Short communication

Phoma medicaginis* stimulates the induction of the octadecanoid and phenylpropanoid pathways in *Medicago truncatulaLARS G. KAMPHUIS^{1,2,3,*}, ANGELA H. WILLIAMS³, HELGE KÜSTER⁴, ROBERT D. TRENGOVE⁵, KARAM B. SINGH^{1,2}, RICHARD P. OLIVER⁶ AND SIMON R. ELLWOOD⁶¹CSIRO Plant Industry, Wembley, Perth, WA 6913, Australia²The University of Western Australia Institute of Agriculture, University of Western Australia, Crawley, WA 6009, Australia³Division of Health Sciences, Murdoch University, Perth, WA 6150, Australia⁴Institute for Plant Genetics, Unit IV – Plant Genomics, Leibniz Universität Hannover, D-30419 Hannover, Germany⁵Separation Science Laboratory, Research and Development, Murdoch University, Perth, WA 6150, Australia⁶Department of Environment and Agriculture, Curtin University, Bentley, Perth, WA 6102, Australia**SUMMARY**

Gene expression changes and metabolite abundances were measured during the interaction of *Medicago truncatula* with the fungal necrotrophic pathogen *Phoma medicaginis* in leaf tissue of susceptible and resistant accessions. Over 330 genes were differentially expressed in plants infected with *P. medicaginis* relative to mock-inoculated plants at 12 h post-inoculation. Of these, 191 were induced in either the resistant or the susceptible accession, with 143 genes repressed. Expression changes were observed in genes involved in the oxidative burst, cell wall strengthening and lipid metabolism, as well as several transcription factors. Genes related to salicylic acid, jasmonate and ethylene responses were up-regulated, as well as genes leading to the production of jasmonic acid. Significant induction of genes in the phenylpropanoid pathway leading to lignin and isoflavonoid biosynthesis occurred. High-pressure liquid chromatography with UV detection (HPLC-UV) identified several phenolic compounds induced by *P. medicaginis*, as well as constitutively higher levels of phenolic compounds, in the resistant *M. truncatula* accession. Differentially regulated genes induced in both the resistant and susceptible accessions, but with different kinetics, and constitutively more highly expressed and induced phenolic compounds provide candidates for functional analysis. Taken together, these results highlight the importance of the octadecanoid and phenylpropanoid pathways in defence against this necrotrophic pathogen.

Medicago truncatula is an important resource for the study of resistance to fungal pathogens (Ameline-Torregrosa *et al.*, 2008; Kamphuis *et al.*, 2008; Pilet-Nayel *et al.*, 2009; Yang *et al.*, 2008), and is a natural host to fungal necrotrophs that are closely related to economically important, but poorly understood, pathogens of crop and pasture legumes (Ellwood *et al.*, 2006a; Infantino *et al.*,

2006; Tivoli *et al.*, 2006). Although interactions between *M. truncatula* and symbiotic microbes have been studied intensively using macro- and microarrays (e.g. Frenzel *et al.*, 2005; Hohnjec *et al.*, 2005, 2006; Kuster *et al.*, 2004), expression profiling with necrotrophic fungi has been limited to *Phymatotrichopsis omnivora*, a soil-borne pathogen with a wide host range (Uppalapati *et al.*, 2009).

Phoma medicaginis var. *medicaginis* causes spring black stem and leaf spot in lucerne (alfalfa, *M. sativa*) and results in serious yield losses in *Medicago* spp. (Graham *et al.*, 1979; Lamprecht and Knox-Davies, 1984; Smith *et al.*, 1988). Different *P. medicaginis* genotypes are present in Australia, and genotype OMT5 shows diverse disease phenotypes, with the *M. truncatula* accession SA27063 being resistant and SA3054 being susceptible (Ellwood *et al.*, 2006c). Resistance to *P. medicaginis* is complex and is controlled by quantitative trait loci with modest effects (Kamphuis *et al.*, 2008). The primary aim of this study was to examine gene expression in *M. truncatula* following *P. medicaginis* inoculation using SA27063 and SA3054. These two accessions are closely related, as judged by intron and simple sequence repeat (SSR) sequencing (Ellwood *et al.*, 2006b; Kamphuis *et al.*, 2007, 2008).

To identify genes in *M. truncatula* that are expressed in response to *P. medicaginis*, SA27063 and SA3054 were infected and sampled at 0, 12, 24 and 48 h post-inoculation (hpi) and the highest transcription of defence-related genes was observed at 12 hpi (data not shown). RNA from this time point was therefore used in the hybridization experiments with Mt16KOL1plus microarrays, as described by Hohnjec *et al.* (2005, 2006). Several thousand *M. truncatula* genes showed significant transcriptional changes at 12 hpi with *P. medicaginis* ($P \leq 0.01$). A greater than two-fold change was observed for 334 differentially expressed genes in either the resistant or susceptible accession (Fig. 1). No genes were identified that were induced in the resistant accession and repressed in the susceptible accession, or vice versa. In the mock-inoculated controls, similar gene expression patterns were

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Fig. 1 Venn diagram of the numbers of induced or repressed genes showing a two-fold or greater change in abundance at 12 h post-inoculation (hpi) with *Phoma medicaginis* OMT5 relative to noninoculated controls. The resistant accession SA27063 is designated R and the susceptible accession SA3054 is designated S.

evident between the resistant and susceptible plants. All raw expression data are available on request.

To identify the developmental and metabolic pathways related to defence and susceptibility, the sequences of differentially expressed genes showing at least a two-fold change in abundance were annotated and assigned to different functional categories for *Phoma*-induced genes (PIGs; Fig. 2A) and *Phoma*-repressed genes (PRGs; Fig. 2B). A complete list of genes and their transcript abundances is given in Tables S1 and S2 (see Supporting Information).

Genes involved in the ethylene (ET), jasmonic acid (JA) (Turner *et al.*, 2002) and salicylic acid (SA) (Wildermuth *et al.*, 2001) pathways were among the PIGs (Table 1). Transcripts of genes encoding proteins involved in the synthesis of JA were strongly induced in the resistant accession and, to a lesser degree, in the susceptible accession. The activation of the ET, JA and SA defence signalling pathway leads to the expression of genes encoding pathogenesis-related proteins (PR proteins) with antimicrobial activity (Van Loon *et al.*, 2006). SA-regulated PR homologues that were strongly induced were mainly PR10-like, and these tended to show greater induction in resistant plants (Table 1). Other SA-regulated PR protein homologues were PR2-like and PR5-like, which were strongly induced in the resistant accession, with no significant change in expression observed in the susceptible accession. JA and ET are known to induce PR4-like antimicrobial proteins (Turner *et al.*, 2002). The microarray results showed that there were PR4 members that were specific to the resistant and susceptible accessions with others expressed in both responses.

A number of PIGs encode proteins that function in the plant secondary metabolic pathways derived from L-phenylalanine, termed the 'phenylpropanoid pathway' (Fig. 2A, Table 2). *trans*-

Fig. 2 (A) Classification of 191 genes found to be at least two-fold induced in *Medicago truncatula* on infection with *Phoma medicaginis* OMT5 at 12 h post-inoculation (hpi). The number of genes allocated to each functional category is indicated. (B) Classification of 143 genes found to be at least two-fold repressed in *M. truncatula* on infection with *P. medicaginis* OMT5 at 12 hpi. The number of genes allocated to each functional category is indicated.

Cinnamate 4-monooxygenase (CA4H) and 4-coumarate:CoA ligase (4CL) genes showed a strong induction only in the resistant accession. 4CL converts coumaric acid into 4-coumaroyl-CoA, which can feed into the lignin biosynthetic pathway, the flavonoid

Table 1 Genes induced on infection with *Phoma medicaginis* OMT5 in the ethylene and jasmonic acid biosynthesis pathways, and genes whose products have antimicrobial properties.

MtGI v5*	MtGI v9†	Annotation	SA27063‡¶	SA3054§¶
Antimicrobial				
TC76640	TC124784	Similar to PR10-like Pprg2 protein (alfalfa)	2.98	1.79
BM815404	BM815404	Class PR10-1 protein	2.91	2.32
TC77137	TC123318	Similar to PR10 (chickpea)	2.88	2.73
TC76511	TC118987	Class PR10-1 protein	2.87	2.38
TC76641	TC119143	Similar to PR10-like Pprg2 protein (alfalfa)	2.61	–
TC76643	TC122461	Similar to PR10-like Pprg2 protein (alfalfa)	2.59	1.66
TC76642	TC119223	Similar to PR10-like Pprg2 protein (alfalfa)	2.51	1.47
TC76518	TC117333 TC137058 TC124769	Similar to PR10-like disease resistance protein Pi49 (garden pea)	2.47	2.10
TC77138	TC141769 TC123804	Similar to PR10 (chickpea)	2.39	2.54
TC76638	TC122461 TC132834	Similar to PR10-like Pprg2 protein (alfalfa)	2.28	–
TC86847	TC121925	Similar to PR5 osmotin-like protein (grapevine)	1.94	1.14
TC78899	TC117363	Similar to PR2 1,3-β-D-glucosidase (chickpea)	1.88	–
BG452227	BG452227	Homologue to PR4A (pea)	1.55	–
TC84433	TC114200	Similar to PR2 β-1,3-glucanase (soybean)	1.50	–
TC85874	TC118357	Homologue to PR2 β-1,3-glucanase (alfalfa)	1.46	–
TC86735	TC132983 TC120244	Similar to PR4 pseudo-hevein (rubber tree)	1.27	1.37
TC87236	TC125088	Homologue to PR4A (garden pea)	1.20	–
TC76694	TC121437	Homologue to PR-5-like thaumatin protein (chickpea)	1.15	–
TC88905	TC119742	Class PR10-1 protein	1.07	1.21
TC77149	TC113538	Similar to PR5-like thaumatin precursor Mdt1 (apple)	1.03	–
TC90394	TC114891 TC125255	Class PR4 chitinase precursor	–	1.13
ET/JA pathway				
TC85808	TC122702 TC130623	Similar to 12-oxophytodienoate reductase (Arabidopsis)	1.96	0.75
TC76875	TC113755	Similar to methyl jasmonate esterase (alfalfa)	1.37	0.85
TC79124	TC113403	1-Aminocyclopropanecarboxylic acid synthase	1.04	0.97
TC85168	TC122728 TC132660	Similar to lipoxygenase (garden pea)	1.04	–
TC91892	TC117550	Similar to lipoxygenase (almond)	0.96	1.13
TC85618	TC126072	Similar to lipoxygenase (CPRD46, cowpea)	0.64	1.24

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‡log₂ expression ratio inoculated/mock-inoculated control SA27063 (resistant) at 12 hpi with *P. medicaginis* OMT5.

§log₂ expression ratio inoculated/mock-inoculated control SA3054 (susceptible) at 12 hpi with *P. medicaginis* OMT5.

¶–, TC is not significantly differentially expressed ($P \leq 0.01$).

biosynthetic pathway and the isoflavonoid biosynthetic pathway. In addition to genes involved in lignin biosynthesis, genes involved in isoflavonoid biosynthesis were strongly induced on infection in both resistant and susceptible accessions (Table 2). Genes leading to the production of flavonoids, flavonols, anthocyanins and tannins were not induced at 12 hpi with *P. medicaginis*.

Among the 143 genes repressed by at least two-fold in either the resistant or the susceptible accessions, most were involved in the synthesis of sugars or amino acids. However, no significant differences were observed between the resistant and susceptible accessions (Table S2).

Reverse transcriptase-quantitative polymerase chain reaction (qPCR) was used to investigate diagnostic SA and JA signalling pathway-related genes identified by the array data and previously shown to be induced in *M. truncatula* by these compounds (Gao *et al.*, 2007). Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Biorad Laboratories, Hercules, CA, USA), according to the manufacturer's description. The

qPCR primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3>), as described by Gao *et al.* (2007), and the primer sequences used in this study are listed in Table S3 (see Supporting Information). qPCR analysis was carried out on a Rotorgene 3000 (Corbett Research, Sydney, Australia) using iQ SYBR Green Supermix (Biorad), and the level of transcript expression was calculated using the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001) with the fold change in gene expression normalized to actin and relative to the mock-inoculated control (0 h).

In the SA pathway, we examined *PR2*, *PR5* and *PR10* homologues; these genes showed no significant induction in mock-inoculated plants (Fig. 3). A small but significant increase was observed for the *PR2* (β-1,3-glucanase) homologue in infected SA3054 compared with mock-inoculated SA27063 plants at 9 hpi (Fig. 3A). *PR2* expression levels declined and returned to basal levels by 24 h. In susceptible plants, the induction of *PR5* was seen at 9 h, reaching a peak at 12 h, with significantly higher levels of expression observed throughout the later stages of the infection

Table 2 Genes of the phenylpropanoid pathway induced on infection with *Phoma medicaginis* OMT5.

MtGI v5*	MtGI v9†	Annotation	SA27063‡	SA3054§¶
General phenylpropanoid pathway				
TC85502	TC122748	Phenylalanine ammonia lyase (<i>M. truncatula</i>)	2.27	1.82
TC85506	BM815220	Similar to phenylalanine ammonia lyase (<i>Rubus idaeus</i>)	1.29	1.45
TC85501	TC113890	Homologue to phenylalanine ammonia lyase (<i>Stylosanthes humilis</i>)	1.69	1.44
TC85505	TC122748	Phenylalanine ammonia lyase (<i>M. truncatula</i>)	1.64	1.60
TC76780	TC134377	<i>trans</i> -Cinnamate 4-monooxygenase (<i>M. truncatula</i>)	1.35	–
TC77196	TC121243	Similar to 4-coumarate:CoA ligase (<i>Amorpha fruticosa</i>)	1.80	–
TC77196	TC121243	Similar to 4-coumarate:CoA ligase (<i>Amorpha fruticosa</i>)	1.19	–
TC77753	TC125942	Similar to 4-coumarate:CoA ligase (soybean)	1.12	–
Isoflavonoid biosynthesis				
TC76770	TC125306	Homologue to chalcone synthase 2 (alfalfa)	3.38	2.75
TC85138	TC118157	Homologue to chalcone synthase 8 (alfalfa)	3.30	2.80
TC85146	TC119414	Homologue to chalcone synthase 2 (alfalfa)	3.09	2.63
TC76765	TC118157	Homologue to chalcone synthase 2 (alfalfa)	3.02	3.11
TC76769	TC117768	Homologue to chalcone synthase 4 (alfalfa)	2.31	2.12
TC85174	TC119020	Homologue to chalcone synthase 4 (alfalfa)	2.25	1.82
TC85150	TC119999	Homologue to chalcone synthase 9 (alfalfa)	2.75	2.57
TC76767	TC120398	Homologue to chalcone synthase 2 (alfalfa)	1.98	1.95
TC85174	TC119020	Homologue to chalcone synthase 4 (alfalfa)	0.55	1.16
TC85186	TC122308	Homologue to chalcone synthase 4 (alfalfa)	1.98	1.94
TC76766	TC120398	Homologue to chalcone synthase 2 (alfalfa)	2.01	2.48
TC85521	TC118229 TC131843	Homologue to chalcone reductase (alfalfa)	2.86	2.11
TC76884	TC133203 TC118248 TC118220	Similar to chalcone reductase (chickpea)	2.24	1.43
TC85521	TC139616	Homologue to chalcone reductase (alfalfa)	2.86	2.07
TC85519	TC113451	Homologue to chalcone reductase (alfalfa)	2.46	2.15
TC85633	TC140281 TC116656	Homologue to chalcone-flavonone isomerase 1 (alfalfa)	2.01	1.03
TC76950	TC114999 TC132525	Similar to chalcone-flavonone isomerase (Arabidopsis)	1.58	0.99
TC77996	TC113279	Similar to isoflavone-7- <i>O</i> -methyltransferase 9 (alfalfa)	1.40	1.46
TC85608	TC118886	Isoflavone 2'-hydroxylase (<i>M. truncatula</i>)	1.23	–
TC85478	TC128705	Isoflavone reductase (<i>M. truncatula</i>)	3.75	2.54
TC85477	TC128258	Isoflavone reductase (<i>M. truncatula</i>)	2.90	2.44
TC86455	TC114432	Similar to isoflavone reductase homologue (<i>Lupinus albus</i>)	1.58	1.20
TC86358	TC113945	Homologue to 6a-hydroxymaackiain methyltransferase (garden pea)	1.49	1.46
TC77308	TC116846	Vestitone reductase (alfalfa)	1.67	1.62
TC87730	TC133180	Weakly similar to UDP-glucose:flavonoid glycosyltransferase (soybean)	1.75	1.36
TC78077	TC133180	Similar to UDP-glucose:flavonoid glycosyltransferase (mungbean)	1.62	–
Lignin biosynthesis				
TC85611	TC136402	Caffeoyl-CoA <i>O</i> -methyltransferase (alfalfa)	2.48	1.73
TC85611	TC117823	Caffeoyl-CoA <i>O</i> -methyltransferase (alfalfa)	1.84	1.39
TC85981	TC114331	Similar to caffeic acid 3- <i>O</i> -methyltransferase 1 (alfalfa)	1.35	1.63
TC85550	TC113727	Homologue to caffeic acid 3- <i>O</i> -methyltransferase (alfalfa)	1.05	0.51
TC92066	TC141438	Homologue to caffeic acid 3- <i>O</i> -methyltransferase (alfalfa)	1.05	–
TC91631	TC139283	Weakly similar to putative cinnamyl alcohol dehydrogenase (apple)	1.06	–
TC85789	TC127837 TC121615	Similar to peroxidase 3 (common bean)	1.61	1.33
TC85974	TC113405	Peroxidase 2 precursor (alfalfa)	1.34	1.10
TC86423	TC136419	Homologue to peroxidase precursor (alfalfa)	1.31	0.67
TC77974	TC133990 TC114005	Similar to peroxidase (soybean)	1.04	–
TC85172	TC119042	Weakly similar to peroxidase 3 (<i>Scutellaria baicalensis</i>)	0.68	1.17

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‡log₂ expression ratio inoculated/mock-inoculated control SA27063 (resistant) at 12 hpi with *P. medicaginis* OMT5.

§log₂ expression ratio inoculated/mock-inoculated control SA3054 (susceptible) at 12 hpi with *P. medicaginis* OMT5.

¶–, TC is not significantly differentially expressed ($P \leq 0.01$).

(Fig. 3B). In contrast, a slight but significant increase in *PR5* expression was seen at 24 hpi in resistant plants, which returned to basal levels by 48 h. *PR10* expression increased strongly on *P. medicaginis* infection in both SA27063 and SA3054 (Fig. 3C). In resistant plants, the increase was seen at 9 h and reached a peak

at 24 h, whereas, in susceptible plants, the increase started at 9 h and reached a peak at 48 h.

Most *PR* genes up-regulated on infection with *P. medicaginis* were *PR10*-like, and these tended to show greater induction in resistant plants. Reverse transcriptase-qPCR revealed relatively

Fig. 3 Regulation of genes representing the salicylic acid signalling pathway and the octadecanoid pathway on *Phoma medicaginis* OMT5 inoculation of SA27063 and SA3054. The relative transcript abundance is shown as a ratio of the relative gene expression for each sample to that in the untreated SA27063 or SA3054, and the values are the means of three biological replicates. Different letters for a given time point indicate significant differences in transcript abundance by Tukey–Kramer honestly significant difference (HSD) test ($P < 0.05$). Light grey bars represent mock-inoculated SA27063; dark grey bars represent SA27063 infected with *P. medicaginis* OMT5; white bars represent mock-inoculated SA3054; black bars represent SA3054 infected with *P. medicaginis* OMT5. 12-OPDA, 12-oxophytodienoate.

low induction of *PR2* and *PR5* genes, and confirmed strong induction of *PR10*. *PR10* genes were also induced in *M. truncatula* by the oomycete *Aphanomyces euteiches* (Colditz *et al.*, 2005, 2007), as well as the fungal pathogens *Colletotrichum trifolii* (Torregrosa *et al.*, 2004) and *Erysiphe pisi* (Foster-Hartnett *et al.*, 2007).

Reverse transcriptase-qPCR of genes in the octadecanoid acid pathway [i.e. lipoxygenases, allene oxide synthases (AOSs) and 12-oxophytodienoate reductases] showed that these genes were induced in resistant and susceptible accessions following *P. medicaginis* infection, but with differences in the kinetics and magnitude of induction (Fig. 3D–F; Table S4, see Supporting Information). Lipoxygenases showed a significant increase in

expression at 9 and 12 h, which returned to basal levels by 24 h (Fig. 3D; Table S4). In all cases, significantly higher lipoxygenase transcript abundances were observed in infected plants at 9 and 12 h, and lipoxygenase transcripts accumulated more rapidly in resistant than in susceptible plants.

AOS is the second enzyme in the octadecanoid pathway. Of the two known AOS genes in *M. truncatula*, TC116308 showed a small change in transcript abundance in infected tissue at 12 h (Fig. 3E), whereas TC110784 showed significantly higher transcript abundances in mock-inoculated and inoculated resistant plants than in mock-inoculated and inoculated susceptible plants at 24 hpi (Table S4). No other significant differences were observed for the AOS genes.

The 12-oxophytodienoate reductase genes were induced in both resistant and susceptible plants following *P. medicaginis* inoculation (Fig. 3F; Table S4). Induction occurred as early as 9 h and was significantly different from that in mock-inoculated plants at 12, 24 and 48 h.

Genes in the octadecanoid pathway were induced significantly by *P. medicaginis* at 12 hpi and confirmed by qPCR. Genes at key branch points in the octadecanoid pathway, hydroperoxide lyases and divinyl ether synthases, did not show any significant changes in expression. JA biosynthesis genes were induced in a compatible response between *M. truncatula* cv. Jemalong A17 and the soil-borne necrotroph *Phymatotrichopsis omnivora*, as early as 3 dpi, when the initial entry of fungal hyphae takes place (Uppalapati *et al.*, 2009). Following infection with the hemibiotroph *C. trifolii*, six lipoxygenase genes were induced in resistant *M. truncatula* plants and only two in susceptible plants (Torregrosa *et al.*, 2004), leading the authors to suggest that resistance might be mediated through the octadecanoid pathway in incompatible interactions. The biological significance of lipoxygenase gene induction following *C. trifolii* infection in resistant *M. truncatula* relative to necrotrophs is unclear, as resistance to anthracnose tends to be dependent on recognition by classical host resistance genes, in common with biotrophs.

Chemical defences against pathogenic microorganisms in Fabaceae include the production of alkaloids, coumarins, stilbenes and, especially, isoflavonoid derivatives. Isoflavonoids possessing pterocarpan and isoflavan skeletons are the most frequently found secondary compounds of legumes with antimicrobial activity (Liu *et al.*, 2003). These isoflavonoid phytoalexins exhibit antimicrobial activity *in vitro* (Blount *et al.*, 1992; Hipskind and Paiva, 2000) and are produced at potentially antimicrobial concentrations in *M. sativa* tissue on infection. Furthermore, there is ample evidence of a correlation between the accumulation of isoflavonoids and resistance to pathogens in *Medicago* spp. (Barbetti, 1995, 2007; Deavours and Dixon, 2005; Deavours *et al.*, 2006; Dixon *et al.*, 2002; He and Dixon, 2000; Liu *et al.*, 2003). In *P. medicaginis*-infected plants, genes involved in the synthesis of flavonoids were induced in both resistant and susceptible plants, although differences in the kinetics and magnitude of induction were observed (Fig. 4). For chalcone isomerase (CHI), at the start of the flavonoid pathway, and the subsequent isoflavone synthase (IFS), a significant increase in transcript abundance was observed in resistant and susceptible plants at 12 hpi (Fig. 4A,B). CHI showed its highest transcript abundance at 12 hpi, whereas the IFS maximum was at 48 hpi. A similar pattern was observed for isoflavone

3'-hydroxylase (Fig. 4E), but the magnitude of transcript abundance was larger than that of IFS. Isoflavone O-methyltransferase (IFMT, Fig. 4C; Table S4) showed similar levels of induction at 12 hpi in resistant and susceptible plants, but, in susceptible plants, significantly higher levels were observed throughout the later stages of the infection, which could either be a prolonged or second induction in transcription. One isoflavone hydroxylase gene (TC100787) showed no significant changes in expression, whereas the other (IF3H homologue TC114906) was strongly induced in susceptible plants from 12 hpi, reaching a maximum abundance at 48 hpi (Fig. 4E; Table S4). An increase in transcript abundance for this gene was also observed in the resistant accession from 48 hpi, but was significantly less than that observed in susceptible plants (Fig. 4E). Isoflavone 2'-hydroxylase (IF2H), the enzyme responsible for the conversion of formononetin into 2'-hydroxyformononetin, showed stronger induction in resistant plants than in susceptible plants, particularly at 24 hpi (Fig. 4D). The subsequent enzymatic step in the pathway involves isoflavone reductase (IFR) conversion of 2'-hydroxyformononetin into vestitone. IFR was strongly induced, reaching a peak at 12 hpi, where susceptible plants showed significantly higher expression than resistant plants (Fig. 4F; Table S4), particularly at 9 hpi. Vestitone reductase was similarly induced as early as 9 hpi following infection, reaching a maximum expression at 12 hpi and remaining induced in the later stages of the infection (24–72 h; Fig. 4G). This is one of two enzymes involved in the conversion of vestitone into the phytoalexin medicarpin (Guo *et al.*, 1994).

As expression levels do not necessarily reflect enzyme activity, high-pressure liquid chromatography with UV detection (HPLC-UV) was employed to differentiate, identify and quantify major foliar flavonoids induced by *P. medicaginis*. HPLC-UV has been used to profile flavonoids in red and white clover (Lin *et al.*, 2000; Wu *et al.*, 2003) and in *M. truncatula* (Farag *et al.*, 2006). UV spectra (200–600 nm) and retention times (RTs) for 22 flavonoid standards are listed in Table S5 (see Supporting Information). Of these, *p*-coumaric acid, daidzein, liquiritigenin, naringenin, coumestrol, formononetin and medicarpin were identified on the basis of their relative RTs and absorbance spectra.

In total, 62 metabolite peaks were resolved (a representative trace file is shown in Fig. S1, see Supporting Information). To identify which compounds showed significant differences in abundance following infection, these were subjected to a three-way analysis of variance (ANOVA) and compared by a Tukey–Kramer honestly significant difference (HSD) test ($P < 0.05$). Forty-six metabolites, including those identified as daidzein (Fig. 5A),

Fig. 4 Regulation of genes representing the isoflavonoid pathway on mock or *Phoma medicaginis* OMT5 inoculation of SA27063 and SA3054. The relative transcript abundance is shown as a ratio of the relative gene expression for each sample to that in the untreated SA27063 or SA3054, and the values are the means of three biological replicates. Different letters for a given time point indicate a significant difference in transcript abundance by Tukey–Kramer honestly significant difference (HSD) test ($P < 0.05$). Light grey bars represent mock-inoculated SA27063; dark grey bars represent SA27063 infected with *P. medicaginis* OMT5; white bars represent mock-inoculated SA3054; black bars represent SA3054 infected with *P. medicaginis* OMT5.

Fig. 5 Abundance of metabolites in *Medicago truncatula* accessions SA27063 and SA3054 mock-inoculated and inoculated with *Phoma medicaginis* OMT5 over a period of 72 h. Metabolite abundances are the mean of three biological replicates. Different letters for a given time point indicate a significant difference in transcript abundance by Tukey–Kramer honestly significant difference (HSD) test ($P < 0.05$). A white star (☆) indicates a significant difference in transcript abundance between treatments (mock vs. *Phoma*) for a given time point ($P < 0.05$), and a black star (★) indicates a significant difference in transcript abundance between accessions (SA27063 vs. SA3054) for a given time point ($P < 0.05$). Yellow and light blue represent SA3054 mock-inoculated and inoculated with *P. medicaginis* OMT5, respectively. Navy and pink represent SA27063 mock-inoculated and inoculated with *P. medicaginis* OMT5, respectively.

Fig. 5 Continued.

liquiritigenin (Fig. 5B) and *p*-coumaric acid (Fig. 5C), did not show significant changes in abundance on infection. In this group, the abundance of *p*-coumaric acid was clearly higher in the susceptible accession than in the resistant accession at 12 hpi (infected or mock-inoculated, Fig. 5C).

Sixteen metabolites showed significant induction following *P. medicaginis* infection. Five of these compounds showed consti-

tutively higher abundance in the resistant accession SA27063 than in the susceptible accession SA3054 (Fig. 5D–H). These were coumestrol (RT = 37.1 min) and unidentified metabolites at RTs of 35.2, 36.5, 42.3 and 42.7 min. Coumestrol and RT = 35.2 min showed an increase at 72 hpi in SA3054 to similar levels present in SA27063 (Fig. 5D,E). From 24 hpi onwards, a second group of compounds, composed of naringenin, formononetin,

RT = 32.0 min, RT = 39.4 min and RT = 40.9 min, showed elevated levels in both infected accessions compared with mock-inoculated controls (Fig. 5I–M). Medicago (Fig. 5P) was increased significantly in infected plants relative to mock-inoculated controls from 48 hpi.

Three unknown metabolites (RT = 28.9 min, RT = 31.0 min and RT = 32.0 min) accumulated more rapidly in the resistant accession SA27063 than in the susceptible accession SA3054 following infection. RT = 28.9 min and RT = 31.0 min showed a significant increase from 48 hpi in SA27063, but at 72 hpi in SA3054 (Fig. 5N,O). The metabolite at RT = 32.0 min showed a rapid increase to 24 hpi in SA27063, whereas, in SA3054, an increase was first observed at 48 hpi (Fig. 5M).

We observed a greater induction of the phenylpropanoid pathway in the susceptible interaction. One explanation for this is that more cells are successfully penetrated by *P. medicaginis* relative to the resistant interaction, resulting in higher isoflavonoid transcript levels. This is consistent with immunolocalization studies in alfalfa, which showed that IFR and vestitone reductase proteins specifically accumulate in leaf cells surrounding *P. medicaginis* lesions (Lopez-Meyer and Paiva, 2002). A second induction in susceptible SA3054 at 48 hpi for CHI, IFMT, IFR and vestitone reductase, which is absent in resistant SA27063, could be a consequence of *P. medicaginis* being able to colonize cells surrounding the initial infection zone. These higher transcript abundances did not result in significantly higher levels of isoflavonoids relative to the resistant plants. In both resistant and susceptible responses, we observed similar levels of accumulation of the flavonoids naringenin, formononetin and medicarpin. This is in agreement with the findings of Deavours and Dixon (2005) in alfalfa, where liquiritigenin, formononetin and medicarpin accumulated on infection of the susceptible cultivar Regen SY with *P. medicaginis*. Induction of the phenylpropanoid pathway was also observed on infection by *Phymatotrichopsis omnivora* in *M. truncatula*, but differed from this study, as genes involved in flavonol biosynthesis were induced (Uppalapati *et al.*, 2009).

The induction of the octadecanoid and phenylpropanoid pathways in *M. truncatula* following *P. medicaginis* infection was detected using microarrays and corroborated by qPCR and HPLC-UV. The results suggest that there are at least two independent defence mechanisms effective against *P. medicaginis*, with a faster induction of lipoxygenase genes in the octadecanoid pathway and constitutively higher abundances of isoflavonoid-like compounds of the phenylpropanoid pathway in resistant plants. This study provides a basis for the functional characterization of several genes and compounds that may represent key components for resistance to fungal necrotrophs in *M. truncatula* and other galeoid legumes. These include precursors to the JA pathway, key enzymes in the phenylpropanoid pathway, constitutively more highly expressed phenolic

compounds and specifically induced compounds in the resistant accession.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 High-pressure liquid chromatography (HPLC) trace file of accession SA27063, 72 h post-inoculation (hpi), biological repeat 1.

Table S1 Differentially expressed transcripts showing a two-fold or greater activation ratio at 12 h post-inoculation (hpi) with *Phoma medicaginis* OMT5.

Table S2 Differentially expressed transcripts showing a two-fold or greater repression ratio at 12 h post-inoculation (hpi) with *Phoma medicaginis* OMT5.

Table S3 Genes and primer sequences used for quantitative polymerase chain reaction (qPCR).

Table S4 Expression profiling of additional genes in the octadecanoid pathway and the phenylpropanoid pathway in response to *Phoma medicaginis* OMT5 in resistant (SA27063) and susceptible (SA3054) *Medicago truncatula* accessions.

Table S5 Metabolites of the phenylpropanoid pathway, their retention times and maximum absorbances as identified by high-pressure liquid chromatography with UV detection (HPLC-UV).

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