Antifungal Proteins and Grain Mold Resistance in Sorghum with Nonpigmented Testa

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Levels of four antifungal proteins (AFPs) were determined in mature caryopses (40−45 days after anthesis) of eight grain mold resistant (GMR) and eight susceptible (GMS) sorghum lines using the immunoblot technique. These 16 lines came from the same cross and were selected for high and low grain mold resistance. The 16 lines were grown in eight environments over three years. In the environments with grain mold incidence, levels of sormatin, chitinases, and ribosomal inactivating proteins (RIP) in the GMR group were higher than those in the GMS group. In a grain mold-free environment, the GMR group had higher RIP and lower β-1,3-glucanase levels than the GMS group. Unlike the GMS group, chitinase, sormatin, and RIP levels in the GMR group were higher in the environments with grain mold than in the mold-free environment. AFPs correlated among themselves and with grain mold resistance. Grain mold infection pressure caused GMR lines to induce and/or retain more AFPs compared to GMS lines. The coexpression of these four AFPs may be a necessary prerequisite for resistance to grain mold in sorghums without a pigmented testa.

Keywords: *Antifungal proteins; sormatin; chitinase; glucanase; ribosomal inactivating proteins; grain mold; Sorghum bicolor (L.) Moench*

INTRODUCTION

In many regions of the world, grain mold is a major disease of sorghum (*Sorghum bicolor* L. Moench). Grain mold is damaging because it reduces sorghum grain yield and quality, which affects the nutritional and market values of the sorghum crop. The disease is caused by an array of fungal species, with the most prominent being *Curvularia lunata*, *Fusarium* spp., *Alternaria* spp., *Phoma sorghina*, and *Dreschlera* spp. (Castor, 1981). In addition to the numerous fungal species that can cause the disease, the environment strongly influences the development of the disease. Wet and humid weather is very conducive to the development of this disease.

Controlling grain mold in sorghum has been very difficult. Chemical control is cost-prohibitive, and biological control mechanisms have not been feasible. The most cost-effective and realistic method to control grain mold is through the use of genetic resistance. However, selection for grain mold resistance is difficult, because numerous genetic factors are reported to influence it. Plant traits such as panicle shape, plant height, and glume structure have been associated with grain mold resistance (Castor, 1981; Rao and Rana, 1989). Caryopsis traits such as endosperm density, a pigmented testa layer, and pericarp color are related with grain mold resistance (Glueck and Rooney, 1980; Esele et al., 1993). However, none of these traits solely explain the variation in grain mold resistance found in sorghum. This indicates that additional factors underlay the visible factors in the expression of grain mold resistance.

One potentially important factor that may enhance grain mold resistance is the content of antifungal proteins (AFPs). Proteins potentially inhibitory to fungal growth have been identified in sorghum endosperm. These proteins have also been detected in pearl millet and corn (Kumari and Chandrashekar, 1994). In sorghum, more intense deposition of protein bodies has been observed in grain with hard endosperm than in soft grains, and extracts of immature and mature hard and soft endosperm were inhibitory to *Fusarium moniliforme* growth (Kumari and Chandrashekar, 1992). Seetharaman et al. (1996) reported that sormatin, chitinase, and glucanase levels increase during caryopsis development, were high at physiological maturity, and decreased at combine harvest maturity of the grain. Ribosomal inactivating protein (RIP) levels were high at 15 days after anthesis (DAA) and then subsequently decreased. A mixture of several AFPs extracted from sorghum caryopsis was most inhibitory against *F. moniliforme*, *C. lunata*, and *A. flavus* (Seetharaman et al., 1997).

Sormatin is a thaumatin-like protein, that is, a small, basic protein (~22 kDa) with potent antifungal activity against a wide variety of fungi in vitro (Seetharaman et al., 1997). Sormatin acts by causing membrane permabilization (Vigers et al., 1991). A high concentration of sormatin has been reported in sorghum seeds (Darnetty et al., 1993; Seetharaman et al., 1996, 1997).

β-1,3-Glucanases have been found in many plants, range in molecular mass from 21 to 31 kDa, and are found in both extracellular and intracellular spaces (Boller, 1985). One β-1,3-glucanase with an estimated molecular mass of 30 kDa was identified in sorghum (Darnetty et al., 1993). Glucanase levels increased during sorghum caryopsis development and peaked at 30 DAA (Seetharaman et al., 1996). The suggested role of β-1,3-glucanases in plant defense is based on their inhibitory effect of the in vitro growth of pathogenic fungi (Mauch et al., 1988).

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Chitinases are enzymes of 25−35 kDa molecular mass that hydrolyze the *N*-acetylglucosamine polymer, chitin (Yun et al., 1997). In vitro studies have established that plant chitinases readily attack and degrade chitin, a compound of fungal cell walls (Boller, 1985). A coordinated induction of expression for three chitinase isoforms was observed in maize seeds in response to infection by the fungus *F. moniliforme* (Cordero et al., 1994). One chitinase of ∼29 kDa and two or three additional chitinases ranging in size from 21 to 24 kDa have been reported in sorghum (Darnetty et al., 1993). Chitinase levels increased during sorghum caryopsis development and peaked at physiological maturity (Seetharaman et al., 1996).

RIPs have molecular masses between 28 and 31 kDa and are stable, basic proteins found in seeds, roots, leaves, and sap of many plants (Darnetty et al., 1993). RIP inhibit protein synthesis in target cells by specific RNA *N*-glucosidase modification of 28 s-RNA (Logeman et al., 1992). Barley RIP antibody had no cross-reacting bands with sorghum proteins (Darnetty et al., 1993). However, maize RIP antibodies cross-reacted with a 30 kDa sorghum protein (Seetharaman et al., 1996).

Some studies clearly indicate that sorghum produces AFPs and these AFPs inhibit grain mold pathogens in vitro, but a relationship between AFP content and grain mold resistance has not been proven in vivo. Seetharaman et al. (1996) found no significant correlation between grain mold incidence and sormatin levels in caryopsis at 15 and 50 DAA, in 17 inbred lines of sorghum, whereas a significant correlation was found between grain mold incidence and sormatin content at 30 DAA. Bueso (1997) found no correlation between AFP content and grain mold resistance in 10 sorghum inbreeds previously documented to differ for grain mold resistance and AFP content. However, in both studies, the genotypes selected for analysis were variable for many phenotypic plant- and caryopsis-based traits known to influence grain mold resistance (Esele et al., 1993). Thus, attempts to correlate grain mold resistance with a single specific trait (i.e., AFP content) using inbred lines with different genetic backgrounds would be a difficult task.

To determine if AFP content influences grain mold resistance, it is necessary to eliminate or minimize variation for traits that are known to influence grain mold resistance. The goal of this research is to determine if AFP content is correlated with grain mold resistance in germplasm with a common genetic background. Specifically, the objectives of the present study were (1) to determine the AFP content in a set of grain mold susceptible (GMS) and resistant (GMR) lines with identical pedigrees in several different environments and (2) to correlate AFP content with grain mold resistance in the sorghum germplasm.

MATERIALS AND METHODS

The experimental germplasm for this study consisted of 16 $F_{2:5}$ lines selected from a recombinant inbred line population developed from the cross of Sureño × RTx430. Sureño is a dual-purpose grain and forage variety with good resistance to grain molding (Meckenstock et al., 1993). RTx430 is a widely adapted sorghum inbred line that is commonly used as the male parent in many U.S. sorghum hybrids, but it is extremely susceptible to grain mold (Miller, 1984). Because both Sureño and RTx430 have a white pericarp and a thin mesocarp and lack a pigmented testa, these traits are eliminated as factors that will influence differences in grain mold resistance between these lines. From the recombinant inbred line population, the eight most GMR and eight most GMS lines were selected on the basis of grain mold resistance data collected from eight environments. Sets of eight GMS and GMR lines were used because RTx430 and Sureño are different for several other phenotypic traits known to influence GMR. Analysis of the set of GMS and GMR should minimize the effects of these traits.

Evaluation Sites. In 1995, the inbred lines were planted at Beeville, TX, on March 25, and at College Station, TX, on April 12. These environments are subsequently denoted BE95 and CW95, respectively. In the last experiment, sprinkler irrigation was used during grain development to enhance the grain mold incidence. In 1996, the test was planted at Halfway, TX, and College Station, TX, under two moisture levels (with and without sprinkler irrigation). These environments are subsequently denoted HW96, CD96, and CW96, respectively. In 1997, the test was planted at Beeville, TX, and College Station, TX (under two moisture levels). These environments are subsequently denoted BE97, CD97, and CW97, respectively.

Mature caryopses from 10 panicles of the same $F_{2:5}$ derived line were harvested at grain maturity (40−45 DAA) in each location to determine the level of AFP. Caryopses were hand-threshed, cleaned, lyophilized, and stored frozen (−10 °C) for up to 1 year. Mature grain from BE97 and CW97 environments was inadvertently stored for 6 months at ambient temperature prior to determination of the AFP content.

Experimental Design. The 16 $F_{2:5}$ lines were grown at each location in a randomized complete bock design with two replications. The rows were 6.3 m in length, with a row spacing of 0.76 m. At all environments but Halfway (HW97), significant levels of grain mold occurred naturally; therefore, inoculation was not necessary. AFP data were ranked and analyzed by nonparametric procedures (Eskridge, 1995). Comparisons between GMR and GMS groups were done using orthogonal contrasts (Lentner and Bishop, 1993). Spearman's rank correlation was used to estimate association among AFP levels (Eskridge, 1995). Because grain mold did not occur in Halfway, this location was used to estimate the AFP content in sorghum caryopses in the 16 lines grown at a location without grain mold incidence.

Disease Evaluation. In all experiments, grain mold was rated at 40−45 days after flowering. Grain mold was recorded on a 1−5 scale, where 1 = seed bright, free from mold damage, and 5 = very susceptible, embryos dead, and endosperm deteriorated (Frederiksen et al., 1991).

Sample Preparation and Immunoblotting. Samples were extracted and immunologically assayed (Western blots) for AFPs using the procedure described by Vigers (1992). Relative amounts of sormatin, glucanase, RIP, and chitinase were calculated by measuring the band intensities of known amounts of pure zeamatin, glucanase, chitinase, and RIP, respectively, in each immunoblot. Blots were scanned using a Hewlett-Packard ScanJet 3C and saved as a *.tif file. The images and band intensities were determined using NIH Image software version 1.4 to obtain pixel density values for each blot. Band intensities were then transformed into concentration values of AFPs (micrograms per caryopsis) by comparing them to the Dorado standard intensity in the same blot, for which AFP concentration was known and established as constant for all blots.

RESULTS AND DISCUSSION

AFP Content in Sorghum Caryopses under Grain Mold Incidence. High levels of grain mold incidence were observed at all environments except Halfway, 1996 (Figure 1). Levels of four AFPs were significantly affected by the 16 $F_{2:5}$ lines in all environments (Figures 2 and 3). Higher levels of sormatin were observed in the GMR lines than in the GMS lines in every location. This strongly suggests that sormatin is associated with grain mold resistance. Orthogonal contrasts showed that the β-1,3-glucanase levels were significantly higher in

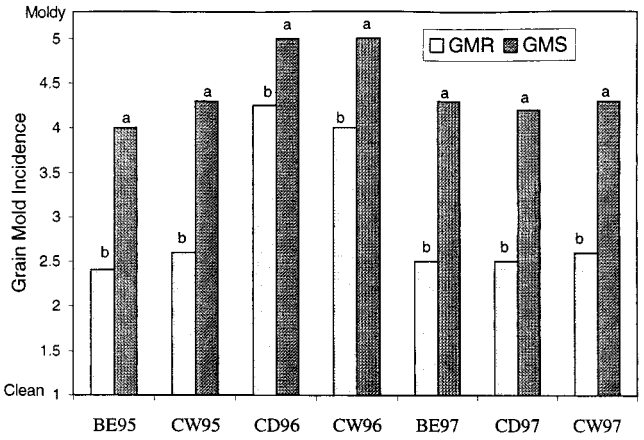

Figure 1. Grain mold incidence (continuous scale: 1 = none; 5 = deteriorated) in mature caryopses of eight GMR and eight GMS lines grown in seven environments. Within environments, means followed by the same letter are not significantly different.

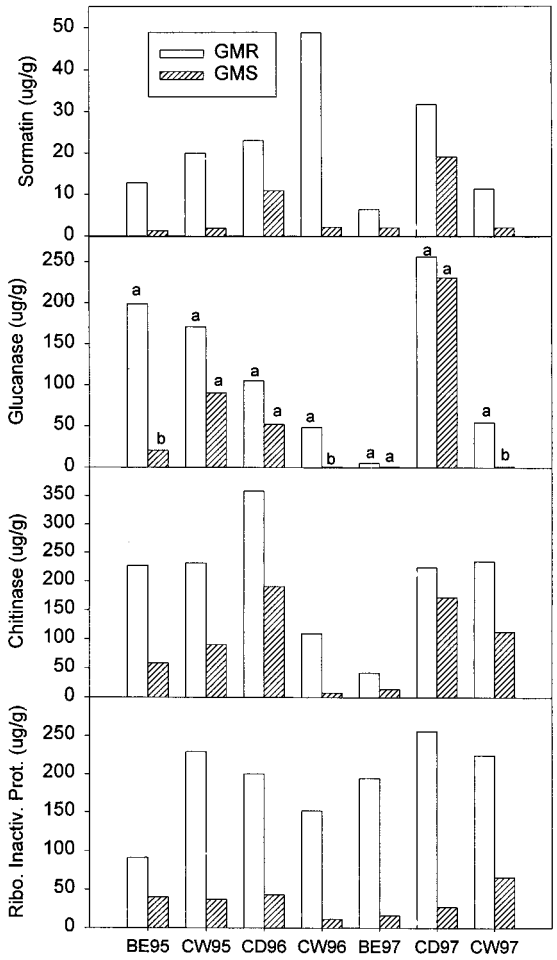

Figure 2. AFP levels (micrograms per gram) in mature caryopses of eight GMR and eight GMS lines grown in seven environments. Resistant lines have more AFP than susceptible lines except for glucanase. Glucanase means followed by the same letter are not significantly different (within environments).

the GMR group in only three of seven environments. This suggests that β-1,3-glucanase may be associated with grain mold resistance in sorghum. However, its function on grain mold resistance may not be as direct as that of other AFPs or the β-1,3-glucanase level in plants may also be a result of other mechanisms of

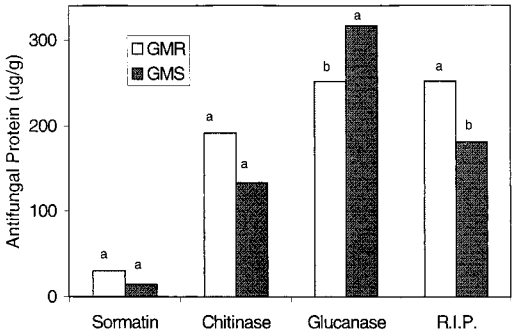

Figure 3. AFP levels (micrograms per gram) in mature caryopses of eight GMR and eight GMS lines grown at Halfway, TX, in 1996. Within each AFP, means followed by the same letter are not significantly different.

response, different from a disease-resistance response. Chitinase concentrations in the GMR lines were 1.5−15-fold higher than the chitinase content in the GMS group and were associated with grain mold resistance. Because levels of RIP in the GMR lines were 1.5−14 times higher than in the GMS lines, RIP may contribute to the resistance mechanisms of sorghum to grain mold.

The GMR lines contained more AFP than did the GMS lines even when caryopses were stored for 6 months at ambient temperature (BE97 and CW97 environments). Similar stability of AFP to degradation has been reported previously. RIP (saporin) is resistant to denaturing agents and proteolytic degradation (Stirpe et al., 1992). Bioactive zeamatin-like protein could be recovered from insect cell culture supernatants stored at 8 °C for >6 months (Malehorn et al., 1994).

Amounts of β-1,3-glucanases, chitinases, RIPs, and sormatin (up to 50 μg/g) were considerably higher than the concentration (3−10 μg mL^{-1}) at which zeamatin is antifungal in vitro (Darnetty et al., 1993). Therefore, it is possible that AFPs present in sorghum caryopses could limit fungal colonization and deterioration of grain either directly or indirectly. Certain fungi may be more susceptible to the hydrolases (chitinases and β-1,3-glucanases), whereas other fungi may be sensitive to sormatin or RIP (Darnetty et al., 1993). Some AFPs have an indirect role in plant resistance (Takeuchi et al., 1990); that is, β-1,3-glucanases may also be involved in the stimulation of the plant defense reaction by releasing elicitors from fungal cell walls that can stimulate phytoalexin accumulation in the host plant.

Caryopsis AFP Content under Grain Mold-Free Environments. Sormatin and chitinase contents were not significantly different ($P < 0.05$) between GMR and GMS lines at Halfway in 1996 (Figure 3). This supports the constitutive expression of sormatin in caryopsis. Sormatin content in caryopses of GMR and GMS lines grown under a grain mold-free environment was higher than that in lines grown in environments with grain mold incidence. There are several possible causes of this result. GMR lines may maintain higher levels of sormatin for longer periods of time than GMS lines under grain mold pressure and/or GMR lines may induce more sormatin production than GMS lines upon infection. Bueso (1997) reported that GMR lines had higher levels of sormatin 1 or 2 weeks after being stressed with grain mold fungal species compared to GMS lines.

Chitinase level in GMR lines was higher under grain mold incidence than in the grain mold-free environment, but the opposite was observed in GMS lines. This suggests that infection of caryopses induces accumula-

Table 1. Correlation Estimates[a] among Four AFPs in Mature Caryopses of Eight GMR and Eight GMS Lines Grown in Eight Environments

	environments with moderate grain mold incidence							low mold
AFP	BE95	CW95	CD96	CW96	BE97	CD97	CW97	HW96
sormatin versus								
glucanase	0.71**	0.44*	0.07	0.59**	0.07	0.42*	0.42**	0.07
chitinase	0.78**	0.58**	0.17	0.73**	0.17	0.29	0.43*	0.17
RIP	0.79**	0.67**	0.32	0.74**	0.32	0.54**	0.71**	0.12
glucanase versus								
chitinase	0.71**	0.37*	0.27	0.44*	0.27	0.43*	0.47**	0.14
RIP	0.69**	0.55**	0.27	0.49**	0.27	0.16	0.44*	0.04
chitinase versus								
RIP	0.78**	0.49**	0.47**	0.63**	0.47**	0.44**	0.60**	0.42*

[a] *, **, significant differences at 0.05 and 0.01 probability levels, respectively.

tion of chitinases in GMR lines but not in GMS lines. A similar increase in chitinase following infection was observed in sorghum by Bueso (1997). Resistant tissues accumulate chitinases more rapidly and in some instances to higher final concentrations than susceptible tomato tissues (Punja and Zhang, 1993).

Variation due to genotypes for β-1,3-glucanases was significant ($P < 0.05$) at HW96 (Figure 2), and the GMS group had higher levels of β-1,3-glucanases (Figure 3). No clear conclusion can be derived from these results. Although β-1,3-glucanase gene expression is induced by fungal attack and elicitors (Lamb et al., 1989), glucanases are also differentially regulated in tissue and during development by various inducers including stresses, hormones, and other chemicals (Felix and Meins, 1987). Therefore, ascertaining the roles of β-1,3-glucanase either in plant development or in defense response has been elusive (Yun et al., 1997).

RIP content in the GMR group was significantly higher than in the GMS group (Figure 3). GMR lines constitutively expressed higher RIP levels than GMS lines. Comparison of RIP content among different environments showed that RIP content was higher in both the GMR and the GMS groups under grain mold-free environments than under grain mold environments. GMR lines, in addition to constitutively expressing higher RIP levels than GMS lines, responded to fungal infection by inducing or maintaining higher levels of RIP.

Association among AFPs. *Correlation among AFPs under Grain Mold Incidence.* Correlation estimates among the AFPs were positive and significant for most of the environments, except for CD96 and BE97 (Table 1). Heavy rains could have decreased AFP content in caryopses from CD96 because AFPs are mobile and leach from the caryopsis (Seetharaman et al., 1996). Samples of the BE97 experiment were left at room temperature for 6 months before analysis. This fact may have differentially affected the levels of the AFPs in the GMR and the GMS groups, so Spearman's correlation analysis failed to find significant correlations.

A significant and positive Spearman's correlation indicated that under grain mold incidence, as one AFP increases in the sorghum caryopsis, so do the others. Coexpression of all AFPs seems to be a strategic biochemical action of the plant's resistance mechanism to grain mold. In vitro studies with *Trichoderma reesei* and *Fusarium sporotrichioides* demonstrated that a combination of barley RIP and chitinase inhibits fungal growth more efficiently than does either enzyme alone (Leah et al., 1991). Additionally, in vivo, transgenic plants that expressed one or more AFPs exhibited increased protection against fungi (Joch et al., 1995; Zhu et al., 1994). Detailed cytological investigation of bean chitinase transgenic plants revealed that fungal invasion was primarily restricted to the cortex of these transgenic plants and that the hyphore of invading fungi exhibited severe morphological alterations (Benhamou et al., 1993). Hydrolytic activity of chitinase or β-1,3-glucanase could result in an increased uptake of RIP into fungal cells, therefore drastically enhancing the inhibition of the growth of invading fungi (Joch et al., 1995). Recently, transgenic plants with one or more AFPs have been successfully created (Grison et al., 1996). This fact may help to elucidate the role in vivo of AFPs in the host−pathogen interaction.

Correlation among AFPs under a Grain Mold-Free Environment. The correlation estimate between chitinase and RIP was positive and significant ($P < 0.05$) (Table 1). No significant correlations among the other AFPs were found. Normal hormonal and plant developmental signals determine levels of basic chitinases and β-1,3-glucanases in healthy plant roots, floral organs, and seed tissue (Leah et al., 1991). Because AFP levels were affected by environment/fungal pressure and by degree of resistance, proteins that exhibit antifungal properties appear to have an important role in sorghum grain mold resistance. Further studies will determine if GMR lines induce higher levels of AFP upon fungal infection by grain molds than the GMS lines and/or if the GMR lines maintain higher levels of AFP during the infection process than the GMS group.

ABBREVIATIONS USED

AFP, antifungal protein; GMR, grain mold resistant; GMS, grain mold susceptible; RIP, ribosome inactivating protein.

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