HOST DEFENSE RESPONSES OF SORGHUM AND JOHNSONGRASS AGAINST *Colletotrichum sublineola* AND IDENTIFICATION OF CANDIDATE DEFENSE RELATED GENES IN SORGHUM

A Dissertation

by

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ABSTRACT

While sorghum (*Sorghum bicolor* L. Moench.) is the fifth most important cereal grown worldwide, johnsongrass (*Sorghum halepense* L. Pers.) 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, three isolates of *Colletotrichum sublineola* P. Henn., which causes sorghum anthracnose, were inoculated onto twenty-six johnsongrass cultivars collected from across the southern U.S. by using an excised leaf method. Each *C. sublineola* isolate caused various degrees of infection when inoculated onto different johnsongrass cultivars. Moreover, three different *C. sublineola* 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 *C. sublineola* 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. In addition, *C. sublineola* isolates were

inoculated on leaf blades and midribs of the johnsongrass cultivars along with two sorghum cultivars, BTx623 and SC748-5 by using an excised leaf method. Some johnsongrass cultivars and BTx623 showed different responses between the two tissues. Based on the results from Real-time qRT-PCR, host resistance gene expression in leaf blades and midribs showed three different patterns in BTx623 inoculated with FSP53 isolate of *C. sublineola*.

Finally, a sorghum mini core collection was scored over several years for response to *Colletotrichum sublineola*, *Peronosclerospora sorghi*, and *Sporisorium reilianum*, the causal agents of anthracnose, downy mildew, and head smut, respectively. The screening results were combined with over 290,000 Single nucleotides polymorphic (SNP) loci from an updated version of a publicly available genotype by sequencing (GBS) dataset available for the mini core collection. GAPIT (Genome Association and Prediction Integrated Tool) R package was used to identify chromosomal locations that differ in disease response. When the top scoring SNPs were mapped to the most recent version of the published sorghum genome, in each case, a nearby and most often the closest annotated gene has precedence for a role in host defense.

CONTRIBUTORS AND FUNDING SOURCES

Dr. Clint Magill orchestrated entire project and guided me in both academic and philosophical ways. Drs. Muthukumar Bagavathian, Louis K. Prom and Gary Odvody have provided academic and technical support which I greatly appreciate. Founding support was provided from AFRI, NIFA, USDA award # 20156800423492.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Sorghum (*Sorghum bicolor* (L.) Moench.) is known as the fifth most important cereal grown in the world (Doggett 1988). It has been hypothesized that the first truly domestic sorghum came from the durras of India (Frederiksen and Odvody 2000). Sorghum is used for various purposes such as food, feed, building material, biofuel, and fiber. Sorghum grows relatively well under extreme heat and drought conditions (Tack et al. 2017); under a changing climate regime sorghum would assume renewed importance as a food and industrial crop, and therefore concerted focus is necessary on such currently marginalized crops to ensure food and nutritional security in a sustainable manner in the years to come.

Unlike sorghum grown for grain, johnsongrass (*Sorghum halepense* L. Pers.) is considered one of the most problematic weeds in the U.S. and world agriculture (Holm, Plucknett, Pancho, and Herberger 1977; Howard 2004). The genomic relationship between cultivated sorghum [*Sorghum bicolor* (L.) Moench, race bicolor, De Wet, 2n=20] and johnsongrass [*S. halepense* (L.) Pers., 2n=40] has been a subject of extensive studies (Hoang-Tang and Liang 1988). Recently, hybridization between cultivated sorghum with johnsongrass showed that several desirable traits from johnsongrass, including resistance to greenbug and chinch bug and adaptability to cold temperatures, were expressed in the resulting

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This dissertation follows the style of Theoretical and Applied Genetics.

progenies (Dweikat 2005).

Colletotrichum sublineola Henn. Ex Sacc. & Trotter is a pathogen causing anthracnose in sorghum. Since it was first reported in 1902 from Toga, West Africa, it has been observed to be widely prevalent under hot, humid conditions in most tropical and subtropical regions of the world (Frederiksen and Odvody 2000). Leaf anthracnose significantly reduced sorghum yields in the susceptible genotypes (Cota et al. 2017). The most economical and environmentally friendly control of anthracnose is the use of resistant cultivars (Frederiksen and Odvody 2000).

This dissertation interpolates material from Ahn et al. (2018) for chapter 2, Ahn et al. (2019b) for chapter 3, and Ahn et al. (2019a) for chapter 4.

Due to genetic similarity to sorghum, it is speculated that johnsongrass is an alternate way to acquire genes for sorghum that can be effective against sorghum diseases such as *C. sublineola* (Ahn et al. 2018). As the first step in testing this hypothesis, in chapter II, I will discuss the response of johnsongrass to *C. sublineola* with three hypotheses:

I. Johnsongrass is an alternate host of *C. sublineola*.

II. Different johnsongrass cultivars will show phenotypically different degree of infection upon *C. sublineola* inoculation.

III. Different johnsongrass cultivars will show different level of expression of defense-related genes upon *C. sublineola* inoculation.

To better evaluate these hypotheses, the host defense responses of twenty-six johnsongrass cultivars were tested for response to *C. sublineola* by using an

excised leaf method (Prom et al. 2016). Expression of commonly expressed host defense-related genes, 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 at 24- and 48-hours post *C. sublineola* inoculation were measured with Real-Time qRT-PCR.

β-(1,3)-glucanase belongs to the class of PR-2 proteins (Katile 2007), and plant β-(1,3)-glucanase hydrolyzes the structural (1,3)- β-glucanase present in the fungal cell wall (Selitrennik off 2001; Katile 2007). Likewise, chitinase, a member of PR-3 proteins, catalyze the hydrolysis of β-1, 4-N-acetylglucosamine linkages present in chitin and chitodextrins fungal cell walls (Krishnaveni et al. 1999; Little and Magill 2003; Katile 2007). Chalcone synthase and flavonoid hydroxylase are involved in phytoalexin and pigment production associated with hypersensitive responses (Lo et al.1999; Boddu et al. 2004). PR10 is a small acidic protein with potential nuclease activity that is activated in host defense of many species (Jain and Khurana 2018). Thaumatin-like proteins (TLPs), members of PR-5 proteins, are antifungal proteins responsive to biotic and abiotic stress in plants (Petre et al. 2011).

Leaf midrib has an important structural role in aerial parts of monocotyledonous plants. In maize, for example, it provides the primary support for the blade and is largely associated with leaf angle (Wang et al. 2015). Leaf architecture directly influences canopy structure, consequentially affecting yield (Strable et al. 2017). In many plants, genes are expressed differently between leaf blade and midrib. As an example, in *Brassica rapa*, the gene *BrLSH2*, which contains

auxin- and cytokinin-responsive elements as well as leaf development-related elements, is specifically expressed in the midrib of Chinese cabbage (Dong et al. 2014). They also found that transcript levels of most *LIGHT-SENSITIVEHYPOCOTYLS* (*LSH*) genes were very high in the midrib but low in the leaf blade in various *Brassica* species (Dong et al. 2014). In tobacco, *tpoxN1*, a peroxidase gene, is quickly induced by wounding in leaves, preferentially in stems and petioles in 1 hr. but was negligible in leaf blade even 8 hours after wounding (Sasaki et al. 2002). These differential gene expressions are expected. Genes involved in the differentiation and development of tissues and organs are temporally and spatially regulated in plant development: the drooping leaf (DL) genes in rice promote midrib formation in the leaf and carpel specification in the flower (Ohmori et al. 2011). DL is initially expressed in the central region of the leaf primordia, midrib, and in the carpel primordia in the meristem (Ohmori et al. 2011). Since leaf blade and midrib develop differently in many plants including both monocots and dicots, it would be reasonable to think that many genes are expressed in different ways between them. In addition to midrib influences on physical formation of crops, differential gene expression between leaf blade and midrib could be a key to understand possible differences in disease severity in response to pathogen inoculation and help improve crop yields. It is hypothesized that johnsongrass and sorghum cultivars inoculated with *C. sublineola* conidia would have different levels of infection between cultivars and also on the leaf blade compared to midrib. It is further hypothesized that there are different levels of host defense related gene

response activities between leaf blade and midrib tissues in some johnsongrass and sorghum cultivars. In chapter III, to test these hypotheses, host defense responses in both leaf blade and midrib was measured by using three isolates of *C. sublineola* in an excised-leaf spot inoculation method on twenty-six cultivars of johnsongrass and two of sorghum (Ahn et al 2019b). Finally, Real-Time qRT-PCR was used to measure expression products of six defense-related genes in both leaf blade and midrib, 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.

Among over 37,000 accessions of sorghum germplasm collection at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) gene bank, a maxi core collection of 2247 accessions was developed in 2001, but this core maxi collection was considered to be too large for many studies. Hence, a sorghum mini core (10% accessions of the core or 1% of the entire collection) was developed from the existing maxi core collection (Upadhyaya et al. 2009). In order to maximize genetic diversity, the mini core was developed by including accessions of all races of sorghum collected from around the globe. The mini core collection was included in a genotyping by sequencing (GPS) project that revealed locations of 265,487 Single Nucleotide Polymorphisms (SNPs) (Morris et al. 2013). The data revealed 14,739 SNPs markers scattered across the genome that could be used to genotype the complete sorghum mini core collection and for genome wide association studies (GWAS). SNPs markers were first used with the mini core

collection to identify prospective genes underlying the traits plant height and maturity (Upadhyaya et al. 2013). The same set of SNPs were used to identify 8 alleles in the mini core collection that are associated with resistance to natural isolates of *C. sublineola* collected from infected plants at ICRISAT in Pantancheru, India. A GWAS analysis of the mini core collection was also conducted for drought tolerance traits (Lasky et al. 2015).

Anthracnose caused by the fungal pathogen *Colletotrichum sublineola* is one of the most devastating diseases in sorghum (Li et al. 2013). Whether measured by DNA–based tests or ability to infect different host cultivars, Colletotrichum is highly variable. In testing virulence of isolates collected from 6 populations from India using 15 host differentials, Thakur et al. (Thakur et al. 1998) found differences in all six, as was also true for Random Amplified Polymorphic DNA (RAPD) Polymerase Chain Reaction (PCR) product electrophoretic patterns. Moore et al. (Moore et al. 2008) defined 13 pathotypes among 87 anthracnose isolates from Arkansas, including 11 when testing pathogenicity using 8 host differentials. Prom et al, found 17 different pathotypes on 10 host differentials from 235 U.S. isolates that also differed in Amplified Fragment Length Polymorphism (AFLP) patterns (Prom et al. 2012). The latter reference also pointed out that two lines resistant to all isolates had shown differential responses in an earlier study in Brazil.

Sorghum downy mildew, caused by *Peronosclerospora sorghi* [(Weston & Uppal) Shaw], can create severe epidemics, resulting in heavy yield loss (Perumal et al. 2008). Since *P. sorghi* causes downy mildew in maize as well, it has been subdivided

into 'sorghum/maize' and 'maize' infecting strains (Perumal et al. 2008). As for anthracnose, there are different pathotypes as defined by ability to infect host plants with different resistance genes, but in this case much less variation has been reported. Race 6 was detected in Texas in 2005 based on its occurrence on previously resistant commercial sorghum varieties (Isakiet and Jaster 2005) and was used exclusively in this study.

Head smut, caused by the soil-borne facultative biotrophic basidiomycete *Sporisorium reilianum* (Kühn) Langdon & Fullerton [syns. *Sphacelotheca reiliana* (Kühn) G.P. Clinton and *Sorosporium reilianum* (Kühn) McAlpine], is an important sorghum disease that has been reported from all parts of the world where sorghum is grown (Prom et al. 2011). It grows through the plant as a dikaryotic hypha formed between compatible mating types and is not generally detected until the time of heading when the fungal sorus replaces the sorghum inflorescence. At present 6 races have been defined in the US, but because of the use of different host differentials, it is not known if races defined elsewhere are unique.

In previous studies, the sorghum mini core collection was scored over several years for response to *C. sublineola*, *P. sorghi*, and *S. reilianum* (Radwan et al. 2011). The screening results of these three common sorghum diseases were combined with over 290,000 Single Nucleotide Polymorphic (SNP) loci from a recently updated version of a publicly available genotype by sequencing dataset available for the mini core collection (Morris et al. 2013) in order to search potential candidate defense related genes against each of the three pathogens. In chapter IV,

top candidate sorghum defense related genes against the three pathogens are discussed.

CHAPTER II

UNDERSTANDING HOST DEFENSE RESPONSES OF JOHNSONGRASS AGAINST *COLLETOTRICHUM SUBLINEOLAolletotrichum sublineola*

INTRODUCTION

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, three isolates of *Colletotrichum sublineola*, which cause anthracnose in sorghum, were inoculated onto twenty-six johnsongrass cultivars collected from across the southern U.S. by using an excised leaf method. Different johnsongrass cultivars showed different degrees of resistance against *C. sublineola*. Moreover, three *C. sublineola* 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

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greenhouse study with conidia of *C. sublineola* 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.

MATERIALS AND METHODS

Plant material preparation

Twenty-six cultivars (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, an excised leaf assay was first used. Virulent strains of *C. sublineola* [(FSP2, FSP35, and FSP53) obtained from Dr. L. K. Prom's collection – Plains Area, SPARC, CGRU, College Station, TX] isolated from sorghum were inoculated to each johnsongrass cultivar by using Prom's excised leaf assay with slight modifications (Prom et al. 2016). In brief, *C. sublineola* 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. sublineola* 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 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.

Table 1: Additional information on johnsongrass cultivars.

Accession	Population	State	Latitude	Longitude	Elevation	Home Habitat
SH1002	VA-1	VA				
SH1030	VA-3	VA	37.169875	-80.429565	608	disturbed
SH1048	TX-1	TX	31.06	-97.3422	200	agricultural
SH1094	TX-2	TX	30.0264	-94.3343	11	agricultural
SH1104	TX-3	TX	33.2797	-96.8927	171	agricultural
SH1126	TX-3	TX				
SH1116	TX-3	TX	33.2797	-96.8927	171	agricultural
SH1136	NM-4	NM	32.1852	-106.7097	1168	roadside
SH1152	GA-14	GA	33.8829	-83.1546	235	undisturbed
SH1154	GA-14	GA	33.8829	-83.1546	235	undisturbed
SH1165	GA-15	GA	33.96	-83.37	195	roadside
SH1201	TX-4	TX	34.330872	-102.97623	1240	undisturbed
SH1229	TX-5	TX	34.315554	-102.77363	1196	roadside
SH1233	TX-5	TX	34.315554	-102.77363	1196	roadside
SH1247	KS-1	KS	38.883333	-97.734444	408	disturbed
SH1281	KS-4	KS	38.706111	-97.427778	378	agricultural
SH1325	CA-1	CA	36.199022	-119.25191	92	agricultural
SH1337	CA-1	CA	36.199022	-119.25191	92	agricultural
SH1350	CA-2	CA	36.308806	-119.38064	92	roadside
SH1409	AZ-2	AZ				
SH1426	AZ-2	AZ	32.251061	-111.00645	703	roadside
SH1450	AZ-3	AZ				
SH1457	AZ-3	AZ	33.103267	-111.97425	353	disturbed
SH1484	AZ-4	AZ				
SH1490	AZ-4	AZ				
SH1493	AZ-4	AZ	32.99968	-112.08694	375	agricultural

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

All johnsongrass cultivars were grown from rhizomes in a greenhouse until they reached the 8-leaf-stage. FSP53 isolate was grown on half strength PDA plate for 10-14 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 located in College Station, TX. 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 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

SH1136 (M-S), SH1152 (R-M), SH1247 (R), and SH1450 (S) were selected for evaluating defense gene expression following inoculation with FSP53. 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. sublineola* (FSP53) spores were diluted into distilled water to

Fig.1 a-e. Rating scale for Table 1 corresponding to ratings 1 to 5. a. No germination, b. Fungal germ tube formed, c. Some acervuli starting to form (fungal bed formed) d. Rare acervuli formed with clear setae. Many acervuli formed. The same scale was used for leaf blade and midrib. Photographs were taken at 10x magnification and are cropped to provide clear images.

a concentration of 10^6 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 10ng/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 dH2O, 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 ºC for 5 min and 95ºC for 10 sec, followed by 40 cycles of 95ºC for 5 sec and 50-55ºC (dependent on primer pairs) for 20 sec, followed by melt curve starting at 65ºC and ending at 95ºC. 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 Table 2. 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.

Table 2: Primer information

Primer Name	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
Actin (Intron spanning)	GGT CGT ACC ACA GGT ATC G	ATG AGG TAG TCG GTG AGG TC
Flavonoid 3' Hydoxylase (Intron spanning)	AGC ACT CCT CCT CAA CCT C	CTT CAG CAC GTC CGG GTG
PR10 (Intron spanning)	CAC CTC ATT CAT GCC GTT C	CTC GAC GAG CGT GTT CTT G
Endo β-glucanase	ATC CCA ACA GCG GTG C	CGA TTC CCT TGG ACT GGT AG
Pathogen Induced Chitinase	CGA ACT ACC CCG GCG GCA AG	TGT TCT GCC GCT GCA GCT CC
Chalcone Synthase-8 Intron	AAG AGG ATA TGC CAC AAG TCG	TCT AGG GAT GGT GCC CAG TA
Thaumatin-like-protein	CCG CAG GAT TAC TAC GAC ATC TC	CGC ATC AGG GCA TTT GG

RESULTS AND DISCUSSION

Susceptibility check of johnsongrass cultivars against sorghum anthracnose by excised-leaf spot inoculation assay

The excised-leaf spot inoculation method was conducted using twenty-six cultivars with three isolates of *C. sublineola*: FSP2, FSP35, and FSP53. 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 3.

Table 3. Disease ratings for three *C. sublineola* Isolates.
(0/0) = (# of leaves formed acervuli/ # of total leaves).

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	5/10	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

Table 3 Continued

Cultivar		FSP2	FSP35	FSP53
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

Table 3 Continued

Cultivar		FSP2	FSP35	FSP53
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

Table 3 Continued

Cultivar		FSP2	FSP35	FSP53
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	3.86	3
	Range	1-4	1-5	1-5
	Score >3	4/9	6/6	6/9
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

Table 3 Continued

Cultivar		FSP2	FSP35	FSP53
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	3.80	1.77

The spot inoculation method was repeated three times, and several leaves from each plant were inoculated in each trial. Upon inoculation with spores from three different *C. sublineola* isolates, johnsongrass cultivars showed different responses. Interestingly, FSP35 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. It has been reported that FSP35 does not lead to formation of acervuli on SC748-5, 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 (Ferreira and Warren 1982). One other hypothesis is that excised-leaf-assay could accelerate progress of *C. sublineola* infection. Though not included in the dissertation, SC 748-5 was spray inoculated in a greenhouse with FSP35 with the result that only germ tube formation was seen. The responses of johnsongrass upon inoculation of FSP2 and FSP53 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 SH1409 and SH1450 being considered moderately susceptible, especially to isolate FSP53. 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. Student's t-test in pairing each cultivar shows that SH1030, SH1201, and SH1247 are the most resistant cultivars (Mean score=1), while SH1350, SH1450, and SH1467 are the opposites against FSP35 (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, I conducted t-tests 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-tests in each paired group revealed 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).

Against FSP2, SH1490, SH1233, and SH1165 are the most resistant cultivars (Mean score=1, 1.125, and 1.286 respectively), while SH1450 and SH1126 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. As opposed to FSP53, 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, results suggest 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 (highly virulent on anthracnose susceptible sorghum cultivar BTx623) showed mild to moderate wilt and discoloration into brown starting

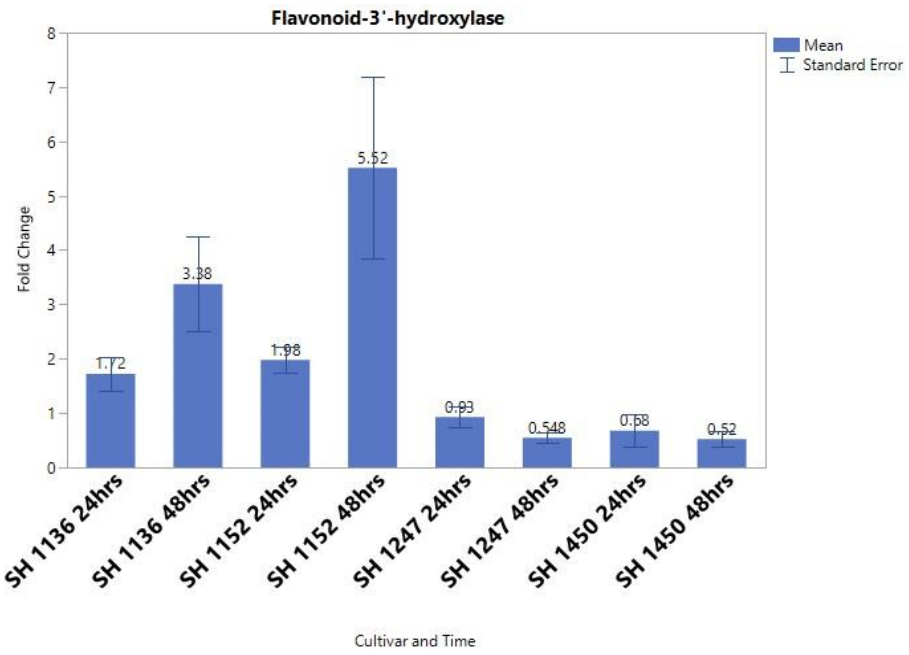
soon after inoculation. These symptoms are assumed to initiate from host recognition of the potential pathogen and induction of hypersensitive type (HR) defense responses. Characteristic anthracnose lesions were found on some leaves including SH1116. Leaves from the twenty-six cultivars tested were collected and brought to the laboratory. Microscopic observation confirmed no acervuli were formed except on SH1116 upon FSP53 inoculation. *C. sublineola* was successfully subcultured from a leaf of SH1116. Ungerminated conidia were easily found under a microscope on leaves of most cultivars. Among all cultivars, three cultivars, including SH1094, SH1337, and SH1350, had higher levels of pigmentation changes typically associated with active defense responses (Lo et al. 1999) than others at 3 wpi. Even though acervuli were formed in SH1116, 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, SH1116 shows that cross infection of *C. sublineola* 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

SH1136, SH1152, SH1247, and SH1450 were selected for evaluating gene expressions based on different responses to FSP53 inoculation in the detached leaf assay. SH1136 (M-S), which is considered a moderately susceptible cultivar, had 67% chance of acervuli formation. SH1152 (M-R), which is considered a moderately

resistant cultivar, had 25% chance of acervuli formation. SH1247 (R) was resistant with 0%, while SH1450 (S) had 72.7% chance of acervuli formation. Since SH1152 and SH1247 showed a higher level of resistance against FSP53, see earlier or higher upregulation of host defense related genes were expected 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 (Boddu et al. 2004; Lo, De Verdier, and Nicholson 1999). Here, expression of CHS 8 was around 4.6-fold upregulated in SH1152 48 hpi which was statistically different from SH1247 and SH1450. SH1136 followed the same pattern of SH1152 with slightly lower upregulation (Fig. 2a). Flavonoid 3' hydroxylase expression had exactly the same pattern of CHS 8 with 5.52-fold upregulation in SH 1152 48hpi which is statistically different from others except SH 1136 48hpi (Fig. 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 (Jain and Khurana 2018) 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 SH1247 and SH1450 were slightly higher that the control zero-time values, but did not show significant changes between 24 and 48 hours.

a



b

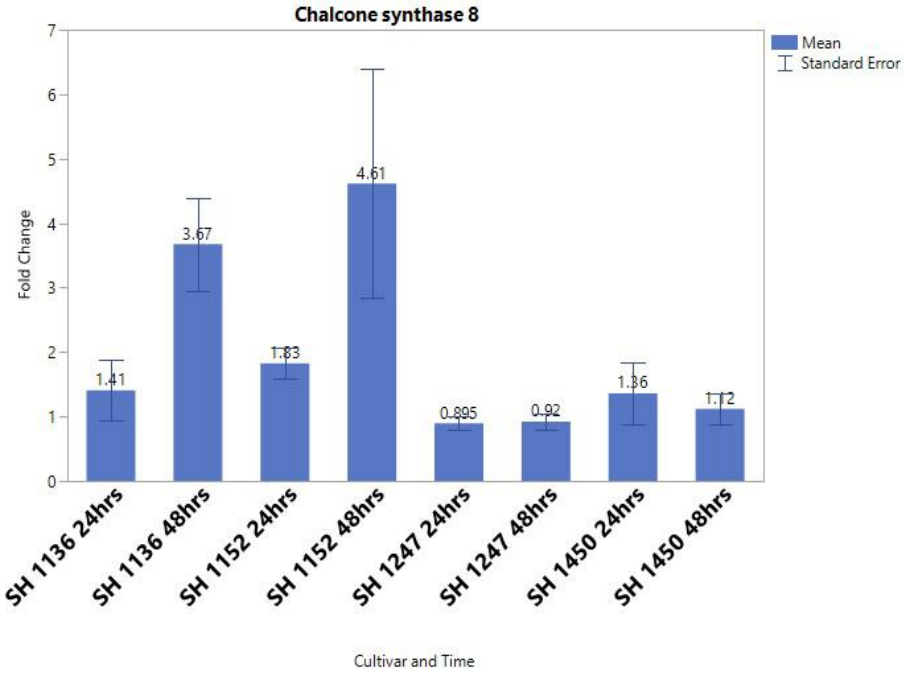
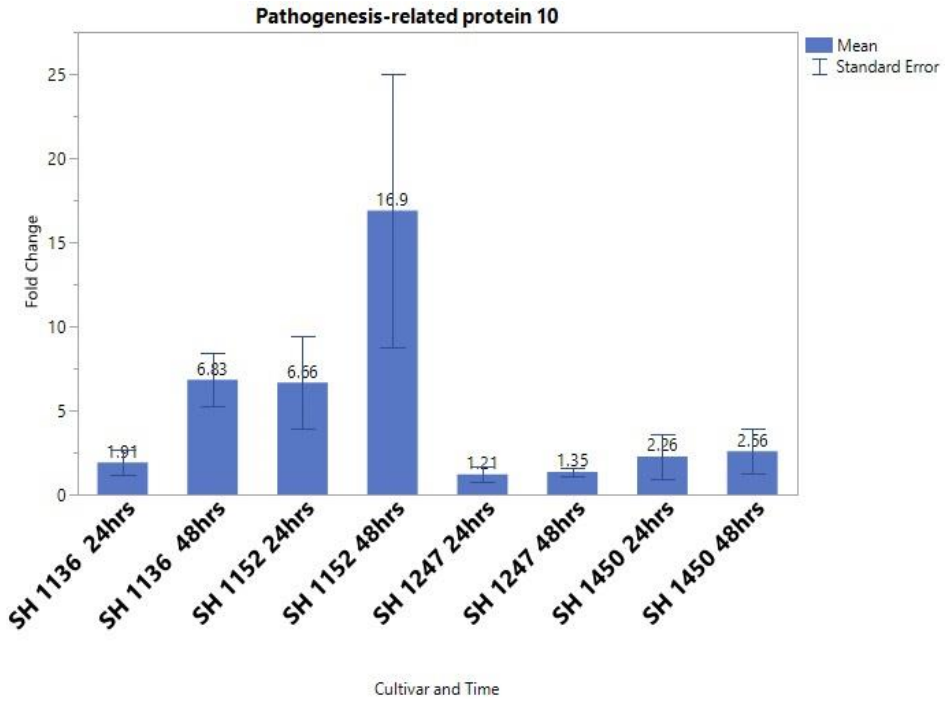


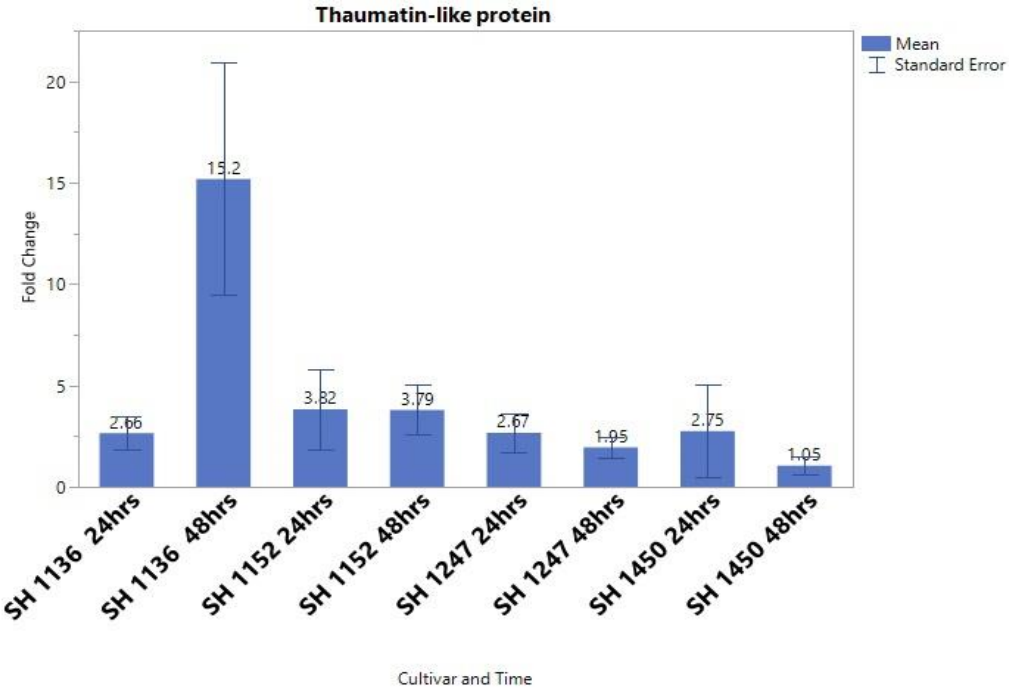
Figure 2 a-d. Expression of host defense related genes.a. CHS8 b. Flavonoid-3'-hydroxylase, c. PR10, and d. thaumatin-like protein at 24 and 48 hours post inoculation.

Figure 2 Continued

c



d



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 (Waniska et al. 2001) whose mRNA appears many times in cDNA libraries made from a sorghum resistant to anthracnose (Zamora et al. 2009).

No significant differential induction was found for the β-1,3-glucanase or chitinase genes tested. These genes that encode enzymes capable of degrading fungal cell walls are typically expressed at high levels in sorghum following inoculation with fungal pathogens (Krishnaveni et al. 1999; Little and Magill 2003). 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 (Chen 2007). 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.

CONCLUSION

As a conclusion, it is clear that SH1152, a moderately resistant cultivar, greatly upregulates chalcone synthase 8 (CHS8), flavonoid-3'-hydroxylase, pathogenesis related protein-10 (PR-10), and thaumatin-like protein 48hpi. Pathogen induced chitinase was highly expressed in SH1152 along with SH1136, which is a moderately susceptible cultivar. Interestingly, SH1247, a highly defensive cultivar, and SH1450, one of the most vulnerable cultivars, were always grouped together in statistical analysis with nearly no upregulation. This could mean that SH1247 did not have to upregulate the specific host defense related genes tested in order to protect itself against *C. sublineola* and could be a novel source of 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, SH1450 could be prone to infection by at least FSP53 isolate because of low or delayed host defense related gene expression.

CHAPTER III

DEFENSE RESPONSES AGAINST THE SORGHUM ANTHRACNOSE PATHOGEN IN LEAF BLADE AND MIDRIB TISSUE OF JOHNSONGRASS AND SORGHUM

INTRODUCTION

Three isolates of *Colletotrichum sublineola* P. Henn. ex Sacc. & Trotter were inoculated on leaf blades and midribs of twenty-six johnsongrass cultivars with two sorghum cultivars, BTx623 and SC748-5 by using an excised leaf method. Phenotypic host responses from no response to severe infection, were observed. Some johnsongrass cultivars and BTx623 showed different responses between the two tissues. Expression of six defense response related genes were measured using Real-time qRT-PCR. Leaf blade and midrib showed different patterns in BTx623 inoculated with *C. sublineola*; high expression of CHS8 and TLP were measured in midrib at 24 and 48 hpi respectively.

MATERIALS AND METHODS

Plant material preparation

Twenty-six cultivars of johnsongrass (Table 1) were provided by Jacob Barney (Virginia Tech University) in the form of rhizomes that were transplanted into plastic

*Reprinted with permission from “Defense responses against the sorghum anthracnose pathogen in leaf blade and midrib tissue of johnsongrass and sorghum” by Ahn E, Prom LK, Odvody G, Magill C, 2019. Physiological and molecular plant pathology, 106, 81-86, Copyright [2019] by Elsevier Ltd.

round pots filled with Sungro professional growing mix soil materials in a greenhouse in College Station, TX, USA. Water and additional nutritional supplements were provided regularly. Six to seven-week-old plants at the 8-leaf stage were used for further steps.

Screening cultivars of johnsongrass against *C. sublineola* by excised-leaf spot inoculation

For the 26-cultivar set grown from rhizomes, an excised leaf assay developed for screening disease response was used (Prom et al. 2016). In a previous study, virulent strains of *C. sublineola* (FSP2, FSP35, and FSP53) isolates elicited various host defense responses on leaf blade of johnsongrass and sorghum cultivars based on the same protocol (Ahn et al. 2018). Here the same cultivars from that study were tested using the same virulent strains of *C. sublineola.* Here also, rather than just scoring resistance versus susceptibility, a 1-5 scale as described below was used to evaluate disease progress.

C. sublineola was inoculated onto half strength PDA plates and grown in an incubator for 10-14 days. A small amount of sterile water was added to the plate, and a sterile spatula was used to scrape and remove colony growth, which includes both mycelia and conidia from the plate. This suspension mixture was filtered through four layers of cheesecloth to remove mycelium and concentrate conidia which were diluted to a final conidial concentration of ~10^6 conidia/mL.

For the excised leaf assay, leaf pieces (approximately 4 cm x 2.5 cm) of each

cultivar were placed, adaxial side up, on a half strength PDA plate, and 5 µl of the spore suspension was inoculated on each side of the leaf blade and one spot on the midrib.

Excised leaves were observed under an Olympus BX60 microscope with 10x magnification at 24, 48, 72, and 96 hrs post-inoculation. At 96 hrs post inoculation, susceptibility was scored using a 1-5 scale: 1 fully resistant without visible fungal infection, 2. fungal germ tube formed, 3. Some acervuli imperfectly formed (fungal bed formed) 4. Some acervuli perfectly formed, and 5. the most susceptible with visible countless acervuli present (Figure 1). The experiment was repeated three times with a different set of plants. In each repeat experiment, three to four leaf segments were evaluated for each cultivar. Scores of leaf blade and midrib were separately recorded and averaged.

Statistical analysis

A parametric two tailed pooled t-test was done with JMP Pro 14 for comparing leaf and midrib results. Although de Winter and Dodou (2010) showed through simulations that t-tests can be used in nearly every case for five-point Likert scale data, the acceptability for using t-tests was verified by showing that using the non-parametric Wilcoxon test resulted in the same results with respect to significance.

RNA extraction and Real-Time Quantitative Reverse Transcription PCR analysis

Of the original group of cultivars, two johnsongrass (SH1136, SH1350) and two sorghum (BTx623, SC748-5) cultivars were selected for evaluating potential differences in gene expression of leaf blade and midrib tissues in response to inoculation with isolate FSP53 inoculation which caused various responses on the cultivars as opposed to FSP35, a strongly infectious isolate on all cultivars. Eight-leaf stage plants were transferred from the greenhouse to a Conviron® CMP3244 growth chamber to minimize environmental interference and four to seven individual plants were used from each cultivar. An aqueous conidial suspension of *C. sublineola* (FSP53) was diluted to a concentration of ~10^6 conidia/ml with an addition of 2-3 drops of Tween 20. The conidial inoculum was pipetted onto a pre-marked leaf surface area and spread using a brush or cotton swabs. The labeled inoculated leaves were detached from the whole plants 1 dpi(=24hpi) and 2 dpi(=48hpi). 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 spectrometer was used to measure RNA concentrations, and RNA isolates were diluted to 10ng/µl with sterile RNAase free dH2O. For Real-Time qRT-PCR analysis, one step SYBR® PrimeScript ™ RT-PCR kit II from TaKaRa Clontech was used, and as the manual suggests, 10 µl of TaKaRa 2x One Step

SYBR RT-PCR Buffer, 0.8 µl of PrimeScript 1 step Enzyme Mix 2, 5.6 µl of sterile dH2O, 0.8 µl of forward primer, 0.8 µl of reverse primer (Table 4), 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 ºC for 5 min and 95ºC for 10 sec, followed by 40 cycles of 95ºC for 5 sec and 50-55ºC (depends on primer pairs and primers listed in *SI Appendix*, Table 2) for 20 sec, followed by melt curve starting at 65ºC and ending at 95ºC.

Expression was measured for previously determined defense response genes (Ahn et al. 2018), including flavonoid-3'-hydroxylase, β-1,3-Glucanase, chitinase, chalcone synthase (CHS), thaumatin-like protein, and PR-10. 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 two tailed pooled t-test with JMP Pro 14. In all cases, fold values are expressed relative to zero-time control samples.

RESULTS AND DISCUSSION

Leaf blade and midrib disease response to three isolates of *C. sublineola* using the excised-leaf spot inoculation assay

Nearly identical degrees of infection between leaf blade and midrib were observed upon FSP35 inoculation of the johnsongrass cultivars. Disease evaluation data are summarized in Table 4. Table 4 also shows the results based on use of t-

test analysis (parametric). Winter and Dodou (2010) have verified that the t-test is valid for 5-point Likert scales and provide a more straightforward representation of the results as compared to non-parametric tests. This was confirmed for the results reported here by comparison to analysis using the non-parametric Wilcoxon test.

The degrees of infection for FSP2 or FSP53 were comparably mild. A few johnsongrass cultivars showed fairly different degrees of susceptibility upon inoculation of the two isolates. As an example, the average susceptibility scores of SH1350 were 1.75 and 2.45 in leaf blade and were 1 and 1.45 in midrib upon FSP 2 and FSP 53 inoculation, respectively. In SH1450, the scores were 2.75 and 3.18 in leaf blade and were 2.08 and 1.82 respectively, upon FSP2 and FSP53 inoculation.

The sorghum cultivars, BTx623, a susceptible cultivar, and SC748-5, a resistant cultivar, were also tested to survey any possible phenotypic difference between leaf blade and midrib upon inoculation of the three isolates of *C. sublineola*. SC748-5, like most of the johnsongrass cultivars, showed similar degrees of infection in leaf blade and midrib after inoculation. In contrast, BTx 623 had a large difference in infection severity between the leaf blade and midrib in response to inoculation with isolates FSP2 and FSP53 (Figure 3).

Table 4. Degree of infection on leaf blade and midrib day-4(=96 hrs) post-inoculation based on detached-leaf-spot assay. Mean±SEM was recorded.

Cultivar	FSP2		FSP35		FSP53	
	Leaf blade	Midrib	Leaf blade	Midrib	Leaf blade	Midrib
(+)BTx623 (sorghum)	4.67±0.33	2±0.47	3.67±0.21	3±0.45	5±0	1.8±0.8
(-)SC748-5 (sorghum)	1±0	1±0	4.14±0.26	3±0.53	1.44±0.44	1.56±0.24
SH1002	2.2±0.42	1.2±0.13	4.17±0.17	4.17±0.17	1.71±0.42	1±0
SH1030	1.7±0.34	1.3±0.21	3.5±0.38	3.5±0.38	1±0	1±0
SH1048	1.83±0.40	1.67±0.49	4±0.32	3.4±0.24	1.17±0.17	1±0
SH1094	1.73±0.23	1.27±0.20	3.88±0.13	3.14±0.55	1.67±0.16	2±0.42
SH1104	1.3±0.21	1.2±0.13	4±0	3.67±0.33	1.43±0.30	1.29±0.29
SH1116	1.73±0.31	1.36±0.24	4±0	4.17±0.17	1.63±0.50	1±0
SH1126	2.5±0.45	2±0.45	4±0	4±0	2.7±0.42	2.7±0.42
SH1136	2.45±0.47	2.45±0.47	4±0	4±0	2.33±0.49	2.67±0.56
SH1152	2.1±0.46	1.4±0.27	4±0	4±0	1.75±0.41	1.5±0.33
SH1154	1.64±0.28	1.18±0.12	4±0	4±0	1.75±0.37	1.25±0.25
SH1165	1.29±0.29	1.44±0.24	3.86±0.26	3.57±0.20	1.75±0.53	1.67±0.33
SH1201	1.91±0.39	1.27±0.20	4±0	4±0	1±0	1.67±0.42
SH1229	2.4±0.38	1.17±0.11	4±0	3.83±0.17	1.88±0.30	1.38±0.18
SH1233	1.13±0.13	1.13±0.13	3.63±0.18	3.88±0.13	1.4±0.27	1.4±0.22
SH1247	1.57±0.30	1±0	3.8±0.2	3.14±0.34	1±0	1±0
SH1281	1.86±0.46	2.43±0.53	3.4±0.24	3±0.32	1.75±0.31	1.88±0.48

Table 4 Continued

Cultivar	FSP2		FSP35		FSP53	
	Leaf blade	Midrib	Leaf blade	Midrib	Leaf blade	Midrib
SH1325	2.18±0.42	1.36±0.28	3.67±0.21	3.83±0.17	1.88±0.52	1±0
SH1337	2±0.42	1.11±0.11	3.25±0.49	2.5±0.57	1.88±0.35	1.13±0.13
SH1350	1.75±0.30	1±0	3.17±0.17	3.17±0.17	2.45±0.55	1.45±0.25
SH1409	2.5±0.5	1.9±0.35	4±0	4±0	3.33±0.76	3.14±0.55
SH1426	1.75±0.33	1.0±0	3.86±0.34	4.29±0.18	3±0.55	1.3±0.3
SH1450	2.75±0.39	2.08±0.31	4.17±0.2	3.67±0.21	3.18±0.42	1.82±0.36
SH1457	1.55±0.25	1.09±0.09	4±0	4±0	1.57±0.43	1.71±0.36
SH1484	1.89±0.39	1.67±0.24	4.17±0.17	3.83±0.17	1.57±0.37	1.57±0.37
SH1490	1±0	1.3±0.21	4±0	3.83±0.17	1.29±0.18	1.29±0.29
SH1493	2.36±0.31	2±0.30	3.8±0.2	4±0	1.29±0.29	1.14±0.14
Total	1.96±0.08	1.46±0.05	3.85±0.05	3.46±0.05	1.94±0.08	1.55±0.08

Figure 3. BTx623 and SH1350 midrib and leaf blade day-4 (=96hpi) based on detached-leaf-spot assay. a. BTx623 Midrib/FSP 2, b. BTx623 leaf blade/FSP2, c. BTx623 Midrib/FSP53, d. BTx623 leaf blade/FSP53. c and d are from the same excised leaf piece. Acervuli formation started from the boundary of midrib and leaf blade, e. SH1350 Midrib/FSP53, f. SH1350 leaf blade/FSP53. Difference of infection rate between midrib and leaf blade is present, but not as great as with BTx623. Photographs were taken at 10x magnification and are cropped to provide clear images.

For isolate FSP2, the average infection score was 4.67 for leaf blade compared to 2.0 for midrib. Similarly, for isolate FSP53, the average score was 5.0 for leaf blade compared to 1.8 for midrib.

Student's t-test analysis revealed different host responses between leaf blade and midrib in BTx623, SH1002, SH1229, SH1325, SH1337, SH1426, and SH1450 (P-values≤0.05, Mean scores are available in Table 4). For isolate FSP 35 inoculation, only SC-748-5, SH1337, and SH1426 showed statistically different responses between leaf blade and midrib. Similarly, for FSP53 inoculation, only differed in a few but different cultivars; BTx623, SH1350, and SH1450 had statistically meaningful response differences between the two tissues. The results clearly indicate different isolates of *C. sublineola* elicited different responses of leaf blade and midrib across different cultivars. Overall, leaf blade was more susceptible than midrib. The results from the Wilcoxon test, a non-parametric analysis, followed the parametric t-test with the same results so far as significance.

Wharton et al. (2001) have shown that *C. sublineola* appressoria penetrate directly through the cuticle and epidermal cell wall of host cells rather than entering via stomata, thus, so differences in stomata frequency in leaf blade and midrib cannot explain the difference.

Student's t-test analysis for overall average scores for johnsongrass isolates revealed significantly different host responses between leaf blade and midrib upon FSP2 inoculation (Mean leaf blade score and midrib score= 1.96 and 1.46 respectively with p-value≤0.0001). Similar results were found following FSP35

inoculation (Mean leaf blade score and midrib score= 3.85 and 3.46 respectively with p-value≤0.0076). Leaf blade and midrib response to FSP53 inoculation followed a similar pattern (mean leaf blade score and midrib score= 1.94 and 1.55 respectively with p-value≤0.0005

The results from Wilcoxon tests followed the parametric t-test; p-values based on comparisons between leaf blade and midrib are≤0.0001 upon FSP 2 inoculation, 0.0275 upon FSP 35 inoculation, and 0.0017 upon FSP 53 inoculation.

Real-Time Quantitative Reverse Transcription PCR analysis

Two johnsongrass cultivars, SH1136 and SH1350, and two sorghum cultivars, BTx623 and SC748-5, were selected for evaluating gene expression based on different phenotypic responses between leaf blade and midrib to FSP 53 inoculation. The average susceptibility scores of SH1136 were 2.33 and 2.67 in leaf blade and midrib respectively. SH1350 was scored 2.45 in leaf blade, while it was scored 1.45 in midrib. Like SH1136, SC748-5 had nearly identical average scores between leaf blade and midrib with 1.44 and 1.56 respectively. BTx623 was extremely susceptible in leaf blade with average score 5. In contrast, midrib was quite resistant with average score 1.8. Because nearly identical average scores for leaf blade and midrib were seen in SH1136 and SC748-5, gene expression levels were also expected to be similar. Conversely, different levels of host defense related gene expression in leaf blade and midrib were expected in SH1350 and BTx623.

In SH1136, SH1350, and SC748-5, no statistically meaningful differences were detected in any of the 6 tested defense response genes: the expression of the

genes upon FSP53 inoculations were nearly identical 1-day (=24hrs) and 2-day (=48hrs) post inoculation. Importantly, three different expression patterns between leaf blade and midrib were observed in BTx623 in response to inoculation with isolate FSP53 of *C. sublineola*. CHS8, which is involved in phytoalexin synthesis (Lo et al. 1999), was highly upregulated in midrib day-1 post inoculation. Midrib CHS8 expression was 23.28-fold upregulated, while leaf blade was 4.11-fold upregulated with P-value=0.0237 based on a two tailed pooled t-test (Figure 4 & Table 5).

The P-value comparing the 2 tissues is 0.00237 based on two tailed pooled t test. At day-2 post inoculation, midrib CHS8 was 34.58-fold upregulated but not statistically significant, while leaf blade CHS8 was 5.40-fold upregulated with P-value=0.1228. Meanwhile, chitinase was slightly upregulated in leaf blade (2.20 mean fold change) but was downregulated in midrib (0.50 mean fold change) at a P-value of 0.0082 based on two tailed pooled t test. Thaumatin-like protein (TLP) at day-2 post inoculation with isolate FSP53 was slightly upregulated (2.61 mean fold change) in midrib but downregulated in leaf blade (0.687 mean fold change) with p-value = 0.0485 based on two tailed pooled t-test. Overall, the aforementioned three genes had statistically significantly different expressions between leaf blade and midrib.

A different pattern was observed for chitinase, which can enhance resistance to fungal pathogens in sorghum (Sharma et al. 2014), as it was slightly upregulated in leaf blade, whereas it was downregulated in midrib day-1 post inoculation.

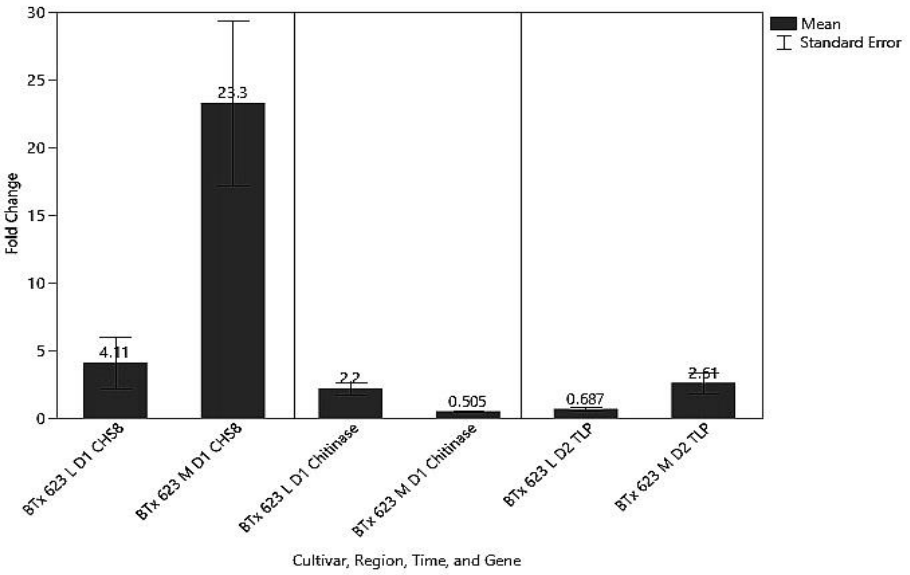


Figure 4. Expression of chalcone synthase 8 (CHS8), chitinase, and thaumatin-like-protein (TLP) in BTx 623 at day-1 (CHS8 & chitinase) and day-2 (TLP) post inoculation with FSP 53. (L = leaf, M = midrib, D1= day-1 post inoculation, D2= day-2 post inoculation).

Table 5. Significantly different host defense related gene expressions between leaf blade and midrib.

Pairs	Gene	Average fold change		P-value
		Leaf blade	Midrib	
BTx 623 Leaf blade D1 vs midrib D1	CHS8	4.11±1.89	23.28±6.08	0.0237
BTx 623 Leaf blade D1 vs midrib D1	Chitinase	2.20±0.43	0.50±0.06	0.0082
BTx 623 Leaf blade D2 vs midrib D2	TLP	0.69±0.12	2.61±0.77	0.0485

Mean fold changes ±SEM are shown.

At day-2 post inoculation with isolate FSP53, thaumatin-like protein (TLP) was slightly upregulated (2.61 mean fold change) in the midrib but downregulated in the leaf blade (0.687 mean fold change) with p-value = 0.0485 based on two tailed pooled t test. TLP is known to be produced in response to pathogen infection (Breiteneder 2004).

CONCLUSION

Overall, twenty-six johnsongrass and two sorghum cultivars inoculated with three selected isolates of *C. sublineola* produced phenotypic differences in disease severity between midrib and leaf blade on the same excised leaf tissue. More significantly, the differences in rates of expression of host resistance genes CHS8, chitinase, and TLP between midrib and leaf blade tissues occurred on BTx623 on day-1 and day-2 post inoculation with isolate FSP53 of *C. sublineola*. Based on the magnitude of difference between the two leaf parts, CHS8 might have an important role for midrib protection against *C. sublineola*. TLP might also be a contributor to the observed susceptibility differences between midrib and leaf blade tissues although not as significant as CHS8. Though it is unclear, leaf blade versus midrib expression of chitinase at day-2 post inoculation could be a good indication that leaf blade and midrib variously express host defense related genes with possible temporal difference.

One other important fact is that midrib versus blade differences in expression of tested genes were only observed in BTx623. The gap between leaf blade and

midrib average score was 3.2 in BTx623 whereas it was much lower in all other comparisons. While the small difference may simply result from equivalent responses, it is also possible that physical characteristics such as thickness of cuticle and wax on midrib compared to leaf blade may differ among cultivars and contribute to differences in response. In addition, there are many other host defense related genes I must further explore.

CHAPTER IV

GENOME WIDE ASSOCIATION ANALYSIS OF SORGHUM MINI CORE LINES REGARDING ANTHRACNOSE, DOWNY MILDEW, AND HEAD SMUT

INTRODUCTION

The mini core collection was included in a genotyping by sequencing (GBS) project that revealed locations of 265,487 Single Nucleotide Polymorphisms (SNPs) (Morris et al. 2013). The data revealed 14,739 SNPs markers scattered across the genome that could be used to genotype the complete sorghum mini core collection and for genome wide association studies (GWAS). In previous studies, the sorghum mini core collection was scored over several years for response to *C. sublineola*, *P. sorghi*, and *S. reilianum* (Radwan et al. 2011), and the screening results of these three common sorghum diseases were combined with over 290,000 Single Nucleotide Polymorphic (SNP) loci from a recently updated version of a publicly available genotype by sequencing dataset available for the mini core collection (Morris et al. 2013) in order to search potential candidate defense related genes against each of the three pathogens. Top candidate defense related genes are discussed here.

MATERIALS AND METHODS

Disease screening and ratings

For anthracnose, each of the 245 mini core accessions were evaluated two times consecutively in September 2007 and January 2008 in the USDA-ARS green house, College Station, Texas as described in (Prom et al. 2009). BTx635 (resistant) and B1? (BTx643) and BTx623 (susceptible) were included as checks in each experiment. The experiments were conducted under randomized block design replicated four times. A mixture of five anthracnose isolates which are aggressively virulent and commonly present in Texas was used as inoculum. The isolates are maintained at the USDA-ARS, Southern Plains Agricultural Research Center, College Station, Texas, USA. An effective spray inoculation technique and disease assessment method was used as previously described by Erpelding and Prom (2004) and Prom *et al.* (2009). Briefly, inoculum for *C. sublineola* from each isolate was pooled following growth on ½ strength PDA plates. The spray inoculation was used at the 8-10 leaf stage (approx. 30 days after planting) to deposit approx. 3-5 ml conidial suspension (10^6 conidia/mL) onto the leaves of each plant. Tween 20 (wetting agent) was added to the inoculum (0.5 ml/L). Immediately after spraying, plants were misted for 30 sec at 30-45 min intervals over a 10 hr/d period for one month. Disease assessments were conducted 30 days post-inoculation and thereafter, on a weekly basis for four weeks. Ratings were based on a scale of 1 to 5 (Erpelding and Prom 2004), where 1 = no symptoms or chlorotic flecks on leaves; 2 = hypersensitive reaction (reddening or red spots) on inoculated leaves but no

acervuli formation and no lesion development on other leaves; 3 = lesions on inoculated and bottom leaves with acervuli in the center; 4 = necrotic lesions with acervuli on inoculated leaves and infection spreading to bottom and middle leaves; and 5 = most leaves dead with abundant acervuli on the flag leaf. Accessions were considered resistant if plants in the row were rated as 1 or 2 and susceptible if rated as 3, 4, or 5. Thus the difference between a resistant and susceptible response as used here was the presence of acervuli on the leaves, which indicates successful reproduction of the pathogen.

For downy mildew, inoculation of germinating seeds of 242 mini core accessions and subsequent scoring for downy mildew was detailed in Radwan et al. (2011). Each experiment was replicated three times using a randomized block design. In each replication, 16-17 inoculated seedlings were transplanted to a half-gallon pot, and allowed to grow in the greenhouse at 25° ± 1°C for 14 days. Each week, 15 to 20 accessions were tested at a time along with susceptible (Pioneer hybrid 84G62) and resistant (Pioneer hybrid 83P67) as checks. The sandwich inoculation technique was used as detailed by Thakur et al. (2007) for downy mildew resistance screening in the greenhouse. Spores were collected from plants systemically infected with virulent pathotype 6 being maintained year-round in the greenhouse on infected susceptible plants (Pioneer hybrid 84G62) and served as the source inoculum. As the conidia production was found to be very difficult during the winter months, the greenhouse inoculation experiments were initiated in June 2009 and completed in May 2010 by skipping five months between October 2009

and February 2010. Susceptibility to downy mildew was evaluated two weeks after transplanting. Plants showing systemic and or local lesions were counted as infected. Disease incidence was determined from the percentage of infected plants in each replication and evaluated for disease symptoms. Accessions with 10% or less downy mildew incidence were considered resistant (Prom et al. 2010).

For head smut, all 245 minicore accessions along with BTx7078 (susceptible) and BTx635 (resistant) as checks were used for head smut resistance screening in the greenhouse. A set of fifty accessions along with two checks were planted in five-gallon pots starting April 2018 and at 15-day intervals the next 50 accessions and two checks were planted. Each experiment was conducted in a randomized block design with three replications. A five-gallon pot with five plants was maintained for inoculation in each replication. A reliable syringe inoculation technique as detailed by Perumal et al. (2007) was used for green house evaluation. Briefly, sporida were grown from teliospores that had been collected from infected sorghum plants in south and central Texas. Cultures were grown on a rotator in liquid culture (PDB) at 26° for 3 days and mixed for hypodermic inoculation. In susceptible interactions, the inflorescence is replaced with sori visible at the time of flowering, the time at which scoring was done. To verify resistance, all apparently healthy inflorescences were cut back to verify that tillers were not infected as occurs with systemic head smut infections. Since 37 photoperiod-sensitive lines did not flower, their main tillers were cut and the plants maintained until day length reduction

induced flowering (mid-November). Lines with less than 10% infected plants (0 most often) were scored as resistant.

GWAS and SNPs Mapping

The SNPs data was extracted from an integrated sorghum SNPs dataset and originally genotyped using GBS (Elshire et al. 2011) (Morris et al. 2013) (Lasky et al. 2015). The missing data were imputed using Beagle 4.1 (Browning and Browning 2016). GWAS was run using a linear mixed model in GAPIT with Model.selection=T, SNP.MAF=0.01 (J. Yu et al. 2005) (Lipka et al. 2012). The Manhattan plots were made using qqman package (Turner 2014). SNPs with high probability of contribution to each of the three diseases responses were tracked to the specific chromosome location based on the sorghum genome sequence, version 3.1.1 available at the JGI Phytozome 12 web site, updated in 2018 (McCormick et al. 2018).

RESULTS AND DISCUSSION

As part of a study supported by the Global Crop Diversity Foundation, the sorghum mini core lines were examined for response to *Colletotrichum sublineola* with a mix of spores from isolates causing disease in Texas. They were also specifically examined for response to race 6 of *P. sorghi* and to a mix of *S. reilianum* isolates collected from several locations in Texas. For anthracnose, 123 of the 245 lines that could be scored were resistant, for downy mildew 52 of the 240 mini core lines tested were resistant and for head smut, 102 of 229 lines were classed as

resistant. All together 459,304 SNPs went through filtering process and generated 299,204 SNPs for anthracnose, 306,615 for downy mildew, and 290,299 for head smut. Because of the very high number of comparisons possible a very high cutoff score is generally employed, However, since there is also a possibility that differential responses to races of the pathogens and that single resistance genes may not be detected if not present in enough accessions of the extremely diverse mini core collection to be detected, I opted to examine the highest scoring SNPs for each pathogen in order to determine if they identified genes known to be involved with host defense responses or had been identified in other disease association studies (Figure 5-7, Table 6-8). The tables also show the distance in base pairs to the nearest genes or physically nearby genes with defined functions.

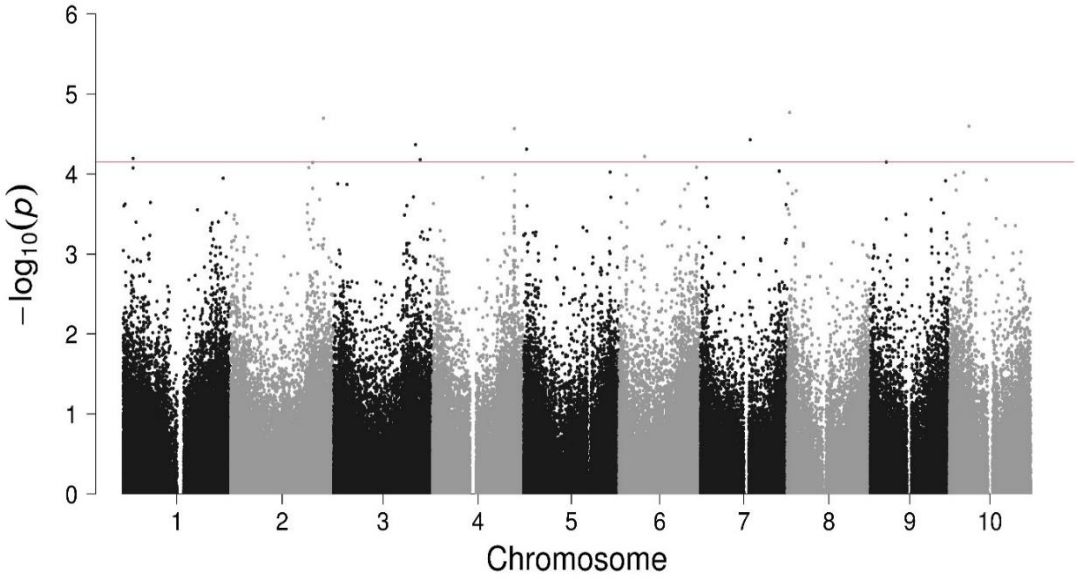


Figure 5. Genome-wide associations with anthracnose in Manhattan plot. The line is a cut-off for top candidate genes listed in table 6.

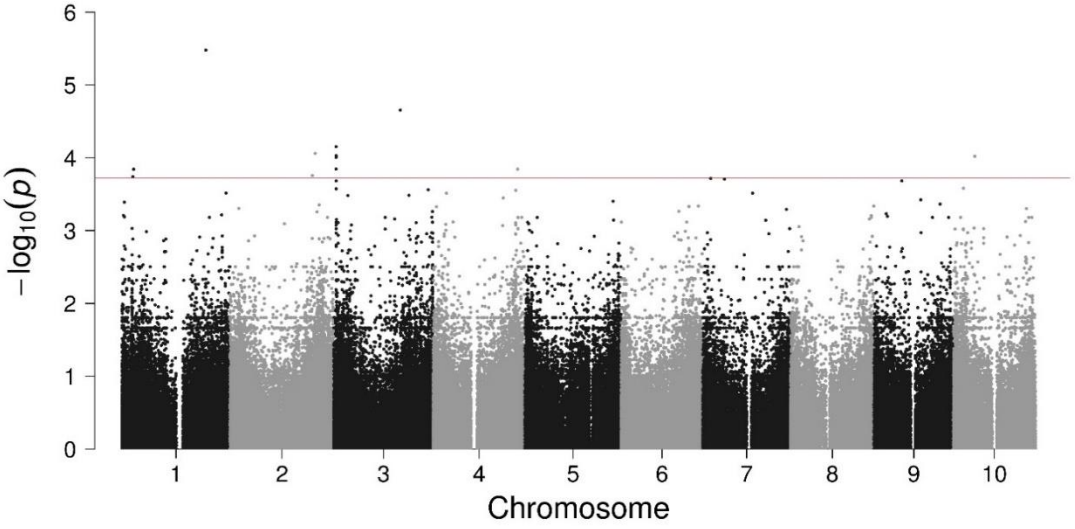


Figure 6. Genome-wide associations with downy mildew in Manhattan plot. The line is a cut-off for top candidate genes listed in table 7.

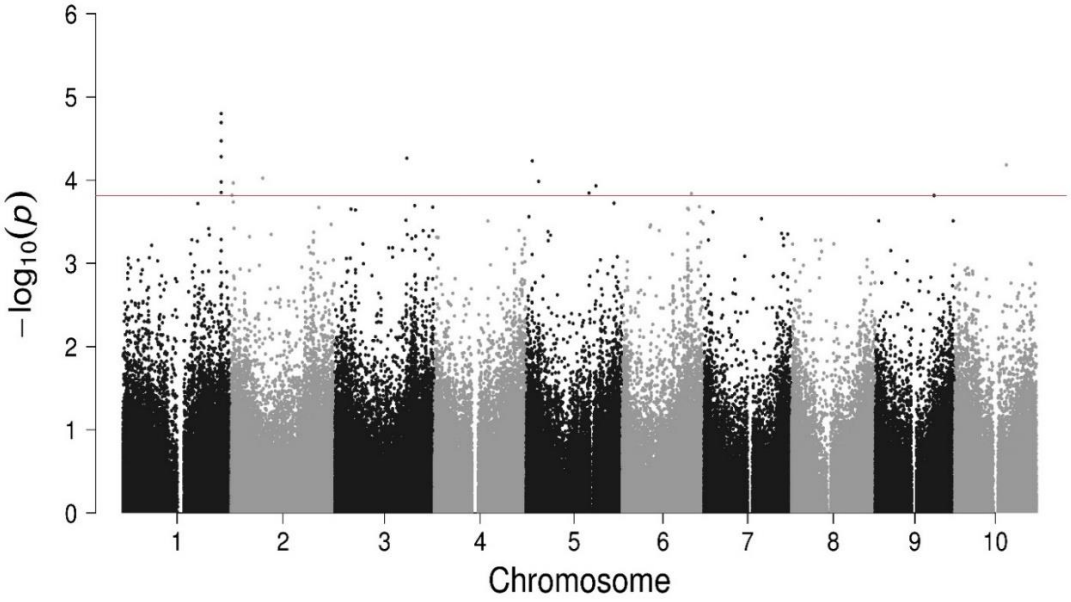


Figure 7. Genome-wide associations with head smut in Manhattan plot. The line is a cut-off for top candidate genes listed in table 8.

Table 6. Top SNPs associated with anthracnose. Top SNPs associated with anthracnose are listed. The tables also show the distance in base pairs to the nearest genes with functions.

Chr.	Location	Nearest gene and function	Base pairs away	P value
8	1802680	Sobic.008G020700 Similar to Zinc finger homeodomain (ZF-HD) protein dimerisation region containing protein	2605	1.70E-05
2	69955660	Sobic.002G330900 F-box domain.	5134	2.01E-05
4	61746687	Sobic.004G273600 Similar to H0215F08.15 protein. RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain). Exodeoxyribonuclease VII.	8004	2.70E-05
7	37557648	Sobic.007G105400 Similar to Ubiquitin carrier protein. Ubiquitin-conjugating enzyme (E2)	28912	3.74E-05
3	61650258	Sobic.003G281500 Similar to Pleiotropic drug resistance protein 15. ABC transporter domain, ABC-2 type transporter domain, Plant PDR ABC transporter associated protein domain, and ABC-transporter extracellular N-terminal domain containing	5220	4.30E-05
5	2211068	Sobic.005G024400 Ribosome Assembly protein WD domain, G-beta repeat, Histone-binding protein RBBP4 or subunit C of CAF1 complex.	0	4.90E-05
6	19369682 & 19369687	Sobic.006G040101 Plant transposase (Ptta/En/Spm family)	54956	6.02E-05
1	7499623	Sobic.001G097400 Similar to Phosphatidylinositol-3-and 4-kinase family protein, expressed. WD domain, G-beta repeat containing	268	6.39E-05
3	65141341	Sobic.003G325100 Similar to Blight resistance protein RGA3-like. NB-ARC domain and leucine rich repeat containing	7468	6.61E-05
9	11921711	Sobic.009G082200 Similar to Putative serine/threonine kinase protein.	26295	7.09E-05

Table 7. Top SNPs associated with downy mildew. Top SNPs associated with downy mildew are listed. The tables also show the distance in base pairs to the nearest or nearby genes (*) with functions.

Chr.	Location	Nearest/nearby genes and function	Base pairs away	P value
1	62708122	Sobic.001G339100 Similar to Glucose-6-phosphate 1-dehydrogenase, pentose phosphate pathway (oxidative branch)	254	3.32E-06
3	49693492	Sobic.003G185000 PF05266 - Protein of unknown function (DUF724) (DUF724), Agenet domain containing protein.	15551	2.21E-05
3	1603221 & 1608048 & 1608046	Sobic.003G018300 Similar to Os01g0213000 protein Ring finger domain containing protein	22460* 17633 17635	7.04E-05
2	63642528	Sobic.002G249000 Similar to AT-hook motif nuclear localized protein 2.	7612*	8.70E-05
10	15963823	Sobic.010G126500 Aspartyl proteases Xylanase inhibitor N-terminal and C-terminal containing protein	54532	9.54E-05
3	1636714	Sobic.003G018600 Similar to Small nuclear ribonucleoprotein homolog	0	9.84E-05
1	8688112	Sobic.001G111300 Uncharacterized protein Protein tyrosine kinase.	4318*	0.000144
4	63086639	Sobic.004G289700 Predicted Ca2+-dependent phospholipid-binding protein C2 domain containing protein	11853*	0.000144
2	61590648	Sobic.002G224300 Predicted protein No apical meristem (NAM) protein containing.	0	0.000175
1	8131244	Sobic.001G105900 Similar to Acc synthase ethylene biosynthesis I pathway Aminotransferase class I and II.	10046	0.000182

Table 8. Top SNPs associated with head smut. Top SNPs associated with head smut are listed. The tables also show the distance in base pairs to the nearest genes with functions.

Chr.	Location	Nearest gene and function	Base pairs away	P value
1	73516778 and 7 more within 2000 bp	Sobic.001G459500 Similar to Leucine Rich Repeat family protein, expressed.	271	1.58E-05
3	53833202	Sobic.003G207500 Minor histocompatibility antigen H13 Signal peptide peptidase	25606	5.44E-05
5	4712627	Sobic.005G049600 Weakly similar to H0607F01.6 protein Protein tyrosine kinase	0	5.86E-05
10	38702488	Sobic.010G143900 Similar to Os06g0470000 protein Glycosyltransferase	85520	6.55E-05
2	23750579	Sobic.002G142900 Similar to Putative uncharacterized protein, Tetratricopeptide repeat-containing protein	5417	9.44E-05
5	9400668	Sobic.005G076301 Coiled-coil domain containing protein lobo homolog Leucine-rich repeat containing protein	0	0.000103
2	1751971	Sobic.002G019000 Similar to Putative uncharacterized protein, Xyloglucan fucosyltransferase containing.	10608	0.000108
5	52227160	Sobic.005G120201 Glutathione S-transferase	1426	0.000117
1	73523579	Sobic.001G459600 Leucine Rich Repeat containing protein	0	0.00014
6	51844045	Sobic.006G160500 Similar to OSIGBa0159I10.13 protein, Aspartyl proteases Xylanase inhibitor N-terminal containing	0	0.000145
2	734336	Sobic.002G007800 Zinc finger containing protein	0	0.000151

Anthracnose

The highest probability for a SNP associated with resistance/susceptibility to anthracnose is associated with a Zinc-finger-homeodomain protein encoded by a gene on chromosome 8. (ZF-HD) proteins have known functions in plant defense through activation of calmodulin isoform 4 (GmCaM4) gene expression in soybean (Park et al. 2007). Next is SNP S02_69955660 on chromosome 2, that is 5134 bases from an F-box domain coding region. F-box proteins are involved in cell death and defense responses in tobacco, tomato (van den Burg et al. 2008), and Arabidopsis (Kim and Delaney 2002). Cuevas et al. also reported that an F-box protein is one of the top candidate genes related to sorghum defense response against *C. sublineola i*n a GWAS using a different set of sorghum cultivars (Cuevas et al. 2018).

A number of plant RNA-binding proteins (RBPs) have known roles in plant immune response regulation (Woloshen et al. 2011). The nearest gene coding region of SNP S04_61746687 on chromosome 4 includes an RNA recognition motif. Moreover, a peroxidase related gene coding region is only 23,915 bps away from the same SNP. Among the proteins induced during host plant defense, class III plant peroxidases are well known (Almagro et al. 2009).

According to Zhou et al., ubiquitin-conjugating enzymes as detected by the SNP on chromosome 7 play an essential role in both positive and negative plant responses to pathogens (Zhou et al. 2017). ABC transporters such as the gene nearest this SNP have been shown to be required for organ growth, plant nutrition, plant development, response to abiotic stresses, and pathogen resistance (Kang et

al. 2011).

Plant ribosomal proteins are known to play a role in non-host disease resistance against bacterial pathogens in *Nicotiana benthamiana* (Nagaraj et al. 2015). In addition, a WD40 repeat is reported to be involved in cell wall formation in plants (Guerriero et al. 2015). The SNP S05_2211068 on chromosome 5 is within the coding region of a gene that may serve either or both of these functions. Also, this SNP is also only 6148 bps away from a leucine-rich-repeat protein coding region which is a common feature of known resistance genes.

Transposable elements (TEs) are known to be able to affect plant gene expression and reduce host defense mechanisms (Sahebi et al. 2018). The nearest annotated coding region of the two SNPs S06_19369682 and S06_19369687 on chromosome 6 is relatively near a region with a transposase signature.

Salicylic acid (SA) has a central role in defense against pathogen attack, and phosphatidylinositol 4-kinase activation is an early response to SA in Arabidopsis (Krinke et al. 2007). The SNP S01_7499623 on chromosome 1 is 268 bps to the right from a coding region which contains a member of the phosphatidylinositol-3- and 4-kinase family and a WD domain.

The majority of disease resistance genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (McHale et al. 2006). The SNP S03_65141341 on chromosome 3 is located close to the coding region similar to resistance gene analog RGA3, a member of the nucleotide-binding site (NBS)-leucine-rich repeat (LRR) gene (Yu et al. 2017). The SNP on chromosome 9 is

comparably close to a coding region of putative serine/threonine kinase, enzymes that are key to signal transduction. Receptor-like kinases (RLKs) are involved in a diverse array of plant responses including development, growth, hormone perception and the response to pathogens (Goff and Ramonell 2007).

Downy mildew

Glucose-6-phosphate dehydrogenase (G6PDH) plays a role in response to abiotic stresses and pathogenesis (Y. Yang et al. 2014). On chromosome 1, the SNP S01_62708122, is statistically the most distinguishable with the lowest p-value.

The *Arabidopsis thaliana* gene *enhanced downy mildew 2* (*EDM2*) encodes a nuclear protein required for RPP7-mediated race-specific disease resistance against *Hyaloperonospora arabidopsidis*, proper floral transition and additional developmental processes (Tsuchiya and Eulgem 2011). By yeast two-hybrid screening for EDM2-interacting proteins, Tsuchiya & Eulgem identified AtEML1, a member of a small group of four Arabidopsis proteins containing an EMSY N-terminal domain, a central Agenet domain, and a C-terminal coiled-coil motif (Tsuchiya and Eulgem, 2011). This suggests the Agenet domain containing protein on chromosome 3 could play a similar role in sorghum

RING finger proteins comprise a large family and play important roles in regulation of growth and development, hormone signaling, and responses to biotic and abiotic stresses in plants (Y. Yu et al. 2011). Among the list of top candidates, 3 SNPs on chromosome 3 are all closest to a RING finger domain protein.

It is reported that AHL20, an AT-hook containing DNA-binding protein,

negatively regulates pathogen triggered immunity (PTI) (Lu et al. 2010) in Arabidopsis, and the SNP S02_63642528 on chromosome 2 is close to a nuclear localized, AT-hook-motif containing protein.

The SNP S10_15963823 on chromosome 10 is near a coding region that contains both aspartyl protease and xylanase inhibitor activity in the N- and C-termini. Aspartyl protease-mediated cleavage of Bcl-2-associated anti-apoptosis gene product of (BAG)6 is necessary for autophagy and fungal resistance in plants (Li et al. 2016). Further, xylanase inhibitor proteins (XIP) are potential defense molecules, which could act to prevent plant cell wall degradation by fungal hydrolytic enzymes (Vasconcelos et al. 2011).

Alternative splicing (AS) functions in a range of physiological processes, including plant disease resistance (S. Yang et al. 2014). The SNP S03_1636714 on chromosome 3 is located on the coding region of a protein similar to small nuclear ribonucleoprotein which is highly involved in AS. Moreover, the SNP is only 5607bp away from a ring-finger domain protein.

Plant receptor protein kinases (RPKs) represent the main plasma membrane pattern recognition receptors (PRRs) that can detect diverse microbe-associated molecular patterns (MAMPs) (Tena et al. 2011).

In a GWAS study with another sorghum collection, Cuevas et al reported a tyrosine-kinase as one of top candidate resistance genes for sorghum against *C. sublineola* (Cuevas et al. 2018). The SNP S01_8688112 on chromosome 1 is only 4318bp away from a tyrosine-kinase coding region.

In Arabidopsis, the C2 domain protein BAP1 negatively regulates defense responses (H. Yang et al. 2006). Similarly, on chromosome 4, I found the SNP S04_63086639 near the coding region of a C2 domain.

NAC (NAM, ATAF1&2, and CUC2) genes play roles in plant growth and development ranging from the formation of shoot apical meristem, floral organ development, reproduction, lateral shoot development, and defense to responses to biotic and abiotic stresses (Solomon and Sang-Keun 2017). The SNP S02_61590648 on chromosome 2 is in the coding region of a 'No Apical mannose binding lectin coding region. Plant mannose-binding lectins (MBLs) are crucial for plant defense signaling during pathogen attack by recognizing specific carbohydrates on pathogen surfaces (Hwang and Hwang 2011).

One of the earliest detectable events during plant-pathogen interaction is a rapid increase in ethylene biosynthesis (Ecker and Davis 1987). It is also known that aminotransferases confer enzymatic resistance to downy mildew in melon (Eckardt 2004). The SNP S01_8131244 on chromosome 1 is nearby a coding region similar to ACC synthase, which is related to ethylene biosynthesis I pathway, and aminotransferase class I and II domains.

Head smut

As previously mentioned most plant and animal immune receptors have a leucine-rich repeat (LRR) domain (Padmanabhan et al. 2009), and LRR proteins are known to take a significant role in plant defenses (Jones and Jones 1997). Three SNP Manhattan plot peaks were associated with LRR protein encoding genes, two

on chromosome 1 and one on chromosome 5 In sorghum NB-LRR resistance genes are found in clusters on several chromosomes, including chromosomes 1 and 5 (Yang and Wang 2016).

Signal peptide peptidase (SPP) plays a crucial role in life processes including immunological response in vertebrates, and SPPs are found in plants (Tamura et al. 2008). On chromosome 3, SNP S03_53833202 is located near a signal peptide peptidase gene. Since these genes are critical for secreting or delivering proteins to correct membrane locations, a role in defense is logical, though not proven.

Plant receptor protein kinases (RPKs) represent the main plasma membrane pattern recognition receptors, perceiving (PRRs) diverse microbe-associated molecular patterns (MAMPs) (Tena et al. 2011). As for downy mildew, but on a different chromosome a SNP was detected in a tyrosine-kinase gene on chromosome 5.

Glycosyltransferases of plant secondary metabolism transfer nucleotide-diphosphate-activated sugars to low molecular weight substrates, and, additionally, it has been suggested that glycosyltransferases have an important role in plant defense and stress tolerance (Vogt and Jones 2000). A SNP on chromosome 10 was nearby to a glycosyltransferase coding region.

It is known that one of the tetratricopeptide repeat (TPR) proteins known as SRFR1 (suppressor of rps4-RLD 1) functions negatively in resistance toward the effector molecule for AvrRps4 in Arabidopsis (Sharma and Pandey 2016). It seems

likely TPR also affects resistance in sorghum since a SNP nearby a TPR coding regions on chromosome 2 was detected.

Cell walls are crucial for disease resistance in plants, and xyloglucan fucosyltransferase is a well-known enzyme involved in plant cell wall biosynthesis (Perrin et al. 1999). On chromosome 2, a SNP near a xyloglucan fucosyltransferase coding region was found.

Glutathione (GSH) is a non-protein thiol compound which has been repeatedly reported to play an important role in plant responses during biotic stresses (Dubreuil-Maurizi and Poinssot 2012). I found a SNP near a glutathione S-transferase.

As described earlier, aspartyl protease-mediated cleavage of BAG6 is necessary for autophagy and fungal resistance in plants (Y. Li et al. 2016). Xylanase inhibitor proteins (XIP) are potential defense molecules, which could act to prevent plant cell wall degradation by fungal hydrolytic enzymes (Vasconcelos et al. 2011). The SNP detected within the coding region of aspartyl proteases and XIP N-terminal on chromosome 6 is congruent with previous studies.

Cuevas et al. reported LRR, tyrosine-kinase, and zinc finger proteins are top candidate resistance genes for sorghum against *C. sublineola* (Cuevas et al. 2018). As mentioned previously, three SNPs were found that are near or within LRR tyrosine-kinases were found. Additionally, zinc finger protein, which plays essential roles in plant responses to biotic and abiotic stress, was directly tagged by a SNP on chromosome 2 (Shi et al. 2014). The results strongly suggest the fact that LRR,

tyrosine-kinase, and zing finger protein are truly involved in sorghum immunity against both facultative biotrophs *C. sublineola*, and *S. reilianum*, and possibly other pathogens as well.

CONCLUSION

In this research I took advantage of disease rating data for up to 245 accessions from the mini core collection and the availability of a publicgenome sequencing project for those same accessions to identify SNPs that may be associated with resistance. A similar study was made earlier (Upadyhaya et al. 2013) using sequenced restriction fragments to identify SNPs associated with anthracnose resistance. In that study 14,739 SNPs identified 8 regions on chromosomes 1, 6, 8 and 10 which could be associated with potential disease-related genes at a p value of 10^{-4} or lower. The genes identified ranged from being 23 bp to 49 kb from the identifying SNP. Here, with the whole genome sequences available, over 290,000 useful SNPS were available for association mapping with anthracnose, so only genes that are essentially adjacent to individual SNPs at a p value of $7 X10^{-5}$ or less were examined for possible roles in disease. Putative host defense genes were found on chromosomes 1, 6 and 8, but they did not appear to be the same genes identified in the earlier study. Here, genes on chromosomes 3, 5, 7 and 9 were also detected. In all but one of the SNPs examined, nearby genes have previously been implicated in disease responses in sorghum or other plants. There are several factors that may explain the differences in the two studies. First, our analysis was

made simply on the basis of resistance (no fungal reproduction, even when lesions are present) or susceptibility rather than the 1-9 scoring system used in the earlier study. In addition, *C. sublineola* is an extremely variable pathogen that typically is controlled by single gene resistance via recognition of pathogen avirulence factors that trigger host recognition involving NB-LRR proteins. As a consequence, the isolates collected from Texas may differ significantly from those in the India study. Also, a few substitutions have been made to replace lines included in the original mini core accessions for reasons such as low seed supply. Here, a more recent update of the annotated sorghum genome sequence was used, and while the update resulted in some re-numbering, generally those changes are not great and none would change chromosomal assignments. Since all the genes identified in both studies have potential roles in host defense, all are deserving of additional analysis.

This is the first GWAS analysis of the mini core collection for downy mildew. In this case, only a single, newly discovered race 6 of the pathogen was used to inoculate plants of each accession. Over 306,000 useable SNPs were detected in the 240 mini core accessions successfully screened, of which 52 were resistant. As was the case with anthracnose the 10 most likely candidates ($p \leq 1.8 \times 10^{-4}$) were very near genes with functions predicting a potential role in host defense.

This is also the first case of using GWAS for head smut. Since symptoms can be scored only after heading, many of the lines could only be scored months after planting and inoculation. Here, over 290,000 SNPs could be scored for the 229 accessions scored of which 102 were resistant. Again, only R vs S responses were

recorded but now R meant less than 10 % of inoculated plants developed symptoms in the primary shoots or tillers.

Unless individual R genes providing race-specific resistance were present in a number of accessions it is unlikely they would be detected by this type of analysis. Especially in the case of anthracnose, where many races have evolved to overcome LRR type R genes, plants have also evolved a large family of such genes. The plasticity of NBS-LRR resistance genes in sorghum is driven by multiple evolutionary processes (Mace et al., 2014). In fact, defense response genes typically occur in families with numerous copies (Yang and Wang, 2016) so it is not surprising those identified differ in location.

Overall, most of the genes identified are involved in aspects of host defense that would be typical of quantitative trait loci with minor effects rather than major genes. Those expected to be more directly involved in host defense, include SNPs nearby encoding regions of zinc finger and LRR related proteins. Both were on the top list for anthracnose and head smut and near the top of the list for downy mildew. Tyrosine kinase related SNPs were on the top lists of downy mildew and head smut, but not on the top list of anthracnose. Overall, the top candidate host-defense related genes were diverse against three different pathogens.

CHAPTER V

CONCLUSION

Different *C. sublineola* isolates (FSP2, FSP35 and FSP53) elicited various responses in twenty-six johnsongrass cultivars. Through real-Time qRT-PCR, expression of five known host defense related genes: 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 24hpi and 48hpi. As a result, SH1152, a moderately resistant cultivar, greatly upregulated chalcone synthase 8 (CHS8), flavonoid-3'-hydroxylase, pathogenesis related protein-10 (PR-10), and thaumatin-like protein 48hpi. Meanwhile, SH1247, 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 it is unnecessary to upregulate the specific host defense related genes tested in order to protect itself against *C. sublineola* in SH1247 which could be a novel source of resistance for sorghum.

When the three *C. sublineola* isolates were inoculated to the twenty-six johnsongrass and two sorghum cultivars (BTx623 and SC748-5), disease severities between midrib and leaf blade on the same excised leaf tissue differed. The differences in rates of expression of host resistance genes CHS8, chitinase, and TLP between midrib and leaf blade tissues occurred on BTx623 on day-1 and day-2 post inoculation with isolate FSP53 of *C. sublineola*. CHS8 might have an important role for midrib protection against *C. sublineola* based on huge gene expression rate

difference.

Finally, the results from pathogenicity tests of *C. sublineola*, *P. sorghi*, and *S. reilianum* (Radwan et al. 2011) in sorghum mini core collection were combined with over 290,000 Single Nucleotide Polymorphic (SNP) loci from a recently updated version of a publicly available genotype by sequencing dataset available for the mini core collection (Morris et al. 2013). Based on the result of genome wide association studies (GWAS), potential candidate defense related genes against each of the three pathogens were detected.

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