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Research article

Transcriptome analysis reveals key genes and pathways for prickle development in *Zanthoxylum armatum*

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ABSTRACT

Zanthoxylum armatum is an economically important tree species. However, well-developed prickles on its stems and leaves pose serious challenges in terms of management and harvesting. To investigate the molecular mechanism underlying prickle development, we sequenced different stages of prickle morphological development and transcriptomes of different tissues in the root tips (Gen), leaf buds (Ya), and fruits of *Z. armatum*. The results revealed that proteins related to cell division and genes related to the growth hormone signaling pathway were highly expressed in the prickle just protrusion (PC1). In addition, a high expression of lignin biosynthesis genes was observed during the developmental onset of lignification (PC2) and prickle lignification (PC3). These findings indicate that phenylpropanoid biosynthesis and plant hormone signal transduction are key pathways for the completion of lignification development in the prickle. During prickle development, ZaMYB2 and ZaWRKY3 were significantly upregulated in PC2 and PC3, suggesting their possible involvement in prickle development. Transcriptome and qRT-PCR analyses revealed differential gene expression of zaPAL3, za4CLL1, zaCOMT1, ZaWRKY3, and ZaCCD31 in the Gen, Ya, newly formed fruit (ZaF1), newly oil-spotted fruits (ZaF2), PC1, PC2, and PC3 of *Zarmatum*. zaCCD31 was highly expressed in leaf buds, whereas Za4CLL1 was highly expressed in root tips. During the lignification of prickles, the relative expression of genes including zaMYB2 increased gradually; however, the relative expression of zaCCD31 decreased during this process. Therefore, we inferred that these genes might be closely related to prickle development. Notably, zaMYB2 was expressed at higher levels in PC2 and PC3 than in PC1 and was not expressed in Gen, Ya, ZaF1, and ZaF2. Therefore, zaMYB2 is a key gene involved in prickle development of *Z. armatum* that exhibited tissue-specific expression. This study establishes a foundation for future analyses of the molecular mechanism underlying prickle development in *Z. armatum*.

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1. Introduction

Zanthoxylum armatum DC. is a small thorny tree of the genus *Zanthoxylum i*n the family Rutaceae, native to temperate and subtropical regions of the world, and is usually found in bushes in valleys and mountains at altitudes of 1000–2500 m. It is cultivated in China, India, Nepal, Vietnam. and Myanmar. The root, stem, leaf, fruit, and seeds of *Z. armatum* have great medicinal value. The volatile oil of the plant has been the subject of pharmacological studies revealing its antioxidant, anti-inflammatory, analgesic, antibacterial and local anesthetic effects [1]. The volatile oil contains alkaloids and terpenoids which are the primary active medicinal ingredients. *Z. armatum* is considered an important spice and condiment in China because of its unique numbness and aroma [2].

Plants rely primarily on epidermal tissue for protection and defense against pathogens and predators and use structures such as epidermal hairs, cuticles, and plant waxes, or biochemical mechanisms such as terpenoids, alkaloids, and phenolics to provide defense. In addition, the epidermal cells of some plants, such as those on *Rosa rugosa* plants, can produce sharp protrusions that are thorn-like in form, called prickles. Branch spines originate from lateral branches and leaf spines from the phloem of leaves, both of which contain vascular bundles, whereas prickles are ectomycorrhizal or cortical tissues lacking the vascular system, similar to trichomes [3]. From a morphological point of view, prickles are multicellular, peculiar forms of epidermal hair that are mainly composed of lignin, cellulose, hemicellulose, and corky matter [4,5]. It is well known that the trunk, branches, and leaves of the *Z. armatum*, are also densely covered with special morphological growths called *Zanthoxylum* prickles (Fig. 1D–E). These prickles first appear as small green bumps on the epidermis of *Zanthoxylum* trees and then gradually lignify as they grow and develop, eventually forming hard prickles that provide their own protective effects. However, this also leads to labor and effort during *Zanthoxylum* harvesting, which inevitably increases the burden and cost of field management. Therefore, cultivation of *Zanthoxylum* without prickles or with reduced prickles has become a feasible solution to improve the industry.

Usually, the development of prickles is considered a process of gradual sclerosis with a continuous accumulation of metabolites, especially phenylpropanoids, including lignin and suberin. During the development of prickles, once the outermost cells are lignified, lignification continues inward and downward until the prickles are fully lignified and mature [6], suggesting that the hardening of prickles is closely related to lignin accumulation. In the study on rose, transcriptome analysis of three different stages of dermatophyte morphogenesis revealed that similar to trichomes, dermatophytes accumulate large amounts of secondary metabolites, particularly lignans and flavonoids, during morphogenesis [7]. In most plants, biosynthesis of lignin polymers occurs mainly through phenylpropane and lignin-specific pathways. Studies have shown that multiple enzymes are involved in the operation of both pathways, among which phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), and coumartae3-hydroxylas (C3H) are key enzymes in the metabolic pathway of phenylpropane; cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) are key enzymes in the synthesis of the lignin-specific pathway and play an important role in

Fig. 1. The situation of prickly prickles in different developmental stages and parts of Z. armatum. (A) PC1: Prickles just protruding. (B) PC2: Prickles starting to develop lignification. (C)PC3: Fully lignified prickles). (D) Prickles on leaves. (E) Prickles on branches.

lignin biosynthesis [8].

In a study on rose prickles, the cloned RrTTG1 gene was expressed significantly less in thorny roses than in less thorny roses, and the RrTTG1 gene was 80% homologous to the TTG1 gene in *Arabidopsis*, cucumber, and tobacco, which are known to regulate epidermal hair development, suggesting that the RrTTG1 gene may play a role in the development of rose prickles [9]. In a study on eggplant prickles, comparative transcriptome analysis revealed differential expression of development-related transcription factors, such as bHLH, ARF, MYB, TCP, and WRKY, during prickle development. The study identified MYB12 and MYB111 as master regulators capable of strongly activating downstream structural gene promoters and confirmed that SmARF16 was the strongest factor in prickle development in eggplant [10]. Studies related to *Zanthoxylum* prickles have identified the homologous structural domain leucine zipper (HD-ZIP) gene family as a plant-specific class of transcription factors that plays important roles in biological processes such as morphogenesis, signal transduction, and secondary metabolite synthesis. In particular, studies targeting HDZ genes has revealed that ZaHDZ39 and ZaHDZ32 genes may be important in the biosynthesis of flavonol, anthocyanin, and mucilage. In addition, genes such as ZaHDZ16 may regulate the orderly and precise division of prickly *Zanthoxylum* cells in specific directions. A previous study identified an R2R3MYB transcription factor, ZaMYB86, that negatively regulates the development of dermal spines. ZaMYB86 interacts with the AaMYB5 protein, which is similar to the structural domain of ZaMYB5 that affects prickle development in *Z. armatum* [11]. However, molecular studies targeting key genes that regulate prickle development in *Z. armatum* leaves are lacking. Therefore, we performed transcriptome sequencing analysis targeting Z. armatum prickle tissue (PC1: prickles just protruding. PC2: prickles starting to lignify. PC3: prickles fully lignified.), root tips, Leaf buds and fruit (ZaF1: fruit samples without oil spots. ZaF2: fruits with fresh oil spots.) to explore molecular clues of prickle development, with the aim of providing potential candidate genes for the subsequent breeding of thornless *Z. armatum* varieties.

2. Materials and methods

2.1. Plant material

Five-year-old live *Z. armatum* in good growth conditions in the arboretum of the Yunnan Academy of Forestry and Grassland Sciences were selected as the research material (The stage of taking materials is *Z. armatum* first fruiting period) . Single plant samples were collected from the leaf buds (Ya) and root tips (Gen), prickles just protruding (PC1) (Fig. 1A), prickles starting to lignify (PC2) (Fig. 1B), prickles fully lignified (PC3) (Fig. 1C) and fruit samples without oil spots (ZaF1), fruits with fresh oil spots (ZaF2). The collected samples were quickly frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA extraction and sequencing library construction

RNA was extracted from seven samples using the TransZol Up Plus RNA Kit (TransGen Biotech, China). Samples were analyzed using 1.5% agarose gel electrophoresis for RNA degradation and contamination, Thermo Nanodrop 2000 for RNA purity (OD260/280 ratio), Qubit (Thermo Fisher Scientific) for precise quantification of RNA concentration, and an Agilent 2100 Bioanalyzer for RNA integrity. The cDNA libraries of the seven purified samples were constructed, and the cDNA libraries were paired-end (PE) sequenced using the Illumina HiSeq 2500 sequencing platform. The reading was PE150 and the depth of the independent sample was 6G.

2.3. Transcriptome data processing and analysis

After removing the sequencing junction, duplicate redundant sequences, and low-quality sequence data, the raw data were obtained as clean reads, and the number of clean reads, total length, Q30, N%, and GC%, were counted. Trinity software (http://trinityrnaseq.github.io/) was used to perform de novo assembly by first concatenating overlapping reads of a certain length into a longer N-free fragment contig and then concatenating different contigs from the same transcript to obtain a non-redundant sequence that cannot be extended at both ends to obtain a single gene library of the species. Single gene sequences were compared with the NR, GO, KEGG, EggNOG, and Swissprot databases using the BLAST software to functionally annotate the unigenes of *Z. armatum*. After predicting the amino acid sequences of the single genes, the HMMER software was used for comparison with the Pfam database to obtain annotation information for the single genes.

2.4. Differential gene expression analysis

Annotated transcripts were selected and reads obtained from the sequencing of each sample were compared with a single gene library using Bowtie and expression levels were estimated by combining with RSEM. DEseq was used for differential screening analysis and a threshold of q value < 0.05 and log2Ratio≥ 1 was set to screen differentially expressed genes. Functional classification of DEGs was performed using MapMan and COG to create the mapping files. Enrichment analysis of DEGs was performed in the GO database using the topGO software. Enrichment factors were used to analyze the enrichment level of DEGs in a pathway, and Fisher's exact test was used to calculate the significance of enrichment. The hypergeometric test was also applied to identify the most significantly enriched pathway in differentially expressed genes compared with the assembled transcriptome background, using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway as the unit.

2.5. Quantitative real-time PCR (qRT-PCR) verification

To verify the results of transcriptome sequencing and the expression pattern of target genes, specific primers were designed and ubiquitin gene was used as internal reference genes to detect the expression of *Z. armatum* genes in PC1, PC2, PC3, Gen, Ya, ZaF1, ZaF27 by real-time fluorescence quantitative PCR. The 25ul reaction system were as follows: 2 × SYBR Green master mixture 12.5 μL, upstream and downstream primers (10μm/l) 0.5 μL, template (cDNA)1 μL, ddH2O 10.5 μL. Genes were quantified in real time using SYBR Green (Invitrogen) and a PCR thermal cycler (ABI 7300; Applied Biosystems, Foster City, CA, USA). PCR reaction procedures: denaturation procedure (95 °C, 10 min), amplification quantification procedure repeated 40 times (95 °C, 15s; 57 °C, 10s; 72 °C, 15s; single fluorescence measurement), melting curve procedure (60 °C–95 °C, heating rate 0.1 °C, continuous fluorescence measurement), and finally cooling to 40 °C. Three independent biological replicates of each sample and three technical replicates of each biological replicate were analyzed by qRT-PCR using the Elongation factor gene as an internal regulator of normal gene expression. The primers are listed in Table S1.

2.6. Gene cloning of ZaMYB2

In this study, specific primers were designed based on the sequence of ZaMYB2 of *Z. armatum*, and the cDNA with the highest expression of the target gene was used as the template for RT-PCR amplification. The resulting product was recovered and purified after electrophoretic detection. The target fragment was cloned into the vector pUMT, overnight transformed into E. coli receptor cells Trans1-T1, and single colonies of positive clones were selected and sequenced to obtain the target gene.

3. Results

3.1. Transcriptome sequencing and de-novo assembly

In this study, we sequenced the transcriptome of three different stages (PC1, PC2, and PC3) of *Z. armatum* prickle development samples, as well as root tip, leaf bud, and fruit samples. A total of 302.6 million raw reads were obtained and 277.9 million clean reads were obtained by removing spliced and low-quality reads resulting in a total of 41.97 billion high quality bases. The average GC percentage was 46.41%, the average Q30 (sequencing error rate less than 0.1%) was 91.8% (Table 1), and all the libraries showed good sequencing quality. After sequence cleaning, reads from all samples were mixed and assembled from scratch using the Trinity software. A total of 400,092 transcripts (N50: 1136) and 177,324 unigenes (N50: 900) were generated, with the total number of sequences greater than N50 in length being 79,411 and 33,310 respectively (Table S2).

3.2. Functional classification

To obtain comprehensive gene function information, all 177,324 unigenes were compared with available protein databases using the BLAST x algorithm with an E-value of 10-5. The results (Table S3) showed that the maximum number of unigenes annotated in the NR database was 83,004 (46.81% of the total), 49,256 (27.78%) unigenes in the GO database, 39,066 (22.03%) unigenes in the KEGG database, 77,632 (43.78%) unigenes in the eggNOG database, 70,304 (39.65%) unigenes in the Swissprot database, and 24,829 (14%) unigenes were annotated in all databases, with a large proportion of unigenes with no annotation in the currently known protein databases, which may represent a new gene in *Z. armatum*.

The results of the NR-annotated matched species (Fig. S1) showed that 83,004 Unigenes of *Z. armatum* had different degrees of homology with known genes of other species, and the two species with a greater distribution of annotated sequences were *Citrus sinensis* and *Citrus clementina,* accounting for 32.68% and 19.18%, respectively. The series annotated to *Citrus* spp. comprised more than 50%, and the remaining nearly 1/2 of the Unigenes were distributed in the other 892 species. From the distribution of similarity of matched sequences, it can be seen (Fig. S1) that 36.59% of the sequences had similarities between 80% and 95%, 25.54% had similarities between 60% and 80%, 17.83% had similarities >95%, and 20.03% had similarity <60%, and from the distribution of similarity, the matches of *Z. armatum* in the NR library of NCBI were high.

GO gene classification is in the use of statistical methods for gene enrichment, covering the cellular components, molecular functions and biological processes of genes, to understand the distribution characteristics of the gene functions of *Z. armatum* at a macro level and to facilitate the understanding of the biological significance of the genes. Functional matching of GO for unigenes of

Table 1
Statistics of transcriptome sequencing results.

Sample	Total Reads	Clean Reads	Clean Data (bp)	Q30 (%)
PC1	45295008	40362278	6094703978	92.55
PC2	46111348	41078908	6202915108	92.55
PC3	43373116	40403808	6100975008	92.84
Ya	48611108	45401420	6855614420	93.02
Gen	42532196	39567318	5974665018	92.54
ZaF1	38657804	35873152	5416845952	90.38
ZaF2	37986206	35246760	5322260760	89.01

Z. armatum revealed that 49,256 (27.78% of total annotations) unigenes annotated in the GO database could be matched to at least one GO term. The analysis showed that 49,256 unigenes were classified into three major functional categories: biological process, cellular component, molecular function, and 67 functional groups (Fig. S2). In the biological process category, the most enriched subcategories were 'metabolic process' and 'cellular process'. The most significantly enriched subcategories in the cellular component category are 'cell' and 'cell part'. In the molecular function category, the most enriched subcategories were 'binding' and 'catalytic activity'. In addition, 996 single genes were identified to have nucleic acid-binding transcription factor activity (Fig. S2).

A total of 77,632 single genes were placed in the EggNOG database for further homology of the gene products and 77,632 single genes were annotated into 24 NOG functional classes. Among them, "Signal transduction mechanisms" (7639) and "Posttranslational modification, protein turnover, chaperones" (7148) were the most distributed genes followed by "Transcription" (5819) and "Translation, ribosomal structure, and biogenesis" (3890). A small number of single genes were annotated as "Extracellular structures" (170) and "Cell motility" (19). Notably, a large proportion of genes were not specifically allocated in the annotation results of the NOG database, for example, "Function unknown" (17,181) and "General function prediction only" (17,280) (Fig. 2).

3.3. DEGs analysis of the development process of *Z. armatum* prickly bark

To analyze the mechanism of prickle development in *Z. armatum*, comparative transcriptome analysis was performed for three different stages of prickle development. Differential analysis of gene expression was performed using DESeq, with an expression difference fold|log2FoldChange| > 1 and a corrected significance *P*-value <0.05 as the screening condition. A total of 4343 differential genes expression (DEGs) were identified in the analysis of PC1 vs. PC2, of which 1749 were upregulated and 2594 were downregulated; 4491 DEGs were identified in the comparison of PC1 vs. PC3, of which 1670 were upregulated and 2821 were downregulated; 1777 DEGs were identified in the comparison of PC2 vs. PC3, of which 525 were upregulated and 1252were downregulated. A total of 252 downregulated (Fig. 3a) and 10,611 DEGs were identified during skin spur development, with 153 DEGs detected in the upregulated three group comparison and 69 DEGs detected in the downregulated 3 group comparison (Fig. 3b). The number of DEGs in PC2 vs. PC3 was significantly less than that in PC1 vs. PC2 and PC1 vs. PC3, indicating that the molecular reactions involved in the developmental stages of PC2 to PC3 started to diminish.

To determine the main metabolic pathways during the development of *Z. armatum* prickles, DEGs of PC1 vs. PC2, PC1 vs. PC3, and PC2 vs. PC3 were used for enrichment analysis. Phenylpropanoid biosynthesis, plant hormone signal transduction, and plant-pathogen interactions were the three most significantly enriched pathways in PC1 and PC2. Ribosomes, phenylpropanoid biosynthesis, and plant hormone signal transduction were the three most significantly enriched pathways in PC1 and PC3. Ribosome, phenylpropanoid biosynthesis, and glycolysis/gluconeogenesis were the three most significantly enriched pathways in PC2 vs. PC3. Phenylalanine biosynthesis, phytohormone signaling, and starch and sucrose metabolism were significantly enriched in all three groups.

The K-means clustering algorithm was used for cluster analysis to analyze the expression patterns of the DEGs during prickle development. A total of nine clusters were obtained, which were roughly divided into four categories: upregulated and downregulated (cluster 2 and cluster 5), downregulated (cluster 1, cluster 7, and cluster 9), upregulated (cluster 3, cluster 4, and cluster 6) and unchanged (cluster 8). KEGG analysis of genes in cluster 3 and cluster 5 showed more biological significance than the results of the remaining seven clusters. The genes in cluster 3 showed an overall upward trend during different development stages of PC1 to PC3,

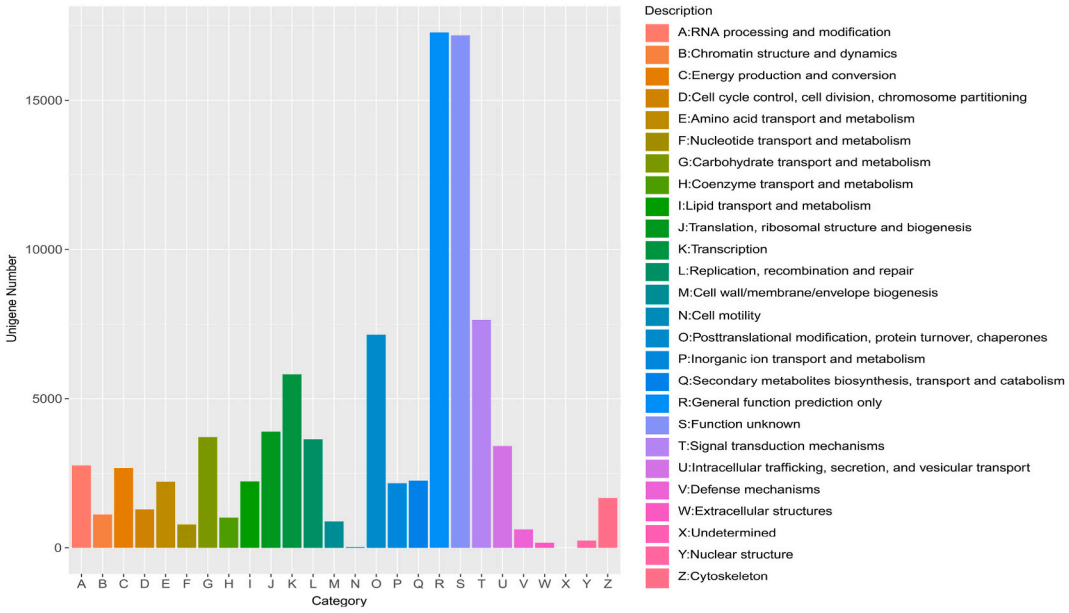

Fig. 2. EggNOG functional classification of Unigenes in *Z. armatum*.

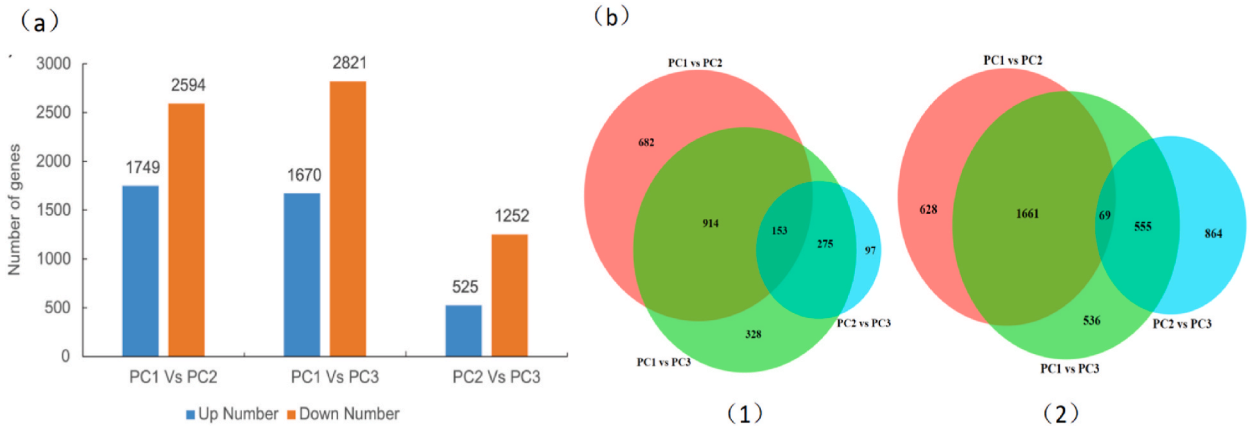

Fig. 3. Differentially expressed genes between different developmental stages of Z. armatum prickles. a: number of genes. b: (1): upregulated genes. (2): downregulated genes.

and the expression level of cluster 3 genes was not expressed or low in the remaining tissues. The concentration of KEGG metabolic pathways related to phenylpropanoid biosynthesis and plant hormone signal transduction was the highest in this cluster. This indicates that related hormones and phenylpropanoid metabolites play important roles in prickle development. Cluster 5 genes increased first and then decreased in different developmental stages of PC1-PC3, rapidly increased in developing cutaneous thorns, and then strongly decreased in mature cutaneous thorns. Gene expression in cluster 5 was significantly enriched in phenylpropanoid biosynthesis, amino sugar, and nucleotide sugar metabolism pathways (Fig. S3).

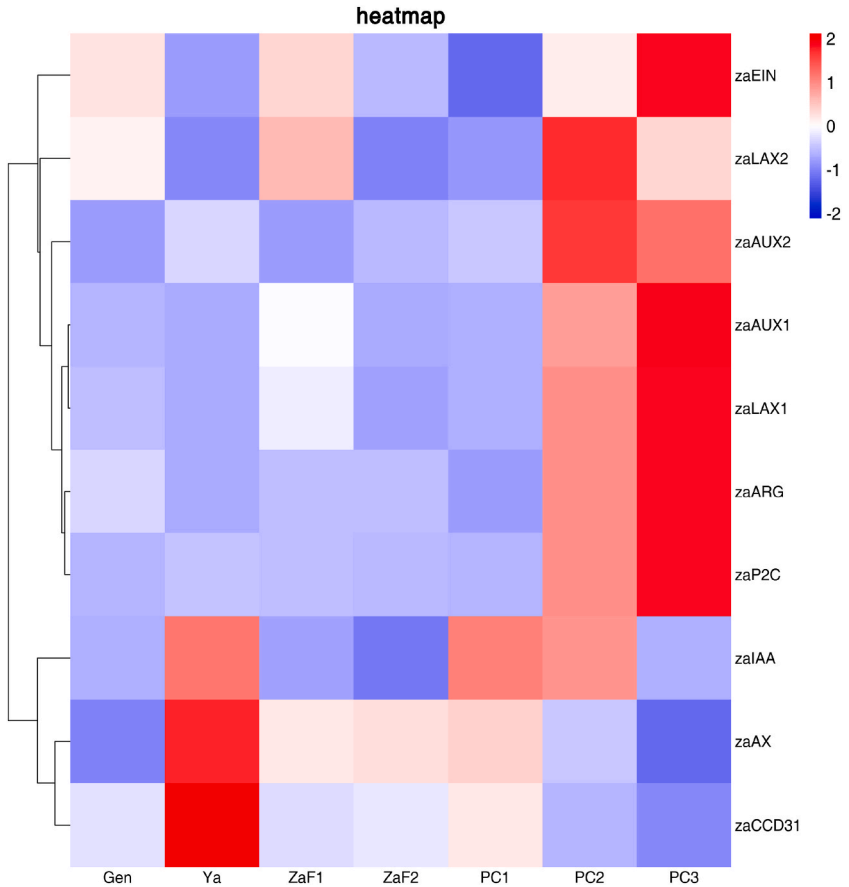

Fig. 4. Heat map of phytohormone-related differential gene expression. (Gen: root tip, Ya: shoot, ZaF1: newly formed fruit, ZaF2: fruit with fresh oil spots, PC1: newly protruding prickles, PC2: prickles starting to develop lignification, and PC3: fully lignified prickles).

3.4. Phytohormone related DEGs

Plant hormones are involved in different plant growth and development processes, and there are growing evidences that certain hormones play a decisive role in plant development. The complexity of plant hormone regulation is reflected in the hormone synthesis, transport, and signaling pathways. To investigate the role of hormones during prickle development, several hormone-related genes were detected at different times. auxin transporter protein 1 (zaAUX1), auxin transporter-like protein 2 (zaLAX1), and auxin transporter-like protein 3 (zaLAX2) are carrier proteins involved in proton-driven auxin influx, mainly in amino acid transport and auxin-activated signaling pathways, and are expressed at different developmental levels in PC1 to PC3. The expression levels gradually increased at different developmental stages, from PC1 to PC3 (Fig. 4; Table S4). The same Probable protein phosphatase (zaP2C), auxin-induced protein 22D (zaAUX2), Indole-3-acetic acid-induced protein ARG7 (zaARG), Ethylene insensitive 3 (zaEIN), and other hormone-related genes were expressed at progressively higher levels during different developmental stages of the skin spur. In contrast, the cytokine-related protein Cyclin-D3-1 (zaCCD31) and the growth hormone signaling pathway-related genes auxin-induced protein 15A (zaAX) and auxin-responsive protein (zaIAA) were highly expressed in PC1 and were downregulated in PC2 and PC3. These results suggest that genes related to plant hormones may be key genes in the development of prickly bark in *Z. armatum*.

3.5. Phenylpropanoid biosynthesis

Phenylpropanoid biosynthesis is one of the most important plant secondary metabolic pathways, producing various metabolites, such as lignin, flavonoid, lignan, and cinnamic acid amide, which play important roles in plant growth, development, and plant environmental interactions [12]. To further analyze the genes involved in prickle development, we explored genes that were progressively upregulated during prickle morphogenesis. Among the progressively upregulated genes, those involved in phenylpropanoid biosynthesis, particularly lignin and flavonoid biosynthesis, were significantly enriched.

The key genes of the flavonoid biosynthetic pathway, chalcone synthase (CHS) and flavonol synthase (FLS) showed higher expression at excessive stages of prickle development, indicating that flavonoids accumulate during prickle maturation. phenylalanine ammonia-lyase (PAL), as the first rate-limiting enzyme in the lignin biosynthesis pathway, PAL gene expression and its abundance directly affect the entire process of lignin biosynthesis, and at different stages of prickle development the three encoding PAL genes all

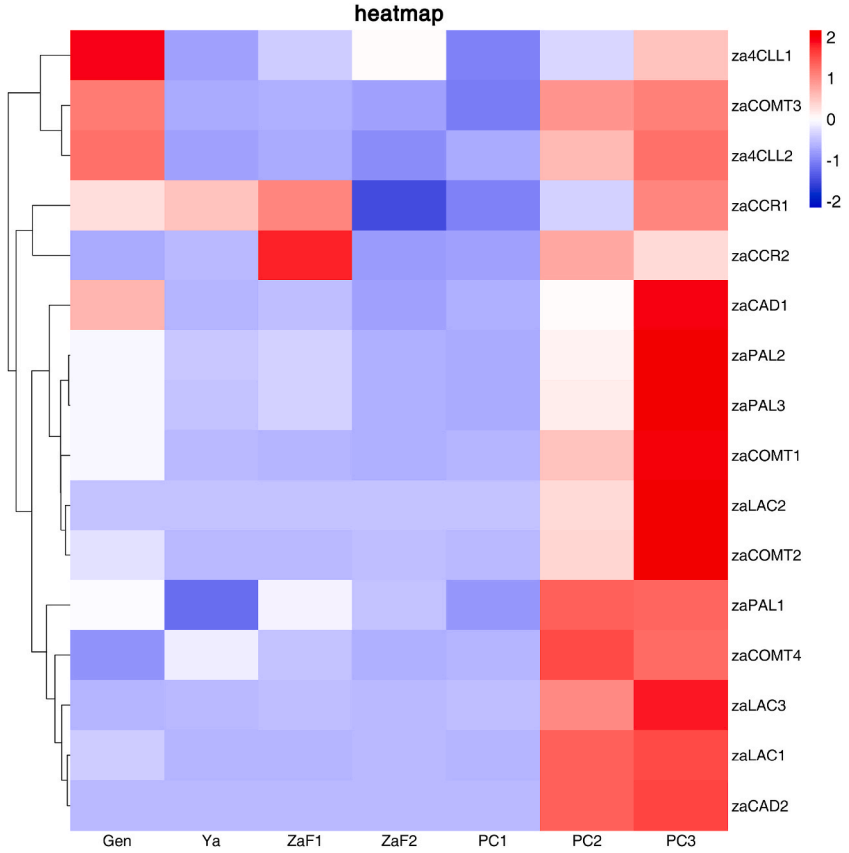

Fig. 5. Heat map of gene expression related to the propane metabolic pathway.. (Gen: root tip. Ya: leaf buds. ZaF1: newly formed fruit. ZaF2: fruit with fresh oil spots. PC1: newly protruding prickles. PC2: prickles starting to develop lignification. PC3: fully lignified prickles.)

showed low expression in PC1 at different stages of bark spur development, Meanwhile some in lignin synthesis cinnamic acid-4-hydroxylase (4CL), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR), caffeate oxygen methyltransferase (COMT), peroxidase (POX), and laccase (LAC) [8] were similarly expressed at low levels in PC1, which may contribute to the slow lignin deposition in the early stages of prickle development. As the prickles gradually harden, the key genes involved in lignin synthesis, cinnamyl alcohol dehydrogenase (zaCAD1, zaCAD2), phenylalanine ammonia-lyase (zaPAL1, zaPAL2, zaPAL3), caffeate oxygen methyltransferase (zaCOMT1, zaCOMT2, zaCOMT3, zaCOMT4), and laccase (zaLAC1, zaLAC2, zaLAC3) were expressed at progressively higher levels in PC2 and PC3 (Fig. 5; Table S5), where key genes (zaCAD1, zaPAL2, zaPAL3, zaCOMT1, zaLAC2, zaCOMT2, zaLAC3) high expression in PC3 may lead to high lignin accumulation during the maturation of the prickles, which is consistent with the lignin accumulation required for prickle hardening.

3.6. Analysis of differentially expressed transcription factors

Transcription factors (TFs) play key roles in plant tissue development [13]. In this study, various TFs were analyzed to identify TFs that may be involved in the development of *Z. armatum* prickles. A total of 29 TFs were found to be differentially expressed during different developmental stages of the prickles, with low expression levels in the early stages of prickle development and gradually upregulated expression levels as the prickles matured, indicating their important roles in the development of prickles. Notably, MYB (zaMYB1, zaMYB2, zaMYB3, zaMYB4, and zaMYB5) and WRKY (zaWRKY1, zaWRKY2, zaWRKY13, zaWRKY4, and zaWRKY5) type transcription factors involved in lignin synthesis and secondary cell wall biogenesis were significantly upregulated in PC2 and PC3 (Fig. 6; Table S6). This indicates that MYB and WRKY transcription factors may be involved in lignification and prickle sclerosis.

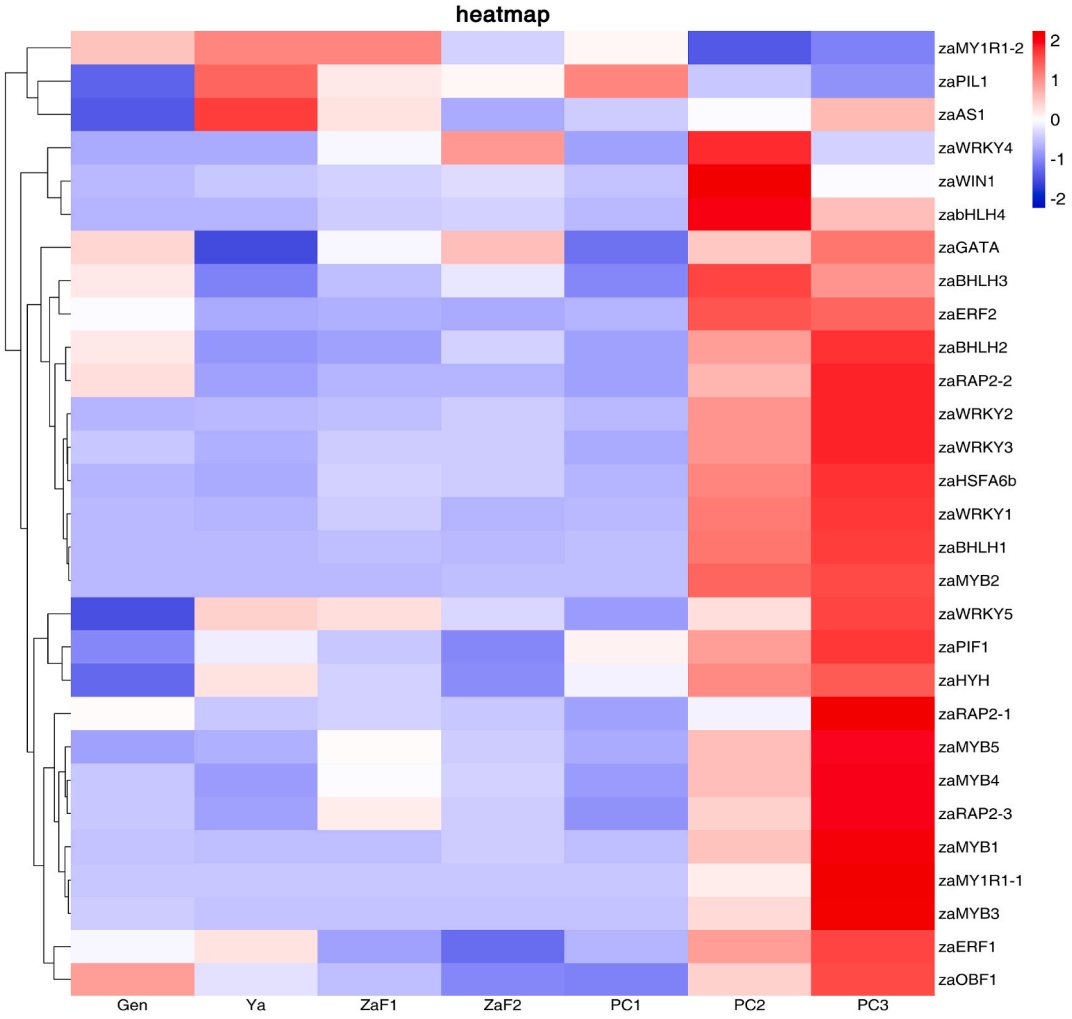

Fig. 6. Heat map of transcription factor expression at different stages and sites of *Z. armatum*.. (Gen: root tip. Ya: leaf buds. ZaF1: newly formed fruit. ZaF2: fruit with fresh oil spots. PC1: newly protruding prickles. PC2: prickles starting to develop lignification. PC3: fully lignified prickles.)

3.7. RNA-seq gene expression data qRT-PCR validation

To confirm the validity of the transcriptome sequencing data and further verify the expression patterns of the candidate genes, qRT-PCR was used to analyze the specific expression of seven genes in different developmental stages and in different parts of *Z. armatum* prickles. qRT-PCR showed (Fig. 7) that the relative expression levels obtained from qRT-PCR analysis of the six genes were consistent with the trend of transcriptome expression profile analysis. zaPAL3, za4CLL1, zaCOMT1, zaWRKY3, and zaMYB2 were higher in PC2 and PC3 than in PC1, and their expression gradually increased with the maturation of prickles, indicating that they may have a positive regulatory role in the process of prickle development. In contrast, the expression level of zaCCD31 gene was gradually downregulated during prickle development, which may play a negative regulatory role in the process of prickle development. The expression of zaMYB2 was much higher in PC2 and PC3 than in PC1 and was not expressed in Gen, Ya, ZaF1, and ZaF2. We suspect that zaMYB2 is a key gene in *Z. armatum* prickle development.

3.8. Gene cloning of ZaMYB2

The ZaMYB2 gene was successfully cloned using ZaMYB2F and ZaMYB2R as specific primers. Analysis of the sequencing results on NCBI ORF Finder software showed that the ZaMYB2 gene has a complete full-length cDNA open reading frame (ORF) of 1032 bp, encoding 343 amino acids (Fig. S4; NCBI accession number: MW417213).

4. Discussion

The prickles are sharp protrusions formed by the epidermis or cortex of the plant body to protect the plant from herbivores, pathogens or mechanical damage, but the dense prickles on the main stem and branches of the *Z. armatum* can affect harvesting and increase cultivation costs [10]. Therefore, breeding thornless or few thorns *Z. armatum* varieties has become an important goal of the breeding program. Continuous reduction of *Zanthoxylum* prickles can be achieved by multiple grafting, but stable inheritance of *Zanthoxylum* traits cannot be ensured [14]. Therefore, research to analyze the developmental molecular mechanism of *Zanthoxylum* prickle hardening and breeding prickle-free or prickle less varieties is a problem waiting to be solved.

Plant hormones play an important role in the development of plant organs [15], and recent studies have shown that many plant hormones are involved in trichome development, such as jasmonic acid (JA), abscisic acid (ABA), growth hormone, cytokinin (CK), and salicylic acid (SA) [16–18]. In the study of *Zanthoxylum* prickles, jasmonic acid, growth hormone, and cytokinin contents were found to be significantly higher in leaves, petioles, and stems with multiple prickles than in leaves, petioles, and stems with few prickles, while zaIAA and zaARF genes were in playing an active role in *Zanthoxylum* prickle development [11]. Using

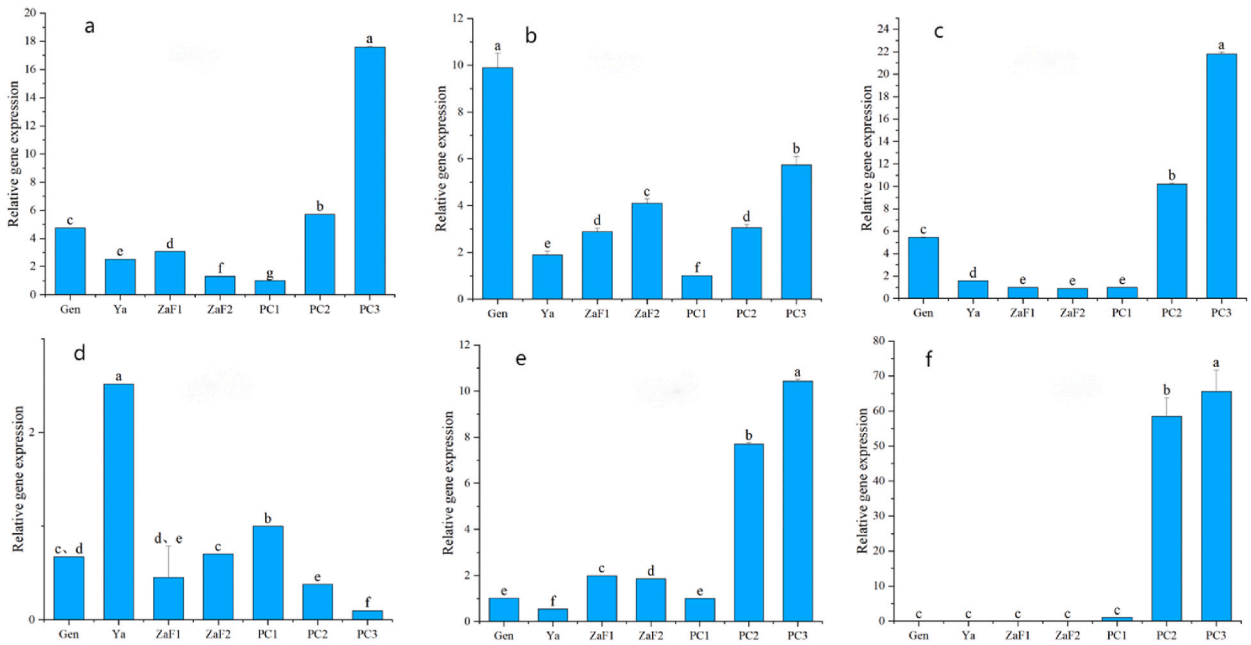

Fig. 7. Relative expression of differentially expressed genes by qRT-PCR. Y-axis: expression of genes at different periods and sites. X-axis: different sites and different developmental stages of prickles in *Z. armatum*.. (a: qRT-PCR results for gene zaPAL3. b: qRT-PCR results for gene za4CLL1. c: qRT-PCR results for gene zaCOMT1. d: qRT-PCR results for gene zaCCD31. e: qRT-PCR results for gene zaWRKY3. f: qRT-PCR results for gene zaMYB2. Gen: root tip. Ya: leaf buds. ZaF1: newly formed fruit. ZaF2: fruit with fresh oil spots. PC1: newly protruding prickles. PC2: prickles starting to develop lignification. PC3: fully lignified prickles.)

phytohormone-related differential gene expression analysis, we found that the carrier protein auxin transporter protein 1 (zaAUX1) and some genes encoding auxin transporter-like proteins (zaLAX1 and zaLAX2), which are involved in proton-driven growth hormone inflow, have gradually increased expression levels during the development of PC1 to PC3, possible positive regulation in the development of Z. *armatum* prickle. In contrast, Cyclin-D3-1 (zaCCD31), a cell cycle protein associated with cell division, was highly expressed in PC1 and downregulation in PC2 and PC3 in *Z. armatum*.

Phenylpropanoid biosynthesis is the main pathway for lignin and flavonoid biosynthesis in plants [12]. PAL, 4CL, CAD, and LAC are key components of the phenylpropanoid metabolic pathway and can regulate the anabolism of lignin, flavonoids, and alkaloids [19]. For example, in a study of lignin biosynthesis in *Vacciniumspp.*, VcMYB4a was found to inhibit lignin synthesis by downregulating the expression of 4CL, COMT, and CAD [20]. The degree of lignification in the stem tissues of dicotyledonous short-stalked grass is positively correlated with the expression level of the PAL gene [21]. The Df4CL1 gene was positively correlated with flavonoid and lignin content in a study of flavonoid and lignin synthesis in tobacco transgenics [22]. In this study, we found that the genes PAL (zaPAL1, zaPAL2, and zaPAL3), 4CL (za4CLL1 and za4CLL2), CAD (zaCAD1 and zaCAD2), COMT (zaCOMT1, zaCOMT3, zaCOMT4, and zaCOMT2), and LAC (zaLAC1 and zaLAC2) were significantly and progressively upregulated in *Z. armatum* prickle development at different stages. The expression patterns of these genes were consistent with the results of RT-qPCR validation, and all were significantly upregulated in PC3 of *Zanthoxylum* prickle development, which was similar to previous studies related to phenylpropanoid biosynthesis in plants. These results were similar, suggesting that they may enhance the regulation of lignin biosynthesis in prickles and are important for lignin accumulation in mature prickles in *Z. armatum*.

Transcription factors such as MADS-box, MYB, AP2/ERF, WRKY, and NAC, which may have important roles in prickle development, were derived from transcriptome analysis of eggplant prickly spur [23,24]. In previous studies on the transcriptome analysis of the morphogenesis of rose prickles, it was found that the development of prickles is regulated by the MBW typical complex, in which the R2R3MYBs protein seems to determine the differentiation of epidermal cells. At the same time, MYB transcription factors related to lignin synthesis and secondary cell wall genesis are gradually up-regulated in expression level during the development of prickles, indicating an important role of MYB transcription factors in the development of prickles [7].In previous studies on *Zanthoxylum* prickle, a negatively regulated R2R3 MYB transcription factor, ZaMYB86, was found to interact with ZaMYB5 protein and affect *Zanthoxylum* prickle formation [11].In the transcription factors (TF) analysis, we found that MYB and WRKY accounted for most of the developmental hardening of the prickles at different times, and their expression levels were significantly increased in prickles PC3 and PC2. We suggest that they may have positive regulatory effects on the developmental hardening of *Z. armatum* prickles. Meanwhile, we found that the ZaMYB2 gene was highly specifically expressed in PC2 and PC3 and showed increased expression from PC1 to PC3. Further validation by PCR showed that the ZaMYB2 gene was specifically expressed in the skin spines, transcriptome analysis was consistent, and ZaMYB2 was not expressed in other tissues. This is consistent with the results of a rose prickle study showing that MYB transcription factors play an important role in the prickle development. Unlike previous studies on *Zanthoxylum* prickles, they derived the gene ZaMYB86 to interact with the ZaMYB5 protein and thus affect the development of prickles based on the comparison of expression between multiple and a few prickles in different parts of *Z. armatum*. We then explored the expression levels of *Z. armatum* prickles at different stages of development and compared them in the roots, leaves, and fruits; the key gene ZaMYB2, which is expressed only in the prickles and gradually upregulated during the lignification of the prickles, was identified. Our finding that ZaMYB2 belongs to the same family as VcMYB4a further illustrates the key role of ZaMYB2 in the lignification of *Z. armatum* prickles. In the future, ZaMYB2 could be used as a candidate gene to obtain thornless *Z. armatum* varieties using RNA interference.

5. Conclusions

In this study, we analyzed the transcriptome at three different stages of *Z. armatum* prickle growth, and sequenced root tips, shoots, and fruits, and the results showed that plant hormone signal transduction and phenylpropanoid biosynthesis are the key pathways in prickle development, and these two pathways play important roles in the developmental hardening of *Z. armatum*. These two pathways play important roles in the development of sclerosis in *Z. armatum* prickle. Second, we identified the ZaMYB2 gene that is specifically expressed only during the lignification of the prickles.

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Data availability statement

Data associated with this study has been deposited at GenBank and Sequence Read Archive (Accession number: SRR26938733, SRR26938732, SRR26938731, SRR26938730, SRR26938729, SRR26938728, SRR26938727), National Center for Biotechnology Information database. https://submit.ncbi.nlm.nih.gov/subs/sra/SUB13998275/overview.

CRediT authorship contribution statement

Yi Wang: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Data curation. **Yuhui Jiang:** Writing – original draft, Data curation. **Fayu Feng:** Investigation. **Yongqing Guo:** Project administration. **Jiabo Hao:** Resources. **Li**

Huyan: Resources. **Chunhua Du:** Investigation. **Liang Xu:** Formal analysis. **Bin Lu:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27222.

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