Transcriptome Analysis Reveals the Molecular Patterns Regulating Prickle Reduction in Grafted *Zanthoxylum armatum*

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Grafting has been found to effectively reduce the prickly nature of *Zanthoxylum armatum* seedlings, but the molecular mechanisms are unclear. A comparative transcriptome analysis was performed on stems (JJ) and leaves (JY) of grafted stems (SJ) and leaves (SY) of non-grafted *Zanthoxylum armatum* seedlings. The authors obtained 3097, 2124, 5995, and 5043 differentially expressed genes from JJ *vs.* SJ, JY *vs.* SY, JY *vs.* JJ, and SY *vs.* SJ with 17 co-expressed genes. The RNA-seq results were confirmed by qRT-PCR analysis. Function annotations showed that many DEGs enriched plant hormone signal transduction, phenylpropanoid biosynthesis, and plant-pathogen interaction. Secondary metabolites associated with stress-related hormones and defense were noticeably up-regulated in grafted plants. However, key enzymes regulating lignin synthesis were slightly down-regulated in grafted plants. Additionally, grafted plants had several noticeable up-regulated stress-response TFs (transcription factors), including NAC (NAM, ATAF1/2, CUC1/2), ERF (ethylene response factor), MYB (v-myb avian myeloblastosis viral oncogene homolog), bHLH (basic helix-loop-helix), and WRKY. This study generated abundant sequences for elucidating the genetic differences between grafted and non-grafted *Z. armatum*.

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INTRODUCTION

Grafting, an ancient asexual plant propagation technology, is widely used in fruits, vegetables, ornamental plants, and other crop species (Li *et al*. 2019). The process involves cutting and adhering the scion and rootstock to each other. The attached rootstock and scion share water, mineral elements, and organic nutrient resources and co-influence their physiological metabolism. The interaction improves scion growth (Kumar *et al*. 2015), enhances plant resistance to pests and diseases (Rouphael *et al*. 2012; Spanò *et al*. 2020), and promotes flowering (Mubayiwa *et al*. 2016) and fruiting (Aslam *et al*. 2020). Additionally, grafting is essential for changing the phenotype of dwarf plants and extending the fruiting period in economically important tree species (Lee *et al*. 2010; Albacete *et al*. 2015).

Graft-union formation is a complex process involving phloem reconnection, restarting of root growth, and finally, xylem reconnection (Melnyk *et al*. 2015). Moreover,

the growth of grafted plants involves various molecular mechanisms. For example, multiple grafting combinations have demonstrated the transportation of macromolecules such as plant hormones, proteins, genetic material, and nutrients (Albacete *et al*. 2015). Auxin is the most crucial hormone besides cytokinin, ethylene, jasmonic acid (JA), and gibberellin for regulating the stress response after grafting (Nanda and Melnyk 2018). In citrus, most of the differentially expressed genes (DEGs) in "shatangju" leaves are involved in the auxin signal transduction and gibberellin biosynthesis pathways in grafted plants (Liu *et al*. 2017). However, most research on the molecules produced from grafting mainly focused on the molecular mechanisms at the healing site, with few studies on the quality and morphological changes post-grafting.

Plant growth and morphology are genetically controlled, and rootstocks can regulate the growth, development, and morphological characteristics of grafted trees by influencing the gene expression pattern in the scion (Prassinos *et al*. 2009; Jensen *et al*. 2010). For instance, the size, shape, secondary metabolites, and DNA methylation patterns in the scion of same-sex and hetero-grafted zucchini fruits are different from non-grafted controls (Xanthopoulou *et al*. 2019). Some studies also report that combining the rootstock and scion of two different pepper genotypes changed the fruit shape, indicating that intraspecific grafting of pepper rootstock and scion affects fruit shape. Moreover, the hormone levels and genes involved in hormone regulation vary between grafted and non-grafted plants (Rouphael *et al*. 2010; Liu *et al*. 2016), thus influencing the phenotype of grafted plants. In eggplants, grafting increased yield and reduced the appearance of calyx prickles (Nina *et al*. 2014). Grafting also reduces the prickles on the main branch of acacia trees by 75 to 88% (Mohamed *et al*. 2014). These examples above confirm that grafting alters plant phenotypes.

Zanthoxylum armatum is a small prickly tree of the Rutaceae family. It is usually distributed in shrubs at an altitude of 1000 to 2500 m, and is planted in China, India, Nepal, Vietnam, Myanmar, and other regions (Kashyap *et al*. 2021). *Z. armatum* has important edible and medicinal value. Because of its special sesame flavor and aroma, it is considered as an important spice and condiment in China (Xu *et al*. 2019). *Z. armatum* peel, root, and tender leaves can be used as medicines, which are commonly used in the treatment of stomachache, respiratory diseases, diarrhea, and toothache (Phuyal *et al.* 2020).

The prickle in *Z. armatum* hamper picking and restrict the development of mechanized *Z. armatum* picking systems (Yang *et al*. 2017). Therefore, many breeding experts are developing thornless *Z. armatum* varieties. Many studies and patents have shown that grafting reduces the prickles in *Z. armatum* Maxim (Dang *et al*. 2020). For example, the 'Yunlin No.1' scion becomes less pricked when grafted onto the *Z. acanthopodium* var. timbor rootstock. Combining prickly-free traits with other important traits through multiple grafting is a time-consuming and expensive process. Moreover, there are no reports on the molecular mechanisms of prickle reduction in *Z. armatum* post-grafting. Transcriptome analysis of grafted plants may reveal specific genes regulating graft-induced physiological responses (Zhao *et al*. 2013). Therefore, the authors sequenced the transcriptome of the tender stems and leaves from grafted and non-grafted trees and seedlings to determine the gene expression and metabolic activities between grafted and non-grafted trees. A quantitative real-time polymerase chain reaction (qRT-PCR) verified the transcriptome results. This study laid the foundation for elucidating the molecular mechanism of less prickle after grafting of *Z. armatum*.

EXPERIMENTAL

Plant Materials

Plant materials were planted in *Z. armatum* Planting Base (E102°44′42.57″, N25°08′50.67″), Botanical Garden, Yunnan Academy of Forestry and Grassland Sciences (Kunming, China). The scions of *Z. armatum* 'Yunlin No.1' were grafted onto the 5-year-old *Z. acanthopodium* var. timbor rootstock (grafted plants), and the non-grafted control was 5-year-old *Z. armatum* 'Yunlin No.1'. Three grafted plants and three non-grafted plants (three replicates) with good growth status and uniform growth environment were selected respectively. Three samples of grafted tender stems (JJ), three samples of leaves (JY), three samples of non-grafted tender stems (SJ) and three samples of leaves (SY) with uniform growth status were taken respectively. A total of 12 samples were frozen in liquid nitrogen and stored in a refrigerator at -80 °C until RNA was extracted.

RNA Extraction and Sequencing Library Construction

Total RNA was extracted using the TransZol Up Plus RNA Kit (Transgen Biotech, Beijing, China). The extracted RNA quality was assayed on a 1.5% agarose gel to determine the RNA quality. Further, RNA purity and quantity were determined using the Thermo Nanodrop 2000 (OD260/280 ratio) and Qubit (Thermo Fisher Scientific, Waltham, MA, USA), while the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) detected the RNA integrity. Three biological replicates were purified to construct cDNA libraries. Then, Shanghai Personalbio Biotechnology Co., Ltd. (Shanghai, China) sequenced the libraries on the Illumina HiSeq^{TM} 2500 platform (Illumina Inc., Hayward, CA, USA) following the paired-end (PE) protocol (PE150, and the independent sample sequencing depth was 6G).

De novo Assembly and Functional Annotation of the Transcriptome

Raw data (raw reads) of fastq format were firstly processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, ploy-N, and low-quality reads. The counts of clean reads, total length, Q20, N%, and GC% were determined accordingly. All cleans reads were de novo assembled using the Trinity assembly program with default parameters to form contigs (Grabherr *et al*. 2011). The functions of *Z. armatum* unigenes were annotated using NCBI non-redundant protein sequences (Nr), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), eggNOG and Swiss-Prot alignments (E-value < 10^{-5}).

Functional Analysis of the DEGs

The clean reads sequenced from each sample were mapped back to the unigene library to calculate the abundance of unigenes. To quantify the gene expression level, FPKM (fragments per kilobase of exon per million mapped reads) was calculated in each sample by RSEM (RNA-Seq by Expect-Maximization). The DEseq approach was used for differential analysis at this threshold |qvalue < 0.001 and log2Ratio≥1|. Enrichment analysis of the DEGs was performed on the GO database using the topGO R package. Essentially, the enrichment factor was used to determine DEGs enrichment levels on each enriched pathway, and the Fisher's exact test established the significance of the enrichment. Hypergeometric tests determined the most noticeably enriched KEGG pathways by the DEGs.

qRT-PCR Analysis of the Differentially Expressed Genes

Eleven DEGs were selected for plant hormone signal transduction, ribosome, phenylpropanoid biosynthesis, and plant-pathogen interaction pathways to verify the transcriptome data. The eleven genes were analyzed using real-time quantitative PCR (qRT-PCR) of the stem and leaf samples from grafted and seedling plants. The primers (Table S1) were designed according to the sequences from transcriptome data, and an elongation factor gene was the internal reference. Reverse transcription was performed using the transScript II one-step gDNA removal and cDNA synthesis superMix kit (Takara Bio, Dalian, China) and subjected to SYBR Green I qRT-PCR analysis on the CFX96 Real-Time PCR detection system (BioRad, Hercules, CA, USA). The SYBR Green I reagent was TransStart Top Green qPCR SuperMix (Transgen Biotech, Beijing, China). The data were standardized using the $2^{-\Delta\Delta Ct}$ method with three replicate experiments.

RESULTS AND DISCUSSION

De novo Assembly of *Z. armatum* Transcriptome Sequences

The transcriptomes of *Z. armatum* leaves and tender stems were sequenced using the Illumina HiSeq high-throughput sequencing platform, and the data obtained were processed as shown in Table S2. The results showed that the average number of Raw Reads in each sample was 43,760,958, and the average number of Clean Reads obtained after filtering was 43,519,333. The Trinity software for *de novo* assembly generated 490,624 transcripts (N50: 1137) and 209,507 unigenes (N50: 886). The sequences exceeding N50 accounted for 98,039 and 40,629. The overall unigenes GC content was 41.02% (Table S2). These data show that the high-throughput sequencing platform produced high-quality data for the transcriptome sequencing of *Z. armatum*, which can be analysed in the next step.

Unigene Functional Annotation, Classification, and Metabolic Pathway Analysis

The 209,507 unigenes were compared with the available protein database using the BLASTx algorithm at E-value 10^{-5} to obtain comprehensive gene function information. Up to 88,175 (42.09% of the total) unigenes were annotated in the NR database, 49,331 (23.55%) in GO, 6,422 (3.07%) in KEGG, 81,051 (38.69%) in eggNOG, 72,551 (34.63%) in Swissprot, and 5,415 (2.58%) in all the databases. Several unigenes were not mapped to any currently known protein database, suggesting that they represent new genes in *Z. armatum* Maxim (Table 1).

Table 1. Functional Annotation of Unigenes Gene in *Z. armatum*

Database	Number	Percentage (%)
NR	88175	42.09
GO	49331	23.55
KEGG	6422	3.07
eggNOG	81051	38.69
Swissprot	72551	34.63
In all database	5415	2.58

The 49,331 *Z. armatum* unigenes (23.55% of the total annotations) matched at least one of the 67 enriched functional GO terms under biological process, cellular component, and molecular function. The most substantially enriched biological processes are 'metabolic process' and 'cellular process', 'binding' and 'catalytic activity' under molecular function, and 'cell' and 'cell part' under the cellular component. The enriching unigenes exceeded 20,000 (Fig. S1).

The 6,422 unigenes enriched 35 KEGG metabolic pathways (Fig. S2) related to metabolism, genetic information processing, environmental information processing, cellular processes, and organismal systems. The five most enriched KEGG pathways included carbohydrate metabolism (438), translation (557), signal transduction (453), transport and catabolism (270), and carbohydrate metabolism (442).

KEGG Metabolic Pathway Enrichment after Grafting *Z. armatum*

The fragments per kilobase of transcript per million mapped reads (FPKM) method was used to calculate the abundance of the screened differential genes, and 16,259 differential genes were identified (Fig. S3). A total of 3,097 DEGs were identified from the JJ *vs.* SJ transcriptome analysis, including 2,061 up-regulated and 1,036 down-regulated genes. The JY *vs.* SY transcriptome generated 2,124 DEGs (1,674 up-regulated and 450 down-regulated), JY *vs.* JJ had 5,995 DEGs (4,010 up-regulated and 1,985 down-regulated), and SY *vs.* SJ generated 5,043 DEGs (2,856 up-regulated and 2,187 down-regulated). The JJ *vs.* SJ and JY *vs.* SY comparisons had 1,629 DEGs, and 17 DEGs were detected across all comparisons (Fig. 1). Thus, different tissues of grafted materials deferred more than different seedlings, suggesting that grafting affects gene expression in stems and leaves, increasing the number of DEGs in the different tissues.

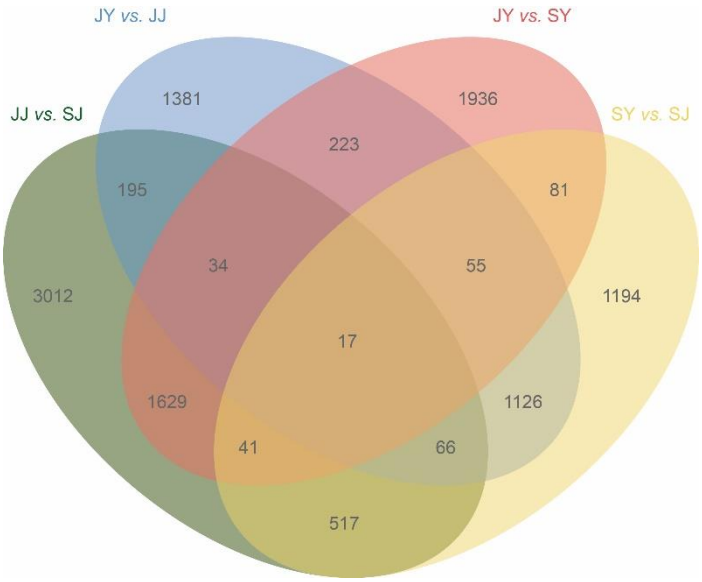


Fig. 1. Venn diagram showing the number of DEGs between four different comparisons

The noticeably enriched KEGG pathways, including ribosome, plant hormone signal transduction, phenylpropanoid biosynthesis, plant-pathogen interaction, and carbon metabolism were in the JJ *vs.* SJ comparison (Table 2). Oxidative phosphorylation, plant-pathogenic interaction, and photosynthesis were the most enriched pathways between

leaves of grafted and non-grafted trees. In addition, plant hormone signal transduction, phenylpropanoid biosynthesis, and plant-pathogen interaction pathways were enriched in the four different comparison combinations. The DEGs for the plant hormone signal transduction and phenylpropanoid biosynthesis pathways were up-regulated in most grafted materials, suggesting that grafting greatly impacted the hormone transduction and lignin synthesis pathways of *Z. armatum*. However, the DEGs for photosynthesis pathways were all down-regulated in the JY *vs.* JJ (23 down-regulated DEGs) and SY *vs.* SJ (28 down-regulated DEGs) comparisons. Therefore, the transcriptome sequencing was accurate as leaves are the main site of photosynthesis (Table 2).

Table 2. Five Most Obvious KEGG Pathways for DEG Enrichment among Different Comparisons

	KEGG Pathway	Up_ Number	Down_ Number	DEG_ number	Total_ number
JJ *vs.* SJ	Ribosome	13	2	15	240
	Plant hormone signal transduction	7	6	13	169
	Phenylpropanoid biosynthesis	4	5	9	60
	Plant-pathogen interaction	4	3	7	92
	Carbon metabolism	2	5	7	188
JY *vs.* SY	Oxidative phosphorylation	7	4	11	109
	Plant-pathogen interaction	0	9	9	92
	Photosynthesis	7	0	7	56
	Phenylpropanoid biosynthesis	2	5	7	60
	Starch and sucrose metabolism	2	4	6	112
JY *vs.* JJ	Plant hormone signal transduction	22	19	41	169
	Ribosome	2	26	28	240
	Photosynthesis	0	23	23	56
	Plant-pathogen interaction	1	16	17	92
	Phenylpropanoid biosynthesis	4	12	16	60
SY *vs.* SJ	Plant hormone signal transduction	17	14	31	169
	Photosynthesis	0	28	28	56
	Phenylpropanoid biosynthesis	7	7	14	71
	Carbon metabolism	3	13	16	90
	Plant-pathogen interaction	4	11	15	41

The DEGs Involved in Plant Hormone Signal Transduction

Plant hormones regulate various aspects of plant development and response to biotic and abiotic stresses (Santner and Estelle 2009). Studies have shown that changing plant hormone contents is one mechanism by which grafting affects physiological processes (Nanda and Melnyk 2018). The transcriptome analysis revealed that the DEGs for plant hormone signal transduction differed between the same tissues of grafted and seedling materials and between different tissues of the same material.

Grafting activates the auxin signaling pathway. Auxin is central in plant growth and development by controlling vascular bundle formation. This study revealed seven differentially expressed proteins: auxin-responsive protein IAA (8, 13, and 26), auxin transporter-like protein 2, ARF6 auxin response factor 6, auxin-induced protein AUX28,

and auxin-induced protein PCNT115. These auxins participated in the IAA signal transduction or IAA biosynthesis pathways and their expression was up-regulated in the grafted material. The expression of IAA8 (DN108930_c0_g1) and ARF6 (DN105277_c0_g1) was twice as high in grafted than in seedling stems. Generally, high auxin levels increase cell numbers in the xylem and phloem tissues of plants (Smetana *et al*. 2019). In this study, multiple auxin signal-related genes were highly expressed in grafted materials, indicating that auxin has a crucial regulatory role in grafted *Z. armatum* (Fig. 2).

Moreover, the expression patterns of abscisic acid, jasmonic acid, gibberellin, and ethylene signal-related genes were similar to the auxin pathway, which were up-regulated in grafted materials. The genes include abscisic acid receptor PYL4(DN101476_c1_g3), allene oxide cyclase 4 (DN100412_c0_g3), aminocyclopropane-1-carboxylate synthase (DN107216_c0_g1), and gibberellin receptor GID1C (DN110971_c3_g1). These results suggest that grafting activates the *Z. armatum* defense mechanism. Moreover, the number of DEGs in the different tissues of grafted plants was higher than in the tissues of seedling materials, indicating that grafting affected the hormone transduction pathways in tender stems and leaves of *Z. armatum*. Meanwhile, the expression of phytohormone-related genes in the shoots and leaves of grafted seedling materials was higher than that of non-grafted materials, indicating that grafting had an effect on the hormone transduction pathway in both shoots and leaves of *Z. armatum* (Fig. 2).

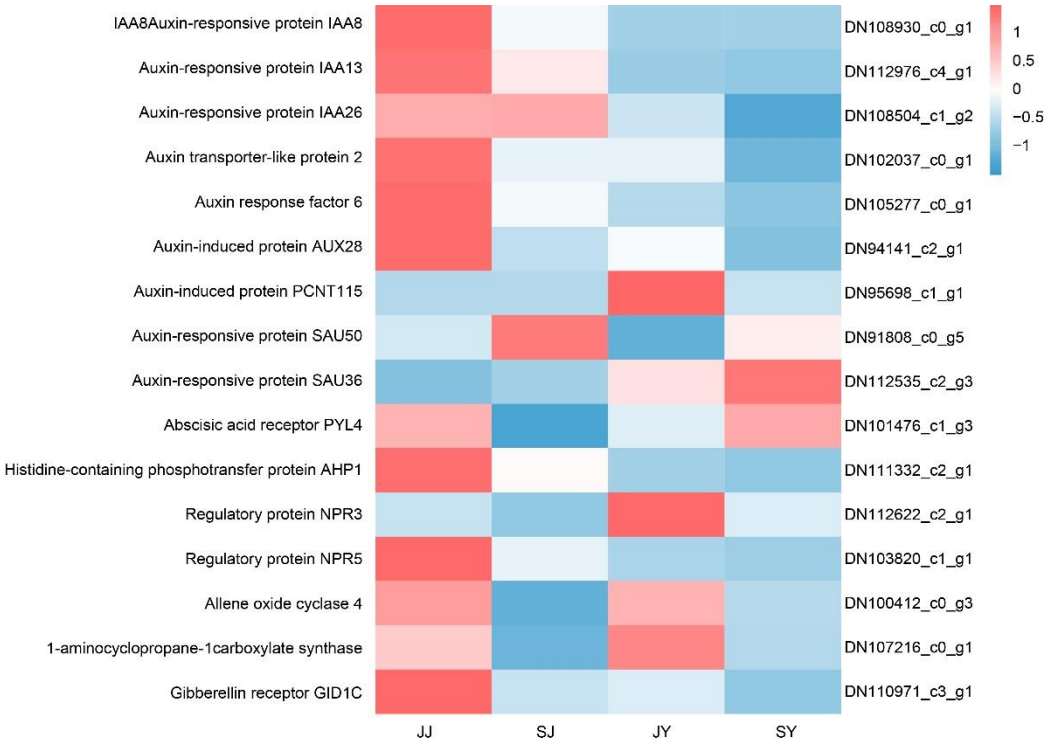


Fig. 2. Heat map of differentially expressed genes related to plant hormone transduction pathway. Horizontally represent genes, and each column represents a sample. Red represents high expression genes, and blue represents low expression genes.

The DEGs Involved in Phenylpropanoid Biosynthesis

Phenylpropane biosynthesis is an important secondary metabolic pathway in plants, producing lignin, flavonoids, lignans, *etc*. This process is widely involved in plant growth

and development and helps to cope with external stress (Vogt 2010). In this study, 60 *Z. armatum* unigenes were annotated to phenylpropanoid biosynthesis. The JJ *vs.* SJ comparison had more DEGs than JY *vs*. SY, indicating that grafting greatly impacted the phenylpropanoid biosynthesis pathway in tender *Z. armatum* stems.

The DEGs enriched the phenylpropanoid biosynthesis pathway in grafted and non-grafted young stems. In the JJ *vs.* SJ treatment, five genes were down-regulated, including two genes encoded by phenylalanine ammonia-lyase (PAL) (DN111284_c0_g1, DN88468_c0_g1), two COMT1 genes (DN109847_c2_g1, DN94980_c1_g1), one 4CL gene (DN102970_c2_g1), and one HST gene (DN107945 _c1_g1). There was upregulation of two genes encoding peroxidase (PER) (DN109806_c1_g1, DN98068_c1_g1) (Fig. 3). The genes mainly involved in lignin synthesis included p-Hydroxyphenyl lignin (H type), guaiacyl lignin (G type), 5-Hydroxyguaiacyl lignin, and syringyl lignin (S type) (Chio *et al*. 2019). The PAL and 4CL genes from the precursor pathway were highly expressed in grafted and non-grafted materials, indicating that these genes were transcriptionally regulated in both plant materials.

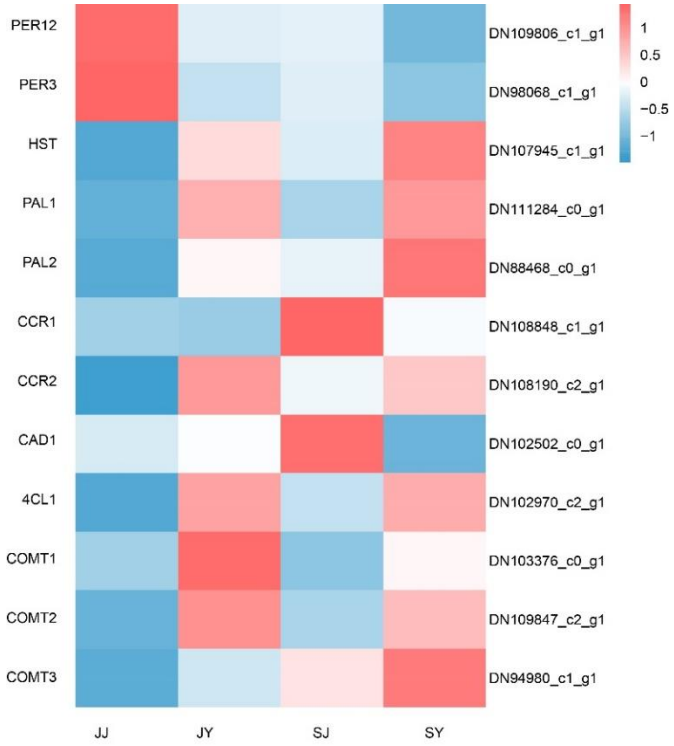


Fig. 3. Heat map of differentially expressed genes related to phenylpropanoid biosynthesis pathway. Horizontally represent genes, and each column represents a sample. Red represents high expression genes, whereas blue represents low expression genes.

Differential Expression of Specific Transcription Factors

A total of 20 highly expressed transcription factors were screened between grafted and non-grafted materials, including family transcription factors such as WRKY, ERF, NAC, MYB, BH, NFYA, AP2, and TCP. Among them, NAC29, NAC35, WRKY16, WRKY33, WRKY40, WRKY70, ERF80, ERF100, MYB26, BH14, and BH92 were up-regulated in grafting materials, and AP2-4, ICE1, GAT21, TCP4, GTE12, and NFYA9 were down-regulated in grafting materials (Fig. 4).

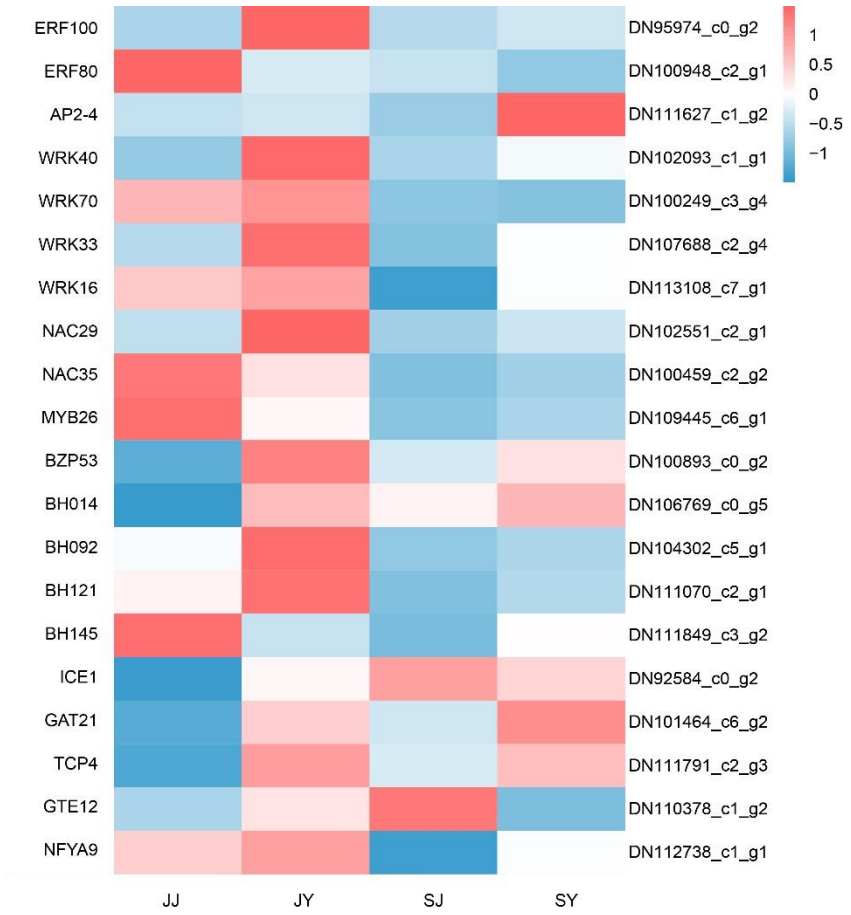
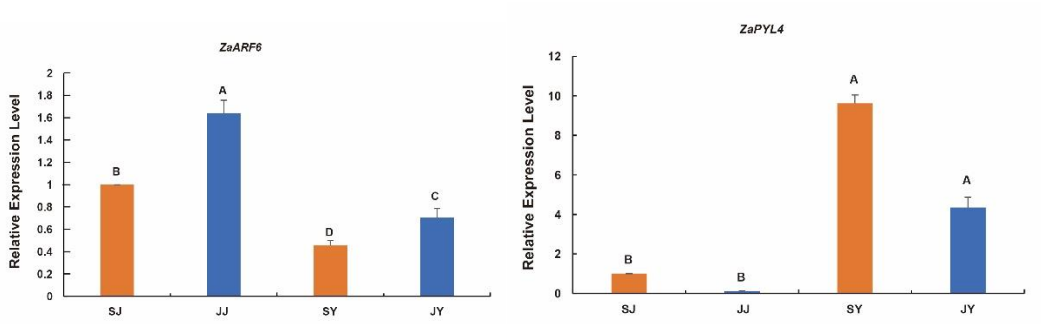


Fig. 4. Differential transcription factor expression heat map. Horizontally represent genes, and each column represents a sample. Red represents high expression genes, whereas blue represents low expression genes.

qRT-PCR Verification Analysis of DEGs

Eleven candidate genes were selected randomly for qRT-PCR analysis to validate the results of RNA-Seq. They represented various functional classes or pathways, including plant hormone signal transduction, phenylpropanoid biosynthesis, plant-pathogen interaction, and TFs. The results showed that the patterns of expression of both methods were consistent, confirming the reliability of the transcriptome results (Fig. 5).



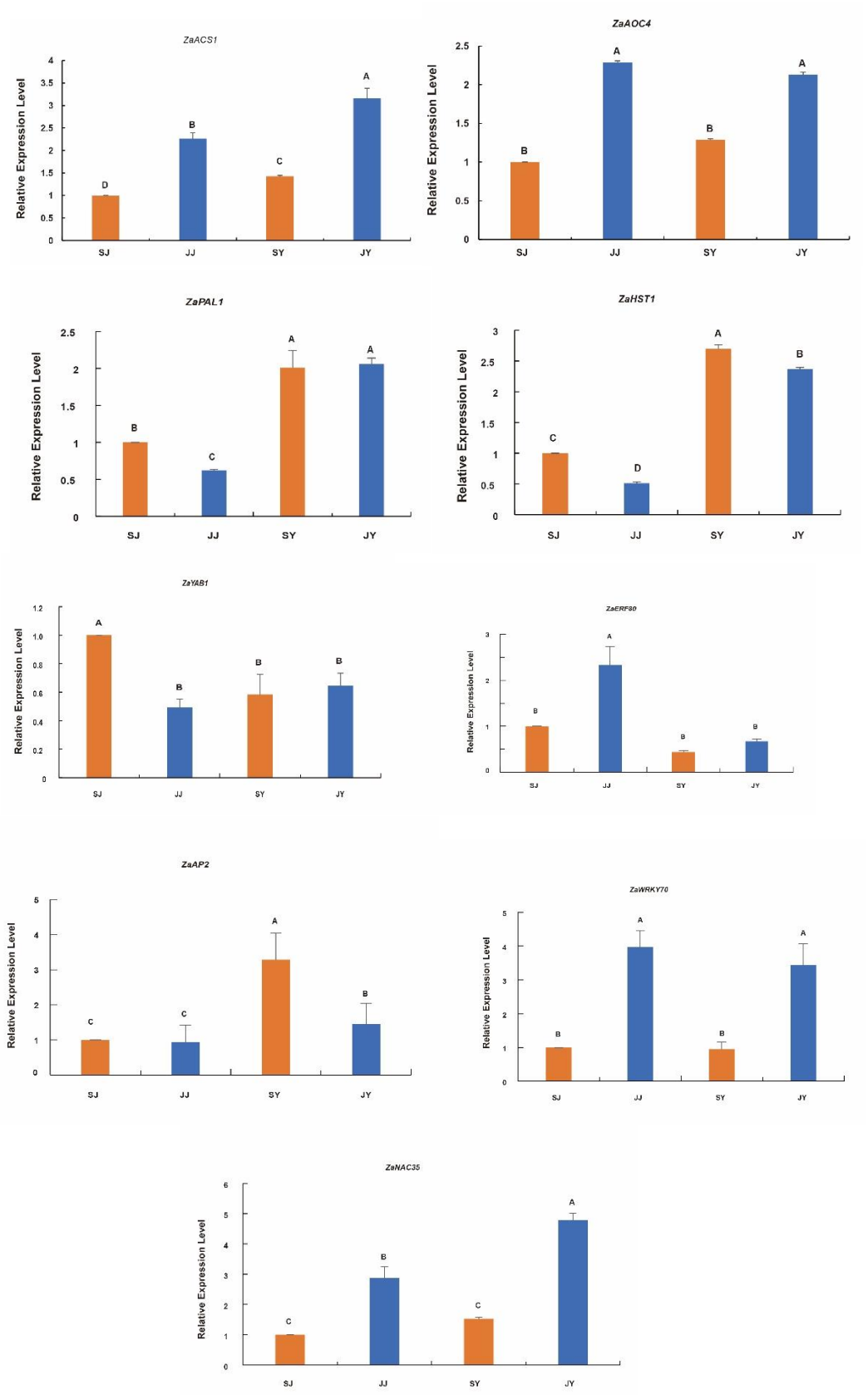


Fig. 5. qRT-PCR verification results of 11 differential genes. x-coordinate is different treatment group; and the ordinate is DEGs expression changes. Error bars: standard error of mean.

Z. armatum is spiny, especially the stem and leaf back, which are difficult to harvest. Previous studies have found that the scions of *Z. armatum* 'Yunlin1 ' were grafted onto *Z. acanthopodium* var. timbor rootstocks, and the prickles were reduced by 50 to 60%. After repeated grafting for 2 to 3 generations, the prickles were reduced by 80 to 90% (Pan *et al.* 2014). It is well known that grafting effectively improves fruit quality, scion phenotypic characteristics, and plant resistance to pests and diseases (Tsaballa *et al.* 2021). In the study of Acacia Species (Mohamed *et al.* 2014), and Rose (Kazaz *et al.* 2013), it was also found that grafting led to a decrease in prickles. Some researcher proposed the decrease is because after grafting, rootstocks regulate growth by transferring metabolites and signaling substances to aboveground parts (Li *et al.* 2019). This phenotypic change in grafted plants implies that the molecular patterns of gene expression have also changed (Tsaballa *et al.* 2021). However, the molecular mechanism of prickle reduction after grafting is still unclear. In this study, transcriptome sequencing of tender stems and leaves of grafted and non-grafted plants was performed by next-generation sequencing technology to explore the differences in gene expression between grafted and non-grafted plants.

Hormonal changes in grafted plants (Rouphael *et al*. 2010; Liu *et al*. 2016) affect their phenotype. In the study of *Z. bungeanum*, the stem apex nodes of differentiated spines and internode samples of undifferentiated spines in tender stems were compared and analyzed by transcriptome sequencing. The results showed that the genes related to auxin and YABBY genes involved in polar axis differentiation were differentially expressed in shoot tips and stem nodes. Among them, the expression levels of YABBY1, YABBY2, and YABBY4 in the shoot tips of prickle differentiation were much higher than those in the internodes of undifferentiated prickle. Therefore, the YABBY gene is considered to be a candidate gene involved in prickle differentiation of *Z. bungeanum*. This study found that auxin response genes such as auxin response protein IAA, auxin transporter-like protein 2, ARF6 auxin response factor 6 were differentially expressed between grafted and non-grafted plants. After grafting, the expression of the axial regulator YABBY1 (DN100270_c0_g1) and the axial regulator YABBY2 (DN106863_c2_g1) genes was down-regulated in the tender stems, and the decrease in the expression level of the YABBY gene may result in a decrease in spines.

In citrus, the expression of genes related to auxin and gibberellin biosynthesis pathway was high in rootstocks of grafted plants, thereby regulating hormone levels and their signaling pathways (Liu *et al*. 2017). Plant hormones, such as gibberellin, auxin, cytokinin, abscisic acid, and ethylene, function through complex interaction and feedback regulation networks (Vanstraelen and Benková 2012). In the study of *Z. armatum*, several DEGs related to the above five hormones were identified. The authors found that DEGs related to hormone signal transduction (such as auxin-responsive protein IAA, Auxin-responsive protein SAU, and ethylene receptor ETR) were more different genes than those related to hormone synthesis pathways (such as gibberellin 20 oxidase, cytokinin, and dehydrogenase). This means that the enhancement of hormone signal transmission and crosstalk may be the main result of grafting of *Z. armatum*, rather than the increase of hormone content.

The prickle is considered effective for resisting herbivore and abiotic stress in plants (Barton 2014). The transcriptome of tender stems and leaves from grafted and seedling materials showed that the expression of two defense-related hormones, ethylene, and salicylic acid, is higher in grafted than non-grafted plants. Usually, ethylene and salicylic acid are synthesized in infected and injured tissues of plants for defense (Bari and Jones 2009). Thus, the increased expression of genes for these defense-related hormones may

reduce the necessity of prickling growth. Abscisic acid receptor PYL4 is also important in ABA-responsive-element stress response (Park *et al*. 2009; Bolt *et al.* 2017). The ABA signaling pathway inhibits the activity of PP2Cs proteins through its receptor PYL protein family (Fuentes *et al*. 2019). In this study, PP2C and PYL4 genes were differentially expressed between grafted and non-grafted materials, consistent with the transcriptome results of peony grafting (Li *et al*. 2019). The expression of PYL4 in grafted young stems was four times higher in young stems of non-grafted plants, indicating that grafting triggered stress in *Z. armatum*.

Phenylpropanoid biosynthesis is one of the most important secondary metabolic pathways in plants, which is mainly used for lignin biosynthesis and flavonoids biosynthesis (Vogt 2010). This study found that some key enzymes in lignin biosynthesis, such as phenylalanine ammonia-lyase (PAL1, PAL2), cinnamoyl-CoA reductase (CCR1, CCR2), cinnamyl alcohol dehydrogenase (CAD1), were down-regulated in grafted plants. The difference is that some genes encoding peroxidase (PER) are up-regulated in graft materials. In addition, most of the calcium-binding protein CML was up-regulated in grafted materials. Among them, the gene annotated as CML41 was 5 times higher in grafted tender stems than in non-grafted tender stems, and 3 times higher in leaves than in non-grafted leaves. The CML gene family is usually considered to be a specific sensor of plant Ca^{2+} and plays an important role in adapting to the environment and responding to various stress responses (Ranty *et al*. 2016). The up-regulation of CML and PER gene expression levels and the down-regulation of some key enzymes in lignin synthesis may have a complex relationship with the reduction of *Z. armatum* spines.

Comparative transcriptome analysis of *Solanum viarum*, eggplant, and raspberry showed that MADS-box, MYB, AP2/ERF, WRKY, and NAC TFs possibly regulate prickle development (Pandey *et al*. 2018; Khadgi and Weber 2020; Zhang *et al.* 2021). In this study, 20 highly expressed transcription factors in grafted and non-grafted materials were identified by homology search and differential expression analysis. Among them, WRKY transcription factors are one of the largest families of transcriptional regulators in plants and form integral parts of signaling webs that modulate many plant processes (Rushton *et al*. 2010). The WRKY family members play important roles in diverse stress responses (Li *et al*. 2020). This study found that genes annotated as WRKY16, WRKY33, WRKY40, and WRKY70 were up-regulated in grafting materials. In addition, some stress-related TFs, such as NAC29, NAC35, ERF80, ERF100, and BH92 (Wang *et al*. 2016; Heyman *et al*. 2018), were also up-regulated in the grafted materials. The increase in the expression level of these transcription factors in the grafted materials may be complicatedly related to the reduction of prickle.

In summary, grafted *Z. armatum* undergoes complex gene network regulation involving multiple signaling pathways. Some genes related to plant hormones and phenylpropanoid metabolism seemingly interacted with specific TFs to reduce prickle formation after grafting. Moreover, some defense-related genes were up-regulated in grafted plants, probably leading to prickle loss. This report is the first to explore the molecular mechanism in grafted *Z. armatum* with abundant sequence resources for elucidating the genetic differences between grafted and non-grafted *Z. armatum*.

CONCLUSIONS

1. In this study, the Illumina HiSeq platform was used for transcriptome sequencing of grafted and non-grafted *Z. armatum* samples. A comprehensive reference transcriptome database was obtained by deep sequencing and assembly.
2. The sequencing data were analyzed to identify several differentially expressed genes (DEGs) in grafted and non-grafted materials, including plant hormone signal transduction, phenylpropanoid metabolism-related genes, and some transcription factors (TFs).
3. This study revealed the differences between grafted and non-grafted *Z. armatum* at the molecular level, providing abundant sequence resources for further determining the molecular mechanism of less pricking after grafting.

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SUPPLEMENTARY INFORMATION

Table S1. Primer Sequences

Primer Name	Sequence (5'-3')
DN105277_c0_G1F	CACAGATGACCTTACAGCCA
DN105277_c0_G1R	TCCTCCATGAGTACTTGTGT
DN101476_c1_G3F	CTCAAGCATACAAACACTTC
DN101476_c1_G3R	TTCGCCAGCCTGTGATCGCC
DN100412_c0_G3F	AGATCAATGAGCGAGACAGA
DN100412_c0_G3R	AGTGACGGCTAAATAAGTGT
DN107216_c0_G1F	ATCCAGGATTCGATCGAGAT
DN107216_c0_G1R	TATTATCTCCGCGATGCTAA
DN111284_c0_G1F	AAGCAAGGTGGTGCTTTGCA
DN111284_c0_G1R	GGAGGTGATCGTGCCACGGA
DN107945_c1_g1F	AGATTGATTGTAACGCTGAG
DN107945_c1_g1R	CACACCAAGTGAGACTCCAC
DN100270_c0_g1F	CAGCTTCACTTGGGACATCC
DN100270_c0_g1R	TGATGATCAACTCCAGGAAC
DN100948_c2_g1F	AGCAGCACTGTGGAGTCTTC
DN100948_c2_g1R	GCGTGGGTCACACAACCAAC
DN111627_c1_g2F	ATTCAACCAACACAGACTCA
DN111627_c1_g2R	TCAAGGTCAATACGTTACTC
DN100249_c3_g4F	GGACAAGAGAGGCTGTTACA
DN100249_c3_g4R	TGTGTACACCTATAGTAACT
DN100459_c2_g2F	GAGCCTAAACGTATAATCGG
DN100459_c2_g2R	TCTTCCTCCATTGCAGCCCT
Elongation factor F	CCCTATTGGGAAGGTTCCAG
Elongation factor R	CCTTGAGAGATTAGCATCGA

Table S2. Statistical Results

Sample	Reads	Clean Reads	Q20 (%)	Transcript	N50 (bp)	Unigenes	N50 (bp)	GC (%)
JJ1	42380248	42150734	96.98	490642	1137	209507	886	41.02
JJ2	44897428	44643408	96.80					
JJ3	46046566	45793464	96.94					
JY1	41530950	41270742	96.79					
JY2	40948210	40695792	96.81					
JY3	45338796	45130884	97.20					
SJ1	43844494	43617910	97.00					
SJ2	40515352	40284680	96.98					
SJ3	46311494	46079062	97.07					
SY1	45451618	45171938	96.76					
SY2	44270696	44006124	96.81					
SY3	43595646	43387268	97.13					

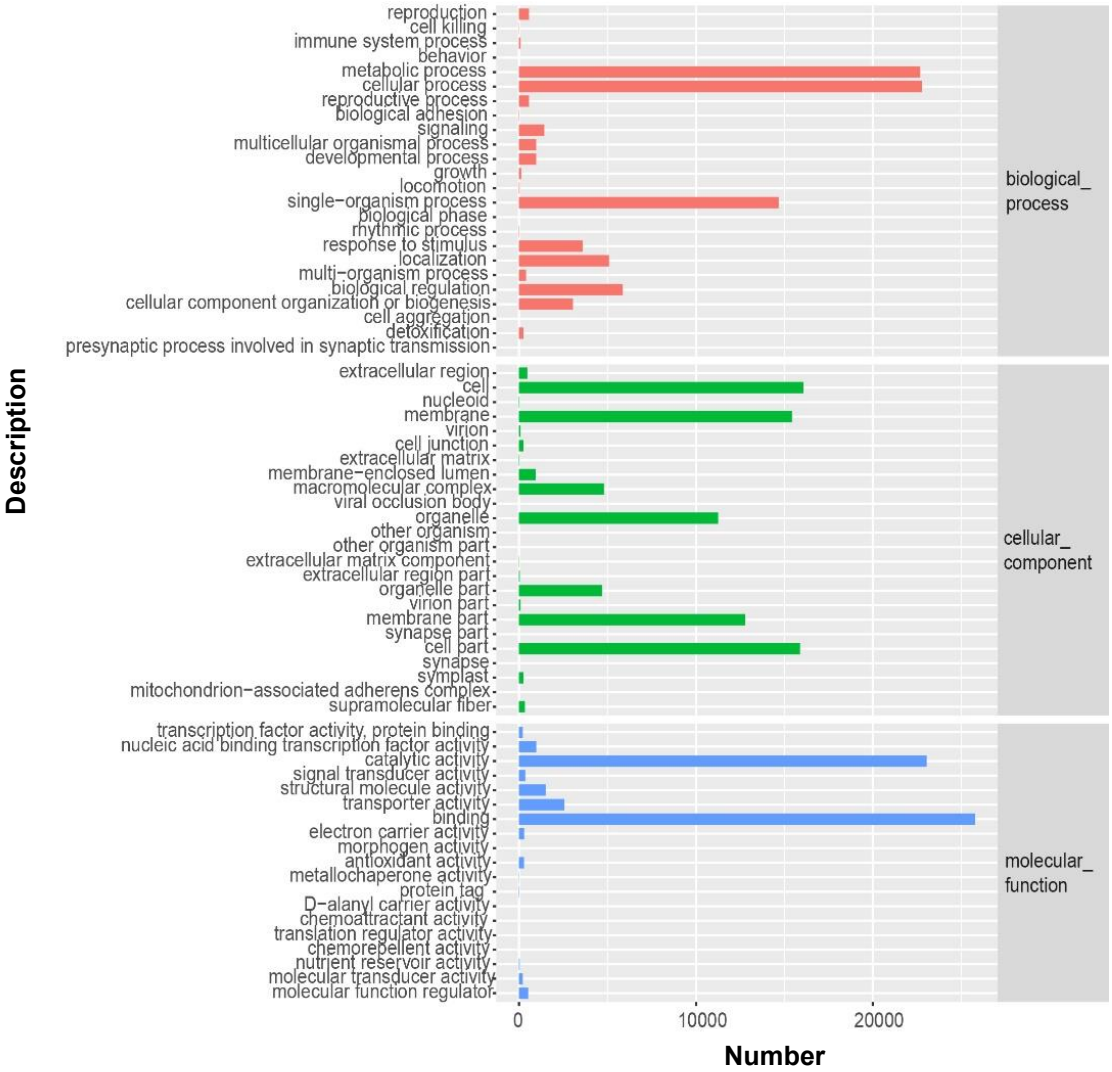

Fig. S1. GO functional classification of *Z. armatum* unigenes

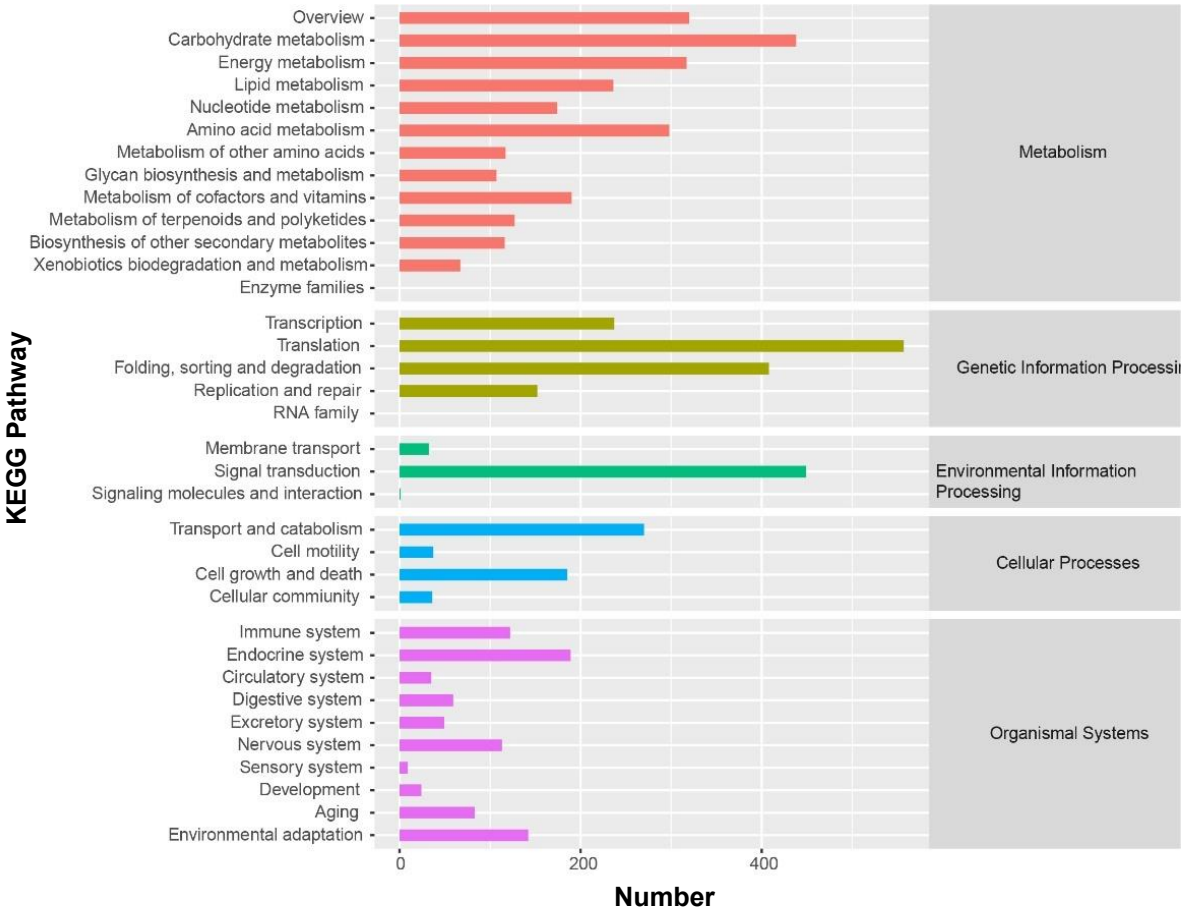

Fig. S2. KEGG annotation statistics of unigenes of *Z. armatum* Maxim

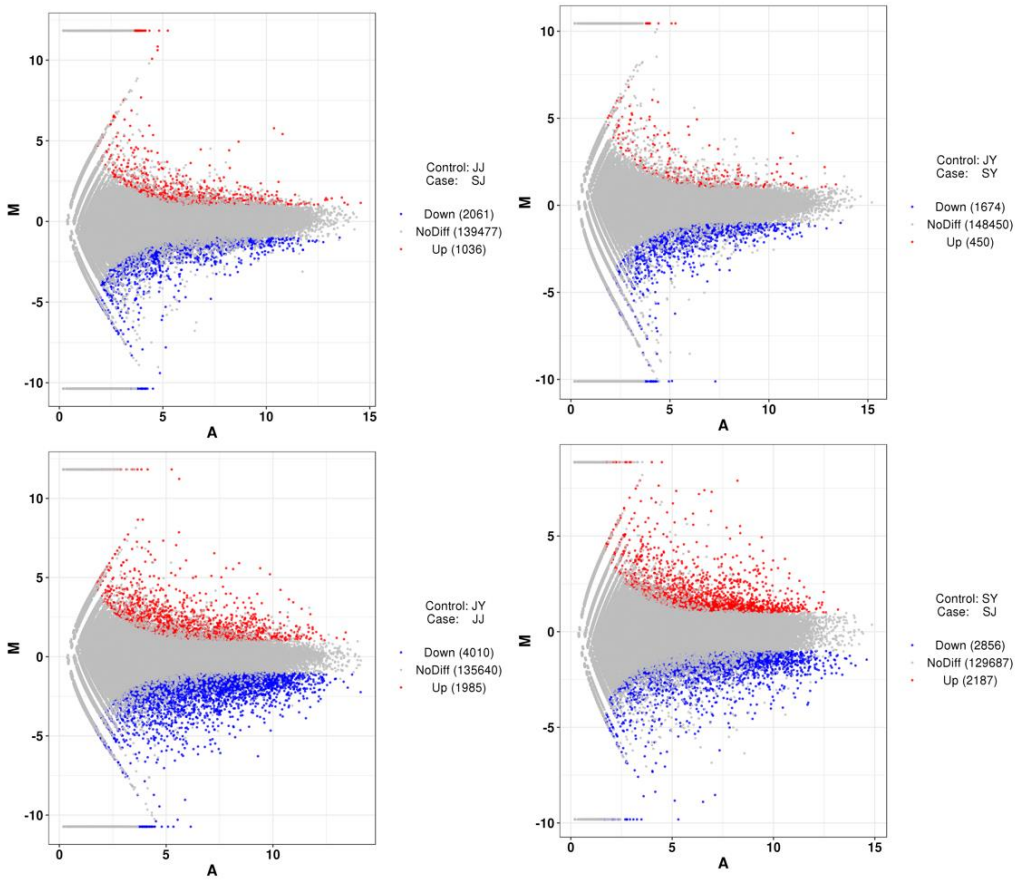

Fig. S3. Volcano plots of DEGs in different comparisons during the girdling process in *Z. armatum*