The Necrotrophic Fungus *Macrophomina phaseolina* Promotes Charcoal Rot Susceptibility in Grain Sorghum Through Induced Host Cell-Wall-Degrading Enzymes

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Accepted for publication 18 February 2018.

ABSTRACT

The cell-wall-degrading enzymes (CWDE) secreted by necrotrophs are important virulence factors. Although not unequivocally demonstrated, it has been suggested that necrotrophs induce hosts to cooperate in disease development through manipulation of host CWDE. The necrotrophic fungus *Macrophomina phaseolina* causes charcoal rot disease in *Sorghum bicolor*. An RNA-seq experiment was conducted to investigate the behavior of sorghum CWDE-encoding genes after *M. phaseolina* inoculation. Results revealed *M. phaseolina*'s ability to significantly upregulate pectin methylesterase-, polygalacturonase-, cellulase-, endoglucanase-, and glycosyl hydrolase-encoding genes in a charcoal rot-susceptible sorghum genotype (Tx7000) but not in a resistant genotype (SC599). For functional validation, crude enzyme mixtures were extracted from *M. phaseolina*- and mock-inoculated charcoal-

rot-resistant (SC599 and SC35) and -susceptible (Tx7000 and BTx3042) sorghum genotype stalks. A gel diffusion assay (pectin substrate) revealed significantly increased pectin methylesterase activity in *M. phaseolina*-inoculated Tx7000 and BTx3042. Polygalacturonase activity was determined using a ruthenium red absorbance assay (535 nm). Significantly increased polygalacturonase activity was observed in two susceptible genotypes after *M. phaseolina* inoculation. The activity of cellulose-degrading enzymes was determined using a 2-cyanoacetamide fluorimetric assay (excitation and emission maxima at 331 and 383 nm, respectively). The assay revealed significantly increased cellulose-degrading enzyme activity in *M. phaseolina*-inoculated Tx7000 and BTx3042. These findings revealed *M. phaseolina*'s ability to promote charcoal rot susceptibility in grain sorghum through induced host CWDE.

The plant cell wall is made up of a complex network of cellulose, hemicelluloses, and pectin (Cosgrove 2005). The cellulose microfibrils are embedded in a matrix of pectin, hemicellulose, lignin, and structural proteins (Cosgrove 2005; Rose et al. 2004). During pathogen infection, plants deposit callose-rich cell wall appositions (i.e., papillae) at penetration sites, accumulate phenolic compounds, and synthesize lignin-like polymers to reinforce the wall (Hückelhoven 2007). Therefore, the plant cell wall is a significant defensive barrier that pathogens encounter before facing intracellular plant defense machinery (Hématy et al. 2009; Lipka et al. 2005; Underwood 2012; Underwood and Somerville 2008).

Although the cell wall poses a significant barrier to pathogen entrance, plant pathogens (especially necrotrophs) have mechanisms to overcome this barrier. Fungal phytopathogens secrete numerous cell-wall-degrading enzymes (CWDE) to disrupt the cell and for absorptive nutrition. These include acetyl esterases, endoglucanases, exo- and endopolygalacturonases, laccases, pectate and pectin lyases, pectin methylesterases (PME), proteases, and xylanases (Di Matteo et al. 2006; Esquerre-Tugayé et al. 2000; Kars et al. 2005; Lebeda et al. 2001). Studies have revealed a positive correlation between specific CWDE and virulence of the necrotrophic pathogen *Botrytis cinerea*, the wilt pathogen *Verticillium dahliae*, and the blotch fungus *Mycosphaerella*

graminicola (Brito et al. 2006; Espino et al. 2005; Fernández-Acero et al. 2010; Kema et al. 2008). When *Arabidopsis thaliana* is infected with *Ustilago maydis*, pathogen genes responsible for the degradation of cellulose, including an endoglucanase, and the degradation of hemicellulose, including an arabinofuranosidase and a xylanase, are upregulated (Martinez-Soto et al. 2013). An endopolygalacturonase gene, *Bcpg1*, is required by *B. cinerea* for virulence on different hosts (ten Have et al. 1998) and *Alternaria citri* for citrus fruit (Isshiki et al. 2001). A *Claviceps purpurea* strain with two deleted polygalacturonase (PG) genes (*cpgg1* and *cpgg2*) is nonpathogenic on rye (Oeser et al. 2002). In the rice blast fungus *Magnaporthe oryzae*, transcript levels of genes encoding cellulases, hemicellulases, and pectate lyases increase during infection relative to a minimal media control (Mathioni et al. 2011).

As a defensive measure, plants protect their cell walls from degradative enzymes through the production of CWDE-inhibiting proteins (Juge 2006). Examples include PG inhibitors (PGIP), PME inhibitors (PMEI), pectin lyase inhibitor protein, *Triticum aestivum* xylanase inhibitor, xylanase inhibitor protein, and xyloglucan endoglucanase-inhibiting protein (De Lorenzo et al. 2001; Juge 2006). The pectin-degrading enzyme inhibitors are common in dicots and noncommelinid monocots while the xylan-degrading enzyme inhibitors are common in grasses (Sarkar et al. 2009). Although inhibitors such as PGIP do not hinder the plant's PG (Cervone et al. 1990), some inhibitors can only inhibit the enzymes of plant origin. For instance, PMEI inhibit PME of plant origin but typically do not inhibit PME produced by plant pathogens (D'Avino et al. 2003; Di Matteo et al. 2006). However, the inhibition of plant CWDE can still contribute to reduced susceptibility to necrotrophs. For example, the overexpression of PMEI in *A. thaliana* limits fungal infection by *B. cinerea* by decreasing plant PME activity and altering the level of pectin methylesterification of the cell wall (Lionetti et al. 2007). Other than deactivating fungal CWDE, inhibitors can also play an important role in eliciting plant defense responses. For example,

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Funding: Financial support of this research was provided by the Kansas Grain Sorghum Commission.

This article is Contribution Number 18-321-J from the Kansas Agricultural Experiment Station, Manhattan.

*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary table is published online.

impaired fungal PG activity by PGIP results in accumulation of long-chain oligogalacturonides, which are capable of eliciting defense responses in plants (Cervone et al. 1989; Ridley et al. 2001).

Macrophomina phaseolina (Tassi) Goid. is an important, soilborne, necrotrophic fungal pathogen that causes numerous diseases in over 500 different plant species (Islam et al. 2012), including food crops (Su et al. 2001), pulse crops (Mayek-Pérez et al. 2001), fiber crops such as jute (De et al. 1992) and cotton (Aly et al. 2007), and oil crops (Wyllie 1998). Charcoal rot disease is an economically important disease in many crops, including sorghum, soybean, maize, and alfalfa (Islam et al. 2012). A major symptom of charcoal rot is degraded pith tissue at the crown of the plant (Edmunds 1964). Root and stalk damage leads to impaired water and nutrient absorption and translocation (Hundekar and Anahosur 2012). Charcoal rot negatively affects chlorophyll content (Bandara et al. 2016), yield (Bandara et al. 2017c), and macronutrients and mineral content (Bandara et al. 2017a) of grain sorghum as well as biofuel traits of sweet sorghum (Bandara et al. 2017b, 2018). Through genome analysis, Islam et al. (2012) showed that the *M. phaseolina* genome contains genes that can encode 219 glycoside hydrolase-related proteins and 16 polysaccharide lyase proteins. Some studies have shown *M. phaseolina*'s ability to produce CWDE under *in vitro* conditions (Ramos et al. 2016).

Although necrotrophs such as *M. phaseolina* use their CWDE as virulence factors during infection and colonization, the potential of necrotroph-infection-associated upregulation of host CWDE and their contribution to enhanced disease susceptibility are poorly described. Here, with the aim of investigating the differentially expressed genes that are associated with host CWDE and their inhibitors, we compare the global transcriptome profiles of charcoal rot-resistant (SC599) and -susceptible (Tx7000) sorghum genotypes in response to *M. phaseolina* inoculation using RNA-Seq technology. Moreover, follow-up functional and biochemical studies in relation to cellulase, PG, and PME activities of known resistant (SC599 and SC35) and susceptible (Tx7000 and BTx3042) sorghum genotypes in response to *M. phaseolina* inoculation are reported.

MATERIALS AND METHODS

Plant material establishment and maintenance, inoculum preparation, inoculation, and sorghum stalk tissue collection. To obtain stalk tissues for the RNA-Seq experiment, a greenhouse experiment (completely randomized design) was conducted with one sorghum genotype resistant (SC599) and one susceptible (Tx7000) to charcoal rot. Seeds treated with the fungicide Captan (N-trichloromethyl thio-4-cyclohexane-1,2 dicarboxamide) were planted in 19-liter Poly-Tainer pots filled with Metro-Mix 360 growing medium (Sun Gro). Initially, three seeds were planted in each pot. The two least vigorous seedlings were thinned at 3 weeks after emergence, leaving one plant per pot. Pots were randomly placed on a bench and kept at 25 to 32°C with a photoperiod of 16 h of light and 8 h of darkness. Plant maintenance was carried out according to the procedures described by Bandara et al. (2015). A previously characterized, highly virulent *M. phaseolina* isolate (obtained from the Row Crops Pathology Lab, Department of Plant Pathology, Kansas State University) was used for inoculation. The protocol described by Bandara et al. (2015) was used to prepare *M. phaseolina* inoculum. Briefly, *M. phaseolina* was grown on potato dextrose agar (PDA) at 30°C for 5 days. Subcultures from PDA were used to initiate liquid cultures (potato dextrose broth) to obtain *M. phaseolina* mycelia. Small hyphal fragments (1 to 2 intact cells/fragment) were obtained by blending mycelia at 16,000 rpm for 8 min and straining the resulting slurry through four layers of sterile cheesecloth. This suspension was centrifuged (3,000 × g, 5 min) and the pellet was resuspended in sterile phosphate-buffered saline (PBS; pH 7.2). Concentrations of hyphal fragments were determined using a hemocytometer and adjusted to 2 × 10⁶ hyphal fragments ml⁻¹ by diluting with PBS. All inoculum preparation steps were performed under aseptic conditions. Plants were inoculated at 14 days after

anthesis using a 1-ml, 26-gauge, 1.5-in. needle, sterile surgical syringe by injecting 0.1 ml of inoculum into the basal internode of the stalk. PBS (pH 7.2) was used as the mock-inoculated control treatment. The stalk tissues of inoculated and mock-inoculated (10 mM PBS) control plants were collected from three biological replicates at 2, 7, and 30 days postinoculation (DPI) (three biological replicates per DPI per treatment per sorghum line; 36 plants total). The tissue harvest times were selected with the intention of investigating differences in early, mid-, and late gene expression. Three harvest times were selected (2, 7, and 30 DPI), considering the downstream cost for cDNA library construction and Illumina sequencing. From each biological replicate, an approximately 8- to 10-cm-long stalk section that contained the inoculation point was collected and frozen in liquid nitrogen. Stalk sections were stored at -80°C until RNA extraction.

To obtain stalk tissues for host-derived CWDE assays, a separate greenhouse experiment (completely randomized design) was conducted with sorghum genotypes resistant (SC599 and SC35) and susceptible (Tx7000 and BTx3042) to charcoal rot under greenhouse conditions similar to those mentioned above. Plant establishment, maintenance, inoculum preparation, and inoculation were also performed as described above. Because the highest differential gene expression was found to occur at 7 DPI (see Results), enzyme assays were deliberately centralized around 7 DPI. Therefore, at 4, 7, and 10 DPI, 15-cm stalk pieces encompassing the inoculation point were cut from five biologically replicated plants, immediately suspended in liquid nitrogen, and subsequently stored at -80°C until used.

RNA extraction, quality check, cDNA library construction, and Illumina sequencing. For RNA extraction, approximately 1 g of stalk tissue was collected 1 cm above the symptomatic area. The symptomatic area is a necrotic lesion. Therefore, to ensure the quality and quantity of the RNA extract, tissue sampling was conducted 1 cm above the lesion border. This tissue sampling strategy also helped to avoid potential fungal RNA contamination. Total stalk RNA was extracted using Triazole reagent (Thermo Scientific), following the manufacturer's instructions, and subsequently treated with amplification-grade DNase I (Invitrogen Corporation). The quality and quantity of RNA extracts were checked using a Nanodrop 2000 instrument (Thermo Scientific). Extracts were diluted with RNase-free water to obtain the required RNA concentration (100 to 200 ng/μl). RNA integrity and quantity were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies Genomics) before cDNA library preparation. RNA from biological replicates was not pooled.

Following the manufacturer's protocol (Illumina Inc.), 36 cDNA libraries were prepared using the Illumina TruSeq RNA sample preparation kit. RNA from each sample was subjected to two rounds of enrichment for poly-A mRNA using "oligodT" attached magnetic beads. Purified mRNA was chemically fragmented and subsequently converted to single-stranded cDNA. cDNA from each library was separately barcoded with adapter indexes and then pooled. Sequencing was performed on a HiSeq 2000 platform (Illumina Inc.) using 100-bp single-end sequencing runs at the Genome Sequencing Facility of Kansas University Medical Center, Lawrence.

Sequence processing, genome alignment, and differential gene expression analysis. Single-end sequence reads were subjected to adapter trimming and quality filtering using a stand-alone adapter trimmer "Cutadapt" (Martin 2011). The Genomic Short-Read Nucleotide Alignment Program (Wu and Watanabe 2005) was used to perform the read alignment to *Sorghum bicolor* (L.) Moench reference genome (BTx623; Sbicolor_v1.4) (Paterson et al. 2009). Differential gene expression analysis was conducted using DESeq2, which employs a negative binomial distribution-based method. Only reads "uniquely" mapped to the *S. bicolor* reference genome were used for differential gene expression analysis. A q value (Benjamini and Hochberg 1995) was determined for each gene to account for multiple tests. To control false discovery rate at 5%, only the genes with q values < 0.05 were

considered to be significantly differentially expressed. DESeq2 analysis was performed to test the null hypothesis of no significant two-way interaction between sorghum line (resistant and susceptible) and treatment (inoculated with *M. phaseolina* and mock-inoculated control) for each gene within each postinoculation stage (i.e., 2, 7, and 30 DPI). To identify the annotated functions of differentially expressed genes, the SorghumCyc (<http://pathway.gramene.org/gramene/sorghumcyc.shtml>) and Phytozome (Goodstein et al. 2012) databases were used.

Preparation of tissue lysates for enzyme assays and 96-well plate measurements. Stalk tissues were retrieved from -80°C storage. In a mortar, approximately 1 g of stalk tissue (1 cm above the symptomatic region) was quickly chopped into liquid nitrogen using a sterile scalpel. Stalk pieces were ground into a powder using a pestle. Approximately 200 mg of tissue powder was transferred into microcentrifuge tubes filled with 1 ml of 1× lysis buffer (10 mM Tris [pH 7.5], 150 mM NaCl, and 0.1 mM EDTA). Samples were centrifuged at $10,000 \times g$ for 10 min at 4°C . Supernatants were transferred into new microcentrifuge tubes and immediately stored at -80°C until used in assays (crude enzyme mixes). All absorption and fluorescence measurements were performed using a 96-well plate reader (Synergy H1 Hybrid Reader; BioTek). All enzyme activities were expressed as per gram of stalk tissue (wet basis). All enzyme activity assay experiments were repeated twice.

Measuring the cumulative activity of cellulose-degrading enzymes. A 2-cyanoacetamide-based protocol, described by Honda et al. (1980), was used with modifications to measure the cumulative activity of cellulose-degrading enzymes in cell extracts. When reducing carbohydrates such as glucose react with 2-cyanoacetamide in a borate buffer, strong fluorescence results. Its excitation and emission maxima are 331 and 383 nm, respectively. The borate-phosphate (BP) buffer (pH 8.0, 5 ml) was prepared from 0.3 M sodium tetraborate and 0.3 M potassium dihydrogen phosphate. Determination of the cellulase enzyme activity of cell extracts was carried out using carboxymethyl cellulose (CMC) as the substrate. CMC is degraded by enzymes such as cellulases, endoglucanases, glucan endo-1,3- β -glucosidases, and glycosyl hydrolases into glucose. Glucose reacts with 2-cyanoacetamide and fluoresces (Honda et al. 1980). The glucose naturally present in each sample was determined first without adding CMC. These baseline values ($0.5 \times$ value) were subtracted from respective samples with added CMC to determine the glucose coming from CMC only. This glucose concentration is proportional to the cellulase activity of the sample. To develop a calibration curve, glucose (Sigma-Aldrich) was dissolved in BP buffer to obtain standard solutions of 0, 200, 400, 600, and 800 mg liter $^{-1}$. Reaction components were mixed in microcentrifuge tubes (in microliters) as shown in Table 1.

Reaction tubes were incubated in a boiling water bath for 30 min. After cooling the tubes to room temperature, 200 μl of the reaction mixture from each tube was transferred to a transparent, flat-bottom, 96-well microplate and fluorescence was measured. Using the calibration curve, the cellulose-degrading enzyme activity of samples were determined and expressed as relative units (RU). One RU was defined as the amount of glucose (milligrams per milliliter) generated by the crude enzyme mixture extracted from 1 g of stalk tissue through CMC hydrolysis.

TABLE 1. Volumes (μl) of reaction components used for 2-cyanoacetamide assays^a

Assay	Glucose standard	Sample	CMC	2-Cyan	BP	1× lysis buffer
For calibration curve	5	0	0	50	200	0
Assessment of						
Original glucose content of the sample	0	5	0	50	200	0
Glucose coming from CMC due to cellulase activity	0	2.5	2.5	50	200	0
Blank	0	0	2.5	50	200	2.5

^a CMC = carboxymethyl cellulose, 2-Cyan = 2-cyanoacetamide, and BP = borate-phosphate.

Measuring polygalacturonase (PG) activity. PG activity of cell extracts was quantified according to the protocol described by Ortiz et al. (2014), with some modifications. This assay is based on the reaction between polygalacturonic acid (PGA) and ruthenium red (RR). Upon precipitation of high-molecular-weight PGA by RR, the optical density (OD) of the remaining RR was determined. The OD reduction (between blank and sample) was used as a measure of PG activity. In this assay, a 0.2% PGA sodium salt stock solution was prepared in citrate phosphate buffer (CPB; 50 mM, pH 5.0). This solution was obtained by dissolving 0.2 g of PGA sodium salt (Sigma-Aldrich) in citric acid solution and then adjusting the pH to 5.0 with dibasic sodium phosphate. PGA, CPB, 1× lysis buffer (see above), and crude enzyme samples were mixed in microcentrifuge tubes according to Table 2 (all in microliters) and kept on ice.

The reaction tubes were then incubated for 20 min at 40°C in a water bath. The tubes were placed on ice after incubation, and 40 μl of RR aqueous solution at 1.125 mg/ml was added to each tube and mixed for 30 s. The mixture was diluted by adding 100 μl of 8 mM NaOH solution, mixed for 30 s, and centrifuged at 4°C and $3,200 \times g$ for 10 min. For OD quantification, a 25- μl aliquot of the supernatant was transferred to a clear, flat-bottom, 96-well microplate containing 175 μl of water, and absorbance was read at 535 nm. Using the calibration curve, the PG activity of samples was determined and expressed as RU. One RU was defined as the amount of PGA (in micrograms) hydrolyzed per minute with a crude enzyme mixture extracted from 1 g of stalk tissue.

Measuring pectin methylesterase (PME) activity. A modified gel diffusion assay (Downie et al. 1998) was used to quantify PME activity. The medium contained 1% agar, 0.05% citrus pectin (Sigma-Aldrich), 10 mM PBS (pH 7.2), and 10 mM EDTA. Each Petri plate (90 mm in diameter) contained 15 ml of medium. Wells with a diameter of 4 mm were made in the center of the agar plates using a sterile cork borer, and the protein samples were loaded into each well. Assay plates were incubated at 30°C for 16 h. The gels were stained with 0.05% (wt/vol) RR for 45 min and destained with water. The hydrolysis of esterified pectin was measured by the radii of red-staining areas and converted to RU (1 RU = 10 mm of dark staining area). The radius of the red-stained zone increased with increasing quantities of PME.

RESULTS

Differential expression of genes related to host CWDE in response to *M. phaseolina* infection. Differential gene expression analysis revealed 15 and 34 CWDE genes (involved in

TABLE 2. Volumes (μl) of reaction components used for polygalacturonase activity assays^a

Assay	0.2% PGA	CPB	1× lysis buffer	Sample
For calibration curve	10	0	10	0
	8	2	10	0
	6	4	10	0
	4	6	10	0
	2	8	10	0
	0	10	10	0
Samples	10	0	0	10
Blank	0	10	10	0

^a PGA = polygalacturonic acid and CPB = citrate phosphate buffer.

cellulose and homogalacturonan degradation) that were differentially expressed between SC599 and Tx7000 in response to pathogen inoculation at 2 and 7 DPI, respectively (Fig. 1; Supplementary Table S1). Moreover, 3 and 13 genes that are related to cellulose biosynthesis were differentially expressed between two genotypes after pathogen inoculation at 2 and 7 DPI, respectively. The heatmap (Fig. 1) depicts the differentially expressed genes involved in cellulose and homogalacturonan degradation and cellulose biosynthesis in charcoal rot-resistant (SC599) and -susceptible (Tx7000) sorghum genotypes in response to *M. phaseolina* inoculation. None of the CWDE or cellulose biosynthetic genes were differentially expressed at 30 DPI.

Of the 15 differentially expressed CWDE genes at 2 DPI, 12 are involved in cellulose degradation (Fig. 1). These included one cellulase, three endoglucanases, two glucan endo-1,3- β -glucosidase precursors, and six glycosyl hydrolase family 17 genes. The net log twofold upregulation of these 12 genes in Tx7000 was 11.5 whereas

the net downregulation of these genes in SC599 was -3.9. The remaining three genes were related to homogalacturonan degradation. These included two PG genes and an invertase or PME1 family protein gene. The net up- and downregulation of the two PG genes in Tx7000 and SC599 were +2.4 and -3.8, respectively. The PME1 family protein gene was significantly upregulated in SC599 whereas that of Tx7000 was not significantly differentially expressed. Also, three cellulose biosynthesis-related genes were differentially expressed at 2 DPI. These included CESA2-cellulose synthase, CSLA4-cellulose synthase-like family A, and CSLE6-cellulose synthase-like family E genes. The net up- and downregulation of these three genes in Tx7000 and SC599 were +3.7 and -1.7, respectively (Fig. 1).

In total, 34 CWDE genes were differentially expressed at 7 DPI. These included 12 cellulose-degradation-related genes including 1 cellulase, 1 endoglucanase, 2 glucan endo-1,3- β -glucosidase precursors, and 8 glycosyl hydrolase family 17 genes. The net log

Fig. 1. Heatmap showing differentially expressed cell-wall-related genes in charcoal rot-resistant (SC599) and -susceptible (Tx7000) sorghum genotypes in response to *Macrophomina phaseolina* at **A**, 2 and **B**, 7 days postinoculation (DPI). Red, green, and black color gradients represent up- and downregulated and nondifferentially expressed genes, respectively, compared with the mock-inoculated control treatment with sterile, phosphate-buffered saline. Cell. = cellulose, Bio. = biosynthesis, Hom. = homogalacturon, and Deg. = degradation.

twofold upregulation of these 12 genes in Tx7000 was 33.2 while the net downregulation in SC599 was 5.5 (Fig. 1). The remaining 22 genes were related to homogalacturonan degradation. These included six PME, nine PG, six PME or invertase inhibitor family protein genes, and a PME domain-containing protein gene. The net up- and downregulation of the six PME genes in Tx7000 and SC599 were +14.3 and -3.1, respectively (Fig. 1). The nine PG genes showed a +3.2 net log twofold upregulation in Tx7000 whereas none were significantly differentially expressed in SC599. The net up- and downregulation of the six PME domain-containing protein genes in Tx7000 and SC599 were +9.8 and -2.5, respectively. The PME domain-containing protein gene was also significantly upregulated in Tx7000 whereas that of SC599 was not significantly differentially expressed (Fig. 1). The 13 cellulose biosynthesis-related genes included 5 cellulose synthase genes (CESA2, CESA3, CESA4, CESA7, and CESA9) and 8 cellulose synthase-like genes (CSLA4, CSLA7, CSLC1, CSLC7, CSLE2, CSLF2, CSLF6, and CSLH1). The net down- and upregulation of the five

cellulose synthase genes in Tx7000 and SC599 were -15.4 and +2.2, respectively, while net down- and upregulation for the eight cellulose synthase-like genes were -4.0 and +1.1, respectively (Fig. 1).

Dynamics of sorghum CWDE after *M. phaseolina* inoculation. The *F* and *P* values from analysis of variance for the enzyme assays conducted in this study are shown in Table 3. The genotype-treatment interaction effect was found to be significant for all enzymes investigated (cellulase, PG, and PME) at 4, 7, and 10 DPI ($\alpha = 0.05$). Compared with the control treatment, *M. phaseolina* inoculation significantly increased the activity of cellulose-degradative enzymes in two charcoal rot-susceptible genotypes at 4 DPI (Tx7000: 77%, $P < 0.0001$; BTx3042: 102%, $P = 0.0006$), 7 DPI (Tx7000: 70%, $P = 0.0098$; BTx3042: 48%, $P = 0.0011$), and 10 DPI (Tx7000: 39%, $P = 0.0196$; BTx3042: 75%, $P = 0.0005$) (Fig. 2). However, pathogen inoculation did not significantly affect the activity of cellulose-degrading enzymes in resistant genotypes SC599 and SC35 at any postinoculation stage.

TABLE 3. *F* statistic and *P* values of analysis of variance for functional assays including cellulase, pectin methylesterase (PME), and polygalacturonase (PG) activity measured with four sorghum genotypes after inoculation with *Macrophomina phaseolina* at three different days postinoculation (DPI) ($\alpha = 0.05$)

DPI	Effect	Cellulase		PG		PME	
		<i>F</i> value	Pr > <i>F</i>	<i>F</i> value	Pr > <i>F</i>	<i>F</i> value	Pr > <i>F</i>
4	Genotype	16.3	<0.0001	2.4	0.0940	8.4	0.0006
	Treatment	20.2	0.0001	8.8	0.0067	8.6	0.0074
	Genotype × treatment	6.4	0.0024	7.4	0.0011	10.7	0.0001
	Genotype	17.3	<0.0001	3.3	0.0365	2.1	0.1239
7	Treatment	11.8	0.0021	5.6	0.0270	1.9	0.1803
	Genotype × treatment	3.0	0.0489	9.2	0.0003	0.7	0.0037
	Genotype	39.7	<0.0001	17.1	<0.0001	2.3	0.1082
	Treatment	20.8	0.0001	7.8	0.0100	5.4	0.0292
10	Genotype × treatment	4.1	0.0175	28.9	<0.0001	5.6	0.0049

Fig. 2. Comparison of the mean cellulase-degrading enzyme activity (in relative units, 1 RU = the amount of glucose [milligrams per milliliter] generated by the crude enzyme mixture extracted from 1 g of stalk tissue through carboxymethyl cellulose hydrolysis) between two treatments (mock inoculated with phosphate-buffered saline [CON] and *Macrophomina phaseolina* [MP]) in charcoal rot-susceptible (S) (BTx3042 and Tx7000) and -resistant (R) (SC599 and SC35) genotypes at three stages (4, 7, and 10 days postinoculation [DPI]). Treatment means bracketed by different letters within each genotype at a given DPI are significantly different. Treatment means with "ns" designations within each genotype at a given DPI are not significantly different at $\alpha = 0.05$. Error bars represent standard errors.

Compared with the control treatment, *M. phaseolina* inoculation significantly increased PG activity in both charcoal rot-susceptible genotypes at 4 DPI (Tx7000: 149%, $P = 0.0007$; BTx3042: 196%, $P = 0.0218$), 7 DPI (Tx7000: 209%, $P = 0.0057$; BTx3042: 102%, $P < 0.0001$), and 10 DPI (Tx7000: 127%, $P = 0.0030$; BTx3042: 139%, $P < 0.0001$) (Fig. 3). Although pathogen inoculation did not significantly affect the PG activity of SC599 at 4 DPI, inoculation significantly decreased PG activity at 7 DPI (–53%, $P = 0.0460$) and 10 DPI (–51%, $P = 0.0147$). Although pathogen inoculation did not significantly affect the PG activity of SC35 at 4 and 7 DPI, inoculation significantly decreased PG activity at 10 DPI (–56%, $P < 0.0001$).

Compared with the control treatment, *M. phaseolina* inoculation significantly increased the PME activity of both charcoal-rot-susceptible genotypes at 4 DPI (Tx7000: 29%, $P = 0.0046$; BTx3042: 62%, $P = 0.0005$), 7 DPI (Tx7000: 26%, $P = 0.0113$; BTx3042: 24%, $P = 0.0136$), and 10 DPI (Tx7000: 58%, $P = 0.0067$; BTx3042: 33%, $P = 0.0202$) (Fig. 4). Pathogen inoculation did not significantly affect the PME activity of SC599 and SC35 at any postinoculation stage, except for the significantly decreased PME activity of SC599 (–22%, $P = 0.0081$) at 4 DPI.

DISCUSSION

Host tissue penetration is a prerequisite for infection and pathogens use physical and chemical means to facilitate penetration. For example, appressoria generate high levels of turgor pressure to breach the phylloplane cuticle and epidermal cell walls (Choi et al. 2011; Park et al. 2009). CWDE usually play a supplementary role in the penetration of pathogens that can produce highly melanized appressoria (Linsell et al. 2011; Yi et al. 2008). For example, because *B. cinerea* does not possess a thick melanin layer in its appressoria, penetration is accomplished by the release of CWDE that digest the host cell wall rather than by physical force (Choquer et al. 2007). In fact, many plant-pathogenic fungi, especially necrotrophs, rely on the manufacture of CWDE to enter

plant tissue (Łaźniewska et al. 2012). Although “appressoria” production by *M. phaseolina* is reported (Ammon et al. 1975), penetration of interior cell walls has been shown to be a result of both mechanical pressure and chemical softening (Ammon et al. 1974). In fact, Islam et al. (2012) showed that the *M. phaseolina* genome contains genes that encode for 219 glycoside-hydrolase-related proteins and 16 polysaccharide lyase proteins. Ramos et al. (2016) showed *M. phaseolina*’s ability to produce CWDE under in vitro conditions. Therefore, CWDE appeared to be virulence factors for *M. phaseolina*. In the current study, we provide gene expression and functional evidence for the ability of *M. phaseolina* to induce host CWDE in charcoal rot-susceptible sorghum genotypes.

Cellulose is an integral component of plant cell walls. Cellulase converts cellulose polymers into simple sugars such as glucose. Three enzyme classes comprise cellulases (endoglucanases, cellobiohydrolases, and β -glucosidases), which act synergistically to break down the cellulose polymer (Yennamalli et al. 2013). These cellulases are also categorized under a broader enzyme group called the glycosyl hydrolases (Yennamalli et al. 2013). In the current study, compared with mock-inoculated controls, the cellulose degradation capacity (including cellulase, endoglucanase, glucan endo-1,3- β -glucosidase precursor, and glycosyl hydrolase family 17) was found to be significantly higher in charcoal rot-susceptible genotypes (Tx7000 and BTx3042) after *M. phaseolina* inoculation whereas the same was significantly lower in the two resistant genotypes (SC599 and SC35). Therefore, pathogen inoculation-associated host CWDE transcripts and cellulose-degradation activity appeared to contribute to be associated with enhanced charcoal rot susceptibility in grain sorghum.

Cellulose deficiency in the primary cell wall elicits jasmonic acid signaling and enhances resistance to some bacteria, fungi, and aphids (Ellis et al. 2002a). Further, mutations of specific *cellulose synthase* genes result in activation of jasmonic acid signaling (Ellis and Turner 2001; Ellis et al. 2002b). Moreover, the oligosaccharides generated by CWDE can act as elicitors to trigger jasmonic acid

Fig. 3. Comparison of the mean polygalacturonase activity (in relative units, 1 RU = the amount of polygalacturonic acid [micrograms] hydrolyzed per minute with crude enzyme mixture extracted from 1 g of stalk tissue) among two treatments (mock inoculated with phosphate-buffered saline [CON] and *Macrophomina phaseolina* [MP]) in charcoal rot-susceptible (S) (BTx3042 and Tx7000) and -resistant (R) (SC599 and SC35) genotypes at three postinoculation stages (4, 7, and 10 days postinoculation [DPI]). Treatment means bracketed by different letters within each genotype at a given DPI are significantly different. Treatment means with “ns” designations within each genotype at a given DPI are not significantly different at $\alpha = 0.05$. Error bars represent standard errors.

pathways (Aziz et al. 2004, 2007; Moscatiello et al. 2006). In the current study, although cellulose synthase and cellulose synthase-like genes showed net upregulation in *M. phaseolina*-inoculated Tx7000 at 2 DPI, a net downregulation was observed at 7 DPI. This revealed the potential cellulose deficiency faced by Tx7000 after pathogen inoculation at 7 DPI. Although not tested, this deficiency could contribute to the enhanced jasmonic acid biosynthetic capacity of Tx7000.

In addition to enhanced cellulose degradation, this study provided gene expression and functional evidence for enhanced homogalacturonan degradation (through upregulated PME and PG activity) in charcoal rot-susceptible sorghum genotypes after *M. phaseolina* inoculation. PME catalyze the demethylesterification of homogalacturonan. If this occurs on noncontiguous sugars (i.e., random demethylesterification), the molecule becomes a substrate for pectin-degrading enzymes, leading to cell wall disintegration (Micheli 2001; Pelloux et al. 2007). Also, the action of PME makes homogalacturonan vulnerable to degradation by hydrolases such as PG, contributing to cell wall softening (Brummell and Harpster 2001; Wakabayashi et al. 2003). Therefore, the increased PME activity observed in *M. phaseolina*-inoculated Tx7000 and BTx3042 may contribute to enhanced charcoal rot disease susceptibility. However, it is important to note that, if PME-mediated demethylesterification occurs on contiguous sugar residues (i.e., blockwise demethylesterification), Ca^{2+} bonds can form between pectin molecules, which results in rigid cell walls (Micheli 2001; Pelloux et al. 2007). If this is the case with Tx7000 and BTx3042, their enhanced PME activity after *M. phaseolina* inoculation should be viewed as a mechanism of cell wall reinforcement which, in turn, impedes the ability of the pathogen to penetrate and should contribute to reduced disease susceptibility. Therefore, more specific experiments are needed to determine the role of PME in the sorghum-*Macrophomina* interaction. However, the net upregulation of PME in Tx7000 after pathogen inoculation, particularly at 7 DPI, indirectly suggests that host-derived PME

results in cell wall disintegration rather than reinforcement. Therefore, PME may contribute to enhanced charcoal rot disease susceptibility whereas PMEI contribute to reduced disease susceptibility. PMEI play a significant role in pathogenesis by influencing the susceptibility of the wall to CWDE (Cole et al. 1998; De Lorenzo et al. 2001; D'Ovidio et al. 2004).

Some CWDE inhibitors may play other roles in defense responses. For example, pepper PMEI protein (CaPMEI1) shows antifungal properties against necrotrophic fungi such as *Fusarium oxysporum* f. sp. *matthiole*, *A. brassicicola*, and *B. cinerea* by delaying their spore germination and hyphal development (An et al. 2008). If this occurs within the sorghum-*Macrophomina* pathosystem, it may be possible that Tx7000 attempts to reduce disease susceptibility by overexpressing PMEI and restricting *M. phaseolina* spread.

The oligosaccharides generated by CWDE can act as elicitors that trigger plant defenses (Aziz et al. 2007). Treatment of plants with these oligosaccharides can induce the production of reactive oxygen species (ROS) (Aziz et al. 2004; Moscatiello et al. 2006). Moreover, the degradation of homogalacturonans (the main component of pectin) by PG results in oligogalacturonide release in *Arabidopsis thaliana* after *B. cinerea* infection and induces a robust NADPH oxidase (AtrbohD)-dependent oxidative burst (Galletti et al. 2008). In the current study, we observed significantly higher CWDE activity in *M. phaseolina*-inoculated charcoal-rot-susceptible sorghum genotypes. It is possible that these genotypes accumulate more oligosaccharides and oligogalacturonides, which results in enhanced ROS production. ROS can, in turn, trigger cell death and contribute to increased susceptibility to a necrotrophic pathogen such as *M. phaseolina* (Häffner et al. 2015).

In summary, our findings showed *M. phaseolina*'s ability to manipulate host CWDE, which may result in induced stalk cell wall degradation in charcoal rot-susceptible sorghum genotypes. Although *M. phaseolina* can produce its own CWDE, its ability to induce host-derived CWDE could be an evolutionarily stable and

Fig. 4. Comparison of the mean pectin methylesterase activity (in relative units, 1 RU = 10-mm length of the diameter of the dark red zone on a gel diffusion assay) among two treatments (mock inoculated with phosphate-buffered saline [CON] and *Macrophomina phaseolina* [MP]) in charcoal rot-susceptible (S) (BTx3042 and Tx7000) and resistant (R) (SC599 and SC35) genotypes at three postinoculation stages (4, 7, and 10 days postinoculation [DPI]). Treatment means bracketed by different letters within each genotype at a given DPI are significantly different. Treatment means with “ns” designations within each genotype at a given DPI are not significantly different at $\alpha = 0.05$. Error bars represent standard errors.

efficient virulence strategy. Because sorghum cannot avoid producing CWDE due to an associated fitness cost, *M. phaseolina* appears to take advantage of deploying the plant's CWDE to promote charcoal rot disease susceptibility in grain sorghum.

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