

Transcriptome Profiling of Rust Resistance in Switchgrass Using RNA-Seq Analysis

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Abstract

Switchgrass rust caused by *Puccinia emaculata* is a major limiting factor for switchgrass (*Panicum virgatum* L.) production, especially in monoculture. Natural populations of switchgrass displayed diverse reactions to *P. emaculata* when evaluated in an Ardmore, OK, field. To identify the differentially expressed genes during the rust infection process and the mechanisms of switchgrass rust resistance, transcriptome analysis using RNA-Seq was conducted in two pseudo-F₁ parents ('PV281' and 'NFGA472'), and three moderately resistant and three susceptible progenies selected from a three-generation, four-founder switchgrass population (K5 × A4) × (AP13 × VS16). On average, 23.5 million reads per sample (leaf tissue was collected at 0, 24, and 60 h post-inoculation (hpi)) were obtained from paired-end (2 × 100 bp) sequencing on the Illumina HiSeq2000 platform. Mapping of the RNA-Seq reads to the switchgrass reference genome (AP13 ver. 1.1 assembly) constructed a total of 84,209 transcripts from 98,007 gene loci among all of the samples. Further analysis revealed that host defense-related genes, including the nucleotide binding site–leucine-rich repeat domain containing disease resistance gene analogs, play an important role in resistance to rust infection. Rust-induced gene (RIG) transcripts inherited across generations were identified. The rust-resistant gene transcripts can be a valuable resource for developing molecular markers for rust resistance. Furthermore, the rust-resistant genotypes and gene transcripts identified in this study can expedite rust-resistant cultivar development in switchgrass.

SWITCHGRASS RUST CAUSED by *Puccinia emaculata* is a potential threat to switchgrass feedstock production for the second-generation biofuels industry. The damaging effects of switchgrass rust have been documented in different parts of the United States, indicating its widespread occurrence and importance (Gustafson et al., 2003; Zale et al., 2008; Hirsch et al., 2010). Rust disease would have a detrimental effect on the biomass yield and quality of switchgrass when this crop is grown on a large scale as abioenergy feedstock. A preponderance of genetic variability for rust resistance within and among switchgrass cultivars and ecotypes has been discovered (Uppalapati et al., 2013). In general, lowland ecotypes collected from the southern United States are moderately resistant and the upland ecotypes are susceptible to rust (Gustafson et al., 2003; Uppalapati et al., 2013). Characterization based on teliospore morphology and internal transcribed spacer primers identified the rust pathogen in Oklahoma as *P. emaculata* (Uppalapati et al., 2013). This was deemed to be important, as previous

Published in The Plant Genome 8
doi: 10.3835/plantgenome2014.10.0075
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Abbreviations: DEGs, differentially expressed genes; FPKM, fragments per kb of transcript sequence per million bp mapped; GO, gene ontology; hpi, hours post-inoculation; RIG, rust-induced genes.

studies had reported the widespread occurrence of rust to be caused by other genera of fungi such as *Uromyces graminicola* (Cornelius and Johnston, 1941).

Host resistance is the most preferred and economical disease management system in crop production. It is our contention that a proactive approach toward the study of rust disease in switchgrass and an integration of resistance into any cultivar development program will avert the potential danger posed by the disease. The identification of rust-resistant genotypes in a population would make a significant contribution toward developing rust-resistant switchgrass cultivars carrying various sources of resistance genes for sustainable biofuel feedstock production. Furthermore, markers developed from potential resistant genes would facilitate the resistant cultivar development process through marker-assisted selection. Identification of resistant genes and understanding their role and patterns of expression during the rust infection process are important preludes for breeding resistance. The establishment of molecular tools is especially important for stacking genes, where several resistance genes are assembled in a genotypic background to ensure durable resistance in case race-specific resistance breaks down. In switchgrass, few studies have been conducted to evaluate germplasm for rust resistance (Gustafson et al., 2003; Uppalapati et al., 2013) or to develop markers for characterization of the rust pathogen (Wadl et al., 2011; Uppalapati et al., 2013).

The cDNA sequencing technology known as RNA-Seq has been applied to analyze the transcriptional landscape of switchgrass, to study diversity (Lu et al., 2013; Childs et al., 2014), and to document unique transcriptional signatures of different tissues (Wang et al., 2012), various features of development (Palmer et al., 2014a, 2014b), and responses to stresses (Meyer et al., 2014). In a study regarding drought and salinity stress responses in switchgrass, many differentially expressed microRNAs were identified that can potentially target genes encoding transcription factors, stress-response proteins and cellulose biosynthesis-related proteins (Xie et al., 2013). In *Panicum hallii* Vasey, a tractable diploid model of the genetic and genomic studies of the allopolyploid switchgrass (Anderson et al., 2011), RNA-Seq analysis enabled the development of a gene expression atlas across tissues and growth stages (Meyer et al., 2012). However, there is a large information gap about rust resistance both in switchgrass and model species that needs to be bridged by research.

Plants respond to a pathogen attack through a variety of signaling pathways consisting of a large number of constitutive as well as inducible genes (Van Verk et al., 2009). This phenomenon has been documented in wheat stripe rust (*Puccinia striiformis*) (Wang et al., 2010) and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) (Caldo et al., 2004), as different sets of genes were expressed during the infection process. There is a lack of information about switchgrass rust resistance gene expression and inheritance. Thus the objectives of this study were to document rust reactions in a switchgrass population, measure the differential gene expression of

resistant and susceptible genotypes concomitant with the rust infection process, and study the inheritance of RIG transcripts across generations.

Materials and Methods

Plant Materials

A three-generation, four-founder population (Li et al., 2014) was developed by crossing selected pseudo-F₁ parents, where the female parent was PV281 and the male parent was NFGA472. PV281 is a pseudo-F₁ from the cross between the lowland ecotypes 'Kanlow' (genotype K5) and 'Alamo' (genotype A4) (Okada et al., 2010); NFGA472 is a pseudo-F₁ from the cross between the Alamo genotype AP13 and upland 'Summer' (genotype VS16) (Missaoui et al., 2005; Serba et al., 2013) populations. Details of the crossing scheme can be viewed in Li et al. (2014). A total of 251 genotypes from the population, a duplicate set of the pseudo-F₁ parents and an Alamo check were evaluated at an Ardmore, OK, field under natural rust incidence conditions during 2009 and 2010. The details of the experimental design, field transplanting, and crop management practices were as described by Serba et al. (2014). The rust score was taken after flowering using a 1 to 9 visual rust severity rating scale (1 = highly resistant; 9 = highly susceptible) as described previously (McNeal et al., 1971; Gustafson et al., 2003; Uppalapati et al., 2013). For this transcriptome study, the two pseudo-F₁ parents and three moderately resistant (AL-NF156, AL-NF207, and AL-NF254) and three susceptible (AL-NF124, AL-NF240, and AL-NF255) progenies were selected based on 2 yr of average field rust scores. Three clonal replicates of each of the two parents and selected progenies were further evaluated in growth chambers by artificial inoculation with rust spores collected from the Ardmore field.

Rust Maintenance and Inoculation Procedures

A pool of switchgrass rust isolates collected from an Ardmore, OK, field over the 2007 to 2010 growing seasons was maintained on a susceptible genotype (VS16). The urediniospore collection, suspension preparation, and inoculation methods followed were as described previously (Uppalapati et al., 2013). In brief, fresh urediniospores were collected from the susceptible genotype using a gelatin capsule spore collector (designed by Cereal Disease Laboratory, St. Paul, MN) and suspended in distilled water with 0.001% Tween 20. Inoculation was performed on ramets at the E2 stage by spraying 1×10^5 spores mL⁻¹ using an artist's airbrush (Paasche Airbrush Co., Chicago, IL) set at 13.79 kPa with a portable air-pump (Gast Mfg Co., Benton Harbor, MI) for uniform spore deposition. The plants were subsequently moved to a growth chamber maintained at 80% relative humidity, 29 : 22°C day/night temperatures, and a 16-h photoperiod, with a photon flux density of 150 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Leaf tissue samples were collected at 0, 24, and 60 hpi. Samples at 24 hpi captured the gene expressed

during early infection (appressorium formation and initial penetration), whereas the 60-hpi samples detected the patterns of gene expression at a later stage of infection, including haustoria formation and invasion.

RNA Isolation and Library Construction

Total RNA was isolated from the leaf tissues of the parents and progenies using TRIzol Reagent (a total RNA isolation reagent) following the manufacturer's instructions (Life Technologies, Grand Island, NY). RNA was eluted in RNase-free water and verified for quality and quantity using a NanoDrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and on an Agilent 2100 Bioanalyzer using an Agilent RNA 6000 Nano Kit according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). RNA samples extracted from leaf samples of three independent biological replicates were pooled for each sample per time point. The cDNA libraries were constructed by using a TruSeq RNA library preparation kit (Illumina, San Diego, CA). In brief, polyA containing messenger RNA was first purified from total RNA using poly-thymine oligo-attached magnetic beads and then chemically fragmented and primed with random hexamer priming for single-stranded cDNA synthesis. A second cDNA strand was synthesized as a replacement strand for the RNA to create a double-stranded cDNA that was ready for TruSeq library construction. The short overhanging double-stranded cDNA fragments were converted to blunt ends. The blunt ends were ligated to multiple sequencing adapters for hybridization into a flow-cell. Finally, RNA libraries were built by polymerase chain reaction amplification, where RNA fragments that have adaptor molecules were enriched by polymerase chain reaction with the primers annealed to the end of the adapters. The RNA libraries were quantified using quantitative polymerase chain reaction, according to the Illumina Sequencing Library Quantitative Polymerase Chain Reaction Quantification Guide. Indexed libraries were normalized to 10 nM, quantified using the Agilent Technologies 2100 Bioanalyzer, and pooled.

RNA-Seq and Expression Analysis

Paired-end (2 × 100 bp) sequencing of the cDNA libraries was conducted using Illumina TruSeq sequencing-by-synthesis chemistry on the HiSeq 2000 platform (Illumina). Reads were quality trimmed from the end to ensure that two consecutive bases had a score of 30 or more; resultant reads less than 30 bp were discarded. The trimmed reads were aligned onto the switchgrass reference genome sequence (<http://www.phytozome.net/panicumvirgatum>; accessed 30 Jan. 2015) with TopHat2 (Langmead et al., 2009; Trapnell et al., 2009; Kim and Salzberg, 2011; Kim et al., 2013) and underlying mapping was performed with Bowtie2 (Langmead et al., 2009; Langmead and Salzberg, 2012), allowing multi-mapped reads and a maximum of two mismatches per read with other default settings. Cufflinks (Trapnell et al., 2010, 2013; Roberts et al., 2011a, 2011b) was used to quantify transcripts and we could identify 84,209 genes as having

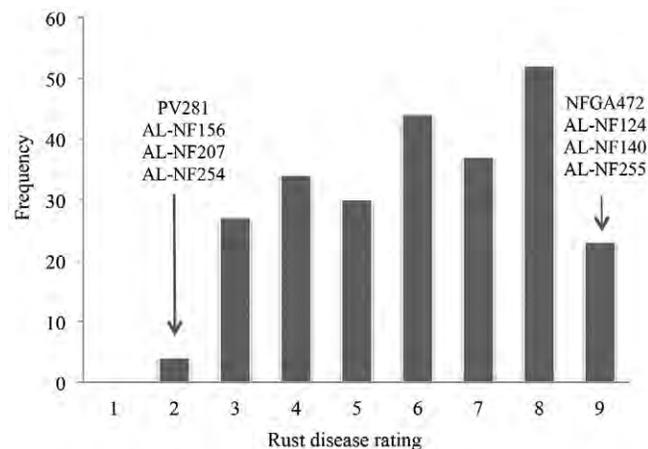


Figure 1. Frequency distribution of mean rust score in the AL-NF switchgrass mapping population under natural infestation in the Ardmore, Oklahoma, field. Rust scoring scale used: 1 = resistant; 3 = moderately resistant; 5 = moderately susceptible; 7 = susceptible; 9 = highly susceptible. Data were averaged across two replications and 2 yr (2009 and 2010) for each genotype.

at least 10 reads (default) aligned. Cuffdiff was used to compare the normalized transcript counts in fragments per kb of transcript sequence per million bp mapped (FPKM) expression levels (Mortazavi et al., 2008) of annotated genes among samples. Differential expression between samples was assessed at 5% false discovery rate. Gene ontology (GO) (Ashburner et al., 2000; Harris et al., 2004) analysis of differentially expressed genes was performed using the Singular Enrichment Analysis tool from AgriGO (<http://bioinfo.cau.edu.cn/agriGO>, accessed 30 Jan. 2015) (Du et al., 2010) with annotations derived from the switchgrass ver. 1.1 genome. The 'plant slim GO' terms of enriched gene functional categories (cut-down versions of the GO ontologies; <http://geneontology.org>, accessed 31 Jan. 2015) were assessed for redundancy using a REVIGO web server (<http://revigo.irb.hr>, accessed 30 Jan. 2015) (Supek et al., 2011) and visualized in a two-dimensional scatter plot for semantic similarity based on multidimensional scaling to a matrix of GO terms (Pesquita et al., 2009).

Results and Discussion

Rust Resistance Characteristics of the Population and the Selected Progenies

The 2-yr average rust score under field conditions demonstrated that the AL-NF population was segregating for rust resistance (Fig. 1). Three progenies were found to be resistant to rust (an average score of 2). Nearly 11% (27 progenies) were moderately resistant (a rust score of 3). About 14% (34 progenies) had a rust score of 4, which is the average of moderately resistant and moderately susceptible scores. A total of 74 progenies (35%) showed a moderately susceptible reaction (rust scores of 5 and 6). The remaining 112 progenies (nearly 45%) were found

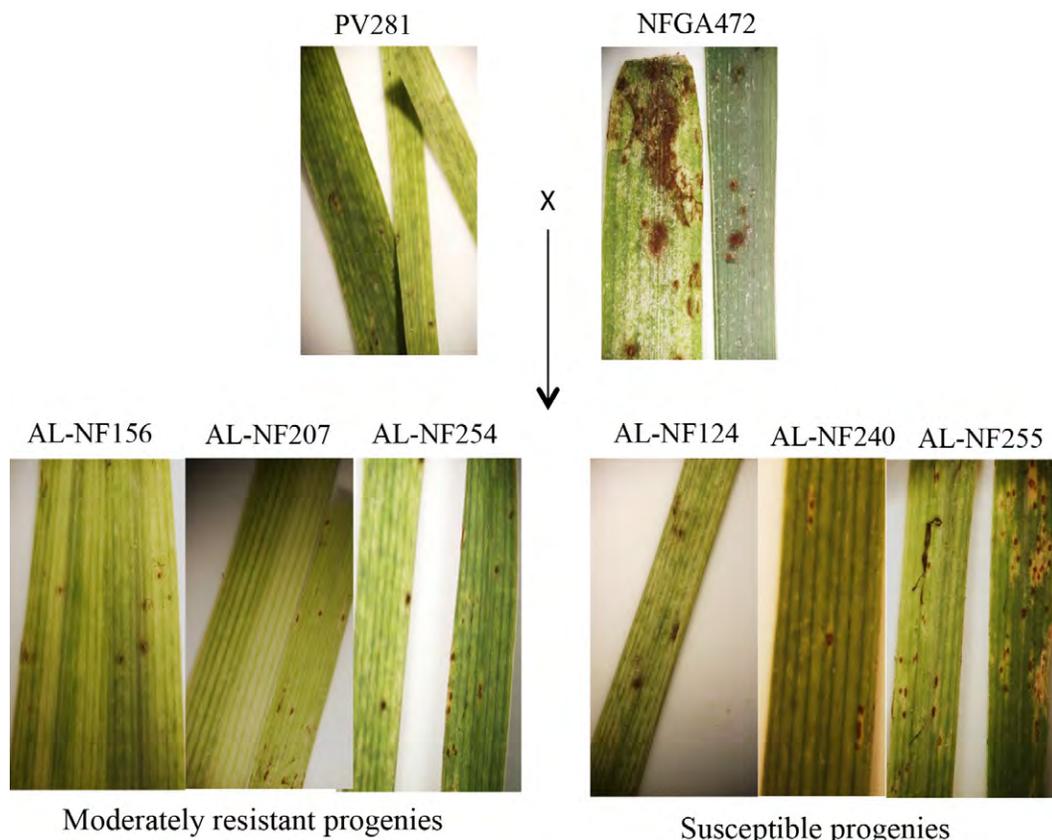


Figure 2. Reaction to inoculation with rust spores of the switchgrass pseudo- F_1 parents and progenies selected for transcriptome analysis. The pictures were taken 14 d after inoculation in the growth chamber study.

to be consistently susceptible to rust (a rust score of ≥ 7), 11% of which were highly susceptible (a rust score of 9 in both years). The female parent, PV281, was found to be moderately resistant, whereas the male parent, NFGA472, was susceptible to rust. Disease development is an effect of the interplay among the host, the pathogen, and the environment (Agrios, 1988). Few of the genotypes that exhibited a resistant reaction (a rust score of 1) in one season had moderate resistance in the other (a rust score of 3). As a result, we designated PV281 and the three genotypes with an average score of 2 as being moderately resistant for this study.

Since the growth chamber evaluation was combined with leaf tissue sample collection for the transcriptome analysis, no structured data were collected in the growth chamber. Nevertheless, visual observation for a rust reaction in the pseudo- F_1 parents and the six selected progenies 14 d after artificial rust inoculation (Fig. 2) proved that the level of resistance of each genotype under growth chamber conditions was similar to the field evaluation result. PV281 maintained the moderate resistance observed under field conditions. NFGA472 showed a highly susceptible reaction. This outcome was similar to previous findings on the rust resistance of different populations under field and controlled conditions (Uppalapati et al., 2013). Among the susceptible progenies, we observed chlorosis around the pustules on AL-NF255 but this did not limit the pustule size and extension of the

disease along the leaf tissue. Programmed cell death is one of the resistance mechanisms that limit the spread of the obligate pathogens along the tissue by limiting food sources for the pathogen (Coll et al., 2011). However, in the case of AL-NF255, we observed massive cell death and coalesced pustules (Fig. 2) that did not limit the pustule size or coalition. In summary, our field evaluation result documented differential responses to rust among AL-NF populations, which was confirmed under artificial inoculation under controlled conditions for selected genotypes.

Transcriptome Sequencing and Mapping

The paired-end (2×100 bp) sequencing on the Illumina HiSeq 2000 platform generated an average of 23.5 million reads with a minimum of 14.7 million and a maximum of 34.1 million reads per sample (Supplemental Fig. 1). The maximum number of reads was obtained for AL-NF254 at 24 hpi (34.1 million) followed by AL-NF207 at 24 hpi (33.5 million) and AL-NF156 at 24 hpi (32.3 million); the lowest number was obtained for NF472 at 60 hpi. Variations in the number of reads were observed among the samples but these variations did not follow any defined trend with either the time points or resistant versus susceptible genotypes. Quality trimming discarded 3.6% of the reads on average. For all the samples together, 334.96 million were mapped out of a total of 466.37 million quality trimmed reads. On average, about 72% of the trimmed reads could be mapped to the

reference genome, whereas the other 28% were discarded from further analysis. For the moderately resistant group of progenies, an average of 14.0, 23.8 and 16.3 million reads were mapped to the reference genome at 0, 24, and 60 hpi, respectively. Likewise, for the susceptible group of progenies, there were 16.4, 17.2 and 18.7 million reads at 0, 24, and 60 hpi, respectively, mapped to the reference genome. The average number of reads and total number of bases obtained for the samples were higher than those obtained for the switchgrass transcriptome analysis conducted in germinating seedlings, emerging tillers, flowers, and dormant seeds (Wang et al., 2012). However, the number of reads per sample we obtained was lower than those generated for seven lowland and upland cultivars of switchgrass (Childs et al., 2014) and flag leaf transcriptome analysis (Palmer et al., 2014a). For PV281 at 24 hpi and NF472 at 60 hpi, only 18% (3.1 : 16.9 million) and 17% (2.4 : 14.3 million) of the reads were mapped, respectively (Supplemental Figure 1). The low percentage of mapped reads for the two parental samples was due to quality trimming, which rejected most of the reads. We assessed the effect of a low percentage of mapping on both depth and coverage. As we considered at least 10 reads to be aligned in Cuffdiff for all samples, the low percentage of mapped reads did not significantly affect the number of transcripts mapped.

Transcript mapping to the reference genome, the AP13 v. 1.1 assembly, identified a total of 84,209 gene transcripts in one or more of the samples. Out of these, 72,614 gene transcripts were expressed in the two parents, PV281 and NFGA472, at one or both time points (24 and 60 hpi). A total of 80,291 gene transcripts were identified in one or more of the three moderately resistant and the three susceptible progenies at one or more of the three time points. A 7677-gene transcript shortfall in the pseudo- F_1 parents, compared to the six progenies combined, may result from the number of genotypes and genotypic differences or else the low percentage of mapped reads for PV281–24 and NF472–60 might have contributed to a certain extent. This result suggests that in our study, about 86% of the 98,007 total loci containing protein-coding transcripts identified for switchgrass (Shu et al., unpublished data, 2014) was covered. This demonstrated a greater or similar coverage of gene transcripts to that seen in previous studies (Wang et al., 2012; Meyer et al., 2014; Palmer et al., 2014a). About 90% of the switchgrass gene space coverage was estimated from de novo assembly of switchgrass transcripts from different tissue types (Wang et al., 2012); however, the number of expressed sequence tags obtained was much lower than the number of transcripts covered in this study. The total number of annotated switchgrass genes available, along with the preliminary release genome sequence, is lower than the 128,058 untranscripts annotated for function and used for the development of the switchgrass Affymetrix cDNA microarray chip (Pvi_cDNAa520831) containing 122, 973 probe sets (Zhang et al., 2013), but the reference we used is RNA-Seq-based and development is in progress.

Gene Ontology of Switchgrass RIG Transcripts

Gene ontology analysis was conducted for the transcripts of differentially expressed genes (DEGs) between the moderately resistant and susceptible progenies for a detailed annotation of their gene function, biological involvement, and target cellular component. The GO analysis was conducted using switchgrass gene annotation (Shu et al., unpublished). Out of 397 DEGs between the moderately resistant and susceptible progenies at 60 hpi, 170 had GO terms. The other 85 gene transcripts had homologs in the rice (*Oryza sativa* L.) genome with GO terms. The number of GO terms associated with a gene transcript ranged from one to nine. The plant slim GO terms based on GO slim ontologies categorized 39, 20, and 19 of the GO terms into biological processes (Supplemental Fig. 2a), cellular components (Supplemental Fig. 2b), and molecular functions (Supplemental Fig. 2c), respectively. The GO terms for cellular component, except the extracellular region formed at 60 hpi and molecular function between the two time points (24 and 60 hpi) analyzed, were consistent (Fig. 3a and Fig. 3b). However, few new functional groups, such as development processes, multicellular organismal processes, reproduction, and response to stimuli, were formed under biological processes at 60 hpi (Fig. 3b). The GO terms of the transcripts differentially expressed in the resistant progeny at 60 hpi formed a gene regulatory network (Supplemental Fig. 3). The transcripts encoding a response to stimuli were highly over-represented in the pool of transcripts at 60 hpi compared to the reference.

Further analysis using the GO terms split the highly over-represented response to stimuli functional group into the response to stimuli and response to stress (Fig. 4). Disease resistance genes encode immune receptors that recognize pathogen effectors and activate defense responses (Jones and Dangl, 2006) through programmed gene expression. Consequently, we deduced that the response to stimulus may include the genes expressed in response to the effect of pathogen inoculation on host cells, whereas the response to stress may represent the genes transcribed to overcome the stress imposed by the pathogen on the host system. The common GO terms in both the response to stimuli and response to stress categories are associated with DNA binding (GO: 0003677), peroxidase activity (GO: 0004601), nucleotide excision repair (GO: 0006289), response to stress (GO: 0006950), response to oxidative stress (GO: 0006979), response to biotic stimuli (GO: 0009607), heme binding (GO: 0020037), and oxidation reduction (GO: 0055114). Transmembrane transport (GO: 0055085), antiporter activity (GO: 0015297), multidrug transport (GO: 0006855), and drug transmembrane transporter (GO: 0015238) activity were specifically observed in the response to stress category (Supplemental Fig. 3). These results indicate that the transcripts were involved in host–pathogen response processes such as recognition of pathogenic proteins on the cell surface, which limit pathogen maturation within the cells and overcome the deleterious effects of the pathogen

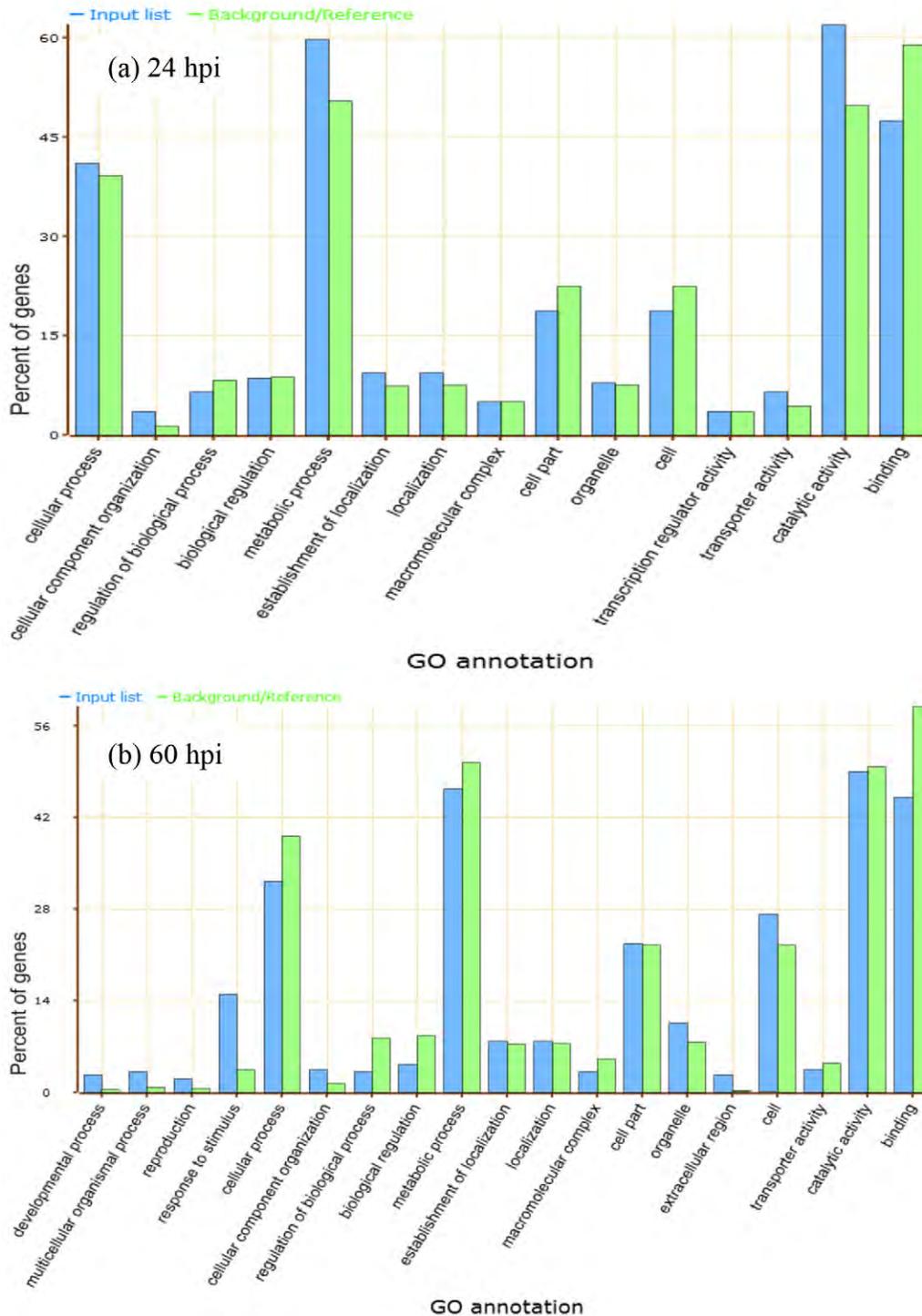


Figure 3. Functional category of annotated switchgrass gene transcripts unique to the moderately resistant progenies at (a) 24 h post-inoculation (hpi) and (b) 60 hpi, compared to the Gene Ontology (GO)-annotated reference. Overrepresentation of transcripts related to response to stimuli and stresses was observed at 60 rather than 24 hpi.

on the cell. Plants have evolved specific resistance protein alleles to detect specific effectors (Postel and Kemmerling, 2009; Zipfel, 2014) that trigger rapid activation of an effective defense against the deleterious effect of the pathogen (Dangl and McDowell, 2006). Plant resistance strategies include processes that limit the spread of damage within the host system by localized cell death

(Lattanzio et al., 2006) and limit pathogen maturation within the cells. The resistant plants overcome the effect of the pathogen while maintaining normal physiological and development processes. The GO terms for cellular components were also formed into a gene network, with an extracellular region formed at 60 hpi that was not involved in the expression network (Supplemental Fig. 4).

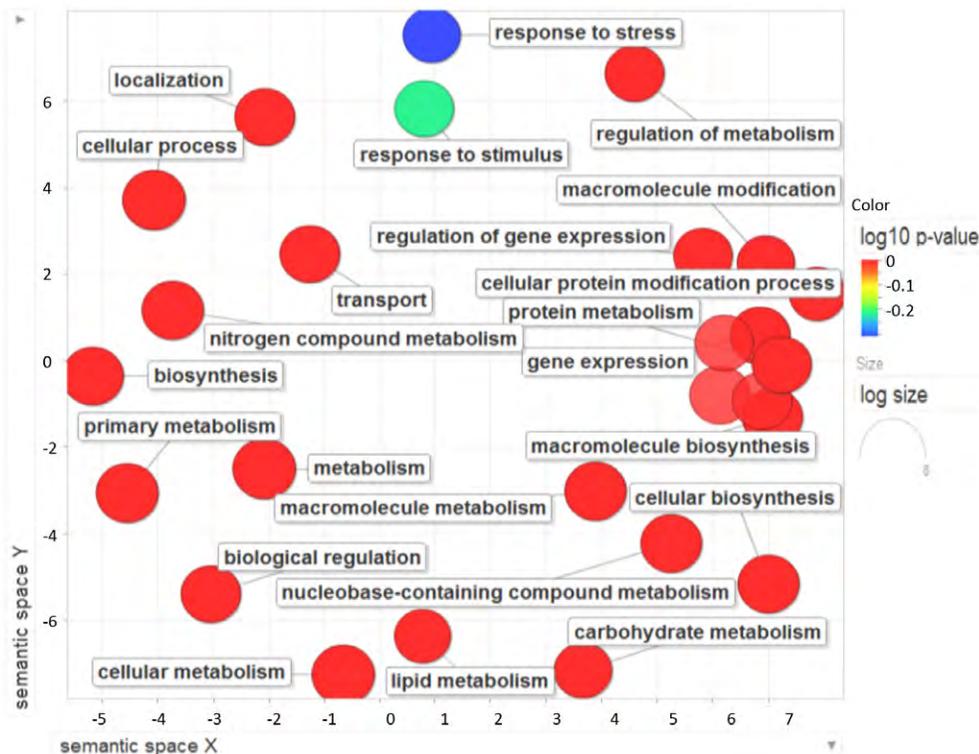


Figure 4. Gene Ontology (GO) analysis of genes upregulated in moderately resistant switchgrass progenies at 60 h post-inoculation (hpi) using REVIGO. The two-dimensional cluster plot shows gene functions based on the GO terms' semantic similarities. Bubble color indicates the p -value for the false discovery rates derived from the AgriGO analysis. The circle size represents the frequency of the GO term in the Uniprot database, where a larger bubble size represents more general terms. Transcripts for responses to stimuli and stress were identified as new clusters.

Differentially Expressed Genes in Host-Pathogen Interaction

Comparison of the pseudo- F_1 female parent (PV281) against the moderately resistant and susceptible progenies revealed relatively high DEGs at both 24 and 60 hpi (Table 1). There was a total of 397 DEGs at 60 hpi compared to 298 at 24 hpi. At 24 hpi, 35% (105 : 298) were upregulated in the moderately resistant progenies. At 60 hpi, the majority (75%, 296 : 397) were commonly upregulated in the three moderately resistant progenies. This massive upregulation of transcripts at 60 hpi suggested widely distributed regulation of transcriptional activity 2 d after rust inoculation. This was similar to what was reported for leaf rust resistance in wheat (*Triticum aestivum* L.), where differential expression between the resistant and susceptible cultivars was found after 48 hpi, with the highest abundance appearing at 96 hpi (Raman et al., 2011). The profusion of the stress-tolerance-related transcripts may be attributed to recognition of the pathogen by the host resistance genes before 60 hpi and initiation of the defense mechanism in host cells carrying the resistance gene against the rust pathogen (Bolton, 2009; Wang et al., 2010). We speculate that the expression of genes by the moderately resistant progenies is caused by massive reprogramming of the moderately resistant plant cells to restrict pathogen invasion. The susceptible plants may be lacking a timely pathogen recognition system for cell reprogramming.

Dramatic upregulation of the genes involved in conferring blast resistance during the early steps of defense perception signaling, such as diterpene phytoalexin biosynthetic enzymes, flavin-containing monooxygenase, class I chitinase and glycosyl hydrolase 17, was observed in rice using RNA-seq analysis (Bagnaresi et al., 2012).

Highly differentially expressed genes (FPKM > 100) between the moderately resistant and susceptible progenies were more prevalent at 60 than 24 hpi (Supplemental Table 1). These gene transcripts were found to encode host defense-related proteins such as nicotinamide adenine dinucleotide'-dependent epimerase or dehydratase family protein (a fourfold change), which are reported to have RNA-binding activities and be involved in guanosine diphosphate-*L*-fucose biosynthetic and cellular metabolic processes (Baker et al., 1998). They were also found to be responsive to reactive oxygen species in *Arabidopsis thaliana* (L.) Heynh. treated with fungal *Alternaria alternata* f.sp. *lycopersici* toxin (Gechev et al., 2004). The latter case implies that the epimerase or dehydratase family proteins have an important function in biotic stress tolerance. The putative serine carboxypeptidase homolog (*SCPI*) (a threefold change) was among the highly expressed genes in the moderately resistant group and known to encode acetyltransferase enzymes, which have been reported to catalyze the production of secondary plant metabolites involved in

Table 1. Differentially expressed genes (DEGs) between parents, between parent and progenies, and between moderately resistant and susceptible genotypes of the PV281 and NFGA472 switchgrass population.

Comparisons	Total DEGs	Upregulated in first [†]	Upregulated in second [†]	Exclusively upregulated [§] in first	Exclusively upregulated in second
PV281 vs. NFGA472	1651	611	1040	915	496
NFGA472 vs. moderately resistant at 24 h	1217	201	1013	795	142
NFGA472 vs. moderately resistant at 60 h	2372	577	1795	950	150
NFGA472 vs. susceptible at 24 h	956	146	810	636	132
NFGA472 vs. susceptible at 60 h	4553	1483	3070	1056	376
PV281 vs. moderately resistant at 24 h	4873	1180	3693	2127	231
PV281 vs. moderately resistant at 60 h	1665	235	1430	867	98
PV281 vs. susceptible at 24 h	3226	775	2451	1612	172
PV281 vs. susceptible at 60 h	3044	900	2144	987	246
Moderately resistant vs. susceptible at 0 h	200	90	110	65	38
Moderately resistant vs. Susceptible at 24h	298	105	193	60	63
Moderately resistant vs. Susceptible at 60h	397	296	101	45	154

[†] First and second refer to the order of the genotype or group presented in the comparison column.

[§] Indicates upregulation in one and no expression in the other.

host defense (Shirley et al., 2001) and serve important ecological functions such as protection against microbial attack (Dixon, 2001). The other highly differentially expressed gene in moderately resistant progenies was the chitinase family protein precursor (*CHIT16*) (threefold change). Chitinases are pathogenesis-related enzymes produced by defensive response genes when resistant plants recognize cognate elicitors and catalyze the cleavage of chitin monomers, which are a common component of the fungal cell wall (Ebrahim et al., 2001).

The other highly expressed proteins were amine oxidases or flavin domain-containing proteins (a fourfold change). The amine oxidases are heterogeneous apoplastic proteins that mostly act as H₂O₂ delivery systems in the cell wall during growth and development and host–pathogen interactions (Tisi et al., 2008). Hydrogen peroxide is a versatile molecule of the reactive oxygen species network and is a key signaling molecule in defense mechanisms (Quan et al., 2008). The developmental and defense responses of H₂O₂ depend on the apoplastic production performed by copper amine oxidases and flavin-containing amine oxidases located in the cell wall that are responsible for the oxidative de-amination of polyamines (Cona et al., 2006). Proteins containing the universal stress protein domain were among the several highly expressed gene products with 246.3 FPKM in the resistance progenies compared to 89.2 FPKM in the susceptible ones (a more than twofold change). As the name implies, the universal stress protein encompasses a conserved group of proteins that are widely spread in living organisms and constitute a biological defense against a wide variety of stresses that may arrest cell growth (Tkaczuk et al., 2013). This group of proteins plays a major role in oxidative stress resistance and helps the plants survive such adverse conditions (Tkaczuk et al., 2013) but the exact physiological role of the universal stress protein is not well understood. Similarly, CCT or B-box zinc finger protein had 216.4 FPKM in the

resistance group but it had only 57.8 FPKM in the susceptible group of progenies (fourfold change). CCT or B-box zinc finger protein is a class of zinc finger transcription factors that plays an important role in plant growth and developmental processes, including biotic and abiotic stress responses (Gangappa and Botto, 2014). It is known to be involved in the wounding response in plants (Taki et al., 2005). It also acts as a chitin-responsive transcription factor that is part of the plant defense reaction (Libault et al., 2007).

Aspartic proteinases also had high expression, especially in the moderately resistant progenies (a fourfold change). Aspartic proteinases are a family of enzymes that are widely distributed among plant species and are involved in protein processing and degradation in stress responses (Simoes and Faro, 2004). Plant aspartic proteinases have a unique conserved region consisting of approximately 100 amino acids, termed ‘plant-specific inserts,’ which is excised during activation and is implicated in membrane interactions including vacuolar targeting and host defense (Romer et al., 2004). The cycloartenol synthase also had fourfold higher expression in the moderately resistant compared to the susceptible progenies and is suggested to be involved in the regulation of triterpenoid biosynthesis and chloroplast differentiation (Babychuk et al., 2008), suggesting its importance in the photosynthetic efficiency of plants, which may help resilience under stress. This group of genes is important for the maintenance of normal physiological and development processes in the resistant plants and effectively overcoming the stress posed by the pathogen.

There were three highly expressed gene transcripts coding glucan endo-1,3-β-glucosidase precursor (fourfold change). Glucan endo-1,3-β-glucosidase plays an important role in plant defenses against pathogen attack through its ability to degrade fungal cell wall polysaccharides (Xu et al., 1992). Phosphoenolpyruvate

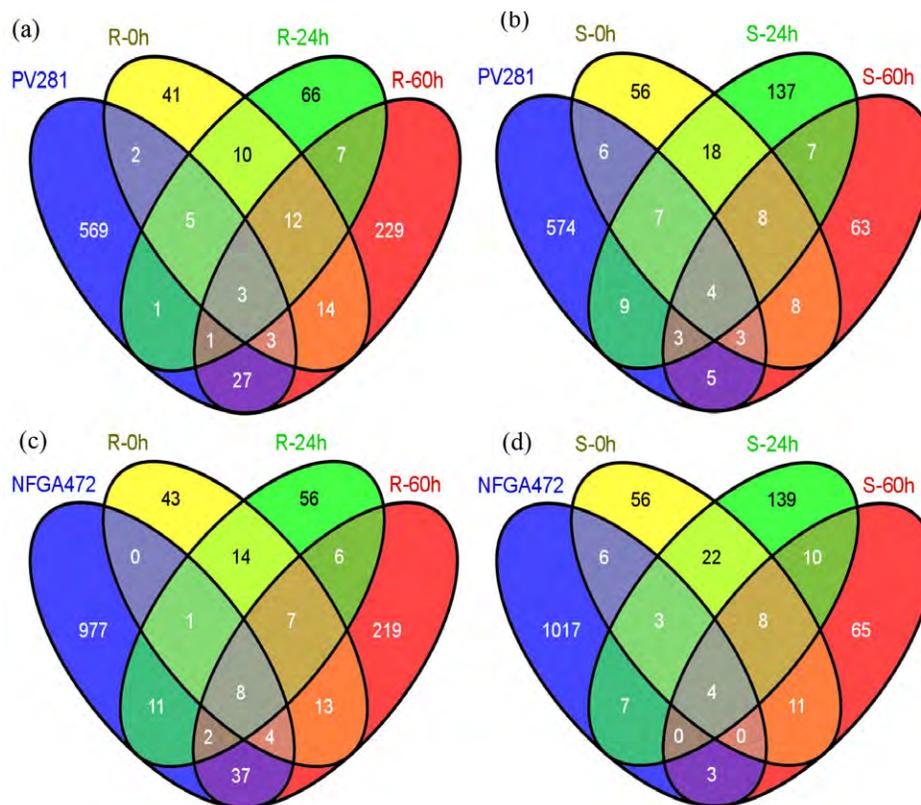


Figure 5. Comparison of switchgrass gene transcripts between pseudo- F_1 parents and progenies at different time points in the rust infection process. Commonly expressed transcripts between either the female or male parent and the progenies indicate the source of inheritance of the differentially expressed gene transcripts. R-0h, R-24h and R-60h, and S-0h, S-24h, and S-60h refer to resistant and susceptible progenies at 0, 24, and 60 h post-inoculation, respectively.

carboxylase also had threefold higher expression in the moderately resistant than in the susceptible progenies. Phosphoenolpyruvate is an important enzyme catalyzing a crucial reaction in the process of carbon fixation (Kai et al., 2003). The enzyme 9-*cis*-epoxycarotenoid dioxygenase (a chloroplast precursor) had more than threefold upregulation in the moderately resistant compared to the susceptible progenies and is known to catalyze the regulatory step in abscisic acid synthesis in plants. Abscisic acid-mediated signaling plays an important role in plant responses to pathogens (Seo and Koshiba, 2002) and is an integral part of the plant defense system. Disease resistance-responsive (dirigent protein), stem-specific protein (TSJT1), and protease inhibitor and LTP family protein precursors (LTPL7 and LTPL124) were also among the highly expressed transcripts with over twofold change in the moderately resistant compared with the susceptible progenies. Gene expression is known to be modulated at any step from transcriptional initiation to RNA processing and during the post-translational modification of a protein. These and other genes with low expression levels, which had a several fold change, were expressed during the host-pathogen interaction process and were apparently responsible for the phenotypic variation in rust resistance among the selected genotypes in particular and in switchgrass in general.

Inheritance of RIG Transcripts across Generations

To prove that some of the transcriptome signatures are heritable across generations, we tracked the expressed genes across generations and time points (Fig. 5a–d). There were a total of 1651 DEGs between the two pseudo- F_1 parents at 24 and 60 hpi combined, 1040 of which were upregulated in NFGA472 and 611 in PV281. There were 34 gene transcripts in common between PV281 and the moderately resistant progenies at 60 hpi (Fig. 5a), some of which were highly expressed. However, only 15 gene transcripts were seen in both PV281 and the susceptible progenies at the same time point (Fig. 5b), most of which had a low in expression level. NFGA472 and the moderately resistant progenies had 51 in common (Fig. 5c) but these were not related to the genes for resistance from the gene annotation. Only seven genes were seen in both NFGA472 and the susceptible progenies (Fig. 5d).

Among the transcripts commonly expressed in the resistant parents and the moderately resistant progenies, switchgrass gene locus *Pavir.J20496* is homologous to the yellow-rust resistance gene (*Yr10*) of wheat, which encodes the nucleotide binding site-leucine-rich repeat domain-containing protein, which is a disease resistance protein (an RPM1-like isoform) in foxtail millet (*Setaria italica* (L.) P.Beauv.) and *Brachypodium distachyon* (L.) P.Beauv. (Klimke et al., 2009). Gene locus *Pavir.Da00825* has an myeloblastosis-like DNA-binding

domain containing a protein homolog in rice that was involved in transcription regulation (Klimke et al., 2009). *Pavir.J38239* was expressed both in the moderately resistant (FPKM = 928) and susceptible (FPKM = 206) progenies, but it had more than fourfold upregulation in the resistant strain. The rice homolog of *Pavir.J38239* is a protein inhibitor or lipid transfer protein family precursor (LTPL24). Lipid transfer proteins function in the transfer of phospholipids between membranes (Kader, 1996) and are suggested to be active plant defense proteins (Garcia-Olmedo et al., 1995). We observed that 112 transcripts had no expression (0 FPKM) in the susceptible progenies but had 0.6 to 23.3 FPKM in the moderately resistant progenies at 60 hpi. Combined over both time points (24 and 60 hpi), the moderately resistant progenies inherited a total of 39 and 53 gene transcripts from the resistant parent (PV281) and susceptible parent (NFGA472), respectively. On the other hand, the susceptible progenies inherited 31 and 17 gene transcripts from PV281 and NFGA472 at both time points, respectively. This shows that the resistant progenies inherited a greater number of transcripts from both parents than from the susceptible progenies. Assessment of the identity of the transcripts from the custom switchgrass annotation table indicated that most of the genes inherited from the resistant parent were related to responses to stress, as mentioned above. However, since information on transcript inheritance is lacking, it was not possible to validate this outcome with related work in the literature.

Potential Rust Resistance Candidate Genes

Among the 296 differentially expressed gene transcripts in the moderately resistant progenies at 60 hpi, 117 and 86 had gene homologs in rice and *A. thaliana*, respectively. The other 109 did not have GO terms in switchgrass annotation, of which 78 did not have homologs in either the *A. thaliana* or rice genomes. Also, protein sequence basic local alignment searching in the database did not hit any matches. We speculated these transcripts represent new candidate genes for rust resistance in switchgrass. Transcripts of unknown function have also been reported in wheat yellow rust and leaf rust resistance studies (Wang et al., 2010; Raman et al., 2011). Out of the identified homologs, 29 are expressed proteins with no known function. Among the functionally known 56 gene transcripts, two transcripts (*Pavir.Ca02314* and *Pavir.Ba00982*) had homologs in rice with the nucleotide binding site–leucine-rich repeat domain, which is characteristic of disease resistance gene analogs. The expression level of these two transcripts in the moderately resistant progenies was low (<1 FPKM) at 60 hpi, but there was no expression in the susceptible progenies. This low expression level at 60 hpi implies that these types of genes are expressed later in the infection process and may be involved in localization of the invasion. For some of the highly expressed gene transcripts like *Pavir.J27411* (Supp. Table 1), a homolog was not found in our

analysis, but with 17% query cover, it hit maize (*Zea mays* L.) ubiquitin-conjugating enzyme. This latter case demonstrates that this transcript is involved in the ubiquitination reaction that may target pathogens through selective protein–protein interaction for degradation of the pathogen (Nandi et al., 2006). Other highly expressed transcripts in the moderately resistant progenies, such as *Pavir.J30830*, *Pavir.Fa00720*, *Pavir.J40613*, *Pavir.J18966*, *Pavir.Ba00481*, and *Pavir.J30296*, did not hit a significant homolog in the database.

The gene locus *Pavir.J26188* was more highly expressed (fivefold change) in the susceptible than in the moderately resistant progenies. The function of this gene transcript could not be identified, since an associated annotation term was not found in switchgrass, *A. thaliana* or rice. It was also mapped on one of the unanchored contigs and its genomic location in the switchgrass genome could not be determined. Basic local alignment nucleotide searches of the cDNA sequence showed that *S. italica* metallothioneins-like protein is the closest gene with 41% query cover and 83% identity ($2e^{-22}$). Metallothioneins are ubiquitous cysteine-rich proteins involved in metal ion homeostasis and detoxification, as well as protection against reactive oxygen species (Coyle et al., 2002). From this BLASTn result, we learned that this gene is also involved in plant defense but the reason why its expression is higher in the susceptible than in the moderately resistant progenies could not be resolved. Other transcripts with more than fourfold change upregulation in the susceptible compared with the resistant progenies were *Pavir.Ga02681*, *Pavir.J28091*, *Pavir.Ib00007*, and *Pavir.Ia02057*. Among these genes, only *Pavir.J28091* hit an uncharacterized hypothetical protein of *Z. mays* (ZEAMMB73_582383) in the NCBI gene database. In *Eucalyptus*, plants susceptible to *Puccinia psidii* showed higher expression of genes linked to generalized stress responses and detoxification that are seemingly incapable of inducing a competent host defense response (Moon et al., 2007). Evidently, genes upregulated in the resistant progenies include those encoding defense mechanisms, the hypersensitive response, and cellular function and maintenance for normal growth and development in plants. Those upregulated in the susceptible plants were genes for general stress response.

Conclusions

In this study, host–pathogen interaction-related transcriptome profiling was done in switchgrass during rust infection. A majority of the differential transcripts appeared at 60 hpi rather than 24 hpi, with a potential increase in the infection process for a certain period of time. Based on GO annotation, the differentially upregulated transcripts in the moderately resistant group are functionally involved in responses to biotic stimuli, response to oxidative stress, DNA binding and repair, and transmembrane transport activity. These transcripts were downregulated or low in expression in

the susceptible progenies compared with the moderately resistant ones, suggesting that the susceptible plants lack a gene expression triggering system in response to pathogen attack and effective control. There were also several novel candidate genes identified that are presumed to be involved in the host-pathogen interactions. Several RIG transcripts that parents and offspring had in common were also identified. These resources are essential for developing molecular markers for rust resistance to expedite breeding rust resistance in switchgrass.

Acknowledgments

The three-generation, four founder population was developed with funds provided by the Department of Energy (DOE) and United States Department of Agriculture. This research work was partially funded by the Bio-Energy Science Center, a U.S. DOE Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science, and the National Science Foundation's Experimental Program to Stimulate Competitive Research (EPS-0814361). The authors thank Stacy Allen of the Genomics and Microarray Core Facility of The Samuel Roberts Noble Foundation for RNA-Seq data generation. We are also thankful to Jackie Kelley for her grammatical editing of the manuscript. Mention of commercial products and companies in this manuscript is solely to provide specific information and does not constitute endorsement by any part herein.

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