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Research

Impaired *Brown midrib12* function orchestrates sorghum resistance to aphids via an auxin conjugate indole-3-acetic acid–aspartic acid

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Summary

- Lignin, a complex heterogenous polymer present in virtually all plant cell walls, plays a critical role in protecting plants from various stresses. However, little is known about how lignin modifications in sorghum will impact plant defense against sugarcane aphids (SCA), a key pest of sorghum.
- We utilized the sorghum *brown midrib* (*bmr*) mutants, which are impaired in monolignol synthesis, to understand sorghum defense mechanisms against SCA. We found that loss of *Bmr12* function and overexpression (OE) of *Bmr12* provided enhanced resistance and susceptibility to SCA, respectively, as compared with wild-type (WT; RTx430) plants.
- Monitoring of the aphid feeding behavior indicated that SCA spent more time in reaching the first sieve element phase on *bmr12* plants compared with RTx430 and *Bmr12*-OE plants. A combination of transcriptomic and metabolomic analyses revealed that *bmr12* plants displayed altered auxin metabolism upon SCA infestation and specifically, elevated levels of auxin conjugate indole-3-acetic acid–aspartic acid (IAA–Asp) were observed in *bmr12* plants compared with RTx430 and *Bmr12*-OE plants. Furthermore, exogenous application of IAA–Asp restored resistance in *Bmr12*-OE plants, and artificial diet aphid feeding trial bioassays revealed that IAA–Asp is associated with enhanced resistance to SCA.
- Our findings highlight the molecular underpinnings that contribute to sorghum *bmr12*-mediated resistance to SCA.

Introduction

Sorghum (*Sorghum bicolor*) is one of the top five cereal crops in the world grown for grain and forage. Additionally, sorghum's potential as bioenergy crop has been attributed to its low nitrogen and water requirements and high plant biomass content (Rooney *et al.*, 2007). Despite being a heat- and drought-tolerant crop, sorghum still harbors a variety of insect pests (Sharma, 1993). Sugarcane aphid (SCA; *Melanaphis sacchari*) is a major and devastating pest of sorghum in the United States (Armstrong *et al.*, 2015; Bowling *et al.*, 2016; Thudi *et al.*, 2024; Vasquez *et al.*, 2024). SCA insert its needle-like stylets in the sieve elements and remove the photosynthates from the plants that can impact plant growth and development massively (Nalam *et al.*, 2019; Mou *et al.*, 2023; Vasquez *et al.*, 2024). SCA excrete sticky honeydew on plants that aids the development of sooty mold and further dwindles the photosynthetic efficiency of plants. Moreover, abundant honeydew on sorghum leaves affects the harvest efficiency negatively (Bowling *et al.*, 2016).

Plant cell walls make a first line of contact between internal and external environments. Lignin is a complex heterogenous polymer composed of three major subunits: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, present in the secondary cell wall of plants. Lignin biosynthesis pathway, which is derived from phenylalanine, leads to the production of the three monolignols: p-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1). These monolignols are further transported to the cell wall and polymerized into lignin subunits through oxidative processes by the action of laccases and peroxidases (Boerjan *et al.*, 2003). Lignin is also present in the cell walls of the plant vasculature, along with pectins, hemicelluloses, cellulose, and structural proteins. Reduced lignin levels can affect plant development and often, plant immunity via multiple mechanisms (Zhao & Dixon, 2014). Plants with altered lignin levels have been known to modify plant defenses and impact pathogen growth through enhanced susceptibility/resistance (Miedes *et al.*, 2014; Zhao & Dixon, 2014). Lignin accumulation has been reported in Chinese cabbage, wheat, Eucalyptus, Elm, and Arabidopsis upon pathogen attack (Parrott *et al.*, 2002; Martin *et al.*, 2007; Menden *et al.*, 2007;

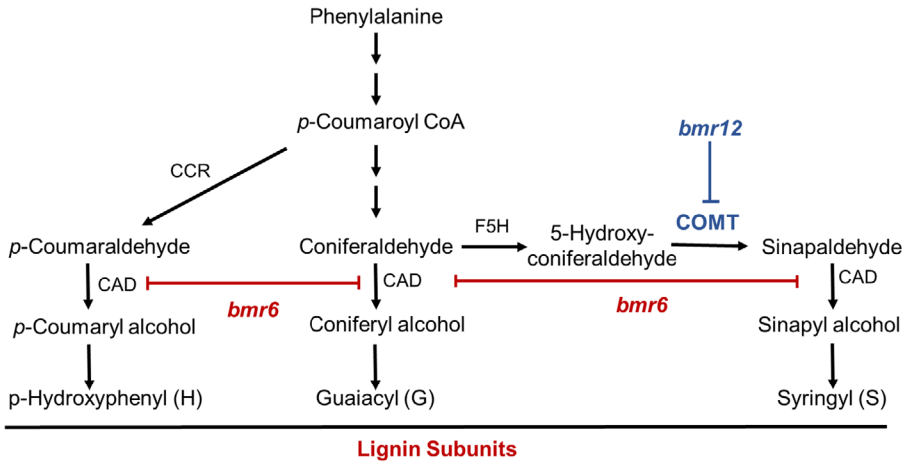

Fig. 1 Monolignol biosynthesis pathway in sorghum based on consensus models from dicot and monocot plants (modified from Sattler *et al.*, 2014). The enzymatic steps are as follows: cinnamyl CoA reductase; ferulate 5-hydroxylase; caffeic acid O-methyltransferase (COMT); and cinnamyl alcohol dehydrogenase (CAD). The *bmr6* and *bmr12* mutant plants are impaired in CAD and COMT enzymatic activities, respectively.

Smith *et al.*, 2007; Zhang *et al.*, 2007; Kim *et al.*, 2020; Wang *et al.*, 2020). The restricted pathogen spread was often associated with lignification levels of plant cells and hypersensitive cell response (Martin *et al.*, 2007; Smith *et al.*, 2007; Kim *et al.*, 2020). Although there are a few reports about plant resistance to aphids via monolignol pathway in dicot plants (Gallego-Giraldo *et al.*, 2018; An *et al.*, 2019), little is known about it in agriculturally important monocot plants.

Alterations in expression levels of genes belonging to monolignol biosynthesis pathway may impact biotic stress responses through modulating plant defense signaling pathways. In Arabidopsis, the plants overexpressing gene encoding for ferulate 5-hydroxylase (F5H) enzyme promoted the development of green peach aphids (*Myzus persicae*) but reduced growth of the pathogen *Pseudomonas syringae* through *cis*-jasmone-mediated defense responses (Gallego-Giraldo *et al.*, 2018). In sorghum, lack of caffeic acid O-methyltransferase (COMT) activity promoted resistance to pathogens by elevating jasmonic acid (JA) and salicylic acid (SA) levels (Khasin *et al.*, 2021). However, Arabidopsis *comt1* mutants impacted asexual sporulation of the oomycete pathogen *Hyaloperonospora arabidopsidis*, a causal agent of downy mildew disease, independent of JA and SA pathway (Quentin *et al.*, 2009). Some reports showed that alterations in monolignol biosynthesis pathway can lead to phytohormonal changes, although the vice-versa holds true as well. For instance, auxin-response factor can fine-tune the expression levels of genes involved in monolignol biosynthesis pathway (Xu *et al.*, 2023; Wang *et al.*, 2024). Moreover, jasmonates and SA can also regulate lignin accumulation in plants through its interacting partners, *Myb* transcription factors, or can modulate other signaling molecules such as reactive oxygen species (Kováčik *et al.*, 2009; Denness *et al.*, 2011; Zhao *et al.*, 2023). The understanding of interplay between phytohormones in response to stress is far from complete since the outcomes can vary between different plant systems. Furthermore, phytohormone conjugation is considered as part of mechanisms that can tightly control the levels of phytohormones and simultaneously, conjugated forms also possess the biological role in plants (Staswick, 2009; Piotrowska & Bajguz, 2011; Luo *et al.*, 2023). For instance, JA-isoleucine (a biological active form of JA) and methyl jasmonate (MeJA) can modulate the plant defenses to insects and pathogens (Xu *et al.*, 1994; Woldemariam *et al.*, 2012; Farooq *et al.*, 2016; Yates-Stewart *et al.*, 2020; Li *et al.*, 2021; Grover *et al.*, 2022c). The conjugated auxin indole-3-acetic acid–aspartic acid (IAA–Asp) has also been shown to promote plant susceptibility to necrotrophic fungus, *Botrytis cinerea*, by regulating the transcription of virulence genes (González-Lamothe *et al.*, 2012). These studies suggest the connection between endogenous plant defense pathways and monolignol biosynthesis pathway (Gallego-Giraldo *et al.*, 2018; Khasin *et al.*, 2021).

Sorghum lines with altered levels of lignin provide a great model to understand the complex interactions between sorghum and SCA. The sorghum *Bmr6* gene encodes for cinnamyl alcohol dehydrogenase (CAD) enzyme, which catalyzes the conversion of hydroxycinnamoyl aldehydes to monolignols (Sattler *et al.*, 2009). The sorghum *Bmr12* gene encodes for COMT enzyme, responsible for catalysis of penultimate step in monolignol biosynthesis pathway, which converts 5-hydroxyconiferaldehyde into sinapyl aldehyde (Sattler *et al.*, 2012). The sorghum *bmr6* and *bmr12* mutants are null alleles of proteins involved in all lignin subunits and S-lignin subunit synthesis, respectively. Previously, sorghum lines with lowered lignin levels were tested against chewing insects, *Helicoverpa zea* and *Spodoptera frugiperda*, and resulted in varied levels of resistance to these insects (Dowd & Sattler, 2015). However, the role of sorghum monolignol pathway in defense against aphids is still unknown.

In this study, we investigated the contributions of *Bmr6* and *Bmr12* genes in providing sorghum defense against SCA. The evidence presented here reveals that *bmr12* and *Bmr12*-overexpression (OE) provided enhanced resistance and susceptibility to SCA, respectively, as compared with wild-type (WT; RTx430) sorghum plants. The transcriptomic and metabolomic analyses in conjunction with insect bioassays further demonstrate that IAA–Asp is a critical component of *bmr12*-conferred resistance to aphids. Our results highlight the complex interaction between *Bmr12* and auxin metabolism in sorghum plants in response to SCA infestation.

Materials and Methods

Plants

Sorghum (*Sorghum bicolor* (L.) Moench) COMT (*bmr12-ref*) and CAD (*bmr6-ref*) mutant near isogenic lines in RTx430 background along with *bmr6 bmr12* double mutants were used in this study (Pedersen *et al.*, 2006). *bmr12-ref* and *bmr6-ref* were referred to as *bmr12* and *bmr6*, respectively, in this study. The seed for these lines were further produced, and experiments were carried out at the University of Nebraska-Lincoln (UNL) glasshouse. Sorghum plants were grown in pots filled with soil mixed with vermiculite and perlite (PRO-MIX BX BIOFUNGICIDE + MYCORRHIZAS, Premier Tech Horticulture Ltd, Canada) with a 16 h : 8 h, 25°C, light : dark, and 50–60% relative humidity. Plants were watered regularly and fertiga once a week. Two-week-old plants at the three-leaf stage (Vanderlip & Reeves, 1972) were used for all the experiments.

Generation of transgenic *Bmr6* and *Bmr12* overexpression lines

The coding regions of *S. bicolor* cinnamyl alcohol dehydrogenase (CAD; *Bmr6*; Sobic.004G071000.1) and caffeate *O*-methyltransferase (COMT; *Bmr12*; Sobic.007G047300.1) were amplified by PCR with the primers SbCAD2_PciI-Fv2 GTAACATGTGGAGCCTGGCGTCCGAGAGG, SbCAD2_XbaI-Rv2 AAATCTAGATCAGTTGCTCGGCGCATCAGCGGCCCA, COMT-F GGTACCATGGGGTCGACGGCGGAG and COMT-R TCTAGATTACTTGATGAACTCGATGGCCC using Turbo *Pfu* polymerase (Agilent, Santa Clara, CA, USA) and pET-30a heterologous expression vectors containing the CDSs of these genes (Sattler *et al.*, 2009; Palmer *et al.*, 2010) as templates. The coding region was subcloned between the E35S CaMV promoter and the 35S CaMV terminator as a *Pci*I-*Xba*I and *Nco*I-*Xba*I fragments, respectively, and submitted to Eurofins Genomics (https://www.eurofinsgenomics.com) for DNA sequencing to confirm DNA sequence fidelity. The E35S::SbCAD/-COMT cassettes in the pZP211 binary vector were transformed into grain *S. bicolor* (RTx430) using *Agrobacterium tumefaciens*. Independent transgenic events were generated for both constructs and Bmr6 and Bmr12 protein accumulation (T3 generation) quantified via immunoblot assay following methods previously described (Sattler *et al.*, 2009, 2012; Tetreault *et al.*, 2018). Homozygous transgenic lines (NN 394-2-4-1, NN 393-4-1-1, ZG 271-1-11A, ZG 270-2-13B), referred to as *Bmr6*-OE1, *Bmr6*-OE2, *Bmr12*-OE1, and *Bmr12*-OE2 were selected based on proteins levels. NN and ZG are abbreviations for the technical staff that conducted transformation experiments.

Aphids

The SCA colony was maintained as previously described (Tetreault *et al.*, 2019; Grover *et al.*, 2022a), and aphids were reared on the susceptible sorghum genotype, BCK60 in a growth chamber with 16 h : 8 h, light : dark, 140 μE m^{-2} s^{-1} light quality, 23°C, and 50–60% relative humidity. The BCK60 sorghum plants for aphid rearing were grown to 7-leaf stage in the glasshouse, then provided to aphid colony as needed and old dried plants were removed. Adult apterous aphids were used for all the experiments.

Gene expression analyses

Two-week-old plants were infested with 10 adult aphids, and leaf samples were collected 5 and 10 d after SCA infestation. Uninfested plants served as controls and the leaf samples were also collected from control plants at the same time as aphid-infested ones. Samples were flash-frozen in liquid nitrogen immediately after collecting. Sorghum leaf tissues (*c.* 100 mg) were ground using a 2010 Geno/Grinder (SPEX SamplePrep) for 30 s at 1400 strokes min^{-1} under liquid nitrogen conditions. The RNA was isolated and purified using the RNA Clean and Concentrator Kit (Zymo, Irvine, CA, USA, https://www.zymoresearch.com/), and on-column DNase treatment was performed. Extracted total RNA was quantified with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNAs were synthesized from 1 μg of total RNA using the High-Capacity cDNA reverse transcriptase kit (Applied Biosystems, Forest City, CA, USA). cDNAs were diluted to 1 : 10 before using them for reverse transcription quantitative polymerase chain reaction. The reverse transcription quantitative polymerase chain reaction was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) on a StepOnePlus Real-Time PCR System (Applied Biosystems). At least three independent biological replicates were used for reverse transcription quantitative polymerase chain reaction, and each biological replicate contained three technical replicates. The gene-specific primers used in this study are listed in Supporting Information Table S1. The 10 μl reaction volume consisted of 1 μl of cDNA (with 1 : 10 dilution factor), 5 μl of SYBR Green, 0.5 μl of sense and antisense 10 μM primers, and 3 μl of nuclease free water. The cycling parameters were 40 cycles each consisting of an initial holding at 95°C for 5 s and annealing at 58°C for 30 s followed by a melt curve analysis. Relative gene expression of transcripts was analyzed using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The mRNA levels were normalized using the internal control tubulin (Scully *et al.*, 2016; Grover *et al.*, 2022d). Fold change was calculated by comparing the normalized transcript level of gene in infested to control samples.

Western blot and immunodetection

Proteins from RTx430 plants were isolated from leaf tissue of 2-wk-old sorghum plants after 0, 6, 24, and 48 h of SCA infestation using an extraction buffer containing protease inhibitor (Sigma-Aldrich, http://www.sigmaaldrich.com/) (Sattler *et al.*, 2009). Protein concentrations were measured using Pierce 660 Protein assay kit as per protocol using BSA as a standard (https://www.thermofisher.com/). Western blot analyses were performed as described previously (Scully *et al.*, 2016). The blot was probed with 1 : 14 000 dilutions of COMT and 1 : 4000 dilutions of APX primary antibodies as loading controls. The

secondary antibody was detected using chemiluminescence with Amersham ECL Western blotting reagent (GE Healthcare, Chicago, IL, USA, http://www.gehealthcare.com/). Each lane on the gel represents protein extract collected from three plants.

Sample fixation, histochemical staining, and microscopy

Leaf tissues were fixed in an ethanol:acetic acid solution (3 : 1 v/v) overnight, then stored in 70% ethanol, embedded in 7% agarose, and 100 μM sections were cut using a Leica VT1200s vibratome (Leica Microsystems, Wetzlar, Germany). Sections were stained for 2 min in 0.5% potassium permanganate solution, followed by 3–4 distilled water rinses, then placed in 3.0% HCl until the deep brown color was discharged from the section then immediately followed with the addition of ammonium hydroxide solution (14.8 M). Sections were imaged using an Olympus BX-51 light microscope (Olympus Co.) at ×80 magnification.

Lignin quantification

The lignin levels in RTx430, *bmr12*, and *Bmr12*-OE1 plants before and after SCA infestation were quantified using the thioglycolic acid (TGA) method as described previously (Moreira-Vilar *et al.*, 2014; Kundu *et al.*, 2018, 2023).

No-choice assays

For no-choice assays, the experimental design was completely randomized design (CRD); that is, all plants were randomly arranged and infested with aphids. For SCA infestation, five adult apterous aphids were released on each plant and covered with tubular clear plastic cages ventilated with organdy fabric on the top of the cage. Total number of aphids, including both adults and nymphs, were counted after 5 and 10 d of aphid release on each plant.

Aphid feeding behavior analysis using Electrical Penetration Graph (EPG) technique

The EPG technique (Walker, 2000) was used to assess the SCA feeding behavior on sorghum plants as previously described (Grover *et al.*, 2019, 2022c; Tetreault *et al.*, 2019). Eight channels of EPG recordings were used simultaneously over an 8-h period of SCA feeding, and at least 12 replications were obtained for each sorghum line. Several feeding behavior parameters such as the duration of pathway phase (inter- and/or intracellular aphid stylet paths during the brief sampling of cells), xylem phase (aphid feeding from xylem tissues), sieve element phase (SEP; aphid feeding from phloem sap and ingestion of nutrients), and nonprobing phases (stationary phase or relatively no aphid stylet movement) were recorded and calculated. The other parameters assessed include time to first probe by aphid (time difference between starting of recording and first insertion of stylet into plant) and time to first SEP (time difference between starting of recording and initiation of SEP) during 8-h recording of waveforms. E1 and E2 phases represent aphid salivation and passive ingestion of phloem sap, respectively, which were together considered as SEP. The EPG analysis software (*Stylet+*, EPG Systems, Wageningen, the Netherlands) was used to analyze the waveforms of SCA feeding on sorghum plants.

RNA-Seq data analysis

Quality assessment of mRNA-seq raw reads was conducted using FastQC (Andrews, 2010), followed by trimming with Trimmomatic v.0.39. Trimming parameters included LEADING:20, TRAILING:20, SLIDINGWINDOW: 4:20, and MINLEN:75 (Bolger *et al.*, 2014). The trimmed reads were aligned to the sorghum reference genome RTx430 v2.1 using Tophat v.2.1.1 (Kim & Salzberg, 2011). For RTx430 samples, the mapping parameters included 0 splicing mismatches (-m 0) and 0 mismatches (-N 0), while *bmr12* and *Bmr12*-OE1 were mapped with 0 splicing mismatches (-m 0) and 1 mismatch (-N 1). Transcript reconstruction was performed using Cufflinks v2.2.1, employing quantification against the reference annotation only (-G), multi-read-correct (-u), and frag-bias-correct (-b). Differential expression analysis of genes (DEGs) utilized Cuffdiff 2.2.1 with the options multi-read-correct (-u) and frag-bias-correct (-b) (Trapnell *et al.*, 2012). DEGs were considered significantly expressed with q-values < 0.05 and fold change $|\log_2(\text{FPKMinfested/FPKMcontrol})| \geq \log_2(2)$ (Schurch *et al.*, 2016) for each infested time point and its corresponding control. Raw reads are available in the NCBI SRA database under the bioproject no. PRJNA1086520.

Gene Ontology analysis

The upset plot for DEGs was created using the UpSetR (https://github.com/hms-dbmi/UpSetR) for 5 and 10 d post infestation (dpi) individually. The Gene Ontology (GO) analysis was conducted for all the DEGs using agriGOv2 (http://systemsbiology.cau.edu.cn/agriGOv2/) (Tian *et al.*, 2017) with *S. bicolor* as reference genome and $P < 0.05$ to determine the enriched GO terms.

Phytohormone and phenolics quantification

For phytohormone and phenolics quantification, samples from RTx430, *bmr12*, and *Bmr12*-OE1 plants were collected. For aphid-infested treatment, 10 adult apterous SCA were introduced in each plant and were covered with tubular clear plastic cages to avoid aphid escape. The aphid-uninfested control plants were also covered with tubular clear plastic cages without aphid infestation. Approximately 100 mg leaf tissue from control and aphid-infested plants at 5 and 10 dpi from each sorghum line was collected and flash-frozen immediately. Sorghum leaf tissues were ground using a 2010 Geno/Grinder (SPEX SamplePrep) for 30 s at 1400 strokes min^{-1} under liquid nitrogen conditions. Subsequently, LC-MS assays and quantification of plant hormones/metabolites were performed at the Proteomics and Metabolomics Facility at the Center for Biotechnology, UNL using deuterium-labeled internal standards as previously described (Chapman *et al.*, 2018; Varsani *et al.*, 2019; Grover *et al.*, 2022c).

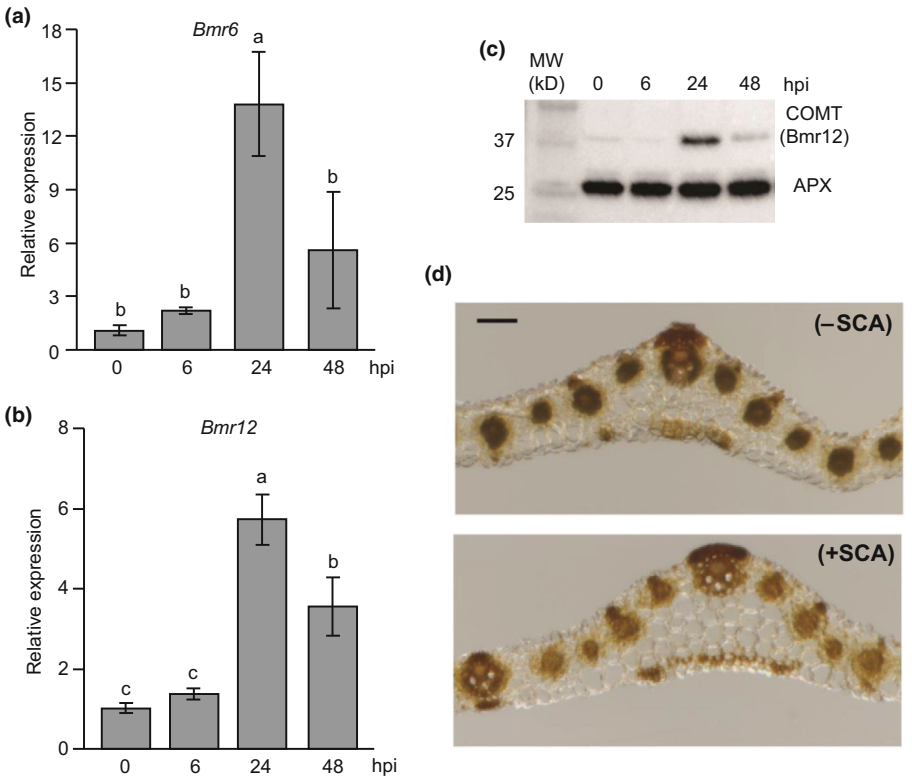

Fig. 2 Time course reverse transcription quantitative polymerase chain reaction analysis of (a) *Bmr6* and (b) *Bmr12* in 2-wk-old wild-type (WT; RTx430) sorghum leaves before (0 h) and after sugarcane aphid (+SCA) infestation (n = 3). (c) Immunoblot detection of Bmr12 (COMT) in 2-wk-old RTx430 sorghum leaves before (0 h) and after SCA infestation. Total proteins extracted from leaves were separated by SDS-PAGE, transferred to membrane, and probed with polyclonal antibodies raised against the recombinant COMT protein. Monoclonal antibodies raised against ascorbate peroxidase (APX) protein were used as a protein loading control. MW, Molecular mass markers in kD. This experiment was conducted twice with similar results. (d) Mäule staining of leaf cross sections after infestation of 2-wk-old RTx430 sorghum plants with 10 adult apterous aphids (+SCA) for 10 d. Leaves of SCA-uninfested (−SCA) plants were used as controls (bar, 100 μm). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ±SE. hpi, h post infestation.

Chemical treatment of plants

IAA–Asp (50 μM) dissolved in 0.1% (v/v) DMSO was used for exogenous application on sorghum plants. Plants that were sprayed with 0.1% (v/v) DMSO and plants that did not receive any treatment were used as the controls. IAA–Asp-treated and control plants were maintained in different glasshouse chambers to avoid the effect of any volatiles emitted from treated plants to control plants and vice-versa. After 24 h of treatment, five adult apterous SCA were introduced in each plant and were covered with tubular clear plastic cages to avoid aphid escape. The aphid counting was performed at 5 and 10 dpi.

Artificial diet feeding trial bioassays

Aphid feeding trial bioassays were conducted as previously described (Grover *et al.*, 2022c). Briefly, five adult SCA were introduced into each feeding chamber and allowed to feed on the diet, and the total numbers of aphids (adults plus nymphs) in each chamber was counted after 3 days. Different concentrations of IAA–Asp dissolved in 0.1% (v/v) DMSO or aphid diet mixed with 0.1% (v/v) DMSO was used as the control for artificial diet feeding assays.

Statistical analyses

The statistical analyses for gene expression, phytohormone, and metabolomics data were performed using PROC GLIMMIX in SAS 9.4 (SAS Institute). For aphid count, a generalized regression model was used for data analysis using negative binomial distribution. For no-choice assays, pairwise comparisons between treatments or sorghum lines were performed by comparing the means using Tukey's honestly significant difference tests ($P < 0.05$). For EPG experiments, nonparametric Kruskal–Wallis test was used to compare the duration of six different feeding parameters/phases among three different sorghum lines using PROC NPAR1WAY procedure, considering the non-normally distributed data.

Results

SCA feeding modulates *Bmr6* and *Bmr12* expression levels and reduces S-lignin accumulation in sorghum wild-type (RTx430) plants

The sorghum *Bmr6* and *Bmr12* genes encode for CAD enzyme, which catalyzes the conversion of hydroxycinnamoyl aldehydes to monolignols, and the COMT enzyme responsible for catalysis of the penultimate step in the monolignol biosynthesis pathway, respectively. We tested the expression level of genes, *Bmr6* and *Bmr12* after SCA infestation at early time points in the RTx430 plants. SCA feeding enhanced the expression level of *Bmr6* and *Bmr12* genes at 24 h post infestation (hpi) and decreased significantly at 48 hpi (Fig. 2a,b). Western blots probed with *Bmr12* (COMT) antibodies correlated with change to *Bmr12* expression (Fig. 2c). Moreover, histochemical analysis using Mäule stain, which preferentially stains S-lignin subunits (Saluja *et al.*, 2021), showed reduced deposition of S-lignin in RTx430 sorghum leaves after SCA infestation for 10 d (Fig. 2d).

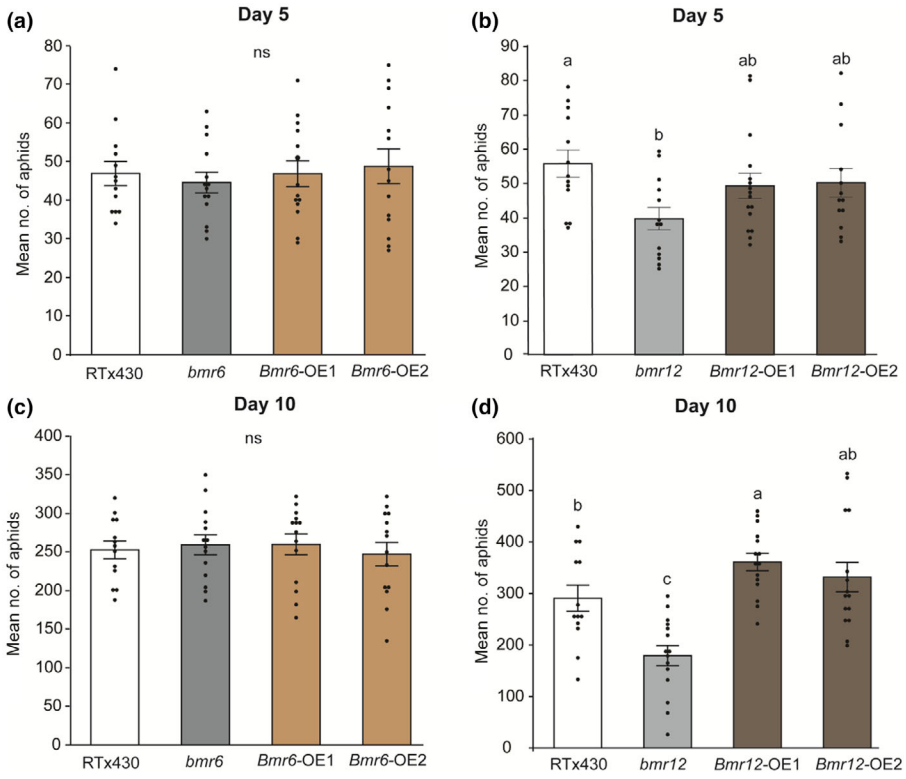

Fig. 3 Total number of sugarcane aphid (SCA) adults and nymphs recovered 5 and 10 d after infestation of 2-wk-old (a and c) wild-type (WT; RTx430), *bmr6*, and *Bmr6*-OE and (b and d) RTx430, *bmr12*, and *Bmr12*-OE sorghum plants with five adult apterous aphids/plant (n = 13–15). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ±SE. ns, no significant differences.

Loss of function of *Bmr12*, but not *Bmr6*, provides enhanced resistance to SCA

CAD (Bmr6) is an important enzyme in synthesis of lignin, and loss of Bmr6 activity results in decreased lignin content of cells (Sattler *et al.*, 2009). We performed a no-choice assay with *bmr6*, *bmr12*, and overexpression lines of *Bmr6* and *Bmr12* genes to test the role of monolignols in providing defenses to SCA. Surprisingly, we found comparable numbers of SCA on *bmr6* and *Bmr6*-OE lines as compared with RTx430 plants at 5 and 10 d after aphid infestation (Fig. 3a,c). On the other hand, we found significantly lower numbers of SCA on *bmr12* and higher numbers of SCA on *Bmr12*-OE1 plants after 5 and 10 d of SCA infestation as compared with RTx430 plants (Fig. 3b,d). These results indicate that loss of *Bmr12* function enhances sorghum resistance to SCA.

Bmr6 is not required for the *bmr12*-conferred enhanced resistance to SCA

To establish whether *Bmr6* is required for the *bmr12*-conferred enhanced resistance, a no-choice bioassay was performed with the *bmr6 bmr12*-stacked mutant plants. SCA numbers on *bmr6 bmr12* plants were comparable with those on the *bmr12* single mutant and were lower compared with the *bmr6* single mutant and RTx430 plants on both 5 and 10 dpi (Fig. 4a,b). These data indicate that the presence of the *Bmr6* allele does not affect *bmr12*-conferred resistance to SCA and *bmr12*-conferred enhanced resistance to SCA does not require Bmr6 (CAD) activity.

Lignin levels were comparable among all three sorghum lines after SCA infestation

SCA feeding on RTx430 sorghum leaves reduced S-lignin deposition (Fig. 2d). Furthermore, Mäule staining of *bmr12* and *Bmr12*-OE1 plants displayed reduced and enhanced deposition of basal S-lignin, respectively (Fig. S1). We also examined the total lignin levels in RTx430, *bmr12*, and *Bmr12*-OE1 plants before and after SCA infestation for 10 d. Our results suggest that basal lignin levels were comparable between RTx430 and *bmr12* plants; however, OE of *Bmr12* resulted in significantly elevated basal lignin levels (Fig. S2). Although SCA feeding for 10 d significantly reduced lignin levels in *Bmr12*-OE1 plants compared with *Bmr12*-OE1 control plants, total lignin levels were comparable among RTx430, *bmr12*, and *Bmr12*-OE1 plants after SCA infestation for 10 d (Fig. S2). These results suggest that lignin accumulation is not a major contributor to the *bmr12*-mediated resistance to SCA.

Aphids spent more time to reach sieve element phase on *bmr12* plants

To assess the feeding behavior of SCA on *bmr12* and *Bmr12*-OE1 compared with RTx430, we utilized the EPG technique. We measured the total time spent by SCA in pathway phase, xylem phase, phloem phase, and no-probing phase on RTx430, *bmr12*, and *Bmr12*-OE1 plants. Also, we measured the total time taken to first probe and reach first SEP during the 8-h recording. Except for the time to reach first SEP, we did not find

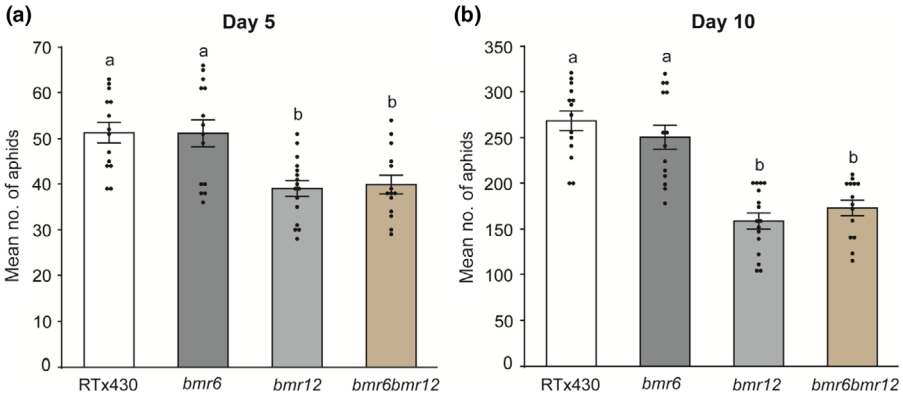

Fig. 4 Total number of sugarcane aphid (SCA) adults and nymphs recovered (a) 5 and (b) 10 d after infestation of 2-wk-old wild-type (WT; RTx430), *bmr6*, *bmr12*, and *bmr6 bmr12* double mutant sorghum plants with five adult apterous aphids/plant. Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ±SE.

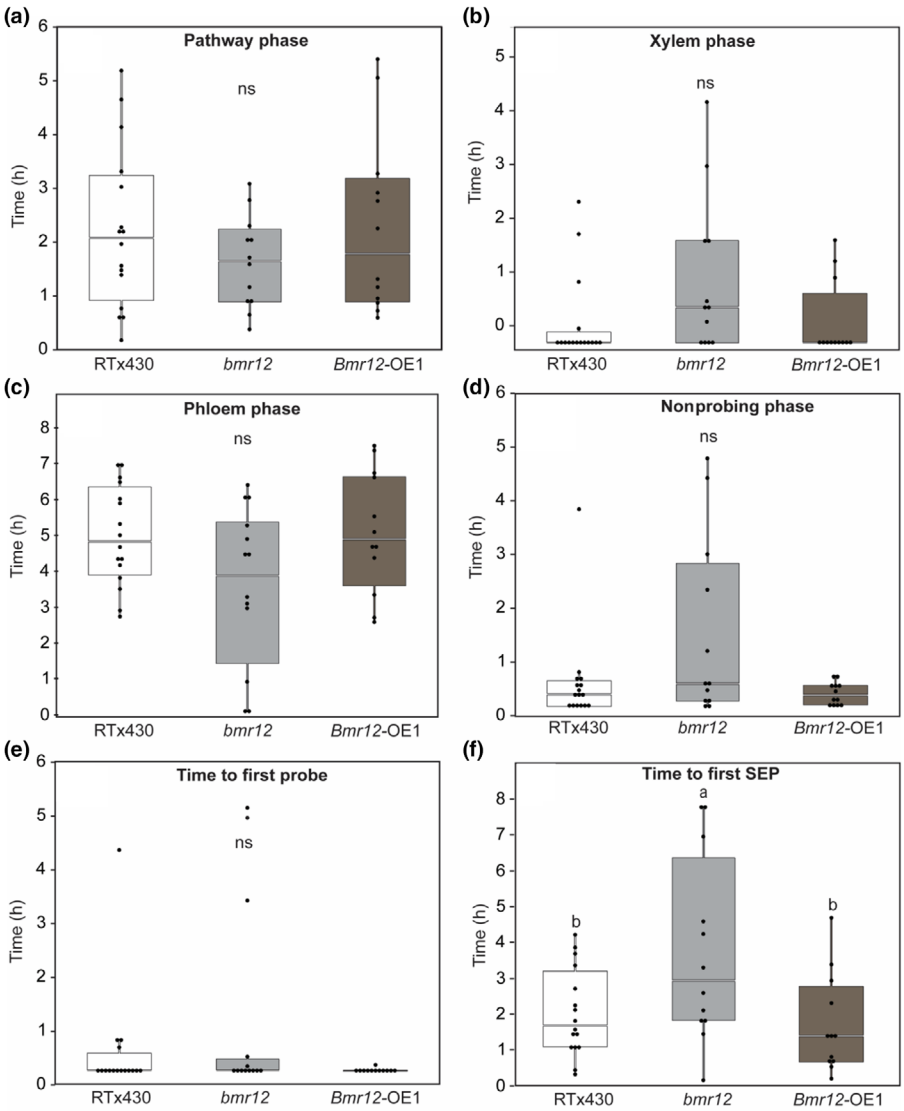

Fig. 5 Total mean time spent by sugarcane aphids (SCA) for different feeding behavior parameters on each sorghum line during an 8-h period of EPG recording (n = 12–15). (a) Pathway phase, (b) Xylem phase, (c) Phloem phase, (d) Nonprobing phase, (e) Time to first probe, and (f) Time to first sieve element phase. Different letters above the bar indicate significant difference based on Kruskal–Wallis test and multiple comparisons ($P < 0.05$). Bars show the mean values obtained for different sorghum lines. Error bars represent ±SE. ns, no significant differences; SEP, sieve element phase.

significant difference in any of the parameters mentioned above for SCA feeding behavior on RTx430, *bmr12*, and *Bmr12*-OE1 plants (Fig. 5a–f). SCA took significantly longer time to reach first SEP in *bmr12* plants, compared with RTx430 and *Bmr12*-OE1 plants during an EPG recording of 8 h (Fig. 5f). Collectively, EPG results indicate that the *bmr12*-conferred enhanced resistance to SCA could be due to the factors present outside the vascular tissues.

SCA feeding alters the transcriptome across all three sorghum lines

The number of DEGs were higher in 10 dpi compared with the 5 dpi across all three lines (Fig. S3a,b; Table S2). At 5 dpi, all three sorghum lines had number of upregulated DEGs almost four times (*c.* 500) than the downregulated ones (*c.* 130) (Fig. S3a). The number of common genes across all three lines during upregulation was 194, which are much higher than at downregulation (24). The number of unique genes for RTx430, *bmr12*, and *Bmr12*-OE1 was 109, 145, and 166 during upregulation and 104, 58, and 82 during downregulation, respectively (Fig. S3a,b). At 10 dpi, the number of upregulated DEGs were in the range of 750–850 in all three lines (Fig. S3b). The number of common genes across all three lines during upregulation was 348 that are twice as downregulated common genes (163). The number of unique genes for RTx430, *bmr12*, and *Bmr12*-OE1 was 162, 216, and 213 during upregulation and 120, 169, and 386 during downregulation, respectively (Fig. S3b).

The GO enrichment analysis was performed in these unique genes of both 5 and 10 dpi (Table S3). The 216 unique genes upregulated for *bmr12* at 10 dpi were enriched with GO terms related to primary metabolism (lipid catabolic process, carbohydrate, and sucrose metabolic process) and defense (response to auxin, hormone, and endogenous stimulus) (Fig. S3c). The 213 unique genes upregulated for *Bmr12*-OE1 at 10 dpi were enriched with GO terms related to primary metabolism (photosynthesis and carboxylic biosynthesis process), amino acid biosynthesis process, and oxidation and reduction process (Fig. S3d). Similarly, the 162 unique genes upregulated for RTx430 plants at 10 dpi were enriched with GO terms related to protein phosphorylation, protein metabolic process, phosphorous metabolic process, and ion transport (Fig. S3e). Our unique gene enrichment analysis revealed that only sorghum *bmr12* line displayed defense-related GO terms in response to SCA infestation.

JA and SA pathways are not involved in *bmr12*-mediated resistance to SCA

Previously, it has been reported that plants with altered levels of lignin could impact biotic defenses through JA and/or SA pathway (Gallego-Giraldo *et al.*, 2018; Khasin *et al.*, 2021). First, we monitored the DEGs related to JA pathway in our transcriptomics data and found 12 genes related to jasmonates. Subsequently, we performed the hierarchal cluster analysis to cluster the genes and treatments. The genes encoding for lipoxygenases, allene oxide synthase, and hydroperoxide lyase (*SbiRTX430.04G008200*, *SbiRTX430.01G129400*, *SbiRTX430.01G473900*, *SbiRTX430.01G129600*, *SbiRTX430.03G416000*, and *SbiRTX430.03G416200*) were significantly upregulated in all three lines at 5 and 10 dpi (Fig. 6a). Whereas, other genes encoding for 12-oxophytodienoate reductase (*OPR*), F-box protein, and jasmonate-zim-domain protein (*SbiRTX430.06G097100*, *SbiRTX430.06G097000*, *SbiRTX430.02G082700*, *SbiRTX430.10G089500*, and *SbiRTX430.06G062000*) were significantly downregulated or unaltered in at least one line. Specifically, one gene, *SbiRTX430.01G508500*, which encodes jasmonate-zim-domain protein was highly abundant in *Bmr12*-OE1 SCA-uninfested plants; however, SCA feeding suppressed the expression of this gene at 10 dpi. These results did not show any specific changes in JA-related genes in *bmr12* plants. To confirm the role of jasmonates in *bmr12*-mediated resistance to SCA, we quantified the levels of OPDA, JA, and JA-Ile at 5 and 10 dpi. No significant changes in OPDA and JA levels were observed irrespective of SCA infestation and time points (Figs 6b–e, S4). However, the constitutive levels of JA-Ile were significantly higher in *Bmr12*-OE1 plants; however, SCA feeding suppressed the JA-Ile levels and reverted to basal levels at 10 dpi and were comparable to other treatments.

Our transcriptome data showed 21 genes related to SA pathway and downstream responses. Several genes (*SbiRTX430.10G021800*, *SbiRTX430.03G119200*, *SbiRTX430.02G024000*, *SbiRTX430.05G180700*, *SbiRTX430.05G180500*, *SbiRTX430.07G191700*, *SbiRTX430.01G421600*, *SbiRTX430.03G119200*, *SbiRTX430.01G421000*, and *SbiRTX430.01G421100*) encoding for pathogenesis-related proteins (PR proteins) were significantly upregulated in all three lines at least at one time point (Fig. 7a). Two genes encoding for SAM-dependent carboxyl methyltransferases (*SbiRTX430.03G290500* and *SbiRTX430.10G107400*) and one gene encoding for PR protein (*SbiRTX430.02G284100*) were specifically upregulated in *bmr12* plants at 5 or 10 dpi. We found one gene encoding for isochorismate synthase (*SbiRTX430.02G186500*) significantly downregulated in *bmr12* and *Bmr12*-OE1 plants at 10 dpi. The transcriptomic data indicated the overall upregulation of SA-related genes. To dissect the role of SA pathway in *bmr12*-mediated resistance to SCA, we further quantified the SA levels in all three lines at 5 and 10 dpi. SCA feeding increased SA levels in all three sorghum lines at 5 dpi but were not significantly altered (Fig. 7b). At 10 dpi, although we found significantly elevated levels of SA in RTx430 plants, SA levels were comparable between *bmr12* and *Bmr12*-OE1 plants before and after SCA feeding for 10 d (Fig. 7c), suggesting that *bmr12*-mediated resistance to SCA is independent of the SA pathway.

No specific alterations in phenylpropanoids were observed in *bmr12* plants upon SCA infestation

Previously, it has been shown that changes in expression levels of monolignol biosynthesis pathway genes can lead to altered levels of phenylpropanoids (phenolic compounds/flavonoids) in plants (Fornalé *et al.*, 2010; Gill *et al.*, 2018; Grover *et al.*, 2022d). Phenylpropanoids can impact the insect growth directly or also act as feeding deterrent/antifeedants (Morkunas *et al.*, 2016; Kariyat *et al.*, 2019; Grover *et al.*, 2022d; Chatterjee *et al.*, 2023) that could possibly contribute to *bmr12*-mediated resistance to SCA. However, our transcriptomic analysis revealed that loss of *Bmr12* function did not alter the expression levels of genes belonging to monolignol biosynthesis pathway before and after SCA infestation (Fig. S5). Similarly, no specific changes in phenylpropanoids were observed in *bmr12* plants before and after SCA infestation (Fig. S6).

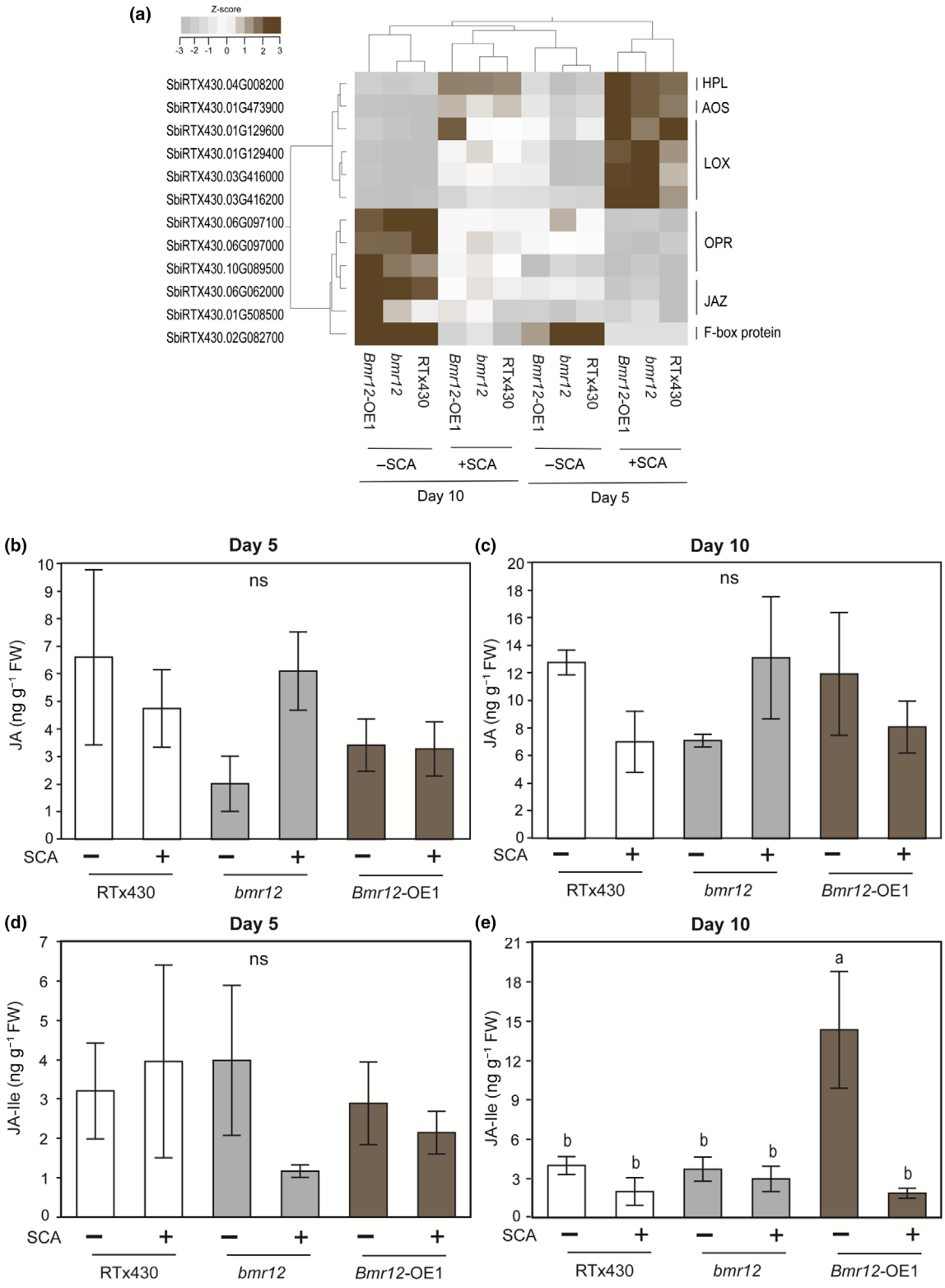

Fig. 6 Impact of sugarcane aphid (SCA) infestation on the jasmonic acid (JA) pathway genes and metabolites on sorghum plants. (a) Heatmap of the relative expression level for the differentially expressed genes related to jasmonic acid (JA) pathway in wild-type (WT; RTx430), *bmr12*, and *Bmr12*-OE1 sorghum lines after 5 and 10 d of sugarcane aphid (SCA) infestation. Color key represents the Z-score standardized values. (b–e) Time course of changes in JA and JA-Isoleucine (Ile) levels before (–SCA) and after (5 and 10 d post infestation (dpi)) SCA infestation in different sorghum lines. (n = 4). Different letters above the bars indicate values that are significantly different from each other (P < 0.05; Tukey's test). Error bars represent ±SE. FW, fresh weight; ns, no significant differences.

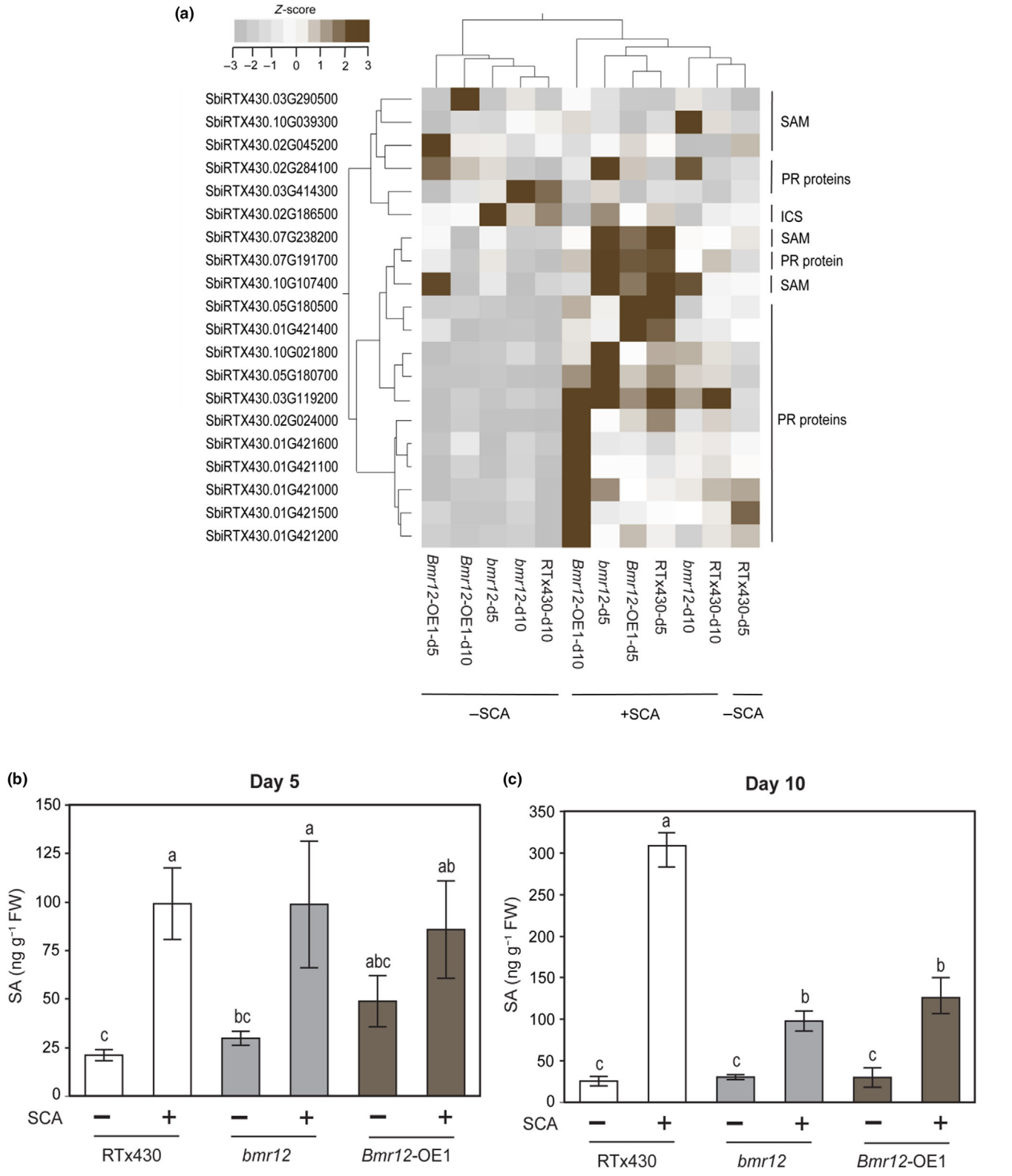

Fig. 7 Impact of sugarcane aphid (SCA) infestation on the salicylic acid (SA) pathway genes and metabolites on sorghum plants. (a) Heatmap of the relative expression level for the differentially expressed genes related to SA pathway in wild-type (WT; RTx430), *bmr12*, and *Bmr12*-OE1 sorghum lines after 5 and 10 d of SCA infestation. Color key represents the Z-score standardized values. (b, c) Time course of changes in SA levels before (−SCA) and after (5 and 10 d post infestation (dpi)) SCA infestation in different sorghum lines. (n = 4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ±SE. FW, fresh weight.

SCA feeding triggers expression of genes related to auxin metabolism and IAA–Asp levels in *bmr12* plants

Several genes related to auxin signaling such as auxin responsive genes, SAUR-like, GH3 family proteins (*SbiRTX430.01G096100*, *SbiRTX430.06G286800*, *SbiRTX430.02G290800*, *SbiRTX430.04G165000*, *SbiRTX430.07G213900*, *SbiRTX430.02G367000*, and *SbiRTX430.08G057800*) were significantly upregulated in all three lines at 5 or 10 dpi. Two genes encoding for auxin metabolism and response factors (*SbiRTX430.04G363800* and *SbiRTX430.10G077200*) were significantly downregulated at 5 and 10 dpi. Three SAUR-like auxin-responsive family proteins (*SbiRTX430.10G282200*, *SbiRTX430.10G268600*, and *SbiRTX430.07G207000*) were significantly upregulated in *bmr12* plants at 10 dpi while no changes were found in RTx430 and *Bmr12*-OE1 plants. Moreover, three genes related to signal transduction and auxin-responsive family proteins (*SbiRTX430.04G251100*, *SbiRTX430.09G246400*, and *SbiRTX430.03G278800*) were significantly downregulated only in RTx430 and *Bmr12*-OE1 plants. Overall, the genes related to auxin metabolism were differentially expressed in *bmr12* plants (Fig. 8a). Upon phytohormone quantification, no significant changes in total IAA levels were observed in *bmr12* plants at both 5 and 10 dpi before and after SCA infestation (Fig. 8b). Although IAA levels remained unaltered in *bmr12* plants, IAA levels significantly decreased in RTx430 and *Bmr12*-OE1 plants at 10 dpi (Fig. 8c). Further, we found that IAA–Asp levels were significantly higher in *bmr12* plants at 5 dpi (Fig. 8d). At 10 dpi, both RTx430 and *bmr12* displayed elevated levels of IAA–Asp; however, IAA–Asp levels were significantly higher in SCA-infested *bmr12* plants as compared with SCA-infested RTx430 plants (Fig. 8e). No changes in IAA–Asp levels were observed in *Bmr12*-OE1 plants before and after SCA infestation for 5 and 10 d. Similarly, no changes in methyl IAA levels were observed across all three sorghum lines and treatments (Fig. S7).

Exogenous IAA–Asp application restores resistance in the *Bmr12* overexpression plants

To confirm the role of IAA–Asp in providing sorghum resistance to aphids, we exogenously applied 50 μM IAA–Asp on all three sorghum lines. Five adult apterous aphids were infested on each plant after 24 h of IAA–Asp treatment. At 5 and 10 dpi, a reduced number of aphids were observed on RTx430 and *Bmr12*-OE1 lines as compared with control plants (Fig. 9a,b). No differences in SCA numbers were observed between the control and IAA–Asp-pretreated *bmr12* plants at 5 and 10 dpi (Fig. 9a,b), indicating that exogenous IAA–Asp application does not result in any increase in heightened resistance to aphids. Notably, our results suggest that exogenous IAA–Asp application restored resistance in *Bmr12*-OE1 plants compared with *Bmr12*-OE1 control plants at 5 and 10 dpi (Fig. 9a,b). We therefore conclude that IAA–Asp is a critical component for *bmr12*-conferred resistance to aphids.

IAA–Asp at higher concentrations can adversely impact SCA reproduction

Recently, it has been suggested that IAA–Asp may be associated with improving tomato defense against *P. syringae* (Yang *et al.*, 2024). Furthermore, since exogenous IAA–Asp application restored SCA resistance in *Bmr12*-OE1 plants compared with *Bmr12*-OE1 control plants, we examined whether IAA–Asp has a direct negative effect on SCA growth and fecundity. To assess this, SCA was reared on an artificial diet containing different concentrations of IAA–Asp for 3 d. Our aphid feeding assays indicate that IAA–Asp at lower concentrations (0.1–10 μM) did not impact SCA proliferation compared with SCA reared on diet alone and the diet mixed with DMSO, the solvent used to make the IAA–Asp stock solution (Fig. 10). However, IAA–Asp included in artificial diet at higher concentrations (25, 50, and 100 μM) resulted in reduced SCA numbers compared with controls (Fig. 10), suggesting that IAA–Asp at elevated levels can adversely impact SCA growth and fecundity.

Discussion

Insect feeding manipulates resource allocation patterns and physiology of host plants (Louis *et al.*, 2012; Louis & Shah, 2013; Nalam *et al.*, 2019). For example, aphid feeding on a plant alters the rate of photosynthesis, source–sink relationships, nutrient allocation, carbohydrate metabolism, and transport (Morkunas *et al.*, 2016; Ponzio *et al.*, 2017; Zogli *et al.*, 2020b; Grover *et al.*, 2022c). Our recent studies on monocot–aphid interactions suggest that aphid feeding on monocot crops alters the production of defensive proteins and secondary metabolites (Varsani *et al.*, 2019; Koch *et al.*, 2020; Pingault *et al.*, 2020, 2021; Zogli *et al.*, 2020a). Importantly, our recent studies have suggested that aphid feeding on sorghum plants can alter the expression of genes/metabolites involved in the monolignol biosynthesis pathway (Tetreault *et al.*, 2019; Kundu *et al.*, 2023; Puri *et al.*, 2023), which also is a potential defense mechanism utilized by plants to disrupt aphid growth and reproduction. SCA feeding induced the expression levels of *Bmr6* and *Bmr12* (Fig. 2), whose gene products are involved in the last steps of monolignol biosynthesis, which is in alignment with our previous studies (Tetreault *et al.*, 2019; Kundu *et al.*, 2023).

In several plants, insect pest herbivory has been shown to induce the expression levels of *CAD* genes (Barakat *et al.*, 2010; Aslam *et al.*, 2022; Kundu *et al.*, 2023; Liu *et al.*, 2023; Yao *et al.*, 2023). However, silencing *CAD* genes in tobacco (*Nicotiana attenuata*) did not influence the relative growth rates of two leaf-chewing herbivores, *Manduca sexta* and *S. exigua* (Joo *et al.*, 2021). Similarly, CAD-reduced poplar tress did not display any interaction with insects in long-term field trials (Pilate *et al.*, 2002). In sorghum, *bmr6* lines displayed similar levels of damage to fall armyworm (*S. frugiperda*) infestation under field conditions (Dowd *et al.*, 2016; Gruss *et al.*, 2022). However, detached leaf assays with *bmr6* plants showed moderate resistance to *S. frugiperda* under laboratory conditions (Dowd & Sattler, 2015). In the current study, we found comparable numbers

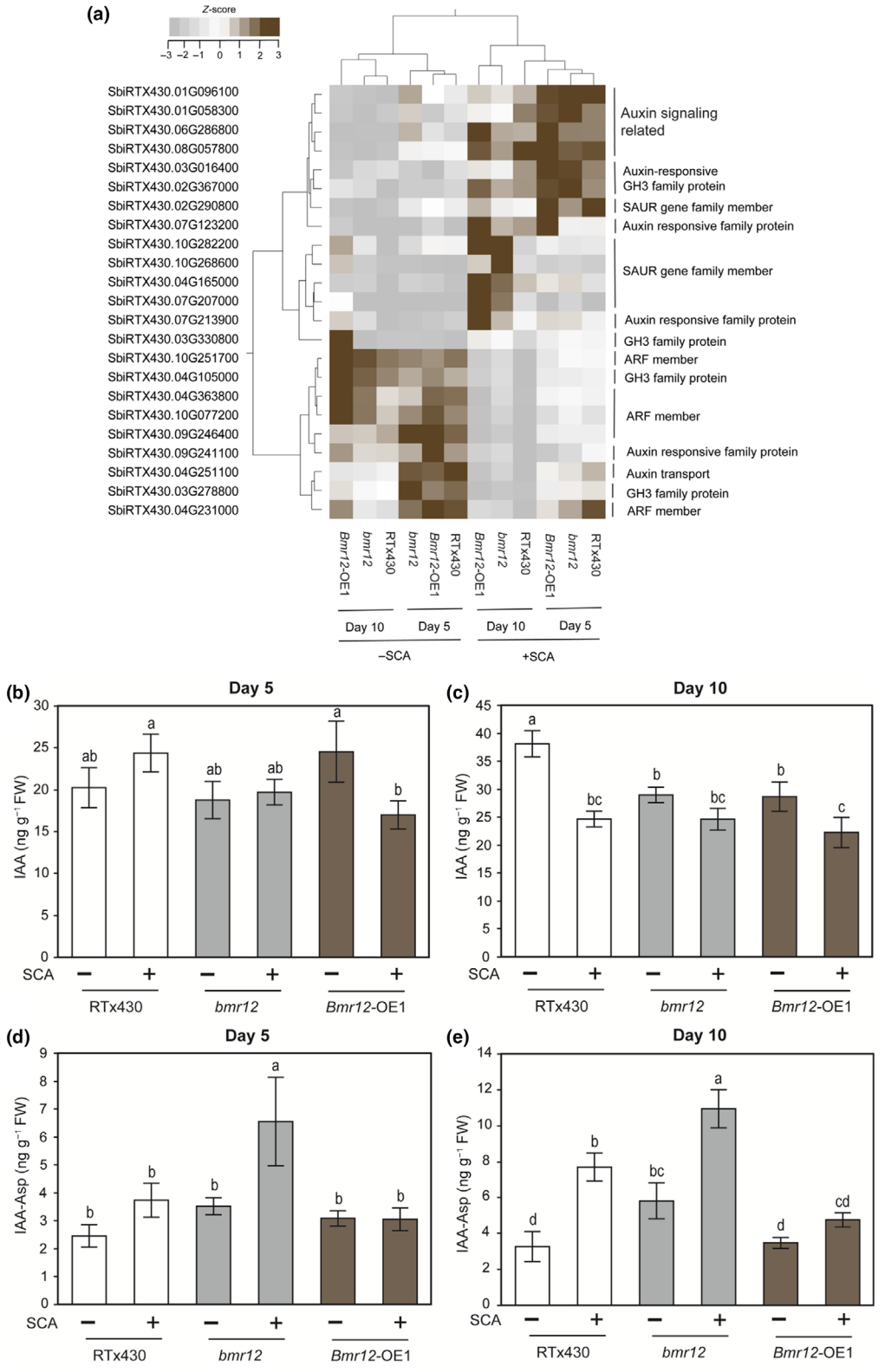

Fig. 8 Impact of sugarcane aphid (SCA) infestation on the auxin pathway genes and metabolites on sorghum plants. (a) Heatmap of the relative expression level for the differentially expressed genes related to auxin metabolism in wild-type (WT; RTx430), *bmr12*, and *Bmr12*-OE1 sorghum lines after 5 and 10 d of SCA infestation. Color key represents the Z-score standardized values. (b–e) Time course of changes in indole-3-acetic acid (IAA) and IAA–Aspartic acid (IAA–Asp) levels before (–SCA) and after (5 and 10 d post infestation (dpi)) SCA infestation in different sorghum lines. (n = 4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ±SE. FW, fresh weight.

of aphids on *bmr6* and *Bmr6*-OE lines (Fig. 3a,c). Additionally, the SCA population size was comparable between *bmr12*- and *bmr6 bmr12*-stacked mutant plants (Fig. 4), thus confirming that Bmr6 (CAD) does not influence the *bmr12*-mediated resistance to SCA.

COMT is one of the core enzymes in monolignol biosynthesis pathway, which can affect the lignin content as well as plant signaling pathways (Weng *et al.*, 2010; Sattler *et al.*, 2012; Khasin *et al.*, 2021; Saluja *et al.*, 2021). Silencing of *COMT* gene in wheat led to enhanced fungal penetration into host cells (Bhuiyan

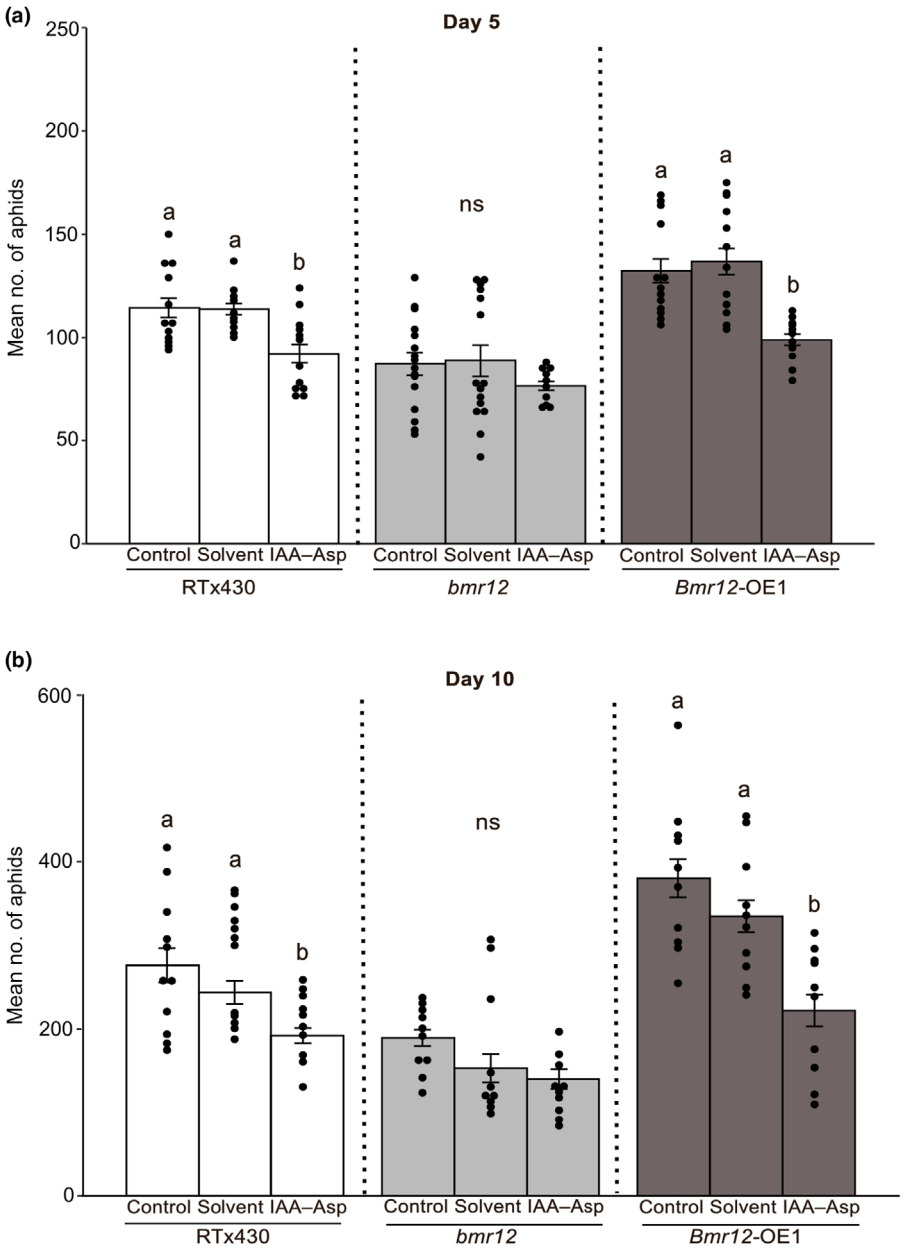

Fig. 9 Total number of sugarcane aphid (SCA) adults and nymphs that were recovered (a) 5 d and (b) 10 d post infestation (dpi) of wild-type (WT; RTx430), *bmr12*, and *Bmr12*-OE1 sorghum lines that were pretreated with indole-3-acetic acid–aspartic acid (IAA–Asp) for 24 h. Plants that were treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls. Sorghum plants were infested with five adult apterous aphids per plant after 24 h of IAA–Asp treatment. Plants treated with DMSO to dissolve IAA–Asp and plants that did not receive any treatment were used as the negative controls (n = 10–15). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ±SE. ns, no significant differences.

et al., 2007). Similarly, *COMT*-silenced wheat plants displayed susceptibility to necrotrophic fungus, *Rhizoctonia cerealis*, compared with control plants, whereas overexpression of *COMT* enhanced resistance to sharp eyespot (Wang *et al.*, 2018). *COMT* expression was also found to be higher in wheat-resistant lines and significantly enhanced after inoculation. On the contrary, our data suggested loss of COMT leads to enhanced resistance to SCA. Aphid proliferation was lower on *bmr12* and higher on *Bmr12*-OE plants as compared with RTx430 plants (Fig. 3d). Our previous sorghum proteomic analysis before and after SCA feeding coupled with aphid feeding behavior analysis on SCA-resistant sorghum plants demonstrated that SCA feeding can suppress the defense-related proteins at early time points (Grover *et al.*, 2022b). We speculate that the SCA attempts to trick the plants by suppressing defenses at early time points and alterations in COMT levels could be a consequence of this strategy.

In Arabidopsis, altered lignin composition has been reported to affect the resistance to pathogens and aphids (Gallego-Giraldo *et al.*, 2018). For instance, overexpression of *F5H*, a gene upstream to *COMT*, in Arabidopsis displayed elevated levels of S-lignin, led to enhanced resistance and susceptibility to *Pseudomonas syringae* and green peach aphids, respectively. Indeed, *Bmr12*-OE1 displayed enhanced susceptibility to aphids compared with RTx430 plants (Fig. 3d). Histochemical analysis using Mäule stain, which preferentially stains S-lignin subunits (Saluja *et al.*, 2021), showed reduced deposition of S-lignin in RTx430 sorghum leaves after SCA infestation for 10 d (Fig. 2d). Additionally, since aphids took significantly more time in

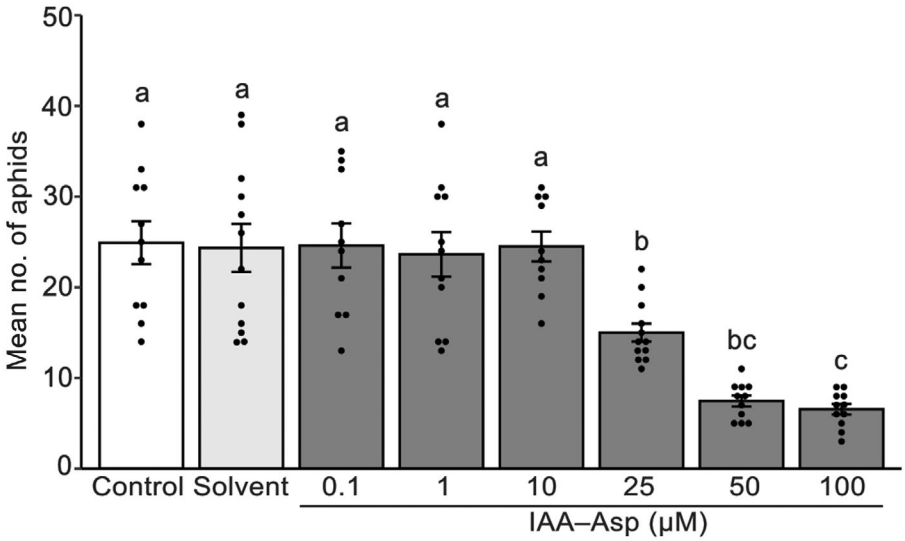

Fig. 10 Comparison of sugarcane aphid (SCA) numbers on artificial diet supplemented with different concentrations of indole-3-acetic acid–aspartic acid (IAA–Asp). Five adult apterous SCA were introduced into each feeding chamber and allowed to feed on the diet. The total numbers of aphids (adults and nymphs) in each chamber were counted after 3 d (n = 10–12). Different letters indicate significant differences between treatments on each day ($P < 0.05$; Tukey's test). Error bars represent ±SE.

reaching the first SEP in *bmr12* plants compared with RTx430 and *Bmr12*-OE1 plants, it is highly plausible that the aphids potentially encountered resistance factors outside the vascular tissues, leading to enhanced resistance to SCA in *bmr12* plants.

Although we report here the role of *Bmr6* and *Bmr12* genes in defense response to aphids, other genes in monolignol biosynthesis pathway have also been shown to modulate plant immunity under biotic stress. For instance, the upstream genes of monolignol biosynthesis pathway such as phenylalanine ammonia-lyase (*PAL*) and caffeoyl-CoA O-methyltransferase (*CCoAOMT*) provided immunity to the sap-sucking brown planthopper (*Nilaparvata lugens*), hemibiotrophic bacterial pathogen (*P. syringae*), biotrophic viral pathogen tobacco mosaic virus, and conferred quantitative resistance to both southern leaf blight and gray leaf spot (Elkind *et al.*, 1990; Pallas *et al.*, 1996; Huang *et al.*, 2010; He *et al.*, 2020). The cinnamoyl-CoA reductase (*CCR*) gene has also been reported to provide *R*-mediated immunity against the hemibiotrophic fungal pathogen *Magnaporthe grisea* (Kawasaki *et al.*, 2006). In Arabidopsis, cinnamoyl-CoA reductase 1 (*CCR1*) is involved in constitutive lignification of plant cells. *CCR1* mutants led to three- to fourfold lesser sinapoyl malate accumulation than WT plants (Mir Derikvand *et al.*, 2008). Some studies reported the possibility of diverting metabolic flux to other branches of phenylpropanoid pathway. For example, Arabidopsis hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT)-RNAi lines had higher flavonoid levels because of metabolic flux from lignin to flavonoid pathway (Gallego-Giraldo *et al.*, 2011a). Therefore, it arises the question whether loss of *Bmr12* function alters the expression level of other genes of monolignol biosynthesis pathway and potentially contribute to *bmr12*-mediated resistance to SCA. However, our transcriptomic and metabolic data revealed that loss of *Bmr12* function did not impact the expression levels of other genes/metabolites in phenylpropanoid pathway (Figs S5, S6), suggesting that loss of function mutations in *Bmr12* potentially alter other defense-related metabolic and signaling pathways beyond monolignol biosynthesis pathway.

Alterations in monolignol pathway can modify plant resistance levels through modulating plant defense signaling pathways and secondary metabolism (Mir Derikvand *et al.*, 2008; Gallego-Giraldo *et al.*, 2011b, 2018; Baxter & Stewart, 2013; Gill *et al.*, 2018; Grover *et al.*, 2022d). Previously, it has been reported that plants with altered levels of lignin provided resistance to pathogens and insects through impacting JA and SA levels of the host plants (Gallego-Giraldo *et al.*, 2018; Khasin *et al.*, 2021). For instance, downregulation of *HCT* gene in alfalfa (*Medicago sativa*) led to constitutive activation of defense responses. The defense activation has been attributed to the release of bioactive cell wall fragments and production of hydrogen peroxide (Gallego-Giraldo *et al.*, 2011b). Downregulation of HCT enzyme in Arabidopsis and alfalfa has exhibited reduced lignin levels along with elevated levels of pathogenesis-related proteins and SA (Gallego-Giraldo *et al.*, 2011a,b). In Arabidopsis, *F5H* overexpression lines exhibited different responses to bacteria and aphids through *cis*-jasmone-mediated responses (Gallego-Giraldo *et al.*, 2018). Recently, it has been reported that sorghum *bmr12* plants had significantly higher levels of transcripts involved in gibberellic acid (GA) biosynthesis and signaling, which further reduces lateral root formation (Saluja *et al.*, 2021). SA has often been associated with GA signaling and responsiveness (Alonso-Ramírez *et al.*, 2009; Gallego-Giraldo *et al.*, 2011a), suggesting that GA–SA hormonal crosstalk could be important in determining growth-defense trade-offs. However, sorghum *bmr12* plants also showed elevated levels of SA, JA, and gibberellic acid 19 (GA19) in response to pathogen and drought stress (Khasin *et al.*, 2021). Surprisingly, we did not observe any correlation between JA/SA levels in these sorghum lines before and after SCA infestation (Figs 6, 7), thus confirming that *bmr12*-mediated resistance to SCA is independent of JA and SA pathways.

It is well-established that auxin/IAA and/or IAA-derived metabolite(s) have diverse roles in plant biotic stresses and are involved in various aspects of plant growth and development (Gallei *et al.*, 2020; Kunkel & Johnson, 2021). Our results

demonstrate that SCA feeding altered the auxin metabolism/-signaling in all three sorghum lines (Fig. 8). Furthermore, SCA feeding enhanced the IAA–Asp levels in *bmr12* plants as compared with RTx430 and *Bmr12*-OE1 plants at both 5 and 10 dpi. A handful of studies have revealed the connection between auxin and lignin levels (Xu *et al.*, 2023; Wang *et al.*, 2024). For instance, auxin signaling can impact the lignin levels in plants (Wang *et al.*, 2024). However, the reverse has not been reported. Our findings revealed that plants pretreated with IAA–Asp displayed a lower number of aphids (Fig. 9), suggesting the critical role of IAA–Asp in *bmr12*-mediated resistance to SCA. Previously, it was reported that the conjugated auxin IAA–Asp promotes plant disease development via activation of virulence genes, not through changes in JA and SA pathways (González-Lamothe *et al.*, 2012). Furthermore, *Manduca sexta* feeding on *Nicotiana attenuata* plants rapidly induced IAA levels and IAA was also required for the JA-dependent accumulation of anthocyanins and phenolamides in the stems (Machado *et al.*, 2016). Thus, it is evident that IAA/IAA-derived metabolite(s) can modulate defenses that are dependent or independent of phytohormonal pathways. Our previous work reported elevated basal IAA levels in SCA-tolerant SC35 sorghum plants; however, aphid feeding suppressed IAA levels in the SCA-tolerant sorghum plants (Grover *et al.*, 2022a). Simultaneously, no changes in IAA–Asp levels were found in SCA-susceptible and tolerant sorghum genotypes (Grover *et al.*, 2022a). Thus, it is highly likely that induction of IAA and IAA–Asp levels may be related to plant growth and defense, respectively. In fact, our artificial diet feeding assays confirmed that IAA–Asp at higher concentrations has a direct negative effect on SCA growth and fecundity (Fig. 10), which suggests IAA–Asp plays a key role in *bmr12*-mediated resistance to SCA.

Insect cues, such as saliva, oral secretions, frass, and honeydew, contain phytohormones that can modulate plant defense responses to insects (Maxwell & Painter, 1962; Dafoe *et al.*, 2013; Acevedo *et al.*, 2019). For example, the gall-inducing caterpillar species significantly induced the IAA in galls in the stem tissues of *Solidago altissima* (Tooker & De Moraes, 2011). Simultaneously, it was also shown that the gall-inducing caterpillars contained high concentrations of IAA (Tooker & De Moraes, 2011), suggesting that IAA and/or IAA-derived metabolites are encountered by the insects in their diets. Similarly, sawfly larvae have been shown to contain high concentrations of IAA levels and are involved in the initial stages of gall formation (Yamaguchi *et al.*, 2012). Furthermore, European corn borer excreted exceedingly high IAA levels in their frass that led to enhanced host plant quality and subsequent improved larval feeding and growth (Dafoe *et al.*, 2013). Similar to caterpillar frass, honeydew, which is the digestive waste of sap-feeding insects, also contained auxins (Maxwell & Painter, 1962). A strong correlation between auxins present in aphid honeydew and their host plants was observed, suggesting the ability of aphids to extract auxins from host plants. The auxin uptake from plants by aphids have also been demonstrated through isotope labeling study (Cambridge & Morris, 1996). Additionally, it was shown that disruption in auxin perception and signaling led to aphid resistance in melon (Sattar *et al.*, 2016). Our data also suggest that SCA feeding altered the auxin metabolism and significantly elevated levels of auxin conjugate IAA–Asp, which potentially contributed to heightened resistance in *bmr12* plants (Figs 8, 9). Recently, it was shown that the transcription factor WRKY75 is involved in tomato defense against *P. syringae* by modulating auxin homeostasis, which promotes the conversion of free IAA to IAA–Asp, thereby enhancing plant defense (Yang *et al.*, 2024). Although the exact mechanism of how the loss of *Bmr12* function alters the IAA–Asp levels after SCA infestation is not known, our feeding trial bioassays with IAA–Asp adversely impacted aphid reproduction, growth, and development (Fig. 10).

In summary, we have uncovered an important role of *Bmr12* in modulating sorghum defense against aphids, independent of *Bmr6*. While the enhanced resistance of *bmr12* plants is linked to altered IAA–Asp levels, EPG data indicated the possibility of resistance factors outside vascular tissues. Future studies are needed to further dissect out the complex interactions between *bmr12* and auxin metabolism pathway that can alter the resistance to SCA. Understanding the complex sorghum defense mechanisms against aphids can accelerate the development of insect control traits through advanced cutting-edge biotechnological tools.

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Competing interests

None declared.

Author contributions

SG and JL conceived and designed the study. SG, D-FM, KS and HP performed aphid bioassays and the aphid feeding behavior experiments. SG, LP and D-FM collected the plant samples and SG performed gene expression studies, Western blots. SG and KS performed histochemical staining experiments. SES contributed reagents, methods development and provided guidance on experiments. SG, D-FM, KS and LP analyzed the data. SG and JL wrote the draft. All authors contributed to the writing and editing of the final manuscript.

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Data availability

All data supporting the findings of this study are available within the paper and/or supporting information. Raw reads are available in the NCBI SRA database under the bioproject no. PRJNA1086520 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1086520?reviewer=pgugbva5nushv1pi5v10hqvrpl).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Mäule staining of leaf cross sections of 2-wk-old wild-type (WT; RTx430), *bmr12* and *Bmr12*-OE1 sorghum lines.

Fig. S2 Lignin levels in RTx430, *bmr12*, and *Bmr12*-OE1 plants before and after sugarcane aphid feeding for 10 d.

Fig. S3 Upset intersection plots of the total number of differentially expressed genes and Gene Ontology terms in RTx430, *bmr12*, and *Bmr12*-OE1 lines.

Fig. S4 Time course of changes in 12-oxo-phytodienoic acid levels before (−) and after (+) sugarcane aphid feeding for 5 and 10 d in different sorghum lines.

Fig. S5 Heatmap of the relative expression level for the differentially expressed genes related to monolignol biosynthesis pathway in RTx430, *bmr12*, and *Bmr12*-OE1 sorghum lines before (−) and after (+) sugarcane aphid feeding for 5 and 10 d.

Fig. S6 Heatmap analysis of quantified phenylpropanoids in RTx430, *bmr12*, and *Bmr12*-OE1 sorghum lines before (−) and after (+) sugarcane aphid (SCA) feeding for 5 and 10 d.

Fig. S7 Time course of changes in methyl indole-3-acetic acid levels before (−) and after (+) sugarcane aphid (SCA) feeding for 5 and 10 d in different sorghum lines.

Table S1 Reverse transcription quantitative polymerase chain reaction primers used to analyze expression levels of genes from the monolignol biosynthesis pathway.

Table S2 Total number of differentially expressed genes in RTx430, *bmr12*, and *Bmr12*-OE1 sorghum lines after 5 and 10 d of sugarcane aphid infestation.

Table S3 Gene enrichment analysis of unique differentially expressed genes identified in RTx430, *bmr12*, and *Bmr12*-OE1 lines in response to sugarcane aphid infestation at 5 and 10 d post infestation.

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