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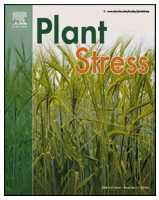

Disruption of the sorghum circadian clock impacts sorghum-sugarcane aphid interaction dynamics and aphid feeding behavior

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ABSTRACT

Circadian clocks play a pivotal role in orchestrating metabolic rhythms in response to environmental stress. *Sorghum bicolor* (sorghum), a multipurpose cereal crop, suffers severe growth and yield reduction due to feeding by sugarcane aphids (SCA; *Melanaphis sacchari*), which are phloem-feeding pests. Previously, it has been shown that sorghum utilizes a multitude of defense mechanisms to curb SCA colonization. However, our understanding of the impact of circadian clock on sorghum-aphid interaction dynamics is limited. To explore this, a time-series transcriptomics was conducted on sorghum plants in disrupted circadian rhythm with and without SCA infestation. Transcriptomic analysis revealed a total of 2,873 differentially expressed genes (DEGs) and weighted gene co-expression network analysis (WGCNA) identified four modules with distinct expression patterns unique to night-time. Further, a total of 946 sorghum circadian genes were identified and among those, 328 circadian genes were unique to SCA-uninfested control groups that belonged to defense responses to insects and wounding. The circadian genes upregulated during the night-time after SCA infestation were related to MYB transcription factors, primary metabolism, and transporters, suggesting that SCA feeding modulates host defenses during the night-time. Aphid feeding modulation of sorghum defense genes during the night-time is in alignment with the electrical monitoring of SCA feeding behavior analysis, which revealed that the SCA spent significantly more time in the salivation phase during the night-time feeding. Our study provides novel insights into the circadian response of sorghum-aphid interaction dynamics and identified sorghum circadian genes in response to SCA infestation.

1. Introduction

The C_4 grass bioenergy model crop, *Sorghum bicolor* (sorghum), is the fifth most important cereal crop grown worldwide. The United States is the top producer of sorghum and is mainly used in animal feed, biofuel production and export trade. Sorghum is also gaining attention from the food industry due to its high nutritional value (de Morais Cardoso et al., 2017). However, sorghum yield is severely hampered due to attack by insect pests and pathogens. Sugarcane aphid (SCA; *Melanaphis sacchari*) is a major sap-feeding sorghum pest that causes grain and biomass yield reduction (Bowling et al., 2016; Gordy et al., 2019). The SCA has become a major pest in sorghum growing regions in the North America, resulting in significant sorghum yield loss (10–50%) (Gordy et al., 2019). Apart from causing yield loss, SCA deposit honeydew on sorghum leaf surface, which promotes sooty mold fungal growth making plants prone to secondary diseases (Gordy et al., 2019).

Previously, it was shown that the SCA-resistant sorghum line utilizes an arsenal of defense responses, including cuticular waxes, sugars, and upregulation of genes related to leucine-rich repeat (LRR) proteins, cell-wall related, pathogenesis-related proteins, jasmonic acid (JA) and salicylic acid (SA) biosynthesis (Cardona et al., 2023a,b; Puri et al., 2023; Grover et al., 2022a,b; Huang et al., 2022). In *Arabidopsis*, most of the defense-related genes regulated during wounding show strong circadian regulation: >40% of genes induced by wounding have peak circadian expression at dusk, and >80% of genes downregulated by wounding have peak expression at dawn (Goodspeed et al., 2012; Walley et al., 2007). Circadian clock is an internal timekeeper, which predicts alteration during light and dark cycle, that helps plants to attain fitness,

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proper growth, and response to biotic and abiotic stress (Kiełbowicz-Matuk et al., 2014). The central circadian clock regulator, *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*), coordinates plant responses to environmental challenges. A study using circadian clock mutants, including *cca1-1*, implicated the circadian clock in plant defense against aphid herbivory (Lei et al., 2019). Interestingly, metabolomic analysis linked *CCA1*-regulated indole glucosinolate production to defense against aphids (Lei et al., 2019), further supporting a previous report that glucosinolate accumulation is under circadian control (Goodspeed et al., 2013). Additionally, circadian clock regulation of JA and SA biosynthesis has been well implicated in *Arabidopsis* against pests and pathogens (Thines et al., 2019). However, the role of sorghum circadian clock in providing defense against aphids is unknown and unexplored.

Similar to plants, insects also use the diurnal cycle (circadian rhythm) to modify their activities to increase their growth and fitness (Salmela and Weinig, 2019; Xu et al., 2011). The insect activities, for example, host finding (Narayandas and Alyokhin, 2006), honeydew excretion (Joschinski et al., 2016) and oviposition (Hodgson and Lane, 1981) are influenced by diurnal rhythm. Herbivorous insect feeding and performance are affected by the diurnal variation of the plant nutrient composition. During night-time lower concentrations of sugar flux have been observed due to lack of photosynthesis, which may play a role in diurnal feeding (Nalam et al., 2021). The diurnal variation in aphid feeding has been reported in bird cherry-oat aphid (*Rhopalosiphum padi*) on wheat, where aphids took significantly longer time in the salivation phase during the night-time (Nalam et al., 2021). Aphid saliva consists of "effectors" that can suppress the plant defense responses (Mou et al., 2023; Nalam et al., 2019; Elzinga and Jander, 2013). It is highly plausible that the longer salivation phase during night-time suppresses the plant defense responses.

Despite the emerging data on the role of individual gene/compound in the interaction between plant circadian clock and pest/pathogen stress, understanding how the plant circadian system responds to biotic stress is limited. In this study, we performed a time-series transcriptomic approach to understand the impact of SCA feeding on the sorghum circadian rhythm. Transcriptomes from 13 timepoints (samples collected every 4 h up to 48 h) from SCA-infested and uninfested (control) plants showed that sorghum genes have distinct circadian oscillation after SCA feeding during the night-time and host plant balance the growth-defense tradeoff. We further identified 946 sorghum circadian genes during sorghum-SCA interaction and SCA feeding disrupts most of the defense-related circadian genes. These results also reveal the candidate genes for the phloem-based plant responses during the night-time aphid feeding, thus providing novel insights into the circadian response of the host plant.

2. Materials and methods

2.1. Plant growth conditions and aphids

Sorghum BTx623 plants were grown in 3.8 cm x 21.0 cm plastic cone-trainers (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 with a 16/8 h light/dark photoperiod, 25 °C, and 50–60% relative humidity at the University of Nebraska-Lincoln as described previously (Tetreault et al., 2019; Puri et al., 2023). Plants were watered regularly and fertigated once a week during the two-week growth period. SCA were maintained on susceptible sorghum genotype BCK60 in the same conditions as above to keep both aphids and plants in the same phase.

2.2. Circadian rhythm disruption and sample collection

The two-week-old sorghum plants (three-leaf stage) grown under 16/8 h light/dark photoperiod conditions were switched to continuous light for 24 h before infestation. After 24 h of constant light entrainment, plants were split into two groups. One group was infested with 10 adult apterous SCA and the other group was kept uninfested (SCA-uninfested control group) as described previously (Puri et al., 2023). The first leaf tissue samples were collected within 30 min of SCA infestation and then every 4 h intervals for 48 h as illustrated in Fig. 1. Three replicates were collected for each aphid-infested and uninfested timepoints and one replicate consisted of three pooled leaf tissue samples. The collected plant tissues were frozen in liquid N_2 and stored at −80 °C until further use for RNA extraction.

2.3. RNA extraction and sequencing

Leaf samples were homogenized using the 2010 Gino Grinder (SPEX Sample Prep, NJ, USA) for 60 s in the presence of liquid nitrogen. RNA was extracted using the RNA extraction kit (Zymo Research, Irvine, CA, USA) following the manufacturer protocol. The quantity of RNA was measured using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Two micrograms of RNA per sample were submitted to the Genewiz facility (NJ, USA) for mRNA-seq library preparation and Illumina RNA sequencing. The sequencing resulted in 150-bp paired end with 20 million reads on average per library. Raw reads are available in the NCBI SRA database under the bioproject number PRJNA716316.

2.4. RNA-seq data analysis

The quality of the mRNA-seq raw reads was assessed with FASTQC (Andrews, 2010) and the reads were trimmed using Trimmomatic v0.39 using the following parameters: LEADING:20 TRAILING:20 SLIDINGWINDOW: 4:20 MINLEN:75 (Bolger et al., 2014). The trimmed reads were mapped on the sorghum reference genome BTx623 v3.1.1 (https://phytozome-next.jgi.doe.gov/info/Sbicolor_v3_1_1) using TOPHAT v2.1.1 (Kim et al., 2013). Parameters for mapping include 0 splicing mismatch (-m 0) and 0 mismatches (-N 0) for the samples collected. The transcripts reconstruction was performed using Cufflinks v2.2.1 with the following parameters: quantification against the reference annotation only (-G), multi-read-correct (-u) and frag-bias-correct (-b). To further evaluate the quality of transcriptomics data and see the effect on circadian genes we evaluated the expression of circadian marker genes in our dataset. The sorghum circadian marker genes information has been used from previous paper (Kebrom et al., 2020). The differential expressed gene (DEGs) analysis was performed with Cuffdiff 2.2.1. The DEGs were considered significantly expressed with the following parameters: q-values < 0.05 and fold-change $|\log_2(\mathrm{FPKM}_{\mathrm{infested}}/\mathrm{FPKM}_{\mathrm{control}}| \geq \log_2(2)$ (Schurch et al., 2016) for each timepoint. The FPKM values of these DEGs were subjected to weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) analysis in R to identify the gene co-expression module and the set of DEGs that have a similar expression pattern.

2.5. Identification and functional annotation of circadian genes

To identify the circadian genes, we use RAIN algorithm to the FPKM values of DEGs from each group, control, and SCA-infested (Thaben and Westermark, 2014). RAIN algorithm is advanced to other algorithms such as, JTK_CYCLE, and can detect strong but asymmetric circadian rhythms in genes, which often are unnoticed by JTK_CYCLE (Thaben and Westermark, 2014). In RAIN genes with Benjamini-Hochberg corrected $P<0.01$ were considered circadian genes (Thaben and Westermark, 2014). These circadian genes from each group were subjected to the WGCNA analysis to identify the gene co-expression module.

The Gene Ontology (GO) analysis was conducted for the DEGs modules and circadian genes modules using PlantRegMap (http://plantregmap.gao-lab.org/) with *Sorghum bicolor* as reference genome and a $P \leq 0.05$ to determine the enriched GO terms in the circadian DEGs. These GO terms were further used and analyzed using R package (ggplot2) and

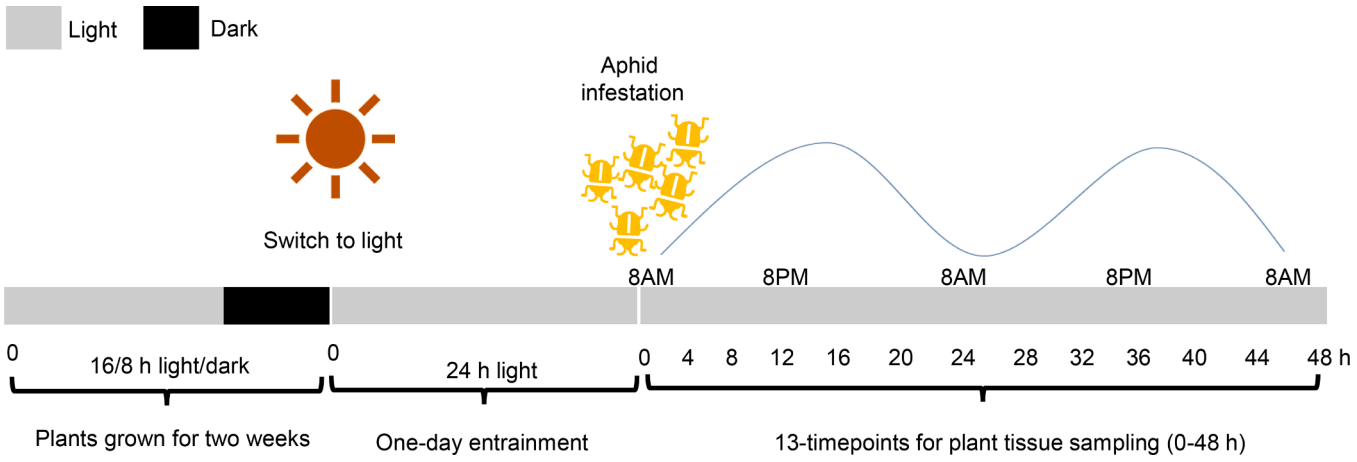


Fig. 1. Experimental setup for the time-series transcriptome sample collection. The two-week-old sorghum BTx623 grown under 16/8 h light/dark photoperiod conditions were switched to continuous light for 24 h for disrupting the circadian oscillations. The plants were split into two groups: Aphid-infested with 10 adult sugarcane aphids (+SCA) and control uninfested (-SCA) plants. Plant tissue samples were collected at every 4 h intervals up to 48 h for both groups.

REVIGO (http://revigo.irb.hr/) to produce non-redundant GO terms represented in Treemap (Supek et al., 2011). For the gene annotation and pathway analysis, the circadian DEGs protein sequence was subjected to BlastKOALA database of KEGG (Kyoto Encyclopedia of Genes and Genomes). Further to annotate all the genes, Sbicolor_454_v3.1.1 annotation file from the phytozome database was used. Heatmaps for these genes were prepared using gplots package using heatmap.2 function in R studio. The gene expression in the heatmap is the $\log_2$ FPKM values of SCA-infested group and a *t*-test was performed with respective control groups for significance level [adjusted *P*-value (* < 0.05, ** < 0.01, *** < 0.001)].

2.6. Monitoring aphid feeding behavior using electrical penetration graph (EPG)

Two-week-old sorghum plants and adult SCA were placed in 24 h light for the light entrainment as performed for the aphid infestation and RNA-seq sampling. EPG was recorded for 8 h for both day and night phases and EPG was performed during plant-insect synchronized day and night phases. EPG was set up for day and night-time feeding behavior assessment at ZT03 and ZT16 h, respectively. The aphid feeding behavior on sorghum plants was conducted as described previously (Grover et al., 2019; Tetreault et al., 2019). Six channels were used for EPG recordings at one time and at least 16 replicates were obtained for day and night each. The waveform recordings obtained were analyzed using the EPG analysis software *Stylet*$^+$ (EPG Systems, Wageningen, The Netherlands). A non-parametric Kruskal–Wallis test was used to compare the duration of different feeding parameters/phases in sorghum plants during day and night phases, using PROC NPAR1WAY procedure, considering the non-normally distributed data (PROC GLIMMIX, SAS 9.3, SAS Institute).

3. Results

3.1. Sorghum transcriptome analysis and circadian marker gene expression before and after SCA infestation

Here, we investigated the impact of circadian clock disruption on sorghum-SCA interaction and identified the genes that displayed circadian rhythm. The principal component analysis (PCA) was conducted on normalized FPKM values of 33,810 genes expressed in at least one condition (Fig. 2A). PCA1 accounted for 6.4% of the variance separated the ZT00 timepoint, both SCA-infested and SCA-uninfested (control), from the remaining timepoints. PCA2 accounted for 5.3% of the variance that showed a separation between day 1 (ZT04 to ZT24, green-dashed circle) and day 2 (ZT28 to ZT48, blue-dashed circle) in both SCA-infested and uninfested conditions (Fig. 2A). The PCA with all three replicates showed a similar trend of clustering for day 1 and day 2 (Fig. S1).

Previously, it was shown that the critical sorghum circadian genes were expressed either during morning or evening time (Kebrom et al., 2020). In our study, we found eight circadian genes that displayed circadian oscillation (Fig. 2B; Table S1). The early daytime regulated gene, for example, *LATE ELONGATED HYPOCOTYL* (*LHY, Sobic.007G047400*) (ZT00, ZT24), and the evening regulated genes, such as *TIMING OF CAB EXPRESSION* (*TOC1, Sobic.004G216700*) (ZT12, ZT36) were highly expressed at the respective times (Fig. 2B). The circadian gene that controls flowering, *GIGANTEA* (*GI, Sobic.003G040900*), did not display any circadian pattern. Besides these two major clock genes mentioned above, other morning and evening-expressed *Arabidopsis* clock genes form interconnected loops that regulate the core clock loop (Hsu and Harmer, 2014). The homologs of these genes in sorghum (Kebrom et al., 2020) showed circadian rhythm in our dataset (Fig. 2B). The morning phase genes such as *REVEILLE* (*RVE2, Sobic.010G275700*) and *LATE NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1* (*LNK1, Sobic.001G352400*) were upregulated during the morning times (ZT00, ZT04, ZT24, ZT48). Similarly, the evening genes *FLAVIN-BINDING, KELCH REPEAT, and F BOX 1* (*FKF1, Sobic.005G145300*) and *JUMONJI C DOMAIN-CONTAINING PROTEIN (JMJ5, Sobic.007G210801*) showed higher expression during the evening times (ZT08, ZT12) compared to other timepoints and displayed the circadian pattern. In all the circadian gene expressions, the expression pattern was similar in both groups (control and SCA-infested). Notably, circadian genes had a strong expression level during the first 24 h (ZT00-ZT24), either high or low, compared to the second day (ZT24-ZT48), potentially due to the circadian disruption using constant light entrainment.

3.2. SCA infestation modulates gene expression modules of DEGs in a disrupted circadian condition

To get a deeper understanding of the genes affected by SCA infestation during the disrupted circadian time course, we characterized the DEGs. In total, 2873 genes were identified as DEGs ($\log_2(FPKM_{infested}/FPKM_{control}| \geq \log_2(2)$ and *q*-value < 0.05) (Table S2). The repartition of the 2873 DEGs is shown in Fig. 3A and the higher number of upregulated DEGs were identified at ZT00 (1060 DEGs). Subsequently, 636 DEGs were found to be differentially expressed after 4 h of SCA feeding (ZT04), which indicates the strong early response from host plants (Fig. 3A). The DEGs have low expression on ZT08 and ZT12 and

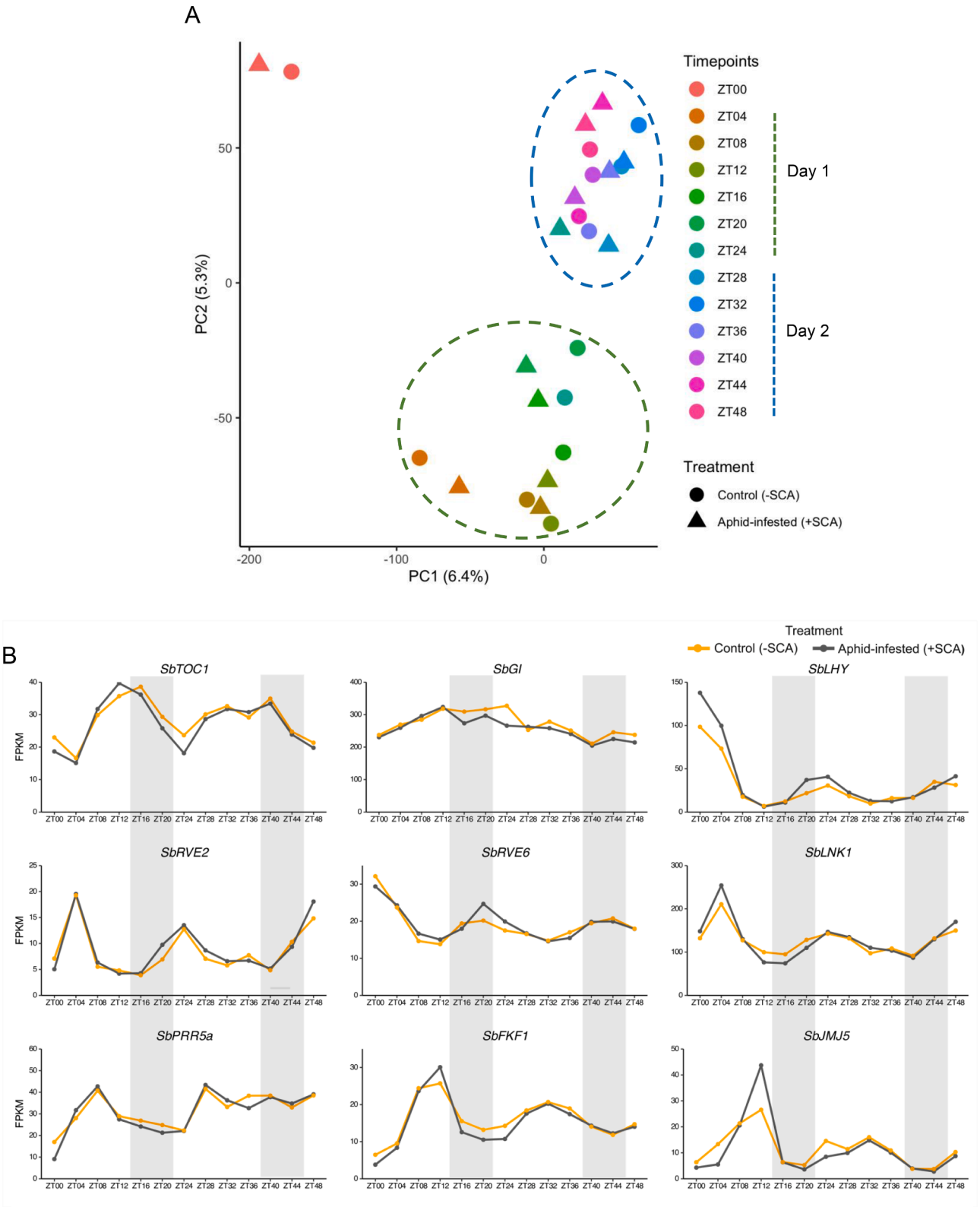


Fig. 2. **(A)** Principal component analysis (PCA) of all the 33,810 genes expressed in at least one condition. Timepoints and treatments are represented with colors and shapes. The green-dashed and blue-dashed lines represents day 1 and day 2 respectively. **(B)** Expression profile of nine circadian clock marker genes during 48 h time course under control sugarcane aphid (-SCA) uninfested (orange) and SCA-infested (+SCA) conditions (gray). Gray panels indicate night-time conditions. The y-axis represents the FPKM values. *TOC1: TIMING OF CAB EXPRESSION, GI: GIGANTEA, LHY: LATE ELONGATED HYPOCOTYL, RVE: REVEILLE, LNK1: NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1, PRR5: PSEUDO-RESPONSE REGULATOR 5, FKF1: FLAVIN-BINDING, KELCH REPEAT, and F BOX 1, JMJ5: JUMONJI C DOMAIN-CONTAINING PROTEIN.*

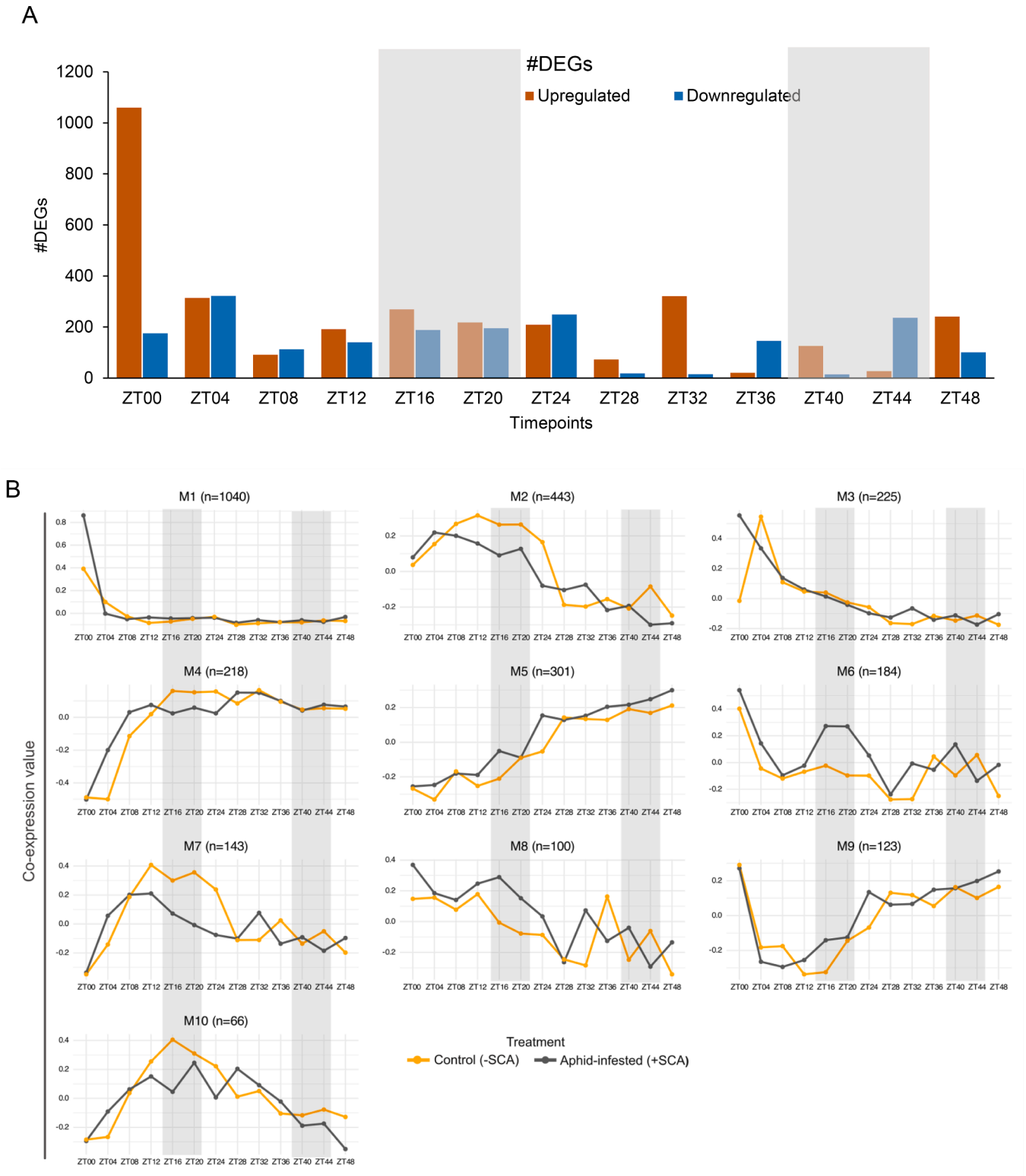


Fig. 3. **(A)** Repartition of the 2873 DEGs ($|\log_2$ (fold-change)$| \geq 1$ and q-value < 0.05) in expression for at least one of the time point (Aphid-infested vs. uninfested control). Gray panels indicate night-time conditions. **(B)** Weighted Gene Correlation Network Analysis (WGCNA) of differentially expressed genes (DEGs) in BTx623 at 13 different timepoints using FPKM values. Modules, M1 to M10, show expression patterns of a set of genes at 13 different timepoints. 'n' indicates the number of genes for each module.

increased during the night-time (ZT16 and ZT20) and early morning of second day (ZT24). On day 1 (ZT00 to ZT20), the ratio of the DEGs up or downregulated for respective timepoints was comparable, while on day 2, the ratio was dissimilar. The number of upregulated DEGs were also low on the second day (ZT28, ZT32 and ZT40) compared to day 1.

Further, the WGCNA analysis was used to group the genes sharing the same expression pattern. The 2873 DEGs were clustered into 10 different modules (M1-M10) (Fig. 3B), with each module having a

distinct gene expression pattern. Among them, M6, M7, M8, and M10 (total of 492 genes) showed a distinct pattern between control and SCA-infested groups. In M6 and M8, the SCA-infested group had higher expression during the day 1 night-times (ZT16 and ZT24). In contrast, M7 and M10 displayed lower expressions for the SCA-infested group during the day 1 night-times compared to the SCA-uninfested control group. Interestingly, during the second day for both SCA-infested and SCA-uninfested control groups, we observed a weak and dissimilar expression pattern.

We also performed the GO term analysis for the four modules (M6, M7, M8 and M10) that showed different expression levels during the night-time. M6 (184 genes) and M8 (100 genes) showed higher expressions in SCA-infested group in comparison to the control group (Fig. 3B). The gene enrichment analysis showed that the aphid feeding induced the genes involved in photosynthesis, response to different light conditions, glycoside biosynthetic process, secondary metabolomic process and ion transport (Fig. 4A and 4B; Table S3). In contrast, M7 (143 genes) and M10 (66 genes) displayed lower expression in SCA-infested group compared to the control group. The GO analysis revealed that the aphid feeding suppressed the genes involved in lipid biosynthesis, wax biosynthesis, secondary metabolic process, lignin biosynthesis and cell wall biogenesis (Fig. 4C and 4D; Table S3).

3.3. Identification of sorghum circadian genes and their modulation after feeding by SCA

We identified the circadian genes using the FPKM values of 2873 DEGs. In this context, we defined the term 'circadian gene' to those genes identified by the RAIN algorithm with high confidence thresholds ($P<0.01$) (Thaben and Westermark, 2014). In total, 946 genes (32.92%) were identified as circadian genes among the 2873 DEGs during sorghum-SCA interaction. Among the 946 genes, 328 and 319 were identified as circadian genes unique for SCA-uninfested and SCA-infested groups, respectively. In addition, 299 genes were common between the two groups (Fig. 5A; Table S4). The WGCNA analysis displayed four modules for the circadian genes in SCA-uninfested control and SCA-infested groups (Fig. 5B; Table S5). The SCA-infested group consisted of three distinct modules (Fig. 5B; Fig. S2) with strong circadian oscillation. M1 (234 genes; represented by black color) showed higher expression during the evening time (ZT08, ZT12, ZT32) and low expression during the night-time. M2 (158 genes; represented by pink color) showed higher expression at each morning time (ZT00, ZT24 and ZT48) and low expression to none in all day and night-time points. M3 (106 genes; represented by yellow color) displayed different expression patterns during the day, evening, and night timepoints for both days.

The SCA-uninfested control group had 328 unique circadian genes

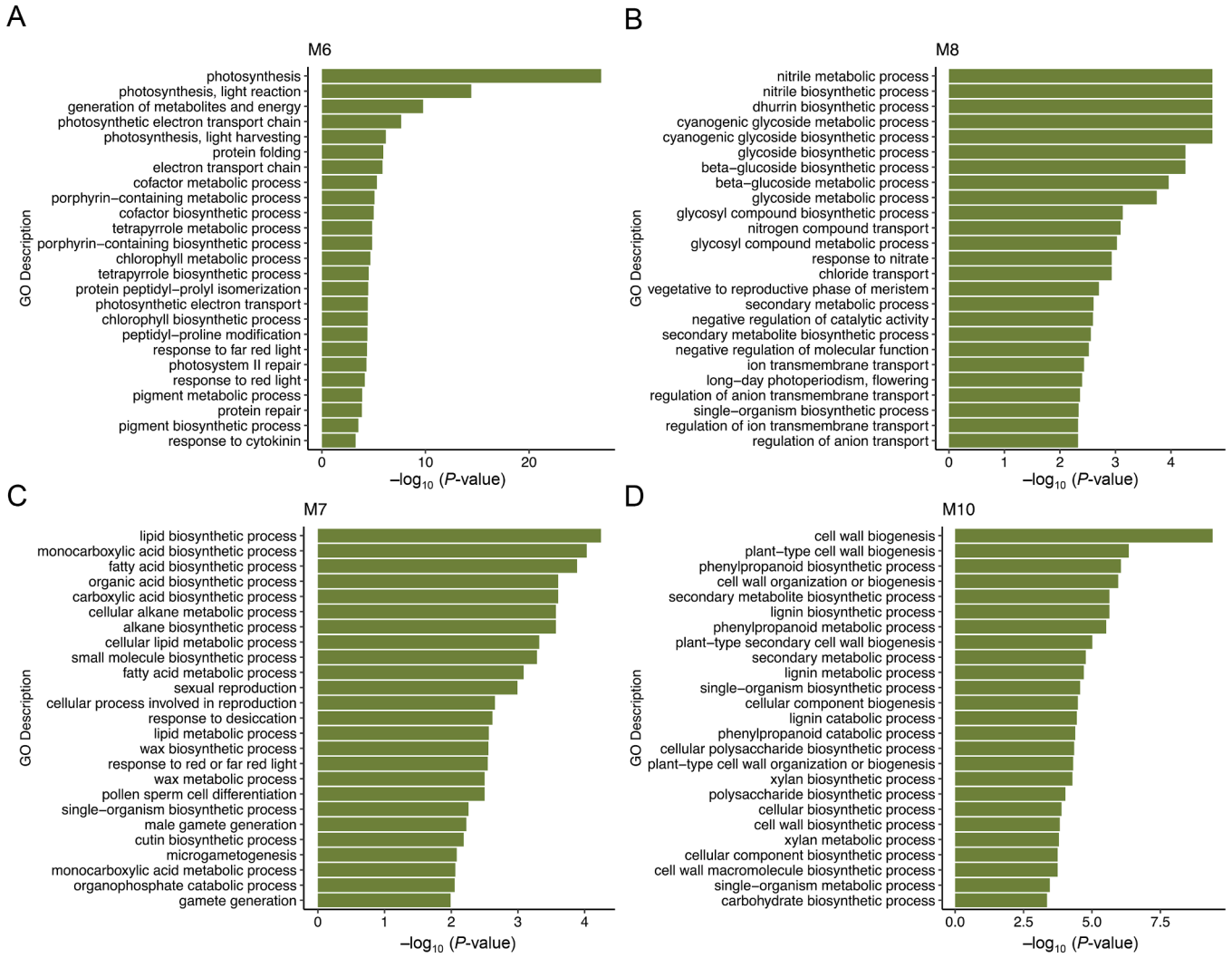


Fig. 4. Gene ontology (GO) terms of distinct WGCNA modules (aphid-infested and uninfested control) for modules (**A**) M6 (**B**) M8 (**C**) M7 (**D**) M10 biological functions. The top 25 GO terms based on *P*-value are represented in a bar graph, each bar represents the $-\log_{10}$ ($P<0.05$) of individual GO term and longer bar reflects most significant GO terms.

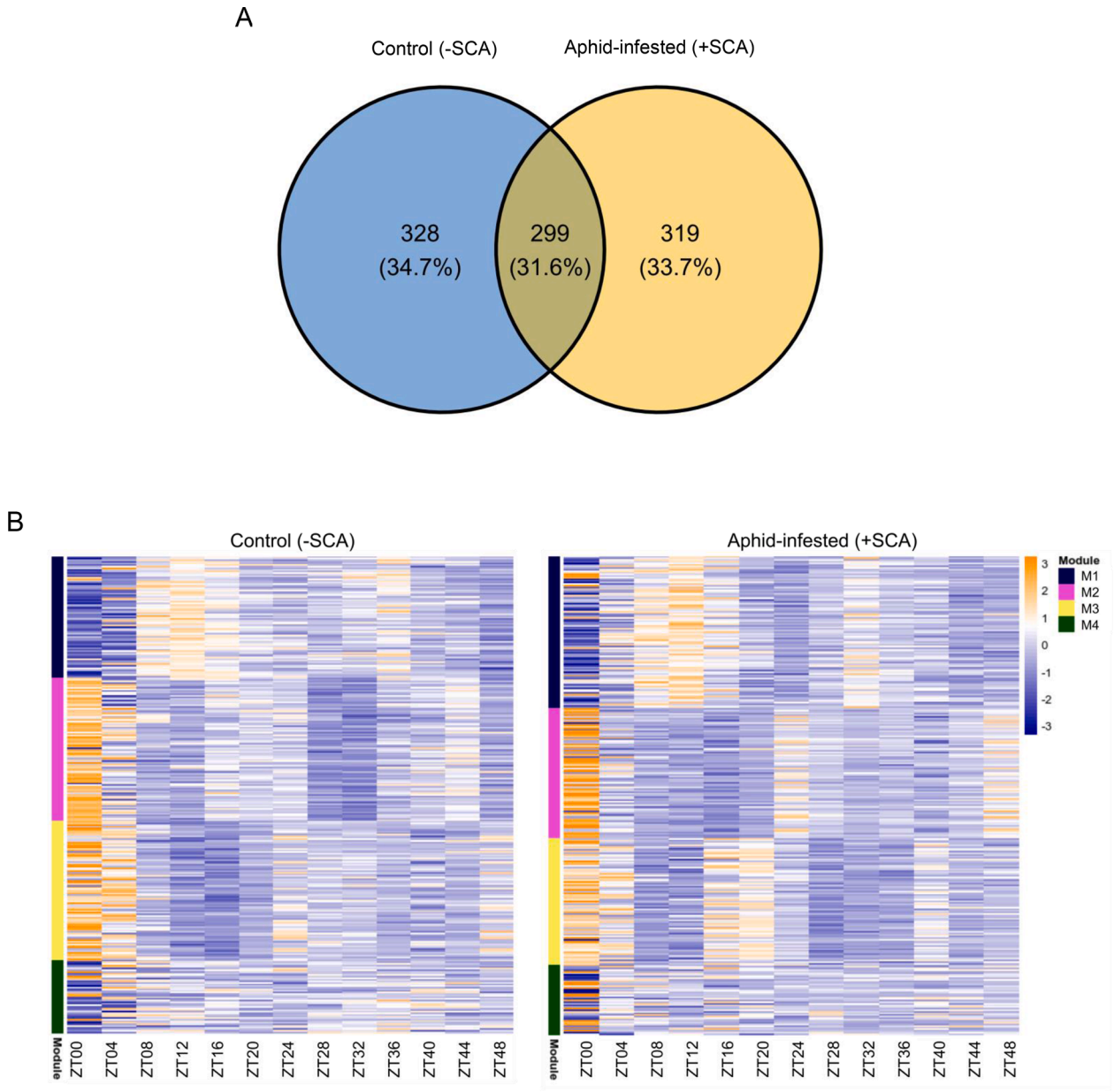


Fig. 5. **(A)** Venn Diagram of Circadian genes identified through the RAIN analysis, using $\log_2$ foldchange (FPKM+1) for all three replicates value of 2873 DEGs. **(B)** Heatmap of the aphid uninfested control circadian genes and aphid infested circadian genes represents the expression values. **(C)** Gene ontology (GO) treemap of overrepresented GO terms related to biological process of circadian genes of control only group (328 genes). Each box represents the $-\log_{10}$ (P <0.05) of individual GO term and bigger size of the box reflects most significant GO terms. Similar functional categories with semantic similarity are represented in similar colored boxes.

that were not present in the SCA-infested groups (Fig. 5A; Table S4), which implies that SCA feeding disrupted the circadian oscillation of these genes. The gene enrichment analysis of these 328 circadian genes revealed that the defense-related GO terms included response to wounding, insect, external stimulus and defense response (Fig. 5C; Table S6). Other GO terms related to defense were secondary and JA metabolic processes. Additionally, GO terms related to these circadian genes were carbohydrate catabolic process, amino acid metabolic process, photosynthesis, starch metabolism and ion transport system (Fig. 5C; Table S6).

3.4. Circadian genes displaying higher expression in night-time are related to primary metabolism and transporters

The SCA-infested M3 (107 genes) showed higher expression of the circadian genes during the night-time (Table S5). The GO enrichment analysis revealed that these circadian genes were related to biological processes such as starch metabolic and biosynthesis process, photosynthesis, light reaction, cellular process and generation of precursor metabolites and energy (Fig. 6A; Table S7). Similarly, these circadian genes were also enriched in different transport mechanisms. The GO enrichment analysis of SCA-infested M1 showed higher expression during the evening time were related to amino acid metabolic processes, such as serine, tryptophan and regulation of reactive oxygen species and superoxide metabolic processes (Fig. S3; Table S8).

To further explore the gene functions of these 107 circadian genes, KEGG analysis was performed and the heatmap for these genes were plotted using $\log_2$ of FPKM (Table S9). Among those, 11 of them belonged to seven transcription factors (TFs) and 31 belonged to primary metabolisms and ion transport system (Fig. 6B and C). These

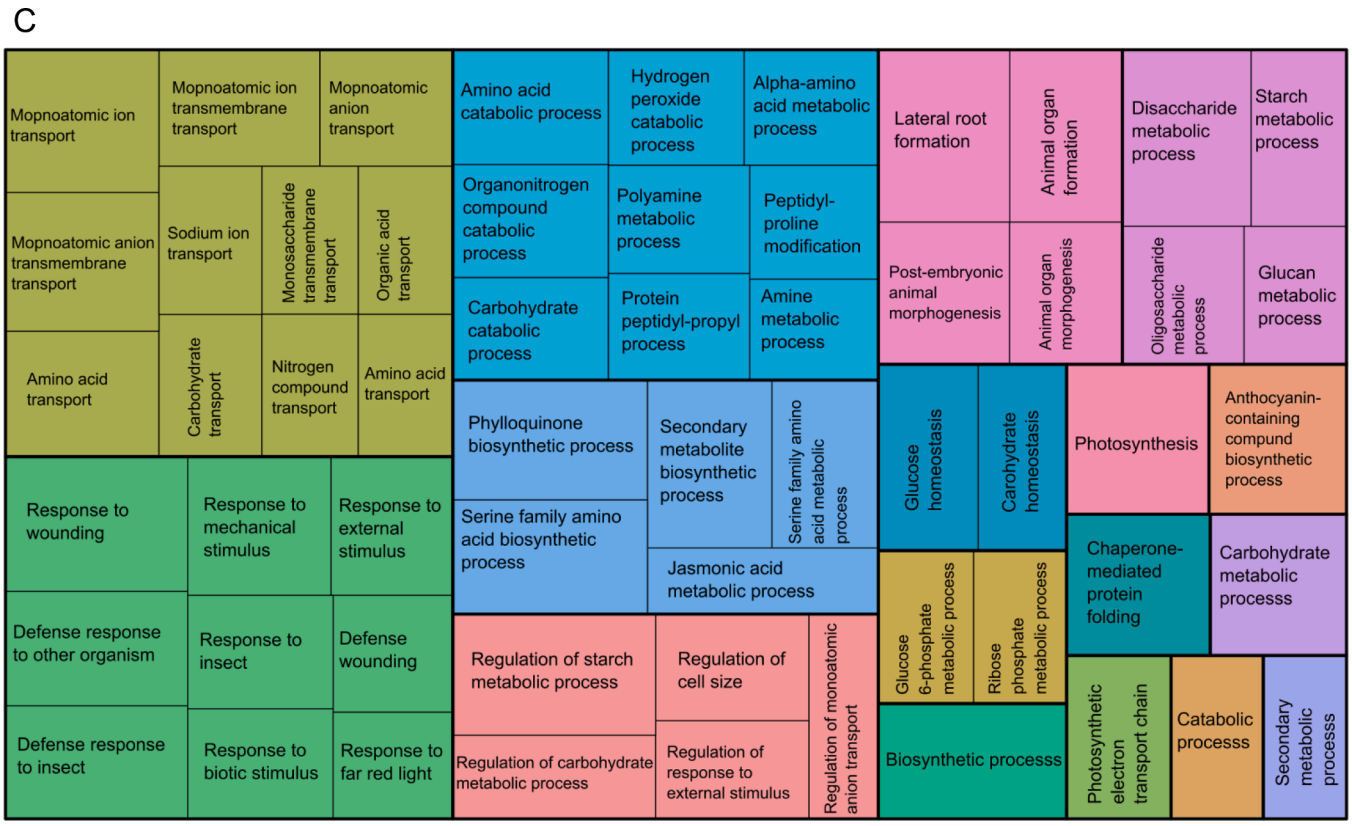


Fig. 5. (*continued*).

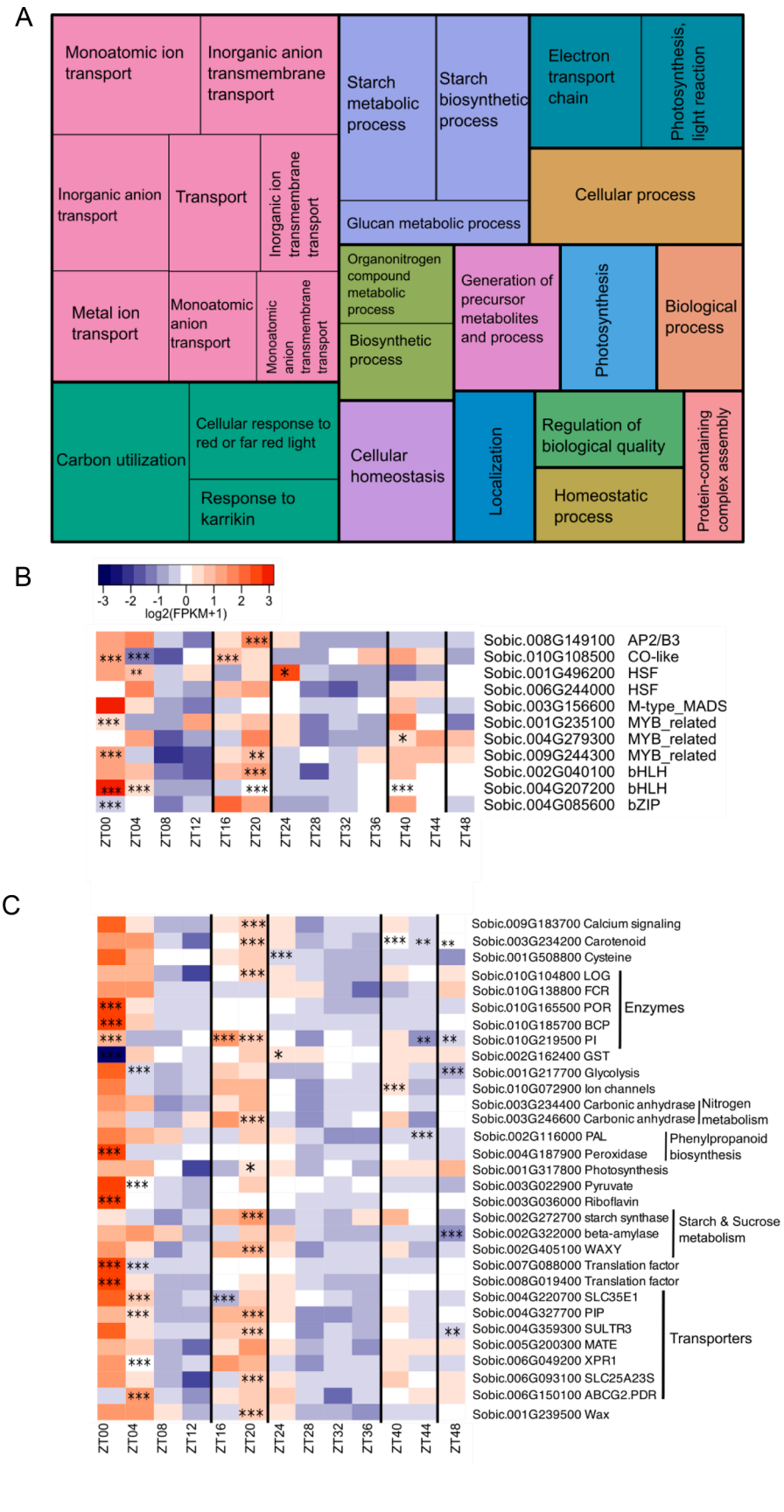
circadian genes were significantly upregulated at early timepoints (ZT00, ZT04), both night-time (ZT16-ZT20, ZT40) and were lowly expressed during the both daytimes (ZT08, ZT28-ZT32). The TFs belonged to heat shock proteins (HSF), MYB-related, basic helix-loop-helix protein (bHLH) and constans-like (CO-like) (Fig. 6B). Three sorghum circadian genes belonged to MYB-related TFs and two of them, *Sobic.004G279300* and *Sobic.009G244300*, were significantly upregulated during the day 1 night-time (ZT20) only. The CO-like TFs, *Sobic.010G108500*, also showed significant upregulation during the day 1 night-time (ZT16) and early morning (ZT00). The bHLH TF, *Sobic.002G040100,* was also significantly upregulated during the early daytime (ZT04) and both night-times (ZT20, ZT40). The primary metabolism identified were photosynthesis, nitrogen metabolism, starch and sucrose metabolism (Fig. 6C). One each of the genes related to photosynthesis, ferredoxin (*Sobic.001G317800*), nitrogen metabolism, and carbonic anhydrase (*can, Sobic.003G246600*), was significantly upregulated during the day 1 night-time (ZT20) only. Among the three circadian genes related to starch and sucrose metabolism, two were starch synthase (*glgA, Sobic.002G272700*) and granule-bound starch synthase (*WAXY, Sobic.002G405100*) genes, which were significantly upregulated during the day 1 night-time (ZT20). Similarly, another wax gene, *peroxygenase* (*PXG, Sobic.001G239500*), also showed significant upregulation during the day 1 night-time (ZT20). In total, seven circadian genes belonging to the plant transporter system were also reported to be crucial for nutrient acquisition, cellular homeostasis and stress tolerance (Li et al., 2021). Three transporters, aquaporin PIP (*PIP, Sobic.004G327700*), sulfate transporter (*SULTR, Sobic.004G359300*) and solute carrier family 25 (*SLC25A23S, Sobic.006G093100*), were significantly upregulated during the day 1 night-time (ZT20). Two of the transporter genes, *PIP* and *ATP-binding cassette, subfamily* (*ABCG2.PDR, Sobic.006G150100*), were also significantly upregulated during the early daytime (ZT04). Most of these genes displayed high or low expressions across the timepoints, however, they were not significantly different from the control groups at the respective timepoints.

3.5. *Aphids exhibit a prolonged salivation phase during night-time feeding*

To monitor the effect of circadian rhythm on SCA feeding behavior on sorghum plants, we utilized EPG to monitor the SCA feeding behavior. Fig. 7A shows the representative EPG waveform patterns produced by SCA feeding on leaves of sorghum BTx623 plants. The different waveforms patterns obtained for 8 h revealed that SCA displayed similar feeding behaviors during the day and night-times in the pathway phase, xylem phase, sieve-element phase (SEP), and non-probing phase (Fig. 7B). Furthermore, our results did not show significant differences in the time taken by the SCA for the first probe and to reach the first SEP. For the detailed SEP activities, we further analyzed E1 (salivation phase), E2 (phloem-sap ingestion phase) and E1/E2 transition phases, and number of potential drops. Our data reveals that SCA spent significantly more time in the salivation phase during night as compared to daytime feeding (Fig. 7C). The aphid salivation precedes phloem-sap ingestion (E2) (Nalam et al., 2018). However, there was no significant difference in the E2 phase during the night-time feeding by SCA (Fig. 7C). Similarly, E1/E2 transition phase and potential drops also exhibited no significant differences between day and night-time feeding by SCA (Fig. 7C and 7D).

4. Discussion

Our time-series transcriptomics study illustrates the impact of circadian clock disruption on sorghum-aphid interaction dynamics. Additionally, we have also identified the sorghum circadian clock genes and their response during SCA infestation. Most of the sorghum circadian marker genes, *SbTOC1, SbLHY, SbRVE2, SbRVE6, SbLNK1, SbPRR5a, SbFKF1* and *SbJMJ5*, showed circadian oscillation even during the circadian disruption (48 h continuous light). Our results are not



(caption on next page)

Fig. 6. **(A)** Gene ontology (GO) treemap of overrepresented GO terms related to biological process of circadian DEGs of aphid-infested group in Module 3 (M3; 106 genes) made by REVIGO program. Each box represents the $-\log_{10}$ ($P<0.05$) of individual GO terms and bigger size of the box reflects most significant GO terms. Similar functional categories with semantic similarity are represented in similar colored boxes. Heatmap of the relative expression level for the circadian genes related to **(B)** transcription factors and **(C)** primary metabolism and transporters in aphid-infested group of M3. Asterisks in the individual cell represent the significant difference between SCA-infested as compared to the respective control groups for adjusted *P*-value (* < 0.05, ** < 0.01, *** < 0.001). The cells without asterisk are non-significant ($P>0.05$). AP2/B3: Aptela2/B3, CO-like: Constans-like, HSF: Heat shock proteins, bHLH: basic helix-loop-helix protein, bZIP: basic leucine zipper domain, LOG: LONELY GUY, FCR: ferric-chelate reductase, POR: NADPH-ferrihemoprotein reductase, BCP: thioredoxin-dependent peroxiredoxin, PI: peptidylprolyl isomerase, *PAL: phenylalanine ammonia-lyase, SLC35E1: solute carrier family 35, PIP; aquaporin PIP, SULTR3; sulfate transporter 3, MATE: multidrug resistance protein, XPR1: xenotropic and polytropic retrovirus receptor 1, SLC25A23S; solute carrier family 25, ABCG2.PDR: ATP-binding cassette, subfamily G, member 2, PDR.*

surprising because a previous study on cold stress and continuous light on *Arabidopsis* plants exhibited oscillation for the circadian clock genes until 48 h (Bieniawska et al., 2008). Similarly, in our study, both the SCA-infested and control groups showed similar oscillation throughout the 48 h continuous light (Fig. 2B). Notably, these marker genes had a strong expression during the early daytimes (ZT00 to ZT24), either high or low, compared to the second daytimes (ZT24 to ZT48), which may be due to the longer constant light entrainment. A circadian disruption study conducted on *Arabidopsis*, as mentioned above, also showed weaker magnitude of circadian oscillation after 48 h of continuous light (Bieniawska et al., 2008).

The WGCNA analysis of the 2873 DEGs revealed that the gene modules were impacted by SCA infestation during the night-time. Among the 10 modules (Fig. 3B), two modules, M6 and M8, showed higher expression at night-time (Z16-ZT20), whereas two modules, M7 and M10, showed lower gene expression at night-time during SCA infestation compared to control groups. We also observed most differences during the night-time compared to daytime because we disrupted the night-time circadian clock of both plants and aphids by exposing them to continuous light. The sorghum plants that were exposed to both stresses, disruption in circadian rhythms and SCA infestation, potentially adapted by modulating the intrinsic plant metabolisms. For example, the GO terms enriched in M6 and M8 were related to the growth and defense mechanisms (Fig. 4A and B), however, the significant level was higher for the growth metabolism GO terms. In contrast, M7 and M10 that showed suppression of gene expressions in SCA-infested groups were mostly related to the defense mechanism GO term (Fig. 4C and D). The suppression of GO-related defense and higher expression of growth-related GO terms during SCA infestation implies that plants may be able to balance the growth-defense trade-offs during the circadian clock disruption. The growth-defense trade-offs is considered as the most fundamental principle of 'plant economics', which can occur due to biotic and abiotic stresses including light, circadian clock, temperature, nutrients, and microbiome (He et al., 2022).

The RAIN algorithm identified a total of 946 circadian genes in sorghum from 2873 DEGs during SCA infestation. RAIN algorithm is advanced compared to other algorithms, such as COSPOT and JTK_CYCLE, as RAIN can detect strong but asymmetric circadian rhythms in genes that often go unnoticed by other methods (Thaben and Westermark, 2014). Among the 946 circadian genes, 328 belonged to control groups and 319 to SCA-infested groups (Fig. 5A). Surprisingly, most of the GO terms in the SCA uninfested control group were related to response to biotic stress, such as insect, wounding and external stimulus (Fig. 5C). The other defense-related GO terms in this group were related to JA metabolic pathway, which is an important pathway for biotic stress. Previously, it was reported that during biotic stress, JA peaks during the middle of the day and SA peaks during the night to defend the host plant against pests/pathogens (Goodspeed et al., 2012). However, we did not observe diurnal pattern of these genes in the SCA-infested group modules, suggesting that the disruption of the circadian oscillation of defense genes potentially benefits the feeding activity of aphids.

In the M3 SCA-infested group, the circadian genes that displayed higher expression during the night-times were mostly related to MYB TFs, photosynthesis, starch and sucrose metabolism and transporters (Fig. 6). Two MYB genes, *Sobic.004G279300* and *Sobic.009G244300*, showed diurnal regulation with significant upregulation during the night-time (Fig. 6B). The MYB transcription factors regulate phloem-based defenses by producing phloem proteins or through regulating ethylene and lignin metabolism (Biswas et al., 2023). The English grain aphid (*Sitobion avenae*) feeding on wheat identified three MYB genes that co-regulate phloem-based defenses through callose synthesis and mannose-binding lectins (Zhai et al., 2017). Similarly, colonization of the chrysanthemum aphid (*Macrosiphoniella sanborni*) was restricted by chrysanthemum MYB19 through the expression of lignin biosynthesis genes (Wang et al., 2017). In our study, the genes related to primary metabolism displayed a diurnal pattern and showed significant upregulation during the day 1 night-time (Fig. 6C). Previously, it was reported that the plant transporters are responsible for nutrient acquisition, cellular homeostasis and stress response (Li et al., 2021). Additionally, these transporters are a class of membrane protein that mobilize metabolites, sucrose and nutrients during growth and stress responses (Larsen et al., 2017). The three transporters identified in this study, *PIP, SULTR3* and *SLC25A23S*, were significantly upregulated in the night-time during SCA feeding (Fig. 6C). We also observed disruption of circadian genes related to defense and upregulation of growth-related circadian genes, photosynthesis, starch and sucrose metabolism and transporters during the night-time. Taken together, these observations suggest that SCA feeding potentially suppressed the plant defenses during the night-time and manipulated the host plant to invest its energy for growth mechanisms, further supporting our hypothesis related to growth-defense trade-offs.

Our feeding behavior analysis revealed that SCA feeding on sorghum plants during the night-time exhibited a longer aphid salivation phase (E1) (Fig. 7C). A similar response has been recorded when bird cherry-oat aphid (*R. padi*) fed on wheat, where aphid took significantly longer time in the E1 phase during the night-time (Nalam et al., 2021). During salivation, aphid secretes the watery saliva, enriched with salivary proteins, peroxidases, pectinases, carbohydrates, phospholipids, and enzymes that can modulate plant defenses (Cherqui and Tjallingii, 2000). In addition, aphid saliva consists of "effectors" that can facilitate the infestation by suppressing the plant defense responses and reallocating sugars (Elzinga and Jander, 2013; Goodspeed et al., 2012). Thus, longer salivation periods may be required during night-time to suppress these plant defense traits activated as part of the innate plant diurnal rhythm (Goodspeed et al., 2012; Jander, 2012). Further support for this observation was obtained from the transcriptomics data, where one of the circadian gene, phloem protein 2 (*PP2, Sobic.003G062500*) and two MYB TFs (*Sobic.006G199800* and *Sobic.008G055800*) (Table S10) showed higher expression during the night-times (ZT16, ZT20) compared to the daytime after SCA infestation. *PP2* gene is conserved as a phloem lectin in plants, which binds to carbohydrates in the insect gut and interfere with physiological processes (Cristofoletti et al., 2006; Vandenborre et al., 2011). The PP2-A1 from *Arabidopsis* when added to a synthetic diet impacted the green peach aphid (*Myzus persicae*) and soybean aphid (*Aphis gossypii*) weight gain (Beneteau et al., 2010). Similarly, PP2-A1 impacted the *A. gossypii* colony size and reproduction rate in cucumber plants (Li et al., 2023). The MYB TFs provide phloem-based defense by producing phloem proteins or through ethylene and lignin metabolisms as mentioned before. In contrast to E1

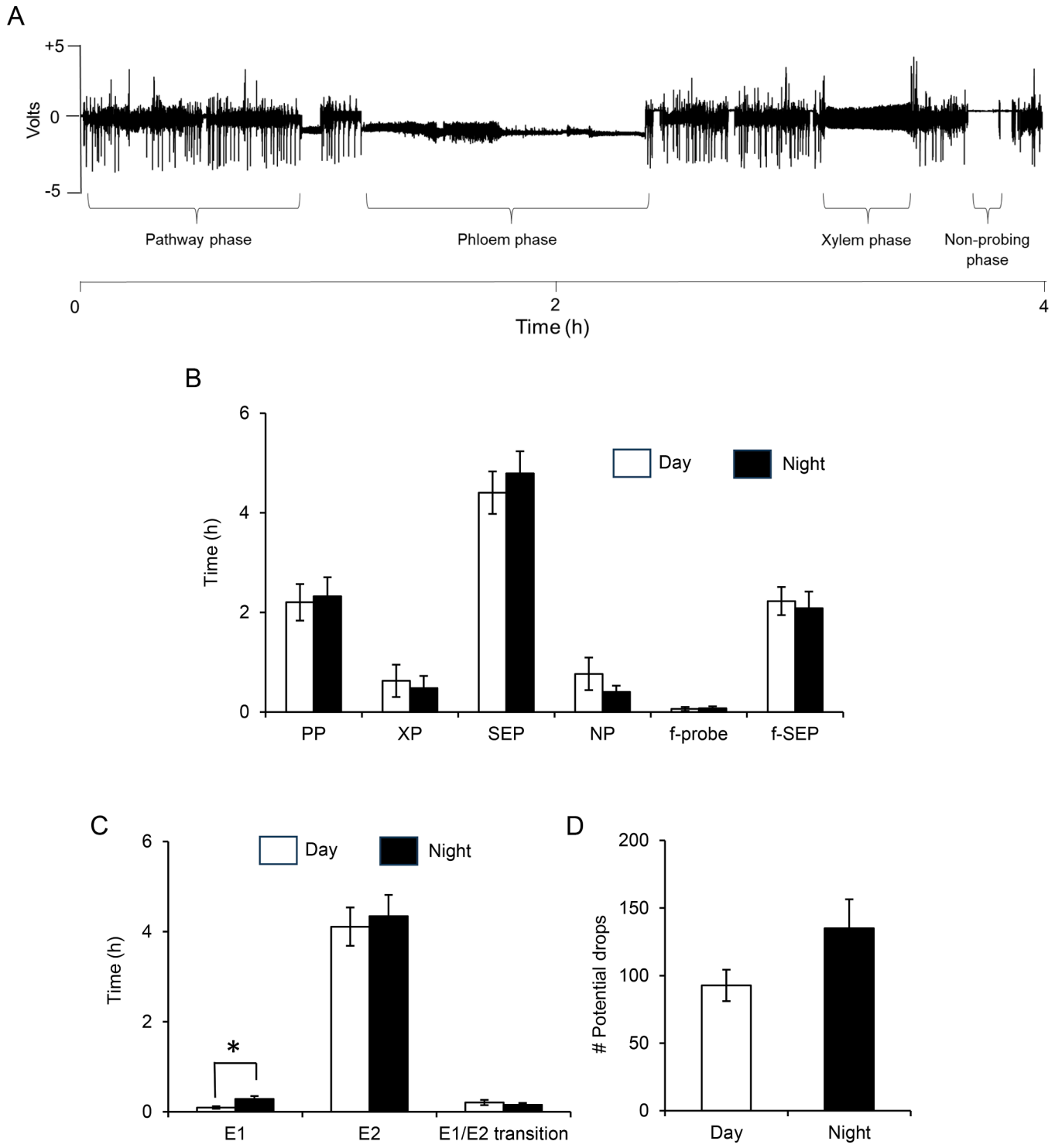


Fig. 7. **(A)** Representative Electrical Penetration Graph (EPG) waveform patterns obtained when sugarcane aphid (SCA) feeds on leaves of sorghum BTx623 plants. The different patterns represent different phases of aphid probing on the sorghum plant. **(B)** EPG monitoring of mean time spent by SCA for various feeding behavior activities on sorghum plants during day and night-times. PP, pathway phase; XP, xylem phase; SEP, the total duration of sieve element phase; NPP, non-probing phase; f-probe, the time to first probe in the plant; f-SEP, the time to reach first SEP. **(C)** E1, aphid salivation phase; E2, phloem sap ingestion phase; E1/E2 transition, a combination of E1 and E2 phases. **(D)** Number of potential drops. Each value is the mean ± SE of 16–17 replications. An asterisk (*) represents significant difference ($P<0.05$; Kruskal–Wallis test) in the time spent by SCA for the indicated activity on the sorghum plants during different phases.

phase, the phloem-sap ingestion phase (E2), an important aphid feeding phase, displayed no significant difference during the diurnal feeding.

Overall, our study demonstrates that circadian clock disruption impacts the sorghum-aphid interaction dynamics. The time-series transcriptomics study revealed that SCA feeding modulates the plant responses during the night-time by suppressing the expression of defense genes. As a counter mechanism, the host plant mobilizes its energy and metabolism for primary growth mechanisms, thus supporting our hypothesis related to growth-defense trade-offs. Further studies are needed to understand the mechanism(s) by which the aphids suppress plant

defenses, for example, salivary effector(s) contributing to suppression of plant defenses, and how plants balance growth-defense trade-offs and regulate circadian rhythms.

CRediT authorship contribution statement

Kumar Shrestha: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Prince Zogli:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Lise Pingault:** Writing – review & editing, Methodology, Formal analysis. **Sajjan Grover:** Writing – review & editing, Methodology, Formal analysis. **Juan Betancurt Cardona:** Writing – review & editing, Methodology, Formal analysis. **Joe Louis:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Supplementary materials

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