Flavonoid Phytoalexin-Dependent Resistance to Anthracnose Leaf Blight Requires a Functional *yellow seed1* in *Sorghum bicolor*

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ABSTRACT

In *Sorghum bicolor*, a group of phytoalexins are induced at the site of infection by *Colletotrichum sublineolum*, the anthracnose fungus. These compounds, classified as 3-deoxyanthocyanidins, have structural similarities to the precursors of phlobaphenes. Sorghum *yellow seed1* (*y1*) encodes a MYB transcription factor that regulates phlobaphene biosynthesis. Using the *candystripe1* transposon mutagenesis system in sorghum, we have isolated functional revertants as well as loss-of-function alleles of *y1*. These near-isogenic lines of sorghum show that, compared to functionally revertant alleles, loss of *y1* lines do not accumulate phlobaphenes. Molecular characterization of two null *y1* alleles shows a partial internal deletion in the *y1* sequence. These null alleles, designated as *y1-ww1* and *y1-ww4*, do not accumulate 3-deoxyanthocyanidins when challenged with the nonpathogenic fungus *Cochliobolus heterostrophus*. Further, as compared to the wild-type allele, both *y1-ww1* and *y1-ww4* show greater susceptibility to the pathogenic fungus *C. sublineolum*. In fungal-inoculated wild-type seedlings, *y1* and its target flavonoid structural genes are coordinately expressed. However, in *y1-ww1* and *y1-ww4* seedlings where *y1* is not expressed, steady-state transcripts of its target genes could not be detected. Cosegregation analysis showed that the functional *y1* gene is genetically linked with resistance to *C. sublineolum*. Overall results demonstrate that the accumulation of sorghum 3-deoxyanthocyanidin phytoalexins and resistance to *C. sublineolum* in sorghum require a functional *y1* gene.

PHYTOALEXINS are chemically diverse antimicrobial compounds that are induced in response to microbes. Examples include isoflavonoids and pterocarpanes in legumes, sulfur-containing indole derivatives in cruciferous plants (TSUJI *et al.* 1992), sesquiterpenoids in solanaceous plants, and coumarins in umbelliferous plants (KNOGGE *et al.* 1987). In rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*), flavonoid compounds have been shown to act as phytoalexins against *Magnaporthe grisea* and *Colletotrichum* spp., respectively (SNYDER and NICHOLSON 1990; KODAMA *et al.* 1992). In sorghum leaves, a suite of reddish-brown flavonoid compounds are induced in the epidermal cells at the site of attempted fungal ingress (SNYDER and NICHOLSON 1990). These pigments belong to the 3-deoxyanthocyanidin class, which includes luteolinidin, 5-methoxy-luteolinidin, apigeninidin, caffeic acid ester of arabinosyl 5-O-apigeninidin, and 7-methoxyapigeninidin (SNYDER and NICHOLSON 1990; LO *et al.* 1996; WHARTON and NICHOLSON 2000).

This work is dedicated to the memory of Professor Ralph Nicholson, Purdue University, for his encouragement and enthusiasm in the study of the genetics and biochemistry of sorghum phytoalexins.

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Biosynthesis of these flavonoid compounds can be induced by inoculation of seedlings with the *Cochliobolus heterostrophus* fungus. Attempted penetration by this nonpathogenic fungus of sorghum leads to extremely rapid induction of 3-deoxyanthocyanidins (LO and NICHOLSON 1998; AGUERO *et al.* 2002). Traditionally, phytoalexin induction and biosynthesis have been studied in such *incompatible* systems for this reason. However, the profile of phytoalexins induced in this interaction can be directly compared to that of a *compatible* interaction (ROGERS *et al.* 1996).

The contribution of flavonoid phytoalexins to resistance against *Colletotrichum sublineolum* in sorghum has been investigated by comparing the response of several sorghum cultivars that differentially produce 3-deoxyanthocyanidins (WHARTON and JULIAN 1996; TENKOUANO *et al.* 1998; LO *et al.* 1999; BASAVARAJU *et al.* 2009). These studies, although performed on non-isogenic lines, indicated that phytoalexin production in the resistant cultivars was not only more rapid but also more intense than in the susceptible lines. In addition, phytoalexin accumulation was associated with the distortion of fungal hyphae and restriction of fungal proliferation in the resistant cultivars. On the other hand, extensive fungal colonization of the tissue was reported on the leaf blades of susceptible sorghum plants (NICHOLSON *et al.* 1987; TENKOUANO *et al.* 1998;

FIGURE 1.—Flavonoid biosynthetic pathway of anthocyanins, phlobaphenes, and 3-deoxyanthocyanidins in sorghum. Phenylalanine undergoes a series of enzymatic steps to give rise to 4-coumaroyl-CoA. CHS then catalyzes the stepwise condensation of one molecule of coumaroyl-CoA and three molecules of malonyl-CoA as the first step in the flavonoid biosynthetic pathway to form naringenin chalcone. This then

Lo *et al.* 1999). Although sorghum 3-deoxyanthocyanidins have been implicated in anthracnose leaf blight (ALB) resistance, the genetic and molecular mechanisms controlling their biosynthesis are unclear.

One of the approaches for elucidating the role of phytoalexins in disease resistance has been the use of phytoalexin-deficient (*pad*) mutants in *Arabidopsis thaliana* (GLAZEBROOK and AUSUBEL 1994; GLAZEBROOK *et al.* 1997; HAMMERSCHMIDT 1999; THOMMA *et al.* 1999). In this work, isolation and characterization of loss-of-function mutants has been performed to dissect the sorghum phytoalexin biosynthetic pathway. The 3-deoxyanthocyanidins have structural similarity with flavan-4-ols, which are precursors of the red phlobaphenes (Figure 1) (KAMBAL and BATE-SMITH 1976; STYLES and CESKA 1977). A parallel pathway has been suggested for the biosynthesis of 3-deoxyanthocyanidins (Figure 1) (LO and NICHOLSON 1998; BODDU *et al.* 2004, 2005). However, direct genetic evidence for the regulation of this branch of the pathway has been lacking because of the unavailability of near isogenic lines. The mutants analyzed in this study are in the *yellow seed1* (*y1*) gene, which encodes an R2R3 MYB transcription factor. We have previously shown that *y1* regulates phlobaphene accumulation and that a *Y1-candystripe* (*Y1-cs*) allele carrying an active transposon in *y1* produces a variegated seed pericarp phenotype (CHOPRA *et al.* 1999). The *Y1-cs* allele also showed partial deficiency in the biosynthesis of 3-deoxyanthocyanidins, providing the first clue to the dual role of this transcription factor (CHOPRA *et al.* 2002). We have now developed near-isogenic mutant and functional *y1* lines and demonstrated an overlap between phlobaphene and 3-deoxyanthocyanidin biosynthesis. The potential contribution of Y1-regulated 3-deoxyanthocyanidins in protecting sorghum plants against anthracnose disease was demonstrated through this pathway.

MATERIALS AND METHODS

Sorghum and fungal stocks: We have used the maize genetics nomenclature to name and describe sorghum [*Sorghum bicolor* (L.) Moench] *y1* alleles. The locus or gene is designated as *y1*, whereas alleles are shown as dominant (functional) or recessive (nonfunctional) followed by a two-letter suffix representing pericarp and glume pigmentation. For example, *Y1-rr* specifies a red pericarp and red glumes

undergoes stereo-specific isomerization by CHI to form naringenin, the common precursor of anthocyanins, phlobaphenes, and 3-deoxyanthocyanidins. The fate of naringenin is determined by the genetic constitution of the plant and the environmental conditions. CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; F3'H, flavonoid 3'-hydroxylase. (B) Seed pericarp and mature leaf phenotypes of sorghum genetic stocks carrying *Y1-cs30*, *Y1-rr3*, and *y1-ww1* alleles.

and *y1-ww* indicates a white pericarp and white glumes. The *Y1-rr3*, *y1-ww1*, and *y1-ww4* alleles used in this study originated from a common progenitor line (CS8110419) that carries a mutable *Y1-cs30* (candy stripe) allele (CHOPRA *et al.* 1999). The *Y1-rr3*, *y1-ww1*, and *y1-ww4* alleles resulted from spontaneous excision of the *candy stripe1* (*cs1*) transposon. The functionally revertant allele, *Y1-rr*, arises with a frequency of ~10%, and the structure of one such allele has been described previously (CHOPRA *et al.* 1999; BODDU *et al.* 2004). The nonfunctional *y1-ww* alleles were obtained with a frequency of 0.1%, and the molecular structure of two *y1-ww* alleles is described here.

Fungal cultures of *C. heterostrophus* [anamorph *Bipolaris maydis* (Nisikado and Miyake) Shoemaker] and *C. sublineolum* (P. Henn, Kabat, and Bubak) were maintained on potato dextrose agar or oatmeal agar under constant illumination at 26° for 7–10 days. The conidial suspensions were prepared in a Tween/water mixture as described previously (LO and NICHOLSON 1998; LO *et al.* 1999). Conidial suspensions were filtrated through cheesecloth and diluted to a concentration of 10⁶ spores·ml⁻¹.

Plant growth and fungal inoculation conditions: To obtain seedlings with uniformly etiolated mesocotyls, seeds were surface sterilized for 1 hr with 10% commercial bleach, washed in running tap water, and then imbibed in sterile water for 12 hr. Seeds were planted in germination paper rolls and incubated in the dark for 5–7 days at 26° in a growth chamber. To inoculate, the conidial suspension was sprayed onto etiolated seedlings followed by incubation in the dark at 26° in a growth chamber with 100% relative humidity. For controls, etiolated seedlings were sprayed with mock inoculum containing a Tween/water mixture only. Triplicate samples of mesocotyls were collected at 0, 3, 6, 9, 12, and 24 hr from both inoculated and control genotypes. At the time of tissue collection, seedlings were first photographed using a dissection scope SMZ1000 (Nikon) connected with a digital camera DXM1200F (Nikon). Mesocotyls were excised 5 mm above the point of attachment to the seed, flash frozen in liquid nitrogen, and stored at -80° for biochemical and gene expression analyses.

Fungal spores were harvested from cultures of *C. sublineolum* maintained on oatmeal agar and rinsed in water. To observe the development of disease symptoms, leaves of 6-week-old greenhouse grown plants were spray inoculated with a suspension of 10⁶ conidia·ml⁻¹ and 0.1% Tween 20 as a wetting agent. The plants were allowed to stand for 1 hr before respraying. Once surface moisture had evaporated from the leaves, the plants were placed in a mist chamber at 29° for 24 hr and transferred to a growth chamber maintained at a temperature of 29° and a light intensity of 20 μ mol·m⁻²·s⁻¹. High relative humidity was maintained by spraying water in the chamber twice a day. Disease progression and symptom development was recorded on 4 and 11 days post inoculation (dpi). Lesion density was measured at 4 dpi, and a percentage of the lesion area was recorded at 11 dpi. At 11 dpi, leaves were excised at the base and scanned. A total of 54 plants were used from each genotype and the lesion area on the image was measured using ImageJ software (ImageJ 1.36b; Wayne Rasband, National Institutes of Health, Bethesda, MD). To determine the effect of genotype on conidiation, the fourth leaf was immobilized on trays and drop inoculated with 10⁵ spores (10 drops/leaf, 5 plants/genotype), and the number of conidia produced 8 dpi was determined (GAO *et al.* 2007). Inoculations of plants were carried out independently a minimum of three times with similar results. An analysis of variance was computed for all these traits, considering the three inbred lines as source of variation. Mean comparisons were made using Fisher's protected LSD (STEEL *et al.* 1996). Statistical analyses were computed with SAS version 9.1 (SAS Institute).

Cosegregation analysis was performed using a testcross population from (*Y1-rr3*×*BT*×623) × *BT*×623. *BT*×623 is an anthracnose-susceptible line of sorghum (PERUMAL *et al.* 2009). Parents, F₁ plants, and 50 testcross progeny plants were genotyped using the *y1* gene fragment F-3 corresponding to intron II as shown previously (BODDU *et al.* 2005). Disease phenotypes were rated resistant or susceptible on the basis of the corresponding disease-response rating of the two parental lines (1, *Y1-rr3*—resistant; 5, *BT*×623—susceptible).

Plasmids and probes: Positions of *y1* gene primers and probes used for gel blot hybridizations are shown in Figure 2C. The *y1*-cDNA probe was prepared by PCR amplification of exons I and II and 70 bp of exon III using Y1-F1, 5'-ACAC ACTGCGAGCTGAGAG-3' and Y1-R3, 5'-CGAGTTCCAGTA GTTCTTGATC-3' as forward and reverse primers, respectively. Flavonoid structural gene probes consisted of DNA fragments obtained from the plasmid pZMC2 containing a maize *c2* cDNA (WIENAND *et al.* 1986), plasmid pZMA1 containing a maize *dfr1* cDNA (SCHWARZ-SOMMER *et al.* 1987), pChi1 containing maize *chi1* cDNA (from Erich Grotewold Ohio State University), and pSbF3'H3 containing a sorghum *f3'h3* gene (BODDU *et al.* 2004). Maize gene probes did not pose any problem with hybridizations because the maize genes used here are highly homologous to sorghum. For all probe preparations, the [α -³²P]dCTP was used as a label during random prime labeling of DNA fragments using prime-a-gene kit (Promega, Madison, WI).

DNA and RNA gel blot analysis: Plant genomic DNA was extracted from sorghum seedlings following the CTAB method (SAGHA-MAROOF *et al.* 1984). DNA was digested using restriction enzymes, reagents, and reaction conditions from Promega. DNA digests were fractionated on 0.8% agarose gels, and gels were blotted onto nylon membranes through standard protocol of capillary transfer (SAMBROOK and RUSSELL 2001). DNA gel blots were hybridized using buffers and conditions described previously (SEKHON and CHOPRA 2009).

Total RNA was isolated from inoculated sorghum seedlings collected at various time points using TriReagent (Molecular Research Center, Cincinnati). For preparation of slot blots, 200 ng of the purified and denatured gene fragment or linear plasmid vector DNA was added per slot and transferred to nitrocellulose membrane using a slot blotter. RNA samples [24 hr post inoculation (hpi)] were reverse transcribed using [³²P]dCTP as the radioactive label and first-strand cDNA was used as a probe. For RNA gel blots, 5 μ g of total RNA per sample was fractionated on 1.2% denaturing gels containing 5% (v/v) formaldehyde. Gels were rinsed in DEPC-treated water and blotted onto nylon membranes (Osmonics, Minnetonka, MN) by standard capillary transfer. RNA blots were hybridized with ³²P-labeled individual DNA probes for *y1*, *chs1*, *chi1*, *f3'h3*, and *dfr1* genes. All blots were exposed to X-OMAT X-ray films (Kodak, Rochester, NY) for 3 days prior to developing. A boiling solution of 0.1% SDS was used for stripping the blot for subsequent hybridizations.

PCR analysis: PCR analysis was performed on genomic DNA extracted from seedlings of *Y1-rr3*, *Y1-cs30*, *y1-ww1*, and *y1-ww4*. Forward primer Y1-F1, 5'-ACACACTGCGAGCTGAGAG-3', and reverse primer Y1-R1, 5'-GACGTGGCCCCGAAGGTAGT TGATCC-3', were used for amplification of a *y1*-DNA fragment corresponding to regions of exon I, intron I, and a part of exon II. In addition, Y1-F2 5'-CAAGAACTACTGGAATTCC CACCT-3' and Y1-R2 5'-AGTACAGTACATGTGAAGAAG-3' primers were used for amplification within exon III. PCR-based analyses of *y1-ww1* and *y1-ww4* alleles were done using several *y1* gene and *cs1* transposon-specific primers (not shown). Two *y1* primers present on the flanking regions of the deletion gave a PCR product. These primers are Y1-F1 in exon I and Y1-4R (5'-GAGCTAATGAGACGTGTC-3') in exon

III. All genomic PCR reactions were performed using standard conditions with an annealing temperature of 58° and 30 cycles of amplification.

Spectrophotometry and thin-layer chromatography: To detect the presence of 3-deoxyanthocyanidins, 100 mg of powdered tissues collected at 0, 24, and 36 hpi were incubated in 0.083% HCl in HPLC-grade methanol at 4° for 24 hr. The clear extracts were collected after centrifugation at 10,000 rpm for 10 min. Total anthocyanidin content was quantified spectrophotometrically at 480 nm using a UV mini-1240 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD), and the concentration was expressed as micromoles of 3-deoxyanthocyanidins using the molar extinction coefficient of luteolinidin ($13,800 \text{ M}^{-1}\text{cm}^{-1}$) (NICHOLSON *et al.* 1987; YAMAOKA *et al.* 1990; AGUERO *et al.* 2002).

For thin-layer chromatography (TLC), 100 mg of the ground tissues were extracted in HPLC-grade methanol and clarified by centrifugation at 14,000 rpm for 10 min. Equal aliquots of the clear extracts were loaded onto a 20×20 cellulose plate (Analtech, Newark, DE). TLC plates were developed in Forestal [$\text{CH}_3\text{COOH}:\text{HCl}:\text{H}_2\text{O}$ (30:3:10, v/v)] as a mobile phase (STAFFORD 1965). Commercially available apigeninidin and luteolinidin ($10 \text{ ng}\cdot\mu\text{L}^{-1}$) (Extrasynthese) were loaded as controls. These experiments were repeated at least three times using independent samples.

HPLC and liquid chromatography–mass spectrometry analysis: For HPLC analysis of compounds accumulating in mesocotyls, 20 mg of tissue was placed in 1 ml of HPLC-grade methanol, and compounds were allowed to leach at 4° for 24 hr in the dark. Extracts were collected by centrifugation at 1000 rpm, filtered through 0.45- μm Acrodisc LC 13-mm syringe filters (Gelman Laboratory, Ann Arbor, MI), and evaporated down to 100 μL . To separate compounds, two reversed phase C18 columns connected in tandem (Supelco, Bellefonte, PA) were used, and a 50- μL sample was applied to the column fitted on a Waters 600 HPLC separator with a Waters 900 detector system. Spectral measurements were taken over a wavelength range of 230–550 nm, which is known to detect all flavonoid compounds (GROTEWOLD *et al.* 1998). Detection of 3-deoxyanthocyanidins was carried out at 480 nm as described previously (SNYDER and NICHOLSON 1990; LO and NICHOLSON 1998). Pure luteolinidin and apigeninidin (Extrasynthese) were dissolved in HPLC-grade methanol and used as standards. To measure 3-deoxyanthocyanidins accumulating during *C. sublineolum* inoculation of greenhouse-grown plants, lesions were dissected and compounds were extracted as described above. Compounds were separated on a $1 \times 150\text{-mm}$ BetaBasic C18 column with a solvent gradient of water containing 0.15% formic acid (solvent A) and methanol (solvent B) with a flow rate of $0.05 \text{ mL}\cdot\text{min}^{-1}$ delivered using a preinjection split. The program of solvent composition was established as the following: 0–5 min (99% solvent A; 1% solvent B) followed by a linear gradient to 50% solvent A, 50% solvent B at 12 min, another linear gradient to 100% of solvent B at 20 min, with a hold at 100% solvent B until 25 min. Liquid chromatography–mass spectrometry (LC–MS) analyses were carried out using a Quattro II mass spectrometer (Micromass, Beverly, MA) interfaced with a Shimadzu LC10ADvp pump. Electro-spray ionization was carried out in the positive ions mode for detection of 3-deoxyanthocyanidin compounds.

RESULTS

Null *y1* alleles have internal deletions: Genetic tests showed that two sorghum mutant stocks with the white pericarp and white glumes phenotype segregated as

recessive alleles of *y1* and were designated as *y1-ww1* and *y1-ww4*. To characterize the cause of their nonfunctionality, gel blots were first hybridized with intron II probe F-3, and results are shown in Figure 2A. In the *Bam*HI digest of *Y1-cs30*, the F-3 probe hybridized to two bands of ~7.5 and 4.9 kb. The 7.5-kb band originates from digestion of *Bam*HI sites at positions 2633 and 3911 within *y1* and the *cs1* transposon, respectively. The 4.9-kb band is generated due to *Bam*HI sites at positions 2633 and 7542 in the *y1* sequence. In the *Bam*HI digest of *Y1-rr3* DNA, the 7.5-kb band is not present because of the absence of the *cs1* element. Interestingly, the F-3 probe did not hybridize to any genomic DNA fragments in the *y1-ww1* *Bam*HI digest. Similarly, bands of expected size were observed in *Hind*III, *Sca*I, and *Sa*I digests of *Y1-cs30* and *Y1-rr3* DNA with fragment F-3. This probe did not detect any homologous sequences in *y1-ww1* DNA digests, indicating that fragment F-3 may be either deleted or altered.

To test whether there is any modification in the *y1*-coding region in the *y1-ww1* line, the DNA gel blot was hybridized with *y1* cDNA (Figure 2A). In *Y1-cs30* and *Y1-rr3* *Bam*HI digests, the probe hybridized to bands of approximate sizes of 5.0 and 4.0 kb, representing *y1* and *y2* genes, respectively. *y2* is a pseudogene present at 8.5 kbp 3' to *y1* (BODDU *et al.* 2006). Interestingly, the cDNA probe hybridized only to the 4-kb *Bam*HI band (*y2* specific) in *y1-ww1*. In the *Hind*III digest, the 6.0-kb *y1*-specific band is absent in *y1-ww1*. Similarly, in the *Sca*I digest, the 1.9-kb band belonging to *y1* is also absent. This is indicated by the low intensity of this band in *y1-ww1* as compared to *Y1-rr3* and *y1-cs30*, both of which have *y1*- and *y2*-specific bands of 1.9 kb. *Sa*I digests could not be resolved very well because of large fragment sizes. Similar gel blot hybridization results were obtained from characterization of the *y1-ww4* allele (data not shown). These results established that the *y1-ww1* and *y1-ww4* alleles have deletions.

To further characterize the extent of the deletion within *y1-ww1* and *y1-ww4*, PCR analysis was performed using two sets of primers (Figure 2B). Primers located in exons I and II gave rise to expected PCR products of 846 bp in both *Y1-cs30* and *Y1-rr3*, while *y1-ww1* and *y1-ww4* did not show this amplification. However, primers corresponding to exon III detected *y1* (1016 bp)- and *y2* (821 bp)-specific PCR products in all four genotypes. These results together with DNA gel blot hybridization further confirmed that *y1* either is deleted or significantly modified in *y1-ww* alleles. To precisely map the deletion within *y1*, PCR amplifications were performed using forward primers in the 5' UTR, exon I, exon II, and reverse primers in the intron II or exon III regions. The primer combination Y1-F1 (5' UTR) and Y1-4R (intron II) gave PCR products of ~2.5 and 3.0 kb in *y1-ww1* and *y1-ww4* DNA, respectively. These PCR primers did not amplify any product from *Y1-rr3* and *Y1-cs30* DNAs. Furthermore, PCR-based investigations and se-

FIGURE 2.—Molecular structure of the *y1-ww1* and *y1-ww4* alleles. (A) DNA gel blot analysis of *Y1-rr3* (R), *y1-ww1* (W), and their common progenitor *Y1-cs30* (C lanes). A genomic DNA gel blot was hybridized with either an intron II-specific fragment of *Y1-rr3* (F-3) or a *y1*-cDNA probe. Molecular weight markers in kilobase pairs are shown between the blots. (B) PCR reactions were carried out using genomic DNA as templates and two sets of primer pairs: I, YF-1 and YR-1; II, YF-2 and YR-2. The positions of these primers are shown in C. Lanes are the following: 1, *Y1-cs30*; 2, *Y1-rr3*; 3, *y1-ww1*; and 4, *y1-ww4*. (C) Line diagram of the structure of *y1* in *Y1-rr3*, *y1-ww1*, and *y1-ww4*. A bent arrow represents the transcription start site. Solid boxes are exons joined by angled lines representing introns. The *csI* transposon inserted in intron II is shown as an inverted triangle. The open triangle in the maps of *y1-ww1* and *y1-ww4* represents the remnants of *csI*. The dashed line in *y1-ww1* and *y1-ww4* indicates sequence deletion. The restriction enzyme sites shown are the following: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; SA, *Sac*I; SL, *Sal*I; SC, *Sca*I; X, *Xho*I. The accession numbers corresponding to *y1* and *csI* sequences are AY860968 and AF206660, respectively.

quencing of the deletion end points revealed that both *y1-ww1* and *y1-ww4* alleles have a common deletion point at the 5' end of the *y1* sequence and harbor a piece of *csI*. As shown in Figure 2C, there is a leftover sequence of *csI* element. Interestingly, *y1-ww1* carries 2291 bp of the leftover transposon whereas *y1-ww4* carries 2818 bp. These leftover pieces correspond to the 3' end of the *csI* present at its original position in

intron II of *y1*. These molecular characterizations indicated that the two *y1* nulls may have resulted from the improper excision of the *csI* transposon from *y1*. The mechanism leading to an interstitial deletion leaving a fractured transposon within the *y1* sequence is not clear and currently under investigation.

Fungal-induced 3-deoxyanthocyanidins are not synthesized in *y1-ww* mesocotyls: On the basis of the

FIGURE 3.—Comparative response of etiolated seedlings of *Y1-rr3* and *y1-ww1* after inoculation with *C. heterostrophus*. (A) Accumulation of reddish-brown phytoalexin compounds in *Y1-rr3* and *y1-ww1* mesocotyls at 0, 24, and 36 hpi with *C. heterostrophus*. The upper mesocotyl in each panel is from the *Y1-rr3* allele while the lower one is from the *y1-ww1* allele. (B) TLC analysis of methanolic extracts prepared from inoculated *Y1-rr3* and *y1-ww1* mesocotyls. Luteolinidin and apigeninidin were loaded as standards.

proposed role of *y1* in the regulation of flavonoid biosynthesis, we tested the hypothesis that a functional *y1* gene is required for the synthesis of 3-deoxyanthocyanidins. Etiolated mesocotyls inoculated with *C. heterostrophus* showed accumulation of reddish-brown pigments in *Y1-rr3* (24 and 36 hpi), while these compounds were barely visible in *y1-ww1* (Figure 3A) and *y1-ww4* (not shown). Thin-layer chromatographic analysis of the *Y1-rr3* extract showed the presence of two bands that comigrated with the standards for luteolinidin and apigeninidin. These bands were not detected in the *y1-ww1* extract (Figure 3B). The acidified methanolic extracts of the inoculated *Y1-rr3* tissues showed absorption spectra characteristic of 3-deoxyanthocyanidins ($\lambda_{\max}$ 480 nm; data not shown). Total 3-deoxyanthocyanidin concentration produced at 0, 24, and 36 hpi indicated that, in response to fungal infection, sorghum seedlings carrying a functional *y1* gene accumulated much higher levels of anthocyanidin compounds than the ones with a nonfunctional *y1-ww1* allele

FIGURE 4.—Spectrophotometry of induced compounds. Quantification of flavonoid compounds from methanolic extracts prepared from *Y1-rr3* and *y1-ww1* mesocotyls harvested at 0, 24, and 36 hpi. Absorbance was recorded at 480 nm. The error bars represent the standard error of three replicates.

(Figure 4). In addition, it revealed that the level of total anthocyanidins increased over time in seedlings carrying a functional *y1* allele.

The identity of the compounds was confirmed by HPLC (Figure 5A). The retention times of primary standards of luteolinidin and apigeninidin were used to identify the induced compounds. We identified luteolinidin (retention time, ~10 min), apigeninidin (retention time, ~11 min), and 5-methoxyluteolinidin (retention time, ~12.8 min) in *Y1-rr3*. *y1-ww1* and *y1-ww4* produced trace amounts of unknown compounds. Extracts of uninoculated mesocotyls did not show induction of any pigments or 3-deoxyflavonoid compounds (data not shown). LC-MS analysis of the *Y1-rr3* extract showed three major and several minor ions (Figure 5B). Major peaks corresponded to luteolinidin [271 mass/charge ratio (m/z)], methoxy-apigeninidin (269 m/z), and methoxy-luteolinidin (285 m/z). One of the observed minor peaks was for a compound with a mass consistent with that of apigeninidin (255 m/z) while the other minor peaks could not be confirmed. In the *y1-ww1* extract, minor peaks that had retention times of ~10 and ~12.8 min were observed. These peaks could be attributed to the presence of other flavonoid compounds such as pelargonidin produced in response to stress. This is in agreement with our spectrophotometric quantification of total anthocyanidins, which were detected in the *y1-ww* lines although at much lower levels (see Figure 4).

***y1* and flavonoid structural genes are coordinately induced after fungal ingress:** To test whether the expression of the flavonoid structural genes was dependant on *y1* functionality, slot blot hybridization showed a significant level of *y1* transcripts in *Y1-rr3* while the signal observed in *y1-ww1* was equivalent to the background level (Figure 6A). Interestingly, *chs1* and *f3'h3* expression were also induced after fungal inoculation in *Y1-rr3* but not in *y1-ww1*. These results provided evidence that *y1* and its putative target genes in the flavonoid pathway are induced after inoculation with *C. heterostrophus* and that the differential induction of

FIGURE 5.—Characterization of the 3-deoxyanthocyanidins from inoculated mesocotyls of *Yl-rr3* and *yl-ww1* in response to *C. heterostrophus*. (A) HPLC chromatograms monitored at 480 nm. Pure luteolinidin and apigeninidin were eluted at ~10 and 11 min, respectively. (B) LC-MS profile of the *Yl-rr3* extract shows three major and a number of minor ions. The major ions at 269.1, 271.1, and 285.2 m/z correspond to methoxy-apigeninidin (MA), luteolinidin (L), and methoxy-luteolinidin (ML), respectively. One of the minor ions at 255 m/z corresponds to apigeninidin (A), while other minor ions have not been identified. The profile of the *yl-ww1* extract shows two minor peaks with mass/charge ratios of 269.1 and 271.1.

flavonoid genes correlates with the allelic constitution at the *yl* locus. Further, gel blots of total RNA isolated at 0, 3, 6, 9, 12, and 24 hpi were hybridized with *yl* and flavonoid structural gene probes (Figure 6B). *yl* transcripts were observed as early as 3 hpi and showed accumulation over time. Neither *chs1* nor *f3'h3* showed any detectable level of expression up to 6 hpi. Their transcripts started to accumulate by 9 hpi and reached a plateau at 12 hpi with a considerably higher level of *chs1* induction than that of *f3'h3*. Interestingly, *dfr1* exhibited much lower levels of induction at 6 and 12 hpi and then showed a sharp increase at 24 hpi. On the other hand, *chi1* showed expression through all time points with upregulation at the later time points (9–24 hpi). These results indicated that the transcriptional machinery of the flavonoid pathway is activated in response to *C. heterostrophus* infection. In addition, our data established that the induction of *yl* expression preceded that of these

candidate target genes. Expression of *yl* and the flavonoid structural genes studied here are coordinately regulated during sorghum–fungus interaction. In the *yl-ww1* plants, there was neither induction of *yl* nor the upregulation of the flavonoid structural genes with time (data not shown).

***Yl-rr3* line shows resistance to anthracnose leaf blight:** We examined fungal infection and disease progression in the *Yl-rr3* and mutant *yl-ww1* and *yl-ww4* lines to determine the role of 3-deoxyanthocyanidins in disease resistance to *C. sublineolum*, the causal agent of sorghum ALB. By 4 dpi, all three lines produced pigmented lesions at the sites of primary infection (SNYDER and NICHOLSON 1990) (Figure 7A). The lesions on the null lines progressed and eventually coalesced, resulting in necrosis of the infected leaves. At this stage, acervuli were observed in the lesions, and this phenotype has been documented (Lo *et al.* 1999). However, the lesions on the *Yl-rr3* line were restricted and the rest of the leaf

FIGURE 6.—Gene expression analysis of *y1* and the flavonoid structural genes. (A) Slot blot hybridization of radioactively labeled first-strand cDNA from RNA isolated 24 hpi. “Empty” means no DNA was added as a control for background hybridization. (B) Temporal expression of *y1* and flavonoid structural genes in *Y1-rr3* mesocotyls after inoculation with *C. heterostrophus*. Blots were probed with cDNA of *y1*, chalcone synthase (*chs1*), chalcone isomerase (*chi1*), flavonoid 3' hydroxylase (*f3'h3*), and dihydroflavonol reductase (*dfr1*). Numbers at the top represent hours after inoculation with *C. heterostrophus*.

tissue remained healthy. The lesion density on *Y1-rr3* was found to be 0.059 cm^{-2} while that on *y1-ww1* and *y1-ww4* was 0.120 cm^{-2} and 0.103 cm^{-2} , respectively. Lesion density on *y1-ww1* was greater than on *Y1-rr3* while differences between *Y1-rr3* and *y1-ww4* were not significant ($P = 0.0451$), (Figure 7B, I). By 11 dpi, a significantly greater area of the leaf was covered in lesions in the *y1-ww1* (55.78%) and *y1-ww4* (61.21%) lines compared to the *Y1-rr3* line (25.08%, $P \leq 0.01$) (Figures 7B, II). Because the inhibition of fungal proliferation would result

in reduced sporulation, the effect of genotype on fungal sporulation was determined 8 dpi. Analysis of variance showed that there was a highly significant difference between *Y1-rr3* and the two null lines ($P \leq 0.01$). The line *Y1-rr3* had an average of 1.96×10^6 conidia/disc while *y1-ww1* and *y1-ww4* had 3.40×10^6 and 3.51×10^6 conidia per disc, respectively (Figure 7B, III). This result shows that the presence of a functional *y1* gene correlates with greater resistance to the fungus *C. sublineolum* in sorghum. Our result thus provides genetic evidence that a sorghum line that produces 3-deoxyanthocyanidins under the control of *y1* is more resistant while two sibling lines that carry a transposon-induced deletion of the *y1* gene are more susceptible to *C. sublineolum*.

The pigments induced in the lesions at 72 hpi were extracted and analyzed. HPLC profiles of extracts from both null lines were similar and showed only two minor peaks corresponding to unknown compounds. The HPLC profile of the *Y1-rr3* line, on the other hand, showed five major peaks with retention times of 13.63, 14.20, 16.24, 18.40, and 18.75 min (Figure 7C). Three of these peaks were identified, on the basis of their retention times, as luteolinidin (13.63 min), apigeninidin (14.2 min), and 7-methoxy-apigeninidin (16.2 min) while the other two have not been identified. Retention times of 3-deoxyanthocyanidins in this experiment are different from the ones observed in mesocotyl extracts (Figure 5A). These differences can be attributed to the use of two different HPLC systems (see MATERIALS AND METHODS).

Cosegregation analysis of *y1* and resistance to *C. sublineolum*: Genetic linkage between the functional *y1* gene and resistance to *C. sublineolum* was tested in a testcross population derived from [$(Y1-rr3 \times BT \times 623) \times BT \times 623$]. The sorghum BT×623 line is susceptible to *C. sublineolum* (Figure 8A) (Lo *et al.* 1999) and carries a nonfunctional allele of *y1* (BODDU *et al.* 2005). Parents, F_1 , and testcross progeny plants were grown in a greenhouse and genotyped at the seedling stage. DNA gel blots carrying *Bam*HI-digested genomic DNA from testcross progeny plants were hybridized with the *y1*-intron II probe F-3. As shown previously, BT×623 carries a partially deleted *y1* gene that gives rise to an 11.5-kb *Bam*HI band hybridizing with the F3 probe, whereas a functional *y1* gene present in *Y1-rr3* shows an ~5.0-kb restriction band (CHOPRA *et al.* 2002; BODDU *et al.* 2005). Results of the cosegregation analysis are shown in Figure 8B; the hybridization pattern of a set of representative samples shows that the heterozygous plants carry both 11.5- and 5.0-kb bands (“R/B” lanes) while the homozygous BT×623 segregants (“B” lanes) show the presence of the 11.5-kb band. Plants were inoculated with *C. sublineolum* (see MATERIALS AND METHODS), and disease lesions were observed 7 dpi. Progeny plants were scored as resistant or susceptible, depending upon the phenotypes in comparison with disease lesions observed in the two parental lines (Figure 8A). Resistant and susceptible plants segregated, as expected, at a 1:1

FIGURE 7.—Fungal infection and plant responses after infection with *C. sublineolum*. (A) Inoculated leaves of the three genotypes showing disease symptoms 4 dpi (a, c, and e) and 11 dpi (b, d, and f). Restriction of the disease is shown in the *Yl-rr3* (b). Development of characteristic ALB symptoms with necrosis of the leaf as the lesions grow and coalesce is shown in the null *yl-ww* lines (d and f). (B) Quantification of lesion density at 4 dpi (I), percentage of infection area at 11 dpi (II), and sporulation at 8 dpi (III). (C) HPLC analysis of the induced 3-deoxyanthocyanidin compounds 72 hpi with *C. sublineolum*. (Top) A chromatogram of pigments extracted from lesions of inoculated *Yl-rr3* leaves showing the accumulation of luteolinidin (L, retention time 13.63 min), apigeninidin (A, 14.2 min), and 7-methoxy-apigeninidin (MA, 16.2 min). (Bottom) A chromatogram of pigments extracted from inoculated plants of the *yl-ww1* allele.

ratio for a testcross (27 resistant/23 susceptible). Genotyping showed that all heterozygous (*Yl-rr3*/BT×623) plants were resistant while homozygous (BT×623) segregants were susceptible. This result further confirms that resistance to *C. sublineolum* is genetically linked to a functional *yl* gene present in *Yl-rr3*.

DISCUSSION

Sorghum responds to the invasion of both pathogenic and nonpathogenic fungi by the induction of 3-deoxyanthocyanidin phytoalexins (NICHOLSON *et al.* 1987; Lo *et al.* 1999). Although extensive studies have demonstrated the antimicrobial nature of these com-

FIGURE 8.—Cosegregation analysis of disease phenotype and *y1* gene in a testcross population of [(*Y1-rr3* × BT×623) × BT×623]. (A) ALB phenotypes of *C. sublineolum*-infected leaves of *Y1-rr3* and BT×623 observed at 7 dpi. (B) Genotype and disease phenotype of testcross progeny plants. “R/B” lanes indicate *Y1-rr3*/BT×623 heterozygotes, and “B” lanes are homozygous for BT×623. The disease phenotype corresponding to each plant is shown below the lane.

pounds and their elevated levels in resistant lines when exposed to pathogens, their biosynthetic pathway and its regulation remains obscure. Furthermore, present evidence for the role of 3-deoxyanthocyanidins in disease resistance is not conclusive since these studies were carried out using non-isogenic lines. The structural similarities between 3-deoxyanthocyanidins and flavan-4-ols, the precursors of the red phlobaphene pigments found in sorghum and maize, suggest that these compounds might be synthesized via a common or overlapping pathway (GROTEWOLD *et al.* 1998; CHOPRA *et al.* 2002; WINEFIELD *et al.* 2005). In a previous study, we showed that a mutable allele of an R2R3 MYB transcription factor encoded by *Y1-cs* is partially defective in the synthesis of 3-deoxyanthocyanidins (CHOPRA *et al.* 2002). In a recent study, we have found that biosynthesis of sorghum flavan-4-ols is under the regulatory control of *y1* (BODDU *et al.* 2005, 2006). In this article, we have characterized a functionally revertant allele and two null alleles of *y1*. The molecular analysis and sequencing of the *y1-ww1* and *y1-ww4* alleles indicated that these have an internal deletion in the *y1* sequence. This deletion

removes most of the Myb domain and thus makes the *Y1* protein nonfunctional. Moreover, expression analysis indicated that these *y1-ww* alleles are loss-of-function alleles and do not show any *y1*-specific transcript even during fungal infection. Biochemical analysis established that these *y1* null alleles are deficient in flavan 4-ols and 3-deoxyflavonoid pigment accumulation, while the functional *Y1-rr3* allele accumulates these compounds. Our disease assays revealed that, as compared to *y1-ww* null alleles, *Y1-rr3* synthesizes the suite of 3-deoxyanthocyanidin compounds in response to infection with both *C. heterostrophus* and *C. sublineolum*. Furthermore, these data indicate that 3-deoxyanthocyanidins play a role in making the plant resistant to ALB.

Phytoalexin accumulation has been shown to be associated with *de novo* protein synthesis of enzymes required for their biosynthesis (DIXON *et al.* 1983; CRAMER *et al.* 1985; BERENBAUM and ZANGERL 1992; DIXON and PAIVA 1995). We have shown that genes coding for enzymes predicted to be involved in 3-deoxyanthocyanidin biosynthesis are induced and upregulated over time in response to infection with *C. heterostrophus*. The activation of biosynthetic genes in metabolic pathways is often coordinated by transcription factors. Chalcone synthase (CHS) and chalcone isomerase (CHI) are two early pathway enzymes that drive the flavonoid pathway toward the formation of naringenin, the common precursor of many flavonoid compounds including anthocyanins, flavones, and phlobaphenes (DONG *et al.* 2001; WINKEL-SHIRLEY 2001a,b). Gene expression analysis in the *y1* mutant and wild-type genotypes further revealed that the transcription of these biosynthetic genes is *y1* dependent. In addition, this investigation revealed that the accumulation of *y1* transcripts preceded (3 hpi) that of the biosynthetic genes, which reached a peak several hours post inoculation. The coordinated induction of flavonoid structural genes reported here supports the idea of a common transcription factor. The cosegregation analysis in our testcross population shows that *y1* is genetically linked to ALB resistance. To shunt naringenin toward 3-deoxyanthocyanidins, it is crucial to induce late-pathway enzymes such as DFR, ANS, and F3'H (see Figure 1). Similarly, induction of camalexin in Arabidopsis has been associated with transcriptional activation of both early pathway genes for the biosynthesis of tryptophan and late-pathway genes such as anthranilate synthase (ZHAO and LAST 1996; SCHUEGGER *et al.* 2007). In rice, the biosynthesis of sakuranetin was shown to be correlated with the accumulation of naringenin and the induction of naringenin *O*-methyltransferase (KODAMA *et al.* 1992; RAKWAL *et al.* 2000, 2001).

In response to infection by the anthracnose fungus *C. sublineolum*, sorghum plants carrying a functional *y1* allele accumulated known 3-deoxyanthocyanidins, resulting in a significant reduction in ALB disease symptoms. This is evinced by the localized red spots on the infected leaves. On the other hand, the *y1-ww1*

and *yl-ww4* lines were deficient in the biosynthesis of 3-deoxyanthocyanidins and exhibited severe symptoms typical of anthracnose disease. The antifungal activity of these 3-deoxyanthocyanidins, and particularly of luteolinidin and methoxy luteolinidin, has been established (NICHOLSON *et al.* 1987). This investigation provides genetic evidence that a functional *yl* confers increased resistance to ALB. The gene expression analysis supports our biochemical data, and both suggest that the functional *yl* controls biosynthesis of sorghum 3-deoxyanthocyanidins. In addition to inducing the requisite pathway genes, *yl* is predicted to contain promoter elements that are responsive to specific stresses such as pathogenesis or herbivory. Therefore, these favorable traits such as resistance to ALB can be introduced to related plant species more economically by transferring a single regulatory gene.

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LITERATURE CITED

- AGUERO, M. E., A. GEVENS and R. L. NICHOLSON, 2002 Interaction of *Cochliobolus heterostrophus* with phytoalexin inclusions in *Sorghum bicolor*. *Physiol. Mol. Plant Pathol.* **61**: 267–271.
- BASAVARAJU, P., N. P. SHETTY, H. S. SHETTY, E. DE NEERGAARD and H. J. L. JØRGENSEN, 2009 Infection biology and defence responses in sorghum against *Colletotrichum sublineolum*. *J. Appl. Microbiol.* **107**: 404–415.
- BERENBAUM, M. R., and A. R. ZANGERL, 1992 Genetics of secondary metabolism and herbivore resistance in plants, pp. 415–438 in *Herbivores: Their Interactions With Secondary Plant Metabolites*, edited by G. A. ROSENTHAL and M. R. BERENBAUM. Academic Press, San Diego.
- BODDU, J., C. SVABEK, R. SEKHON, A. GEVENS, R. L. NICHOLSON *et al.*, 2004 Expression of a putative flavonoid 3'-hydroxylase in sorghum mesocotyls synthesizing 3-deoxyanthocyanidin phytoalexins. *Physiol. Mol. Plant Pathol.* **65**: 101–113.
- BODDU, J., C. SVABEK, F. IBRAHEEM, A. D. JONES and S. CHOPRA, 2005 Characterization of a deletion allele of a sorghum Myb gene, *yellow seed1*, showing loss of 3-deoxyflavonoids. *Plant Sci.* **169**: 542–552.
- BODDU, J., C. H. JIANG, V. SANGAR, T. OLSON, T. PETERSON *et al.*, 2006 Comparative structural and functional characterization of sorghum and maize duplications containing orthologous Myb transcription regulators of 3-deoxyflavonoid biosynthesis. *Plant Mol. Biol.* **60**: 185–199.
- CHOPRA, S., V. BRENDL, J. B. ZHANG, J. D. AXTELL and T. PETERSON, 1999 Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. *Proc. Natl. Acad. Sci. USA* **96**: 15330–15335.
- CHOPRA, S., A. GEVENS, C. SVABEK, K. V. WOOD, T. PETERSON *et al.*, 2002 Excision of the *CandyStripe1* transposon from a hypermutable *Y1-cs* allele shows that the sorghum *Y1* gene controls the biosynthesis of both 3-deoxyanthocyanidin phytoalexins and phlobaphene pigments. *Physiol. Mol. Plant Pathol.* **60**: 321–330.
- CRAMER, C. L., J. N. BELL, T. B. RYDER, J. A. BAILEY, W. SCHUCH *et al.*, 1985 Co-ordinated synthesis of phytoalexin biosynthetic enzymes in biologically-stressed cells of bean (*Phaseolus vulgaris* L.). *EMBO J.* **4**: 285–289.
- DIXON, R. A., and N. L. PAIVA, 1995 Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**: 1085–1097.
- DIXON, R. A., P. M. DEY and C. J. LAMB, 1983 Phytoalexins: enzymology and molecular biology. *Adv. Enzymol. Relat. Areas Mol. Biol.* **55**: 1–136.
- DONG, X. Y., E. L. BRAUN and E. GROTEWOLD, 2001 Functional conservation of plant secondary metabolic enzymes revealed by complementation of Arabidopsis flavonoid mutants with maize genes. *Plant Physiol.* **127**: 46–57.
- GAO, X. Q., W. B. SHIM, C. GÖBEL, S. KUNZE, I. FEUSSNER *et al.*, 2007 Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. *Mol. Plant Microbe Interact.* **20**: 922–933.
- GLAZEBROOK, J., and F. M. AUSUBEL, 1994 Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proc. Natl. Acad. Sci. USA* **91**: 8955–8959.
- GLAZEBROOK, J., M. ZOOK, F. MERT, I. KAGAN, E. E. ROGERS *et al.*, 1997 Phytoalexin-deficient mutants of Arabidopsis reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics* **146**: 381–392.
- GROTEWOLD, E., M. CHAMBERLIN, M. SNOOK, B. SIAME, L. BUTLER *et al.*, 1998 Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. *Plant Cell* **10**: 721–740.
- HAMMERSCHMIDT, R., 1999 Phytoalexins: What have we learned after 60 years? *Annu. Rev. Phytopathol.* **37**: 285–306.
- KAMBAL, A. E., and E. C. BATE-SMITH, 1976 A genetic and biochemical study on pericarp pigments in a cross between two cultivars of grain sorghum, *Sorghum bicolor*. *Heredity* **37**: 413–416.
- KNOGGE, W., E. KOMBRINK, E. SCHMELZER and K. HAHNBROCK, 1987 Occurrence of phytoalexins and other putative defense-related substances in uninfected parsley plants. *Planta* **171**: 279–287.
- KODAMA, O., J. MIYAKAWA, T. AKATSUKA and S. KIYOSAWA, 1992 Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. *Phytochemistry* **31**: 3807–3809.
- LO, S. C. C., and R. L. NICHOLSON, 1998 Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls: implications for a compensatory role in the defense response. *Plant Physiol.* **116**: 979–989.
- LO, S. C., I. WEIERGANG, C. BONHAM, J. HIPSKIND, K. WOOD *et al.*, 1996 Phytoalexin accumulation in sorghum: identification of a methyl ether of luteolinidin. *Physiol. Mol. Plant Pathol.* **49**: 21–31.
- LO, S. C. C., K. DE VERDIER and R. L. NICHOLSON, 1999 Accumulation of 3-deoxyanthocyanidin phytoalexins and resistance to *Colletotrichum sublineolum* in sorghum. *Physiol. Mol. Plant Pathol.* **55**: 263–273.
- NICHOLSON, R. L., S. S. KOLLIPARA, J. R. VINCENT, P. C. LYONS and G. CADENA-GOMEZ, 1987 Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. *Proc. Natl. Acad. Sci. USA* **84**: 5520–5524.
- PERUMAL, R., M. A. MENZ, P. J. MEHTA, S. KATILÉ, L. A. GUTIERREZ-ROJAS *et al.*, 2009 Molecular mapping of *Cg1*, a gene for resistance to anthracnose (*Colletotrichum sublineolum*) in sorghum. *Euphytica* **165**: 597–606.
- RAKAWAL, R., G. K. AGRAWAL, M. YONEKURA and O. KODAMA, 2000 Naringenin 7-O-methyltransferase involved in the biosynthesis of the flavanone phytoalexin sakuranetin from rice (*Oryza sativa* L.). *Plant Sci.* **155**: 213–221.
- RAKAWAL, R., K. SHII, G. K. AGRAWAL and M. YONEKURA, 2001 Protein phosphatase inhibitors activate defense responses in rice (*Oryza sativa*) leaves. *Physiol. Plant.* **111**: 151–157.
- ROGERS, E. E., J. GLAZEBROOK and F. N. AUSUBEL, 1996 Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*-pathogen interactions. *Mol. Plant Microbe Interact.* **9**: 748–757.
- SAGHA-MAROOF, M. A., K. M. SOLIMAN, R. A. JØRGENSEN and R. W. ALLARD, 1984 Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* **81**: 8014–8018.

- SAMBROOK, J., and D. RUSSELL, 2001 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SCHUEHEGGER, R., T. RAUHUT and E. GLAWISCHNIG, 2007 Regulatory variability of camalexin biosynthesis. *J. Plant Physiol.* **164**: 636–644.
- SCHWARZ-SOMMER, Z., N. SHEPHERD, E. TACKE, A. GIERL, W. ROHDE *et al.*, 1987 Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. *EMBO J.* **6**: 287–294.
- SEKHON, R. S., and S. CHOPRA, 2009 Progressive loss of DNA methylation releases epigenetic gene silencing from a tandemly repeated maize *Myb* gene. *Genetics* **181**: 81–91.
- SNYDER, B. A., and R. L. NICHOLSON, 1990 Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science* **248**: 1637–1639.
- STAFFORD, H. A., 1965 Flavonoids and related phenolic compounds produced in the first internode of *Sorghum vulgare* Pers. in darkness and in light. *Plant Physiol.* **40**: 130.
- STEEL, R. G. D., D. A. DICKEY and J. H. TORRIE, 1996 *Principles and Procedures of Statistics: A Biometrical Approach*. McGraw-Hill Higher Education, New York.
- STYLES, E. D., and O. CESKA, 1977 Genetic control of flavonoid synthesis in maize. *Can. J. Genet. and Cytol.* **19**: 289–302.
- TENKOUANO, A., F. R. MILLER, R. A. FREDERICKSEN and R. L. NICHOLSON, 1998 Ontogenetic characteristics and inheritance of resistance to leaf anthracnose in sorghum. *Afr. Crop Sci. J.* **6**: 249–258.
- THOMMA, B., I. NELISSEN, K. EGGERMONT and W. F. BROEKAERT, 1999 Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J.* **19**: 163–171.
- TSUJI, J., E. P. JACKSON, D. A. GAGE, R. HAMMERSCHMIDT and S. C. SOMERVILLE, 1992 Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv. *synringae*. *Plant Physiol.* **98**: 1304–1309.
- WHARTON, P. S., and A. M. JULIAN, 1996 A cytological study of compatible and incompatible interactions between *Sorghum bicolor* and *Colletotrichum sublineolum*. *New Phytol.* **134**: 25–34.
- WHARTON, P. S., and R. L. NICHOLSON, 2000 Temporal synthesis and radiolabelling of the sorghum 3-deoxyanthocyanidin phytoalexins and the anthocyanin, cyanidin 3-dimalonyl glucoside. *New Phytol.* **145**: 457–469.
- WIENAND, U., U. WEYDEMANN, U. NIESBACH-KLÖSGEN, P. A. PETERSON and H. SAEDLER, 1986 Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. *Mol. Gen. Genet.* **203**: 202–207.
- WINEFIELD, C. S., D. H. LEWIS, E. E. SWINNY, H. B. ZHANG, H. S. ARATHOON *et al.*, 2005 Investigation of the biosynthesis of 3-deoxyanthocyanins in *Sinningia cardinalis*. *Physiol. Plant.* **124**: 419–430.
- WINKEL-SHIRLEY, B., 2001a Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* **126**: 485–493.
- WINKEL-SHIRLEY, B., 2001b It takes a garden. How work on diverse plant species has contributed to an understanding of flavonoid metabolism. *Plant Physiol.* **127**: 1399–1404.
- YAMAOKA, N., P. C. LYONS, J. HIPSKIND and R. L. NICHOLSON, 1990 Elicitor of sorghum phytoalexin synthesis from *Colletotrichum graminicola*. *Physiol. Mol. Plant Pathol.* **37**: 255–270.
- ZHAO, J. M., and R. L. LAST, 1996 Coordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in *Arabidopsis*. *Plant Cell* **8**: 2235–2244.

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