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Research Paper

SNPs that identify alleles with highest effect on grain mold ratings after inoculation with *Alternaria alternata* or with a mixture of *Alternaria alternata*, *Fusarium thapsinum* and *Curvularia lunata*

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Abstract. In a recent study, sorghum association panel (SAP) lines were evaluated for grain mold resistance against *Alternaria alternata* individually and co-inoculated with *Fusarium thapsinum*, and *Curvularia lunata*. Several accessions were found to be resistant to both treatments. A genome-wide association study based strictly on mold rating scores for each SAP line revealed a number of SNPs closely linked or in genes known to play roles in host defense. In that analysis mold ratings were used directly, without accounting for how much change was made in each rating versus water treated controls. Here, the same datasets for mold scores and more that 79,000 SNP alleles were used, but the untreated control values for each cultivar were subtracted from the treated sample ratings. The new dataset is thus designed to identify host genes that had the most impact in changing the mold rating over background. Our goal was to determine if the same genes would be identified, and if not, did the functions of the new genes also make them candidates for roles in mold resistance. As before, when the newly found top-scoring SNPs were mapped to the published sorghum genome, the nearest annotated gene has precedence for a role in host defense.

Keywords: Sorghum grain mold, fungal species, *Alternaria alternata*, *Fusarium thapsinum*, *Curvularia lunata*, GWAS, SNP, QTL.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important cereal crops and in the drier tropics, it supplies the daily calorie needs of hundreds of millions of people (Frederiksen and Odvody, 2000). The productivity and profitability of the crop is impacted by numerous biotic stresses, including grain mold, caused by a consortium of pathogenic fungal species: this disease complex is considered the most important disease of sorghum (Nida *et al*., 2019). When frequent rains occur at maturity and grains are not harvested, yield losses can reach 100% on susceptible lines (Singh and Bandyopadhyaya, 2000; Navi *et al*., 2005). Although resistant lines have been identified, there is limited information on the stability or gene(s) responsible for the resistance in these sources (Singh and Bandyopadhyaya, 2000; Prom *et al*., 2020). A recent study identified sorghum grain mold resistance loci, including a MYB gene, through genome wide association mapping (Nida *et al*., 2019). More recently, Prom *et al*. (2020) evaluated grain mold resistance of 377 sorghum association panel (SAP) lines by inoculating them with *Alternaria alternata* alone, a mixture of *A*. *alternata*, *Fusarium thapsinum*, and

Curvularia lunata, and untreated control sprayed with sterile water (Prom *et al*., 2020). *A*. *alternata*, *F. thapsinum*, and *C. lunata* are common pathogens causing grain mold in sorghum (Singh and Bandyopadhyay, 2000; Prom *et al.*, 2020). At 50% flowering, panicles were inoculated with spore suspension using a hand-held spray bottle until run-off and bagged for 24 hours to enhance infection and disease development. The Prom *et al*. (2020) study included genome-wide association study (GWAS) using TASSEL software to map over 79,000 single-nucleotide polymorphic (SNP) loci. Chromosomal locations of SNP alleles associated with differences in grain mold response were determined for each of the treatments. Although that study identified many SNPs closely linked to candidate genes for grain mold resistance the results may not identify those genes whose expression is associated with the greatest improvement in mold ratings versus controls. In order to find the SNPs more directly associated with the fungal treatments, here we report the use of GWAS based on the gaps between average scores of *A. alternata* and of a mixture of *A*. *alternata*, *F. thapsinum*, and *C. lunata* inoculation minus the average scores of the untreated water sprayed control for the same cultivar. The results generated new candidates for genes that lead to mold score improvement. When the newly found top-scoring SNPs were mapped to the published sorghum genome, in all case, the nearest annotated gene has precedence for a role in host defense.

MATERIALS AND METHODS

GWAS and SNP mapping

Phenotype data from Prom *et al.* (2020) were used. Average scores of SAP lines' grain mold response against *A. alternata* inoculated alone and with a mixture of *A*. *alternata*, *F. thapsinum*, and *C. lunata* inoculation were subtracted from average scores of SAP lines' grain mold response under untreated water sprayed control, respectively. With the subtracted averages, GWAS analysis was conducted as described in a recent study (Prom *et al.*, 2019).

In brief, the 2018 updated version of 79,000 single-nucleotide polymorphic (SNP) loci from a publicly available genotype by sequencing dataset available for the SAP lines (link: https://www.morrislab.org/data) was used as genotype data (Morris *et al.*, 2013). TASSEL version 5.2.55 (Bradbury *et al*., 2007) was used to conduct a mixed linear model (MLM) association analysis based on average scores of *A*. *alternata* inoculated score – untreated score and a mixture of the three pathogens inoculated score – untreated score for the detection of SNPs activated/affected by the two treatments compared to untreated control for grain mold response.

In order to reduce potential false positive associations, SNPs with more than 20% unknown alleles were removed as were those with minor allele frequency (MAF, below 5%) as described for GWAS analysis of stalk rots (Adeyanju *et al*., 2015). As a result, 79,034 SNP markers were used for GWAS. Principal component analysis was used to visualize the population structure which was followed by calculation of kinship data based on the TASSEL 'centered IBS' method. Optimum compression level and P3D variance component estimation were used as MLM options. SNPs with high probability of contribution to the scores for grain mold response were tracked to the specific chromosome location based on the sorghum genome sequence, version 3.1.1 available at the JGI Phytozome 12.5.1 web site, updated in 2018 (McCormick *et al.*, 2018). For each of the prospective genes, the average disease rating score for all SAP lines with either of the two prevalent bases was determined and verified to differ significantly ($p < 0.05$) using JMP statistical software from SAS®, version 14, and any SNPs that failed to pass t-test were excluded from the list.

RESULTS AND DISCUSSION

When mapped back to the published genome, the top ten SNPs for both treatments *A. alternata* alone and the mixture of *A*. *alternata*, *F. thapsinum*, and *C. lunata* are nearest to genes with functions that have previously been implicated in various resistance/stress responses or identified in other disease association studies (Figures 1 and 2). Tables 1 and 2 show the distance in base pairs to the nearest gene, the bases that create the SNP, the fraction of the population with each SNP allele, and average scores of differences between treated – untreated scores per each SNP allele. For the average scores, positive numbers indicate higher treated scores, and negative numbers indicate lower treated scores compared to untreated scores.

SNP S10_7369881 on chromosome 10 and SNP S07_55256111 on chromosome 7 are closely linked to MYB family protein coding genes. The MYB transcription factor gene family is large and involved in controlling various processes, including responses to biotic and abiotic stresses, development, differentiation, metabolism, defense etc. (Ambawat *et al.*, 2013). A recent GWAS with 1425 Ethiopian sorghum landraces identified a major grain mold resistance region on chromosome 2 that included a MYB transcription factor gene as well as genes affecting synthesis of flavonoid in developing seeds (Nida *et al*., 2019). Protein BLAST of the protein showed most similarity to MYB20 which in Arabidopsis has been shown to activate lignin biosynthesis and to decrease flavonoid biosynthesis (Geng *et al.*, 2020), both of which are relevant to pathogen interactions.

A betaine aldehyde dehydrogenase gene was detected

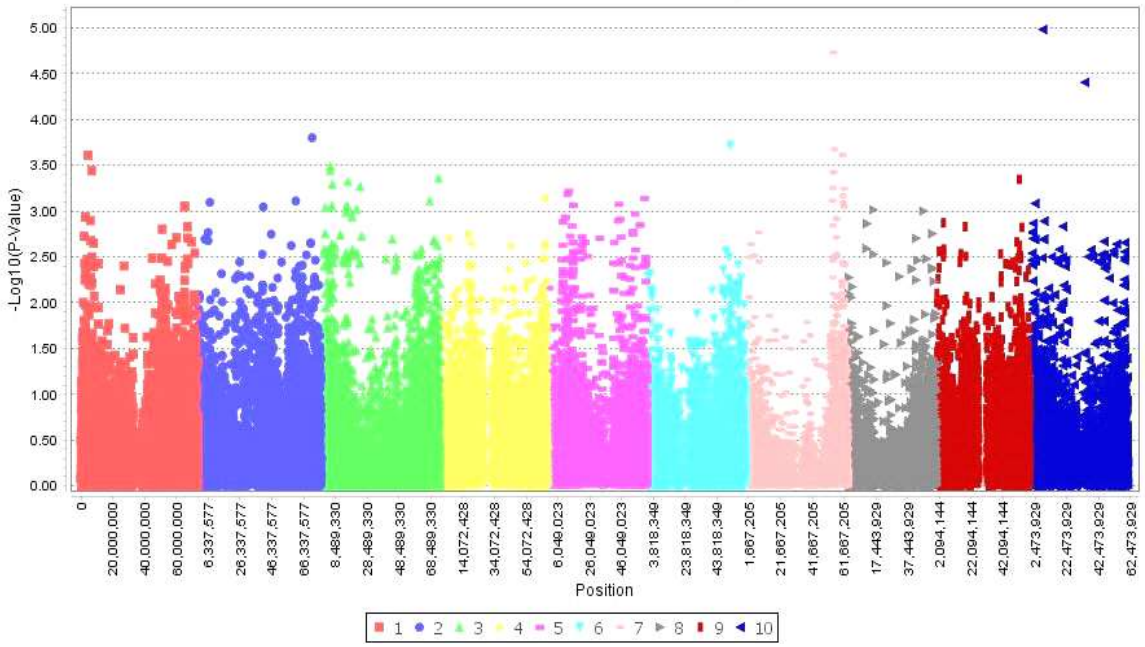

Figure 1. Manhattan plot showing locations of SNP-detected QTLs associated with response to *A. alternata* treated grain mold score - untreated grain mold score on the ten chromosomes of *Sorghum bicolor*.

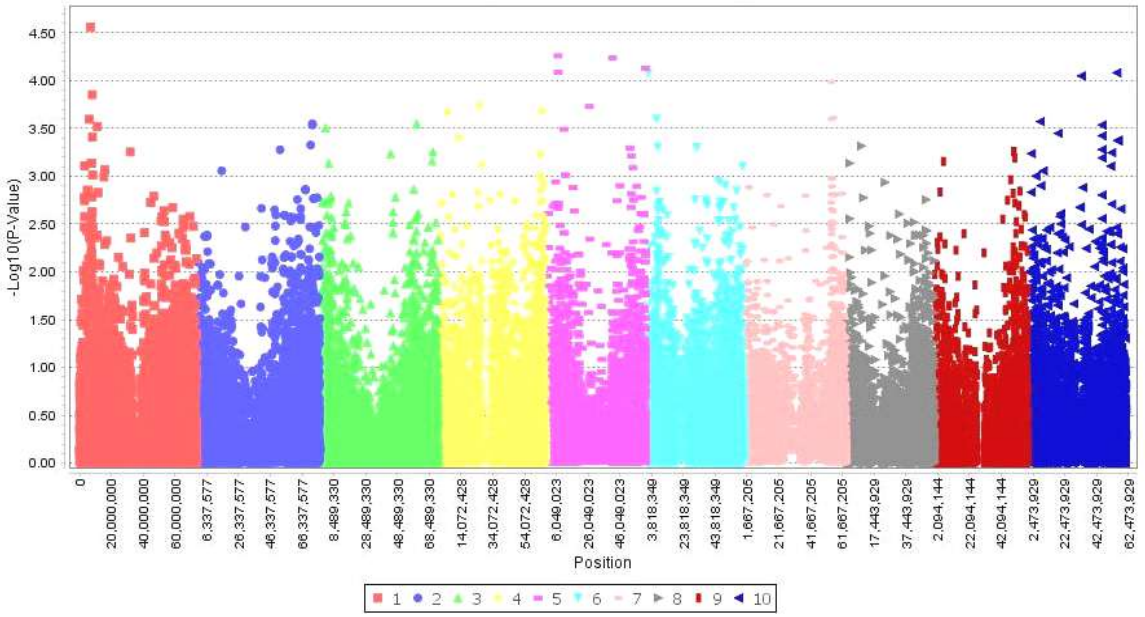

Figure 2. Manhattan plot showing locations of SNP-detected QTLs associated with response to a mixture of *A. alternata*, *F. thapsinum*, and *C.lunata* treated grain mold score - untreated grain mold score on the ten chromosomes of *Sorghum bicolor*.

by two SNPs (SNP S07_54752149 and SNP S07 54751485). The enzyme adds glycine to betaine and the product plays an important role in osmoregulation. As such, it plays a multifunctional role in controlling abiotic stresses as well as production of fragrance in seed such as Basmati rice (Golestan *et al.*, 2018). Glycine-betaine has also been shown to increase hypersensitive responses and thus to enhance resistance to downy mildew in pearl millet (Lavanya and Amruthesh, 2017). Also, in a wheat substitution line developed to incorporate resistance to powdery mildew, a proteomic analysis conducted during seed development showed enhanced

Table 1. Genes nearest to the most significant SNPs with *A. alternata* treated grain mold score - untreated grain mold score comparisons.

Chr.	Location	Nearest gene and function	Base pairs away	SNP base %	TASSEL *P* value	Average score of *A. alternata* treatment score-control score/SNP
10	7369881	Sobic.010G085700 F25A4.19 Protein (MYB family)	1501	A:67% G:33%	1.0444×10^{-5}	A:0.133 G:0.032
7	54752149	Sobic.007G130800 Similar to betaine aldehyde dehydrogenase	521	A:22% G:78%	1.8548×10^{-5}	A:-0.103 G:0.175
10	33802706	Sobic.010G141001 DNA topoisomerase II	105086	C:79% T:21%	3.9438×10^{-5}	C:0.154 T:-0.030
2	71714764	Sobic.002G353800 Similar to homeodomain leucine zipper protein 14	6045	A:46% G:54%	1.5861×10^{-4}	A:0.205 G:0.033
7	55256111	Sobic.007G132600 MYB related protein	16083	C:9% T:91%	2.1209×10^{-4}	C:-0.230 T:0.160
1	4354046	Sobic.001G057600 Asparagine synthetase	8582	C:59% T:41%	2.4578×10^{-4}	C:0.013 T:0.264
1	6693923	Sobic.001G086500 MADS box Transcription factor 14	5657	A:10% G:90%	3.6284×10^{-4}	A:0.382 G:0.061
7	54751485	Sobic.007G130800 Similar to betaine aldehyde dehydrogenase	0	A:24% G:76%	3.7742×10^{-4}	A:-0.077 G:0.171
3	73389833	Sobic.003G431400 Hypothetical protein with Trypsin-like peptidase domain (Trypsin_2)	0	C:78% T:22%	4.351×10^{-4}	C:0.166 T:-0.048
9	52200920	Sobic.009G165900 Protein tyrosine kinase Serine/threonine protein kinase	767	C:7% T:93%	4.5216×10^{-4}	C:0.332 T:0.083
3	16284054	Sobic.003G153000 Galactosyltransferase	31966	G:10% T:90%	4.7158×10^{-4}	G:-0.041 T:0.137

levels of betaine aldehyde dehydrogenase (Zou *et al.*, 2020).

Topoisomerase II enzymes are essential for relieving supercoiling of replicating DNA and for successful separation of daughter molecules (McClendon and Osheroff, 2007) and also for transcription of long mRNAs (Joshi *et al.*, 2012). They function by creating transient double strand breaks: failure to either make or repair the breaks can be lethal to the cell. Flavone-based phytoalexins synthesized by sorghum on exposure to pathogens have been shown to include compounds that inhibit topoisomersase II activity (Cantero *et al*., 2006; Du *et al*., 2010). Whether involved with inhibition of pathogen growth or with triggering apoptosis, it can be predicted that differences in activity of the topoisomerase alleles identified here as SNPs could have an effect on grain mold ratings.

Homeodomain-leucine zipper proteins (SNP S02_71714764) participate in diverse and sometimes overlapping events ranging from stress responses to morphogenesis and development (Elhiti and Stasolla, 2009). Gene Ontology (GO) analysis of this protein places it in the DNA-binding and transcription factor categories (JGI Phytozome 12.5.1).

In pepper (*Capsicum annuum*) asparagine synthetase 1 (SNP S01_4354046) is essential for plant defense to microbial pathogens (Hwang *et al.*, 2011). Similarly, Seifi *et al.* (2014) suggested that activation of asparagine synthesis both provided a source of nitrogen and hastened senescence of tomato plants that are susceptible to *Botrytis cinerea,* the necrotrophic gray mold pathogen of many hosts (Seifi *et al.*, 2014).

Table 2. Genes nearest to the most significant SNPs with a mixture of *A. alternata*, *F. thapsinum*, and *C. lunata* treated grain mold score - untreated grain mold score comparisons.

Chr.	Location	Nearest gene and function	Base pairs away	SNP base %	TASSEL *P* value	Average score of *A. alternata*, *F. thapsinum*, and *C. lunata* treatment score- control score/SNP
5	7106146	Sobic.005G064300 Aspartic protease	5031	A:10% C:90%	5.4887×10^{-5}	A:-0.110 C:0.295
5	41339258	Sobic.005G113300 Similar to Serine hydroxymethyltransferase	81972	C:51% T:49%	5.7821×10^{-5}	C:0.192 T:0.297
5	7154499	Sobic.005G064600 No apical meristem (NAM) protein	30592 36033	A:9% G:91%	8.166×10^{-5}	A:-0.149 G:0.289
7	54751485	Sobic.007G130800 Similar to betaine aldehyde dehydrogenase	0	A:24% G:76%	1.029×10^{-4}	A:0.102 G:0.305
4	25476069	Sobic.004G134700 Similar to drought-induced protein 19 (Di19)	330389	A:27% G:73%	1.8212×10^{-4}	A:0.380 G:0.174
5	26775247	Sobic.005G110442 Glutathione peroxidase	41315	A:21% G:79%	1.8611×10^{-4}	A:0.095 G:0.279
4	64915267	Sobic.004G312300 Protein of unknown function (DUF1644)	6418	C:88% G:12%	2.0621×10^{-4}	C:0.234 G:0.436
7	55922399	Sobic.007G13440 Similar to Putative UVB-resistance protein UVR8	20093	A:16% C:84%	2.4558×10^{-4}	A:0.101 C:0.273
7	54752149	Sobic.007G130800 Similar to betaine aldehyde dehydrogenase	521	A:22% G:78%	2.517×10^{-4}	A:0.079 G:0.296
6	7023593	Sobic.006G032300 Similar to HECT ubiquitin-protein ligase 3	7029	A:10% G:90%	2.5211×10^{-4}	A:0.600 G:0.221
10	6934391	Sobic.010G081300 FALZ-Related Bromodomain-containing protein	401	A:16% G:84%	2.6844×10^{-4}	A:0.107 G:0.265

MADS box genes (SNP S01_6693923) were first defined based on abnormal floral development resulting from mutations in these transcription factor genes in *Arabidopsis* and later found to be involved in regulating other events such as lignin formation (Cosio *et al*., 2017). Subsequently they have been shown to interact as homo- or heterodimers (de Folter *et al*., 2005), and have also been shown to be present in the genomes of many pathogenic fungi where function is essential for growth and pathogenicity (de Folter *et al*., 2005; Mohammadi *et al*., 2020). Consequently, interaction among host and pathogen factors may also occur. However, at least in one case, a direct effect of a host MADS box and pathogenicity has been established. MADS1, a novel

MADS-box protein, is involved in the response of *Nicotiana benthamiana* to bacterial harpin (Xoo), an elicitor of the hypersensitive response (Zhang *et al*., 2015).

SNP S03_73389833 is located in the coding region of trypsin-like peptidase domain of a hypothetical protein. Proteins with this feature are known to play a role in plant defense of trypsin inhibitors against herbivores and spider mites (Arnaiz *et al.*, 2018; Major and Constabel, 2008).

Protein kinases (SNP S09_52200920) play a central role in signaling during pathogen recognition and the subsequent activation of plant defense mechanisms (Romeis, 2001).

Galactosyltransferases are involved in several pathways that involve cell wall synthesis, including the amount of pectin made in cell walls (Liwanag *et al*., 2012). Pectin levels affect penetration frequency of *Colletotrichum higginsianum* in *Arabidopsis* leaves (Engelsdorf *et al*., 2017). The sorghum enzyme identified here is most similar to hydroxyproline-O-galacosyltransferases in other plants, an enzyme that converts proline to hydroxyproline in arabinogalactan-protein components of plant cell walls (Basu *et al*., 2015).

The nearest coding region from SNP S05_7106146 is an aspartic protease. Aspartic proteases have been implicated directly in diseases resistance. The CDR1 gene in *Arabidopsis* encodes a secreted form of aspartic protease that confers resistance to *Pseudomonas syringae* (Xia *et al*., 2004). Also, incorporating a constitutively expressed potato aspartic protease into Arabidopsis provides resistance to *Botrytis cinerea* (Frey *et al*., 2018).

In *Arabidopsis*, SHMT1, a serine hydroxymethyltransferase (SNP S05_41339258) plays a role in the photorespiratory pathway that influences resistance to biotic and abiotic stress through regulation of cell death (Moreno *et al*., 2005), and in soybean, duplication of a serine hydroxymethyltransferase gene results in resistance to cyst nematodes (Wu *et al*., 2016).

The same betaine aldehyde dehydrogenase on chromosome 7 as was detected by two SNPs in the *Alternaria*-only inoculation was also detected here.

In plants, the main families of transcription factors responsible for the regulation of genes responsive to pathogens are categorized into a few families such as WRKY, MYB, bZIP, and the recently added no apical meristem (NAM) (Alves *et al.*, 2014). NAM is the closest protein from SNP S05_7154499. This SNP is also fairly close to Sobic.005G064400 that contains an aspartic protease coding region (36033 bp away).

In *Arabidopsis*, dehydration-responsive element binding protein 1 (DREB1) and drought-induced protein 19 (SNP S04_25476069) induces pathogenesis-related genes (Ali *et al.*, 2018; Tsutsui *et al.*, 2009). The number of base pairs from the SNP to the coding region is fairly large, but a recent study found that activated genes are often up to 2 Mbps away from the associated SNP, and are not necessarily the closest genes to the SNP (Brodie *et al.*, 2016).

Some plant glutathione S-transferases (GSTs) display glutathione peroxidase (SNP S05_26775247) activity and these GSTs can detoxify toxic lipid hydroperoxides that accumulate during infections (Gullner *et al.*, 2018).

Over-expression of a DUF1644 protein gene (SNP S04_64915267), SIDP361, enhanced tolerance to salt stress in transgenic rice (Li, 2016).

It is reported that UVR8 mediates UV-B-induced *Arabidopsis* defense responses against fungal pathogen *Botrytis cinerea* by controlling sinapate accumulation (Demkura and Ballare, 2012).

A recent study revealed that proteasome-associated HECT-type ubiquitin ligase (SNP S06_7023593) activity is required for plant immunity (Furniss *et al*., 2018).

FALZ-Related Bromodomain-containing protein (SNP S10_6934391) was listed as a candidate gene for resistance to *Cercospora sojina* K. Hara in soybean (Pham *et al.*, 2015).

As in the previous study, all the annotated genes nearest the highest scoring SNPs detected here have reported roles in host defenses or stress responses. Interestingly, none of the top ten genes was from the same group as those reported by Prom *et al.* (2020) where only mold scores were used. In that study there was sufficient difference among the lines that presumably arose from natural inoculation to identify other defense related genes. This study analyzed the same data from a different point of view; SNPs seen here reflect changes from the control generated when lines were challenged with *A. alternata* alone or in combination with *F. thapsinum* and *C. lunata* compared to untreated water sprayed control. Eleven accessions, including PI576130, PI656036, PI656051, and PI533871 were resistant when inoculated with *A. alternata* alone while 7 accessions, including PI576130, PI656036, and PI566819 were resistant when challenged with *A. alternata*, *F. thapsinum* and *C. lunata* mixture (Prom *et al.*, 2020). Mycoflora analysis revealed that *A. alternata* was present in 23% of the seeds obtained from panicles inoculated with *A. alternata* alone, and 34.8% (*F. thapsinum*), 16.7% (*C. lunata*), and 12% (*A. alternata*) of the seeds obtained from panicles inoculated with the mixture (Data not shown). As such, it is predicted that the alternate alleles will reflect differences in enzyme activity or level of expression related to mold response. The newly detected SNPs identify more candidate genes that may be useful for future breeding efforts to enhance resistance to grain mold.

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