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Brief Report

Genome-Wide Investigation of Knotted Related Homeobox Genes and Identification of a Fiber-Growth-Repressed Knotted Related Homeobox Gene in Ramie

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Abstract: The KNOX transcription factor plays crucial roles in regulating fiber growth in plants. Although the genome of ramie, an important fiber crop in China, is available, knotted related homeobox (*KNOX*) genes have not been systematically explored in this crop. In this study, seven members of the *KNOX* gene from the ramie genome were identified and assigned to two groups, Class I and II. The intron–exon structure, conserved domain architecture, cis-regulating elements, and expression pattern showed distinct differences among the seven KNOX regulators. One of the genes, *Bnt07G011994*, encodes an ortholog of *Arabidopsis* fiber-growth-related KNAT7, and is differentially expressed among barks undergoing different stages of fiber growth. The overexpression of *Bnt07G011994* dramatically decreases the fiber number in transgenic *Arabidopsis*, indicating a negative role played by this gene in modulating fiber growth. Further transcriptome analysis of transgenic *Arabidopsis* revealed that the overexpression of *Bnt07G011994* resulted in an expression change in 14 pectin biosynthesis-/metabolism-related genes. These findings provide a useful foundation for further investigating the function of *KNOX* genes in ramie, and provide an important insight into the involvement of the ramie *KNOX* gene in fiber growth.

Keywords: ramie; *KNOX* gene; fiber growth; expression pattern; pectin

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1. Introduction

The cell wall is an important structure in plant cells, including the intercellular layer, primary wall, and secondary wall. The primary wall exists in all plant cells, and is mainly composed of pectin, cellulose, hemicellulose, and other polysaccharides [1], whereas the secondary wall is composed of cellulose, hemicellulose (xylan and glucomannan), and lignin [2]. Fibers are typical type of sclerenchymatous cells, and comprise primary walls and thickened secondary cellular walls [2]. Fiber growth is mainly involved in primary wall formation, and the thickness of secondary walls in plants. Studies on *Arabidopsis* have identified numerous genes that participate in the biosynthesis of secondary walls, and they are coordinated to express via a NAC-MYB-based regulatory network [3].

Ramie (*Boehmeria nivea* L. Gaud) is an ancient fiber crop, and has been cultivated in China for over 4700 years [4]. Unlike cotton, ramie fibers are bast fibers extracted from the stem bark, indicating a potential difference in the growth of fibers between these two crops. Recent genetic and molecular studies have identified hundreds of genes/QTLs

for fiber yield traits, which provided important insights into the mechanism underpinning the growth of bast fiber in ramie [5,6]. In addition, numerous non-coding RNAs, such as small RNAs and circRNAs, have been identified, and are suggested to have a role in the epigenetic regulation of growth in ramie fibers [7–10]. Recently, several studies focusing on the proteomes of ramie stem barks have provided important insights, by proposing a putative model for the mechanism of fiber growth [11], and revealing the roles of phosphorylation and ubiquitination modification in the modulation of fiber growth in this fiber crop [12,13].

Homeobox genes encode the proteins with a homeodomain, consisting of three helical structures. Transcription factors from the three-amino-acid-loop-extension (TALE) family comprise a type of homeobox protein. Unlike the typical homeodomain that comprise 60 amino acids residues, the homeodomain of TALE possesses 63 amino acids residues, and there are three extra amino acids residues between the first and second helices of its domain, as a result [14]. There are two subfamilies, knotted-like homeobox (KNOX) and BEL-like homeobox, in TALE. The proteins in the KNOX and BLH subfamilies show distinct difference in their domains and motifs. For example, BEL1-like proteins contain a highly conserved SKY-box in the N-terminus, and the VSLTLGL motif and the BELL domain at the C-terminal end, except the homeodomain [15,16], whereas KNOX proteins generally comprise the domains of KNOX1, KNOX2, ELK, and homeodomain [17,18].

The KNOX genes play crucial roles in regulating the development of various green plant lineages, for example, maintaining a pluripotent shoot apical meristem of the growing tip [19,20]. Notably, KNOX proteins have also been shown to be involved in the control of fiber growth via a reduction in the deposition of the secondary cellular walls in *Arabidopsis* [20–22]. For example, *KNAT1* and *STM* are two class-I KNOX genes in *Arabidopsis*, and the loss of their function causes diminishing fiber levels [20]. A recent study indicated that KNOX regulators could suppress the expression of lignin biosynthetic genes by directly binding to the promoters of these genes, or indirectly forming heterodimers with BELL proteins, and then negatively mediating lignin biosynthesis [23]. The *Arabidopsis* KNAT7 gene negatively regulates the fiber growth [24]. It has been proposed to form a functional complex with ovate family protein 4 (OFP4), thereby regulating secondary wall biosynthesis [25]. Bhargava et al. have found that KNAT7 interacts with MYB75, and modulates secondary wall deposition in *Arabidopsis* [26], indicating several mechanisms for the involvement of KNAT7 in fiber growth. A varied number of KNOX genes has been identified in plants. For example, among 72 *TaTALE* genes, 36 are KNOX genes in bread wheat [27], whereas only the 22, 19, and 13 *KNOX* genes have been identified in the genome of the apple [28] and the pear [29]. In particular, only 5–9 *KNOX* genes exist in the genome of *Arabidopsis* and some orchid species [30]. However, a genome-wide identification of KNOX genes in the ramie genome has not been performed in this species. Therefore, in this study, we systemically identified *KNOX* genes from the ramie genome, and one of them, *Bnt07G011994*, was further analyzed regarding its function via overexpression in *Arabidopsis*.

2. Materials and Methods

2.1. Mining KNOX Genes from Ramie Genome

The KNOX protein sequences of flax, *Arabidopsis*, and rice were downloaded from the PlantTFDB database (http://planttfdb.gao-lab.org/; accessed on 1 July 2023) [31], and were used as a reference for any queries during the process of detecting the KNOX proteins from the ramie genome via the BLASTP method, with an e-value of 10^{-10}. Additionally, the Pfam Blast search was carried out to search the KNOX (PF03790 or PF03791), with an e-value of 10^{-10}. Subsequently, the preliminarily identified proteins were subjected to conserved domain analysis via the CDD program in NCBI (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; accessed on 1 July 2023) [32] and MEME (http://meme-suite.org/tools/meme; accessed on 1 July 2023) [33]. After verification, only proteins containing specific conserved domains were reserved for subsequent analysis.

2.2. Chromosomal Location, Conserved Motifs, and Gene Structures Analysis

The location of the *KNOX* genes on the chromosomes was visualized with the use of TBtools [34], according to the annotated information about the chromosomal location in the ramie genome [35], which was downloaded from the National Genomics Data center of China (https://ngdc.cncb.ac.cn/; accessed on 1 July 2023). Jalview software (v 2.11.2.0) was used to investigate the domains of the ramie KNOX proteins, using multiple sequence alignments [36]. Two online tools, MEME [33] and batch CD-Search [32], were used to analyze the conserved motifs. The protein structures and motifs were then visualized via TBtools (v1.045) [34]. To determine the gene structure, the genomic and CDS sequences of the ramie KNOX genes were aligned into the ramie genome (NCBI Genbank accession: PRJNA663427), to infer the exon–intron organization and intron phases.

2.3. Synteny Analysis, Duplication, and Cis-Regulatory Elements

The segmental duplication and collinearity within the species, and between ramie and other species (*Arabidopsis* and *Oryza sativa*), were analyzed using the Multiple Collinearity Scantoolkit (MCscanX), in the software TBtool (v1.045) [34], with the default parameters. The visualization of the results was carried out using the program Advanced Circos and Multiple Synteny Plot plugin of TBtools [34]. Further, based on the distance between the ramie KNOX genes, tandem and segmental duplication events were determined [37]. Cis-regulatory elements within the 1.5 kb upstream region of each ramie KNOX gene were predicted using the PlantCARE database [38], with default parameters, and they were then visualized via the TBtool software (v1.045) [34].

2.4. Expression Pattern of Ramie KNOX Genes

The RNA sequencing (RNA-seq) of the leaves, roots, and stems from domesticated variety 'Zhongsizhu 1' (ZSZ1) and wild ramie 'Qingyezhuma' (QYZM) was performed as part of a recent study [35]. In addition, the sections of bark from the top and middle stems of 'Zhongzhu 1', an elite variety for fiber production, were sequenced for their RNAs [35]. Based on these reported RNA-seq data, the fragments per kilobase per million read values of each ramie *KNOX* gene were extracted, and were used to estimate the expression of the corresponding gene in various tissues. The expression heatmap was visualized via an online tool (https://cloud.oebiotech.cn/task/detail/heatmap/; accessed on 6 July 2023).

2.5. Overexpression of Bnt07G011994

The full-length sequence of *Bnt07G011994* was obtained via amplification from a cDNA library, using a high-fidelity thermostable DNA polymerase employing the following primer sequences: 5′-ATGAATCATGAGCCTGGATTG-3′ (forward primer) and 5′-TTAGGTGATCATTACCTGTTTCAGC-3′ (reverse primer). Then, the full-length sequence was inserted into the PBI121 vector, in which its expression was driven by the CaMV 35S promoter. Subsequently, the heat-shock method was used to introduce the plasmid construct into the *Agrobacterium tumefaciens* strain GV3101, and the generated *Agrobacterium* was used to transfer *Bnt07G011994* into *Arabidopsis*, using the floral dip method [39]. Transgenic plants were grown in a greenhouse at 22 °C under a 9 h dark/15 h light cycle. After 40 days, stem cells from the transgenic plants were sectioned, stained with Safranin O-Fast Green, and examined via transmission light microscopy.

2.6. Subcellular Localization

PCR amplification for the full-length sequence of *Bnt07G011994* was executed. Then, the amplified gene sequence and the cDNA sequence of enhanced green fluorescent protein (EGFP) were fused via an in-frame method, and a fusion expression of the vector PEZR (K)-LN was performed. The heat-shock method (37 °C) introduced the plasmid construct into cells of the *Agrobacterium tumefaciens* strain GV3101. Subsequently, the transformant of a transient expression in the epidermal cells of tobacco was performed, according to the

method of Sparkes et al. [40]. After 48 h incubation, the fluorescence signal in the transfected tobacco leaves was observed, using the Leica TCS SP5 spectral confocal microscope.

2.7. RNA Sequencing for Wild and Bnt07G011994-Overexpressed Arabidopsis

Wild and *Bnt07G011994*-overexpressed *Arabidopsis* were grown in a greenhouse under the growth conditions of 22 °C, and a 9 h dark/15 h light cycle. When the stems reached about 10 cm in length, the stems of each individual were collected as a sample. The collected samples were frozen in liquid nitrogen, and stored in a refrigerator under −80 °C. Subsequently, the total RNAs were extracted for each sample, using an E.Z.N.A. Plant RNA Kit (OMEGA Bio-Tek, Norcross, GA, USA), according to the protocol of the manufacturer. Thereafter, these RNAs were individually used to construct cDNA libraries, with a fragment length of approximately 300 bp (±150 bp), using a NEBNext UltraTM RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA), following the manufacturer's instructions. Paired-end sequencing for the constructed cDNA library was carried out using a HiSeq PE Cluster Kit v4 cBot (Illumina, San Diego, CA, USA) on the Illumina sequencing platform, according to the manufacturer's instructions. After filtering the low-quality reads, clean reads were aligned with the *Arabidopsis* genome (GenBank accession: GCA_000001735.2 TAIR10.1), using hisat2 software (version: 2.2.1.0) [41], with the default parameters. The expression level of each *Arabidopsis* was quantified via the estimation of the fragments per kilobase per million reads (FPKMs) [42]. Differentially expressed genes between the stems of wild and *Bnt07G011994*-overexpressed *Arabidopsis* were determined using the DEseq program (version: 1.18.0) [43], and the genes with more than a two-fold change were deemed to be significant DEGs ($p < 0.01$). The GO functional categories enriched by differentially expressed genes were detected using GOseq (v3.15) [44], according to the Wallenius non-central hypergeometric distribution.

3. Results

3.1. Identification and Characterization of Ramie KNOX Genes

Eight, thirteen, and twelve KNOX transcription factors from *Arabidopsis*, flax, and rice were used as a reference for queries and, consequently, a total of seven KNOX genes were identified in the ramie genome, and they were distributed across eight chromosomes (Figure 1a). A distinct difference in the gene structure and putative protein structure was observed among the seven KNOX members (Figure 1b). All seven KNOX members comprised the KNOX1 and Homeobox_KN domain; additionally, Bnt07G011994 and Bnt13G018705 contained the KNOX2_superfamily domain, whereas Bnt01G001191, Bnt09G014495, Bnt10G015309, Bnt01G001412, and Bnt09G014495, possessed the KNOX2 domain (Figure 1b). Phylogenetic analysis indicated that ramie KNOX members could be classified into two major groups, Class I and II, comprising four and three members, respectively (Figure 2a). Bnt01G001412, Bnt10G015309, Bnt09G014495, and Bnt09G014495 belonged to Class I, whereas Bnt07G011994, Bnt01G001191, and Bnt13G018705 were grouped into Class II. Notably, one segmental duplication event between *Bnt01G001191* and *Bnt13G018705* was observed (Figure 2b). Sequence evolutionary analysis indicated that three ramie *KNOX* genes were the orthologous genes of rice and/or *Arabidopsis* (Figure 2c), with a conserved gene-encoding sequence.

The expression analysis of ramie *KNOX* genes in wild 'Qingyezhuma' (QYZM) and cultivated 'Zhongsizhu 1' (ZSZ1; data from the reported transcriptomes [36]) indicated that all seven genes were differentially expressed in leaves, roots, and stems (Figure 2c). Two *KNOX* genes (*Bnt01G001412* and *Bnt09G014495*) were not expressed in leaves, whereas three were differentially expressed in the roots of the two varieties (Figure 2d), indicating a difference in their expression pattern. We further analyzed the cis-regulating elements in the promoter region of ramie *KNOX* genes, and revealed a wide diversity among them (Figure 2e), which should be responsible for the difference in their expression pattern.

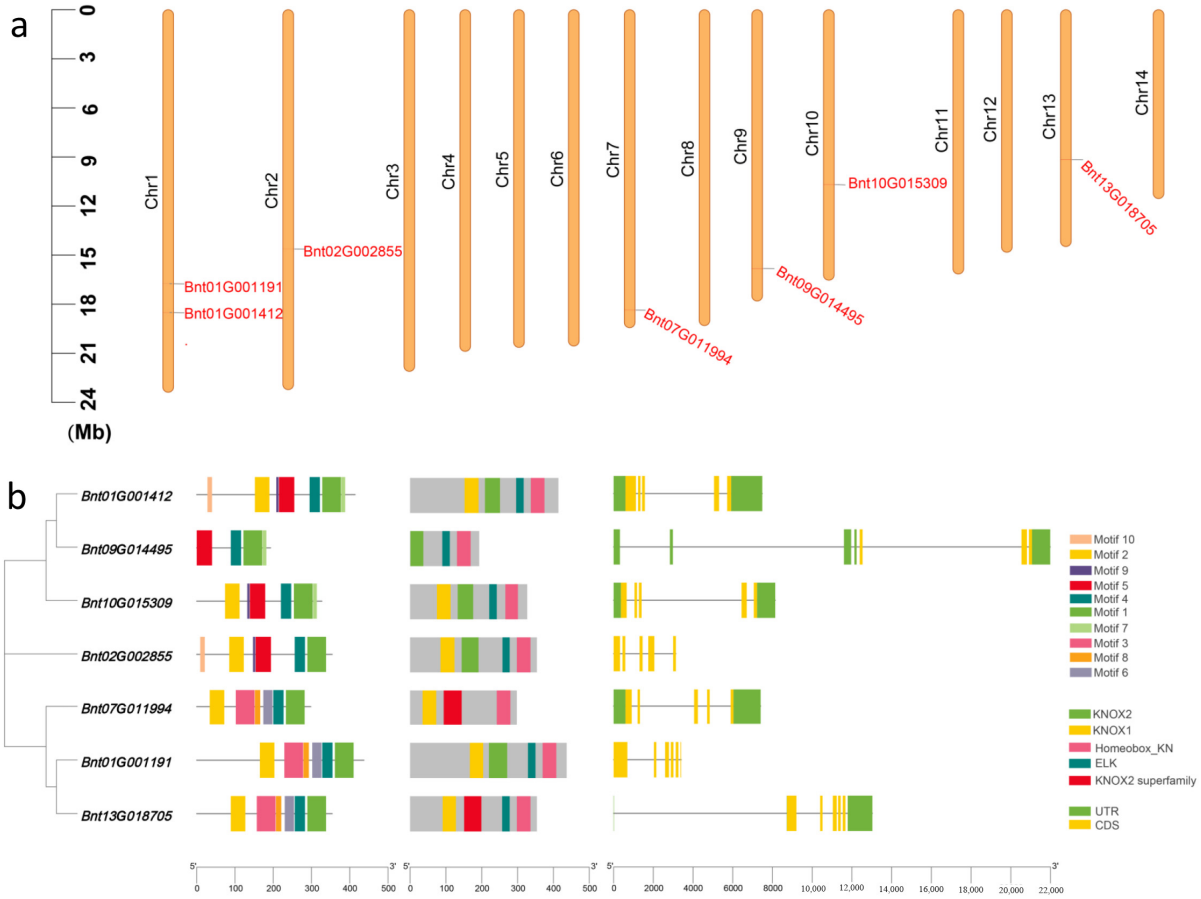


Figure 1. Identification of ramie *KNOX* genes. (**a**) Schematic representation of the chromosomal distribution of ramie *KNOX* genes. Vertical bars indicate the ramie chromosomes, and the scale on the left represents the chromosome length (Mb). (**b**) The domain, gene-structure, and motif distribution of ramie *KNOX* genes; I and II indicate the conserved domain structures and amino acid motifs of seven *KNOX* genes. The black lines represent the relative sequence lengths of the protein. III shows the introns and exons, which are represented by gray lines and rectangles, respectively.

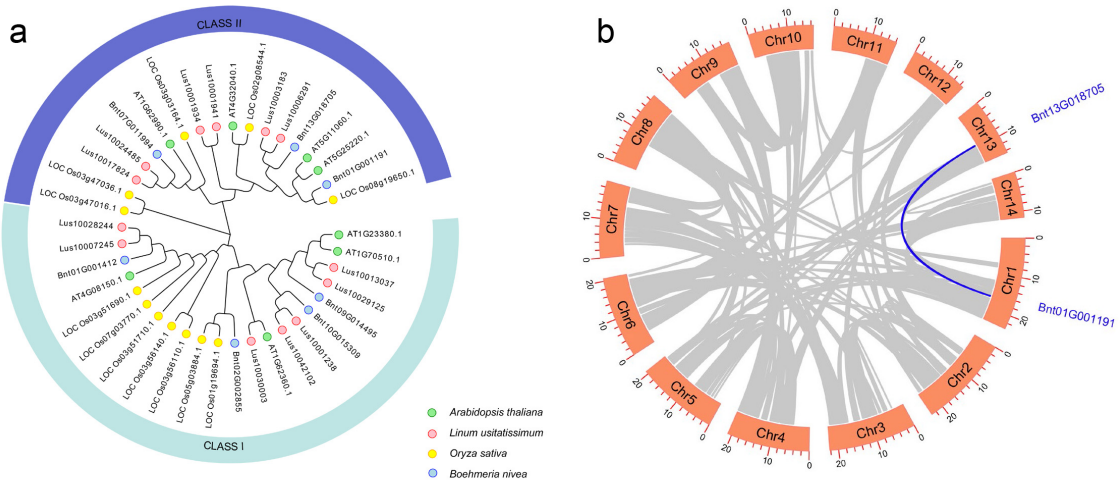


Figure 2. *Cont.*

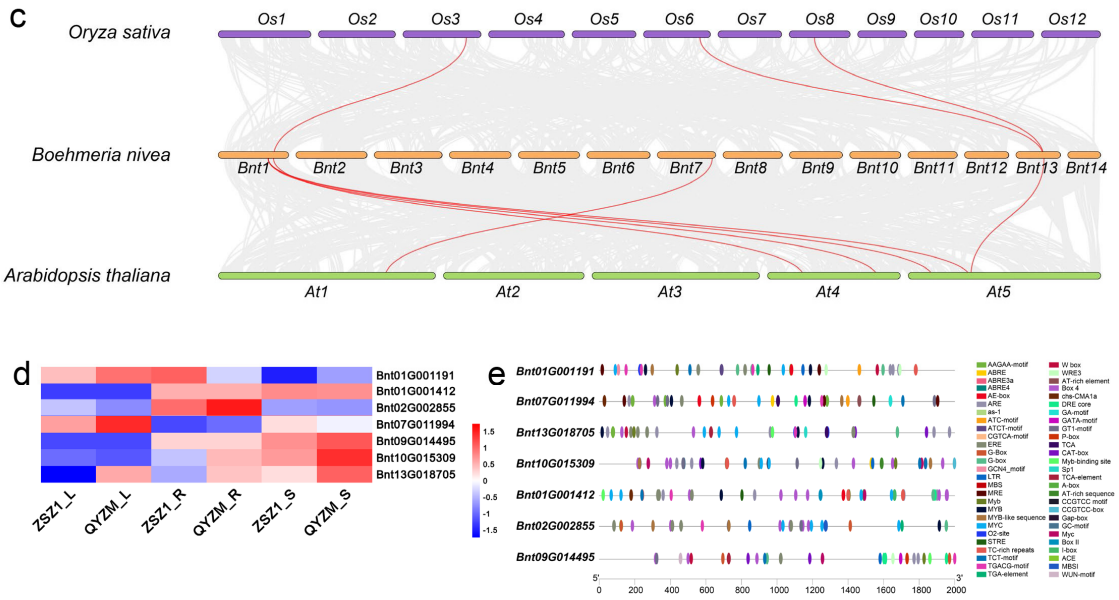


Figure 2. Characterization of ramie *KNOX* genes. (**a**) Phylogenetic tree of the KNOX proteins of *Arabidopsis*, rice, and ramie. The MEGA 5 program was used to construct the unrooted tree via the neighbor-joining method, and the bootstrap test was performed on 1000 replicates. (**b**) The segmental duplication event of the ramie *KNOX* genes. The gray lines represent all the synteny blocks in the ramie genome, and the blue line indicates duplicated *KNOX* gene pairs. (**c**) Gene duplication and synteny analysis of *KNOX* genes in the genome of ramie, rice (*Oryza sativa*), and *Arabidopsis*. The background of the collinear blocks between ramie and other plant genomes is indicated by gray lines, while the syntenic *KNOX* gene pairs are highlighted using red lines. (**d**) Expression heatmap of the *KNOX* genes based on their fragments per kilobase of transcript per million mapped reads value in the leaves (L), roots (R), and stems (S) of cultivated Zhongsizhu 1 (ZSZ1) and wild Qingyezhuma (QYZM). (**e**) The cis-regulating elements in the promoter region of the ramie *KNOX* genes.

3.2. Overexpression of Bnt07G011994 in Arabidopsis

The KNOX transcription factor Bnt07G011994 is a ramie ortholog of KNAT7 (i.e., AT1G62990.1), which is a key repressor for fiber growth [45]. Bnt07G011994 showed a wide expression in the roots, leaves, and stems of QYZM and ZSZ1 (Figure 2c), implying its necessity in ramie growth and development. However, we found that *Bnt07G011994* was differentially expressed in two barks sections from the top and middle stem of 'Zhongzhu 1', where the fiber growth was at different stages, indicating its potential role in fiber growth (Figure 3a). To validate the association between *Bnt07G011994* and fiber growth, we performed an overexpression analysis, and obtained four lines of transgenic *Arabidopsis*. Because fibers possess secondary cell walls, one of the main components of which is lignin, and the lignin can be stained red using the method of Safranin O-Fast Green, we performed a section for the stems of the overexpressed *Arabidopsis* and the wild control, and then strained them. Consequently, although more xylem regions could be observed in the transgenic *Arabidopsis*, fewer cells were stained red in the section of the stems of the overexpressed *Arabidopsis* than in those of the wild type, implying that the overexpression resulted in a significant decrease in the xylem fibers (Figure 3b). Therefore, these results indicate that *Bnt07G011994* represses fiber growth, which is similar with its *Arabidopsis* ortholog, KNAT7.

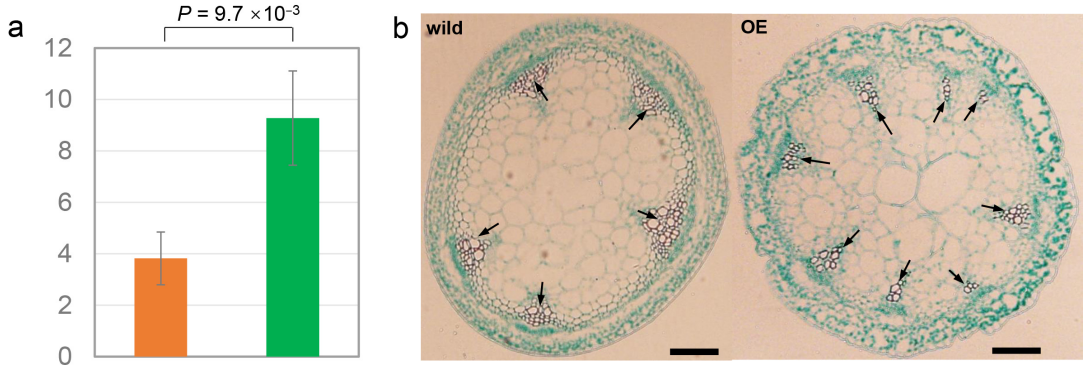
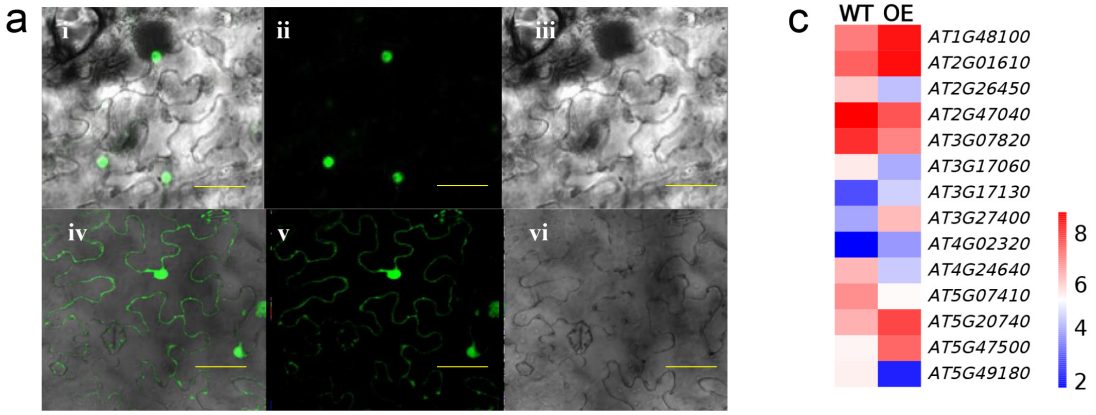


Figure 3. Characterizing the function of *Bnt07G011994*. (**a**) *Bnt07G011994* showed a significant expressed difference between the barks from the section of the top (orange column) and middle (green column) stem, where fibers underwent different developmental stages ($p = 9.7 \times 10^{-3}$). (**b**) The light microscopy findings for the transected stems of the wild control and the *Bnt07G011994*-overexpressing (OE) *Arabidopsis*. Fiber cells in the xylem regions are indicated via arrows. The scale bar represents 200 μm.

3.3. Bnt07G011994 Regulated the Expression of Pectin-Biosynthetic Genes in Transgenic Arabidopsis

The investigation into the subcellular location of the Bnt07G011994 protein revealed that Bnt07G011994 is expressed in the cell nucleus (Figure 4a), indicating that this *KNOX* gene functions as a transcriptional regulator. Furthermore, to obtain insights into the potential functional mechanism of *Bnt07G011994*, we performed RNA sequencing for the *Bnt07G011994*-overexpressed *Arabidopsis*, and the wild control (Table S1). After their transcriptomes were compared, a total of 251 differentially expressed genes were identified (Figure 4b). Of them, 14 encoded a putative pectin methylesterase or pectin lyase (Figure 4c). Further enrichment analysis of these 251 genes indicated that these genes were dramatically enriched in the GO function term, in the pectin catabolic process (GO:0045490, $p = 9.63 \times 10^{-14}$), cell wall modification (GO:0042545, $p = 1.16 \times 10^{-8}$), and pectate lyase activity (GO:0030570, $p = 4.48 \times 10^{-6}$). Interestingly, there were numerous transcription factors reported to regulate secondary wall biosynthesis [2]; however, none of them showed an expression change in the *Bnt07G011994*-overexpressed *Arabidopsis*, indicating that this *KNOX* gene was in a downstream position in the regulatory network of fiber formation in the overexpressed *Arabidopsis*. Moreover, none of the genes reported to be involved in cellulose, lignin, and hemicellulose were found to show a differential expression in the *Bnt07G011994*-overexpressed *Arabidopsis*. Because pectin is a main component of the primary cell wall which is important for fiber growth [46], we speculated that Bnt07G011994 regulates pectin biosynthesis, and thereby controls fiber growth.



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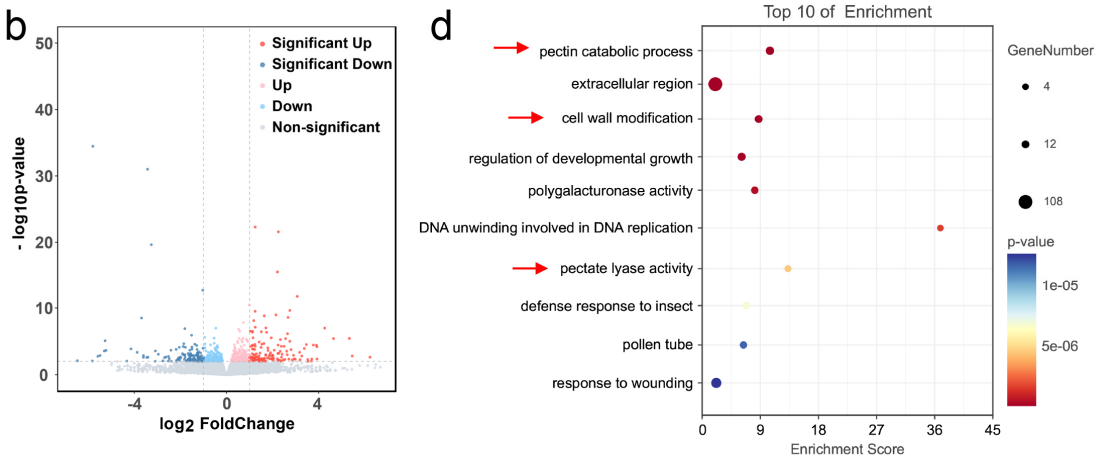

Figure 4. Molecular features of *Bnt07G011994*. (**a**) The subcellular localization of the *Bnt07G011994*-EGFP fusion protein in the epidermal cells of tobacco leaves (i–iii). p35S: EGFP was used as a control (iv–vi). i and iv, bright-field images; ii and v, green fluorescent protein (GFP) images; and iii and vi, merged images. The bar scale = 50 μm. (**b**) The genes that were differentially expressed between the stems of the wild *Arabidopsis* and the *Bnt07G011994*-overexpressed *Arabidopsis*. The parameters are $p < 0.01$, and the change fold is >2. (**c**) Heat map for the expression of 14 pectin biosynthesis-/metabolism-related genes in the wild *Arabidopsis* (WT) and the *Bnt07G011994*-overexpressed (OE) *Arabidopsis*. (**d**) The top 10 GO terms enriched by differentially expressed genes. The red arrows indicate terms associated with cell wall formation.

4. Discussion

In the present study, seven KNOX members were identified, and their sequence evolution, intron–exon structure, conserved domain architectures, and tissue-expressed pattern were comprehensively characterized. The number of ramie *KNOX* genes was far smaller than those of wheat [27], apple [28], and pear [29], probably resulting in a small ramie genome. Similarly, *Arabidopsis* and some orchid species possess a similar small genome, and only 5–9 KNOX genes were identified in their genome [30]. Of these seven *KNOX* genes, one of the KNAT7-orthologous genes, *Bnt07G011994*, was found to repress fiber growth in transgenic *Arabidopsis*. Additionally, a ramie KNOX member, whole_GLEAN_10029667 (whose ID was designated as *Bnt01G001412* in the reference genome, *tenacissima* [35]), has been found to dramatically repress fiber formation in *Arabidopsis*, and its function is modulated via phosphorylation modification [12]. However, because it remains a challenge to transform genes in ramie, the function of these two *KNOX* genes had to be verified via their overexpression in *Arabidopsis*. Actually, plants have evolved a conserved regulatory mechanism for fiber growth [3]. For example, a cotton-fiber-controlled MYB gene, *GhMYBL1*, regulated the secondary wall biosynthesis of xylem fibers in the *Arabidopsis* stem [47]. In ramie, one ramie MYB gene (*whole_GLEAN_10015497*) and two ramie NAC genes (*Bnt03G004997* and *Bnt08G012573*) that encoded the orthologs of the *Arabidopsis* regulators of fiber growth have been shown to be fiber-growth-associated genes, and their overexpression dramatically increases the fiber number in transgenic *Arabidopsis* [13,35,46]. Therefore, our results support the theory that the function of *Bnt07G011994* and its *Arabidopsis* ortholog KNAT7 should be conserved, in the control of fiber growth. These findings can enhance future investigations into *KNOX* gene function in the control of fiber formation in ramie.

Fiber growth is a complex process, and numerous enzymes for catalyzing the biosynthesis of cell wall components are involved [48–51]. Pectin has been identified as one of the important components in cell walls. Pectin generally secretes from the Golgi complex in highly methylesterified forms, and these forms must be further modified by pectinases, including pectin methylesterases, for catalyzing the demethylesterification of acidic pectin and methanol [52]. Pectin lyase is a depolymerizing enzyme for degrading pectin as oligose.

Therefore, the pectin methylesterases and lyase are two pivotal enzymes in pectin biosynthesis and metabolism. This study revealed that the KNOX gene *Bnt07G011994* could promote the expression of pectin biosynthetic/metabolic genes in *Arabidopsis*, based on transcriptome analysis of transgenic *Arabidopsis*, indicating that it potentially facilitated pectin biosynthesis. However, ramie fibers are mainly composed of cellulose, with a ratio of 65–75%, following by hemicellulose (14–16%), pectin (4–5%), and lignin (0.8–1.5%) [53]. A recent study identified the methylesterase-encoding pectin *Bnt14G019616* in ramie, and a natural mutant of its promoter caused a great increase in the expression level, thereby negatively controlling fiber growth [46], and suggesting that a high ratio of pectin might repress fiber growth. Therefore, *Bnt07G01199* promoted the biosynthesis of pectin by fibers and, thereby, the high pectin content might repress the growth of fiber. This finding provides important insights into the regulation of the KNOX gene *Bnt07G011994* in fiber growth.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13092297/s1, Table S1: Differentially expressed genes between wild and *Bnt07G011994*-overexpressed *Arabidopsis*.

Author Contributions: Y.W. performed the bioinformatic analysis. J.C. performed the overexpression experiment. C.L. (Chan Liu) and F.L. carried out the subcellular localization. Y.T. and C.L. (Chuanyan Li) performed the RNA-seq analysis. Y.G. managed the project, and X.X. contributed novel reagents. X.Z. revised the manuscript. T.L. designed this study, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw sequence reads of transcriptome and the expression data have been deposited in the NCBI GEO database under the accession number GSE234285, and the other data are contained within the article or in Supplementary Materials.

Conflicts of Interest: The authors declare no competing interests.

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