



Research Article

Integrative analysis of microstructure, morphological structure, characteristics, and tissue-specific transcriptome provide insights into mechanisms of pectin polysaccharide biosynthesis in *Premna puberula* Pamp.

Dao-Ping Wang^{1,†}, Xue-Li Liu^{2,†}, Zi-Nian Wang³, Hong-Ying Li³, Xiang Tan³, Yu Xia¹, Ning-Xian Yang^{1,3}

¹ State Key Laboratory of Discovery and Utilization of Functional Components in Traditional Chinese Medicine, Natural Products Research Center of Guizhou Province, Guizhou Medical University, Guiyang 550014, China

² Key laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), College of Life Sciences; Institute of Agro-bioengineering, Guizhou University, Guiyang 550025, China

³ The Key Laboratory of Environmental Pollution Monitoring and Disease Control, Ministry of Education, Guizhou Provincial Engineering Research Center of Ecological Food Innovation, School of Public Health, Guizhou Medical University, Guiyang 561113, China

[†] Dao-Ping Wang and Xue-Li Liu contributed equally to this work.

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Abstract: *Premna puberula* Pamp. is a kind of medicinal and edible plant that is widely distributed in Southwest of China. The content of pectin in different tissues is different, but the reason for the difference is unknown. In this study, we detected and found that pectin content varies greatly in roots, stems and leaves of *P. puberula* and we carried out microstructure of leaves, stems and roots, detected histochemical localization, galacturonic acid (GalA) content, degree of esterification (DE), scanning electron microscopy (SEM), X-ray diffraction (XRD) of pectin and binding transcriptomics to investigate the pectin polysaccharide biosynthesis and related genes from different tissues at the molecular level. A total of 26.04 Gb clean data of the high-quality reads were successfully mapped to the reference genome of *P. puberula* and assembled into 107,250 nonredundant genes. We mapped all of the genes to reference pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify pectin biosynthesis pathways in which the genes might be involved, which provide understanding of the regulation of *P. puberula* pectin at the molecular level. The pectin-related key enzyme genes were identified, and these studies have important value for understanding the biosynthesis mechanism of pectin in different tissues.

Keywords: *Premna puberula* Pamp.; pectin; structure; characteristics; transcriptome

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1 Introduction

Pectin is made up of polygalacturonic methoxylate and is used in a variety of applications, such as food (thickening agent, gelling agent and stabilizer) industries and biofuels. Further, this polymer has beneficial uses in human health where it helps to lower serum cholesterol levels, influence secondary wall formation in woody tissues and fibers^[1,2], restore intestinal function, stabilize blood pressure and remove heavy metal ions from the body^[3]. In addition, pectin structure and synthesis had been reviewed over the past decades^[4], studies have shown that at least 67 transferases are involved in the synthesis of pectin^[5], such as galacturonosyltransferase (GAUT), the researchers also reported transcription factors (TFs) such as: WRKYGOK (WRKY)^[6], basic

helix loop helix (bHLH)^[7], apetala-2-like transcription factor gene (AP2)^[8] and myeloblastosis (MYB)^[9]. To date, however, molecular studies of pectin in *Premna* have not been reported, and the biosynthetic metabolic pathways and key enzyme genes of pectin polysaccharides are fundamentally unknown.

Premna puberula Pamp. belongs to the species of *Premna* (Verbenaceae), is a kind of perennial medicinal and edible plant that is widely distributed in southwest of China and grows on the slopes and jungles of 700–1,800 m above sea level^[10]. The roots, stems and leaves of *P. puberula* could be used as medicine for the functions of clearing away heat and detoxification, wound bleeding, detumescence, nameless swelling, hemostasis, snake bite and so on^[11]. Of these bioactive compounds, polysaccharides are the primary substances, which principally consist of pectin and

coarse fibre^[12], and it is abundant in pectin^[13]. Histochemical localization technology has been widely used in the study of chemical components such as polysaccharides, flavones, saponins, and alkaloids. In recent years, histochemical localization of *Dendrobium officinale* polysaccharide^[14] and *Artemisia argyi* polysaccharide^[15] had been reported. However, the histochemical localization of pectin polysaccharides in roots, stems and leaves of *P. puberula* is yet unknown. Thus, understanding the location of pectin in different tissues might provide a better understanding of the role and sites of synthesis and accumulation of these compounds.

With the rapid development of molecular biology, there are more and more researches on the synthesis and regulation mechanism of Chinese traditional medicine^[16,17]. Chen *et al.*^[18] and Cheuk *et al.*^[19] performed the transcriptome sequencing of flowers, roots, stems and leaves of the 6-month-old *D. officinale*, showing that transcriptomes from four organs in order to explore biological mechanisms in the organ-specific distribution of polysaccharides in *D. officinale*. Up to the present, the whole genome sequencing of *P. puberula* has not been completed, so it is difficult to differentiate and analysis of key genes involved in the pectin. For the advantages of cost-effective, efficient, and no need of a reference genome, RNA-Seq together with digital gene expression (DGE) tag profiling have been extensively applied in characterizing non-model organisms. The transcriptome profiling of multitudinous plants under any given conditions have been used to identify differentially expressed genes (DEGs). Nevertheless, deciphering the transcriptome profiles of *P. puberula* reflecting different tissues is a significant step to comprehend the function and mechanism on pectin metabolic pathways at transcriptional level.

In this study, we examined the pectin contents of leaves, stems and roots of *P. puberula* and found that pectin content varies greatly in different tissues. To understand the reasons for these differences, we carried out histochemical localization of pectin in the mature stages of *P. puberula*, then detected morphological and structural characteristics. Finally, we applied transcriptomics to investigate the pectin mechanisms and their biosynthesis-related genes from different tissues. Our results demonstrated that the pectin content in *P. puberula* and the expression of related genes on the pectin metabolic pathway were tissue-specific, meanwhile, the relationship between them was ultimately analyzed. Ultimately, these results not only provide sufficient information resources for the further study of pectin, but also provide new perspective to the molecular mechanism of key genes regulating the synthesis and metabolism of *P. puberula*.

2 Materials and methods

2.1 Plant materials

P. puberula plants were artificially cultivated in the botanical garden of Guizhou University, Guiyang, China in July, 2020, with the light intensity 180–230 mol/(m²·s) and the relative humidity was 70%–85%, and the plants were collected and their identify was authenticated by Professor Xin-Yuan Xiong. Finally, the roots, stems and leaves were collected from 2-year old *P. puberula* plants and mixed evenly to create a single sample with three biological replicates for anatomic analysis and RNA extraction. Then leaves were taken from the top of trees for detection pectin structure.

2.2 Anatomic analysis

2.2.1 Paraffin section making

Dehydrate and wax the fresh tissue after fixing it. The melted wax is placed in the embedding frame and labeled accordingly. It is then cooled on a freezer, and after the wax has cured, the wax block is removed from the embedded frame and repaired.

2.2.2 Microscopic analysis of leaves, stems and roots

In July 2020, root, stem and leaves of *P. puberula* were taken and fixed in FAA (V(formalin):V(ethanol):V(acetic acid):V(water) = 100:500:5:350) after washing. Anatomical study of leaves, stems and roots of *P. puberula* was carried out referring to the methodological instructions of Murzabulatova *et al.*^[20], the fixed material was sliced and positioned through a series of steps, including dehydration, transparency, waxing, embedding, slicing and sticking, followed by dewaxing, dyeing and sealing, the thickness of section was (10 ± 2) μm. Microphotographs of sections were observed on the Nikon Eclipse E1 (Nikon, Japan) microscope and image acquisition was carried out with its supporting information acquisition system Nikon DS-U3 (Nikon, Japan).

2.2.3 Histochemical localization of polysaccharides

Histochemical study was carried out according to method of Márcia *et al.*^[21]. Ruthenium red and solid green were used to stain the paraffin sections of the pectin.

2.3 Preparation of pectin samples

2.3.1 Pectin extraction and purification

The root, stem and leaves of *P. puberula* were dried in the laboratory oven at 40 °C circulating air for 24 h, and powered samples (10.0 g) were extracted with hydrochloric acid solution at 90 °C for 2 h with pH 2, the liquid-solid (LS) ratio of 50:1 mL/g according to Liu *et al.*^[22]. Briefly, the acid extract was concentrated with 95% ethanol at room temperature for overnight precipitation. Then the sediment was collected and dried to constant weight at 40 °C. There were three biological replicates for each tissues in the experiment.

2.3.2 Acid hydrolysis and preparation of derivatives

The hydrolysate of pectin and its derivatives were prepared referring to the method of Yang *et al.*^[23]. In brief, a 20-mg pectin samples were accurately weighed and placed in an explosion-proof bottle. The sample was sealed with 8 mL 4 mol/L trifluoroacetic acid (TFA) and hydrolyzed at 120 °C for 2 h, after which the excess acid hydrolysate was removed with 2 mL methanol. The whole process was repeated 3 times, and the final dried sample was used for detection of monosaccharide composition.

2.4 Physicochemical property of pectin

2.4.1 The galacturonic acid (GalA) content

The GalA content in pectin samples from *P. puberula* were examined by carbazole method with some modifications, using a 96-well microplate reader (SpectraMax Versamax, Germany) at 530 nm. Then the GalA solutions were applied to create a standard calibration curve.

2.4.2 Monosaccharide analysis

Monosaccharide composition was detected by gas

chromatography-mass spectrometer (GC-MS). A total of 10 mg pectin sample was hydrolyzed with 8 mL of 8 mol/L TFA for 3 h at 120 °C, and the excess acid was co-distilled with CH₃OH and the hydrolysate was dried at 60 °C. The hydrolysate was mixed with hydroxylamine hydrochloride (20 mg), inositol (10 mg) and pyridine (1.2 mL) at 90 °C for 30 min, then cooled to room temperature. Then 2 mL acetic acid drying solution was added and incubated under the same conditions. Monosaccharides were analyzed by 5975C-7890A system (Agilent, USA) with a DB-5 capillary column (30 m × 0.25 mm, 0.25 µm).

2.5 Structure characterization of pectin

2.5.1 Morphological of pectin

Trace amounts of dried pectin powder of leaves, stems and roots from *P. puberula* were attached to the sample table by double-sided conductive adhesive. And the micromorphology of pectin was observed and photographed by scanning electron microscope (SEM) after spraying the gold (COXEM, EM-30, PLUS⁺, Korea). The magnification was 500 times and 5,000 times, respectively^[24].

2.5.2 X-ray diffraction (XRD)

The relative crystallization index of pectin was detected by using an XRD diffractometer (Empyrean, Netherlands). The samples were tested under the following conditions: diffraction angle (2θ) was between 5° and 80° with Cu Kα radiation generated at 45 kV and 40 mA, and sampling interval of 0.02° at 25 °C.

2.5.3 Fourier transformed infrared analysis (FTIR)

The pectin powder samples were placed directly on the attenuated total reflection (ATR) crystal and tested by pressure tower. Fourier transform infrared spectrometry (Nicolet iS50, Thermo Fisher Scientific, USA) was applied to analyze the structural characteristics of pectin according to the previous study by Wang *et al.*^[25]. The spectral resolution was 4 cm⁻¹, and the spectral scanning range was 4,000⁻¹–500 cm⁻¹ with 100 scans.

2.5.4 The degree of esterification (DE)

DE of pectin was calculated from FTIR according to the method depicted by Yang *et al.*^[23], and the DE was computed by the following equation:

$$DE(\%) = \frac{A_{1740}}{A_{1740} + A_{1630}}$$

where A_{1740} and A_{1630} represents the peak area at 1,740 and 1,630 cm⁻¹, respectively.

2.6 Transcription analysis of roots, stems and leaves

2.6.1 RNA extraction and creation of cDNA library

A total amount of 1 µg RNA per sample was used as input material for the RNA sample preparations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends *via* xonuclease/polymerase activities. In order to

select cDNA fragments of preferentially 240 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.6.2 Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2000 platform and paired-end reads were generated.

2.6.3 Quality control and transcriptome assembly

Raw data of fastq format were firstly processed through in-house perlscripts. Then clean data were obtained by removing reads containing adapter, reads including ploy-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. The left files (read 1 files) from all libraries/samples were pooled into one big left.fq file, and right files (read 2 files) into one big right.fq file. Transcriptome assembly was accomplished based on the left.fq and right.fq using Trinity^[26] with min_kmer_cov set to 2 by default and all other parameters set default.

2.6.4 Identification of DEGs and functional annotation

Gene function was annotated based on the following databases: NR (NCBI nonredundant protein sequences), Pfam (Protein family), KOG/COG/eggNOG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes), GO (Gene Ontology).

Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by EBSeq program package through empirical Bayesian approach. Differential expression analysis of two samples was performed using the EBSeq R package. *P*-value was adjusted using *q* value^[27]. *Q*-value 1 was set as the threshold for significantly differential expression.

2.6.5 GO and KEGG enrichment analysis

GO enrichment analysis of the DEGs was implemented by the top GO R packages based Kolmogorov-Smirnov test. KEGG^[28] is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>). We used the methods Sun *et al.*^[29] reported to test the statistical enrichment of differential expression genes in KEGG pathways.

2.7 Statistical analysis

Statistical analysis of plant pectin content was conducted using SPSS 19.0 (IBM, Chicago, IL, USA). Statistical results were gained by one-way analysis of variance (ANOVA) followed by Tuckey's test to speculate significant treatment effects at significance of *P* < 0.05. And the data supplied are means ± standard errors.

3 Results

3.1 Microstructure and histochemical localization of pectin in leaves, stems and roots

Polysaccharides were distributed in vascular bundle and cortical parenchyma cells at different ages, and the cell wall staining was deep in these parts, and a small amount was distributed in other parts.

Ruthenium red is a cationic stain, which can bind to the acidic glycoylation structure and precipitate at the structure in the presence of osmic acid^[30]. It can not only fix the polysaccharide in situ to prevent its loss during sample preparation, but also outline the existence structure of polysaccharide in the particle through the precipitation particles with increased electron density. As shown in Fig. 1, the content of pectin in leaves was the highest and was most widely distributed, followed by stem and root was least. In addition, the color of the outer wall and intercellular space of the cells with thicker veins was red in the leaves' cells, indicating that pectin was distributed intensively. While the color of the cell wall of phloem cells and epidermal cells was purple, declaring that pectin and cellulose were contained in the site. Pectin polysaccharides are most abundant in primary cell walls and intermediate lamellae of plants, which was accordance with previous report. As reported, using histochemical method to locate pectin can reflect the distribution and content of pectin polysaccharides^[31], and the result of pectin histochemical localization was consistent with the pectin content determined.

3.2 The pectin contents analysis in different tissues

Pectin was determined in three different tissues including leaves

(Pp_L), stems (Pp_S) and roots (Pp_R). It can be seen from Fig. 2, the content of pectin was 19.21% for leaves, follows was 3.52% for stems, and the lowest content of pectin was 0.92% for roots, which indicates that the pectin content in different tissues of *P. puberula* varied significantly ($P < 0.01$), and the pectin content in leaves was significantly higher than that in stems and roots. This is basically consistent with the research conclusion of Li *et al.*^[32], the high pectin content in the leaves of *P. puberula* may be related to the light area and light intensity.

The high pectin content in the leaves of *P. puberula* may be related to the light area and light intensity. As the main site of photosynthesis, leaves provide a large number of substrates for the biosynthesis of pectin compounds, so the synthesis and accumulation of pectin have great advantages over other tissues, while the metabolic activity of stems, as the supporting and guiding tissues of plants, and leaves are significantly reduced. However, as the main absorption organ of the plant, the root system only absorbed a small part of small molecules from the soil, and its pectin content was also maintained at a very low level.

3.3 The physicochemical property of pectin

3.3.1 The GalA and DE

Total GalA 70.42%, 75.92%, 72.25%, in line with GB 25533–2010 Food Safety National Standard of Food Additive Pectin standard. The DE of pectin was determined by FTIR, and the results were 57.14%, 53.33%, and 55.56%, respectively, which shown that it belonged to high methoxy (HM) pectin. Our consequence was in accordance with Cheng *et al.*^[33], which is related to the source and extraction method of pectin.

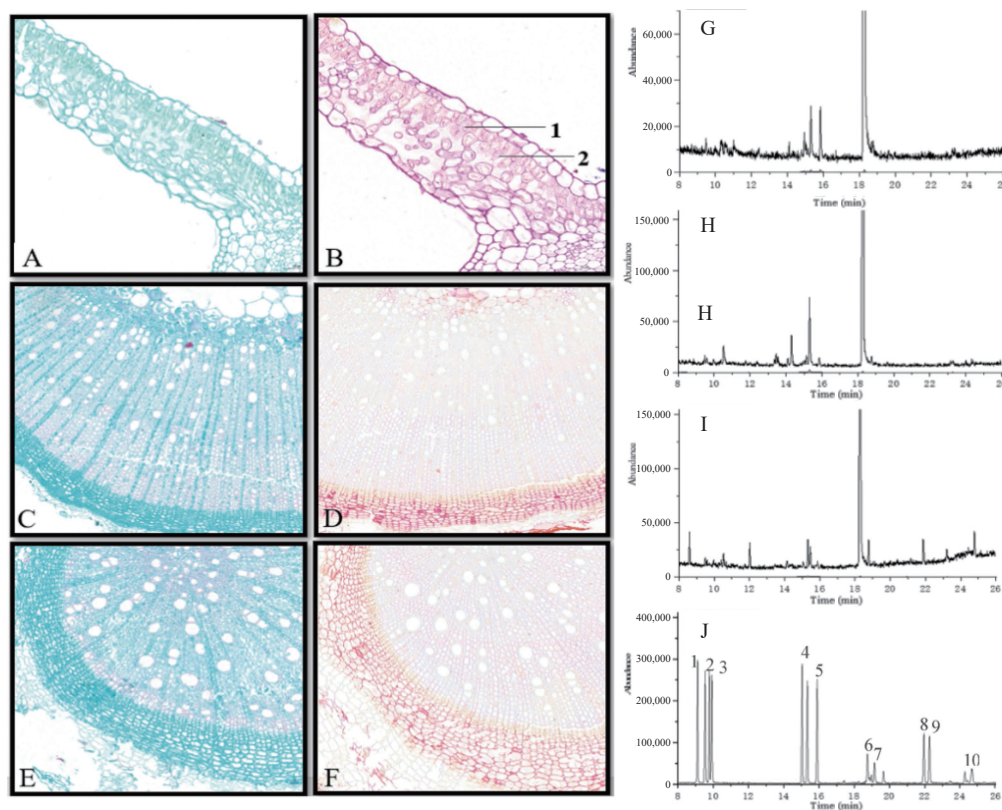


Figure 1 Microstructure (400×), histochemical localization and monosaccharides content of *P. puberula* pectin. (A, C, E) Sanguine solid green staining for leaves, stems and roots. (B, D, F) Ruthenium red showed characteristic red color (arrow) for cellulose and pectates at cellular walls of leaves, stem and root. (G–J) Monosaccharides content of *P. puberula* pectin in roots, stems, leaves and standards.

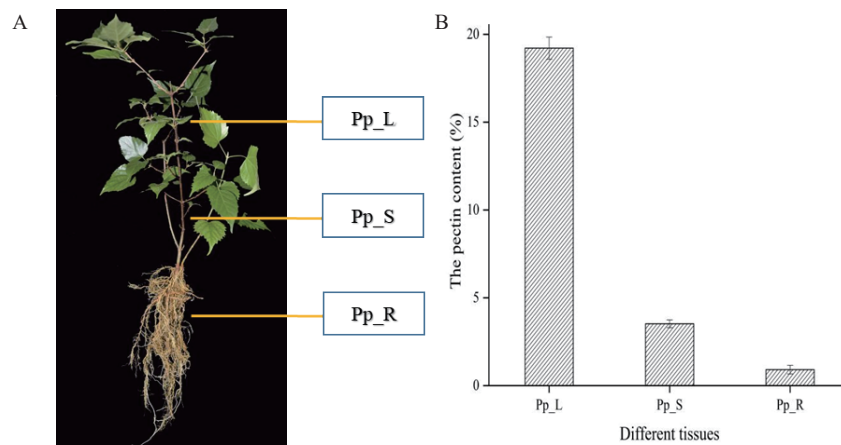


Figure 2 Pectin content in roots, stems and leaves of *P. puberula*. (A) Samples of *P. puberula*. (B) The pectin content in leaves, stems and roots.

3.3.2 Monosaccharide composition

The monosaccharide composition of polysaccharides extracted from different tissues of *P. puberula* was analyzed, and the results were shown in Table 1. The GalA content of *P. puberula* leaves was 74.16%, the polysaccharide component is mainly composed of common pectin neutral sugars, such as galactose, rhamnose and arabinose. The top 5 monosaccharides contents in pectin of leaves were Glu (7.93%), Ara (3.89%), Gal (3.76%), Rha (3.59%) and Man (3.18%). Moreover, other pectin-constituting monosaccharides such as Fru (1.75%) and Xyl (1.1%) were present in small amounts. In addition, the presence of rhamnose and GalA, coupled with higher galactose content and more arabinose, means that pectin is composed of the RG-I skeleton, replaced by arabinose as a side chain^[34]. In addition, due to the low ratio of rhamnose- GalA (0.05), the content of RG-I fragment in polysaccharide was relatively small.

Table 1 Number of genes related to pectin biosynthesis.

EC	Definition	Number of genes
1.1.2.22	UGD	5
5.1.3.6	GAE	7
2.4.1.43	GAUT	13
4.1.1.35	AXS	11
5.1.3.2	GALE	7

3.4 Characterization of pectin

3.4.1 SEM analysis of *P. puberula* pectin from leaves, stems and roots

The pectin morphology structure was observed with scanning electron microscopy (COXEM, EM-30, PLUS⁺, Korea) at 500× and 5,000×, respectively showed in Figs. 3A-F. The morphology structure of leaves, stems and roots were basically the same. When the magnification is 500 times, the pectin shows thick-flaky and block-shaped, while magnifying 5,000 times, the surface of pectin was flocculent and flaky. Further, the surface of leaves pectin was smoother than that of stems and roots pectin, the differences in surface morphology may be due to the varying degree of branching in the pectin^[35].

3.4.2 XRD analysis of *P. puberula* pectin from leaves, stems and roots

The XRD pattern of pectin (Figs. 3G₁-G₃) has several sharp

and intense peaks at 15.0°, 20.8°, 29.6°, 34.4°, and 38.2°, respectively, which represent the systematic mostly lacturonic structure of a large number of carboxyl groups in pectin. The results were basically consistent with the experimental results of Kazemi *et al.*^[36], demonstrating that it has a certain crystallinity. Further, the crystallinity of pectin varies significantly due to the influence of sources and chemical bonds^[37], showing that pectin from roots, stems and leaves were similar with the crystal structure.

3.4.3 Functional group analysis

FTIR spectra displayed the primary functional groups of polysaccharides. As demonstrated in Fig. 4, typical bands appeared in the spectrum, confirming that the presence of pectin. The absorption band at 3,300 cm⁻¹ was owing to the O-H stretching of hydroxyl groups, and the band of 2,860 cm⁻¹ was attributed to C-H stretching including -CH, -CH₂ and -CH₃ bending vibrations of pectin^[38] and demonstrated two characteristic peaks at 1,740 and 1,630 cm⁻¹ in connection with ester carbonyl stretching bands and free carboxyl stretching vibrations, respectively^[39]. Further, two intense overlapping signal peaks around 1,120 and 1,030 cm⁻¹ were shown that a higher amount of C-O bending vibration^[40].

3.5 Transcriptome analysis of rhizomes and leaves

3.5.1 Illumina sequencing and reads mapping

We utilized the transcriptomes from three tissues to explore biological mechanisms in tissue-specific distribution of pectin in *P. puberula*. One-way ANOVA showed that there were significant differences in pectin content between the three different tissues of *P. puberula*.

To obtain a comprehensive understanding of different tissues at the transcriptome level in *P. puberula*, three cDNA libraries were prepared and subjected to RNA-Seq analysis on the Illumina HiSeq platform. The libraries were named as follows: root cDNA libraries: Pp_R, stem cDNA libraries: Pp_S1, leaves cDNA libraries: Do_L. After removing low quality sequences and trimming adapter sequences, the proportion of the clean reads were 26.04 Gb, and the Q30 base percentage was 93.61% or above. A total of 107,250 unigene were obtained after assembly. There were 18,839 unigene with a length of more than 1 kb.

3.5.2 Identification of DEGs

To survey gene expression differences among the tissues of

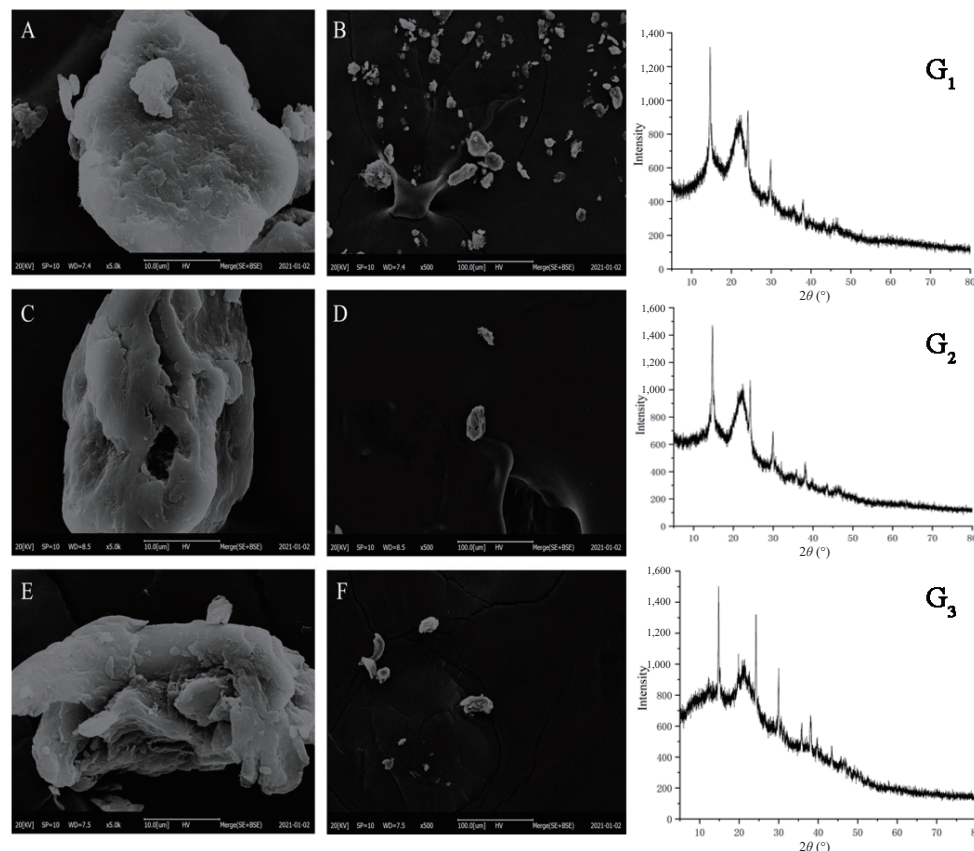


Figure 3 SEM images and XRD images of *P. puberula* pectin. (A, C, E) SEM of pectin from leaves, stems, and roots (5,000×); (B, D, F) SEM of pectin from leaves, stems, and roots (500×); (G₁-G₃) XRD of pectin from leaves, stems, and roots.

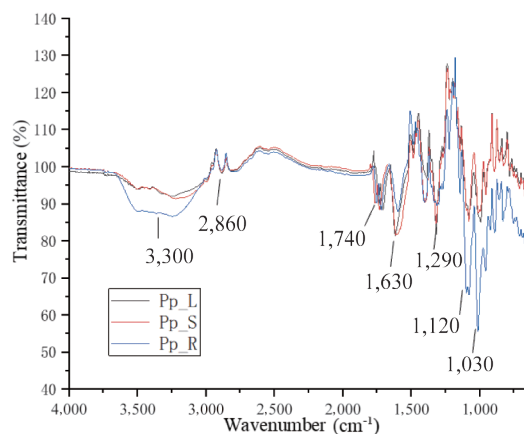


Figure 4 FTIR spectrum of *P. puberula* pectin.

P. puberula, FPKM were computed for each sample to normalize the expression. The Venn diagram shows the overlapped DEGs in different groups (Fig. 5A). Further, in all three comparison groups (Pp_L vs. Pp_S, Pp_R vs. Pp_L and Pp_R vs. Pp_S), 401 DEGs were confirmed in common. In total, the number of DEGs between three different tissues were as follows: 4,733 between Pp_L vs. Pp_S (48.6% up-regulated and 51.4% down-regulated), 5,336 between Pp_R vs. Pp_L (45.7% up-regulated and 54.3% down-regulated), 5,163 between Pp_R vs. Pp_S (47.3% up-regulated and 52.7% down-regulated) (Fig. 5B).

3.5.3 GO enrichment analysis of DEGs

To better comprehend the functions of DEGs, we carried

out GO category enrichment analysis using Fisher's test with P -value < 0.01 as a threshold. GO significant enrichment is displayed in Fig. 5C. In brief, GO terms are primarily concentrated in biological processes. Despite GO terms have more significant terms in different comparison groups, there are only three GO terms in common, which are biological process, cellular component and molecular function.

3.5.4 TFs involved in pectin

TFs are protein molecules with special structure that could regulate gene expression function, which was known as trans-acting factor. We searched the complete TFs against the iTAK database (<http://itak.feilab.net/cgi-bin/itak/index.cgi>). All transcriptome sequences mapped TFs (2,817) were classified into 65 TF families. And the top 5 TF families were C2H2 (122), AP2/ERF-ERF (91), MYB-related (88), RLK-pellk_DLSV (84) and bHLH (83) (Fig. 6A).

3.5.5 Detection of candidate genes involved in biosynthesis of pectin

Pectin is produced by the Golgi apparatus and secreted between the cell membrane and the primary cell wall. To understand the mechanisms regulating the biosynthesis of pectin in *P. puberula*, we sketched proposed approach for pectin biosynthesis in *P. puberula*, the number of genes related to pectin biosynthesis is displayed in Table 1.

3.5.6 Analysis of expression of genes on pectin synthesis pathway

In the pectin pathway, UDP-glucose 6-dehydrogenase (UGD)

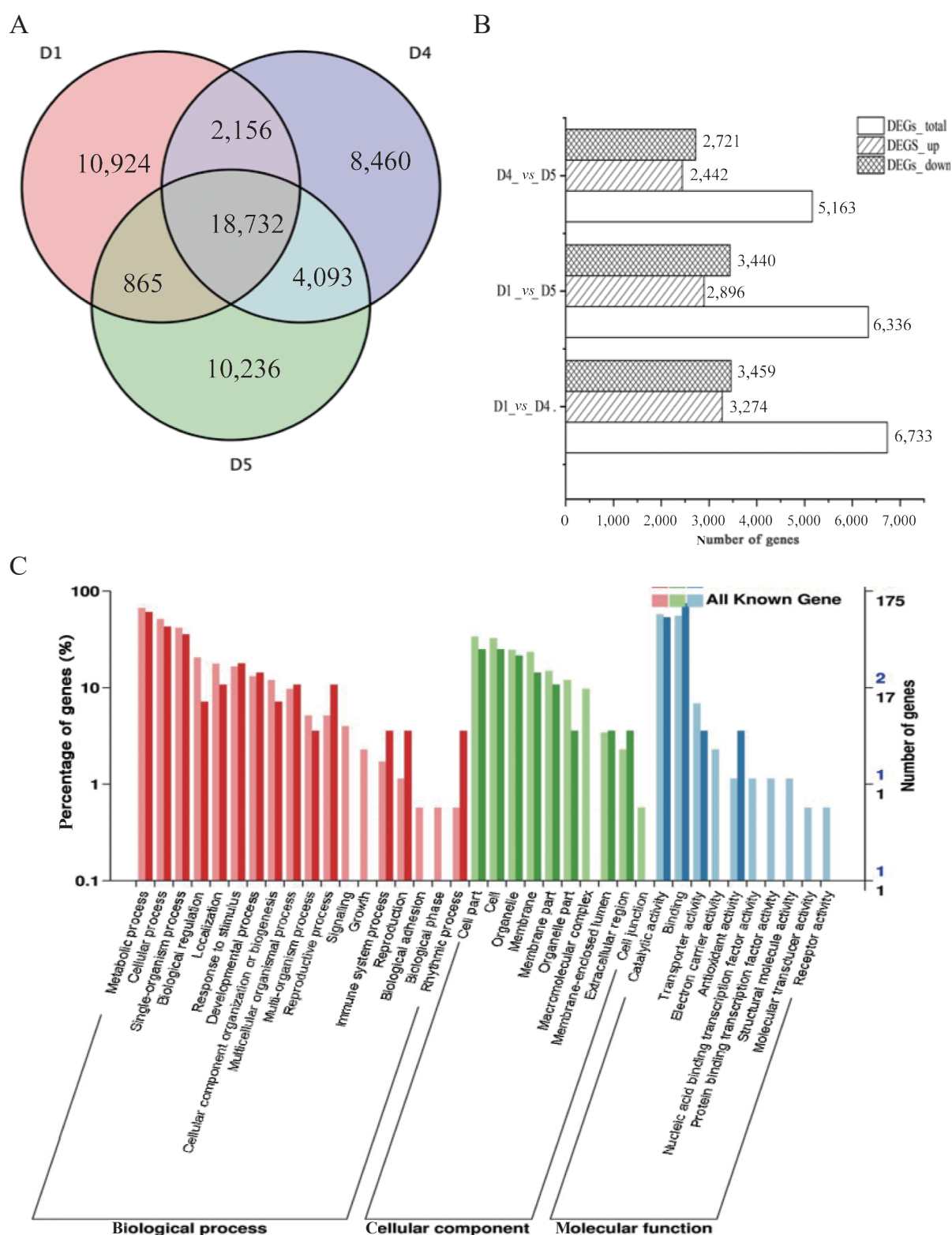


Figure 5 (A, B) Venn diagram of DEGs in different tissues. (C) GO enrichment analysis of *P. puberula* pectin in different tissues.

catalyzes the first step in the biosynthesis of pectin. We identified 5 genes annotated as UGD, which were nearly expressed in all of the tissues, and one gene was specifically expressed in stems and roots, respectively, further, 2 genes are highly expressed in stems, 3 genes in roots according to the KEGG pathway. Besides, we found 5 UDP-glucuronate 4-epimerase (GAE) genes annotated, which is the key enzyme to catalyze the conversion of

UDP- α -D-glucuronic acid (UDP-GlcA) and UDP- α -D-GalA (UDP-GalA) of which 1 gene was expressed specifically in leaves, and one was expressed highly in leaves, 2 genes in stems and 2 genes in roots. In this pathway, α -1,4-galacturonosyltransferase (GAUT) catalyzes the formation of pectin from UDP-GalA. And like the GAE, which were expressed in all tissues. Moreover, 2 genes are expressed highly in leaves and 10 genes in stems (Fig. 6B).

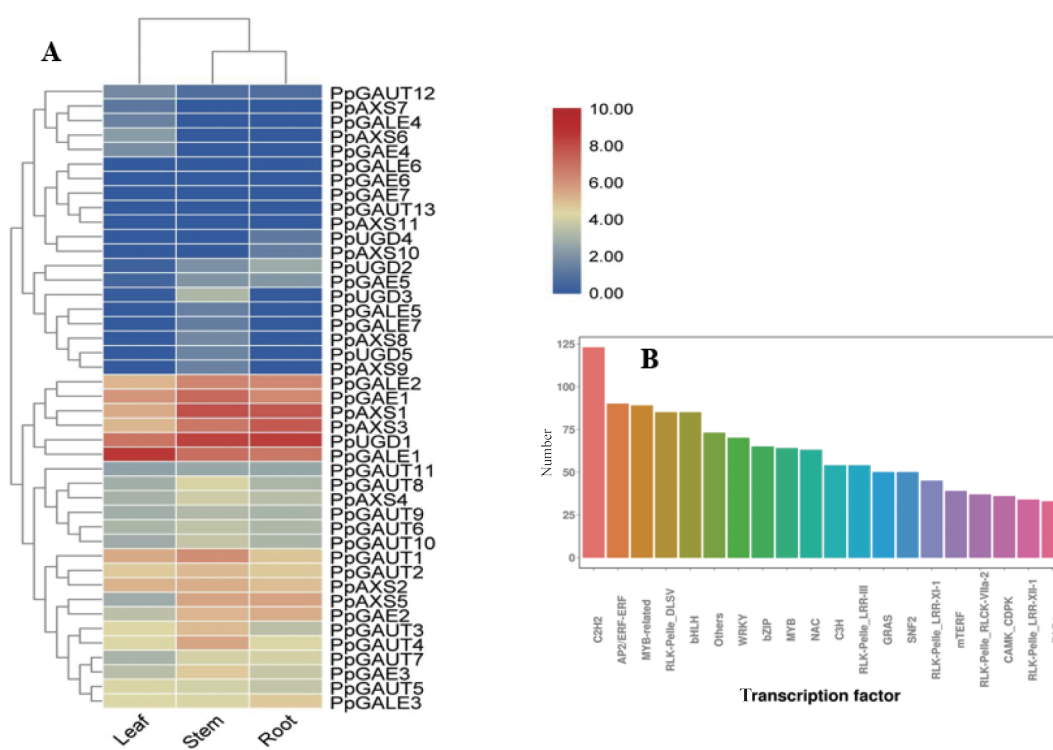


Figure 6 (A) Clustering of different genes for pectin synthesis. (B) TF families identified in *P. puberula*.

4 Discussion

4.1 The content of pectin in leaves was the highest, and the genes related to pectin synthesis were up-regulated

Pectin is composed of a backbone of α -(1-4)-D-GalA, presents in the primary cells wall and middle lamella of dicotyledons, which is the most complex polymer in cell walls^[41]. Further, pectin is a practical food additive on account of its various functional properties as stabilizing, thickening, and emulsifying^[42]. The histochemical localization of pectin can be used to interpret and study its distribution at the level of organs and tissues. Our experiment found that the content of pectin in leaves was significantly higher than that in stems, and the content of pectin in roots was the lowest, which is consistent with Caffall *et al.*^[43], their studies had found that there was pectin in the cell wall, pectin and cellulose are cross-distributed in the cell wall. Pectin polysaccharides can be stained with ruthenium red, and the content of pectin polysaccharides varies from plant to plant and from plant to plant. Further, the genes which primary regulate pectin demonstrated remarkable up-regulated in leaves, including UGD (1.1.2.22), GAE (5.1.3.6), GAUT (2.4.1.43), UDP-glucuronate decarboxylase (AXS) (4.1.1.35) and UDP-glucose 4-epimerase (GALE) (5.1.3.2). These up-regulated high expression genes might be helpful to the accumulation of pectin in mature leaves, which might be the mainly reason for the different content of pectin in roots, stems and leaves. The key enzyme types associated with pectin biosynthesis found in this transcriptome study were not significantly different from those found in other plants, such as AXS, UGD, and GALE, which was broadly in line with the genes found in the transcriptome that are involved in pectin synthesis in *Arabidopsis thaliana* and *Ramie*^[44,45]. Meanwhile, there were certain differences with other species, such as *Solanum lycopersicum*^[46], which may be related to different species, sample growth period and growth environment, so that some genes have different expression levels.

4.2 Pectin synthesis pathway and related genes

Pectin is mainly found in the primary cell walls of plants, whose synthesis pathway and related structural genes have been successfully proved^[47]. The biosynthesis of pectin requires a variety of nucleotide sugars as activating sugars as donor substrates^[48]. More than 60% of pectin is polyhomologous GalA, which is synthesized in Golgi body and transferred to the cell wall^[49]. GAE is a key enzyme that catalyses the conversion of UDP-GlcA to UDP-GalA. Moreover, UDP-GalA was used as the substrate of GAUT to synthesize pectin^[50]. GALE, AXS, GAE and UGD were also remarkably up-regulated in the stems and leaves of *P. puberula*, which was similar to previous literature. In tomatoes, GAUT4 gene expression level is higher, in the development of the organ after the gene silence, the nutrition of plant growth and palisade tissue expansion were significantly increased^[51].

4.3 TFs activate and regulate gene expression

TFs are crucial to the regulation of gene expression^[52], which can activate and regulate genes by identifying and binding *cis*-acting elements in gene promoter regions^[53]. TFs are essential^[54], and a large number of studies have shown that pectin biosynthesis is regulated by complexes composed of MYB and bHLH^[55]. Therefore, it is very important to search for TFs related to pectin synthesis. In the present study, 122 genes encoding C₂H₂, 88 genes encoding MYB-related, 91 genes encoding AP2/ERF-ERF and 83 genes encoding BHLH were found.

4.4 Enzyme genes in the pectin synthesis pathway

Pectin is a polysaccharide rich in GalA, which can be divided into two classes according to its structure, one is 1,4-D-GalA main chain and the other is polyrhmannogalacturonic acid II. Since pectin is a complex polysaccharide, its synthesis requires the involvement of a large amount of glycosyltransferase^[56]. Pectin is generally synthesized in the Golgi body and then transferred to the

cell wall^[57], and about 67 enzymes have been reported that may be involved in pectin synthesis. Of these, at least 50 are Glyco-Transf (GIS). Chen *et al.*^[58] demonstrated that GTS is closely related to cell wall thickness, cellulosic and xylose content. GAE is a catalytic agent for the conversion of UDP-glucuronic acid (UDP-GICA, GT8) to glucan. Glucuronic acid (UDP-A-D-GalA) is the key enzyme for conversion between UDP-GalA, and GAUT uses UDP-GalA as substrate to synthesize pectin finally.

In this study, biennial *P. puberula* was used for transcriptome sequencing on the Illumina HiSeq platform. The Illumina HiSeq platform has particular advantages over the Roche 454, SOLiD, and HeliScope platforms. It analyzes the structure and expression levels of transcripts and can find rare and previously undiscovered transcripts. Further, the accuracy of sequencing has also improved^[59]. We have generated 530 million of the high-quality reads, and approximately 70.73% of the sequences could be mapped onto the reference genome sequence of *P. puberula*. A total of 24,927 genes were assembled from nine libraries. Moreover, 8,456 DEGs were also identified in all three of the different tissues.

It should be noted that this study has determined the content, SEM, XRD and histochemical localization of pectin in the root, stem and leaves of *P. puberula*, and the relevant genes in the pectin synthesis pathway were analyzed and verified by qRT-PCR. Despite the limitations, these findings are valuable in light of understanding the mechanisms of pectin biosynthesis of different tissues, and the cloning and functional verification of key genes in the pectin synthesis pathway were sought to be further studied.

5 Conclusion

We detected and found that the content of pectin in the roots, stems and leaves of *P. puberula* was very different. Through the microstructure, histochemical localization, GalA content, DE, SEM and XRD of the leaves, stems and roots, combined with transcriptomic detection, the biosynthesis and related genes of pectin polysaccharides in different tissues were studied at the molecular level. A total of 26.04 Gb of clean data high quality reads were successfully mapped to the reference genome of adolescent pseudoblue and white and assembled into 107,250 non-redundant genes. The KEGG pathway was further analyzed to identify the pectin biosynthesis pathways that these genes might be involved in, thereby understanding the regulation of pectin at the molecular level. In the next step, we will study the pectin accumulation rules of leaves at different developmental stages of *P. puberula*, and use combined omics to predict the relevant pathways of efficient pectin accumulation, with the aim of searching for differential genes and verifying their functions.

Conflicts of interest

The authors declare that there are no conflicts of interest, Ningxian Yang is an Editorial Board Member of Food & Medicine Homology, but was not involved in the processing, peer review or decision making of this article.

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