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RESEARCH ARTICLE

Transcriptomic analysis of the defense response in "Cabernet Sauvignon" grape leaf induced by *Apolygus lucorum* feeding

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Abstract

To investigate the molecular mechanism of the defense response of "Cabernet Sauvignon" grapes to feeding by *Apolygus lucorum*, high-throughput sequencing technology was used to analyze the transcriptome of grape leaves under three different treatments: feeding by *A. lucorum*, puncture injury, and an untreated control. The research findings indicated that the differentially expressed genes were primarily enriched in three aspects: cellular composition, molecular function, and biological process. These genes were found to be involved in 42 metabolic pathways, particularly in plant hormone signaling metabolism, plant-pathogen interaction, MAPK signaling pathway, and other metabolic pathways associated with plant-induced insect resistance. Feeding by *A. lucorum* stimulated and upregulated a significant number of genes related to jasmonic acid and calcium ion pathways, suggesting their crucial role in the defense molecular mechanism of "Cabernet Sauvignon" grapes. The consistency between the gene expression and transcriptome sequencing results further supports these findings. This study provides a reference for the further exploration of the defense response in "Cabernet Sauvignon" grapes by elucidating the expression of relevant genes during feeding by *A. lucorum*.

KEYWORDS
Apolygus lucorum, Cabernet Sauvignon, defense response, differentially expressed gene, metabolic pathway, transcriptome sequencing

1 | INTRODUCTION

"Cabernet Sauvignon" (*Vitis vinifera* cv.) is one of the main varieties cultivated in China's wine-producing regions (Wei et al., 2022). As a significant agricultural pest in China (Pan et al., 2013), *Apolygus lucorum* (Meyer-Dür) has caused increasing harm to wine and grape cultivation areas in recent years, becoming one of the most important control targets for grapes (Pan et al., 2021). Currently, most of the cultivation areas use broad-spectrum chemicals to kill *A. lucorum* (Stevens et al., 2012), which pollutes the environment and also leaves residue that is harmful to humans and other animals (Wangari Nderitu

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et al., 2020). In the long term, the effective way to fundamentally limit the damage caused by *A. lucorum* is to improve the resistance of crops to pests, such as by identifying resistance genes and cultivating new resistant varieties (Liu et al., 2019). Currently, the exploration of plant-induced insect resistance formation and the optimization of plant defense against pests have become relevant to the research and practice of ecological regulation in agriculture and forestry (Kumari et al., 2022).

Numerous studies have demonstrated that plants exhibit resistance to insect feeding through physical factors as well as physiological and biochemical changes. In response to invasion by insects, bacteria, and other pathogens, plants have developed a complex defense system by undergoing material metabolism reactions (Barah et al., 2013; Gong et al., 2020; Jiang et al., 2019). Generally, induced resistance in plants can only reduce the level of damage or increase their tolerance within a certain range. The extent of induced resistance is directly proportional to the intensity of the stimulation. Thus, the stronger the stimulation, the greater the induced resistance within a certain range. After reaching the peak, the resistance of plants tends to either remain unchanged or decrease (Sobral et al., 2021). Plant defense responses to insect feeding are triggered by elicitors contained in insect oral secretions (Shinya et al., 2016), and specific elicitors trigger different levels of plant defense responses (Acevedo et al., 2018). Among the phloem-feeding insects, the most representative aphid uses piercing mouthparts to ingest phloem sap, and its saliva can interfere with plant defenses (Will et al., 2007). Studies have shown that *A. lucorum* disturbs the cellular processes of plants during feeding through glutathione peroxidase (Dong et al., 2020), and the polygalacturonase in its salivary enzyme may also cause damage to plants (Liu et al., 2021). In recent years, RNA sequencing (RNA-seq) technology has been increasingly used to comprehensively investigate the expression and functionality of plant genes in response to insect pest stress (Kiani et al., 2021; Qiu et al., 2017), especially in the identification of resistance of different cotton varieties to *A. lucorum* (Chen et al., 2022; Livak & Schmittgen, 2001; Niu et al., 2020). Using RNA-seq technology, it was found that the jasmonic acid (JA) pathway played a major role in the defense of *Bacillus thuringiensis* (*Bt*) cotton against the *A. lucorum*, and it was further confirmed that the salicylic acid (SA) pathway did not play a significant role in the defense of *A. lucorum* after feeding and even showed an inhibitory effect. A series of genes related to the biosynthesis of phenylpropanoid, α-linolenic acid (LA), flavone, and the pathway of plant hormone signal transduction may be involved in the regulation of Bt cotton to the defense response of *A. lucorum* (Chen et al., 2022). Silencing of the *LIM* gene in cotton results in strong resistance to *A. lucorum* (Liang et al., 2021). These studies provide insight into the resistance levels of various host plants against *A. lucorum*, as well as the selection and adaptation mechanisms of *A. lucorum* to different host plants (Wang et al., 2019). However, it is important to note that the defense response of plants to insect feeding is a complex process that varies depending on the plant and insect species and the type of feeding stimulus (Chen et al., 2017; Zhang et al., 2022).

To date, there have been no reports on the transcriptome sequencing and analysis of grapes after predation by *A. lucorum*. This study used RNA-seq technology to systematically reveal the defense response mechanism of "Cabernet Sauvignon" grapes to damage by *A. lucorum*. Bioinformatics analysis on all differentially expressed genes was conducted to clarify the defense response molecular mechanism of "Cabernet Sauvignon" grapes and determine the response of the key functional genes of *A. lucorum*. The findings should provide a theoretical basis for the molecular breeding based of insect-resistance genes.

2 | MATERIALS AND METHODS

2.1 | Test materials

The "Cabernet Sauvignon" grape seedlings were provided by Langes Winery (Qinhuangdao) Co., Ltd. The potted cuttings were cultivated, and only the new shoots were kept. The green *Lygus* was provided by the Institute of Plant Protection, Hebei Academy of Agriculture and Forestry Sciences (T:26°C, L:D = 16:8 h, RH: 60–70%) and was fed with fresh green beans to obtain the test population. "Cabernet Sauvignon" grape seedlings exhibiting similar growth in the same year were selected as standard test plants. The test was divided into two treatments: *A. lucorum* infestation (AI) and puncture injury (PI), and the leaves of healthy and normal untreated plants in the same batch were used as the control check (CK). Samples were taken 4 days after treatment. See Table S1 for sample information.

2.1.1 | *Apolygus lucorum* infestation (AI)

Healthy "Cabernet Sauvignon" grape plants were selected, and five third-instar nymphs from *A. lucorum* were placed on the tender leaves of the stem tip. The plants were then covered with insect-proof nets, and the insect net was removed after allowing them to feed for 4 days.

2.1.2 | Puncture injury (PI)

Healthy plants exhibiting the same size and growth as the damaged plants were pricked with an insect needle 0 about 100 times on the back of the grape stem tip and tender leaves, following which they were covered with an insect net. The insect net was removed after 4 days.

2.1.3 | Control check (CK)

The tender leaves of healthy plants exhibiting the same size and growth trend as the damaged plants were covered at the stem tip with insect prevention nets, which were removed after 4 days.

2.1.4 | Sampling process

Three pieces of young leaves from the top to the bottom of each test plant were collected, wrapped in tinfoil, quenched with liquid nitrogen, and frozen at −80°C for further experimentation. Three replicates were tested for each test plant.

2.2 | Total RNA extraction method

The total RNA was extracted from the "Cabernet Sauvignon" grape leaves using Invitrogen's TRIzol® Reagent Kit following the kit instructions. The RNA degradation and contamination were monitored on 1% agarose gels. The quality of the extracted RNA samples was evaluated using a NanoDrop2000. Only high-quality RNA samples ($OD_{260/280} = 1.8–2.2$, $OD_{260/230} \geq 2.0$, RIN ≥ 8.0, 28S ≥ 1.0, >1 μg) were used to construct a sequencing library.

2.3 | Construction and sequencing of the library

After the sample RNA was tested, the Illumina NovaSeq6000 sequencing platform was used to sequence the mRNA of nine grape leaf test samples. The Illumina TruSeq™ RNA sample preparation Kit was used to construct a library for subsequent data analysis.

2.4 | Quality control and read mapping

To ensure the accuracy of subsequent biological information analysis, the original sequencing data were first filtered to obtain high-quality sequencing data (clean data) to ensure the integrity of subsequent analyses. The specific steps and sequence are as follows: (1) remove reads from adapter sequences, and remove reads that do not have inserted fragments due to self-ligation of the adapter. (2) Trim low-quality (quality value less than 20) bases at the end of the sequence (3′ end) if there are still quality values less than 20 in the remaining sequence. If there are 10 bases, the entire sequence will be removed; otherwise, it will be retained. (3) Remove reads containing N (module bases). (4) Discard sequences less than 30 bp in length after adapter and quality trimming. The raw paired-end reads were trimmed and quality controlled using fastp (http://github.com/OpenGene/fastp) with default parameters. The clean data (reads) obtained after quality control were compared with the reference genome to obtain mapped data (reads) for subsequent transcript assembly and expression calculations. Additionally, the transcriptome sequencing results were compared. The quality assessment of the results primarily involved evaluating sequencing saturation, gene coverage, and the distribution of reads in different regions of the reference genome and analyzing the distribution of reads across various chromosomes. The clean reads were separately aligned to the reference genome in orientation mode using HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml) software. The mapped reads of each sample were assembled with StringTie (http://ccb.jhu.edu/softwara/stringtie/) using a reference-based approach.

2.5 | Differentially expressed gene (DEG) analysis

The grape genome database (http://plants.ensembl.org/Vitisvinifera/Info/Index) was used as the reference sequence, and the reference genome version was IGGP-12x. The expression level of a gene was calculated from the reads count of the clean reads that were mapped to the genomic region. The software RSEM (http://deweylab.github.io/RSEM/) was used to quantitatively analyze the expression levels of genes and transcripts for subsequent analysis of the differential expression of transcripts between different samples. Using DESeq2 software (http://bioconductor.org/packages/stats/bioc/DESeq2/), the DEGs between groups were analyzed and compared based on the conditions of $p < .05$ &|log2FC| ≥ 1. The multiple test correction method BH was used, whereby the multiple difference between upregulation and downregulation was ≥2, serving as the screening criterion for DEGs in this experiment. At different levels (|log2FC| ≥ 2, |log2FC| ≥ 5), functional genes related to insect resistance in the "Cabernet Sauvignon" grape leaves were identified through screening conditions such as gene function, expression level, and expression differences. Goatools software was used to perform Gene Ontology (GO, http://www.geneontology.org/) enrichment analysis on the DEGs in gene sets using Fisher's exact test. To control the calculated false positive rate, four multiple test methods (Bonferroni, Holm, BH, and BY) were used to correct the *P* value. The corrected *P* value was set at a threshold of .05, indicating significant enrichment of this GO function. KOBAS (http://kobas.cbi.pku.edu.cn/home.do) was used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and the principle GO functional enrichment analysis was calculated. The GO and KEGG tables were selected for different groups, as well as the corresponding difference result tables. Biological process (BP), cellular component (CC), and molecular function (MF) were not distinguished at the graph level. The different groups were selected, and the significance level was set to $p \leq .05$, with only the top 10 enriched GO terms or KEGG pathways shown. Note that if the total number of genes in the selected GO term/KEGG pathway exceeded 200, the top 10 genes with significant differences (|log2FC|) in each GO term/KEGG pathway were selected for plotting.

2.6 | Real-time quantitative PCR (RT-qPCR) analysis

In the annotated KEGG database, 20 key DEGs, including *ACS6* and *calcium-dependent protein kinase 25* (*CPK25*), were selected from the three metabolic pathways of the hormone signal transmission pathway, plant-pathogen interaction pathway, and MAPK signaling pathway. *Actin* and *GAPDH* were selected as grape reference genes based on the literature, and the primer sequences of the reference genes were synthesized by Shanghai Biotechnology Company.

Reliability verification was performed using qRT-PCR. Table S2 shows the primers. A 96-well plate was used for the RT-qPCR reaction. Tables S3–S5 show the reaction system for the mRNA reverse transcription to cDNA. The reaction conditions were set to 37°C for 15 min, 85°C for 5 s, and then cooling to 4°C. The amplification conditions were set to 95°C for 3 min; 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s for 35 cycles; and then 72°C for signal detection. The $2^{-(\Delta\Delta Ct)}$ algorithm was used to analyze the gene expression of different samples. Multiple differences in the expression levels of the selected target genes in the RT-qPCR quantitative experiment were calculated under different treatments, and Excel (Microsoft Corp., Redmond, WA, United States) was used to analyze the correlation between the RT-qPCR and RNA-seq sequencing results.

3 | RESULTS

3.1 | Total RNA quality analysis and sequencing quality analysis

The 28S and 18S bands of RNA extracted from nine "Cabernet Sauvignon" grape leaf samples were clearly visible and intact (Figure 1a). The OD_{260}/OD_{280} value should be between 2 and 2.2, indicating the purity of the sample. Similarly, the OD_{260}/OD_{230} value should mostly be greater than 1.5, indicating the absence of contaminants. Additionally, the RIN value should be greater than 6.0, indicating the integrity of the RNA (Table S6). The results showed that the RNA extracted from the nine samples reached level B or above, and the quality met the requirements for cDNA library construction. The Illumina Nova Seq6000 sequencing platform was used to sequence the transcriptome of the AI, PI, and CK samples, and a total of 64.26 Gb clean data were obtained. The Q30 base percentage of each product was more than 92%, and the GC content was more than 46%. This indicates that the sequencing results are good and that the gene library data are reliable, meeting the requirements for subsequent data assembly and bioinformatics analysis.

3.2 | Analysis of DEGs

The PCA analysis results (Figure 1b) showed that the gene expression profile of AI was significantly different from those of PI and CK in terms of principal components, forming an independent population, while PI and CK grouped in terms of their gene expression. After screening ($|\log2FC| \geq 1$), a total of 938 DEGs were obtained between AI and CK, of which 762 were upregulated and 176 were downregulated. A total of 973 DEGs were identified through the comparison of PI and CK, of which 432 were upregulated and 541 were upregulated. A total of 1,603 DEGs were obtained between AI and PI, of which 1,008 were upregulated and 595 were downregulated. There were 58 DEGs shared among the three treatments. These genes play an important role in the defense response of "Cabernet Sauvignon" grape leaves to *A. lucorum* predation and may be involved in multiple biological metabolic pathways and processes such as protective enzyme regulation, hormone signal transduction, protein ubiquitination, phosphorylation, primary and secondary metabolism, and the stress response. Therefore, further research is needed.

A volcano plot was generated to analyze the differential gene expression among the three treatments (Figure 1c). The plot revealed that, in comparison to CK, AI and PI exhibited more pronounced changes in gene expression. Specifically, the number of upregulated genes was significantly higher than that of downregulated genes in the two treatment groups. Moreover, the change in gene expression range for PI was considerably smaller than that of AI, with a relatively equal number of upregulated and downregulated genes observed in PI. The molecular responses of the grape leaves to *A. lucorum* feeding and puncture injury were significantly different. This suggests that "Cabernet Sauvignon" grape exhibits specific defense responses when exposed to *A. lucorum* feeding and puncture injury, though there are notable variations in their defense mechanisms.

Cluster analysis was conducted at different levels for the gene set of interest, analyzing the highly significant DEGs among AI, PI, and CK (Figure 2a). The biological replicates of each of the three treatments clustered into one category, with the expression patterns of the control and puncture damage treatment being more similar, while the gene expression patterns of the leaves subjected to *A. lucorum* feeding were significantly different from the other two categories. This conclusion is consistent with the results of the PCA analysis. There was a strong response at the gene level, with most DEG expression levels upregulated compared to the control. When Sum = 3, all but one gene showed upregulated expression (Figure 2b). When $|\log2FC| \geq 5$, all DEGs showed upregulation (Figure 2c; Table S7).

Following annotation using multiple databases, it was observed that the gene products involved in different metabolic pathways exhibited significant variation. This study primarily investigated DEGs associated with resistance pathways, such as oxidative stress defense, environmental adaptability, information transduction, and secondary matter metabolism. A total of 29 DEGs, including 14 named genes, were the main focus of this experiment (Table S8). These genes have been previously validated to have close associations with defense mechanisms, aging, and stress tolerance. To identify relevant resistance genes, this study specifically examined plant-pathogen interactions, plant hormone signal transduction, MAPK kinase plant signaling pathways, and biosynthetic metabolic pathways of secondary metabolites.

3.3 | Functional and enrichment analysis of DEGs

Using BLAST2GO software and $|\log2FC| \geq 2$ as the screening condition, GO functional analysis was performed on the DEGs among the three treatments. Overall, the DEGs between the three treatments were mapped to a total of 42 GO functional pathways in BP, MF, and CC functional classifications (Table S9), and the number of genetic changes in grape leaves subjected to feeding by *A. lucorum* was the highest. However, the effect of puncture injury on the grape leaves at

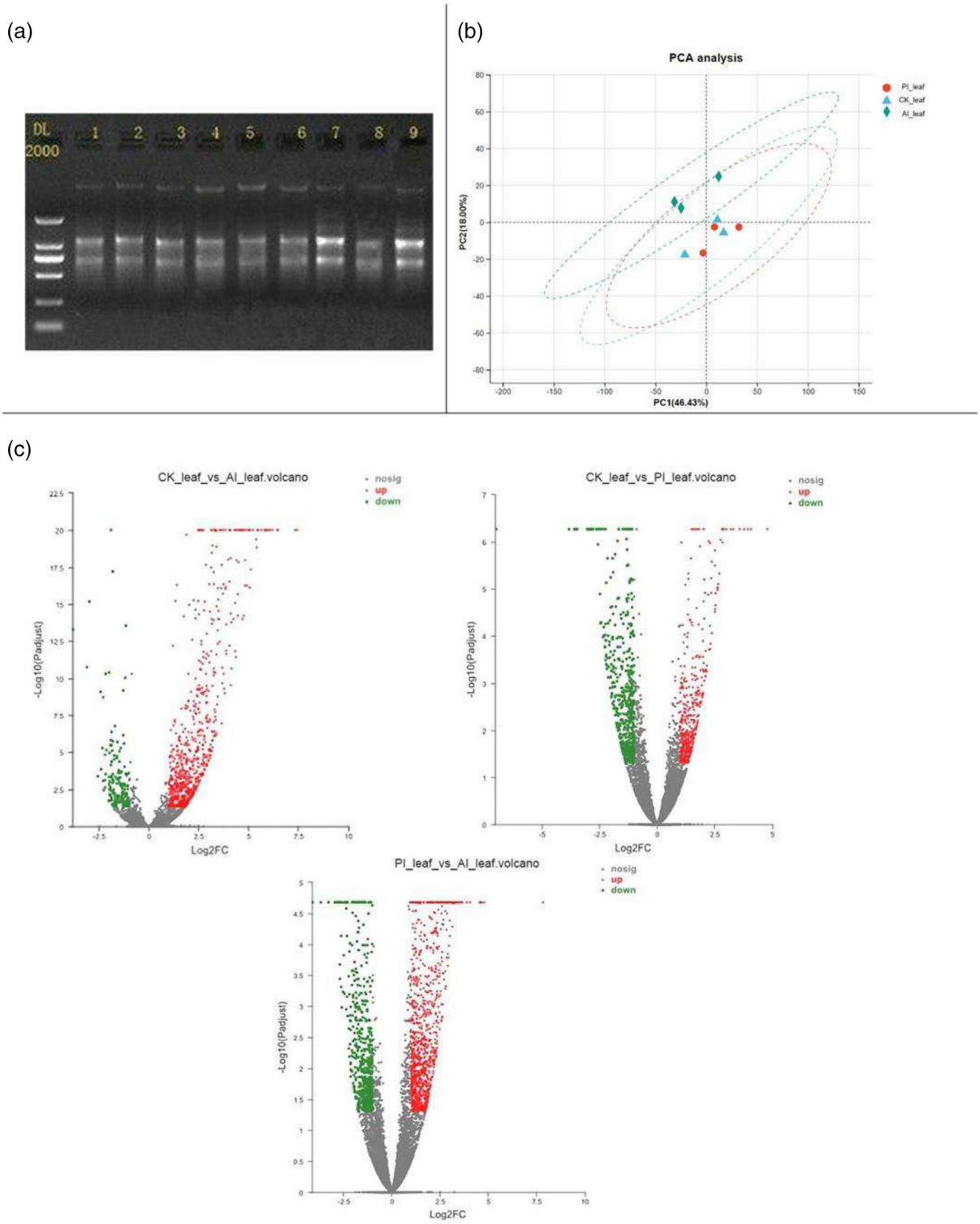

FIGURE 1 RNA quality analysis on nine "Cabernet Sauvignon" grape leaf samples and analysis of differentially expressed genes (DEGs) among the three treatments. (a) RNA quality detection showing clear and complete bands for all nine samples. (b) PCA analysis was performed to examine the differential genes among the three treatments. (c) Volcano maps were created to visualize the DEGs across the different treatments.

the molecular level was relatively small. In the functional classification of biological processes, nine pathways were collectively enriched into the secondary functional classification (Figure 3a, Lev2). Among them, most DEGs were enriched through metabolic processes, with the AI treatment reaching 158 DEGs. The PI treatment only enriched 28 DEGs, with significant differences. The second category was cellular process, with AI treatment enriching 131 DEGs. The PI treatment enriched 26 DEGs, with significant differences. In the single

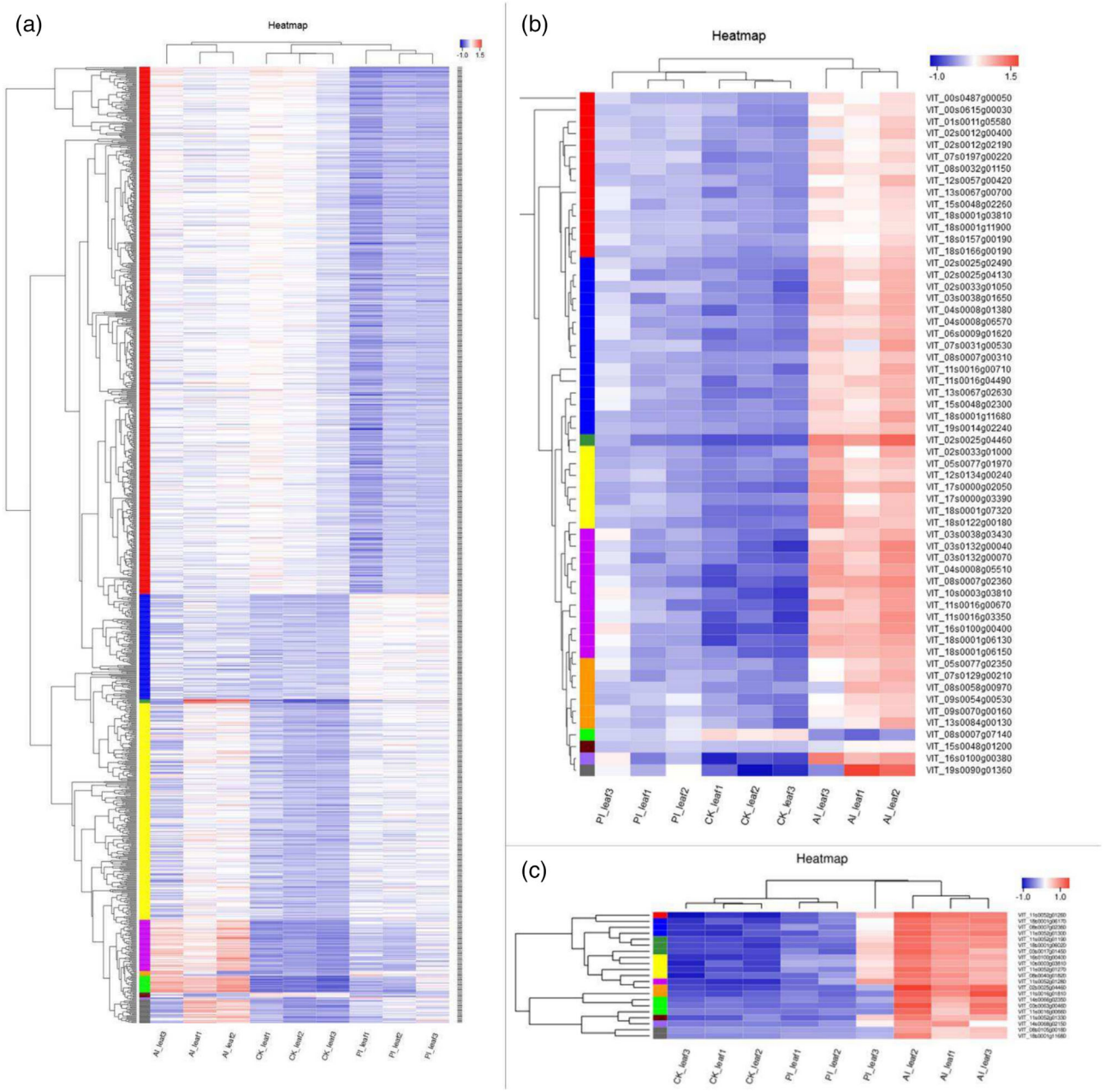

FIGURE 2 Cluster analysis was conducted on differentially expressed genes (DEGs) using the K-means algorithm. (a) Clustering pattern analysis of DEGs(p adjust < .05). *Note*: Red represents upregulated genes, and blue represents downregulated genes. (b) Clustering pattern analysis of DEGs when Sum = 3. (c) |Log2FC| ≥ 5 pairs of screened DEGs cluster analysis with a difference of more than 5

organization process category, the AI treatment enriched 81 DEGs, while the PI treatment only enriched 19 DEGs, with significant differences detected between the two treatments. In this functional category, there was a higher occurrence of DEGs involved in metabolic biological processes such as detoxification, reproduction, and negative biological regulation in the AI treatment compared with the PI treatment. This suggests that insect-feeding stimulation triggers the activation of breeding, reproduction, detoxification, and antioxidant processes in "Cabernet Sauvignon" grape leaves. These processes are influenced by specific genes, such as the *PAD4* gene, which negatively regulates biological processes in "Cabernet Sauvignon" grapes.

Similarly, in the categories of CC and MF, the number of DEGs under the PI treatment differed significantly from AI. From the graph, it can be seen that feeding by *A. lucorum* induced a strong response in the leaves at the cellular level, which led to a series of phenotypic changes in the grapes, such as leaf shrinkage, color deepening, and aging, to resist insect feeding. The *ATRBOHB* homologous gene, which is the focus of this experiment, is known for its differential expression in the antioxidant activity functional category. This gene family, known as NADPH oxidase mutant, is primarily responsible for producing reactive oxygen species (ROS), which are an injury signal and have been found to play a crucial

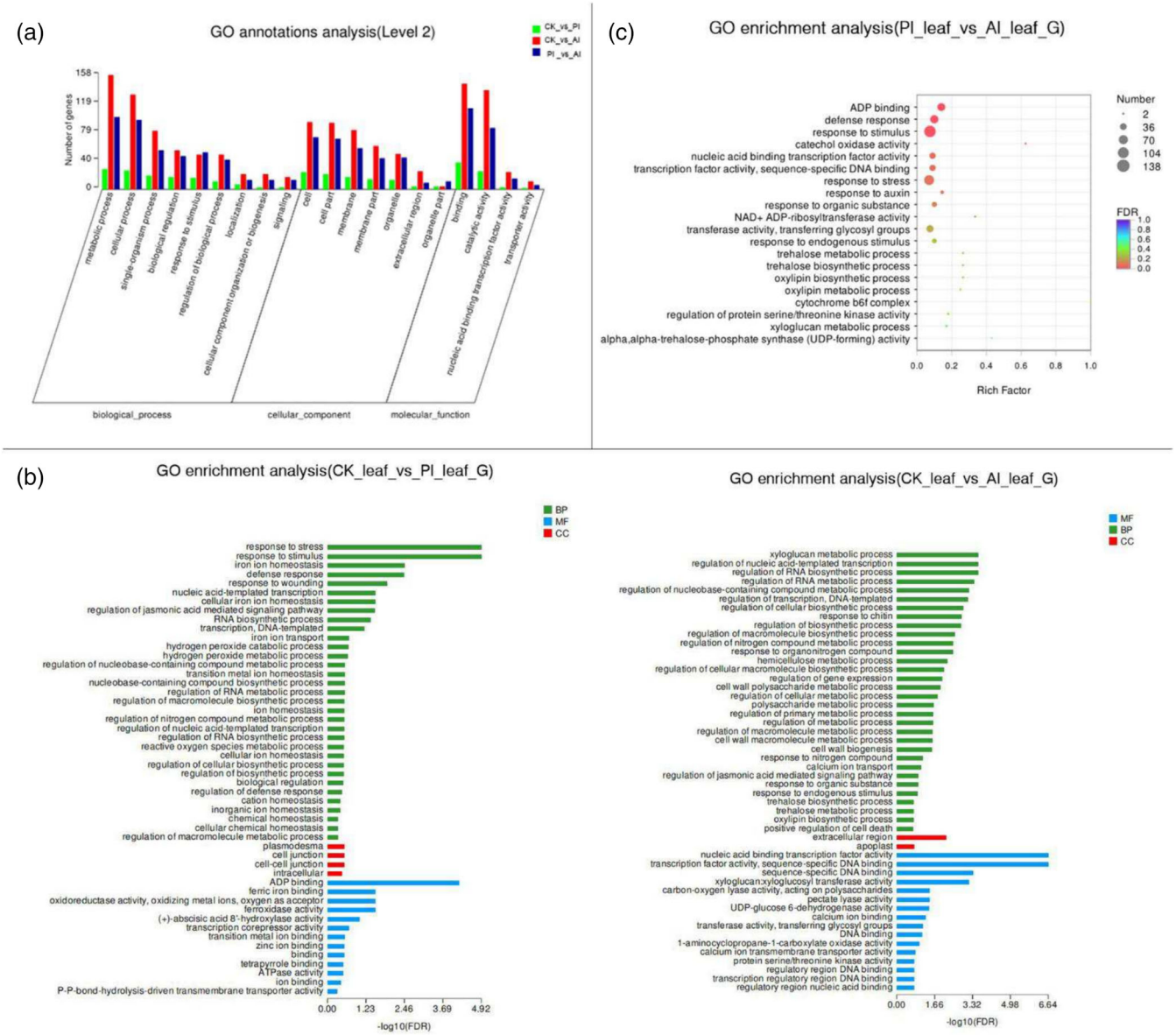

FIGURE 3 GO functional classification and metabolic pathway enrichment. (a) GO annotation of differentially expressed genes (DEGs) in various treatments (|Log2FC| ≥ 2). *Note*: The *x* axis represents the functional categories of GO, and the *y* axis represents the number of genes associated with each function. (b) GO enrichment analysis of DEGs between the PI and CK treatments and the AI and CK treatments. (c) GO enrichment analysis of DEGs between the AI and PI treatments

role in various signal transduction and metabolic pathways (You & Chan, 2015).

Through GO functional enrichment analysis of the DEGs among the AI, PI, and CK treatments (|log2FC| ≥ 1), it was found that the DEGs between the feeding and puncture injury treatments of *A. lucorum* were enriched in different metabolic pathways in the GO database. Among the first 20 pathways with an abundance of DEGs between PI and CK (Figure 3b), the defense response induced by acupuncture injury may be initiated by JA and hydrogen peroxide as signaling substances. It is worth noting that multiple pathways involving iron ions were also mapped, including iron homeostasis, intracellular iron, ferric iron binding, iron oxidase activity, and iron ion transport. The DEGs between the AI and CK treatments were enriched in the GO database, and the metabolic pathways were significantly different from those between PI and CK. Notably, there was high enrichment significance in 44 nucleic acid-binding transcription factor genes, 44 DNA sequences specific to binding genes, and 10 xyloglucan metabolism genes. Previous studies have demonstrated the close relationship between the xyloglucan pathway and induced resistance in plants (Claverie et al., 2018). The significant enrichment of these metabolic pathways shows that the induced metabolic pathway of the feeding treatment was completely different from that of the puncture injury. Nucleic acid transcription and synthesis were induced in the "Cabernet Sauvignon" grape leaves,

participating in the process of large fraction biosynthesis and regulating the metabolism of xyloglucan, organic nitrogen compounds, and hemicellulose. From the perspective of signal pathways, feeding by *A. lucorum* also induced the regulation of the JA-mediated signal pathway. The enrichment of metabolic pathways such as calcium ion transport, calcium ion binding, and calcium ion transmembrane transport protein activity also indicated the significant role of calcium ion signaling in the entire induction event and in the transport of carrier binding. The DEGs related to secondary metabolic pathways that can produce toxic or insect-resistant metabolites include nitrogen-containing compound response, trehalose synthesis and metabolic processes, lipid oxide synthesis, UDP glucose-6-dehydrogenase activity, ACC oxidase activity, and STK kinase activity.

The DEGs of AI and PI were found to be significantly enriched in various metabolic pathways associated with plant resistance responses (Figure 3c). Among the top 20 enriched pathways, the DEGs were predominantly involved in response to external stimuli, defense responses, ADP binding biological pathways, and stress response, with highly significant differences.

Based on the above analysis, it can be seen that the response modes of "Cabernet Sauvignon" grapes in the AI and PI treatments were significantly different. Feeding by *A. lucorum* induced the histiocytes of "Cabernet Sauvignon" grape leaves to react violently and produce signaling substances to initiate growth and defense response to the invasion. However, physical damage is manifested in the regulation of ion balance in the body by leaf tissue, especially the involvement of calcium ions in multiple metabolic pathways, which is worthy of attention.

Using the KEGG database, the DEGs in the three treatment groups were assessed under the condition of |log2FC| ≥ 2 (Figure 4a). The metabolic pathway analysis revealed that the DEGs under AI were significantly associated with five functional categories: metabolism, genetic information processing, environmental information processing, cellular processes, and biological systems. Among these, metabolic processes were most mapped, with a total of nine types mapped. By contrast, the DEGs under PI were classified into four functional categories, which did not include any cellular processes. Additionally, only five categories in the metabolic process were mapped, resulting in a significant reduction in the number of DEGs in each functional category.

The response of "Cabernet Sauvignon" grape leaves to insect bite and puncture injury differed significantly, particularly in genes related to energy release and volatile terpenes. These genes were not observed to be activated in response to puncture injury, suggesting that "Cabernet Sauvignon" grapes exhibit distinct responses to different types of damage. The findings from the KEGG functional analysis align with the results obtained from the GO functional analysis, indicating that the response of "Cabernet Sauvignon" grape leaves to *A. lucorum* infestation primarily involves metabolic processes.

To assess the effect of the exposure of "Cabernet Sauvignon" grape leaves to feeding by *A. lucorum*, GO enrichment chord plots and KEGG enrichment chord plots were generated for DEGs among the AI, PI, and CK treatments (Figure 5, |log2FC| ≥ 2). The results indicated that, compared with the CK treatment, 55 DEGs were annotated to the top 10 metabolic pathways by the GO database in the AI treatment (Figure 5a). Among these, 51 DEGs were significantly upregulated, and three DEGs were significantly downregulated. Similarly, the KEGG database annotated a total of 56 DEGs in the AI treatment, which were enriched in six KEGG pathways, namely, plant hormone signal transduction, plant-pathogen interaction, MAPK plant signaling pathway, PPG biosynthesis, fatty acid elongation response, and linoleic acid metabolism (Figure 5d). Out of these 56 DEGs, 54 were significantly upregulated, and the remaining two showed significant downregulation. On the contrary, in the PI treatment, only eight DEGs were annotated to the top 10 metabolic pathways by the GO database (Figure 5b). Among these eight DEGs, two were significantly downregulated, and six were significantly upregulated. Similarly, the KEGG database annotated seven DEGs in the PI treatment, which were enriched in cutin, imine, and wax biosynthesis and the PPG biosynthesis KEGG pathway (Figure 5e). Out of these seven DEGs, five were significantly upregulated, and two were significantly downregulated. Overall, the comparison revealed that the molecular response triggered by the stress of puncture injury in "Cabernet Sauvignon" grapes was much simpler than the response caused by the feeding stimulus of *A. lucorum*. Between the AI and PI treatment, a total of 64 DEGs were annotated by the GO database to the top 10 metabolic pathways (Figure 5c). Out of these 64 DEGs, 49 were upregulated, and 15 were significantly downregulated. Additionally, the KEGG database annotated 29 DEGs, which were enriched in various pathways including plant-pathogen interaction, stilbenoid, diarylheptanoid and gingerol biosynthesis, plant hormone signal transduction, flavonoid biosynthesis, circadian rhythm-plant, and tyrosine metabolism (Figure 5f). Among these 29 DEGs, 23 were significantly upregulated, and 6 were significantly downregulated in a total of six KEGG pathways. These findings indicate that "Cabernet Sauvignon" grapes exhibit distinct molecular responses to the two treatments.

By conducting KEGG enrichment analysis on the DEGs obtained from pairwise comparisons of each treatment (|log2FC| ≥ 2, Table S10), it can be seen that the enrichment of DEGs in the KEGG metabolic pathway was significantly different between the three treatments. Table S10 shows 42 pathways enriched with DEGs among the three treatment groups, with significant mapping to the map 04075 pathway of 19 DEGs, the map 04626 pathway of 18 DEGs, and the map 04016 pathway of seven DEGs. The DEGs induced by AI were significantly enriched in plant hormone signal transduction (map 04075), plant-pathogen interaction (map 04626), and MAPK signaling pathway-plant (map 04016), whereas the DEGs induced by PI were only significantly enriched in carotenoid biosynthesis (map 00906). The DEGs between the AI and PI treatments were mainly enriched in the plant hormone signal transduction (map 04075) and plant-pathogen interaction (map 04626) metabolic pathways, while four DEGs were enriched in the MAPK signaling pathway (map 04016).

In the plant hormone signaling pathway (map 04075, Figure 6a), there were 543 annotated genes categorized into

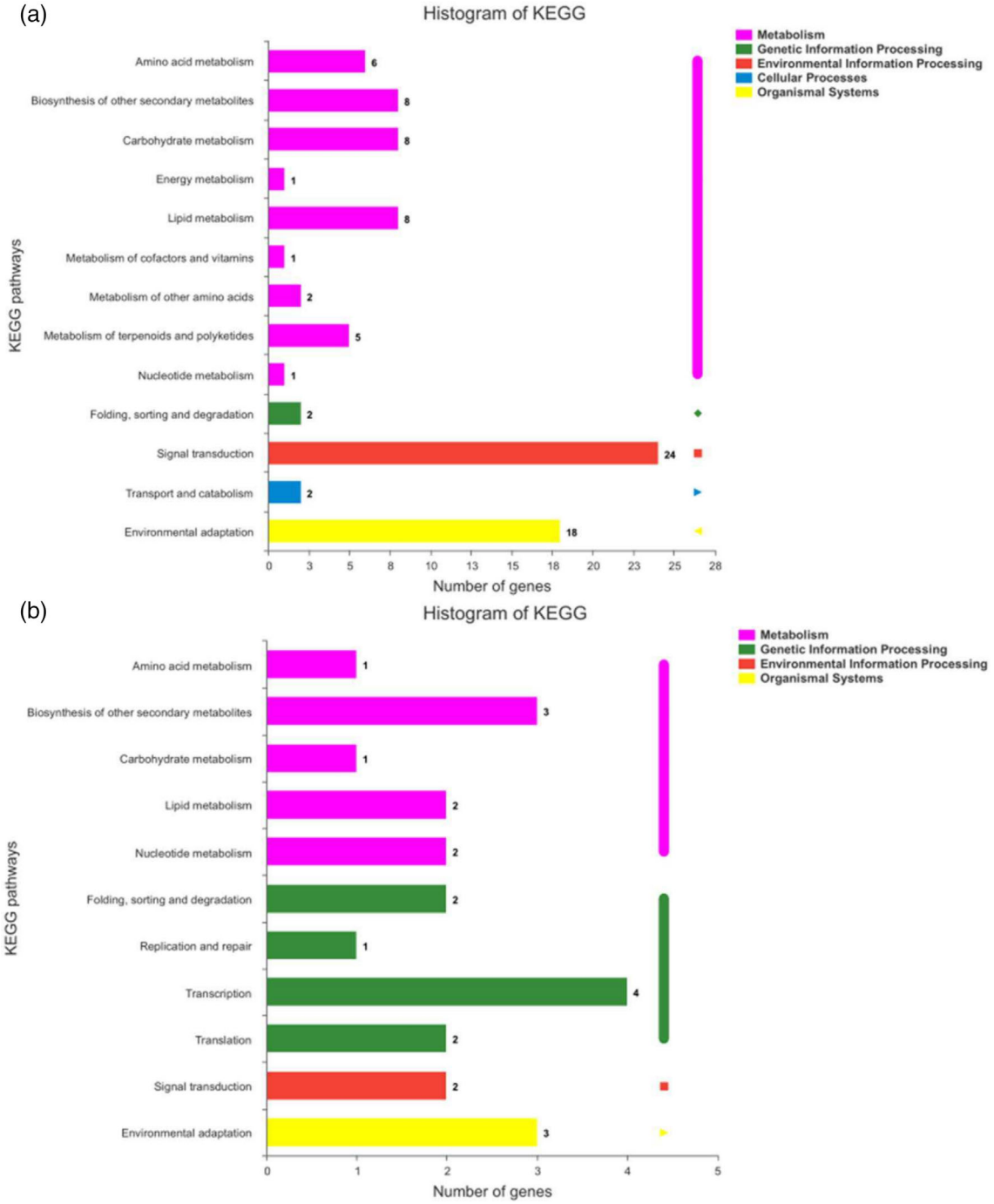

FIGURE 4 KEGG functional analysis and enrichment analysis were performed on the differentially expressed genes (DEGs). A histogram of KEGG function annotations was generated to compare the DEGs between AI&CK and PI&CK.

42 categories. Some of these categories are associated with biological processes related to plant resistance, such as the ethylene-mediated cysteine and methionine pathways. Additionally, genes homologous to *ETR*, *CTR1*, *MKK4-5*, *MPK6*, and *ERF1* were annotated. The DEGs between the AI and CK treatments were mapped to the α-LA metabolism pathway mediated by JA. Functional genes, such as *JAZ* and *MYC2*, which are homologous to each other, were annotated. *JAZ* indirectly initiates ubiquitin-mediated proteolysis, while *MYC2* transcription activates the synthesis of monoterpenoids and indole alkaloids, leading to the production of defense volatiles and toxic substances. Additionally, the homologous genes of the *IAA* family have been annotated in the tryptophan metabolism pathway mediated by auxin. These genes regulate the expansion and elongation of "Cabernet Sauvignon" grape leaf cells, enhancing their growth potential. Moreover, in the brassinosteroid biosynthetic and metabolic pathway, the expression of *XTH*

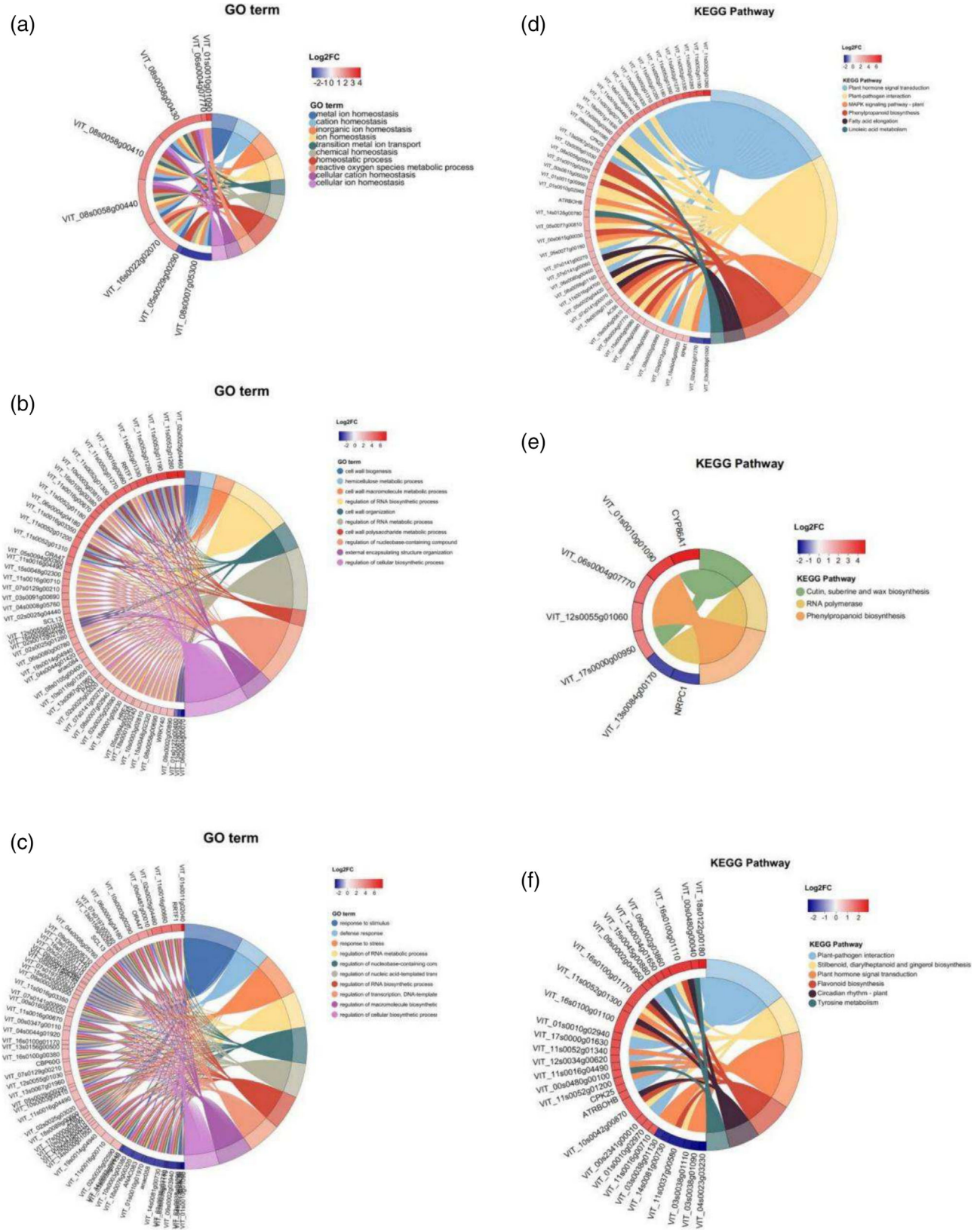

FIGURE 5 Legend on next page.

FIGURE 5 Enrichment chord plot analysis was conducted to compare differentially expressed genes (DEGs) between the treatments. (a–c) GO enrichment chord plots (|log2FC| ≥ 2; $p < .05$). (a) DEGs between the PI and CK treatments, (b) DEGs between the AI and CK treatments, and (c) DEGs between the AI and PI treatments. Similarly, (d)–(f) depict the KEGG enrichment chord plot analysis of DEGs between different treatments (|log2FC| ≥ 2; $p < .05$). (d) KEGG enrichment of DEGs in "Cabernet Sauvignon" leaves compared with the AI and CK treatments, (e) KEGG enrichment of DEGs in "Cabernet Sauvignon" leaves compared with the PI and CK treatments, and (f) comparison of the AI and PI treatments. *Note*: The genes/transcripts on the left are arranged in descending order of log2FC, indicating the expression difference between upregulated and downregulated genes/transcripts. A larger log2FC value indicates a greater expression difference for upregulated genes/transcripts, while a smaller log2FC value indicates a greater expression difference for downregulated genes/transcripts. The closer the value of log2FC is to 0, the smaller the differential expression of genes/transcripts.

homologous functional genes, which are involved in enhanced cell division, was significantly increased. These findings suggest that feeding by *A. lucorum* induces the production of JA as a signaling factor, resulting in defense responses characterized by cell division and growth enhancement.

In the plant-pathogen interaction pathway (map 04626, Figure 6b), a total of 540 resistance-related genes were identified. These genes were classified into 32 categories, including *CNGC*, *CALM*, *RBOH*, and *NOA1* homologous genes. These genes play a role in initiating the pathogen-associated molecular patterns (PAMPs) basic immunity response in plants and can also cause allergic reactions (HR). Additionally, several other homologous genes, such as *FLS2*, *BAK1*, *MEKK*, *WRKY*, *PTI6/1*, *glpK*, and *PR1*, were annotated. These genes are involved in phytoalexin accumulation, miRNA production, and defense mechanisms. Notably, *FLS2* kinase acts as an endosome to trigger endocytosis. The DEGs treated with AI were analyzed to identify their association with biological processes related to the calcium ion pathway and allergic response. This analysis revealed that Ca^{2+} signaling plays a crucial role in regulating the high expression of *CPK25*, *ATRBOHB*, *CALM*, and *CML* homologous genes. *ATRBOHB* indirectly induces the production of ROS, leading to the activation of PAMP and triggering pattern-triggered immunity (PTI) in plants, which can cause allergic reactions and thickening of leaf cell walls. *CALM* and *CML*, on the contrary, contribute to the closure of leaf stomata. Furthermore, Ca^{2+} acts as a messenger and indirectly influences the *MPK4* gene, leading to its incorporation into the nucleus and activation of the *WRKY33* homologous functional gene. This activation results in the upregulation of defense genes, including PR1 expression, and the accumulation of toxins in "Cabernet Sauvignon" grape leaves.

In the MAPK plant signaling pathway (map 04016, Figure 6c), a total of 289 genes have been identified as being associated with resistance, which are further classified into 62 categories. Among these genes, *FLS2*, *BAK1*, *MKS1*, *WRKY*, *SUMM2*, and *PR1* homologs play a crucial role in triggering cell death and late-stage defense responses. This leads to the production of indole alkaloid phytoalexins and ethylene compounds. Additionally, cell death results in the accumulation of H_2O_2 and ROS, which initiate defense mechanisms against pathogens. Protein kinase, *WRKY*, *NME*, and *PR1* homologous genes are involved in this defense process. Furthermore, the ethylene pathway involves *ETR*, *copA*, *TMEM222*, *CTR1*, *XRN4*, *EIN3*, *ERF1*, and *CHIB* homologous genes, which collectively induce the "Cabernet Sauvignon" response through stomatal closure, root growth, and wound response. Similarly, *PYL*, *PPLC*, *SNRK2*, and *CAT* homologous genes in the abscisic acid (ABA) pathway trigger plant stress resistance. Finally, *CALM* and *RBOH* homologous genes are annotated in the plant injury immune transmission chain. The AI-treated DEGs were categorized into five groups of homologous functional genes, all of which were upregulated. These groups included homologous genes of *WRKY33* and *ACS6*. In the immune transmission chain, *FLS2*, a membrane receptor, activates the LRR receptor protein kinase and BAK1 receptor kinase. This activation indirectly affects the *MAPKKK* kinase family genes, leading to phosphorylation and generation of MAPKK and MAPK kinase. The MAP kinase substrate is then phosphorylated and activated, which acts on the *WRKY* transcription factor, ultimately resulting in the transcriptional activation of DNA expression and synthesis of phytoalexins. Simultaneously, MAPK kinase phosphorylation activates ACS6, leading to the synthesis of ethylene. Similarly, in the transmission chain that uses JA as a signal, the homologous gene of *MYC2* showed significant upregulation, inducing the growth of "Cabernet Sauvignon" roots. Mediated by ABA, the transmembrane binding of the homologous genes of the ABA receptor *PYR/PYL* family genes caused downregulated expression, indirectly affecting plant stress resistance and triggering the stress response in "Cabernet Sauvignon" grape leaves.

A significant number of resistance-related enzymes were annotated in the 13 secondary metabolism biosynthetic pathways, including flavones and flavonoids, phenylpropanoids, stilbene, anthocyanins, isoflavones, indole alkaloids, isoquinoline alkaloids, betaine, glucosinolates, monomycin, caffeine metabolism, and other biosynthetic pathways. This suggests that when the young leaves of "Cabernet Sauvignon" are damaged by puncturing and feeding by *A. lucorum*, a defense mechanism is activated. This mechanism involves the production of secondary metabolites that affect the growth and development of *A. lucorum*, thereby preventing it from causing harm. Additionally, volatile small molecule substances are produced to deter *A. lucorum*, warn neighboring plants, or attract natural enemy insects to aid in defense. However, further verification is needed for these findings.

Based on the above analysis, it can be concluded that the defense mechanism of "Cabernet Sauvignon" grape leaves under feeding by *A. lucorum* is relatively complex. Multiple metabolic pathways are activated simultaneously, and multiple reactions work together to resist the harm caused by *A. lucorum*.

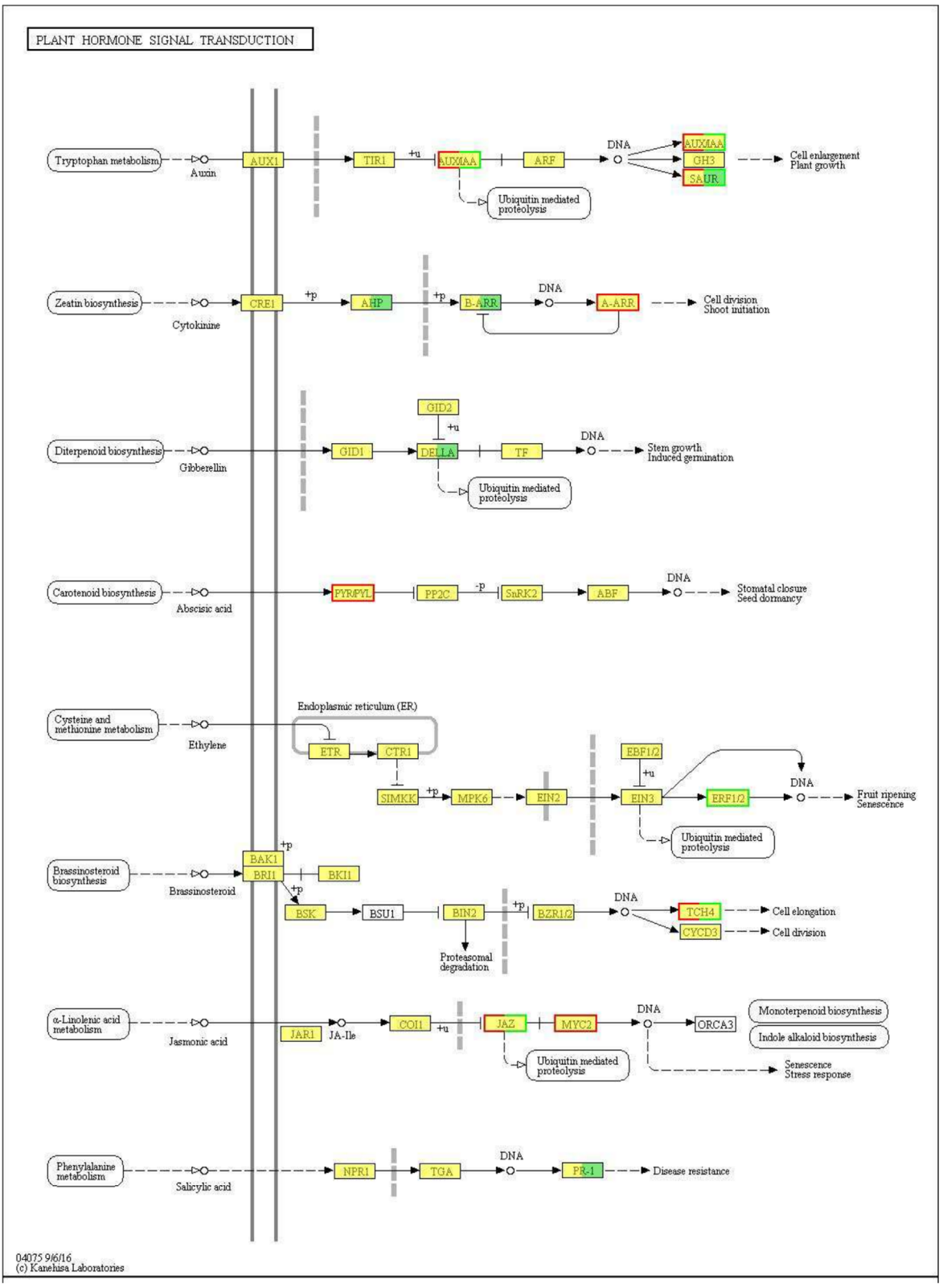

FIGURE 6 Compared with the CK treatment, the signal pathway map of DEGs in the AI treatment was significantly localized. Map 04075 is the plant hormone signal transfer pathway, and homologous genes such as *JAZ* and *MYC2* have been annotated. Map 04626 is the interaction metabolic pathway of plant pathogens, which is mainly mapped to the expression of defense genes related to Ca2+ and allergic reactions. Map 04016 is the MAPK signaling pathway, in which the phosphorylation of MAPK kinases causes the stress response of "Cabernet Sauvignon." *Note*: "" represents known genes/transcripts, "" represents upregulated genes, "" represents downregulated genes, "" represents up + downregulated genes, "" represents new genes/transcripts, and "" represents known + new genes/transcripts.

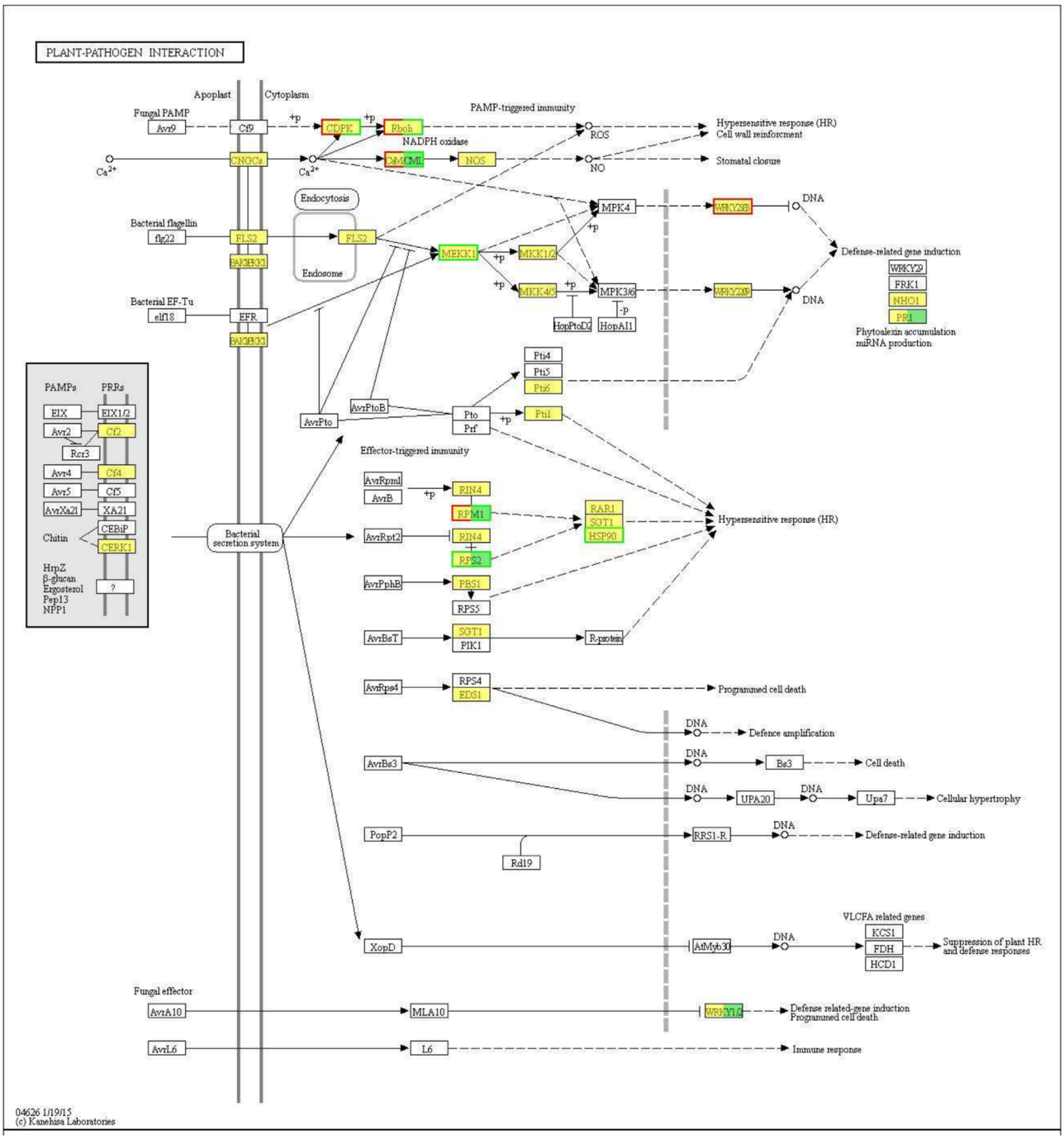

FIGURE 6 (Continued)

3.4 | Real-time qPCR verification of RNA-seq data

To further validate the reliability of the RNA-seq results, RT-qPCR was conducted on 20 DEGs. The PCR amplification electrophoresis plot (Figure 7a) demonstrated the successful amplification of specific bands for all 20 DEGs with no presence of primer dimer or other products. Moreover, the dissolution curve of the internal reference genes *Actin* and *GAPDH* (Figure 7b) exhibited a single signal peak, indicating good specificity and confirming that the analysis met the requirements for subsequent analysis. The RT-qPCR detection results for the selected 20 DEGs were largely consistent with the RNA-seq results (Table S11). The linear regression analysis of the RNA-Seq and RT-qPCR data (Figure 7c) indicated a significant correlation between the two methods, confirming the accuracy and reliability of the transcriptome sequencing data ($R^2 = .9718$ for *GAPDH*; $R^2 = .9702$ for *ACTIN*, $p < .00001$).

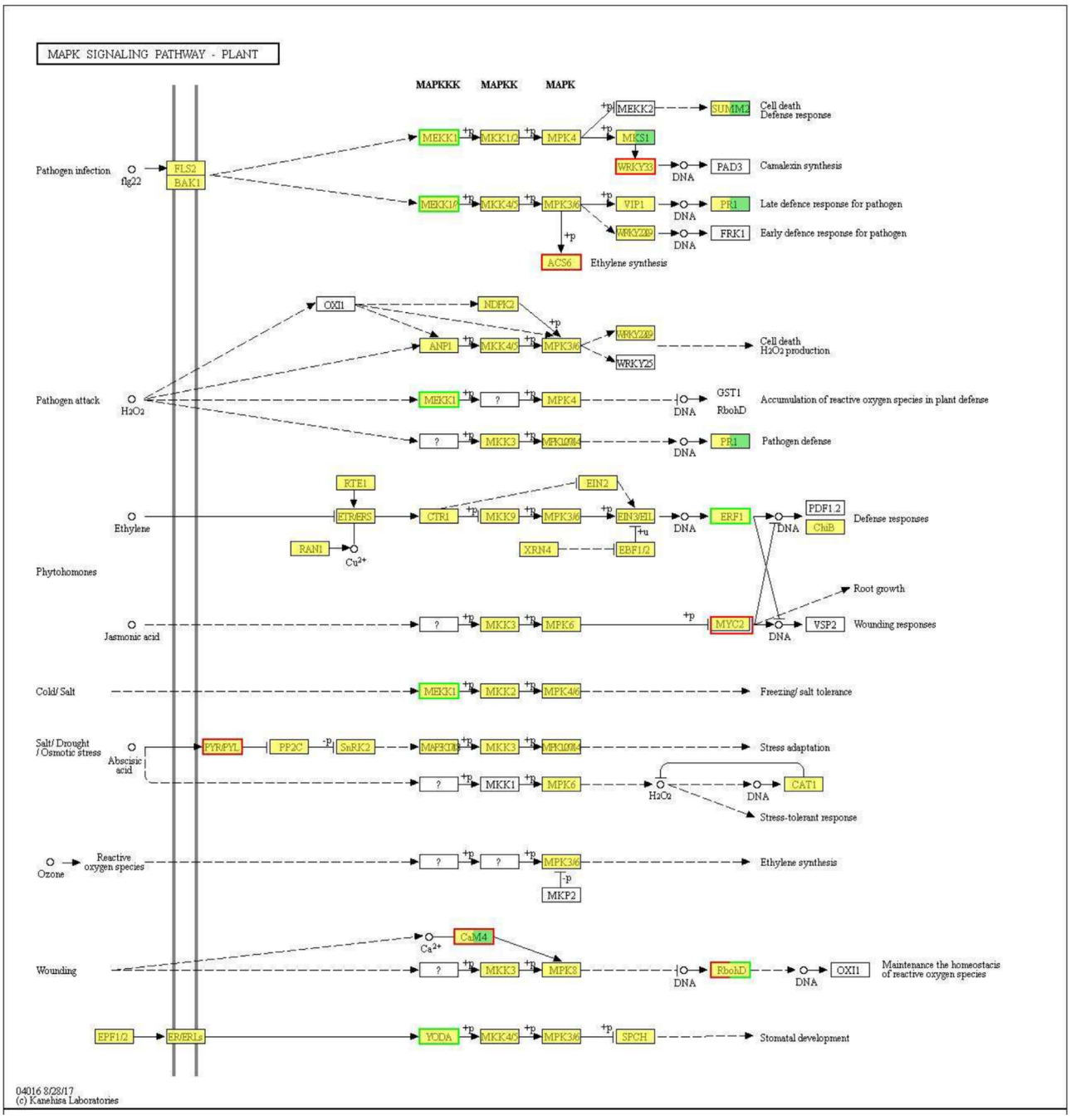

FIGURE 6 (Continued)

4 | DISCUSSION

To investigate the molecular mechanism of the response of "Cabernet Sauvignon" grapes to *A. lucorum*, this study used RNA-seq technology to analyze transcriptome data and obtain gene expression data. The DEGs were then compared with the GO and KEGG databases for enrichment analysis, functional category annotation, and mapping of metabolic pathways involved in regulation. The genes that showed significant differences were found to be upregulated and played a crucial role in the defense response of "Cabernet Sauvignon" grape leaves to *A. lucorum*.

Plants have evolved a series of defense responses to resist pest invasion (Guo et al., 2018; Wu & Baldwin, 2010). Multiple research reports have indicated that when plants are subjected to injury stress, they undergo a process of strengthening and transmit various plant hormone substances (Pieterse et al., 2012) such as JA (Mao et al., 2017), SA (Maruri-Lopez et al., 2019), ethylene, and ABA. This induces downstream genes, including *MYC* (Wang et al., 2021), *JAZ*

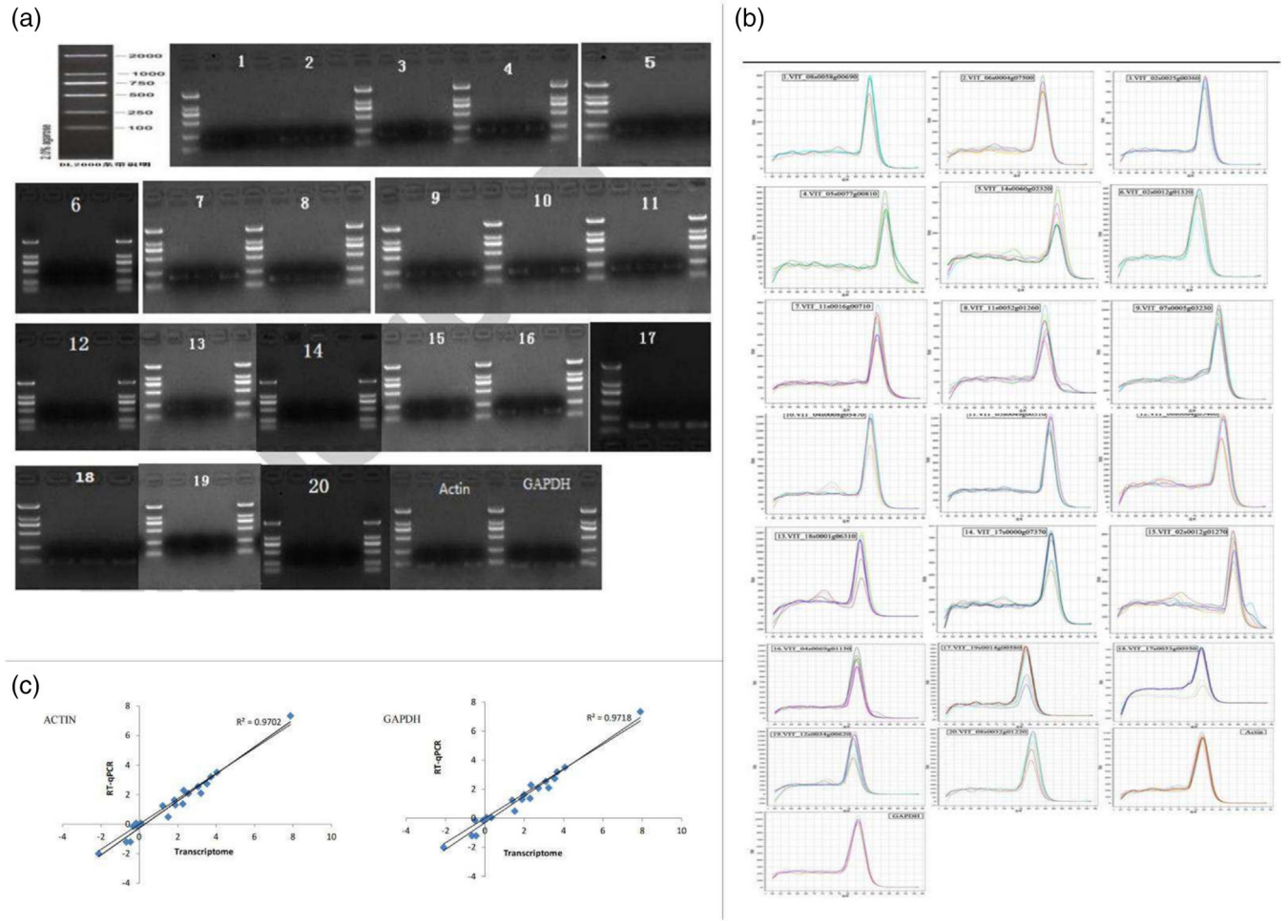

FIGURE 7 Validation map for RT-qPCR includes the following: (a) PCR amplification electrophoresis; (b) melting curves of 20 differential genes and internal reference genes; and (c) linear regression analysis of the RNA-seq and RT-qPCR data

(Howe & Yoshida, 2019), and *ERF* transcription factors (Müller & Munné-Bosch, 2015), to regulate plant metabolic reactions, ultimately initiating defense-related mechanisms (Robert-Seilaniantz et al., 2011; War et al., 2012). The GO and KEGG analysis results of the DEGs in this study indicated that under feeding by *A. lucorum*, "Cabernet Sauvignon" grapes undergo regulation by signaling substances and related genes, resulting in the formation of a unique defense mechanism. *JAZ* indirectly triggers the process of ubiquitin-mediated proteolysis (Howe & Yoshida, 2019), while *MYC2* transcription plays a role in activating the synthesis of monoterpenes and indole alkaloids. Additionally, the grape leaves exhibited a stress response and synthesized defense volatiles and toxic substances. This indicates that the response of the grape leaves to *A. lucorum* feeding stimuli was triggered by JA as a signaling substance, which initiates defense and produces toxic substances to resist *A. lucorum*. However, DEGs were found in the phenylalanine metabolism pathway mediated by SA. The PI treatment also induced the defense response of "Cabernet Sauvignon" grape, which may be initiated by JA and H_2O_2 as signaling molecules in response to physical injury. This study discovered that both the AI and PI treatments activated the *JA* signal transduction pathway and induced the expression of downstream enzymes such as peroxidase and polyphenol oxidase. However, the main difference between the puncture and feeding by *A. lucorum* was that "Cabernet Sauvignon" grape responded to the latter by significantly increasing the expression of xylan glycosyltransferase and hydrolase genes, brassinolide, and *IAA* genes in its leaves. This response indicates that grape activates its defense mechanism while also improving its tolerance by stimulating its own cell division to strengthen, elongate, and grow.

In the top 20 enriched pathways, metabolic pathways involving calcium ion transport, binding, and transmembrane transporter activity were found to be enriched. Compared with the CK treatment, the expression levels of DEGs such as *CPK25*, *ATRBOHB*, *CALM*, and *CML* were significantly upregulated in the plant-pathogen interaction pathway (map 04626) and MAPK plant signaling pathway (map 04016) in "Cabernet Sauvignon" leaves under AI treatment. External stresses, such as insect feeding, have been shown to affect the processes within plant cells as well as the growth and reproduction of plants (Schuman & Baldwin, 2016) and also affect plant intracellular processes and plant growth and reproduction. The plant cell wall

framework is formed by the interaction of xylan and microfilaments. The *XTH* gene, which is the focus of this experiment, plays an important role in its lysis and polymerization, affecting the structural changes of plant cells, and calmodulin kinase regulates the cell cycle (Maris et al., 2009). In this experiment, the expression of *CPK25*, plant growth hormone response protein, and non-specific LRR receptor protein kinase in the *CPK* family gene increased significantly in the PI treatment, while the expression of the *CIPK20* gene in the *CIPK* family gene decreased in the AI treatment. CIPK is an interactive protein kinase of CBL, both of which participate in Ca^{2+} signal transduction under abiotic stress (Verma et al., 2021). *WPK4* has been confirmed to participate in the signal pathway of cytokinin synthesis under light and nutrient stress (Ikeda et al., 1999). The expression of the *CIPK* gene was significantly downregulated under the AI treatment, and its function and mechanism still need further study. Among the DEGs in the leaves of "Cabernet Sauvignon" grapevine, four DEGs under the AI treatment were annotated in the plant auxin mediated tryptophan metabolism pathway. Among them, the auxin-responsive protein (*IAA*) family gene (Fendrych et al., 2016) and *SAUR* protein family plant auxin (Markakis et al., 2013) response genes were significantly upregulated. Under the action of the above genes, the cell expansion and growth potential of "Cabernet Sauvignon" grape were increased. The *ARR-A* family gene in the biosynthesis pathway of zeatin mediated by cytokinin was annotated, which can cause plant cell division and bud germination (Xie et al., 2018).

The antioxidant activity of plants is closely related to their resistance response. This experiment focused on the *ATRBOHB* gene and peroxidase genes. The *ATRBOHB* gene family is a NADPH oxidase mutant, which proven to be the main source of ROS (Muller et al., 2009). ROS, as an injury signal, has been shown to participate in many important signal transduction and metabolic pathways (Sies & Jones, 2020). Therefore, it is speculated that the high expression of this gene is related to *A. lucorum* infestation, which was specifically manifested by increased antioxidant activity and defense enzyme activity. The expression of the peroxidase gene was significantly upregulated in both the AI and PI treatments, which indicated that physical injury also causes an antioxidant activity response of "Cabernet Sauvignon" grape leaves, though the upregulation was more significant in the AI treatment than in the PI treatment. This also indicated that the molecular mechanisms of regulation of the two treatments were not completely consistent and had unique characteristics. Feeding by *A. lucorum* inevitably caused damage to "Cabernet Sauvignon" grapes, the cell membrane was damaged and oxidized, and the content of malondialdehyde, the final product of lipid oxidation, increased significantly. The LA decomposition pathway was also activated. Under the action of lipase, LA was dissociated, and the octadecanoic acid pathway was activated. Under the action of lipoxygenase and malodiene oxide synthase, an enzymatic reaction took place to generate jasmonates. Jasmonates have been proven to be related to plant insect resistance and are important signaling molecules (Tang et al., 2020). This is consistent with previous research on the initation of defense reactions by JA for signaling molecules.

The *PAD4* gene is closely related to plant defense against insects, especially stinging insects (Louis & Shah, 2015). In this experiment, the expression of the *PAD4* gene was significantly increased in the AI treatment. Previous studies have shown that *PAD4* is the upstream gene of the SA signaling pathway, encoding a nucleocytoplasmic protein of plant antitoxin, and participating in the process of plant resistance and defense. Some studies have shown that the *RPM1* gene plays an obvious role in the process of plant resistance to stress (Mackey et al., 2002). The *RPM1* gene in this experiment also showed highly significant upregulation in the AI treatment. In the biological process of the hypersensitive response, cell wall thickening, and stomatal closure, Ca^{2+} is used in signal transduction to regulate the expression of *CNGC*, cause Ca^{2+} to enter the cytoplasm, and act on *CPK25*, *ATRBOHB*, *CALM*, and *CML* homologous genes to regulate their expression. The indirect action of the ATRBOHB enzyme produced ROS, which triggered PAMP and caused anaphylaxis and cell wall thickening of the "Cabernet Sauvignon" grape leaves. Several homologous genes of *CALM* and *CML* were highly expressed, regulating NOA1 and indirectly producing NO, causing stomatal closure in the leaves of "Cabernet Sauvignon." At the same time, Ca^{2+} also acted as a messenger, indirectly acting on the *MPK4* mitogen-activated protein kinase gene, incorporating the nuclear excitation *WRKY33* homologous sequence and the functional gene *WRKY* transcription factor, so as to make it highly expressed and activate its transcription, thereby inducing the expression of defense-related genes in grape leaves. In the plant MAPK signal pathway, the DEGs in the leaves of "Cabernet Sauvignon" grapevine under *A. lucorum* feeding mainly included *FLS2*, *BAK1*, *MAPKKK* kinase family, *WRKY33*, *WRKY* transcription factor, and the *ACS6* gene, which were all upregulated.

The presence of flavonoids, tannins, phenols, alkaloids, lignin, terpenes, and other metabolites in plants plays a crucial role in their ability to resist insect feeding (Ritter & Schulz, 2004). Through the analysis of 13 secondary metabolic biosynthesis pathways enriched in KEGG metabolic pathways obtained from the three different treatments, the DEGs were annotated in phenylpropanoid, terpenoid, and isoquinoline alkaloid biosynthesis, as well as phenylalanine, linoleic acid, porphyrin, and chlorophyll metabolism pathways under *A. lucorum* feeding. The defense mechanism of "Cabernet Sauvignon" grape leaves stimulated by *A. lucorum* feeding is relatively complex, with multiple metabolic pathways activated simultaneously, working together to resist insect invasion.

Through the GO and KEGG databases, the functional categories of the DEGs and metabolic pathways involved in regulation were annotated. The DEGs were found to be enriched in various metabolic pathways including plant hormone signal transduction, plant-pathogen interaction, plant antioxidant, MAPK kinase pathway, and other metabolic pathways. Many genes related to hormone signal transduction, glucose metabolism, and the calcium signal pathway were stimulated by *A. lucorum* feeding. This study provides preliminary clarification on the expression of related genes during the response of "Cabernet Sauvignon" grapes to *A. lucorum* feeding, serving as a reference for

further exploration of the resistance mechanism of "Cabernet Sauvignon" grapes to this pest.

AUTHOR CONTRIBUTIONS

Conceptualization: Suhong Gao and Tianhua Sun. *Data curation*: Heng Yao and Suhong Gao. *Formal analysis*: Heng Yao and Suhong Gao. *Funding acquisition*: Baojia Gao and Changkuan Lu. *Investigation*: Suhong Gao and Guona Zhou. *Visualization*: Suhong Gao and Heng Yao. *Writing—original draft preparation*: Heng Yao. Writing—review and editing: Heng Yao, Suhong Gao, Wenshu Chen, and Y.Z. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

PEER REVIEW

The peer review history for this article is available in the supporting information for this article.

DATA AVAILABILITY STATEMENT

No data were used or generated during the research for this study. Therefore, there are no data available for sharing.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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