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Response of Sorghum Enhanced in Monolignol Biosynthesis to Stalk Rot Pathogens

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Abstract

To increase phenylpropanoid constituents and energy content in the versatile C$_4$ grass sorghum (*Sorghum bicolor* [L.] Moench), sorghum genes for proteins related to monolignol biosynthesis were overexpressed: SbMyb60 (transcriptional activator), SbPAL (phenylalanine ammonia lyase), SbCCoAOMT (caffeoyl coenzyme A [CoA] 3-*O*-methyltransferase), Bmr2 (4-coumarate:CoA ligase), and SbC3H (coumaroyl shikimate 3-hydroxylase). Overexpression lines were evaluated for responses to stalk pathogens under greenhouse and field conditions. Greenhouse-grown plants were inoculated with *Fusarium thapsinum* (Fusarium stalk rot) and *Macrophomina phaseolina* (charcoal rot), which cause yield-reducing diseases. *F. thapsinum*-inoculated overexpression plants had mean lesion lengths not significantly different than wild-type, except for significantly smaller lesions on two of three *SbMyb60* and one of two *SbCCoAOMT* lines. *M. phaseolina*-inoculated overexpression lines had lesions not significantly different from wild-type except one *SbPAL* line (of two lines studied) with mean lesion lengths significantly larger. Field-grown *SbMyb60* and *SbCCoAOMT* overexpression plants were inoculated with *F. thapsinum*. Mean lesions of *SbMyb60* lines were similar to wild-type, but one *SbCCoAOMT* had larger lesions, whereas the other line was not significantly different than wild-type. Because overexpression of *SbMyb60*, *Bmr2*, or *SbC3H* may not render sorghum more susceptible to stalk rots, these lines may provide sources for development of sorghum with increased phenylpropanoid concentrations.

Keywords: fungi, sorghum, resistance, monolignol biosynthesis, *Fusarium thapsinum*, *Macrophomina phaseolina*

The C$_4$ grass sorghum (*Sorghum bicolor* [L.] Moench) is being used for feed, forage, food, and feedstock for bioenergy platforms. Grain and sweet sorghum are currently being used for grain- and juice-based ethanol production to supplement corn or sugarcane, respectively (Teixeira et al. 2017; K. Zhang et al. 2017). However, biomass from forage or energy sorghums, or bagasse from sweet sorghum, also has huge bioenergy potential (Ameen et al. 2017; Barcelos et al. 2016). Lignocellulosic ethanol can be effectively produced from sorghum biomass, particularly if processes reduce the negative impacts of lignin, which can impair enzyme access to hemicellulose, one of two cell wall polysaccharides (Scully et al. 2016).

However, lignin and the phenolic intermediates of the phenylpropanoid pathway incorporated into the cell wall have greater energy potential than the two cell wall polysaccharides, cellulose and hemicellulose (Boateng et al. 2008). Alternatives to lignocellulosic ethanol for energy production involve thermochemical conversion of biomass through pyrolysis to biocrude oil, liquid and gaseous

(syngas and hydrogen) (Azadi et al. 2013), or using cofiring technologies to increase efficiency and environmental quality of fossil fuel-generated electricity (Madanayake et al. 2017). It would be advantageous to increase phenylpropanoid content of biomass in order to increase its energy content for thermoconversion platforms. However, there has been increasing interest recently in developing ways to valorize lignin for a range of applications (Ragauskas et al. 2014; Wu et al. 2017; Xie et al. 2015).

The monolignol biosynthesis pathway produces three major lignin subunits (*p*-coumaryl, coniferyl, and sinapyl) that yield, respectively, three forms of lignin: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Fig. 1). Specific steps of the monolignol pathway have been characterized in sorghum (Bout and Vermerris 2003; Eudes et al. 2017; Saballos et al. 2012; Sattler et al. 2009; Scully et al. 2016, 2018; Walker et al. 2013). Three steps linked to the brown midrib phenotype are particularly well-studied. Sorghum *brown midrib* (*bmr*) mutants have reddish-brown leaf midribs instead of white or green midribs of wild-type sorghum, and this mutant phenotype has long been associated with reduced lignin content and altered lignin composition (Palmer et al. 2008; Pillonel et al. 1991; Saballos et al. 2012). *Bmr6* and *Bmr12* encode a cinnamyl alcohol dehydrogenase (CAD) and a caffeic acid *O*-methyltransferase (COMT) that catalyze the last step synthesizing all three monolignols, and the penultimate step synthesizing sinapyl aldehyde, respectively (Fig. 1). *Bmr2* encodes for 4-coumarate:coenzyme A [CoA] ligase (4CL), which catalyzes the formation of 4-coumaroyl CoA, a substrate in the synthesis of both flavonoids and monolignols (Saballos et al. 2012). Recently, sorghum overexpressing the transcription factor, *SbMyb60*, or the monolignol biosynthesis pathway enzyme, caffeoyl CoA 3-*O*-methyltransferase (*SbCCoAOMT*), were characterized (Scully et al. 2016, 2018; Tetreault et al. 2018) (Fig. 1). The *SbMyb60* transcription factor activates many of the genes encoding enzymes in the monolignol biosynthesis pathway (Scully et al. 2016, 2018). Overexpression of *SbMyb60* also significantly increased lignin content and altered cell wall composition, and it activated genes involved in biosynthesis of aromatic amino acids and S-adenosyl methionine

(SAM), which are substrates and a cofactor, respectively, necessary for monolignol biosynthesis. In addition, *SbMyb60* overexpression affected expression of genes involved in nitrogen assimilation and carbon metabolism, presumably to redirect carbon and nitrogen toward monolignol biosynthesis (Scully et al. 2018). The enzyme SbCCoAOMT is a SAM-dependent *O*-methyltransferase that catalyzes the formation of feruloyl-CoA required for synthesis of both G- and S-lignin (Tetreault et al. 2018). Interestingly, overexpression of *SbCCoAOMT* did not affect lignin content nor alter lignin composition; however, biomass total energy availability was significantly increased owing to increased soluble and cell wall-bound ferulic and sinapic acids.

Lignin and other phenylpropanoid metabolites have long been implicated in plant defense (Naoumkina et al. 2010; Nicholson and Hammerschmidt 1992; Walter 1992) as induced or extant physical barriers within the cell walls to limit pathogen ingress (Uloth et al. 2015; Wuyts et al. 2007; Y. Zhang et al. 2017) and antimicrobial or signaling molecules directly or indirectly inhibiting pathogen progression (Chang et al. 2015; Gunnaiah and Kushalappa 2014; Naoumkina et al. 2010). However, the role of these plant constituents became more complex in sorghum when *bmr6*, *bmr12*, and *bmr6 bmr12* double mutant lines, significantly reduced in lignin content (Oliver et al. 2005; Pedersen et al. 2008), were compared with near-isogenic wild-type lines for responses to sorghum fungal stalk pathogens. None of the *bmr* lines were shown to be more susceptible to the pathogens, and in some cases the *bmr* lines were more resistant to a given pathogen, under greenhouse or field conditions (Funnell and Pedersen 2006; Funnell-Harris et al. 2010, 2014, 2017, 2018). Analyses of cell wall-bound and soluble phenolic compounds showed that *bmr* lines accumulated significantly more phenolic compounds derived from monolignol biosynthesis than near-isogenic wild-type lines (Palmer et al. 2008). These phenolic acids were shown to be inhibitory to sorghum fungal pathogens in vitro at biological concentrations (Funnell-Harris et al. 2014). However, soluble extracts from *bmr6* stalks did not inhibit in vitro growth of the virulent stalk pathogen *Fusarium thapsinum* Klittich, J.F. Leslie, P.E.

Nelson & Marasas 1997 (= *Gibberella thapsina* Klittich, J.F. Leslie, P.E. Nelson & Marasas 1997), which suggested that cell wall-bound phenolics or these compounds acting as signals to trigger defense-related pathways are also involved in the increased resistance observed in the *bmr*-pathogen interactions (Funnell-Harris et al. 2017). Therefore, overexpression of monolignol biosynthetic genes or the pathway transcriptional activator, *SbMyb60*, could alter how sorghum plants respond to stalk pathogens leading to either increased or decreased resistance.

Sorghum stalk diseases are huge problems worldwide that cause reduced grain, biomass, and sugar yield and quality (Bandara et al. 2017b, 2018; Rajewski and Francis 1991), and the increased propensity of infected plants to lodge further reduces yield (Claflin and Giorda 2002; Mughogho and Rosenberg 1984). Fusarium stalk rot, which is caused by several *Fusarium* species (Bandara et al. 2017c; Claflin and Giorda 2002; Funnell-Harris et al. 2017; Kelly et al. 2017; Mughogho and Rosenberg 1984) and degrades the stalk pith, likely negatively affects translocation of water and nutrients (Claflin and Giorda 2002; Reed et al. 1983). Charcoal rot, which is caused by *Macrophomina phaseolina* (Tassi) Goid. 1947, also triggers deterioration of the stalk, and the remaining vascular bundles become covered in dark mycelia with small black sclerotia as it progresses, which gives the disease its name (Kumari et al. 2015; Mughogho and Rosenberg 1984; Rao et al 1980). Because of the numerous pathogens causing sorghum stalk rots and their multiple hosts, classical gene-for-gene resistance is not possible (Bandara et al. 2016; Rao et al. 1980; Tesso et al. 2010). Development of sorghum with antimicrobial cell wall components or enhanced defense signaling may be an alternative breeding approach.

For the current study, the genes encoding phenylalanine ammonia lyase, 4CL, and coumaroyl shikimate 3-hydroxylase (*SbPAL*, *Bmr2*, and *SbC3H*, respectively; Fig. 1) in sorghum were overexpressed in the sorghum line RTx430. Phenylalanine ammonia lyase (PAL) (EC 4.3.1.24) catalyzes the reversible first step of the pathway, the non-oxidative deamination of phenylalanine, to produce *trans*-cinnamic acid (Fig. 1), which is also used for production of flavonoids and
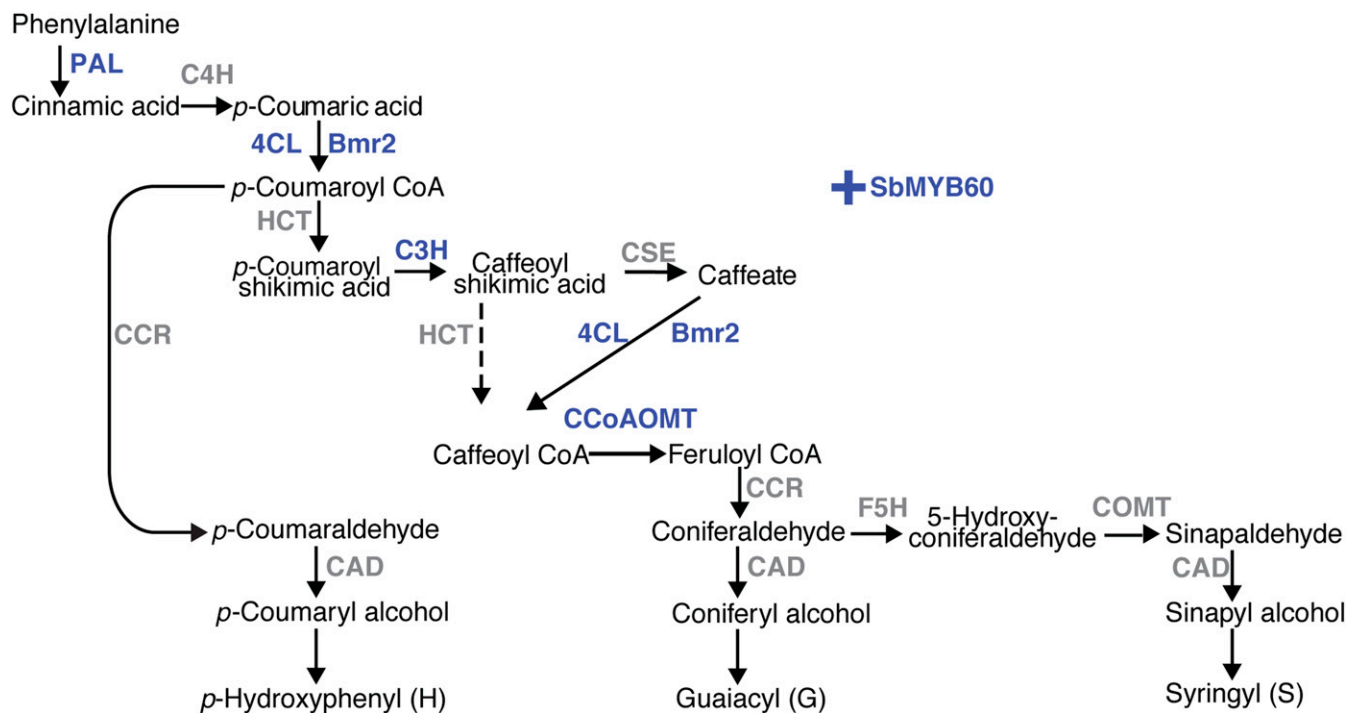
Fig. 1. Schematic of the monolignol biosynthesis pathway, the subunits that are incorporated into the lignin polymer. In sorghum, the transcription factor SbMyb60 controls expression of genes for the enzymes in the pathway. Sorghum (RTx430) was transformed with overexpressed *SbMyb60*, and *SbPAL*, *SbCCoAOMT*, *Bmr2*, and *SbC3H*, which encode for phenylalanine ammonia lyase (PAL), caffeoyl coenzyme A (CoA) 3-*O*-methyltransferase, 4-coumarate:CoA ligase (4CL), and *p*-coumarate 3-hydrolyase (C3H), respectively (highlighted in purple). C4H = cinnamate 4-hydroxylase; CSE = caffeoyl shikimate esterase; HCT = hydroxycinnamoyl-CoA shikimate transferase; CCR = cinnamoyl-CoA reductase; F5H = ferulate 5-hydroxylase; COMT = caffeic acid O-methyltransferase; and CAD = cinnamyl alcohol dehydrogenase.

other phenylpropanoids (Cui et al. 2014). The coumaroyl shikimate 3-hydroxylase enzyme (C3H) (EC 2.1.1.104) is one of three membrane-bound cytochrome P450 monolignol enzymes and central for the formation of G- and S-lignin (Fornalé et al. 2015; Reddy et al. 2005). PAL, 4CL, and C3H have been implicated in defense responses to pathogens (Bhuiyan et al. 2009; Franke et al. 2002; Moerschbacher et al. 1986; Reinold and Hahlbrock 1996; Steiner et al. 2009).

Transgenic lines overexpressing *SbPAL*, *Bmr2*, *SbC3H*, *SbMyb60*, or *SbCCoAOMT*, along with the wild-type line, RTx430, were assessed for responses to the stalk rot pathogens *F. thapsinum* and *M. phaseolina* under greenhouse conditions. Additionally, the *SbMyb60* and *SbCCoAOMT* overexpression lines were assessed for response to *F. thapsinum* under field conditions. These assays were conducted to test the hypothesis that transgenic sorghum lines overexpressing genes in the monolignol biosynthesis pathway will have increased resistance to sorghum stalk pathogens.

Materials and Methods

Fungi. The *F. thapsinum* isolates H03-11S-9 and M05A_1J_3b were identified by using colony, conidia, and conidiophore morphology (Funnell-Harris et al. 2013) and comparing the sequence of the 5′ region of the *translation elongation factor 1-α* gene to the *Fusarium*-ID database (http://www.fusariumdb.org/index.php) (Geiser et al. 2004) for previous studies (Funnell-Harris et al. 2010, 2018). Isolate H03-11S-9, from a field in Lincoln, NE, was used to perform greenhouse inoculations at University of Nebraska, Lincoln, whereas isolate M05A_1J_3b was used for field inoculations at Mead, NE, the same location from which it was obtained. In a previous greenhouse assessment, the two isolates had similar virulence levels (Funnell-Harris et al. 2018). The *M. phaseolina* isolate (MP01-001), a kind gift from G. Odvody (Texas A & M AgriLife Research and Extension Center, Corpus Christi, TX), was hyphal-tip purified before use. Medium for maintenance of working stocks was one-half strength potato dextrose agar (PDA) prepared using potato dextrose broth (Becton Dickinson and Co., Sparks, MD) and amended with 100 μM/liter of ampicillin (Sigma-Aldrich, St. Louis, MO). To prepare inoculum, agar discs (5 mm in diameter) from the growing edge of 4-day-old cultures on PDA were inoculated into sterile full-strength potato dextrose broth (PDB) (one disc per 5 ml of broth) with sterile toothpicks, previously treated to remove toxins and other inhibitors of fungal growth (Funnell-Harris et al. 2017, 2018; Jardine and Leslie 1992). The broth-and-toothpick cultures were incubated for 2 weeks at 25°C before inoculations.

Overexpression constructs and transgenic plant analyses. Overexpression constructs were transformed into sorghum RTx430, a parental line commonly used in hybrid production (http://archive.gramene.org/db/diversity/diversity_view?action=view&object=div_passport&id=3919&db_name=diversity_sorghum). Generation of constructs and transgenic lines with overexpression of *SbMyb60* (Sobic.004G2738000) and *SbCCoAOMT* (Sobic.010G052200.1) has been previously described (Scully et al. 2016; Tetreault et al. 2018). For *SbMyb60*, the coding region plus one intron was used, whereas for *SbCCoAOMT* only the coding region was used. For *SbPAL* (Sobic.004G220300.1) (Jun et al. 2018), *SbC3H* (Sobic.009G181800.1), and *Bmr2* (Sobic.004G062500.1) (Saballos et al. 2012), preparation of constructs and *Agrobacterium tumefaciens*, and transformation of sorghum, were similar to *SbCCoAOMT* (Tetreault et al. 2018). The coding regions of *SbPAL* and *SbC3H* were amplified from genomic DNA. The coding region of *Bmr2* was amplified from the pET30a-Bmr2 vector (Saballos et al. 2012). Each coding region was cloned into a cassette between the cauliflower mosaic virus (CaMV) E35S promoter and the 35S CaMV terminator (Scully et al. 2016). Each cassette was incorporated into the binary vector pPZP211, which includes antibiotic resistance and selection markers streptomycin and neomycin, respectively (Hajdukiewicz et al. 1994). Primers for amplification of genes and modification for insertion into overexpression cassettes and detection in genomic DNA of transgenic plants are listed in Supplementary Table S1, along with primers used for reverse transcription quantitative PCR (RT-qPCR) (see below).

Two independent lead events each of *SbPAL* (out of nine independent events) and *Bmr2* (out of four independent events) were chosen based on visual estimates of protein abundance on Western blots (Supplementary Fig. S1), as previously described (Tetreault et al. 2018). To identify two lead events of expression of *SbC3H* (out of eight) that encode for *p*-coumarate 3-hydroxylase, a membrane-associated cytochrome P-450, RT-qPCR was conducted to measure transcript levels of this gene, as well as for *SbPAL* and *Bmr2* (Supplementary Fig. S2). Once T_2 independent lead events were identified, each was selfed (if necessary) until homozygous lines were obtained for each overexpression construct. For RT-qPCR, total RNA was extracted from ground leaf samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified with a Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). The Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Corporation, Indianapolis, IN) was used to produce cDNA using 500 ng of RNA from each sample. qPCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using transgene-specific primers to each cDNA and conditions as previously described (Scully et al. 2016). Primers specific to sorghum *α-tubulin* were used as standards. Relative expression was computed using the ΔΔCt method with *α-tubulin* for normalization. No template and no reverse transcription controls were included to verify the absence of DNA contamination. Variation was estimated using the GLM procedure and mean values compared using Tukey's Studentized Range (HSD) test (SAS 2013). Within individual gene and wild-type comparisons were considered significantly different at $P < 0.05$.

For each lead event, available energy (Cal/g) was determined for stalk tissues from 5- to 6-week-old greenhouse-grown plants using bomb calorimetry as previously described (Scully et al. 2016). Acid detergent lignin (g/kg) was determined for *SbPAL* and *SbC3H* lead events as previously described (Vogel et al. 1999). Results are shown in Supplementary Table S2.

Greenhouse stalk inoculations of lines overexpressing genes in monolignol biosynthesis. Lines used in greenhouse assessments of responses to stalk pathogens *F. thapsinum* and *M. phaseolina* included lead events for each overexpression construct. Plants overexpressing *SbCCoAOMT* were event numbers ZG 234-1-28b and ZG 234-3-9a (Tetreault et al. 2018) and will be referred to as SbCCoAOMT_28b and SbCCoAOMT_9a, respectively; overexpressing *SbMyb60* were events ZG 129-4-10a, ZG 129-4-15a, and ZG 124-1-2a (Scully et al. 2016) and will be referred to as SbMyb_10a, SbMyb_15a, and SbMyb_2a, respectively; overexpressing *SbPAL* were events ZG 162-1-4a and ZG 136-2-3b and will be referred to as SbPAL_3b and SbPAL_4a, respectively; overexpressing *Bmr2* were events ZG 300-1-2a and ZG 300-1-6a and will be referred to as Bmr2_2a and Bmr2_6a, respectively; and plants overexpressing *SbC3H* were events ZG 177-2-1a and ZG 170-3-1a and will be referred to as SbC3H_2b and SbC3H_3a, respectively. Included in all assays was the wild-type line, RTx430. The Myb_10a line is maintained as heterozygous for the transgene owing to profound delays in flowering when in the homozygous state (Scully et al. 2016); all other lines are homozygous for the transgene.

Greenhouse inoculations were conducted at the University of Nebraska Plant Growth Facilities, throughout the years of 2016 to 2018. Each set of lead events were compared for responses to *F. thapsinum*, *M. phaseolina*, and a broth (PDB) control with the wild-type line RTx430. Greenhouse-produced seeds were sown into 25.4-cm-diameter pots containing pasteurized soil mix (one part sand, one part coarse vermiculite, one part top soil, and two parts shredded peat moss). Seedlings of SbMyb_10a were screened using PCR to detect the presence of the transgene. All lines were culled to one plant per pot. During head emergence (when the spikelet emerges from the collar of the flag leaf) heads of all lines, including nontransgenic wild-type, were covered with pollination bags to prevent pollen release. Because of secondary effects of overexpression on some of the lines, development, especially timing of flowering, was significantly affected. In particular, SbMyb60_10a flowered nearly 20 days later than the wild-type (Scully et al. 2016). Therefore,

SbMyb60_10a was planted in advance of the other lines so that lines were inoculated at the same time. Staggered planting of the lines is described in Supplementary Table S3, along with days after planting (DAP) for inoculations; time of year greatly affected the number of DAP to boot stage. Within 3 weeks of boot stage (when leaves are fully expanded and the head is near complete development but still enclosed in the flag-leaf sheath) at the base of each stalk, the middle of the second internode above the soil was inoculated with one of the two fungi or the PDB control, essentially as previously described (Funnell-Harris et al. 2014). A surface-disinfested awl was used to make a small hole, into which a fungus-infested toothpick, or a control toothpick, was inserted. At 32 days after inoculation (DAI) the stalk was split longitudinally, and the length of the resulting red-to-purple discoloration resulting from wounding and pathogen ingress (defined as the lesion) was measured. This methodology has been commonly used in several other studies, including with plants of varying heights (Bandara et al. 2017a; Funnell-Harris et al. 2014; Tesso et al. 2005). In addition to measurements of lesion lengths, plant heights after flowering and stalk diameters at site of inoculation (middle of second internode) were determined. A total of six plantings were performed.

Data analysis. The experimental design was randomized complete block (RCB). There were three (*SbMyb60* lines) or two (all other constructs) lead events and one wild-type line and three treatments in each of eight replicated blocks, with two (*SbPAL* lines) or three (all other lines) repetitions of each experiment. Individual plants or pots were considered the experimental unit. Replications within each experiment were blocked by location in the greenhouse. Analyses for mean lesion lengths, plant heights, and stalk diameters were performed. Replication within a repetition was treated as a random effect. Repetition, line, and inoculum were considered fixed effects. The data were analyzed using the PROC MIXED procedure of SAS/STAT software (SAS 2013). Datasets were analyzed for Levene's homogeneity of variance, and appropriate adjustments were incorporated using the REPEATED/GROUP option. Least squares means (LSM) and standard errors (SE) are reported. Differences of LSM were considered significant at $P \le 0.05$. Pearson correlations were generated for the response variables lesion length, height, and stalk diameter using the PROC CORR procedure in SAS/STAT software.

Table 1. Plant height and basal stalk diameter at maturity of greenhouse-grown wild-type and transgenic sorghum plants overexpressing monolignol pathway-associated genes

Transgenic line[z] RTx430	Plant height (cm)	Stalk diameter (mm)
SbPAL_3b	106.2 ± 2.4 b	15.3 ± 0.3 a
SbPAL_4a	110.3 ± 1.5 b	14.8 ± 0.3 a
Wild-type	138.8 ± 4.7 a	15.2 ± 0.3 a
SbMyb_2a	107.6 ± 1.9 b	15.2 ± 0.2 a
SbMyb_10a	83.9 ± 1.9 c	13.9 ± 0.3 b
SbMyb_15a	79.3 ± 1.9 c	12.3 ± 0.2 c
Wild-type	126.0 ± 2.0 a	14.2 ± 0.2 b
SbCCoAOMT_9a	113.8 ± 1.9 b	15.3 ± 0.2 a
SbCCoAOMT_28b	105.8 ± 2.0 c	14.2 ± 0.2 b
Wild-type	126.0 ± 2.0 a	14.2 ± 0.2 b
Bmr2_2a	98.0 ± 1.8 b	11.9 ± 0.2 a
Bmr2_6a	82.9 ± 2.2 c	8.8 ± 0.2 b
Wild-type	102.4 ± 1.5 a	11.9 ± 0.2 a
SbC3H_2b	80.9 ± 0.9 c	12.0 ± 0.2 a
SbC3H_3a	89.6 ± 1.2 b	10.4 ± 0.2 b
Wild-type	97.6 ± 1.1 a	11.6 ± 0.2 a

[z] The overexpressed monolignol pathway-associated genes were *SbMyb60* that encodes for the transcription factor that upregulates genes for enzymes in the monolignol biosynthesis pathway; *SbPAL* for phenylalanine ammonia lyase; *SbCCoAOMT* for caffeoyl coenzyme A (CoA) 3-*O*-methyltransferase; *Bmr2* for 4-coumarate:CoA ligase; and *SbC3H* for coumaroyl shikimate 3-hydroxylase. Letters indicate comparisons between transgenic lines carrying the same construct and the wild-type (below); values with different letters are significantly different at $P \le 0.05$.

Field response of lines overexpressing *SbMyb60* and *SbCCoAOMT* to *F. thapsinum*. The *SbMyb60* and *SbCCoAOMT* overexpression lead events and the wild-type line were planted at University of Nebraska Plant Biotechnology Field Facility, at Mead, NE, during the growing seasons of 2017 and 2018; for each year there was a repetition of the entire experiment. Seeds (160) of each transgenic line and the wild-type were mechanically planted in 6.1-m rows in three RCBs within a 0.05-ha plot; each of the three RCBs is considered a replication within each year. Two-and-one-half months before the 2017 planting, debris from alfalfa planted 3 years before was plowed under. The field was disked and cultivated, 2 and 1 days, respectively, before planting on June 1. Atrazine (1.1 kg/ha; 2-chloro-*n*-ethyl-*N′*-[1-methyl-ethyl]-1,3,5-triazine-2,4-diamine) was applied at planting for weed control. Overhead irrigation was applied on June 7 (1.3 cm), 9 (3.2 cm), and 10 (0.6 cm) and on July 10 (2.5 cm), 11 (1.9 cm), and 20 (3.1 cm). Rainfall totals for June, July, August, and September were 9.0, 8.0, 10.8, and 8.6 cm, respectively. For the 2018 planting (June 6), the previous crops had been corn (2017) and alfalfa (2016). The field was cultivated 2 days before planting. Atrazine was applied at planting, and then, 1 month after planting, the plots were fertilized with urea (90.8 kg/ha). Irrigation was applied June 7 (1.3 cm), June 14 (2.5 cm), and August 17 (2.5 cm). Rainfall totals for June, July, August, and September were 17.8, 5.3, 6.3, and 12.9 cm, respectively.

All lines were planted at the same time. After emergence, SbMyb_10a plants were again screened for presence of the transgene using PCR. Because *M. phaseolina* was imported into Nebraska from Texas, only *F. thapsinum* M05A_1J_3b, isolated from a spore trap at Mead, NE (Funnell-Harris et al. 2017), was inoculated onto the field-grown plants. Inoculations were conducted as previously described (Funnell-Harris et al. 2018). In each row (or replication), five plants were randomly chosen and marked with red loop-lock tags (27.9 × 2.5 cm; Gempler's, Janesville, WI) and numbered 1 through 5 for pathogen inoculations, and five plants were similarly marked with blue tags for control inoculations. Plants with different inoculations were interspersed within the row. At boot stage, plants were covered with pollination bags to prevent pollen release to the surrounding area. Staggered planting was not possible; therefore, all lines were inoculated at the same time, and lesion lengths were measured on the same day. For inoculations, most of the randomly chosen plants had flowered, with the notable exception of SbMyb_10a plants. The second internode of each stalk was wound inoculated as described above. At 32 DAI the stalk was split longitudinally, and the lesion length was measured. Along with lesion length, maturity and stalk diameters of individual inoculated plants were measured. Because staggered planting was not possible, plant maturity was assessed at three positions on the head (top, middle, and bottom) and was based on a 1 to 8 scale: 1 = vegetative; 2 = vegetative to anthesis; 3 = anthesis; 4 = milk; 5 = milk to soft dough; 6 = soft dough; 7 = hard dough; and 8 = mature. Row heights of six repetitions also were recorded.

Data analysis. Because there were three replications per year, the number of plants per each of six lines per treatment (*F. thapsinum* and control) per year was 15. The entire experiment was conducted during two years or growing seasons (repetitions). The data were analyzed using PROC MIXED procedure of SAS/STAT software (SAS 2013). Replication (row) and repetition (year) were considered random effects. Plant line and treatment were considered fixed effects. The dataset was analyzed for Levene's homogeneity of variance and appropriate adjustments incorporated using the REPEATED/GROUP option. The LSM and SE were reported; differences in LSM were considered significant at $P \le 0.05$. Pearson correlations were generated for the response variables lesion length, plant stage, and stalk diameter using the PROC CORR procedure in SAS/STAT software.

Results

Stalk rot inoculations of monolignol overexpression lines in the greenhouse. Heights and basal stalk diameters were taken of transgenic and wild-type plants, because these dimensions could affect lesion length (Table 1). It had been shown that plant heights of all

three *SbMyb60* lead events were significantly less than the wild-type, but plant heights were not significantly different for *SbCCoAOMT* overexpression events (Scully et al. 2016; Tetreault et al. 2018). In the current study, plant heights of the *SbMyb60* lines at maturity also were significantly less than the wild-type; however, the plant heights at maturity of both *SbCCoAOMT* lines were significantly shorter than the wild-type (Table 1). Plant heights for *SbPAL*, *Bmr2*, and *SbC3H* overexpression lines also were significantly shorter than wild-type (Table 1). Stalk diameters of *SbPAL* lead events were similar to the wild-type line; however, the other overexpression constructs had at least one line with a significantly different stalk diameter from the wild-type. For *SbMyb60* overexpression lines, it had been shown that stalk diameters of SbMyb_10a and SbMyb_2a were similar to the wild-type, but SbMyb_15a stalks had significantly smaller mean diameters (Scully et al. 2018). In the current study, the mean stalk diameter of SbMyb_15a was significantly less than the wild-type, but the diameter of SbMyb_2a was greater (Table 1). SbCCoAOMT_9a had a greater mean stalk diameter, whereas Bmr2_6a and SbC3H_3a had smaller stalk diameters than the wild-type (Table 1). In no case was there a significant interaction of plant height ($P \geq 0.17$) or stalk diameter ($P \geq 0.11$) with inoculum (i.e., treatments were randomly applied).

When *SbPAL* overexpression lines and the wild-type were compared for mean lesion lengths following inoculations with stalk pathogens, effects of repetition, line, and inoculum were significant (Table 2). There were no significant differences between the overexpression lines and wild-type when inoculated with *F. thapsinum* ($P \geq 0.41$) (Fig. 2A). When the *SbPAL* lines were inoculated with *M. phaseolina*, SbPAL_4a had significantly larger mean lesion lengths than the wild-type and SbPAL_3b ($P \leq 0.05$). Mean lengths of discolorations as a result of control inoculations were significantly less than mean lesion lengths as a result of pathogen inoculations ($P < 0.01$) (Fig. 2A). Correlations of lesion length on individual lines compared with plant height or stalk diameter were not significant (Table 3); coefficients were significant when inoculating all three lines with *F. thapsinum* for both measurements ($P \leq 0.02$) but not with *M. phaseolina* ($P \geq 0.06$) or the control ($P \geq 0.26$).

Inoculations of *SbMyb60* and *SbCCoAOMT* overexpression and wild-type lines with stalk pathogens were conducted in the same repetitions. Effects of repetition, line, and treatment were significantly different (Table 2). For *SbMyb60* overexpression lines and the wild-type, mean lesion lengths of *F. thapsinum*-inoculated plants were shorter for SbMyb_10a and SbMyb_15a ($P \leq 0.03$), but there were no significant differences when comparing lead event, SbMyb_2a and the wild-type ($P = 0.24$) (Fig. 2B). There were no significant differences in mean lesion lengths between each overexpression line and wild-type when inoculated with *M. phaseolina* ($P \geq 0.20$). For inoculations of *SbCCoAOMT* overexpression lines and wild-type with *F. thapsinum*, significantly smaller mean lesion lengths resulted for SbCCoAOMT_9a ($P = 0.03$) but not SbCCoAOMT_28b ($P = 0.21$) (Fig. 2C). After inoculation with *M. phaseolina*, there were

no significant differences between mean lesion length resulting on SbCCoAOMT_9a ($P = 0.21$) or SbCCoAOMT_28b ($P = 0.07$) compared with the wild-type. Mean lengths of discoloration following control inoculations were significantly shorter than mean lesion lengths as a result of pathogen inoculations for all lines ($P < 0.01$) (Fig. 2B and C). When correlation analyses were conducted, comparing lesion length with either plant height or stalk diameter in individual lines, significant coefficients were found for SbMyb_10a and for SbCCoAOMT_28 for both measurements (Table 3). Across all *SbMyb60* and *SbCCoAOMT* transgenic lines and the wild-type, correlation coefficients were significant for inoculations with either *F. thapsinum* or *M. phaseolina* ($P < 0.01$) but not the control ($P \geq 0.58$). To indicate whether plant height may have influenced lesion measurements for each treatment of each *SbMyb60* overexpression event, Pearson correlation coefficients were generated for individual lines (SbMyb_2a, SbMyb_10a, SbMyb_15a, and wild-type) comparing lesion lengths with plant height for each inoculum (*F. thapsinum*, *M. phaseolina*, and control). Again, correlations were significant for pathogen inoculations of SbMyb_10a plants, *F. thapsinum* ($r = 0.5288$; $P = 0.01$), and *M. phaseolina* ($r = 0.7266$; $P < 0.01$), but not the control inoculation ($r = 0.3050$; $P = 0.16$). Inoculation of wild-type plants with *F. thapsinum* also resulted in significant correlations of height with lesion length ($r = 0.4383$; $P = 0.04$). All other line-treatment combinations did not result in significant correlations ($P \geq 0.07$).

When *Bmr2* overexpression lines were inoculated with stalk pathogens, there were significant differences in repetition or treatment but not line, or the interactions of line with repetition and/or inoculum (Table 2). There were no significant differences in mean lesion lengths between the lead events, Bmr2_2a and Bmr2_6a, and the wild-type line when inoculated with *F. thapsinum* ($P \geq 0.30$) or *M. phaseolina* ($P \geq 0.49$) (Fig. 2D). Mean lengths of discolorations following control inoculation were significantly less than mean lesion lengths following pathogen inoculations ($P < 0.01$) (Fig. 2D). There were no significant correlations of mean lesion length on individual lines with plant height or stalk diameter (Table 3). Additionally, there were no significant correlations with plant height or stalk diameter when considering each inoculum ($P \geq 0.13$).

For inoculations of *SbC3H* overexpression lines and the wild-type, there was a significant effect of repetition and treatment but not of line, and their interactions also were not significant (Table 2). There were no significant differences between SbC3H_2 or SbC3H_3 and the wild-type for both *F. thapsinum* ($P \geq 0.32$) and *M. phaseolina* ($P \geq 0.23$) inoculations (Fig. 2E). Mean discoloration lengths as a result of control inoculation were significantly less than mean lesion lengths from pathogen inoculations ($P < 0.01$) (Fig. 2E). There were no significant correlations of mean lesion length on individual lines with plant height or stalk diameter (Table 3). Also, there were no significant correlations of plant height or stalk diameter with each inoculum ($P \geq 0.07$).

Table 2. Analysis of variance of fixed effects (repetition [R], transgenic or wild-type line [E], and treatment [T]) and interactions for inoculation of transgenic lines carrying an overexpressed gene from monolignol biosynthesis with *Fusarium thapsinum*, *Macrophomina phaseolina*, and control compared with wild-type[z]

| | Summary statistics from type 3 tests of fixed effects | | | | | | | |
| | *SbPAL* | | *SbMyb60, SbCCoAOMT* | | *Bmr2* | | *SbC3H* | |
Effect	*F* value	*Pr > F*	*F* value	*Pr > F*	*F* value	*Pr > F*	*F* value	*Pr > F*
R	10.69	<0.01	17.15	<0.01	3.62	0.03	9.08	<0.01
E	2.69	0.01	2.72	0.02	1.00	0.37	2.09	0.13
R × E	2.94	0.01	2.67	<0.01	1.33	0.26	1.09	0.36
T	249.97	<0.01	205.31	<0.01	61.70	<0.01	55.10	<0.01
R × T	4.69	0.01	16.68	<0.01	1.64	0.17	0.30	0.88
E × T	1.37	0.17	1.60	0.11	0.65	0.63	0.48	0.75
R × E × T	1.27	0.23	1.21	0.25	1.40	0.20	1.09	0.38

[z] The response variable was lesion length (mm). Overexpression was accomplished using the 35S promoter from the cauliflower mosaic virus. *SbMyb60* encodes for a transcription factor, *SbPAL* encodes for phenylalanine ammonia lyase, *SbCCoAOMT* encodes for caffeoyl coenzyme A (CoA) 3-*O*-methyltransferase, *Bmr2* encodes for 4-coumarate:CoA ligase, and *SbC3H* encodes for coumaroyl shikimate 3-hydroxylase. The *SbMyb60* and *SbCCoAOMT* lines were assessed together.

Field response of lines overexpressing *SbMyb60* and *SbCCoAOMT* to *F. thapsinum*. Because all lines were planted at the same time, plant heights, growth stages, and stalk diameters were noted for field-grown plants. The transgenic lines overexpressing *SbMyb60* were significantly shorter than the wild-type line, whereas the lines overexpressing *SbCCoAOMT* were not significantly different in height than the wild-type (Table 4). Because plant maturity can affect responses to stalk pathogens (Bandara et al. 2016), plant stage was noted for individual plants, based on a 1 to 8 scale. The wild-type lines were in the milk to soft dough stages (Table 4). Only SbCCoAOMT_28b was at a similar stage as the wild-type, whereas all other lines were at earlier stages, especially the *SbMyb60* overexpression lines. Mean stalk diameter of line SbCCoAOMT_9a was not significantly different from wild-type; however, diameters of all other transgenic lines were either significantly larger (SbMyb_2a; $P < 0.01$) or smaller (SbMyb_10a, SbMyb_15a, SbCCoAOMT_28a; $P \leq 0.01$) than wild-type (Table 4).

When inoculated with *F. thapsinum*, effects of line, inoculum, and repetition (year) were significant (Table 5). For the lead events of *SbMyb60* overexpression lines, all had mean lesion lengths not significantly different than the wild-type ($P \geq 0.11$) (Fig. 3). For *SbCCoAOMT* overexpression lines, SbCCoAOMT_9a had a mean lesion length significantly larger than wild-type ($P = 0.02$), whereas SbCCoAOMT_28b had a larger mean lesion length but not significantly different from wild-type ($P = 0.08$). Mean lesion lengths for all *F. thapsinum* inoculations were significantly greater than responses of control inoculations on the same line ($P < 0.01$) (Fig. 3); however, the wound response of SbMyb_10a was significantly shorter than that of wild-type ($P = 0.01$). Pearson correlation coefficients were generated comparing lesion length with either growth
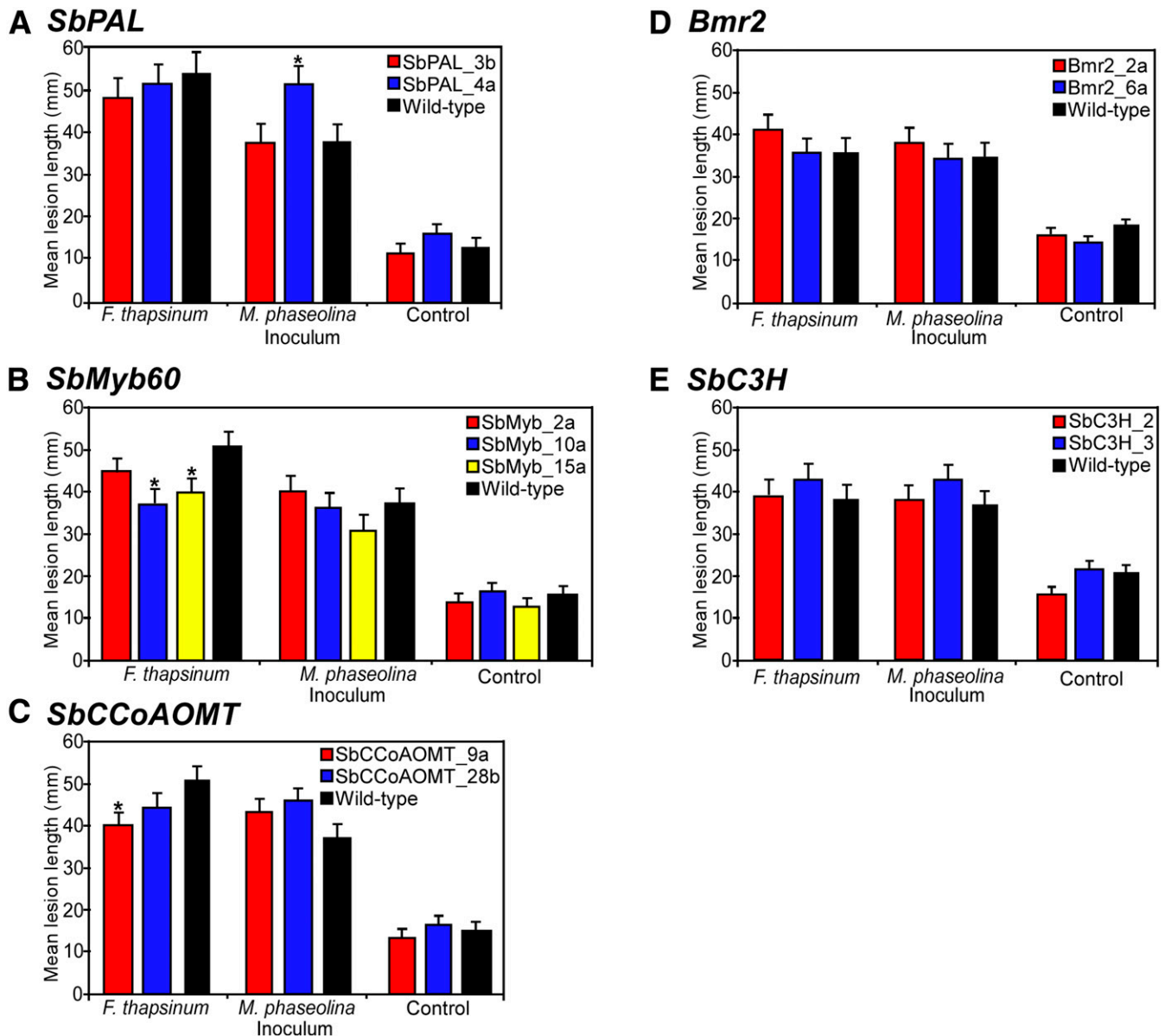
Fig. 2. Lesion lengths (mm) resulting on transgenic sorghum lines overexpressing genes involved in the monolignol biosynthesis pathway to the stalk pathogens *Fusarium thapsinum* (Fusarium stalk rot) and *Macrophomina phaseolina* (charcoal rot). Each gene was overexpressed using the cauliflower mosaic virus E35S promoter by transformation into sorghum line RTx430 (wild-type). Greenhouse-grown plants of each transgenic line and the wild-type were wound inoculated within 3 weeks of boot stage with one of the two stalk pathogens or control (potato dextrose broth). Thirty-two days after inoculation, stalks were split longitudinally, and lengths of lesions or discoloration as a result of wounding were measured. **A,** Lines overexpressing the gene encoding for phenylalanine ammonia lyase (*SbPAL*). **B,** Lines overexpressing the gene encoding the transcription factor that regulates genes in the monolignol pathway (*SbMyb60*). **C,** Lines overexpressing the gene encoding for the enzyme caffeoyl coenzyme A (CoA) 3-*O*-methyltransferase (*SbCCoAOMT*). **D,** Lines overexpressing the gene encoding for the enzyme 4-coumarate:CoA ligase (*Bmr2*). **E,** Lines overexpressing the gene encoding for *p*-coumarate 3-hydrolylase (*SbC3H*). Asterisks indicate that mean lesion length is significantly different from that on wild-type plants at $P \leq 0.05$. Control inoculations resulted in responses significantly smaller than those following fungal inoculations ($P < 0.01$).

stage or stalk diameter. The only case in which lesion length was associated with maturity was with SbMyb_10a (Table 6). There were no significant correlations of lesion length with stalk diameter.

Discussion

Sorghum genes encoding a transcription factor and four different enzymes in the monolignol biosynthesis pathway were overexpressed in transgenic plants, and these overexpression lines, along with the wild-type, were assessed for response to stalk pathogens. Because such lines could be valuable in future efforts to develop sorghum as a dedicated bioenergy crop in thermal processes (Sanna 2014) or a source for renewable biochemicals (Arevalo-Gallegos et al. 2017; Gupta et al. 2016), it was necessary to screen these lines for responses to stalk pathogens. In nearly every case, except for one *SbPAL* line challenged with *M. phaseolina* (greenhouse) and one *SbCCoAOMT* line challenged with *F. thapsinum* (field), the transgenic lines were not more susceptible to both stalk pathogens than wild-type. Thus, increased expression of steps in monolignol biosynthesis should not render plants more susceptible to stalk pathogens. On the other hand, increased resistance was observed in two *SbMyb60* overexpression lines and one *SbCCoAOMT* overexpression line when inoculated with *F. thapsinum* in the greenhouse

Table 3. Pearson correlation coefficients (*r*) and significance levels (*P*) for comparisons of lesion lengths with plant heights and stalk diameters of greenhouse-grown transgenic sorghum plants overexpressing genes associated with the monolignol biosynthesis pathway[z]

	Pearson correlation coefficient significance level	
Construct line	Plant height	Stalk diameter
SbPAL		
SbPAL_3b	0.0253	0.1199
	0.8657	0.4170
SbPAL_4a	−0.0863	0.0963
	0.5598	0.5196
Wild-type	0.0881	−0.0091
	0.5606	0.9514
SbMyb and *SbCCoAOMT*		
SbMyb_2a	0.1133	0.1840
	0.3468	0.1244
SbMyb_10a	0.2483	0.4218
	0.0368	0.0003
SbMyb_15a	0.1106	−0.0231
	0.3621	0.8493
SbCCoAOMT_9a	0.0167	0.1566
	0.8896	0.1889
SbCCoAOMT_28b	0.4082	0.2698
	0.0005	0.0239
Wild-type	0.0203	0.1003
	0.8665	0.4089
Bmr2		
Bmr2_2a	0.1445	−0.0639
	0.2258	0.5939
Bmr2_6a	0.0365	0.2114
	0.7627	0.0812
Wild-type	−0.0113	0.0498
	0.9251	0.6780
SbC3H		
SbC3H_2b	−0.1742	−0.2035
	0.1463	0.0887
SbC3H_3a	0.0340	0.1796
	0.7783	0.1340

[z] *SbMyb60* encodes for a transcription factor, *SbPAL* encodes for phenylalanine ammonia lyase, *SbCCoAOMT* encodes for caffeoyl coenzyme A (CoA) 3-*O*-methyltransferase, *Bmr2* encodes for 4-coumarate:CoA ligase, and *SbC3H* encodes for coumaroyl shikimate 3-hydroxylase. The *SbMyb60* and *SbCCoAOMT* lines were assessed together. Transgenic lines and wild-type line, RTx430, were inoculated with the stalk pathogens *Fusarium thapsinum* or *Macrophomina phaseolina* or with a broth control. Coefficients are considered significant when $P \leq 0.05$.

(Fig. 2); however, the hypothesis that increasing either expression of steps in monolignol biosynthesis or the entire pathway activity would result in increased resistance to stalk pathogens was disproven for the most part.

In previous research, sorghum *bmr* lines were as resistant as near-isogenic wild-type or even more resistant, depending on the line and pathogen (Funnell-Harris et al. 2014, 2017). Such observations were attributed to accumulation of phenolic compounds (soluble and cell-wall bound) (Palmer et al. 2008) that are potentially inhibitory to the pathogen (Boutigny et al. 2009; Ferruz et al. 2016; Funnell-Harris et al. 2014, 2017). This may be one explanation as to why, in the current study, *SbMyb60* overexpression lines had mean lesion lengths similar to wild-type, or even significantly smaller than wild-type, in both greenhouse and field assessments to stalk pathogens (Figs. 2B and 3). It had been previously shown that *SbMyb60* overexpression resulted in significantly greater accumulation of lignin in cell walls and of wall-bound metabolites derived from the monolignol pathway, which include ferulic and caffeic acids that were previously shown to inhibit *M. phaseolina* and *F. thapsinum* growth in vitro, respectively (Funnell-Harris et al. 2014; Scully et al. 2016). Additionally, genes associated with SAM metabolism, one of three cofactors necessary for monolignol biosynthesis, were also upregulated in these lines, which suggests *SbMyb60* not only activates monolignol synthesis but also the substrate and a cofactor required for this pathway (Scully et al. 2018). SAM, a cofactor for many methyltransferases in animals, fungi, plants, and bacteria (Liscombe et al. 2012), also serves as a substrate for ethylene synthesis in plants (Amir 2010; Roje 2006). The ethylene signaling pathway has been associated with response to pathogens and is necessary for defense against many soil-borne necrotrophic pathogens (Berrocal-Lobo

Table 4. Heights, maturity, and stalk diameters at time of lesion measurement on field-grown plants[z]

| Line	Row height (cm)	Maturity	Stalk diameter (mm)
SbMyb_2a	127.08 ± 1.50 a	3.88 ± 0.06 a	21.78 ± 0.45 a
SbMyb_10a	120.00 ± 1.50 b	1.53 ± 0.18 b	15.00 ± 0.48 d
SbMyb_15a	107.08 ± 1.50 c	3.80 ± 0.09 a	14.48 ± 0.32 d
SbCCoAOMT_9a	133.75 ± 1.50 d	5.15 ± 0.11 c	18.85 ± 0.33 bc
SbCCoAOMT_28b	134.58 ± 1.50 d	5.65 ± 0.12 d	18.25 ± 0.37 c
Wild-type	136.25 ± 1.50 d	5.81 ± 0.09 d	19.42 ± 0.27 b

[z] *SbMyb60* and *SbCCoAOMT* overexpression lines with wild-type were planted during the growing seasons of 2017 and 2018. Row heights were determined in six replicate plots in each season. Maturity at time of lesion measurement was assessed at three positions on the head (top, middle, and bottom), using a 1 to 8 scale: 1 = vegetative; 2 = vegetative to anthesis; 3 = anthesis; 4 = milk; 5 = milk to soft dough; 6 = soft dough; 7 = hard dough; and 8 = mature. Stalk diameters were also determined. Least squares means (LSM) and standard errors are shown. LSM with different letters are significantly different at $P \leq 0.05$.

Table 5. Analysis of variance of fixed effects (year [Y], transgenic or wild-type line [E], and treatment [T]) and interactions for inoculation of field-grown transgenic lines overexpressing *SbMyb60* or *SbCCoAOMT* with *Fusarium thapsinum* or control compared with wild-type[z]

	Summary statistics from type 3 tests of fixed effects	
Effect	*F* value	*Pr > F*
Y	7.17	0.01
E	10.02	<0.01
Y × E	1.61	0.16
T	229.71	<0.01
Y × T	3.23	0.07
E × T	2.26	0.05
Y × E × T	1.72	0.14

[z] The response variable was lesion length (mm). The constructs were *SbMyb60*, which encodes for a transcription factor, and *SbCCoAOMT*, which encodes for caffeoyl coenzyme A 3-*O*-methyltransferase.

and Antonio 2004; Geraats et al. 2003; Okubara and Paulitz 2005; Zhang et al. 2007). *Arabidopsis thaliana* lines that expressed *SbSAM synthase* from a wild potato (*Solanum brevidens*) had enhanced SAM biosynthesis along with upregulation of ethylene biosynthetic genes (Kim et al. 2015). Overexpression of *SbSAM synthase* also induced three *Pathogenesis-related* genes in *A. thaliana*, which supports its role in plant defense. Expression of yeast spermidine synthase (*ySpd-Syn*), which requires SAM as a substrate, in tomato (*Solanum lycopersicum*), resulted in increased susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* but not to two other pathogens (the necrotrophic pathogen *Alternaria solani* and the hemibiotrophic bacterial pathogen *Pseudomonas syringae*), and larvae of a chewing insect (*Manduca sexta*); resistance to *B. cinerea* was rescued by exogenous application of ethylene precursors that included SAM (Nambeesan et al. 2012). In cotton (*Gossypium barbadense*), the gene for the ethylene response-related factor GbERF1-like was both downregulated and overexpressed, resulting in increased susceptibility and resistance, respectively, to the necrotrophic fungus *Verticillium dahliae* (Guo et al. 2016). Following inoculation of both overexpression and wild-type lines, transcripts of monolignol
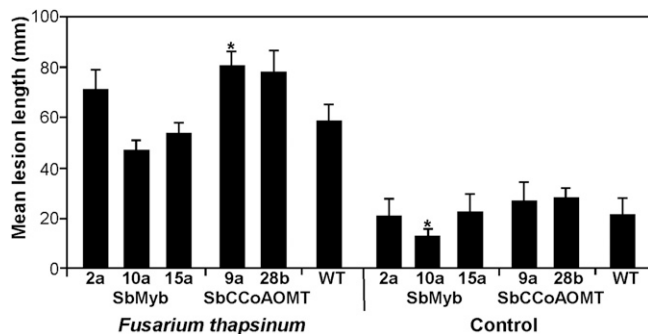
Fig. 3. Mean lesion lengths (mm) of field-grown transgenic sorghum plants overexpressing the gene for transcription factor SbMyb60 and the gene for the enzyme caffeoyl coenzyme A 3-O-methyltransferase (SbCCoAOMT), along with the wild-type line, RTx430, inoculated with *Fusarium thapsinum*. Each gene was overexpressed using the cauliflower mosaic virus E35S promoter by transformation into sorghum line RTx430 (wild-type). Plants of each transgenic line and the wild-type were wound inoculated at flowering, and then 32 days after inoculation stalks were split longitudinally, and the lengths of lesions or discolorations as a result of wounding were measured. Field inoculations were conducted over 2 years (2017 and 2018). Asterisk indicates that mean lesion length or wound discoloration of transgenic line is significantly different from that on wild-type plants ($P \leq 0.02$). Responses of control inoculations were significantly less than those following fungal inoculation ($P < 0.01$).

Table 6. Pearson correlation coefficients (*r*) and significance levels (*P*) for comparisons of lesion lengths with plant maturity and stalk diameters of field-grown plants overexpressing the genes *SbMyb60* and *SbCCoAOMT*[z]

Construct line	Pearson correlation coefficient significance level	
	Maturity	Stalk diameter
SbMyb_2a	−0.1382	−0.1311
	0.2919	0.3180
SbMyb_10a	0.3925	0.1095
	0.0028	0.4176
SbMyb_15a	−0.0085	−0.0446
	0.9489	0.7376
SbCCoAOMT_9a	0.0521	−0.0775
	0.6927	0.5564
SbCCoAOMT_28b	0.0948	0.0694
	0.4710	0.5983
Wild-type	0.1932	−0.1318
	0.1463	0.3196

[z] Transgenic lines and wild-type line, RTx430, were inoculated with the stalk pathogen *Fusarium thapsinum* or with a broth control. Coefficients are considered significant when $P \leq 0.05$.

biosynthetic genes that included *PAL*, *C3H*, and *CCoAOMT* accumulated, and total lignin (as determined by the Klason method) increased. Additionally, there was a significantly greater accumulation of lignin in uninoculated overexpressing plants and significantly lower accumulation in downregulated lines compared with the wild-type, which possibly affected responses to *V. dahliae*. Taken together, these previous studies indicate a connection between SAM, ethylene, the monolignol biosynthesis pathway and responses to pathogens (Bhuiyan et al. 2007; Jin et al. 2017).

In the current study, pleiotropic effects of overexpression of *SbMyb60* in sorghum lines previously observed (Scully et al. 2016, 2018) were reflected in differential responses of the three overexpression lines to the two stalk pathogens in greenhouse assessments, especially following inoculations with *F. thapsinum* (Fig. 2). Pearson coefficients indicated there were no significant correlations between plant height or stalk diameter and lesion length (or response from control inoculations) for SbMyb_2a and SbMyb_15a, but for SbMyb_10a, there were significant correlations (Table 2). SbMyb_10a had the highest relative expression level of *SbMyb60* in stalks, highest expression levels of genes encoding for PAL, 4CL, cinnamyl-CoA reductase (CCR), CCoAOMT, and CAD, highest levels of *p*-coumaric acid, syringic acid, and vanillic acid, and highest levels of all three forms of lignin, compared with SbMyb_2a, SbMyb_15a, and the wild-type lines (Scully et al. 2016). Therefore, multiple factors may have contributed to reduced lesion lengths in the SbMyb_10a line, which includ̄ed the anatomical changes resulting from overexpression of *SbMyb60* in these plants.

It has been previously shown that overexpression of *SbCCoAOMT* increased soluble and wall-bound hydroxycinnamic acids, although lignin and polysaccharide contents did not change (Tetreault et al. 2018). In *A. thaliana*, overexpression of the lipid transfer protein gene, *AZELAIC ACID INDUCED 1* (*AZI1*) resulted in increased transcription of *CCoAOMT* and *CCR*, enzymes acting in consecutive steps that lead to G and S lignin, but no other genes in the monolignol pathway were induced (Gao et al. 2015). The AZI1 protein is associated with generation or transmission of the defense signal, salicylic acid, necessary for systemic acquired resistance (Jung et al. 2009), which implies a role for CCoAOMT and CCR and/or their products in induced resistance. In the current study, greenhouse inoculations of the *SbCCoAOMT* lines with *F. thapsinum* resulted in lesions not significantly different (SbCCoAOMT_28b) or smaller (SbCCoAOMT_9a) than wild-type (Fig. 2C). However, in field inoculations using another *F. thapsinum* isolate, lesion lengths were significantly greater (SbCCoAOMT_9a), and it could be argued that lesions resulting on SbCCoAOMT_28b ($P = 0.08$) also were larger than those on wild-type (Fig. 3). Additionally, greenhouse inoculations with *M. phaseolina* resulted in mean lesion lengths that tended to be larger than that on the wild-type, and again it could be argued that line SbCCoAOMT_28b had increased susceptibility to *M. phaseolina* compared with the wild-type ($P = 0.07$). Therefore, it appears that overexpression of *SbCCoAOMT* in sorghum may actually negatively affect resistance responses to the two stalk diseases examined in this study. In maize, the AvrE family type II effector and pathogenicity factor, WtsE, from the bacterial pathogen *Pantoea stewartii* ssp. *stewartii*, alone could increase flux through the phenylpropanoid pathway, by increasing expression of *PAL* and *4CL*, but resulted in increased susceptibility, which the authors propose as "misregulated induction" of the phenylpropanoid pathway (Asselin et al. 2015). Thus, in the current study, plants with overexpression of *SbCCoAOMT* grown under greenhouse conditions with increased susceptibility to *M. phaseolina* and under field conditions with increased susceptibility to *F. thapsinum* may have been owing to changes in accumulation of intermediates and flux through the monolignol synthesis pathway, which altered defense responses.

In field inoculations with *F. thapsinum*, *SbMyb60* overexpression lines appeared to respond similarly to the pathogen as in greenhouse studies, but for *SbCCoAOMT* overexpression lines, results in the greenhouse and the field were not consistent (Figs. 2C and 3). Differences in responses between field- and greenhouse-grown transgenic plants have been observed with decreased fitness or increased disease

susceptibility in field-grown transgenic switchgrass (*Panicum virgatum*) and increased accumulation of deoxynivalenol in field-grown barley (*Hordeum vulgare*) engineered to detoxify the mycotoxin compared with greenhouse-grown plants (Baxter et al. 2015, 2018; Manoharan et al. 2006).

The lines overexpressing *SbPAL*, *Bmr2*, and *SbC3H* have not been as thoroughly studied as *SbMyb60* and *SbCCoAOMT*, because the original project goal was to increase energy availability for bioenergy, and these lines did not display increases in lignin or energy. However, it is currently unknown whether cell wall components have been altered. Previously, constitutive expression of heterologous *PAL* transgenes in tobacco (*Nicotiana tabacum*) and *A. thaliana* resulted in plants resistant to multiple pathogens (Kim and Hwang 2014; Way et al. 2011). *PAL* is well-known to be associated with resistance to pathogens (Ganapathy et al. 2016; Wojtasik et al. 2016). *4CL* has also been associated with defense responses to pathogens (Reinold and Hahlbrock 1996), whereas *C3H* has been shown to be involved in responses during colonization by beneficial microorganisms (Battini et al. 2016). In the current study, when plants overexpressing each of these enzymes were inoculated with the two stalk pathogens, there were no significant differences in mean lesion lengths compared with the corresponding wild-type, except for one *SbPAL* overexpression line, SbPAL_4a, which was significantly more susceptible to *M. phaseolina* than the wild-type (Fig. 2A, D, and E). Mean lesion lengths, used to determine the response to *M. phaseolina* inoculation, compared between individual transgenic lines from the same construct, were variable not only for *SbPAL* but also for *SbMyb60* ($P \geq 0.06$) lines. Because *SbMyb60* is a transcriptional activator of multiple genes, this is not surprising. However, responses of the same lines to *F. thapsinum* inoculation seemed to be more consistent ($P \geq 0.10$). Differential responses between events (lines) with the same construct to multiple pathogens have been previously reported (Ghanta et al. 2011; Goto et al. 2015).

In summary, lines overexpressing genes involved in the monolignol biosynthesis pathway, *SbMyb60* (Scully et al. 2016), *SbCCoAOMT* (Tetreault et al. 2018), *SbPAL*, *Bmr2*, or *SbC3H* (this work), were inoculated with two pathogens under greenhouse conditions, which are associated with the greatest stalk disease problems of sorghum in the United States: *F. thapsinum* and *M. phaseolina*. Additionally, the *SbMyb60* and *SbCCoAOMT* transgenic lines, along with wild-type, were assessed in the field for response to *F. thapsinum*. *SbMyb60* overexpression lines responded similarly to the pathogens, and two of the three lines exhibited increased resistance to *F. thapsinum* in greenhouse inoculations. Although *SbCCoAOMT* lines exhibited smaller mean lesion lengths after inoculation with *F. thapsinum* in the greenhouse, these lines appeared to be more susceptible in the field study; these same lines tended to exhibit increased susceptibility to *M. phaseolina*. *SbPAL*, *Bmr2*, and *SbC3H* overexpression lines were all as resistant as the wild-type lines to *F. thapsinum*. For *M. phaseolina*, *Bmr2* and *SbC3H* overexpression lines were as resistant as the wild-type lines, but one *SbPAL* lead event was more susceptible than the wild-type to this pathogen. To our knowledge, this is the first examination of response to necrotrophic pathogens from any crop overexpressing genes in the monolignol biosynthesis. This research provides valuable information for using sorghum lines with increased expression of monolignol biosynthesis genes as feedstock for thermochemical conversion of biomass (Carvalho et al. 2017; Habyarimana et al. 2016) or production of renewable products from the phenylpropanoid pathway in pharmaceuticals or cosmetics (Hauck et al. 2013; Massey et al. 2014; Parveen et al. 2014).

Acknowledgments

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