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# Reprogramming of sorghum proteome in response to sugarcane aphid infestation

Sajjan Grover [a,1], Juan Betancurt Cardona [a], Prince Zogli [a], Sophie Alvarez [b,2], Michael J. Naldrett [b,3], Scott E. Sattler [c,4], Joe Louis [a,d,*,5]

[a] Department of Entomology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA
[b] Proteomics and Metabolomics Facility, Nebraska Center for Biotechnology, University of Nebraska-Lincoln, Lincoln, NE 68588, USA
[c] Wheat, Sorghum, and Forage Research Unit, US Department of Agriculture-Agricultural Research Service, Lincoln, NE 68583, USA
[d] Department of Biochemistry, University of Nebraska-Lincoln, Lincoln NE 68583, USA

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ABSTRACT

Sugarcane aphid (SCA; *Melanaphis sacchari* Zehntner) is a key piercing-sucking pest of sorghum (*Sorghum bicolor*) that cause significant yield losses. While feeding on host plants, complex signaling networks are invoked from recognition of insect attack to induction of plant defenses. Consequently, these signaling networks lead to the production of insecticidal compounds or limited access of nutrients to insects. Previously, several studies were published on the transcriptomics analysis of sorghum in response to SCA infestation, but no information is available on the physiological changes of sorghum at the proteome level. We used the SCA resistant sorghum genotype SC265 for the global proteomics analysis after 1 and 7 days of SCA infestation using the TMT-plex technique. Peptides matching a total of 4211 proteins were identified and 158 proteins were differentially expressed at day 1 and 7. Overall, proteome profiling of SC265 after SCA infestation at days 1 and 7 revealed the suppression of plant defense-related proteins and upregulation of plant defense and signaling-related proteins, respectively. The plant defense responses based on proteome data were validated using electrical penetration graph (EPG) technique to observe changes in aphid feeding. Feeding behavior analyses revealed that SCA spent significantly longer time in phloem phase on SCA infested plants for day 1 and lesser time in day 7 SCA infested sorghum plants, compared to their respective control plants. Overall, our study provides insights into underlying mechanisms that contribute to sorghum resistance to SCA.

## 1. Introduction

Despite being an environmentally hardy crop, sorghum (*Sorghum bicolor*) has been found very vulnerable to sugarcane aphids (SCA; *Melanaphis sacchari*) (Armstrong et al., 2015; Bowling et al., 2016). Since 2013 SCA has acquired the status of "a key pest" by causing significant sorghum economic yield losses and expanded its range to include most of the sorghum growing areas of the United States (Bowling et al., 2016; Brewer et al., 2017; Medina et al., 2017). As such, sorghum production in the United States is currently facing a serious threat from SCA, which can cause crop yield losses between $62 and $432 per hectare as reported in 2015 (Bowling et al., 2016). SCA is a piercing-sucking type insect that penetrates its stylets into plant tissues and ingests the phloem sap (Singh et al., 2004). While feeding on plants, aphids secrete saliva into the plants and some aphids are also known to have toxins in their saliva that kills the plant tissues (Ma et al., 1990; Stone et al., 2000). Although the composition of SCA saliva is unknown, rapid withdrawal of plant photosynthetic assimilates during SCA feeding may result in death of sorghum plants (Armstrong et al., 2015). During sorghum anthesis, SCA infestation may result in complete loss of seed formation

* Corresponding author at: Department of Entomology and Department of Biochemistry University of Nebraska-Lincoln, Lincoln, NE 68583, USA.
*E-mail address:* joelouis@unl.edu (J. Louis).
[1] 0000-0003-4391-0584
[2] 0000-0001-8550-2832
[3] 0000-0002-6899-5652
[4] 0000-0002-6814-4073
[5] 0000-0001-7137-8797

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or poor seed quality (Bowling et al., 2016; Brewer et al., 2017). Besides direct feeding damage, SCA deposits honeydew, a digestive waste product of aphids, over the leaf surface that promotes the growth of sooty mold and reduces the photosynthetic efficiency of plants (Singh et al., 2004). SCA honeydew stickiness on sorghum leaves also makes mechanical harvesting difficult.

Chemical insecticides have maintained the "status quo" of available SCA management strategies so far but rapid development of biotypes in other aphids on sorghum, fast growth of SCA and their rapid dispersal rate in the United States as seen in previous years call for an alternative pest management strategy (Haar et al., 2019; Calvin et al., 2021). The inherent ability of plants to activate defenses in response to insect attack provides us with a tool to manage insect-pest populations in an eco-friendly manner. Deployment of resistant sorghum genotypes against SCA has always been considered as a powerful strategy for sustainable management (Limaje et al., 2018; Paudyal et al., 2019). Although, the dominant source of SCA resistance, *RMES1* locus on the short arm of chromosome 6, has been identified and characterized, still the underlying mechanism of SCA resistance is largely unknown (Wang et al., 2013). Among host plant resistance categories, antibiosis and antixenosis contributes to the reduction in aphid population on plants. Antibiosis affects the insect biology adversely using chemical characteristics of the plant, whereas antixenosis manipulates the insect behavior and does not let insects feed well on plants using the physical and/or chemical characteristics of the plant (Smith and Resistance, 2005). Plants can synthesize a variety of secondary metabolites, which affect insect growth negatively or can act as insect-deterrent compounds (Wink, 2018).

Plants have evolved to utilize *R* genes to recognize herbivore-associated molecular patterns (HAMPs) (Uemura and Arimura, 2019), thereby turning on the plant defenses against aphids (Mithöfer and Boland, 2008). Plant cell wall is the first contact between plants and insects, consequently, insect feeding alters the cell wall metabolism/configuration (Kirsch et al., 2020; Reymond, 2021). Insect feeding can activate cell wall receptors responsible for activating plant immunity (Abdul Malik et al., 2020; Gust and Nürnberger, 2020). Also, the insect attack can trigger the oxidative burst, and the growing body of evidence suggests that oxidative burst can modulate the plant immunity (Xu et al., 2021). Upon recognition of aphid attack, there are dynamic changes occurring in plants from activation of signaling pathways, which further contributes to the downstream defenses (Erb et al., 2012). The plant hormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and cytokinins (CKs) contribute to the signaling associated defense activation against aphids (Schäfer et al., 2015; Florencio-Ortiz et al., 2020; Johnson et al., 2020; Yates-Stewart et al., 2020). Several studies have reported SA accumulation in response to aphid attack and enhanced the levels of pathogenesis-related genes (Florencio-Ortiz et al., 2020; Johnson et al., 2020; Hogenhout and Bos, 2011; Palmer et al., 2017; Koch et al., 2020; Wang et al., 2020). JA is known to provide defense against chewing herbivores (Schmiesing et al., 2016; Lu et al., 2018; Nouri-Ganbalani et al., 2018; Qi et al., 2018; Ma et al., 2020; Hunter et al., 2020), but its role in providing defenses to piercing-sucking type insects has not been much documented. SCA feeding triggered the expression of SA receptor transcripts in sorghum resistant hybrid (Kiani and Szczepaniec, 2018). ABA has been shown to promote aphid colonization and attenuate plant defenses, which benefits aphids (Hillwig et al., 2016; Chapman et al., 2018). CKs are known to be a major regulator of plant growth, which trades off with plant defense (Giron et al., 2013).

Upon insect attack, the activation of signal transduction pathways further brings concomitant changes in the secondary metabolism of plants (Zogli et al., 2020a; Iqrar et al., 2021; Jogawat et al., 2021). Benzoxazinoids, phenolic compounds, flavonoids, protease inhibitors have been widely studied secondary metabolites involved in the complex plant defense responses to insects. The capacity of these secondary metabolites to affect insect physiology directly draws attention towards understanding the defense mechanisms in different plant-insect systems. For example- DIMBOA-Glc (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside) has been reported as a key defense metabolite in maize (Israni et al., 2020; Zhang et al., 2021). Flavonoids have been shown to affect insect growth in maize and sorghum (Byrne et al., 1997; Sharma et al., 2012; Dowd et al., 2018; Kariyat et al., 2019). More often, secondary metabolites have been suggested as a biomarker to identify the resistant genotypes in breeding programs (McPherson et al., 2014; Lanubile et al., 2017; Maia et al., 2020; Yele et al., 2021). Phenolic metabolites can provide direct toxicity to insects and reduce their survival (Florencio-Ortiz et al., 2020; Delvas et al., 2011; Gesteiro et al., 2021). Defense proteins are mainly proteinases that target the insect gut, which can cause insect mortality. In maize inbred line Mp708, a 33-kD Cys protease (Maize insect resistance1-Cys Protease [Mir1-CP]) accumulates rapidly at the site of insect infestation and disrupts the peritrophic matrix of caterpillars (Fescemyer et al., 2013). Mir1-CP has also been found to affect the corn leaf aphid fecundity, though the underlying mechanism is unknown (Louis et al., 2015).

Nested association mapping (NAM) population has been recently developed in sorghum to dissect the complex traits (Bouchet et al., 2017). This panel provides great opportunities to exploit natural variation for resistance against sorghum insect pests. Previously, we identified SC265 and Segaolane as resistant and susceptible genotypes, respectively to greenbugs, *Schizaphis graminum* (Grover et al., 2019). Recently, we found that SC265 also provides enhanced resistance to SCA and significantly diminished aphid feeding from the phloem sap (Grover et al., 2020; Grover et al., 2022). In our recent review (Zogli et al., 2020a), we emphasized the importance of "omic" approaches to capture the dynamic changes occurring in plants upon insect attack. Proteomics based assessment of insect-plant interactions (Coppola et al., 2013; Duceppe et al., 2012; Guan et al., 2015; Muneer et al., 2018; Truong et al., 2015; Ferry et al., 2011) have been reported. Besides using 2-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) for protein profiling, recent innovations like tandem mass tag (TMT) of proteins followed by MS/MS, has been used to study plant-insect interactions (Wu et al., 2019; Zhang et al., 2019). These techniques provide great opportunities to dissect the complex plant-insect molecular networks (Zogli et al., 2020a). To investigate the plant defense responses in sorghum SC265 against SCA, TMT-plex proteomics technique was utilized to profile the proteome of the sorghum genotype at early and late-time points. The Electrical Penetration Graph (EPG) technique is a robust tool that could be utilized to understand the effects of plant resistance on aphid feeding (Zogli et al., 2020a; Grover et al., 2019; Tjallingii, 1985; Tetreault et al., 2019; Varsani et al., 2019) and thus, the proteome dataset was further validated using the EPG technique to understand the overall defense status of sorghum to SCA. The proteome profiling of SC265 after SCA infestation at days 1 and 7 revealed the suppression of plant defense-related proteins and upregulation of plant defense and signaling-related proteins, respectively. Furthermore, EPG data analyses revealed that SCA spent a significantly longer time in phloem phase of SC265 plants infested with SCA for 1 day and lesser time in phloem phase of SC265 plants infested with SCA for 7 days, compared to their respective control plants.

## 2. Materials and methods

### 2.1. Plants and insects

Sorghum NAM founder line, SC265, was obtained from USDA-GRIN global germplasm, USA. The seeds for this line were further propagated at the University of Nebraska-Lincoln greenhouse. For experiments, the seeds were grown Cone-Tainers (Ray Leach SC10; Stuewe & Sons, Inc., Tangent, OR) which were filled with soil mixed with vermiculite and perlite (PRO-MIX BX BIOFUNGICIDE + MYCORRHIZAE, Premier Tech Horticulture Ltd., Canada) in the greenhouse. The greenhouse conditions were set to be 16-h-light/8-h-dark photoperiod, 25 °C, and 50–60%

relative humidity. For all the experiments, two-week-old plants (3-leaf stage (Vanderlip and Reeves, 1972)) were used. Plants were watered regularly and fertigated once in a week.

Sugarcane aphid colony was founded from a single wingless aphid collected from sorghum fields at the Louisiana State Agricultural Center Dean Lee Research Station, Alexandria, LA, in July 2014. A single parthenogenic female from the above colony was reared on susceptible sorghum genotype, BCK60 in the growth chamber (Thermo Scientific). The growth chamber conditions were 16:8 h light: dark cycle and temperatures were maintained at 25 °C. BCK60 plants were grown in greenhouse till panicle initiation growth stage (Vanderlip and Reeves, 1972) and replaced with degenerated plants in growth chamber.

### 2.2. SCA growth assay and infestation setup

Two-week-old sorghum plants were infested with 5 adult apterous aphids. Plants were covered with tubular clear plastic cages to avoid aphid escape. The cages were ventilated with organdy fabric on the sides and top of cage for proper aeration. The cages were removed and aphids including both nymphs and adults, were counted to see the effect of sorghum SC265 genotype on aphid reproduction on each day for next 7 days.

For proteome profiling, the first fully leaf of SC265 plants from the top were infested with 10 adult aphids and clip caged. The leaf samples were collected from the clip-caged area after 1 and 7 days of infestation after carefully removing the aphids. For controls, plants were also clip caged with no aphids. For collection of samples after 7 days, clip cages were removed from the plants and covered with clear plastic cages to avoid aphid crowding in one area of the plant. We collected three biological replicates per treatment and each biological replicate (~150 mg) consisted of leaf samples from three plants after carefully removing the aphids from the plants using fine paint brush. The samples were immediately flash frozen in the liquid nitrogen and stored at − 80 °C until further use.

### 2.3. Protein extraction, digestion and TMT10plex labeling

Proteins from sorghum leaves were extracted according to (Zogli et al., 2020b) then redissolved in 8 M Urea, 0.1 M tris HCl, pH 7.6 containing 5 mM DTT and 1x complete, EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Protein amounts were quantified using the CB-X™ protein assay (G-Biosciences, St Louis, MO) and 160 µg of each sample was reduced at 37 °C for 2 h and then alkylated with 15 mM iodoacetamide (30 min at RT in darkness), then quenched with an equimolar amount of DTT. Samples were diluted to 4 M urea and digested with 3.2 µg Lys-C (1:50 enzyme: substrate (E:S)) at 25 °C for 8 h. The urea was then diluted to 1 M and trypsin digestion carried out for 16 h at a 1:50 ratio E:S. A further aliquot of trypsin (1:100 E:S) was added and digestion carried out for a further 4 h. Digests were acidified with 20% TFA to pH 3, then desalted using 50 mg Sep-Pak® C18 reverse-phase SPE columns (Waters Corp, Milford, MA). Eluted samples were dried down and redissolved in 100 mM TEAB. An additional sample mix of all 12 samples was made to act as a pooled channel (labeled with 126) between the two TMT sets. Seven samples for each set were labeled using TMT-10-plex reagent (ThermoFisher Scientific). For each sample, 70 µg of desalted peptides was labeled with 400 µg of TMT 10-plex reagent. The samples were combined into two 7-plex (126, 127 N, 127 C, 128 N, 128 C, 129 N, 129 C) experiments, acidified to 1% formic acid and desalted using 50 mg Sep-Pak® C18 reverse-phase SPE columns (Waters Corp, Milford, MA). 200 µg of each set was sub-fractionated offline into 96 fractions using high pH reverse phase C18 chromatography (ACQUITY UPLC® BEH™ C18, 1.7 µm, 2.1 ×100 mm, Waters Corp) at pH 10.0 and then recombined to give a total of 12 fractions (Yang et al., 2012).

### 2.4. LC-MS/MS analysis

Each fraction was analyzed by LC-MS/MS on an RSLCnano system (ThermoFisher Scientific) coupled to a Q-Exactive HF mass spectrometer (ThermoFisher Scientific). The samples were first injected onto a trap column (Acclaim PepMap™ 100, 75 µm x 2 cm, ThermoFisher Scientific) for 3.0 min at a flow rate of 5 µL/min, 1.5% acetonitrile, 0.2% formic acid before switching in line with the main column. Separation was performed on a C18 nano column (Acquity UPLC® M-class, Peptide CSH™ 130 A, 1.7 µm, 75 µm x 250 mm, Waters Corp) at 260 nL/min with a linear gradient from 5% to 35% over 96 min. The LC aqueous mobile phase contained 0.1% (v/v) formic acid in water and the organic mobile phase contained 0.1% (v/v) formic acid in 80% (v/v) acetonitrile. Mass spectra for the eluted peptides were acquired on a Q Exactive HF mass spectrometer in data-dependent mode using a mass range of $m/z$ 375–1500, resolution 120,000, AGC target $3 \times 106$, maximum injection time 60 ms for the MS1 peptide measurements. Data-dependent MS2 spectra were acquired by HCD as a Top20 experiment with a normalized collision energy (NCE) set at 33%, AGC target set to $1 \times 10^5$, 45,000 resolution, intensity threshold $1 \times 10^5$ and a maximum injection time of 86 ms. Dynamic exclusion was set at 45 s and the isolation window set to 1.2 $m/z$ to reduce co-isolation.

### 2.5. Electrical penetration graph

To determine whether SC265 early and late defenses affects SCA feeding behavior, we preinfested the SC265 plants for 1 day and 7 days and further used for EPG recordings. Two-week-old plants were infested with 10 apterous adult aphids using a clip-cage as previously described (Grover et al., 2020; Varsani et al., 2019). The control plants were also clip-caged without aphids to avoid any variation. After day 1, aphids were carefully removed from plants with a fine paint brush and the plants were used for the EPG recording. For 7-day treatment plants, clip-cages were removed after two days of infestation and then caged with tubular clear plastic ventilated with organdy fabric on the top and sides. Similarly, as for day 1, aphids were carefully removed from the plants with a fine paint brush after 7 days of infestation, and plants were used for the EPG recordings.

The adult apterous aphids used for EPG recordings were starved for one hour prior to the start of the experiment. The aphid wiring and experimental procedure were followed as described in (Nalam et al., 2018). After wiring, one single aphid was placed in the third fully developed leaf in the middle of the adaxial lamina in each of the potted SC265 genotype plants for each treatment. Subsequently, a stiff copper wire was introduced into the pot close to the potted plant. Feeding behavior and EPG recording was measured by a Giga-8 EPG model (EPG Systems, Wageningen, The Netherlands) with a $10^9$ Ω resistance amplifier that was connected to each of the plant electrode and an adjustable plant voltage were used for measuring feeding behavior of SCA on SC265 genotype. Both insects and plants were contained in a Faraday cage to avoid external electrical noise. Recordings were performed in eight plants (eight channels) at the same time and during 8 h under constant light at 22–24 °C and 40–45% RH. In total, we analyzed 14–15 replicates for each treatment. EPG acquisition software (*Stylet*$^+$, EPG Systems, Wageningen, The Netherlands) was used to record waveforms of SCA feeding on sorghum plants. Seven categorized EPG parameters were considered in this analysis: pathway phase, xylem phase, sieve elements phase, non-probing phase, time to first probe, time to first sieve element phase and number of potential drops.

### 2.6. Statistical analyses

The SCA growth assay data were analyzed using mixed model analyses and replications were considered as random effects (PROC GLIMMIX, SAS 9.3, SAS Institute). One-way analysis of variance (ANOVA) was used to compare the number of aphids at different time points. For

EPG data, non-parametric Kruskal–Wallis test was used to compare the duration of different feeding parameters/phases among different treatments using PROC NPAR1WAY procedure, considering the non-normally distributed data.

For proteomics study, data were analyzed in Proteome Discoverer 2.4 software (ThermoFisher Scientific) connected to Mascot 2.6.1 database search engine (Perkins et al., 1999) (Matrix Science), which searched the common contaminants database cRAP (116 entries, www.theGPM.org) and the SbicolorRT_2.1 database (https://phytozome-next.jgi.doe.gov/info/SbicolorRTx430_v2_1). Methionine oxidation, protein N-terminal acetylation, asparagine/glutamine deamidation, cysteine carbamidomethylation were set as variable modifications, whilst TMT10plex (K) and TMT10plex (N-term) were specified as fixed modifications. A maximum of two trypsin missed cleavages were permitted and the precursor and fragment mass tolerances were set to 10 ppm and 0.06 Da, respectively. Peptides were validated by Percolator with a 0.01 posterior error probability (PEP) threshold. The data were searched using a decoy database to set the false discovery (FDR) rate to 1% (high confidence). The protein quantification was processed using the Reporter Ion Quantifier node in Proteome Discoverer with the co-isolation threshold set to 50% and the average S/N to 10. The peptides were quantified using the peak intensity of the reporter ion in the MS2 spectrum. The peak abundance was normalized for differences in sample loading using total peptide amount where the peptide group abundances are summed for each sample and the maximum sum across all runs is determined. The pooled sample was used to normalize each TMT experiment based on the same sample. The protein ratios, expressed as $\log_2$ fold change, are calculated as the median of all possible pairwise peptide ratios calculated between replicates for each sample. To compensate for missing values in some of the replicates, the low abundance resampling imputation mode was selected. The significance of differential expression is tested using a *t*-test, which provides *P*-values for all the calculated ratios. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD029691. For functional annotation, enrichment analysis was performed using AgriGO gene ontology analysis tools (Du et al., 2010) to identify the enriched GO terms.

## 3. Results

### 3.1. SCA growth rate is higher at early time points of infestation

The level of plant defenses is correlated with the insect growth and survival on the plants. Based on the aphid count of each day, we measured the aphid rate of increase per day on SC265 genotype for next 7 days. Our results showed that the highest rate of increase for SCA was found on first two days of aphid infestation and later it decreases (Fig. 1). These results suggested induced plant defenses from SC265 may interfere with the reproductive capacity of SCA.

### 3.2. Identification and enrichment analysis of differentially expressed proteins

Proteomics analysis was used to identify the proteins potentially involved in SC265 defenses against SCA at early and late time points. Proteome profiling of SC265 at day 1 and day 7 after aphid infestation identified and quantified a total of 4211 sorghum proteins. Out of these 4211 proteins, 158 proteins were identified as differentially expressed proteins (DEPs) on both days using a $\log_2$ fold change Infected/Control ($\log_2$FC (Infested/Control)) and an adjusted *P*-value $\leq 0.05$ cutoff criteria. A principal component analysis of 158 proteins was performed where PC1 accounted for 48.76% of the variance, separating the day 1 and day 7 DEPs and PC2 accounted for 21.52% of the variance, which could not separate the control and SCA infested samples on day 1, but partially separated the treatments on day 7 (Fig. 2a). A total of 41 and 59

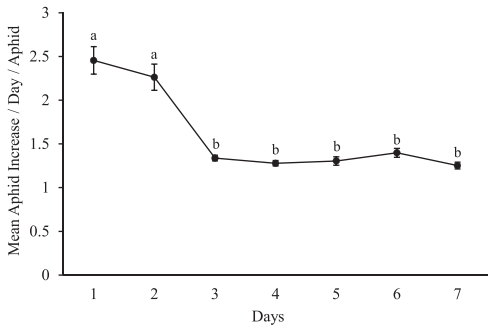


**Fig. 1.** Sugarcane aphid (SCA) rate of increase was higher on first two days after infestation. Total number of aphids (adults and nymphs pooled together) per SC265 plant counted for 7 days after infestation of two-week-old sorghum plants with 5 adult apterous aphids per plant (n = 11). All the infested plants were contained in cages. Different letters indicate significant difference between aphid rate of increase on different days ($P < 0.05$). Error bars represent ± SEM.

proteins were found upregulated and downregulated, respectively on day 1 (Fig. 2b). On day 7, a total of 59 and 13 proteins were found upregulated and downregulated, respectively. There were 13 proteins common in with the downregulated proteins on day 1 and the proteins upregulated on day 7. Enrichment analysis revealed that most of the upregulated proteins on day 1 are related to peptide biosynthetic process, amide biosynthetic process, cellular amide metabolic process, organonitrogen compound biosynthetic process, organonitrogen compound metabolic process (Table 1). Enrichment analysis of downregulated proteins on day 1 revealed that most of the proteins have functions related to stress response, carbohydrate metabolic process, hydrolase activity and catalytic activity. However, upregulated proteins on day 7 have functions related to stress response, single-organism metabolic process and lipid metabolic process. These results suggested that SCA feeding remodels the sorghum proteome differently at early and late time points.

### 3.3. SCA feeding suppressed plant defense, signal transduction, oxidative stress related and secondary metabolism proteins at early time point

The pathogenesis-related proteins SbiRTX430.01G421600, SbiRTX430.01G421000, SbiRTX430.02G024000 and SbiRTX430.06G210100 were suppressed by SCA feeding at 1 day (Fig. 3a). Also, SCA feeding suppressed few defense-related proteins, SbiRTX430.06G008000, SbiRTX430.05G196500, SbiRTX430.
05G231300, SbiRTX430.04G270100, which are a cysteine proteinase, serine carboxypeptidase-like 27, PATATIN-like protein 4, eukaryotic aspartyl protease, respectively (Fig. 3a).

Dehydrins are believed to protect cellular components from dehydration stress (Hundertmark et al., 2011). One dehydrin protein, SbiRTX430.09G120700, was also found to be downregulated. Cysteine-rich repeat secretory proteins are known to be involved in signaling in response to stress conditions (Shingaki-Wells et al., 2011; Raineri et al., 2015). We found downregulation of protein, SbiRTX430.01G439500, homolog of Cysteine-rich repeat secretory proteins after day 1. Other signaling related proteins, SbiRTX430.07G095400 (cysteine-rich receptor-like protein kinase), SbiRTX430.06G006100 (Zinc finger C3HC4-type RING family protein), SbiRTX430.05G084000 (zinc-binding dehydrogenase family protein) were also suppressed by SCA feeding (Fig. 3b). Other downregulated proteins, SbiRTX430.02G356300 and SbiRTX430.01G149200, belonging to the osmotin family that are secretory in nature and responsive to abiotic/biotic stress, therefore, is considered a PR protein (Abdin et al., 2011). SbiRTX430.06G105900, homolog of Arabidopsis AT2G14095, is involved in cell death program (Olvera-Carrillo et al., 2015). SbiRTX430.02G143200, homolog of dynamin-like protein,

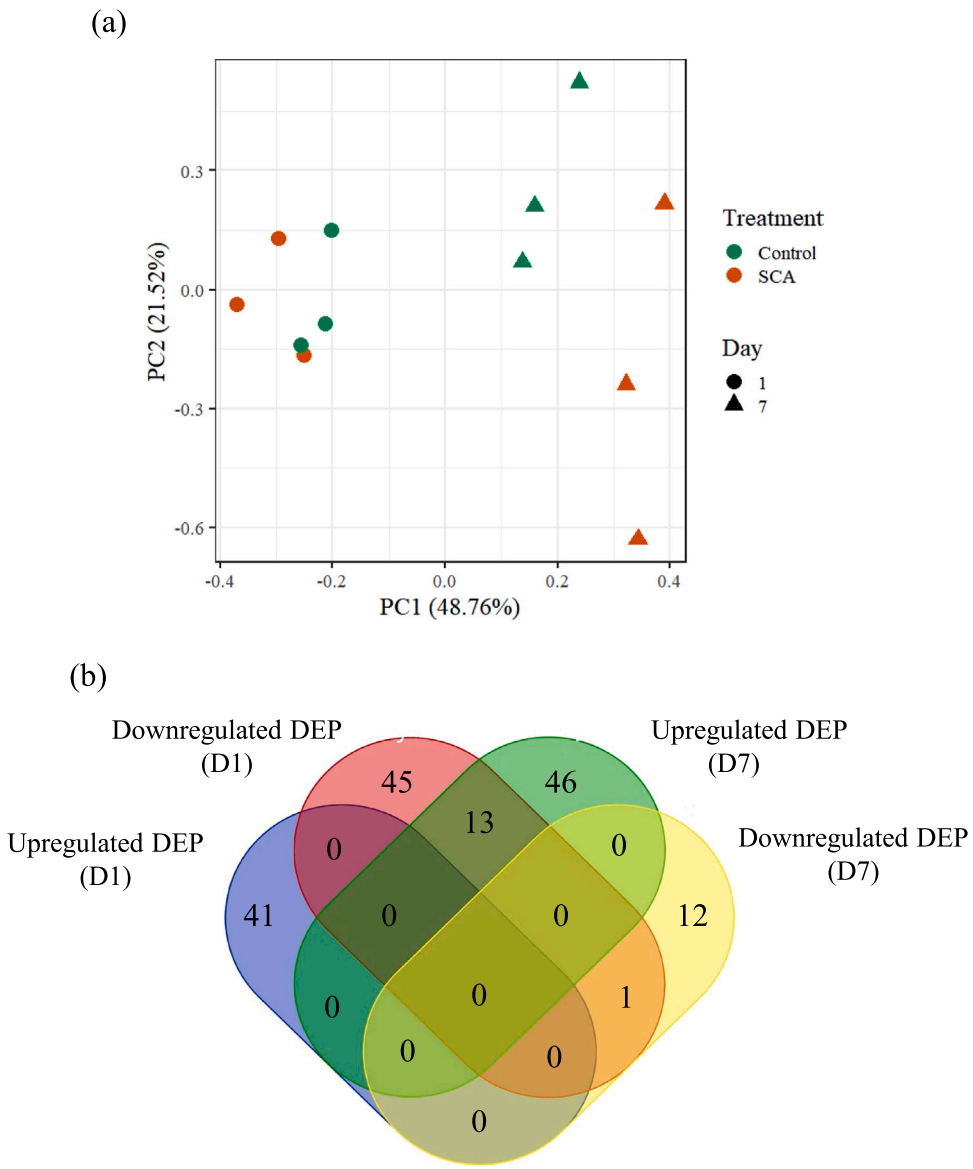


**Fig. 2.** (a) Principal component analysis (PCA) of all the 158 proteins expressed differentially on at least one day. Sugarcane aphid (SCA) uninfested plants and SCA infested samples were represented as different colors and different days as different shapes. (b) Venn diagram of upregulated and downregulated differentially expressed proteins detected in aphid infested relative to control (aphid uninfested) in SC265 plants after day 1 and day 7 of SCA infestation. Numbers within regions in venn diagram indicate common and unique proteins within each sector.

associated with programmed cell death was also suppressed by aphid infestation (Tang et al., 2006). SbiRTX430.03G443500, homolog of Sec14p-like phosphatidylinositol transfer family protein, was also downregulated. Sec14 is crucial for coordinating the lipid signaling interface with innate plant immunity responses (Huang et al., 1861). GDSL-like lipases are known to play role in plant immune responses to pathogens (Lai et al., 2017). SbiRTX430.08G083200, a GDSL-like Lipase, was found to be down regulated during aphid infestation. The early suppression of immune response-related proteins may suggest that SCA secretes suppressors of plant immune responsive proteins during the early stages of aphid feeding.

### *3.4. SCA feeding decreased the cell wall and amino acid protein abundance at one day of aphid infestation*

SCA feeding also suppressed the proteins related to amino acid metabolism, SbiRTX430.01G120500 (glutamine synthase) and SbiRTX430.01G268300 (alanine aminotransferase 2) (Fig. 3b). Tyrosine transaminase protein SbiRTX430.02G041500 was also found to be downregulated. Besides affecting amino acid metabolism, glutamate also triggers calcium based long-distance defense signals via the phloem (Toyota et al., 2018). SCA feeding also suppressed cell-wall related proteins such as SbiRTX430.10G050500, proline-rich cell wall protein-like (Fig. 4a). Chitinases, SbiRTX430.02G056100, SbiRTX430.02G056000, SbiRTX430.05G087200, were also downregulated. Beta-1,3-glucanase proteins, SbiRTX430.08G157900, SbiRTX430.03G455000, SbiRTX430.03G454500, SbiRTX430.02G332500 were also suppressed by SCA feeding. SbiRTX430.06G226500, homolog of Arabidopsis PICC, was found to be downregulated, which is crucial for callose deposition (Wang et al., 2019). Peroxidases, SbiRTX430.10G172600 and SbiRTX430.02G239000 were also downregulated. SbiRTX430.01G217500, its homolog in rice belongs to pectin lyase family, confers cold tolerance to plants (Xiao et al., 2018). SbiRTX430.08G202700 homolog of AT5G40010, is known to act as plastidial transporter of precursors for lignin synthesis (Vahabi et al., 2015). The protein, SbiRTX430.10G182700, plant L-ascorbate oxidase was also found to be downregulated after 1 day of infestation. Another protein related to oxidative stress, NADPH: quinone oxidoreductase, SbiRTX430.03G462400, was also downregulated (Fig. 4a). SCA feeding suppressed three proteins in the flavonoid pathway, SbiRTX430.03G450700 and SbiRTX430.01G331800, and SbiRTX430.03G112200, which were a leucoanthocyanidin dioxygenase, flavonol synthase/flavanone 3-hydroxylase and isoflavone reductase,

**Table 1**
Enrichment analysis of significantly enriched PFAM domains, KEGG metabolic pathways, and molecular function GO terms among differentially expressed proteins (DEPs). *P*-value and false discovery rates (FDR) are also listed in the table. Significant GO terms are reported here.

| GO terms | Description | Number of GO terms in input list | Number of GO terms in reference genome | *P*-value | FDR |
|---|---|---|---|---|---|
| **Enrichment analysis of upregulated DEPs at day 1** | | | | | |
| GO:0043043 | peptide biosynthetic process | 15 | 428 | 1.5E-17 | 4.4E-16 |
| GO:0043604 | amide biosynthetic process | 15 | 428 | 1.5E-17 | 4.4E-16 |
| GO:0043603 | cellular amide metabolic process | 15 | 442 | 2.3E-17 | 4.4E-16 |
| GO:0006518 | peptide metabolic process | 15 | 438 | 2E-17 | 4.4E-16 |
| GO:0006412 | translation | 15 | 424 | 1.3E-17 | 4.4E-16 |
| GO:1901566 | organonitrogen compound biosynthetic process | 16 | 634 | 1.2E-16 | 1.8E-15 |
| GO:1901564 | organonitrogen compound metabolic process | 16 | 825 | 6.7E-15 | 9.1E-14 |
| GO:0044271 | cellular nitrogen compound biosynthetic process | 16 | 1694 | 3.7E-10 | 4.4E-09 |
| GO:0034645 | cellular macromolecule biosynthetic process | 15 | 1721 | 5.9E-09 | 5.8E-08 |
| GO:0009059 | macromolecule biosynthetic process | 15 | 1723 | 6E-09 | 5.8E-08 |
| GO:0010467 | gene expression | 15 | 1752 | 7.6E-09 | 6.6E-08 |
| GO:0044249 | cellular biosynthetic process | 16 | 2124 | 1E-08 | 8.2E-08 |
| GO:1901576 | organic substance biosynthetic process | 16 | 2173 | 1.4E-08 | 1.1E-07 |
| GO:0034641 | cellular nitrogen compound metabolic process | 16 | 2276 | 2.8E-08 | 1.8E-07 |
| GO:0009058 | biosynthetic process | 16 | 2276 | 2.8E-08 | 1.8E-07 |
| GO:0006807 | nitrogen compound metabolic process | 16 | 2439 | 7.7E-08 | 4.6E-07 |
| GO:0044267 | cellular protein metabolic process | 15 | 2145 | 1.2E-07 | 6.7E-07 |
| GO:0019538 | protein metabolic process | 15 | 2595 | 1.5E-06 | 7.9E-06 |
| GO:0044260 | cellular macromolecule metabolic process | 15 | 3759 | 0.00016 | 0.00081 |
| GO:0009987 | cellular process | 18 | 5485 | 0.00023 | 0.0011 |
| GO:0044237 | cellular metabolic process | 16 | 4625 | 0.00046 | 0.0021 |
| GO:0043170 | macromolecule metabolic process | 15 | 4242 | 0.00067 | 0.0029 |
| GO:0071704 | organic substance metabolic process | 17 | 5560 | 0.0012 | 0.0049 |
| GO:0044238 | primary metabolic process | 16 | 5300 | 0.0024 | 0.0095 |
| GO:0003735 | structural constituent of ribosome | 14 | 297 | 4.9E-18 | 2.7E-17 |
| GO:0005198 | structural molecule activity | 14 | 316 | 1.1E-17 | 3.1E-17 |
| GO:0005840 | ribosome | 14 | 295 | 4.5E-18 | 2E-16 |
| GO:1990904 | ribonucleoprotein complex | 14 | 347 | 4E-17 | 6.1E-16 |
| GO:0030529 | intracellular ribonucleoprotein complex | 14 | 347 | 4E-17 | 6.1E-16 |
| GO:0043232 | intracellular non-membrane-bounded organelle | 14 | 538 | 1.5E-14 | 1.3E-13 |
| GO:0043228 | non-membrane-bounded organelle | 14 | 538 | 1.5E-14 | 1.3E-13 |
| GO:0044444 | cytoplasmic part | 14 | 666 | 2.6E-13 | 1.9E-12 |
| GO:0005737 | cytoplasm | 14 | 795 | 2.7E-12 | 1.8E-11 |
| GO:0032991 | macromolecular complex | 14 | 989 | 4.9E-11 | 2.8E-10 |
| GO:0005623 | cell | 16 | 1999 | 4.2E-09 | 1.9E-08 |
| GO:0044464 | cell part | 16 | 1999 | 4.2E-09 | 1.9E-08 |
| GO:0043226 | organelle | 14 | 1423 | 5.7E-09 | 2.2E-08 |
| GO:0043229 | intracellular organelle | 14 | 1421 | 5.6E-09 | 2.2E-08 |
| GO:0005622 | intracellular | 15 | 1895 | 2.2E-08 | 7.8E-08 |
| GO:0044424 | intracellular part | 14 | 1815 | 1.3E-07 | 4.1E-07 |
| **Enrichment analysis of downregulated DEPs at day 1** | | | | | |
| GO:0006950 | response to stress | 7 | 483 | 0.00014 | 0.0057 |
| GO:0005975 | carbohydrate metabolic process | 7 | 607 | 0.00055 | 0.011 |
| GO:0050896 | response to stimulus | 8 | 872 | 0.00094 | 0.013 |
| GO:0016798 | hydrolase activity, acting on glycosyl bonds | 8 | 396 | 0.000004 | 0.00033 |
| GO:0004553 | hydrolase activity, hydrolyzing O-glycosyl compounds | 7 | 363 | 0.000024 | 0.00096 |
| GO:0016787 | hydrolase activity | 13 | 2042 | 0.00062 | 0.017 |
| GO:0003824 | catalytic activity | 26 | 6982 | 0.0019 | 0.038 |
| **Enrichment analysis of upregulated DEPs at day 7** | | | | | |
| GO:0006950 | response to stress | 8 | 483 | 0.000017 | 0.00043 |
| GO:0044710 | single-organism metabolic process | 14 | 2199 | 0.00035 | 0.0044 |
| GO:0050896 | response to stimulus | 8 | 872 | 0.00094 | 0.008 |
| GO:0006629 | lipid metabolic process | 5 | 424 | 0.0034 | 0.021 |

respectively (Fig. 4b).

### 3.5. SCA feeding induced proteins are mostly related to photosynthesis and plant growth at one day of aphid infestation

We found very few proteins related to plant defense and signaling after day 1 of SCA infestation. The proteins related to photosynthesis, SbiRTX430.06G271700, SbiRTX430.07G096800 (electron transport chain), SbiRTX430.03G140800 (chlorophyll biosynthesis), SbiRTX430.04G335800 (photosynthetic efficiency), SbiRTX430.01G030000 (number of chloroplast and leaf pigment) were found to be upregulated (Fig. 5a). On the other hand, chlorophyll hydrolyzing enzymes, SbiRTX430.08G141900 and SbiRTX430.03G024700 were also found to be upregulated. SbiRTX430.02G180800, homolog of RPL36aA in Arabidopsis, is important for leaf development (Casanova-Sáez et al., 2014). SbiRTX430.07G019600, homolog of Arabidopsis RPS13A (Ito et al., 2000), is involved in vascular network development. SCA feeding upregulates the SbiRTX430.02G286200, acyl career protein 2 involved in fatty acid biosynthesis. Homolog of SbiRTX430.09G010900 in Arabidopsis is chloroplastic protein involved in thylakoid FtsH complex (Lopes et al., 2018). SbiRTX430.10G116600, homolog of Arabidopsis CURT1a is involved in the chloroplast thylakoid membrane transport, was also found to be upregulated. SCA feeding also induced the thioredoxin family protein, SbiRTX430.10G054300. SbiRTX430.04G135400 is a homolog of plasma-membrane associated cation-binding protein 1 (PCaP1) in Arabidopsis. PCaP1 binds through calmodulin in calcium dependent manner, so it could be involved in regulating intracellular signaling (Huang et al., 2017). SbiRTX430.06G174800 is a member of the pentatricopeptide repeat (PPR) protein family, which known to play role under stress conditions (Chen et al., 2018). SbiRTX430.03G452700 is a RNA binding


**Fig. 3.** Heatmap analysis of differentially expressed (a) pathogenesis related proteins, protease inhibitors and oxylipins, and (b) stress-signaling related proteins in SC265 plants after day 1 (D1) and day 7 (D7) of sugarcane aphid (SCA) infestation. Color key represents the Z-score standardized values. Asterisks in the cells represent significant differences compared to the respective control based on the adjusted *P*-value < 0.05.

protein, and also a positive regulator of SA immunity (Qi et al., 2010). The few proteins related to defense, SbiRTX430.05G196100 (oxylipin biosynthesis) and SbiRTX430.09G06620, homolog of Arabidopsis breast basic conserved 1, involved in MAPK signaling, were found upregulated.

### *3.6. SC265 exhibits upregulation of pathogenesis-related proteins, oxylipins, and protease inhibitors after 7 days of aphid feeding*

Consistent with the phytohormonal data (Grover et al., 2022), proteomics profiling also exhibited enhanced accumulation of SA-marker proteins such as pathogenesis-related proteins after SCA feeding

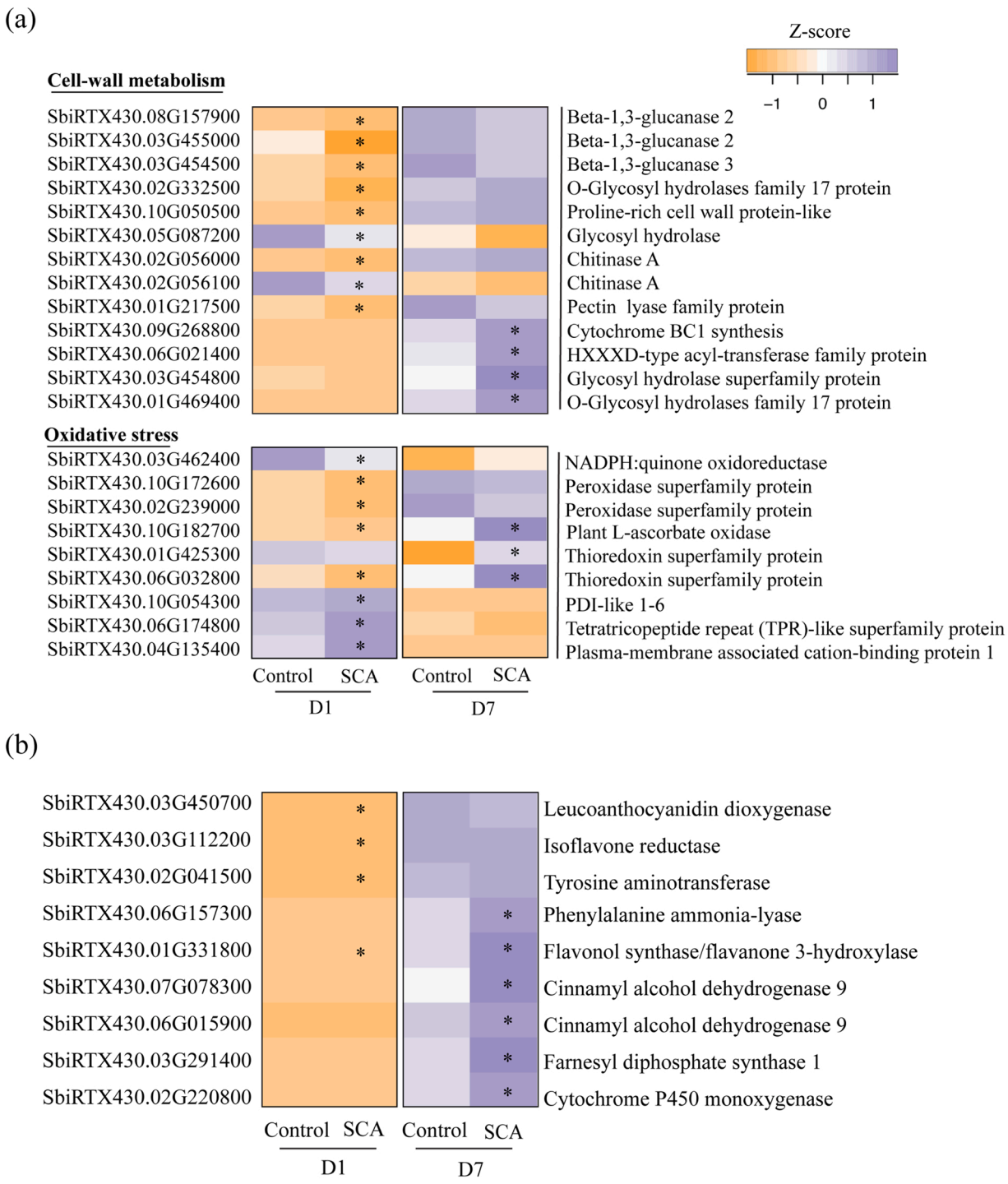


**Fig. 4.** Heatmap of differentially expressed proteins related to (a) cell wall metabolism and oxidative stress and (b) secondary metabolism related proteins in SC265 plants after day 1 (D1) and day 7 (D7) of sugarcane aphid (SCA) infestation. Color key represents the Z-score standardized values. Asterisks in the cells represent significant differences compared to the respective control based on the adjusted *P*-value $< 0.05$.

(Fig. 3a). SA is known to promote the resistance to aphids and pathogens, which also elevates the expression level of PR genes (Florencio-Ortiz et al., 2020; Johnson et al., 2020; Shah, 2003). Sorghum protein SbiRTX430.10G021800, a homolog of Arabidopsis pathogenesis-related protein, was found to be upregulated in SC265 after 7 days of infestation. Four other proteins, SbiRTX430.01G421000, SbiRTX430.01G421300, SbiRTX430.01G421200, and SbiRTX 430.01G421500 found to be upregulated are the homologs of pathogenesis-related proteins in rice. SA has also been found to be upregulated in SC265 after 7 days of SCA infestation (Grover et al., 2022). The upregulation of these proteins is in alignment with upregulated SA levels upon SCA infestation.

Oxylipins are defense signaling molecules in plants (Eckardt, 2008). The biosynthesis of oxylipins involves the formation of fatty acid hydroperoxides by lipoxygenases (Mosblech et al., 2009). SCA feeding led to the upregulation of SbiRTX430.01G129400 and SbiRTX430.03G416000, which are homologs of *Atlox1* (lipoxygenase 1) (Fig. 3a). Upon insect attack, plants activate phospholipases, which release fatty acids from plasma membranes including linolenic acid, the precursor of JA and other oxylipins (Shah, 2014; Ruan et al., 2019). SCA

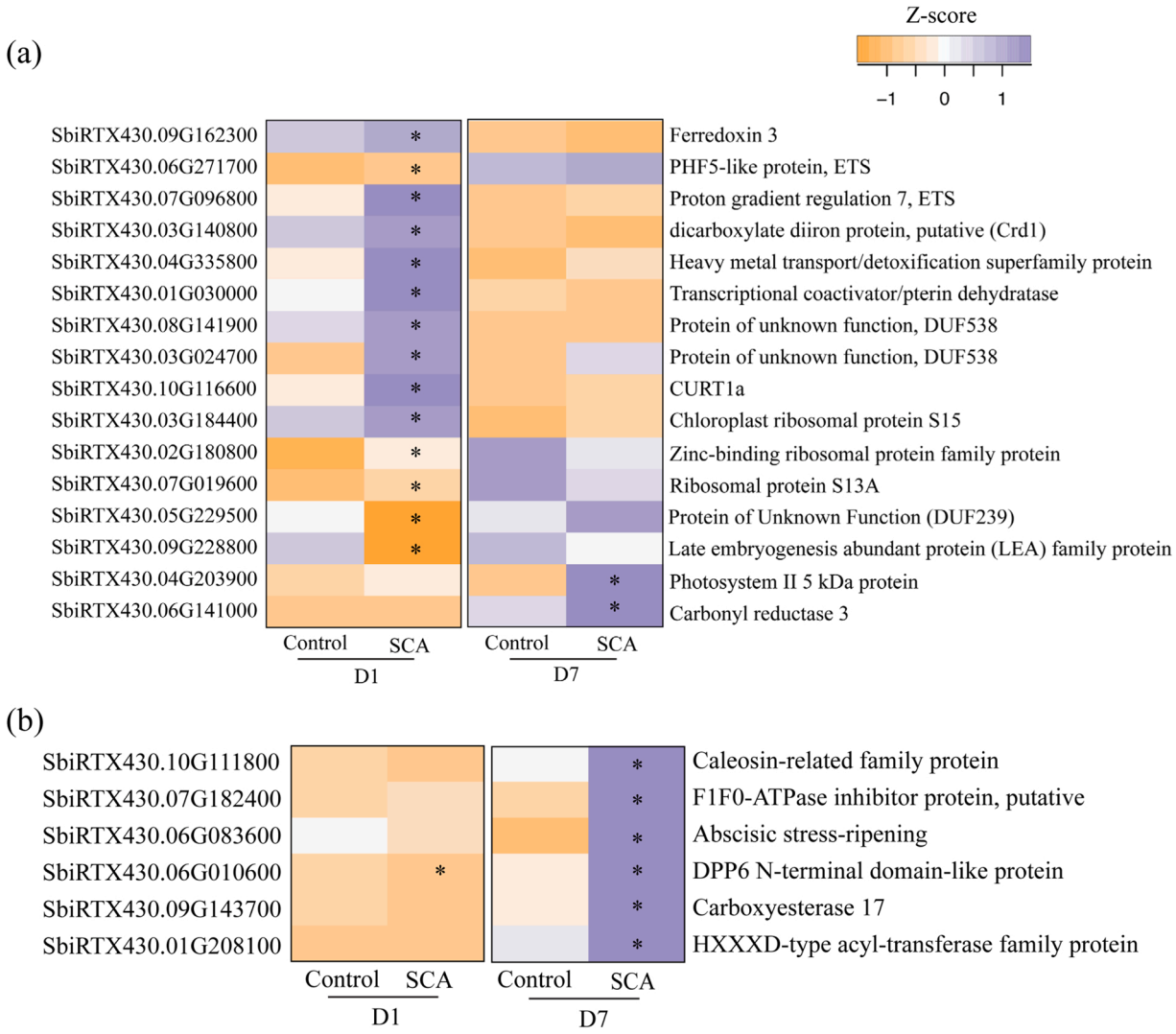


**Fig. 5.** Heatmap of differentially expressed proteins (a) photosynthesis and plant growth related, and (b) abscisic acid related proteins in SC265 plants after day 1 (D1) and day 7 (D7) of sugarcane aphid (SCA) infestation. Color key represents the Z-score standardized values. Asterisks in the cells represent significant differences compared to the respective control based on the adjusted *P*-value $< 0.05$.

feeding triggered the expression of two more phospholipases, SbiRTX430.09G015400 (Chen et al., 2011) and SbiRTX430.03G465700 that are known to be involved JA synthesis (Shah, 2014; Ellinger et al., 2010). SbiRTX430.10G197000, *SPX1* gene, may also be involved in phospholipase activity. The proteins encoded by the gene, SbiRTX430.05G196400 (homolog of Arabidopsis PLA II A) that is not known to produce JA (Yang et al., 2007) was also upregulated.

Protease inhibitors (PPIs) are the small proteins that are part of plant defense responses to insects (Solomon et al., 1999; Hartl et al., 2011). PPIs are well known to inhibit insect growth by interfering with digestive physiology of chewing type insects through preventing digestion of dietary proteins (Johnson et al., 1989; Vila et al., 2005), whereas PIs might inhibit the secreted proteases present in aphid saliva/gut during feeding (Furch et al., 2015; Losvik et al., 2018). After 7 days post infestation (dpi), SC265 showed significant upregulation of five proteases/protease inhibitors such as cystatin B (SbiRTX430.03G354400), cysteine proteinases superfamily protein (SbiRTX430.06G008000), PATATIN-like protein 4 (SbiRTX430.05G231300), serine carboxypeptidase-like 27 (SbiRTX430.05G196500), serine protease inhibitor, potato inhibitor I-type family protein (SbiRTX430.09G009700) (Fig. 3a). Therefore, the upregulation of PPIs 7 dpi may be a defense mechanism used by the resistant sorghum genotype to deal with prolonged feeding by aphids.

### 3.7. SCA feeding upregulated stress signaling-related proteins and cell wall metabolism after 7 days of aphid infestation

Calcium sensor proteins such as calmodul2c1in play an important role in SA accumulation (Wang et al., 2011; Zhang et al., 2014). On day 7 of SCA infestation, we found upregulation of SbiRTX430.05G022600, homolog of Arabidopsis calmodulin 5 (*AtCAM5*), which enhances the activity of calmodulin binding partners (Fig. 3b) (Lv et al., 2019). The second most upregulated protein on day 7, SbiRTX430.02G093500 has been annotated as Mannose-binding lectin superfamily protein (Fig. 3b), which has been known to activate SA pathway and downstream defenses in pepper (Hwang and Hwang, 2011). We found three upregulated proteins, SbiRTX430.06G006000, SbiRTX430.06G006100, and SbiRTX430.06G010500, belonging to Zinc finger (C3HC4-type FRING finger) family proteins. SbiRTX430.01G216200 was also induced upon SCA infestation, annotated as plant basic secretory protein (BSP) family protein, known to travel from roots to shoots and vice-versa through phloem (Thieme et al., 2015). Several proteins have been reported to be activated or translocated in the presence of $Ca^{2+}$ including cytosolic phospholipase A2 (cPLA2), phospholipase C (PLC), calmodulin etc. de

Silva et al., (2011). Two upregulated proteins found in this study, SbiRTX430.02G141000 and SbiRTX430.02G313300, belong to Calcium-dependent lipid-binding (CaLB domain) family, which might be important for signal transduction. Another upregulated protein, SbiRTX430.01G385100 belongs to α-N-acetylglucosaminidase family, involved in the hydrolysis of UDP-N-acetylglucosamine, important for intracellular signaling in plants (Ronceret et al., 2008). Sugar residues in proteoglycan complexes such as glucosamine and N-acetyl-glucosamine carry important signaling and regulatory functions, present in cell walls (Ronceret et al., 2008). We observed an upregulation of protein, SbiRTX430.01G385100, α-N-acetyl-glucosaminidase, reported to be involved in the catabolism of these sugar residues (Ronceret et al., 2008).

SCA feeding modulates few proteins related to cell wall metabolism. SCA feeding upregulates acyl transferase: SbiRTX430.06G021400, glucan endo-1,3–β-glucosidase: SbiRTX430.01G469400 and SbiRTX430.03G454800 (Fig. 4a). SbiRTX430.09G268800, homolog has been annotated as Outer Mitochondrial membrane protein of 66 kDa in Arabidopsis. Overexpression of *AtOM66* led to increased SA content, accelerated cell death rates and plants more tolerant to the biotrophic pathogen, but more susceptible to the necrotrophic fungus (Zhang et al., 2014).

### 3.8. SCA feeding for 7 days upregulated proteins involved in oxidative metabolism, secondary metabolism and abscisic acid related proteins

Few proteins from phenylpropanoid pathway were upregulated after SCA infestation (Fig. 4b). SbiRTX430.06G157300, phenyl ammonia lyase, and two proteins, SbiRTX430.07G078300 and SbiRTX430.06G015900, (pathway homologs of cinnamyl alcohol dehydrogenase 9, *AtCAD9* gene), belonging to the phenylpropanoid group of proteins, were also found to be upregulated. These enzymes catalyze different hydroxylation and desaturation steps in plants for example flavanone 3β-hydroxylase (F3H) in the biosynthesis of flavonoids, catechins and anthocyanidins (Damme et al., 2008). In rice, this sorghum protein is a homolog of flavanol synthase. Another SbiRTX430.02G220800 protein, which encodes for a flavonoid 3'-hydroxylase in Arabidopsis was found to be upregulated (Han et al., 2010). Farnesyl diphosphate synthase 1, SbiRTX430.03G291400, was found to be upregulated and is involved in synthesis of plant terpenoids. Another protein, SbiRTX430.01G331800 was found to be induced after SCA feeding, which represents 2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein in Arabidopsis (*AtDMR6*).

Thioredoxins (Trxs) are known to be involved in plant tolerance of oxidative stress. Trxs protect the plants from oxidative damage by detoxification of lipid hydroperoxides and repair of oxidized proteins through reductases (Santos and Rey, 2006). Thioredoxins, SbiRTX430.01G425300 and SbiRTX430.06G032800 were found to be upregulated (Fig. 4a), implicating the redox regulation in SCA response to aphid feeding. Plant L-ascorbate oxidase (SbiRTX430.10G182700) was also found to be upregulated in response to SCA infestation.

The protein SbiRTX430.07G182400, an ortholog of Arabidopsis F1F0-ATPase inhibitor protein, is known to be involved in plant growth and response to abscisic acid (Chen et al., 2020), was upregulated (Fig. 5b). Also, SbiRTX430.06G083600, which is suggested to be involved in abscisic acid signaling was also found to upregulated. Another upregulated protein, AtCLO4 (At1g70670), homolog of SbiRTX430.10G111800, is a stress-responsive and caleosin-like protein that is capable of binding to $Ca^{2+}$, which also act as a negative regulator of ABA (Kim et al., 2011). Other ABA dependent proteins such as DPP6 N-terminal domain-like transmembrane protein, SbiRTX 430.06G010600, were also found to be upregulated.

### 3.9. SCA feeding suppressed proteins related to photosynthesis, growth and development, and detoxification after 7 days of infestation

Glutathione-S-Transferases (GSTs) are antioxidant enzymes that remove xenobiotic compounds and toxic metabolites (Gullner et al., 2018). The GST proteins, SbiRTX430.01G543000 and SbiRTX430.01G327200, were found to be downregulated in SC265 after 7 days of SCA infestation (Fig. 6). SbiRTX430.03G465000, an inner envelope proteins of chloroplast that might be associated with light-harvesting systems of the thylakoid membranes or release of stress related factors (Kwon et al., 2013; Richardson et al., 2017; Mamaeva et al., 2020) was also downregulated.

SCA feeding-suppressed protein, SbiRTX430.04G316400, a homolog of AT3G50790 belonging to hydrolases family, is late embryogenesis abundant (LEA) protein, which is also found to be involved in trichome initiation (Morohashi and Grotewold, 2009). SCA feeding also impacts the protein related to sugar metabolism. SbiRTX430.10G152700 encodes for glucan, water dikinase 1 and phosphoglucan, water dikinase, which are chloroplastic enzymes that degrades the leaf starch (Pirone et al., 2017). SbiRTX430.10G199200 belongs to AAA-type ATPase family proteins, which are responsible for diverse cellular activities of cell physiology (Zhang et al., 2014) and this is annotated as nuclear pore forming protein in Arabidopsis (Janská et al., 2014). SbiRTX430.03G302700 protein function is mainly related to signaling and it functions to initiate signaling and provide tolerance against abiotic stress in Arabidopsis (Guo et al., 2002). Another downregulated protein, SbiRTX430.06G264500 is purine biosynthesis protein involved in synthesis of precursors of cytokinins and secondary metabolites (Berthomé et al., 2008). SbiRTX430.01G549700 is a glycerol kinase protein important for glycolysis.

### 3.10. SCA spent more time feeding on SC265 plants that were preinfested with SCA for one day

To determine whether the correlation exists between accumulation of defense-associated proteins and SCA feeding behavior, we monitored the feeding behavior of aphids on SC265 healthy and SCA-infested plants for 1 and 7 days using the EPG technique (Supplemental Fig. S1). We found significant differences in the total sieve element phase (SEP) after 1-day of infestation compared with the non-infested plants. SCA spent more time feeding on the phloem sap from SCA infested plants for 1 day compared to control plants (Fig. 7a). Likewise, SCA spent less time in non-probing activity in 1-day infested plants compared to healthy plants. We did not find any significant differences in the mean time spent by SCA in pathway, xylem phase, time to first probe and time to first SEP (Fig. 7a & b). On the contrary, SCA spent less mean time in SEP phase of SCA infested plants for 7 days compared to the uninfested plants. Additionally, SCA spent significantly longer time in the pathway phase and non-probing phase in the plants infested 7-days prior the EGP recording (Fig. 7c). However, SCA reached the sieve elements almost two times slower in SCA 7 days infested plants compared to controls (Fig. 7d). However, there were no significant differences found in the time spent by SCA in the xylem phase and time to first probe.

## 4. Discussion

The present study provides a global analysis and overview of sorghum proteome in response to SCA infestation. We utilized sorghum resistant line, SC265, to understand the changes in sorghum physiological mechanisms at the cellular level. Overall, we identified the DEPs related to protease inhibitors, phospholipases, pathogenesis related

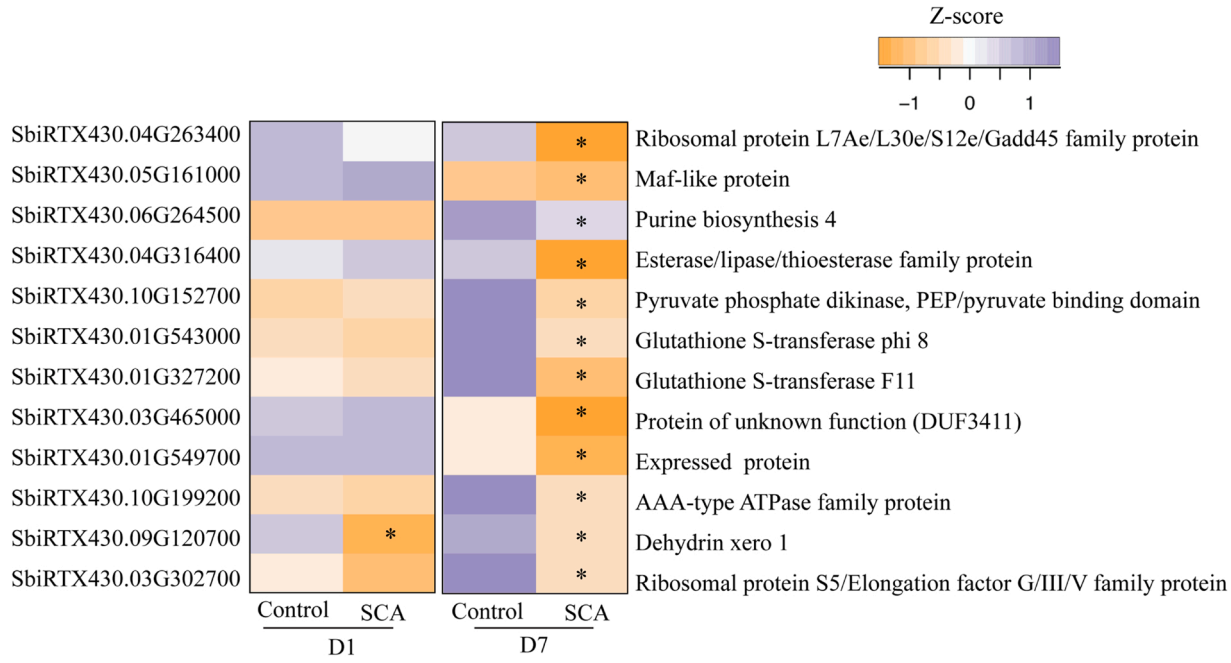


**Fig. 6.** Heatmap of downregulated proteins in SC265 plants after 7 days of sugarcane aphid (SCA) infestation. Color key represents the Z-score standardized values. Asterisks in the cells represent significant differences compared to the respective control based on the adjusted *P*-value < 0.05.

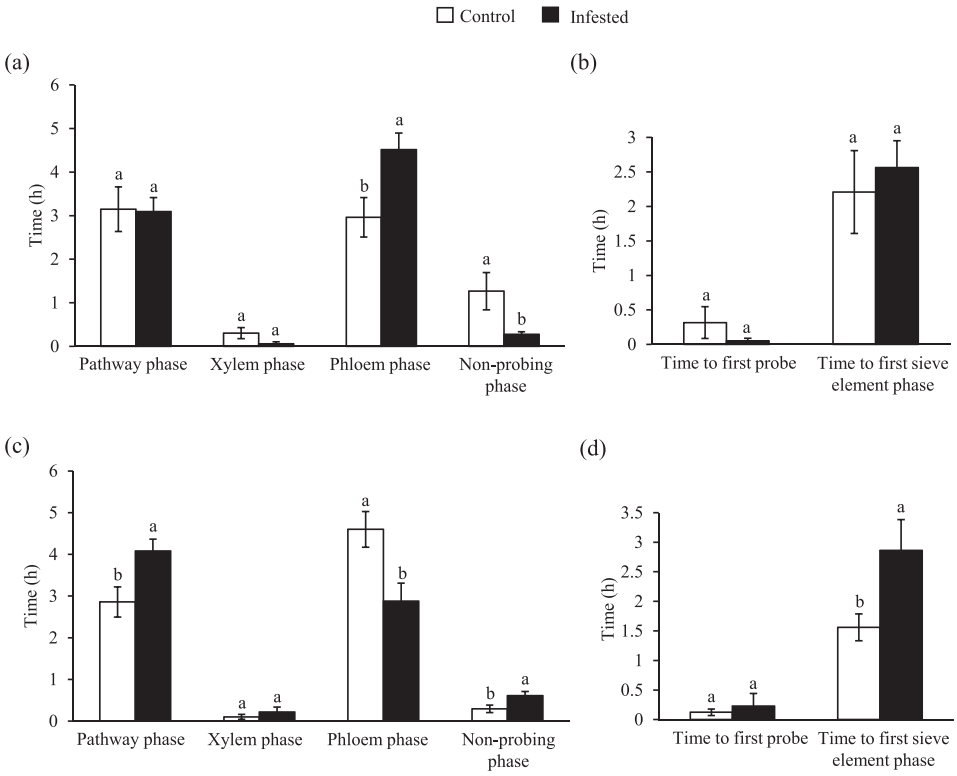


**Fig. 7.** Mean time spent by sugarcane aphids (SCA) for various feeding behavior activities (total duration of pathway phase, xylem phase, phloem phase, and non-probing phase) on SC265 plants after (a) 1 day and (c) 7 day of SCA preinfestation. Mean time spent by SCA for the first probe and to reach the first sieve element phase on SC265 plants after (b) 1 day and (d) 7 day of SCA pre-infestation. SCA uninfested plants were used as the control plants. Each value represents mean ± SE (n = 13–15). Different letters above the bars represent significant differences from each other ($P < 0.05$; Kruskal-Wallis test) in the time spent by SCA for the indicated activity on SC265 plants.

proteins, signal transduction, calcium binding, zinc finger family proteins, cell-wall metabolism, oxidative stress, and secondary metabolism. Most of the defense signaling related proteins were suppressed at day1 and induced at day 7. To validate the defense proteome trend, we utilized the EPG technique using control and SCA-infested plants for one day and seven days. Reduced phloem feeding in aphids has been often associated with enhanced resistance to aphids in plants (Grover et al., 2019; Tetreault et al., 2019; Varsani et al., 2019; Diaz-Montano et al., 2007; Koch et al., 2015; Grover et al., 2020). We have previously shown that greenbugs feeding and reproduction were lower in SC265 (Grover et al., 2019). EPG data strongly indicates the suppression of plant defenses at day 1 by enhanced SCA feeding in phloem phase of SCA pre-infested plants, whereas plant defenses were upregulated on day 7 since SCA spent lesser time in phloem phase of the SCA-infested plants for 7 days compared to control plants.

Proteomics analyses of SC265 showed the downregulation of several

defense and signaling related proteins on day 1. Furthermore, SCA feeding triggers JA levels at 1 hpi and 1 dpi, but JA-Ile only at 1 hpi (Grover et al., 2022). The simultaneous occurrence of induction of JA-Ile levels and suppression of defense and signaling related proteins indicates the possibility of SCAs trying to suppress the plant defenses by inducing JA-Ile levels. Previously, it was shown that the sorghum resistant hybrid DKS 37–07 revealed higher expression of non-expressor of pathogenesis-related gene 1 (NPR1) as well as gene encoding jasmonate ZIM-domain (JAZ) family, which act as JA repressors (Kiani and Szczepaniec, 2018). Similarly, transcriptome analyses of sorghum susceptible line BCK60 showed the higher expression of genes involved in JA synthesis and lower expression of pathogenesis-related genes as compared to resistant line, RTx2783 (Tetreault et al., 2019). Our proteomics analyses demonstrated the downregulation of pathogenesis-related proteins, protease inhibitors (PATATIN-like protein 4, serine carboxypeptidase-like 27, cysteine proteinases superfamily protein), oxidative stress-protection (plant L-ascorbate oxidase, thioredoxin superfamily protein, NADPH:quinone oxidoreductase, peroxidases) at 1 dpi. Moreover, SCA feeding also suppressed secondary metabolism (flavonoid proteins) and several signaling related proteins. In pepper, the proteomics analysis showed the suppression of defense related proteins in response to aphids (Florencio-Ortiz et al., 2021). The extensive crosstalk between the plant hormone signaling pathways not only fine tunes the plant transcriptional network, but also manipulates the resistance to herbivores (Johnson et al., 2020; Ma et al., 2020; Ruan et al., 2019). It is also widely established that plant hormones such as jasmonates can antagonize the SA based defense responses (Caarls et al., 2015). These data clearly indicate that SCAs may trick the plants by activating JA pathway, to suppress early plant defense responses.

Higher constitutive and induced SA levels in SC265 could be responsible for providing defense against SCA (Grover et al., 2022). Increased SA levels have been associated with enhanced resistance against piercing-sucking type insects, which cause minimal injury while feeding on plants (Moran and Thompson, 2001; Mohase and van der Westhuizen, 2002; Li et al., 2006). One of the upregulated proteins at 1 dpi, RNA-binding protein-defense related 1, known to be a positive regulator of SA immunity, has been shown to provide resistance to the pathogen *Pseudomonas syringae (*Qi et al., 2010*)*. At day 7, proteomics analysis also showed the elevated expression of pathogenesis-related (PR) proteins, which is in line with previously reported elevated SA levels. Some oxylipin related proteins, such as phospholipases and lipoxygenases, were upregulated at 7 dpi, which are involved in providing defenses whether or not they are involved in JA-mediated defenses. It is more likely that upregulated oxylipin synthesis related proteins might provide defense against SCA independent of the JA pathway because no changes in JA/JA-Ile were observed for the time period. Lipoxygenases have been divided into 9-and 13-LOX categories based on their ability to incorporate oxygen at either C-9 or C-13 positions of fatty acid (Liavonchanka and Feussner, 2006). *Atlox1*, 9-position specific locus which does not lead to JA biosynthesis but known to report cell death responses against microbial pathogens, was also found upregulated at 7 dpi (Hwang and Hwang, 2011). Homolog of Arabidopsis PLA II A, which was found upregulated at 7 dpi is not known to produce JA, but it promotes cell death and differentially affecting resistance to different pathogens (Camera et al., 2009). Intracellular phospholipase plays an important role in oxylipin biosynthesis. *SPX 1* protein was upregulated at 7 dpi found to be induced by the overexpression of MYB transcription factors that also binds with the cis elements of phospholipases gene promoter in Arabidopsis (Nguyen et al., 2016).

Plant inter- and intracellular signaling is crucial for activating plant defenses. For example, secondary messengers, $Ca^{2+}$ and reactive oxygen species are known to fine-tune cellular signaling networks and activate plant immunity to stress (Marcec et al., 2019). Zinc finger proteins also play an important role in plant-pathogen interaction (Noman et al., 2019). In this report, evidence suggests that SCA feeding triggered several signaling proteins such as calmodulin, mannose binding lectin, calcium-dependent lipid binding and Zinc C3HC4-type RING finger family proteins, which are known to be involved in plant defenses. Moreover, plant L-ascorbate oxidase, which is involved in ROS metabolism and plant defenses, was upregulated (Felton and Summers, 1993; Pignocchi et al., 2003). Ascorbic acid is an antioxidant which detoxifies the reactive oxygen species produced due to biotic/abiotic stress. Ascorbate oxidase catalyzes the oxidation of ascorbic acid and converts it into dehydroascorbate (Horemans et al., 2000). Oxidation of ascorbic acid has been shown to induce defenses against root-knot nematodes by activating JA and ethylene pathways and primed the plants with generation of hydrogen peroxide upon nematode infection (Singh et al., 2020). C3HC4-type RING zinc finger genes are responsive to ascorbic acid in Arabidopsis (Gao et al., 2011).

SCA feeding leads to upregulation of cell wall metabolism and stress signaling-related proteins at day 7. Callose is an important defense mechanism in plants for aphids and pathogens (Luna et al., 2011). Callose deposition can occlude the sieve-elements of plants and reduce the phloem sap access to aphids (Varsani et al., 2019). Aphid-induced upregulation of callose-degrading β-1,3-glucanase genes can counteract the callose defense mechanism (Kim et al., 2020). The Arabidopsis homolog of SbiRTX430.03G454800 (β-1,3-glucanase) was found to be upregulated at 7dpi, and is known to recognize nematode effector and induce plant defenses (Hamamouch et al., 2012). SCA feeding upregulated the glucan endo-1,3–β-glucosidase proteins on day 7, which could be one of the aphid's abilities to weaken the SC265 defenses or plant's trick to induce defenses. Another glucan endo-1,3–β-glucosidase, SbiRTX430.01G469400 has been referred to as SA-induced cell wall degradation enzyme in Arabidopsis (Coppola et al., 2018). SbiRTX430.06G021400, annotated as HXXXD-type acyl-transferase family protein, has been reported to be involved in cell wall metabolism (Rautengarten et al., 2012) and differentiation of water-conducting tracheary elements (Pyo et al., 2007). Moreover, PPIs are an important component of plant evolutionarily customized defenses that can directly affect the insect digestion system (Clemente et al., 2019; Singh et al., 2020). Several PI proteins were found to be upregulated after 7 dpi of SCA and it is plausible that oxylipins/SA might be involved in regulating these proteins.

ABA is known to be mainly involved in abiotic stresses and has also been associated with aphid herbivory (Hillwig et al., 2016; Studham and MacIntosh, 2012; Danquah et al., 2014). Proteomics data showed the upregulation of several ABA dependent and signaling proteins including the negative regulators of ABA in SC265 at 7 dpi, which could explain our finding of unaltered ABA hormone levels in SC265. In Arabidopsis, green peach aphid (*Myzus persicae*) feeding triggers ABA responses that suppress effective plant defenses (Hillwig et al., 2016). In soybean, ABA responses has also been found induced in susceptible genotype after soybean aphid infestation (Studham and MacIntosh, 2012). Similarly, we have also observed higher ABA basal levels in SCA-susceptible line, SC1345 (Grover et al., 2020). Some reports documented the importance of ABA in activating the MYC arm of JA pathway and enhance the JA based defenses (Vos et al., 2013; Long et al., 2019). However, this is highly unlikely in the case of SCA-sorghum interactions because no changes in JA levels were observed at 7 dpi (Grover et al., 2022).

Besides early signaling, SCA feeding also altered secondary metabolism. Plant terpenoids have been known to play a role in plant-insect interactions (Bhatia et al., 2015). Farnesyl diphosphate (FDP), a common substrate for the biosynthesis of an array of terpenoids, is synthesized by the cytosolic mevalonic acid (MVA) pathway (Lombard and Moreira, 2011). Overexpression of *FPS1* in Arabidopsis led to premature senescence in plants, but it is not evident that if elevated FDP also altered the terpenoid profiles and how it affects aphid performance. In our study, we have also observed the upregulation of protein, SbiRTX430.03G291400 that encodes for *FPS1* in Arabidopsis. Suppression of *FPS* genes triggers the genes related to JA pathway (Manzano et al., 2016). Lignin biosynthetic pathway gene, cinnamyl alcohol dehydrogenase, *AtCAD9,* has been found induced in response to SCA

infestation, which is expressed in vascular tissues (Kim et al., 2020). Flavonoid pathway also seems to be induced upon SCA infestation. Flavonoids can also deter insect growth, behavior and development (War et al., 2012). SCA feeding also suppressed the protein which degrade starch upon 7 days of infestation. It has been reported that green peach aphid infestation of Arabidopsis led to increase in starch content of plants, which explains the blockage of sugar export to the plant sinks (Singh et al., 2011) and it can retard plant growth (Stettler et al., 2009). Downregulation of AAA-type ATPase protein at 7 dpi also suggests an aphid-induced reduced growth of plants.

In our study, the molecular shift of plant defense suppression at early time points to defense activation at late time points is very intriguing and crucial to understand in the future. Similar kind of trend has not been reported before. Most of the studies conducted has been mainly focused on single factors such as plant defense machinery or insect elicitors. Though there are several papers published on insect effectors showing their capability to suppress plant defenses (Furch et al., 2015; Will et al., 2007; Bruessow et al., 2010; van Bel and Will, 2016), but the duration of insect effectors efficacy has not been really studied at multiple time points. The insect cues consist of several ingredients with different functions and how those ingredients interact with plant molecular machinery derive the outcome of plant-insect interactions.

## 5. Conclusion

In this study, we provide an overview of sorghum proteome reprogramming in response to SCA attack. Overall, this research shows that the resistance mechanism in SC265 results from the sequential induction of defense pathways such as constitutive and induced levels of SA, phospholipases, calcium signaling and zinc finger related proteins, flavonoids, several proteinases, and protease inhibitors. In addition, SCA feeding triggered the suppression of several defense related proteins at 1 dpi. This indicates the possibility of aphids using JA as a part of counter defenses to plants. This aphid counterattack was only observed at early time points after SCA infestation, suggesting that sorghum plants possess the ability to adjust their defense response over time and regain control. This scenario has not been reported before to the best of our knowledge and needs to be explored. This study laid the foundation to understand underlying molecular defense mechanisms to SCA and would also contribute knowledge towards development of novel pest management strategies by further validating the function of proteins identified in this study.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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*Author contributions*

S.G., P.Z. and J.L. designed the study. S.G. and P.Z. collected the plant samples. S.A. and M.J.N. performed the proteomics analysis and analyzed the data. S.G. interpreted the proteomics data. S.G. and J.B.C. performed and analyzed the aphid bioassay and aphid feeding behavior experiments. S.G. and J.L. wrote the draft. J.L. provided all the funding. All authors contributed to the writing and editing of the final manuscript.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111289.

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