs* respond to the abiotic stress from the mock challenge. In contrast, all bacterial challenges induce higher levels of GST protein relative to mock infiltration, although increases were not usually evident until 4 hpi. An exception to this was the *GSTF8* transcript, which showed rapid induction kinetics within 2 hpi, and GSTF8 was significantly elevated by 4 hpi. The dynamics of *GSTF8* supports previous studies in our laboratory which suggests that initial transcriptional re-programming in response to PAMP recognition acts to prime the host and transcription may continue to increase in specific interactions (de Torres et al., 2003). GSTF8 also responded differentially with respect to the avirulent challenge, in which the GSTF8a isomer increased 4 hpi, although at this time point no significant increase in *GSTF8* was observed.

3.3. Function of Prxs

Prxs constitute a newly described family of antioxidant enzymes. In contrast to other peroxidases, Prxs do

not use redox cofactors such as metal or prosthetic groups, but reduce hydrogen peroxides and alkyl peroxides to water and alcohols (Schroder and Ponting, 1998). The seven *Prx* expressed in *Arabidopsis* shoots are divided into three classes containing two catalytic cysteine residues: (a) 2-cys Prx; (b) Prx Q; (c) Prx II (Horling et al., 2003; Dietz, 2003). Heterogenously expressed Prxs reduce peroxide (Horling et al., 2003) however, their affinity for peroxide is orders of magnitude lower than that of ascorbate peroxide, catalase and glutathione peroxidase (Hofmann et al., 2002; Hillas et al., 2000).

All three Prxs discussed here are localised to the chloroplast (Baier and Dietz, 1997; Horling et al., 2003). A possible role for 2-cys prx in defence signalling lies in their ability to reduce reactive nitrogen peroxides, such as peroxynitrite (Bryk et al., 2000), generated during incompatible interactions. The rate of reaction of bacterial 2-cys Prx is sufficient to detoxify peroxynitrite fast enough to prevent the oxidation of other susceptible molecules (Bryk et al., 2000). A similar protective role against RNS for *Arabidopsis* 2-cys Prx A is supported by complementation studies in yeast (Sakamoto et al., 2003). Antisense mediated suppression of 2-cys Prx leads to increased transcription of other antioxidant systems, possibly mediated through a more highly oxidised ascorbate pool (Baier et al., 2000). In common with GSTF2 and F8, heterologously expressed 2-cys Prx reduces linolenic acid hydroperoxide and also phosphatidylcholine dilinoleoyl hydroperoxide (Dietz, 2003).

The occurrence of the lower MW form of PrxA (and possibly PrxB-L in the mixed spot) is strongly decreased in the response to the *hrp* mutant but less so in reactions to bacterial strains with functional TTSS. If targeted proteolysis is responsible for the occurrence of PrxA-L, then this process is inhibited during the basal defence response and this suppression is overcome by TTSS effectors. C-terminally truncated forms of Prx such as PrxA-L and B-L are more resistant to overoxidation by peroxide (Koo et al., 2002).

4. Concluding remarks

Attempts to quantify changes in plant pathogen interactions as undertaken here are technically challenging. Both compatible and incompatible interactions rely upon the co-ordinated responses of two biological entities. Changes in protein abundance are inherently less than those observed for corresponding genes, and the functionality of proteins are likely dependent upon post-translational modifications as much as increased abundance. Therefore, the complexity of proteomic data is huge, biological reproducibility challenging and reconstructing the sequence of events leading to the observed phenotype difficult. Here we have discussed data from only the most abundant soluble proteins, and are currently using enhanced IEF resolution, subcellular fractionation and enrichment of phosphoproteins to further investigate aspects of the *Arabidopsis* defence-related proteome.

What possible roles could GSTs and Prxs have in defence responses? Both ROS and NOS act in parallel or synergistically to modulate or modify the redox status of the cell. Hydroxyl radicals in particular, are capable of abstracting protons from lipid bilayers leading to the propagative formation of damaging peroxyl radicals. GSTs scavenge lipid hydroperoxide derivatives, but are also reported to act as potential redox "sensor-receptors" in mammals. Our data indicate that specific GSTs and lipid Prxs may play an essential role in modulating host responses to both pathogens and non-pathogenic bacteria. Glutathione peroxidases will, for example, reduce free radicals, and GSTs will conjugate glutathione to electrophilic compounds. Therefore, the induction of GSTs following bacterial challenge is consistent with a role in scavenging lipid peroxides. The different cellular addresses of the GSTs identified here suggest the discrimination of different degradation product in their respective subcellular compartments. By contrast, all Prxs identified here were localised to the chloroplast, suggesting the chloroplast, whose membranes are rich in polyunsaturated fatty acids (Gutteridge and Halliwell, 1990) are a likely site of generation of lipid peroxides which may have important signalling functions during elaboration of defence responses. Studies in our laboratory measuring low level biophoton generation (Havaux, 2003) in the defence response support the chloroplasts as an important site of lipid peroxidation (M. Grant, unpublished).

5. Experimental

5.1. General procedures

All chemicals were purchased from Sigma (Gillingham, Dorset, UK) except where specified. Maintenance of bacteria, growth of plants and treatments are detailed in de Torres et al. (2003). To minimise biological variation, plants were inoculated with each suspension in an alternating block pattern so every treatment contained the same number of plants from identical positions. Leaf samples were snap frozen in liquid nitrogen after inoculation and stored at −70 °C before extraction of total soluble protein. The complete experiment was repeated three times providing three replicate protein gels for each inoculation and time point.

5.2. Protein extraction

Leaf tissue was ground to a powder in liquid nitrogen and extraction buffer added 1.5 ml/g fresh wt (50 mM

Tris-MES pH 8.0; 0.5 M sucrose; 1 mM $MgCl_2$; 10 mM EDTA pH 8.0; 10 mM EGTA pH 8.0; 1% (v/v) 5 mM DTT; 1 mM ascorbic acid, plant protease inhibitor cocktail). The homogenate was filtered through two layers of miracloth and centrifuged at 16,000*g* for 10 min at 4 °C. Supernatants were passed through mesh filters (100 μm) into pre-cooled tubes and further centrifuged at 34,000*g* for 30 min, 4 °C. Supernatants were desalted into 5 mM Tris pH 6.8 (Amersham-Pharmacia PD 10 columns), protein concentration determined relative to BSA (Bio-Rad Bradford assay) and aliquots freeze-dried and stored at −70 °C.

5.3. First dimension-isoelectric focusing (IEF)

Analytical and preparative samples were prepared using 75 and 600 μg of protein respectively for IEF. Proteins were solublised in IEF buffer (7 M Urea (Amersham-Plus One); 2 M thiourea; 4% (w/v) CHAPS (VWR-Biochemical); 0.5% (v/v) Triton 100; 0.002% (w/v) Bromophenol Blue (Amersham-Plus One); 0.25% (v/v) TBP; 0.05% (v/v) 3–10 NL Ampholytes (Amersham)) for 1 h at room temperature, vortexing every 15 min. Insoluble matter was pelleted by centrifugation at 20,000*g* for 10 min. IEF strips (18 cm, non-linear pI 3-10, Amersham) were rehydrated for 12 h prior to IEF (programme: 500 V for 1 h, 1000 V for 1 h, 3500 V for 25,000 V h, maximum 50 uA/strip). After focusing, the strips were either immediately equilibrated for SDS–PAGE or stored at −70 °C.

5.4. Second dimension – SDS–PAGE

Focused IEF strips were equilibrated in DTT buffer for 15 min [6 M Urea (Amersham-Plus One); 50 mM Tris–HCl pH 8.8; 2% (w/v) SDS (Amersham-Plus One); 20% (v/v) Glycerol (Amersham-Plus One); 2% (w/v) DTT (VWR-AnalaR); 0.002% (w/v) Bromophenol Blue (Amersham-Plus One)]. IEF strips were then placed in iodoacetamide buffer for 10 min [as above but DTT replaced by 2.5% (w/v) iodoacetamide]. Acrylamide gels were prepared (12.5%, 1 mm thick) and run at 18 °C, 20 mA/gel for 6 h 47 min (Protean II xi system, Bio-Rad). Gels were run in simultaneous sets of four, one from each treatment, to reduce variation. Triplicate 2D gels were run for each treatment.

5.5. Image analysis

Analytical gels were silver stained (Santoni et al., 1994). Gel images from each time point were analysed together using PDQuest software (Bio-Rad). Spot detection parameters were; sensitivity 39.33, size scale 7, minimum peak 567, vertical streaking 89, horizontal streaking 57, large spot size 56 × 89. The analysis area was cropped to 10–55 kDa and saturated spots were removed. Spots were selected for identification based on significant difference between treatment pairs (*t*-test, $P < 0.05$).

5.6. Protein identification

For analysis by mass spectrometry, preparative gels were run and proteins stained with Coomassie G250. Protein spots were excised manually. Proteins within the gel-excised spots were first reduced, carboxyamidomethylated, and then digested to peptides using trypsin on a MassPrepStation (Micromass, Manchester, UK). The resulting peptides were applied to LC-MS/MS. The liquid chromatographic separation was achieved with a PepMap C18, 180 mm i.d., 15 cm column (LC Packings, Amsterdam). The mass spectrometer was a QTof (Micromass). Fragmentation data were used to search the National Center for Biotechnology Information non-redundant database using the MASCOT search engine (http://www.matrixscience.com), to distinguish between proteins from *A. thaliana*, *P. syringae* pv. *tomato* or contamination from *Homo sapiens*. Probability-based MASCOT scores were used to evaluate identifications. Only matches with $P < 0.05$ for random occurrence were considered significant. Manual sequence assignment was assisted using the peptide-sequencing feature of BioLynx (Micromass).

5.7. Transcriptome analyses

The Affymetrix data was based upon triplicate GeneChip experiments from leaves sampled at 2 and 4 hpi using exactly the same inocula and growth conditions as for proteomics profiling. RNA was isolated as previously described (de Torres et al., 2003). Total RNA was cleaned up by RNA-easy (Qiagen) according to the manufacturers instructions. cRNA was synthesized and hybridised to Affymetrix *Arabidopsis* Ath1 GeneChips at the Nottingham *Arabidopsis* Stock Centre (http://affymetrix.arabidopsis.info/). The raw GeneChip data (see http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid = 59). was analysed using Affymetrix Microarray Suite 5.0 (MAS 5.0) and globally scaled for chip to chip comparison. The probe sets representing the GST and Prx genes were all scored ''present'' by the MAS 5.0 indicating their expression was above background.

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