Theor Appl Genet (2009) 118:359–370
DOI 10.1007/s00122-008-0905-1

ORIGINAL PAPER

Differential expression of genes in soybean in response to the causal agent of Asian soybean rust (*Phakopsora pachyrhizi* Sydow) is soybean growth stage-specific

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Received: 20 May 2008 / Accepted: 23 September 2008 / Published online: 14 October 2008
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Abstract Understanding plant host response to a pathogen such as *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust (ASR), under different environmental conditions and growth stages is crucial for developing a resistant plant variety. The main objective of this study was to perform global transcriptome profiling of *P. pachyrhizi*-exposed soybean (*Glycine max*) with susceptible reaction to the pathogen from two distinct developmental growth stages using whole genome Affymetrix microarrays of soybean followed by confirmation using a resistant genotype. Soybean cv. 5601T (susceptible to ASR) at the V_4 and R_1 growth stages and *Glycine tomentella* (resistant to ASR) plants were inoculated with *P. pachyrhizi* and leaf samples were collected after 72 h of inoculation for microarray analysis. Upon analyzing the data using Array Assist software at 5% false discovery rate (FDR), a total of 5,056 genes were found significantly differentially expressed at V_4 growth stage, of which 2,401 were up-regulated, whereas 579 were found differentially expressed at R_1 growth stage, of which 264 were up-regulated. There were 333 differentially expressed common genes between the V_4 and R_1 growth stages, of which 125 were up-regulated. A large difference in number of differentially expressed genes between the two growth stages indicates that the gene expression is growth-stage-specific. We performed real-time RT-PCR analysis on nine of these genes from both growth stages and both plant species and found results to be congruent with those from the microarray analysis.

Communicated by D. A. Lightfoot.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-008-0905-1) contains supplementary material, which is available to authorized users.

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Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the most important row crops in the USA, which annually contributes more than 19 billion dollars to the national economy (Soytech Inc. 2006). A number of biotic and abiotic factors have made soybean production more challenging. At present, one of the threats to this crop is Asian soybean rust (ASR), which is caused by *Phakopsora pachyrhizi* Sydow, a fungal pathogen that overwinters in a number of alternate host plants including Kudzu in the southern U.S. since fall of 2004. It has been shown that ASR can cause complete crop failure under the most severe conditions (Gupta et al. 1999; Hartman et al. 1991; Rahangdale and Raut 2003; Ramteke et al. 2003; Tschanz and Shanmugasundaram 1984) This disease poses a serious threat to the soybean industry in U.S., which needs to be managed before it becomes epidemic.

No commercial soybean cultivars are available in the U.S. that are confirmed to be ASR-resistant (Schneider

et al. 2006), although there are efforts to address the problem by conventional as well as molecular approaches (Hyten et al. 2007; Monteros et al. 2007). One approach at the molecular level is by global gene expression analysis in soybean in response to *P. pachyrhizi* (DeMortel et al. 2007a, b; Panthee et al. 2007). Global gene expression analysis has emerged as an important tool to understand how plants respond to biotic and abiotic stresses in order to identify genes associated with specific traits. Furthermore, it is important to target appropriate environmental conditions and growth stages to identify differentially expressed genes appropriate for agronomic conditions relevant to field production. Microarray analysis has been performed to aid the development of resistant varieties by identifying resistance genes (DeMortel et al. 2007a; Panthee et al. 2007). However, these studies did not consider the growth stage in the analysis, although it is clear that there is developmental regulation in gene expression (Davies et al. 1997; Tian et al. 2003). Therefore, one objective of this study was to include growth stage as a factor to evaluate soybean gene expression in response to *P. pachyrhizi* infection. Recently, DeMortel et al. (2007a) concluded that timely expression of resistance genes could prevent ASR development, indicating that growth stage-dependent gene expression is an important factor for a resistant genotype.

Isoflavone synthesis is associated with responses of plants to disease. In soybean seed in particular, isoflavone accumulation is development-dependent (Dhaubhadel et al. 2007). Induction of isoflavone related genes at different growth stages indicates that disease response of the host plant might also vary among growth stages. Therefore, evaluation of gene expression in response to *P. pachyrhizi* at various growth stages could be helpful to identify candidate resistance genes.

Identification of resistance genes is the first step toward the development of resistant varieties and gauging endogenous up-regulation of genes in response to disease-causing agents might give indications that there could be relevant defense genes. Microarray analysis is a useful technology for assaying transcriptional responses for biotic and abiotic plant stresses (Bohnert et al. 2001; Kawasaki et al. 2001; Moy et al. 2004; Schenk et al. 2000). This technology has been used for such studies including soybean root rot caused by *Phytophthora sojae* (Moy et al. 2004). This technology has also been used to identify the homologs of soybean cyst nematode in *Arabidopsis* (Puthoff et al. 2003).

The main objective of this study was to perform a global transcriptome profiling of *P. pachyrhizi*-exposed soybean with resistance and susceptible reaction to the ASR from two distinct developmental growth stages using whole genome Affymetrix microarrays of soybean and evaluate the differences between growth stages for gene expression. Expression patterns of selected genes were confirmed in *G. max* and *Glycine tomentella* by real-time RT-polymerase chain reaction (PCR) experiments.

Materials and methods

Plant materials

Soybean [*G. max* (L.) Merr] cv. 5601T (*PI 630984*) and *G. tomentella* (*PI 446998*) plants were grown in a greenhouse in potting mix at the University of Florida, North Florida Research and Education Center in Quincy, Florida. Six 4-l pots were planted with four seeds per pot in the third week of August and culled to three plants per pot. Growing environments were approximately 28/20°C and 14/10-h photoperiod. Cultivar 5601T was chosen because this is a USDA check for germplasm evaluation in the southeastern U.S. and is highly susceptible to *P. pachyrhizi* (http://www.cropsoil.uga.edu/soylab/rustresistance.html). *G. tomentella* was analyzed since it has been reported to be ASR resistant (Hartman et al. 1992; Schoen et al. 1992). All plants were kept in the same chamber of the greenhouse until the V_4 (advanced vegetative growth stage approximately 2-week before bloom) and R_1 (first bloom) growth stage (Fehr and Caviness 1977).

Inoculation of plants, sampling and RNA isolation

Seven week-old V_4 stage plants and 9-week-old R_1 stage plants were inoculated with *P. pachyrhizi*. Seeds were planted at staggered times so that both sets of plants could be inoculated at once. Inoculum of *P. pachyrhizi* was collected from a nearby soybean field at Quincy, Florida that was naturally and severely infected. The identity of *P. pachyrhizi* spores were confirmed using light microscopy from field collections prior to inoculation. Three out of six pots from each growth stage were transferred to the adjacent chamber with identical growing conditions of the greenhouse so that no cross-contamination would occur. Inoculation of plants, sample collection and RNA isolation was done as described elsewhere (Panthee et al. 2007). Post-inoculation time for sampling was chosen on the basis of results from past studies (Puthoff et al. 2003; Schenk et al. 2000).

DNA isolation and polymerase chain reaction

DNA was isolated from the same soybean plants (control and inoculated) by a cetyl-trimethyl-ammonium bromide (CTAB) method (Stewart and Via 1993). PCR was performed on control as well as inoculated samples using *P. pachyrhizi*-specific primers (Frederick et al. 2002) to confirm the presence of *P. pachyrhizi* in the samples from

inoculated plants using a Master Cycler (Eppendorf AG, Hamburg, Germany). Bands were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV light. All primers were acquired from Integrated DNA Technologies (IDT DNA Inc., Coralville, IA, USA).

Microarray analysis

Soybean whole-genome GeneChip Arrays containing over 35,611 *G. max* unigenes were purchased from Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) and the microarray experiment was performed as described before (Panthee et al. 2007). Microarray preparation and hybridization was performed at the University of Tennessee Knoxville Affymetrix Core Facility. Each microarray experiment was replicated three times with an independent biological sample (i.e. sample from an individual pot of control and inoculated plants) for statistical analysis for a total of 12 chips.

Real-time RT-PCR

Real-time RT-PCR as described by Yuan et al. (2006) was performed on a subset of nine differentially expressed genes from each growth stage in three replications each of four biological samples as confirmation and for the expansion of biological sample sizes. Real-time RT-PCR was also performed on all these selected genes from *G. tomentella* RNA samples with three replications. For real-time RT-PCR analysis of gene expression level, the primers were designed for amplicons of about 25 bp for each gene using Primer Express 2.0 software (Applied Biosystems). A list of primers used for real-time RT-PCR is listed in Table 1. A tubulin gene (Shen et al. 2006) was used as an internal control. Primer titration and dissociation experiments were performed to ensure that no primer dimers or false amplicons would be produced that would interfere with the amplification. A standard curve was developed from three serial diluted concentrations of cDNA.

Microarray and real-time RT-PCR data analysis

Data analysis was carried out using the Array Assist software from Stratagene as described before (Panthee et al. 2007). The CEL file was created from the DAT image file of the chip using the Gene Chip operating software from Affymetrix. Each probe set consists of 11–20 probe pairs which are summarized into a signal value using the GC-RMA algorithm available in Array Assist. GC-RMA is a model based algorithm which takes into consideration of the multiple chips present in the experiment (Wu et al. 2004). The algorithm performs a background adjustment taking into consideration probe sequence information to correct for non-specific binding followed by normalization and expression measure calculation. The expression values were then converted into $\log_2$ scale. Probes that do not differ in expression across all the experiments by $\leq$2-fold were filtered out. This resulted into 17,203 probe sets, which were subjected to two-way ANOVA using growth stage as the first factor and ASR treatment as the second factor. Individual effect of each factor and their interaction was tested using Array Assist. Furthermore, an unpaired *t* test at an individual growth stage (i.e. V_4 and R_1) was performed to determine the number of genes with differential expression with respect to *P. pachyrhizi* infection. The genes determined to be up-regulated or down-regulated in the test sample relative to the control sample (determined on the basis of unpaired *t* test) were examined by setting a cut-off value of 2.0 and less than 0.05 probability level for the signal log ratio for the data from each growth stage separately. A Venn diagram was created on the basis of two-way

Table 1 Selected genes and their primer sequence used for real-time RT-PCR to verify the expression of gene in microarray analysis

Probe set ID	Gene title	Primers	
		Forward	Reverese
Gma.8490.1.S1_at	Thiamin biosynthetic enzyme	GGGATGAAGGCACTTGACATG	CAGGCACAACCTCCCTAGTGA
GmaAffx.93603.1.S1_s_at	Ferritin light chain	GGCTCTTGCTCCATCCAAAG	TTCTGAGCACCCCCAACACT
Gma.51.1.S1_at	Phytoene desaturase	TGTTCTTTCTGGGAAGCTTTGTG	TTTCTGGCCCCGAGTAGCTA
Gma.15625.1.S1_x_at	Asparagine synthetase 2	ACAACCTTGATCCCTCTGGTAGAG	CACCCTTGTTGTTCTGGTTTTCA
Gma.16877.1.S1_at	β-Amyrin synthase	GGAAGCCGATCAAATGTTGTACA	CCGCCTGTCCAGCATGA
Gma.17702.1.S1_at	At5g18230 gene	CGTTTCTCCCTCCATTTTTCC	CGCTCAGGCTGAAGAGTGATAA
Gma.117.1.S1_at	Syringolide-induced protein 19-1-5	CCCAAGAACCAGGCAATGAG	TCCCCTTGTGGCCCAAT
Gma.15307.1.S1_at	Sali5-4a protein	GGAACTAACCCTGTTTGCCATT	GGCAGTGTCCACAGATAAATTGG
Gma.6633.1.S1_x_at	Sali3-2	AAGTCCGTGAAACAACAGCTTTC	AGCAAGTGCCTGAGTTTTGGTT

ANOVA, and common genes between V_4 and R_1 growth stages. Cluster analysis was performed on the common genes between V_4 and R_1 growth stages using GeneSpring GX 7.3.1 software (Agilent Technologies). Real-time RT-PCR data analysis was performed with the *t* test method as described by Yuan et al. (2006). A list of known or unknown genes was generated by performing BLASTn search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) from Affymetrix and Soybase (http://www.soybase.org) websites. Genes with $E \leq 1 \times 10^{-4}$ were retained for further analysis and are reported. Genes involved in various biological functions and processes were categorized on the basis of information obtained from BLASTn search.

Results

All inoculated samples were PCR-positive whereas controls were negative for *P. pachyrhizi*, which was a crucial determination since ASR symptoms were not visible even in the inoculated plants when leaf samples were collected for nucleic acid extraction. However, infection was later verified on the basis of foliar symptoms appearing on soybean plants and also a hypersensitive reaction in *G. tomentella* (Fig. 1). Scatter plots of signals from averaged rust inoculated versus control samples from both V_4 and R_1 growth stages revealed the normal distribution of data with up- or down-regulation of genes (Fig. 2). The original datasets are available at http://web.utk.edu/~genome.htm (the University of Tennessee Microarray Database site). The same set of data will also be available publicly at Gene Expression Omnibus of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo).

Differentially expressed genes: growth stages versus *P. pachyrhizi* treatment

When analyzed by two-way ANOVA using growth stage and rust treatment as two different factors with three replications, there were 2003 genes with significant differential expression with respect to growth stage. Overall, in the *P. pachyrhizi* treatment, there were 7,370 genes expressed differentially in soybean without considering the growth stage (Table 2). However, there was a significant interaction between growth stage and *P. pachyrhizi* treatment, in which 3,998 genes were expressed differentially in all possible combinations indicating that gene expression pattern in response to *P. pachyrhizi* inoculation was growth stage (V_4 or R_1) specific (Fig. 3). Details of number of differentially expressed genes with respect to growth stage and ASR treatment at various levels of probability are given in Table 2. Gene expression pattern at particular growth stage is more meaningful in biological sense from the gene identification perspective. Therefore, the discussion will be focused toward gene expression at V_4 and R_1 in response to *P. pachyrhizi* treatment.

Fig. 1 **a** Leaves of soybean cv. 5601T. (a) *Phakopsora pachyrhizi* inoculated showing foliar symptoms, and (b) mock inoculated showing no symptoms of Asian soybean rust. **b** Leaves of *Glycine tomentella*. (a) *Phakopsora pachyrhizi* inoculated showing no foliar symptoms, and (b) mock inoculated also showing no symptoms of Asian soybean rust

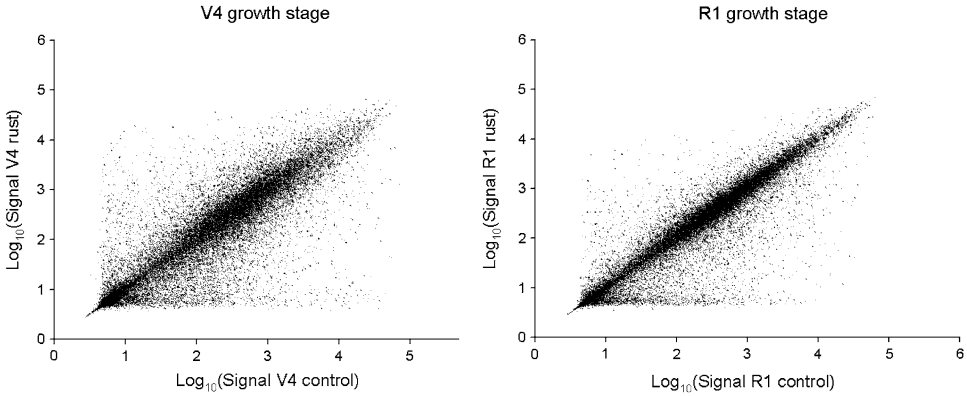

Fig. 2 Scatter plot of differentially expressed genes in response to *Phakopsora pachyrhizi* at V_4 and R_1 growth stage of soybean

Table 2 Number of significantly differentially expressed genes at different corrected probability levels with Benjamini–Hochbedrg false discovery rates as revealed by two-way analysis of variance

Factor	Corrected probability levels				
	$P < 0.05$	$P < 0.02$	$P < 0.01$	$P < 0.005$	$P < 0.001$
Growth stage (factor A)	2,003	893	483	272	83
Rust treatment (factor B)	7,370	5,213	3,864	2,803	1,183
Interaction (A × B)	3,998	2,133	1,146	611	150
Expected number of genes by chance	860	344	172	86	17

Factor A = growth stage, factor B = rust treatment

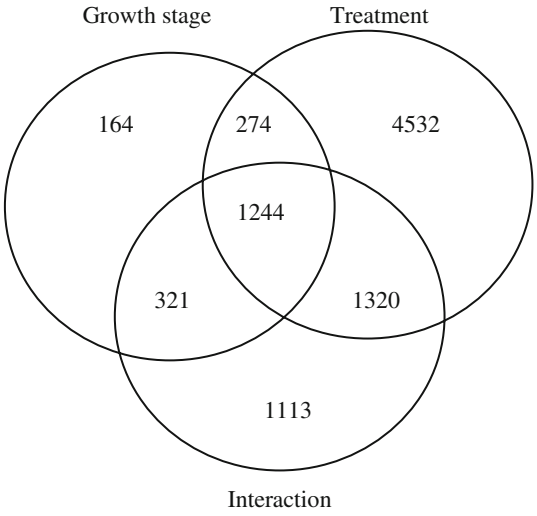

Fig. 3 Venn diagram showing commonality of differentially expressed genes with respect to growth stage, rust treat treatment and their interaction in soybean as revealed by two-way analysis of variance

Differential gene expression: *P. pachyrhizi* treatment at individual growth stage

Upon analyzing the data by Array Assist using Benjamini–Hochbedrg false discovery rate (FDR) at 5% probability level at V_4 growth stage, a total of 5,056 genes were expressed differentially, of which 2,401 were up-regulated (Table S-1 in Electronic supplementary material). To make the selection more stringent and to get more accurate information about the expressed gene, we used $E \leq 1 \times 10^{-4}$ value as a cutoff point so that the reported gene list would be more conservative. Thus, 4,186 differentially expressed known genes, of which 1,986 were up-regulated, were identified in the analysis. A total of 870 unknown genes at V_4 growth stage provided a large pool to identify genes of interest specific to ASR resistance.

There were 579 genes expressed significantly at R_1 growth stage, of which 264 were up-regulated (Table S-2). Out of this, there were 462 differentially expressed known genes, of which 208 were up-regulated. There were nearly ten times more genes expressed differentially at V_4 growth stage as compared to R_1. This indicated that transcript response is largely dependent on the specific soybean growth stage with respect to *P. pachyrhizi* inoculation. This difference accounts for the large number of genes expressed from the same gene family and also some of the specific genes expressed at V_4 growth stage. For example, there were 54 transcripts from cytochrome P450 family at V_4 growth stage whereas there were only 15 at R_1 growth stage. WRKY transcription factors were represented by 28 and 11 transcripts at V_4 and R_1, respectively. There were 24 and 9 lipoxygenase transcripts expressed differentially at the two growth stages, respectively. However, there were also unique genes expressed differentially at V_4 and R_1 growth stages. Details of such comparisons are presented in

Table S-3. This is novel information and is expected to be helpful to concentrate on V_4 growth stage for further investigation of gene expression for gene discovery.

Unique genes at the V_4 growth stage

There were 1,848 differentially expressed unique known genes at V_4 growth stage, of which 849 were up-regulated (Table S-4). Many of these genes have been reported to have defense, stress, metabolic or transport related roles in the plant system in response to biotic or abiotic stress. For example, 12-oxo-phytodienoic acid reductase and 4-coumarate-CoA ligase-like protein are involved in jasmonic acid (JA) biosynthesis, which has been reported to be involved in defense systems in plants.

Unique genes at R_1 growth stage

More than 52% of total differentially expressed genes at R_1 growth stage were either unknown or their biological function was unknown. Out of those 48% known genes, we found 131 unique genes at R_1 growth stage, of which 79 were up-regulated (Table S-5). Important known up-regulated genes were glucan endo-1,3-beta-D-glucosidase, glycosyl hydrolases, HEV1.2, isoflavone reductase, L-lactate dehydrogenase, *O*-methyltransferase, pectin methylesterase, peroxisomal small heat shock protein Acd31.2, phospholipase PLDb1, plasma membrane Ca^{++}-ATPase, protein phosphatase 2C ABI1, putative cytochrome P450/ABA 8′-hydroxylase CYP707A1, and a disease resistance response protein.

Common genes between V_4 and R_1 growth stages

Despite the large differences between two growth stages in terms of gene expression, many genes were from the same gene families. Among the differentially expressed genes, there were 333 common genes between V_4 and R_1 growth stages (Table 3, Fig. 4), of which 125 were up-regulated and 208 were down-regulated (Table S-6). Out of 333 genes, we found 280 differentially expressed known genes

Table 3 Number of significantly differentially expressed genes at V_4 and R_1, and common genes between these two growth stages of soybean in response to *Phakopsora pachyrhizi*

Growth stage	Up-regulated	Down-regulated	Total
V_2	15 (13)	9 (8)	24 (21)
V_4	2,401 (1,986)	2,655 (2,200)	5,056 (4,186)
R_1	264 (208)	315 (254)	579 (462)
Common	125 (107)	208 (173)	333 (280)

Number given within the parenthesis is the number of known genes selected with $E \leq 1 \times 10^{-4}$ value from BLAST search

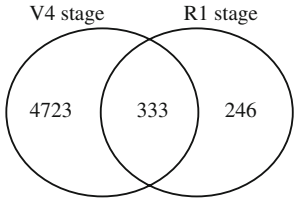

Fig. 4 Venn diagram showing commonality of differentially expressed genes with respect to *Phakopsora pachyrhizi* inoculation at V_4 and R_1 growth stage in soybean

common between V_4 and R_1 growth stage, of which 107 were up-regulated.

All differentially expressed genes from V_4 and R_1 growth stage were classified into 13 categories on the basis of gene ontology (GO) process and function description including genes with unknown biological function, and unknown genes (Fig. 5), and detailed lists are in Tables S-1 and S-2.

Defense related genes

On the basis of GO process description, genes associated with cell death defense response, JA biosynthesis, defense response lignin biosynthesis, defense response signal transduction, response to pathogen, salicylic acid biosynthesis were grouped under defense category. There were 193 genes at V_4, and 40 at R_1 growth stage differentially expressed under this category, of which 36 were common between these two growth stages (Fig. 5). Detailed information of all these genes is given in Table S-4.

Stress related genes

There were 224 and 27 stress related associated with response to heat, cold, starvation, oxygen, salt and other abiotic stresses that were differentially expressed genes at V_4 and R_1 growth stages, respectively, of which 13 were common (Fig. 5). Stress associated up-regulated genes at V_4 were MYB29 protein, MYBR5, phosphatase-like protein, universal stress protein family, and dehydrin, whereas those at the R_1 growth stage were L-lactate dehydrogenase, peroxisomal small heat shock protein Acd31.2, and protein phosphatase 2C. Common up-regulated stress related genes were ACC oxidase, α-glucan water dikinase, homeodomain-related, hypoxia induced protein conserved region, and a putative TFIIIA (kruppel)-like zinc finger protein.

Metabolic processes

Genes associated with metabolism of compounds such as carotenoid, glucosinolates, carbohydrates, protein, glycine betaine, nucleotide base, riboflavin, abscissic acid and other compounds were grouped under this category. We found 226 differentially expressed genes involved in metabolic processes at V_4 and 19 at R_1 growth stage, of which

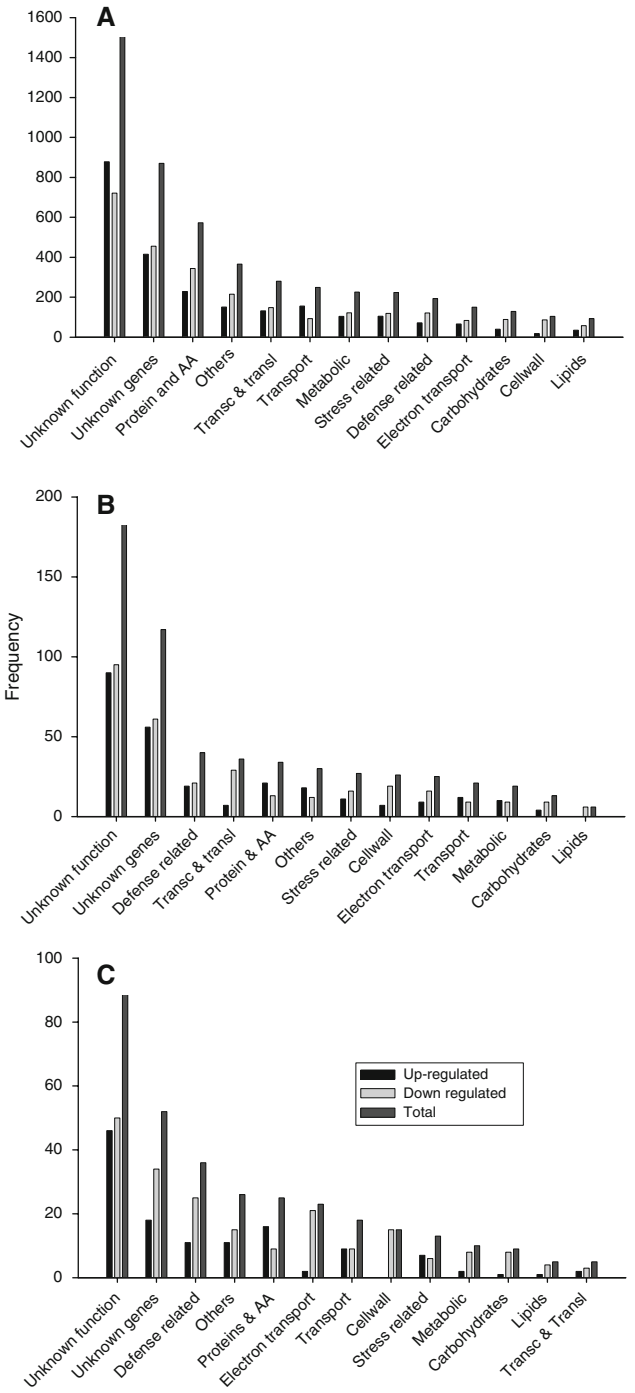

Fig. 5 Differentially expressed genes at **a** V_4 growth stage, **b** R_1 growth stage, and **c** common between V_4 and R_1 growth stage in response to *Phakopsora pachyrhizi* in soybean classified on the basis of their function

10 were common (Fig. 5). Major up-regulated common genes related to metabolic processes identified in this experiment were phytoene dehydrogenase and epoxide hydrolase. Details on genes involved in metabolic processes of secondary metabolic compounds at V_4 growth stage can be found in Tables S-1 and S-2.

Cell-wall related genes

Genes involved in cell wall modification, and lignin and cellulose biosynthesis were grouped in this category. We found 104 and 26 genes differentially expressed at V_4 and R_1 growth stage, respectively, out of those, 15 were common (Fig. 5). We found differentially expressed genes such as SF21D2 splice variant protein, galactoside 2-alpha-L-fucosyltransferase, xyloglucan fucosyltransferase, Class 1 chitinase, and expansin-like protein at V_4 growth stage whereas an *O*-methyltransferase was detected at R_1 growth stage (Tables S-1, S-2).

Electron transport

Genes involved in electron carrier activity were included in this category. We detected 150 differentially expressed genes at V_4 and 25 at R_1 growth stage, of which 23 were common. Most of the transcripts were from P450 and WRKY gene family, which were common between V_4 and R_1 growth stage (Fig. 5). Those common electron transporters were a blue (Type 1) copper domain, CYP82C1p, cytochrome b5, members of cytochrome p450 gene family, F3M18.8, glutaredoxin and TGF-beta receptor. Some of those at V_4 were cytochrome b561, cytochrome P450 71D10, flavoprotein β-subunit-like, ferredoxin–thioredoxin reductase, ferrodoxin NADP oxidoreductase, glycolate oxidase, polyamine oxidase, putative NADH dehydrogenase, and thioredoxin domain 2.

Other categories

Other genes associated with biosynthesis and metabolism of carbohydrates, lipids, protein and amino acids, signal transduction, transcription and translation, and other physiological and biochemical processes were also categorized and are presented in Tables S-1 and S-2.

Cluster analysis

We performed cluster analysis with GeneSpring GX (Agilant Technologies, Inc.) on the basis of fold induction value of 333 common genes between V_4 and R_1 growth stage. It produced seven distinct clusters, which are shown as a heat map and dendrogram (Fig. 6). These results did not match with the functional categories.

Number of transcripts expressed differentially

While there was a large difference between V_4 and R_1 growth stage in terms of gene expression in all categories in response to *P. pachyrhizi*, we examined if this difference was because of unique single genes or member of the gene

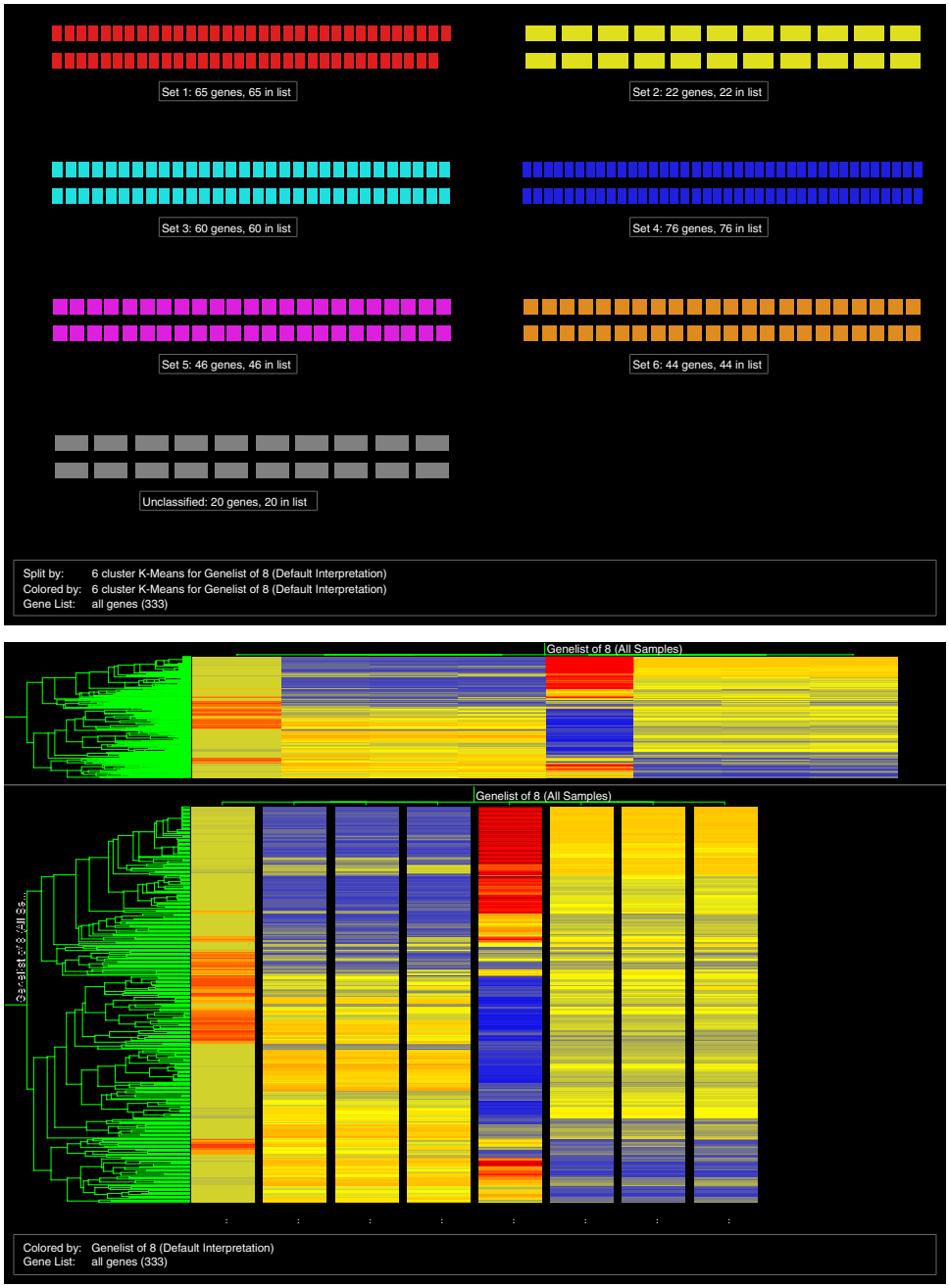

Fig. 6 Cluster analysis of differentially expressed common genes between V_4 and R_1 growth stage in soybean in response to *Phakopsora pachyrhizi*

family. Cytochrome gene family members were found to be the most differentially expressed both at V_4 and R_1 growth stage, 54 and 15, respectively, 14 of those were common between the two growth stages. The second most common gene family transcripts were ferritin and lipoxygenase (9), followed by proline rich protein and cellulose synthase, six each. Although number of ferritins was similar at V_4 (10) and R_1 (9) growth stage, there were 24 lipoxygenases at V_4, and only 9 at R_1 growth stage. Observed was a similar trend in other gene families such as GST, heat shock protein, proline rich proteins, cellulose synthase, alcohol dehydrogenase and others (Table S-3). There were also a large number of unique genes found at V_4 growth stage with a single transcript expressed differentially (Table S-3). This indicated that genes from V_4 growth stage did express significantly more in number either from the same gene family or a single transcript.

Confirmation of differentially expressed genes by real time RT-PCR

A randomly selected list of genes from up- and down-regulated genes, confirmed by real-time RT-PCR using RNA from *G. tomentella* indicated that list of genes reported as a

differentially expressed are showing the similar pattern in both the species. Although the extent of expression was slightly different in microarray and real-time RT-PCR, the direction of expression was the same (Fig. 7). The difference at the level of expression might be because of variation in the number of transcripts in different gene family.

Discussion

Growth stage specificity for differential gene expression

We studied the gene expression pattern in soybean at V_4 and R_1 growth stage in response to *P. pachyrhizi* infection and found a distinct difference between these two growth stages. These are the growth stages during which ASR develops under natural conditions, although it can infect at any growth stage, and the present experiment showed that most of the genes are expressed differentially at V_4 growth stage. Assessment of expression of a selected list of genes in *G. tomentella* (an ASR resistant wild relative of soybean) indicated the validity of our microarray experiments toward the detection of differentially expressed genes. Combining these data with the global transcript analysis at the V_2 growth stage reported earlier (Panthee et al. 2007) gives clearer picture about the potential molecular responses to *P. pachyrhizi* throughout much of the soybean life cycle.

The large number of up-regulated genes, especially defense-related, at the V_4 growth stage relative to V_2 and R_1, is not unexpected given that the V_4 is the growth stage most prone to *P. pachyrhizi* infestation. In developmentally regulated defense and stress tolerance systems, such results are congruent with other studies (Kus et al. 2002; Yen et al. 2001). Significant differences in terms of number of differentially expressed genes at different time interval after inoculation was observed by DeMortel et al. (2007a). While they took samples from a wide range of post-inoculation times they did not consider growth stage as a factor.

Targeting an appropriate growth stage to evaluate gene expression pattern with respect to a growth stage specific disease or other physiology is quite common. Pasquer et al. (2005) chose growth stage 32 in wheat to evaluate the gene expression pattern in response to fungicides commonly used to control powdery mildew commonly occurring at this growth stage. Facts of developmental regulation of genes have been already established in various plants (Cassab et al. 1985; Coenen et al. 2002; Guenther et al. 2003; Horiguchi et al. 1998). We verified this fact in soybean and identified V_4 as an appropriate growth stage for further gene discovery studies in response to *P. pachyrhizi*. Heat shock and proline rich proteins found as up-regulated genes in the present experiment have already been reported as developmentally regulated genes (Dong and Dunstan 1996; Rojas et al. 2002). Stress tolerant gene orthologues reported in wheat were also found to be up-regulated in the present experiment. The congruent findings of the present experiment with the past findings indicated that *P. pachyrhizi*-induced genes in soybean may have similar function in *Arabidopsis* or in monocots. This also indicated that growth stage specific up-regulated genes detected in the present experiment are in agreement with the past findings.

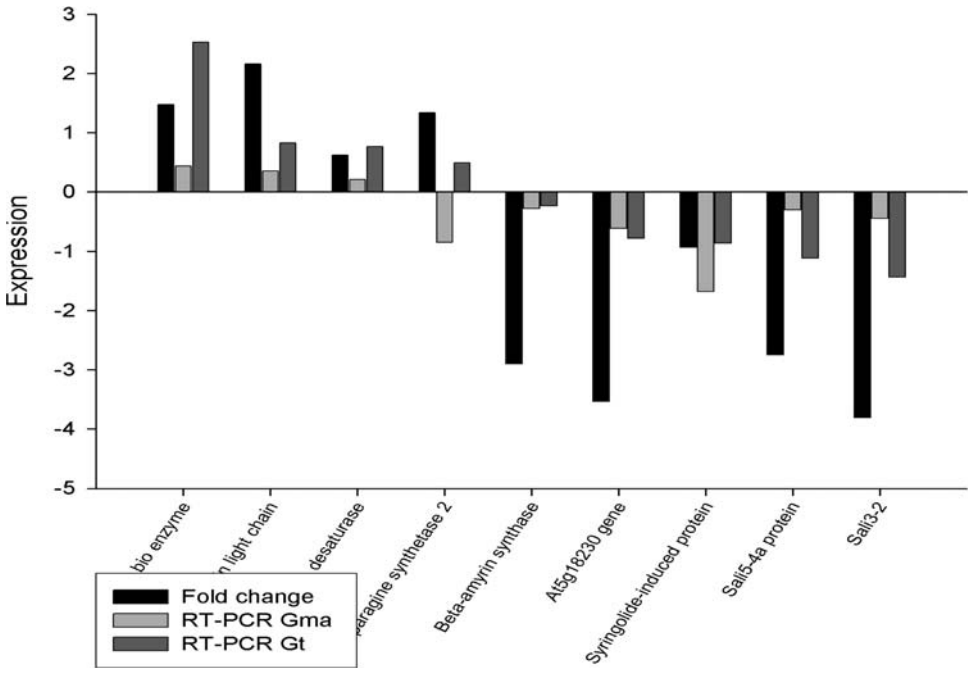

Fig. 7 Comparison of level of expression ($\log_{10}$) in selected genes in soybean (*Glycine max*) and its wild relative (*Glycine tomentella*) in response to *Phakopsora pachyrhizi* by real-time RT-PCR and microarray analysis

Pathway-specific genes leading to biotic tolerance in plants

We found a number of up-regulated genes associated with compounds involved in plant defense. The phenylaline pathway has been reported to have defense role in plants (Subramanian et al. 2005; Winkel-Shirley 2001). There are mainly four enzymes involved in the biosynthesis of isoflavone through phenylaline pathway-chalcone synthase, chalcone isomerase, chalcone reductase and isoflavone synthase (Jung et al. 2000; Winkel-Shirley 2001; Yu et al. 2003). We found three genes, other than isoflavone synthase, up-regulated at V_4 growth stage in the present experiment (Table S-1). This indicated that most probably phenylaline pathway is activated to induce resistance reaction to ASR in soybean. Similar findings have been reported elsewhere (DeMortel et al. 2007a). Furthermore, members of P450 have also been shown to be involved in isoflavonoid biosynthesis pathway (Akashi et al. 1999; Shimada et al. 2000). A number of P450 members found differentially expressed in the present study are expected to have similar roles in plant defense. The up-regulation of these genes in soybean or other plant species and the analysis of isoflavones would further confirm the findings, which is lacking in this experiment. Chalcone synthase was up-regulated at both growth stages but did not observe the other two genes differentially expressed at R_1. DeMortel et al. (2007a) reported the over-expression of cytochrome P450 hydroxylases (CYPs), and *O*-methyltransferases (OMTs), which are involved in producing phytoalexins. The CYPs have also been reported as genes associated with phenylpropanoid pathway (Dhaubhadel et al. 2007). We found both genes up-regulated in the present study.

Another important compound, JA, which is involved in plant defense involves four key enzymes—lipoxygenase, allene oxide synthase, allene oxide cyclase and 12-Oxo-PoA-reductase in its biosynthesis pathway (León and Sánchez-Serrano 1999; Wasternack et al. 1998). We found the first three genes differentially expressed at the V_4 growth stage (Table S-1) whereas lipoxygenase and allene oxide cyclase were also detected at R_1 (Table S-2). We also found 4-coumarate-CoA ligase up-regulated at V_4, which has also been reported to play an important role in JA biosynthesis (Schilmiller et al. 2007).

A hormone-like compound involved in plant defense system is salicylic acid (Chong et al. 2001; Ribnicky et al. 1998; Wildermuth et al. 2001). We found at least three salicylic acid-associated genes to be up-regulated at the V_4 growth stage: UDP-glucose-salicylic acid glucosyltransferase, *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, and salicylic acid-binding protein 2 (Table S-1), and one at R_1 growth stage: UDP-glucose:salicylic acid glucosyltransferase (Table S-2). We also found a number of serine/threonine-specific phosphatases, especially at V_4 growth stage, which are required for the hypersensitive reaction in plants (Luan 2003; Martin et al. 2003). The hypersensitive reaction is one of the resistance mechanisms in plants that limits the pathogens spread by immediately killing infested plant cells.

Other important genes associated with biotic tolerance in plants

Large number of genes in stress, defense and secondary metabolite category indicates that there may not be a single mechanism to defend soybean against *P. pachyrhizi* infection in soybean. Genes related to isoflavone, carotenoid, glucosinolates, terpene biosynthesis are of general nature related to secondary metabolites and plant defense, whereas ABC transporter, P450, GST genes are related to disease resistance and detoxification processes (Halkier and Gershenzon 2006; Martin et al. 2003; Rea 2007; Schuler and Werck-Reichhart 2003). Detection of these genes in the present experiment indicates that we detected a number of the general defense-related genes.

The ATP binding cassette (ABC) transporter family has more than 125 member genes and is believed to be involved to confer drug resistance in prokaryotes and eukaryotes (Higgins 1992). It has also been reported that some of the members are involved in plant defense since they are capable of transporting anti-fungal secondary metabolites such as sclareol (Jasinski et al. 2001; Rea 2007). We found at least nine ABC transporters at V_4 and four at the R_1 growth stage. Similarly, the WRKY gene family has been reported to be involved in plant defense (Martin et al. 2003). We found 28 WRKY-related transcripts at V_4 and 11 at R_1 growth stages, respectively, mostly involved in gene regulation. DeMortel et al. (2007a) also reported 46 WRKY transcripts expressed differentially in response to *P. pachyrhizi* in soybean. Comparing the findings from gene expression analysis in peanuts (*Arachis hypogae*) in response to *Cercosporidium personatum* (Luo et al. 2005), rice, potato and pepper (Kawasaki et al. 2001; Lee et al. 2004; Tian et al. 2006) with the present finding indicated that most of the up-regulated genes found in this study are general defense-related genes, similar to what we reported previously (Panthee et al. 2007).

In conclusion, we found distinct differences between growth stages for gene expression in soybean in response to *P. pachyrhizi*. A similar study using a resistant genotype is worth conducting to confirm these findings although we verified a limited number of genes by real-time PCR in the present study.

Acknowledgments This study was supported by Tennessee Soybean Promotion Board and funds from the Tennessee Agricultural Experiment Station. Julia Gouffon at the Affymetrix Core Center of the University of Tennessee, is sincerely acknowledged for her help in

conducting the microarray experiment. We appreciate the collaborations and conversations with Vince Pantalone, Kurt Lamour, and Mitra Mazarei. Minimum information about a microarray experiment (MIAME) guidelines were followed in this study.

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