The CsLAZY1 Mediates Shoot Gravitropism and Branch Angle in Tea Plant (Camellia Sinensis)

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Research Article

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Abstract

Background: The tea plant (Camellia sinensis) architecture not only affects tea quality and yield, but also influences the efficiency of automatic pruning of tea plants. However, the molecular mechanism of branch angle that is an important aspect of plant architecture is poorly understood in tea plant.

Results: In the present study, three CsLAZY genes were identified from the tea plant genome data through sequence homology. Phylogenetic tree displayed that the CsLAZY genes have high sequence similarity with LAZY genes from other plant species, especially those in woody plants. The expression patterns of the three CsLAZYs in eight tissues were surveyed, and we further verified the expression levels of the key CsLAZY1 transcript in different tissues among eight tea cultivars, demonstrating that CsLAZY1 was highly expressed in stem. Subcellular localization analysis showed that CsLAZY1 protein was localized in the plasma membrane. Remarkably, CsLAZY1 was transferred into Arabidopsis thaliana to investigate its potential role in regulating shoot development, demonstrating that the over-expressed plants responded more effectively than the wild types under gravity processing in light and dark. The results indicate that CsLAZY1 plays an important role in regulating shoot gravitropism in tea plant.

Conclusions: The results provide evidence that CsLAZY1 may play a critical role in regulating shoot gravitropism, and further affecting stem branch angle in tea plant.

Background

The tea plant (Camellia sinensis) is an economic crop with significant importance, which leaves can be used to produce most traditional caffeinated teas that is the second most popular beverage worldwide [1]. The productivity of tea plant is greatly affected by the architecture of tea plant. A well-designed tree architecture should minimize competition with adjacent crops for environmental resources, such as light. In densely planted stands, a relatively wide branch angle may help the plant to escape some diseases by decreasing humidity, but it occupies more space and increases the extent of shade [2], thereby an optimum tree architecture may contribute to increasing the yield and yield stability of crops [3]. Tea plant architecture depends on geometric, environmental factors and tea estate elevations influencing growth, including plucking patterns like manual plucking and shear harvesting and monsoon seasons [4]. According to the degree of branch angle, tea plants are classified into three types of plant architectures: open type, half open type, and erect type.

Plant architectures are significantly associated with plant hormones, including gibberellic acid (GA), auxin, and strigolactones (SLs). GA has been thought to promote upward growth and inhibit bending, and it was highly likely to be responsible for the weeping trait [5, 6]. Previous researches also suggested that genes associated with auxin and ethylene probably play crucial roles in shoot elongation [7, 8]. SLs are a group of newly identified plant hormones that are essential for shoot branch/tiller angle, they can inhibit auxin biosynthesis and attenuate rice shoot gravitropism, mainly by decreasing the local indoleacetic acid (IAA) content [9].
Many environmental signals, including light and gravity, can influence plant architecture [5, 10]. The branch angle is an important factor in determining plant structure and is regulated by specific genes. Until now, many genes and transcription factors associated with branch angle have been identified. For instance, over-expression of OsPIN2 leads to increased tiller numbers, altering OsPIN2 expression by genetic transformation which can be directly used for modifying rice architecture [11]. The OsTAC1 controls tiller angle in rice [2], and changes in TAC1 have since been linked to upright tiller or branch angles in other plant species including Arabidopsis [15], rice [14], poplar [3], peach [12, 17], and apple [16]. The OsTAC4 participates in the regulation of rice tiller angle, and it influenced the endogenous auxin content, ultimately leading to reduced gravitropism and a tiller-spreading phenotype [13, 18].

In many plant species, LAZY1 plays an important role in plant branch angle. For example, the rice lazy1 mutant displays a tiller-spreading phenotype because the gravitropism was reduced [19]. In Arabidopsis, a total of six LAZY genes were identified, and mutating AtLAZY1 caused a large change in branch angle while the primary inflorescence stem remained vertical [20]. The other lazy mutations reversed the growth angle of lateral branches and roots, LAZY genes regulate the direction of polar auxin transport in response to gravity through the control of asymmetric PIN3 expression in the root cap columella [21]. In apple and poplar, evidence showed that LAZY genes affect the vascular tissues of transgenic plants to modify branch angle [3, 16].

Although LAZY genes have been indicated to play an important role in modifying branch angle in a variety of plant species, the potential function of homologous genes in tea plant (Camellia sinensis) is still in mystery. Branch angle is an important trait of tea plant, which can influence the plant architecture and mechanical picking of tea leaves. In this study, three LAZY genes were identified in tea plant and their expression levels in distinct tissues were characterized. The CsLAZY1 was predominately expressed in stem and was located in the plasma membrane. It was showed that over-expressed CsLAZY1 responded more effectively than the wild type under gravity processing. Our results provide new candidate genes to breed new varieties with an ideal tea plant architecture.

Results
Identification, Conserved Domain and Sequence Feature Analysis of CsLAZYs

A total of six LAZY genes were identified in Arabidopsis thaliana [20]. Subsequently, the six AtLAZYs genes were used as queries through Basic Local Alignment Search Tool (BLAST) analysis against the tea plant genome (http://tpia.teaplant.org/Blast.html) [22]. Initially, a total of 15 candidate unique genes were obtained in tea plant, and multiple sequence alignments of all LAZY genes were performed among tea plant, Arabidopsis and rice (data not shown). As a result, only 3 unique genes contained regions of conserved sequence V that possessing an ethylene-responsive element-binding factor-associated amphiphilic repression (EAR) motif (LxLxL) (Figure 1A), and it is an indispensable conserved domain of LAZY [20, 23]. Thereafter, the obtained three genes are referred to as CsLAZY1 (CSS025254), CsLAZY2 (CSS049138) and CsLAZY3 (CSS020288), which were located in different scaffolds (Table 1). Their
amino acid lengths are 399 aa (CsLAZY1), 367 aa (CsLAZY2) and 251 aa (CsLAZY3), respectively. Furthermore, the molecular weights (Mw) of CsLAZY1 to CsLAZY3 were 44.2, 41.2 and 29.0, respectively, and their isoelectric points (pl) were 6.55, 6.18 and 6.47, respectively (Table 1).

Subsequently, the exon/intron organization in the coding sequences of each CsLAZY gene was performed (Figure 1B), which contained 5 (CsLAZY1), 5 (CsLAZY2), and 4 (CsLAZY3) exons, respectively. In terms of intron and exon length, CsLAZY1 is the longest while CsLAZY3 is the shortest. The coding sequence of CsLAZY1 was cloned and sequenced, demonstrating that the cloned cDNA was totally consistent with the genomic reference sequence.

### Evolution and Phylogenetic Analysis of LAZY Genes

Previous studies and numerous fully sequenced plant genomes make it possible to perform a comparative genomic analysis of LAZY genes across a broad range of plant species. The LAZY genes have been identified in many species that play a similar role, so we performed iterative BLAST searches to determine the phylogeny of LAZY1 genes. The LAZY1 genes were identified from 20 distinct plant species, and homology analysis of LAZY1 among algal, lowland species, monocots, and dicots provided further insight into the evolutionary processes of this gene family. Phylogenetic analyses showed these LAZY1 genes were highly conserved among algae, monocots and dicots, and LAZY1 gene evolved from primitive organisms despite their overall relatively low sequence similarities (Figure 2A). Furthermore, we analyzed them in evolutionary trees with other species including Populus tomentosa, Vitis vinifera, Solanaceae lycopersicon, Oryza sativa, Arabidopsis thaliana, and Camellia sinensis. The complete LAZY gene families including 23 members from six plant species were used for phylogenetic analysis, displaying that the three CsLAZY genes were distinctly classified into two clades of class I and class II (Figure 2B). CsLAZY1 and CsLAZY2 were grouped into class I categories, and they both have high sequence similarity with the protein sequences of VvLAZY1, PtLAZY1 and PtLAZY2. CsLAZY3 was grouped into class II and showed the highest sequence similarity with VvLAZY3 gene.

### Analysis of cis-elements in promoters and tissue expression patterns

To explore the potential difference in non-coding regions of CsLAZYs, a 2-kb flanking sequence upstream of the translation start codon was obtained, and several putative cis-regulatory elements in the promoter were identified using the PLACE and PlantCARE databases (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The light-sensitive cis-elements comprised the largest part of all elements, including the Box 4, TCT-motif, ATC-motif, ATCT-motif, G-box, I-box, chs-CMA1a, MRE, and ACE (Figure 3A). The hormone-sensitive cis-elements have CGTCA-motif, GARE-motif, TCA-element, ERE, ABRE, TATC-box, and TGACG-motif, while only one type of cis-element contained in the promoter of each gene. It is noteworthy that the promoter region of CsLAZY1 contained many hormone cis-elements, including MeJA, GA, SA and ABA hormone responsive elements, implying that CsLAZY1 may plays an important role in tea plant by responds to hormones.
To understand the potential role of $CsLAZY$s in tea plant, we downloaded RNA-Seq data of eight tissues from the tea plant genome database, displaying that the expression levels of the three $CsLAZY$ genes were obvious specificity in tissues (Figure 3B). For instance, $CsLAZY1$ was mainly expressed in stem, followed by in bud and leaf, while basically not expressed in fruit and root. In comparison, $CsLAZY2$ was mainly expressed in flower, and $CsLAZY3$ showed the highest expression level in the second leaf. Due to stem bending is one of the main causes of the branch angle, therefore $CsLAZY1$ was probably played a vital role in regulating branch angle of tea plant.

Expression pattern of $CsLAZY1$ in tissues among different tea varieties

To further verify the tissue expression pattern of $CsLAZY1$, we examined the tissue expression level of $CsLAZY1$ in different varieties. A total of eight varieties were selected, which possessed different branch angles, including four open type varieties (Benshan, Foshou, Yaoshanxiulv, and Tieguanyin) and four erect type varieties (Echa 5, Fuzao 2, Longjingchangye, and Zhenghedabaicha). The expression level of $CsLAZY1$ transcript varied significantly among the four tissues (leaf, bud, root and stem) (Figure 4). It was showed that $CsLAZY1$ transcript was not detected in root of the eight tea varieties, and it had the highest expression level in stem, followed by in leaf. Unexpectedly, $CsLAZY1$ had the highest expression level in leaf, followed by in stem in the cultivar of Tieguanyin.

Subcellular localization of $CsLAZY1$ protein

To obtain insight into the molecular function of $CsLAZY1$ protein, we constructed $CsLAZY1$-GFP and Pk7WG2 35S-GFP fusion protein expression vectors to examine its subcellular localization. Transient expression in Arabidopsis protoplasts showed that $CsLAZY1$ protein was localized in the plasma membrane (Figure 5A). Besides, we transferred the $CsLAZY1$ protein to Agrobacterium to infect tobacco leaves, and obtained the identical result, namely $CsLAZY1$ protein was localized in the plasma membrane (Figure 5B).

Over-expression of $CsLAZY1$ in Arabidopsis

To further investigate the role of $CsLAZY1$ in shoot gravitropism, we transferred $CsLAZY1$ into Arabidopsis thaliana. The expression of $CsLAZY1$ was detected using real-time PCR assay in over-expressed (OE) plants but not detected in wild-type (WT) plants, and the three OE lines were named as OELAZY1-11, OELAZY1-20, and OELAZY1-24, respectively (Figure 6A). Subsequently, gravitropism assays through time-lapse imaging were implemented to survey the response to reorientation of the WT and three OE lines. All the seedlings with main stem of 5-10 cm were treated in 90° inverted gravity processing. In light, images were collected by computer-controlled cameras at 0, 30, 60, 90 and 120 minutes of inversion (Figure 6B), and the angle of the hypocotyls were measured from the images. Obviously, the OE plants bended upward slightly at 30 minutes, while no bend was seen from the WT plants. After 90 minutes of inversion, OE plants were reached the maximum bending angle, while the WT bended upward slightly at 90 minutes (Figure 6B). Compared to the WT plants, significant difference of bending angle was observed from OE plants after 30 minutes treatment (Figure 6C).
In dark, images were collected at 0, 30, 60, 90, 120, 150 and 180 minutes of inversion, and the angle of the hypocotyls were measured from the images. Both the WT and OE plants in dark bended upward later than their corresponding plants in light, demonstrating that the OE plants bended upward slightly after 60 minutes, while the WT plants bended upward slightly after 90 minutes (Figure 7A). In comparison, significant difference of bending angle was observed from OE plants after 90 minutes (Figure 7B). Consistent with the expression patterns, evidence indicate that *CsLAZY1* may play a vital role in respond to gravitropism in stem of tea plant.

**Discussion**

Agricultural productivity is affected by various environmental factors resulting in lower crop yield. The plant architecture is one of the major constraints, and branch angle plays a vital role in the formation of plant architecture [24]. Accumulated evidence indicates *LAZY1* plays a crucial role in plant responses to gravitropism, and then regulates the branch angle [3, 20, 21, 25]. For tea plant, branch angle is a critical factor which can greatly influences the productivity and efficiency of mechanical plucking. Nevertheless, the molecular mechanism for controlling branch angle of tea plant is scarcely revealed until now. In the present study, we identified three *CsLAZY* genes in tea plant, and analyzed their phylogenetic relationship, gene structures and tissue-specific expression patterns. Subsequently, the biological function of candidate *CsLAZY1* was investigated, including its subcellular localization, tissue-specific expression patterns in different varieties, and heterogenous overexpression analysis which obviously responds differently to gravity.

The three *CsLAZY* genes have different tissue expression patterns (Figure 3B). *CsLAZY3* was distinguished from the other two *CsLAZY* genes by clustering into a different subclade, which had specific high expression level in the second leaf. *CsLAZY2* showed high sequence similarity with *CsLAZY1*, and had the highest expression level in flower, indicating that *CsLAZY2* may plays an important role in the development of flower. In comparison, *CsLAZY1* showed the highest expression level in stem, far higher than in the other seven tissues (Figure 3B), and similar tissue-specific expression patterns can be observed in several other woody plants, such as in poplar [3], peach [12] and apple [16]. To identify whether *CsLAZY1* had different tissue-specific expression patterns in distinct tea varieties, we selected two types of tea plant including open-type (Benshan, Foshou, Yaoshanxiulv and Tieguanyin) and erect-type (Echa 5, Fuzao 2, Longjingchangye, Zhenghedabaicha) based on branch angle, while no obvious difference of the expression pattern was observed in distinct tea varieties (Figure 4). In poplar, the transcript level of *LAZY1* displayed similar expression profiles in different tissues between narrow-crown and broad-crown poplars [3]. The results indicate that the uneven expression of the *LAZY1* gene in stem affected the curvature of the stem [21, 31]. Besides, subcellular localization of *CsLAZY1* gene was analyzed, demonstrating that the CsLAZY1 protein is located in the membrane, which is consistent with the previous researches in *Arabidopsis* [20, 21, 29]. However, *OsLAZY1* is located in the nucleus in rice, and *OsBRXL4* regulates shoot gravitropism and rice tiller angle by affecting nuclear localization of *LAZY1* [33].
*LAZY* genes that share common domain sequences usually have a common origin, and thus have similar functions [12, 20, 23]. Homology analysis of *LAZY-like* genes in poplar and functional investigation of *PzLAZY* suggested that *PzLAZY* may be involved in altering branch angle [3]. Among *LAZY* genes, an EAR motif located in the conserved region V, which plays a role in controlling the hormonal systems and being related with gravitropic response of plants. For instance, the binding of TOPTLESS proteins to the EAR motif of AUX/IAA proteins can repress auxin-responsive genes [30]. The obtained three *CsLAZY* genes from tea plant shared five limited regions of sequence and highly conserved in the region V (Figure 1A), indicating that they may play roles in the development of tea plant. Phylogenetic analysis showed that *CsLAZY1* had high sequence similarity with *LAZY1* from other woody plants including in grape, poplar and peach, indicating that *LAZY1* had higher conservation in the process of evolution within woody plants.

Furthermore, we obtained heterotopic OE Arabidopsis plants, while no difference of phenotypes between OE plants and wild types was observed, and this is also consistent with a previous study [34]. It is noteworthy that OE plants had obviously distinct phenotype with the wild types in response to gravity (Figure 6-7). It was speculated that *CsLAZY1* may play roles in altering branch angle by acting on the transportation of phytohormones. In *Arabidopsis thaliana*, six *AtLAZY* genes were participated in the early gravity signaling for shoot gravitropism [20, 21, 27-29]. *AtLAZY1* leads to the asymmetric distribution of auxin thus altering rice tiller angle, and *AtLAZY1* mediate gravity signaling in statocytes downstream of amyloplast displacement, leading to the generation of asymmetric auxin distribution in gravity-responding organs [28, 31, 32]. In rice, *OsLAZY1* controls rice tiller angle by regulating shoot gravitropism through inhibition of polar auxin transport (PAT) [19, 26, 27]. We also analyzed the cis-elements in promoters of *CsLAZYs*, demonstrating that MeJA, GA, SA and three ABA hormone responsive element were existed in the promoter of *CsLAZY1* (Figure 3A). Collectively, *CsLAZY1* may play roles in response to gravitropism and altering branch angle by acting on the transportation of phytohormones.

**Materials And Methods**

**Plant materials**

A total of nine five-year-old tea plant cultivars (*Camellia sinensis* var. ‘Shuchazao’, ‘Benshan’, ‘Foshou’, ‘Yaoshanxiulv’, ‘Tieguanyin’, ‘Echa 5’, ‘Fuzao 2’, ‘Longjingchangye’, and ‘Zhenghedabaicha’) from the Tea Plant Cultivar and Germplasm Resource Garden in Guohe Town (Anhui Agricultural University) were used for collection of various tissues (leaf, bud, root, and stem). All the samplings were collected with permission by our institution (State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University). All tissues were sampled according to the demands of each experiment, and they were immediately frozen in liquid nitrogen and stored at -80°C until utilization. Study protocol must comply with relevant institutional, national, and international guidelines and legislation.

**Identification and Molecular Cloning of *CsLAZYs***
The nucleotide and deduced amino acid sequences of 6 AtLAZY genes from Arabidopsis were obtained from TAIR (The Arabidopsis Information Resource) database (https://www.arabidopsis.org/). A genome-wide search of 6 AtLAZY genes was carried out using Basic Local Alignment Search Tool (BLAST) analysis with the 6 AtLAZY genes used as queries against the tea plant genome (http://tpia.teaplant.org/Blast.html) [35]. All non-redundant protein sequences were compared with AtLAZYs and OsLAZYs, and the genes possessed pivotal conserved domains were selected. To verify the coding regions of CsLAZYs, the gene-specific primers were designed for amplification of CsLAZY genes with cDNA templates from leaves of Camellia sinensis var. ‘Shuchazao’.

Multiple sequence alignment of LAZY protein sequences were performed using the ClustalW program. The phylogenetic trees were generated by MEGA 6.0 with the Neighbor joining (NJ) algorithm. Bootstrap analysis with 1000 replicates was used to evaluate the significance of the nodes, and p-distance model was used to ensure that the divergent domains could contribute to the topology of the NJ tree.

Alignment of amino acid sequences was performed using T-COFFEE (http://tcoffee.org/) [36]. Based on the gene structure display server (GSDS 2.0, http://gsds.cbi.pku.edu.cn/index.php) program, we determined the exon/intron organization of CsLAZYs through comparing the coding sequences to their corresponding genomic sequences.

**Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from tea leaves using the RNAprep pure Plant Kit (cat DP432, Tiangen, Beijing) according to the manufacturer’s protocol. The quality and quantity of each RNA extract was detected using agarose gel electrophoresis and the Nanodrop 2000 (Thermo Fisher Scientific, US). The first-strand cDNA was synthesized from total RNA using the PrimeScript RT Reagent Kit (cat RR036A, Takara, Japan) following the manufacturer’s protocol. A total of 10 ul reaction, including 5 ul TB Green Enzyme, 1.2 ul cDNA, 3.2 ul water and 0.6 ul primer, was used for qRT-PCR, and the detailed process was performed as described previously [37, 38]. The CsGAPDH gene was selected as the internal control, and the relative gene expression values were analyzed using the $2^{-\Delta \Delta Ct}$ method [39]. All reactions were run in triplicate technical replicates for each sample, and three biological replicates were performed. The relevant primers are listed in Additional file 1.

**Subcellular localization of CsLAZY1**

The CsLAZY1 plasmid fused with GFP was constructed by Gateway Technology, and the ORFs of CsLAZY1 with 25 bp vector adapter were amplified by RT-PCR. PCR products were inserted into the pdonor207 vector by BP clone enzyme mix, followed by were transferred into PK7WGF2 through LR reactions. The resultant plasmids of empty vector and pk7WGF2-LAZY1 were transformed into Arabidopsis protoplast cells, and the protoplasts were examined after overnight transformation. Besides, the resultant vectors were also transformed into Agrobacterium GV3101 competent cells, and the construct and empty vector were transiently introduced into tobacco leaves by injection. The tobacco
leaves were held for 48 h at 25 °C in the dark after transformation, followed by the tobacco leaves and protoplasts were examined using an Olympus FV1000 confocal microscope (Olympus, Japan).

**Arabidopsis Transformation**

The full-length cDNA sequences were ligated into PBI121 driven by CaMV35S, and then transferred into Agrobacterium strain GV3101. *Arabidopsis* (Col) was transformed using the floral dip method as described previously [40]. Transformed plants were selected on the basis of their resistance to kanamycin, and 4-weeks-old homozygous T3 plants were used for further experiments. Three transgenic lines were treated at 90° inverted gravity processing for analysis of bending angle.

**Abbreviations**

OE: over-expressed; WT: Wild type; qRT-PCR: Quantitative real-time polymerase chain reaction; IAA: Indoleacetic acid; ABA: Abscisic Acid; SA: Salicylic acid; GA: Gibberellin; MeJA: Methyl Jasmonate; SLs: Strigolactones; BRXL4: Brevis Radix Like 4; PIN: PIN-FORMED.

**Declarations**

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**Authors’ contributions**

XBX dealt with the experiments and wrote the manuscript. XZM, LJ, RG, JYZ, HX, LL, YLA, and CZ were involved in the experiments and data analysis. SRL and CLW conceived the project, designed the research and revised the paper. All authors have read and approved the manuscript.

**Availability of data and materials**

The data sets supporting the results of this article are available at the SRA database of NCBI (https://www.ncbi.nlm.nih.gov/) under project accession number PRJNA387105.

**Ethics approval and consent to participate**
The tea cultivars used in this study were planted and grown at Anhui Agricultural University, Hefei, Anhui province, China. No specific permits were required for our collection of plant materials. This study did not require ethical approval or consent, as no endangered or protected plant species were involved. Study protocol must comply with relevant institutional, national, and international guidelines and legislation.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publish**

Not applicable

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Tables
Table 1 Characterization of CsLAZYs in tea plant

| Gene name | Gene ID   | Genomic position    | CDs (bp) | ORF (aa) | MW (kDa) | pI  |
|-----------|-----------|---------------------|----------|----------|----------|-----|
| CsLAZY1   | CSS025254 | Scaffold308520-312877 | 1200     | 399      | 44.2     | 6.55|
| CsLAZY2   | CSS049138 | Scaffold357075-360635 | 1104     | 367      | 41.2     | 6.18|
| CsLAZY3   | CSS020288 | Scaffold190296-193383 | 756      | 251      | 29       | 6.47|