

# Transcriptional profiling of *Zea mays* roots reveals roles for jasmonic acid and terpenoids in resistance against *Phytophthora cinnamomi*

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**Abstract** *Phytophthora cinnamomi* is a soil-borne plant pathogen that has caused widespread damage to vulnerable native ecosystems and agriculture systems across the world and shows no sign of abating. Management of the pathogen in the natural environment is difficult and the options are limited. In order to discover more about how resistant plants are able to defend themselves against this generalist pathogen, a microarray study of plant gene expression following root inoculation with *P. cinnamomi* was undertaken. *Zea mays* was used as a resistant model plant, and microarray analysis was conducted using the Affymetrix GeneChip Maize Genome Array on root samples collected at 6- and 24-h post-inoculation. Over 300 genes were differentially expressed in inoculated roots compared with controls across the two time points. Following Gene Ontology enrichment analysis and REVIGO visualisation of the up-regulated genes, many were implicated in plant defence responses to biotic stress. Genes that were up-regulated included those involved in phytoalexin biosynthesis and jasmonic acid/ethylene biosynthesis and other defence-related genes including those encoding glutathione S-transferases and serine-protease inhibitors. Of particular interest was the identification of the two most highly up-regulated genes, terpene synthase11 (*Tps11*) and kaurene synthase2 (*An2*), which are both involved

in production of terpenoid phytoalexins. This is the first study that has investigated gene expression at a global level in roots in response to *P. cinnamomi* in a model plant species and provides valuable insights into the mechanisms involved in defence.

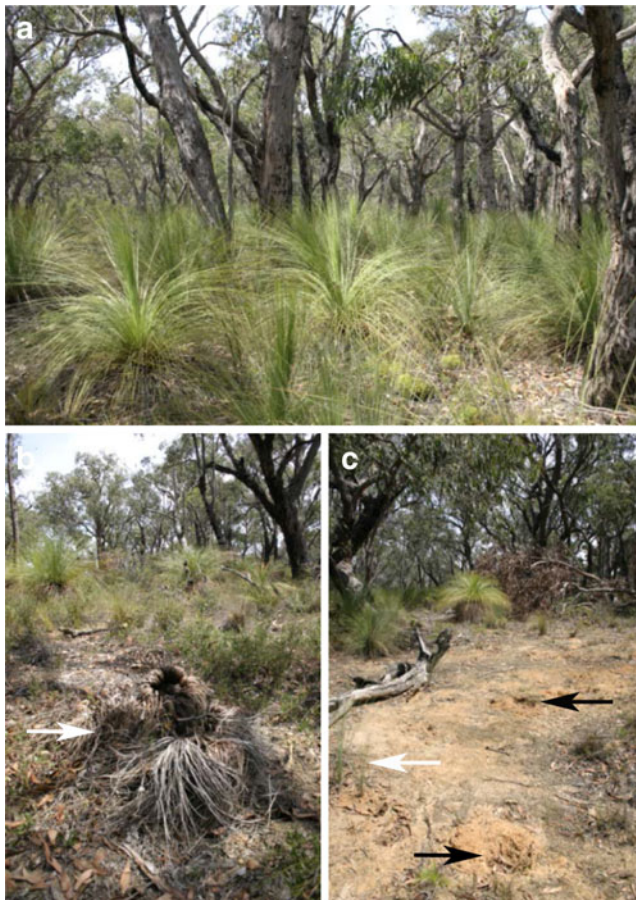
**Keywords** *P. cinnamomi* · Resistance · Microarray · *Z. mays*

## Introduction

The soil-borne oomycete *Phytophthora cinnamomi* Rands is the causal agent of disease in a wide range of hosts from diverse environments including the forests of the Mediterranean (Vettrano et al. 2005; Gómez-Aparicio et al. 2012) and the eastern USA (Balci et al. 2007; Nagle et al. 2010), and in a range of horticultural species, for example, macadamia (Mbaka et al. 2009) and avocado (Zentmyer 1984; Acosta-Muñiz et al. 2011) and the species-rich ecosystems of Australia (Cahill et al. 2008). In Australia, invasion of native vegetation communities across vast tracts of the continent has resulted in irreparable changes to their floristic and structural components (Fig. 1). The disease and its impacts are so serious that *P. cinnamomi* is listed by the Australian Government as a key-threatening process to Australia's biodiversity (Environment Australia 2001). It has been estimated, for example, that for the South-West botanical province of Western Australia over 3,000 of the 5,710 described plant species are either susceptible or highly susceptible to this generalist pathogen (Shearer et al. 2004). Resistance to *P. cinnamomi* is rare in ecosystems where the pathogen is invasive. Currently, there are no effective, long-term methods of eradicating the pathogen from either soil or plants without subsequent vegetation damage and loss

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**Fig. 1** Disease caused by *P. cinnamomi* in an Australian open forest ecosystem. **a** Healthy, uninfected vegetation at Anglesea, south-west Victoria, Australia with an intact over-storey of eucalypt species and an understorey dominated by the iconic Australian species, *Xanthorrhoea australis*. **b** Recent disease spread that has resulted in collapse and death of *X. australis* (arrow) and associated understorey species. **c** Older diseased site, with large areas of bare ground where susceptible plants have died and decomposed. Where *X. australis* was present depressions in the soil are all that remain (black arrows). Over time these bare sites become colonised by resistant species such as the sedges *Lomandra filiformis* and *Lepidosperma semiteres* (white arrow) resulting in a complete change in structural and floristic diversity (Cahill et al. 2008)

(Dunstan et al. 2010), although the use of the systemic chemical, phosphite, provides some protection against disease (Eshraghi et al. 2011).

The pathogen invades plant root systems predominantly via a motile zoospore stage that is released from asexual sporangia that are initiated during conducive environmental conditions. Root infection occurs also through root-to-root contact (Cahill et al. 2008). The biflagellate zoospores exhibit chemotaxis towards roots where they encyst at the zone of elongation and form a single germ tube. *P. cinnamomi* produces effectors that allow it to establish within host cells (Hardham and Cahill 2010; Horta et al. 2010) and in the case of the polygalacturonases enable penetration into the epidermis through degradation of anticlinal cell

walls within a very short time after cyst germination (Hardham 2005). Hyphae then penetrate both inter- and intracellularly to invade successive layers of the cortex and move into the vascular tissue to colonise both phloem and xylem and at the same time move laterally along the root in all cell layers (Cahill et al. 1989). In susceptible species, colonisation and degradation of root cells leads to lesion formation, root rot and then, over time, the inhibition of water and nutrient uptake (Dawson and Weste 1984; Aberton et al. 2001) that lead to chlorosis of leaves and then plant death (Weste and Marks 1987; Wilson et al. 2000; Laidlaw and Wilson 2003; Cahill et al. 2008).

Resistance to *P. cinnamomi* is still quite poorly understood. For the species that do restrict colonisation of roots by the pathogen, for example, grasses and sedges, there are some responses that have been found to be associated with resistance including the involvement of reactive oxygen species (ROS), cell wall fortification, an accumulation of resistance-related proteins and antimicrobial secondary metabolites, alterations in phytohormones levels, and induction of defence-related genes (Cahill et al. 1986; Rookes et al. 2008; Sánchez-Pérez et al. 2009; Panjehkeh et al. 2010; Acosta-Muñiz et al. 2011; Eshraghi et al. 2011). In contrast, the interactions of host-specific *Phytophthora* species with their hosts, for example, *Phytophthora infestans* with potato (Fry 2008) and *Phytophthora sojae* with soybean (Tyler 2007), are very well characterised. The shortfall in knowledge of interactions with *P. cinnamomi* can now be addressed by the availability of fast throughput genomic and proteomic approaches. For example, two recent studies of the interaction of *P. cinnamomi* with *Persea americana* roots have provided new insights into the interaction through the use of protein profiling (Acosta-Muñiz et al. 2011) and EST sequencing and gene expression profiling (Mahomed and van den Berg 2011).

In this paper, we have used gene expression profiling to examine the response to *P. cinnamomi* induced in roots of *Z. mays* and show that this host rapidly responds to the pathogen. We have also found in this model system that resistance resulted from an active defence response and that it was associated with the strong up-regulation of key defence-related genes, including those related to oxidative stress, hormone biosynthesis and secondary metabolite synthesis. This new information provides insights into the fundamental molecular components that comprise a resistance response against this intractable oomycete pathogen.

## Materials and methods

### Plant growth and maintenance

*Z. mays* ('Early Leaming', Lower Beechmont, QLD, Australia) seeds were germinated and grown in a soil-free

plant growth system as described in Gunning and Cahill (2009) within a controlled-temperature cabinet under high-pressure sodium lights at a Photosynthetic Photon Flux of 250–300  $\mu\text{molm}^{-2}\text{s}^{-1}$  with a 14/10-h light/dark photoperiod at 22 °C. Each box within which plants were grown contained 1 L of one third strength hydroponic nutrient solution (THC, Excel Distributors, Doncaster East, Victoria, Australia), the plants were top misted with tap water at 2-day intervals and the nutrient solution refreshed every 5 days. The apical region of the primary root of each plant was removed, using a scalpel, 5 days after seeding to encourage lateral root growth.

#### Pathogen growth, maintenance and plant inoculation

*P. cinnamomi* isolate DU67 (A2 mating type; Deakin University culture collection) was used for inoculation of *Z. mays* roots (Allardyce et al. 2012). The isolate was maintained on 10 % CV8 agar plates at 25 °C in the dark and subcultured every 10 days. Pathogen virulence was maintained by passing the isolate through *Lupinus angustifolius* radicles every 12–16 weeks.

Zoospores of *P. cinnamomi* were produced using a method based on that of Byrt and Grant (1979), and the suspension diluted to a density of  $1 \times 10^5$  spores  $\text{mL}^{-1}$  with  $\text{sdH}_2\text{O}$  prior to use as inoculum. Lateral roots were inoculated when *Z. mays* seedlings were 10 days old. For microarray experiments, a minimum of five 20- $\mu\text{L}$  droplets were applied contiguously to lateral roots, starting 0.5 cm from each root tip. Control plants were treated in an identical manner except for the use of  $\text{sdH}_2\text{O}$  as the inoculum. For microscopic analysis of the interaction over time, a single 20- $\mu\text{L}$  drop of zoospores was applied 0.5 cm from root tips.

#### Microscopic analysis of the interaction of *Z. mays* roots with *P. cinnamomi*

Using fine forceps, epidermal peels were taken from directly beneath the point of inoculation from control and inoculated *Z. mays* at half hourly intervals to 2-h post-inoculation (hpi) then 3 and 6 hpi and then from 24 hpi every 24 h until 120 hpi. The peels (one peel per root from a minimum of three roots per time point) were then mounted in distilled water on a microscope slide and viewed with light and epifluorescence microscopy (365 nm excitation and 420 nm emission) (Axioskop2, Zeiss, Clayton, Victoria, Australia). At the same time points  $\text{H}_2\text{O}_2$  production was examined using 3'3' di-aminobenzidine (DAB; 1  $\text{mgmL}^{-1}$  DAB, pH 3.5) (Thordal-Christensen et al. 1997) and additional defence responses such as the production of callose and lignin were assessed as previously described (Mauch-Mani and Slusarenko 1996; Rookes et al. 2008).

#### Microarray analysis—RNA preparation, labelling, hybridisation and scanning

*Z. mays* plants were arranged in a randomised block design (Nettleton 2006) within the growth cabinet and inoculated as described above. All replicates were conducted at the same time of day across each experimental repeat to exclude variations due to differences in circadian rhythm. Root tissue was collected from the inoculated root zones and snap frozen in liquid nitrogen prior to RNA extraction at 6 and 24 hpi. Three experimental repeats were conducted; each consisted of 60 seedlings in total, with 15 control and 15 inoculated plants at each of the two time points. All harvested root tissue within each treatment group at the two time points was pooled for each RNA extraction.

Total RNA was isolated and purified from root tissue using a TRIzol® Plus RNA Purification System (which includes a DNase 1 treatment step) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). A minimum of 15  $\mu\text{g}$  of purified total RNA per sample was prepared for microarray analysis. The 12 samples of purified total RNA were processed at the Australian Genome Research Facility, (AGRF Melbourne Node, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria). Each biological replicate was then labelled, hybridised and scanned according to prescribed protocols for the GeneChip Maize Genome Array (Affymetrix, Santa Clara, CA; [www.affymetrix.com](http://www.affymetrix.com)).

#### Bioinformatics analysis

Bioinformatic analysis of raw data was conducted using Partek® Genomics Suite™ software (Partek Incorporated, St. Louis, MO). All raw data, comprising 12 separate .CEL files, were imported into Partek® Genomics Suite™ software for gene expression data, which allowed statistical interpretation and visualisation of data. All data were normalised using Robust Multi-chip Average software, to generate an expression profile from Affymetrix data (Irizarry et al. 2003). Differentially expressed genes were defined by a fold difference of  $>1.5$  and were considered statistically significant if they had a *p* value cut-off of  $<0.05$ . Genes were annotated according to Affymetrix Maize Array Annotation file release 32.

#### Analysis of differentially regulated transcripts and promoter analysis

Gene ontology (GO) enrichment analysis of up- and down-regulated transcripts was carried out using AgriGO Singular Enrichment Analysis (Du et al. 2010) using a minimum of two mapping entries and the remainder of the parameters as specified by the programme defaults. The GO enrichment data were transferred to REVIGO (Supek et al. 2011) for reduction

and visualisation. The allowed similarity value was set at 0.5 and the *Z. mays* genome database was used as the source for the GO term sizes. For analysis of the promoter regions of genes encoding up-regulated transcripts, the 500-bp upstream regions were downloaded from the *Z. mays* Genomic Database (Dong et al. 2004; Lawrence et al. 2004) and analysed using Regulatory Sequence Analysis Tools (RSAT; van Helden 2003). Background frequencies were calculated from input data using a higher order Markov model order value of 2. Significantly over-represented 6–8 mers were assembled to identify consensus motifs.

#### Data submission to public repository

All supporting information including raw gene expression data (as .CEL files), normalised data, experimental design details, array annotation and experimental protocols were submitted to the Gene Expression Omnibus (GEO; Platform Accession Number: GPL4032) in accordance with the Minimum Information About a Microarray Experiment (MIAME) standard by the Microarray Gene Expression Data society (Brazma et al. 2001; Rayner et al. 2006). The records were assigned the accession number GSE27626.

#### Validation of microarray analysis using semi-quantitative reverse transcription-PCR

Semi-quantitative RT-PCR was conducted to validate microarray results using the same RNA samples. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed in a reaction containing Tetro reverse transcriptase, reaction buffer, and 2 µM random hexamers as per the manufacturer's specifications (Bioline, Alexandria, NSW, Australia). Primers were designed to examine the relative expression of three genes (*An2*, *Gst30* and *Opr1*) that were identified as being up-regulated in the microarray analysis as well as *Gapc2* that encodes glyceraldehyde 3-phosphate dehydrogenase for use as a control (Table S3). PCR reactions were carried out with GoTaq green master mix and reagents (Promega, Madison, WI). PCR reactions contained 1 µL of cDNA from the RT reaction and 0.5 µM of the appropriate oligonucleotides. PCR cycles consisted of an initial denaturing step of 3 min at 94 °C, followed by repetitions (18–34 cycles, depending on the primer set) of the following three steps: a 30-s denaturation step at 94 °C, 30-s annealing step ranging between 50 and 52 °C and a 1-min elongation step at 72 °C. Initial reactions were performed to determine the appropriate cycle number for analysis to be conducted within the exponential phase of the PCR reaction (data not shown). PCR products were analysed on 0.5×TBE-agarose gels and visualised using ethidium bromide staining. Digital analysis of gel images was used to calculate fold change differences (Image J software, US

National Institutes of Health, Bethesda, MD <http://imagej.nih.gov/ij/>, 1997–2011).

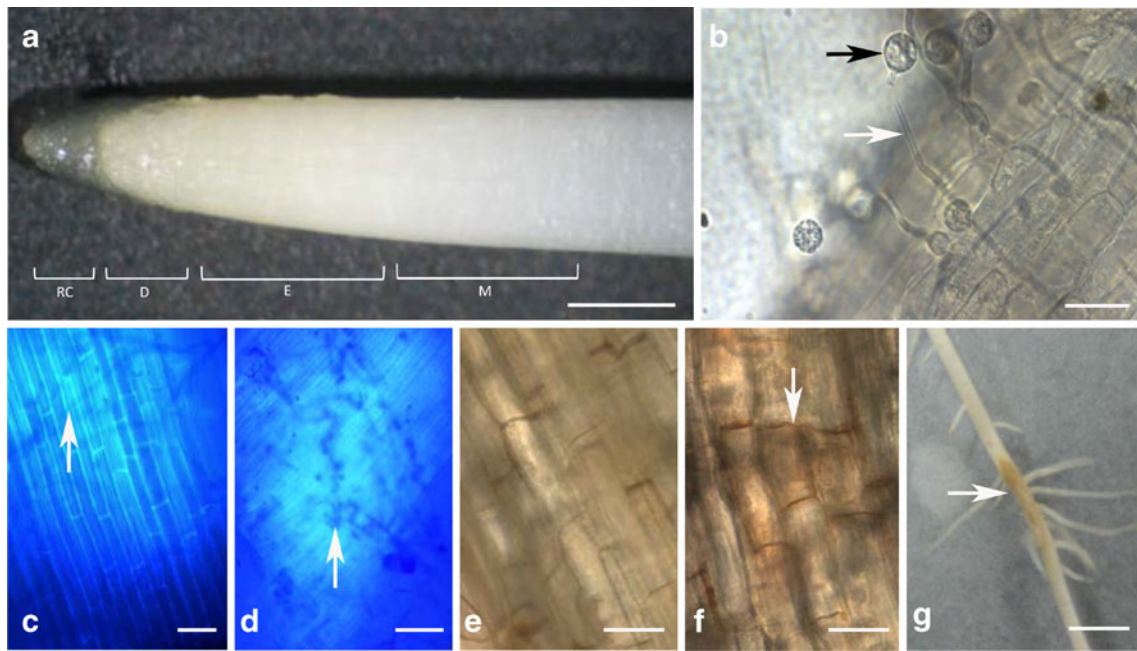
## Results

### Timing of penetration of root cells and response to infection

When grown under the conditions described, the roots of *Z. mays* were healthy and white with a clearly differentiated root cap, and the various zones of differentiation, elongation and maturation could be discerned (Fig. 2a). Following inoculation, *P. cinnamomi* zoospores were attracted to the root surface within 30 min and within 1 h had shed their flagella and encysted, subsequently forming germ tubes which penetrated the unsterilised primary root tissue within 3 h (Fig. 2b). At 6 hpi, cell wall thickening occurred directly below the point of inoculation and there was autofluorescence of epidermal cell walls (Fig. 2c). Larger, discrete areas of autofluorescence associated with hyphal penetration, as well as cell wall thickening, were apparent by 24 hpi (Fig. 2d). The production of hydrogen peroxide was examined using DAB staining. In control roots there was little staining but there was intense brown staining consistent with H<sub>2</sub>O<sub>2</sub> production concentrated in the intracellular regions of epidermal cells associated with areas of germ tube penetration (Fig. 2e, f). At 24 hpi, restricted brown lesions were found beneath the point of inoculation (Fig. 2g). An overview of observed *Z. mays* defence responses following challenge by *P. cinnamomi* is provided in Table S1. Based on these observations and previous studies (Hinch et al. 1985; Rookes et al. 2008; Allardyce et al. 2012), 6 and 24 hpi were selected for subsequent microarray analysis.

### Differential gene expression in *Z. mays* roots following inoculation with *P. cinnamomi* zoospores

Transcriptional profiling of *Z. mays* roots in response to infection with *P. cinnamomi* was conducted using the Affymetrix GeneChip® maize genome array, representing 13,339 genes. Levels of gene expression at 6 and 24 hpi in inoculated plants were compared with those of control plants. Over the two time points examined, a combined total of 317 genes were identified as being differentially expressed ( $p < 0.05$ , 1.5 fold cut-off) in inoculated root tissue, of which 257 were annotated (Table S2). By 6 hpi, 234 genes were differentially expressed in inoculated roots, of which 186 were annotated. Of these differentially expressed genes, 212 were up-regulated while 22 genes were down-regulated. By 24 hpi, the number of differentially expressed genes in inoculated roots was reduced to 83, 71 of which were annotated. Of the genes that showed differential expression at 24 hpi, 46 genes were up-regulated while 37 were down-regulated.



**Fig. 2** General appearance of *Z. mays* roots and response to infection by *P. cinnamomi*. **a** Uninoculated root tip region showing locations of the root cap (RC) the zone of differentiation (D), zone of elongation (E) and zone of maturation (M). Zoospores of *P. cinnamomi* are chemotactically attracted to the zone of elongation. Scale bar=3mm, **b** zone of elongation observed under the light microscope 180 min post-inoculation with *P. cinnamomi* zoospores. Zoospores formed walled cysts (black arrow) which extended germ tubes (white arrow) towards the root followed by their penetration of the epidermis. Scale bar=

20  $\mu$ m. **c, d** Root epidermal peels taken 6 (**c**) and 24 h (**d**) after inoculation that show autofluorescence at the site of inoculation. Hyphae can be observed in association with the autofluorescence (arrows). Scale bar=10  $\mu$ m. **e, f** Epidermal peel of control (**e**) and inoculated (**f**) root 6 h after inoculation stained with DAB. Intense DAB staining (arrow) was associated with cells and cell walls following inoculation and indicated the production of hydrogen peroxide. Scale bar=10  $\mu$ m. **g** Restricted lesion beneath the point of inoculation (arrow). Scale bar=10 mm

#### Overview of functional categories and promoter analysis of up-regulated genes

The web-based AgriGO Singular Enrichment Analysis tool was used to obtain significantly over-represented GO terms from the up-regulated gene datasets. The REVIGO web server was then used to summarise identified GO terms and visualise the data. At 6 hpi, REVIGO assigned 247 GO terms to Biological Process, 48 to Cellular Component and 99 to Molecular Function. Data were displayed as scatterplots for biological process and molecular function (Fig. 3; Fig. S1). Following analysis for biological process, several clusters of GO terms with related function were identified. At 6 hpi, a tight cluster of frequently represented biological process GO terms were associated with defence responses and responses to biotic stimulus and other organisms (Fig. 3a). A further cluster was associated with glutathione metabolism and related metabolic processes. Other categories of enriched biological process GO terms included those related to carbohydrate metabolism and terpenoid metabolism. Of the GO terms related to molecular function (Fig. 3b), those for glutathione transferase activity and other transferase activities were enriched, while others related, presumably, to activities around the cell wall included pattern binding, polysaccharide binding and chitinase activity. With fewer genes up-regulated at 24 hpi, REVIGO summarised 69 GO terms to biological

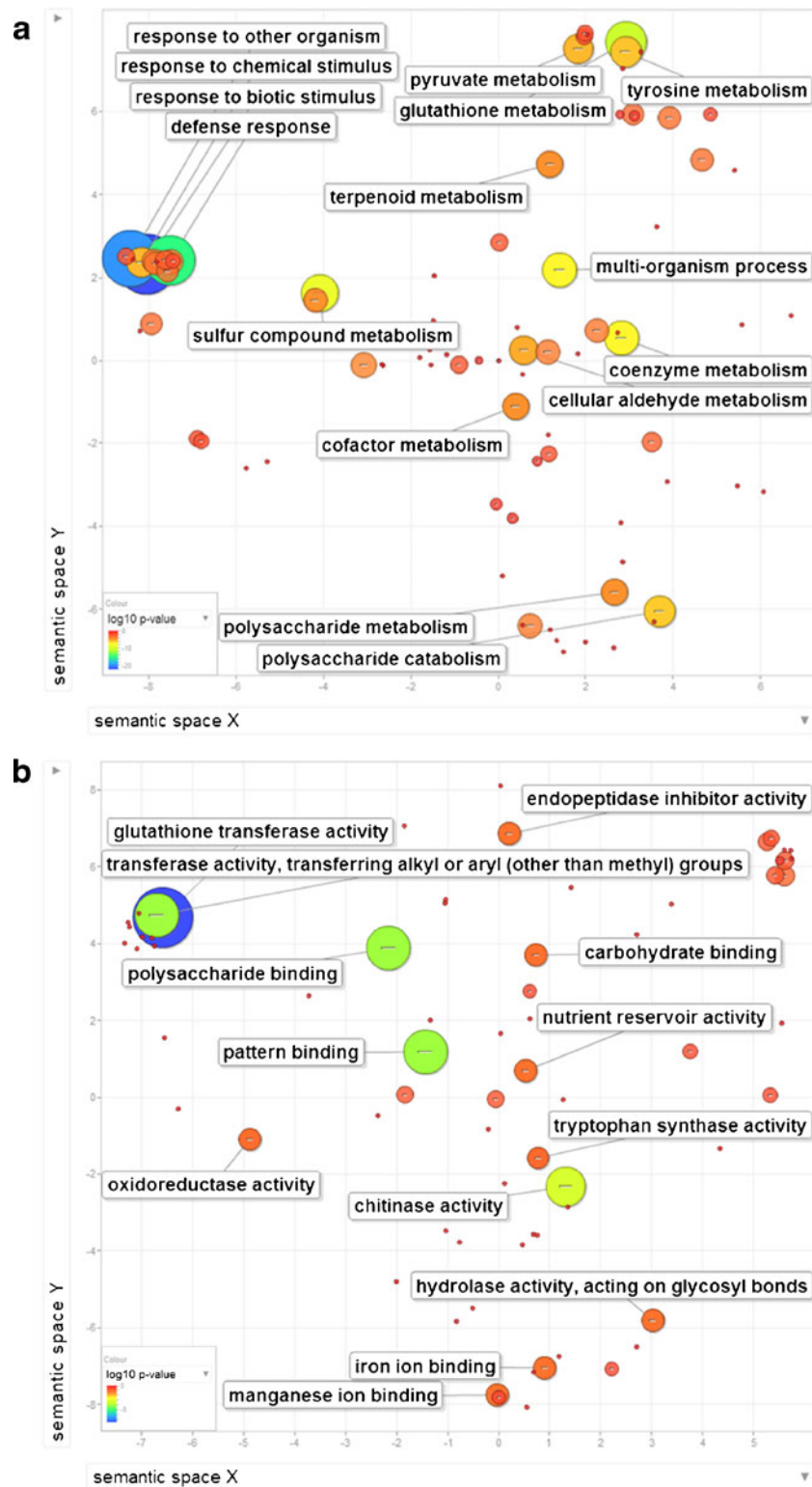
process, 24 to cellular component and 47 to molecular function. While a smaller set of GO terms were identified at 24 hpi in comparison with the 6 hpi dataset, the categories of terms across the two time points were similar (Fig. S1).

Promoter analysis revealed three motifs that were enriched in the genes that were up-regulated at 6 h (Table 1). Interestingly, the RY repeat motif that is associated with genes that are related to ABA response was found to be prominent, followed by the GCC-Box that is related to ethylene (Et) and jasmonic acid (JA) signalling, and then the A-Box motif related to sugar (carbohydrate) signalling. REVIGO and promoter analysis of down-regulated genes was also performed but due to the small number of genes that were down-regulated at the two time points the analysis tools provided limited information (data not shown).

Principal categories of genes that were found to be up-regulated in roots in response to *P. cinnamomi* at 6 and 24 h after inoculation

#### *Highly expressed genes encoding enzymes of secondary metabolite synthesis and defence-related proteins*

The two most highly expressed genes at 6 hpi were both associated with the biosynthesis of terpenoid phytoalexins: *Tps11* (Zm.14496.1.A1\_at) that encodes terpene synthase was



**Fig. 3** Genes that were up-regulated at 6 h after inoculation were subjected to GO enrichment analysis, then summarised and visualised as a scatter plot using the REVIGO Web server. **a** Summarised GO terms related to biological processes and **b** molecular function. GO terms are represented by circles and are plotted according to semantic

similarities to other GO terms (adjoining circles are most closely related). Circle size is proportional to the frequency of the GO term, while colour indicates the log<sub>10</sub> *p* value (*red* higher, *blue* lower). GO terms with a log<sub>10</sub> *p* value of  $-4.3$  or less for biological process and  $-0.96$  or less for molecular function are labelled

**Table 1** Common promoter motifs found in the up-regulated gene dataset at 6 hpi

Motif	Probability	<i>E</i> value	Significance ( $-\log E$ )	Name	Description
catgcatg	2.60E-10	9.50E-06	5.02	RY repeat	ABA responses/seed storage
gccgcc	9.10E-09	3.70E-05	4.43	GCC-BOX	Ethylene/jasmonic acid signalling
gtacgta	1.30E-08	2.00E-04	3.7	A-BOX	Sugar signalling

Upstream regions of genes were analysed using RSAT and significantly over-represented 6–8 mers were identified and ranked according to significance of occurrence. Motif descriptions according to Higo et al. (1998)

up-regulated 18.1-fold, and *An2* (ZmAffx.12.1.S1\_at) which encodes *ent*-copalyl diphosphate synthase and is associated with kauralexin production (Schmelz et al. 2011) was up-regulated 10-fold (Table 2; Table S2). By 24 hpi, the expression levels of both these genes decreased to the level of controls. A number of key defence-related genes also showed high up-regulation (>5-fold) at 6 hpi including *Wip1* (Zm186.1.S1\_at), that encodes a wound-induced proteinase inhibitor, *Pr5* (Zm.6659.1.A1\_at) that encodes the pathogenesis-related protein 5 and *Glp2* (Zm.503.1.A1\_at) which encodes the germin-like protein 2. Both *Wip1* and *Glp2* remained up-regulated at 24 hpi, although to lesser levels (3.3- and 3.4-fold, respectively).

#### *Glutathione S-transferase transcripts involved in detoxifying reactive oxygen species*

Of the annotated genes up-regulated at 6 hpi in *P. cinnamomi* inoculated *Z. mays* roots, seven were glutathione *S*-transferases (GSTs), which have previously been found to be induced under stress conditions, including pathogen attack, and serve to protect plant cells and tissues from oxidative stress caused by the generation of reactive oxygen species (Marrs 1996; Sappl et al. 2009) (Table S2). The GST genes included *Gst30* (Zm864.1.S1\_at) which was up-regulated 6.8-fold, *Gst8* (Zm.539.1.A1\_at) up-regulated 3.9-fold, *Gst24* (Zm.627.1.A1\_at) up-regulated 3.6-fold and *Gst7* (Zm.246.1.A1\_at), *Gst25* (Zm.545.1.S1\_at), *Gst23* (Zm.561.1.A1\_at) and *Gst15* (Zm.545.1.S1\_at), all of which were up-regulated between 1.6–2.0-fold. None of the GST-encoding genes were up-regulated at 24 hpi.

#### *Biosynthesis of defence-related plant hormones*

Several genes that encode enzymes involved in JA biosynthesis from linolenic acid (Turner et al. 2002) were up-regulated at 6 hpi, including *Opr1* (Zm.10676.1.S1\_at) that encodes 12-oxo-phytodienoic acid reductase 1 (induced 5.2-fold), *Aos1* (Zm.6107.1.A1\_at), allene oxide synthase 1 (1.6-fold) and a lipoxygenase gene, *LOC542495* (Zm.445.1.S1\_at; 1.5-fold) (Fig. 4). An Et biosynthesis gene, acc oxidase *Aco31* (Zm.8714.1.A1\_at) that catalyses the final step in the Et biosynthetic pathway, was up-regulated 4.1-fold at 6 hpi; whereas, the ethylene-responsive-factor-like protein *Erf1*

(Zm.11441.1.S1\_at) was down-regulated by 24 hpi (−3.1-fold). In contrast, there was no change in the expression of genes associated with salicylic acid (SA) biosynthesis or SA-signalling pathways.

#### *Genes encoding pathogenesis-related proteins and other defence-associated genes*

A number of proteins directly involved in defence against invading pathogens were differentially expressed at both 6 and 24 hpi in inoculated root tissue (Table S2). These included protease inhibitors that contribute to plant defence by inhibiting proteases produced by invading microorganisms. At 6 hpi, several protease-inhibitor type genes were induced including genes encoding two Bowman–Birk serine proteinase inhibitors (*Wip1*, Zm.186.1.S1\_at, Zm.17271.6.S1\_x\_at) that were up-regulated by 7.7- and 3.4-fold, respectively. Two thaumatin-like, pathogenesis-related protein encoding genes were also up-regulated at 6 hpi, namely *Pr5* (Zm.6659.1.A1\_at; 7.5-fold) and *Sip1* (Zm.281.1.S1\_s\_at; 4.2-fold) which has 100 % amino acid sequence identity to the anti-fungal protein, zeamatin. By 24 hpi, four putative serine-protease inhibitor genes were up-regulated, including *Wip1* (Zm.186.1.S1\_at; 3.3-fold) and the putative Bowman–Birk serine protease inhibitor gene *LOC100037814* (Zm.4270.2.A1\_a\_at; 2-fold) both of which were also up-regulated at 6 hpi, and two trypsin inhibitor genes, *LOC541688* (Zm.375.1.S1\_at) at 2.7-fold and the subtilisin-chymotrypsin inhibitor gene *Sci1* (Zm.301.1.A1\_a\_at) at 2.2-fold. A number of genes encoding chitinase proteins were up-regulated over both time points, with a propensity towards their expression at 24 hpi.

#### *Validation of differential gene expression using semi-quantitative RT-PCR*

To verify the results of the microarray analysis, semi-quantitative RT-PCR was performed to examine expression levels of three genes (*An2*, *Gst30* and *Opr1*) that were shown in the microarray analysis to display differential expression in inoculated root tissue at 6 hpi. The *Gapc2* gene was used as an internal control to confirm even RNA loading and reaction efficiency across all cDNA samples. All three genes that displayed differential gene expression in the microarray analysis were shown to exhibit similar gene

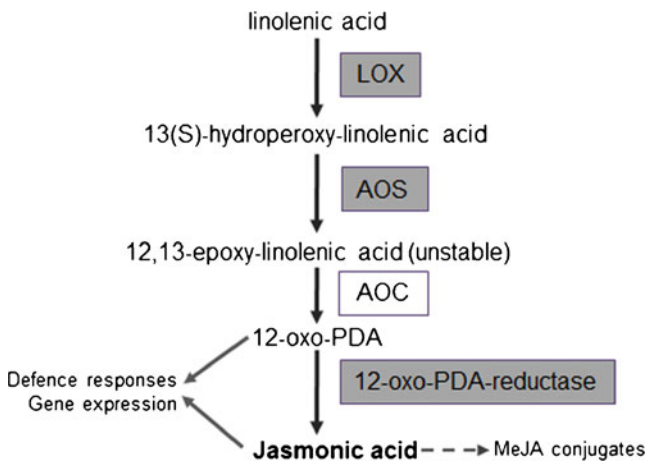
**Table 2** The 20 most highly up-regulated annotated genes in *Zea mays* roots at 6- and 24-h post-inoculation with *Phytophthora cinnamomi*

Probe set ID	Fold change	Gene title	Gene symbol
6-h dataset			
Zm.14496.1.A1_at	18.1	Terpene synthase11	tps11
ZmAffx.12.1.S1_at	10.0	Kaurene synthase2	an2
Zm.8610.1.A1_at	9.3	LOC100281076	umc2600
Zm.9297.1.A1_at	9.0	Hypothetical protein LOC100275777	LOC100275777
Zm.2136.1.S1_at	8.0	LOC100192761	TIDP3705
Zm.186.1.S1_at	7.7	Bowman–Birk-type wound-induced proteinase inhibitor Wip1	LOC100281975
Zm.10304.1.S1_at	7.7	Hypothetical protein LOC100383937	LOC100383937
Zm.6659.1.A1_at	7.5	Pathogenesis related protein-5	PR-5
Zm.503.1.A1_at	7.1	Germin-like protein 2	GLP2
Zm.8726.1.A1_at	7.0	LOC100284970	cl1774_-2(594)
Zm.9093.1.A1_at	6.8	Hypothetical protein LOC100384236	LOC100384236
Zm.864.1.S1_at	6.8	Glutathione transferase30	gst30
Zm.7909.1.A1_at	6.0	Hypothetical protein LOC100273458	LOC100273458
Zm.7654.1.A1_at	5.5	Erg28-like protein	LOC100284209
Zm.9486.1.A1_at	5.5	CHY1	LOC100283469
Zm.10586.1.A1_at	5.3	Hypothetical protein LOC100191582	LOC100191582
Zm.9644.1.A1_at	5.3	VQ motif family protein	LOC100281024
Zm.847.1.S1_at	5.3	Hypothetical protein LOC100274400	LOC100274400
Zm.10676.1.S1_at	5.2	12-oxo-phytodienoic acid reductase1	opr1
Zm.9932.1.A1_at	5.0	Hypothetical protein LOC100278059	LOC100278059
24-h dataset			
Zm.503.1.A1_at	3.4	Germin-like protein 2 <sup>a</sup>	GLP2
Zm.186.1.S1_at	3.3	Bowman–Birk-type wound-induced proteinase inhibitor Wip1 <sup>a</sup>	LOC100281975
Zm.1450.1.S1_at	3.1	Hypothetical protein LOC100275439	LOC100275439
Zm.9486.1.A1_at	3.0	CHY1 <sup>a</sup>	LOC100283469
Zm.375.1.S1_at	2.7	Trypsin inhibitor	LOC541688
Zm.9845.1.S1_at	2.4	LOC100272820	TIDP2793
Zm.1506.1.A1_at	2.3	Defence-related protein	inra2(prp)
Zm.16805.3.S1_x_at	2.3	Basic endochitinase C	LOC100281825
Zm.16805.3.S1_a_at	2.3	Hypothetical protein LOC100191592	LOC100191592
Zm.301.1.A1_a_at	2.2	Subtilisin-chymotrypsin inhibitor CI-1B and subtilisin-chymotrypsin inhibitor homolog1	LOC100281906, sci1
Zm.7654.1.A1_at	2.2	Erg28-like protein <sup>a</sup>	LOC100284209
Zm.2301.1.A1_at	2.2	LOC100281319	TIDP3344
Zm.10103.1.A1_at	2.1	LOC100275728, hypothetical protein LOC100277770, hypothetical protein, LOC100303893, and hypothetical protein LOC100381311	AY106736, LOC100277770, LOC100303893, and LOC100381311
Zm.1921.1.S1_at	2.1	Inorganic phosphate transporter 3	LOC732717
Zm.12518.1.A1_at	2.0	Hypothetical protein LOC100191976	LOC100191976
Zm.4270.2.A1_a_at	2.0	LOC100037814	umc1246
Zm.7566.1.A1_at	2.0	Nitrilase-associated protein	LOC100282364
Zm.2136.1.S1_at	1.9	LOC100192761 <sup>a</sup>	TIDP3705
Zm.2227.1.A1_at	1.9	LOC100191593	pco080661a
Zm.16805.3.S1_at	1.9	Basic endochitinase C	LOC100281825

Annotations are according to Affymetrix Maize Array Annotation file release 32

<sup>a</sup> Genes in the 24-h dataset that were also up-regulated at 6-h post-inoculation



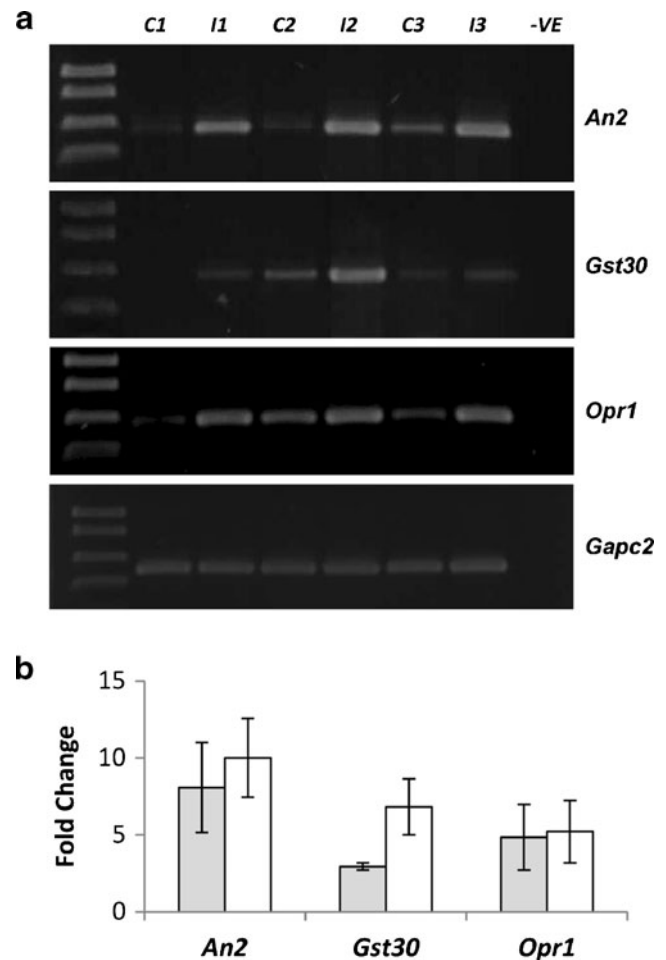


**Fig. 4** Schematic representation of the JA biosynthetic pathway including the genes found to be up-regulated in this study (grey boxes) at 6 hpi in inoculated *Z. mays* roots. Membrane derived linolenic acid is oxidised to 13-hydroperoxy-linolenic acid via the enzyme lipoxygenase (*LOX*). Allene oxide synthase (*AOS*) converts 13-hydroperoxy-linolenic acid to an unstable epoxide 12,13-epoxy-linolenic acid which rapidly becomes 12-oxo-phytodienoic acid (*12-oxo-PDA*) and defence responses may be initiated at this point (Turner et al. 2002). The enzyme 12-oxo-PDA reductase is involved in the final step of the conversion of 12-oxo-PDA to JA. JA may then be further catabolised to its methyl ester (*MeJA*). Genes up-regulated: *LOX*, 1.5-fold; *AOS*, 1.6-fold; and 12-oxo-PDA-reductase, 5.2-fold. *AOC* not differentially expressed (white box)

expression patterns in semi-quantitative RT-PCR reactions (Fig. 5a). There was some variation between the three experimental repeats but the pattern of differential gene expression was consistent. Average fold change differences in expression of the four genes were calculated through image analysis and were found to correspond with the microarray analysis results (Fig. 5b).

**Discussion**

This study provides the first insights into global gene expression changes of a plant in response to challenge by the pathogen *P. cinnamomi*. By analysing the changes in gene expression in the resistant monocot species, *Z. mays*, our analysis provides important new information on the molecular basis of resistance to this pathogen. Changes in the *Z. mays* transcriptome in response to *P. cinnamomi* infection demonstrates that the plant both recognises and responds rapidly to the pathogen by inducing a suite of key defence-related genes by 6 hpi. By 24 hpi, several defence-related genes showed reduced expression levels, or their expression had returned to basal levels, reflecting the rapid, transient nature of this response. Our observations show the rapidity with which encystment, germination and then penetration occurs (within 3 h of inoculation) in this system and that resistance requires a rapid host response. Furthermore, the up-regulation of genes associated with the production of



**Fig. 5** Semi-quantitative RT-PCR was used to validate the microarray analysis for untreated controls (*C1–C3*) and for the 6-h inoculated root samples (*I1–I3*) for each of three biological replicates. **a** The expression levels of *An2*, *Gst30* and *Opr1* were up-regulated following inoculation. **b** Comparison of fold change for each gene between the semi-quantitative RT-PCR data (grey bar) and microarray data (white bar). Data presented are the mean of the three independent replicates, and error bars represent standard error of the mean

plant-derived pathogen-specific toxins, as well as inhibitors of pathogen effectors, including a number of serine-protease inhibitors, indicates that *Z. mays* responds to penetration by *P. cinnamomi* in an active and controlled manner.

Phytoalexins have been previously recorded as being involved in the resistance responses of *Z. mays* and accumulate in response to specific elicitors derived directly from pathogens or *in vitro* elicitation (Shen et al. 2000; Doehlemann et al. 2008). Of all the up-regulated genes identified in this study, the two most highly expressed were *Tps11* and *An2* which are both associated with the biosynthesis of terpenoid phytoalexins. *Tps11* encodes (s)-beta-macrocarpene synthase, which is putatively involved in zealexin production (Huffaker et al. 2011), while *An2* encodes *ent*-copalyl diphosphate synthase and is associated with kauralexin production. Both genes have recently been shown to be highly expressed

in *Z. mays* following inoculation with several fungal pathogens including *Fusarium graminearum*, and it has been proposed that terpenoid phytoalexins are important components of defence (Huffaker et al. 2011; Schmelz et al. 2011). It is clear from these recent studies and our own that terpenoid phytoalexins are likely to be central to the *Z. mays* defence response and that these metabolites may offer alternatives for plant breeding programs and transgenic approaches towards developing disease resistant lines.

It is now well established that the SA pathway is usually associated with resistance to biotrophic pathogens, while the JA/Et pathways are important for defence against necrotrophs (Glazebrook 2005; Trusov et al. 2009; Antico et al. 2012). Our study, which showed the up-regulation of key genes in the JA and Et biosynthesis pathways at 6 hpi, including the gene encoding 12-oxo-PDA reductase, the enzyme that catalyses the final conversion of 12-oxo-PDA to JA, also the gene encoding the enzyme involved in the final step in Et biosynthesis (1-aminocyclopropane-1-carboxylate oxidase) indicates that the JA/Et pathways are important in the development of resistance of *Z. mays* to *P. cinnamomi*. Promoter analysis also revealed JA/Et-related motifs (GCC-BOX) as prominent within the up-regulated gene set and also, interestingly, an ABA-related motif (RY Repeat) that may indicate a role, as yet undetermined, for ABA in this interaction. Previous studies have suggested that the JA/Et pathways are induced in defence against *P. cinnamomi* (Rookes et al. 2008; Mahomed and van den Berg 2011). The results described here further substantiate JA/Et involvement in the development of resistance to *P. cinnamomi* and also demonstrates that the defence response exhibited by *Z. mays* is consistent with that of plants challenged by necrotrophic pathogens.

GSTs are a family of enzymes implicated in stress responses from both exogenous and endogenous origins, including pathogen defence (Marrs 1996). During defence, GSTs neutralise toxic components released by both pathogens and the plants themselves by facilitating conjugation of the toxicant with glutathione. Following conjugation, the compounds are then transported to the apoplast or sequestered within vacuoles (Marrs 1996; Dixon et al. 2002). A suite of seven GST genes were up-regulated at the early time-point of 6 hpi in this study. GST gene expression has been shown to be transcriptionally regulated by a broad range of stress conditions, including the production of reactive oxygen species, such as H<sub>2</sub>O<sub>2</sub> during pathogen attack (Tenhaken et al. 1995; Marrs 1996; Lamb and Dixon 1997; Sappl et al. 2009).

A number of other genes also associated with oxidative stress were observed to be up-regulated in the *Z. mays*–*P. cinnamomi* interaction. For example, a gene (*Glp2*) encoding a germin-like protein was up-regulated at 6 hpi and to a lesser degree at 24 hpi. GLPs are purported to have strong anti-oxidant activity and are extremely resistant to

degradation by both pathogen-derived proteases and H<sub>2</sub>O<sub>2</sub> (Kukavika et al. 2005; Łażniewska et al. 2010). Several GLPs have superoxide dismutase activity but a clear link between GLPs and H<sub>2</sub>O<sub>2</sub> production is yet to be established (Christensen et al. 2004; Łażniewska et al. 2010). While there is little information on the role of GLPs in defence to necrotrophs in *Z. mays*, they have been reported to be induced following pathogen invasion in other monocots (Membré et al. 2000; Zimmermann et al. 2006).

Inducible defence-related proteins that are present within healthy tissue at low levels have been identified in many plant species following infection by necrotrophic fungi and oomycetes (van Loon et al. 2006; Meier and Gehring 2008; Aswati-Nair et al. 2010). In our study two thaumatin-like protein (TLP) genes were up-regulated at 6 hpi, namely those encoding the pathogenesis-related protein PR5, which was highly up-regulated (7.5-fold), and the stress-induced protein 1 (4.2-fold) with 100 % sequence identity to zeamatin, a 22-kDa TLP which has been reported to have potent antifungal activity in vitro (Schimoler-O'Rourke et al. 2001). TLPs exhibit multiple enzymatic activities, including  $\beta$ -1,3 glucanase activity, which specifically targets the cell walls of pathogenic organisms, including fungi and oomycetes (Liu et al. 2010). Early up-regulation of TLPs in *Z. mays* roots may therefore form an integral part of an arsenal of defence genes and their products that work synergistically to limit *P. cinnamomi* invasion.

Our study has identified a range of key defence-related genes that are up-regulated in *Z. mays* roots in response to infection by *P. cinnamomi*. While some of these genes encode products that have been identified during resistance of plants to other root pathogens (Okubara and Paulitz 2005), our analysis has revealed new components of defence against a broad host range oomycete that include GSTs, the PR5 protein and enzymes involved in phytoalexin synthesis as well as GLPs and serine-protease inhibitors whose role in resistance is less well characterised. Furthermore, our results demonstrate that resistance to *P. cinnamomi* in *Z. mays* requires the simultaneous involvement of JA/Et pathways, terpenoid biosynthesis, ROS-related genes and the production of other potentially fungitoxic molecules. This new knowledge has opened up the potential for manipulation of molecular pathways and defence responses in other species and provides a sound molecular platform for further research on plant resistance to *P. cinnamomi*.

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