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ORIGINAL PAPER

Transcriptome analysis of resistant and susceptible genotypes of *Glycine tomentella* during *Phakopsora pachyrhizi* infection reveals novel rust resistance genes

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Abstract Soybean rust, caused by *Phakopsora pachyrhizi*, is a destructive foliar disease in nearly all soybean-producing countries. To identify genes controlling resistance to soybean rust, transcriptome profiling was conducted in resistant and susceptible *Glycine tomentella* genotypes triggered by *P. pachyrhizi* infection. Among 38,400 genes monitored using a soybean microarray, at 5% false discovery rate, 1,342 genes were identified exhibiting significant differential expression between uninfected and *P. pachyrhizi*-infected leaves at 12, 24, 48, and 72 h post-inoculation (hpi) in both rust-susceptible and rust-resistant genotypes. Differentially expressed genes were grouped into 12 functional categories, and among those, large numbers relate to basic plant metabolism. Transcripts for genes involved in the phenylpropanoid pathway were up-regulated early during rust infection. Similarly, genes coding for proteins related to stress and defense responses such as glutathione-*S*-transferases, peroxidases, heat shock proteins, and lipoxygenases were consistently up-regulated following infection at all four time points. Whereas, subsets of genes involved in cellular transport, cellular communication, cell cycle, and DNA processing were down-regulated. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) on randomly selected genes from the different categories confirmed these findings. Of differentially expressed genes, those associated with the flavonoid biosynthesis pathway as well as those coding for peroxidases and lipoxygenases were likely to be involved in rust resistance in soybean, and would serve as good candidates for functional studies. These findings provided insights into mechanisms underlying resistance and general activation of plant defense pathways in response to rust infection.

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Introduction

Soybean (*Glycine max* L.) is a major economic crop that is a valuable source of protein, oil, and various nutrients, including antioxidants and isoflavones. Moreover, soybean is an excellent source of nutrients in the animal feed market. The United States is the world's leading soybean producer and exporter.

Soybean rust (SBR), a serious foliar disease incited by the fungal pathogen *Phakopsora pachyrhizi*, has the potential of causing significant yield loss (over 50%) in soybean (Hartman et al. 2004; Hartman et al. 2005). Although SBR has been identified on soybean plants in Hawaii in 1994 (Killgore and Heu 1994), the disease has not been reported in the continental United States until 2004 (Schneider et al. 2005) when it was first reported in Louisiana, and the occurrence of the disease has now been racked throughout the year (USDA 2009). Presence of SBR in the continental US has become a major focus of soybean growers and the soybean industry, as it can have a major impact on both total soybean production and production costs. Conservative predictions have indicated potential yield losses of over 10% in nearly all US soybean growing areas with potential losses of up to 50% in the Mississippi delta and southeastern coastal states (Yang 1995). In 2008, SBR has been detected in 392 counties representing 16 states in the United States (USDA 2009).

Host plant resistance to *P. pachyrhizi* has been first reported in the 1960s based on field evaluations in Taiwan. Since then, resistance to *P. pachyrhizi* has been investigated in controlled genetic studies, and five single dominant genes conditioning resistance to a limited number of rust isolates, *Rpp 1*, *Rpp 2*, *Rpp 3*, *Rpp 4*, and *Rpp5*, have been identified (Hartwig 1995; Garcia et al. 2008). It has become apparent that none of the five rust resistance genes nor any of the soybean cultivars grown today provide strong and durable resistance to SBR (Bonde et al. 2006; Patzoldt et al. 2007). Recently, Meyer et al. (2009) have reported that *Rpp4-C4* is a single candidate gene for resistance to SBR. Nevertheless, it is clear that other sources of durable genetic resistance, particularly those found in *Glycine* germplasm accessions, must be identified and exploited. Several wild perennial soybean species, including *Glycine tomentella*, *G. tabacina*, and *G. argyrea*, have been reported as resistant to SBR (Hartman et al. 1992). Single resistance genes of at least four distinct loci have been detected in lines of *G. canescen* (Burdon 1988), and a single major gene for resistance to *P. pachyrhizi* has been identified in *G. argyrea* (Jarosz and Burdon 1990). Therefore, elucidating the mechanism of active defense response against SBR in these species may identify novel genes for rust resistance that can be transferred to *G. max* using the tools of genetic engineering.

Many plants, including soybean, accumulate isoflavonoid phytoalexins as part of their defense response to pathogen attack (Dixon 1986, 1999; Lozovaya et al. 2004; Liu et al. 2006). Some plant defense-response genes are activated either as a result of plant–pathogen interaction or upon treatment of plant tissues with either pathogen- or plant-derived elicitors (Dixon 1986; Ebel and Grisebach 1988; Baldridge et al. 1998).

Previous reports have noted an increase in phenylalanine ammonia-lyase (PAL), a key enzyme of general phenylpropanoid metabolism, upon fungal infection in plants, such as wheat, barley, potato, and parsley (Fritzemeier et al. 1987; Jahnen and Hahlbrock 1988; Zierold et al. 2005; Bhuiyan et al. 2009). Concomitant with PAL, 4-coumarate-CoA ligase is quickly induced and accumulates in small confined areas around fungal penetration sites in infected parsley and potato leaves (Fritzemeier et al. 1987; Schmelzer et al. 1989). In sorghum and in legumes, chalcone synthase gene transcripts/proteins are localized within areas of inoculation along with accumulation of defense-related phytoalexins (Dixon et al. 1983; Hahlbrock and Scheel 1989; Lo et al. 1999).

The overall goal of this study was to identify key rust resistance genes in *G. tomentella* by investigating global gene expression profiles of *P. pachyrhizi*-infected leaves of susceptible (PI441101) and resistant (PI509501) genotypes at different time points, including 12, 24, 48, and 72 h post-inoculation (hpi). Selected groups of genes were then subjected to quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).

Materials and methods

Plant material

An SBR-resistant *Glycine tomentella* genotype (PI509501) (R) and an SBR-susceptible *G. tomentella* genotype (PI441101) (S) were selected based on previous greenhouse inoculation studies and detached leaf assays (G. L. Hartman, unpublished). Seeds of these two genotypes (gift from T. Hymowitz, University of Illinois) were scarified, and germinated on moist filter paper. After 3 days, 21 seedlings of each genotype were transplanted to plastic pots (2–3 seedlings per 15 cm diameter pot) containing LC-1 potting mix (Sungro Horticulture, Bellevue, WA), and fertilized with N–P–K Osmocote (Scott Miracle Company, Marysville, OH). All seedlings were maintained in a growth chamber (Controlled Environments, Winnipeg, Canada) under a 14 h photoperiod (100 μmol m^{-2} s^{-1}) and 26°C day/22°C night temperature regime.

Inoculum preparation

Soybean rust spores, originally collected from a soybean field in 2007 at Quincy, FL (designated FL07-1), were purified, and proliferated on detached leaves of soybean cv. Williams 82 in water agar supplemented with 2 mg/L 6-benzyladenine (BA) (Sigma, St. Louis, MO) in 100 mm diameter Petri plates.

Spores were collected from infected detached leaves using a custom-made mini-cyclone vacuum spore collector (Barnant Company, Barrington, IL). Dry spores were suspended in 0.01% Tween-20 (Sigma), vortexed for 30 s, and the spore concentration was estimated by microscopic observation using a hemacytometer (Hausser Scientific, Horsham, PA). The spore suspension was diluted to 35 spores per microliter prior to inoculation, and 10 μL droplets of the suspension were spotted on water agar to assess spore germination.

Inoculation of leaf tissues, sampling, and RNA isolation

When young immature plants were 5 weeks old, 240 single leaflets from randomly selected trifoliates of each genotype were excised using a razor blade. Ten leaflets per genotype were placed in 90 mm Petri dishes containing 20 mL of 1.5% water-agar medium supplemented with 2 mg/L BA. For each genotype, 120 leaflets (12 Petri dishes) were either mock-inoculated or inoculated with *P. pachyrhizi*. Leaflets were individually sprayed with a urediniospore suspension amended with a 0.01% (v/v) Tween-20 solution using an air paint-brush (Paashe Airbrush Co, Lindenhurst, IL) driven by a small compressor (Badger Co., Franklin Park, IL) at 20 psi, at a rate of ~1 mL per three leaflets. For mock inoculations, sterile water with a 0.01% (v/v) Tween-20 solution was sprayed. Following inoculation, leaflets were incubated in a controlled environment chamber (Percival Scientific, Perry, IA) at 14/10 h of day/night photoperiod (36 μmol m^{-2} s^{-1}) at 22°C. After 12, 24, 48, and 72 hpi, 10 randomly selected leaflets per genotype were collected from inoculated and mock-inoculated treatments within each genotype, and immediately frozen in liquid nitrogen. Total RNA was extracted as previously described (Gasic et al. 2004). All remaining leaflets that were not sampled were incubated as previously described and scored for rust symptoms at 12 days post-inoculation (dpi).

Microarray hybridization

Isolated total RNA (15 μg) from R and S genotypes at each time point was reverse-transcribed, and then labeled with Cy3/Cy5 dyes using the Alexa Fluor Reactive Dye Decapacks for Microarrays Applications, as previously described (Soria-Guerra et al. unpublished). cDNA from mock-inoculated S or R genotypes was used as a reference in the assay. Each of the microarray experiments was performed in duplicate (with different RNA samples) using a 70-mer long-oligo soybean microarray representing ~38,400 genes covering wide developmental stages and physiological conditions (Gonzalez and Vodkin 2007), along with a dye-swap. Two biological replicates along with three technical replicates at each time point were used.

Slides were prehybridized in a solution containing 20% formamide, 6× SSC, 0.1% SDS, and 5× Denhardt's solution, with 25 μg mL^{-1} tRNA (Sigma, St. Louis, MO) for 45 min at 42°C. These were then washed five times in sterilized distilled water and once in isopropanol, and dried by centrifugation at 400*g* for 3 min. cDNA probes were dissolved in 42 μL of 1× hybridization solution (Ambion, Austin, TX), denatured for 1 min in boiling water, and cooled to 42°C. Hybridizations were done at 42°C for 16 h using the Maui chamber system (BioMicro Systems, Salt Lake City, UT). Post-hybridization washes were done by placing slides into Coplin jars with gentle agitation. Washes were done once in 1× SSC and 0.2% SDS at 42°C for 5 min, 0.1× SSC, 0.2% SDS at 25°C for 5 min, and twice in 0.1× SSC for 5 min. Finally, slides were dipped in 0.01× SSC, and dried by centrifugation at 400*g* for 3 min.

Slides were scanned using a Genepix 4000 B fluorescence reader (Axon Instruments, Concord, ON) using Genepix 3.0 image acquisition software adjusted for Cy3 and for Cy5.

Data analysis

Data processing included removal of spots flagged by the scanning software GeneSpring and log2 transformation of intensities after subtracting the background. Log-transformed values were normalized using a global LOWESS transformation (Cui et al. 2003; Smith et al. 2007) to remove dye bias within the microarray. A two-stage approach was used to adjust for technical sources of variation (Wolfinger et al. 2001; Smith et al. 2007). At first, global dye and microarray effects were removed across all microarray elements or transcripts. Then, each transcript within each microarray was described using a model including fixed effects of dye, genotype, infection status, time point, along with two- and three-way interactions among genotypes, infection status, and time point. The microarray slide was a random effect assumed to be identical and independently distributed (iid) having a normal distribution with a mean of zero and variance of α^2. Genes were regarded as significantly differentially expressed if $P \leq 0.05$.

Functional categories of identified genes were assigned based on the database of the Munich Information Center from Protein Sequence (MIPS) (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp).

Verification of changes in gene expression using qRT-PCR

To validate microarray results, 21 genes that were either up- or down-regulated were verified by qRT-PCR. Gene-specific oligonucleotide primers were designed against genes that were selected from the microarray analysis based on their functional identities and expression profiles using a Fast PCR program (http://www.biocenter.helsinki.fi/bi/Programs/download.htm).

RNA samples from susceptible and resistant genotypes, initially isolated for microarray analysis, were used. Prior to synthesis of cDNA, any residual genomic DNA was removed by treating samples with DNase I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized with Oligo (dT) primer using SuperScript III RT (Invitrogen).

Polymerase chain reactions were carried out in 96-well in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems). Each 25 μL real time PCR reaction mixture contained 10.5 μL dd_2 water, 0.5 μL 200 nM each of forward and reverse primers, 12.5 μL of 2 × SYBR Green I Master, and 100 ng of cDNA. PCR reaction conditions were as follows: 95°C for 10 min, and followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Following amplification, a melting curve analysis was run using the program for one cycle at 95°C for 5 s, 65°C for 1 min, and 95°C with 0 s hold in the step acquisition mode, and followed by cooling at 40°C for 10 s.

A negative control without a cDNA template was run with each analysis to evaluate the overall specificity. For each gene, three technical replicates and two biological replicates were used at each sampling time point. A total of 21 primers were designed for amplicons between 150 and 220 bp for each gene. A list of primers used for real-time RT-PCR is listed in Supplemental Table 1. A soybean tubulin gene (Shen et al. 2006) was used as an internal control to normalize the total amount of cDNA in each reaction. The quantification of gene expression was performed using the relative quantification ($\Delta\Delta C_T$) method and comparing data with internal controls.

Each sample was replicated three times, and the resultant data were analyzed, using the mock inoculation conditions at each time point as calibrators, with the aid of SDS software from 7300 Real Time PCR System (Applied Biosystems).

Results

Symptoms of infection assays

Leaflets of *G. tomentella* genotype PI441101 (S) exhibited typical rust lesion development with sporulating uredinia; while, *G. tomentella* genotype PI509501 (R) had no lesions or uredinia and produced no spores.

Differentially expressed genes

To identify genes associated with resistance response to SBR, the differential gene expression between uninfected and *P. pachyrhizi*-infected leaves during the course of infection in susceptible and resistant genotypes of *G. tomentella* were compared using a soybean microarray. Data from infected leaflets from each genotype were compared to their corresponding mock-inoculated leaflets over four time points (12, 24, 48, and 72 hpi), and representing different stages of infection in *G. tomentella*. A total of 1,342 genes were found to be differentially expressed over the four time points in R and S genotypes at $P \leq 0.05$ (Supplemental Table 2). Of these, ~70% were up-regulated and ~30% were down-regulated at all time points (Supplemental Table 2). Most of the up-regulated genes belonged to those with metabolic and defense-related functions.

Using a false discovery rate (FDR) at 5%, 943 and 925 genes were up-regulated in R and S genotypes, respectively, at 12 hpi. Of these, 526 and 518 were unknown genes, respectively. Known up-regulated genes included PAL, cytochrome P450, chalcone synthases, WRKY transcription factors, isoflavone reductases, lipoxygenases, and *S*-adenosylmethionine decarboxylases encoding genes. A total of 399 and 417 genes were down-regulated in R and S genotypes, respectively; including only 130 and 140 known genes. Among these, most genes were related to cell cycle and DNA processing, cell transport, protein fate, and metabolism, such as DNA methyltransferases, DNA-methyladenine glycosylases, lipases, hydrolases, and PHD finger proteins (Fig. 1; Table 1). There were 57 differentially expressed genes between R and S genotypes at this early stage (12 h) post-inoculation. Of these, 18 had metabolic-related functions, including three glycosyl transferases. Also, there were 12 differentially expressed genes implicated in cell rescue and defense, such as chitinases, oxidases, heat shock proteins, and lipoxygenases. It is noteworthy to indicate that most of these genes were up-regulated in the R genotype and down-regulated in the S genotype (Table 1).

There were 915 and 950 genes up-regulated in R and S genotypes, respectively, at 24 hpi (second time point) at FDR 5%. Of these, 395 and 417 genes were known genes,

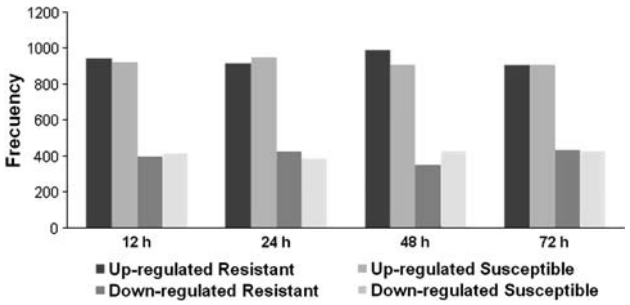

Fig. 1 Distribution of up- and down-regulated genes at 12, 24, 48, and 72 h in response to *Phakopsora pachyrhizi* infection in resistant and susceptible *Glycine tomentella* genotypes

respectively. Among the up-regulated genes, transcripts of PAL, cytochrome P450, disease-resistance protein (TIR-NBS-LRR class), isoflavone reductases, lipoxygenases, chalcone synthases, cinnamoyl CoA dehydrogenases, WRKY family transcription factors, and dihydroflavonol 4-reductase family were found in both genotypes. Examples of down-regulated genes included Myb family transcription factors, hydrolases, heat shock transcription factors, ferredoxin-related, and anexins (Fig. 1 and Supplemental Table 2). In summary, there were 54 significantly differentially expressed genes between the two genotypes at 24 hpi. Most of these genes were the same as those detected at 12 hpi. However, there were also unique differentially expressed genes at this time point, including three genes involved in cell cycle and DNA processing, two genes related to energy, and three genes implicated in regulation of metabolism. All of these genes were down-regulated in the R genotype and up-regulated in the S genotype (Table 1).

At 48 hpi (third time point), 988 and 912 genes were up-regulated in R and S genotypes, respectively (Supplemental Table 2). Of these, 432 and 391 were known genes, respectively, using FDR at 5%. Moreover, there were 354 and 430 genes down-regulated in R and S genotypes, respectively. Overall, genes detected at this time point (48 hpi) in both genotypes were the same as those identified in the previous two time points (12 and 24 hpi). Furthermore, those 53 differentially expressed genes identified between R and S genotypes at 48 hpi were almost the same as those detected at 24 hpi (54 genes), except for a single change in a gene involved in the regulation of metabolism (details of such comparisons are presented in Table 1).

At 72 hpi, at FDR of 5%, 904 and 910 genes were up-regulated and 438 and 432 were down-regulated in R and S genotypes, respectively. Of these, 392 and 390 genes were known up-regulated genes, and 155 and 157 were known down-regulated genes in R and S genotypes, respectively (Fig. 1; Supplementary Table 2). Although the numbers of up- and down- regulated genes were very similar between the two genotypes, higher numbers of genes (98 genes) were differentially expressed at this time point, almost twofold than at all other previous time points. Moreover, unlike all other post-inoculation stages, there was an increase in genes associated with transcription, regulation of metabolism, protein with binding function, metabolism, and cellular communication. Among differentially expressed genes, six transcripts related to kinases were down-regulated in the R genotype and up-regulated in the S genotype. In contrast, cinnamoyl CoA dehydrogenase was represented by three transcripts up-regulated in the R genotype and down-regulated in the S genotype (Table 1).

Functional annotation of regulated genes

All 1,342 genes, from S and R genotypes, found to be differentially expressed at $P \leq 0.05$, were assigned into 12 functional categories by searching the MIPS database (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp). This revealed that in both genotypes, classes of metabolism, cell rescue and defense, protein with binding function, and cellular transport were overrepresented (Fig. 2). Details of differentially expressed genes with respect to time and genotype are given in Supplemental Table 2.

Responses in resistant versus susceptible genotypes to *P. pachyrhizi*

Cell cycle and DNA processing related genes

Genes involved in mitotic cell cycle, cell cycle control, and DNA synthesis and replication were grouped in this category. Overall, an increase in up-regulated genes was observed in this category at both 24 and 48 hpi compared with genes expressed at 12 and 72 hpi. At 24 hpi, higher numbers of genes were up-regulated in the S genotype in comparison with the R genotype, and among those included DNA polymerases alpha catalytic subunit, DNA-binding proteins, and lipases class 3 family proteins (Fig. 3a).

Defense-related genes

On the basis of MIPS Functional Catalogue, genes associated with stress response, DNA repair, resistance proteins, defense-related proteins, detoxification, cell death, degradation of exogenous proteins, and other cell rescue activities were grouped under the defense category, which formed the second largest group. Over all time points, larger numbers of these genes were up-regulated in R genotype compared with the S genotype (Fig. 3b).

As shown in Fig. 4, expression of genes, such as PAL, chalcone synthases, and UDP-glycose—flavonoid

Table 1 Genes showing differential expression in response to *Phakospora pachyrhizi* during four time points (12, 24, 48, and 72 hpi) in resistant (R) and susceptible (S) *Glycine tomentella* genotypes

Clone ID	Function	Fold change		Description
		R	S	
12 hpi				
Gm-r1021-853	Protein fate	2.40	−0.50	Synaptosomal-associated protein SNAP25
Gm-r1070-4082	Various	1.58	−0.78	Protease inhibitor, putative (DR4)
Gm-c1051-2231	Metabolism	1.50	−0.37	Annexin 4; calcium ion binding
Gm-r1021-2080	Defense	1.33	−0.39	Pathogenesis-related protein 3—kidney bean
Gm-c1054-1664	Cell communication	1.28	−1.26	S-locus protein kinase, putative (ARK3)
Gm-r1070-8283	Defense	1.14	−0.95	Gibberellin-regulated family protein
Gm-c1004-1294	Metabolism	1.06	−0.01	HMG-CoA synthase 2
Gm-r1070-1996	Metabolism	0.98	−0.69	Anthocyanin 3′ glucosyltransferase
Gm-c1052-4710	Cellular transport	0.92	−1.94	Metal-dependent phosphohydrolase HD domain
Gm-c1049-5674	Binding function	0.88	−2.00	Iron ion binding/isopenicillin-N synthase
Gm-c1015-2572	Metabolism	0.86	−0.05	UDP-glycose:flavonoid glycosyltransferase
Gm-r1088-2153	Defense	0.84	−1.81	Functional candidate resistance protein KR1
Gm-c1019-932	Cellular transport	0.72	−1.53	F-box family protein (FKF1)
Gm-r1088-6566	Binding function	0.67	−0.06	Iron ion binding/isopenicillin-*N* synthase
Gm-c1066-3452	Various	0.65	−0.68	R 1 protein [*Glycine max*]
Gm-c1073-5208	Defense	0.61	−0.27	Cf-4/9 disease resistance-like protein
Gm-c1010-902	Defense	0.56	−0.18	L-ascorbate oxidase
Gm-c1081-4257	Cellular transport	0.56	−1.03	Nucleoside transporter
Gm-r1088-3720	Transcription	0.37	−0.19	Scarecrow-like transcription factor 8 (SCL8)
Gm-J03211	Defense	0.32	−0.87	Seed lipoxygenase-2 (L-2)
Gm-r1021-1280	Cellular transport	0.30	−0.12	Secretory carrier membrane protein
Gm-c1049-2011	Binding function	0.29	−0.26	Protein binding
Gm-c1049-916	Cellular transport	0.26	−0.03	Anion channel/voltage-gated chloride channel
Gm-r1070-3721	Metabolism	0.24	−0.54	Pseudo-response regulator, putative
Gm-r1070-4537	Binding function	0.16	−0.66	Calmodulin-binding protein
Gm-r1088-7998	Protein fate	0.15	−0.11	Leucine-rich repeat family protein
Gm-c1041-698	Cell cycle	0.13	−0.02	Lipase class 3 family protein c
Gm-c1049-6284	Metabolism	0.09	−0.16	Starch synthase, isoform V
Gm-r1088-551	Cellular transport	0.06	−2.27	ATPase, coupled to movement of substances
Gm-r1083-2584	Defense	0.05	−0.46	Cytochrome P450
Gm-c1009-3814	Defense	0.03	−0.05	Chitinase
Gm-c1028-3902	Metabolism	0.01	−0.22	Argininosuccinate lyase
Gm-r1021-2837	Metabolism	0.01	−0.91	7-*O*-methyltransferase
Gm-r1070-5141	Cell communication	−0.01	0.69	Protein kinase, putative
Gm-c1062-5425	Various	−0.01	0.06	Auxin down-regulated ADR6
Gm-r1088-4420	Energy	−0.01	0.73	NADPH cytochrome P450
Gm-r1088-6307	Cellular transport	−0.04	0.55	Homeobox-leucine zipper protein 13 (HB-13)
Gm-r1021-136	Metabolism	−0.07	0.38	ADP-ribosylation factor-like protein
Gm-c1048-6490	Metabolism	−0.12	0.59	Phosphoric diester hydrolase
Gm-r1089-7232	Defense	−0.14	1.02	Abscisic acid-induced protein-like
Gm-c1043-1378	Cell cycle	−0.16	0.38	DNAJ heat shock family proteins
Gm-r1088-535	Transcription	−0.19	0.15	AP2 domain-containing transcription factor
Gm-r1089-897	Cell cycle	−0.20	0.60	Cyclin, putative similar to mitotic cyclin a2-type
Gm-r1089-6291	Metabolism	−0.20	2.20	Secretory carrier membrane protein
Gm-r1089-5264	Metabolism	−0.21	0.54	NADH dehydrogenase-related

Table 1 continued

Clone ID	Function	Fold change		Description
		R	S	
Gm-AF363021	Metabolism	−0.21	0.16	Cytosolic glutamine synthetase gamma2
Gm-r1088-1288	Defense	−0.28	0.11	Small heat shock protein
Gm-c1028-4632	Metabolism	−0.37	0.20	Ubiquitin-protein ligase/zinc ion binding
Gm-r1089-303	Transcription	−0.45	0.11	RNA helicase
Gm-r1089-6301	Protein fate	−0.46	0.07	No apical meristem (NAM) family protein
Gm-r1083-3409	Metabolism	−0.58	0.37	Cysteine protease 14
Gm-r1089-7578	Defense	−0.64	0.00	Disease-resistance responsive family protein
Gm-r1088-6428	Metabolism	−0.73	0.01	Glycosyl transferase family 8 protein
Gm-r1088-6704	Metabolism	−0.74	0.07	Serine/threonine kinase-like protein
Gm-c1046-806	Cell communication	−0.76	0.23	Preprotein translocase secY subunit, chloroplast
Gm-c1062-2042	Defense	−0.79	0.26	Laccase, putative/diphenol oxidase,
Gm-c1031-168	Metabolism	−0.95	0.01	Phospholipase A1
24 hpi				
Gm-c1012-580	Defense	1.73	−1.50	Lipoxygenase
Gm-r1089-2810	Metabolism	1.70	−0.39	Secondary cell wall-related glycosyltransferase
Gm-r1021-2080	Defense	1.13	−0.51	Pathogenesis-related protein 3—kidney bean
Gm-r1089-7578	Defense	0.92	−0.08	Disease-resistance responsive family protein
Gm-r1088-5019	Cellular transport	0.88	−0.70	Xyloglucan:xyloglucosyl transferase,
Gm-r1089-1323	Transcription	0.85	−0.29	Transcription factor LFY
Gm-c1028-6512	Metabolism	0.79	−0.21	Hhydrolase, hydrolyzing *O*-glycosyl compounds
Gm-c1065-5118	Metabolism	0.74	−0.08	Glycine hydroxymethyltransferase
Gm-r1070-8283	Defense	0.51	−0.53	Gibberellin-regulated family protein
Gm-r1083-1259	Various	0.50	−0.20	Auxin-binding protein ABP19
Gm-r1089-897	Cell cycle	0.41	−0.26	Cyclin, similar to mitotic cyclin a2-type
Gm-c1054-4973	Defense	0.31	−0.15	Isoflavone reductase homolog 1
Gm-r1089-7232	Defense	0.30	−0.33	Abscisic acid-induced protein-like
Gm-c1062-2042	Defense	0.21	−1.17	Laccase, putative/diphenol oxidase,
Gm-c1016-11319	Transcription	0.19	−1.46	Scarecrow-like transcription factor 8 (SCL8)
Gm-r1021-3191	Protein fate	0.08	−0.50	Hydroxyproline-rich glycoprotein family protein
Gm-c1049-7973	Metabolism	−0.03	0.24	1-phosphatidylinositol-4-phosphate 5-kinase
Gm-c1012-640	Metabolism	−0.04	0.16	Nicastrin-related
Gm-r1089-1568	Metabolism	−0.05	0.62	Phosphatidylinositol-4-phosphate 5-kinase
Gm-M58336 J05	Energy	−0.05	0.50	Ferritin light chain
Gm-r1021-136	Metabolism	−0.08	0.34	ADP-ribosylation factor-like protein
Gm-c1031-168	Metabolism	−0.08	0.70	Phospholipase A1
Gm-c1037-587	Defense	−0.08	0.38	Avr9/Cf-9 rapidly elicited protein 236
Gm-r1088-7336	Cell communication	−0.11	0.91	Protein kinase family protein
Gm-r1089-5157	Metabolism	−0.12	0.46	SET domain-containing protein (TXR7)
Gm-c1010-902	Defense	−0.13	0.29	L-ascorbate oxidase
Gm-r1089-6207	Metabolism	−0.16	0.93	NADH dehydrogenase-related
Gm-c1043-1378	Cell cycle	−0.18	0.23	DNAJ heat shock family protein s
Gm-r1088-5805	Metabolism	−0.19	0.28	Trypsin and protease inhibitor family protein
Gm-c1062-1635	Cellular transport	−0.20	0.00	High mobility group (HMG1/2) fam profiles
Gm-c1052-919	Cell cycle	−0.20	0.15	Carboxylic ester hydrolase/hydrolase
Gm-c1040-4196	Protein fate	−0.21	0.10	Endoribonuclease L-PSP family protein
Gm-c1028-6035	Various	−0.21	0.04	Isoleucyl-tRNA synthetase, putative
Gm-c1049-916	Cellular transport	−0.23	0.03	Anion channel/voltage-gated chloride channel

Table 1 continued

Clone ID	Function	Fold change		Description
		R	S	
Gm-r1089-5784	Metabolism	−0.24	0.25	Aminopeptidase M similar to SP
Gm-c1068-4575	Defense	−0.27	0.14	Heat shock transcription factor 34
Gm-c1037-823	Metabolism	−0.27	0.09	Adenylosuccinate Synthetase
Gm-r1089-303	Transcription	−0.30	0.19	RNA helicase
Gm-r1083-828	Metabolism	−0.31	0.01	Protein phosphatase-2C; PP2C
Gm-r1070-3540	Metabolism	−0.32	0.11	Hydroxycinnamoyl transferase
Gm-r1088-5195	Cellular transport	−0.37	0.09	Peptidase family-like protein
Gm-r1070-5512	Metabolism	−0.38	0.74	MutT-like protein
Gm-r1088-863	Metabolism	−0.39	0.17	Phosphoglycerate dehydrogenase-like protein
Gm-c1051-4286	Cell communication	−0.39	0.16	Peptidase/subtilase
Gm-r1070-1807	Cell cycle	−0.48	0.01	DNA-dependent ATPase, putative
Gm-c1020-259	Energy	−0.50	0.15	Dynein light chain
Gm-c1049-5674	Protein fate	−0.50	0.09	Iron ion binding/isopenicillin-N synthase
Gm-r1070-6415	Cellular transport	−0.58	0.02	Phytochrome E
Gm-c1087-2411	Protein fate	−0.58	0.36	Small GTP-binding protein
Gm-X53958	Defense	−0.60	0.50	Chalcone synthase, CHS3
Gm-r1088-7101	Protein fate	−0.61	0.34	Calmodulin-binding protein
Gm-c1066-2122	Cell communication	−0.69	0.06	Serine/threonine kinase
Gm-c1033-634	Metabolism	−0.74	0.07	Kinase
Gm-c1018-405	Protein fate	−1.04	0.43	Binding/carnitine:acyl carnitine antiporter
48 hpi				
Gm-J03211	Defense	1.59	−1.19	Lipoxygenase
Gm-r1089-1017	Cell cycle	1.56	−0.06	DNA polymerase alpha catalytic subunit
Gm-r1083-1367	Cell cycle	1.30	−0.14	Lipase class 3 family protein c
Gm-r1070-4537	Cell communication	1.08	−0.10	Calmodulin—soybean
Gm-r1070-3540	Metabolism	1.06	−0.23	Hydroxycinnamoyl transferase
Gm-r1070-8283	Defense	1.02	−0.70	Gibberellin-regulated family protein
Gm-c1015-462	Defense	0.81	−0.03	Lipoxygenase
Gm-c1017-2578	Protein fate	0.77	−0.01	CBS domain-containing protein
Gm-c1012-640	Metabolism	0.69	−0.40	Nicastrin-related
Gm-c1031-168	Metabolism	0.68	−0.17	Phospholipase A1
Gm-c1087-2411	Protein fate	0.66	−0.26	Small GTP-binding protein
Gm-c1018-1491	Metabolism	0.62	−0.12	Neoxanthin cleavage enzyme
Gm-c1028-6035	Various	0.53	−0.43	Isoleucyl-tRNA synthetase, putative
Gm-c1032-1400	Metabolism	0.52	−0.32	Haloacid dehalogenase-like hydrolase
Gm-c1068-4575	Defense	0.52	−0.08	Heat shock transcription factor 34
Gm-r1089-7578	Defense	0.50	−0.04	Disease-resistance responsive family protein
Gm-c1066-2122	Metabolism	0.47	−0.65	Serine/threonine protein kinase, putative
Gm-r1070-7636	Metabolism	0.44	−0.71	Trypsin and protease inhibitor family protein
Gm-r1088-8596	Metabolism	0.44	−0.31	1-deoxy-D-xylulose 5-phosphate synthase
Gm-M58336 J05	Energy	0.43	−0.30	Ferritin light chain
Gm-r1089-5784	Metabolism	0.40	−0.12	Aminopeptidase M similar to SP
Gm-r1089-303	Transcription	0.40	−0.01	RNA helicase
Gm-r1088-2303	Defense	0.39	−0.02	Cytochrome P450 monooxygenaseCYP93D1
Gm-c1049-773	Metabolism	0.38	−0.87	Short-chain dehydrogenase/reductase (SDR)
Gm-r1083-4181	Defense	0.37	−0.64	Glutathione S-transferase GST 15
Gm-r1021-3058	Various	0.36	−0.62	Auxin down-regulated ADR6

Table 1 continued

Clone ID	Function	Fold change		Description
		R	S	
Gm-c1023-4394	Cellular transport	0.34	−0.23	SDAT(At)
Gm-r1088-5527	Metabolism	0.34	−0.41	Protein phosphatase-2C; PP2C
Gm-c1069-6061	Metabolism	0.30	−0.20	1-deoxy-D-xylulose 5-phosphate synthase 2
Gm-r1088-6704	Cell communication	0.29	−0.02	CBL-interacting protein kinase 1
Gm-r1070-4972	Defense	0.29	−0.99	Cytochrome P450
Gm-r1070-1807	Cell cycle	0.28	−0.52	DNA-dependent ATPase, putative
Gm-c1087-2217	Cell communication	0.24	−0.94	Transducin family protein/WD-40
Gm-c1074-6478	Defense	0.24	−0.34	Isoflavone reductase homolog 1
Gm-c1062-1635	Cellular transport	0.24	−0.14	High mobility group (HMG1/2)
Gm-c1067-211	Protein fate	0.17	−0.09	AFC1 (Arabidopsis FUS3-complementing gen 1)
Gm-r1089-8518	Cell communication	0.16	−0.36	Peptidase/subtilase
Gm-r1088-1417	Cellular transport	0.14	−0.42	Sugar transporter family protein
Gm-r1070-5781	Protein fate	0.12	−0.03	Chorismate mutase CM2
Gm-r1070-4082	Various	0.11	−4.60	Protease inhibitor, putative (DR4)
Gm-c1045-4102	Protein fate	0.10	−0.09	Calmodulin-binding protein
Gm-r1089-1101	Protein fate	0.06	−0.46	ATP binding/kinase/protein kinase
Gm-r1089-3156	Metabolism	0.04	−0.58	Annexin
Gm-c1037-823	Metabolism	0.02	−0.61	Adenylosuccinate Synthetase
Gm-c1007-2143	Protein fate	0.01	−0.43	Nucleic acid binding/transcription factor
Gm-r1070-5285	Metabolism	0.01	−0.20	Hydroxymethylglutaryl coenzyme A synthase
Gm-c1048-6490	Metabolism	0.01	−0.02	Phosphoric diester hydrolase
Gm-r1088-6307	Cellular transport	−0.04	0.41	Homeobox-leucine zipper protein 13 (HB-13)
Gm-r1021-435	Metabolism	−0.08	0.00	7-*O*-methyltransferase
Gm-c1054-5425	Metabolism	−0.09	0.29	Transferase, transferring glycosyl groups
Gm-r1021-2294	Protein fate	−0.10	0.42	Hydroxyproline-rich glycoprotein family
Gm-c1045-853	Energy	−0.12	0.05	NADPH oxidase
Gm-r1088-1288	Defense	−0.20	0.27	Small heat shock protein
72 hpi				
Gm-r1089-6800	Metabolism	2.53	−0.73	Polygalacturonase
Gm-c1067-4455	Defense	2.23	−0.19	Resistance protein
Gm-r1089-1323	Transcription	1.92	−2.56	Transcription factor LFY
Gm-c1063-3688	Defense	1.67	−1.48	Disease-resistance protein RPG1-B
Gm-c1016-10754	Binding function	1.66	−0.17	Squamosa promoter binding like-protein
Gm-c1013-2358	Metabolism	1.41	−0.74	Kinase
Gm-r1021-2390	Metabolism	1.36	−0.04	Cynnamoyl CoA dehydrogenase
Gm-r1021-2390	Metabolism	1.33	−0.51	Cynnamoyl CoA dehydrogenase
Gm-c1008-2599	Defense	1.22	−1.97	Disease-resistance responsive family protein
Gm-c1042-470	Protein fate	1.20	−0.60	Dynamin-like protein 4 (ADL4)
Gm-c1065-5118	Metabolism	1.18	−0.36	Glycine hydroxymethyltransferase
Gm-r1089-4070	Metabolism	1.17	−0.35	Triacylglycerol lipase
Gm-r1070-8283	Defense	1.06	−0.45	Gibberellin-regulated family protein
Gm-r1088-1855	Binding function	1.06	−1.00	Nucleic acid binding
Gm-J03211	Defense	0.97	−0.56	Lipoxygenase
Gm-r1021-2390	Metabolism	0.87	−0.73	Cynnamoyl CoA dehydrogenase
Gm-c1012-2563	Metabolism	0.85	−1.01	CDPK-related protein kinase
Gm-r1083-2584	Defense	0.81	−0.03	Cytochrome P450
Gm-c1028-6512	Metabolism	0.79	−0.07	Hydrolase, hydrolyzing *O*-glycosyl compounds

Table 1 continued

Clone ID	Function	Fold change		Description
		R	S	
Gm-c1062-2042	Defense	0.79	−0.48	Laccase, putative/diphenol oxidase,
Gm-c1074-7133	Defense	0.74	−0.45	Cytochrome P450
Gm-c1015-8113	Protein fate	0.66	−2.21	Leucine-rich repeat family protein
Gm-r1088-2303	Defense	0.62	−0.16	Cytochrome P450 monooxygenaseCYP93D1
Gm-r1089-8309	Cell communication	0.57	−0.50	Protein kinase, putative
Gm-c1016-1131	Transcription	0.53	−0.17	Scarecrow-like transcription factor 8 (SCL8)
Gm-r1021-4055	Defense	0.48	−0.20	Disease-resistance responsive family protein
Gm-r1088-4878	Cell cycle	0.44	−0.65	Lipase class 3 family protein c
Gm-r1083-4068	Cellular transport	0.44	−0.31	Sugar transporter family protein
Gm-c1012-640	Metabolism	0.40	−0.47	Nicastrin-related
Gm-c1061-1803	Metabolism	0.39	−0.15	Catalytic/protein phosphatase type 2C
Gm-r1089-7578	Defense	0.38	−1.08	Disease-resistance protein (TIR-NBS-LRR class)
Gm-c1082-3537	Metabolism	0.37	−0.89	Beta-glucosidase
Gm-r1070-4082	Various	0.37	−1.77	Protease inhibitor, putative (DR4)
Gm-r1070-5285	Metabolism	0.35	−0.04	Hydroxymethylglutaryl coenzyme A synthase
Gm-r1088-7095	Binding function	0.34	−1.29	Protein binding
Gm-c1066-2122	Metabolism	0.32	−0.55	Serine/threonine protein kinase
Gm-r1089-5157	Metabolism	0.24	−0.02	SET domain-containing protein (TXR7)
Gm-c1062-1635	Cellular transport	0.24	−0.27	High mobility group (HMG1/2) family protein
Gm-c1031-168	Metabolism	0.21	−0.37	Phospholipase A1 [Nicotiana tabacum]
Gm-c1013-4276	Binding function	0.20	−1.30	ATP binding/protein kinase/protein serine
Gm-c1007-2319	Metabolism	0.20	−0.01	Poly (ADP-ribose) glycohydrolase (PARG)
Gm-r1089-303	Transcription	0.17	−0.31	RNA helicase
Gm-r1070-7636	Metabolism	0.13	−0.93	Trypsin and protease inhibitor family protein
Gm-r1021-2461	Metabolism	0.12	−1.00	Cynnamoyl CoA reductase
Gm-c1074-6478	Defense	0.12	−0.30	Isoflavone reductase homolog 1
Gm-r1089-5166	Metabolism	0.09	−0.09	Glycosyl transferase family 8 protein
Gm-c1069-3688	Defense	0.08	−0.44	Cytochrome P450
Gm-c1087-2411	Binding function	0.07	−0.03	Small GTP-binding protein
Gm-r1088-5527	Metabolism	0.07	−0.62	Protein phosphatase-2C; PP2C
Gm-r1089-5784	Metabolism	0.03	−0.46	Aminopeptidase M similar to SP
Gm-r1088-4158	Binding function	−0.02	0.13	ATP binding/kinase/protein kinase
Gm-c1043-1378	Cell cycle	−0.04	0.25	DNAJ heat shock family protein s
Gm-r1083-1773	Binding function	−0.05	0.13	Binding/transporter
Gm-r1089-6291	Cellular transport	−0.07	0.61	Secretory carrier membrane protein
Gm-r1088-165	Cell communication	−0.11	1.41	Receptor protein kinase PERK1-like protein
Gm-r1070-1727	Metabolism	−0.12	0.02	Ca(2+)-dependent nuclease
Gm-r1089-897	Cell cycle	−0.12	0.36	Cyclin, putative to mitotic cyclin a2-type
Gm-c1048-6490	Metabolism	−0.12	0.74	Phosphoric diester hydrolase
Gm-c1039-1571	Cell communication	−0.14	0.29	Protein kinase family protein
Gm-r1088-1754	Metabolism	−0.17	1.15	Catalytic/ubiquitin-protein ligase
Gm-r1088-6704	Cell communication	−0.18	0.47	CBL-interacting protein kinase 1
Gm-r1070-2245	Defense	−0.19	0.19	Disease-resistance responsive family protein
Gm-c1016-6201	Various	−0.20	0.21	Vesicle-associated membrane family protein
Gm-c1019-5809	Metabolism	−0.24	1.12	Serine-type endopeptidase
Gm-c1052-919	Cell cycle	−0.24	0.51	Carboxylic ester hydrolase
Gm-c1073-4126	Protein fate	−0.31	1.31	Calcineurin B-like protein 10

Table 1 continued

Clone ID	Function	Fold change		Description
		R	S	
Gm-r1088-1288	Defense	−0.35	0.13	Small heat shock protein
Gm-r1070-6882	Cellular transport	−0.37	0.63	Carbohydrate transporter/sugar porter
Gm-c1036-107	Cellular transport	−0.38	1.00	Amino acid transport protein
Gm-c1045-853	Energy	−0.39	0.36	NADPH oxidase
Gm-c1062-8855	Metabolism	−0.39	1.09	Digalactosyldiacylglycerol synthase 2
Gm-r1089-1101	Binding function	−0.39	0.87	ATP binding/kinase/protein serine
Gm-c1007-2143	Binding function	−0.41	0.26	Nucleic acid binding/transcription factor
Gm-r1089-667	Metabolism	−0.50	0.76	Ferredoxin-related contains Pfam profile
Gm-r1088-1609	Binding function	−0.51	1.06	GTP binding/translation initiation factor
Gm-c1028-4632	Metabolism	−0.57	1.66	Ubiquitin-protein ligase/zinc ion binding
Gm-r1089-5564	Binding function	−0.57	0.97	Calmodulin-binding protein
Gm-c1028-3902	Metabolism	−0.59	0.20	Argininosuccinate lyase
Gm-c1049-7973	Metabolism	−0.61	0.90	1-phosphatidylinositol-4-phosphate 5-kinase
Gm-c1049-2011	Binding function	−0.67	0.54	Protein binding
Gm-r1088-551	Cellular transport	−0.67	0.42	ATPase, coupled to transmembrane movement
Gm-r1089-8293	Transcription	−0.69	0.24	WRKY-type DNA binding protein 1
Gm-c1079-1547	Cellular transport	−0.81	0.38	Hhigh affinity sulphate transporter
Gm-r1088-5659	Defense	−0.90	0.09	Cytochrome P450
Gm-r1070-6415	Cellular transport	−0.91	0.80	Phytochrome E
Gm-c1036-3396	Cellular transport	−0.94	0.52	Monosaccharide transporter 4
Gm-c1040-4196	Protein fate	−0.95	0.67	Endoribonuclease L-PSP family protein
Gm-c1016-4934	Cell communication	−0.96	0.34	Myc-like anthocyanin regulatory protein
Gm-r1070-6764	Cell communication	−0.97	0.16	Protein kinase-like protein
Gm-X53958	Defense	−1.08	1.06	Chalcone synthase, CHS3
Gm-r1070-5512	Metabolism	−1.11	0.60	MutT-like protein
Gm-c1020-259	Energy	−1.14	0.28	Dynein light chain
Gm-r1088-6177	Defense	−1.14	0.09	Allene oxide cyclase
Gm-r1088-7998	Metabolism	−1.17	0.24	Kinase
Gm-r1088-5909	Cellular transport	−1.31	0.22	RHM1; NAD binding
Gm-r1089-7787	Cell communication	−1.34	0.38	Receptor protein kinase PERK1-like protein
Gm-r1088-376	Transcription	−1.41	0.02	RNase PD2
Gm-r1089-7694	Cell communication	−1.44	0.33	Zinc finger (C2H2 type)

Induction ratio was expressed as fold change

glycosiltransferase was high at 12 hpi (early infection) in both genotypes. However during 24–72 hpi, expression of these genes decreased. For dihydroflavonol-4-reductases, an increase in transcript levels was observed at 24 hpi, and then these dropped down to levels observed at 12 hpi. For 4-coumarate-CoA ligase genes, expression levels did not vary over all time points (Fig. 4).

Genes involved in cellular communication

A number of genes associated with intracellular and intercellular communications, morphogenesis, and receptor proteins were also differentially expressed. An increase in up-regulated genes was observed in both genotypes through 12, 24, and 48 hpi. However, differences in transcriptional levels of these groups of genes between the two genotypes were detected at 72 hpi (Fig. 3c). At this time point, most genes were induced in the S genotype, including those coding for protein kinases, receptor-like protein kinase, and zinc fingers.

Cellular transport, transport facilitation, and transport routes

Genes related to nuclear, chloroplast, mitochondrial, vacuolar, extracellular, and vesicular transport, along with

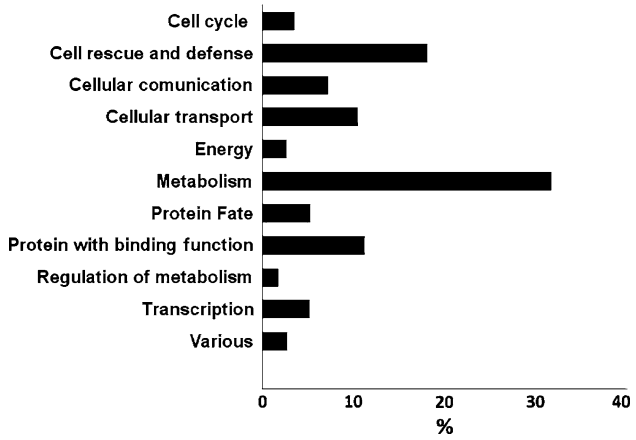

Fig. 2 Functional classification of differentially expressed genes

transported compounds and transport mechanism were included. Lower numbers of up-regulated genes were observed at 12–72 hpi in the R genotype. However, transcriptional profiles of these up-regulated genes in the S genotype were very similar at 12, 24, and 72 hpi, but lower levels at 48 hpi (Fig. 3d). Among these, carbohydrates, amino acids, and glutathione-conjugate transporters were predominant.

Energy

Genes involved in glycolysis and gluconeogenesis, electron transport and membrane-associated energy conservation, respiration, fermentation, photosynthesis, and other energy generation activities were included in this category. In general, through all time points, the numbers of genes up-regulated in the S genotype were higher than those found in the R genotype, whose expression profiles were very similar at all time points (Fig. 3e). The most common genes identified belonged to rubisco small chain precursor, NADPH cytochrome P450, and NADPH oxidases.

Metabolic processes

A number of genes associated with metabolism were differentially expressed. Genes associated with metabolism of compounds such as amino acids, nitrogen, sulphur, nucleotides, phosphates, carbohydrates, lipid, fatty acids, vitamins, prosthetic groups, and secondary metabolism were included. These genes formed the largest category consisting of 173 genes among 547 known genes. Details of differentially expressed genes are given in Supplemental Table 2. In the R genotype, a high number of up-regulated genes was detected through 12, 24, and 48 hpi, but this dropped off at 72 hpi. Expression profiles remained almost constant during all four time points in the S genotype (Fig. 3f).

Activities of many genes involved in metabolic processes are also affected upon pathogen infection, leading to significant changes in the content of several metabolites. Among those are secondary metabolites that play essential roles in many plant defense responses, and several genes associated with synthesis of chemicals that may serve as both antimicrobials and antioxidants.

Protein fate related genes

This included genes associated with protein folding, stabilization, protein targeting, sorting, translocation, modification, assembly of protein complexes, proteolysis, storage, and other protein-destination activities. In general, a higher number of up-regulated genes was observed in the R genotype compared to the S genotype, primarily during the first three time points. These genes included those associated with pentatricopeptide (PPR) repeat-containing protein-like, pectate lyase, and hydroxyproline-rich glycoprotein family protein (Fig. 3g).

Protein with binding function

A number of genes associated with binding functions to protein, peptide, nucleic acids, polysaccharide, lipid, amine, sulfate, fatty acid, metal, calcium, ATP, GTP, NAD/NADP, and oxygen were found (Fig. 3h). Overall, a lower number of up-regulated genes in the R genotype was observed at 12, 24, and 72 hpi; whereas, a higher number of genes was observed at 12 and 24 hpi in the S genotype. Moreover, a lower number of genes was detected in the S genotype at both 48 and 72 hpi. Among these latter genes, nucleic acid binding, DNA binding, calmodulin-binding protein, and zinc ion binding were found (Fig. 3h). Some of these genes play roles in modulating the rate of transcription of specific target genes (Zhang and Wang 2005).

Regulation of metabolism-related genes

Genes involved in regulation of protein activity, enzymatic activity regulation, and enzyme regulators were included. There were no changes in the number of genes between R and S genotypes at 12, 48, and 72 hpi. However, at 24 hpi higher numbers of up-regulated genes were observed in the S genotype than in the R genotype. Among those were subtilase family protein and nicastrin-related transcripts (Fig. 3i).

Transcription processes

Genes related to RNA synthesis, RNA processing, and RNA modification were grouped in this category. For both genotypes, there was a higher number of up-regulated genes at 12 hpi, but transcript levels decreased and

Table 2 Validation of microarrays-bases gene expression by real-time RT-PCR in susceptible and resistant *Glycine tomentella* genotypes at 12, 24, 48, and 72 h

Clone ID	12 h		24 h		48 h		72 h		Function
	M[a]	q[a]	M[a]	q[a]	M[a]	q[a]	M[a]	q[a]	
Resistant genotype—PI509501									
Gm-r1083-1367	3.53	2.14	2.86	1.36	4.27	2.32	4.33	2.36	Lipase
Gm-c1019-932	0.72	0.68	3.66	2.17	3.27	2.66	2.94	1.26	F-box protein
Gm-r1070-4537	−0.72	−0.44	−1.02	−0.83	1.08	−0.63	−0.58	−0.30	Calmodulin
Gm-r1089-7694	1.37	1.13	0.88	0.57	0.25	0.19	0.74	0.59	Zinc finger
Gm-c1079-1547	2.15	2.23	−2.68	−2.39	−2.81	−1.88	−2.83	−1.34	Sulphate transporter
Gm-c1004-2866	3.55	2.46	2.55	2.26	1.79	2.09	2.15	2.11	Chalcone synthase
Gm-r1089-7578	0.61	0.32	0.86	0.56	1.94	1.50	0.27	0.40	Disease-resistance protein
Gm-c1063-3688	1.55	1.38	1.77	1.59	3.15	2.14	1.89	1.20	Disease-resistance protein
Gm-c1008-2599	−0.49	−0.26	0.88	0.69	0.78	0.66	0.48	0.20	Disease-resistance-protein
Gm-r1088-2153	0.84	0.56	0.87	0.42	2.45	2.10	0.91	0.90	Resistance protein KR1
Gm-c1004-7748	2.69	2.11	1.15	1.41	1.06	1.20	0.99	0.79	Phenylalanine ammonia-lyase
Gm-r1070-2093	5.13	2.35	4.37	2.38	5.31	2.88	5.01	2.65	Chlorophyllase 1
Gm-r1083-1305	4.29	2.29	4.28	2.37	2.85	1.67	3.19	2.63	Acid phosphatase-like protein
Gm-c1051-2231	1.50	1.10	−1.20	−1.06	−1.23	−1.45	−1.69	−1.95	Calcium ion binding; Annexin 4
Gm-r1089-7995	3.26	2.10	2.75	2.17	1.81	1.76	1.75	1.19	L-iditol 2-dehydrogenase
Gm-r1089-9011	3.95	2.25	4.37	2.41	4.76	2.51	4.01	2.42	L-ascorbate peroxidase 1
Gm-r1088-8178	2.11	1.43	1.24	1.16	1.87	1.14	0.66	0.56	Lipoxygenase LOX2
Gm-r1089-4657	3.43	2.17	3.76	1.99	4.49	2.40	4.48	2.55	Oxidoreductase, 2OG-Fe(II)
Gm-r1089-6666	3.53	1.28	3.19	1.30	2.24	1.91	2.61	1.36	Pentatricopeptide (PPR) repeat
Gm-r1089-8200	3.31	2.17	3.22	2.47	1.72	1.49	1.85	1.10	Leucine-rich repeat receptor
Gm-r1089-8678	2.01	2.31	2.29	1.95	2.86	1.98	2.29	1.67	WRKY family transcription factor
Susceptible genotype—PI441101									
Gm-r1083-1367	3.06	2.72	2.32	1.17	3.08	2.92	2.48	2.31	Lipase
Gm-c1019-932	−1.53	−1.33	2.40	2.16	2.07	1.56	3.02	2.06	F-box protein
Gm-r1070-4537	−0.95	−0.97	−0.63	−0.42	−0.10	−0.23	−0.44	−0.42	Calmodulin
Gm-r1089-7694	1.05	0.95	1.13	1.19	0.59	0.46	1.36	0.51	Zinc finger
Gm-c1079-1547	1.82	1.14	−1.85	−1.21	−3.48	−2.75	−2.79	−2.16	Sulphate transporter
Gm-c1004-2866	3.06	2.24	2.90	0.14	1.75	1.97	3.29	2.74	Chalcone synthase
Gm-r1089-7578	−0.27	−0.23	1.51	1.03	1.88	1.25	2.12	1.86	Disease-resistance protein
Gm-c1063-3688	2.05	1.36	1.73	1.37	2.82	1.75	1.74	1.12	Disease-resistance protein
Gm-c1008-2599	−0.52	−0.38	1.13	0.86	0.42	0.20	−0.20	−0.45	Disease-resistance-protein
Gm-r1088-2153	−1.81	−1.41	1.18	1.11	2.05	1.78	2.92	1.63	Resistance protein KR1
Gm-c1004-7748	2.54	1.71	1.91	1.53	1.04	0.97	1.19	0.87	Phenylalanine ammonia-lyase
Gm-r1070-2093	5.32	2.45	3.22	1.89	4.72	2.56	4.98	2.34	Chlorophyllase 1
Gm-r1083-1305	3.30	1.02	2.56	1.61	4.06	2.03	3.71	2.01	Acid phosphatase-like protein
Gm-c1051-2231	−0.37	−0.47	−0.57	−0.76	−2.59	−1.49	−2.00	−1.41	Calcium ion binding; Annexin 4
Gm-r1089-7995	3.73	2.61	3.75	2.71	2.27	1.80	3.33	1.32	L-iditol 2-dehydrogenase
Gm-r1089-9011	4.59	2.33	5.13	2.56	4.19	2.09	3.73	2.47	L-ascorbate peroxidase 1
Gm-r1088-8178	1.48	1.63	1.65	0.98	2.02	1.78	0.94	0.45	Lipoxygenase LOX2
Gm-r1089-4657	4.76	2.36	3.40	2.06	4.01	2.48	2.84	1.69	Oxidoreductase, 2OG-Fe(II)
Gm-r1089-6666	1.98	1.95	2.83	2.43	2.97	2.50	3.54	2.59	Pentatricopeptide (PPR) repeat
Gm-r1089-8200	2.80	1.75	3.59	2.37	1.81	1.58	2.74	1.79	Leucine-rich repeat receptor
Gm-r1089-8678	1.55	1.38	2.40	2.13	3.25	2.08	2.23	2.31	WRKY family transcription factor

[a] Fold change was determined based on M microarray experiment and q qRT-PCR

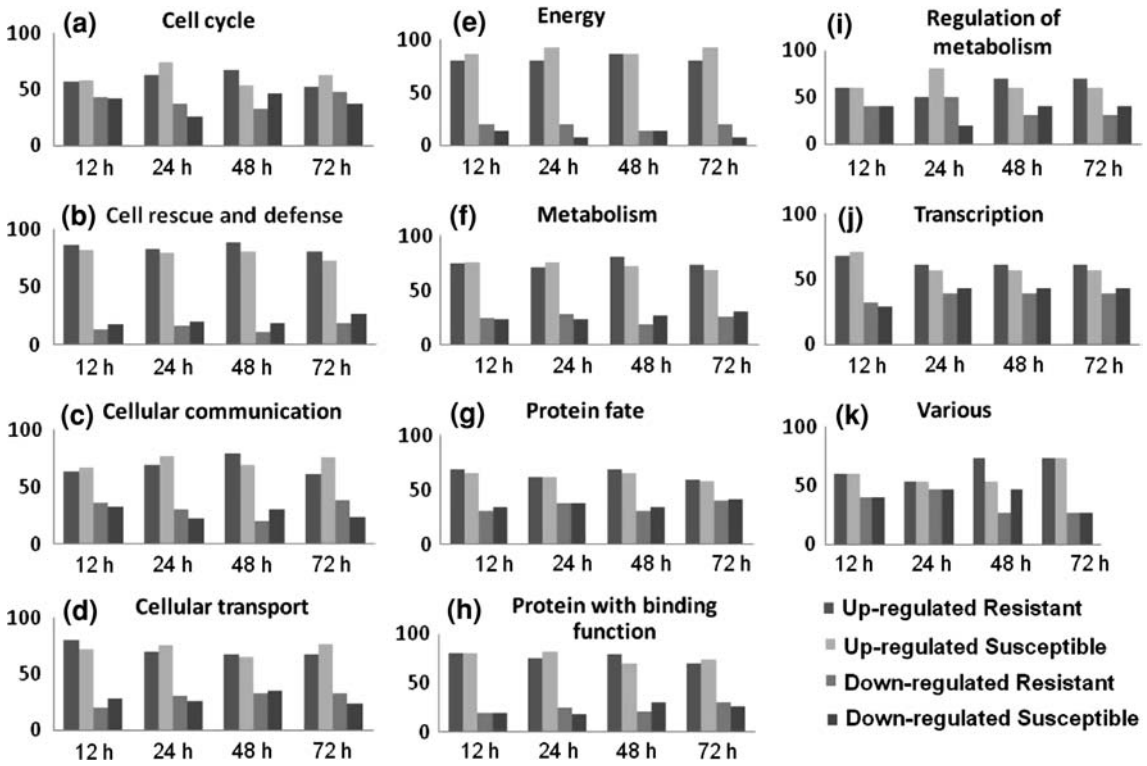

Fig. 3 Distribution of the expression ratio of differentially expressed genes in susceptible and resistant *Glycine tomentella* genotypes in each functional category

remained constant for all other time points (Fig. 3j). Among genes involved in this category, 14% belonged to the WRKY family transcription factors. This family of transcription factors have been reported to be important in plant response not only to rust infection, but also in host defense responses, by presumably changing host gene transcription to modulate defense responses (Zhang and Wang 2005; Eulgem 2005; Van de Mortel et al. 2007).

Other categories

Various other genes associated with plant hormonal regulation, aminoacyl-tRNA-synthetases, storage facilitating proteins, and transmembrane signal transduction were also identified (Fig. 3k). Among those, an auxin-regulated protein increased at both 48 and 72 hpi in the S genotype. It has been previously reported that this gene was significantly up-regulated upon virus infection, thus, it could be involved in plant resistance response (Abe et al. 2008).

Confirmation of differentially expressed genes by qRT-PCR

Quantitative real-time RT-PCR was conducted using at least one representative gene from each of the functional categories identified and listed above.

Although levels of expression were slightly different between microarray and real-time RT-PCR analyses, for most cases, the fold change was generally higher in the microarray analysis than that observed for qRT-PCR, and this was true for all genes analyzed (Table 2). Differences at the level of expression between the two methods have been reported in several studies (Ithal et al. 2007; Panthee et al. 2007; Mentewab et al. 2005). These were likely attributed to differences in sensitivity between the two different methodologies, possibly due to cross-hybridization of closely related gene families in microarray experiments, thus affecting the number of host cells responding to infection in separate experiments (Mentewab et al. 2005; Ithal et al. 2007; Panthee et al. 2007).

Discussion

Phakopsora pachyrhizi is one of the most important foliar pathogens that affect soybean. Characterizing SBR resistance in *G. tomentella*, a wild perennial relative of *G. max*, could identify new genes for resistance that ultimately could be transferred to soybean to develop SBR-resistant soybean varieties.

While microscopic studies have provided comprehensive information about infection and development of SBR

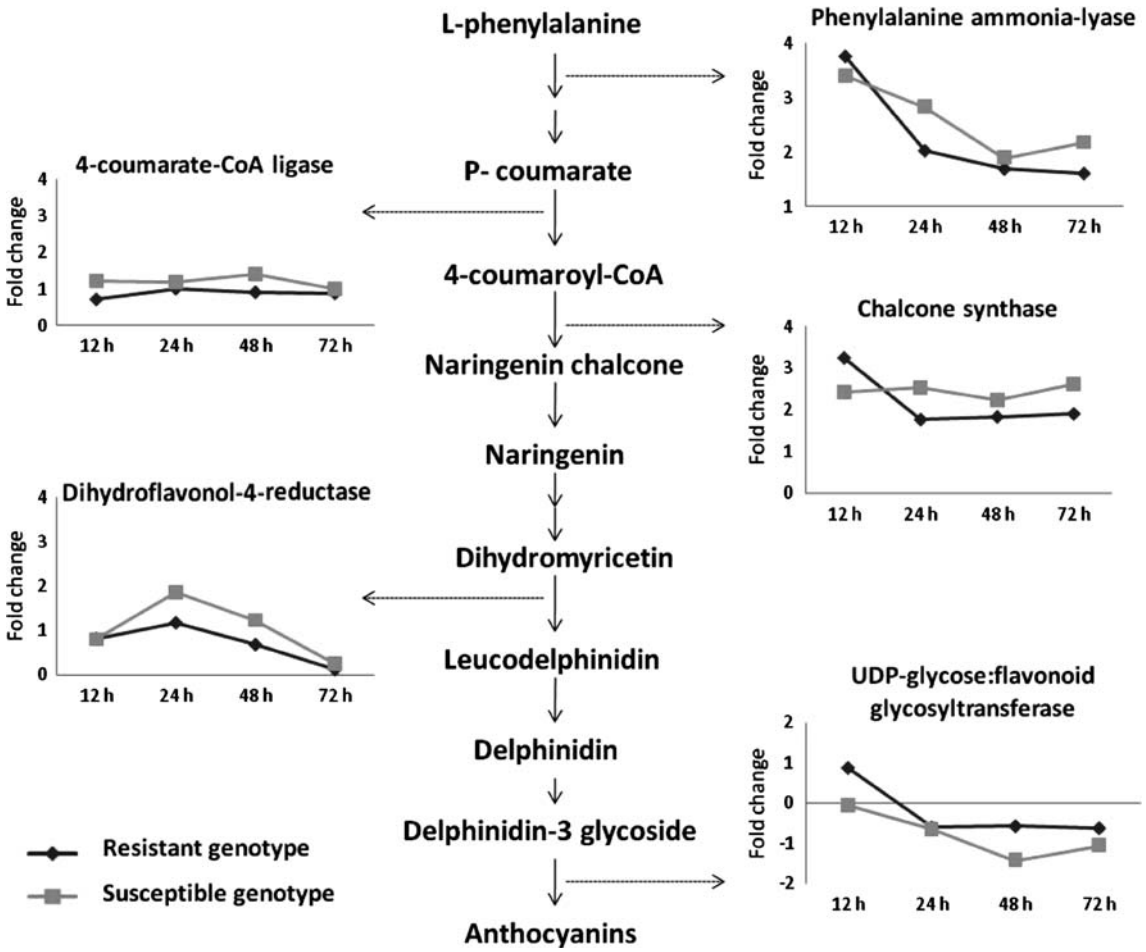

Fig. 4 Expression profiles of selected genes in the flavonoid biosynthetic pathway in susceptible and resistant *Glycine tomentella* genotypes. The expression pattern of five genes in response to *Phakopsora pachyrhizi* from 12 to 72 h. Induction ratio was expressed as fold change

infection within resistant and susceptible soybean genotypes (Koch et al. 1983; Staples 2001; Voegele and Mendgen 2003), currently there is only modest information about molecular events related to susceptible and resistant reactions. Global gene expression analysis is a useful and valuable tool for identifying genes associated with specific traits (van de Mortel et al. 2007; Panthee et al. 2009).

We designed an experimental approach to determine the effects of *P. pachyrhizi* infection on the abundance of *G. tomentella* transcripts in both susceptible and resistant genotypes using a soybean microarray. Four different sampling time points, including 12, 24, 48, and 72 hpi, were chosen to coincide with crucial stages of fungal infection. Successful infection was verified by the appearance of visual symptoms on inoculated susceptible plants (data not shown). Direct comparisons with previous microarray studies of rust infection often cannot be compared directly due to differences in pathosystems, microarray platforms, time point selection, tissue sampling methods, and analysis. Nevertheless, there are some interesting similarities with earlier studies.

Van de Mortel et al. (2007) infected soybean resistant and susceptible genotypes with *P. pachyrhizi*, and profiled soybean mRNA from 6 to 168 h after infection (hai). They reported that most genes with differential expression patterns were significantly induced early in the infection process. At 12 hai, differential gene expression changes were evident in both genotypes, and mRNA expression of these genes returned back to levels found in mock-inoculated plants by 24 hai. This suggested a non-specific innate response to SBR. Panthee et al. (2007) analyzing a transcriptome profile of *P. pachyrhizi*-exposed young soybean plants using an Affymetrix microarray reported similar observations. Among 112 genes that were differentially expressed, most were grouped in defense- and stress-related categories, including peroxidases, glutathione *S*-transferases, and lipoxygenases. It was reported that peroxidases were repressed by as much as fourfold. In contrast, in this

study, peroxidases were induced by more than threefold in the R genotype. This finding suggested these genes played important roles in the antioxidant defense of plant cells (Tripathi et al. 2009). Recently, Panthee et al. (2009) using an Affymetrix GeneChip array investigated susceptible and resistant soybean genotypes, and reported up-regulation of lipoxygenase genes. Likewise, Choi et al. (2008) reported on the up-regulation of 300 genes and down-regulation of 258 genes over 6–48 hai in *P. pachyrhizi*-infected leaves of soybean using a cDNA array with approximately 7,883 probes, and concurred on the importance of peroxidases and lipoxygenases.

In this study, several differentially expressed genes are associated with the flavonoid biosynthetic pathway reported to be involved in plant defense response through production of various defense-related secondary metabolites including phytoalexins and anti-microbial compounds, such as diadzein, glyceollin, and tannins, among others (Hahlbrock and Scheel 1989; Chang et al. 1995; Abbasi et al. 2001; La Camera et al. 2004). Choi et al. (2008) have reported high levels of expression of isoflavone reductase at 24 and 48 hai. In this study, expression of this gene increased at 12 hpi in the R genotype, but this increase was delayed until 24 hpi in the S genotype. It has been widely reported that genes in the phenylpropanoid synthesis pathway are among the most powerful antioxidants in plant cells, and they are notably up-regulated in soybean in response to challenge by *P. syringae* (Zou et al. 2005; Zabala et al. 2006). Similarly, van de Mortel et al. (2007) have reported that there are at least ten genes involved in the phenylpropanoid synthesis in R and S genotypes. Moreover, Panthee et al. (2009) have indicated that there are four enzymes involved in this pathway. In this study, five genes involved in phenylpropanoid synthesis are differentially expressed, including PAL and chalcone synthases, both have been reported to be consistently induced in plants in response to pathogen infection (Dhawale et al. 1989; La Camera et al. 2004; Zou et al. 2005; Zabala et al. 2006). Up-regulation of genes in the multibranched phenylpropanoid pathway leads to accumulation of toxic metabolites such as phytoalexins and anti-microbial compounds including pathogenesis-related proteins and cell wall components such as hydroxyproline-rich glycoproteins and lignin and its precursors (Schmelzer et al. 1984; Winkel-Shirley 2001; Zabala et al. 2006).

In this study, the enzyme 4-coumarate-CoA ligase, another enzyme involved in the phenylpropanoid synthesis, is up-regulated in both R and S genotypes at all time points, with higher levels of expression in R compared to S genotype. This is similar to findings reported by Panthee et al. (2009). This enzyme has been reported to play a role in the biosynthesis of jasmonic acid, which is effective in defensive processes (Fritzemeier et al. 1987; Howe 2004; Schilmiller et al. 2007).

Among those abundant genes whose expression change in infected plants are those pathogenesis-related (PR) genes associated with the development of systemic acquired resistance and encode anti-microbial proteins. In this study, these genes were up-regulated in the R genotype during the first three time points, and as expected, these were down-regulated in the S genotype. PR proteins have deleterious activities toward structural components of pathogens; for example, both β-1,3-glucanases and chitinases attack fungal cell walls (Fritig et al. 1998). In this study, the level of expression of the β-1,3-glucanase gene in the R genotype was more than double that of the S genotype at 12 hpi; whereas, the chitinase gene was up-regulated in the R genotype and down-regulated in the S genotype. Recently, Panthee et al. (2009) indicated that transcript responses were largely dependent on the specific soybean growth stage and their interactions with *P. pachyrhizi*, and a larger number of genes were differentially expressed at V4 (approximately 2 weeks before bloom). These findings were similar to those observed in this study whereby most of the up-regulated genes belonged to those involved in the general defense-related response.

In this study, genes encoding glutathione *S*-transferase, a detoxification enzyme that plays an important role in pathogen-resistance in plants mainly via peroxide and xenobiotic detoxification (Sugiyama and Sekiya 2005; Luo et al. 2005; Rouhier et al. 2008) have been found to be up-regulated in the R genotype. This is similar to findings reported previously by Choi et al. (2008) and Panthee et al. (2007).

Phyto-oxylipins are assumed to play critical roles in plant defense response as they act as signaling molecules and/or protective compounds such as antibacterial and wound-healing agents. Evidence for accumulation of fatty acid derivatives in plant–microbe interactions has been widely reported (Blee 2002; La Camera et al. 2004). Phyto-oxylipins are produced during the metabolism of unsaturated fatty acids through their oxidation catalyzed by cytochrome P450, lipoxygenase, and α-dioxygenase (Rancé et al. 1998; Sanz et al. 1998; Tijet et al. 1998). In this study, cytochrome P450 monooxygenase is up-regulated to a higher level in R than in S genotypes. Moreover, lipoxygenase is also differentially expressed, and that α-dioxygenase transcriptional activity is higher in R than in S genotypes as early as 12 hpi. It is reported that these three enzymes, cytochrome P450 monooxygenase, lipoxygenase, and α-dioxygenase, play important roles in preventing the potentially harmful effects of free fatty acid accumulation that could be induced by lipases in the early responses of plants against stress and pathogens (Croft et al. 1993; Blee 2002).

For genes involved in cellular communication, an increase in transcripts at all first three time points in R and S genotypes has been observed in this study. Among these are genes encoding for protein kinases, receptor-like protein kinase, serine/threonine kinases, and zinc fingers. Garcia-Brugger et al. (2006) has reported that genes encoding for kinases and MAPKS cascades as well as oxidative stress-associated genes are all related with the establishment of innate immune responses at particular stages during early pathogen infection processes.

Many WRKY proteins are involved in defense against pathogenic bacteria, fungi, viruses, and oomycetes (Yang et al. 1999; Beyer et al. 2001; Chen et al. 2002; Deslandes et al. 2002). Choi et al. (2008) have demonstrated that MYB transcription factors are up-regulated at 12 and 24 hai, and down-regulated at 48 hai; whereas, WRKY transcription factors are up-regulated at 12 hai and down-regulated at both 24 and 48 hai. Similar findings have been observed in this study whereby up-regulation of WRKY transcription factors is noted at 12 hpi in the R genotype, but only increased at 24 and 48 hpi in the S genotype, while, MYB transcription factors genes are down-regulated in both genotypes at all time points. This is also similar to findings reported by Van de Mortel et al. (2007) and Panthee et al. (2009). Previously, it has been reported that WRKY genes may be activated by the same physiological or environmental stimulus, such as pathogen attack, wounding or senescence, and that these are possibly involved in multiple pathways leading to an array of physiological responses (Cheong et al. 2002; Deslandes et al. 2002; Zhang and Wang 2005). All of these findings suggest that transcription factors have both positive and negative regulatory functions that control expression of additional genes involved in defense pathways to prevent infection.

Based on the above findings, genes associated with the flavonoid biosynthesis pathway as well as those coding for peroxidases and lipoxygenases are likely to be involved in rust resistance in soybean, and serve as good candidates for functional studies. Among genes involved in the flavonoid biosynthetic pathway, those coding for PAL and chalcone synthases are differentially expressed in *G. tomentella* genotypes tested in this study, with higher levels of expression detected in the R genotype. Although 4-coumarate-CoA ligase is up-regulated in both R and S genotypes at all time points, the observed higher levels of expression in R compared to S genotypes, also support the likelihood of its involvement in rust resistance. Moreover, peroxidases are induced by more than threefold in the R genotype, while, lipoxygenases are notably up-regulated in the R genotype 24 and 48 hpi, thereby highly involved in rust resistance responses. All of the above-listed genes are undergoing functional studies in our laboratory. However, it is also important to point out that the majority of differentially expressed genes identified in this study have unknown functions. Therefore, this large pool of genes should be investigated further as they may also play specific roles in rust resistance in soybean.

In conclusion, in this study, we have identified a comprehensive list of transcripts regulated in R and S *G. tomentella* genotypes at four time points following *P. pachyrhizi* infection. These findings provide new insights into the complex changes in plant gene expression occurring globally in response to SBR. Some of these genes can serve as potential targets for genetic improvement of soybean plants for enhanced rust resistance.

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