Expression of pathogenesis-related protein PR-10 in sorghum floral tissues in response to inoculation with *Fusarium thapsinum* and *Curvularia lunata*

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SUMMARY

Differences in grain mould disease levels among different sorghum varieties grown in the same environment imply that host genes play a role in controlling disease severity. The fungi most often recovered from naturally infected sorghum grain, *Fusarium thapsinum* and *Curvularia lunata*, were used to inoculate a set of resistant and susceptible cultivars at anthesis in both field and glasshouse trials. In the field, 12 cultivars were inoculated with a mixture of *F. thapsinum* and *C. lunata* and, in the glasshouse, individual panicles from four selected cultivars were inoculated with spore suspensions of *C. lunata*, *F. thapsinum*, a mixture of the two or water to serve as a control. Based on grain mould severity ratings and germination tests on the seed from the field trial, Tx2911, Sureno, SC719-11E and SC650-11E displayed a high level of resistance to grain mould. To determine whether resistant and susceptible lines differed in response to the pathogens, PR-10 mRNA levels were measured using real-time reverse transcriptase-polymerase chain reaction. PR-10 is a protein with antifungal properties that has been associated with defence responses in sorghum and other plant species. In field tests, most, but not all, cultivars showed significant induction of normalized relative quantities of PR-10 after dual inoculation with spores of both *C. lunata* and *F. thapsinum*. Under glasshouse-controlled conditions, glumes of inoculated plants showed the clear induction of PR-10 mRNA, and the response was greater in resistant (Tx2911 and Sureno) than in susceptible (RTx430 and SC170-6-17) cultivars. Inoculation with spores from a single mould-inducing pathogen generally induced greater responses than when spores were combined. For RTx430, SC170-6-17 and Sureno, the response to *C. lunata* was greater, whereas Tx2911 showed a stronger response to *F. thapsinum*. The results

indicate that the induction of PR-10 in sorghum glumes may be a factor useful in breeding programmes designed to combine multiple factors for resistance.

INTRODUCTION

Grain mould in sorghum [*Sorghum bicolor* (L.) Moench] is a major problem; damage caused by this fungal disease includes a reduction in yield (loss of seed mass), grain density and germination (Castor, 1981; Ibrahim *et al.*, 1985; Maiti *et al.*, 1985). Mouldy grain is a complex of infection of developing grain by parasitic and saprophytic fungi and discoloration caused by weathering. These factors combine to reduce grain and seed quality (Murthy 2000). The disease is more prevalent when moist weather conditions prevail after flowering. Some sorghum cultivars often escape grain mould because they are photoperiod sensitive, with flowering timed so that grains mature only after the rains have ceased. Other types of damage that arise from grain mould relate to storage quality, food and feed processing quality, and market value (Hodges *et al.*, 1999). Several disease-causing fungi can produce mycotoxins that are harmful to human and animal health and reproduction (Bandyopadhyay *et al.*, 2002).

Grain mould-resistant cultivars with a red or white pericarp and corneous hard endosperm typically show less growth of mould fungi on grain surfaces. Grain with a corneous hard endosperm is also more resistant to internal fungal colonization, but it cannot suppress late-season surface infection of fungi such as *Cladosporium*, *Alternaria* and *Curvularia* (Glueck and Rooney, 1980). These late infections are generally referred to as grain weathering.

Fusarium spp. are filamentous fungi that are widely distributed on plants and in the soil (Correll *et al.*, 1986). The species most often associated with grain mould of sorghum is

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F. thapsinum, often previously defined as *F. verticillioides* until the recent acceptance of the change of the name *F. moniliforme* to *F. verticillioides* according to Summerell *et al.* (2003). It is one of the two most commonly reported grain mould pathogens in sorghum on a global scale (Klittich *et al.*, 1997). The other comes from the genus *Curvularia*, the species of which are commonly found as parasites or saprobes (saprophytes) on graminaceous hosts. *Curvularia lunata* (teleomorph *Cochliobolus lunatus* = *C. lunatus*) is most prevalent on sorghum.

Grain mould severity, kernel weight, seed germination and seed mycoflora are important factors in determining the reaction of sorghum germplasm to grain mould (Erpelding and Prom, 2006). Mechanisms of resistance of sorghum to pathogens can be divided broadly into two types: constitutive features of the structure of the plant organ or tissue, and inducible systems which respond when the plant is challenged by infection, damage or chemical elicitors (Chandrashekar and Satyanarayana, 2006; Forbes *et al.*, 1992; Glueck and Rooney, 1980; Mansuetus, 1990). In constitutive resistance, structural features, such as the thickness of the cuticle and cell wall, may limit penetration into the cells, and texture (endosperm hardness) may hinder the progress of the fungi within the cells themselves (Chandrashekar *et al.*, 2000). The inducible systems are composed of receptors which recognize the challenge, coupled to signal transduction pathways that lead to resistance through the effects of response genes (Esele *et al.*, 1993; Menkir *et al.*, 1996).

In some cases, such as antifungal proteins, the same type of component may be present constitutively in some tissues and form part of an inducible response in others (Chandrashekar *et al.*, 2000). Waniska *et al.* (2001) found wide variation in the amount of antifungal protein present in different sorghum cultivars. PR-10 proteins are small, primarily acidic, intracellular proteins of about 16 kDa (parsley PR protein), at least some of which have antifungal properties. PR-10 gene family members are induced by pathogen attack in a wide variety of plant species (Liu *et al.*, 2003), including pea (Fristensky *et al.*, 1988), bean (Walter *et al.*, 1996), potato (Matton and Brisson, 1989) and asparagus (Warner *et al.*, 1992). A relationship between the expression of PR-10 protein and disease resistance was first demonstrated in pea (Riggleman *et al.*, 1985). PR-10 expression has previously been shown to be induced as part of the active host defence of sorghum against foliar fungal pathogens (Lo *et al.*, 1999). The induction of other pathogen response genes has been reported in sorghum floral tissues exposed to grain mould fungi, but no indication of a role in resistance was evident (Little and Magill, 2003).

The objectives of this study were to determine the effect of *F. thapsinum*, *C. lunata* and a mixture of the two species on grain mould severity, kernel weight, seed viability and seed mycoflora analysis. In addition, we used real-time polymerase chain reaction (PCR) to quantify mRNA levels of antifungal protein PR-10

gene in floral tissues (glumes and gynoecia) in control and treated plants in response to sorghum infection by *F. thapsinum* and *C. lunata*.

RESULTS

The grain mould severity rating and kernel weight were significantly affected by cultivar and cultivar by treatment interaction. Disease severity in the resistant cultivars Tx2911, Sureno, SC103-12E, SC279-14E, SC719-11E and SC650-11E ranged from 1.4 to 2.0 and, in the susceptible cultivars RTx430, BTx623, BTxARG1 and SC414-12E, from 4.6 to 5.0; all remaining cultivars were intermediate, ranging from 2.5 to 3.5. Cultivars showed significant differences with regard to 100-kernel weight, but treated samples were not significantly different from their respective controls. Seed quality, as measured by germination tests, of most of the cultivars was poor (less than 75%) in both control and inoculated plants. The mixed spore treatment caused a significant reduction in seed germination rates of susceptible cultivars to an average of 35%, in comparison with the sterile water control with an average of 51.7%. The germination rates for the treated grain mould-resistant cultivars Tx2911 (45%), Sureno (55%), SC719-11E (67.5%) and SC650-11E (57%) were significantly higher than those of the other cultivars (Table 1).

The results of seed mycoflora analysis from field studies showed that *F. thapsinum* was present on all the lines tested in all treatments. It was recovered at a high level from plants inoculated with *F. thapsinum*, but was also found on control plants. Overall, *F. thapsinum* was the most frequently recovered species at 45%, followed by *C. lunata* at 34%. *Alternaria* spp. were recovered at 13% overall, but were predominantly recovered from seeds of control plants. *Fusarium semitectum* appeared at a lower frequency (3%). *Aspergillus* spp. and *Rhizopus* spp. were also present at lower levels (less than 5%). Generally, seeds from the natural infection control plants showed the largest number of different fungal species (Table 2).

Real-time PCR analysis

Field study

Significant differences among cultivars were seen in real-time reverse transcriptase (RT)-PCR cycle threshold (C_t) values for PR-10 expression in glumes and gynoecia in response to inoculation with a mixture of *C. lunata* and *F. thapsinum* for all 12 cultivars evaluated in the field during the 2006 growing season (Table 3). For glumes, treatment effects were detected for 90EON343, BTx399, BTxARG-1, SC650-11E, S719-11E, Sureno and Tx2911 (low C_t values in the treated samples indicate higher levels of mRNA). Treatment effects were not detected in BTx623, RTx430, SC103-12E, SC279-14E and SC414-12E. Both lists include susceptible and resistant cultivars.

Table 1 Mean percentage disease severity, kernel weight and seed germination of 13 sorghum lines inoculated with a mixture of *Curvularia lunata* and *Fusarium thapsinum* and water-sprayed control at College Station, TX, research farm during 2006.

Cultivar	Disease severity	100-kernel weight (g)		Seed germination (%)	
		Inoculated	Control	Inoculated	Control
90EON343	3.40 cd	2.58 a	2.66 a	10.00 d	62.50 bc
BTx623	5.00 a	2.04 a–c	2.08 a–c	27.50 c	32.50 de
BTx399	3.80 bc	2.41 ab	2.48 ab	18.85 cd	53.80 b–d
BTxARG-1	4.60 ab	1.94 b–f	1.96 b–f	57.50 ab	42.50 cd
RTx430	4.60 ab	1.81 d–h	1.83 d–h	2.50 e	37.50 de
SC103-12E	2.00 de	2.03 a–c	2.06 a–c	30.00 c	52.50 b–d
SC279-14E	3.40 cd	2.63 a	2.64 a	17.20 cd	7.50 f
SC414-12E	4.90 a	2.14 a–e	2.17 a–e	27.50 c	40.00 de
SC650-1E(T)	2.00 de	2.14 a–e	2.19 a–e	57.50 ab	90.00 a
SC719-11E	2.00 de	2.41 ab	2.50 ab	67.50 a	95.28 a
Sureno	2.00 de	2.33 ab	2.30 ab	55.00 ab	63.50 bc
Tx2911	1.40 d–g	2.13 a–e	2.16 a–e	45.00 bc	45.00 de
SC170-6-17	4.00 bc	2.06 a–c	2.08 a–c	34.08 c	49.6 b–d
Mean	3.31	2.20	2.24	34.62	51.70

Means within a column followed by the same letter(s) are not significantly different at the 5% probability level. Mean comparison was conducted using Tukey's studentized range test.

For gynoecia, the C_i values were in the range 20.36–30.64 and high C_i values were detected in cultivars 90EON343, RTx430, SC279-14E, SC650-11E and Sureno (Table 3). In general, there was much less PR-10 mRNA present (an average of over 25 rounds of amplification before detection vs. 22 for control glumes), and there was no consistent pattern of induction (average C_i value of 25 in treated and untreated, in comparison with 20.9 in treated glumes).

An analysis of the results using qBase software, which calculates expression [normalized relative quantities (NRQs) = $2^{\Delta\Delta C_i}$] values and permits the use of a comparative standard to verify mRNA levels if the samples are equivalent (actin was used in this case), indicated that PR-10 expression was highest in glumes of Tx2911 and Sureno (180 and 14 times relative fold induction, respectively). 90EON343, BTx399, S719-11E and BTxARG-1 showed higher expression of PR-10 mRNA than the remaining cultivars, and each was induced when inoculated with a mixture of *C. lunata* + *F. thapsinum* spores (Table 4 and Fig. 1). Expression in cultivars BTx623, SC103-12E and SC414-12E was lower in the inoculated samples than in the respective controls. The effect for BTx623 was statistically significant, suggesting that repression rather than induction occurs in this cultivar, an observation that requires further examination.

Glasshouse study

In the glumes of all four cultivars, *C. lunata* inoculation resulted in significantly higher expression of PR-10 mRNA at 48 h post-inoculation in comparison with the controls (Table 5). The response to *Fusarium* inoculation was not as great, but all except SC170-6-17 recorded significantly lower C_i values than the controls. Surprisingly, only in Tx2911 did the mixed spore treatment elicit a greater response than for the individual treatments or controls. In gynoecia, although the amount of mRNA detected

following inoculations with a single pathogen differed from the control, the C_i value was lower in the control (21.57) than in the other three treatments in the susceptible cultivar RTx430 (Table 3). Non-significant differences in C_i values were recorded between controls and mixed *C. lunata* + *F. thapsinum* treatment in cultivars SC170-6-17 (moderately susceptible) and Sureno (resistant). Taken together, as in the field study, inconsistent results were observed in gynoecia with different inoculation treatments.

In glumes, NRQs of 35.98 in the cultivar Sureno inoculated with *C. lunata* and 31.45 in the other resistant cultivar Tx2911 inoculated with a mixture of *C. lunata* and *F. thapsinum* spores represented the highest PR-10 mRNA expression (Table 6 and Fig. 2). Both resistant lines showed higher PR-10 expression than SC170-6-17 and RTx430. PR-10 expression was generally greater when treatments involved a single pathogen (and *C. lunata* was greater than *F. thapsinum*), rather than a mixed inoculum. Overall, the expression of the PR-10 protein gene in glumes was greater in resistant cultivars, 48 h after inoculation, than in susceptible cultivars. The variability among multiple extractions was sufficiently low such that standard deviations did not overlap between control and inoculated samples. Data for gynoecia were variable with no consistent pattern of induction detected (Table 6). The highest level of relative expression of PR-10 mRNA of 32.31 was seen in the control samples from susceptible cultivar RTx430, followed by the resistant cultivar Sureno inoculated with *C. lunata* (14.4); the lowest (1.00) was observed on the resistant cultivar Tx2911 inoculated with *F. thapsinum*.

DISCUSSION

This study was designed to compare cultivars that differed in susceptibility to grain mould disease-causing fungi in both the

Table 2 Relative frequency of recovery (%) of fungal species from eight selected sorghum cultivars inoculated with *Curvularia lunata*, *Fusarium thapsinum*, a mixture of both and control from natural infection (not bagged) at College Station, TX, research farm during 2006.

Cultivar	Treatment	Fungal species					
		Ft	Fs	Cl	Alter	Asp	Rhi
90EON343 (MR)	Cl	45	0	55	0	0	0
	Ft	97	0	3	0	0	0
	Cl + Ft	28	4	53	15	0	0
	N	58	0	2	10	0	30
BTx623 (S)	Cl	45	0	55	0	0	0
	Ft	93	0	7	0	0	0
	Cl + Ft	95	0	5	0	0	0
	N	13	8	18	58	0	3
BTx399 (S)	Cl	12	0	88	0	0	0
	Ft	53	20	0	2	2	23
	Cl + Ft	63	0	37	0	0	0
	N	53	0	4	17	3	23
BTxARG-1 (S)	Cl	10	0	90	0	0	0
	Ft	43	0	57	0	0	0
	Cl + Ft	38	0	55	7	0	0
	N	14	8	18	58	0	2
RTx430 (VS)	Cl	25	0	75	0	0	0
	Ft	85	0	15	0	0	0
	Cl + Ft	93	3	4	0	0	0
	N	30	5	27	25	0	13
SC414-12E (MS)	Cl	3	0	97	0	0	0
	Ft	100	0	0	0	0	0
	Cl + Ft	—	—	—	—	—	—
	N	30	0	30	40	0	0
Sureno (R)	Cl	15	10	72	3	0	0
	Ft	93	0	0	7	0	0
	Cl + Ft	38	4	55	3	0	0
	N	7	35	0	45	3	10
Tx2911 (HR)	Cl	12	0	85	0	3	0
	Ft	63	0	2	12	23	0
	Cl + Ft	17	0	35	38	7	3
	N	20	0	12	53	3	12
Mean		45	3	34	13	1	4

Alter, *Alternaria* spp.; Asp, *Aspergillus* spp.; Cl, *Curvularia lunata*; Cl + Ft, mixture of *C. lunata* and *F. thapsinum*; Fs, *Fusarium semitectum*; Ft, *Fusarium thapsinum*; N, natural infection; Rhi, *Rhizopus* spp.

VS, very susceptible; S, susceptible; MS, moderately susceptible; MR, moderately resistant; R, resistant; HR, highly resistant.

field and glasshouse. Significant cultivar by treatment interaction of grain mould severity indicates that sorghum cultivars respond differently when treated with a mixture of *C. lunata* and *F. thapsinum*. In brief, the cultivars Tx2911, Sureno, SC719-11E, SC103-12E, SC279-14E and SC650-11E were resistant and RTx430, SC170-6-17, BTx623, BTxARG1 and SC414-12E were susceptible. This result confirms previous research by Eese *et al.* (1993) and Prom *et al.* (2003), which showed that, based on grain mould severity ratings, Sureno is relatively mould resistant, whereas RTx430 is mould susceptible. The absence of a treatment effect for 100-kernel weight for the selected cultivars in this study suggests that kernel weight may not be useful in

Table 3 C_t values for pathogenesis-related protein 10 (PR-10) in sorghum glumes and gynoecia of 12 cultivars evaluated in the field (2006) in response to a mixture of *Curvularia lunata* and *Fusarium thapsinum* inoculation and water-sprayed control.

Cultivar	Glumes		Gynoecia	
	Inoculated*	Control	Inoculated*	Control
90EON-343	21.05 b-d†	24.31 d-g	30.64 a†	26.71 ab
BTx623	19.62 de	22.87 b-d	23.32 b-d†	26.84 ab
BTx399	21.85 a†	23.85 c-f	24.21 bc	24.74 bc
BTxARG-1	21.05 b-d†	24.31 ab	22.30 b-c†	25.94 bc
RTx430	22.94 ab	23.87 a-c	30.04 a†	26.14 ab
SC103-12E	19.32 de	19.29 g	20.36 d	20.95 d
SC279-14E	20.73 b-d	20.16 fg	28.98 ab†	23.48 b-d
SC414-12E	22.10 ab	22.35 b-e	23.69 b-d†	26.29 ab
SC650-11E(T)	20.15 a-c†	21.61 e-g	26.82 b†	23.08 b-d
SC719-11E	20.35 b-d†	22.20 c-f	25.57 b-c†	27.99 a
Sureno	21.80 a-c†	25.03 a	25.81 b-c†	23.34 b-d
Tx2911	19.73 c-e†	23.05 a-d	23.07 b-c†	25.36 bc
Average	20.89	22.44	25.40	25.07

Means within a column followed by the same letter(s) are not significantly different based on Tukey's standardized range test at the 5% probability level.

*Samples were collected 48 h after inoculation with a mixture of spores from cultures of *C. lunata* and *F. thapsinum*.

†Significant when tested against respective control using T grouping of the least-squares means at the 5% probability level.

assessing grain mould susceptibility. The overall reduction in seed quality was probably caused by grain weathering. Even so, Tx2911, Sureno, SC719-11E, SC103-12E, SC279-14E and SC650-11E showed significantly higher germination rates, indicating greater seed viability, after inoculation.

From naturally inoculated (unbagged) plants, *Alternaria* spp. were recovered most frequently from five of the eight cultivars and on an overall basis (Table 2). However, *F. thapsinum* was also recovered from all plants that were inoculated and bagged at anthesis with *C. lunata*, and *C. lunata* was generally also isolated from plants inoculated with *F. thapsinum*. As *Alternaria* was only rarely isolated from these plants, the implication is that *F. thapsinum* and *C. lunata* are early invaders that are the primary cause of grain mould, whereas the other species recovered, *Alternaria*, *F. semitectum*, *Rhizopus* and *Aspergillus*, are probable saprophytes or weak pathogens that invade after the grain has been formed (grain weathering). Hepperly *et al.* (1982) identified *F. moniliforme*, *C. lunata* and *Alternaria* spp. in Mayaguez, Puerto Rico and Prom (2004) identified *Alternaria* spp., *F. semitectum* and *C. lunata* in College Station, Texas, as the most frequently recovered fungal species from naturally infected grain sorghum. Together, these studies clearly indicate that many fungal species interact differentially over years, locations and cultivars, making selection for grain mould resistance extremely complicated. The fungal species *F. thapsinum*, *C. lunata* and *Alternaria* spp. appear to be most critical for the evaluation of sorghum germplasm mould resistance in central Texas.

Table 4 Expression [normalized relative quantities (NRQs) = $2^{\Delta\Delta C_T}$] of pathogenesis-related protein 10 (PR-10) mRNA in glumes of sorghum cultivars inoculated with a mixture of *Curvularia lunata* + *Fusarium thapsinum* and water-sprayed control in field experiment, 2006.

Cultivar	Expression		Relative fold induction
	Inoculated ($\pm 1SD$)	Control ($\pm 1SD$)	
90EON-343	29.19 $\pm$ 8.25	2.57 $\pm$ 0.22	11.3
BTx623	2.37 $\pm$ 0.62	13.90 $\pm$ 3.44	—
BTx399	168.31 $\pm$ 34.51	18.60 $\pm$ 8.26	9.0
BTxARG-1	178.83 $\pm$ 63.22	45.33 $\pm$ 11.58	3.9
RTx430	25.85 $\pm$ 9.82	11.00 $\pm$ 3.26	2.3
SC103-12E	47.34 $\pm$ 4.49	55.42 $\pm$ 10.93	—
SC279-14E	97.17 $\pm$ 17.99	52.70 $\pm$ 9.83	1.8
SC414-12E	5.40 $\pm$ 1.04	6.42 $\pm$ 0.82	—
SC650-11E(T)	77.84 $\pm$ 6.95	2.96 $\pm$ 0.13	26.3
SC719-11E	46.12 $\pm$ 2.51	10.46 $\pm$ 3.19	4.4
Sureno	13.97 $\pm$ 0.97	1.03 $\pm$ 0.36	13.5
Tx2911	374.81 $\pm$ 37.77	2.08 $\pm$ 0.17	180.1

Fig. 1 Histogram showing the expression (normalized relative quantities) of pathogenesis-related protein 10 (PR-10) mRNA in glumes of 12 sorghum cultivars inoculated with a mixture of *Curvularia lunata* + *Fusarium thapsinum* (T) vs. control (C) (field experiment, 2006).

Fusarium semitectum, *Aspergillus* spp. and *Rhizopus* spp. were present at low levels (less than 5%), suggesting that these fungal species are not major causal agents for grain mould on sorghum grown in Texas. Earlier studies by Erpelding and Prom (2006) reported that *Bipolaris* spp., *Alternaria* spp. and *C. graminicola* were recovered at low frequencies from the majority of the sorghum genotypes evaluated at Isabela, Puerto Rico.

Real-time RT-PCR with SuperScript™ III was used to quantify the responses in sorghum floral tissues 48 h after inoculation

with fungal spores, in comparison with mock-inoculated controls. Because SuperScript™ III is not significantly inhibited by ribosomal and transfer RNA, it was used to synthesize cDNA from total RNA.

The expression of PR-10 during kernel development in maize was found to increase five-fold between 7 and 22 days after pollination, and was induced on *A. flavus* infection in resistant but not susceptible genotypes (Chen *et al.*, 2006). PR-10 expression is regulated by a variety of stress responses. In rice, a PR-10

Treatment*	RTx430	SC170-6-17	Sureno	Tx2911
Glumes				
<i>C. lunata</i>	22.51 bc	21.52 c	19.47 d	21.36 bc
<i>F. thapsinum</i>	23.49 bc	22.49 ab	20.51 bc	20.33 bc
<i>C. lunata</i> + <i>F. thapsinum</i>	24.17 a	22.91 ab	20.84 bc	19.59 d
Control (sterile water)	25.02 a	23.25 a	25.59 a	22.58 a
Gynoecia				
<i>C. lunata</i>	25.71 a	21.50 cd	19.81 e	21.57 cd
<i>F. thapsinum</i>	24.21 ab	21.00 de	23.55 bc	23.55 bc
<i>C. lunata</i> + <i>F. thapsinum</i>	22.80 bc	22.93 bc	24.17 ab	20.46 de
Control (sterile water)	21.57 cd	23.68 ab	25.64 a	24.89 a

Means within a column followed by the same letter(s) are not significantly different based on Tukey's standardized range test at the 5% probability level.

*Samples were collected 48 h after inoculation with a mixture of spores from cultures of *C. lunata* and *F. thapsinum*.

Inoculum	RTx430 ($\pm$ 1SD)	SC170-6-17 ($\pm$ 1SD)	Sureno ($\pm$ 1SD)	Tx2911 ($\pm$ 1SD)
Glumes				
<i>C. lunata</i>	6.45 $\pm$ 0.36	8.03 $\pm$ 1.34	35.98 $\pm$ 4.69	11.31 $\pm$ 0.71
<i>F. thapsinum</i>	2.29 $\pm$ 0.13	3.31 $\pm$ 0.87	18.51 $\pm$ 4.88	15.27 $\pm$ 2.69
<i>C. lunata</i> + <i>F. thapsinum</i>	1.88 $\pm$ 0.07	3.19 $\pm$ 0.12	15.51 $\pm$ 1.66	31.45 $\pm$ 4.17
Control (sterile water)	1.42 $\pm$ 0.11	1.71 $\pm$ 0.11	1.00 $\pm$ 0.07	4.26 $\pm$ 0.19
Gynoecia				
<i>C. lunata</i>	1.20 $\pm$ 0.08	4.66 $\pm$ 1.00	14.40 $\pm$ 1.50	8.76 $\pm$ 0.39
<i>F. thapsinum</i>	7.43 $\pm$ 0.67	3.27 $\pm$ 0.13	1.91 $\pm$ 1.59	1.00 $\pm$ 0.13
<i>C. lunata</i> + <i>F. thapsinum</i>	2.48 $\pm$ 0.19	3.27 $\pm$ 0.13	1.15 $\pm$ 0.08	13.42 $\pm$ 2.98
Control (sterile water)	32.31 $\pm$ 2.74	1.68 $\pm$ 0.31	1.46 $\pm$ 0.40	3.10 $\pm$ 0.27

protein was first characterized as a probenazole-inducible protein (PBZ1). Hashimoto *et al.* (2004) found a protein, RSOsPR-10, a novel rice PR-10 protein that was induced by salt and drought stresses. Constitutive expression of a pea PR-10 gene in *Brassica napus* has been reported to enhance seed germination and growth in the presence of NaCl (Srivastava *et al.*, 2004). PR-10 can protect seedlings of *B. napus* from salinity, which may have significance in the genetic engineering of water-deficit stress tolerance in plants.

The biological function of PR-10 has been proposed to involve a possible ribonuclease activity based on significant amino acid sequence homology between a ginseng ribonuclease and parsley PR-10 proteins (Moiseyev *et al.*, 1997). Some members of the PR-10 gene family encode proteins that have RNase activity (Moiseyev *et al.*, 1997; Somssich *et al.*, 1986). Certain PR-10 proteins are induced by abiotic stresses, such as drought and salinity (Dubos and Plomion, 2001; Park *et al.*, 2004), whereas others respond to the hormone abscisic acid and show tissue-specific expression in pollen and root (Tiwari *et al.*, 2003). A PR-10 protein from white birch has been identified as a major pollen allergen (Bet v1) (Breiteneder *et al.*, 1989). In the present study, the transcription of PR-10 was shown to be induced in glumes of inoculated plants of most cultivars tested in the field. In gynoecia, the results were opposite in five cultivars (90EON-343, RTx430, SC279-14E, SC650-11E and Sureno); these cultivars

showed higher induction with lower C_t values in controls than in inoculated plants. In general, PR-10 mRNA levels in sorghum gynoecia imply that factors other than the induction of PR-10 influence grain mould susceptibility (Table 3). In field tests, most genotypes showed significant induction of NRQs of PR-10 after dual inoculation with spores of both *C. lunata* and *F. thapsinum* (Table 4 and Fig. 1). Although the lack of induction of some grain mould-resistant cultivars (SC103-12E and SC279-14E) implies that PR-10 is not always a resistance factor, it is equally likely that different cultivars may rely more on other defence response pathways, so that the creation of new combinations through the appropriate crosses could lead to enhanced resistance.

As many of the lines from the field study, especially those showing high mRNA induction in controls and cultivars showing the greatest level of PR-10 induction, are considered to be resistant varieties, further analysis was conducted under glasshouse conditions. One highly resistant (Tx2911), one resistant (Sureno), one susceptible (RTx430) and one moderately susceptible (SC170-6-17) cultivar were used for these experiments. Data were collected from plants inoculated with *C. lunata* alone, *F. thapsinum* alone or a mixture of spores from both fungi, as well as sterile water as a control. In glasshouse tests, resistant cultivars showed a greater induction of PR-10 than did susceptible cultivars. An unexpected observation from the glasshouse studies was that inoculation with spores from a single grain

Table 5 C_t values for pathogenesis-related protein 10 (PR-10) in sorghum glumes and gynoecia of four cultivars evaluated in the glasshouse (2007) in response to *Curvularia lunata*, *Fusarium thapsinum*, a mixture of both and water-sprayed control.

Table 6 Expression [normalized relative quantities (NRQs) = $2^{\Delta\Delta C_t}$] of pathogenesis-related protein 10 (PR-10) mRNA in glumes from four glasshouse-grown cultivars (2007) in response to *Curvularia lunata*, *Fusarium thapsinum*, a mixture of both and water-sprayed control.

Fig. 2 Histogram showing the expression (normalized relative quantities) of pathogenesis-related protein 10 (PR-10) mRNA in glumes of four sorghum cultivars inoculated with different treatments (glasshouse experiment, 2007).

mould-inducing pathogen induced greater responses (lower C_i values) than combined inoculation (Table 5). For three of the four cultivars (RTx430, SC170-6-17 and Sureno), response was highest following inoculation with *C. lunata* but, for Tx2911, maximum expression in terms of NRQs was induced by a mixture of *C. lunata* and *F. thapsinum* (Table 6 and Fig. 2). This suggests that the fungi may present different elicitors and that cultivars may show differential responses to these fungi; the lower levels of induction when both pathogens are present implies that the concentration of spores (or elicitors) may also be important. In the mixed treatments, only half the number of spores of either type were present. Taken together, under glasshouse-controlled conditions, glumes of inoculated plants showed a clear induction of PR-10 mRNA, and the response was greater in resistant than susceptible cultivars.

The observations that response levels were much greater and induction was more consistent in glumes than in gynoecia are compatible with the role of glumes providing a protective covering. The increased levels of PR-10 mRNA sometimes seen in water-inoculated controls (RTx430 in Table 6) suggest that the relatively low but variable levels of PR-10 mRNA in gynoecia are not simply a result of the higher levels of fungal penetration into female floral tissue in susceptible cultivars vs. resistant cultivars. It is possible that mRNA stability differs between the cultivars,

perhaps as a function of the ability of the RNAlater preservative to enter into the gynoecia. Other explanations, such as differential rates of development, are also possible.

The mechanism whereby PR-10 proteins contribute to overall host defence still remains unknown; however, the higher levels of expression seen in the glumes of resistant sorghum cultivars in this study suggest an active role in preventing fungal colonization and grain mould. The observation that resistant cultivars vary in the level of induction suggests that it may be possible to combine this trait with other factors to develop varieties with improved grain mould resistance.

EXPERIMENTAL PROCEDURES

Field study

Field experiments were conducted in 2006 at the Texas A&M Agricultural Research Farm located near College Station, TX, USA. Twelve sorghum cultivars, with varying degrees of susceptibility to grain mould (Table 7), were selected for this study and evaluated in a randomized block design replicated three times. Each replicate consisted of a single, 5.2 m row, with 76 cm row spacing and a plant spacing of 5–8 cm within rows.

Table 7 Sorghum cultivars and their characteristics included in the grain mould field evaluation conducted at College Station Research Farm, TX, USA during the 2006 growing season.

Cultivar	Pericarp colour	Plant colour	Grain mould reaction	Pigmented testa	Reference
90EON343	Red	Tan	MR	No	D.T. Rosenow, Texas Tech Univ., Lubbock, Tx
BTx623	White	Purple	S	No	W.L. Rooney, Texas A&M Univ., College Station, Tx
BTx399	Red	Purple	S	No	Stephens and Karper (1965)
BTxARG-1	White	Tan	S	No	Miller <i>et al.</i> (1992)
RTx430*	White	Purple	VS	No	Miller (1984)
SC103-12E	Red	Purple	R	Yes	Rosenow <i>et al.</i> (1997)
SC279-14E	Red	Purple	R	No	D.T. Rosenow, Texas Tech Univ., Lubbock, Tx
SC414-12E	White	Purple	MS	No	
SC650-11E(T)	Red	Purple	MR	No	Feliciano <i>et al.</i> (2003)
SC719-11E	Red	Purple	R	Yes	Waniska <i>et al.</i> (2001)
Sureno*	White	Tan	R	No	Meckenstock <i>et al.</i> (1993)
Tx2911*	Red	Purple	HR	No	Rooney <i>et al.</i> (2000)
SC170-6-17*†	White	Red	MS	No	D.T. Rosenow, Texas Tech Univ., Lubbock, Tx

*Selected cultivars used for further grain mould glasshouse evaluation in 2007.

†Used only for glasshouse evaluation in 2007.

VS, very susceptible; S, susceptible; MS, moderately susceptible; MR, moderately resistant; R, resistant; HR, highly resistant.

Isolates of *F. thapsinum* and *C. lunata*, developed and maintained as described by Little and Magill (2003), were used for inoculation. Three sorghum panicles at mid-anthesis in each replication were arbitrarily chosen and inoculated with a freshly prepared mixture of *C. lunata* and *F. thapsinum*. In addition, three plants at mid-anthesis from each replication were sprayed with sterile water as a control. Inoculum was sprayed at all angles on the panicles until the solution began to drip from the individual spikelets. The panicles were immediately covered with pollinating bags for 48 h to maintain high relative humidity and favour initial infection and colonization.

Glasshouse study

Sorghum cultivars Sureno, Tx2911, Tx430 and SC170-6-17 (Table 7) were evaluated in the glasshouse during 2007 under a randomized complete block design with split-plot arrangements. Five gallon pots with Sun Gro Metro Mix 360® 200 series soil mix (Bellevue, WA, USA) were used to raise five plants per pot. The four cultivars were main plots and the treatments were subplots. The fungal treatments consisted of individual panicles inoculated with *C. lunata*, *F. thapsinum* or a mixture of *C. lunata* and *F. thapsinum*. Untreated control panicles were sprayed with sterile water. For each treatment, three panicles per cultivar were used. Each treated or control panicle was considered as a replicate. Plants were maintained at 27–32 °C with regular irrigation and fertilization. The inoculation protocol was similar to that described in the field experiments.

Disease severity and seed viability analysis

At maturity, treated and control panicles from the field and glasshouse experiments were harvested and hand threshed.

Seeds from each panicle were used to estimate disease severity via visual rating, seed germination and 100-kernel weight. Visual grain mould severity was based on a 1–5 scale following Isakeit *et al.* (2008): 1, seed bright with no mould and no discoloration; 5, seed covered entirely with mould and deteriorated, and appeared dead. For germination, test seeds were plated onto a No. 1 Whatman® Schleicher and Schuell 90-mm circle filter paper (Cat #1001090, Kent, UK) in a Petri dish humidified with sterile water and kept at room temperature (25 °C). Twenty seeds were used per cultivar per replication. One week after plating, the seeds were evaluated for germination. The 100-kernel weight was determined by counting 100 kernels and recording the weight.

Seed mycoflora analysis

Eight cultivars with differential grain mould reaction were used for seed mycoflora analysis (Table 2). Seeds were collected from inoculated plants grown under field condition in 2006. Seed mycoflora was determined for 30 seeds per treatment for each variety, and the analysis was completed in triplicate. The identification of fungal species was based on the conidia, conidiophores, colony morphology and colour, according to descriptions provided by Booth (1971). Seed mycoflora analysis and 100-kernel weight were carried out as described in detail by Prom *et al.* (2003) and Prom (2004).

Data for grain mould incidence were analysed using Proc Glimmix (SAS Institute Inc. 2002–03, SAS® User's Guide, Statistical Analysis, version 9.1., SAS Institute, Cary, NC, USA). Mean comparisons were conducted using Tukey's studentized range test at the 5% probability level.

RNA extraction and purification

Forty-eight hours after inoculation, spikelet samples from both the field and glasshouse were collected and kept in RNAlater® (Ambion, Foster City, CA, USA). Glumes and gynoecia were separated using sterilized dissecting forceps whilst in RNAlater, and approximately 100 mg of sample were ground in liquid nitrogen for total RNA extraction using a Qiagen RNeasy® plant mini kit (Valencia, CA, USA). For routine DNase treatment, 1 µL rDNase I (2 U) (Ambion® DNA-free™ DNase) was used for up to 10 µg of RNA in a 50-µL reaction volume. The solution was incubated at 37 °C for 20–30 min before the addition of 0.1 vol of inactivating reagent, and mixed as directed in the Ambion protocol. After 2 min, the solution was centrifuged at 10 000 *g* for 1.5 min and the RNA was transferred to a fresh tube. After quantification using a Thermo Scientific NanoDrop™ spectrophotometer (Waltham, MA, USA), the samples were kept at –80 °C. The total RNA extracted is approximately 80%–85% ribosomal RNA (rRNA: 5S, 18S and 28S), 10%–15% transfer RNA (tRNA) and 1%–5% messenger RNA (mRNA).

Sample preparation and real-time PCR

The RNA samples were calibrated to a final concentration of 200 ng/µL. The protocols used for real-time PCR were as described for Invitrogen SuperScript™ III Platinum® SYBR® Green one step QRT-PCR (Invitrogen, Carlsbad, CA, USA). A Cepheid Smart Cycler® II System (Sunnyvale, CA, USA) was used for real-time PCR analysis.

The SuperScript™ III kit contains 2 × SYBR Green mix, which consists of a proprietary buffer system, SYBR Green I, MgSO₄, dNTPs and stabilizers, and SuperScript™ III reverse transcriptase with reduced RNase H activity but increased thermal stability.

PCRs were carried out in 25-µL reactions with 200 ng of RNA, 12.5 µL of 2 × Reaction Mix, 0.5 µL of SuperScript™ III RT/Platinum® *Taq* mix, 0.5 µL each of 25 pM/µL of forward (PR10rtF: CCGACGCCTACAACCTAAATCTG) and reverse (PR10rtR: CATACACCACACACGCATAGAG) primers and 10 µL of RNase-free water. Sample tubes were spun before loading in the 16 wells of the Cepheid Smart Cycler®. The PCR cycle was as follows: 50 °C for 15 min for cDNA synthesis, 95 °C for 2 min for initial *Taq* activation, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Post-amplification melting curves covered 65–95 °C. The results of RT-PCR in the form of fluorescence curves and threshold cycle (*C_t*) values were analysed using qBase software developed by Hellemans *et al.* (2007). Further, the conversion of quantification cycle values (*C_q*) into NRQs, as $NRQ = 2^{\Delta\Delta C_t}$, was calculated according to the method developed by Livak and Schmittgen (2001) and Pfaffl (2001). For PR-10 mRNA levels, mean comparisons of real-time RT-PCR analysis cycle threshold (*C_t*) values among cultivars vs. inocula-

tion treatments and inoculation treatments vs. respective controls of each cultivar were conducted using T grouping of the least-squares means at the 5% probability level.

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