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Article

Transcriptomic Analysis and Physiological Characteristics of Exogenous Naphthylacetic Acid Application to Regulate the Healing process of Oriental Melon Grafted onto Squash

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Abstract: The plant graft healing process is an intricate development influenced by numerous endogenous and environmental factors. This process involves the histological changes, physiological and biochemical reactions, signal transduction, and hormone exchanges in the grafting junction. Studies have shown that applying exogenous plant growth regulators can effectively promote the graft healing process and improve the quality of grafted plantlets. However, the physiological and molecular mechanism of graft healing formation remains unclear. In our present study, transcriptome changes in the melon and cucurbita genomes were analyzed between control and NAA treatment, and we provided the first view of complex networks to regulate graft healing under exogenous NAA application. The results showed that the exogenous NAA application could accelerate the graft healing process of oriental melon scion grafted onto squash rootstock through histological observation, increase the SOD, POD, PAL, and PPO activities during graft union development and enhance the contents of IAA, GA_3, and ZR except for the IL stage. The DEGs were identified in the plant hormone signal-transduction, phenylpropanoid biosynthesis, and phenylalanine metabolism through transcriptome analysis of CK vs. NAA at the IL, CA, and VB stage by KEGG pathway enrichment analysis. Moreover, the exogenous NAA application significantly promoted the expression of genes involved in the hormone signal-transduction pathway, ROS scavenging system, and vascular bundle formation.

Keywords: oriental melon; squash; graft; exogenous naphthylacetic acid; transcriptomic analysis; endogenous hormone; signal transduction; ROS scavenging; vascular bundle formation

1. Introduction

Grafting is a widely used technology to help horticultural plants overcome their limiting growth and reproduction factors [1]. In cultivation, the most frequently consumed horticulture plants, such as melon, watermelon, tomato, eggplant, and cucumber, are often grafted [2-3]. Successful grafting is a complex process involving the initial adhesion of scion and rootstock, callus tissue formation, and vascular bundle connection during the graft healing process. Callus tissue formation at the graft interface initiates the grafting process, and a lack of callus tissue formation can lead to grafting failure [2]. In addition, the callus cells differentiating into vascular tissue to re-connect the xylem and phloem at the graft junction is an essential process for successful grafting [4]. A functional vascular connection is an important signal for the establishment of successful graft healing [5-6]. However, the reconnection of the rootstock and the scion has tissue asymmetry. The phloem junction occurs at the graft junction before the xylem [7].

As we all know, complex physiological metabolites occur during the graft healing process [8]. Even though the studies on the histological analysis and physiological and

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biochemical changes of graft healing have been reported [9-10], the comprehensive understanding of the molecular mechanism of successful graft healing remains insufficient. The genome-wide transcriptome analysis could help to clarify the specific and underlying molecular mechanisms of grafting-depended biochemical processes. Some studies showed that transcriptomics efficiently analyzed the graft healing process of the different species. In the graft healing process of grapevine, transcriptional changes were examined via whole-genome microarray analysis, and the report revealed many genes associated with cell wall modification, hormone signaling, secondary metabolism, and wound responses [10]. Transcriptomic analysis of graft healing in Litchi showed nine annotated unigenes that participated in the auxin signaling pathway had higher expression levels in the compatible grafts than incompatible ones [9]. In the graft healing process of hickory, the transcriptomic analysis revealed 112 candidate uniqueities, which participated in the auxin and cytokinin signaling pathways [11]. Genome-wide transcriptome analysis of tissues above and below graft junctions revealed that the inter-tissue communication process occurred independently of functional vascular connections and acted as a signal to activate vascular regeneration [12].

Phytohormones play an essential role in plant wound healing and vascular formation [7]. Through the exogenous auxin application to callus, the formation of xylem and phloem was enhanced effectively [13-14]. The application of exogenous phytohormones, such as heteroauxin and zeatin, positively accelerated the healing by increasing the vascular bundle formation rate [15]. In the grafted seedlings cultivation practice, studies showed that applying exogenous plant growth regulators could effectively promote the healing of grafted seedlings, shorten the healing period of grafting, and improve the quality of grafted seedlings. Some plant growth regulators, like an auxin-based plant growth regulator, naphthylacetic acid (NAA), commonly treat scions or rootstocks to accelerate graft healing. However, the physiological and molecular mechanism of graft healing by exogenous NAA application is not comprehensive. The main goals of the present study aimed to characterize the anatomical development stages of graft healing formation and clarify the physiological and transcriptomic changes in the graft junction of oriental melon grafted onto squash under NAA application, and provide a theoretical and practical basis for further improving the efficiency of commercial grafted melon seedlings cultivation.

2. Materials and Methods

2.1 Experimental materials

In the present study, we took the oriental melon cultivar (YinQuan No.1, *Cucumis melo* var. Makuwa) to graft onto the squash cultivar (ShengZhen No.1, *C. moschata*) by splicing graft method when the scion's first-true-leaf fully expanded and rootstock's cotyledon development stage, using the one-cotyledon method [16]. During the grafting process, the grafted seedlings of scions dipped in the NAA solution (40 mg·L^{-1}) were the treatments (NAA), and dipping in the distilled water were the controls (CK). Grafted seedlings were transplanted in the nutritional bowl (12 cm×12 cm) and moved into the healing chamber for grafted seedlings cultivation at Shenyang Agriculture University. The management methods of grafted seedlings for CK and NAA were consistent [17].

2.2 Paraffin sectioning and microscopy

The samples of 0.3-0.5cm stem above and below the graft junction were fixed, softened, dehydrated, infiltrated, and embedded in paraffin during graft union development [18]. Transverse serial sections (≈10μm thick) were stained with pH4.4 toluidine blue [19], and mounted using synthetic resin (Permount). Sections were examined using a light microscope (Lecia RM 2245, Germany). After observing the paraffin section, the IL, CA, and

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VB stage of CK and NAA was respectively screened, and the corresponding samples carried out the other experiments for measuring the activities of enzymes involved in ROS scavenging (SOD, POD, PAL, and PPO), endogenous hormones contents (IAA, GA_3, and CTK), and RNA-seq assay.

2.3 Determination of SOD, POD, PAL, and PPO activities

The graft junction tissues (≈0.5 g) of CK and NAA at the IL, CA, and VB stage with three biological replicates were ground with a pestle in an ice-cold mortar with 4 ml 50 mM phosphate buffer (pH 7.0). The homogenates were centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was used to measure enzyme activities [20]. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium. POD activity was measured as the increase in absorbance at 470 nm caused by guaiacol oxidation [21]. PAL activity was measured [22]. The reaction mixture was 0.4 ml of 100 mM Tris-HCl buffer (pH 8.8), 0.2 ml of 40 mM phenylalanine, and 0.2 ml of enzyme extract. The reaction mixture was incubated for 30 min at 37℃ and the reaction was ended by adding 25% trichloroacetic acid. The absorbance of the supernatant was measured at 280 nm. PPO activity was spectrophotometrically determined at 398 nm [23]. The reaction mixture contained 2.8 ml 0.1% catechol solution, and 0.2 mL enzyme extract in a total volume of 3 ml.

2.4 Determination of hormones content by ELISA

We sampled the graft junction tissues of CK and NAA at the IL stage, CA stage, and VB stage with three biological replicates. The content of IAA, GA_3, and ZR was measured by the Enzyme-Linked Immunosorbent Assay (ELISA), and the detailed protocol for determining the hormone content was previously described [24]. GC-MS and HPLC validated the accuracy of ELISA kits (China Agricultural University).

2.5 RNA Extraction, Library Construction, and Sequencing

Total RNA extraction, library construction, and RNA-Seq were performed by Biomarker Technology Co. (Beijing, China, https://www.biocloud.net/). RNA concentration was measured using NanoDrop 2000(Thermo), and the detailed protocol was previously described [25]. The raw sequencing data had been uploaded to the NCBI Sequence Read Archive (SRA) with the accession number PRJNA655799 and PRJNA689873.

2.6 Differential Expression Analysis

We respectively performed Melon (DHL92) v3.6.1 Genome and Cucurbita moschata (Rifu) Genome (http://cucurbitgenomics.org) to analyze the raw sequencing data of graft junction of CK and NAA at IL, CA, and VB stage. Differential expression analysis of two conditions/groups was performed using the DEseq. DEseq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *p*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value <0.01 found by DEseq were assigned as differentially expressed [26].

2.7 Enrichment Analysis of GO Enrichment and KEGG Pathway

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GO seq R packages based on Wallenius non-central hyper-geometric distribution [27], which can adjust for gene length bias in DEGs. KEGG is a database resource for understanding high-level functions and utilities of the biological

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system, such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways[28].

2.8 Quantitative real-time PCR (qRT-PCR)

Some differentially expressed genes were selected to validate the accuracy of RNA-Seq data using qRT-PCR preparation with three biological replicates for each sample was conducted as described above. According to the manufacturer's instructions, the first-strand cDNA synthesis kit was performed using a Prime-Script™ II First Strand cDNA synthesis kit (Takara Bio, Dalian, China). The primer sets for each unigenes were designed by Primer Premier 5.0. qRT-PCR was carried out on Yena Real-Time PCR System (qTOWER3/qTOWER3 touch, Germany) with SYBR Premix Ex Taq™ II kit (Takara). Expression was calculated as $2^{-\Delta\Delta Ct}$ and normalized to that of the reference gene Actin.

2.9 Statistical Analysis

The data were displayed with the mean ± standard error in triplicate and analyzed by a-way variance (ANOVA, SPSS 22.0 software). Significant analysis was performed by Duncan's multiple range tests (p<0.05). The figures were produced by PRISM 8.0 software.

3. Results

3.1 Effects of exogenous NAA application on histological changes of graft junction

A significant adhesion was observed in the graft junction of oriental melon grafted onto squash at 2 DAG (Figure 1A, 1D). The isolation layer (IL)was generally formed by the protoplasm of destructed parenchyma cells and dead cells on the wound interface [29]. The results showed that exogenous NAA application could not promote the formation of an isolated layer. With the graft junction development, the isolation layer gradually disappeared. Callus tissue (CA) provides a pathway for the communication between scion and stock [30]. The graft junction with exogenous NAA treatment formed a callus tissue at 5 DAG (Figure 1E), while the callus formation of CK was observed at 6 DAG (Figure 1B). The vascular connection between the grafted partners was a mark of grafting success [2, 31]. At 8 DAG (Figure1F), the graft junction with exogenous NAA treatment formed vascular bundles (VB), and the new vascular bundle formation of CK occurred at 9 DAG (Figure 1C). The results suggested that exogenous NAA application could shorten the graft healing process of oriental melon seedlings grafted onto squash.

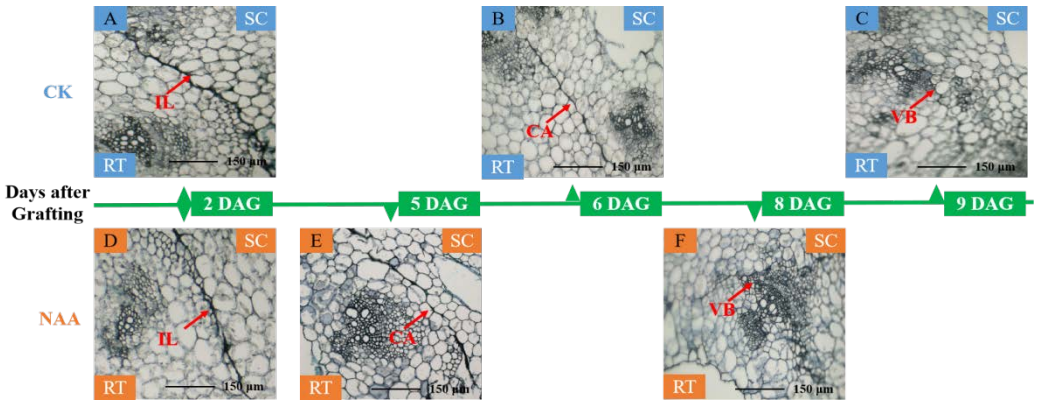

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Figure 1 Histological changes of graft junction using paraffin sectioning and microscopy method during graft union development. (A; D), isolation layer stage (IL stage); (B; E), callus tissue stage (CA stage); (C; F), vascular bundles stage (VB stage). SC, scion. RT, rootstock. DAG, days after grafting.

3.2 Effects of exogenous NAA application on the activities of the related enzyme involved in ROS scavenging of graft junction during graft union development

In higher plants, mechanical wounding generates ROS production, and the antioxidant enzyme activities in the graft junction interface may be responsible for the degradation of the grafting zone [32-35]. Along with the graft union development, SOD activities of CK and NAA treatment first rose and then fell and presented the highest activity at the CA stage. SOD activity of NAA treatment was significantly higher than CK at the IL and CA stage (Figure 2A). POD activity was significantly higher than CK at the CA stage and reached the highest activity (3718 $U \cdot g^{-1} \cdot h^{-1}FW$) under NAA treatment (Figure 2B). In CK, POD activity (3183 $U \cdot g^{-1} \cdot h^{-1}FW$) was the highest value, and no significant difference compared with NAA treatment at the VB stage. As shown in Figure 2C, NAA treatment increased PAL activity during the graft healing process. PAL activity was significantly higher than CK except for the CA stage and reached the highest activity (306.67$U \cdot g^{-1} \cdot h^{-1}FW$) at the VB stage. PPO activity had a similar change trend in CK and NAA, which increased. Under NAA treatment, the value was significantly higher than CK at the IL stage. There were no significant differences in PPO activity at the CA and VB stages between CK and NAA treatment.

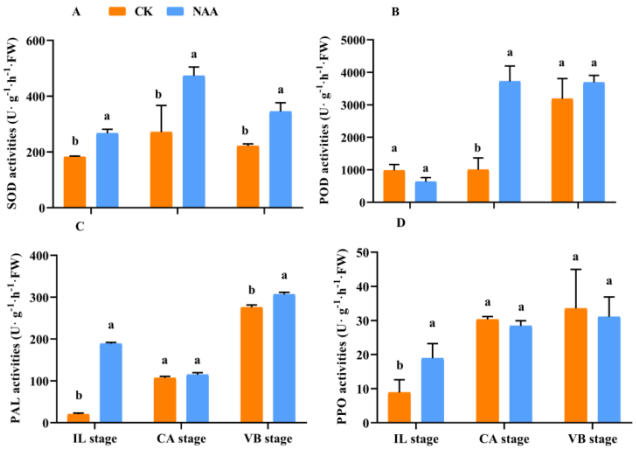

Figure 2 Effects of exogenous NAA application on SOD, POD, PAL, and PPO activities during graft union development. (A) SOD; (B) POD; (C) PAL; (D) PPO. Different letters indicate significant differences (p<0.05). Values are means ± SD, n=3.

3.3 Effects of exogenous NAA application on endogenous hormones contents of graft junction during graft union development

Some major endogenous hormones, IAA, CTK, and GA, relate to callus formation development and vascular bundle reconnection [36-38]. Our results indicated that exogenous NAA application accelerated the graft healing process by promoting the content of three endogenous hormones at three critical stages. During the early stage of graft healing, a block in auxin basipetal transport was generated due to vascular damage, and the content showed a decreased trend. As the healing process was completed,

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auxin was accumulated in the wound interface and the content gradually increased. Under NAA treatment, the auxin content was lower than CK at the IL stage and was significantly higher than CK at the CA and VB stages (Figure 3A). The GA_3 content was significantly increased in CK at the CA and VB stage, while there was no significant difference at the IL stage. When exogenous NAA was applied, the changing trend of GA_3 content was consistent with CK and significantly higher than CK at the VB stage (Figure 3B), which was 1.25 times that of CK. The content of ZR gradually increased during the graft healing process and reached the highest value (9.744149ng^{-1}g·FW) in CK at the VB stage. Under NAA treatment, the trend of ZR content was consistent with CK, and the ZR content was significantly higher than CK at the CA and VB stages, which was 1.3 and 1.2 times that of CK, respectively (Figure 3C).

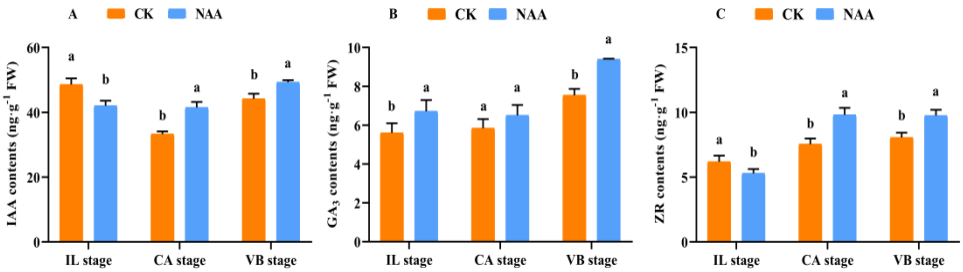

Figure 3 Effects of exogenous NAA application on endogenous hormone contents of IAA, GA, and ZR during graft union development. (A) IAA; (B) GA_3; (C) ZR. Different letters indicate significant differences (p<0.05). Values are means ± SD, n=3.

3.4 Identification of differentially expressed genes (DEGs) of graft junction under exogenous NAA application during graft union development

In order to further study the molecular mechanism of exogenous NAA application to regulate the graft healing process of oriental melon grafted onto squash, we performed transcriptomic analysis of graft junction tissue at the IL, CA, and VB stage under exogenous NAA application. We identified the DEGs in the melon genome (Figure 4A) and Cucurbita moschata (Rifu) genome (Figure 4B), respectively, according to the criteria of at least two-fold change and FDR<0.01. 5324 DEGs were discovered by analyzing CK vs. NAA in the melon genome (Figure 4A), with 1621 up-regulated and 1414 down-regulated at the IL stage; 261 up-regulated and 296 down-regulated at the CA stage; with 815 up-regulated and 917 down-regulated at the VB stage. 6602 DEGs were identified by analyzing CK vs. NAA in the cucurbita moschata (Rifu) genome (Figure 4B), with 696 up-regulated and 914 down-regulated at the IL stage; with 512 up-regulated and 1477 down-regulated at the CA; with 1574 up-regulated and 1429 down-regulated at the VB stage.

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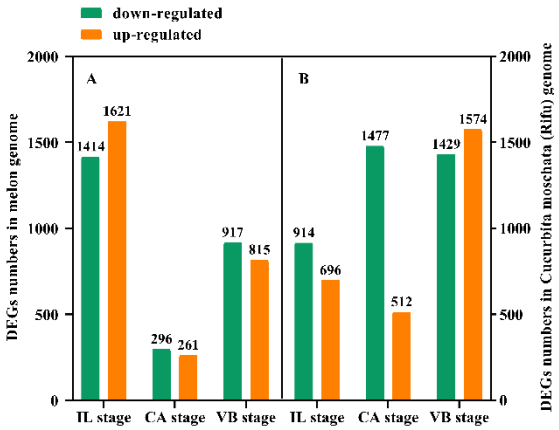

Figure 4 The numbers of DEGs of graft junction at the IL, CA, and VB stage under exogenous NAA application. (A), CK *vs.* NAA in Melon genome; (B), CK *vs.* NAA in Cucurbita moschata (Rifu) genome.

3.5 Gene ontology and pathway enrichment Analyses DEGs induced by exogenous NAA during graft union development

We used the Gene Classification System (GO) to further analyze the identified DEGs at the IL, CA, and VB stage under exogenous NAA treatment. The results showed that most DEGs were classified into three categories: biological process, cellular component, and molecular function (Figure 5). At the IL, CA, and VB stage (Figure 5A, 5C, 5E), there most highly enriched terms in the Melon genome were metabolic process (1270, 206, 693 genes), single-organism process (1142, 196, 609 genes) and cellular process (829, 153, 454 genes) within the biological process category; cell (804, 152, 509 genes), cell part (824, 124, 432 genes) and membrane part (801, 119, 424 genes) within the cellular component; binding (1031, 186, 586 genes), catalytic activity (1041, 200, 605 genes), and transporter activity (162, 42, 108 genes) within the molecular function category. Furthermore, at the IL, CA, and VB stage (Figure 6B, 6D, 6F), there most abundant terms in the Cucurbita moschata (Rifu) genome were metabolic process (540, 836, 1163 genes), single-organism process (453, 709, 847 genes) and cellular process (504, 768, 1129 genes) within the biological process category; cell (321, 538, 843 genes), cell part (321, 538, 843 genes) and organelle (222, 366, 638 genes) within the cellular component; binding (395, 561, 769 genes), catalytic activity (494, 725, 845 genes), and transporter activity (79, 80, 114 genes) within the molecular function category.

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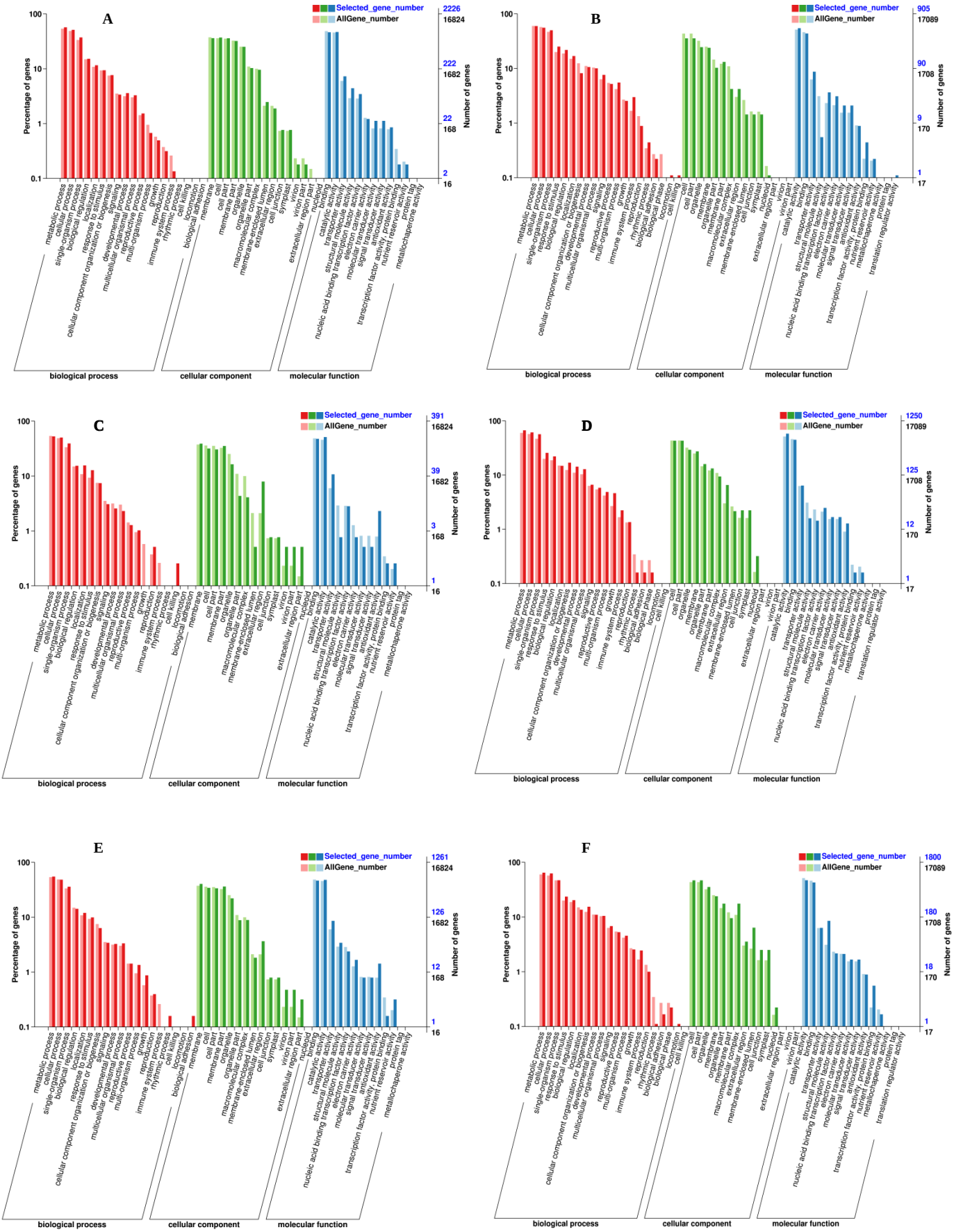

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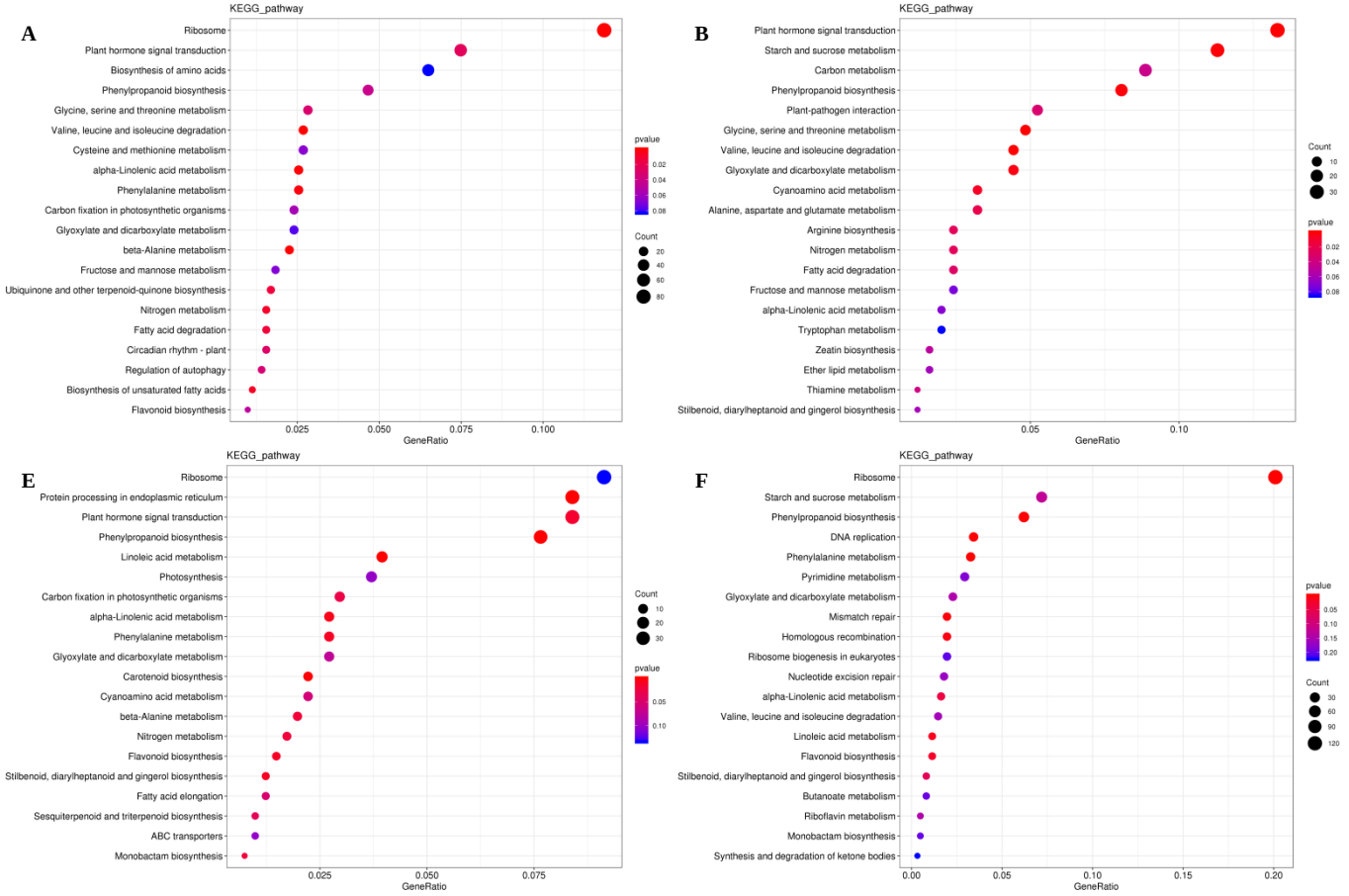
Figure 5 GO functional classification and enrichment analysis of DEGs during graft union development. (A, C, E) CK *vs.* NAA at the IL, CA, and VB stage using Melon genome. (B, D, F) CK *vs.* NAA at the IL, CA, and VB stage using Cucurbita moschata (Rifu) genome.

The DEGs were also subjected to KEGG pathway enrichment analysis. The top 20 pathways, which highest enrichment level based on the numbers and enrichment levels of the annotated DEGs, were shown in Figure 6. The results were consistent with the results of GO functional analysis. It was noteworthy that plant hormone signal-transduction, phenylpropanoid biosynthesis, and phenylalanine metabolism were the overlapping pathways identified at the IL, CA, and VB stage under exogenous NAA treatment, respectively. At the IL stage (Figure 6A, 6B), 53 DEGs, 18 DEGs (using Melon genome), and 33 DEGs, 20 DEGs (using Cucurbita moschata genome) were involved in plant hormone signal transduction and phenylpropanoid biosynthesis, respectively. At the CA stage (Figure 7C) and VB stage (Figure 6E), we found that 12 DEGs, 10 DEGs, and 34 DEGs, 21 DEGs were enriched in plant hormone signal transduction and phenylpropanoid biosynthesis using Melon genome. However, 45 DEGs, 20 DEGs (Figure 6D), 38 DEGs, 20 DEGs (Figure 6F) were involved in phenylpropanoid biosynthesis and phenylalanine metabolism using Cucurbita moschata genome.



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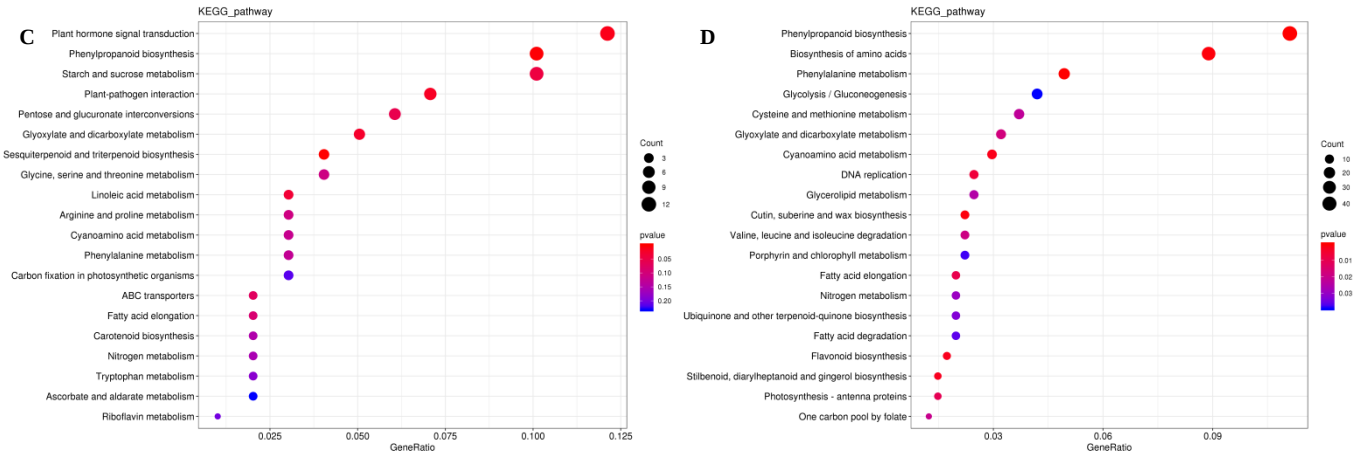

Figure 6 KEGG pathway enrichment analysis of DEGs during graft union development. (A, C, E) CK *vs.* NAA at the IL, CA, and VB stage using Melon genome. (B, D, F) CK *vs.* NAA at the IL, CA, and VB stage using Cucurbita moschata (Rifu) genome.

3.6 Exogenous NAA activated hormone signal-transduction pathway during graft union development

Plant hormones of scion-rootstock communication are critical for successful graft [7]. In our study, IAA, GA_3, and ZR contents of graft junction increased under exogenous NAA application during graft union development (Figure 3), and most DEGs were enriched in the hormone signal-transduction pathway (Figure 6). So we analyzed the unigenes that participated in the hormone signal-transduction under exogenous NAA application (Figure 7). At the IL stage, four unigenes encoding auxin efflux carrier (MELO3C019102.2, CmoCh11G003520, CmoCh04G021920, CmoCh11G003180), three unigenes encoding auxin response factors (ARFs) (MELO3C003768.2, MELO3C025777.2, MELO3C019801.2), five unigenes encoding AUX/IAA (MELO3C007691.2, MELO3C024699.2, MELO3C004382.2, CmoCh10G006330, CmoCh09G004620), two unigenes encoding GH3 (MELO3C008672.2, MELO3C017825.2) were significantly up-regulated in auxin signaling. And three unigenes encoding type-B ARR protein (MELO3C012031.2, CmoCh15G008650, CmoCh05G000700), one unigene encoding CRE1 (CmoCh15G009250) and two unigenes encoding AHP (MELO3C024439.2, CmoCh14G013260) in cytokinin signaling were also greatly up-regulated. At the CA stage, one unigene encoding ARF (MELO3C033303.2) in auxin signaling, one unigene encoding type-B ARR protein (CmoCh14G016360) and four unigenes encoding AHP (CmoCh14G016980, CmoCh17G002800, CmoCh14G013260, CmoCh06G014530) were significantly up-regulated. At the VB stage, one unigene encoding auxin efflux carrier (CmoCh07G011410), one unigene encoding ARF (CmoCh08G001220), three unigenes encoding AUX/IAA (MELO3C025308.2, CmoCh07G006010, CmoCh14G018090), four unigenes encoding GH3 (MELO3C016616.2, MELO3C007597.2, CmoCh03G009710, CmoCh16G003520) in auxin signaling, and one unigene encoding type-B ARR protein (CmoCh15G008650), two unigenes encoding AHP (MELO3C024439.2, MELO3C015359.2) were significantly up-regulated, respectively. However, we found that exogenous NAA application did not significantly activated unigenes expression involved in GA signaling.

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Figure 7 Expression patterns of DEGs involved in hormone signal-transduction. The values of log2 fold change were shown in the heat map.

3.7 Exogenous NAA application activated expression of genes involved in Reactive Oxygen Species (ROS) Scavenging during graft union development

The efficient antioxidant defense system plays a vital role in the graft healing process and is necessary for successful grafting. Under exogenous NAA application, we found many DEGs involved in ROS scavenging, including unigenes encoding peroxidase (POD), ascorbate peroxidase (APX), peroxiredoxin (Prx), superoxide dismutase (SOD), and phenylalanine ammonia-lyase (PAL) were activated during graft union development (Figure 8). At the IL stage, eleven genes expression of POD, one gene expression of APX, two genes expression of Prx, one gene expression of SOD, and two genes expression of PAL were up-regulated by exogenous NAA application. At the CA stage, three genes expression of POD were up-regulated. Moreover, at the VB stage, five genes expression of POD, one gene expression of APX, two genes expression of Prx, two genes expression of SOD, and one gene expression of PAL were up-regulated.

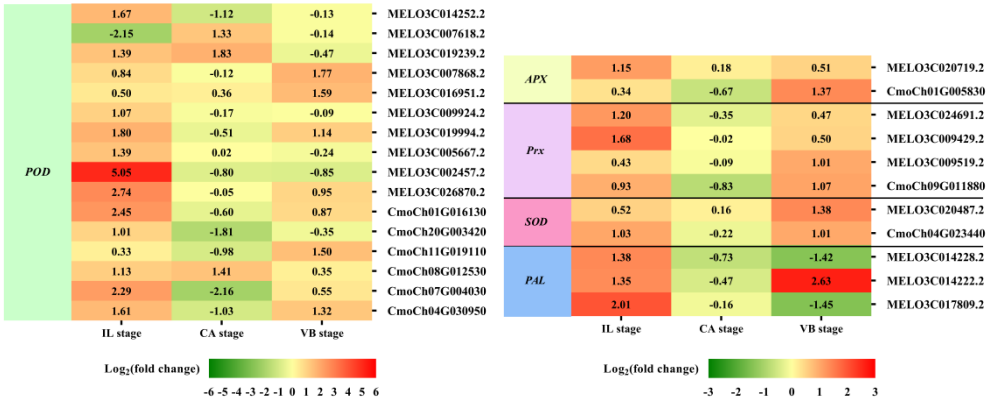

Figure 8 Expression profiles of DEGs involved in ROS scavenging. The values of log_2 fold change were shown in the heat map.

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3.8 Exogenous NAA application activated expression of genes related to vascular bundle formation during graft union development

It is well-known that the vascular bundle re-connection is a mark of successful grafting [2]. In our study, exogenous NAA application accelerated the healing of oriental melon scion grafted onto the squash rootstock. In order to clarify the genes related to the vascular bundle formation process, including the cell elongation, vascular cell differentiation, and developing vascular cell trigger programmed cell death (PCD), we performed the heat map analysis of different unigenes expression (Figure 9), such as expansion, tubulin, cellulose synthase, cinnamoyl-CoA reductase (CCR), hydrolytic enzyme (aspartic proteinase, cysteine proteinase), nuclease (exonuclease, ribonuclease), metacaspases and MYB transcription factors, using transcriptome data (Figure 10). Enzymes such as expansions are necessary for cell growth and cell wall architecture [39]. The results showed that six unigenes encoding expansion (MELO3C017181.2, MELO3C025095.2, CmoCh11G001730, CmoCh01G005150, CmoCh03G004350, CmoCh10G002140) were up-regulated at the VB stage. Tubulin is involved in cell expansion by guiding nascent cellulose microfibrils deposition during vascular development [40-41]. We found that the expression of five tubulin genes (CmoCh03G009120, CmoCh07G010840, CmoCh14G000910, CmoCh07G006190,CmoCh16G003240) were also elevated by exogenous NAA application at the VB stage, but CmoCh03G009120, CmoCh07G010840, CmoCh14G000910, and CmoCh07G006190, except CmoCh16G003240, were down-regulated at the CA stage. We identified four unigenes, encoding cellulose synthase (MELO3C016270.2, CmoCh16G005790, CmoCh09G010200, CmoCh14G009470) implicated in cellulose synthesis, were significantly up-regulated at the VB stage. One unigene encoding CCR (MELO3C017061.2) involved in the phenylpropanoid pathway was up-regulated at the IL and VB stages. Furthermore, five unigenes encoding aspartic proteinase (MELO3C020328.2, CmoCh15G014930, CmoCh07G004000, CmoCh04G013980, CmoCh18G008610), two unigenes encoding cysteine proteinase (MELO3C027001.2, CmoCh03G010910), one unigene encoding exonuclease (CmoCh13G010360), and tow unigenes encoding ribonuclease (MELO3C023673.2, CmoCh03G010620), all of them involved in xylogenesis [41-44], were up-regulated at the VB stage. Meanwhile, one unigene encoding metacaspases (CmoCh11G014810) that participated in PCD was also up-regulated at the VB stage. Additionally, sixteen unigenes encoding MYB transcription factors involved in the phenylpropanoid biosynthesis pathway were identified and significantly up-regulated by exogenous at the VB stage.

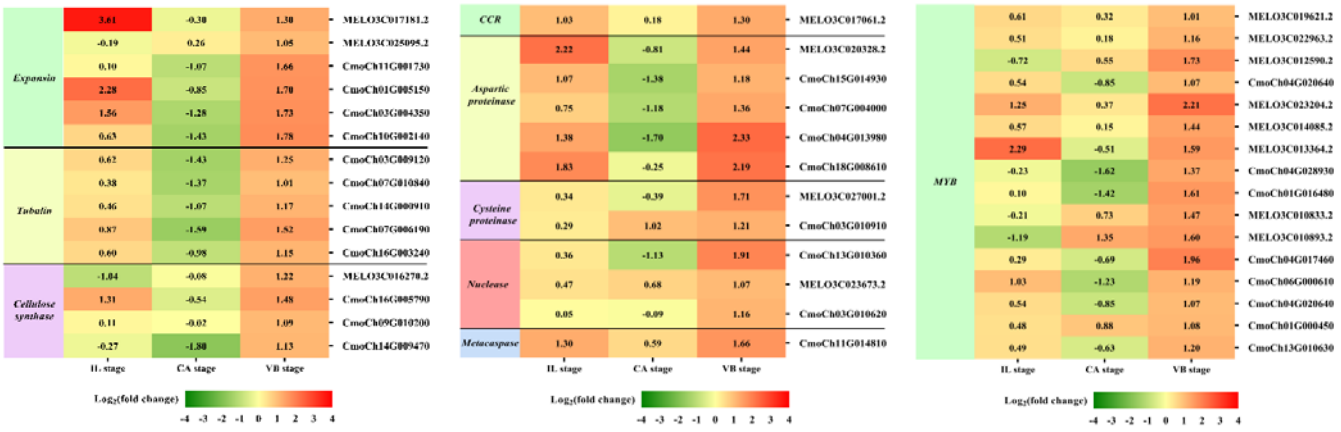

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Figure 9 Expression profiles of DEGs involved in vascular bundle formation. The values of log_2 fold change were shown in the heat map.

3.9 Validation of RNA-Seq data by qRT-PCR

To verify the DEGs related to hormone signal and phenylpropanoid biosynthesis identified using RNA-Seq, we performed qRT-PCR assays with independent samples collected from graft junction tissues at different graft healing stages. The expression levels of these selected ten genes were identified between the two data sets (Figure 10). The result showed that the expression of ten genes detected by qRT-PCR matched the trend of their FPKM value change in RNA-Seq.

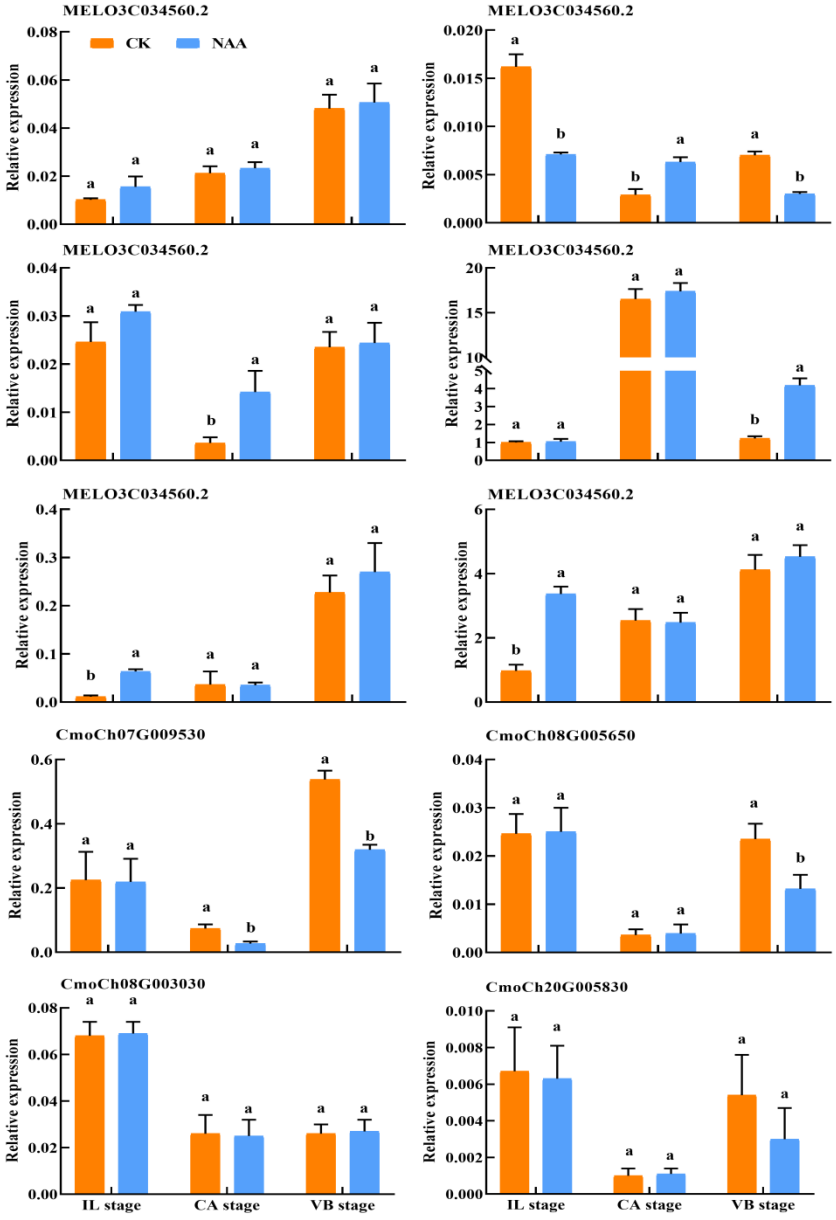

Figure 10 Verification of differentially expressed genes by qRT-PCR. Different letters indicate significant differences ($p<0.05$). Values are means ± SD, n=3.

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4. Discussion

Some reports showed that exogenous auxin application could promote callus formation [14,45]. We found that exogenous NAA application (40 mg·L^{-1}) accelerated the callus formation time and effectively promoted the time of vascular bundles connection of oriental melon scion grafted onto squash rootstock. Our results are consistent with the findings of Lu and Song [15], who indicated that exogenous plant hormone application could accelerate the rate of plant graft healing.

During the graft union development, the antioxidant defense system, including SOD, PAL, POD, and PPO, was essential for the graft healing process [46-47]. PAL and POD enzyme was associated with lignin biosynthesis and tubular molecular formation [48-50]. In addition, PPO not only protected enzymes in plants but also played an important role in lignin biosynthesis. The higher the PPO activity, the longer the existence time of the graft interface between rootstock and scion, which affected the survival of graft healing. [51-53]. In our study (Figure 2), the NAA treatment significantly enhanced SOD activities of graft junction during the graft union development (Figure 2A). POD and PPO activities were remarkably increased by NAA treatment at the CA and IL stages, respectively. (Figure 2B, 2D). PAL activities were significantly higher than the control at the IL and VB stage under exogenous NAA application (Figure 2C). The results indicated that the exogenous NAA treatment effectively removed the damage caused by reactive oxygen species to the graft interface.

Furthermore, The induction of PPO activities increased some black and brown substances that form an isolated layer produced by the oxidation of phenolics when the plant was cut. The higher the PPO activities, the longer the graft interface's existence time between rootstock and scion, which affected the survival of graft healing [54-55]. In our study, NAA treatment had higher PPO activities compared with the control at the IL stage while had lower PPO activities at the CA and VB stage, potentially because the isolated layer disappeared, better formation of callus and the vascular bundle was beneficial to improve the healing speed and survival rate.

IAA, CTK, and GAs are the primary hormones related to callus formation, vascular bundle development, and reconnection [36-38]. The appropriate concentration of IAA was added to the undifferentiated tissues, which could promote the formation of vascular tissues [13, 45, 56]. In our study, the IAA content decreased first and then increased during the graft healing process, consistent with Mo *et al*. [41]. When exogenous NAA was applied at the grafting interface, the content of IAA was significantly higher than control at the CA and VB stage (Figure 3A). Our results indicated that the increase of IAA content could promote the development of the xylem and phloem. ZR participates in cell division and vascular differentiation [36, 57]. Our results showed that the ZR content increased first and then decreased with the increase of grafting days in control plants and reached the highest value at the CA stage. Under exogenous NAA treatment, the ZR content was significantly higher than control at the CA stage, which promoted callus formation and accelerated the graft healing process (Figure. 3C). GAs can accumulate in developing xylem tissues of poplar trees [58-59]. This finding coincided with our results of significantly increased GA_3 content during the VB stage (Figure 3B). When NAA was exogenous applied, the content of GA_3 was significantly higher than control at the CA and VB stage. The possible reason is that the higher the GA_3 content, the shorter the time for the vascular bundle formation. Our results indicated that after exogenous NAA application, IAA, ZR, and GA_3 were higher than control at the CA and VB stage, suggesting that endogenous regulation was performed under exogenous NAA application. Changes in hormone content affected the graft healing process of oriental melon scion grafted on squash rootstock.

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RNA-seq technology has been used to explore the potential transcriptional regulatory mechanisms of the graft healing process [9-10, 12, 41, 60-61]. In order to further analyze the potential molecular networks of exogenous NAA regulating graft healing of oriental melon scion grafted onto squash rootstock, we performed the transcriptome analysis of graft junction at the IL, CA, and VB stage by CK vs. NAA using Melon genome and Cucurbita moschata (Rifu) genome, respectively, and deeply excavated the DEGs involved in hormone signal-transduction, ROS scavenging, and vascular bundle formation during graft union development (Figure 7-9). Some reports showed that plant hormone signal-transduction, phenylpropanoid biosynthesis, and phenylalanine metabolism was crucial for graft healing [11, 41, 62]. Our study similarly found that plant hormone signal-transduction, phenylpropanoid biosynthesis, and phenylalanine metabolism were the overlapping pathways identified at the IL, CA, and VB stage by KEGG pathway enrichment analysis under exogenous NAA treatment.

Previous studies have shown that some genes involved in auxin, cytokinin, and gibberellin signaling are critical regulators for graft union development [10-11, 41, 63]. Auxin signaling was transmitted via transcriptional regulation of auxin early responsive gene families, including AUX/IAA, Gretchen Hagen 3 (GH3) [64-65]. Several auxin-responsive genes were thought to be regulated during the graft healing formation at the transcriptional level [3, 11]. Auxin signaling via auxin response factors (ARFs) might be essential for graft union development [66]. Additionally, cytokinin played an essential role in cell division and vasculature differentiation via the two-component regulatory pathway to active type-B ARR transcription factors [37, 57, 63, 66-68]. In our study, exogenous NAA application significantly promoted the expression of genes involved in auxin signaling, including genes encoding auxin efflux carrier (one melon gene, four cucurbita genes), encoding ARFs (four melon genes, one cucurbita gene), encoding AUX/IAA (four melon genes, four cucurbita genes), and GH3 (four melon genes, two cucurbita genes) during the graft healing process. Meanwhile, genes involved in cytokinin signaling, type-B ARR (one melon gene, three cucurbita genes), CRE1 (one cucurbita gene), AHP (two melon genes, four cucurbita genes), were up-regulated by exogenous NAA. However, we found no significant effects on gibberellin signaling by exogenous NAA application. Moreover, exogenous NAA had different impacts on the melon scion and squash rootstock during graft union development, although exogenous NAA could accelerate graft healing. Besides the hormones, an efficient antioxidant defense system was also crucial in achieving successful grafting [32, 35, 41]. In the present investigation, exogenous NAA not only promoted the expression of sixteen genes (ten melon genes, six cucurbita genes) encoding POD and two genes (one melon gene, one cucurbita gene) encoding APX but also accelerated the expression of three genes (three melon genes, one cucurbita gene) encoding Prx, two genes (one melon gene, one cucurbita gene) encoding SOD, and three genes (three melon genes) encoding PAL. Exogenous NAA application improved the abilities of ROS scavenging during graft union development.

Correspondingly, the new vascular tissue formation between scion and rootstock marks the success of grafting [2]. This process included a complex physiological and biochemical reaction, such as cell elongation, vascular cell differentiation, and the development of vascular cells that trigger programmed cell death [39]. Under exogenous NAA application, the vascular bundle connection between melon scion and squash rootstock was earlier than the control. Moreover, through the transcriptome analysis of CK vs. NAA at the IL, CA, and VB stage, most genes involved in vascular bundle formation were activated by exogenous NAA (Figure 9). During cell elongation, expansion participated in cell growth and cell wall architecture [69]. As expected,

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six genes expression (two melon genes, four cucurbita genes) were significantly up-regulated by exogenous NAA at the stage. Previous studies indicated that tubulin genes were involved in cell expansion and played an essential role in cell division and elongation [40-41]. The expression of five cucurbita tubulin genes was enhanced by exogenous NAA at the VB stage. Differentiating vascular cells will conduct with the deposition of cellulose hemicellulose and lignin in the secondary cell wall after the cell elongation. We identified that four cellulose synthase genes (one melon gene, three cucurbita genes) are involved in cellulose synthesis [70], and one melon cinnamoyl-CoA reductase (CCR) gene is implicated in the phenylpropanoid biosynthesis pathway [53] were up-regulated by exogenous NAA at the VB stage. We also identified sixteen MYB transcription factors (eight melon genes, eight cucurbita genes), participated in the phenylpropanoid biosynthesis pathway as transcriptional regulators [71], and were significantly improved by exogenous NAA at the VB stage. Hydrolytic enzymes, including aspartic proteinase, cysteine proteinase, and nucleases (exonuclease, ribonuclease), were reported to operate the xylogenesis [42-44]. In our study, ten genes encoding them were respectively detected with up-regulation under exogenous NAA application at the VB stage. We also detected one cucurbita gene encoding metacaspase involved in plant programmed death and vascular bundle differentiation [41, 72] with higher expression under exogenous NAA application. Obviously, exogenous NAA application strongly triggered the expression of genes involved in vascular bundle formation during the graft healing process of oriental melon grafted onto squash.

Abbreviations:

NAA: exogenous naphthylacetic Acid; **DAG:** day after grafting;

DEGs: differentially expressed genes; **ROS:** reactive oxygen species; **SOD:** superoxide dismutase;

POD: peroxidase; **PAL:** phenylalanine ammonia-lyase; **PPO:** polyphenol oxidase;

IL: isolated layer; **CA:** callus; VB: vascular bundle;

IAA: indole-3-acetic acid; **GA:** gibberellin; **ZR:** zeatin riboside;

ELISA: Enzyme-Linked Immunosorbent Assay; **HPLC:** high-performance liquid chromatography;

GC-MS: gas-chromatography-mass spectrometry; **RNA-seq:** transcriptome sequencing

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

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Data Availability Statement: The sequencing data are available at NCBI SRA: PRJNA655799 and PRJNA689873. The data supporting this study's findings are available from the corresponding authors upon reasonable request.

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