Transcriptional Analysis of Soybean Root Response to *Fusarium virguliforme*, the Causal Agent of Sudden Death Syndrome

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Sudden death syndrome (SDS) of soybean can be caused by any of four distinct *Fusarium* species, with *F. virguliforme* and *F. tucumaniae* being the main casual agents in North and South America, respectively. Although the fungal tissue is largely confined to the roots, the fungus releases a toxin that is translocated to leaf tissues, in which it causes interveinal chlorosis and necrosis leading to scorching symptoms and possible defoliation. In this study, we report on an Affymetrix analysis measuring transcript abundances in resistant (PI 567.374) and susceptible (Essex) roots upon infection by *F. virguliforme*, 5 and 7 days post-inoculation. Many of the genes with increased expression were common between resistant and susceptible plants (including genes related to programmed cell death, the phenylpropanoid pathway, defense, signal transduction, and transcription factors), but some genotype-specific expression was noted. Changes in small (sm)RNA levels between inoculated and mock-treated samples were also studied and implicate a role for these molecules in this interaction. In total, 2,467 genes were significantly changing in the experiment, with 1,694 changing in response to the pathogen; 93 smRNA and 42 microRNA that have putative soybean gene targets were identified from infected tissue. Comparing genotypes, 247 genes were uniquely modulating in the resistant host, whereas 378 genes were uniquely modulating in the susceptible host. Comparing locations of differentially expressed genes to known resistant quantitative trait loci as well as identifying smRNA that increased while their putative targets decreased (or vice versa) allowed for the narrowing of candidate SDS defense-associated genes.

Soybean (*Glycine max* [L.] Merrill) is a major world crop and is the second most cultivated crop in the U.S.A. following maize. Soybean production contributes billions of dollars annually to the national economy, contributing approximately 17 billion dollars in 2008 (United Nations Food and Agriculture Organization website). Limitations on maximum production

are largely due to disease pressures that reduce yield. The disease called sudden death syndrome (SDS) has often ranked in the top five yield-suppressing diseases of soybean from 1996 to 2007 and has ranked within the top 10 diseases of soybean in 11 of the 12 years of the survey (Wrather and Koenning 2009). SDS disease of soybean can be caused by any of four distinct *Fusarium* species, with *F. virguliforme* and *F. tucumaniae* being the main casual agents in North and South America, respectively (O'Donnell et al. 2010). This soil-borne pathogen colonizes roots, causing reduced root mass, vascular browning, necrosis, and root rot (Roy et al. 1997). Although the fungal tissue is largely confined to the root, the fungus releases a toxin that is translocated to leaf tissues, in which it causes interveinal chlorosis and necrosis leading to symptoms of scorching and possible defoliation (Jin et al. 1996). Symptoms may be most dramatic in the leaves, but reductions in yield are attributed to both root infection and foliar symptoms (Luo et al. 2000; Rupe et al. 1989).

Several reports have been made to characterize the plant response to infection by *F. virguliforme*, formerly known as *F. solani* f. sp. *glycines*. Biochemically, the isoflavone phytoalexin glyceollin and its precursor daidzein as well as lignins increased in infected roots of two partially resistant plant introductions (PI) compared with the highly susceptible variety Spencer, and increased phenolic levels were highest in the area around the infection site (Lozovaya et al. 2004). A gene expression analysis of 191 genes, selected based on putative roles in defense, identified a defensive role for genes associated with phenolics, phenylpropanoids, general defense, cell-wall modification, and signal transduction in soybean roots during a 10-day infection period (Iqbal et al. 2005). In this study, the defense response was clearly being initiated by the third day postinoculation. A study looking at the response of *Arabidopsis thaliana* to *F. virguliforme*, using a cDNA array representing 10,560 genes, suggested a possible role of an SnRK1 in the defense regulation as well as products downstream of phenylalanine ammonia lyase (Yuan et al. 2008).

Small interfering RNA (smRNA) molecules have been identified to play a regulatory role in many plant processes, including defense. Increasing evidence indicates that smRNA play important roles in the response of plants to biotic and abiotic stresses (Sunkar et al. 2007). Levels of smRNA expression were altered in plants infected with virus (Bazzini et al. 2007) or in plants expressing suppressors of posttranscription gene silencing (Chapman et al. 2004; Chen et al. 2004; Kasschau et al. 2003; Mlotshwa et al. 2005). Expression of 10 of the 11 analyzed micro (mi)RNA families was significantly repressed in the galled loblolly pine (*Pinus taeda*) stem infected with the fungus *Cronartium quercuum* f. sp. *fusiforme* (Lu et al. 2007).

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In addition, in response to FLS2 signaling, *Arabidopsis* miR393 targets the TIR1 F-box protein that normally binds auxin and, therefore, suppresses expression of auxin responsive genes (Navarro et al. 2006). Because auxin responses often enhance susceptibility, targeting TIR1 for silencing enhances defense. The smRNA nat-siRNAATGB2 was shown to be induced by *avrRpt2*, in an *RPS2*-specific manner, to repress *PPRL*, a putative negative regulator of *RPS2*-mediated resistance (Katiyar-Agarwal et al. 2006). To date, we are not aware of any published reports in soybean for a role of smRNAs in defense to pathogens, including *F. virguliforme*.

As for any plant disease, control by genetic resistance would be the most valuable approach. Moderate levels of genetic resistance have been identified by screening the United States Department of Agriculture soybean germplasm collection, but no genotypes have been identified with complete resistance (Hartman et al. 1997; Mueller et al. 2002). SDS resistance quantitative trait loci (QTL) have also been identified in a variety of soybean cultivars, such as Forrest and Pyramid (Iqbal et al. 2001; Njiti et al. 2002). The discovery of partially resistant genotypes has allowed for identification and mapping of QTL associated with resistance to SDS (de Farias-Neto et al. 2007; Iqbal et al. 2001; Njiti et al. 2002). Resistance loci playing a role in minimizing leaf or root damage may differ, and positive identification of the specific genes underlying resistance QTL has been elusive (Kazi et al. 2008; Triwitayakorn et al. 2005).

In this manuscript, we used the soybean Affymetrix chips containing oligo probes representing approximately 38,000 transcripts, making it the largest gene expression survey of soybean response to SDS reported to date. Soybean microarrays (Clough and Vodkin 2004; Vodkin et al. 2004, 2007) and Affymetrix gene expression chips are valuable resources to study soybean response to diverse pests and interacting microbes, as has been shown in reports to soybean aphid (Li et al. 2008), Japanese beetles (Casteel et al. 2008), *Sclerotinia sclerotiorum* (Calla et al. 2009), *Phytophthora sojae* (Moy et al. 2004; Zhou et al. 2009), *Pseudomonas syringae* (Zou et al. 2005), *Phakopsora pachyrizi* (Choi et al. 2008; Panthee et al. 2007, 2009; van de Mortel et al. 2007), *Bradyrhizobium japonicum* (Brechenmacher et al. 2008; Libault et al. 2010), *Soybean mosaic virus* (Babu et al. 2008; Bilgin et al. 2008), and *Heterodera glycines* (Alkharouf et al. 2006; Ithal et al. 2007; Klink et al. 2007, 2009, 2010).

Although no soybean genotypes have been identified as having complete resistance to SDS, PI567.374 has good partial resistance to SDS, based on both greenhouse and field studies (Hartman et al. 1997) and has therefore been selected as the resistant (R) genotype in this study. The aim of this study was to i) identify the early specific responses of soybean roots from the partially resistant PI567.374 compared with the highly susceptible (S) genotype Essex, ii) characterize the molecular mechanisms of soybean resistance to this pathogen, iii) map the differentially expressed transcripts to the soybean genome, and iv) identify and characterize smRNA involved in R or S reactions of soybean roots to *F. virguliforme*.

RESULTS AND DISCUSSION

Disease progressed normally (Fig. 1), as typical SDS leaf symptoms of interveinal chlorosis and root rot were apparent at the end of the 15-day experiment. At 5 days postinoculation (dpi), there were no visible foliar symptoms on either cultivar, but both cultivars displayed slight root-rot symptoms along the main tap root. By 7 dpi, chlorotic foliar symptoms were beginning to be visible on the S but not the R cultivar, and root rot was spreading to lateral roots for both cultivars. Foliar chlorotic symptoms were not apparent on the R plants until 15

dpi. To assess progression of this host-pathogen interaction, we monitored expression of a *F. virguliforme* ribosomal RNA gene (GenBank number AF124995) and the soybean defense responsive gene *PR5* (GenBank number AI930611) by real-time quantitative reverse-transcribed polymerase chain reaction (qRT-PCR). Based on the fungus progression, *PR5* gene expression in both R and S genotypes (Fig. 2), and preliminary microarray results (unpublished data), 5- and 7-dpi root samples were chosen for analysis, as these were the earliest time-points showing gene expression changes in this interaction.

Differential gene expression and real-time qRT-PCR validation.

The Affymetrix microarray analysis identified 2,467 transcripts (false discovery rate (FDR)-corrected *P* value <0.01 and a fold change of at least ± 1.5) as being differentially expressed in the roots at 5 and 7 dpi (Supplementary Table 1). For simplicity, we will use the term 'gene' for a probe set representing a given transcript. These 2,467 genes were classified into 19 functional categories according to guidelines established by Calla and associates (2009). Assignment of the genes associated on the Affymetrix chip to the soybean genome (Schmutz et al. 2010) were obtained from SoyBase, and the sequence of the corresponding gene models (The Phytozome website) were compared with public databases to make final decisions on gene annotation calls and functional assignments. To validate the microarray results, 24 genes were selected (Supplementary Table 2) for qRT-PCR using RNAs from 5, 7, and 9 dpi. Nine of these genes were unique in their transcriptional abundance in R, and one was unique in S. Ten genes were highly abundant in both R and S. In addition, four genes were selected that differed between R and S with or without inoculation. The qRT-PCR identified similar trends of increased and decreased abundance in the microarray results (Supplementary Figs. 1 and 2); in a few cases, however, the qRT-PCR expression ratios were greater than the microarray ratios reflecting the more sensitive nature of qRT-PCR.

General summary of *F. virguliforme* induced gene expression changes.

Of the 2,467 genes that were differentially expressed at some points in the experiment, 1,694 genes were significantly different in response to *F. virguliforme* infection (Supplementary Table 3). Most of these genes (1,262 transcripts) increased in abundance, comparing infected tissue to mock control, while 432 transcripts decreased. These 1,694 genes were classified into functional categories (Fig. 3A and B). Although plant gene annotations are constantly improving, many (17% of increasing, 30% of decreasing transcripts) of the genes fell into the "unknown" and "no hits" categories. The number of transcripts in the "signaling," "secondary metabolism," "defense," and "DNA/RNA" categories accounted for half of the transcripts that increased or decreased in abundance due to the fungal infection (50.5 and 51.5%, respectively). These results demonstrate that the Affymetrix experiment successfully identified transcripts modulating during these early timepoints, with many genes reflective of plant defense responses and regulation. Genes responding to infection and related to defense, signaling, secondary metabolism, cell wall, gene regulation, and oxidative stress will be further discussed as genes in these categories are of great importance in determining the outcome of disease development.

Defense. At 5 and 7 dpi, the soybean roots in this disease study were expressing numerous genes associated with defense mechanisms against invading pathogens. A total of 102 transcripts (11.48%) with high homology to defense-related genes were differentially expressed in the study, giving strong evi-

dence of the initiation of an attempt to defend. Genes differentially expressed in the defense category included several genes annotated as being related to fungal cell-wall degradation, *PR* proteins, putative *R* genes, apoptosis, and programmed cell death (PCD).

Fungal cell-wall degradation by host chitinases is a typical plant defense to pathogens (Uppalapati et al. 2009). In this study, 12 chitinase transcripts were highly increased in both R and S genotypes. Another hallmark of triggering host defenses is the transcriptional accumulation of pathogenesis-related

Fig. 1. Sudden death syndrome disease symptoms on roots and leaves of susceptible (Essex) and partially resistant (PI567.374) genotypes upon infection by *Fusarium virguliforme* 5, 9, 15, and 21 days postinoculation.

(PR) genes. In our current study, 31 transcripts related to PR genes increased in abundance in both R and S. Of these 31 transcripts, 12 genes belonged to the protein family PR-5 (osmotin or thaumatin-like proteins), eight genes to PR-10, and seven genes to PR-1. The role of PR proteins as antifungal proteins has been reported in several plant species (Abad et al. 1996; Velazhahan and Muthukrishnan 2003). In addition, there were two genes annotated as photoassimilate-responsive protein 2, one gene belonging to LURP1 (late upregulated in response to *Hyaloperonospora parasitica*), and one gene belonging to defensin (PDF1-2), which is known to induce by jasmonic acid (JA). The LURP1 gene was reported to be required for full *Arabidopsis* basal defense to *Hyaloperonospora parasitica*, mediated by R proteins RPP4 and RPP5 (Knoth and Eulgem 2008).

There were several genes annotated as an aspartyl protease, an enzyme associated with the onset of PCD, that were differentially expressed between infected and mock-infected roots (Solomon et al. 1999). The initiation of PCD pathways might be to the benefit of *F. virguliforme*, as this pathogen is a necrotroph. In all, 24 soybean transcripts annotated as possible R genes were regulated during soybean root infection (Table 1). Of these 24, five transcripts decreased, while 19 increased in abundance. These genes belong to coiled coil nucleotide binding site-leucine rich repeat (CC-NBS-LRR) (five transcripts), toll interleukin 1 receptor (TIR)-NBS-LRR (15 transcripts), red finger NBS-LRR (one transcript), and NBS-LRR (two transcripts) classes, as well as mildew resistance locus O 12 (one transcript). The increase of expression of so many R genes during a defensive response might be a reflection of fatal efforts by the plants to recognize and resist the pathogen and might work to the benefit of this necrotrophic pathogen by mediated hypersensitive response (HR)-like PCD. Another proposed role of R genes is as a host receptor of fungal toxins (Lorang et al. 2007; Nagy and Bennetzen 2008). In addition, Wolpert and associates (2002) reported that host-specific toxins such as victorin, produced by *Cochliobolus victoriae*, evoke susceptibility by inducing molecular and biochemical responses similar to those induced by avirulence factors.

Signaling. Depending on the nature of a pathogen lifestyle (biotrophic or necrotrophic), specific hormone levels tend to be altered, promoting resistance or susceptibility (Glazebrook 2005; Veronese et al. 2006). Some plant hormones affect plant defense due to their role in triggering cell death, while others regulate the activation of protective mechanisms, including the synthesis of antimicrobial compounds (Frick and Schaller 2002; Gechev et al. 2004; Wi et al. 2010). Consequently, many genes involved in the regulation of PCD and hormone signaling are also components of the plant stress and defense response. The roles of the plant hormones ethylene and JA as positive regulators of resistance to necrotrophic fungi have been reported from genetic data included in *Arabidopsis* and tomato mutants (Abuqamar et al. 2008; Berrocal-Lobo et al. 2002; Thomma et al. 1999). In addition, several studies suggest that ethylene plays a critical role during plant resistance to necrotrophic fungi (Calla et al. 2009; Zhao et al. 2007). However, the effectiveness of a given hormone-responsive arsenal varies. For example, recent work addressing the interaction between *F. oxysporum* and *Arabidopsis* (Thatcher et al. 2009), reported that, in response to this pathogen, not only were JA-mediated defense responses activated to restrict pathogen growth but also nondefensive JA-mediated responses that promoted pathogen-induced symptom development. In this study, 23 of the 25 genes annotated as being related to ethylene, increased in abundance in response to infection (Table 2). Likewise, all nine differentially expressed JA-related genes increased in abundance (Table 2). Several other plant hormone-related

genes also responded to infection in the study. Three genes for abscisic acid showed increased abundance. The differential genes related to auxin as well as genes related to cytokinin were mixed in their expression responses. Gibberillic acid-related genes tended to be unchanging or reduced in abundance.

Calcium (Ca^{2+}) is another major signaling compound affecting the early events in plant-pathogen interactions and is firmly established as an ubiquitous intracellular second messenger in plants (Hetherington and Brownlee 2004; Rudd and Franklin-Tong 2001; Sanders et al. 2002). In response to increasing the level of Ca^{2+} , the cell possesses an array of proteins that can respond to these changes such as calmodulin (CaM) (Luan et al. 2002; Snedden and Fromm 2001), Ca^{2+} -dependent protein kinases (Cheng et al. 2002; Harmon et al. 2001), and CaM-binding proteins (Reddy et al. 2002; Zhang and Lu 2003). In our current study, of the 38 differentially expressed genes annotated as being related to Ca^{2+} , 26 increased in at least one of the 5- or 7-dpi samples. Several members of CaM, CaM-binding protein, and protein kinase families increased in abundance in response to *F. virguliforme* infection, suggesting a role of Ca^{2+} signaling during soybean-*F. virguliforme* interactions.

Gene regulation. A total of 110 transcription factors (TF) were identified as being differentially expressed in this study. These TF belong to 19 different families, with the main ones represented being WRKY, MYB, and NAM/NAC factors. The transcripts of 18 members of WRKY TF family increased in abundance in soybean roots infected by *F. virguliforme* (Table 3). The WRKY superfamily of TF plays a central role in defense regulation by acting as positive or negative regulators (Eulgem and Somssich 2007; Pandey and Somssich 2009). WRKY proteins are defined by a 60-amino acid DNA-binding

Fig. 2. Real time reverse transcription-polymerase chain reaction detection of relative transcript accumulation of **A**, *Fusarium virguliforme* ribosomal RNA gene and **B**, soybean defense responsive gene PR5 at different time-points (5, 7, 9, 11, 13, and 15 day postinoculation [dpi]). In each histogram the mean of three biological replicates were represented $\pm$ standard deviation ($n = 3$).

module containing a putative zinc finger and the amino acid sequence WRKYGQK. The cognate *cis* element for WRKY proteins (W box) contains the consensus (T)TGAC(C/T) (Eulgem et al. 2000). W boxes are often overrepresented in promoters of defense-associated genes (Eulgem et al. 2004; Navarro et al. 2004; Zipfel et al. 2004). In addition, a recent study (Mohr et al. 2010) indicated that WRKY transcription factors can regulate expression of surveillance genes at the top of the defense-signaling cascade, including the positive regula-

tion of an *R* gene by one or more WRKY proteins. The numerous genes belonging to MYB TF were mixed in their expression behavior but mostly increased. The transcripts of 18 members of the NAM/NAC TF family increased in abundance in soybean roots infected by *F. virguliforme* (Table 3). The NAC are a family of genes specific to plants and play a role in defense and abiotic stress responses as well as in a diverse set of developmental processes (Collinge and Bolter 2001; Hegedus et al. 2003; Olsen et al. 2005; Tran et al. 2004; Xie et

Fig. 3. Different functional categories of differentially expressed transcripts using cut-off false discovery rate-corrected *P* value < 0.01 with log₂ expression value of ≥ 0.58 and ≤ -0.58. **A**, Transcripts increased in abundance when comparing soybean roots infected by *Fusarium virguliforme* to mock-infected. **B**, Transcripts decreased in abundance when comparing soybean roots infected by *F. virguliforme* to mock-infected.

al. 1999). A NAC TF has been implicated in contributing to lateral root development (Xie et al. 2010), suggesting a possibility that expression of NAM/NAC TF might lead to lateral roots to compensate for root lost due to disease.

Oxidative stress. The 109 genes annotated in the oxidation category were differentially expressed in this study, reflective of changes affecting the oxidative state of the tissue during this early stage of the interaction. A total of 15 peroxidase gene

Table 1. Resistant gene transcripts that differentially expressed between infected and mock^a

Affymetrix ID	PI567374		Essex		Annotation ^b
	5 dpi	7 dpi	5 dpi	7 dpi	
GmaAffx.61216.1.S1_at	-0.17	-0.85	-0.25	0.07	CC-NBS-LRR
GmaAffx.73978.1.S1_at	-0.53	-0.61	-0.24	-0.41	CC-NBS-LRR
Gma.1622.1.A1_s_at	1.69	2.69	2.48	2.95	CC-NBS-LRR
Gma.9560.1.A1_at	0.54	0.86	0.89	1.19	CC-NBS-LRR
Gma.7895.1.A1_at	-0.03	-0.41	0.51	-0.87	CC-NBS-LRR
GmaAffx.37942.1.S1_at	-0.84	-1.05	0.04	0.06	NBS-LRR
GmaAffx.8873.1.S1_at	0.85	2.09	0.82	0.60	Red finger-NBS-LRR
Gma.9827.1.S1_at	-0.02	-0.02	1.71	-0.70	NBS-LRR
Gma.8292.1.S1_at	1.04	0.99	-0.02	0.11	TIR-NBS-LRR
Gma.18007.1.S1_at	0.85	1.31	1.18	1.53	TIR-NBS-LRR
GmaAffx.87942.1.S1_at	-0.04	-0.02	-0.62	0.65	TIR-NBS-LRR
GmaAffx.37095.2.S1_at	0.81	1.15	1.09	1.72	TIR-NBS-LRR
GmaAffx.37095.2.S1_x_at	0.64	1.10	1.14	1.58	TIR-NBS-LRR
Gma.8478.1.S1_at	0.64	0.88	0.77	1.03	TIR-NBS-LRR
GmaAffx.37095.2.S1_s_at	0.40	1.04	0.84	1.43	TIR-NBS-LRR
GmaAffx.79005.1.S1_at	0.35	0.66	0.20	0.38	TIR-NBS-LRR
GmaAffx.46846.1.S1_at	0.35	0.17	0.07	0.75	TIR-NBS-LRR
Gma.1043.1.S1_at	-0.34	-0.67	0.00	-0.81	TIR-NBS-LRR
GmaAffx.91232.1.S1_at	0.42	1.27	1.45	1.78	MLO12
GmaAffx.93642.1.S1_s_at	2.10	2.37	3.03	2.85	TIR-NBS-LRR
GmaAffx.91396.1.S1_at	3.13	3.81	3.93	3.62	TIR-NBS-LRR
Gma.2523.1.S1_s_at	3.12	3.60	4.18	3.47	TIR-NBS-LRR
GmaAffx.2799.1.A1_at	1.31	1.96	2.06	1.91	TIR-NBS-LRR
GmaAffx.91396.1.A1_at	0.64	2.28	1.16	3.02	TIR-NBS-LRR

^a Genes with increased and decreased transcripts are highlighted with dark and light gray shading, respectively.

^b CC = coiled coil, NBS = nucleotide binding site, LRR = leucine-rich repeat, and TIR = toll interleukin 1 receptor.

Table 2. Ethylene- and jasmonic acid-related transcripts that differentially expressed between infected and mock-infected^a

Affymetrix ID	Soybean gene ID	PI567374		Essex		Specific
		5 dpi	7 dpi	5 dpi	7 dpi	
GmaAffx.93151.1.S1_x_at	Glyma14g05350.1	1.80	3.19	3.24	3.37	Ethylene
Gma.5064.1.S1_at	Glyma14g05350.1	1.79	3.42	3.41	3.27	Ethylene
Gma.2776.2.S1_a_at	Glyma06g24130	0.96	2.34	1.82	2.29	Ethylene
Gma.17141.1.S1_at	Glyma07g05420.2	0.01	1.43	0.16	3.02	Ethylene
GmaAffx.93151.1.S1_at	Glyma14g05350.2	2.33	3.89	3.74	3.85	Ethylene
GmaAffx.93182.1.S1_at	Glyma02g43580.1	1.69	2.86	2.94	2.72	Ethylene
GmaAffx.93182.1.S1_s_at	Glyma02g43600	1.64	2.49	2.61	2.45	Ethylene
GmaAffx.93151.1.S1_s_at	Glyma14g05360.1	1.30	2.23	2.28	2.24	Ethylene
GmaAffx.79089.1.S1_at	Glyma07g15480.1	0.81	0.63	1.22	0.50	Ethylene
GmaAffx.92536.1.S1_s_at	Glyma02g43560.5	0.79	2.23	1.40	2.24	Ethylene
GmaAffx.90053.1.S1_at	Glyma07g15480.1	0.77	0.85	1.16	0.35	Ethylene
Gma.2776.1.S1_at	Glyma02g43560.4	0.73	2.14	1.49	2.23	Ethylene
Gma.2776.2.S1_s_at	Glyma02g43560.5	0.65	2.05	1.32	2.16	Ethylene
GmaAffx.91731.1.S1_at	Glyma15g11930.1	-0.26	-0.51	-0.17	-0.71	Ethylene
Gma.3591.1.S1_x_at	Glyma15g11930.1	-0.17	-0.87	-0.31	-0.47	Ethylene
Gma.1332.1.S1_s_at	Glyma08g02130.1	0.21	0.83	1.36	1.47	Ethylene
Gma.17998.1.S1_s_at	Glyma18g46920.1	0.87	1.44	1.11	1.06	Ethylene
Gma.15605.1.S1_at	Glyma15g16260.1	1.00	0.94	1.34	0.95	Ethylene
GmaAffx.87012.1.S1_at	Glyma03g31940.1	1.63	1.65	3.55	2.70	Ethylene
Gma.12330.1.S1_s_at	Glyma02g01960.1	0.49	0.43	1.45	0.52	Ethylene
GmaAffx.51274.1.S1_at	Glyma03g31940.1	1.07	1.49	2.84	1.94	Ethylene
GmaAffx.4445.1.A1_at	Ambiguous Hit	0.11	0.62	-0.08	0.28	Ethylene
GmaAffx.23351.1.S1_at	Glyma02g00870.1	3.42	4.23	3.68	4.37	Ethylene
GmaAffx.15015.1.S1_at	Glyma10g00980.1	1.52	2.31	1.50	2.49	Ethylene
GmaAffx.53033.1.S1_at	Glyma20g34570	1.09	1.59	1.80	1.77	Ethylene
GmaAffx.83910.1.S1_at	Glyma15g35410.1	2.01	3.44	2.73	3.21	Jasmonic acid
GmaAffx.6142.1.S1_at	Glyma13g16950.1	0.81	1.62	1.43	2.17	Jasmonic acid
GmaAffx.18868.1.S1_s_at	Glyma15g35410.1	1.11	2.40	2.41	2.92	Jasmonic acid
GmaAffx.93442.1.S1_at	Glyma13g16950.1	1.02	2.18	1.73	2.63	Jasmonic acid
Gma.1426.1.A1_at	Glyma01g22160.1	0.54	1.11	0.91	1.07	Jasmonic acid
GmaAffx.22456.1.S1_at	Glyma04g03740.1	2.92	4.93	3.39	5.83	Jasmonic acid
GmaAffx.83504.1.A1_at	Glyma04g03740.1	2.30	3.84	2.56	4.85	Jasmonic acid
GmaAffx.22250.1.S1_at	Glyma02g11020	0.49	0.89	1.27	1.31	Jasmonic acid
GmaAffx.22456.1.S1_x_at	Glyma04g03740.1	2.95	4.49	3.27	5.85	Jasmonic acid

^a Genes with increased and decreased transcripts are highlighted with dark and light gray shading, respectively. dpi = days postinoculation.

transcripts increased in abundance in both R and S genotypes. There were 15 glutathione-S-transferases (GST) differentially expressed, with all but two increasing in transcript abundance following infection. One GST, Glyma13g19130.1, increased only in S and another, Glyma08g18640.1, decreased in R at 5 dpi but increased in S for the same timepoint. Four ferritin genes increased for both R and S, but one, Glyma07g19060, showed an increase only in R at 7 dpi. Five genes annotated as tyrosinase/polyphenol oxidase, and all increased across R and S except for one, Glyma13g25260.1, which was unaffected in all samples but increased almost five times in R in the 7 dpi sample. Interestingly, two genes for superoxide dismutase (SOD) decreased in expression in the R samples but were unchanged in S. Most of the transcripts related to the oxidative state involved scavenging reactive oxygen species that might initiate HR or PCD. As necrotrophic fungi feed on dead cells, expression of HR or PCD would most likely enhance pathogen growth and colonization (Govrin and Levine 2000). While plant mutants with enhanced cell death have increased resistance to biotrophic pathogens, these mutants stimulate the susceptibility to necrotrophic fungi (Kachroo et al. 2001; Veronese et al. 2004). In this study, several transcripts related to apoptosis, proteases, and markers of cell death and senescence, such as *hxr203j* and *SRG1*, were induced, suggesting the possible initiation of PCD (Pontier et al. 1998).

Secondary Metabolism. Secondary metabolites form an important component of the plant defense arsenal. In this study, the main classes of differentially expressed genes related to secondary metabolites were alkaloids, indoles, and phenylpro-

panoids. In addition, many genes annotated as cytochrome P450 genes (often involved in biosynthesis of phytoalexins) with unknown substrates were differentially expressed. The uncharacterized cytochrome P450 genes tended to be strongly induced in general, with 48 of 78 of these genes showing an increased abundance in at least one of the infected samples and the other 30 genes being either reduced or unchanged. The phenylpropanoid pathway was represented by 96 differentially expressed genes, with all but 11 increasing in transcript abundance in response to *F. virguliforme*. Of the 11 alkaloid-related differentially expressed genes, all but one, a hyoscyamine 6-dioxygenase hydroxylase, were induced to higher levels in response to infection.

The phenylpropanoid biosynthetic pathway is one of the most important defense pathways employed by soybean against pathogens (Calla et al. 2009; Moy et al. 2004; Zabala et al. 2006; Zhou et al. 2009; Zou et al. 2005). Genes in the phenylpropanoid pathway can be classified into three branches: i) isoflavonoid, ii) flavonoid and anthocyanin, and iii) lignin biosynthesis. In our study, differentially expressed genes involved in all three subpathways were identified.

Transcripts coding for enzymes in the isoflavonoid branch seemed to be mainly increased in abundance in both genotypes. For example, three isoflavone reductase (IFR) genes increased in abundance in both genotypes. The average fold change of IFR in S plants was 1.65 (5 dpi) and 2.42 (7 dpi) while that in R plants was 1.56 (5 dpi) and 2.79 (7 dpi). IFR are the enzymes directing the synthesis of isoflavanones from isoflavones, such as glyceollin from daidzein. Other examples

Table 3. Examples of transcription factors that are differentially expressed between infected and mock-infected^a

Affymetrix ID	Soybean gene ID	PI567374		Essex		Annotation
		5 dpi	7 dpi	5 dpi	7 dpi	
Gma.5331.1.S1_a_at	Glyma13g35550	0.64	1.78	1.78	2.16	NAM
GmaAffx.30791.1.S1_at	Glyma18g13570.1	0.64	2.01	1.06	1.20	NAM
GmaAffx.93509.1.S1_s_at	Glyma13g35550.1	0.63	1.60	1.50	2.13	NAM
GmaAffx.11178.1.A1_at	Glyma18g53950.1	0.62	0.69	0.65	1.03	NAM
GmaAffx.90496.1.S1_s_at	Glyma12g22880.1	0.62	1.38	1.99	1.71	NAM
GmaAffx.51821.1.S1_x_at	Glyma07g35530.1	-0.16	1.88	0.12	2.30	NAM
GmaAffx.57970.1.S1_at	Glyma12g22880.1	0.44	1.65	2.73	1.39	NAM
GmaAffx.78191.1.S1_at	Glyma09g31650.1	0.16	1.46	0.22	0.86	NAM
GmaAffx.41649.1.S1_at	Glyma16g04740.1	0.31	1.30	-0.05	1.70	NAM
Gma.5331.1.S1_at	Glyma13g35550.1	0.56	1.13	1.27	1.88	NAM
GmaAffx.51821.1.S1_s_at	Glyma07g35630.1	0.12	1.02	0.48	1.69	NAM
GmaAffx.51821.1.S1_at	Glyma07g35630.1	0.23	0.91	0.37	2.30	NAM
GmaAffx.57970.2.S1_at	Glyma06g38410.1	0.45	0.84	1.18	1.33	NAM
GmaAffx.90028.1.S1_s_at	Glyma04g38560.1	0.53	0.84	1.25	1.27	NAM
Gma.1649.1.A1_at	Glyma06g38410.1	0.19	0.75	0.93	1.84	NAM
Gma.5519.1.S1_at	Glyma04g38560.1	0.38	0.70	1.18	1.10	NAM
Gma.5519.2.S1_at	Glyma06g16440.2	0.27	0.48	0.99	1.01	NAM
GmaAffx.81791.1.S1_at	Glyma17g23740.1	0.18	0.06	0.63	0.43	NAM
GmaAffx.73813.1.S1_at	Glyma04g38990.1	-0.13	-0.77	-0.11	-0.27	NAM
GmaAffx.61116.1.S1_at	Glyma16g02960.1	0.00	1.05	0.82	1.48	WRKY55
GmaAffx.72524.1.S1_at	Glyma13g38630.1	0.49	0.81	0.66	1.25	WRKY36
Gma.4342.1.S1_at	Glyma13g38630.1	0.51	0.84	0.96	1.20	WRKY36
GmaAffx.29220.1.S1_at	Glyma06g46420.1	0.02	0.69	0.04	1.00	WRKY36
Gma.4400.1.S1_at	Glyma19g26400.1	2.02	2.77	3.20	2.76	WRKY53
GmaAffx.73009.1.S1_s_at	Glyma06g15220.1	1.61	1.04	2.13	1.82	WRKY21
GmaAffx.6438.2.S1_at	—	0.84	1.16	1.04	1.95	WRKY62
GmaAffx.14300.1.S1_at	Glyma13g38630.1	0.77	0.64	1.41	1.22	WRKY36
GmaAffx.73009.2.S1_at	Glyma06g15220.1	0.12	1.47	-0.06	0.31	WRKY61
Gma.4281.1.S1_at	Glyma18g44560.1	0.47	1.26	1.72	1.97	WRKY74
Gma.6156.1.S1_at	Glyma18g47350.1	0.21	1.18	0.67	0.53	WRKY64
GmaAffx.41719.1.S1_at	Glyma17g33890.1	-0.38	0.80	-0.01	1.81	WRKY30
GmaAffx.12883.1.S1_at	Glyma17g33890.1	-0.16	0.48	0.95	2.49	WRKY30
GmaAffx.6438.7.S1_at	Glyma11g29720.1	0.33	0.58	0.95	1.01	WRKY19
GmaAffx.6438.3.S1_s_at	Glyma11g29720.1	0.29	0.54	0.87	1.05	WRKY19
GmaAffx.6438.3.S1_at	Glyma11g29720.1	0.43	0.56	0.73	1.07	WRKY19
GmaAffx.73009.1.S1_x_at	Glyma06g15220	0.10	-0.15	0.08	1.38	WRKY21
GmaAffx.73009.1.S1_at	Glyma04g39650	-0.02	-0.15	0.02	1.36	WRKY59

^a Genes with increased and decreased transcripts are highlighted with dark and light gray shading, respectively. dpi = days postinoculation.

of transcripts increased in abundance and involved in the isoflavonoid branch were 2'-dyoxydihydrodaidzein reductase and daidzein 7-O-methyltransferase. These results suggest that soybean roots were expressing genes required for synthesizing increased amounts of isoflavanones during the dynamic interaction with *F. virguliforme*, consistent with published metabolite data related to soybean-*F. virguliforme* interaction (Lozovaya et al. 2004). Transcripts of genes belonging to the flavonoid branch of this pathway were also detected as differentially expressed in abundance. For example, the transcripts of flavanone 3-hydroxylase, flavanol reductase and oxidoreductase, and 2OG-Fe(II) oxygenase increased in abundance in both genotypes; however, some transcripts decreased in abundance in the S genotype. Activation of the lignin branch is important in plant defense, especially against root-infecting pathogens, as the lignifications of cell wall play an important role. This study revealed 21 differentially expressed genes related to lignin, four of them belonging to cinnamoyl CoA reductase (CCR), the enzyme responsible for diverting the phenylpropanoid pathway into the branch of lignin synthesis. The CCR genes all increased in both genotypes, but two genes (Glyma18g06510.1 and Glyma11g29460.3) decreased or had no change in the S genotype. In addition, other genes involved in the lignin synthesis pathway increased clearly in abundance in the R genotype, while some decreased or had no change in the S genotype, such as caffeic O-methyltransferase, caffeic acid O-methyltransferase homolog, cinnamate 4-hydroxylase, ferulic acid 5-hydroxylase 1, ferulate-5-CoA reductase, and laccase. Altogether, this study reflects that multiple branches of the phenylpropanoid pathway were activated during this host-pathogen interaction.

Cell wall. As one of the main damages of SDS is root rot, it is not surprising that many genes related to cell-wall modification and reinforcement were modified in their expression patterns during pathogen invasion. Pathogens deploy several enzymes to break the cell-wall barrier, the most common being exo- and endo-polygalacturonases (PG), pectin lyases and pectin methyl, and acetyl esterases (Prade et al. 1999). PG can also be produced by the host, and it is interesting to note that the only identified differentially expressed transcript of PG decreased in abundance in the R genotype in response to *F. virguliforme* infection. Plant hosts may also produce inhibitors of cell wall-degrading enzymes, such as PG inhibitory proteins (PGIP) belonging to the super-family of leucine-rich repeat proteins and forming part of the plant defense system in the plant cell wall (Juge 2006). In our study, there were five genes of PGIP induced by fungal infection. It has been proposed that inhibition of PG by PGIP may amplify defense responses by allowing an increase in defense-activating oligogalacturonide fragments (De Lorenzo and Cervone 1997; Federici et al. 2006), but PGIP can also directly inhibit pathogen invasion. Overexpression of two PGIP genes led to enhanced *Arabidopsis* resistance to *Botrytis cinerea*, whereas antisense expression increased disease symptoms (Ferrari et al. 2003). In soybean, four PGIP genes have been identified; however, only a single member has been shown to be capable of inhibiting a known fungal PG (D'Ovidio et al. 2006). In this study of soybean response to *F. virguliforme*, we identified a total of 70 cell wall-related genes as being differentially expressed in response to the pathogen, including transcripts for proteins involved in synthesis of cellulose (β -glucosidase), hemicellulose (xyloglucosyl transferase), and pectin (pectinesterase and PG).

Specific R and S responses to *F. virguliforme*.

Unique transcripts with high abundance in the R genotype totaled 123 genes (Supplementary Table 4), while unique transcripts that decreased in abundance totaled 124 genes. In con-

trast, in the S genotype, the unique transcripts that increased in abundance totaled 170 (Supplementary Table 5), while unique transcripts that decreased in abundance totaled 198. Examples of transcripts that were unique in the R genotype and increased in abundance were those related to cell wall, defense, oxidation, and gene regulation. Two peroxidase genes increased in transcript abundance in the R genotype while, in the S genotype, there was a decrease in one and no change in the other. Peroxidases play an important role as antioxidants; in addition, they are used by the cell as lignin-modifying agents, through cross-linking of cell-wall components. Peroxidases were found to increase in abundance in transcriptomic and proteomic studies in, respectively, soybean and *Brassica napus* infected by *S. sclerotiorum* (Calla et al. 2009; Liang et al. 2008). Other oxidation genes that were uniquely increased in the R genotype were a 2OG-Fe(II) oxygenase, FAD-linked oxidoreductase 1, laccase, and polyphenol oxidase, while the transcripts of an alcohol oxidase and SOD decreased in abundance. Alcohol oxidase was reported as a potential extracellular source of hydrogen peroxide (H_2O_2) in brown rot decay of wood (Daniel et al. 2007), while SOD plays a role in the dismutation of superoxide to H_2O_2 that might reflect differences between genotypes in modulating reactive oxygen species (ROS) levels, which, if excessive, might trigger PCD. Laccase has been implicated as a possible key defense gene to SDS (Iqbal et al. 2005). Accelerated cell death 6 (ACD6) increased in abundance in R plants at 5 and 7 dpi. ACD6 was discovered in *Arabidopsis* in a mutant with accelerated cell death (Dong 2004). Plant mutants with enhanced cell death have increased resistance to biotrophic pathogens but susceptibility to necrotrophic fungi (Kachroo et al. 2001; Veronese et al. 2004). If low levels of ACD6 accelerate cell death, then the enhanced expression of ACD6 in the R genotype might indicate an effort to reduce PCD. Unique genes were also identified that were related to the phenylpropanoid pathway and increased in the R genotype, for example, cinnamoyl-CoA reductase, ferulic acid 5-hydroxylase, *N*-hydroxycinnamoyl/benzoyltransferase, and flavanone 3-hydroxylase. It is interesting to note that one of the PR genes was uniquely induced in R plants at 7 dpi, photoassimilate-responsive protein 2, a new class of PR proteins (Herbers et al. 1995).

Two soybean genes annotated as WRKY transcription factors were increased in abundance in the S genotype, while no change occurred in the R genotype. The WRKY TF family is a big family of TF that can either negatively or positively regulate plant resistance. As these WRKY TF transcripts were higher in S than R plants, it would suggest that these TF might be employed as negative regulators of resistance. Additionally, three peroxidase genes decreased in abundance in the S genotype, while they either increased or had no change in the R genotype.

Specific difference in R and S without inoculation.

A comparison was made between the R and S genotypes without inoculation to determine constitutive differences between the genotypes (Supplementary Table 6). The constitutively differentially expressed transcripts between mock-infected R plants and mock-infected S plants reflects the diversity of genetic background of both genotypes, in which 1,010 transcripts increased in abundance and 818 transcripts decreased in abundance in the R plants. These dramatic changes include genes spread across most of the functional categories, including 129 transcripts belonging to defense. Of these 129 transcripts, there were 44 transcripts from R genes with high abundance in R mock-infected plants. Out of 44 transcripts, 16 decreased in abundance, while 28 increased in abundance. Another example of a functional category that was constitutively differentially expressed was the DNA/RNA category. For ex-

ample, several WRKY and NAM/NAC TF were more abundant in R than in S plants.

To verify the array results related to the mock-inoculated plants, qRT-PCR was employed using specific primers from four genes. Two of these genes are increased in abundance in R mock-infected plants (Glyma02g36640.1 and Glyma06g46280.2), while the two others decreased (Glyma02g12800.1 and Glyma08g41510.1). qRT-PCR failed to amplify the first two genes from the S genotype, while it failed to amplify the other two genes from the R genotype, even with a second primer pair and after increasing the number of PCR cycles to 45.

Possible detoxification genes.

F. virguliforme produces a toxin that is translocated to leaf tissues, in which it causes interveinal chlorosis and necrosis. As this fungus has never been detected in leaves, it is most likely that the pathogen produces its toxin in the roots and it is translocated through the vascular system to the leaves (Baker and Nemec 1994; Jin et al. 1996). Several potential compounds have been isolated from the *F. virguliforme* filtrate and proposed as putative SDS toxins, but none has yet been genetically confirmed (Baker and Nemec 1994; Brar and Bhattacharyya 2007; Ji et al. 2006; Jin et al. 1996). In our study, we identified several genes related to possible detoxification.

ABC transporters are well known for their ability to confer drug resistance in bacteria, animal, and plant cells. A loss of *Arabidopsis* ABC transporter AtPDR8 led to increased cell death after pathogen infection (Kobae et al. 2006). In our current study, the transcripts of 10 genes of ABC transporter increased in abundance. Five aldo/keto reductase (AKR) genes increased in abundance upon *F. virguliforme* infection. An AKR can protect plants against toxic carbonyls and oxidation stress, for example, overexpressing the *Arabidopsis* *ALDH3* gene encoding AKR protein showed improved tolerance against drought, heavy metals, methyl viologen, and H₂O₂ (Ramanjulu et al. 2003). Other potential detoxification agents of fungal toxin include GST and P450 genes, and numerous genes annotated as GST or P450 increased in abundance during infection.

Association of differentially expressed transcripts to SDS resistance QTL.

Ten published (Austecinio et al. 2007; de Farias-Neto et al. 2007; Iqbal et al. 2001; Kazi 2005; Kazi et al. 2008; Njiti et al. 2002) simple sequence repeat (SSR) markers linked to QTL involved in soybean resistance to *F. virguliforme* were used in this study. As QTL can vary from population to population, only markers that were consistent between at least two populations were used. Four, three, and two of SSR markers were mapped to linkage group D2 (chromosome 17), C2 (chromosome 6), and G (chromosome 18), respectively. To identify which differentially expressed genes were located near one of the SDS-resistant QTL SDS markers, the Affymetrix reference sequence of each differentially expressed transcript were blasted against soybean genome sequence. The chromosome location of each of these genes was noted and compared with those of SSR markers that were similarly blasted to the soybean genome (Supplementary Table 7). Nine out of 10 SSR markers were noted to have at least one differentially expressed gene within 500 kb. Sat-222, Satt389, Satt528, and Satt311 SSR markers all mapped to LG D2 and were linked to six, two, two, and one genes, respectively. Examples of the genes linked to Sat-222 were a GDSL-motif lipase/hydrolase, two ABC transporter-like genes, a NAM, and a calmodulin-related gene. These last two genes belong to the constitutively differentially expressed transcripts between the R and S geno-

types. Formamidase, calmodulin, and an unknown expressed protein were linked to Satt389, Satt311, and Satt528 and also mapped to LG D2. A GDSL-motif lipase/hydrolase and a lipid transfer protein were linked to Satt277 (LG C2) and Satt130 (LG G) within the physical distance of 111 and 85 kb, respectively. These two genes are involved in cuticle deposition of plant aerial parts (Yeats et al. 2010) and are suggestive of a similar functional role of these two proteins during soybean root resistance to *F. virguliforme*. Two transcription factors (WRKY and MYB73), hsr203j, transporters, and other genes that may be involved in signal transduction pathway were linked to Satt371 and map to LG C2. Genes encoding for an inositol polyphosphate 5-phosphatase II, a no hit, a calmodulin, and an unknown expressed protein were the closest genes to Sat-222, Satt389, Satt311, and Satt528 map to LG D2. On LG G, differentially expressed genes for a lipid transfer protein, a peroxidase, a WRKY TF, a glucosyltransferase, a naringenin 3-dioxygenase, and a cinnamoyl-CoA reductase genes were located within 460 kb of marker Satt130. Another SSR marker, Satt038, maps to LG G, and genes related to an asparagine synthetase 3, a no hit, and a G protein-coupled receptor gene mapped within 240 kb. Interestingly, two genes related to calmodulin were linked to Satt277 and Satt311 but mapped to different linkage groups (on LG C2 and LG D2, respectively). It is also of interest to note that Kazi and associates (2008) reported that there are two separate QTL conferring resistance to leaf scorch and root resistance. Satt277 mapped to LG C2 and Satt130 mapped to LG G, which contain QTL involved in leaf scorch resistance, while Satt038 mapped to LG G, which contains QTL involved in root resistance (Kazi et al. 2008). Satt277 (LG C2) and Satt130 (LG G) are linked to ABC transporter, peroxidase, calmodulin, and cinnamoyl-CoA reductase genes that may be involved in detoxification, transporter, and signal transduction pathways to decrease the effect of toxin that is produced by *F. virguliforme*. On the other hand, Satt038 (LG G) is linked to asparagine synthetase 3 (ASN3) and G protein-coupled receptor genes that may be involved in root resistance to the fungal infection. Twenty-six transcripts, out of 54 that mapped less than 500 kb of SSR markers, were from constitutively differentially expressed transcripts between R and S genotypes, reflecting a role of genetic background of each genotype used in this study.

SmRNA sequence analysis.

Over 91 million raw sequence reads were obtained from deep sequencing (Supplementary Table 8). The sizes of the smRNA after adapter trimming ranged from 14 to 33 nucleotides, with the majority in the range of 19 to 26 nucleotides. Adapter-trimmed sequences were compared to identify duplicates and to obtain the number of unique sequences and occurrences of each. At this stage, all sequences presented more than five times were carried forward for subsequent comparisons. Sequences from four libraries were merged to calculate the expression ratio of each sequence. All the duplicate sequences were removed from the four libraries, in addition to the sequences less than 19 bp and more than 26 bp, leaving 30,841 sequences for analysis (Supplementary Table 9). These sequences were blasted against the soybean genome using the Novocraft program, the miRBase database, and the R-FAM database to identify matches to the soybean genome, conserved miRNA, transfer RNA (tRNA), and ribosomal RNA (rRNA). Table 4 summarizes the sequence abundance and percentage of each category. The most abundant sequences were those belonging to smRNA (25,433 sequences) with 82.46% of the total sequences. tRNA, rRNA, and miRNA are represented, respectively, by 157 sequences (0.51%), 447 sequences (1.45%), and 917 sequences (2.97%) of the total sequences. In addition,

there were 3,887 sequences (12.60%) that did not fit any of above categories. It is interesting to mention that 297 sequences perfectly matched to the *F. virguliforme* genome (*unpublished data*), which suggests that some of these smRNA might have originated from the pathogen genome. Length distribution and abundance of the sequences were calculated using 26,350 sequences, after removing those related to rRNA, tRNA, and unmatched sequences to soybean genome. The most abundance of small RNA in this study were 21 (29.8%), 22 (25.7%), and 20 (14.9%) bp, while 26 bp (1.9%) was the lowest (Fig. 4).

Putative targets of smRNA.

SmRNA were blasted against the sequences corresponding to the differentially expressed transcripts from the Affymatrix study. A total of 93 genes were identified as putative targets for differentially expressed smRNA (Supplementary Table 10). Of the 93 gene targets, 10 putative target genes belong to defense-related, eight belong to DNA/RNA, eight belong to primary metabolism, and nine belong to signaling, while 14 had no known function. The number of smRNA that targeted each gene varied, the maximum being for an RNase H domain-containing protein gene (Glyma13g12070.1). Comparison of the expression data from smRNA with those from Affymatrix data showed mostly negative correlations between smRNA accumulation and target gene mRNA abundance, suggesting a possible functional role of these smRNA. Invertase (Glyma13g42530.1), kinesin motor domain (Glyma06g01130.1), glycosylphosphatidylinositol-anchored (Glyma16g26480.1), TIR-NBS-LRR (Glyma16g24940.1), amino acid permease (Glyma19g22590.1), senescence-related gene 1 (Glyma01g06820.1), and ethylene-insensitive root 1 (Glyma15g25690.2) were examples of target genes that had mRNA increased in abundance while smRNA transcripts decreased. TIR-NBS-LRR (Glyma07g07100.3) and CCAAT-box-binding TF (Glyma10g28900.1) were examples of targeted genes that had their mRNA decrease in abundance while their smRNA transcripts accumulated. Some differences between R and S genotypes were noted. For example orcinol *O*-methyltransferase (Glyma0335s00200.1) mRNA decreased while its smRNA increased in abundance in S plants only. CCAAT-box binding transcription factor (Glyma10g28900.1) decreased while its smRNA increased in abundance in R plants only.

Identification of miRNA and their putative gene targets.

SmRNA sequences were blasted against the miRBase database to identify conserved and previously known miRNA. There were 831 (2.69%) conserved miRNA from this study that belong to 23 different families derived from different plant species. Of the 831 conserved miRNA, 415 miRNA increased in abundance with $\log_2 \geq 1.5$ and 182 miRNA decreased in abundance with $\log_2 \leq -1.5$. Out of 30,841 smRNA, 42 novel miRNA were identified (0.14%). Most of these novel miRNA matched perfectly to one or two locations of the soybean genome and derived from intergenetic regions of the genome. Most of the mature sequences of novel miRNA had a length of

21 and 22 bp. Expression data from these novel miRNA as a ratio between infected and mock plants reveal that 29 miRNA increased in abundance with $\log_2 \geq 1.5$ and 10 miRNA decreased in abundance with $\log_2 \leq -1.5$. Putative miRNA target genes were identified from those differentially expressed in the Affymatrix experiment in addition to the soybean genome sequence. Table 5 summarizes the soybean target genes from the Affymatrix arrays. There were seven members of a laccase gene family that were targeted by SDS_39254 miRNA, which belongs to mir397 of conserved miRNA, while the transcript accumulation of miRNA was induced, mRNA of target genes either increased (Glyma03g15800.1, Glyma03g15800.3, Glyma01g26750.1, and Glyma18g42520.1) or decreased (Glyma11g14600.1, Glyma18g38660.1, and Glyma08g47380.1) in abundance. While the transcript accumulation of PGIP1 (Glyma15g26790.1) as a target gene coincided with increasing SDS_36340 (gma-MIR1507c) in abundance as a novel miRNA in S plants, the transcript accumulation of CC-NBS-LRR gene coincided with increasing of SDS_42227 (gma-MIR5041) in abundance as a novel miRNA in R and S plants.

As the differentially expressed transcripts from the Affymatrix experiment belonged to limited numbers of soybean genes, we decided to blast the novel and known miRNA identified in this study against the whole soybean-genome sequence. There were 38 miRNA (Supplementary Table 11) that targeted genes of soybean. Of 38 miRNA, 20 were novel, reflecting the important roles of those miRNAs during soybean root responses to *F. virguliforme* infection. The target genes included defense, signaling, and transcriptional regulation-related genes. For example SDS_13582, SDS_40501, and SDS_4437 miRNA (belonging to mir-156) target different members of squamosa promoter-binding protein-like 6. SDS_42227 (gma-MIR5041) and SDS_526 (gma-MIR1516), as novel miRNAs, target different members of CC-NBS-LRR and TIR-NBS-LRR R genes, respectively. As an example of potential functional roles for miRNA during signaling transduction pathways, different gene members belonging to auxin response factor, F-box domain, and protein tyrosine kinase were targeted by miRNA.

Overall conclusions.

Previous gene expression (Iqbal et al. 2005) and metabolite (Lozovaya et al. 2004) studies suggested increased expression of genes related to defense, phenolics, phenylpropanoids, cell wall, and signal transduction as well as increases in lignins and the isoflavone chemicals daidzein and glyceollin play roles in defense to SDS. Here, we presented data that support and extend these earlier findings. The data presented indicate that PCD pathways and ROS levels were being modulated and that some genes that can modulate these responses differed between

Table 4. Classification and abundance of smRNAs from the four investigated libraries^a

SmRNA class	Abundance	%
tRNA	157	0.51
rRNA	447	1.45
miRNA	917	2.97
smRNA	25,433	82.46
Unmatched	3,887	12.60
Total	3,0841	100.00

^a smRNA = small RNA, tRNA = transfer RNA, rRNA = ribosomal RNA, miRNA = microRNA, and unmatched = sequences did not match to soybean genome sequence.

Fig. 4. Small RNA distribution.

the R and S genotypes. These results suggest that the host is modulating PCD and ROS induction, as PCD and ROS imbalances could benefit this necrotrophic pathogen. In addition to presumably trying to avoid PCD and trying to maintain a proper ROS level, the host is also apparently combating the pathogen with expression of numerous PR genes and genes known to be involved in phytoalexin pathways. By correlating this list of differentially expressed genes to QTL maps and identifying smRNA whose expression is opposite to that of genes differentially expressed on the microarrays, we highlighted candidate SDS defense-associated gene candidates that will warrant future functional analyses to verify their putative role in enhancing defense.

MATERIALS AND METHODS

Plant and fungus material.

Soybean [*Glycine max* (L.) Merrill] seed of two genotypes, PI 567.374 (partially resistant) and Essex (highly susceptible), were sown 0.5 cm from the surface in 24-cm-tall plastic cone pots filled with soilless growing medium (Sunshine Mix, LC1, Sun Gro Horticulture Inc., Bellevue, WA, U.S.A.) containing an approximate 2- to 3-cm layer of *F. virguliforme*-infested sorghum in the middle, between two 10-cm-deep layers of soil mix, thus ensuring that seedling roots would contact the inoculum soon after seed germination. Plants were watered from the bottom and were grown in a growth chamber at 22°C (night) and 26°C (day) with a 14-h day length, with approximate light intensity of 110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Inoculum preparation.

Sorghum colonized with *F. virguliforme* (isolate Mont-1) was used as an inoculum to inoculate soybean roots. *F. virguliforme* cultures grown on potato dextrose agar were cut into small pieces and were added to 2-liter flasks containing approximately 1,000 ml of sterilized sorghum grain. Flasks were incubated at 25°C for 14 days and were shaken by hand every other day to promote even distribution of mycelial growth. Inoculum (4 cm³) was added to each cone pot, whereas sterile sorghum grain was used for mock inoculations.

Sample collection and RNA extraction.

Whole roots were collected at 5, 7, 9, 11, 13, and 15 days after sowing seeds, and these dates were considered equivalent

to days postinoculation for the purpose of this study. Each collected sample consisted of pooled roots from seven different plants. The entire experiment was repeated three times on different dates, using new preparations of inoculum to obtain three true independent biological replications. The three biological replicates showed consistency in symptom development as well as in levels of gene expression of a *F. virguliforme* ribosomal gene (GenBank number AF124995) and soybean *PR-5* (GenBank number AI930611), using RT-PCR primer pairs GGCTGAAGTGGCAACTTGA and CAAAGCTTCATTCA ATCCTAATACAATC and gaggttctccaatggacag and caaccctgcactgtgttta, respectively. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) and verified for quality as previously described in detail (Zou et al. 2005).

Affymetrix analysis.

For Affymetrix Soybean Genome GeneChip analysis, 6 μg of total RNA were used following the manufacturer's recommended protocols (Affymetrix, Santa Clara, CA, U.S.A.). Fragmentation of biotin-labeled cRNA was confirmed using an Agilent 2100 Bioanalyzer before hybridization. Microarray hybridizations were performed at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign, following manufacturer recommended protocols, and chips were scanned with a GeneChip model 3000 7G Plus high-resolution scanner. Individual scans were analyzed as previously described (Libault et al. 2010). Each probe set was summarized into a single value using the GC-RMA algorithm (Wu and Irizarry 2005; Wu et al. 2004), and expression values were then converted into log₂ values. Microarray data is publicly available from the University of Illinois Department of Crop Sciences (Urbana, IL, U.S.A.) website.

Statistical analysis.

Statistical analysis was performed as a service by the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. Affymetrix's Soybean Genome GeneChips database contains over 37,500 soybean-related probe sets as well as over 23,300 probe sets designed from two common soybean pathogens. Because these pathogens were not present in this study, their probe sets were removed prior to all data processing and analyses, using R1 packages from the Bioconductor project 2. The experiment was a 2 $\times$ 2 factorial de-

Table 5. microRNA (miRNA) target genes from differentially expressed transcripts of Affymetrix arrays^a

smRNA_ID	miRBase ID	Affymetrix ID	Target		Affymetrix data					
					miRNA data		R I_v_M		S I_v_M	
			Matching soy gene	Annotation	S	R	5 d	7 d	5 d	7 d
SDS_39254	MIR397	Gma.13624.1.A1_at	Glyma18g42520.1	Laccase	1.70	2.03	0.35	1.20	-0.39	0.21
SDS_39254	MIR397	GmaAffx.31832.1.S1_at	Glyma11g14600.1	Laccase	1.70	2.03	-0.13	-1.11	-0.65	-1.28
SDS_39254	MIR397	GmaAffx.31978.1.S1_at	Glyma18g38660.1	Laccase	1.70	2.03	-0.03	-0.05	-0.34	-0.70
SDS_39254	MIR397	GmaAffx.51444.1.S1_at	Glyma08g47380.1	Laccase	1.70	2.03	-0.35	-0.24	-0.77	-1.00
SDS_39254	MIR397	GmaAffx.3562.1.S1_s_at	Glyma03g15800.1	Laccase	1.70	2.03	4.07	5.13	6.02	5.37
SDS_39254	MIR397	GmaAffx.50828.1.S1_at	Glyma03g15800.3	Laccase	1.70	2.03	4.62	5.59	6.36	5.91
SDS_39254	MIR397	GmaAffx.60533.1.S1_at	Glyma01g26750.1	Laccase	1.70	2.03	1.38	1.86	2.01	1.80
SDS_14391	gma-MIR396g	GmaAffx.51847.1.A1_at	...	...	-0.29	0.00	-0.21	-0.47	-0.02	0.08
SDS_23723	gma-MIR396f	GmaAffx.51847.1.A1_at	...	...	0.00	-1.17	-0.21	-0.47	-0.02	0.08
SDS_20372	mir-172	GmaAffx.63114.1.A1_at	Glyma08g19140.1	RNA binding protein	0.00	3.83	0.07	0.44	0.05	-0.39
SDS_36340	gma-MIR1507c	Gma.9956.1.S1_at	Glyma15g26790.1	Polygalacturonase inhibiting	-0.84	2.35	0.44	1.36	0.45	0.93
SDS_36340	gma-MIR1507c	GmaAffx.91749.1.S1_s_at	Glyma15g26790.1	Polygalacturonase inhibiting	-0.84	2.35	0.19	1.41	0.35	1.26
SDS_42227	gma-MIR5041	Gma.1622.1.A1_s_at	Glyma05g17470.1	CC-NBS-LRR	2.17	2.46	1.69	2.69	2.48	2.95

^a Genes and miRNAs that increased or decreased are shaded in dark or light gray, respectively. S= susceptible. R= resistant. I= infected. M= mock. d = days. CC = coiled coil, NBS = nucleotide binding site, LRR = leucine rich repeat, and smRNA = small RNA.

sign, with two treatments (infected and mock) and two time-points (5 and 7 dpi). All arrays of this experiment were analyzed together in a two-way analysis of variance (ANOVA) model with effects for treatment and time, and the comparisons of interest were tabulated as contrasts from the ANOVA model. The model was fit using the limma 4 package, which uses an empirical Bayes correction 5. The false discovery rate (FDR) method 6 was used to correct the raw *P* values for multiple hypothesis testing, and gene probe sets with FDR *P* values < 0.01 were considered statistically significant. An additional criterion of a fold change of ± 1.5 was also applied for a gene to be considered significantly differentially expressed in this study.

RT-PCR and qRT-PCR.

RNA was treated with DNase I (Invitrogen) to remove genomic DNA contamination. DNase-treated RNA (2 μ g) was reverse-transcribed using the SuperScript III First-Strand synthesis system for RT-PCR (Invitrogen). A 'minus' reverse transcriptase PCR reaction, in which no reverse transcriptase enzyme was added during the cDNA synthesis reaction, was used to test each mRNA sample for genomic DNA contamination. The transcriptional expression of each gene was analyzed using real time PCR. cDNA was diluted to a final concentration of 30 ng/ μ l, and then, 2 μ l were used in a 20- μ l PCR reaction containing 8.9 μ l of Brilliant QPCR master mix (Stratagene, La Jolla, CA, U.S.A.) and 0.2 μ M of each primer. PCR was carried out under the following conditions: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 20 s at melting temperature, and 30 s at 72°C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60 to 95°C. In addition, PCR products were sequenced to verify that the correct amplicon was being produced. The real-time PCR data were analyzed using the delta-delta method (McMaugh and Lyon 2003), which establishes the change in magnitude of target gene transcription subjected to different treatments (i.e., infected) relative to transcription levels in controls (mock-infected) after normalization. Three constitutive controls were initially selected for data normalization, a soybean β -actin (GenBank number AI507761) and the recently reported soybean constitutive genes (Libault et al. 2008) cons6 (GenBank number CD397253) and cons15 (GenBank number AW396185). However β -actin was found to be the most consistent one; therefore, results presented here were relative to β -actin only. Three biological replicates (the same RNA used for the Affymetrix analysis) with three technical replicates of each treatment were performed, and the data was calculated as the average of three biological replicates.

Determining location of differentially expressed genes on the soybean genome.

The soybean genome, version Glyma1 (the Phytozome database), was downloaded and formatted as a reference database. Ten SSR markers (Satt371, Satt307, Satt277, Satt038, Satt130, Satt528, Satt574, Satt311, Sat_222, and Satt389), genetically mapped to QTL and linked to *F. virguliforme* resistance, that were previously identified and confirmed by at least two populations (Austecclinio et al. 2007; de Farias-Neto et al. 2007; Iqbal et al. 2001; Kazi 2005, 2008; Njiti et al. 2002) were selected and their sequences retrieved from the National Center for Biotechnology Information (NCBI). To determine their position on the soybean genome, SSR marker sequences were blasted against the soybean genome using the "blastall" program from NCBI on a local server. Similarly, the available soybean sequences from genomic (g)DNAs selected as highly significant in the present microarray study were blasted against

the soybean genome to identify their locations. The locations of SSR markers and gDNA sequences were then compared with each other to identify those located on the same chromosome and <500 kb apart.

SmRNA sequencing and sequence analysis.

Next generation sequencing (SOLiD) was carried out to generate sequence reads from four different libraries. These libraries included PI 567.374_Roots_Mock, PI 567.374_Roots_Infected, Essex_Roots_Mock, and Essex_Roots_Infected. Equal RNA amounts (5 μ g) from different timepoints (5, 7, and 9 dpi) and from three biological replicates were mixed together and were used for each library. To enrich total RNA for the smRNA fraction (>200 nt) mirVana miRNA isolation kit was used (AM1560; Ambion, Austin, TX, U.S.A.).

SmRNA purification, sequencing, and preliminary analysis were conducted by Cofactor Genomics, St. Louis, MO, U.S.A. The returned sequences were coded in color space format, and Perl script was used to return them to familiar nucleotide bases. After removing the adaptor (CGCCTTGCCGTACAG CAG), the sequences were counted and mapped to the soybean genome (Glyma1.01), to obtain the corresponding matching chromosome, position, and strand direction. Sequences between 19 and 26 bases in length were used for further analysis. The raw abundance values for sequence reads in each library were normalized into corresponding transcripts per million (TPM), using $\text{TPM abundance} = (\text{raw abundance value} / \text{sum_use}) * \text{n_base}$ (Joshi et al. 2010), where n_base is one million, sum_use is the number of reads matched to the soybean genome in each study. Expression ratios were calculated between infected and mock-infected for R and S plants and the fold change ratios were converted to log₂ values.

SmRNA target gene prediction and miRNA identification.

Rfam databases were used to remove putative noncoding structural RNA. The Novocraft program was used to identify the target gene prediction among the differentially expressed transcripts from the Affymetrix study. Conserved miRNA were identified by searching against the miRBase to find homologs of known miRNA. To identify novel miRNA, sequences mapping to more than 20 loci were removed, as these are likely to be siRNA mapping to a genomic repeat. All genomic loci left after this filtering were considered to be potential origins of miRNA and were subjected to further analysis. Each locus was checked to assess the stability of a secondary hairpin loop structure that is highly typical of the miRNA genesis process. Secondary structure prediction was done by the M-Fold software. Seventy-five bases from either side of the location of each small RNA mapping was retrieved from the Glyma1 assembly and was submitted to M-Fold with the parameters label = 10 and temp = 25. Secondary structures predicted were screened manually for the presence of a hairpin loop involving the initial locus of smRNA mapping in a stable pairing with a sequence downstream. All loci producing stable hairpins were screened against known soybean repeats (as annotated by the Joint Genome Institute) to remove smRNA from repeat loci. A plant smRNA regulator target analysis server was used to identify miRNA target genes from significant array transcripts and from soybean genome sequence.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Cofactor Genomics website: www.cofactorgenomics.com
M-Fold software: mfold.rna.albany.edu/?q=mfold
miRBase database: www.mirbase.org
The Novocraft program: www.novocraft.com/main/index.php
Rfam databases: fam.janelia.org
The Phytozome database: www.phytozome.net/soybean
SmRNA regulator target analysis server:
plantgrn.noble.org/psRNATarget
SoyBase database: soybase.ncgr.org
United Nations Food and Agriculture Organization website:
faostat.fao.org/site/339/default.aspx
University of Illinois Department of Crop Sciences website:
cropsci.illinois.edu