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Exogenous Salicylic Acid Enhances Strawberry Resistance to *Colletotrichum siamense* Causing Crown Rot by Activating Defense Genes and Lignin Biosynthesis

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ABSTRACT

Crown rot, caused by *Colletotrichum siamense*, is a devastating hemibiotrophic fungal disease that poses a significant threat to the strawberry industry. Salicylic acid (SA) is known to play a critical role in plant defense responses to biotic stress. However, its contribution to mitigating strawberry crown rot remains unclear due to the microbial isolate-specific sensitivity and cultivar/tissue-specific responses in strawberries. In this study, we aimed to investigate how exogenous supply of SA influenced crown rot in strawberry. Exogenous SA application significantly reduced *C. siamense* infection in strawberry crowns, evidenced by the lesion size and pathological analysis. Transcriptomic data showed that for each sample of SA pretreatment and mock, owing to nearly 50 million reads, the ratio of Q20 ranged from 98% to 99%, and 91.63%-94.29% of the reads mapped to the reference genome. The SA pretreatment up-regulated genes encoding MLO-like protein 2, receptor-like kinase, peroxidase, and caffeic acid 3-O-methyltransferase involved in lignin biosynthesis. The SA pretreatment also down-regulated chalcone isomerase, naringenin 3-dioxygenase, bifunctional dihydroflavonol 4-reductase, anthocyanidin synthase, and anthocyanidin reductase expressions involved in flavonoid biosynthesis during *C. siamense* infection. Consistent with gene expression changes, the SA pretreatment remarkably enhanced peroxidase activity and lignin content and decreased flavonoid content and chalcone isomerase activity after *C. siamense* inoculation. The results suggest that exogenous SA enhanced strawberry resistance to crown rot caused by *C. siamense* by up-regulating defense-related genes and lignin biosynthesis.

KEYWORD: 'Benihoppe' strawberry, transcriptome, susceptibility, lignin, enzyme activity.

INTRODUCTION

Colletotrichum is a hemibiotrophic pathogen, using a composite strategy that comprises biotrophic and necrotrophic processes. Crown rot caused by *C. siamense* is a serious disease in strawberries (*Fragaria* × *ananassa*), especially in China (Ji *et al.*, 2022; Shu *et al.*, 2022).

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Promoting resistance to crown rot caused by *C. siamense* is a very meaningful work for strawberry production. Induced resistance refers to the phenotypic state in which an exogenous stimulus conditions a plant to reduce its susceptibility to future biotic challenges (De Kesel *et al*., 2021). Understanding the effects of exogenous stimuli (especially chemical compounds) on the induced resistance of cultivated strawberries to *C. siamense* infection is important for disease control.

Salicylic acid (SA) is a key hormone involved in plant defenses against biotrophic and hemibiotrophic pathogens, as it activates systemic acquired resistance (Esmailzadeh and Soleimani, 2008). SA can change enzyme activity, increase defense genes, enhance several defense responses, and/or generate free radicals (De Kesel *et al*., 2021). Exogenous SA reduced the incidence of potato purple top disease caused by *phytoplasma* (biotrophic) in tomatoes (*Lycopersicon esculentum*) (Wu *et al*., 2012) and decreased the severity of citrus canker disease caused by *Xanthomonas axonopodis* (biotrophic) in oranges (*Citrus sinensis*) (Wang and Liu, 2012). Similar to biotrophic pathogen, exogenous SA reduced disease incidence of *Fusarium* wilt caused by *F. oxysporum* (hemibiotrophic) in chickpea (*Cicer arietinum*) and tomato (*L. esculentum*), respectively (Saikia *et al*., 2003; Jendoubi *et al*., 2017); decreased disease severity of rice blast caused by *Magnaporthe grisea* (hemibiotrophic) (Daw *et al*., 2008); and anthracnose caused by *C. gloeosporioides* (hemibiotrophic) in tea flower (***Camellia*** *oleifera*) (Wang *et al*., 2006).

A study also showed that SA is involved in the strawberry response to *Colletotrichum* invasion (Grellet-Bournonville *et al*., 2012; Amil-Ruiz *et al*., 2016). Genes involved in SA biosynthesis and free SA release from MeSA were up-expressed for a very early burst of free SA under *C. fructicola*-inoculated strawberry leaves in less-susceptible cultivar ‘Jiuxiang’ and susceptible cultivar ‘Benihoppe’ (He *et al*., 2019). Furthermore, after an early SA burst, fast free SA quenching was caused by effectors (CfShy1) of *C. fructicola* interfere with accumulation (He *et al*., 2019). Exogenous SA pretreatment reduced susceptibility and elevated internal SA levels in both varieties, which were sharply reduced in the susceptible cultivar upon inoculation (Zhang *et al*., 2016). In addition to its regulating endogenous SA biosynthesis, studies have shown that exogenous SA promotes the biosynthesis of lignin (i.e., a physical barrier against pathogens) and flavonoids (i.e., antioxidants and signal molecules for resistance) to enhance plant resistance (Dempsey *et al*., 2012; Lee *et al*., 2019; Hou *et al*. 2024). Although the application of SA pretreatment reduced the susceptibility to anthracnose caused by *C.*

gloeosporioides in leaves (Zhang *et al*., 2016), the effects of exogenous SA on strawberries in response to *C. siamense* crown infection remain unknown. Furthermore, variations in defense genes and resistance related to secondary metabolites, e.g., lignin and flavonoids, affected by SA have not been investigated.

The aims of this study were to use lesion size and pathological analysis to test the effects of exogenous SA on strawberry resistance to crown rot caused by *C. siamense*, use RNA-seq and qRT-PCR to examine gene expression profiles to identify SA-induced physiological responses to antagonize *C. siamense* infection, and measure the physiological index to understand the potential factors due to the effects of SA on strawberry crown rot.

MATERIALS AND METHODS

Materials and experiment design

The aseptic strawberry seedlings (cv. Benihoppe) were transplanted into pots with seedling substrates (Pindstrup, 5-20 mm) in a growth chamber (25/15 °C, 16 h light/8 h dark). Seedlings were watered thrice per week and fertilized weekly with 30 ml Hoagland nutrient solution (Li *et al*., 2023). Seedlings were prepared to evaluate the effects of SA on strawberry crown rot after 3 months of growth.

This experiment was designed as SA (dissolved in distilled water) and mock (distilled water) pretreatments. Seedlings were sprayed with an atomizer until thoroughly wetted with 5 mM SA, which was applied twice (8 and 1 d before pathogen inoculation) as described by Desmedt *et al*. (2021). Subsequently, 10 µl of the spore suspension of *C. siamense* SCR-7 (10^4 conidia·ml^{-1}) and mock (sterilized water) were squeezed to crowns after being stabbed with sterilized toothpick (Luo *et al*., 2021). Samples were collected on days 0 and 4 post-inoculation. The experiment comprised four treatments: 0 d post-inoculation with *C. siamense* SCR-7 with SA pretreatment (SA0DPI) and mock seedlings (Mock0DPI), and 4 d post-inoculation with *C. siamense* SCR-7 with SA pretreatment (SA4DPI) and mock seedlings (Mock4DPI). The crowns of 10 seedlings were mixed as one biological replicate. Each treatment contained six biological replicates for the observation of infection and physiological index measurements. Two of the six biological replicates from each treatment were mixed as a new biological replicate for transcriptome analysis and qRT-PCR; three biological replicates were used in these two analyses.

Infection observation of SA and mock pretreatment

The length and width of the lesions were measured using a straight edge. Pathological analyses were performed as described by Shu *et al*. (2022). Next, 10 µl wheat germ agglutination storage solution and 20 µl propidium iodide stock solution were added to 970 µl 0.2% Tween-phosphate buffer saline solution and mixed thoroughly (dye preparation). A Carnot fixative was used to fix the crown samples. The crowns were transferred into a 10% KOH solution, and the tube was sealed with Parafilm to prevent collapse. The sample was then incubated at 85 ℃ for 4 h (fix). The crowns were washed twice or thrice with phosphate buffer saline and sealed with anti-fluorescence quenching, stored at 4°C in the dark, and imaged using a fluorescence microscope (Photographing) (Nikon E400, Melville, NY).

Transcriptome analysis and qRT-PCR

Total RNA was extracted from freeze-dried samples by using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) per the manufacturer's protocol. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and verified using RNase-free agarose gel electrophoresis. The fragments were purified using agarose gel electrophoresis, enriched using PCR amplification to create a cDNA library for each sample, and sequenced using Illumina HiSeq2500. For obtaining high-quality clean reads, raw reads from transcriptome sequencing were filtered using Fastp (version 0.18.0). The strawberry 'Camarosa' Genome v2.0 was used as the reference genome. The FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variation by using StringTie software. FPKM data were directly used to estimate differentially expressed genes (DEGs) between samples. FDR < 0.05 and |log2FC| > 1 were used as thresholds to identify significant DEGs. Based on these DEGs, eukaryotic orthologous group (KOG) analysis, gene ontology (GO) enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed as described by Shu *et al*. (2022).

qRT-PCR was performed, according to the method described by Luo *et al*. (2020), on three independent biological samples with three technical replicates each. **DEGs involved in lignin and flavonoid biosynthesis, MLO-like protein 2, leucine-rich repeat receptor-like serine/threonine-protein kinase and cysteine-rich receptor-like protein kinase, were selected for RNA-seq verification by using a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, USA), and the primers used for qRT-PCR are shown in**

supplementary file, Table S1. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Rao *et al.*, 2013), where actin-1 was used as the reference gene (Zhang *et al.*, 2018).

Measuring flavonoid and lignin contents

The flavonoid content was determined using a plant flavonoid content assay kit (Solarbio Beijing, China) per the manufacturer's instructions and a UV-5200 spectrophotometer (Shanghai Metash Instruments, China) at 470 nm, as described by Lu *et al*. (2023). The lignin content was determined using a lignin content assay kit (Solarbio, Beijing, China) per the manufacturer's instructions and a spectrophotometer at 280 nm, as described by Ning *et al*. (2023).

Measuring chalcone isomerase and peroxidase activities

Chalcone isomerase (CHI) activity was determined, as described by Li *et al*. (2023), using a CHI test kit (TongWei, Shanghai, China). Peroxidase (POD) activity was determined using a peroxidase activity assay kit (Solarbio, Beijing, China) per the manufacturer's instructions, and a spectrophotometer at 470 nm as described by Zhang *et al*. (2023).

Statistical analysis

Significant differences between treatments were determined using Duncan's Multiple Range Tests at $P = 0.05$ with SAS 8.1 (SAS Institute, Inc., Cary, NC, USA). Different letters indicate significant differences between groups.

RESULTS

Effects of SA on *C. siamense* infection

SA pretreatment decreased the severity of crown rot caused by *C. siamense* (Figure 1-A), reducing lesion size (Figure 1-B). Consistent with the size of the lesion, the density of hyphae in the SA-treated crowns was lower than that in the mock-treated crowns (Figure 1-C). The lesion size and pathological analysis suggested that SA pretreatment inhibited the infection of *C. siamense* to strawberry crown.

Effects of SA on physiological responding to *C. siamense* infection

The transcriptomic data showed that the total number of reads per sample was approximately 50 million. The Q20 ratio of each sample ranged from 98% to 99%, and the ratio of N bases

was less than 0.04%. The GC content of each sample was approximately 48% (supplementary file, Table S2). All clean reads were compared with the reference genome, and most of the reads mapped to the reference genome ranged from 91.63% to 94.29%, the ratio of mapped reads to the sense strand and anti-sense chain was nearly 35%-38%, and nearly 75% of the mapped reads of each sample were uniquely mapped to the genome (supplementary file, Table S3). Furthermore, principal component analysis based on RNA-seq showed that three biological replicates of each treatment were gathered together, and the four treatments were relatively dispersed (supplementary file, Figure S1). These results confirmed the reliability of our data, and the transcriptomic data were uploaded to the NCBI Sequence Read Archive as PRJNA1021273.

The SA pretreatment regulated transcripts in strawberry crowns. The SA0DPI vs. Mock0DPI showed 990 up-regulated and 520 down-regulated genes. The number of DEGs in SA4DPI vs. Mock4DPI was lower than that in SA0DPI vs. Mock0DPI, which included 448 significantly up-regulated and 434 down-regulated genes (Figure 2).

GO enrichment analysis revealed that most DEGs were enriched in biological processes. The metabolic process, biological regulation and cellular process clustering into biological process; organelle, cell and cell part clustering into cellular component; transporter activity, catalytic activity and binding clustering into molecular function contained the most differentially expressed transcripts in SA0DPI vs. Mock0DPI. Response to stimulus clustering into biological process and catalytic activity clustering into molecular function was increased even more in SA4DPI vs. Mock4DPI than that in SA0DPI vs. Mock0DPI (Figure 3).

The enrichment map of GO showed regulation of cellular macromolecule biosynthetic process, regulation of cellular biosynthetic process, cell wall organization or biogenesis, xylan biosynthetic process, and plant−type secondary cell wall biogenesis enriched most DEGs in SA0DPI vs. Mock0DPI (Figure 4-A). By contrast, flavonoid metabolic process, chalcone isomerase activity, and salicylic acid catabolic process were enriched in most DEGs in SA4DPI vs. Mock4DPI (Figure 4-B). KOG function classification showed posttranslational modification, protein turnover, chaperones, general function prediction only, and signal transduction mechanisms were mapped for most DEGs in SA0DPI vs. Mock0DPI and SA4DPI vs. Mock4DPIs (Figure 5-A). An enrichment map of KOG function classification showed FxaC_14g15990, FxaC_11g32110, FxaC_13g23280, FxaC_11g26150, FxaC_12g20520, and FxaC_13g23960 transcripts enriched in cell wall/membrane/envelope biogenesis, or

extracellular structures were significantly differentially expressed in SA0DPI vs. Mock0DPI (Figure 6-A). FxaC_16g02820, FxaC_26g03630, and FxaC_18g45220, which were enriched in secondary metabolite biosynthesis, transport, and catabolism, were significantly and differentially expressed in SA4DPI vs. Mock4DPI (Figure 6-B).

The expression level of genes enriched in oxidative phosphorylation and protein processing in endoplasmic reticulum pathways were suppressed by SA based on KEGG enrichment analysis of SA0DPI vs. Mock0DPI (Figure 7-A), and the genes involved in pathways e.g. phenylpropanoid biosynthesis and flavonoid biosynthesis were all suppressed in SA4DPI vs. Mock4DPI (Figure 7-B). An enrichment map showed that the expression of genes encoding CHI, naringenin 3-dioxygenase (F3H), anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR) involved in flavonoid biosynthesis KEGG pathways were all suppressed by SA, but the expressions of *POD* and *caffeic acid O-methyltransferase* (*COMT*) genes in lignin biosynthesis were elevated (Figure 8-A). **Additionally, defense related genes like *MLO-like protein 2* (FxaC_25g37891), *leucine-rich repeat receptor-like serine/threonine-protein kinase* (FxaC_10g18870) and *cysteine-rich receptor-like protein kinase* (FxaC_12g47880) were up-regulated by SA**. The qRT-PCR results were in accordance with the transcriptomic data for the expression of structural genes involved in flavonoid biosynthesis and lignin biosynthesis (Figure 8-B).

Effects of SA on flavonoid and lignin biosynthesis under *C. siamense* infection

For further analysis, this study performed POD and CHI activity assays. The results indicated that on day 0 after *C. siamense* inoculation, there was no significant difference in lignin and flavonoid content, so did POD and CHI activities, between the SA and mock pretreatments. The flavonoid content of the mock was 6.72 mg g^{-1} FW, which was higher than that of the SA pretreatment (5.54 mg g^{-1} FW) 4 d post *C. siamense* inoculation. As confirmed by flavonoid content, SA pretreatment significantly down-regulated CHI activity 4 d after *C. siamense* inoculation. Different from flavonoids and CHI activity, the SA pretreatment remarkably promoted the POD activity (122.32 U g^{-1} FW in Mock4DPI and 131.14 U g^{-1} FW in SA4DPI, respectively) and lignin content (67.11 mg g^{-1} FW in Mock4DPI and 74.82 mg g^{-1} FW in SA4DPI, respectively) after 4 d of *C. siamense* inoculation (Figure 9).

DISCUSSIONS

SA is an important signal for pathogen-associated molecular pattern triggered immunity and

effector-triggered immunity (Saleem *et al*., 2021; Hou *et al*., 2023). Endogenous SA-mediated disease-resistance stress responses usually prevent biotrophic or hemibiotrophic pathogen infections through a hypersensitive response, causing rapid cell death for systemic acquired resistance (Koo *et al*., 2020). The results of this study showed that SA pretreatment decreased the severity of crown rot caused by *C. siamense*. As expected, a decrease in lesion size and hyphal density were observed (Figure 1). Two reasons may explain why *C. siamense* infection was inhibited by SA-pretreated crowns. First, *C. siamense* is a hemibiotrophic pathogen that uses a composite strategy, including biotrophic and necrotrophic processes for pathogenesis (Pokotylo *et al*., 2022). Similar to other biotrophic and hemibiotrophic pathogens, the SA pretreatment inhibited the infection of *C. siamense* to strawberry crown, which may be due to *C. siamense* having biotrophic processes of pathogenesis. Second, 'Benihoppe' strawberry is susceptible to crown rot caused by *C. siamense*, and it cannot accrue hypersensitive response (rapid cell death) as a resistant plant responding to pathogen does (Saleem *et al*., 2021); thus, that reason that the SA pretreatment inhibited the infection of *C. siamense* to strawberry crown might be because of defense genes and secondary metabolites.

Studies have suggested that endogenous SA-mediated disease-resistant stress responses usually prevent pathogen infection by activating PR proteins, ROS-scavenging enzymes (polyphenol oxidase and peroxidase), enzymes involved in defense (chitinase), and secondary metabolism (phenylalanine ammonia-lyase) (Wang and Liu 2012; Kaltdorf and Naseem, 2013). Our enrichment map showed that SA suppressed expression of *CHS, CHI, F3H, ANS,* and *ANR* involved in flavonoid biosynthesis and elevated expression of *POD* and *COMT* in lignin biosynthesis (Figure 3-8). SA significantly down-regulated CHI activity and flavonoid content but promoted POD activity and lignin content after *C. siamense* inoculation (Figure 9), which verified the gene expression results. These results suggest that lignin biosynthesis is important for SA to enhance the resistance to strawberry crown rot caused by *C. siamense*. Existing studies have suggested that lignin acts as a physical barrier against pathogens (Cesarino, 2019) and is important for disease resistance (Xiao *et al*., 2021; Onohata and Gomi, 2020). *Arabidopsis* knockout mutants of *COMT* and *cinnamyl alcohol dehydrogenase*, which have low lignin content, showed a reduction in basal resistance and/or effector-triggered resistance against various microbial pathogens, including the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* and the biotrophic fungal pathogen *Blumeria graminis* (Quentin *et al*., 2009; Huang *et al*., 2010; Tronchet *et al*., 2010).

Peroxidase is located in the monolignol pathway (synthesized from *p*-Coumaroyl-CoA) (Bonawitz and Chapple, 2010; Lee *et al*., 2019) and is promoted by SA pretreatment, which suggests that it plays an important role in inducing resistance. By contrast, flavonoids, the most well-described secondary metabolites in plant defense systems synthesized from *p*-Coumaroyl-CoA (Sarbu *et al*., 2019; Li *et al*., 2021), were significantly down-regulated by the SA pretreatment after *C. siamense* inoculation in our study. Thus, the balance of lignin and flavonoids from *p*-Coumaroyl-CoA biosynthesis requires further research in strawberry on the response to *C. siamense* infection.

CONCLUSIONS

The effects of exogenous SA on strawberry crown rot caused by *C. siamense* were investigated. The SA pretreatment inhibited the infection of *C. siamense* to strawberry crown not only by promoting MLO-like protein 2 and receptor-like kinase-encoding gene expression but also by improving POD activity and lignin content owing to the up-regulation of *POD* and *COMT* genes in lignin biosynthesis. However, SA pretreatment reduced CHI activity and flavonoid content due to the suppression of *CHS*, *CHI*, *F3H*, *ANS*, and *ANR* involved in flavonoid biosynthesis during *C. siamense* infection. Thus, exogenous SA enhanced strawberry resistance to crown rot caused by *C. siamense* by up-regulating the expression of defense genes and balancing lignin and flavonoid biosynthesis.

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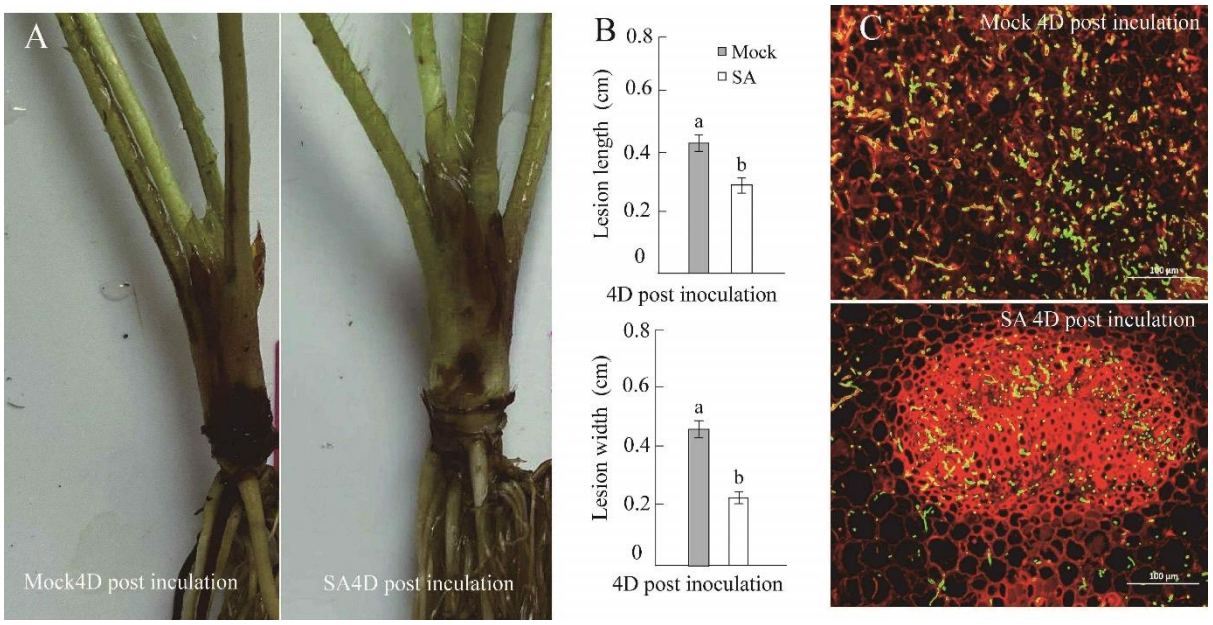


Figure 1. Effect of SA on *Colletotrichum siamense* SCR-7 infection in strawberry crown. **(A)** The lesions after 4 days of pathogen inoculation on strawberry crowns subjected to SA and mock pretreatments. **(B)** The lesion length and width after 4 days of SCR-7 inoculation on strawberry crown treated with SA and mock. (**C**) The hypha after 4 days of SCR-7 inoculation in strawberry crown (green) of SA and mock treatment, respectively. Data (Means±SE, n= 6) followed by different letters above the bars among treatments indicate significant differences.

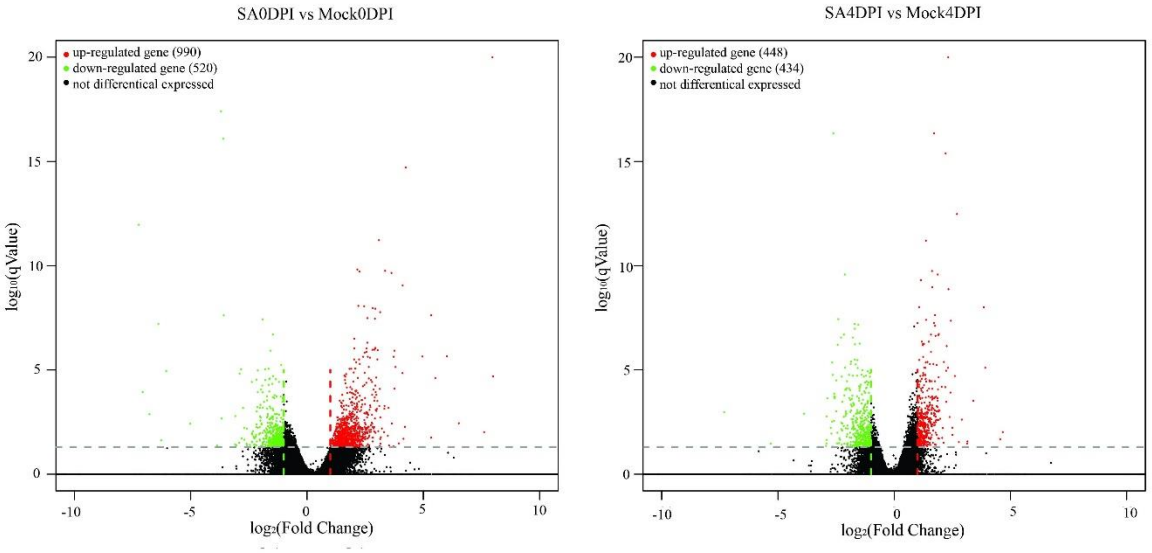


Figure 2. Effect of salicylic acid (SA) on differentially expressed transcript number in *Colletotrichum siamense* infected strawberry crown.

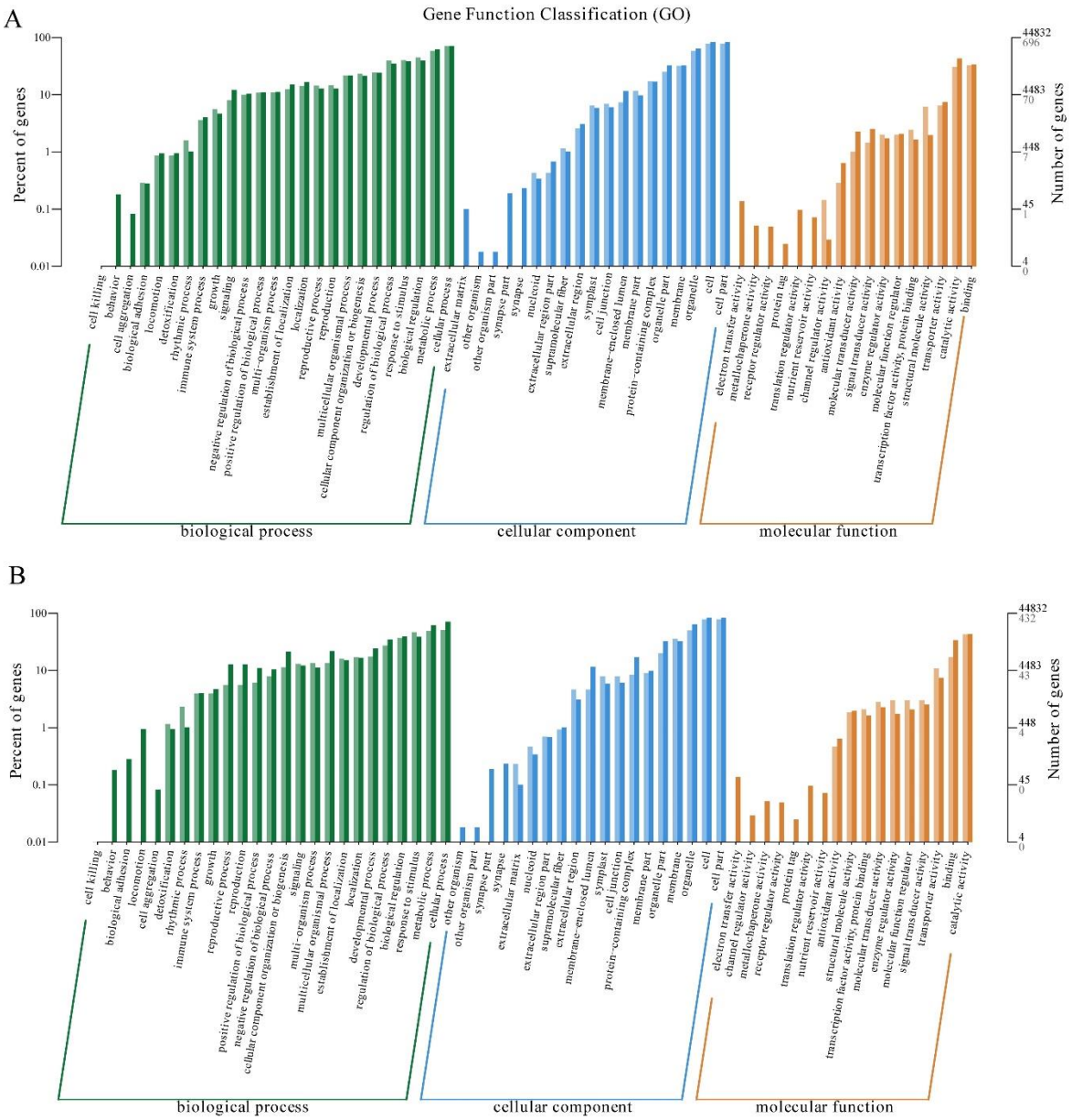


Figure 3. The gene function classification (GO) of differentially expressed transcripts in strawberry crown caused by salicylic acid (SA). A was analyzed based on the differentially expressed transcripts in SA vs mock treatment at 0 day after *Colletotrichum siamense* SCR-7 inoculation. B was analyzed based on the differentially expressed transcripts in SA vs mock treatment at 4 days after *Colletotrichum siamense* SCR-7 inoculation.

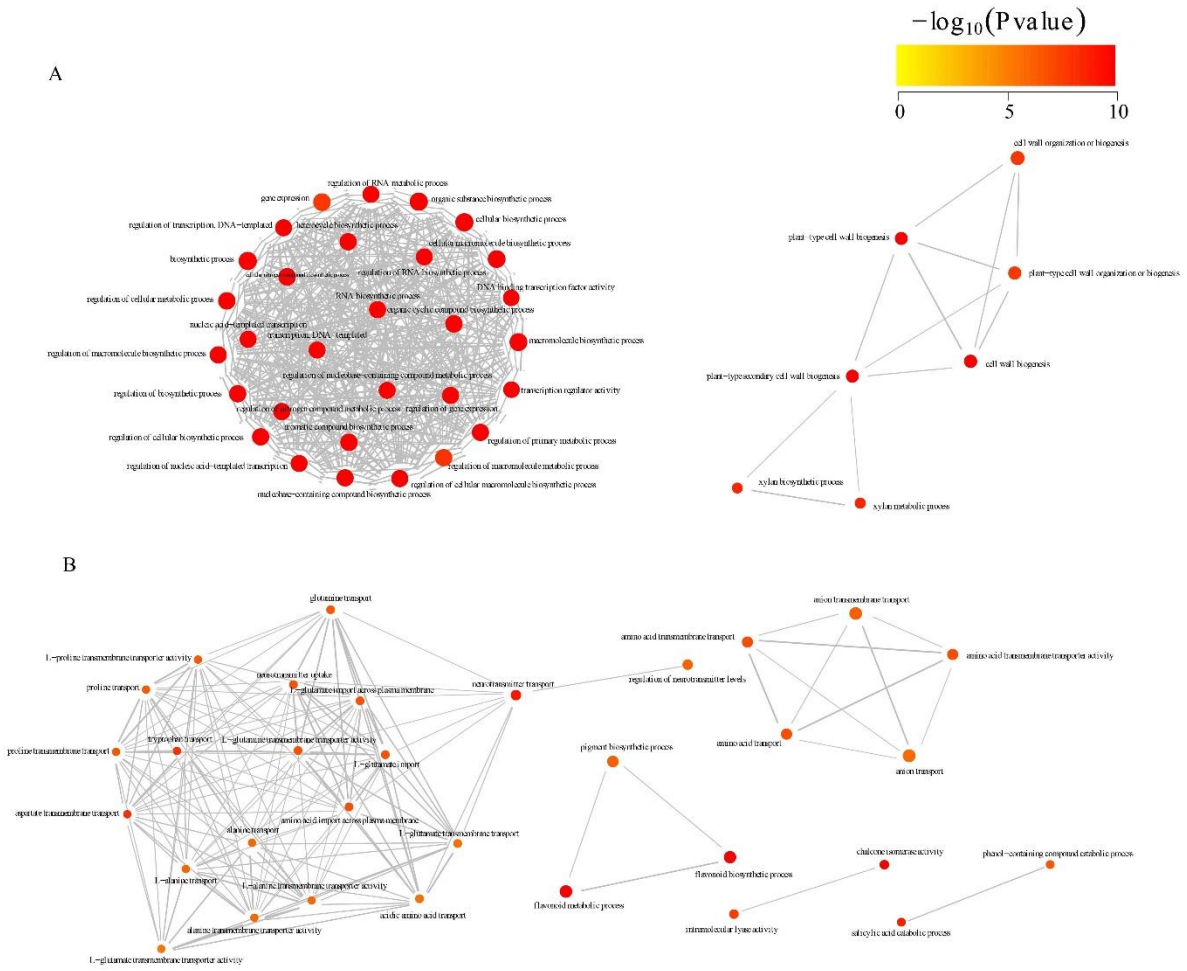


Figure 4. The GO enrichment map of differentially expressed transcripts in strawberry crown caused by salicylic acid (SA). A was analyzed based on the differentially expressed transcripts in SA vs mock treatment at 0 day after *Colletotrichum siamense* SCR-7 inoculation. B was analyzed based on the differentially expressed transcripts in SA vs mock treatment at 4 days after *Colletotrichum siamense* SCR-7 inoculation.

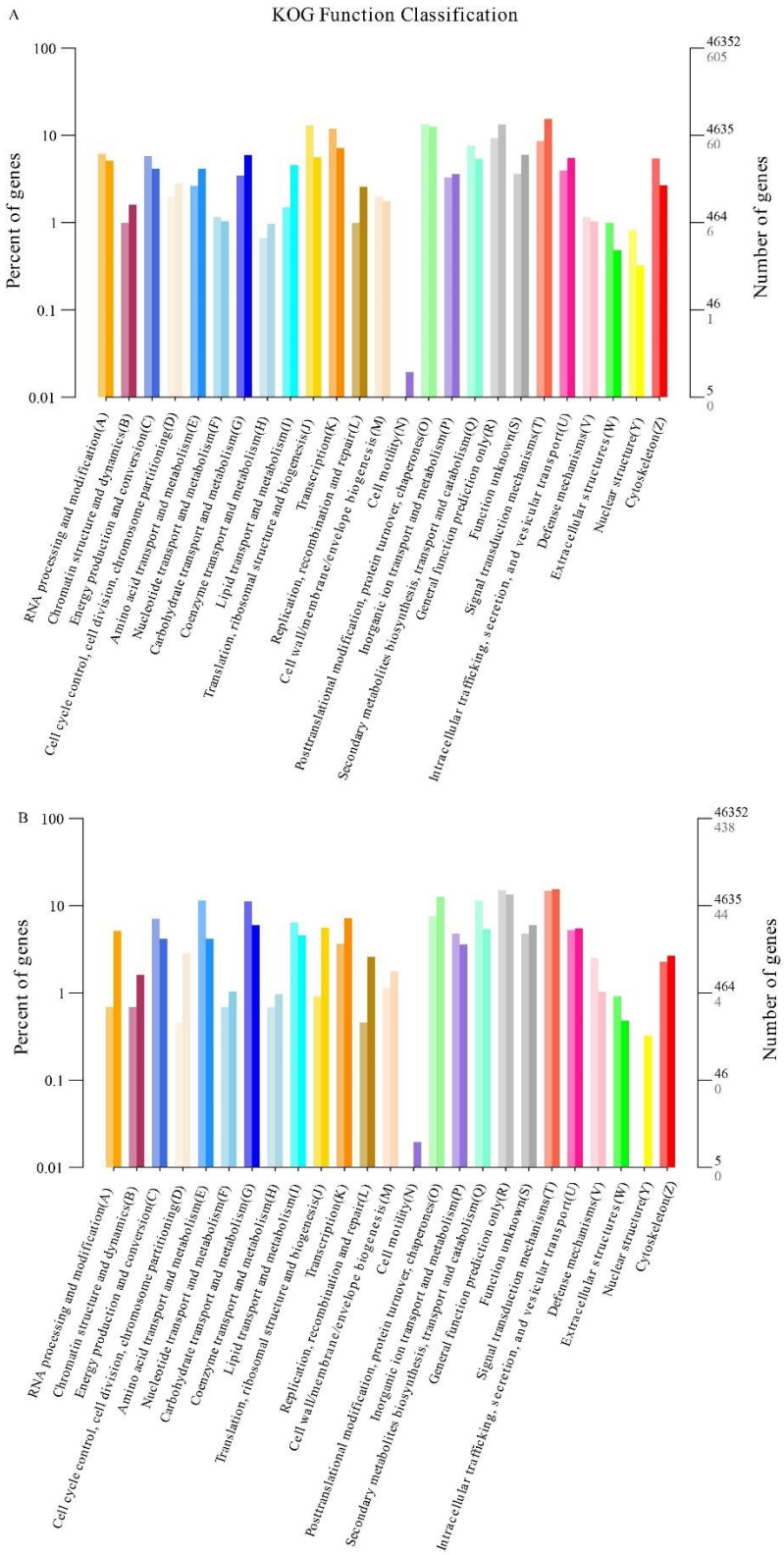
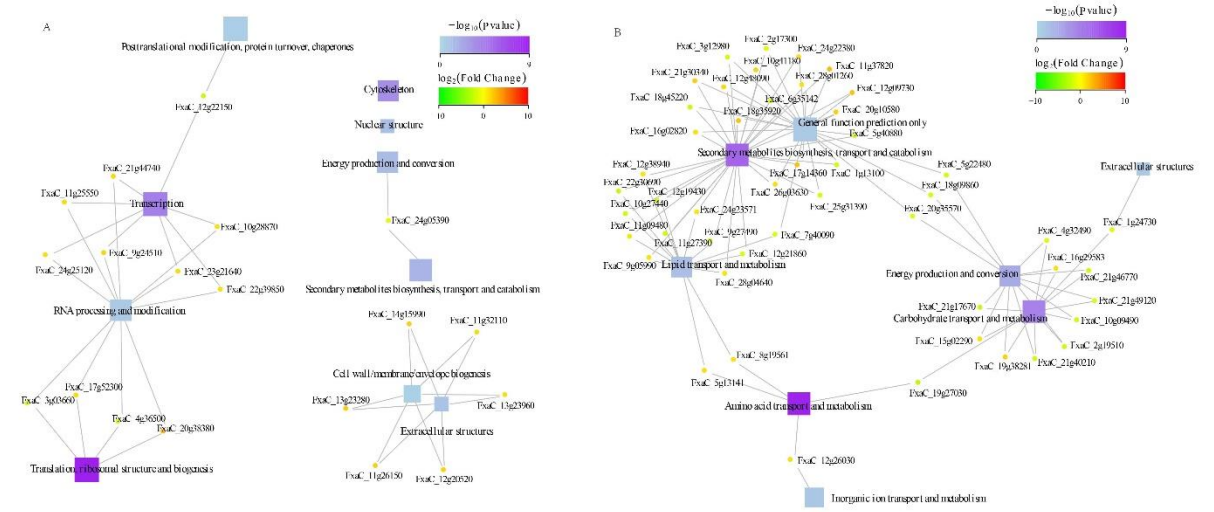


Figure 5. The KOG function classification of differentially expressed transcripts in strawberry crown caused by salicylic acid (SA). A was analyzed based on the differentially expressed transcripts in SA vs mock treatment at 0 day after *Colletotrichum siamense* SCR-7 inoculation. B was analyzed based on the differentially expressed transcripts in SA vs mock treatment at 4 days after *Colletotrichum siamense* SCR-7 inoculation.



435

436 **Figure 6.** The KOG enrichment network of differentially expressed transcripts in strawberry crown caused by
437 salicylic acid (SA). A was analyzed based on the differentially expressed transcripts in SA vs mock treatment at
438 0 day after *Colletotrichum siamense* SCR-7 inoculation. B was analyzed based on the differentially expressed
439 transcripts in SA vs mock treatment at 4 days after *Colletotrichum siamense* SCR-7 inoculation.

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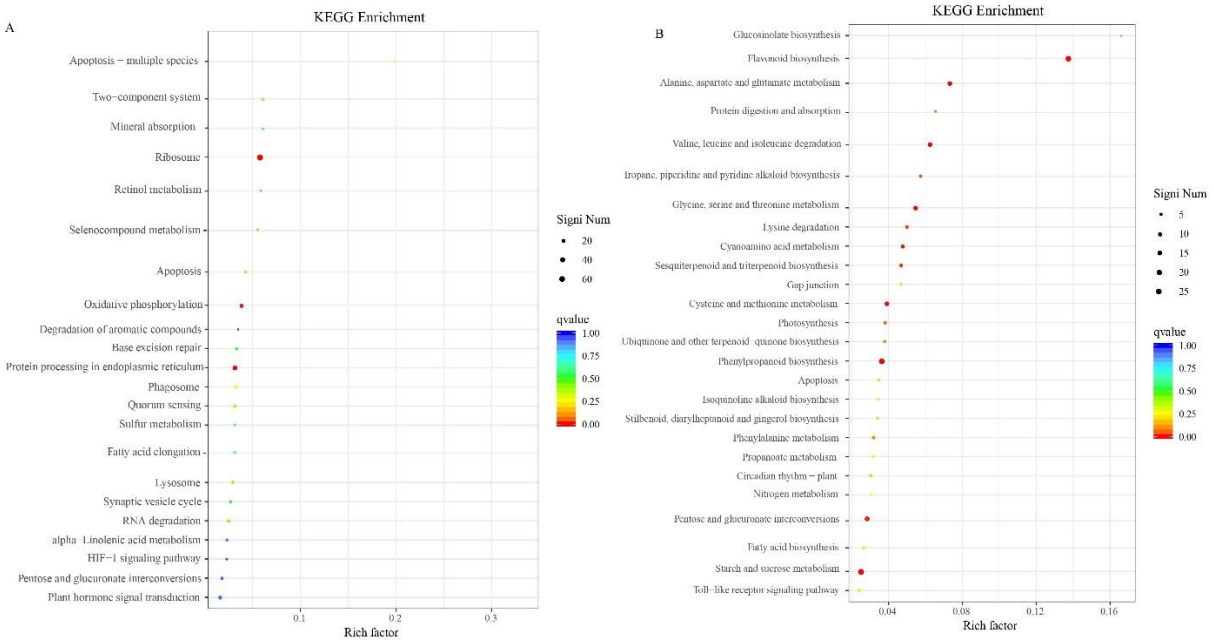

441

442 **Figure 7**. The KEGG enrichment map of differentially expressed transcripts in strawberry crown caused by
443 salicylic acid (SA). A was analyzed based on the differentially expressed transcripts in SA vs mock treatment at
444 0 day after *Colletotrichum siamense* SCR-7 inoculation. B was analyzed based on the differentially expressed
445 transcripts in SA vs mock treatment at 4 days after *Colletotrichum siamense* SCR-7 inoculation.

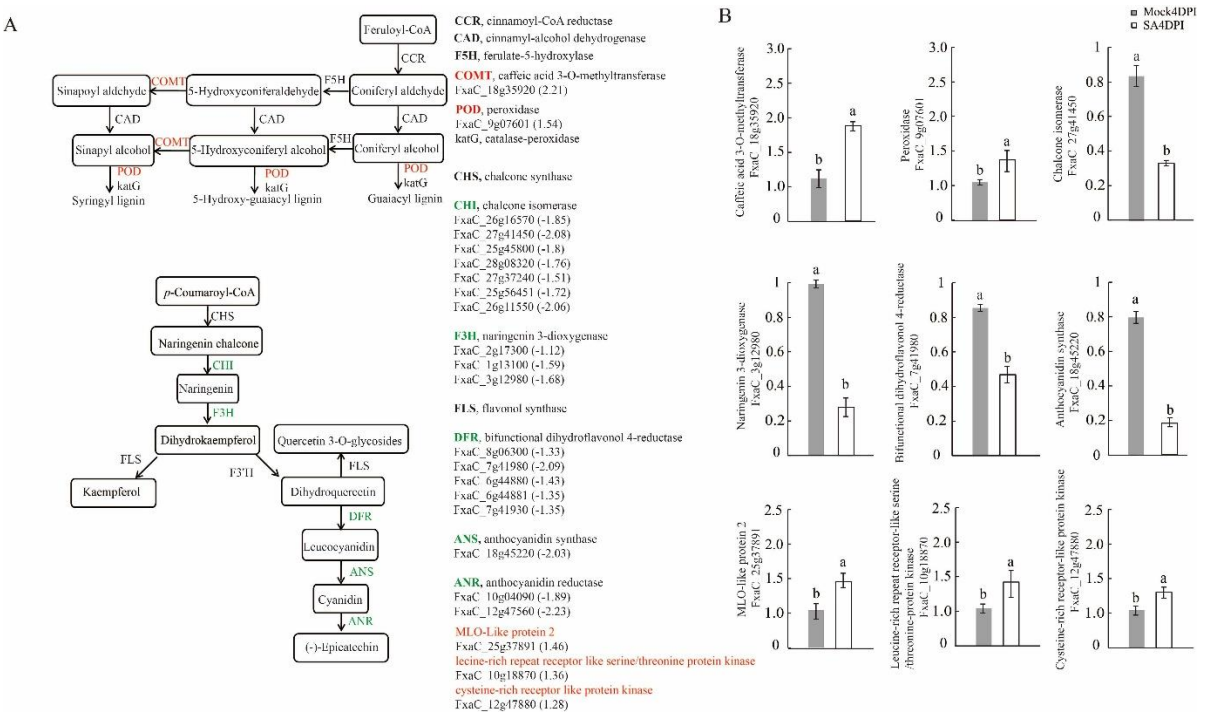

Figure 8. Effect of salicylic acid (SA) on potential genes involved in resistance to crown rot caused by *Colletotrichum siamense* SCR-7. A The variation in the expression of structural genes related to 'lignin biosynthesis' and 'flavonoid biosynthesis' in SA vs mock treatment 4 days after *Colletotrichum siamense* SCR-7 inoculation. B qRT-PCR results of structural gene expression related to 'lignin biosynthesis' and 'flavonoid biosynthesis' 4 days after *C. siamense* SCR-7 inoculation. Data (Means±SE, n= 6) followed by different letters above the bars among treatments indicate significant differences at the 5% level.

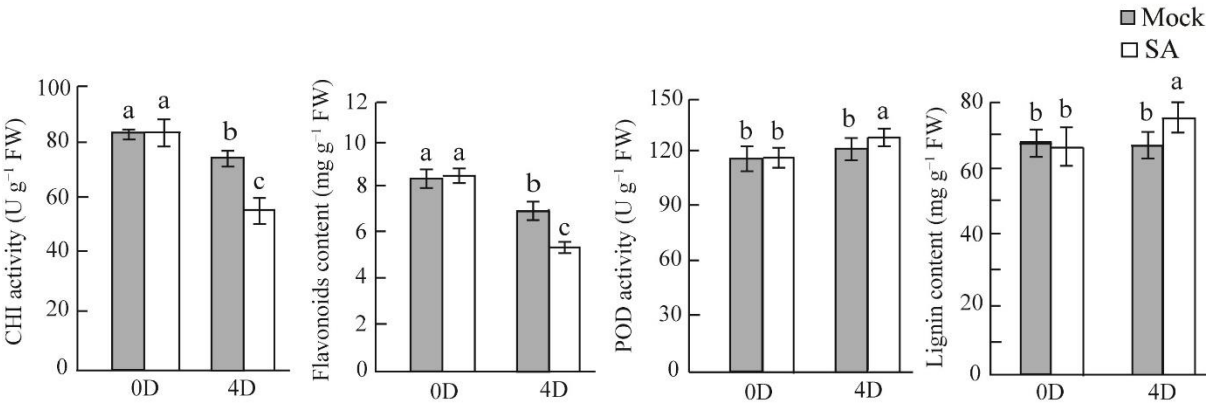

Figure 9. Effect of salicylic acid (SA) on flavonoid and lignin contents and the activities of chalcone isomerase (CHI) and peroxidase (POD). Data (Means±SE, n= 6) followed by different letters above the bars among treatments indicate significant differences at the 5% level.