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Antifungal Proteins in Commercial Hybrids and Elite Sorghums

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The antifungal proteins (AFP) in the caryopsis of commercial and elite sorghums grown in several environments were related to grain mold resistance. Previous studies revealed that improved grain mold resistance was correlated with higher levels of AFP at combine harvest maturity [50 days after anthesis (DAA)] or with better retention of AFP after physiological maturity (30 DAA). Commercial hybrids and public parental lines and hybrids were grown in College Station, TX, during 2000, 2001, and 2002. Samples of caryopses were collected at 30 and 50 DAA, and caryopsis proteins were extracted, blotted, identified with immunoassays, and quantified for two AFP (chitinase and sormatin). Sorghums varied in amounts of AFP and their ability to retain AFP after physiological maturity. The environment in 2002 was conducive for fungal deterioration of grain, and a wide range of AFP and grain molding was observed at harvest maturity. High levels of AFP and low levels of grain molding were observed in non-mold-conducive environments in 2000 and 2001. Commercial and elite sorghums with higher levels and increased retention of AFP had less grain molding in the mold-conducive environment.

KEYWORDS: Chitinase; sormatin; antifungal proteins; grain mold; plant resistance

INTRODUCTION

Grain molding of sorghum results from colonization by fungi (*Fusarium thapsinum*, *Curvularia lunata*, *and Alternaria alternata*) of the developing grain and is associated with warm, humid environments during caryopsis development (*1*). Some of these colonizing fungi also produce mycotoxins, which are harmful to humans and livestock. Initially, fungal growth causes surface discoloration and, if environmental conditions favor mold development, the pathogen eventually breaks down the components of the grain. These changes decrease milling and processing yields and the quality of sorghum for feed or food. Although genetic improvement of sorghum has improved the level of mold resistance in sorghum, it does not result in complete control of the disease. Unfortunately, control of grain mold by using fungicides and crop management strategies is not economically feasible (*2*).

Genetic control of grain mold resistance is a complex interaction of qualitatively and quantitatively inherited traits (*3, 4*). Several simply inherited caryopsis traits are known to contribute to grain mold resistance, specifically tannins, harder endosperm, and red pericarp color (*3, 5*). In addition, antifungal proteins (AFP) are implicated in improving grain mold resistance (*6*). By selecting in grain molding environments, breeders have improved tolerance to grain molding and weathering. However, it is not complete and is overcome in hot and humid environments (*2*).

Several proteins that are constitutively expressed in developing cereal caryopses have antifungal properties, either in vivo or in vitro. Studies on sorghum grains (*6*−*8*) have identified three proteins of 18, 26, and 30 kDa that affected hyphal growth of *F. thapsinum*. The 18 kDa component removes cell wall polysaccharides, whereas the other two increase leakage of cytoplasmic contents. The 18 kDa protein could be an enzyme acting on cell walls, whereas the 26 and 30 kDa components could be related to permeatins. High levels of AFP were detected in hard-endosperm, grain mold resistant sorghums (*9*).

Positively charged, water-soluble proteins belonging to the permeatin group (designated sormatin), chitinase, β-1,3-glucanase, or ribosome-inactivating proteins (RIP) have been identified in sorghums (*5*). The synthesis or extractability of sorghum antifungal proteins increased for several AFP until physiological maturity and then decreased during desiccation of the grain (*7*).

The levels of AFP in sorghum caryopses appear to be high enough to inhibit fungal growth. Sunitha et al. (*7*) reported that the level of AFP in sorghum was 7 μg/caryopsis, whereas 6.8−15 μg/dose was required for inhibitory activity to *Fusarium* sp. An antifungal protein fraction mixture (3.6 μg/dose) containing sormatin, chitinase, glucanase, and RIP inhibited spore germination of *F. thapsinum*, *C. lunata*, and *Aspergillus flavus* (*7*). Hyphal rupture at the growing tips was observed for *F. thapsinum* at 7.0 μg/dose and for *C. lunata* at 7−36 μg/dose, but was not observed for *A. flavus* even at 165 μg/dose. The amount of AFP in physiologically mature sorghum caryopses could inhibit germination and growth of *F. thapsinum* and perhaps *C. lunata*, depending upon how the proteins are concentrated in the pericarp during imbibition (*10*).

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Table 1. Average Sorghum Antifungal Proteins (30 and 50 DAA) and Average Mold Rating at 50 DAA for Each Harvest Year[a]

	2000	2001	2002
grain mold rating[b] (at 50 DAA)	1.4 b	1.1 c	3.0 a
cultivars (total no.)	97	97	42
Tan Plant Hybrid Test	36	40	18
State Performance Hybrid Test	13	32	0
Grain Weathering Trial	39	26	24
others	10	0	0
chitinase			
30 DAA[b] (μg/g)	23 b	56 a	12 c
50 DAA[b] (μg/g)	25 b	52 a	10 c
change in chitinase[b,c] (%)	–3 a	–12 b	–42 c
sormatin			
30 DAA[b] (μg/g)	1.7 c	2.9 a	2.1 b
50 DAA[b] (μg/g)	1.9 b	3.7 a	1.2 c
change in sormatin[b,c] (%)	7 a	17 a	–120 b

[a] Field laboratory at College Station, TX. [b] Means within a row with the same letter are not significantly different ($p = 0.05$). [c] Change in AFP content from 30 to 50 DAA.

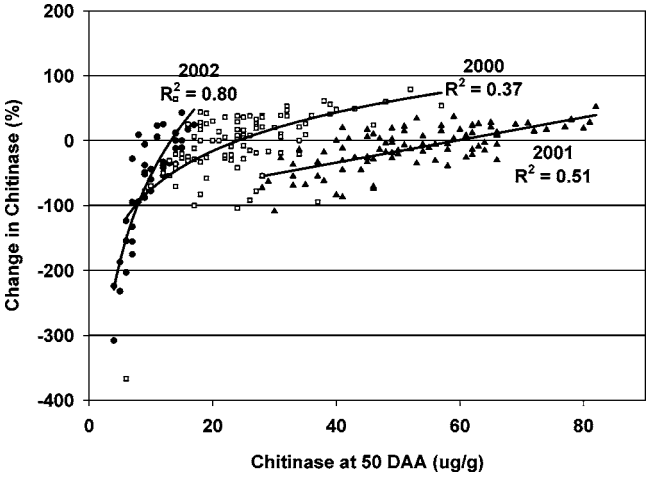

Figure 1. Effect of environment and genotype on chitinase in sorghum caryopsis at combined harvest maturity (50 DAA) and change in chitinase from 30 to 50 DAA ($p = 0.05$).

Sorghum produces AFP in response to fungal infection. Sunitha et al. (*8*) found that sormatin-like proteins were induced after fungal infection. Bueso et al. (*11*) also found that sormatin was induced by infection in vivo in resistant sorghums when the sorghum caryopses were stressed with grain mold fungi at anthesis and sampled at physiological maturity. Also, periodic misting of panicles in the field, similar to in vitro imbibition, decreased levels (or extractability) of AFP in susceptible sorghums at physiological maturity.

Rodriguez et al. (*12*) compared the levels of four AFP in eight mold resistant and eight susceptible lines derived from progeny of a susceptible by resistant cross. Natural infection with grain molds in eight environments over three years resulted in the induction and/or retention of more AFP in the resistant lines, suggesting that the coexpression of four AFP may be required to confer resistance in lines with a nonpigmented testa. The β-1,3-glucanase levels in resistant lines, however, did not increase as much as those of other AFP. Chitinase, sormatin, and RIP concentrations were 1.5−14-fold higher in the resistant lines compared to susceptible lines and were associated with grain mold resistance.

Due to the nature of the research, previous studies evaluated small numbers of sorghum genotypes (*11*, *12*). A larger and more diverse collection of elite and commercial materials needs to be evaluated to determine if these observations apply to sorghum grown using normal agronomic practices and typical environments. In the current study, the amounts and retention of AFP in caryopses during development were quantified in a diverse collection of elite and commercial materials over several years. This research should indicate the applicability of AFP in host plant resistance to grain molding.

MATERIALS AND METHODS

Plant Materials. Sorghum caryopses were collected at two stages of maturity in 2000, 2001, and 2002 (**Table 1**). Sorghum genotypes known to be resistant and susceptible to grain mold were included along with the elite breeding lines and commercial hybrids. Caryopses were sampled at physiological maturity [30 days after anthesis (DAA)] and at combine-harvest maturity (50 DAA). Samples were stabilized by immediately placing them in an ice chest in the field and transferring them to a −60 °C freezer upon arrival in the laboratory. All sorghum samples were lyophilized to remove moisture and to increase analysis efficiency.

In 2000 and 2001, 97 sorghum genotypes were sampled, and 33 of these genotypes were sampled in both years. These genotypes came from the Grain Weathering Test (GWT), the State Hybrid Performance Test (STPT), and the Tan Plant Hybrid Trial (TPHT). In 2002, 42 sorghum genotypes were sampled, and these samples were taken from the GWT and TPHT plots in 2002 (**Table 1**). Twenty-two genotypes were sampled in all three years of the study.

Grain mold ratings were assessed in the field by visually estimating severity, based on a 1−5 scale where 1 = no mold, 2 = 1−10% molded grain, 3 = 11−25% molded grain, 4 = 26−50% molded gain, and 5 = >50% molded grain (*13*).

Determination of AFP Content. For sorghums grown in 2000, ground samples (0.1 g) were mixed with 1 mL of extraction buffer with agitation for 1 h to extract soluble proteins (*11*, *15*). After centrifuging, the supernatant was collected and mixed with sample buffer, boiled, and loaded into 15% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane (Western blotting), rinsed, and blocked. Blocked membranes were soaked with the respective antibody (chitinase or sormatin) (*10*, *11*, *15*). After >2 h of incubation, membranes were rinsed and incubated in affinity-purified goat anti-rabbit IgG antibody for >1 h. Blots were then rinsed and proteins visualized. The buffer-extracted proteins from samples in 2001 and 2002 were directly loaded into the nitrocellulose membrane using the dot-blot manifold. The Western and dot-blot procedures yielded similar results (data not included). Thus, AFPs were quantified more efficiently using the dot-blot procedure.

Statistical Analysis. Analysis of variance was used to determine year effects. Mean comparisons were conducted using Fisher's least significant difference test at the 5% probability level (SAS Institute, Cary, NC). Relationships between gain mold rating and AFP were calculated using Pearson's simple correlation test.

RESULTS

Grain mold ratings were significantly different across years (**Table 1**). Grain mold ratings of individual sorghum genotypes at combine-harvest maturity in 2000 and 2001 ranged from 1.0 (no mold) to 2.0 (1−10% molded grain) (**Table 1**). Environmental conditions during the growing seasons did not promote grain molding or weathering of sorghum, which were considered clean and relatively mold-free. In 2002, significantly higher grain mold ratings were observed, as hot and humid conditions were favorable for mold development. The grain mold ratings ranged from 1.0 to 5.0, and this environment was excellent for discriminating among genotypes for their respective level of grain mold resistance.

The average AFP contents varied across years (**Table 1**). Both chitinase and sormatin were higher in 2001 and lower in 2002

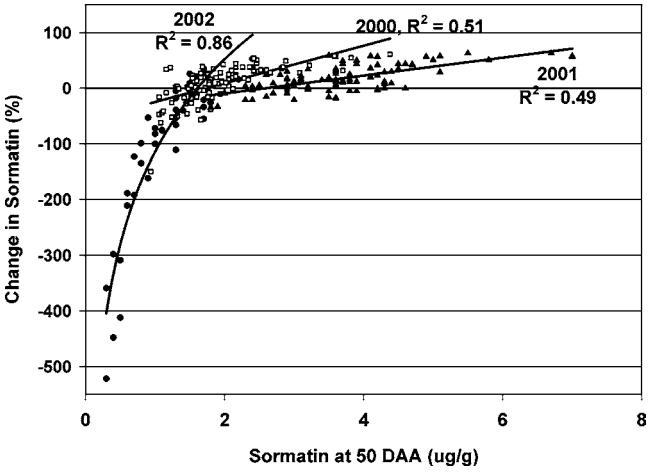

Figure 2. Effect of environment and genotype on sormatin in sorghum caryopsis at combined harvest maturity (50 DAA) and change in sormatin from 30 to 50 DAA ($p = 0.05$).

(**Figures 1** and **2**). Hence, AFP contents were higher when there was less mold infection and caryopsis deterioration and lower for all sorghums when susceptible sorghums were mold damaged. The chitinase and sormatin contents of individual sorghum genotypes varied widely as well (**Figures 1** and **2**).

Some sorghums had more AFP at 50 DAA than at 30 DAA as indicated by a positive change in AFP (**Figures 1** and **2**), whereas other sorghums retained similar levels of AFP or had lower levels of AFP. The range of changes in chitinase from 30 to 50 DAA was larger in 2002 than in 2000 or in 2001 (**Figure 1**). The range of changes in sormatin from 30 to 50 DAA was also larger for 2002, but those of 2000 and 2001 were similar (**Figure 2**).

To illustrate the effects of environment and genotype on AFP and grain mold ratings, data from the 22 genotypes evaluated in all three years were evaluated (**Figures 4** and **5**). The same trends in AFP levels and changes at 50 DAA observed in the complete data set (**Figures 1** and **2**) were observed in the subset data (**Figures 4** and **5**). Thus, fewer AFP at 50 DAA and increased loss of AFP from 30 to 50 DAA occurred in the mold-conducive environment.

Table 2 shows that mold resistance is increased (lower grain mold ratings) when hybrids have RTx436 as the male parent. On the other hand, hybrids have higher grain mold ratings if they have RTx430, a cultivar known to be susceptible to fungal colonization, as the male parent. Consequently, more AFP are retained from 30 to 50 DAA in hybrids having RTx436 as the male parent than in hybrids with RTx430.

Sorghum genotypes more resistant to mold infection in 2002 are listed in **Table 3**. All cultivars, except A8PR1059*BRON139, had AFP levels at 50 DAA ranging from 8.4 to 16.9 μg/g chitinase and from 0.9 to 2.2 μg/g sormatin. The A8PR1059*-BRON139 hybrid had low chitinase and sormatin levels, were later flowering with a more humid environment during grain development, and had a red pericarp color.

DISCUSSION

Sorghums at 30 DAA, (physiological maturity) in each year of the study were either mold-free to only slightly infected. A wide range of mold ratings, however, was observed among the sorghums at 50 DAA (combine-harvest maturity) in 2002 (**Figure 3**). Sorghums with higher mold ratings had large negative values for change in chitinase and sormatin levels from 30 to 50 DAA. Mold ratings of sorghums at 50 DAA correlated

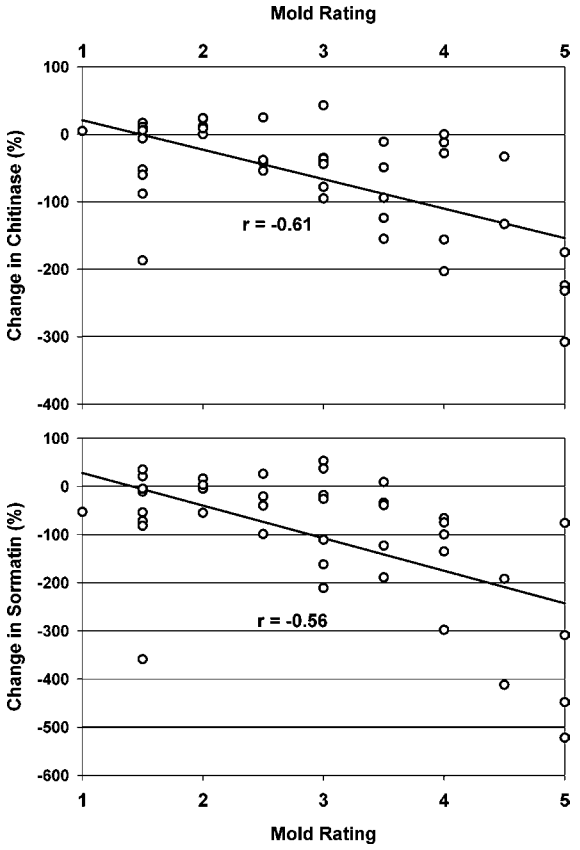

Figure 3. Scatter plots of the change in chitinase (top) or sormatin (bottom) from 30 to 50 DAA with grain mold rating of 2002 sorghums ($p = 0.05$).

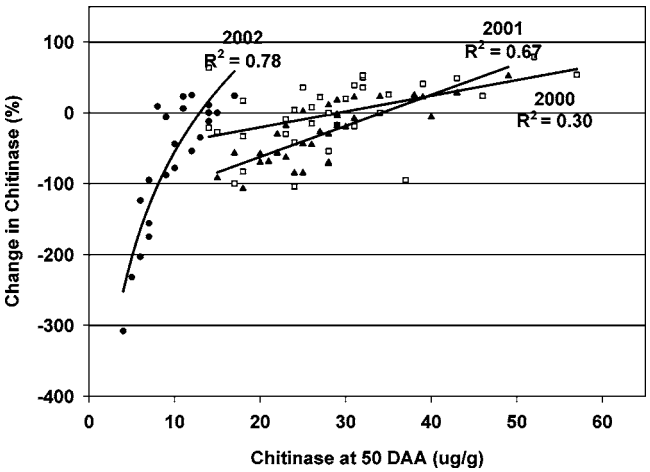

Figure 4. Comparison of specific sorghums evaluated each year for chitinase at 50 DAA and change in chitinase from 30 to 50 DAA ($p = 0.05$).

negatively with the changes in both chitinase ($r = -0.61$; $p = 0.05$) and sormatin ($r = -0.56$; $p = 0.05$) contents. Thus, the ability of sorghums to resist fungal invasion was directly and positively influenced by their retention/production of AFP. Hence, AFP losses are somehow related to diminished resistance (or susceptibility) to grain molding and are observed only in mold-conducive environments during the later stages of grain maturity.

AFP in sorghum caryopses were retained at a higher rate in environments that are not favorable for grain mold development. However, in environments conducive for grain mold development (specifically, 2002), AFP in many sorghum caryopses were not retained from 30 to 50 DAA. The correlation between grain

Table 2. Effect of Antifungal Proteins on Grain Molding in Selected Sorghum Genotypes at 50 DAA

genotype	seed color	plant color	grain mold rating	sormatin (μg/g)	change in sormatin[a] (%)	chitinase (μg/g)	change in chitinase[a] (%)
ATx631*RTx436	white	tan	1.5	1.0	–72	10.9	6
ATx635*RTx436	white	tan	2.5	0.8	–99	12.2	25
ATx623*RTx430	white	purple	4.5	0.7	–192	11.7	–33
ATx2752*RTx430	red	purple	4.5	0.5	–412	6.5	–133
Tx430	white	purple	5.0	0.4	–448	5.2	–232
ATx378*RTx430	white	purple	5.0	0.3	–522	6.5	–175

[a] Change in chitinase/sormatin from 30 to 50 DAA.

Table 3. Sorghums More Resistant to Mold Infection at Combined Harvest Maturity in 2002

cultivar	seed color	plant color	grain mold rating	sormatin (μg/g)	change in sormatin[a] (%)	chitinase (μg/g)	change in chitinase[a] (%)
Monsanto D69	white	tan	1.0	0.9	–53	10.9	5
96C5986	white	purple	1.5	1.6	21	15.5	17
99CA2244	red	tan	1.5	1.6	–11	9.4	–6
99GWO92	red	tan	1.5	1.7	35	14.3	11
99L-GWO50	red	tan	1.5	1.3	–54	9.3	–52
A8PR1059*BRON139	red	tan	1.5	0.3	–359	4.6	–187
ATx631*RTx436	white	tan	1.5	1.0	–72	10.9	6
ICSV1089BF	white	tan	1.5	1.3	–5	8.7	–88
Tx2911	red	purple	1.5	1.0	–82	10.3	–60
90B2662	red	purple	2.0	2.2	16	10.8	23
90L19178	white	purple	2.0	1.4	53	13.5	43
R4317	red	purple	2.0	1.3	–5	14.6	0
SC650-11E(t)	red	purple	2.0	1.7	–55	16.9	24
SC719011E	red	purple	2.0	1.3	3	8.4	9
av			1.6	1.3	–41	11.3	–18

[a] Change in chitinase/sormatin from 30 to 50 DAA.

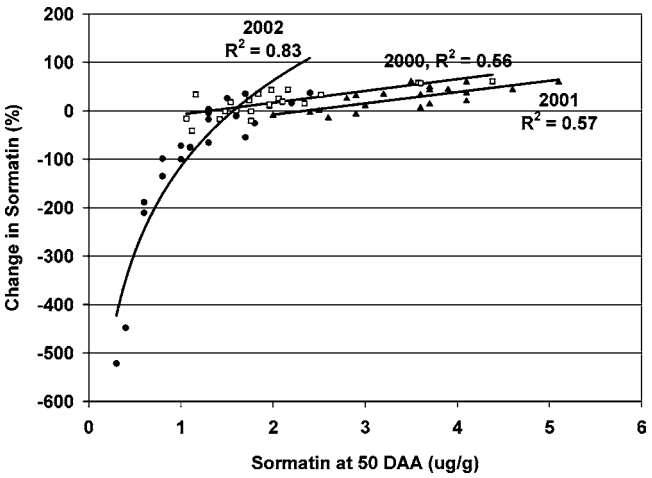

Figure 5. Comparison of specific sorghums evaluated each year for sormatin at 50 DAA and change in sormatin from 30 to 50 DAA ($p = 0.05$).

mold rating and AFP content was significant in 2002; grain mold ratings were not significantly correlated to AFP contents of caryopses at 50 DAA in 2000 or 2001. Thus, an environment favorable for grain mold is required to assess the role of AFP in plant host resistance against grain mold pathogens. Previous papers support the conclusion that environment significantly affects the levels of AFP in resistant and susceptible sorghums (*12*).

The amount of antifungal proteins in sorghum caryopses is not sufficient to predict their resistance to fungal infection in the field (*11*). Instead, the percent change in AFP (from 30 to 50 DAA) significantly and negatively correlates to the observed grain mold ratings (**Figure 3**). Hence, when environmental and fungal stresses are imposed on sorghum cayopses, grain mold resistance is correlated with the retention or increased production of AFPs. This hypothesis is consistent with the results of the present study. Greater losses of AFP in caryopses correspond to more fungal colonization or weathering of sorghum (**Figures 3**−**5**). The absolute values of the AFP are not as important as the retention of AFP during the two weeks after physiological maturity. This is probably why early studies were not able correlate AFPs with grain mold resistance (*15*).

Table 3 shows that cultivars more resistant to mold had an average mold rating of 1.6, whereas for all the cultivars, it was 3.0 (**Table 1**). Consequently, the average chitinase loss from 30 to 50 DAA for all cultivars (**Table 1**) was more than twice that of the more resistant cultivars (**Table 3**). The average sormatin loss for all cultivars was 3 times more than that of the mold resistant cultivars. Thus, the severity of mold infection is directly related to loss of AFP from 30 to 50 DAA.

Table 2 shows some indication that in certain hybrids the relationship between mold resistance and AFP is similar to that of their male parent. Therefore, more research in needed to determine the inheritance of AFP.

Sorghums with red pericarp contain phenolic compounds, some of which are known to contribute to mold resistance (*16*). The most resistant cultivar, Monsanto D69, however, has white pericarp color. Of the more resistant genotypes, nine cultivars have red pericarp and five have white pericarp color (**Table 3**). Moreover, three of the most susceptible genotypes, ATx2752*-RTx430, SC279-14E, and Mycogen 1506, have red pericarp. Thus, having a red pericarp alone does not ensure mold resistance.

The grain mold resistance of sorghum appears to be related to AFP, a more predominant factor than the presence of phenolic compounds in red pericarp sorghums. This study supports the

significant and positive influence of AFP in caryopses to limit fungal colonization and weathering. Sorghums can therefore be classified as mold resistant or susceptible on the basis of their AFP content and retention. This provides breeders/producers with a basis for developing and selecting sorghums with grain mold resistance.

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