Comparative transcriptomics of stem bark reveals genes associated with bast fiber development in Boehmeria nivea L. Gaud (ramie)

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Abstract

Background: Boehmeria nivea L. Gaud (Ramie) produces one of the longest natural fibers in nature. The bark of ramie is the raw material for fiber and primarily includes the entire phloem tissue of the plant stem. Therefore, identifying the molecular regulation of phloem development is important for the understanding of bast fiber biosynthesis and improvement in fiber quality in ramie. Results: In this study, we collected top bud (TB), bark from internode elongating region (ER) and bark from internode fully elongated region (FER) from the ramie variety Zhongzhu No. 1. Histological study indicated that these samples contain phloem tissues at different developmental and maturation stages, with a higher degree of maturation of phloem tissue in FER. RNA sequencing (RNA-seq) was performed to identify differentially expressed genes (DEGs) among these three samples. The de novo transcriptome was assembled and unigenes were identified. The DEGs among TB, ER and FER were analyzed, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) studies revealed clear differences in gene expression between ER and FER. Some unigenes for secondary cell wall biosynthesis were up-regulated in both ER and FER, and other cell wall components or cell wall modification related genes showed differential expression between ER and FER. In addition, ethylene activating pathway and gibberellins biosynthesis varied among samples in a stage dependent pattern. Conclusions: Both morphological study and gene expression analysis supported a burst of phloem and vascular development processes during the fiber maturation in the ramie stem. Two types of phytohormons, ethylene and gibberellin, might be involved in. Our findings provide novel insights into the phloem development and fiber maturation in ramie, which could be useful for fiber improvement in ramie and other fiber crops.

Background

Natural plant fibers can be collected from the seeds of cotton, leaves of pina, fruits of coconut, stalk of bamboo, and bast of ramie (*Boehmeria nivea* L. Gaud). Among these fibers, ramie fiber is one of the longest and strongest natural fibers. Ramie produces fibers from its stem bark, which is originated from phloem tissue. Besides ramie, the well-known bast fiber crops include flax (*Linum usitatissimum*) and hemp (*Cannabis sativa*). In recent years, significant progress has been made in bast fiber study by using genome and transcriptome analysis in flax, hemp and ramie [1-5]. Ramie has a vigorous vegetative growth, and its stem undergoes obvious elongation and thickening processes. Ramie stem produces primary phloem fibers, which is initiated from the shoot apical meristem (SAM) resembling the process in flax. Ramie also produces secondary phloem fibers in stem which is similar to hemp or tension woodof poplar (*Populus tremula*). The secondary phloem fibers are originated from the vascular cambium, which is typical for dicotyledonous plants with secondary stem thickening through the activity of the vascular cambium. A comparison between flax and hemp showed that the fiber elongation process is similar in these two species, but morphological study suggested that the intrusive growth is a major determinant of the structure of the final fiber bundle in hemp [6].

Although the developmental process of fiber in ramie still requires detailed study, the production of both primary and secondary phloem fibers is believed to depend on secondary cell wall synthesis. The studies on the composition of secondary cell wall, i.e. the proportion of cellulose, hemicelluloses and lignin, indicated that these cell wall components vary among different plant species and even in the same cell type in different parts of the model plant Arabidopsis [7-9]. In fact, secondary cell wall formation is a complex process involving signaling events that trigger the secondary cell wall biosynthetic program, leading to transcriptional activation of secondary cell wall biosynthetic genes, which results in the biosynthesis of secondary cell wall components, targeted transport of enzymes and secondary cell wall materials, and patterned deposition and assembly of secondary cell wall. Gene transcriptional regulatory network and signaling cascade integrating signals for secondary cell wall biosynthesis were proposed in recent years. NAC and MYB transcription factors were considered as the master switches, and their downstream transcription factors took part in the weaving of the network [10-14]. In one of the proposed models, at least three layers of regulators could be directly involved in regulating secondary cell wall biosynthetic genes, which are NAC (NO APICAL MERISTEM, ATAF1, ATAF2, and CUP-SHAPED COTYLEDON 2) domain master regulators in the tier 3, two MYB domain regulators in the tier 2 and many other regulators in the tier 1 [14].

In an effort of studying the molecular response during the phloem fiber cell development, we adopted a method to collect samples with different degrees of phloem maturation from ramie's shoot. Fiber development in ramie stem is a continuous and systematic process along the stem tissue, it is therefore difficult to define a "snap point" to mark the transition from elongation to fiber thickening, a process resulting in changes in fiber mechanical properties[15]. However, we noticed that the stems of ramie have relative fixed number of nodes, and the internode regions show obvious elongation until fully elongated during stem growth. Thus, to collect the tissues with phloem at different developmental stages, we truncated the stems of ramie into three parts, top bud (TB), internode elongating region (ER) and internode fully elongated region (FER). Histological study confirmed the different developmental stages of phloem in these three parts of the stems. RNA-seq analysis of the gene expression profiles in the top buds, barks of ER and FER revealed key genes and pathways that are possibly responsible for the distinct secondary phloem formation and fiber development in ramie.

Results And Discussions

Different segments of stem bark exhibit distinct morphological features

Ramie fibers continuously develop along the stem during plant's growth, while the internodes of the stem show obvious elongation only until the plant is fully elongated. To analyze the developmental stages of fiber formation and the gene expression profiles, three parts of ramie's shoot were harvested including top bud (TB), internode elongating region (ER) of stem and internode fully elongated region (FER) of stem

(Fig.1A). The top buds and the barks from both ER and FER regions were peeled off from the woody center pillar for histological analysis and RNA extraction. The strategy for the subsequent RNA-seq data analysis is illustrated in Fig. 1B. The cross and longitudinal sections of TB, ER and FER were performed and images were collected and analyzed (Fig.1C and D). In the TB sample, ramie has amphicribral vascular bundle, which is unlike the flax or hemp plants but is more similar to woody plant with continuous cambia within and outside the vascular bundles (Fig. 1C). Its vascular structure is characterized by multiple layers of primary phloem which has no obvious boundary between vascular bundles. In ER and FER, clear differences were observed between these two regions (Fig. 1D). Firstly, FER has thicker bark than ER, and FER barks consist of more enlarged cells and more layers of phloem tissues. Secondly, fiber cells show thicker cell wall in FER without an increase in cell size; the thickness of the fiber cell wall is about 5.38 μm in FER vs. 1.87 μm in ER (Supplemental table 1). Thirdly, the cell wall of the fibers from FER phloem contains more lignin than that from ER, which is indicated by stronger red color resulting from the staining of safranin dye (Fig. 1b and 1d). The differences among these three samples indicate different developing stages of phloem fiber cells. Therefore, we used these samples for gene expression profiling attempting to identify genes important for fiber development in ramie.

Assembly of *de novo* transcriptome and identification of unigenes in ramie using RNA-seq data

Thirty-three RNA samples were collected and subjected to the next generation sequencing (NGS), and the RNA-seq data, including the 9 submitted SRA files (SRR9112644-SRR9112651), were analyzed. More than 5G clean bases information from each sample was obtained, thus the total analyzed clean bases were about $1.7E^{+11}$. The genome size of Zhongzhu No. 1 is approximately 340 Mb [3, 4]. Therefore, the depth of the RNA-seq data used in this study was expected to be enough for a high quality *de novo* assembly of transcriptome for the coding sequences specific from the top bud and stem bark tissues. The 10 species with the most matching reads comparing with our RNA-seq data were shown in Fig. 2A. Among all the reads generated, 3048 reads match with those in *Boehmeria nivea*, and the highest matching ratio (28%) was found to be with *Morus notabils*. The whole genome sequencing analysis supported a closest evolutionary relationship between ramie and *Morus notabils* [4]. Overall, there were 59486 unigenes assembled with the length longer than 300 bp, 47016 unigenes longer than 500 bp, and 31395 unigenes longer than 1000 bp. The GC content distribution of all unigenes was shown in Fig. 2B, and two peaks appeared between the range of 30% and 45%. The detailed size distribution of all unigenes was illustrated in Fig. 2C. The sequence of each unigene was subsequently processed by blast to NR, SWISSPROT and KOG data base, respectively, and the annotations were obtained according to the most similar protein or gene with $e<1e^{-5}$.

Identification of the expression patterns by analyzing the differentially expressed genes (DEGs) among TB, ER and FER

DEGs among the three tissues were identified following the scheme shown in Fig. 1B. When compared with TB, there were 4138 unigenes up-regulated and 6638 unigenes down-regulated in the ER, and 3853 unigenes up-regulated and 5075 unigenes down-regulated in the FER (Fig. 3A). The VEN diagram showed that the DEGs among these 3 samples were grouped in 6 distinct clusters (Fig. 3B). The heatmaps of the expression of these clustered genes were shown in Fig. 4A, and the schematic map of the expression patterns and GO analysis were illustrated in Fig. 4B.

Cluster 1 and 2 contain the most DEGs with 4354 and 2046 respectively, which were up- or down-regulated unigenes in ER and FER, respectively, comparing with TB. These two clusters might contain tissue-specific DEGs which could result in the differences between the top bud and stem bark samples. The cluster 1 DEGs consist of the unigenes with higher expression level in TB but lower expression level in both bark regions. GO analysis showed that these DEGs are involved in meiotic chromosome segregation and cell division, which could be explained by the active gene expression required in the vigorous dividing cells of the SAM region of the top bud. Other DEGs involved in stomatal or leaf development in the cluster 1 could be due to the remaining young emerging leaves in the TB samples, while no leaves were present in the ER and CER samples (Fig.4B). GO analysis of the DEGs in cluster 2 indicated that up-regulated transcription factors or transcription processes and the plant-type secondary cell wall biogenesis are in the top categories. Fifty-five unigene contigs of cell wall components or cell wall biogenesis involving factors were identified in the cluster 2 (Table S2). Among the cell wall related DEGs were Cellulose Synthase A, Catalytic Subunit 3 and 8 (CesA 3 and 8) (Table S2). There were 9 CesA genes orthologous to Arabidopsis were identified in our RNA-seq data, but only CesA 3 and CesA 8 were up-regulated in both ER and FER, which indicated that these 2 genes might be involved in ramie's secondary cell wall biogenesis during fiber development. Coincidently, CesA 8 from Arabidopsis was found to play a role in secondary cell wall synthesis in flax [1]. In addition to cellulose synthases, Fasciclin-like Arabinogalactan Protein (FLA) and beta-galactosidase (BGAL) have also been reported to be involved in the formation of cellulose rich gelatinous fibers [1] [16-18]. Among the DEGs in the cluster 2, FLA 11/12 and BGAL 3/9 were up-regulated in ER and FER (Table S2). In addition, several pectinesterase/pectinesterase inhibitors (PEMs) and the enzymes for the synthesis of other cell wall components, such as glucuronoxylan glucuronosyltransferase, galacturonosyltransferase, endochitinase, callose synthase, xyloglucan glycosyltransferase, xyloglucan endotransglucosylase, etc. were also found to be among the DEGs in the cluster 2 (Tab. S2). These enzymes or protein factors were reported to play distinct roles in secondary cell wall biogenesis or modulation [2, 7-9]. Our findings suggest that fiber cell formation and development in ramie require gene expression to produce enzymes and protein factors for secondary cell wall biosynthesis, which is the characteristic of the bark tissue in both ER and FER.

DEGs between ER and FER barks with opposite regulating tendency comparing with TB were clustered in the cluster 3, 4, 5 and 6 (Fig. 4A and 4B). In cluster 3 and 4, there were 93 unigenes down-regulated and 476 unigenes up-regulated only in the FER. In cluster 3, there was only a small amount of unigenes responsive for membrane construction that was down-regulated in FER. However, in cluster 4, relatively more unigenes were up-regulated in FER. Among them, ethylene signaling pathway genes were the most enriched unigenes. There were totally 39 transcription factors only up-regulated in FER, and 18 out of the 39 were ethylene activating unigenes (Tab. S3). Differentially expressed unigenes only in the ER were clustered in cluster 5 and 6. Interestingly, some phloem development related unigenes were found to be down-regulated only in ER when compared with those in both TB and CER (Fig.4B). According to the GO analysis of DEGs in cluster 3 to 6, phytohormons such as ethylene and gibberellin might play distinct roles in these two bark stages, as some of the gibberellin-responsive genes were only up-regulated in the ER stage while some of the ethylene signaling pathway related genes were only activated in the FER stage. Furthermore, higher expression of several DEGs (FER *vs.* ER) involved in the phloem development suggested a more vigorous secondary phloem development in the relatively mature stem or bark from FER.

In addition to the expression patterns analyzed among TB, ER and FER, DEGs between TB and ER or FER were also analyzed and GO analyses were performed. The top 10 items of three GO terms were shown in Fig. S1 and S2. When compared with TB sample, barks from ER and FER showed distinct features in gene expression patterns. Therefore, DEG identification between ER and FER is essential to uncover the differences between the barks in different fiber developmental stages.

Up-regulating of secondary phloem formation, ethylene activating pathway and secondary cell wall biosynthesis in FER comparing with ER

There were 1628 up-regulated unigenes and 757 down-regulated unigenes identified in ramie's bark of FER when compared with ER (Fig.5). GO analysis shown in Fig. 6 revealed the top 10 up-regulated biological processes including phloem development, response to chitin, ethylene-activated signaling pathway, DNA replication, salicylic acid mediated signaling, defense response, protein transmembrane transport and vasculature development, and the top 10 down-regulated biological processes including cytoplasmic translation, tricarboxylic acid cycle, indole glucosinolate metabolic process, plant-type secondary cell wall biogenesis, etc.. Processes such as phloem development, vasculature development and DNA replication were up-regulated in the FER, which suggests that the secondary phloem formation was activated in the bark of the relative mature FER rather than in ER.

The activation of ethylene signaling pathway was evidenced by the up-regulation of the genes in this pathway in FER (Tab. S3). Overall 21 unigenes or contigs of 14 *Ethylene Respond Factors* (*ERFs*) were up-regulated in FER, which include *ERF1*, *ERF1A*, *ERF1B*, *ERF2*, *ERF3*, *ERF5*, *ERF17*, *ERF22*, *ERF53*, *ERF61*,

ERF71, *ERF109*, *PAR2-13* and *PAP2-4* (Tab. S5). Most of these ethylene response related DEGs were transcriptional activators involved in the regulation of gene expression by stresses and disease resistance pathways. This may indicate that FER bark is more required for ramie resistance to stress conditions comparing with ER, thus FER requires more and stronger fiber development. Therefore, it is possible that ethylene, together with stress factors, promote phloem tissue development in FER.

According to our gene expression pattern analysis, a number of unigenes involved in secondary cell wall synthesis were up-regulated in both ER and FER (Figure 4, Tab. S2). Although 20 secondary cell wall related unigenes were down-regulated in FER (Fig. 6B), as many as 84 unigenes for secondary cell wall synthesis were up-regulated in FER when compared with ER (Tab. S6). These results indicated that, although secondary cell wall biogenesis is strengthened in both ER and FER, FER and ER displayed differences in the expression of secondary cell wall synthesis genes and more enzymes might be required in FER for the thickening of the fiber cell wall (Fig. 1D). In addition to *CesA 3/8*, *FLA11/12*, *BGAL 3/9*, *PEM 13/28/35* and several other factors that are up-regulated in both ER and FER, several additional *PEMs*, *BGALs*, an *FLA 8*, subtilisin-like proteases (*SBTs*), some leucine-rich repeat extension-like proteins (*LRXs*), *peroxidase 47/64*, laccase-4 (*IRX12*), *pathogenesis-related protein 5* etc. displayed higher expression level in FER than in ER. FLAs contain a cell adhesion fasciclin (FAS) domain. Expression of some *FLAs* has been shown to be correlated with the onset of secondary wall cellulose synthesis in Arabidopsis stem, and with tension wood formation in the stem and branch in *Populus tremula* (L.) [19]. Mutations in *FLA* genes resulted in altered stem biomechanics with reduced tensile strength and elasticity, as well as altered cell wall architecture and composition [20]. While pectin is another important component in ramie's fiber cell wall, PEMs act via demethylesterification of cell wall pectin, and was also shown to carry out endohydrolysis of the N-glycosidic bond at one specific adenosine on the 28S rRNA and inactivate the ribosome [21], which may account for the decreased expression of translation factors in FER (Fig. 6B, Fig. 7B). Myb 26, a transcription factor functioning in fine-tune regulation of fiber or secondary cell wall synthesis [14], showed a higher expression in ER than in FER, which suggests that this transcription factor may function in secondary cell wall biogenesis at early stages.

Differences in starch and sucrose metabolism, phenylpropanoid biosynthesis and GA metabolism between FER and ER

The KEGG analysis of total DEGs from FER vs. ER revealed additional information to the GO analysis. The KEGG analysis indicated that these DEGs are involved in the pathways of starch and sucrose metabolism, citrate cycle, nitrogen metabolism, cysteine and methionine metabolism, ribosome, diterpenoid biosynthesis, phenylpropanoid biosynthesis, DNA replication, cell cycle, etc. (Fig. 8 and Tab. S7).

The secondary cell wall synthesis is a very complex biological process involving the biosynthesis of multiple components or species-specific secondary metabolisms. However, starch and sucrose metabolisms are the important pathways linked with the secondary cell wall synthesis. From the KEGG analysis, we found that the expression of 23 unigenes encoding 11 kinds of enzymes in the starch and sucrose metabolisms differed between ER and FER. These enzymes include sucrose synthase (EC2.4.1.13), sucrose-phosphate synthase (EC2.4.1.14), bata-amylase EC3.2.1.2, endoglucanase (EC3.2.1.4), bata-glucosidase (EC3.2.1.21), glucan endo-1, 3-beta-glucosidase (EC3.2.1.39), glucose-6-phosphate isomerase (EC5.3.1.9), phosphoglucomutase (EC5.4.2.2), UTP-glucose-1-phosphate uridylyltransferase (EC2.7.7.9), trehalose phosphatase (EC3.1.3.12) and trehalase (EC3.2.1.28) (Fig. 8 and 9). Most of these enzyme-encoding unigenes were up-regulated in FER, which suggests that multiple pathways for free D-glucose production might be enhanced in FER. And other sugar producing processes such as sucrose-6P, maltose and dextrin might also be enhanced in FER. The increase in these sugar precursors could be important in providing building materials for the secondary cell wall biogenesis in ramie.

More lignin accumulation in FER was observed by the staining with safranin dye (Fig. 1d), which might be due to the up-regulation of enzymes responsible for phenylpropanoid biosynthesis in this region according to our KEGG analysis (Fig.8 and 10). In the pheylpropanoid biosynthesis pathway (KO00940), 16 up-regulated unigenes encode enzymes including peroxidase (EC1.11.1.7), trans-cinnamate 4-monooxygenase (EC1.14.13.11), Anthranilate N-methyltransferase (EC2.1.1.68), Flavonoid 3',5'-methyltransferase (EC2.1.1.104), beta-glucosidase (EC3.2.1.21), caffeylshikimate esterase and vinorine synthase, some of which were among the DEGs contributing to secondary cell wall synthesis (Fig.10 and Tab. S6). Most enzyme-encoding genes involved in the biosynthesis of lignin were up-regulated in FER, supporting the observation of more lignin accumulation in FER. Similarly, up-regulation of several PEMs may account for differential accumulation of pectin in FER compared with ER (Tab. S6).

Interestingly, in the diterpenoid biosynthesis pathway, unigenes encoding enzymes such as Ent-kaurenoic acid oxidase 2 (EC1.14.13.79) and gibberellins 20 oxidase (EC1.14.11.12) for converting the precursors to active GA isoforms were up-regulated, while the transcripts of the enzyme gibberellins 2-beta-dioxygenese 8 (EC1.14.11.13) for inactivation of GAs were decreased in FER (Fig.11). These results suggest that a higher concentration of active GA might be needed in FER than in ER, and GA could be another phytohormone related to the development of bast fiber in ramie.

Discussions

Both the morphological observation and GO analysis of DEGs comparing ER with FER indicated secondary phloem formation in FER which is the relative mature stem bark. In gene expression detail, contigs of five protein encoding genes involving 13 assembled unigenes possibly responsible for secondary phloem development in FER were identified. These proteins include sieve element occlusion A (SEOA), SEOB, Myb family transcription factor Altered Phloem Development (APL), protein DA1-related 2 (DAR 2) and UPF0503 (Tab.1). SEOA and SEOB are two phloem filament proteins, and their orthologs in

Arabidopsis are required for the formation of phloem filaments. Phloem filaments could only be detected in the presence of both SEO proteins in Arabidopsis [22-24]. APL transcription factor is required for phloem identity, and has a dual role both in promoting phloem differentiation and in repressing xylem differentiation during vascular development [23-25]. The downstream targets of APL include NAC45 and NAC86, which are involved in enucleation, organelles reorganization and cytosol degradation during sieve element cell differentiation [24, 26]. Consistently, NAC86 is also significantly up-regulated in the FER samples with a change of more than 4-fold. DAR 2 is involved in root phloem development and is an essential component for early phloem development and long-distance delivery of phloem content in Arabidopsis [23, 27]. The unigene CL252Contig2 is likely to encode the protein UPF0503, whose ortholog in Arabidopsis is the polarly localized membrane-associated protein OCTOPUS (OPS), which is initially expressed in provascular cells, and upon vascular cell type specification, it becomes restricted to the phloem cell lineage. The *ops* mutants display a reduction in the complexity of the cotyledon vascular pattern and exhibit discontinuous phloem differentiation, whereas OPS overexpressors show accelerated progress of cotyledon vascular patterning and phloem differentiation [23, 24, 28]. The expression of some DEGs related to phloem or vascular development, including APL and DAR2, which termed as Phloem 1 and Phloem 2, was further confirmed by RT-qPCR. The results showed significantly increases of these DEGs in the FER (Fig.7A and Tab. S4).

In addition, we also identified 4 unigenes including WUSCHEL-related homeobox 4 (WOX4) and other three ethylene-responsive transcription factors (ERFs) (Table 1) that are involved in vasculature development. WOX4 functions downstream of the dodeca-peptide TE differentiation inhibition (TDIF) factors and its receptor PHLOEM INTERCALATED WITH XYLEM (PXY) and promotes cambial cell proliferation [29-31]. Mutation of *WOX4* represses procambium proliferation in the hypocotyl of 7-day-old seedlings in *Arabidopsis*[29-31]. The significant up-regulation of *ERF109*, *WOX4* and *ERF1A* in FER was also confirmed by RT-qPCR, which named as Vascul1, Vascul2 and Vascul3, respectively (Fig. 7 and Tab. S4). The up-regulated ethylene activating pathway in FER might be a parallel event to WOX4-PXY signal for phloem or vascular development. PXY is a receptor kinase and functions in ordered and coordinated cell divisions in the procambium [31, 32]. Twelve members of AP2/ERF family, including *ERF109*, *ERF11*, *ERF104*, *ERF018*, *ERF1*, *ERF2*, *ERF5* and *ERF6*, were identified to be up-regulated in *pxy* mutant, which was thought to compensate the loss of function of *PXY* gene in Arabidopsis. Especially, loss of function mutations of both *ERF 109* and *ERF 018*, together with the *pxy* mutation, significantly reduced vascular cell numbers [32]. ERF1 was also found to be a key factor for vascular cell division responding to ethylene signal [32]. Additionally, the base of stems of Arabidopsis exhibits larger fold changes in the gene expression of *ERF109*, *ERF11*, *AtERF1* and *ERF018* than that observed in the middle of stems [32]. Since the FER is closer to the base position of the whole stem of ramie, it is likely that the genes activating ethylene pathway are required for the mechanical and physiological properties of this region. Moreover, ethylene is induced during tension wood formation in poplar, and several factors in the ethylene signaling pathway were identified to be up-regulated during tension wood formation [33]. Therefore, the activation of ethylene pathway or ERFs expression could be an important molecular basis for ramie's fiber differentiation and development. Thus, up-regulation of ethylene pathway and increased *WOX4*

expression could partially explain the burst of secondary phloem formation or cambia activity in the relatively mature part of ramie stem.

GA is another hormone function in cellulose rich fiber development in plant. The KEGG analysis suggest that a higher concentration of active molecules of GA in FER than in ER. But this did not mean the GA functions were simply strengthened along the stem from the top to the base. To the contrary, we found some of the GA responsive gene expression was higher in the region of TB and ER than that in the FER in our RNA-seq data and RT-qPCR confirm (Fig. 8B). We speculated that was because the perception of GA was as same essential as the concentration of this signal. Besides the distribution or concentration of GAs, the response to GA isoforms must differ in different parts of ramie due to the variation of receptor distributions or signal perception patterns. Recently, researches in ramie also support GA was essential to the bast fiber development at a molecular level or when being applied with other nutrients respectively [4, 34]. And overexpression of Arabidopsis thaliana gibberellic acid 20 oxidase (AtGA20ox) gene enhance the vegetative growth and fiber quality in kenaf (Hibiscus cannabinus L.) plants [35, 36]. But further studies and more evidences are needed to explore the detailed mechanism for GA regulation to ramie stem development.

Conclusions

In summary, our findings revealed a burst of phloem formation and secondary cell wall synthesis in the fully elongated regions of the stem with relatively mature fibers in the stem bark of ramie. We found that two types of phytohormons, ethylene and gibberellin, might be involved in the development of ramie's phloem fiber tissues (Fig. 12). However, more studies are needed to elucidate the molecular basis of secondary phloem development. Nonetheless, our study could provide valuable genomic data and gene expression profiles related to the fiber formation in ramie for improvement of this important fiber crop.

Methods

Materials

ZhongZhu No. 1 ramie variety was planted in the green house, originated from cutting propagation of plant in farm of Institute of Ramie, Hunan Agricultural University, Changsha, China. The plant was identified by Ramie's Germplasm Resource Description Standards GB/T 2659 of Crop Germplasm Resource Infrastructure in China (www.cgris.net). And Yucheng Jie was responsible for identification of the ramie variety ZhongZhu No.1. Ramie's shoot including CAM and its connected stem with green surface was harvested. The shoot was then truncated into 3 sections: top bud (TB), internodes elongating region (ER) of stem and internodes fully elongated region (FER) of stem. The elongating or fully elongated regions were judged by the length between adjacent internodes. When the length between the internodes was 2 cm longer than the upper ones but 2 cm shorter than the lower ones, this section was

considered as the elongating region. Below the elongating region was considered as fully elongated region. All the leaves and flowers associated with the shoot were removed, and the bark was peeled from these two stem fragments. The top bud and two bark fragments, which termed as TB, ER and FER were used for further experiments (Fig. 1A).

Tissue sections and images collection

Tissue materials of TB, ER and FER were fixed in FAA (formaldehyde, acetic acid, 70% ethanol) until further processing. The fixed materials were then embedded by paraffin. Microscope slides with transverse or longitudinal sections were prepared using a rotation microtome and following standard protocols. The sections were stained with safranin-fast-green or aniline blue, and the stained sections were then scanned by Pannoramic 250/MIDI (Wuhan servicebio technology company). Images were collected by caseviewer software provided by Wuhan servicebio technology company.

RNA extraction and RNA sequencing

Total RNA was extracted from TB, ER and FER using TRIzol reagent kit (Invitrogen, Carlsbad, CA, US) following the manufacturer's protocol. The mRNAs were enriched by magnetic beads coated with Oligo (dT), and then randomly fragmented by ultrasound. The platform of Illumina HiSeq 2500 was adopted to carry out RNA sequencing (RNA-seq) by OE biotech company (Shanghai). The sequencing data have been deposited in the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) with accession number SRP 199269.

RNA-seq data analysis

De novo transcriptome and unigenes were assembled in OE biotech company (Shanghai) by paired-end method according to Trinity software (trinityrnaseq_r20131110)[37]. If necessary, TIGR Gene Indices clustering tools (TGICL) were then used to get rid of sequence redundancy [38]. Unigene annotation was added by applying Basic Local Alignment Search Tool (BLAST) to search databases of NR, SWISSPROT and KOG [39]. The expression of unigenes was calculated and subsequently normalized to RPKM [40]. The uniform screening conditions for DEGs were $p<=0.05$, and fold change >2 or fold change < 0.5. Then the DEGs were submitted to perform GO and KEGG analyses [41]. VENN diagram was made using a website tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). And the K-means clustering algorithm was adopted to analyze the expression pattern.

RT-qPCR

Total RNA was extracted from TB, ER and FER using TRIzol reagent kit (Invitrogen, Carlsbad, CA, US) following the manufacturer's protocol. After TURBO DNase I (Ambion) treatment, 2 μg of RNA was subjected to reverse transcription reaction using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech). Then cDNAs were used as templates for qPCR with Green qPCR SuperMix (TransGen Biotech) in a CFX96 real-time PCR detection system (Bio-RAD). Ubiquitin-conjugating enzyme E2 (CL1514Contig1) was chosen as a reference according to our RNA-seq data. All of the primers are listed in Table S4. All the reactions were done in triplicates.

Abbreviations

TB: Top Bud; ER: internode Elongating Region; FER: internode fully elongated region; DEG: differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; SAM: shoot apical meristem; NGS: next generation sequencing; RNA-seq: RNA sequencing; CesA: Cellulose Synthase A; FLA: Fasciclin-like Arabinogalactan Protein; BGAL: beta-galactosidase; PEMs: pectinesterase/pectinesterase inhibitors; ERFs: ethylene respond factors; SBTs: subtilisin-like proteases; GAs: gibberellin acids; AtGA20ox: Arabidopsis thaliana gibberellic acid 20 oxidase; BLAST: Basic Local Alignment Search Tool.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The sequencing data are deposited in NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) with accession number of SRP199269.

Competing interests

The authors declare that they have no competing interests.

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Not Applicable.

Authors' Contributions

JX, JL and DY conducted the experiments, YZ designed the experiment and performed the data analysis, YJ provided materials and helped designing experiments, DX and HS drafted and revised the manuscript. All authors have read and approved the manuscript.

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Conflict of interest

No conflict of interest.

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Table

Table 1 could not be inserted here due to technical limitations. It can be found in the supplemental files.

Figures

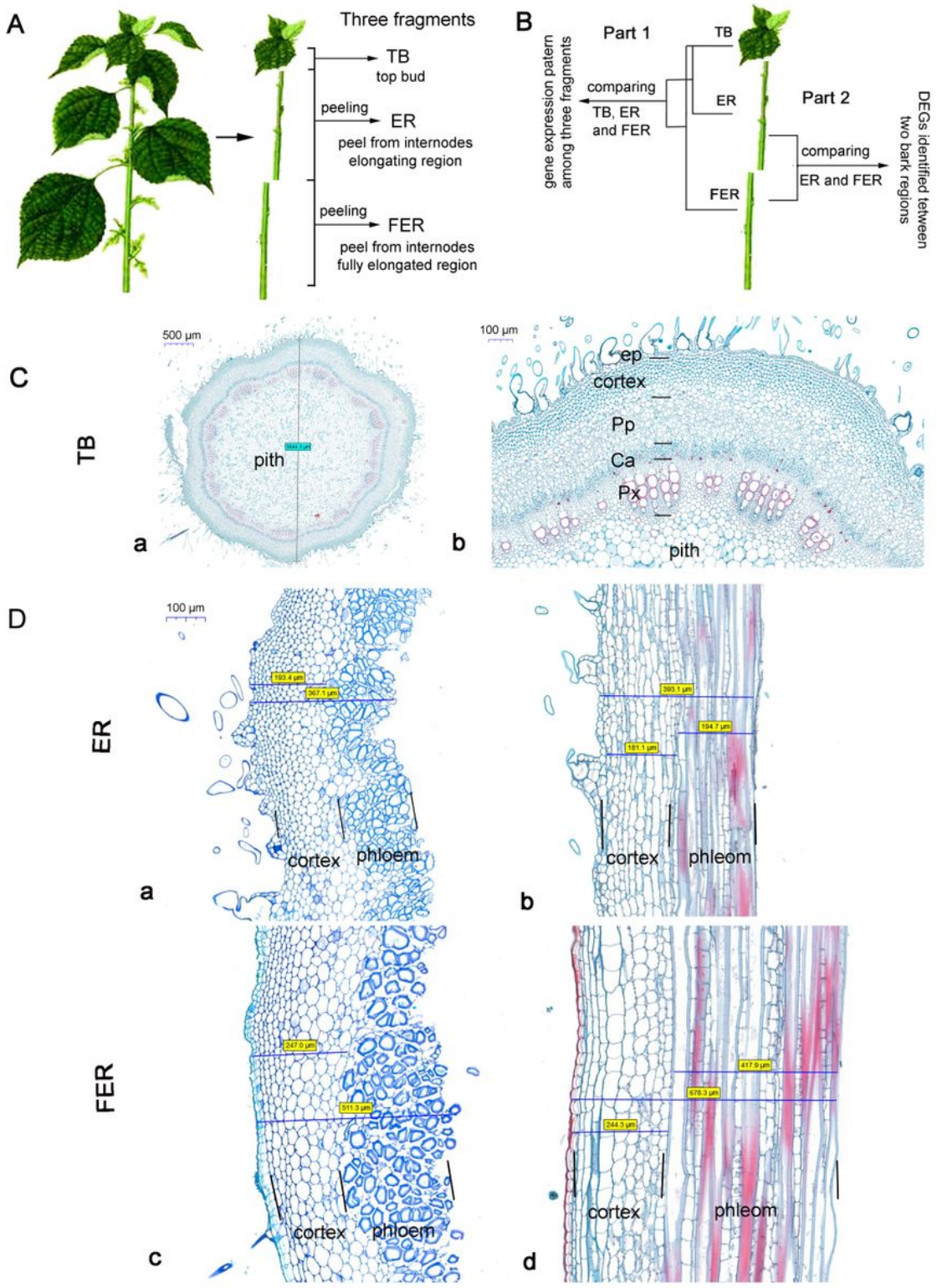
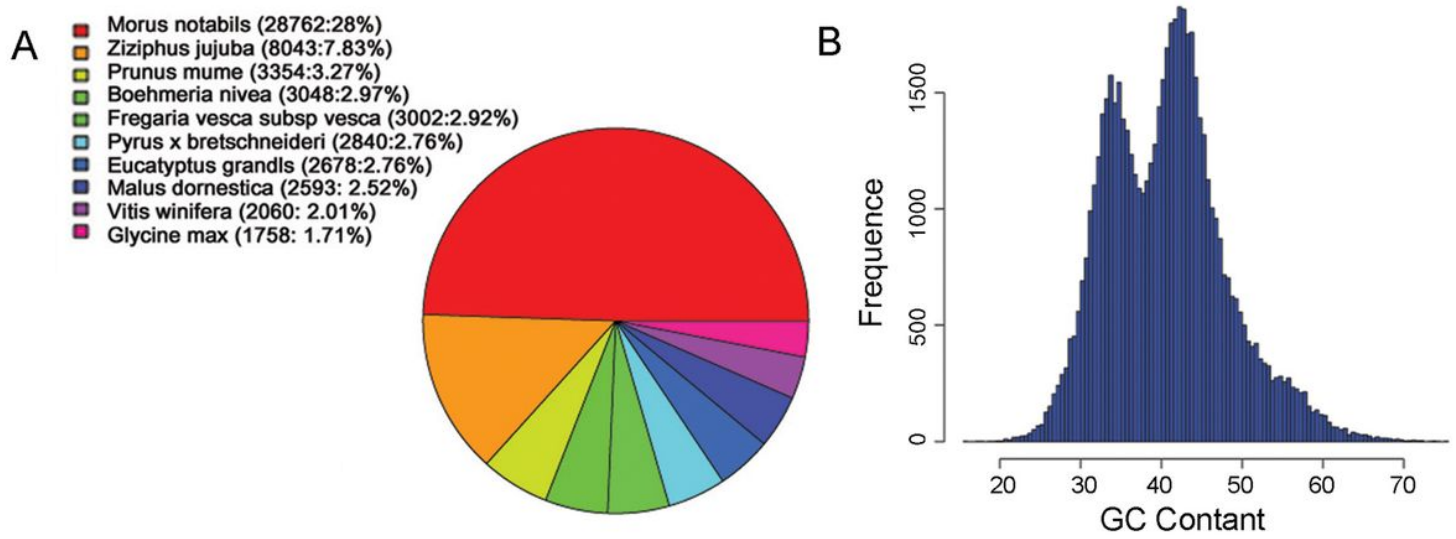


Figure 1

Ramie materials and transcriptome comparison strategy among samples. A. Truncation of ramie shoots. The shoots were cut into three sections including top bud, elongating region (ER) stem and fully elongated region (FER) stem. The leaves were removed, and the ER and FER samples were collected by peeling the bark from the central woody column of the stem. B. The strategy of DEG identification by comparing the transcriptomes between different samples. C. Cross section of TB with 2 times

magnification (a), 10 times (b). ep: epidermal layer; Pp: primary phloem; Ca: cambia; Px: primary xylem. D. Cross and lengthwise sections of ER (a and b) and FER (c and d) samples magnified 40 times.



C

	All≥300bp	≥500 bp	≥1000 bp	N50	Total Length	Max Length	Min Length	Average Length
Unigene	59486	47016	31395	2296	90959127	20133	301	1529.08

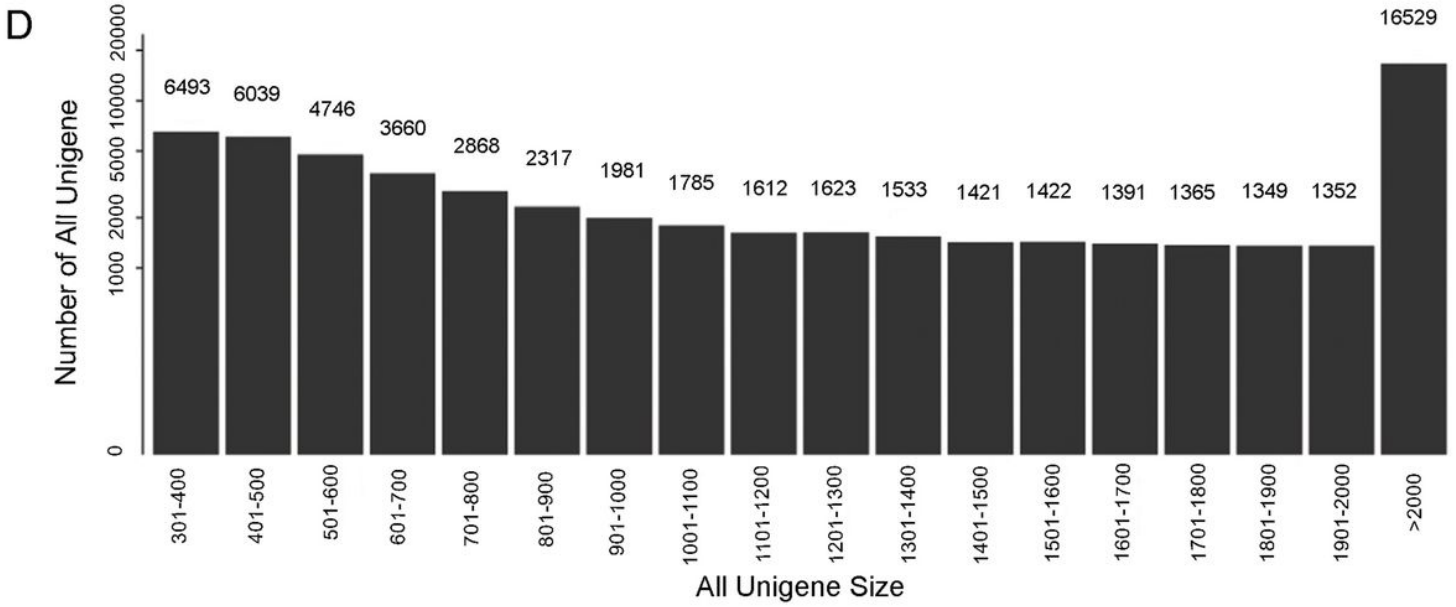


Figure 2

Characterization of the transcriptome and assembled unigenes in ramie. A. 10 species with the most matching reads to our data. Different colors represent different species, and the area was corresponding to the quantity of matching reads in the organism. The amount and the percentage of matching reads were indicated in the brackets. B. GC content distribution of all unigenes. C. Size distribution of all assembled unigenes.

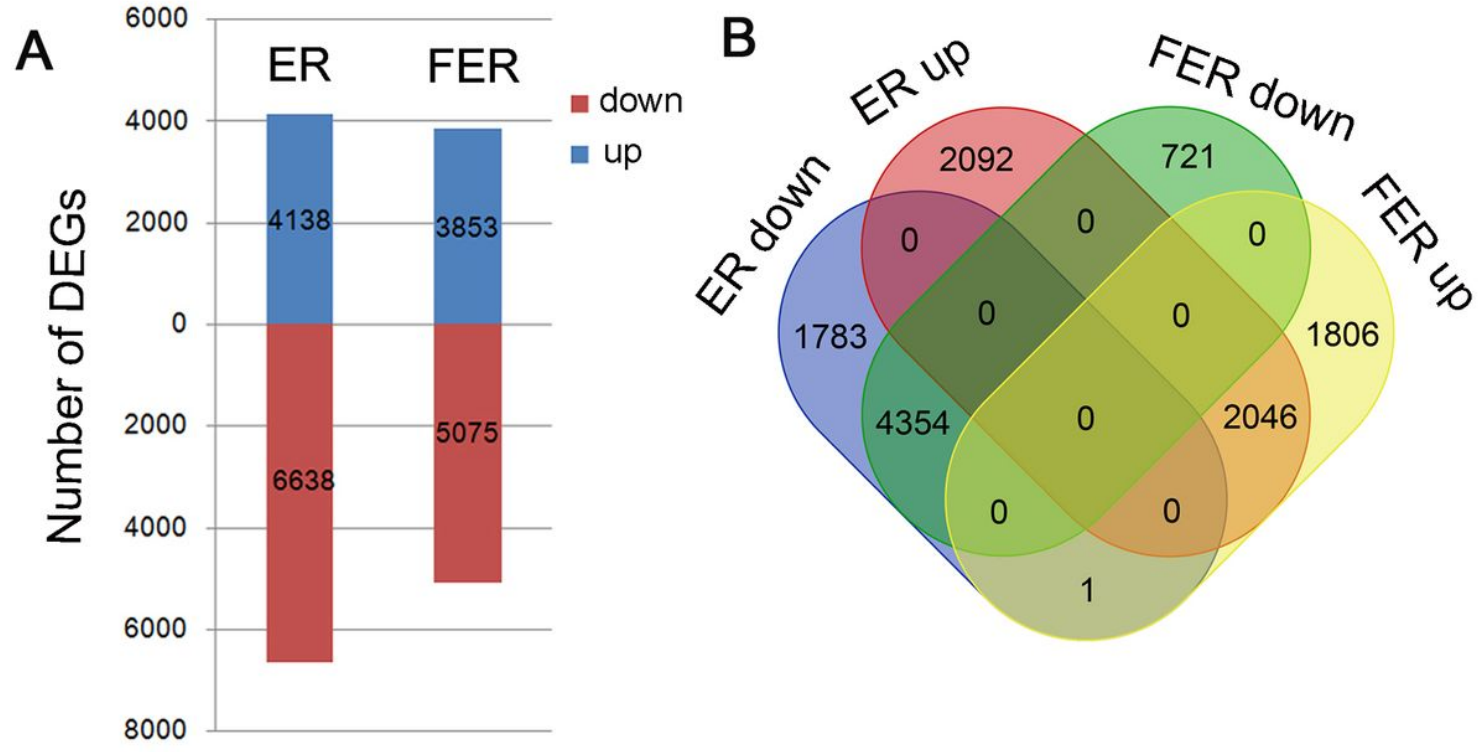


Figure 3

Differentially expressed genes between ER or FER stem section and top bud. A. The number of DEGs identified by comparing ER or FER with TB. DEGs of ≥2 fold changes with P-value less than 0.05 were included. B.VEN diagrams of DEGs in ER and FER comparing with TB.

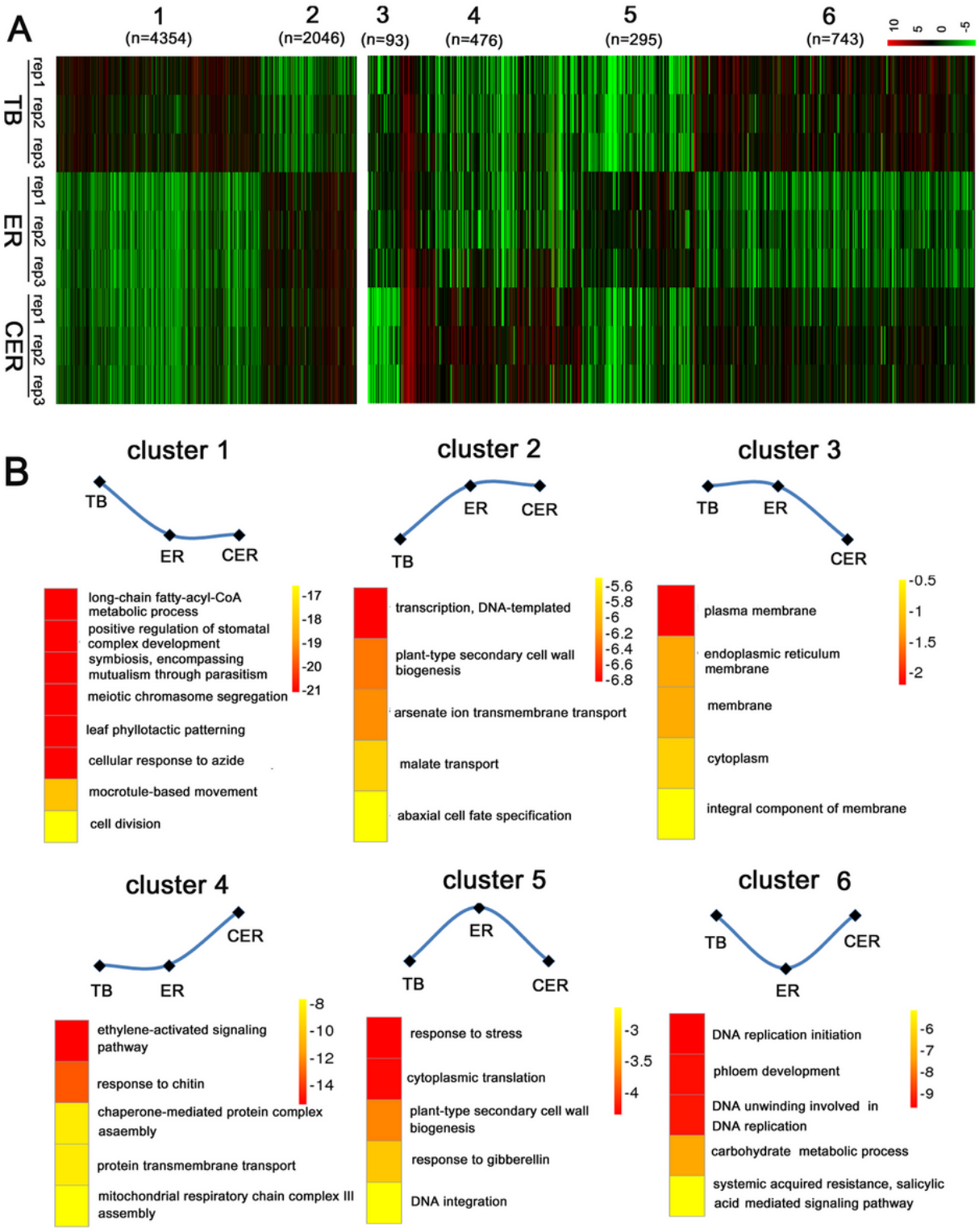


Figure 4

Gene expression patterns among TB, ER and FER regions. A. The heatmaps of six clusters of DEGs among TB, ER and FER. The red color indicates upregulation, while green color represents downregulation. B. Schematic curves for gene expression patterns among TB, ER and CER, and GO analysis of DEGs in each cluster.

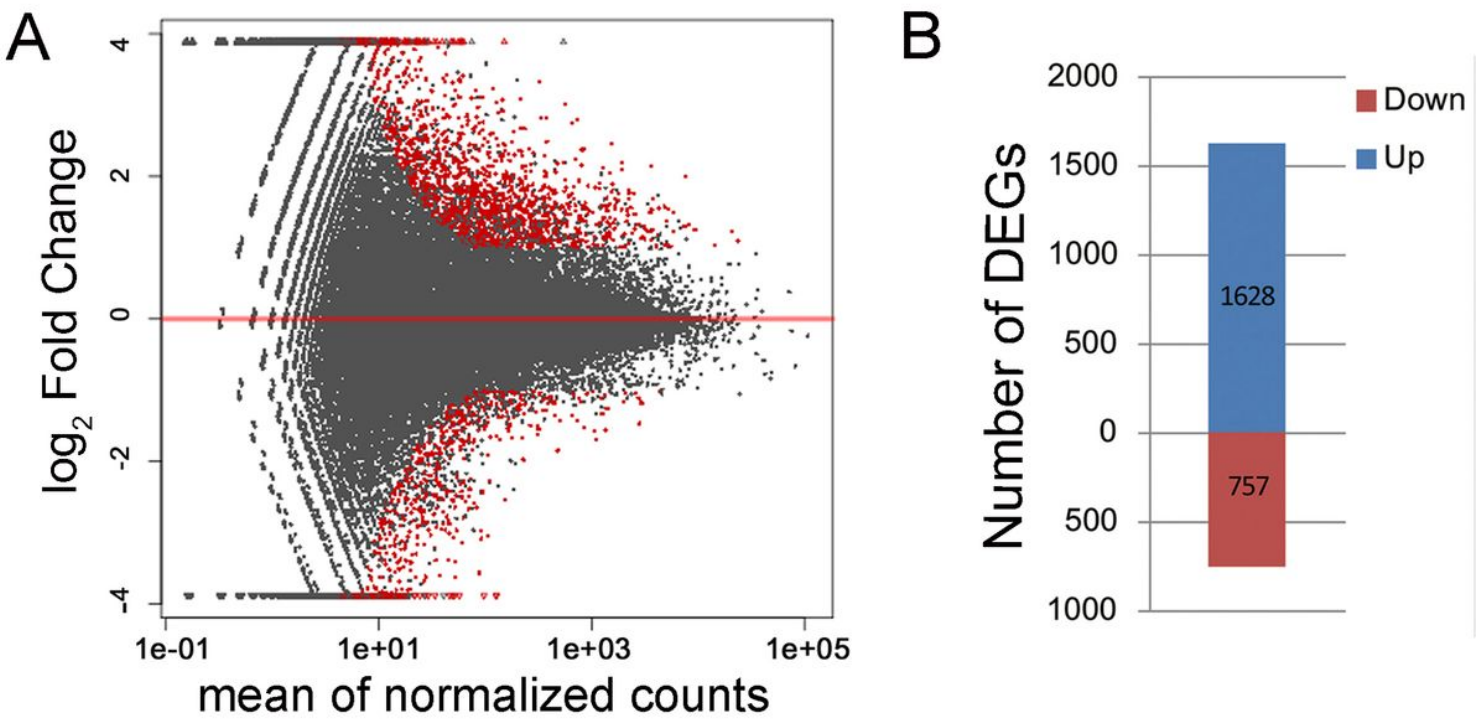


Figure 5

The DEGs between FER with ER. A. Fold change distribution of DEGs of ER vs. FER. The X axis is the normalized average expression value of all identified unigenes, and the Y axis is log2Fold Changes. The red color indicates significant DEGs with more than 2 fold changes. B. The DEG number of FER vs. ER. The number of up-regulated DEGs in FER was 1628, while the number of down-regulated DEGs was 757.

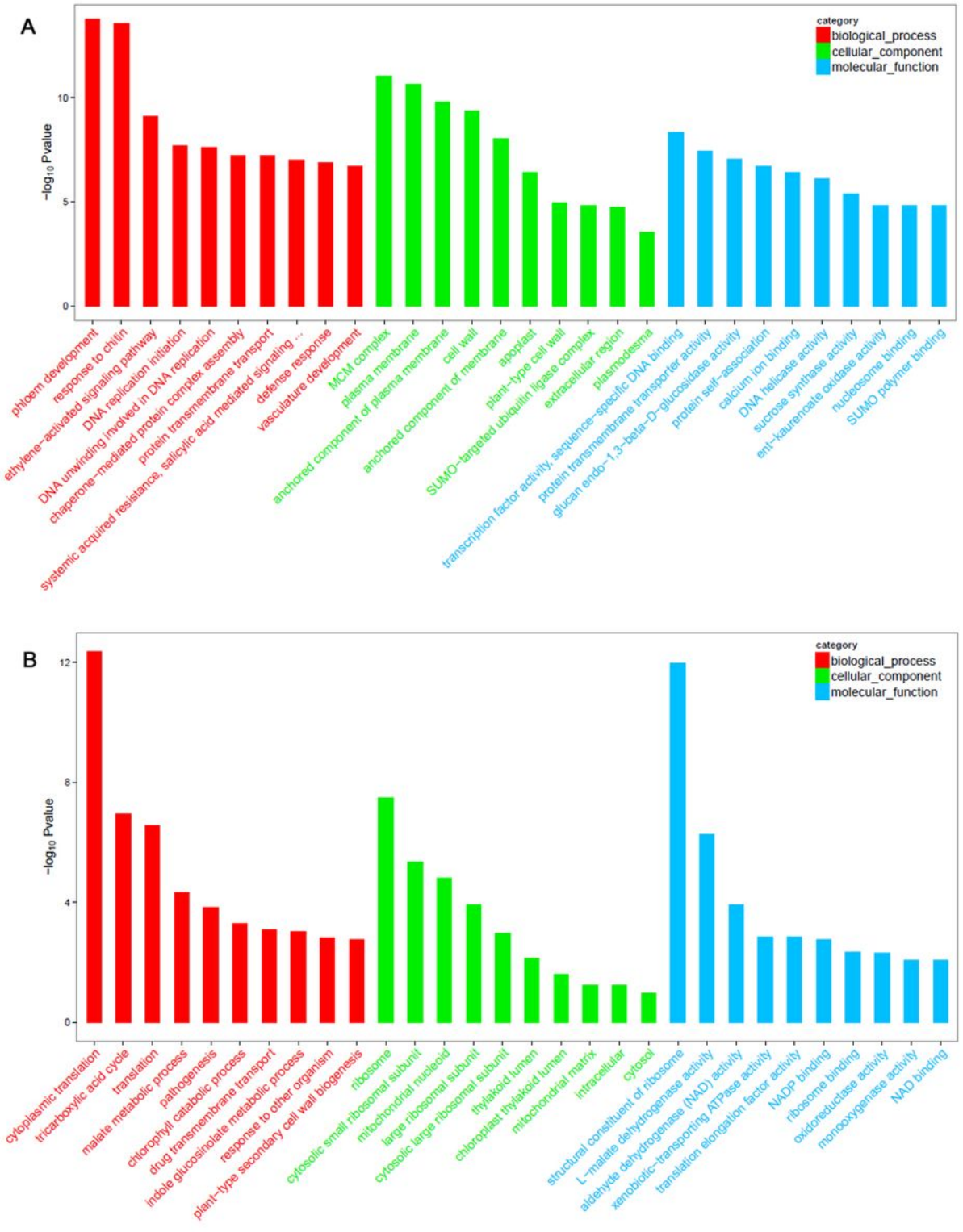


Figure 6

GO analysis of the DEGs between FER and ER bark of ramie. A. GO analysis of the up-regulated genes in FER comparing with ER. B. GO analysis of the down-regulated genes in FER comparing with ER. Top ten items were presented. Different colors represent different GO terms, e.g. Red for biological process, green for cellular component and blue for molecular function

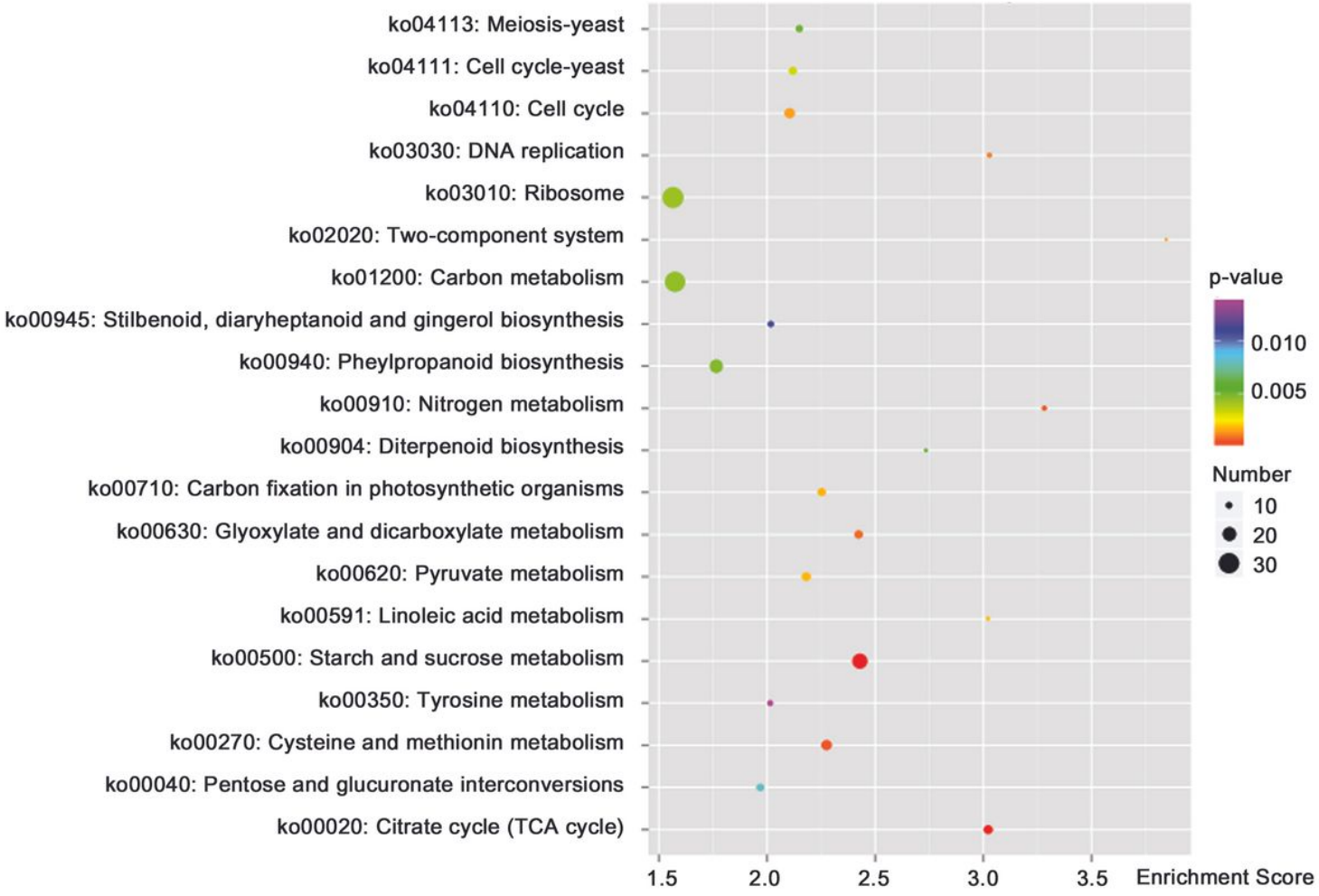


Figure 7

KEGG enrichment of the DEGs between FER and ER. Top 20 categories are shown. The X axis is enrichment score, and the areas of the bubbles indicate the DEG numbers, and the color variation of the bubbles from purple to red indicates decreasing P value.

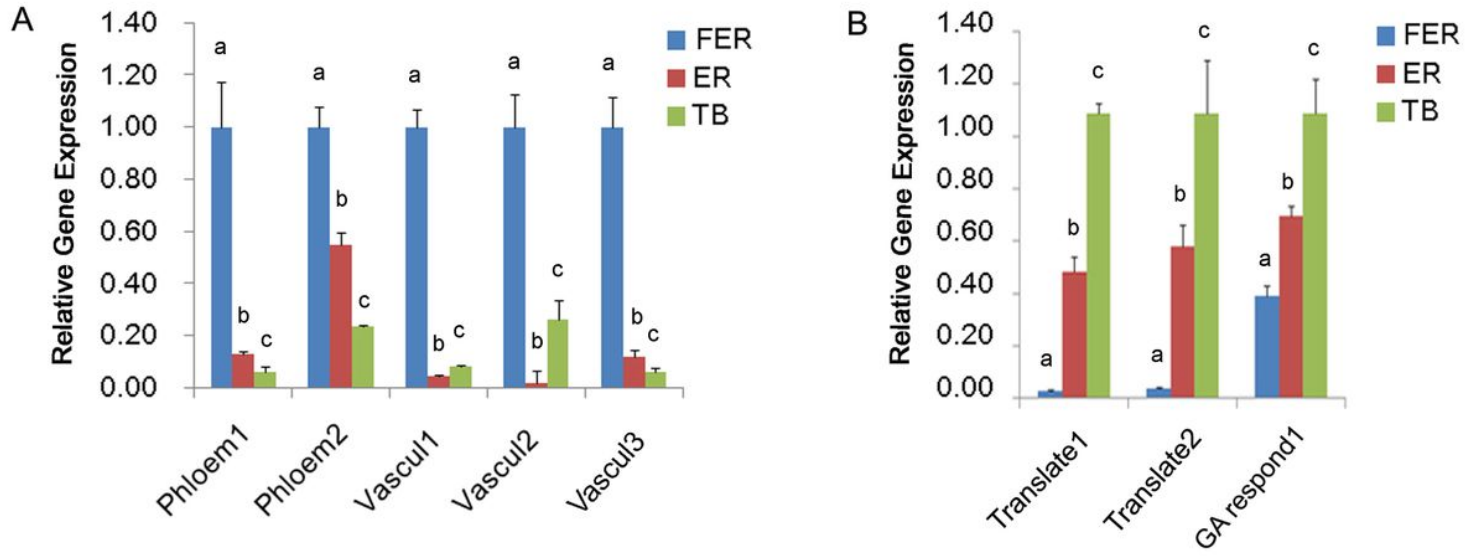
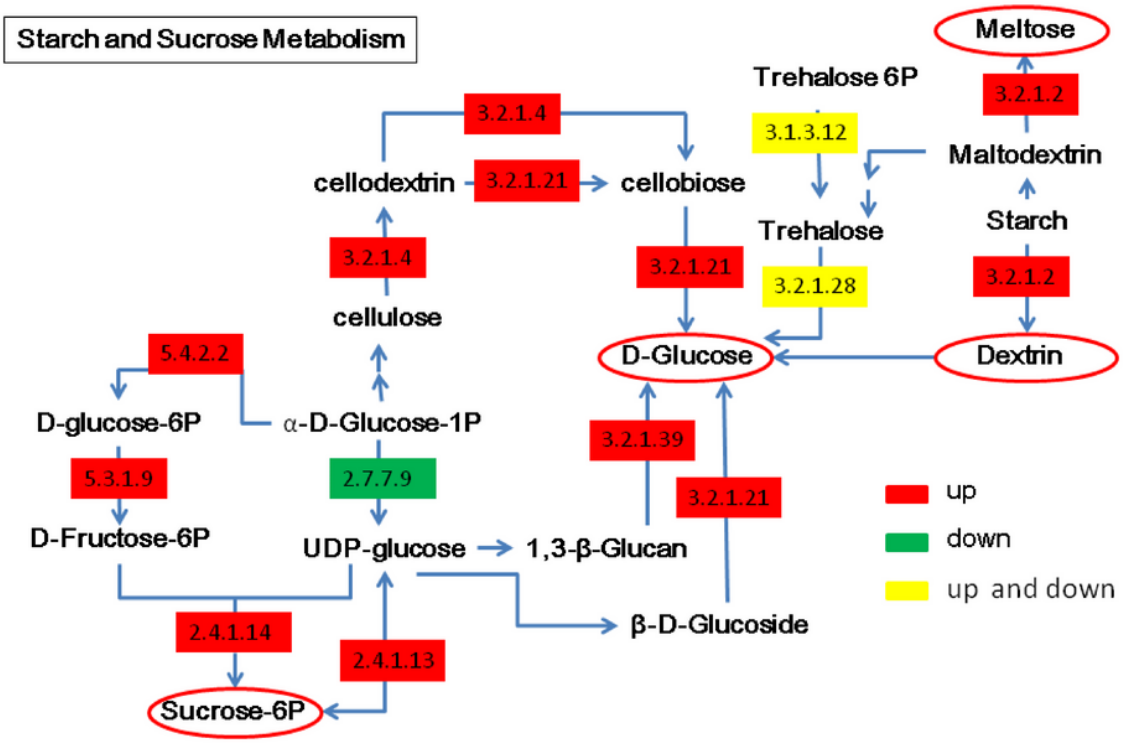


Figure 8

RT-qPCR detection of the selected DEGs among TB, ER and FER. A. Increased expression levels of some of the phloem and vasculature development related DEGs in the FER sample. B. Decreased expression levels of some of the translation and GA responsive factors in the FER sample. Error bars represent the value ±SD (n=3). The lowercase letters above the columns indicate the differences among samples by significance test.



2.4.1.13 CL25176Contig1: Sucrose synthase 7l FC=4.96
CL2876Contig1: Sucrose synthase 6; FC=7.54
CL35518Contig1: Sucrose synthase 7; FC=5.78

2.4.1.14 CL11248Contig1:Probable sucrose-phosphate synthase; FC=2.17
CL14166Contig1: Probable sucrose-phosphate synthase 1; FC=2.22

3.2.1.2 CL23372Contig1: Beta-amylase 1; FC=2.27

3.2.1.4 CL24271Contig1: Endoglucanase 8; FC=2.52
CL4763Contig1: Endoglucanase 24; FC=6.52
comp7326_c0_seq1_4: Endoglucanase 24; FC=3.01

3.2.1.21 CL279Contig2: Vicianin hydrolase; FC=7.06
comp39529_c0_seq1_3: Beta-glucosidase; FC=2.08
CL12993Contig1: Beta-glucosidase 44; FC=2.08
CL3213Contig2: Beta-glucosidase 45; FC=2.95

3.2.1.39 CL12826Contig1: Glucan endo-1,3-beta-glucosidase 6; FC=2.09

5.3.1.9 CL2080Contig1: Glucose-6-phosphate isomerase, cytosolic 1; FC=2.03

5.4.2.2 comp30066_c0_seq1_3: Phosphoglucomutase; FC=13.17

2.7.7.9 CL8067Contig1:
Probable UTP--glucose-1-phosphate uridylyltransferase; FC=0.14

3.1.3.12 comp50998_c0_seq15_4: Probable trehalose-phosphate phosphatase J; FC=2.24
CL3652Contig2: Probable trehalose-phosphate phosphatase J; FC=2.48
comp32417_c0_seq1_4: Probable trehalose-phosphate phosphatase 4; FC=8.27
CL18916Contig1: Probable trehalose-phosphate phosphatase 4; FC=0.14

3.2.1.28 comp48904_c0_seq12_5: Trehalase TRE1; FC=7.32
CL4476Contig1: Probable trehalase; FC=0.10

Figure 9

Different regulation in the starch and sucrose metabolism pathways between elongating and fully elongated regions of ramie stem bark. Multiple processes of free D-Glucose production might be enhanced in FER. Other sugar production processes through sucrose-6P, maltose and dextrin might also e increased in FER.

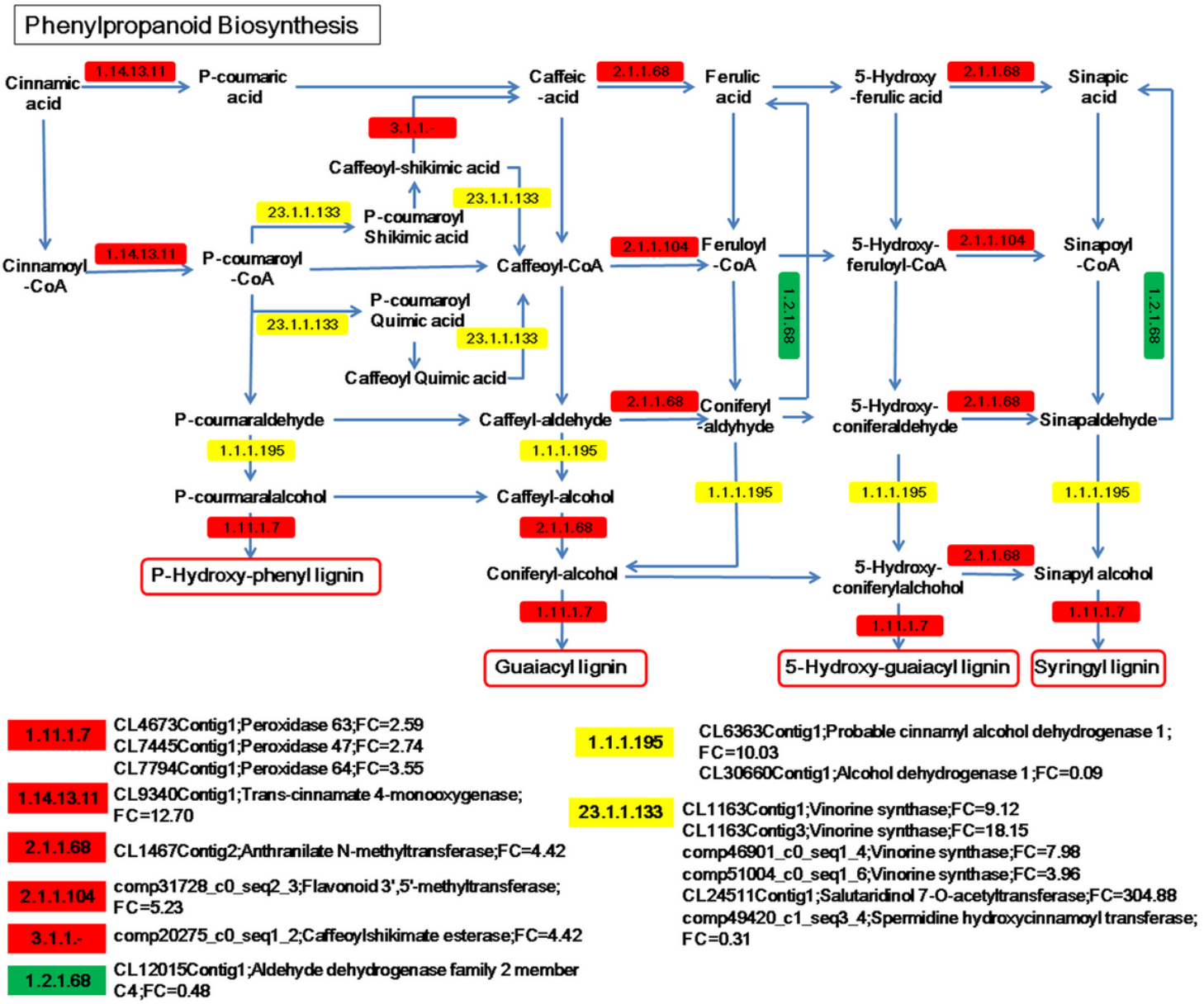


Figure 10

Up-regulation of lignin synthesis related genes of the phenylpropanoid biosynthesis pathways in FER. The unigenes encoding lignin synthesis enzymes including P-hydroxy-phenyl lignin, guaiacyl lignin, 5-Hydroxy-guaiacyl lignin and syringyl lignin were all up-regulated in FER. Red color stands for up-regulation, green color represents down-regulation and yellow means up or down regulation.

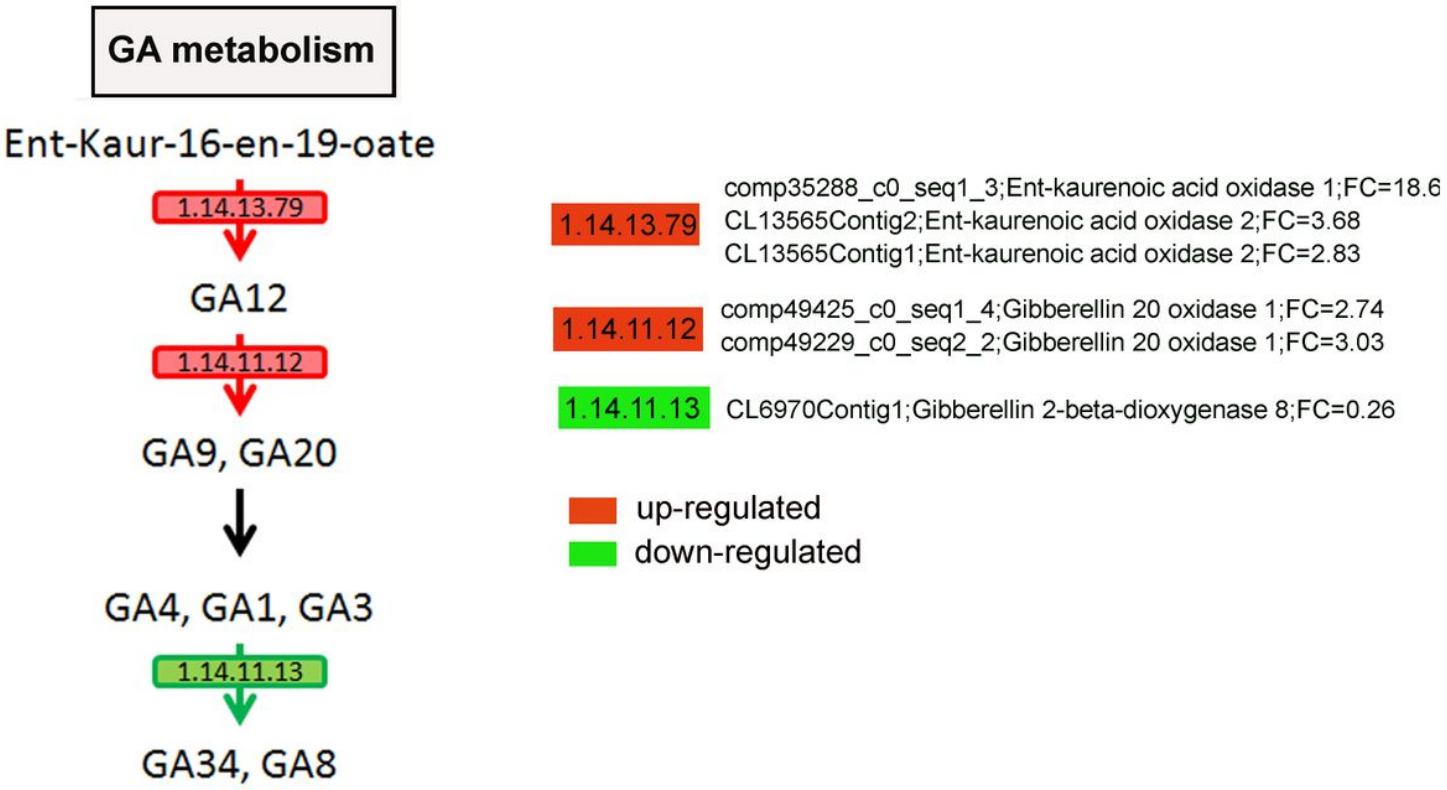


Figure 11

Altered expression of the key genes in the GA metabolism pathway. The unigenes encoding enzymes of Ent-kaurenoic acid oxidase (EC.1.14.13.79) and Gibberellin 20 oxidase (EC.1.14.11.12) were up-regulated in FER. And gibberellins 2-beta-dioxygenase (EC.1.14.11.13) was down-regulated in FER.

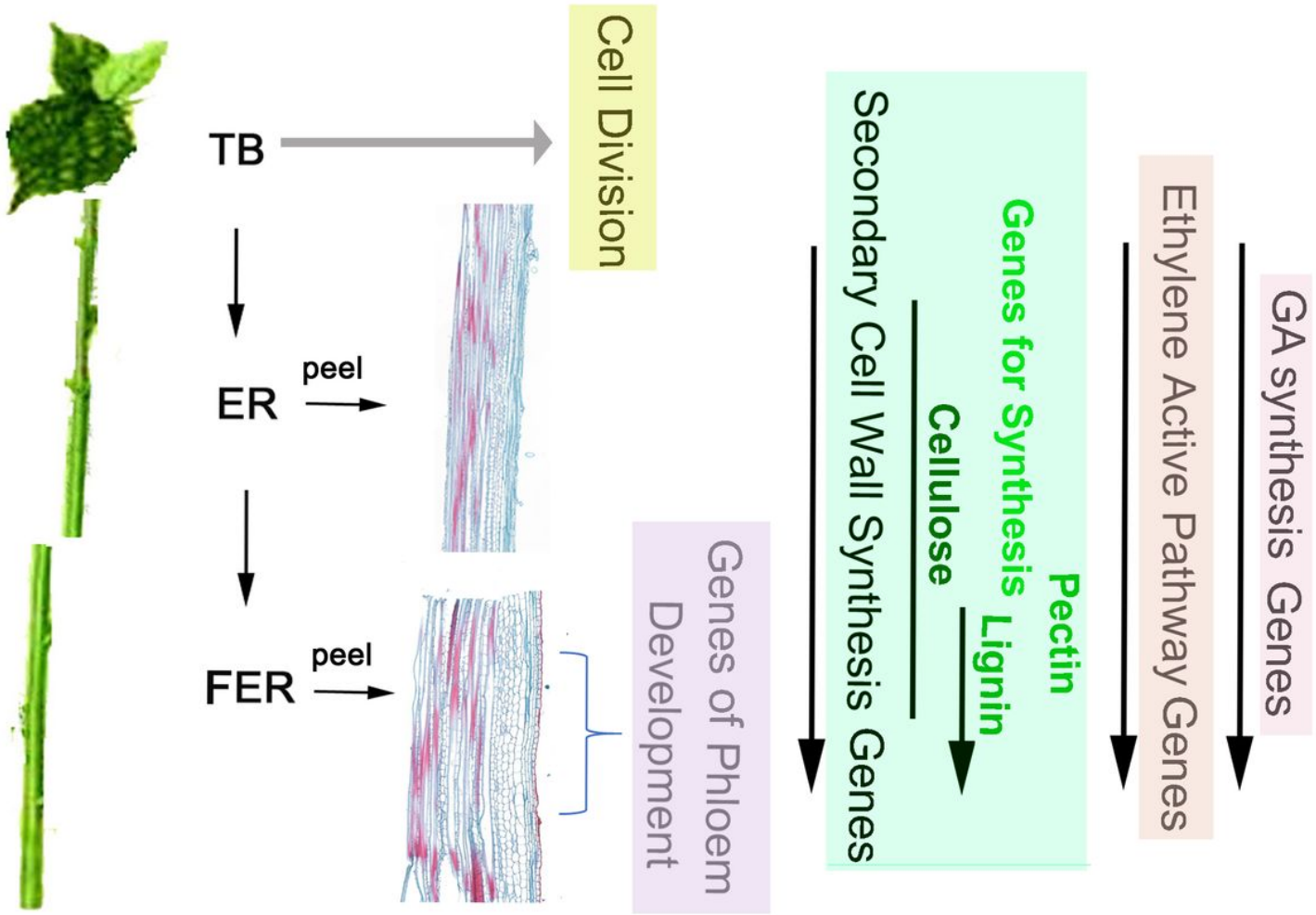


Figure 12

A schematic showing gene expression in TB, ER and FER in Ramie. Gene activation in FER for phloem development is clearly evidenced in our study. Secondary cell wall synthesis genes are up-regulated in both ER and FER but more activated in FER. The genes in the ethylene pathway and gibberellins acids biosynthesis are activated during the maturation of ramie stem.

Supplementary Files

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

- supplement1.pdf
- supplement2.pdf
- supplement3.pdf