



Sorghum defense responses to sequential attack by insect herbivores of different feeding guilds

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Abstract

Main conclusion Insect herbivores of different feeding guilds induced sorghum defenses through differential mechanisms, regardless of the order of herbivore arrival on sorghum plants.

Abstract Sorghum, one of the world's most important cereal crops, suffers severe yield losses due to attack by insects of different feeding guilds. In most instances, the emergence of these pests are not secluded incidents and are followed by another or can also co-infest host plants. Sugarcane aphid (SCA) and fall armyworm (FAW) are the two most important destructive pests of sorghum, which belongs to sap-sucking and chewing feeding guilds, respectively. While the order of the herbivore arriving on the plants has been found to alter the defense response to subsequent herbivores, this is seldom studied with herbivores from different feeding guilds. In this study, we investigated the effects of sequential herbivory of FAW and SCA on sorghum defense responses and their underlying mechanism(s). Sequential feeding on the sorghum RTx430 genotype by either FAW primed–SCA or SCA primed–FAW were monitored to unravel the mechanisms underlying defense priming, and its mode of action. Regardless of the order of herbivore arrival on sorghum RTx430 plants, significant defense induction was observed in the primed state compared to the non-primed condition, irrespective of their feeding guild. Additionally, gene expression and secondary metabolite analysis revealed differential modulation of the phenylpropanoid pathway upon insect attack by different feeding guilds. Our findings suggest that priming in sorghum plants upon sequential herbivory induces defense by the accumulation of the total flavonoids and lignin/salicylic acid in FAW primed–SCA and SCA primed–FAW interaction, respectively.

Keywords Defense priming · Fall armyworm · Flavonoids · Phenylpropanoid pathway · Sequential herbivory · Sorghum · Sugarcane aphid

Introduction

Plants and insects have been co-evolving for > 350 million years, thereby driving the evolution of plant defenses to herbivores (Whitney and Glover 2013). Under natural conditions, plants are attacked by a wide range of insect pests of varying damage levels during their lifespan. Plants possess constitutive defenses and are also able to induce defenses depending on the feeding behavior of the attacking herbivores (Poelman and Kessler 2016; de Bobadilla et al. 2022). It has been established that plant defense outcomes are driven not only by the combination of insect attackers, but also by the order of herbivores arriving on the plants (Kafle et al. 2017; Mertens et al. 2021). The occurrence of herbivores may vary in time, space, and order, depending on several other environmental factors, thus affecting the

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defense type and status of the plants (Ohgushi 2016; Ashra and Nair 2022; Hilker et al. 2023). Plants recognize the herbivore-associated molecular patterns (HAMPs) and activate the cascade of defense signaling pathways (Basu et al. 2018; Steinbrenner et al. 2020). Thus, the specific defense mechanisms to one herbivore may improve or compromise the resistance to subsequent herbivores (Frost et al. 2008; Weeraddana and Evenden 2019; Puri et al. 2023). Differential defense signaling pathways, resource allocation trade-offs, changes in plant biochemistry and physiology, and different HAMPs to specific herbivores may alter the plant resistance to secondary herbivores (Ohgushi 2005; Koornneef and Pieterse 2008; Poelman et al. 2010).

HAMPs arise from insect saliva, regurgitant, frass, honeydew, ovipositional fluids and herbivore-associated endosymbionts (Felton et al. 2014; Basu et al. 2018). These molecules are recognized by plants to induce defense mechanisms against insects or they may also have the ability to suppress plant defenses (Will et al. 2007; Chuang et al. 2014; Felton et al. 2014; Ray et al. 2015; Acevedo et al. 2018). The outcome of interactions between plant defenses and insect counter-defenses varies between different plants and herbivores. For instance, insects with chewing type of mouth parts cause extensive damage to plants and also release a wide array of cues to manipulate plants defenses (Acevedo et al. 2015). The plant responses to chewing-type insects are primarily regulated by jasmonic acid (JA) signaling pathways and generally result in induced resistance to subsequent herbivory by similar feeding guilds (Howe 2004; Ankala et al. 2009; Shivaji et al. 2010). However, insects with piercing–sucking-type mouth parts cause minimal apparent injury to the plants while removing photosynthates from the plants by ingesting sap (Carena and Glogoza 2004; Nalam et al. 2019; Zogli et al. 2020; Mou et al. 2023). The plant defenses to sap-sucking insects are mainly regulated by salicylic acid (SA) pathway and may result in induced resistance to feeding by subsequent sap-feeders (Engelberth et al. 2011; Ding and Ding 2020; Grover et al. 2022a, b; Puri et al. 2023). In this context, the sequential herbivory by insects of different feeding guilds may compromise the resistance to secondary herbivore due to antagonistic crosstalk between JA and SA signaling pathways (Thaler et al. 2002; Koornneef and Pieterse 2008). However, these mechanisms are highly convoluted and can induce resistance to herbivores independent of the generally established JA–SA crosstalk (Cooper and Goggin 2005; Kuśnierczyk et al. 2011; Onkokesung et al. 2016; Lortzing et al. 2019).

Phenylpropanoid pathway generates a wide range of secondary metabolites based on the intermediates of monolignol and flavonoid pathways such as phenolic acids, flavonoids, and condensed tannins (Vogt 2010; Fraser 2011; Singh et al. 2021). Insect herbivory has been shown to alter the expression level of genes belonging to

phenylpropanoid pathway and ultimately, metabolite modulations in plants (Alon et al. 2013; Baxter and Stewart 2013; Singh et al. 2021; Grover et al. 2022c). For instance, flavonoids and phenolic acids have been reported to be effective against both chewing- and piercing–sucking-type insects (Alon et al. 2013; Dowd and Sattler 2015; Kariyat et al. 2019; Grover et al. 2022c; Chatterjee et al. 2022). Moreover, this complex pathway also provides building blocks for macromolecules such as lignin. Lignin deposition in cell walls has been well documented in plants in response to insect and pathogen attack (Bonawitz and Chapple 2010; Funnell-Harris et al. 2010; Baxter and Stewart 2013; Gallego-Giraldo et al. 2018).

Sorghum has gained a significant attention due to its versatility and resource efficiency as a food, fodder and bioenergy crop (Taylor et al. 2006; Stamenković et al. 2020; Ciampitti and Prasad 2020). However, sorghum is attacked by a wide range of insect pests such as fall armyworm (*Spodoptera frugiperda*), greenbugs (*Schizaphis graminum*), sugarcane aphids (*Melanaphis sacchari*), corn leaf aphids (*Rhopalosiphum maidis*), chinch bugs (*Blissus leucopterus*), to name a few (Guo et al. 2011; Okosun et al. 2021). Fall armyworm (FAW) is a chewing-type insect pest of sorghum that feeds on leaves voraciously while leaving the midveins behind (Venkateswarlu et al. 2018; Montezano et al. 2018; Fajemisin et al. 2023). Among piercing–sucking-type insects, sugarcane aphid (SCA) is a key pest on sorghum that utilizes its needle-like structures, called stylets, to pierce the plant tissues and ingest the phloem sap (Scott Armstrong et al. 2015). SCA secretes vast amount of honeydew that promotes the growth of sooty mold, which affects the photosynthetic ability of plants and renders plant difficult to harvest using agricultural machinery. The sequential herbivory studies in sorghum have been only limited to different aphid pests that demonstrate the ability of aphids to manipulate the defenses for subsequent aphid attack (Bayoumy et al. 2016; Michaud et al. 2017; Puri et al. 2023). Interestingly, it was also shown that the feeding guild of the initial attacker acts as the prime factor for determining the resistance/susceptibility to the subsequent attacker (Rodriguez-Saona et al. 2010). Another study reports the importance of the prevalent attacker in the field to be the critical factor determining cross-resistance (Mertens et al. 2021). However, limited knowledge is available on the effects of sequential herbivory of leaf-chewing and piercing–sucking pests on sorghum defense responses. Our study investigates whether the attack of chewing insect, FAW, alters the plant defenses to piercing–sucking-type insect, SCA, and vice versa. Thus, the objective of our study was to determine the sequential herbivory outcomes of sorghum by different feeding guilds and possible underlying mechanism(s).

Materials and methods

Plant growth conditions

The sorghum RTx430 from the NAM founder lines (Bouchet et al. 2017) used in this study was obtained from USDA-GRIN global germplasm (USA) (Grover et al. 2022b). All sorghum plants were grown in 3.8 cm × 21.0 cm plastic cone-tainers (Hummert International, Earth City, MO) filled with a mix of vermiculite and perlite (PRO-MIX BX BIOFUNGICIDE + MYCORRHIZAE, Premier Tech Horticulture Ltd., Canada) in a greenhouse at the University of Nebraska-Lincoln with a 16-h-light/8-h-dark photoperiod, 25 °C, and 50–60% relative humidity. Plants were watered regularly and fertigated when needed. Sorghum plants at the 3-leaf stage (Vanderlip and Reeves 1972) (two-week-old plants) were used for all the experiments.

Insect rearing

FAW larvae were obtained from Benzon Research Inc. (Carlisle, PA) and reared on artificial diet in a growth chamber with 16-h-light/8-h-dark photoperiod, 23 °C, and 50–60% relative humidity as described previously (Grover et al. 2022c). The SCA colony was maintained on a susceptible sorghum genotype, BCK60 and were replaced with new plants every two weeks in a plant growth chamber (Conviron F7, Controlled Environments Ltd.) under a 16:8-h light:dark cycle; temperatures were maintained at 26 °C as described previously (Grover et al. 2022b). As

needed, apterous aphids were transferred to experimental plants with a fine-bristled paint brush.

Sequential herbivory bioassay setup

Two-week-old sorghum plants were used for all the bioassays. For the initial infestation, each plant was infested with either single third-instar FAW larva or with ten adult apterous aphids for 2 days and was covered with tubular clear plastic cages to avoid FAW/aphid escape. The cages were ventilated with organically fabric on the sides and top of the cage for proper aeration. All the plants were randomly arranged. After two days of infestation, the FAW/SCA were removed with a fine paint brush. For the later infestation, ten adult apterous aphids or pre-starved (overnight) and pre-weighed (in the range of 60–70 mg) single third-instar FAW larva were placed in each plant for the next four days. Four days post infestation, the total number of aphids, including both nymphs and adults, were counted. For FAW, the final weight of caterpillars was recorded. For non-primed states, plants were directly subjected to SCA/FAW without any pre-infestation. An illustration of the experimental design used in this study has been shown in Fig. 1. For each treatment, there were 18–20 replicates.

Sampling of plant materials

Experiments were designed to investigate the priming-mediated changes in the expression of the phytohormone signaling and phenylpropanoid pathway genes upon infestation with insects of different feeding guilds. After infestation, SCA were mostly detected on the lower side of the leaves whereas FAW were feeding on the young leaf tissues in whorl region of the sorghum plant, which were



Fig. 1 Schematic representation of the sequential herbivory experiment. The sequential experiment was conducted with sugarcane aphids (SCA; *Melanaphis sacchari*) and fall armyworm (FAW; *Spodoptera frugiperda*) insects of piercing-sucking and chewing feeding guilds, respectively. Adult apterous SCA and third-instar FAW were

used for the experimental purpose. Blue-colored arrow indicates first treatment from 0 to 2 days. Yellow colored arrow indicates second treatment from second to sixth day. The null symbol indicates that plants were not infested with insects for the respective days

collected for sampling. Four to six biological replicates at each of the five conditions (control, only SCA, FAW-primed SCA (FAW-SCA), only FAW, and SCA-primed FAW (SCA-FAW)) were collected in liquid nitrogen and stored at -80°C until used for gene expression studies, and quantification of total flavonoids and lignin.

Gene expression studies

Two-week-old sorghum RTx430 leaf tissues (~100 mg) were used for RNA extraction from control or infested plants. Leaf tissues (80–100 mg) were ground using 2010 Geno/Grinder® (SPEX SamplePrep, NJ, USA) for 40 s at 1400 strokes/min. The homogenized leaf tissue was added to 1 ml of Sigma-Aldrich TRI reagent (St. Louis, MO, USA). RNA was extracted and purified using the RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA) and DNase treatment was performed. Extracted total RNA was quantified using a Nanodrop 2000c Spectrophotometer (Thermo Scientific™). Complementary DNAs (cDNAs) were synthesized from 1 µg of total RNA using the High-Capacity cDNA reverse transcriptase kit (Applied Biosystems Inc., Foster City, CA). cDNAs were diluted to 1:10 before using them for RT-qPCR. The gene-specific primers used in this study are listed in Supplemental Table S1. The RT-qPCR was performed with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories., Hercules, CA) on a StepOnePlus Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). Three–four independent biological replicates, each with three technical replicates, were used for RT-qPCR. Relative gene expression of transcripts was analyzed using $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001). The mRNA levels were normalized using tubulin as the internal control. The fold change was calculated by comparing the normalized transcript level of target gene in control and infested conditions in RTx430 plants.

Quantification of flavonoids and lignin

Total flavonoids were estimated using spectrophotometer as described previously (Tetreault et al. 2021). Briefly, 100 mg of flash frozen leaf samples were extracted in thrice the volume of chilled 80% methanol for overnight at 4°C . To the supernatant, 10% AlCl_3 was added to a final concentration of 1% and total amount of flavonoids were measured spectrophotometrically at 420 nm. The lignin was quantified using the thioglycolic acid (TGA) method as described previously (Moreira-Vilar et al. 2014; Kundu et al. 2018). Briefly, the pellet from 5 mg of lyophilized leaf powder extracted in 500 µL pure ethanol was dried overnight at room temperature. The pellet was then treated with 500 µL of 2 N HCl and 0.1 mL of TGA at 95°C for 6 h, followed by washing and resuspension in 500 µL of 1 N NaOH. After overnight

incubation, the supernatant was acidified with 250 µL of concentrated HCl and kept overnight at 4°C to collect the lignin thioglycolate pellet that was further dissolved in 500 µL of 1 N NaOH and lignin was measured spectrophotometrically at 280 nm. The lignin content was represented as the absorption values (A_{280}) using 1 N NaOH as the blank.

Statistical analysis

For the bioassay, aphid count data were analyzed using negative binomial distribution and FAW larval percent weight gain data was analyzed using student's t test ($P < 0.05$). R statistical environment of 'stat' package (<https://www.r-project.org/>) was used for performing statistical analyses. One-way ANOVA with Fisher's LSD ($P < 0.05$) followed by Bonferroni correction was performed for gene expression analysis and to evaluate metabolite analysis where differences across the treatments were evaluated.

Gene accession IDs

The genes described in this study are listed here with their accession numbers: *PR10* (Sobic.001G037970); *LOX1* (Sobic.001G125900); *CAD* (Sobic.004G071000); *COMT* (Sobic.007G047300); *FNSII* (Sobic.002G000400); *FNR* (Sobic.006G226800); and α -*Tub* (Sobic.001G107200).

Results

Plant defense induction was independent of the arrival sequence of the herbivore feeding guild

To understand the impact of FAW herbivory on SCA reproduction, we infested FAW-primed and undamaged/non-primed sorghum plants with SCA. The total number of aphids were counted from non-primed and FAW primed RTx430 plants on the fourth day (96 hpi). Our results demonstrate that SCA reproduced significantly lesser on sorghum RTx430 plants in the FAW-primed condition compared to the non-primed condition (Fig. 2a, $P < 0.001$). On the other hand, SCA-primed FAW-fed sorghum was monitored by recording the percent weight gain of the third-instar FAW larvae after four days (96 hpi). Our results show a significant reduction in the weight gain of FAW larvae fed on SCA-primed RTx430 plants compared to the non-primed plants (Fig. 2b, $P < 0.05$). Thus, our results suggest that priming-mediated plant defense is not dependent on the

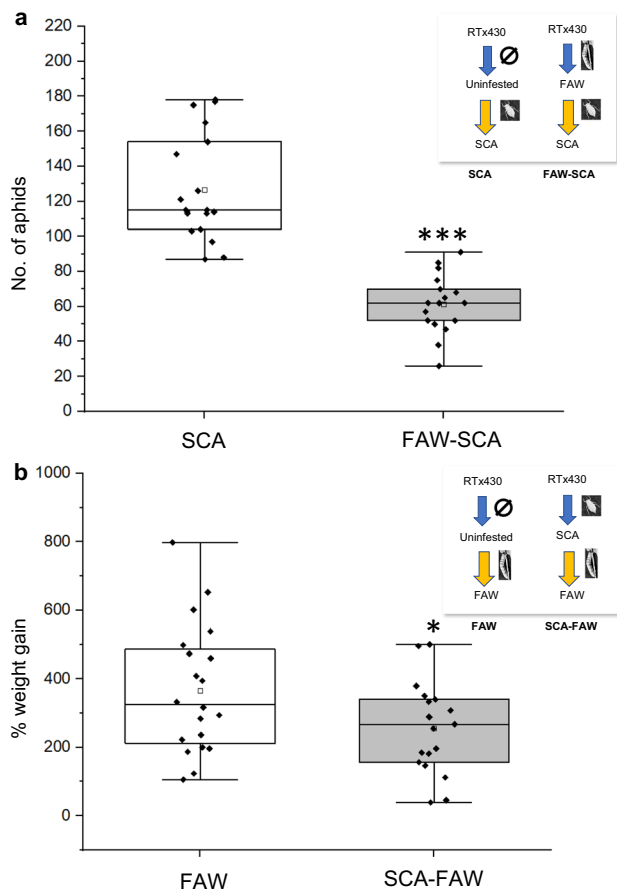


Fig. 2 Priming in plants induces the plant defense irrespective of the insect feeding guilds. **a** Sugarcane aphid (SCA) performance on non-primed (only SCA) and primed (FAW–SCA) sorghum RTx430 plants for four days. Ten adult apterous aphids were placed on each plant. The total number of aphids (mean ± SE) was counted after four days of infestation. **b** Fall armyworm (FAW) larval weight after feeding on non-primed (only FAW) and primed (SCA–FAW) sorghum RTx430 plants for four days. For all experiments, pre-weighed third-instar larvae were placed on plants of each condition. The larval weight (mean ± SE) was measured after four days of feeding. Primed plants were treated with the first insect for two days followed by the second insect for four days ($n=18–20$). This experiment was repeated twice with similar results. Asterisk indicates significant difference at * $P<0.05$ and *** $P<0.001$

sequence of the feeding guild of the insect that pre-infested the plants.

Defense-related phytohormonal signaling genes were significantly induced in SCA–primed FAW-infested plants

To determine the molecular basis of defense induction in SCA/FAW-primed RTx430 sorghum plants, we monitored the expression level of JA and SA marker genes. We analyzed the expression levels of the key phytohormone signaling genes (*PR10* and *LOX1*) in control,

FAW- and SCA-infested plants after 48 h. SCA feeding for 48 h induced the expression of *PR10* compared to FAW-infested plants (Fig. 3a). We also analyzed the expression levels of defense genes in control, FAW, SCA, FAW–SCA and SCA–FAW treatments after four days of sequential herbivory (Fig. 1). Our results displayed significant induction of the *PR10* gene in FAW–SCA and SCA-infested sorghum plants compared to control plants (Fig. 3b, > 35- and 30-fold, respectively). However, significantly higher induction was observed only in SCA–FAW-fed plants compared to control plants (Fig. 3b, > 15-fold). To test the modulation of the JA biosynthesis gene, *LOX1* transcript was monitored. Feeding by SCA alone did not alter the expression of *LOX1*; however, FAW feeding for 48 h significantly induced the expression of *LOX1* compared to uninfested control plants (Fig. 3c). No significant difference in *LOX1* expression was observed between the FAW–SCA and SCA-fed sorghum plants (Fig. 3d). However, > 45-fold induction of the *LOX1* transcript was observed in non-primed FAW-fed plants compared to nearly 33-fold induction in SCA–FAW-fed plants at 96 hpi (Fig. 3d). Taken together, our results suggest that priming differentially induced sorghum defenses irrespective of their feeding guild.

SCA herbivory resulted in elevated levels of lignin

To gain insights on the role of the lignin pathway genes in defense priming, we monitored the relative transcript abundance of the two key genes: *CAD*, cinnamyl alcohol dehydrogenase; and *COMT*, caffeic acid O-methyl transferase in non-primed (Fig. 4a, c) and SCA/FAW-primed RTx430 plants at 96 hpi (Fig. 4b, d). Feeding by SCA alone or FAW alone for 48 h did not alter the expression level of *CAD* and *COMT* transcripts compared to control plants (Fig. 4a, c). No significant difference in the expression of *CAD* was observed in the FAW–SCA- and SCA-fed sorghum plants at 96 hpi (Fig. 4b). However, > 1.5-fold induction of the *CAD* transcript was observed in FAW-fed plants compared to > sevenfold induction in SCA–FAW-fed plants at 96 hpi (Fig. 4b). There was significant downregulation of the *COMT* transcript in the FAW–SCA-fed plants compared to the FAW non-primed SCA-fed RTx430 plants at 96 hpi (Fig. 4d, > three- and > five-fold, respectively). Interestingly, significant upregulation of the *COMT* transcript was observed in the SCA–FAW-fed plants compared to the FAW-fed RTx430 plants at 96 hpi (Fig. 4d, > six- and > twofold, respectively). Collectively, our results suggest that priming with SCA significantly induced the transcript accumulation of the *CAD* and *COMT* genes in SCA–FAW-fed plants compared to the FAW-fed RTx430 plants. Using the thioglycolic acid method, we further determined the lignin content in sorghum RTx430 plants after different treatments. Lignin content was significantly reduced in the FAW–SCA-fed RTx430 plants at 96 hpi compared to

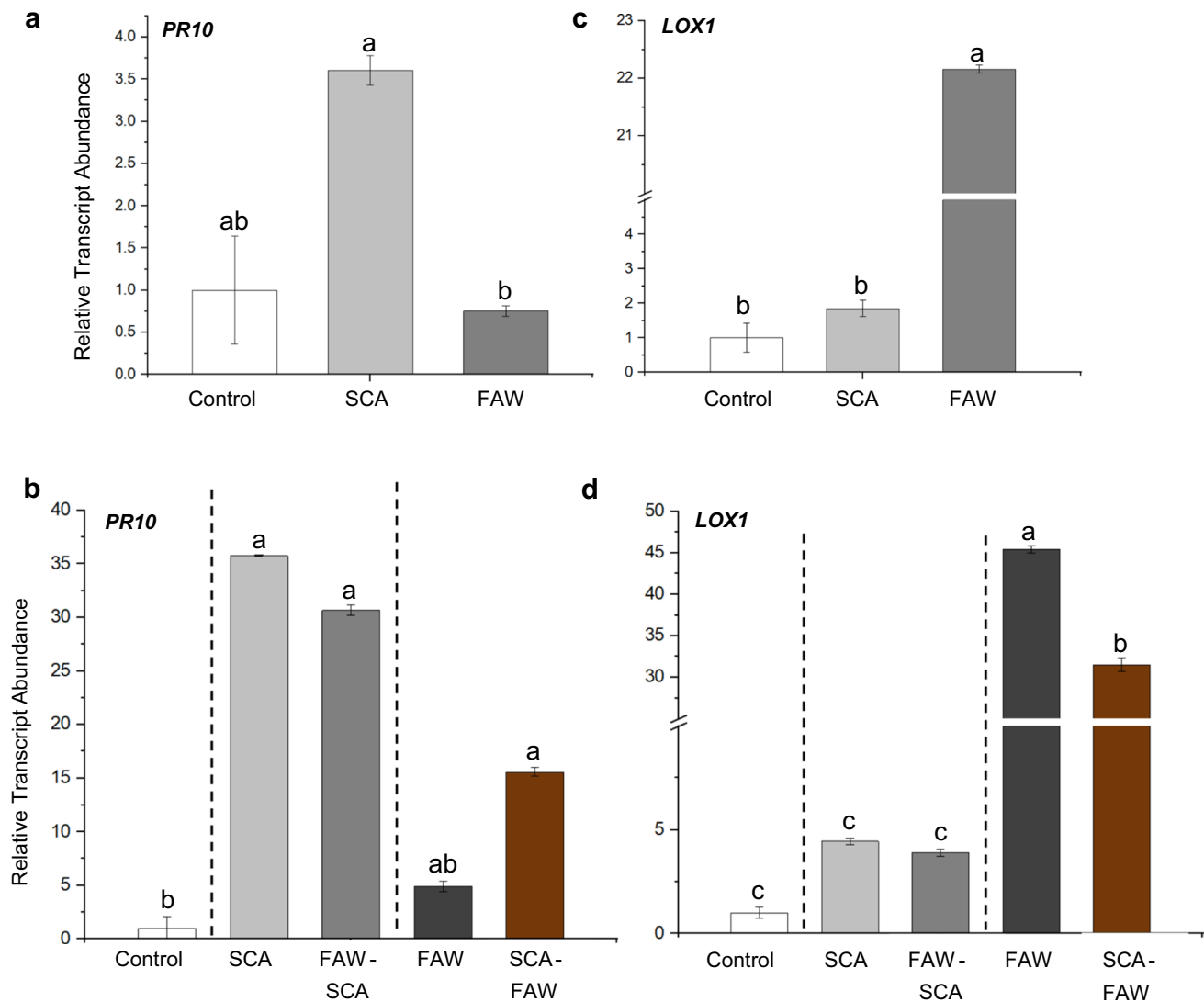


Fig. 3 Herbivory-induced salicylic acid and jasmonic acid pathway genes were differentially regulated in sugarcane aphid (SCA)-primed fall armyworm (FAW)-infested sorghum plants and vice versa. Expression of **a** *PR10* and **c** *LOX1* in sorghum RTx430 plants after two days of individual feeding by SCA and FAW. Expression of **b** *PR10* and **d** *LOX1* after four days of only SCA, only FAW, SCA-

FAW and FAW-SCA feeding. Uninfested plants were used as controls. Primed plants were treated with the first insect for two days followed by the second insect for four days. $n=3-4$ for all panels. Error bars represent $\pm$ SE. Different letters indicate significant difference relative to each other ($P < 0.05$)

SCA-fed plants, with (Fig. 5, $> 63 \mu\text{g}$ and $> 50 \mu\text{g}/\text{mg}$ of dry leaf tissue respectively). In contrast, lignin content was significantly induced in the SCA-FAW-fed plants compared to the FAW-fed RTx430 plants at 96 hpi (Fig. 5, $> 50 \mu\text{g}$ and $> 60 \mu\text{g}/\text{mg}$ of dry leaf tissue, respectively). Together, our data suggest that SCA feeding induced the lignin contents; whereas, feeding by FAW suppressed lignin levels after infestation for 48 h in RTx430 plants.

Pre-infestation with FAW followed by SCA herbivory increased total flavonoid content

To study the role of the flavonoid pathway genes in defense priming, we monitored the relative transcript abundance of the two key genes: *FNR*, flavanone 4-reductase; and *FNS*, flavone synthase II in non-primed (Fig. 6a, c) and SCA/FAW-primed RTx430 plants at 96 hpi (Fig. 6b, d). Feeding

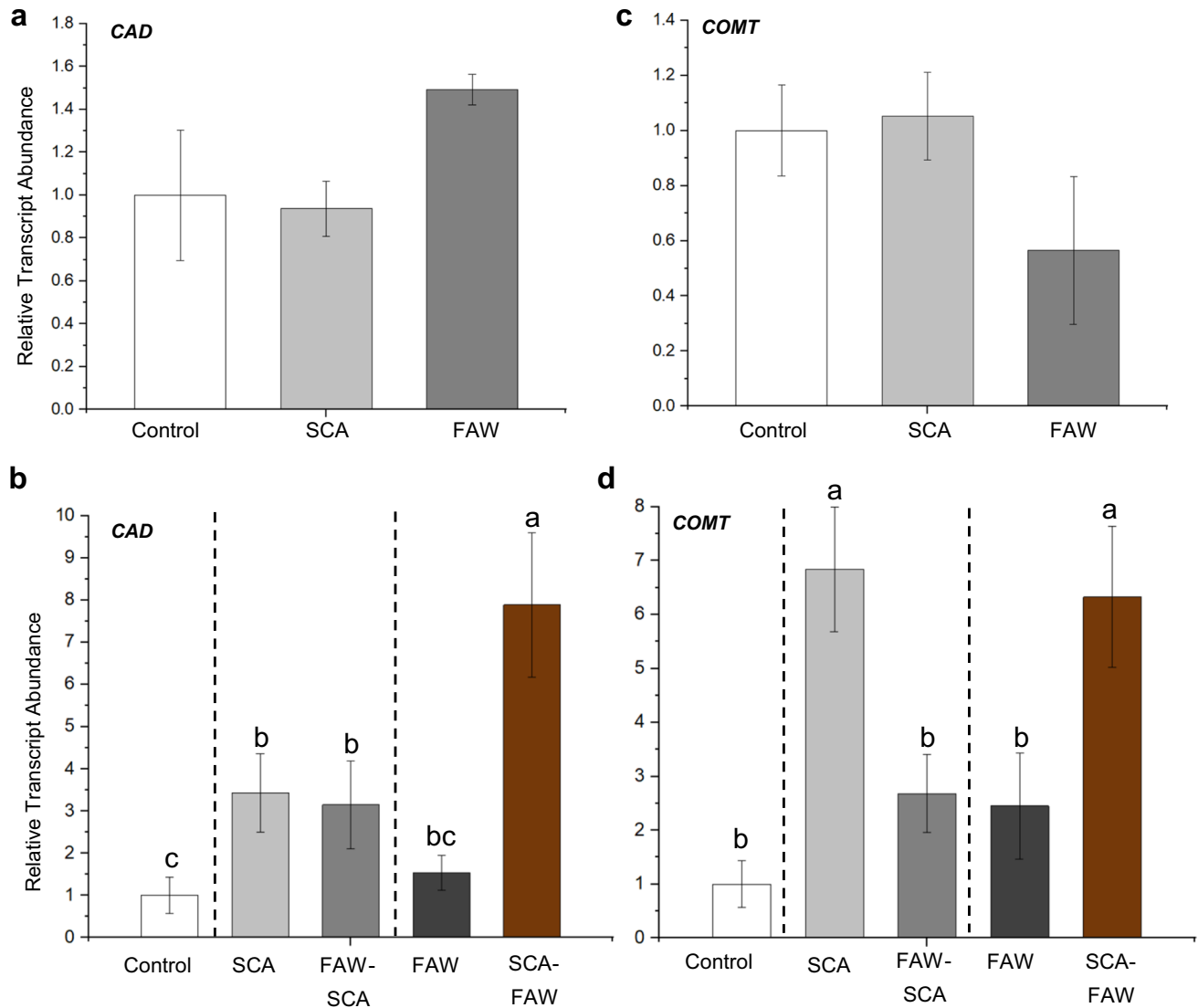


Fig. 4 Herbivory-induced monolignol pathway associated genes were differentially regulated in sugarcane aphid (SCA)–primed fall armyworm (FAW)–infested sorghum plants and vice-versa. Expression of **a** *CAD* and **c** *COMT* in sorghum RTx430 plants after two days of individual feeding by SCA and FAW. Expression of **b** *CAD* and **d** *COMT* after four days of only SCA, only FAW, SCA–FAW and FAW–SCA

feeding. Uninfested plants were used as controls. Primed plants were treated with the first insect for two days followed by the second insect for four days. $n=3-4$ for all panels. Error bars represent $\pm$ SE. Different letters indicate significant difference relative to each other ($P < 0.05$)

by SCA alone or FAW alone for 48 h did not alter the expression level of *FNR* and *FNS* transcripts compared to control plants (Fig. 6a, c). We found a significant > 58 -fold induction for the *FNR* transcript in the SCA-fed RTx430 plants (Fig. 6b). Additionally, *FNR* was significantly induced to > 70 -fold when primed with FAW (Fig. 6b). Although not significantly different, a similar trend in the expression of *FNS* was observed in FAW–SCA-fed RTx430 sorghum plants compared to SCA-fed plants

(Fig. 6d). Moreover, accumulation of total flavonoids was significantly high in FAW–primed SCA-fed plants compared to the non-primed SCA-fed plants (Fig. 7). No significant differences were observed in the accumulation of total flavonoids in the SCA-FAW- and FAW-fed plants, which aligns with our gene expression results (Figs. 6d, 7). Taken together, our data suggest that FAW feeding for 48 h induced the expression of flavonoid pathway genes and total flavonoids in RTx430 plants.

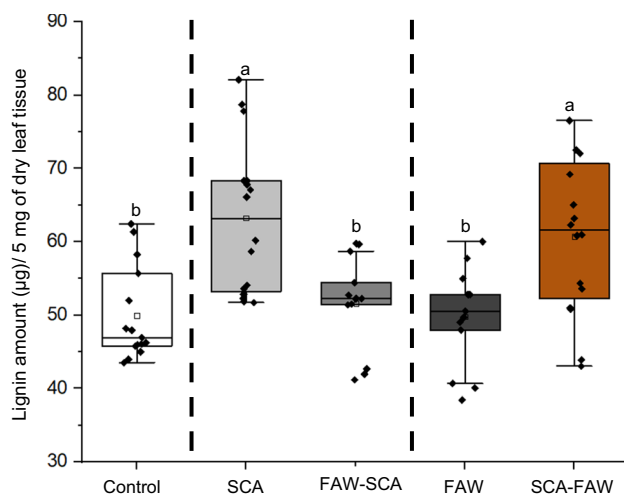


Fig. 5 Herbivory-mediated lignin accumulation is significantly suppressed in fall armyworm (FAW)-primed sugarcane aphid (SCA)-infested sorghum plants. Quantification of lignin content in sorghum foliage after four days of feeding by only SCA, only FAW, SCA-FAW and FAW-SCA. Uninfested plants were used as controls. Primed plants were treated with the first insect for two days followed by the second insect for four days. $n=6$. Error bars represent $\pm$ SE. Different letters indicate significant difference relative to each other ($P < 0.05$)

Discussion

In the present study, we investigated the effects of sequential attack of the sap-feeding pest, SCA and the leaf-chewing pest, FAW and vice versa in sorghum. Here, we show that defense priming is induced in RTx430 plants by the attack of either SCA or FAW, irrespective of the insect feeding guilds (Fig. 2). A similar trend was observed in two different varieties of milkweed, when it was pre-infested with monarch caterpillar, *Danaus plexippus*, followed by infestation with oleander aphids, *Aphis nerii* (Ali and Agrawal 2014). To further understand the underlying mechanisms in plant defense induction, we studied diverse parameters responsible for defense including, expression of defense responsive biosynthesis and signaling genes, and the metabolite quantification such as flavonoids and lignin.

SA plays a crucial role in providing defense against a wide range of piercing-sucking insects and pathogens (Grover et al. 2022a; Kundu and Sahu 2021). To delineate the role of the SA pathway, we analyzed the expression of the PR-protein encoding gene, *PR10*. Our results demonstrated a comparable level of induction of *PR10* in SCA and FAW-SCA-fed plants. Previously, we have shown that SCA induced the expression of *PR10* as early as 48 hpi (Fig. 3a,

Puri et al. 2023). JA together with other phytohormones (e.g., abscisic acid, ethylene, gibberellic acid) acts as a prime defense molecule against a diverse range of leaf-chewing insects (Kundu and Vadassery 2021). Here, the JA biosynthesis marker gene, *LOX1*, showed significantly reduced expression in SCA-FAW-fed plants compared to FAW-fed plants (Fig. 3d), indicating a plausible role of SCA feeding suppression of the JA pathway. Alternatively, plants may be limiting the availability of JA to SCA, as it was previously shown that JA promotes aphid susceptibility in sorghum (Grover et al. 2022b). Similarly, it has been shown that infestation by beet armyworm (*S. exigua*) caterpillar alone induced the JA pathway, however, beet armyworm infestation together with potato aphids suppressed the entire JA machinery, including the *LOX* gene, in tomato (Rodriguez-Saona et al. 2010).

Defense-induced lignification has been reported to provide immunity against a wide range of insects and pests of diverse feeding guilds (Yadav and Chattopadhyay 2023; An et al. 2019; Joo et al. 2021; Yan et al. 2023). Induced expression of the *CAD* and *COMT* genes from the monolignol biosynthetic pathway in SCA-FAW plants suggests SCA feeding-induced lignification. Simultaneously, we also observed FAW-mediated suppression of these genes in SCA-fed plants (Fig. 4b, d). Our results were further supported by lignin quantification in all the five treatments. SCA feeding induced the accumulation of lignin in SCA-FAW-fed plants. Additionally, we observed significant suppression in the accumulation of lignin in FAW-primed SCA-fed plants (Fig. 5). These results are in alignment with our previous work, which suggests that FAW attack induces the suppression of the accumulation of phenolic intermediates of the monolignol biosynthetic pathway in the susceptible sorghum plants (Grover et al. 2022c). Several lignin mutants of sorghum, for example *bmr6* and *bmr12*, have been reported to be susceptible to a wide range of insects under field conditions (Dowd et al. 2016). Overexpression of *MIM396* by sequestering microRNA396 in alfalfa resulted in enhanced resistance to common cutworm (*S. litura*) by increasing the lignin content along with flavonoids and glucosinolates (Yan et al. 2023). Additionally, overexpression of another R2R3-MYB transcription factor, *CmMYB15*, in chrysanthemum induced defense against chrysanthemum aphid (*Macrosiphoniella sanborni*) by targeting the lignin biosynthesis pathway (An et al. 2019).

Flavonoids are secondary metabolites that have been associated with plant defenses against insects (Grover et al. 2022c; Chatterjee et al. 2022; Puri et al. 2023; Sahu et al. 2021). Our results further provide evidence in this direction

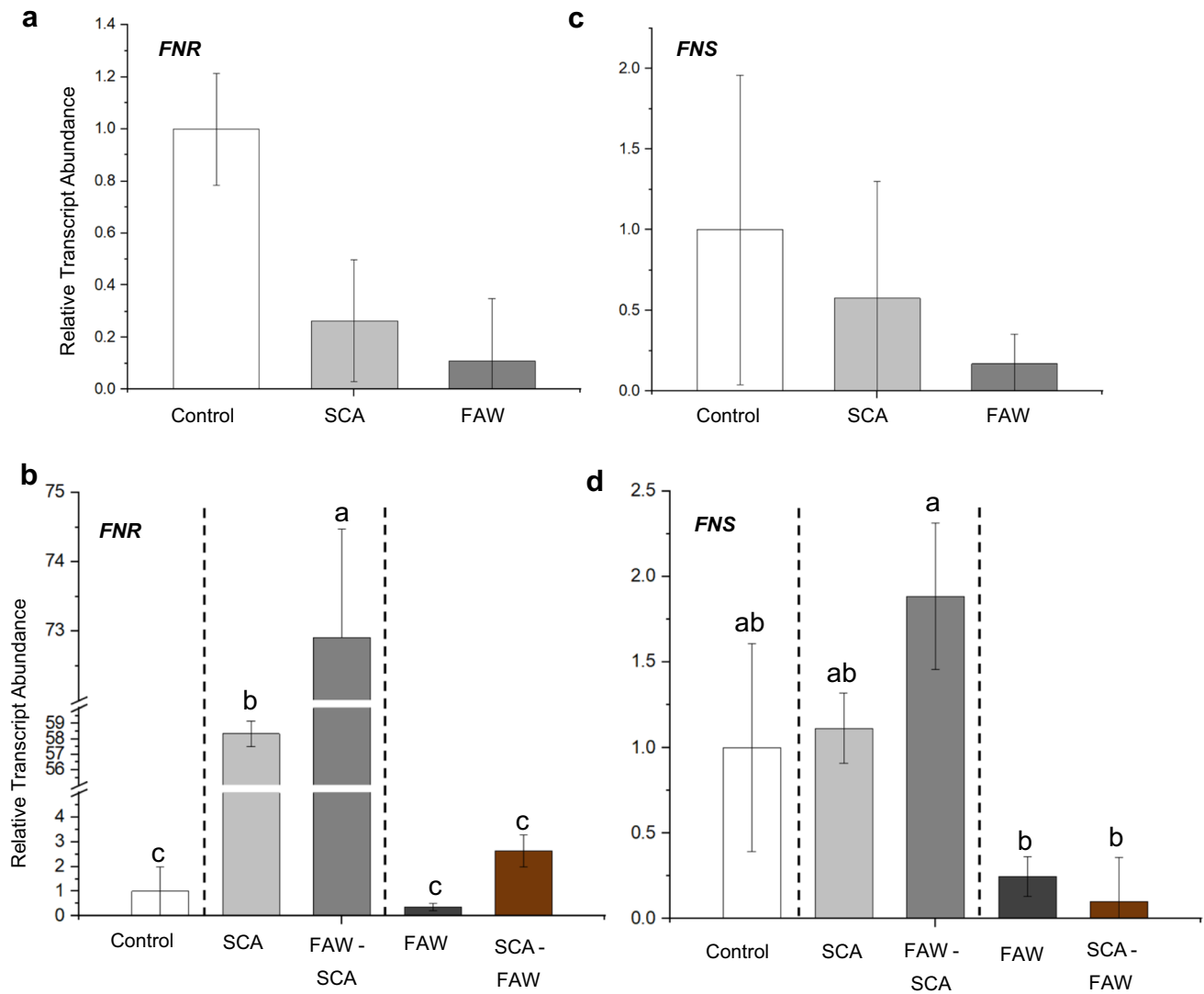


Fig. 6 Herbivory-induced flavonoid pathway associated genes were significantly induced in sugarcane aphid (SCA)-primed fall armyworm (FAW)-infested sorghum plants and vice-versa. Expression of **a** *FNR* and **c** *FNS* in sorghum RTx430 plants after two days of individual feeding by SCA and FAW. Expression of **b** *FNR* and **d** *FNS* after four days of only SCA, only FAW, SCA-FAW and FAW-SCA

feeding. Uninfested plants were used as controls. Primed plants were treated with the first insect for two days followed by the second insect for four days. $n=3-4$ for all panels. Error bars represent $\pm$ SE. Different letters indicate significant difference relative to each other ($P < 0.05$)

with FAW feeding induced expression of *FNR* transcript in SCA-fed plants (Fig. 6b). This further supported our previous work, which proposed that the FAW-mediated diversion of the metabolite synthesis towards the flavonoid pathway in the FAW-resistant sorghum line, SC1345 (Grover et al. 2022c). Similarly, another study suggests that sorghum and maize flavonoids, specifically 3-deoxyanthocyanidins (3-DAs) are detrimental for the growth and the survival of FAW, as it disrupts the peritrophic matrix of the insect

midgut, resulting in early mortality (Chatterjee et al. 2022). In alignment with our transcript data, FAW feeding induced higher accumulation of flavonoids in SCA-fed plants. Also, we did not observe any significant difference in the accumulation of flavonoids in SCA-FAW-fed plants (Fig. 7).

Taken together, we suggest, as summarized in Fig. 8, FAW feeding-induced accumulation of flavonoids negatively impact subsequent colonization/growth of SCA compared to its non-primed condition. However, priming

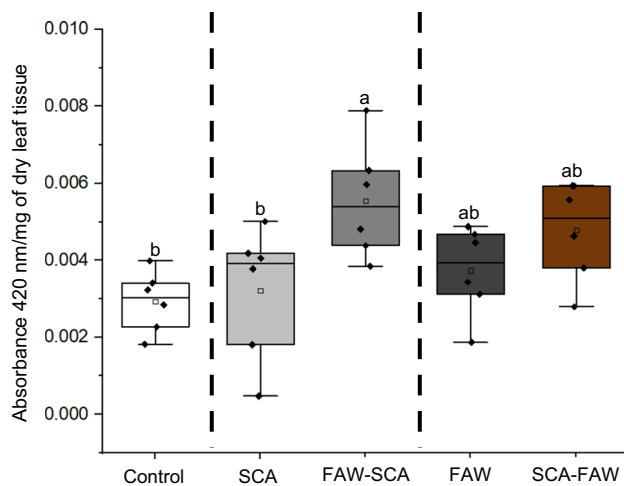
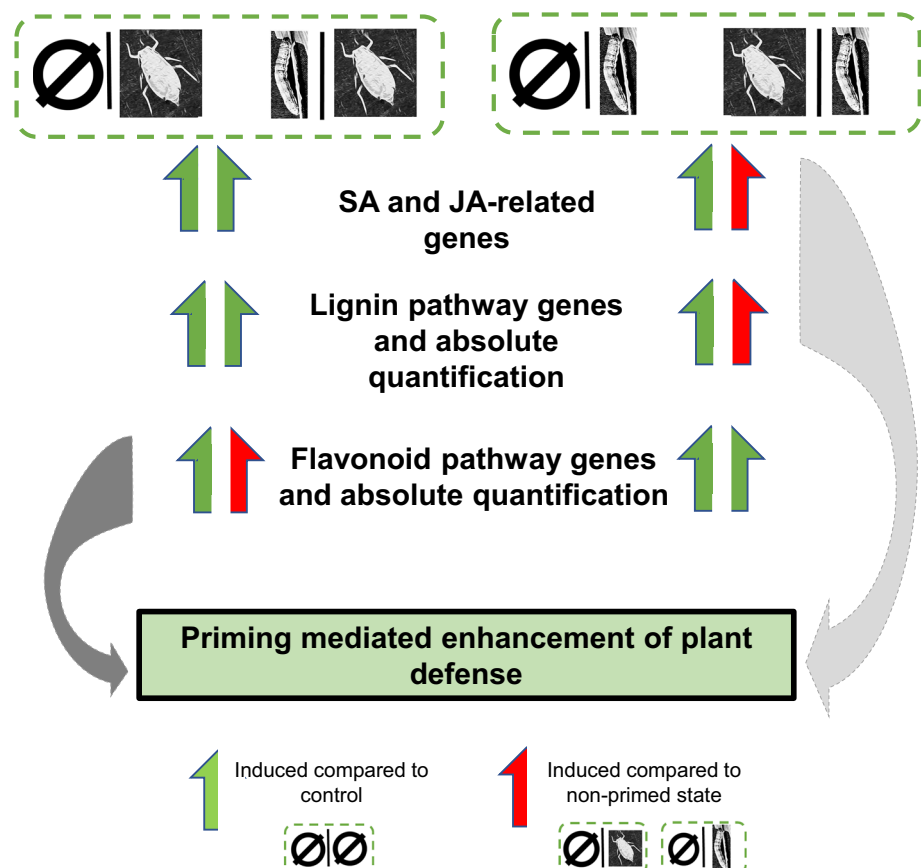


Fig. 7 Herbivory-mediated sorghum flavonoids is significantly induced in fall armyworm (FAW)-primed sugarcane aphid (SCA)-infested sorghum plants. Total flavonoids estimated using spectrophotometer in sorghum foliage after four days of only SCA, only FAW, SCA-FAW and FAW-SCA feeding. Uninfested plants were used as controls. Primed plants were treated with the first insect for two days followed by the second insect for four days. $n=6$. Error bars represent $\pm$ SE. Different letters indicate significant difference relative to each other ($P < 0.05$)

by SCA resulted in the induction of SA and accumulation of lignin, which contributed to reduced FAW larval weight gain in SCA-FAW plants. Thus, pre-infestation of sorghum with SCA resulted in reduced damage by FAW and vice versa. Moreover, irrespective of the order of herbivore arrival on sorghum RTx430 plants, significantly induced defense phenotype was observed in the primed condition compared to the non-primed condition, independent of their feeding guild. Our results also suggest that FAW priming in sorghum plants results in induced defense by the accumulation of the total flavonoids and JA in SCA-infested plants. However, lignin/SA was induced in SCA primed-FAW interaction upon sequential attack. Future studies are essential to elucidate the role of different insect elicitor molecules triggering/suppressing the diverse underlying molecular mechanisms in the host system.

Fig. 8 Model depicting the role of herbivory-induced lignin and flavonoids in sorghum interactions with sugarcane aphid (SCA) and fall armyworm (FAW). SCA feeding on the FAW-primed plants significantly induced the expression of the flavonoid pathway genes followed by its accumulation compared to the non-primed plants, thus indicating the significance of the flavonoid pathway in providing defense against SCA in FAW-primed plants. On the other hand, FAW feeding on the SCA-primed plants displays higher expression of jasmonic acid (JA) and monolignol pathway associated genes. Thus, priming resulted in induced defenses in sorghum plants irrespective of their feeding guild, however, through differential mechanisms depending on their mode of feeding behaviors. Arrows indicate activation in all instances, while their shades denote the intensity of the response



Author contribution statement JL, SG and PK conceived and designed the study. PK, SG, AP and JDRV conducted the experiments. JL and RK contributed reagents and provided guidance on experiments. PK analyzed the data and prepared the figures together with JL and SG. PK, SG and JL prepared the original draft. All authors read, reviewed, and approved the manuscript.

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Data availability All data supporting the findings of this study are available within the paper and its supplementary data published online.

Declarations

Conflict of interest The authors declare no competing interests.

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