Antifungal Proteins and Other Mechanisms in the Control of Sorghum Stalk Rot and Grain Mold

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Research on antifungal proteins and other mechanisms that provide the biochemical basis for host-plant resistance to stalk rot and grain molds is reviewed in this paper. Stalk rot caused by *Fusarium* species leads to substantial yield loss due to poor grain filling and/or lodging. A transgenic sorghum expressing high levels of chitinase exhibited less stalk rot development when exposed to conidia of *F. thapsinum*. Grain mold of sorghum is associated with warm humid environments and results from colonization by several fungi (*F. thapsinum*, *Curvularia lunata*, and *Alternaria alternata*) of the developing caryopsis. The roles of several biochemical mechanisms (tannins, phenolic compounds, red pericarp, proteins, hard endosperm, and antifungal proteins) on grain mold resistance are discussed. Resistance mechanisms related to these compounds appear to be additive, and pyramiding of genes is a feasible approach to limit grain deterioration. Several experimental approaches are proposed to extend current findings.

Keywords: *Antifungal proteins; grain mold; stalk rot; fungal pathogens*

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is used for fodder, feed, food, and beverage. Sorghum is cultivated in tropical and temperate zones and is exposed to a broad range of diseases. Sorghum plants are attacked by fungal, bacterial, and viral pathogens causing root, stalk, foliar, panicle, and caryopsis diseases. We will review biochemical mechanisms to control fungal diseases of the root and stalk before turning to the fungal diseases of the caryopsis.

FUNGAL ROOT AND STALK ROT OF SORGHUM

Fungi cause many severe diseases, including root and stalk rot as a result of infection by *Fusarium moniliforme*, *Fusarium thapsinum*, or *Colletotrichum graminicola*, seedling diseases induced by *Pythium* sp., foliar diseases such as sooty stripe incited by the causal pathogen *Ramulispore sorghi*, and head smut caused by *Sporisorium reilianum*. *Fusarium* stalk rot caused >$80 million lost grain yield in 1996 in Kansas. Yield loss due to stalk rot is attributed directly to lower kernel weight and weakened peduncles or indirectly to lodging and stalk breakage. *Fusarium* sp. are spread by wind, rain, machinery, and insect damage. The fungus penetrates plants directly or via natural openings, such as stomata. It survives as spores or resting structures in soil or plant debris or as hyphae within living plants.

Pathogenesis-related (PR) roteins are inducible plant defenses that restrict the spread of the pathogen in incompatible interactions and allow for systemic acquired resistance. Most research has been carried out with leaves and stalks where there is induction of synthesis mechanisms. A review of such studies made mostly on tobacco was thoroughly covered by Linthorst (*1*). Many members of this group of proteins have in vitro antifungal activity and selectively target cellular components of the pathogen. Included in this group are chitinases and β-1,3-glucanases, which attack the cell walls of fungi, and thaumatin-like proteins (TLP) that affect the permeability of fungal membranes. Genes or cDNAs for these proteins have been isolated from cereals including rice, barley, and wheat (*2*−*4*). Several major cereals (rice, maize, wheat, and barley) have been transformed successfully by both biolistic and *Agrobacterium*-mediated techniques (*5*). Constitutive overexpression of these genes in transgenic rice and wheat plants results in improved resistance to fungal pathogens (*6*, *7*).

Sorghum has been recalcitrant to transformation; however, a gene coding for a rice chitinase was incorporated into an elite sorghum inbred line, Tx430, by a biolistic transformation protocol. Six primary transgenic plants were obtained and were shown to contain the transgene by Southern blot analysis (*8*). The chitinase transgene was inherited in a Mendelian fashion by progeny from these primary transgenic plants and was expressed in the T1 and T2 generations. We have identified one transgenic line that does not segregate for the transgene locus. The chitinase transgene continues to be expressed in T2 and T3 generation plants as measured by western blot analysis of leaf extracts using a chitinase antibody. Plants from the T2 and T3 generations were tested for resistance to infection by inoculating the stalks of mature plants with conidia of *F. thapsinum* (112 conidia mL^{-1}). The development of stalk rot symptoms was significantly reduced in trans-

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10.1021/jf010007f CCC: $20.00
Published on Web 09/27/2001

genic sorghum plants with higher expression of rice chitinase, whereas plants with low or no expression of rice chitinase showed normal disease progression. The constitutive overexpression of rice chitinase in sorghum plants seems to offer protection against the stalk rot disease.

Alternatives to the biolistic procedure have been explored because the biolistic procedure often leads to a high copy number of transgenes, which sometimes result in gene silencing in advanced generation progeny. The gene encoding a rice TLP was introduced into inbred line C-401 using the *Agrobacterium* strain LBA 4404. Several independent transformants appeared to have one or a few copies of the *tlp* gene with good expression of TLP as shown by western blot analysis of leaf extracts using an antiserum.

More TLP (such as osmotin) protects the plants against fungal pathogens as well as against salt and drought stress (*9*). The T2 generation transgenic sorghum plants with a high level of rice TLP were tested for resistance to water stress. Preliminary data indicate that the transgenic plants (1-month-old seedling) with the *tlp* gene also show tolerance to water stress.

FUNGAL CARYOPSIS DISEASES OF SORGHUM

Grain mold of sorghum results from colonization of fungi (*F. thapsinum*, *C. lunata*, and *A. alternata*) of the developing grain and is associated with warm, humid environments during caryopsis development. The major colonizing fungi also produce mycotoxins, which are harmful to humans and livestock (*10*−*12*). Fungal growth causes surface discoloration, initially, and continues, provided warm moist conditions, to break down the components of the grain. These changes decrease milling and processing yields and quality of sorghum for feed or food. Plant breeding efforts have been only partially successful (*13*), whereas control of grain mold by using fungicides and crop management strategies is beyond the means and abilities of many farmers.

Plant traits such as panicle shape, plant height, and glume structure have been associated with grain mold resistance (*14*, *15*). Caryopsis traits such as endosperm hardness, a pigmented testa layer, and red pericarp color are correlated with grain mold resistance (*16*−*19*). Although many sorghums with tannins and higher levels of phenol-based pigments are more resistant to molds, these compounds cause dark colors, astringency, and/or decreased nutritional value in foods or feeds (*20*, *21*). Accordingly, sorghums for food use are grown in environments that are not conducive to deterioration, with a correspondingly lower yield, or cultivars resistant to grain mold are selected, which yield less desirable food. Hence, grain mold resistance is necessary in food-type sorghums.

A review of strategies used by the sorghum grain to combat infestation by fungi is presented here. Included are discussions on fungal colonization, caryopsis structure, phenolic compounds, and antifungal proteins (AFP). We hope our observations lead to the development of newer strategies in the fight against grain mold.

Fungal Invasion. Forbes et al. (*12*) reviewed colonization events in mold-resistant and -susceptible cultivars. During the initial period of flower invasion, *C. lunata* can infect the apical part of the ovary wall from the colonized lemma, palaea, lodicules, filaments, pollen grain, and decaying style (*22*). Mycelium penetrates the pericarp and ramifies through the cross and tube cells within 5−10 days. Generally, the pericarp is not colonized. The placental sac offers a niche for fungal growth that subsequently invades the endosperm and sometimes the embryo as well. *F. thapsinum* appears later and grows beneath the pericarp and then attacks the floury endosperm. Differences in infection patterns of *C. lunata* and *F. thapsinum* are currently being studied (L. Prom, unpublished data).

Mycotoxins. Mycotoxins are less of a problem in sorghum than in maize. Lower levels of fumonisin were found in sorghum (0.7−36.1 ppm) than in maize (5−4000 ppm) (*23*, *24*). Normal sorghums used to prepare Indian foods contain very little fumonisin A or B, which are produced by *F. thapsinum* (*25*). Leslie et al. (*26*) reported that the F mating population of the fungus, which is dominant on sorghum, produces little or no fumonisin, whereas the A mating population, which is abundant on maize, produces more fumonisin. Klittich et al. (*27*) observed that *F. thapsinum* does not produce fumonisin.

Aflatoxins are not found at significant levels in sorghum brought directly in from the field (G. Odvody, personal communication). High levels of mycotoxins, including aflatoxins, are associated with high-moisture storage of sorghum and other cereals. Maize may have excessive levels of aflatoxins, whereas sorghum is aflatoxin-free in similar drought-prone environments. Some sorghums contain another type of toxin, deoxyvarenol, which is an emetic and toxic to rats (*28*−*30*). *Alternaria* also produces toxins, which are confused with metabolites from other fungi (*31*). Ergosterol levels correspond to nonspecific mold colonization of sorghum and have been used to monitor grain molding, but they have not been linked to animal health problems (*32*).

Ergot, *Claviceps africana*, has recently infested sorghums in the Western hemisphere (*33*). The sclerotia contain alkaloids that may cause problems when fed to animals (*34*).

Grain Structure and Development in Sorghum. The structure of the mature grain of sorghum, like all cereals, is composed of the triploid storage endosperm and diploid zygotic embryo surrounded by the pericarp (a maternal tissue) and testa. The testa in some sorghum species is pigmented and contains condensed tannins. The thickness of the testa varies from 8 to 40 μm (*35*), with the thickest area being below the style and the thinnest on the side of the kernel. Many sorghums containing high levels of tannins have a high degree of resistance to molds (*36*); however, most commercial hybrid and feed- and food-type sorghums are virtually tannin free (*37*). The pigmented testa is seen as a dark layer between the light endosperm and the pericarp when the caryopsis is scraped to remove the pericarp.

The endosperm, the bulk of the caryopsis, is inside the pericarp and testa (Figure 1). The first layer, the rectangular aleurone cells, have thick walls and contain oil and protein bodies. Then, three layers of starchy endosperm cells can be recognized. The peripheral area is inside the aleurone cells and comprises several layers of dense cells that are rich in protein but contain only small starch granules. Inside this area is the corneous (horny) area, which contains angular starch granules embedded in a continuous protein matrix. In the center is a white, opaque, floury area in which the protein is loosely packed with air spaces and the starch granules are more spherical in shape (*38*).

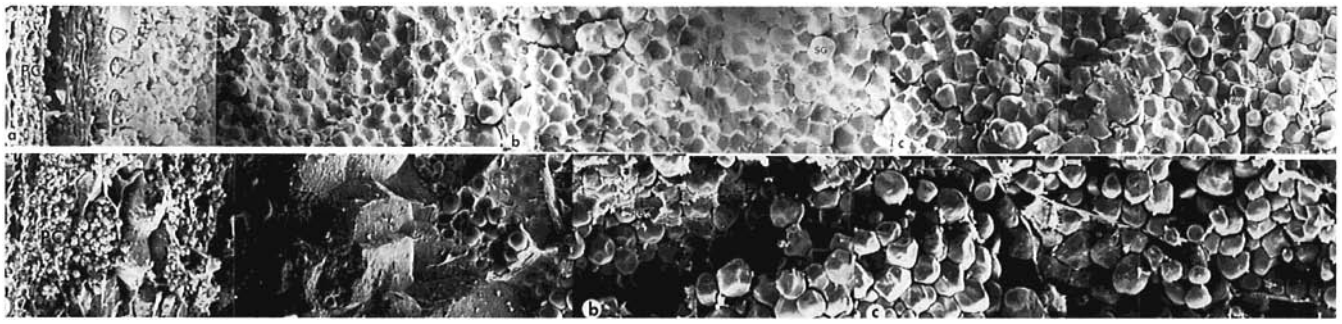

Figure 1. SEM photomicrograph montage of mature hard (top, Mishimba) and soft (bottom, NS283) sorghum grain. PE, pericarp; SG, starch granule; outside (left) to inside (right) of caryopsis.

Table 1. Categorization of Cultivars, Plant Maturity, Caryopsis Properties, and Amounts of Sormatin and Chitinase in Sorghums Varying in Mold Resistance

cultivar	AFP content[a]	mold resistance[a]	maturity[b]	pericarp color	pigmented testa	spreader gene[c]	endosperm hardness[d]	sormatin[e] (μg/caryopsis)	chitinase[e] (μg/caryopsis)	molding[f] 50 DAA
SC719-11E	low	high	early	red	yes	yes	medium	13	101	1.4
Malisor 84-7	high	mod.	medium	white	no	no	high	111	400	1.2
R9025	high	high	medium	red	no	no	low	83	166	1.2
Sureno	high	mod.	medium	white	no	no	high	50	386	1.2
Hegari*Dobbs	high	mod.	medium	white	yes	no	medium	222	562	1.8
IS2319	low	low	late	white	yes	no	low	83	456	5.0
E35-1	low	low	late	white	no	no	medium	17	271	4.8
RTX2536	low	low	medium	white	no	no	low	32	246	3.8
RTX430	high	low	medium	white	no	no	low	153	528	3.0
BTX638	high	low	medium	red	no	no	medium	271	448	1.6

[a] Antifungal protein (AFP) content and grain mold resistance from Bueso et al. (*59*). [b] Early maturity = <60 days after planting, medium = 61−80%, late = >81%. [c] When spreader (S) gene is dominant, tannins accumulate in pericarp and testa. [d] Hard = <30% abraded during decortication, medium = 30−50%, soft = >51%. [e] Sormatin and chitinase in control caryopses at 30 days after anthesis. Sormatin and chitinase had coefficients of variation between 11 and 23% for the different cultivars, locations, and age of caryopsis. [f] Mold rating (1 = no mold; 5 = >50% of surface molded) at 50 days after anthesis at College Station, TX.

McDonough and Rooney (*39*) reported that the pericarp is completely formed 6−9 days after anthesis (DAA) and, thereafter, begins to compress. Cells in the testa layer are apparent 3−6 DAA, whereas the aleurone layer took longer (6−12 DAA) to develop. Ovary walls contain simple and compound starch granules at anthesis. Simple starch granules and protein bodies begin to develop in endosperm cells by 3−6 DAA. Protein bodies at this stage were covered by a thin filamentous webbing, which later develops into a distinctive protein matrix. Shull et al. (*40*) noted that hard-endosperm sorghums that are more resistant to molds deposit protein much earlier than do floury-endosperm sorghums (Figure 1).

Grain Texture and Resistance to Grain Molds. The proportions of the outer translucent layer (also called glassy, horny, vitreous, or corneous) and the inner opaque, white area (also called soft or floury) vary among cultivars (*41*). Grain hardness is related to the relative areas of corneous and floury endosperm. Endosperm hardness attenuates growth of grain mold (*16*−*19*, *42*). Molds more quickly deteriorate the endosperm structure of susceptible cultivars compared to resistant cultivars, even though they may have similar endosperm hardnesses in a mold-free environment (*43*).

A clue to the resistance mechanism related to endosperm texture comes from studies of mutant lines of maize. The opaque2 mutation increases lysine but has a soft texture, susceptible to infection by molds (*44*, *45*), and reduced levels of α-zeins and ribosome-inactivating protein (RIP) (*37*). A DNA-binding protein encoded by the opaque2 locus (*46*−*48*) regulates the levels of both γ-zein and RIP. The homologous RIP from barley has antifungal properties (*49*), whereas Dowd et al. (*50*) suggests that maize RIP may be insecticidal. Decreased levels of RIP may contribute to susceptibility to molds. The hard-textured types of opaque2 maize contain the same lower levels of γ-zein, as do the unmodified opaque-2 lines, whereas their γ-zein levels are increased (*51*, *52*).

The corneous endosperm of sorghum is enriched in kafirins, especially γ-kafirins (*42*, *53*, *54*). The γ-kafirins contain more cysteine and form extensive intrachain disulfide bonds, which may contribute both to hard texture and to resistance to fungal infection (*55*, *56*). The prolamins in the protein bodies are remarkably resilient to proteolysis by a fungal protease until their disulfide bonds are reduced (Mazhar and Chandrashekar, unpubublished results).

The cell wall composition also varies between the corneous and floury endosperm and between hard and soft grains (*57*, *58*). Because the endosperm structure is modified during fungal colonization of the caryopsis, cell wall structure and composition need to be investigated to increase resistance to grain molds.

A combination of factors has recently been shown to correspond to grain mold resistance (*59*). Sorghums with red pericarp and/or tannins (Table 1) did not have to have a hard endosperm to exhibit resistance, but white pericarp sorghums needed a hard endosperm before some level of resistance was observed. Otherwise, sorghums with low and medium endosperm hardness exhibited the most deterioration. Harder grain had lower mold ratings when stressed with sprinkling or inoculation with fungal pathogens.

Tannins and Phenolic Compounds. Most sorghums do not contain tannins but all sorghums contain phenols and most contain flavonoids. Sorghums are classified as type I (no tannins), type II (tannins in pigmented testa), or type III (tannins in pigmented testa

Table 2. Antifungal Proteins of Sorghum, Maize, and Millets

species	size/isoform	fungal species inhibited	effective/inhibitory dose	refs
chitinase				
sorghum	24, 28, and 33 kDa	*T. viridae*	1 μg	*94*
		F. moniliforme	5 μg	
		R. solani	1 μg	
maize	p*I* 5.9/4.7			*69, 3, 75*
pearl millet[a]	24 kDa, p*I* 9.8	*T. reesi*, *F. moniliforme*	250−800 μg/mL	*118*
ribosome-inactivating protein				
maize	30 kDa	in vitro protein synthesis	0.7 μg/mL	*117, 87, 50*
sorghum	32−34 kDa	in vitro proteinsynthesis		*87*
thaumatin-like proteins				
maize	22 kDa		0.1 μg/mL	*3, 73, 74*
	22, p*I* 9.1	*T. reesi*, *R. solani*	10 μg	*72*
sorghum	26 and 30 kDa	*F. moniliforme*, *F. oxysporum*, *Curvularia lunata*	6.8 μg	*76, 77*
general proteins				
sorghum	12, 16, and 20 kDa	*A. flavus*	15 μg/mL	*93*
	18 kDa	*F. moniliforme*	15 μg/mL	*76, 74*
		F. moniliforme	mixture of RIP, chitinase, glucanase, and sormatin	*80*

[a] The pearl millet protein is a cysteine proteinase inhibitor (*118*).

and pericarp). Type II and III sorghums have dominant B1 and B2 genes and a pigmented testa (seed coat); type III sorghums have a dominant spreader (S) gene and more tannins than do type II sorghums (*38*).

Phenolic compounds from three major categories, that is, phenolic acids, flavonoids, and tannins, have been analyzed in sorghum (*21, 60*). Phenolic acids are derivatives of benzoic or cinnamic acids. Flavonoids consist of two units: a C6−C3 fragment from cinnamic acid and a C6 fragment from malonyl-CoA. The major groups of flavonoids in sorghum are the flavans: flavan-3-en-3-ols with a double bond between C3 and C4 and the C3 hydroxylated anthocyanidins. Tannins are polymers of five to seven flavan-3-ol units (catechin) linked through acid labile carbon−carbon bonds (*61*−*63*). Tannin sorghums contain proanthocyanidins as part of their phenolic compounds but do not contain tannic acid or hydrolyzable tannins. Phenolic acids and compounds increase during caryopsis development with a maximum at physiological maturity (and a decrease afterward). These decreases may be due to decomposition or insolubility.

Assabgui et al. (*64*) reported a good correlation between the content of ferulic acid in maize kernels with resistance to *F. graminearum*, whereas ref *65* reports greater levels of *p*-coumaric acid in some white pericarp, non-tannin sorghums that were susceptible to molding. The conversion of *p*-coumaric acid to ferulic acid might be deficient in the susceptible cultivars. Free or bound phenolic acids in control and inoculated caryopses correctly classified sorghums into resistant, intermediate, and susceptible groups (Rodriguez-Ballesteros et al., unpubublished results).

Sorghum cultivars resistant to fungal attack contained both a greater variety and larger amounts of free phenolic acids; this was especially true for tannin sorghums (*62, 65*). Red pericarp sorghums are more resistant to mold than are white pericarp sorghums and contain more flavon-4-ols (*17, 19, 22, 36, 66, 67*). A cause and effect relationship has not been established for flavan-4-ols nor have their enzyme systems been studied.

No phytoalexin has been detected in the caryopsis of sorghum. Clive et al. (*68*) isolated cDNA clones from a sorghum mesocotyl library after infection with *Colletotrichum sublineolum* that appear to align partly with ribonuclease sequences in the database. The level of chalcone synthase, an enzyme that is involved in the synthesis of phytoalexin, was greater when mesocotyls were treated with *Cochilobolus heterostophus*, which does not infect sorghum, than with *C. sublineolum*, which does.

Antifungal Proteins (AFP) in Sorghum Caryopsis. Several proteins are constitutively expressed in developing cereal seeds and have antifungal properties either in vivo or in vitro. Sorghum grain has not been studied in as much detail for antifungal proteins as the more widely cultivated cereals (*3, 69*−*75*). However, it is probable that homologues of many of the proteins, particularly those present in maize, are also present in sorghum. In the past few years, several types of bioactive and antifungal proteins have been identified and characterized in sorghum, maize, and millet (Table 2).

Sunitha and co-workers (*76, 77*) identified three proteins of 18, 26, and 30 kDa, which affected hyphal growth of *F. moniliforme*. The 18 kDa component removes cell wall polysaccharides, whereas the other proteins are involved in leakage of cytoplasmic contents. The authors concluded that the 18 kDa protein could be an enzyme acting on cell walls and that the 26 and 30 kDa components could be related to permeatins. More AFP were in hard endosperm, grain mold resistant sorghums (*54, 77*).

Seetharaman et al. (*78*) identified positively charged, water-soluble proteins belonging to the permeatin (which they called sormatin), chitinase, β-1,3-glucanase, and RIP groups. The synthesis or extractability of antifungal proteins increased for several AFPs until physiological maturity and then decreased during desiccation of the grain (*78, 79*).

The level of AFP in sorghum caryopis was 7 μg; 6.8−15 μg was required for inhibitory activity to *Fusarium* sp. (*76*). An AFP fraction containing sormatin, chitinase, glucanase, and RIP was inhibitory to spore germination of *F. thapsinum*, *C. lunata*, and *Aspergillus flavus*, all at 360 ppm (*80*). Hyphal rupture at the growing tips was observed for *F. thapsinum* at 70 ppm and at 70−360 ppm for *C. lunata* but not for *A. flavus*. The amount of AFPs in sorghum was estimated at 260 ppm in physiologically mature caryopses at 30−35 mg/caryopsis

weight (*80*), whereas about 70 and 300 ppm was enough to inhibit *F. thapsinum* and *C. lunata*, respectively.

Proteinase Inhibitors. Higher levels of serine proteinase inhibitors were observed in developing hard versus soft endosperm sorghums (*42*). The maize tryspin inhibitor was implicated for resistance to *A. flavus* (*81*, *82*).

Ribosome-Inactivating Protein. Type 1 RIP barley grain inhibits the growth in vitro of a number of fungi (*Trichoderma reesi*, *Botrytis cinerea*, and *Rhizoctonia solani*), and this activity is enhanced synergistically by β-1,3-glucanase or endochitinase (*49*). Expression of RIP in transgenic tobacco increased resistance to the soilborne fungal pathogen *R. solani* (*83*), which was enhanced by the coexpression of barley endochitinase (*84*). Transgenic wheat plants expressing the barley RIP gene were not protected against *Erysiphe graminis* (*85*).

Walsh et al. (*86*) showed that the maize RIP was synthesized as a proprotein with a region being removed during germination. Hey et al. (*87*) demonstrated that the connecting hinge between the two domains of maize RIP inhibited enzymic activity on ribosomal RNA and was removed during activation. Sorghum contains protein bands that reacted with maize RIP antibody and had similar molecular weights. Two cross-reacting protein bands of ~30 kDa varied in intensity with sorghum using the same RIP antibody (*88*). The antibody also detected the late deposition of the proprotein in the endosperm during development, and the proprotein was split as in maize. The RIP in maize is located in the aleurone and the scuetellum using tissue prints and antibodies (*89*). Seetharaman et al. (*78*) observed that the RIP was most extractable from caryopses at 15 DAA and decreased subsequently.

Thionins/AMPs. A new family of low molecular weight proteins from sorghum caryopsis that inhibits α-amylase from insects was identified (*90*). These proteins, subsequently called γ-thionins and defensins, each comprised 47 amino acid residues with four disulfide bonds (*91*). A thionin-like peptide fraction identified as MBP-1 was isolated from maize and was found to inhibit spore germination and hyphal elongation of *F. moniliforme* and *F. germinarium* in vitro (*92*).

Chitinase and Glucanase. Several chitinases (three in the 21−24 kDa range and 28 kDa) and one β-1,3-glucanase (30 kDa) were reported in sorghum (*93*). Three chitinases were reported in sorghum caryopses (24, 28, and 33 kDa) that inhibited the growth of *Trichoderma viride* and *F. thapsinum* (*94*). Maximum accumulation of chitinase [28 and 33 kDa (*78*); 27 and 28 kDa (Venkatesha et al., unpublished data)] was observed at 30 DAA. The caryopsis contained less 27 and 28 kDa chitinase after 24 h of imbibition (during germination), but these proteins continued to be present even up to 10 days after imbibition. Several additional chitinase isoforms [18, 26, and 34 kDa (*78*); 18.2, 20, and 24 kDa (Venkatesha et al., unpublished data)] appeared during germination. Putative clones for chitinase have been derived from a cDNA library of developing caryopses as well as from genomic DNA. Sequence analysis revealed very high homology between cDNA and genomic clones, which, in turn, showed higher homology with the chitinases a and b of maize (Venkatesha et al., unpublished data). An immunoblot study revealed the polymorphic nature of chitinases in caryopses of 24 sorghums, and the amplification and cloning of chitinase genes from a few of the sorghums are being investigated (Venkatesh et al., unpublished observations).

Proteins Acting against *Aspergillus*. The extent of invasion of the germ in resistant lines might depend on resistance mechanisms in the endosperm (*95*). More β-1,3-glucanase activity was observed in the caryopsis and callus of maize resistant to *A. flavus* in response to infection (*96*), whereas Chen et al. (*81*, *82*) reported the presence of higher levels of 14 kDa trypsin inhibitor in maize resistant to *A. oryzae*. Three unidentified proteins in sorghum caryopsis inhibited the growth of *A. flavus* (*97*).

Two protein fractions were extracted from maize with one showing growth inhibition of *A. flavus* and the other inhibiting aflatoxin formation with little effect on fungal growth (*98*). Maize protein extracts from resistant kernels were found to have greater antifungal activity against *A. flavus* than did susceptible kernel extracts in vitro (*99*).

Variability and Relative Importance of AFP. A significant inverse correlation coefficient between sormatin in caryopses at 30 DAA with sorghum grain mold rating at harvest time was first observed by Seetharaman et al. (*78*) using 32 sorghum culitvars. Extractability of AFPs increased during caryopsis development and decreased prior to grain drying (*77*, *78*), whereas deterioration of the caryopsis significantly increases after physiological maturity.

Sorghum AFP leached from immature caryopses but was retained in the pericarp of mature caryopses during water imbibition (*78*). If AFPs are mobile and "bound" to the pericarp during imbibition, then AFPs could be concentrated in <10% of the caryopsis. This would increase AFP concentration ~10-fold, thereby increasing their antifungal potential. Moreover, increased quantities of these proteins were observed after treatment of flour with protease.

Variations in levels of AFP within the endosperm were observed, with more AFP being located in the corneous endosperm of resistant sorghums that contain more protein bodies (*42*, *54*, *79*) and different cell wall composition (*57*, *58*). Protein bodies and AFPs appear to be concentrated near cell walls in the corneous endosperm.

The distribution of AFP within the caryopsis also varies among sorghums (Waniska et al., unpublished data). More sormatin was found in the endosperm (versus pericarp or embryo) of Dorado (resistant, hard endosperm sorghum) and in the pericarp (versus endosperm or embryo) of TX2536 (susceptible, intermediate-endosperm-texture sorghum). More chitinase was found in the endosperm (versus pericarp or embryo), whereas more β-1,3-glucanase was found in the pericarp (versus endosperm or embryo). Soaking decreased the extractability of both chitinase and glucanase from physiologically mature caryopses.

Induction of Antifungal Proteins. Sormatin-like proteins were induced after fungal infection (*77*). Bueso et al. (*59*) determined 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 sprinkling of panicles in the field, similar to in vitro imbibition, decreased levels (or extractability) of AFP in susceptible sorghums at physiological maturity.

Rodriguez-Herrera et al. (*43*) compared the levels of four AFPs using eight mold resistant and eight susceptible lines derived from a susceptible by resistant cross, grown in eight environments over three years. Infection with grain mold resulted in the induction and/or retention of more AFPs in the resistant lines (Figure 2), suggesting that the coexpression of four AFPs 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 other AFPs. 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.

Puncturing the germ induced chitinase synthesis in mature Dorado caryopsis but not in TX2536 caryopses (Waniska et al., unpublished observations). Puncturing and soaking induced sormatin mobility and accumulation in caryopses tissues of Dorado and TX2536.

Infection of maize seeds with *F. moniliforme* resulted in induction and accumulation of antifungal proteins, and such response was found to be more rapid in germinating embryos (*100*). Antifungal activity of maize proteins upon imbibition and germination was attributed to fractions that reacted to zeamatin and RIP anti-sera (*101*).

Genetic Markers Associated with Grain Mold. Studies of other cereals demonstrate that it is possible to identify biochemical or molecular markers, which can be exploited to follow resistance in breeding programs. Mingeot and Jacquemin (*102*) found high polymorphism for one marker, which was subsequently shown to encode a thaumatin-like protein, observing many patterns in ~48 cultivars of wheat analyzed. Farris et al. (*103*) reported the use of 508 genetic markers including a large number of candidate genes in screening a population of 114 recombinant inbred lines between a hard red spring wheat and a synthetic hexaploid wheat (derived from *Triticum turgidum* and *Aegilops tauschii*). The oxalate oxidase gene was a good marker for tan spot resistance using a pathotype avirulent to Lr23. A peroxidase gene was linked to both resistance and Lr23. A phenylalanine ammonium lyase gene and a thaumatin gene appeared to be linked to the resistance genes Lr27 and Lr31. The disease resistance genes appeared clustered on the 7BL chromosome. Markers encoding chalcone synthase and a chitinase were associated with karnal bunt resistance. Ittu et al. (*104*) showed that resistance to *Fusarium* head blight was associated with the gliadin loci, Gli-B1 and Gli-D1, which is reminiscent of the positive relationship between prolamins and resistance to grain mold in sorghum. de la Pena et al. (*105*) similarly found QTL associated with resistance to *Fusarium* head blight in barley. The disease-resistance genes (R-genes) map to areas very close to known genes for resistance (*106*, *107*). Mutations in resistance genes may also account for differences between susceptible and resistant varieties. Klein et al. (*108*) have identified five QTLs associated with grain mold. Work is underway to determine the location of genes for AFP content in sorghum and their location relative to mapped grain mold resistance QTLs (*108*).

Resistance Genes. Resistance (R) genes present in the host plant may be activated on infection by pathogenic fungi, leading via signal transduction pathways to defense responses, such as the production of phytoalexins and antifungal proteins (discussed above). The

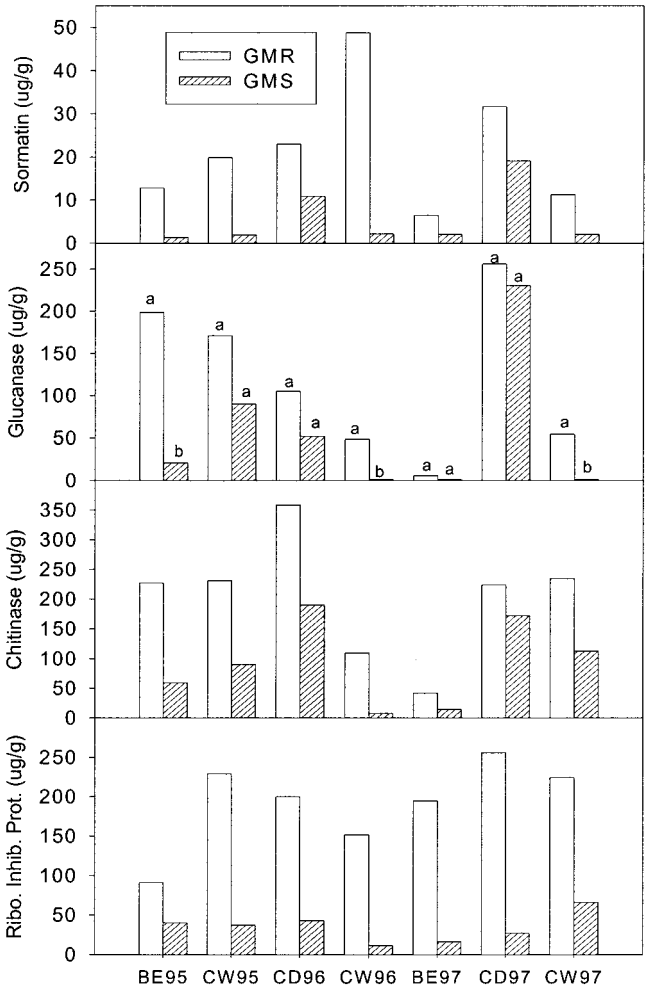

Figure 2. Antifungal protein levels (micrograms per gram) in mature caryopses of eight grain mold resistant (GMR) and eight grain mold susceptible (GMS) lines grown at seven environments. Resistant lines have more AFP than susceptible lines except for glucanase. For glucanase, means followed by the same letter are not significantly different (within environments) (*43*).

relationship between the R genes and pathogens may be extremely specific, resulting in responses only to specific species or races of pathogens. We have shown above that sorghum grain protects itself in a very elaborate manner using several mechanisms. The synthesis of the endoplasmic reticulum, the prolamins, the antifungal proteins, and the cell wall may be coordinated by the R genes.

In recent years R genes have been cloned from a number of plant species, using either mapping approaches ("map-based" cloning) or transposable elements to facilitate gene identification and isolation. This has shown that R proteins share common features, most notably the presence of leucine-rich repeats which may be combined with protein kinase domains. Moreover, resistance genes appear to be clustered in maps (*109*, *110*). This has facilitated the isolation of further R genes, using easier PCR-based strategies.

R genes have not so far been isolated from sorghum. However, their isolation by PCR-based technology should be facilitated by the availability of R gene sequences from other species including lettuce (*111*), tomato (*112*), soy (*107*, *110*), rice (*113*), potato (*114*), and maize (*106*). Both conserved and variable sequences are observed between different R genes, and the former could be used

to design PCR primers or oligonucleotides to isolate part or whole of the disease resistance genes from sorghum (*115*). Once the genes or fragments thereof are available, their relationship with resistance to grain mold could be investigated by assaying for the expression of these genes in developing seed and their linkage with resistance in a breeding program.

Studies of other systems have shown that overexpression of R genes may lead to broad-spectrum resistance with high levels of antifungal proteins and other defense-related compounds (*116*). Transgenic plants have been made with both the antifungal proteins and the kinases and leucine-rich repeat proteins that are involved in signal reception and transduction. Transgenic plants made with specific antifungal proteins may be able to resist certain fungi, whereas a broad-spectrum resistance may occur when kinases are used. Overexpression of such genes in the tissues of developing panicle of sorghum at the time of susceptibility to grain mold infection could lead to increased resistance.

Prospects for the Future. Transformation of crop plants is a promising and powerful tool to protect them from biotic and abiotic stresses and will be of immense value in preventing yield losses, which is an important component of yield stabilization in areas where these stresses are endemic. Even though sorghum is a hardy plant capable of withstanding biotic and abiotic stresses to some extent, further increases in resistance to stresses could result in yield enhancement. This strategy may be important for dealing with the ever-increasing world requirement for food and feed. In the future, other agronomically useful genes, such as the genes involved in the biosynthesis of cellulose and lignin, which strengthens the stalks, may be utilized to prevent lodging. Additionally, genes that confer resistance to insects could be introduced into sorghum to reduce yield loss from insect feeding and to reduce opportunistic fungal infections at the wounding sites. Genes for phytase can reduce the phytic acid content of plants to increase their nutritional value to animals and to reduce phosphate pollution due to excretion of phosphates by livestock. Additionally, transgenic sorghum plants could be a source of pharmaceuticals and biopolymers and thus serve to increase the commercial value of the sorghum crop. We have started a long-term project to introduce these genes in various combinations into sorghum inbreds in order to improve the resistance to stalk rot.

CONCLUSIONS AND RECOMMENDATIONS

Current sources of resistance provide only partial protection against stalk rot and grain mold fungi, the most important of which are *Fusarium*, *Alternaria*, and *Curvularia*. Grain mold resistance can derive from a combination of characteristics (pigmented testa, phenolic compounds, red pericarp, endosperm texture, cell wall, and AFP), which act additively or synergistically. Different resistance mechanisms could be linked by the R genes. Some of the sources of resistance have a negative impact on end use properties. Hence, there may be a tradeoff between protection and quality.

Pigmented Testa and Red Pericarp. Because both red pericarp and tannins are related to grain mold resistance, utilization of red pericarp sorghums (grown on a tan plant) and tannin sorghums are reasonable short-term solutions to decrease grain molding. Determination of the causative agents in these sorghums, for example, nonpigmented phenols, other compounds, and tannins, against fungi is needed. Then, the enzymes, their regulation, and their molecular biology need to be investigated.

Grain Hardness. Hardness corresponds to high levels of γ-kafirin. The biochemical basis for this effector needs to be understood and the existence of modifier genes (as in QPM) established. The effect of introducing more gamma genes into sorghum on mold resistance needs to be studied. In addition, hardness is currently associated with high levels of antifungal proteins, and this linkage needs to be understood and then broken.

Antifungal Proteins. A link between AFP and resistance is clearly established in both stalk rot and grain molding, although only partial protection is obtained. In the short term it will be possible to select for high levels of characterized AFP (sormatin, RIP, β-1,3-glucanase, and endochitinase) in developing, mature, and infected grain by direct analysis or by using antibodies or molecular markers. More information is required on the endogenous sorghum AFP to identify new components, determine activity and synergism, determine location and amounts in developing and mature tissues, determine initiation of synthesis, and relate the above to resistance.

Marker-Assisted Selection. The development of molecular markers for the sorghum genome (e.g., SSRs) is essential to underpin the selection of resistance and the combination of resistance with yield and end use quality. It will also facilitate dissection of the various aspects of resistance, for example, the separate effects of hardness and AFPs. A more direct approach could also be adopted using markers based on characterized antifungal proteins (e.g., thaumatin) and putative R genes (based on the other species).

Transformation. Transformation is an essential prerequisite for long-term improvement and must continue to be supported. Transformation should initially express sormatin, β-1,3-glucanase, and endochitinase under a strong endosperm specific promoter. Further transformation may require specific promoters to control the level and tissue specificity of expression, for example, glume, developing endosperm, or ovary wall/pericarp.

ACKNOWLEDGMENT

We are very grateful for the hard work and dedication of legions of former graduate students and colleagues who have provided so much inspiration and reliable information for our programs. The long-term support of the Texas and Kansas Agricultural Experiment Stations, private industry, INTSORMIL, and CFTRI are appreciated. The permission accorded by the Director of CFTRI to present this paper is acknowledged by A.C. and R.T.V.

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Received for review January 2, 2001. Revised manuscript received August 9, 2001. Accepted August 10, 2001.

JF010007F