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Responses of Johnsongrass Against Sorghum Anthracnose Isolates

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Abstract

Johnsongrass is a creeping perennial weed that interferes with crop productivity. Due to genetic similarity to sorghum, Johnsongrass is considered to have potential as an alternate source of pathogen resistance genes for sorghum. In order to test this hypothesis, sorghum isolates of anthracnose (*Colletotrichum sublineolum*) were inoculated onto twenty-six Johnsongrass cultivars collected from across the southern US by using an excised leaf method. Upon inoculation with a *C. sublineolum* sorghum isolate, different Johnsongrass cultivars showed different degrees of infection. Moreover, three different *C. sublineolum* isolates caused different responses on the same Johnsongrass cultivar. Expression of early defense response related genes, including β-1,3-glucanase, chalcone synthase 8 (CHS8), pathogen induced chitinase, flavonoid-3'-hydroxylase, pathogenesis related protein-10 (PR-10), and thaumatin-like protein, were measured 24 hrs and 48 hrs post inoculation in selected Johnsongrass cultivars by Real-Time qRT-PCR. The results revealed that levels of defense responses varied among cultivars but were not sufficient to establish a basis for resistance. When the same Johnsongrass cultivars were inoculated in a greenhouse study with conidia of *Colletotrichum sublineolum* isolate FSP53 from sorghum, some showed evidence of a hypersensitive response. However, successful reproduction of the pathogen as detected by formation of acervuli and setae was seen only on SH1116 and on only one leaf of this cultivar.

Keywords: *Sorghum halepense*; *Sorghum bicolor*; pathogens

Introduction

Sorghum (*Sorghum bicolor,* L. Moench) is the fifth most important cereal grown worldwide [1], and the yield of sorghum production is a matter for sustainability of human-beings due to explosively increasing population in the world. Sorghum is considered to be relatively hardier under extreme heat and drought conditions compared with other major crops and thus has received much attention as a potential adaptation strategy for farmers [2]. Unlike sorghum grown for grain, Johnsongrass (*Sorghum halepense* L. Pers), which is highly related to sorghum, is considered one of the most noxious weeds in the U.S. and world agriculture [3,4]. In North America, it invades agricultural fields and natural grasslands, including those dominated by functionally similar warm season perennial C4 grasses [5]. It was introduced into the US for use as a forage grass sometime in the 1800s [6] and despite its current weed status, seed can still be purchased. *S. halepense* has been shown to have originated from a cross between *S. bicolor* (2N=20) and *S. propinquum* (2N=20) followed by chromosome doubling so Johnsongrass can be considered a tetraploid or amphidiploid species. Due to genetic similarity to sorghum, it is possible that Johnsongrass is an alternate rich source of genes for sorghum that can be effective against sorghum diseases. As a first step in testing this hypothesis, the response of Johnsongrass to sorghum isolates of anthracnose were tested with three hypotheses:

I. Johnsongrass is an alternate host of sorghum anthracnose.

II. Different Johnsongrass cultivars will show phenotypically different degree of infection upon sorghum anthracnose inoculation.

III. Different Johnsongrass cultivars will show different level of expression of defense-related genes upon sorghum anthracnose infection.

To better evaluate these hypotheses, we surveyed the host defense responses of twenty-six newly obtained Johnsongrass cultivars were tested for response to sorghum anthracnose isolates by using an excised leaf method. Expression of defense-related genes, such as β-1,3-glucanase, chalcone synthase 8 (CHS8), pathogen induced chitinase, flavonoid-3'-hydroxylase, pathogenesis related protein-10 (PR-10), and thaumatin-like protein, in selected Johnsongrass cultivars upon *C. sublineolum* inoculation was measured with Real-Time qRT-PCR.

Materials and Methods

Plant material preparation

Twenty-six cultivars (Supplementary Table 1) were provided by Jacob Barney (Virginia Tech University) in the form of rhizomes that were transplanted into plastic round pots filled with Sungro® professional growing mix soil materials for growth in a greenhouse. Water and additional nutritional supplements were provided regularly.

Screening varieties of Johnsongrass against sorghum anthracnose using an excised leaf assay

For the 26-cultivar set grown from rhizomes, a detached leaf assay was first used. Virulent strains of *C. sublineolum* (FSP 2, FSP 35, and FSP53) isolated from sorghum were inoculated to each Johnsongrass cultivar by using Prom's excised leaf assay with slight modifications [7]. In brief, *C. sublineolum* was grown on half strength PDA plate and stored in an incubator for 10-14 days. A small amount of sterile water was added on the plate, and *C. sublineolum* on the plate was scraped with a spatula. The suspension was filtered through four layers of cheesecloth to remove mycelium, followed by dilution to the final conidia concentration of ~10^6 conidia/mL For the excised leaf assay, leaf pieces of each cultivar that had been grown in pots in a greenhouse

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Cultivar		FSP2	FSP35	FSP53
Sorghum bicolor (+)BTX 623	Average score	4.67	3.67	5
	Range	2-5	3-4	5
	Score >3	8-9	6-6	5/5
sorghum bicolor (-)SC 748-5	Average score	1	4.14	1.44
	Range	1	3-5	1-5
	Score >3	0/9	7-7	1/9
SH 1002	Average score	2.2	4.5	1.71
	Range	1-4	4-5	1-4
	Score >3	5-9	6/6	1/7
SH 1030	Average score	1.7	3.63	1
	Range	1-4	1-4	1
	Score >3	2-10	7/8	0/6
SH 1048	Average score	1.83	4	1.17
	Range	1-3	3-5	1-2
	Score >3	2/6	5/5	0/6
SH 1094	Average score	1.73	3.88	1.67
	Range	1-3	3-4	1-2
	Score >3	2/12	8/8	0/8
SH 1104	Average score	1.3	4	1.43
	Range	1-3	4	1-3
	Score >3	1/10	6/6	1/7
SH 1116	Average score	1.73	4	1.63
	Range	1-4	4	1-5
	Score >3	3/11	6/6	1/8
SH 1126	Average score	2.5	4	2.7
	Range	1-4	4	1-5
	Score >3	5/10	5/5	7/10
SH 1136	Average score	2.45	4	2.33
	Range	1-5	4	1-4
	Score >3	5/11	6/6	4/6
SH 1152	Average score	2.1	4	1.75
	Range	1-5	4	1-4
	Score >3	3/10	6/6	2/8
SH 1154	Average score	1.64	4	1.75
	Range	1-3	4	1-3
	Score >3	3/11	6/6	3/8
SH 1165	Average score	1.29	3.86	1.75
	Range	1-3	3-5	1-5
	Score >3	1/7	7/7	2/8
SH 1201	Average score	1.91	4	1
	Range	1-4	4	1
	Score >3	4/11	5/5	0/6
SH 1229	Average score	2.45	4	1.88
	Range	1-4	4	1-3
	Score >3	6/11	6/6	2/8
SH 1233	Average score	1.13	3.63	1.4
	Range	1-2	3-4	1-3
	Score >3	0/8	8/8	2/10
SH 1247	Average score	1.57	3.8	1
	Range	1-3	3-4	1
	Score >3	1/7	5/5	0/6
SH 1281	Average score	1.86	3.4	1.75
	Range	1-4	3-4	1-3
	Score >3	2/7	5/5	2/8
SH 1325	Average score	2.18	3.67	1.88
	Range	1-5	3-4	1-5
	Score >3	4/11	6/6	2/8
SH 1337	Average score	2	3.25	1.88
	Range	1-4	1-4	1-3
	Score >3	3/9	6/8	3/8

SH 1350	Average score	1.75	3.17	2.45
	Range	1-4	3-4	1-5
	Score >3	3/12	6/6	6/11
SH 1409	Average score	2.5	4	3.33
	Range	1-4	4	1-5
	Score >3	4/8	6/6	4/6
SH 1426	Average score	1.75	1.75	1.75
	Range	1-4	1-4	1-4
	Score >3	3/12	3/12	3/12
SH 1450	Average score	2.75	4.17	3.18
	Range	1-4	4-5	1-5
	Score >3	8/12	6/6	8/11
SH 1457	Average score	1.55	4	1.57
	Range	1-3	4	1-4
	Score >3	2/11	6/6	1/7
SH 1484	Average score	1.89	4.17	1.57
	Range	1-4	4-5	1-3
	Score >3	3/9	6/6	2/7
SH 1490	Average score	1	4	1.29
	Range	1	4	1-2
	Score >3	0/11	6/6	0/7
SH 1493	Average score	2.36	3.8	1.29
	Range	1-4	3-4	1-3
	Score >3	6/11	5/5	1/7
Overall ave score JG CVs	--	1.89	3.8	1.77

SCORE:
1- No spore germination.
2- Some spore germination started.
3- Some acervuli imperfectly formed (fungal bed formed).
4- Some acervuli perfectly formed.
5- Many acervuli perfectly formed.
(0/0)= (# of leaves formed acervuli/ # of total leaves).

Table 1: Disease ratings for 3 Sorghum Colletotrichum isolates.

for approximately one month were placed on a half strength PDA plate, adaxial side up, and 5 µl of the spore suspension was inoculated onto each side of a leaf piece. Detached leaves were observed under an Olympus BX60 microscope at 24 hrs, 48 hrs, 72 hrs, and 96 hrs post-inoculation. After 96 hrs post inoculation, susceptibility was scored in 1-5 scale and percentage of infected leaves. Figure 1 provides visual documentation of the scoring system used.

Susceptibility check of Johnsongrass cultivars against sorghum anthracnose by greenhouse spray inoculation

All Johnsongrass cultivars were grown from rhizomes in a greenhouse for four weeks before inoculation. FSP 53 isolate was grown on ½-strength PDA plates (1/2 PDA) for ten to fourteen days. Each plate was flooded with water and the conidia loosened with a spatula were collected in sterile water. The spores were diluted to a concentration of ~10^6 conidia/mL with distilled water and a few drops of TWEEN 20®. The Johnsongrass cultivars were inoculated by spraying in a greenhouse. The inoculated plants were immediately covered with plastic bags for one week in order to prevent desiccation of inoculum. Inoculated leaves showing any potential signs of lesions were collected and brought to the laboratory every week from week 3 to week 5 post inoculation. Leaves were observed under an Olympus BX60 microscope to confirm any acervulus formation.

RNA extraction and real-time quantitative reverse transcription PCR analysis

SH 1136 (M-S), SH 1152 (R-M), SH 1247 (R), and SH 1450 (S) were selected for evaluating defense gene expression following inoculation with FSP 53. Plants were transferred from the greenhouse to a Conviron® CMP3244 growth chamber to minimize environmental interference. Four to eight individual plants from each cultivar were used. *C. sublineolum* (FSP 53) spores were diluted into distilled water to a concentration of 1000,000 conidia/ml, with a few drops of Tween 20. The conidia dilution was pipetted onto a pre-marked leaf surface and spread using a brush or cotton swabs. The labeled inoculated leaves were detached at '0 time' for controls and 1 dpi and 2 dpi. Immediately after detachment, the protocol from QIAGEN® RNeasy mini handbook (2001) was used in RNA extraction from collected leaf samples. After RNA isolation, a NanoDrop ND-1000 instrument was used to measure RNA concentrations, and RNA isolates were diluted to 10 ng/µl with sterile RNAase free dH2O. For Real-Time qRT-PCR analysis, a one-step SYBR® PrimeScript ™ RT-PCR kit II from TaKaRa Clontech was used as the manual suggests. Each reaction (10 µl of TaKaRa 2x One Step SYBR RT-PCR Buffer, 0.8 µl of PrimeScript 1 step Enzyme Mix2, 5.6 µl of sterile dH_2O, 0.8 µl of forward primer, 0.8 µl of reverse primer, and 2 µl of diluted RNA template) was added into a sterile Cepheid SmartCycler® 25 µl tube. The tube was placed into Cepheid SmartCycler®, and exposed to 42°C for 5 min and 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 50-55°C (dependent on primer pairs) for 20 sec, followed by melt curve starting at 65°C and ending at 95°C. Expression levels of previously determined defense response genes, including flavonoid-3'-hydroxylase, β-1,3-glucanase, chitinase, chalcone synthase (CHS), thaumatin-like protein, and PR-10, were measured. The primers used are shown in Appendix Table 2.

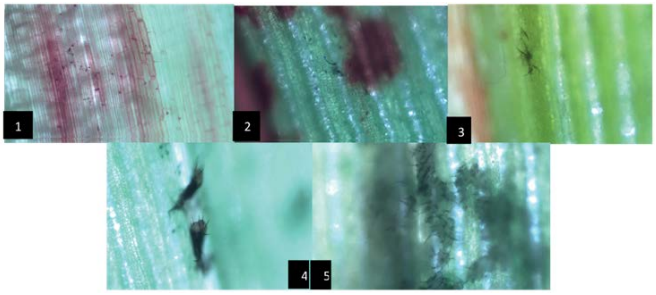

Figure 1: Rating scales for Table 2: **1.** No germination, **2.** Germination started, **3.** Some acervuli imperfectly formed (fungal bed formed) **4.** Some acervuli perfectly formed **5.** Many acervuli perfectly formed.

Expression of actin mRNA amplified using intron spanning primers was measured as a background check, and the ΔΔCt method was used to compare levels of each mRNA. Finally, 2^ΔΔCts were computed to Log_2(Expression Fold Change) transformation and statistically analyzed by using each paired t-test with JMP version 14. In all cases, fold values are expressed relative to zero time control samples.

Results and Discussion

Susceptibility check of Johnsongrass cultivars against sorghum anthracnose by detached-leaf spot inoculation assay

The detached-leaf spot inoculation method was conducted using twenty-six cultivars with three isolates of *C. sublineolum*: FSP 2, FSP 35, and FSP 53. Fungal infections were observed under a microscope at 96 hrs post inoculation, and the degrees of infection were graded between 1 and 5. Disease evaluation data are summarized in Table 1. The spot inoculation method was repeated three times, and several leaves from each plant were inoculated in each trial. Upon inoculations of three different *C. sublineolum* isolates, Johnsongrass cultivars showed different responses. Interestingly, FSP 35 isolate was successfully able to cause infection in all Johnsongrass cultivars tested. Moreover, the presumed resistant sorghum check (SC748-5) was also infected, and acervuli formation was observed. In previous work FSP 35 did not lead to formation of acervuli on SC 748-5, which was also identified as resistant to all races tested in whole plant greenhouse inoculations. Potential explanations for the difference seen here could involve the high density inoculation in the spot test or perhaps age of the plants from which the leaf samples were excised. The sorghum plants were inoculated during growth stages 3-4, *versus* the 8-leaf stage in the earlier experiments and sorghum age has been identified as a factor for successful resistance against sorghum anthracnose [8]. The responses of Johnsongrass upon inoculation of FSP 2 and FSP 53 were similar to each other. The average score on the majority of cultivars was below 3, meaning failure of the *C. sublineola* to propagate, a common definition of resistance. However, there was often a range of responses when comparing observations on different leaves from the same cultivar, leading for example to cultivars SH 1450 and SH 1409 being considered moderately susceptible, especially to isolate FSP 53. Isolate FSP35 was clearly more virulent on Johnsongrass than the other two with an overall average rating of 3.8 versus 1.89 and 1.77. However, even for this isolate, only 5 cultivars included ratings that ranged up to 5, indicative that many perfectly formed acervuli were observed. While only Johnsongrass cultivar 1426 had an average score below 3 (1.75) implying it is better able to defend against this isolate, most other cultivars also limited development of FSP35 to some degree. Upon FSP 53 inoculation, Student's t-tests pairing each cultivar show that SH 1030, SH 1201, and SH 1247 are the most resistant cultivars (Mean score=1), while SH 1350, SH 1450, and SH 1467 are the opposites (Mean score=2.91, 3.18, and 3.33 respectively with p-value=<0.001 for the pairs). Based on home habitat where Johnsongrasses were collected, we conducted t-test in pairing each group. Johnsongrasses collected from roadside had mean susceptibility score 2 which was significantly different from Johnsongrasses collected from disturbed habitat with mean susceptibility score 1.21 (p-value=0.0068) and Johnsongrasses collected from agricultural habitat with mean susceptibility score 1.55 (p-value=0.0271). In addition, based on home state, t-test in each paired group revealed the fact that Johnsongrasses collected from CA (Mean susceptibility score=2.30) is grouped differently from Johnsongrasses collected from TX, KS, and VA (Mean susceptibility score=1.62, 1.43, and 1.38). Unlike FSP 53, upon FSP2 inoculation SH 1490, SH 1233, and SH 1165 are the most resistant cultivars (Mean score=1, 1.125, and 1.286 respectively), while SH 1450 and SH 1126 are the most susceptible cultivars (Mean score=2.75 and 2.50 respectively with p-value=<0.001). There was no statistical difference detected based on habitat and home state with FSP 2 inoculation. Upon FSP 35 inoculation, SH 1426 was the only one with exceptionally resistant phenotypic responses (Mean score=1.75), while SH 1450, SH 1002, and SH 1484 were the most vulnerable cultivars (Mean score=4.20, 4.17, and 4.17 respectively with p-value=<0.001). As oppose to FSP 53, Johnsongrasses collected from roadside had mean susceptibility score 3.20 which was significantly resistant compared to Johnsongrasses collected from other three habitat types (Roadside vs undisturbed p-values=0.0011, roadside vs disturbed p-value=0.0207, and roadside vs agricultural p-value=0.0025). In sum, it indicates that environmental, spatial, and biological factors sculpt host defense system in Johnsongrass.

Susceptibility check of Johnsongrass cultivars against sorghum anthracnose by greenhouse spray inoculation

The leaves of twenty-six Johnsongrass cultivars sprayed with sorghum isolates FSP35 and FSP53 which was highly virulent on anthracnose susceptible sorghum cultivar BTX623 showed mild to moderate wilt and discoloration into brown starting soon after inoculation. These symptoms are assumed to initiate from host recognition of the potential pathogen and induction of hypersensitive type (HR) defense responses. Characteristic anthracnose lesions were found on some leaves including SH 1116. Leaves from the twenty-six cultivars tested were collected and brought to the laboratory. Microscopic observation confirmed no acervuli were formed except on SH 1116 upon FSP53 inoculation. *C. sublineolum* was successfully subcultured from a leaf of SH 1116. Ungerminated conidia were easily found under a microscope on leaves of most cultivars. Among all cultivars, three cultivars, including SH 1094, SH 1337, and 1350, had higher levels of pigmentation changes typically associated with active defense responses [9] than others at 3 WPI. Even though acervuli were formed in SH 1116, as a whole plant, it appeared reasonably healthy since lesions were found on only one leaf. This observation is in accord with the detached leaf assay in that the same isolate gave disease ratings ranging from 1-5 when used to inoculate this cultivar. Still, SH 1116 shows that cross infection of *C. sublineolum* can occur between Johnsongrass and sorghum even though the overall response to the 3 races as defined on sorghum host differentials were not highly virulent to these Johnsongrass cultivars.

Real-time quantitative reverse transcription PCR analysis

Johnsongrass cultivars SH 1136, SH 1152, SH 1247, and SH 1450 were selected for evaluating gene expressions based on different responses to FSP 53 inoculation in the detached leaf assay. SH 1136

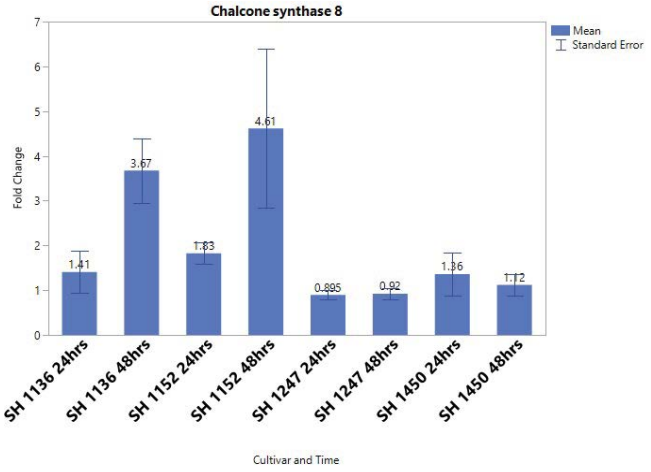

Figure 2a: Expression of CHS8 at 24 and 48 hours post inoculation.

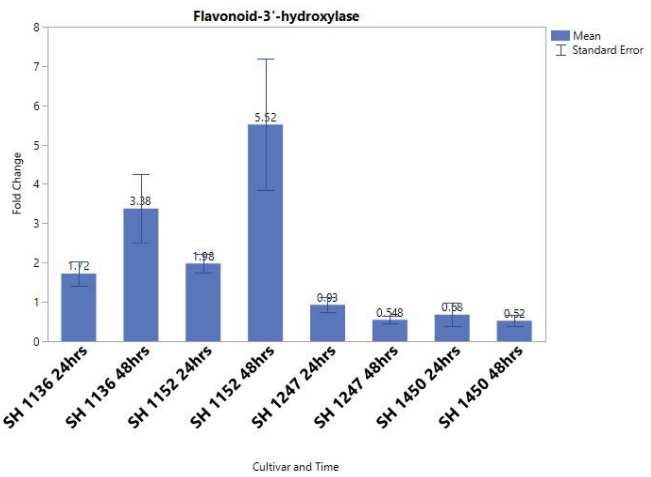

Figure 2b: Expression of Flavonoid-3'-hydroxylase 24 and 48 hours post inoculation.

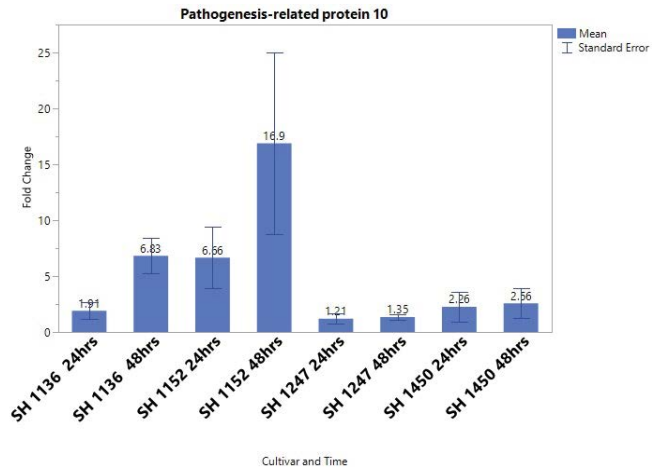

Figure 2c: Expression of PR10 at 24 and 48 hours post inoculation.

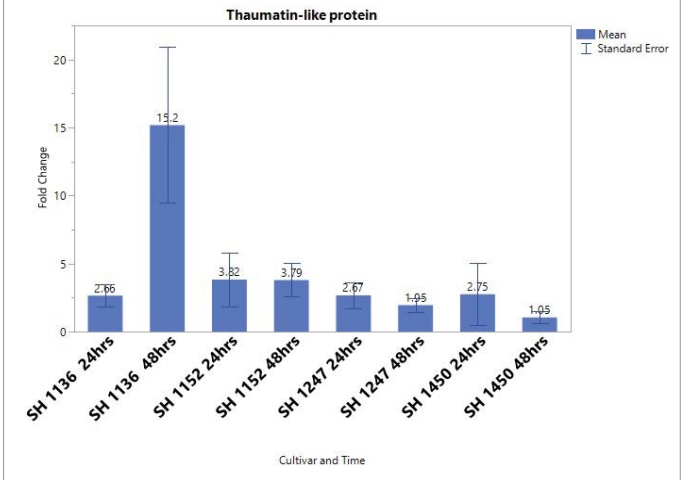

Figure 2d: Expression of thaumatin-like protein 24 and 48 hours post inoculation.

(M-S), which is considered a moderately susceptible cultivar, had 67% chance of acervuli formation. SH 1152 (M-R), which we considered a moderately resistant cultivar, had 25% chance of acervuli formation. SH 1247 (R) was resistant with 0%, while SH 1450 (S) had 72.7% chance of acervuli formation. Since SH 1152 and SH 1247 showed a higher level of resistance against FSP 53, we expected to see earlier or higher upregulation of host defense related genes compared to the other two cultivars. Each of the genes evaluated has previously been demonstrated to be activated as part of a variety of defense responses in sorghum. Examples include the enzymes chalcone synthase and flavonoid hydroxylase that are involved in phytoalexin and pigment production associated with hypersensitive responses [10]. Here, expression of CHS 8 was around 4.6-fold upregulated in SH 1152 48 hpi which was statistically different from SH 1247 and SH 1450. SH 1136 followed the same pattern of SH 1152 with slightly lower upregulation (Figure 2a). Flavonoid 3' hydroxylase expression had exactly the same pattern of CHS 8 with 5.52-fold upregulation in SH 1152 48 hpi which is statistically different from others except SH 1136 48 hpi (Figure 2b). Thus, both genes coding enzymes in the flavonoid phytoalexin pathway were significantly induced, but in only two cultivars and not in SH 1247 as had been predicted. Levels of mRNA for PR10, a small acidic protein with potential nuclease activity that is activated in host defense of many species [11] followed the same pattern as the aforementioned genes. The amount of mRNA present in SH 1136 and SH 1152 both increased significantly between 24 and 48 hpi. As with the flavonoid pathway genes levels for SH 1247 and SH 1450 were slightly higher that the control zero time values, but did not show significant changes between 24 and 48 hours (Figures 2c and 2d). Only the SH 1136 samples extracted 48 hpi measured a dramatic increase in mRNA levels of thaumatin-like protein. Thaumatin is a protein with antifungal properties [12] whose mRNA appears many times in cDNA libraries made from a sorghum resistant to anthracnose [13]. No significant differential induction was found for the β-1,3-glucanase or chitinase genes tested (data not included). These genes that encode enzymes capable of degrading fungal cell walls are typically expressed at high levels in sorghum following inoculation with fungal pathogens [14,15]. A possible explanation for the failure to detect altered expression in some genes lies with the primers used. All were designed for, and have worked well with targeted members of their respective gene families with *S. bicolor*. Although *S. bicolor* is one of the species that is a part of the *S. halapense* tetraploid genome, there is good evidence that

polyploid formation can alter expression of equivalent genes from the donor parents [16].

Conclusion

Thus, it is possible that other chitinase or glucanase family members are induced but not detected in *S. halepense* with the primers used in this study. (Sequence data for the orthologous genes in *S. propinquum* are not available at this time). As a conclusion, it is clear that SH 1152, a moderately resistant cultivar, greatly upregulates chalcone synthase 8 (CHS8), flavonoid-3'-hydroxylase, pathogenesis related protein-10 (PR-10), and thaumatin-like protein 48 hpi. Pathogen induced chitinase was highly expressed in SH 1152 along with SH 1136, which is a moderately susceptible cultivar. Interestingly, SH 1247, a highly defensive cultivar, and SH 1450, one of the most vulnerable cultivars, were always grouped together in statistical analysis with nearly no upregulation. This could mean that SH 1247 did not have to upregulate the specific host defense related genes tested in order to protect itself against sorghum anthracnose and could be a novel source of anthracnose resistance for sorghum. However, the average score of 1 in the detached leaf assay suggests at least some degree of hypersensitive response was initiated. Alternatively, SH 1450 could be prone to infection by at least this sorghum anthracnose isolate because of low or delayed host defense related gene expression.

Acknowledgements

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