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Comprehensive transcriptome analysis of grafting onto Artemisia scoparia W. to affect the aphid resistance of chrysanthemum (Chrysanthemum morifolium T.)

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Abstract

Aphid (Macrosiphoniella sanbourni) stress drastically influences the yield and quality of chrysanthemum, and grafting has been widely used to improve tolerance to biotic and abiotic stresses. However, the effect of grafting on the resistance of chrysanthemum to aphids remains unclear. Therefore, we used the RNA-Seq platform to perform a de novo transcriptome assembly to analyze the self - rooted grafted chrysanthemum (Chrysanthemum morifolium 'Hangbaiju') and the grafted Artermisia-chrysanthemum (grafted onto Artemisia scoparia) transcription response to aphid stress.

Background

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is an ornamental plant with a high economic value worldwide (Shinoyama et al. 2012; Suvija and Suresh 2016); it has a long cultural history in China, where it has been used in tea and medicine, and has diverse functions, including preventing tumorigenesis and reducing blood stress (Liu et al. 2012b; Yue Chen et al. 2018). However, *C. morifolium* is susceptible to aphids (*Macrosiphoniella sanbourni*), from seedling establishment to flowering, which reduces plant vegetative and reproductive growth (Davies et al. 2004). In addition, severe aphid infestations can also result in serious economic losses in chrysanthemum production worldwide.

Studies have shown that grafting has been used in horticulture in China since before 2000 BC (Liu 2006; Mudge et al. 2009). Additionally, grafting is still widely used today to improve the abiotic stress tolerance of many types of plants, for example, in the cultivation of grape vines, apples, *Prunus* spp. and vegetables, by regulating many metabolic pathways and stress response processes (Lee et al. 2010; Gregory et al. 2013). Furthermore, previous research has suggested that the herbaceous genus Artemisia provides a useful rootstock for enhancing abiotic stress tolerance in chrysanthemum (Deng et al. 2012; Zhang et al. 2013; Yue Chen et al. 2018; Wang Xi et al. 2014). However, the exact mechanisms and functions of this rootstock under biotic stress remain unclear. During grafting, a scion and rootstock are cut and adhered to each other and then undergo a series of morphological, physiological and biochemical changes (Mahunu et al. 2013). The inherent genetic characteristics, tissue structures, and physiological and biochemical resistance mechanisms are intertwined, thereby enabling the grafted plants to exhibit enhanced resistance to pests and diseases (Weimin Fang et al. 2009).

Extensive efforts have been made to explicate the mechanism of aphid resistance in chrysanthemum. Numerous transcription factors have been reported to act as regulators of the response to aphid herbivory in chrysanthemum (Xia et al. 2014; Li et al. 2015; Wang et al. 2017). However, the gene expression profiles in response to aphid feeding are rarely reported in grafted chrysanthemum. During aphid infestation, a series of plant defense responses, including transcriptional regulation, plant hormone signal transduction, and the expression of different kinds of defense genes, are induced (Smith and Boyko 2007). Moran (Moran et al. 2002) observed that genes associated with signaling, pathogenesis-related (PR) responses, and calcium-dependent signaling are the essential components of the aphid response profile in *Arabidopsis thaliana.* The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene

(ET) play key roles as signaling molecules during both abiotic and biotic stresses, including plant-aphid interactions (Lazebnik et al. 2014; Morkunas et al. 2011; Walling 2009). In interactions between *Myzus persicae* and *A. thaliana*, the SA signaling pathway is activated, and the expression of genes such as PR genes (i.e., β-1,3-glucanase and chitinases) associated with the signaling pathway increases (Moran et al. 2001). Plants can also activate the biosynthesis of secondary metabolites (alkaloids, terpene, phenolics, and flavonoids) that have antixenotic or antibiotic properties to deter herbivore growth, development, and reproduction (Leszczynski et al. 1989).

In the present study, we aimed to identify the differentially regulated aphid-responsive and defense-related genes between the self-rooted grafted chrysanthemum (Cm / Cm) and grafted Artemisia-chrysanthemum (Cm / As) during aphid infestation. Our findings will enrich our knowledge of the mechanisms underlying the improvements in aphid resistance caused by grafting.

Methods

Study sites and sampling

Plant materials

The cultivar *Chrysanthemum morifolium* 'Hangbaiju' is a national geographical indication product in Tongxiang, Zhejiang Province, China. The material has been deposited in Chinese Virtual Herbarium, and the deposition number is JJF00030415. The materials were collected in 2010 in Tongxiang, authenticated by Professor Chengshu Zheng (Shandong Agricultural University) and then preserved in the Chrysanthemum Germplasm Resource Preservation Center at Shandong Agricultural University. In the present study, 'Hangbaiju' was obtained from the Chrysanthemum Germplasm Resource Preservation Center, and plants were subsequently verified by Shandong Agricultural University scientists based on Cheng-shu Zheng. The seeds of *Artemisia scoparia* were sown on March 10th in plastic pots (19 cm in diameter × 17 cm in depth) filled with a 2:1:1 (v/v/v) mixture of garden soil, peat and vermiculite. On May 1st, healthy *A. scoparia* and chrysanthemum plants 30 cm in height and with a 6-mm stem diameter were used as rootstocks, and apical shoots with a length of 14~16 cm and a stem diameter of 4 mm from healthy chrysanthemum were used as scions. Insert grafting was performed as described by Lee (Lee 1994). On July 9th, morphologically uniform Cm / Cm and Cm / As plants were selected for the aphid infestation treatment. The plants were arranged in a fully randomized manner, and the experiment was repeated three times, with each replicate consisting of 20 plants.

Aphid infestation

Aphids (*M. sanbourni*) were collected from field-grown chrysanthemum plants, and second-instar nymphs were fostered and used to inoculate the plants. The third fully expanded leaves from the tip of the stem of the Cm / Cm and Cm / As plants were infested with twenty aphids (four hours of starvation) transferred by a soft brush. Twenty plants per treatment were repeated three times. The infested plants were caged

with a transparent ventilated plastic net. Aphids were counted 1 d, 3 d, 5 d, 7 d, 9 d and 10 d after aphid inoculation.

The third fully expanded leaves of seedlings for each treatment were harvested at 0 h, 6 h, and 96 h. Before harvest, aphids were removed with a soft brush, which enabled the aphids to be removed from the leaves by flushing the plants with deionized water. Harvested materials were immediately frozen in liquid nitrogen and stored at −80 °C for the following experiments. The samples collected at defined time points of each treatment were pooled for RNA-Seq.

RNA extraction and transcriptome sequencing

Each leaf was mixed with liquid nitrogen and ground into a fine powder. RNA integrity was assessed using the RNA Nano6000 Assay Kit of the Agilent Bioanalyzer2100 system (Agilent Technologies, CA, USA). To account for the gene expression variations among leaves of the same plant, RNA from two leaves of the same plant were equimolarly pooled and used as a single sample for transcriptome sequencing. The transcriptome of each treatment was analyzed at three time points (0 h, 6 h and 96 h) with three biological replicates for each time point. In total, eighteen samples were used for transcriptome sequencing. According to three time points, the samples were divided into six groups: A0, A1, A2, B0, B1 and B2 (Table 2).

A total amount of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample.

Transcriptome assembly and gene functional annotation

Raw reads were processed to remove reads containing adaptors, reads with more than 10% ambiguous bases (N), or reads of low quality (more than 50% bases with small Qphred ≤ 5). All downstream analyses were based on the resulting clean reads. Clean reads were assembled using Trinity (version r 2012-10-05) with minkmercov = 2 (Grabherr et al. 2011). The longest assembled transcript of a gene was taken as unigene.

Functional annotations of unigenes were based on the following databases: NCBI nonredundant protein sequences (Nr), NCBI nonredundant nucleotide sequences (Nt), Protein family (Pfam; http://pfam.xfam.org/) (Finn et al. 2013), euKaryotic Ortholog Groups (KOG)/Clusters of Orthologous Groups of proteins (COG; http://www.ncbi.nlm.nih.gov/COG/) (Koonin et al. 2004), Swiss-Prot (a manually annotated and reviewed protein sequence database; http://www.ebi.ac.uk/uniprot) (Boeckmann et al., 2003), Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) (Kanehisa et al. 2000), and Gene Ontology (GO; http://geneontology.org/). NCBI BLAST 2.2.28+ was used for the alignments of unigenes to Nt, Swiss-Prot, and KOG. The E-value threshold was set to 1E−5 in the alignments to Nt and Swiss-Prot. For the alignments to KOG, the E-value threshold was 1E-3. hmmscan in

HMMER 3.0 was used to search Pfam (Finn et al. 2011). The GO annotations were performed with Blast 2 GO v2.5 (Götz et al. 2008) based on the Nr and Pfam annotations. KEGG Automatic Annotation Server (KAAS; http://www.genome. jp/kegg/kaas/) was used for the KEGG annotations (Moriya et al. 2007).

Differential expression analysis

RSEM (Li et al. 2011) was used to calculate the read count of each unigene in a sample and transform it into expected number of fragments per kilobase of transcript per million fragments mapped (FPKM) (Trapnell et al. 2010). In RSEM analysis, Bowtie was used with a mismatch of 2. The resulting FPKM values were used to represent the gene expression levels of unigenes in different samples. To examine the effects of the different aphid infestation times on the gene expression patterns of leaves in the Cm / Cm and Cm / As plants, the density distributions of FPKM values were compared among different aphid infestation time groups. Differential gene expression analyses between different groups were performed using DESeq (Anders and Huber 2010). Here, the q-value was adopted to adjust the P-values in multiple hypothesis tests (Yekutieli and Benjamini 1999). DEGs were determined based on a threshold of q-value ≤ 0.05 and an absolute value of fold change ≥ 2. Hierarchical clustering and a Venn diagram were used to illustrate the differential gene expression patterns between different aphid stress time points. According to the functional annotations of unigenes, putative functions of the differentially expressed genes (DEGs) were inferred to discover candidate genes for aphid infestation.

Validation of RNA-Seq data by qRT-PCR

The expression of 15 DEGs in chrysanthemum was analyzed by qRT-PCR with three replicates to verify the RNA-Seq data. Using the Prime Script™RT reagent qRT-PCR Kit with gDNA Eraser (Takara, Dalian, China), genomic DNA was removed from total RNAs (300 ng RNA of each sample), and cDNA was synthesized. The PCR mixture contained 12.5 μL SYBR® Premix Ex Taq™II (Tli RNaseH Plus) (Takara, Dalian, China), 9.5 μL ddH_2O, 1 μL of each gene-specific primer (10 μM) and 1 μL cDNA template. The qRT-PCR assays were performed in a CFX96Touch™RT-PCR Detection System (Bio-Rad, USA) with the following program: 94 °C for 2 min; 40 cycles of 94 °C for 20 s, 57 °C for 20 s and 72 °C for 30 s. Relative expression values of genes were calculated by the formula of 2 (38-value of Cp) (Hua Xu et al. 2018). Cp indicates the intersection point and the cycles during which the signals were higher than the threshold. If the value of Cp exceeded 38, the data had high uncertainty. The relative expression value of actin was used as an internal control. Three technical replicates were performed for all fifteen selected genes.

Determination of physiological indexes of chrysanthemum during aphid infestation

Leaves of seedlings under aphid stress were harvested at 0 h, 6 h, 24 h, 48 h, 72 h, and 96 h to determine the physiological indexes. The activities of phenylalanine ammonia lyase (PAL) and catalase (CAT) were determined by the "Plant Physiology Experiment Instruction" (Junfeng Gao et al. 2006), and the content of soluble sugar was determined by the "Plant Physiology Experimental Technique" (Xiangsheng Kong et al. 2008). The flavonoid content was determined according to Jia (Jia et al. 1999). The activity of

lipoxygenase (LOX) was measured as described by Zhang et al. (2017). Each determination included three biological and technical replicates.

Statistical analyses

Statistical analyses of aphid numbers, enzymes and metabolites were performed by ANOVA (SAS Institute, Cary, NC, USA) using SPSS v. 21.0 (SPSS Inc., Chicago, IL, USA), and the determinations were expressed as the means ± standard error (SE) from a minimum of three replicates. Differences between means were compared using Tukey's multiple range tests at $P < 0.05$ or $P < 0.01$.

Results

Aphid population statistics

As shown in Fig. 1 and Table 1, aphid settlement was similar in the Cm / Cm and Cm / As plants in the first 3 d after aphid infestation. On the 5th day, the aphid number between the Cm / Cm and Cm / As plants showed a significant difference (Table 1). Then, the aphids on Cm / As slowly increased, whereas the aphid number on Cm / Cm exhibited a steady and continuous increase from the 5th day to the 10th day. Thus, we hypothesized that the aphid-responsive genes that mediate defense events in the first 4 d of aphid feeding may be crucial to chrysanthemum resistance mechanisms. To unravel the molecular mechanisms involved in aphid resistance, three sampling points during a 4-d experimental period were selected for transcriptome analysis.

Physiological changes in chrysanthemum under aphid stress

As shown in Fig. 5a, a higher concentration of soluble sugar was observed in Cm / As than in Cm / Cm at the same time point after infestation. Furthermore, flavonoid accumulation was observed in the leaves of Cm / As and Cm / Cm, and a higher concentration of flavonoids was observed in Cm / As than in Cm / Cm (Fig. 6b). As shown in Fig. 6c, the activity of PAL in Cm / As was significantly increased by aphid infestation and substantially higher than that in Cm / Cm. Additionally, the CAT activity in Cm / As and Cm / Cm decreased rapidly by 69.5% and 83.7% (Fig. 7b) within 6 h of infestation with aphids, and the activity of LOX (Fig. 7d) was significantly increased by aphid infestation and higher in Cm / As than in Cm / Cm.

Transcriptome sequencing and assembly

After the low-quality reads were removed, 126.66 Gb high-quality sequences were obtained from the transcriptome sequencing of the leaves of the Cm / Cm and Cm / As plants under aphid-infestation conditions, ranging from 6.25 to 8.16 Gb per sample. The average error rates of the sequences were 0.03% and more than 91% (Additional file 1: Figure S1). The assembled sequence data for these raw reads were deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) under the accession number SRP217705. The sequencing data were assembled into 147307 transcripts with lengths ranging from 201 to 38240 bases (mean

length = 825 bases, and median length = 579 bases). As a result, 400234 unigenes were obtained (mean length = 894 bases, and median length = 657 bases). The total length of the unigenes was 35.8 Mb (357942294 bases). (Additional file 1: Figure S2).

Gene annotation and functional classification

In total, 400232 unigenes (59.0% of the total unigenes) were annotated in at least one of the databases used in our study. Those unigenes were mostly annotated in the Swiss-Prot database with good matches (Table 3). According to the GO classification, the largest number of annotations was in the Biological Process (BP) category, for which the top 3 GO terms were cellular process, metabolic process and single-organism process; the second largest number of annotations was in the Cellular Component (CC) category, for which the top 3 terms were cell, cell part and organelle; and the third largest number of annotations was in the Molecular Function (MF) category, for which the top 3 terms were binding, catalytic activity and transporter activity. For KOG classification, the top 10 classes were (O) Posttranslational modification, protein turnover, chaperones, (R) General function prediction only, (J) Translation, ribosomal structure and biogenesis, [A] RNA processing and modification, [U] Intracellular trafficking, secretion, and vesicular transport, [C] Energy production and conversion, [T] Signal transduction mechanisms, [K] Transcription, [I] Lipid transport and metabolism, [G] Carbohydrate transport and metabolism, and [E] Amino acid transport and metabolism. According to the KEGG classification, the largest amount of total annotations were involved in different metabolism pathways, among which carbohydrate metabolism was the most abundant, followed by the “overview” group, energy metabolism, amino acid metabolism and lipid metabolism. (Additional file 1: Figure S3)

Differential expression and gene functional classification

We compared the DEGs among the multiple groups of samples. As shown in Fig. 2b, there was a large number of DEGs in B1 compared to B0, with 457 genes upregulated and 163 genes downregulated. However, there were 219 upregulated genes and 188 downregulated genes (Fig. 2a) among the DEGs in A1 compared to A0. From the overall expression of DEGs induced under aphid stress, the number of upregulated genes was higher than that of downregulated genes. The results indicated that most of the genes in chrysanthemum leaves were activated, and few genes were inhibited after stimulation. In total, the number of different genes in Cm / As was approximately twice that in Cm / Cm.

Furthermore, the results showed that there were 1337 DEGs, among which 680 were upregulated and 667 were downregulated, in the Cm / As compared to the Cm / Cm. Among them, there were 504 genes with different expression in B0 compared to A0, of which 134 genes were upregulated and 370 genes were downregulated (Fig. 2c); 620 genes were differentially expressed in B1 compared to A1, of which 457 genes were upregulated and 163 genes were downregulated (Fig. 2d); and 223 genes were differentially expressed in B2 compared to A2, with 89 upregulated genes and 134 downregulated genes (Fig. 2e). A total of 1337 DEGs were identified, as shown in Fig. 3. According to the GO classification (Fig. 3a), the largest number of annotations was in the BP category, for which the top 3 GO terms were single-organism process, establishment of localization and regulation of biological; the second most abundantly

annotated category was MF, for which the top 3 terms were transporter activity, ion transmembrane transporter and substrate-specific transmembrane; and the third most abundantly annotated category was CC, for which the top term was MHC protein complex. The results of the KEGG pathway classification are shown in Fig. 3b, mainly including photosynthesis, trans-linolenic acid metabolism, pantothenic acid and CoA biosynthesis, plant hormone signal transduction pathways, phenylpropane biosynthesis, betaine synthesis, monogamous biosynthesis, flavonoid biosynthesis, starch and sucrose metabolism, and plant-pathogen interactions. The results indicated that grafting chrysanthemum onto *A. scoparia* resulted in enhanced resistance to aphids, which was closely related to the regulation of metabolic pathways, secondary metabolic biosynthesis, immune response and signal transduction.

Verification of DEGs using qRT-PCR

To validate the results of Illumina RNA-Seq, fifteen genes from the library of the grafted and self-rooted seedlings after aphid infestation were chosen randomly for qRT-PCR. For comparison, clustering heat maps between the RNA-Seq and qRT-PCR data were generated. As shown in Fig. 4, the qRT-PCR results revealed that the expression tendency of these genes showed significant similarity with the Illumina RNA-Seq data, suggesting the reproducibility and accuracy of the RNA-Seq results.

Discussion

Genes related to sucrose metabolism

Sucrose has a central position in plant metabolism as the first free sugar formed during photosynthesis (Koch 2004; Van den Ende 2013) and the major form of translocated sugar in the phloem (Shiratake 2007; Van Bel and Hess 2008). Soluble sugars, such as sucrose, glucose and fructose, can be donors of carbon skeletons for secondary metabolism and signaling molecules that regulated the expression of genes encoding flavonoid biosynthesis enzymes (Morkunas et al. 2007, 2011a). Sucrose metabolism plays an important role in the defense against insect herbivores, such as aphids. In our study, as shown in Fig. 5a, the level of soluble sugar increased after 6 h of infestation and reached a maximum at 24 h in the Cm / As plants. At the same time point, a higher concentration of soluble sugar was observed in Cm / As than in Cm / Cm.

The multiple roles of plant sucrose transporters, especially the central role of sucrose loading into the phloem, suggest that sucrose transport is strongly regulated by biotic stresses (Sauer 2007; Wahl et al. 2010; Lemoine et al. 2013). In the present study, nine genes involved in the sucrose metabolism pathway were differentially expressed between Cm / As and Cm / Cm. As shown in Fig. 5b, three INV genes (Cluster-21353.120200, Cluster-21353.176307 and Cluster-21353.133225), one PYG gene (Cluster-21353.277981) and one PGM gene (Cluster-21353.147957) were strongly upregulated in Cm / As (B1) compared to Cm / Cm (A1) at 6 h, and these genes have been shown to be involved in glucose synthesis (Fig. 5c). In addition, there were four downregulated genes in B1 compared with A1, including two OTS genes (Cluster-21353.199573 and Cluster-21353.175120) and two BPGM genes (Cluster-21353.178805 and Cluster-21353.224391), which have been shown to be involved in glucolysis (Fig. 5c). The results

suggested that grafting chrysanthemum onto *A. scoparia* accelerated the synthesis of soluble sugars, which increased the carbon metabolism burden of aphids and thus prevented aphids from feeding on the leaves. The details of these genes are shown in Additional file 2: Table S1. These genes may play an important role in promoting the synthesis of soluble sugars at the early stage in response to *M. sanbourni* infestation in the Cm / As seedlings.

Genes related to secondary metabolism

Secondary metabolites, for example, flavonoids, terpenes, phenolics and alkaloids, have antixenotic or antibiotic properties and thus function in the defense against insect herbivores, such as aphids (Leszczynski et al. 1989). Among secondary metabolites, flavonoids are responsible for many key functions, which are critical for plant survival (Gould and Lister 2006). Flavonoids are also catalyzed by a number of enzymes; for example, PAL, which has been well studied in plant responses to biotic and abiotic stresses (Mauch-Mani et al. 1996). In the present study (Fig. 6c), the activity of PAL in Cm / As was significantly increased by aphid infestation and higher than that in Cm / Cm, which promoted the accumulation of flavonoids. Studies on insect-plant interactions have revealed important contributions of flavonoids (Simmonds and Stevenson 2003). In Vigna (Lattanzio et al. 2000), there was a positive relationship between resistance or susceptibility properties against aphids and the flavonoid glycoside content. The content of flavonoids in susceptible lines was lower than that in resistant lines (Jing He 2016). In the present study, after *A. sanbourni* infestation, flavonoid accumulation was detected in the leaves of Cm / As and Cm / Cm. The level of flavonoids increased after 6 h of infestation, and a higher concentration of flavonoid was observed in Cm / As than in Cm / Cm (Fig. 6b). Additionally, genes related to flavonoid synthesis were also identified. Eight genes were differentially expressed between Cm / As and Cm / Cm (Supplementary Fig. 6e, d), including one CHS gene (Cluster-25249.0), which was downregulated in B1 compared to B0, and one malonyl-CoA gene (Cluster-21353.290401), which was strongly upregulated in B1 compared to A1 and may play an important role in Cm / As defense towards aphid feeding at the early stage of infestation. In addition, three flavonoid 3'-monooxygenase (F3'H) genes (Cluster-21353.144221, Cluster-21353.9823 and Cluster-21353.344000), two flavonoid 3'5'-hydroxylase (F3'5'H) genes (Cluster-21353.241984 and Cluster-21353.339941) and one flavonoid 3-O-glucosyltransferase gene (Cluster-21353.169006) were strongly upregulated in B2 compared to A2, which could play an important role in Cm / As defense towards aphid feeding in the later stage of infestation (Fig. 6d).

In addition, among secondary metabolites, alkaloids act as a defense against insects and other herbivores (Reinhard et al. 2006; Michael 2008; Ganzera et al. 2010). For example, the reduced alkaloid content in sweet lupins led to a high susceptibility to insect herbivores, e.g., aphids (Wink 1985, 1988; Wink and Witte 1991). In this study, only two alkaloid synthesis-related genes, STR genes (Cluster-21353.170595 and Cluster-21353.170592), were detected in Cm / As and Cm / Cm, both of which were downregulated at 96 h after aphid infestation.

Plants produce a vast array of volatiles that mediate their interaction with the environment and that consist of terpenoids and isoprenoids, which are synthesized through the condensation of C5 isoprene units. Monoterpenes and sesquiterpenes represent the C10 and C15 terpene classes, respectively. These compound classes have typical characteristics, such as volatility, flavor/aroma, and toxicity, and hence play important roles in plant defense, plant-to-plant communication, and pollinator attraction (Pichersky and Gershenzon 2002). A previous study found that the increased content of monoterpenoids and sesquiterpenoids in the leaves of the hybrid between chrysanthemum and *Artemisia vulgaris* enhanced the resistance of the plant to aphids (Deng et al. 2010). In the present study, we obtained several DEGs related to terpenoid synthesis (Fig. 6a). There were three genes related to monoterpene synthesis, one basil synthase gene (Cluster-21353.197680), one myrcene synthase gene (Cluster-21353.180080) and three isoprenoid genes (Cluster-21353.97990, Cluster-21353.154989 and Cluster-21353.47033). All of these genes were strongly upregulated in B1 compared to A1, indicating their potential roles in the defense responses against aphids in the early stage after grafting on *A. scoparia*. Furthermore, five genes involved in diterpenoid biosynthesis, including one gene (Cluster-21353.173831), were strongly upregulated in B1 compared to A1, and four genes (Cluster-21353.18772, Cluster-21353.318315, Cluster-21353.283531 and Cluster-21353.113455) were strongly upregulated in B2 compared to A2 and may play important roles in responding to aphids in the later stage of infestation.

The details of these genes discussed above are shown in Additional file 3: Table S2, which illustrates the involvement of secondary metabolites during aphid herbivory in Cm / As and Cm / Cm leaves, indicating that using the *A. scoparia* rootstock could alleviate aphid stress in chrysanthemums by altering these gene profiles, which play potential roles in defense responses to aphids.

Plant hormone signaling pathway involved in plant-aphid interaction

JA, SA and ET are three major phytohormones involved in the regulation of signaling networks related to aphid-infestation defense responses. As shown in Fig. 7a, hormone transduction pathway-related genes were expressed at different time points between Cm / As and Cm / Cm. A total of 21 DEGs were involved in several plant hormone signal transduction pathways, including cytokinins (CKs), ET, JA and SA, in this study. The details of these genes discussed above are shown in Additional file 4: Table S3.

The JA pathway and SA pathway are two main pathways involved in plant-induced defense (Durrant et al. 2004). Phytohormonal crosstalk between JA- and SA-mediated signaling pathways is thought to underlie plant-mediated interactions among multiple insect species and the behavioral responses of parasitoids and predators (Zhang et al. 2013; Stam et al. 2014; Wei et al. 2014). The activation of SA signaling in response to aphid feeding (Moran et al. 2002; Mewis et al. 2006; Kusnierczyk et al. 2011) may suppress JA-dependent indirect defense responses. In our study (Fig. 7a), one LOX gene, which is a key enzyme in JA synthesis, was downregulated in B1 compared to B0, whereas the SABP genes, which are key enzymes in SA synthesis, were strongly upregulated in B1 compared to B0.

Exogenous SA treatment can enhance plant resistance to pathogenic bacteria and induce the expression of disease-related genes at the same time (White et al. 1979; Ward et al. 1991). SABP2 catalyzes the

conversion of MeSA into SA, which is essential for inducing tobacco systemic acquired resistance (SAR) (Gaffney et al. 1993). In this study, three SABP2 genes (Cluster-21353.21806, Cluster-21353.149100 and Cluster-21353.197082) were upregulated by aphid infestation in B1 compared to A1 in the early stage. In addition, within 6 h of infestation with aphids, the CAT activity in the Cm / As and Cm / Cm plants decreased rapidly by 69.5% and 83.7%, respectively (Fig. 7b). This result might be due to the change in the conformation of CAT, which binds to the SA-binding protein (SABP1) (Yuquan Li et al. 2003), inhibiting the activity of CAT and activating the expression of disease course-related protein genes. We suggest that grafting chrysanthemum onto *A. scoparia* inhibited the activity of CAT through the upregulation of SABP2 genes after aphid infestation, and then the self-feedback mechanism was initiated, which amplified the signal transduction in the cell and ultimately induced the expression of the disease-related protein genes (Fig. 7g). Furthermore, in the SA-mediated SAR reaction of Arabidopsis, NPR1 is a crucial regulatory protein that impacts the SAR pathway downstream of the SA signaling pathway. During SAR, NPR1 induces PR gene expression, and even after induction by SA, BTH or INA, NPR1 expression levels increase (Ryals et al. 1997; Glazebrook et al. 1996). However, in this study (Fig. 7a), two NPR1 genes were downregulated in B1VSA1, suggesting that the NPR1 gene had different regulatory mechanisms on aphid stress after grafting onto *A. scoparia.*

JA affects not only the growth of many plants but also the resistance of plants to pathogen infection. LOX and oxo-phytodienoic reductase (OPR) are not only key enzymes for JA synthesis but also important substances that induce defense mechanisms in plants (Consatbel et al. 1999; Bottcher et al. 2009; Dave et al. 2011). As the receptor in the JA signaling pathway, COI1 (COR-insensitive1) can interact with SKP1 (S-phase kinase-associated protein1) (Yan et al. 2009). LOX genes were significantly upregulated by *M. persicae* feeding on *A. thaliana* leaves (Moran and Thompson 2001), *M. nicotianae* feeding on *Nicotiana attenuata* leaves (Voelckel et al. 2004), and *M. euphorbiae* feeding on tomato leaf tissues (Fidantsef et al. 1999). In our study (Fig. 7e), the overall expression level of one LOX gene (Cluster-21353.151969) in the Cm / As plants was higher than that in the Cm / Cm plants at the same time point. Additionally, the activity of LOX (Fig. 7d) in Cm / As was significantly increased by aphid infestation and higher than that in Cm / Cm, which promoted the accumulation of JA. Compared with A2, B2 exhibited one OPR gene (Cluster-21353.142219) that was strongly upregulated. In addition, one COI1 gene (Cluster-21353.180331) and one MYC gene (Cluster-21353.197996) were strongly upregulated in B1 compared to A1 and responded rapidly to aphid stress in the early stage of infestation. In addition, protease inhibitors (PIs) are widely found in organisms, regulate many important biological activities in plants and have a protective effect against insects (Zhongyi Cheng et al. 2003). These genes above all activated the expression of cytochrome P450 genes and protease inhibitor genes, which were downstream response genes (Fig. 7g) responding to aphid stress. The details of these genes discussed above are shown in Additional file 5: Table S4. In this study, four cytochrome P450 genes (Cluster-21353.342863, Cluster-21353.153679, Cluster-21353.345312 and Cluster-21353.344914) were upregulated by aphid infestation in B1 compared to A1. Protease inhibitors were divided into four categories, serine protease inhibitors, metal carboxypeptidase protease inhibitors, cysteine protease inhibitors and aspartic acid protease inhibitors, according to the active sites of the active enzymes (Zhuhua Wang et al. 2001). In our study,

two cysteine protease inhibitor genes (Cluster-21353.197535 and Cluster-21353.189320) and one phospholipase A2 inhibitor gene (Cluster-21353.200786) were significantly upregulated by aphid infestation in B1 compared to A1. As important enzymes, the acetylcholine receptor inhibitor gene and the phospholipase A2 inhibitor gene can inhibit the nerve conduction of insects, which may play an important role in responding to aphids in the early stage of infestation after grafting onto *A. scoparia*.

Some studies have proven that aphid infestation markedly increases the production of ET in the leaves of plants (Argandona et al. 2001; Divol et al. 2005; Moran et al. 2002 and Boyko et al. 2006). Ethylene-inducing xylanase (EIX) induces ET production and is an effective elicitor of plant defense responses in specific cultivated species of *Nicotiana tabacum* and *Lycopersicon esculentum*. In the present study, one EIX gene (Cluster-21353.186218) was strongly upregulated in B1 compared to A1, which may stimulate the accumulation of ET. CTR1 is a negative regulatory component downstream of the ET receptor and the first cloned gene in the ET signal pathway (Kieber et al. 1993; Ecker 1995). In this study (Fig. 7a), one CTR gene (Cluster-21353.234433) was upregulated in B1 compared to A1 at an early time point after aphid infestation. Research shows that EIN2 has important effects in the ET signal pathway, and the loss of EIN2 gene function leaves the plant completely insensitive to ET (Alonso et al. 1999). In this study (Fig. 7a), one EIN2 transcription factor gene was downregulated in B1 compared to B0, but it was highly expressed in A1. ET-responsive factor (ERF) encodes a transcriptional activator to promote several downstream ET-responsive genes. Additionally, MYC and ERF transcription factors, participate in the ET and JA signaling pathways and activate defense-related genes. In our study, nine ERF genes were involved; among them, four genes (Cluster-21353.197997, Cluster-21353.197998, Cluster-21353.176859 and Cluster-21353.148870) were strongly upregulated in B1 compared with A1 (Fig. 7a).

These DEGs were identified in the comparison of Cm / As and Cm / Cm, as discussed above, which illustrates the involvement of the plant hormone signaling pathway during aphid herbivory, indicating their potential roles and the complex connections in the defense responses against aphids after grafting chrysanthemum onto *A. scoparia*.

Plant-pathogen interaction

Two layers of plant immunity are well defined: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl. 2006). As the first line of the innate immune response, PTI is triggered by the perception of herbivore-associated molecular patterns (HAMPs) in the case of herbivory and microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) by cognate plasma membrane-localized pattern recognition receptors (PRRs) in the case of microbial infection (Zipfel 2014). The downstream immune signaling pathways are activated by different PRRs, including the receptor of kinases and the activation of various kinases, such as mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) (Molotto et al. 2008). In this study, four genes in the MAPK signaling pathway were recognized (Fig. 8): one ROBH gene (Cluster-21353.196945) and one MAPK gene (Cluster-21353.172651), which were upregulated in B1 compared to A1, and one

WRKY2209 gene (Cluster-21353.216549) and one ROBH gene (Cluster-21353.177651), which were upregulated in B2 compared to A2.

Ca^{2+} regulates many important physiological processes. Transcriptome and metabolome changes in *Arabidopsis* were surveyed at 6 h, 12 h, 24 h and 48 h after *B. brassicae* infestation, revealing that reactive calcium is involved in early signaling (Grabherr et al. 2011). Plant defenses against phloem-feeding insects involve multiple signaling cascades, and molecular genetic studies on the model plant *A. thaliana* have demonstrated that leucine-rich repeat (LRR) family receptor-like kinases and calcium signaling proteins are partially activated by phloem feeding (Foyer et al. 2014). MHCII molecules can mediate the transmission of reverse signals and influence and regulate many physiological processes in immune cells (Xingguang Liu et al. 2009). Studies have shown that CDPK2 and MHCII can regulate each other in two major antigen presenting cells (Argandona et al. 2001). In the present study (Fig. 8), four CDPK genes (Cluster-21353.147746, Cluster-21353.90102, Cluster-21353.147746 and Cluster-21353.90102) and three MHCII genes (Cluster-21353.251721, Cluster-21353.196511 and Cluster-21353.177618) were specifically upregulated in B1 compared to A1, which may play an important role in the early stage of responding to *M. sanbourni* infestation in Cm / As seedlings.

Two cloned aphid resistance genes, Mi-1.2, confer resistance to the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Kaloshian et al. 1997; Rossi et al. 1998), and Vat mediates resistance to the cotton aphid *Aphis gossypii* Glover (Pauquet et al. 2004; Brotman et al. 2002), all of which belong to the NBS-LRR family. Similarly, we found four such DEGs (Cluster-21353.336850, Cluster-21353.79718, Cluster-21353.149283 and Cluster-21353.102248) between the Cm / Cm plants and the Cm / As plants, but these DEGs require further cloning and functional identification to confirm the presence of the NBS-LRR region.

Furthermore, in our study (Fig. 8), there were many DEGs related to tyrosine protein kinases and serine/threonine protein kinases in the interaction between the plants of the Cm / Cm and the Cm / As. Eight PAK genes and six PTK genes were specifically upregulated in B1 compared to A1. These protein kinase genes may be closely related to grafting to improve the aphid resistance of *C. morifolium*. (The details of these genes discussed above are shown in Additional file 6: Table S5)

Conclusions

This study showed that the number of aphids was significantly lower in Cm / As than in Cm / Cm 10 d after aphid infestation. The results indicated that grafting chrysanthemum onto *A. scoparia* resulted in enhanced resistance to aphids. In addition, the content of soluble sugars and the accumulation of flavonoids in Cm / As were higher than those of Cm / Cm at the same time point under aphid stress. Transcriptome results showed that grafting onto *A. scoparia* affected aphid-responsive and defense-related gene expression after feeding, mainly in the processes of sucrose metabolism, plant hormone signaling, secondary metabolism and plant-pathogen interaction. Intercommunication typically occurred between the different defense pathways to which these genes belonged, thereby providing the Cm / As

seedlings the ability to integrate environmental, developmental and defense-related signals and to finetune their defense responses to aphids.

Abbreviations

CHS, Chalcone synthase gene; qRT-PCR, Quantitative real-time-polymerase chain reaction; Ct, Threshold cycle; cDNA, Complementary DNA; NR, NCBI non-redundant protein sequences; NT, NCBI non-redundant nucleotide sequences; GO, Gene Ontology; KOG/COG, Clusters of orthologous groups of proteins; KEGG, Kyoto Encyclopedia of Genes and Genomes; Swiss-Prot, A manually annotated and reviewed protein sequence database; Pfam, Protein family; KO, KEGG ortholog database; MF, Molecular function; CC, Cellular component; BP, Biological process; DEGs, Differentially expressed genes; FPKM, Fragments Per Kilobase per Million fragments; RNA-Seq, High-throughput RNA-sequencing.

Declarations

Ethics approval and consent to participate

Chrysanthemum materials of this study were provided by plant tissue culture room of Shandong Agricultural University. No field permission was necessary to collect the plant samples in this study. This experimental research on chrysanthemum complied with institutional, national, or international guidelines.

Consent to publish

Not applicable.

Availability of data and materials.

The assembled sequence data for the raw reads were deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA, http://www. ncbi. nlm. nih. gov/Traces/sra) under the accession number SRP217705.

Competing interests

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

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Authors' contributions

XS conceived the idea and supervised the project. FL and XZ participated in the design of the study and in interpreting the data, and manuscript preparation. XZ performed the experiments and the data analysis, and drafted the manuscript. SZ, JY and JF, contributed analysis tools and participated in the data analysis. All authors carefully read and approved the final version of the manuscript.

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Tables

Due to technical limitations, tables are only available as a download in the supplemental files section.

Additional Files

Additional file 1: Figure S1. Composition of raw reads in the eighteen RNA libraries. **Figure S2.** Unigene Transcript length distribution. **Figure S3.** Functional classification and pathway assignment of DEGs by GO and KEGG.

Additional file 2: Table S1. Genes involved in sucrose metabolism to aphid infestation responses.

Additional file 3: Table S2. Genes involved in secondary metabolism to aphid infestation responses.

Additional file 4: Table S3. Genes involved in Plant hormone signaling pathway to aphid infestation responses.

Additional file 5: Table S4. Genes involved in down stream responses.

Additional file 6: Table S5. Genes involved in Plant-pathogen interaction to aphid infestation responses.

Figures



Figure 1

Representative pictures for aphid settlement at 3 d, 7d and 10d after aphid inoculation on selfrooted grafted chrysanthemum (Cm / Cm) and the grafted Artermisia-chrysanthemum (Cm / As) .

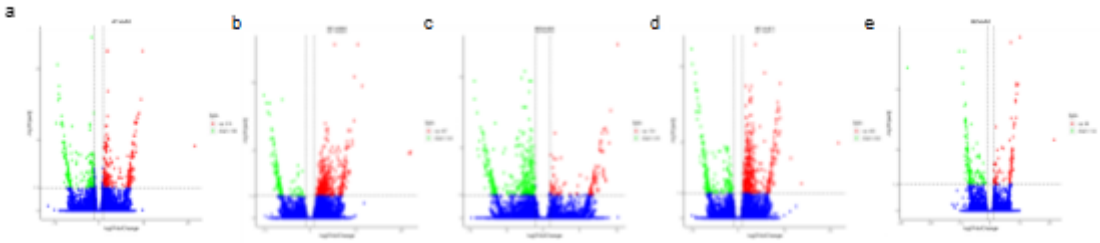


Figure 2

Volcano plots of differentially expressed sequences in selfrooted grafted chrysanthemum and the grafted Artermisia-chrysanthemum. a The up-regulated or down-regulated genes in A1 compared to A0. b The up-regulated or down-regulated genes in B1 compared to B0. c The up-regulated or down-regulated genes in B0 compared to A0. d The up-regulated or down-regulated genes in B1 compared to A1. e The up-regulated or down-regulated genes in B2 compared to B2. (The scattered points represent the genes, the

blue dots represent the genes with no significant difference, the red dots represent the up-regulated genes with significant differences, and the green dots indicate the down-regulated genes with significant differences).

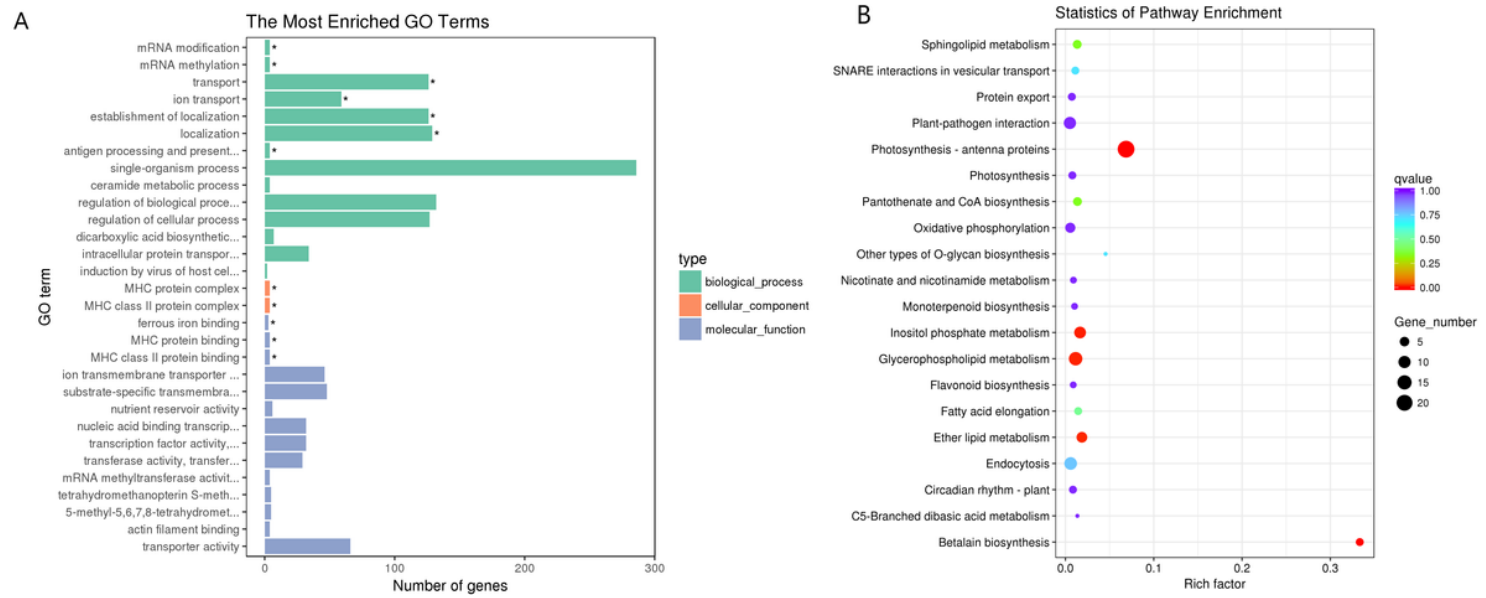


Figure 3

Enrichment analysis of differently expressed genes. a Functional enrichment categories of DEGs in Gene Ontology (Corrected P-value≦0. 05). b Scatterplot of enriched KEGG pathway of DEGs (Top 20).

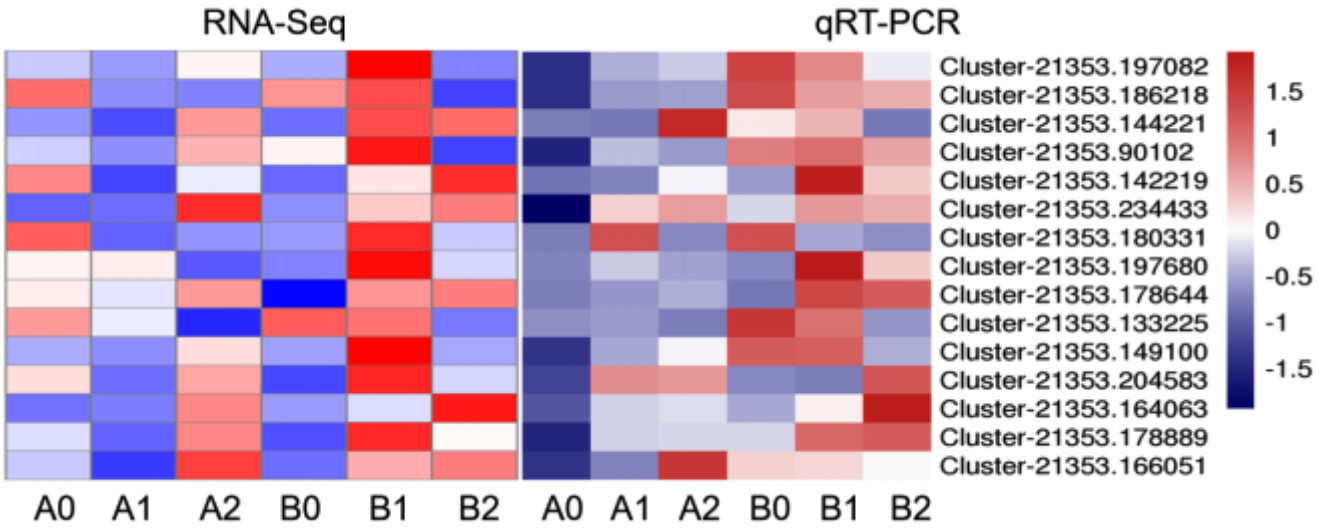


Figure 4

Comparisons of the expression changes of selected genes during aphid stress in qRT-PCR and RNA-Seq analysis were used in heat map analysis. Different colors indicate different levels of genes expression: from red to green, the value ranges from large to small.

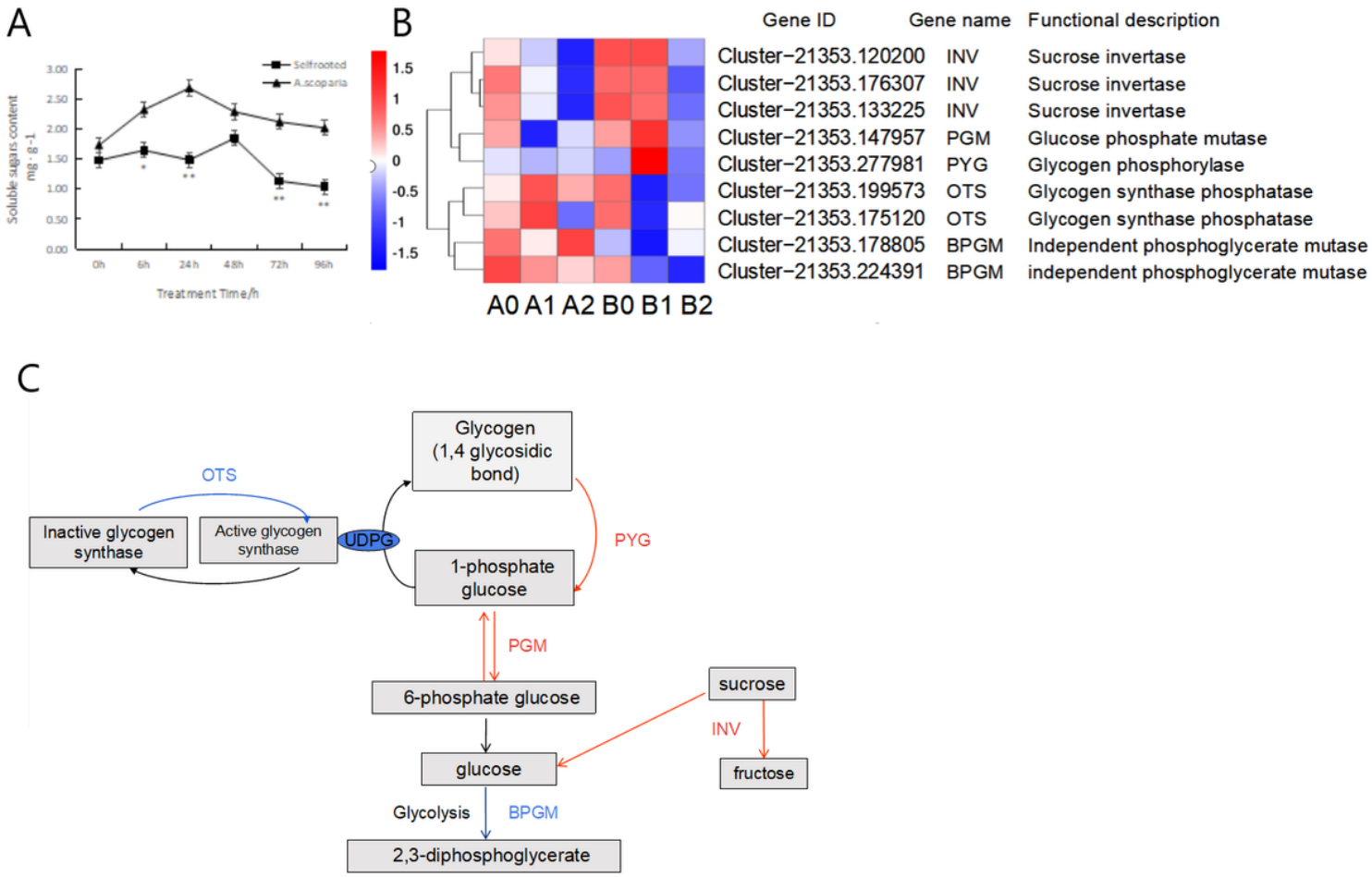


Figure 5

Sucrose metabolism related genes. a Effect of aphid stress on content of total sugars. b Heat map of the DEGs involved in sucrose metabolism. c The metabolic pathway analysis of unigenes involved in sucrose metabolism (Red and blue lines indicate up-regulated and down-regulated genes in Cm/As compared to Cm/Cm, respectively).

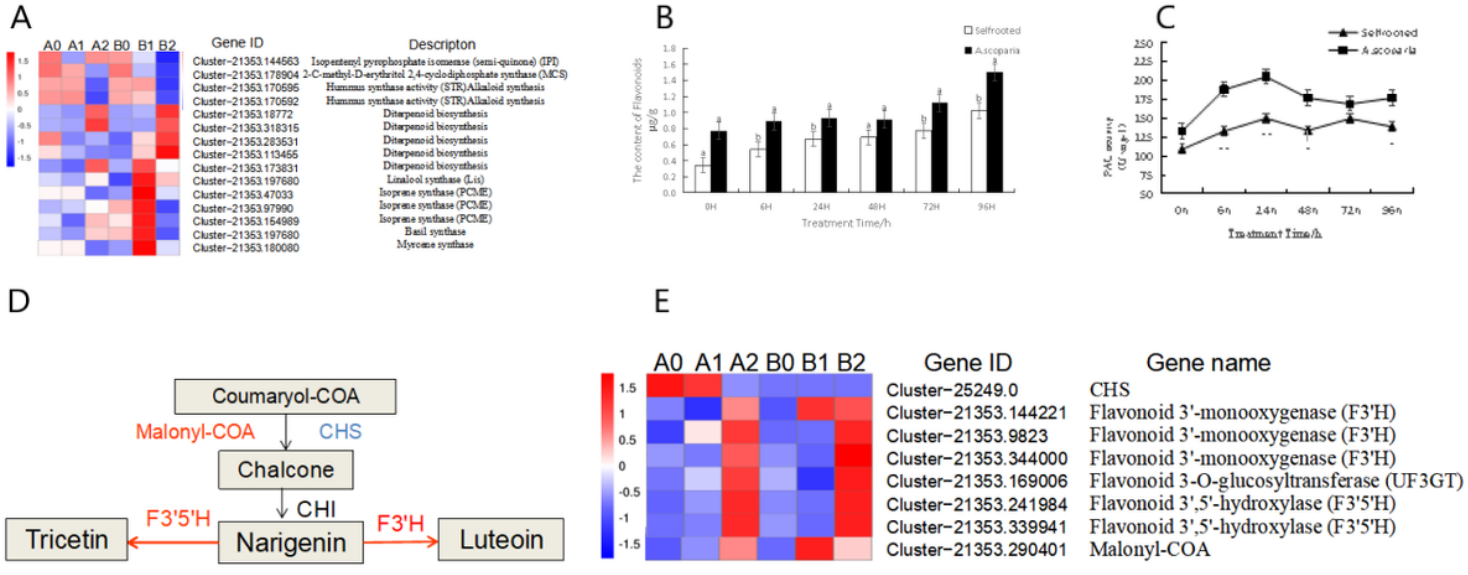


Figure 6

a. Heat map of the genes involved in synthesis of terpenoids and nitrogen-containing secondary organisms. b Effect of aphid stress on content of flavonoids in Cm/As and Cm/Cm. c PAL activity in leaves of Cm/As and Cm/Cm. d The metabolic pathway analysis of unigenes involved in flavonoid biosynthesis of Cm/As. Red and blue lines indicate up-regulated and down-regulated genes in Cm/As compared to Cm/Cm, respectively. e Heat map of the genes involved in flavonoids pathway under aphid stress.

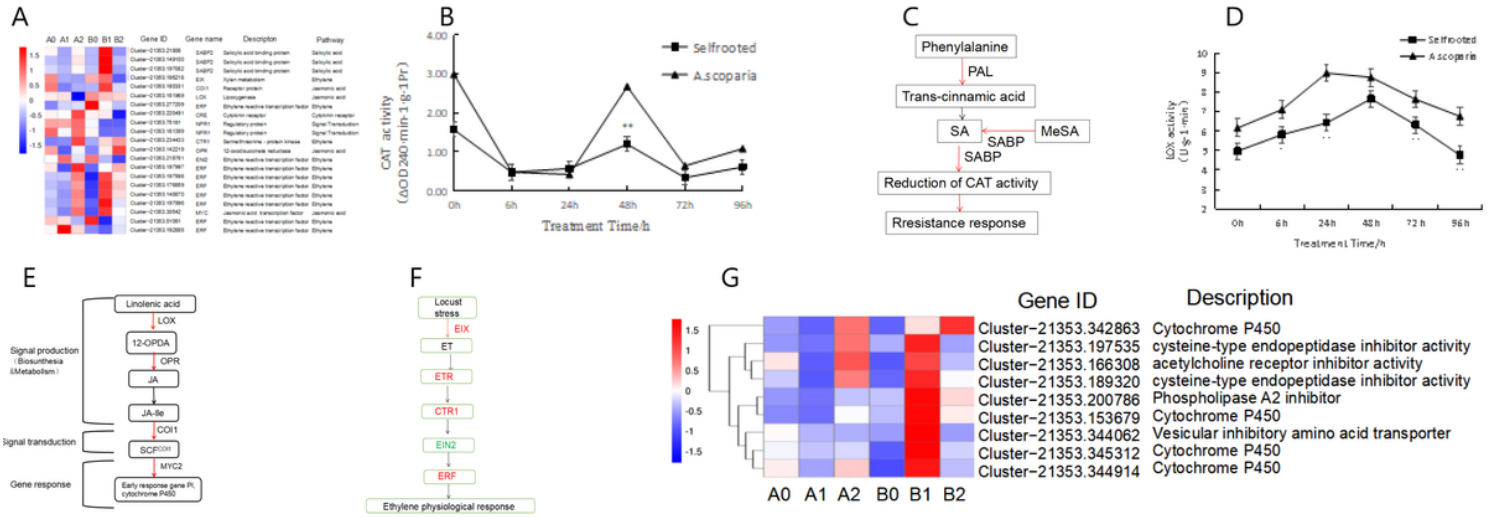


Figure 7

Plant hormone signal transduction between Cm/As and Cm/Cm. a Heat map of the DEGs involved in plant hormone signal transduction between Cm/As and Cm/Cm under aphid stress. b CAT activity in leaves of Cm/As and Cm/Cm. c The metabolic pathway analysis of unigenes involved in SA of Cm/As. d LOX activity in leaves of Cm/As and Cm/Cm. e The metabolic pathway analysis of unigenes involved in JA biosynthesis of Cm/As. f The metabolic pathway analysis of unigenes involved in ET biosynthesis of Cm/As. Red and blue lines indicate up-regulated and down-regulated genes in Cm/As compared to Cm/Cm, respectively. g Heat map of the DEGs involved in down stream response genes.

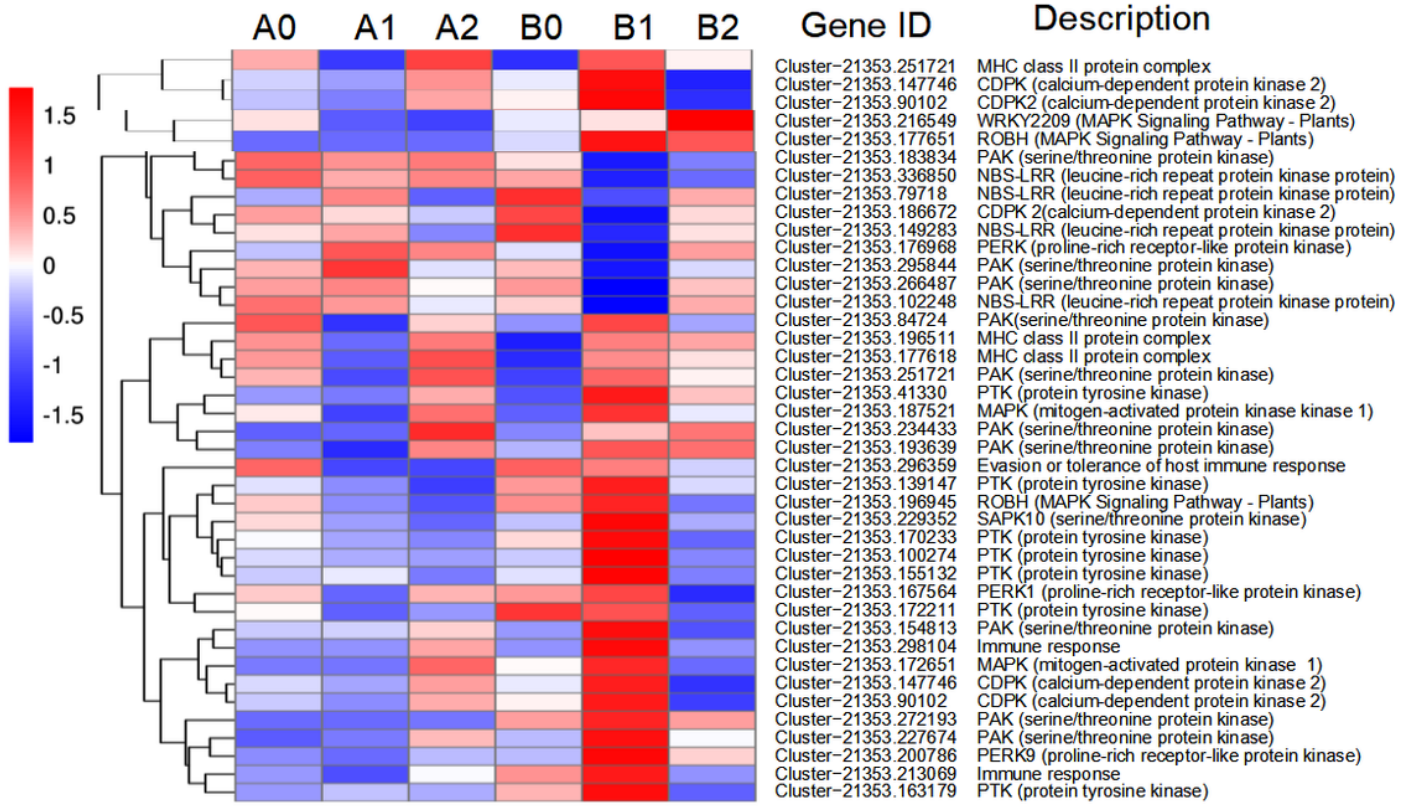


Figure 8

Heat map of the DEGs involved in plant-pathogen interaction.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xls
- Table2.xls
- Table3.xls
- Additionalfile1.docx
- TableS4.xls
- TableS2.xls
- Table1.xls
- TableS5.xls