A regulatory network driving shoot lignification in rapidly growing bamboo

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One-sentence summary
Multi-omics and other approaches reveal the regulatory network of miRNA-mediated ‘Transcription Factor-enzyme genes’ for lignification in moso bamboo, including 11 miRNAs, 22 TFs and 36 enzyme genes.

List of author contributions
Z.M.G. and K.B.Y. designed the experiments; K.B.Y. C.L.Z. and Y.F.L. collected samples; K.B.Y. and L.C.L. performed the experiments. K.B.Y. and X.P.L. analyzed the data. K.B.Y. and Z.M.G. wrote the manuscript. Z.M.G. obtained funding and is responsible for this article. Z.M.G. agrees to serve as the author responsible for contact and ensures communication.

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Abstract
Woody bamboo is environmentally friendly, abundant, and an alternative to conventional timber. Degree of lignification and lignin content and deposition affect timber properties. However, the lignification regulatory network in monocots is poorly understood. To elucidate the regulatory mechanism of lignification in moso bamboo (Phyllostachys edulis), we conducted integrated analyses using transcriptome, small RNA, and degradome sequencing followed by experimental verification. The lignification degree and lignin content increased with increased bamboo shoot height, whereas Phenylalanine Ammonia-Lyase (PAL) and Laccase (LAC) activities first increased and then decreased with shoot growth. Moreover, we identified 11,504 differentially expressed genes (DEGs) in different portions of the 13th internodes of different height shoots; most DEGs associated with cell wall and lignin biosynthesis were up-regulated, whereas some DEGs related to cell growth were down-regulated. We identified a total of 1,502 miRNAs, of which 687 were differentially expressed. Additionally, in silico and degradome analyses indicated that 5,756 genes were targeted by 691 miRNAs. We constructed a regulatory network of lignification, including 11 miRNAs, 22 transcription factors and 36 enzyme genes, in moso bamboo. Furthermore, PeLAC20 overexpression increased lignin content in transgenic Arabidopsis (Arabidopsis thaliana) plants. Finally, we proposed a reliable miRNA-mediated ‘MYB-PeLAC20’ module for lignin monomer polymerization. Our findings provide definite insights into the genetic regulation of bamboo lignification. In addition to providing a platform for understanding related mechanisms in other monocots, these insights could be used to develop strategies to improve bamboo timber properties.

Keywords: Phyllostachys edulis, lignification, lignin biosynthesis pathway, regulatory network, genetic elements

Introduction
With increasing urbanization, industrialization and socio-economic development, there is unabated environmental deterioration and resource demand. The ever-growing demand of non-renewable fossil-based materials has prompted the idea of using renewable lignocellulosic
biomass (Lin et al., 2020; Kalogiannis et al., 2020; Zuo et al., 2020). However, considering the depletion of forest resources and the increase of the protection of existing resources, there is a need to develop and utilize non-wood biomass. In this context, bamboo has become a promising lignocellulosic feedstock due to its abundance and short growth cycle (Qu et al., 2020; Zuo et al., 2020), and it is an essential alternative to wood because of its outstanding properties, such as excellent mechanical and tensile strength and high elasticity (Rasheed et al., 2020). The mechanical properties of bamboo are highly dependent on the lignification degree of the fibrous cell wall, which follows lignin biosynthesis and deposition in the secondary cell wall (SCW). Although lignification helps to improve the mechanical properties of bamboo, the presence of lignin is the major cause of lignocellulosic biomass recalcitrance in efficient industrial processing (Vanholme et al., 2012), and breaking the strong binding of lignin was the most problem to be solved for the utilization of lignocellulosic biomass (Dos Santos et al., 2018; Yu et al., 2019; Xu et al., 2020). The content and distribution of lignin is the foundation of its excellent mechanical property and the important basis of bamboo processing techniques. Therefore, feasible strategies to regulate the components of lignin in bamboo are needed, and a clear understanding of the regulation network of lignin biosynthesis and accumulation is important for improving the mechanical properties of bamboo; these strategies will help facilitate the development and utilization of bamboo resources.

Lignin is an aromatic cell wall polymer and second only to cellulose in terms of abundance in vascular plants, and it is deposited mostly in the walls of secondarily thickened cells and is essential for mechanical support, stress responses, and the formation of wood (Ployet et al., 2018; Ralph et al. 2019; Kushwah et al., 2020). Lignin biosynthesis is initiated by the deamination of phenylalanine into cinnamic acid through PAL, followed by catalysis with various enzymes and eventual oxidation by LAC or peroxidases to form the lignin polymer (Meents et al., 2018; Vanholme et al., 2018). Considerable progress has been made in understanding the genetic regulation of lignin biosynthesis, where a large number of compounds belonging to the NAC, MYB, and other transcription factor (TF) families have been found to form a complex transcriptional regulatory network (Hussey et al., 2013; Zhong
and Ye, 2015; Rao et al., 2019), in which the NACs and MYBs are the two top-level master switches that sequentially regulate downstream TFs (Lin et al., 2017; Geng et al., 2019; Gui et al., 2019; Wang et al., 2020). Additionally, these downstream TFs, such as CCCH Zinc Finger (CCCH) transcription factor, KNOX transcription factor, and APETALA2 / ethylene response factor (AP2 / ERF) transcription factor, further regulate the enzyme genes to form the hierarchical and non-hierarchical regulation networks for lignification (Chai et al., 2014; Wang et al., 2019; Wessels et al., 2019). Moreover, many miRNAs have been identified in this network, and their target genes, including many transcription factors and enzyme genes that play important roles in xylem development and lignocellulosic biosynthesis, have been well studied (Zhao et al., 2015; Sun et al., 2017). Previous studies have identified several genetic factors of lignin biosynthesis in rice (Oryza sativa), a representative monocotyledon, that regulate enzyme gene expression (Zhong et al., 2011; Ko et al., 2013a; Ko et al., 2013b). However, a comprehensive hierarchical transcriptional regulatory network of lignin in monocots is still lacking.

Although several approaches have been used to study the genetic regulations associated with lignin, RNA-seq, based on next-generation sequencing, has become the routine approach used for studies on bamboo (Li et al., 2018; Zhang et al., 2018). A total of 54, 39, 26, and 33 genes involved in the bamboo lignin biosynthesis pathway have been identified in Bonia amplexicaulis, Guadua angustifolia, Olyra latifolia, and Raddia guianensis, respectively (Guo et al., 2019). Although several studies have been performed on bamboo miRNAs (Wang et al., 2016; Cheng et al., 2019), there are no studies on genome-wide lignin biosynthesis-related miRNA identification performed in a tissue-specific manner. A few studies on MYB-, NAC-, and enzyme gene-related lignin biosynthesis have been performed to date (Li et al., 2017; Shan et al., 2019; Yang et al., 2019). However, the knowledge of the genetic factors that regulate moso bamboo lignin biosynthesis—particularly a comprehensive understanding of the regulatory network for lignification—remains poor. In the present study, an integrated approach using transcriptome, small RNA and degradome sequencing analyses was applied, along with morphological and physiochemical analyses, to investigate the mechanism of
lignification in moso bamboo shoots during fast growth. The differentially expressed genes (DEGs), miRNAs, and their targets were identified, and those involved in lignification were further analyzed and validated.

Results

Morphological and physiochemical changes in bamboo shoots during fast growth

Bamboo shoots are immature culms in which lignin accumulates gradually following the cessation of cell enlargement, leading to lignification occurring with an increase in the height. Representative samples of the 13th internode from shoots of varying heights of moso bamboo were investigated. Histological analysis showed that increased lignification was found along with the growth of bamboo shoots, which was indicated by an increased number of stained cells in the vascular bundle and a deeper degree of staining (Fig. 1A). The lignification negligibly varied between the 1.0 m and 2.0 m shoots, but a rapid lignifying process occurred in the 2.0 m to 4.0 m shoots, and after that, lignification continued to occur in the taller shoot samples. The lignin content in all three portions of the older shoot internodes was higher than that in the corresponding portions of the younger ones (p < 0.01) (Fig. 1B). Moreover, the lignin content in the top portion was higher than that in the other portions in the 1.0 m to 4.0 m shoots, but a similar lignin content was observed in all three portions of the 6.0 m shoots. Finally, the maximum lignin content appeared in the lower portion (122.67 mg/g), followed by that in the middle and top portions (99.40 and 90.78 mg/g) in the 8.0 m shoots, which corresponded well with the degree of lignification.

Since PAL and LAC are the two key enzymes of lignin biosynthesis, the activity of PAL and LAC in the representative samples of the 13th internodes of the bamboo was analyzed. The PAL activity in the 1.0 m and 2.0 m shoots was significantly lower than that in the higher shoots, and it significantly increased in the 4.0 m shoots to more than twice that in the 2.0 m shoots. Finally, the PAL activity in all three portions of the 8.0 m shoots showed a decreasing trend compared to that in the 6.0 m shoots, and the largest decline was found in the top portion (Fig. 1C). The LAC activity in all three portions showed a sharply rising trend in the
1.0 m to 2.0 m shoots, and the LAC activity in all three portions was similar in the 6.0 m and 8.0 m shoots, but a slight declining trend was observed in the 6.0 m to 8.0 m shoots (Fig. 1D). These results indicated that the lignification degree in all three portions of the same internode was not consistent, but all three portions demonstrated a similar trend of lignification—lignification deepening gradually with shoot height increasing.

**Detection of DEGs during shoot lignification, based on transcriptome sequencing**

A total of 4,418.03 million raw reads were generated from 13 samples, and 96.28% (4,253.87 million) of the reads representing high-quality reads were processed for further analysis after quality control. On average, 94.84% of the clean reads were mapped on the reference genome of moso bamboo (Zhao et al. 2018) (Supplemental Table S1). A gene was considered to be expressed in a given sample if its FPKM (Fragments per Kilobase Million) was ≥ 1. Out of the 44,970 genes detected, a total of 31,516 genes were expressed in at least one sample, with the number of expressed genes initially increasing and then decreasing as the shoot height increased (Supplemental Fig. S1A).

A total of 25,655 genes were identified as DEGs, which were expressed to be substantially different in at least one comparison. The number of DEGs ranged from 113 (52 up-regulated and 61 down-regulated) in S-8-M VS S-8-L to 17,584 (6,641 up-regulated and 10,943 down-regulated) in S-2-M VS S-4-M. On comparing the same portions of the 13th internode in shoots of varying heights, the number of DEGs initially increased and then decreased, with a peak in S-2 VS S-4. The differences among the number of DEGs in the top portions of the shoots of varying heights were smaller than those observed for the other portions (Supplemental Fig. S1B). Moreover, the differences in the number of DEGs between the top and lower portions were larger than those observed for the other comparisons, and the differences among the number of DEGs in all the three portions were observed to gradually decrease with an increasing shoot height in comparison to those observed for the different portions in the same internode (Supplemental Fig. S1C). Compared to the prior height shoots, a total of 13,924 DEGs were detected in all the portions. A total of 17,487 DEGs were found by comparing the different portions of the same internode. Overall, there were 11,504 DEGs
shared among the shoots of varying height and different portions of these shoots, suggesting that there is an active and dynamic internal mechanism functioning at the transcriptome level during the fast growth of bamboo shoots.

**Functional enrichment analysis of the DEGs**

Out of 11,504 DEGs, 8,381 DEGs were assigned to a total of 5,610 GO terms, including biological process (3,389 GO terms), molecular function (1,582 GO terms), and cellular component (639 GO terms). The biological processes with a high DEG number were cellular process (GO:0009987), metabolic process (GO:0008152), single-organism process (GO:0044699), biological regulation (GO:0065007), and regulation of biological process (GO:0050789). Within the cellular component category, a large number of DEGs were categorized as cell (GO:0005623), cell part (GO:0044464), organelle (GO:0043226), membrane (GO:0016020), and membrane part (GO:0044425). As for the molecular function, the most representative terms were binding (GO:0005488), catalytic activity (GO:0003824), transporter activity (GO:0005215), and structural molecule activity (GO:0005198) (Fig. 2A).

The DEGs were found to represent a total of 123 pathways by mapping to the KEGG reference pathways. With the exception of the ribosome pathways (ko03010), the phenylpropanoid biosynthesis pathway (ko00940) was one of the enriched pathways, with most of the DEGs participating in lignin biosynthesis. Additionally, many DEGs were mapped to the biosynthesis of secondary metabolites (ko01110) and metabolic pathways (ko01100), with most of these involving the secondary metabolites necessary for growth and development. The DEGs related to carbohydrate and cellulose biosynthesis were significantly enriched in flavonoid biosynthesis (ko00941) and starch and sucrose metabolism (ko00500) (Fig. 2B).

These results further support that the lignification process occurs with the shoot height increasing.

**Expression profiles of the DEGs during lignification**

After clustering analysis, 11,504 DEGs were clustered into four major clusters based on similar expression profiles among the samples (Supplemental Fig. S2). With the shoot height
increasing, a trend of down-regulation was observed for the DEGs in Cluster I, in which DEGs, such as those encoding centromeric protein E (PH02Gene06389), DNA binding protein (PH02Gene20121), cell division control protein 45 (PH02Gene18663), and so on, were involved in cell organization, cell division, cell cycle, and cell development (Fig. 3). In Cluster II, the DEGs showed a trend of increased expression and were identified to be related to the deposition and formation of SCW, biosynthesis of secondary metabolites, various transport pathways and signaling pathways, and as those dramatically enriching lignin biosynthesis, transport of nitrogen compounds and transmembrane transport of amino acids, like the PAL gene (PH02Gene27016) and LAC gene (PH02Gene03534). The DEGs in Cluster III showed a trend of initially increasing and then decreasing expression, and were involved in cell wall organization or biogenesis and cell wall macromolecule biosynthesis processes. A large number of DEGs in this cluster were found to be associated with xylem biosynthesis, catabolic and metabolic processes, as well as hemicellulose and cellulose metabolism processes (Fig. 3). Cluster IV included genes with complex and diverse expression profiles (Supplemental Fig. S2B). Furthermore, KEGG analysis results were consistent with those of the GO enrichment analysis, as phenylpropanoid biosynthesis was one of the significantly enriched KEGG pathways in Cluster II (Fig. 2C; Supplemental Fig. S3).

**TFs participating in the regulation of lignin biosynthesis**

TF-mediated transcriptional regulation plays vital roles in the plant lignification process. In this study, 3,823 TFs belonging to 59 families were differentially expressed in at least one comparison, among which the top ten families with the most members were MYB (483), MYB-related (377), AP2-EREBP (303), NAM, ATAF, and CUC (NAC) (275), Basic helix-loop-helix (bHLH) (276), WRKY (191), GRAS (148), C2H2 (125), MADS (111), and CCCH (104) (Supplemental Fig. S1D). After removing low-abundance genes, 30 candidate TF genes were found in moso bamboo, which were homologous to the TFs found in the three tiers of the lignin regulatory networks in Arabidopsis (Supplemental Table S2). Almost all the TF genes shared a similar trend of up-regulated expression, initially increasing and then decreasing, but only a few increased continuously with increasing shoot height (Fig. 4). Four
NST/SND TFs were identified as the master regulators, and these showed a high expression in older shoots. However, MYBs as subordinate regulators showed a similar expression trend with their target gene (NST/SND). The expression of four MYBs in tier two of the lignin regulatory network increased gradually from young shoots to older ones, with a low level in the 1.0 m and 2.0 m shoots. However, the expression gradually increased from the top to the lower portion of the same internode. The TF genes belonging to tier one of the lignin regulatory network showed similar expression profiles, suggesting that all of them had spatiotemporal specificity during the lignification process.

**Enzyme genes involved in lignin biosynthesis**

A total of 280 enzyme genes belonging to 12 families involved in lignin biosynthesis in moso bamboo were found and 153 DEGs were detected, out of which 48 genes were homologous with those well-studied in *A. thaliana* (Supplemental Table S2). The expression analysis based on the heatmap showed that the expressions of almost all these genes were extremely low in the S-1 samples, specifically expression of LACs and PRXs, which participate in the final stage of lignin biosynthesis, were barely detected (Fig. 4). This result was consistent with the absence of lignified cells in the S-1 samples. Thereafter, these genes were activated with higher levels, especially in the 4.0 m shoots, which also displayed a substantially fast lignification. In the 8.0 m shoots, expression of 43 DEGs declined substantially in all the portions. Notably, the expression of upstream genes occurred earlier and stronger than that of downstream genes in the lignin biosynthesis pathway.

**Identification of miRNAs by sequencing**

To identify the miRNAs related to lignification in moso bamboo during fast growth, 13 small RNA libraries were constructed and sequenced. A total of 588.71 million clean reads were obtained after filtering the low-quality reads (Supplemental Table S1). The length distribution of the small RNA reads indicated that 21 nt (46.94%) was the most abundant, followed by 24 nt (20.51%), 22 nt (15.85%), and 23 nt small RNA (9.92%) (Supplemental Fig. S4; Supplemental Table S3). To find the miRNAs in moso bamboo, the filtered reads were
searched against the miRNAs in the miRbase database. A total of 1,502 miRNAs were obtained, out of which 1,223 miRNAs were matched to known miRNAs and 279 miRNAs were predicted to be unknown miRNAs (Supplemental Table S3). Further analysis showed that the identified miRNAs were grouped into 237 families by similarity-based clustering, and the members of each family varied obviously. Only 25 out of the 237 families had more than 10 members, among which Ped-miR166 was the largest family with 59 members, followed by Ped-miR159 (58) and Ped-miR369 (46). By contrast, 160 families had only one or two members, such as Ped-miR161, Ped-miR173, Ped-miR1023, and Ped-miR1024. Furthermore, 94 unknown miRNAs of moso bamboo were clustered with some known miRNAs in other species, indicating that they might be recently evolved members of the families.

**Differentially expressed miRNAs (DEMs) during lignification**

To detect the miRNAs involved in the lignification process, the expression patterns of the identified miRNAs were further analyzed. The results showed that most of the identified miRNAs were expressed in more than one sample. Besides, a total of 687, including 441 known and 246 unknown miRNAs, were found across different comparisons, and these had expression trends similar to that of the DEGs as a whole. According to the transcript per million (TPM) of the DEMs among 13 libraries, we merged the DEMs of similar height shoots together to perform cluster analysis, which were clustered into four major clusters according to similar expression profiles (Supplemental Fig. S5-6). As shoot height increased, the DEMs in cluster I showed a downward trend, while those in cluster II had an upward trend, and the trend of a large amount of DEMs initially increased and then decreased in cluster III. Similar to the DEGs, DEMs with complex expression profiles were clustered in cluster IV. In addition, some DEMs had exactly opposite expression trends compared to those of the DEGs, suggesting that they might have a potential negative regulatory relationship.

**miRNA target prediction via in silico and degradome sequencing analyses**

According to the identified miRNAs and the annotated mRNA sequences of moso bamboo, we identified 25,350 targets for 1,430 (1188 known and 242 unknown) miRNAs using the
TargetFinder software. A total of 30,378 miRNA-target pairs, consisting of 610 miRNAs and 7,940 genes, were identified. Furthermore, a total of 61.53 million clean tags were obtained from degradome sequencing and used to identify the cleavage sites (Supplemental Table S4). The results showed that a total of 135 genes were targeted by 262 miRNAs, constituting a total of 1,216 miRNA-target pairs. The cleavage sites in 135 targets with a $P$-value $\leq 0.05$ were detected and presented in the form of target plots. Through in silico and degradome sequencing analysis, a total of 7,972 genes were identified as the targets for 718 (532 known and 186 unknown) miRNAs. The miRNAs, together with their target genes involved in multiple biological processes, build the corresponding regulatory networks, such as the regulation of nitrogen compound metabolic process and organic cyclic compound metabolic process (Supplemental Fig. S7). In silico and degradome sequencing shared 103 genes targeted by miRNAs, providing reliable insights into targeted gene expression regulation by miRNAs in moso bamboo shoots during fast growth, which also enabled us to find the miRNA-mRNA pairs involved in regulation of shoot lignification.

**Co-expression network of genes involved in lignification**

The interactions of miRNA-mRNA can be either coherent or non-coherent (Garg et al., 2019). Out of 30,378 miRNA-target pairs, 16,467 coherent pairs (which were collectively constituted by 5,756 genes and 691 miRNAs) were identified with the opposite expression tendency. Some coherent pairs were validated by degradome sequencing, such as PH02Gene04996 and PH02Gene45333, which were cleaved by Ped-miR397a.1 (Supplemental Fig. S8). To investigate the gene regulatory network of lignin biosynthesis in bamboo shoots, we performed a weighted gene co-expression network analysis (WGCNA). The DEGs were clustered into 14 major tree branches, where each one represented a module (labeled with different colors) (Supplemental Fig. S9A). Modules are clusters of genes with high correlations, and genes within the same module are co-expressed. Further analysis revealed that the green, bisque4, maroon, dark grey and coral1 modules had a high positive correlation with lignification (Supplemental Fig. S9B), indicating that the genes in these modules might play an important role in the biosynthesis of lignin in shoots.
Combining the analysis of the lignin biosynthesis pathway (Fig. 4), WGCNA, and coherent miRNA-target pairs, we found 22 TFs and 36 genes were retained, and PeCCoAOMT2 and PeCCoAOMT3 were replenished in the network. Further analysis showed that the SCW NAC binding element (SNBE), AC element, and SCW MYB-responsive element (SMRE) were found in the promoters of four MYBs and 36 key enzyme genes (Supplemental Table S5). By integrating all the results, we constructed a regulatory network of the genetic elements in the lignin biosynthesis pathway (Fig. 5), including 11 miRNAs and 22 TFs associated with 36 enzyme genes. Three NST/SND TFs were identified as the master regulators that promote this regulatory network by regulating PeMYB103, with 17 downstream TFs directly regulating enzyme genes from 12 families (Fig. 5). In addition, the co-expression analysis supported that all the 22 TFs were significantly co-expressed with at least one of the key enzyme genes in the lignin biosynthesis pathway (Supplemental Fig. S10). These results indicate that there might be a regulatory relationship between the TFs and the key enzyme genes during bamboo shoot lignification, which provides useful information for the analysis of the genetic regulation of lignin biosynthesis.

**Validation of the genetic elements in the regulation network for lignification**

In order to verify the lignification regulation network, RT-qPCR, degradome sequencing, and yeast one-hybrid assays were used. The expression levels of 11 miRNA-mRNA pairs in the network were examined by RT-qPCR, and opposite expression trends of miRNA and mRNA in each pair were observed, which was similar to the results of the high-throughput sequencing (Fig. 6A). Some of the miRNA-mRNA pairs, such as Ped-miR397b.1 / Ped-miR397b.3-PeLAC35 (PH02Gene45333) and Ped-miR397b.1 / Ped-miR397b.3-PeLAC10 (PH02Gene04996) were further validated by degradome sequencing. In addition, most miRNA-mRNA pairs were coupled at the late stage of rapid growth (6.0 m and 8.0 m), which was consistent with the results of the degradome sequencing (Supplemental Fig. S8). Besides, we had identified PeLAC20 targeted by Ped-miR397 in a previous study (Li et al., 2017). Additionally, the RT-qPCR results of some key TFs showed that their expression patterns were similar to their targeted lignin biosynthesis genes, with trends initially increasing and then
decreasing or continuously increasing along with increasing shoot height of moso bamboo (Supplemental Fig. S11). These results indicated that the enzyme genes were positively regulated by the TFs, which was supported by the analysis of regulatory elements in the promoter sequences of the enzyme genes (Supplemental Table S5) and the results of the yeast one-hybrid assays (Fig. 6B). These results provide evidence for the relationship between the genetic elements in the regulatory network of bamboo lignification.

Furthermore, a total of 43 LAC genes (PeLAC1 ~ PeLAC43) were identified in the genome of moso bamboo. PeLAC20, sharing a higher amino acid sequence similarity with AtLAC4, which contributed to the constitutive lignification of Arabidopsis stems (Supplemental Fig. S12) (Berthet et al., 2011), was selected and its function was investigated by using the overexpression vector of PeLAC20 (Supplemental Fig. S13A). Four independent active transgenic Arabidopsis plant lines overexpressing PeLAC20 were obtained and validated by semi-quantitative PCR (Supplemental Fig. S13B). Subsequently, two transgenic lines (L1 and L3) were selected for lignin analysis, and the results showed that the stems of L1 and L3 had vascular bundles that stained deeper and contained more lignin than Col-0 (Fig. 7A-B), indicating that the overexpression of PeLAC20 could promote lignification in Arabidopsis.

Besides, GS115 of Pichia pastoris harboring PeLAC20 exhibited a dark green color on a substrate containing ABTS, which was characterized by the copper ion-binding protein (LaFayette et al., 1999) (Supplemental Fig. S14). The highest enzyme activity of the recombinant protein was 2.031 U/mL after methanol induction for 48 h.

Moreover, to confirm the interaction of PeMYB4.1 and PeMYB20/85.2 with the PeLAC20 promoter, the whole promoter and promoter fragments containing either native or mutated AC-elements were used separately for yeast one-hybrid assay and electrophoretic mobility shift assay (EMSA). PeMYB4.1 and PeMYB20/85.2 can bind to PeLAC20 promoter as validated by yeast one-hybrid assay on a triple-dropout medium, indicating that PeMYB4.1 and PeMYB20/85.2 could bind to the PeLAC20 promoter in vivo. (Fig. 7CD). EMSA result showed that the binding efficiency was gradually reduced by the addition of increasing amounts of unlabeled native fragment probes (cold probe) (Fig. 7E). Therefore, a reliable
miRNA-mediated ‘MYB-PeLAC20’ module for lignin monomer polymerization has been proposed for the regulation of lignification in bamboo shoots (Fig. 8).

**Discussion**

The culm is the most utilized part of bamboo, and its morphogenesis undergoes three important periods, namely the primary shoot growth which happens underground, the fast shoot (immature culm) growth which happens aboveground, and the continuous lignification of the fibrous cell wall in the culm (Wei et al., 2018; Wei et al., 2019; Zhu et al., 2020).

Importantly, the fast growth of bamboo shoots is accompanied by distinct lignification which is dominated in the fiber bundles. The lignified bamboo fibers possess high intensity, toughness, high rigidity and stability, which are the main factors reflecting the physical and mechanical properties of bamboo culm. Generally, lignification of bamboo is believed to be a process of dynamic lignin deposition, which begins after the formation of the primary cell wall. The fast growth of bamboo is mainly attributed to each internode, whose lignification contributes to the properties of bamboo. Therefore, systematically investigating the lignification in a representative single internode of the shoots aboveground during fast growth will elucidate the regulatory mechanism of bamboo lignification.

**Dynamic changes of bamboo shoots undergoing lignification**

Based on the comprehensive analyses of morphology, anatomy, and physiology, together with multi-omics sequencing, the lignification process in bamboo shoots during fast growth could be divided into three phases, and a special transition process of lignification was discovered in a single internode. In phase I, the lignification proceeded at a very slow rate from 1.0 m to 2.0 m shoots, in which most of the cells were dividing and elongating instead of lignifying, and all the activities of enzymes involved in lignin biosynthesis were at low levels. In phase II, the lignification was at a faster rate in shoots from 2.0 m to 6.0 m, in which cell division and elongation tended to end, and cell wall thickening was predominant, along with the activities of enzymes involved in lignin biosynthesis and deposition increasing. In phase III, the lignification continued further in the shoots taller than 6.0 m at a relatively lower rate than that
of Phase II, and the shoots gradually grew into culms with higher lignin content, in which the cell extension had completed and the activities of lignin biosynthesis-related enzymes were maintained at high levels.

The lignification rate in shoots during fast growth went through a process of rising first and then falling, which was in accordance with the analyses of histochemistry and lignin content (Fig. 1). The tendencies of PAL and LAC activities were similar, along with the fluctuating lignification rate, which was verified by the change in the numbers of DEGs. Although there is no clear boundary between the three phases involved in the completion of elongation and the initiation of lignification, these processes are simultaneous, and two distinct characteristics of lignification were found. One was the change of lignification from a relatively stable state at a low rate initially in the 2.0 m shoots, to a rapidly deepened state in the 4.0 m shoots, and the second was that the lignification process gradually deepened, with a slowly falling rate in the shoots higher than 4.0 m. Interestingly, the number of DEGs in the S-2 VS S-4 was more than the other comparisons, and it dropped sharply in the S-6 VS S-8, indicating these two stages are very important in the lignification process of bamboo shoots.

Moreover, we found that a similar biological process occurred spatiotemporally in the three portions of the same internode during lignification, which led to the three portions being in different degrees of lignification at the same time. In phase I and II, the degree of lignification decreased from the top to the lower portion, which was consistent with the findings of cell elongation that occurred and completed sequentially from the top to lower portion in the same internode (Cui et al., 2012). However, the degree of lignification was opposite in the 8.0 m shoots, in which that of lower portion was higher than that of the middle and top portions, supported by the lignin content analysis (Top < middle < lower) (Fig. 1). This may be related to the senescence and shedding of sheaths. The sheath initially functions as the protector and carbohydrate supplier for bamboo shoots (Chen et al., 2020), then it gradually ages as the degree of internode lignification increases, and it finally sheds when the internode is strong enough to support the whole bamboo culm. However, the relationship between lignification
and sheath senescence is still unclear, and further studies are needed to establish this relationship.

**Spatiotemporal expression changes of genes encoding enzymes involved in lignin biosynthesis**

The spatiotemporal expression of genes contributes to the dynamic changes of biological processes. The expression of genes related to cell division kept decreasing in moso bamboo shoots during fast growth, but the lignification-related genes were continuously upregulated from phase I to phase III (Fig. 3), which was consistent with a previous study done using a single internode of *Bambusa multiplex* (Wei et al., 2019). Lignification depends on the biosynthesis of lignin, which involves multiple metabolic pathways (Pascual et al., 2016). The most important pathway among the aforementioned pathways is the phenylpropanoid metabolic pathway (ko00360 and ko00940) (Cao et al., 2019; Su et al., 2019), which is supported by our GO and KEGG pathway analyses (Fig. 2; Supplemental Fig. S3). Great progress has been made in the study of lignin biosynthesis during the last decade, and 10 genes families associated with lignin biosynthesis have been identified and two oxidase families have been discovered (Vanholme et al., 2018; Adams et al., 2019). In this study, 280 genes involved in lignin biosynthesis were identified and characterized, which was more than the number involved in Arabidopsis (112), Poplar (*Populus trichocarpa*) (193), and rice (126), indicating that some gene families might undergo gene expansion events (Hamberger et al., 2007; Carocha et al., 2015) (Supplemental Table S6). These expanded genes in the different families of moso bamboo might account for its fast lignification and excellent mechanical properties.

Furthermore, the comparison of DEGs suggested that lignin biosynthesis in moso bamboo was more complex than in other species. Forty-eight DEGs in the moso bamboo lignin biosynthesis pathway had high expression levels, indicating that they were the main transcripts and candidates for lignin biosynthesis. Furthermore, their expression patterns were consistent with those observed during the dynamic change trend of shoot lignification. For example, PAL catalyzes the deamination of L-phenylalanine into trans-cinnamic acid and...
directs the carbon flux from primary metabolism into the phenylpropanoid metabolism (Gho and Jung 2019; Lu et al., 2019). The PePALs were up-regulated, which was consistent with the findings in Populus (Tsai et al., 2006). The transcriptional levels of five differently expressed PePALs were higher than the other DEGs in the early stage of bamboo shoot growth, and it was possible that they were activated to provide a large amount of cinnamic acid, as it is the entry point into the phenylpropanoid pathway, for downstream lignin biosynthesis (Vogt, 2010). The gene expression profiles perfectly synchronized with the changing trend of lignin content and enzyme activities. Enzyme genes dynamically expressed with high spatiotemporal specificity, followed by the dynamic changes of enzyme activities, leading to spatiotemporal lignification differences within a single internode of bamboo shoot.

**Universality and particularity of bamboo TFs and miRNAs in regulating lignification**

To date, a multi-tier regulatory network for lignification has been identified in Arabidopsis, including diverse miRNAs and TFs that can activate or inhibit the expression of enzyme genes related to lignin biosynthesis at both the transcription and post-transcriptional levels, resulting in dynamic lignification changes (Zhong and Ye, 2015; Lin et al., 2018; Hossain et al., 2018; Quan et al., 2019). The NST/SND TFs acted as the master switches for the initiation of lignin biosynthesis through regulating the MTB TFs (Takata et al., 2019; Zhang et al., 2019). Our study showed that six NACs and 13 MYBs interacted with enzyme genes involved in lignin biosynthesis, and most of the upstream regulators exhibited a strong correlation of expression with their putative target genes (Supplemental Fig. S10). The downstream TFs and enzyme genes had similar expression patterns compared to those of the upstream TFs (Fig. 4; Supplemental Fig. S11), suggesting that they had a regulatory relationship and affected lignification, which was indirectly verified by RT-qPCR and the yeast one-hybrid assay (Fig. 6; Supplemental Fig. S11). This result was consistent with the NACs that are the key regulators to initiate fiber cell SCW thickening in Arabidopsis (Mitsuda et al., 2005; 2007). Moreover, the enzyme genes targeted by miRNAs were also validated by degradome sequencing and RT-qPCR.
Nine MYBs that were associated with the lignification of bamboo shoots were identified in this study to positively and directly regulate the spatiotemporal expression of enzyme genes. Besides, four PeMYB4s were identified as subordinate regulators with high expression levels in bamboo shoots. This could be explained by the metabolic feedback regulation, which was consistent with previous studies done using Arabidopsis (Jin et al., 2000; Wang and Dixon 2012). Our results indicated that these TFs collectively regulate lignification in shoots during fast growth in moso bamboo, which contributed to the flexibility and complexity of the regulatory network. In addition, AtMYB26 plays an essential role in regulating SCW formation as the epistatic regulator of NAC (NST, VND, and SND) (Zhao et al., 2014), and the AtMYB26 and AtVND6/7 homologous genes in moso bamboo shared low sequence similarity (<40%) with their corresponding homologs in Arabidopsis, and their expression levels were extremely low in the bamboo shoots. A possible reason for this might be that these genes are mainly expressed in other tissues but not in the shoots. The findings from rice and A. thaliana support the hypothesis that AtMYB26 and AtVND6/7 are specifically expressed in the anther endothecium nuclei and flower tissues (Yao et al., 2012; Yang et al., 2017). These results suggested that the regulatory relationship between MYBs and NACs in the shoot is not clear.

Furthermore, some miRNAs play multiple important roles in the lignification process at the post-transcriptional level by regulating the targets of TFs and lignin biosynthesis genes (Shafrin et al., 2015; Sun et al., 2018; Fan et al., 2019; Quan et al., 2019). MiR397-mediated lignin biosynthesis via regulating LAC genes is a common mechanism in plants (Wang et al., 2014; Yu et al., 2020), and PeLAC35 targeted by Ped-miR397a.1 was specifically validated in this study, indicating that miR397 is conserved in the regulatory network of lignin biosynthesis in both herbaceous and woody plants (Xue et al., 2019). It has been reported that miR164 plays a key role in growth and development (Wang et al., 2019), stress responses (Fang et al. 2014) and lignification (Shan et al., 2019) by inhibiting the expression of TFs. Ped-miR164a.1 was identified as the inhibitor of LAC in regulating lignification (Fig. 5). Additionally, other enzyme genes were also targeted by miRNAs in the network, suggesting
that they could play a vital role in mediating lignin biosynthesis. However, no TF genes specifically targeted by conserved miRNAs like miR397 and miR164 were detected in this study. In contrast, the target sites of two miRNAs (Ped-miR528-3p and Ped-miR399j-5p) were discovered in PeNST/SND1.2 and PeMYB20/85.2, and that of unknown_Ped-miR_44 was found in PeSND2/3.1 and PeSND2/3.4 (Fig. 5), which provided insights into these genetic factors in bamboo lignification.

A regulatory model for bamboo lignification

In summary, the present study identifies the key regulatory factors involved in bamboo shoot lignification by integrating mRNA and miRNA expression data along with degradome sequencing analysis, which provides a reference for the study of lignin regulation in other monocots. Based on a series of comprehensive analyses, a complex regulatory network, which is a reliable regulatory model for lignification in bamboo, was successfully established, including 11 miRNAs that could target 22 TFs and 36 key enzyme genes (Fig. 5). Lignin manipulation is one of the major techniques for the improvement of the properties of wood (Sakamoto et al., 2020). This manipulation can be achieved by suppressing endogenous genes for lignin biosynthesis. We proposed a regulatory model of miRNA-mediated ‘MYB-PeLAC20’ for lignin monomer polymerization, through which lignin biosynthesis promotes shoot lignification that leads to changes in the physical and mechanical properties in moso bamboo. This model will be helpful to elucidate the regulatory mechanism of lignification, particularly in shoots during fast growth. There were more genes related to lignin biosynthesis in the regulatory network in moso bamboo compared to rice and Arabidopsis, which might contribute to the faster biomass accumulation and lignification in bamboo. These findings provide valuable insights for understanding the coordinated networks of miRNAs, TFs, and enzyme genes in the lignin biosynthesis pathway of moso bamboo, and might facilitate the improvement of bamboo quantity and quality through genetic engineering in the future.

Materials and Methods
The moso bamboo (*Phyllostachys edulis*) shoots were harvested from a wild bamboo forest in Nanchang, Jiangxi province, China, in April 2018. The top, middle, and lower portions of the 13th internode of five bamboo shoots with different heights (1.0, 2.0, 4.0, 6.0, and 8.0 m) were selected as the experimental material (Supplemental Fig. S15) (Chen et al., 2018). The samples were frozen immediately in liquid nitrogen and stored at -80°C for RNA isolation and enzyme activity assays. Meanwhile, another two parts of the same samples were collected and fixed in formalin-acetic acid-alcohol (FAA) and dried for observation of the sections and determination of lignin content, respectively. The samples were designated as the shoot-height-portion. For example, S-2-T, S-2-M, and S-2-L represented the top, middle, and lower portions of the 13th internode from the 2.0 m shoots, respectively. Additionally, S-1 represented mixed samples since the top, middle, and lower portions of the 13th internode from the 1.0 m shoots were indistinguishable.

**Histological staining and measurement of lignin content and enzyme activity**

The shoot samples fixed in FAA were sectioned and stained with toluidine blue, as described by Yang et al. (Yang et al., 2019). The dried shoot samples were finely ground and filtered with a 40-mesh sieve for lignin content analyses using the acetyl bromide method (Moreira-Vilar et al., 2014). In addition, the harvested shoots (0.1 g) frozen at -80°C were crushed with a prechilled mortar and pestle using an appropriate volume of prechilled extraction buffer. After uniform homogenization and centrifugation at 13000 rpm for 15 min at 4°C, the supernatant containing the crude enzymes was collected and used for the enzyme activity analyses using the kit, following the manufacturer’s instructions (Suzhou Grace Biotechnology Co., Ltd., China).

**RNA isolation, library construction, RNA-seq, small RNA, and degradome sequencing**

The total RNA from 13 samples was isolated using the TRIzol for transcriptome, small RNA, and degradome sequencing, and three biological replicates were used. The total RNA was checked for quality and purity using the Agilent 2100 Bio analyzer (Agilent, USA) and
NanoDrop 2000 (NanoDrop, USA), which were also used for the sequencing. The library construction and processing of data are described in detail in the Supplemental Materials and Methods.

Validation of the DEGs and DEMs by reverse transcription quantitative PCR (RT-qPCR) analysis

To validate the expression of the genes targeted by the miRNAs obtained from high-throughput sequencing, the Primer Premier 5.0 software was used to design the specific primers based on the candidate genes and miRNA sequences (Supplemental Table S7). The RT-qPCR reactions were performed with the SYBR Green Master Mix in a qTOWER 2.2 system (Analytik Jena, Germany) with four technical replicates, using PeNTB (Fan et al., 2013) and U6 snRNA (Ding et al., 2011) as the endogenous controls for the genes and miRNAs, respectively. The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 62°C for 10 s. The expression levels were calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001).

Yeast one-hybrid assay

The coding sequences (CDS) of PeMYB20/85.2, PeMYB103.3, and PeMYB4.1 were recombined into the pGADT7-Rec2 vector, and the three AC-elements, three SMRE element regions, as well as the PeLAC20 promoter sequences were inserted into the pHIS2 vector. The primers that were used to amplify the CDS and promoter fragments are listed in Supplemental Table S8. Two recombinant vectors were co-transformed into the Y187 strain. The transformants were screened on Leu-, Trp-, and His-deficient medium supplemented with 20 mM of 3-amino-1,2,4-triazole (3-AT) for 3 days.

Electrophoretic mobility shift assay (EMSA)

The EMSA was performed using a chemiluminescent EMSA kit (Beyotime, China) following the manufacturer’s instructions. The promoter fragment of PeLAC20 containing the native or mutant AC element was labelled with biotin on both ends of the probe. The sequences of the
DNA probes are listed in Supplemental Table S8. The EMSA was conducted by incubating the labelled probe with purified PeMYB4.1 and PeMYB20/85.2 proteins at 25°C for 1.0 h and separated by 6% native polyacrylamide gel electrophoresis (PAGE) in 0.25 × Tris / Borate / EDTA buffer. Non-labelled probes were used as cold competitors.

**Construction of PeLAC20 plant expression vectors and transformation**

The CDS and the 2,000 bp promoter of PeLAC20 were amplified from moso bamboo using the cDNA and genomic DNA as templates with the primers shown in Supplemental Table S8. The CDS of PeLAC20 was cloned into the multiple cloning sites of pCAMBIA1301 driven by the CaMV 35S promoter. To obtain transgenic Arabidopsis plants, two recombinant vectors were transformed into the Arabidopsis (Col-0) plants using the floral dip method mediated by strain GV3101 (Zhang et al., 2006).

**Screening of the transgenic Arabidopsis plants and function verification**

The transgenic plants overexpressing PeLAC20 were selected using Murashige and Skoog medium with 50 mg·L⁻¹ hygromycin and further verified by RT-PCR. The stems of 6-week seedlings collected from both transgenic and wild-type Arabidopsis plants were used for lignin content analysis using the acetyl bromide method and histological staining with 1% (v/v) of phloroglucinol (Pomar et al., 2002), respectively. Images were taken with a light microscope (Olympus CX31, Tokyo, Japan).

**Phylogenetic analysis**

Clustal W was used for multiple sequence alignment, and the MEGA 6.0 program (http://www.megasoftware.net/mega.php) was used to construct a neighbor-joining (N-J) phylogenetic tree with the following parameters: N-J tree method, complete deletion, and bootstrap analysis with 1,000 replicates.

**Statistical analysis**

Statistical analyses were conducted using the SPSS 17.0 software (SPSS, Chicago, IL, USA). The tests for variance and significant differences were performed to identify the differences.
by one-way ANOVA. The data are presented as the mean ± SD from three biological replicates.

Accession numbers

The sequencing data have been deposited in NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) with the BioProject ID: PRJNA673565 and PRJNA682693. The accession numbers of PeLAC20, MYB20/85.2, MYB4.1 and MYB103.3 are PH02Gene23801, PH02Gene24105, PH02Gene05111 and PH02Gene26889, respectively.

Supplemental Data

Supplemental Figure S1. An overview of genes in different samples.
Supplemental Figure S2. Heatmap and cluster analysis of DEGs based on their expression profiles.
Supplemental Figure S3. Top 20 pathways of KEGG functional enrichment with DEGs.
Supplemental Figure S4. Length distribution of unique small RNAs in 13 small RNA libraries.
Supplemental Figure S5. Cluster analysis of DEMs based on their expression profiles.
Supplemental Figure S6. Expression profiles of representative DEMs in Cluster I ~ Cluster IV.
Supplemental Figure S7. Annotation and pathway analysis of the genes targeted by miRNAs.
Supplemental Figure S8. Target plots (t-plot) of miRNA targets identified by degradome sequencing.
Supplemental Figure S9. Weighted gene co-expression network analysis (WGCNA).
Supplemental Figure S10. The correlation analysis of genes, lignin content and enzyme activities based on Pearson’s correlation coefficients.
Supplemental Figure S11. The expression profiles by RT-qPCR of key TFs and enzyme genes during lignification.
Supplemental Figure S12. Phylogenetic tree constructed on the basis of LAC sequences of Arabidopsis (triangles) and moso bamboo (circles).
Supplemental Figure S13. Expression analysis of PeLAC20 in transgenic plants

Supplemental Figure S14. Expression analysis of PeLAC20 in Pichia pastoris strain GS115.

Supplemental Figure S15. Schematic illustration of sample collection from moso bamboo.

Supplemental Materials and Methods

Supplemental Table S1. Summary of transcriptome and small RNA sequencing data generated for 13 samples.

Supplemental Table S2. Summary for moso bamboo TFs and lignin biosynthetic genes (homologous to those of Arabidopsis) involved in lignin biosynthesis regulatory networks.

Supplemental Table S3. Detailed information of the miRNAs identified in moso bamboo.

Supplemental Table S4. Statistics of degradome sequencing and identified splicing sites.

Supplemental Table S5. Numbers of cis-acting elements found in the promoters of 12 families and MYB TFs.

Supplemental Table S6. The members of 12 families involved in lignin biosynthesis pathway.

Supplemental Table S7. Primer sequences for gene and miRNA cloning and expression analysis.

Supplemental Table S8. Pearson’s correlation coefficients among the expression of 22 TFs and 36 key lignin biosynthetic genes, as well as lignin content and enzyme activity.

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**Conflict of interest**

The authors declare that they have no conflict of interests.

**Figure legends**

**Figure 1.** Morphological and physiochemical changes in bamboo shoots during fast growth.

A: Transverse sections of vascular bundle in bamboo shoots with different heights (Bars: 200 μm). B: Lignin content. C: PAL activity. D: LAC activity. B-D, Different letters represent significant difference among different samples at $p < 0.01$. Average of three biological replicates along with standard deviation (SD) has also been given. A-D, S-1, S-2, S-4, S-L and S-8 represent shoots with height of 1.0, 2.0, 4.0, 6.0 and 8.0 m, respectively. T, M and L represent the top, middle and lower portions of the 13th internode. FC, fiber cells; MX, metaxylem; PC: parenchyma cells.

**Figure 2.** Annotation and pathway analysis of the identified DEGs.

(A) GO classification of 11,504 DEGs. GO terms are summarized in three main categories of cellular component, molecular function, and biological process. Top 20 pathways of KEGG functional enrichment among all DEGs (B) and DEGs in Cluster II (C). B and C, RichFactor represents the value of enrichment factor, which is the quotient of foreground value (the number of DEGs); the larger the value, the more significant enrichment. Coloring indicates $q$-value with higher value in red and lower value in blue. Lower $q$-value indicates the more significantly enriched; point size indicates DEG number.

**Figure 3.** Clustering DEGs expression profiles in different portions of the 13th internode.
The clustering was performed with log2FPKM change for each gene. The DEGs with similar expression trends have been grouped together and the selected representative genes from Cluster I ~ Cluster III were depicted in the form of heat maps. Gray indicates that the gene was not detected. S-1, S-2, S-4, S-L and S-8 represent shoots with height of 1.0, 2.0, 4.0, 6.0 and 8.0 m respectively. T, M and L represent the top, middle and lower portions of the 13th internode.

**Figure 4. The transcription levels of key genes involved in the transcriptional regulatory network of lignin biosynthesis in moso bamboo.**

Gray boxes indicate the major TFs and enzyme genes in moso bamboo that are homologous to those involved in transcriptional regulatory network for lignin biosynthesis in Arabidopsis. Purple and green boxes indicate TFs involved in transcriptional regulatory network for lignin biosynthesis in Arabidopsis and cis-elements. Red font indicate enzymes involved in lignin biosynthesis. Red-blue scales indicate relative expression levels. The color scale at the top shows log2FPKM. The lignin biosynthesis pathway is shown within the blue dashed box. Upper middle: Positive regulation is indicated by blue arrows. Feedback regulation is represented by red edge.

**Figure 5. The proposed regulatory network involved in lignin biosynthesis pathway in moso bamboo.**

The green circles represent the enzyme genes, and blue and yellow circles represent TFs and miRNAs associated with the enzyme genes in regulating the lignin biosynthesis pathway. The green and blue lines represent positive and negative regulation respectively.

**Figure 6. Validation of the genetic elements in the network of lignin biosynthesis in moso bamboo.**

(A) A combined view of the expression levels of selected coherent pairs of differentially expressed miRNAs and their target genes in the network. The dashed line and solid line represent the expression of miRNAs and the corresponding target genes, respectively. Average of three biological replicates along with standard deviation (SD) has also been given. (B)
PeNACs can bind to SNBEs validated by yeast one-hybrid assay on a triple-dropout medium. (C) PeMYBs can bind to AC-elements and SMREs validated by yeast one-hybrid assay on a triple-dropout medium.

**Figure 7.** *PeLAC20* is regulated by MYB and participated in the lignin biosynthesis.

Lignin staining (A) and content (B) analysis in Arabidopsis stem overexpressing *PeLAC20*. Col-0: Wild type; L1 and L3: Transgenic lines over-expressing *PeLAC20*; co: cortex; xy: xylem; if: interfascicular fiber; ph: phloem. B, Different letters represent significant difference among different samples at \( p < 0.05 \). Average of three biological replicates along with standard deviation (SD) has also been given. The tests for variance and significant differences were performed to identify the differences by one-way ANOVA. (C) Positions of probes (AC-II elements) in the promoter region of the *PeLAC20*. Green boxed region represent AC-II element region. (D) Yeast one-hybrid assay, PeMYB4.1 and PeMYB20/85.2 bind to *PeLAC20* promoter. (E) Electrophoretic mobility shift assay (EMSA) showed that PeMYB4.1 and PeMYB20/85.2 bind directly to the AC-II elements in *PeLAC20* promoter.

**Figure 8.** A miRNA-mediated ‘MYB-*PeLAC20’ model for lignin monomer polymerization in the shoots of moso bamboo.

High expression of *Ped-miR399j-5p* and *Ped-miR397* resulted in the downregulation of *PeMYB20/85.2* and *PeLAC20* respectively, while low expression of *Ped-miR399j-5p* and *Ped-miR397* caused upregulation of *PeMYB20/85.2* and *PeLAC20* in secondary growth. *PeMYB4.1* and *PeMYB20/85.2* directly bind to the promoter of *PeLAC20* and activate its transcription. Triangles indicate the expression changes, grey-white indicates the expression level from high to low. Green arrows indicate positive regulation. Black dashed arrows represent multiple processes, and black solid arrows represent the secondary cell wall thickening direction.

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