



Transcriptome and Phytohormone Analysis Reveal the Regulatory Mechanism of Internode Elongation in New Shoots of Tea Plants (*Camellia sinensis*)

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Abstract

Exploiting mechanical picking of fresh leaves is an urgent task in the tea industry. The internode length of new shoots is one of the key traits determining the suitability of tea plants for mechanical picking. In this study, we explored the mechanisms regulating tea internode elongation through transcriptome sequencing and endogenous hormone analysis in two cultivars with different internode length. The results indicated that internode elongation is associated with the increased parenchyma cell diameter in the internode. One *CsDELLA* gene (*CsDELLA2*), three *CsMYC* transcription factors (TFs) (*CsMYC1.2*, *CsMYC1.3*, and *CsAMS.2*), two *CsJAZ* TFs (*CsJAZ1* and *CsJAZ3*), and three secondary cell wall-related genes (*CsEXP1*, *CsCESA1* and *CsXTH1*) have been identified as potential candidate genes. These results provide candidate genes for obtaining tea plants with longer internodes which are suitable for mechanical harvesting.

Keywords DELLA · Gibberellins (GAs) · Internode · Jasmonate (JA) · New shoots · Phytohormones · Tea plant (*Camellia sinensis*) · Transcriptome sequencing

Introduction

Tea plant (*Camellia sinensis* L.), an important economic beverage crop, is widely cultivated worldwide. China is the largest tea producer in the world. With the increasingly severe problems of population aging and labor shortage, realizing full mechanized picking of fresh leaves is an urgent task for the high-quality development of the tea industry. Internode length, used as the core parameter for picking point recognition and feature extraction, directly affects the integrity of machine-picked tea bud leaves (Liu et al. 2024; Zheng et al. 2023). At present, the studies on the regulatory mechanisms of plant internode elongation are relatively limited. The internode length largely depends on cell length, which is regulated by the biosynthesis and expansion of cell wall (Oh et al. 2014; Wang et al. 2024). The secondary cell wall-related genes, such as *xyloglucan endo-hydrolase* (*XTH*), *β-expansin* (*EXP*), and *cellulose synthase* (*CESA*), are involved in cell wall relaxation and elongation (Wang et al. 2024). Plant hormones play important roles in determining cell wall extension and subsequently cell elongation

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during plant growth and morphogenesis (Oh et al. 2014; Wang et al. 2024).

Jasmonate (JA) is a fatty acid derivative, which together with its active precursor 12-oxo-phytodienoic acid (12-OPDA), the methyl ester derivative of JA (MeJA), and the JA-isoleucine conjugate (JA-Ile) are collectively known as JAs (Wang et al. 2020; Ma et al. 2024). JA ZIM-domain (JAZ) and basic helix–loop–helix (bHLH) MYC proteins are key transcriptional repressors and activators of JA signaling, respectively (Hou et al. 2010; Zhang et al. 2024). The F-box protein CORONATINE INSENSITIVE1 (COI1) perceives JA signals and recruits JAZ proteins for ubiquitination and subsequent degradation (Wang et al. 2020; Qi et al. 2014; Wasternack and Song, 2017). JAZ proteins connect early responsive MYC TFs and later respond to downstream genes (Wang et al. 2020). JA biosynthesis is catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC) and OPDA reductase (OPR) in turn. Subsequently, two JA isomers are produced with the help of acyl-CoA oxidase (ACX) (Wang et al. 2020; Ma et al. 2024). Upon stress or developmental signals, the bioactive JA-Ile is rapidly synthesized under the catalysis of jasmonoyl-isoleucine synthetase (JAR1), and JA can also be converted into MeJA by JA carboxyl methyltransferase (JMT) (Wang et al. 2020; Zhang et al. 2024). In tea plants, CsMYC2 can regulate multiple physiological processes through transcription activating *CsLOX7* and *CsAOS2* or by interacting with *CsJAZ2*, *CsJAZ3*, *CsJAZ7*, and *CsJAZ8* (Zhang et al. 2024; Chen et al. 2021; Zhu et al. 2024).

JA exerts a negative regulatory role in cell and internode elongation (Wang et al. 2024). For example, exogenous application of the JA mimic coronatine (COR) inhibits *ZmXTH1* expression, shortens parenchyma cells and basal internodes at maize jointing stage (Wang et al. 2024; Ren et al. 2023). Overexpression of *CYP94C2b*, a cytochrome P450 gene that encodes an enzyme promoting JA-Ile inactivation, increased cell diameter and internode length in rice (Wang et al. 2024). AtMYC2/3/4 proteins repress the expression of cell elongation related-genes, such as *EXP2*, through activating *HY5* expression in *Arabidopsis* (Yi et al. 2020). MYC2 regulates hypocotyl elongation during the plant response to red/far-red light (Zhang et al. 2024; Zhu et al. 2024).

Gibberellins (GAs) are a large group of diterpenoid phytohormones (Fukazawa et al. 2014, 2017; Qin et al. 2014; Yoshida et al. 2014; Chen et al. 2017). Although approximately 136 forms of GAs have been identified, only a few of them, including GA₁, GA₃, GA₄, and GA₇, are biologically active (Davière and Achard 2016; Xue et al. 2022). DELLA proteins are key repressors of almost all GA responses (Fukazawa et al. 2014, 2017, 2021; Qin et al. 2014; Yoshida et al. 2014; Li et al. 2016; Lan et al. 2014). Endogenous

GA levels are regulated by GA biosynthesis and deactivation (Chen et al. 2023). Gibberellin 2-oxidase (GA2ox) plays an important role in GA catabolism (He et al. 2024), while GA20ox and GA3ox catalyze the final step of GA biosynthesis (Fukazawa et al. 2017; Fukazawa et al. 2021). In *Arabidopsis*, 14 early GA-responsive genes, including *AtGA20ox2*, *AtGA3ox1* and GA receptor gene *AtGID1b*, have been identified as the target genes of DELLA (Fukazawa et al. 2017, 2021; Park et al. 2013). GAs play major roles in regulating stem and internode elongation (Xue et al. 2022; He et al. 2024; Davière et al. 2014; Nagai et al. 2020). For instance, *AtGA20ox1* and *AtGA20ox2* act redundantly to promote internode elongation (Fukazawa et al. 2021). Rice *Osga3ox2* and *Osga20ox2* mutants both exhibited a semidwarf phenotype due to decreased active GA contents in the elongating stem (Chen et al. 2023; He et al. 2024). Deepwater conditions promoted internode elongation by increasing *GA20ox2* expression and promoting cell expansion in rice internode (Davière et al. 2014; Nagai et al. 2020). However, in poplar and rice, overexpression of *GA2ox1* reduces active GA content and internode length (Chen et al. 2023; He et al. 2024; Cao et al. 2024).

The internode length is a core agronomic trait that significantly influences crop yield, lodging resistance and suitability for mechanized harvesting in agricultural production. Currently, research on internode development and its regulatory mechanisms mainly focuses on crops. For example, the lodging resistance of crops, such as corn and rice, is closely related to the internode length of the stem base (Wang et al. 2024; Ren et al. 2023), and the internode length in cotton is strongly associated with mechanized picking efficiency (Zhan et al. 2021). In tea production, the internode length is a key factor affecting the crushing rate during mechanical-harvesting, however, its regulatory mechanisms have not yet been reported. This study suggested that the elongation of parenchyma cells is the direct cause leading to internode elongation with new shoot development and the differences in internode length between two tea cultivars. Furthermore, three secondary cell wall-related genes, including *CsEXPI*, *CsCESA1*, and *CsXTH1*, were involved in regulating internode elongation at the transcriptional level. Both JA and GA signaling pathways participate in the regulating of internode elongation of tea plants. Furthermore, *CsDELLA2* gene, three *CsMYC* TFs, and two *CsJAZ* TFs were candidate genes. Understanding these mechanisms provides a theoretical foundation for improving the mechanical picking suitability of tea plants by regulating its internode elongation.

Materials and Methods

Plant Materials and Methods

Mature tea plants (*C. sinensis* L.) of two cultivars ‘Longjing 43’ (‘LJ43’) and ‘Zhongcha 102’ (‘ZC102’) with significantly different internode lengths, both of which were selectively bred from Longjing population species, were used in this study. The tea plants were cultivated in the tea garden of Shengzhou experimental base, Tea Research Institute, Chinese Academy of Agricultural Sciences, located in Shaoxing, Zhejiang Province, China (longitude 120°48'E and latitude 29°44'N, 70 m above sea level), and under uniform soil and agronomic management conditions.

The tea plants were lightly pruned after spring tea picking in late April. The first new shoots emerging just below the trimmed cuts were selected for the study. When two, three, and five true leaves on the new shoots had emerged, the internode length of each node was measured, respectively, and the average internode length was calculated. The samples of the mixed internodes were collected and immediately frozen with liquid nitrogen then stored at -80 °C for later analysis.

Histological Analysis of Internodes

The first internodes located between the fish leaf and first true leaf was selected for paraffin sections. The samples were collected and immediately fixed in formalin-glacial acetic acid-alcohol (FAA) solution. Subsequently, they were dehydrated with a series of ethanol and stained with 1% safranin. After embedding in paraffin, all the cross and longitudinal sections were cut and stained with 0.1% fast green. The internode anatomical structure was observed, and the images were collected using an OLYMPUS BX53 microscope (Olympus Optical Co., Ltd, Japan). The images were captured using an Eclipse E100 microscope (Nikon). ImageJ software (<https://imagej.net/software/imagej/>) was used to measure the cell number and cell diameter.

RNA Extraction, cDNA Library Construction, and Sequencing

RNA-seq sequencing was carried out using 18 samples, which included 3 biological replicates, of two cultivars at 2-leaf, 3-leaf, and 5-leaf stages. The cDNA library construction and RNA sequencing were carried out by Novogene Biotechnology Co., Ltd. (Beijing, China). In brief, a sequencing library was generated using a NEB-Next® Ultra™ RNA Library Prep Kit (NEB, USA). RNA sequencing was conducted on a HiSeq 4000 (Illumina) platform. The genome of ‘LJ43’ was selected as the reference genome and

the clean reads were aligned to it using HISAT v2.0.5 software. The FPKM method was used to normalize the gene expression using StringTie v1.3.1 software. The DEGs in the RNA-seq dataset were identified by DESeq2 ($|\log_2$ fold change|>1, FDR<0.05) based on read counts. Cluster Profile software was used for GO functional enrichment analysis, and the KEGG database was utilized for the pathway enrichment analysis. OmicShare tools were used to graphically display the results of the GO and KEGG analyses.

Verification of RNA-Seq Results Using Quantitative real-time PCR (qRT-PCR)

The total RNA was extracted using an RNA extraction kit (Tiangen Biotech, Beijing, China) and the genomic DNA was removed. The quality, purity, concentration and integrity of RNA were assessed. Reverse transcription was carried out using PrimeScript™ RT-PCR Kit (TaKaRa, Otsu, Japan). The gene-specific primer sequences are listed in Table S4. The qRT-PCR assay was carried out using the ABI 7500 qRT-PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix. The qRT-PCR conditions were as follows: 95°C for 30 s, subsequently 40 cycles of 95 °C for 5 s. The relative gene expression was calculated (Livak and Schmittgen 2001).

Determination of Endogenous Phytohormone Contents

The measurement of endogenous phytohormone contents was carried out by Nanjing Ruiyuan Biotechnology Co., Ltd., China. In brief, 1.0 g of fresh internode samples were ground in liquid nitrogen. Fifty microliters (50 µL) of internal standard solution and 1 ml of a water-acetonitrile solution (containing 1% formic acid) were added sequentially. The samples were subjected to vortex mixing, extraction, and centrifugation. Then, 800 µl supernatant was collected and air-dried with nitrogen gas. The samples were reconstituted in 100 µl water-acetonitrile (1:1, v/v), followed by centrifugation. The detection of the supernatant was analyzed using Agilent 1,290 Infinity LC Ultra Performance Liquid Chromatography (UHPLC, Agilent) and 5,500 QTRAP mass spectrograph (MS, AB SCIEX; UHPLC-MS).

Statistical Analysis

The data were analyzed with Statistica 6 (Statistica, SAS Institute Inc., Cary, NC, USA). Specifically, the internode length and cell diameter in each cultivar among 3 time points, as well as the FPKM values and relative gene expression in cv. ‘LJ43’ among 3 time points were analyzed by one-way ANOVA, and significant differences ($P < 0.05$) were further

analyzed via Duncan's multiple range test. The differences of the internode length and cell diameter between two cultivar at each time point, the hormone contents between two cultivar were assessed using Student's *t*-tests. OmicShare tools were used to graphically display the correlation coefficients between gene FPKM values as well as between gene FPKM values and internode length. The co-expression correlation network based on FPKM values was established and visualized with Cytoscape 3.8.0.

Results

Phenotypic and Endogenous Hormone Analysis of Tea Internodes during New Shoot Development

Tea plants of two cultivars 'Longjing 43' ('LJ43') and 'Zhongcha 102' ('ZC102') were lightly pruned after spring tea picking. From the end of May to mid-June, when the apical buds and the second, third and fifth leaves below the apical buds grew out, the internode lengths of all the nodes were measured. The results showed that the average internode lengths of 'LJ43' were significantly greater than that

of 'ZC102' at all three developmental stages (Fig. 1A, B, D). The first internodes located between the fish leaves and the first true leaves, were selected for paraffin sectioning. The results showed that the diameters of parenchyma cells in longitudinal-sections increased with internode elongation in both cultivars. Furthermore, the cell diameters in 'LJ43' were both significantly greater than those in 'ZC102' at 2 L- and 3 L-stage (Fig. 1C, E). On the other hand, the histological observation of internode cross-sections revealed that the cell numbers increased with internode elongation in the two cultivars, respectively. However, different from cell diameters, there were no significant differences in cell numbers between two cultivars at each stage (Fig S1). Thus, it can be inferred that the increased diameters of internode cells, rather than the cell numbers, may be the primary factor contributing to the differences in internode length between the two cultivars at a given developmental stage, or among different stages in a certain cultivar.

To investigate the roles of phytohormones in regulating tea internode elongation, mixed internode samples from two cultivars at the 2-leaf stage were sampled for measuring endogenous hormone contents. The results showed that the contents of two forms of active GAs, including GA₁

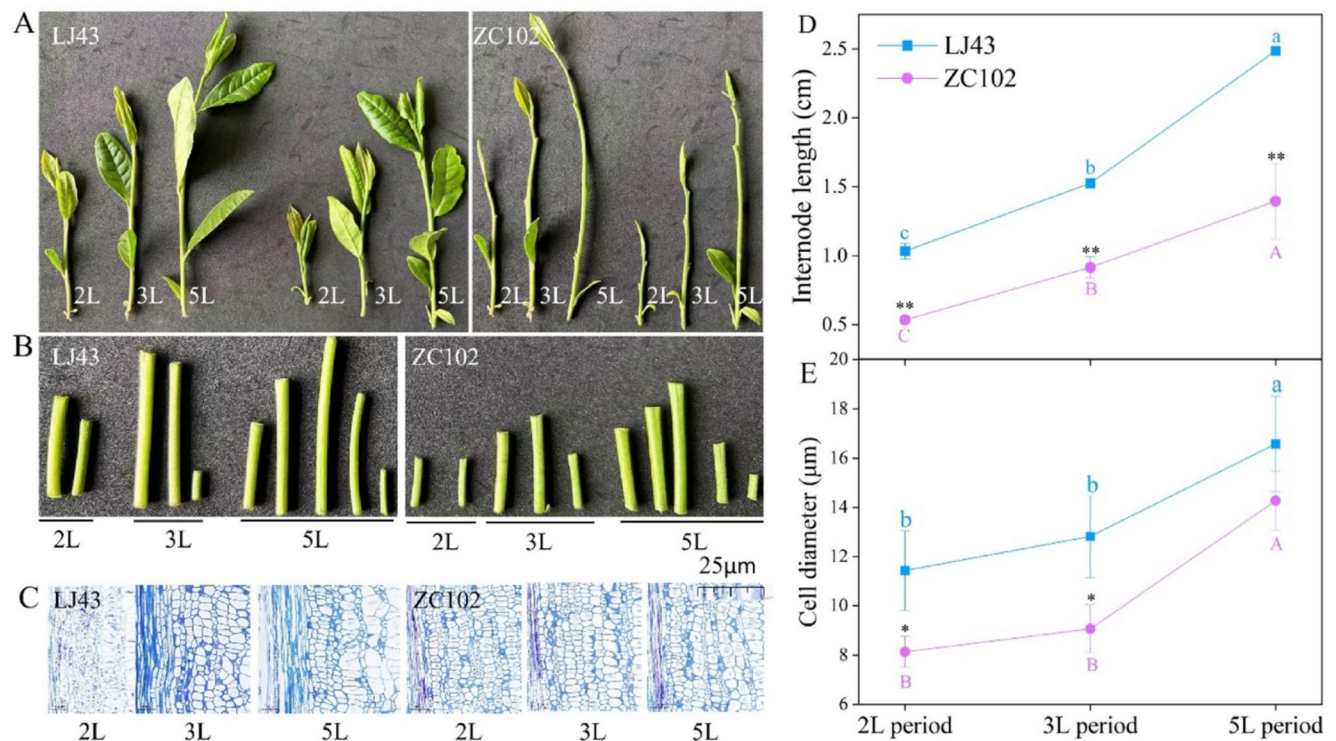


Fig. 1 Phenotypic analysis of new shoots, internodes, and anatomical structures of internode longitudinal sections in two cultivars 'LJ43' and 'ZC102' at 3 developmental stages. The new shoots (A), internodes of all the nodes (B), internode longitudinal sections (C), average internode length (D), and cell diameter based on the internode longitudinal sections (E) in two cultivars at three stages. Note: 2L, 3L, and 5L indicate 2-, 3-, and 5-leaf stage, respectively. Error bars indicate

standard deviation from the mean of 15 and 3 biological replicates for internode length (D) and cell diameter (E), respectively. In Figs. D and E, letters indicate significant differences among different stages in each cultivar ($P < 0.05$, Duncan's multiple range test); asterisks indicate significant differences in ZC102 as compared with LJ43 (control) at the same time point ($P < 0.05$; Student's *t*-test)

and GA₇, in the internode of ‘LJ43’ were all significantly higher than those in ‘ZC102’, while no significant difference was observed in the contents of GA₃ and GA₄ (Fig. 2a; Fig. S2). On the other hand, the contents of two forms of active JAs, including JA-ILE and OPDA, in the internode of ‘LJ43’ were both significantly lower than those in ‘ZC102’, whereas no significant differences were detected in the contents of JA and MeJA (Fig. 2b; Fig. S2). Taken together, the above results suggested that the differences of the contents of endogenous active GAs (GA₁, and GA₇) and JAs (JA-ILE, and OPDA) may contribute to the variation in internode length between the two cultivars at 2-leaf stage. Thus, the endogenous GA and JA signaling pathways may be involved in regulating internode elongation in tea new shoots.

Transcriptome Sequencing of the Internodes in Two Tea Cultivars across Three Developmental Stages

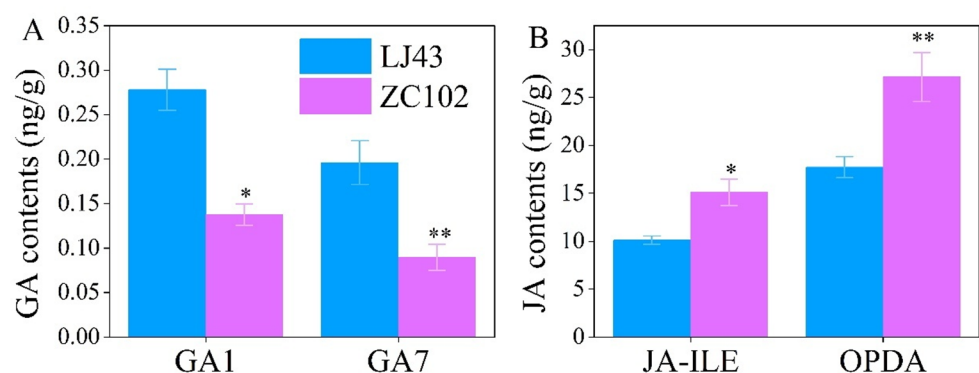
RNA-seq analysis was carried out using internode samples from two cultivars at 2-leaf, 3-leaf, and 5-leaf stages. The quality of the sequencing data was listed in Table S1. Correlation heat-map analysis showed that the minimum R² value among three biological replicates was 0.773 (Fig. S3A). Principal component analysis (PCA) diagram revealed that samples from different groups were well separated, while samples within the same group were closely clustered. Furthermore, the three developmental stages of ‘LJ43’ and ‘ZC102’ were clustered together along the horizontal axis, respectively (Fig. S2B). Thus, the above results indicated that sequencing data were of high quality. The number of DEGs varied between two stages in a certain cultivar as well as between two cultivars at a certain stage. It can be seen that the number of DEGs between the 2L- and 3L- stages of each cultivar, and between the 3L- and 5L- stages in ‘LJ43’ was relative less. However, the number of DEGs at the same certain stage between two cultivars was the highest (Fig. S4). The Venn diagram of gene co-expression also showed that for the 3 stages of ‘LJ43’ and ‘ZC102’, the total number of DEGs was 27,238 and 26,018, respectively. There were

22,158, 22,307, and 22,888 DEGs co-expressed between the two cultivars at 2-, 3-, and 5-leaf stage, respectively (Fig. 3). Gene Ontology (GO) enrichment analysis showed that these DEGs are involved in cellular biosynthetic process, plant-type cell wall organization biogenesis, cell wall organization biogenesis, and hormone metabolic process. From the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, it can be seen that these DEGs are involved in plant hormone signal transduction (Fig. 4). As the potential roles of cell wall-genes, GA and JA pathways in regulating the internode length of tea plants (unpublished data), the secondary cell wall-genes, JA- and GA-related genes were analyzed subsequently.

Secondary Cell Wall-related Genes Associated with Internode Elongation in Tea Plants

We analyzed the correlation coefficients between the average internode length and the FPKM values of secondary cell wall-related genes in the internodes of two cultivars at three stages. The results showed that the average internode length exhibited strong positive correlations with the FPKM values of *CsEXPI*, *CsCESA1* and *CsXTH1*, respectively (Fig. 5A, B). The correlation coefficients were shown in Table S2. RNA-Seq results showed that, the FPKM values of these three genes all gradually increased in ‘LJ43’ from 2-leaf to 5-leaf stage. Subsequently, the FPKM values of DEGs were verified by quantitative real-time PCR (qRT-PCR). The results of qRT-PCR showed that the expression levels of *CsEXPI* and *CsCESA1* both showed a trend of initial increase followed by a decreasing, while *CsXTH1* expression levels showed a trend of gradual increase in ‘LJ43’ from the 2-leaf to 5-leaf stage (Fig. 5C-E). Thus, the trends of the FPKM values and gene expression levels detected by qRT-PCR were generally consistent, suggesting that the RNA-seq results were reliable. Based on the above results, it can be concluded that the three cell wall-related genes, including *CsEXPI*, *CsCESA1* and *CsXTH1*, were all associated with tea internode elongation. Subsequently, the average internode length and these 3 cell wall-related genes

Fig. 2 The contents of plant hormones GAs and JAs in the internodes of two cultivars ‘LJ43’ and ‘ZC102’ at 2-leaf stage. Error bars indicate standard deviation from the mean of 3 biological replicates. Asterisks indicate significant differences of each indicator in ZC102 as compared with LJ43 (control) ($P < 0.05$; Student’s *t*-test)



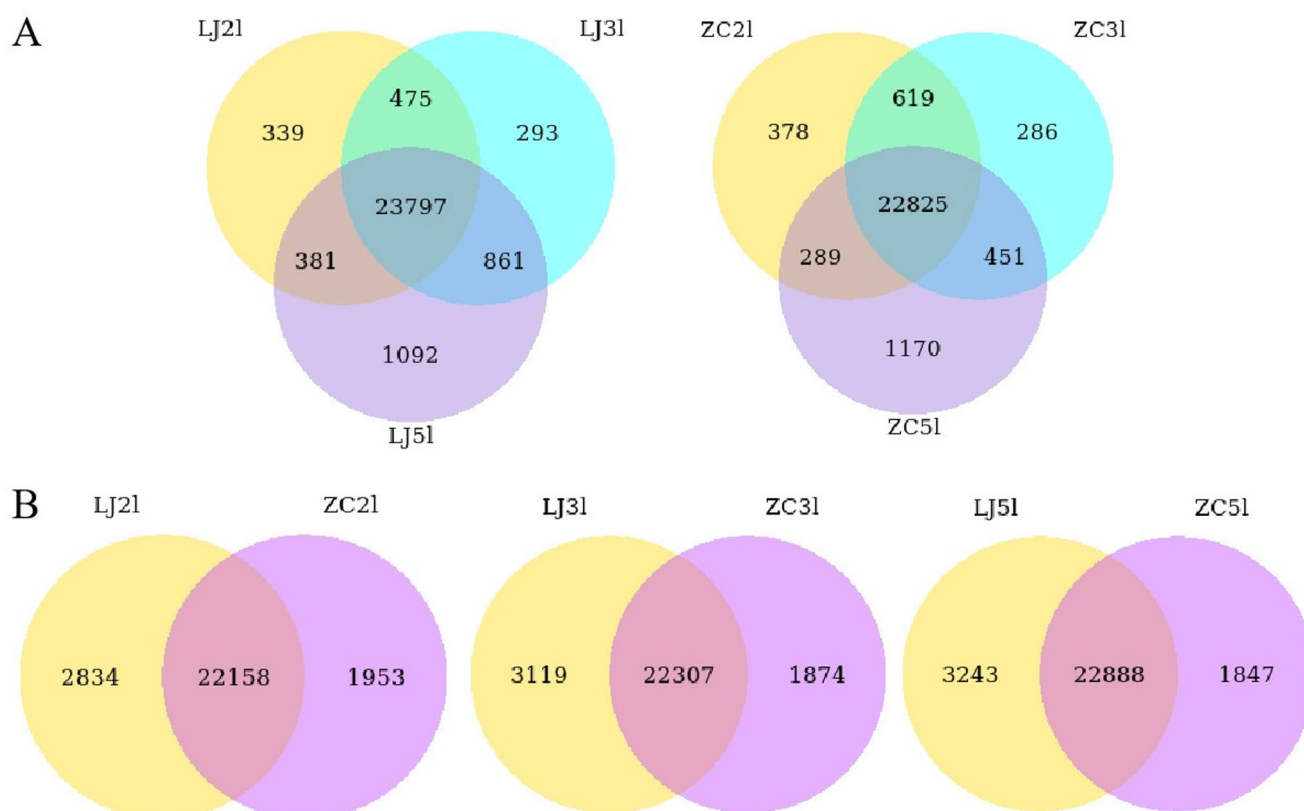


Fig. 3 The co-expression venn diagrams showing DEGs in the internodes at 3 development stages of each cultivar (A) and at each development stage of two cultivars (B)

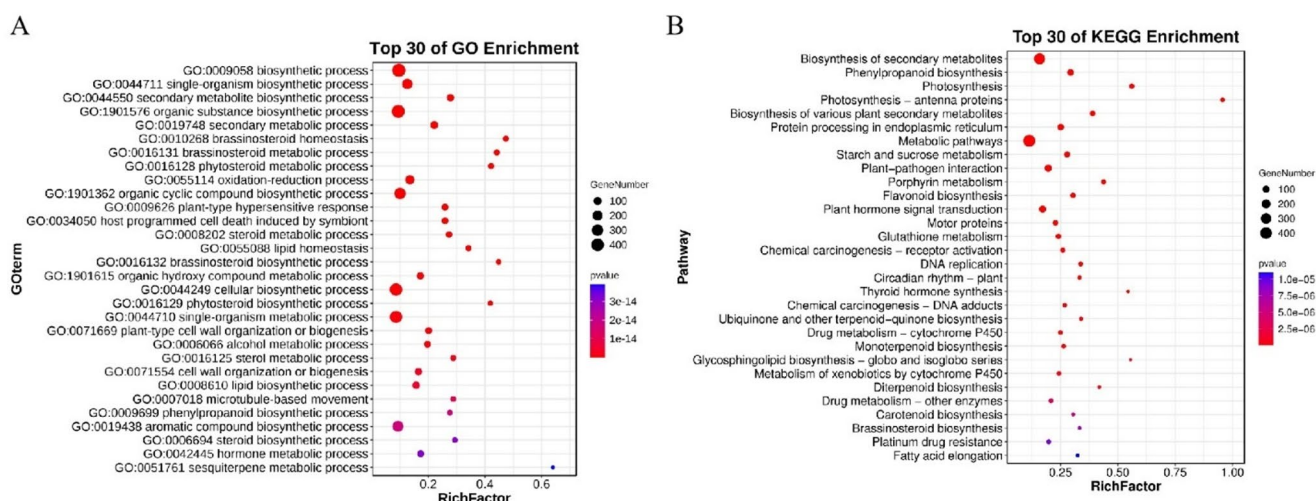


Fig. 4 GO and KEGG term enrichment analysis for DEGs in the internodes of two cultivars ‘LJ43’ and ‘ZC102’ at 3 developmental stages

were used for screening GA- and JA-related genes, which may be the candidate genes regulating internode elongation in ‘LJ43’, through correlation analysis.

GA- and JA-related Genes Associated with Internode Elongation in Tea Plants

To explore the regulatory mechanisms of GA and JA signals in internode elongation, the correlation analysis between the FPKM values of GA- and JA-related genes and internode length or the FPKM values of the 3 cell wall-related genes

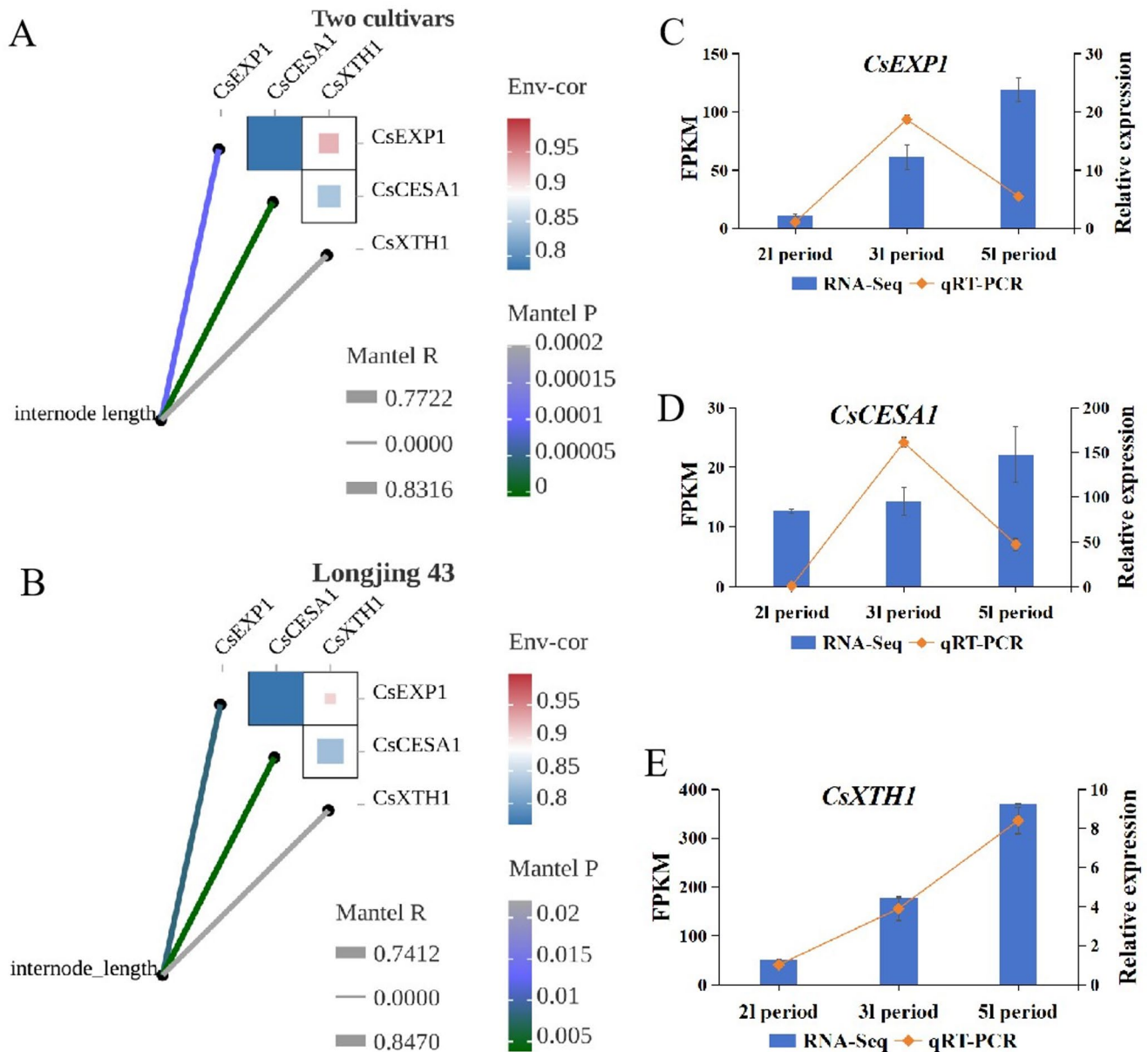


Fig. 5 The correlation analysis between the FPKM values of three cell wall-related genes, including *CsEXP1*, *CsCESA1*, and *CsXTH1*, and the average internode length in two cultivars ‘LJ43’ and ‘ZC102’ (A, B). The FPKM values and relative gene expression detected by qRT-

PCR of three cell wall-related genes in ‘LJ43’ (C, D, E). In Figs. C–E, error bars indicate standard deviation from the mean of 3 biological replicates for the gene FPKM value (the main coordinate axis) and relative gene expression (subordinate axis), respectively

of LJ43 was performed. Based on the correlation analysis, 27 GA- and JA-related genes were screened and the gene IDs were listed in Table S3. Among them, 8 signal transduction genes and 11 biosynthetic genes in GA or JA pathways were selected for further study. The correlation between their FPKM values and the internode length or the FPKM values of 3 cell wall-related genes was visualized, and the FPKM values of these 19 GA and JA pathway genes were verified by qRT-PCR.

It can be seen that, in LJ43, there was a highly negative correlation between the FPKM values of *CsDELLA2*,

CsGID1d, *CsMYC1.2*, *CsAMS.2*, and *CsMYC1.3* and internode length as well as the FPKM values of the 3 cell wall-related genes, while there was a highly positive correlation between the FPKM values of *CsCOI1a*, *CsJAZ1*, *CsJAZ3* and internode length or the FPKM values of 3 cell wall-related genes (Fig. 6A). During internode elongation of ‘LJ43’, the FPKM values of *CsDELLA2*, *CsGID1d*, *CsMYC1.2*, *CsAMS.2* and *CsMYC1.3* all showed trends of gradual decrease, whereas the FPKM values of *CsCOI1a*, *CsJAZ1* and *CsJAZ3* all showed trends of gradual increase. The results of qRT-PCR showed that with internode elongation,

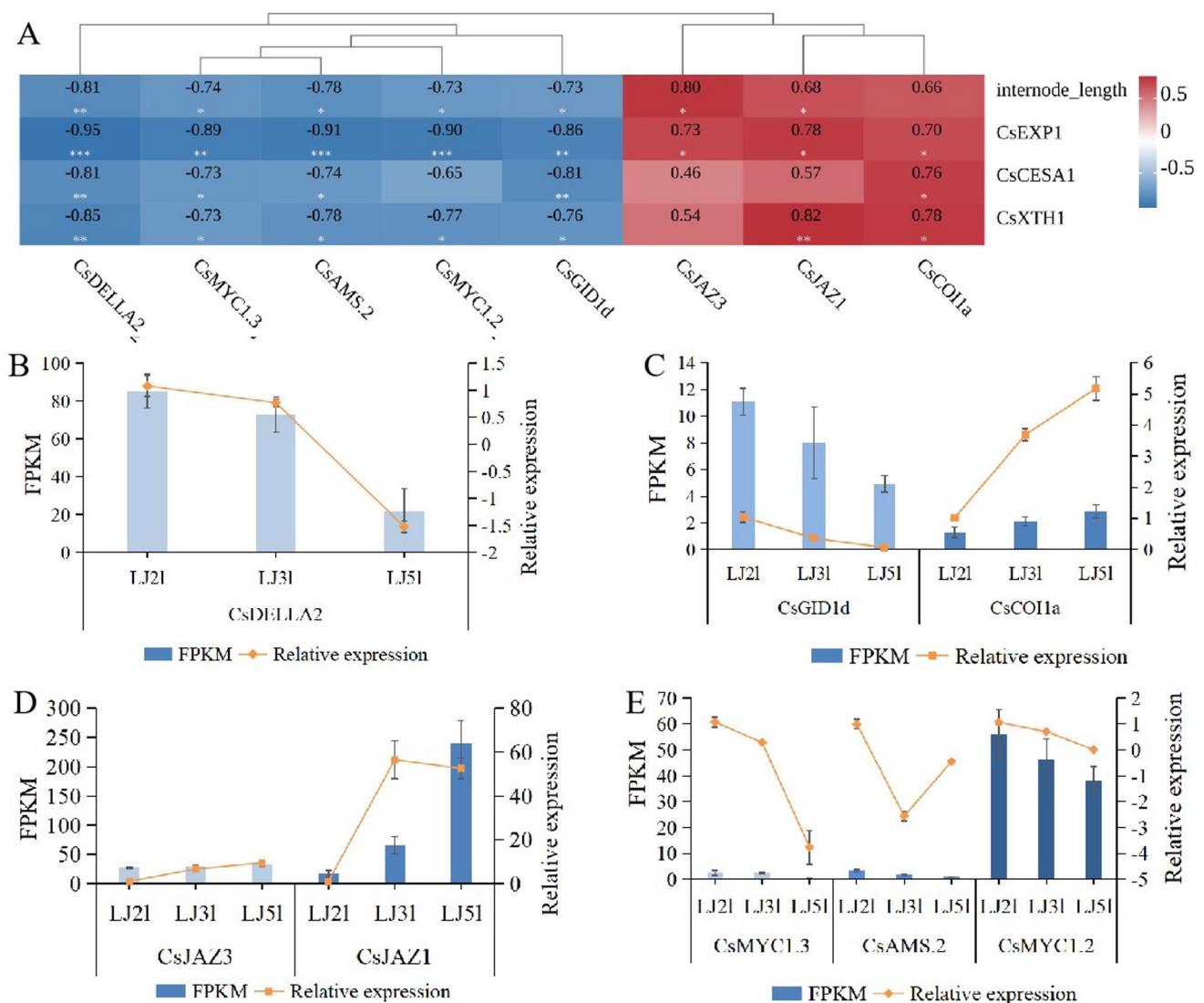


Fig. 6 The correlation analysis between the FPKM values of 8 JA/GA signal transduction genes and 3 cell wall-related genes or the internode length of 'LJ43' (A). The FPKM values and relative gene expression detected by qRT-PCR of 8 JA/GA signal transduction genes in the

internodes of 'LJ43' at 3 developmental stages (B, C, D, E). In Fig. B-E, error bars indicate standard deviation from the mean of 3 biological replicates for the gene FPKM value (the main coordinate axis) and relative gene expression (subordinate axis), respectively

the expression of *CsDELLA2*, *CsGID1d*, *CsMYC1.2*, and *CsMYC1.3* all gradually decreased, while the expression of *CsCOI1a* and *CsJAZ3* all gradually increased. On the other hand, with internode elongation, the expression of *CsJAZ1* increased at first and then decreased, while the expression of *CsAMS.2* decreased at first and then increased (Fig. 6B-E). Thus, the change trends of the expression levels of these 8 JA/GA signal transduction genes detected by qRT-PCR were all basically consistent with the RNA-seq results.

GA and JA Biosynthetic Genes Associated with Internode Elongation in Tea Plants

The correlation between the FPKM values of 11 biosynthetic genes in GA and JA pathways and the internode length or the FPKM values of the 3 cell wall-related genes in 'LJ43' was visualized. It can be seen that it showed a highly negative correlation between the FPKM values of *CsLOX2*, *CsLOX5*, *CsOPR1*, and *CsRUG3* and the internode length as well as the FPKM values of the 3 cell wall-related genes. However, it showed a highly positive correlation between the FPKM values of *CsKO1*, *CsKO2*, *CsKSI1*, *CsGA3ox2*, *CsGA3ox4*, *CsGA3ox5*, and *CsAOPI.2* and the internode length as well as the FPKM values of the 3 cell

Fig. 7 The correlation analysis between the FPKM values of 11 JA/GA biosynthetic genes and 3 cell wall-related genes or the internode length of ‘LJ43’ (A). A correlation network diagram of the FPKM values of *CsDELLA2* (red), 3 *CsMYCs* (light purple), 2 *CsJAZs* (light grey), 3 JA biosynthetic genes (yellow), 7 GA biosynthetic genes (green), and 3 cell wall-related genes (light blue) (B). Each node represented a gene. The blue line indicates a negative correlation, and the red line indicates a positive correlation. The correlation range was 0.72–0.97, and the thicker the line is, the stronger the correlation is

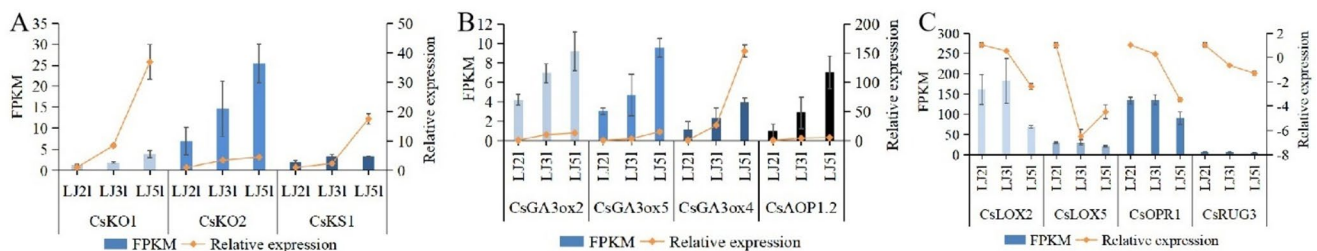
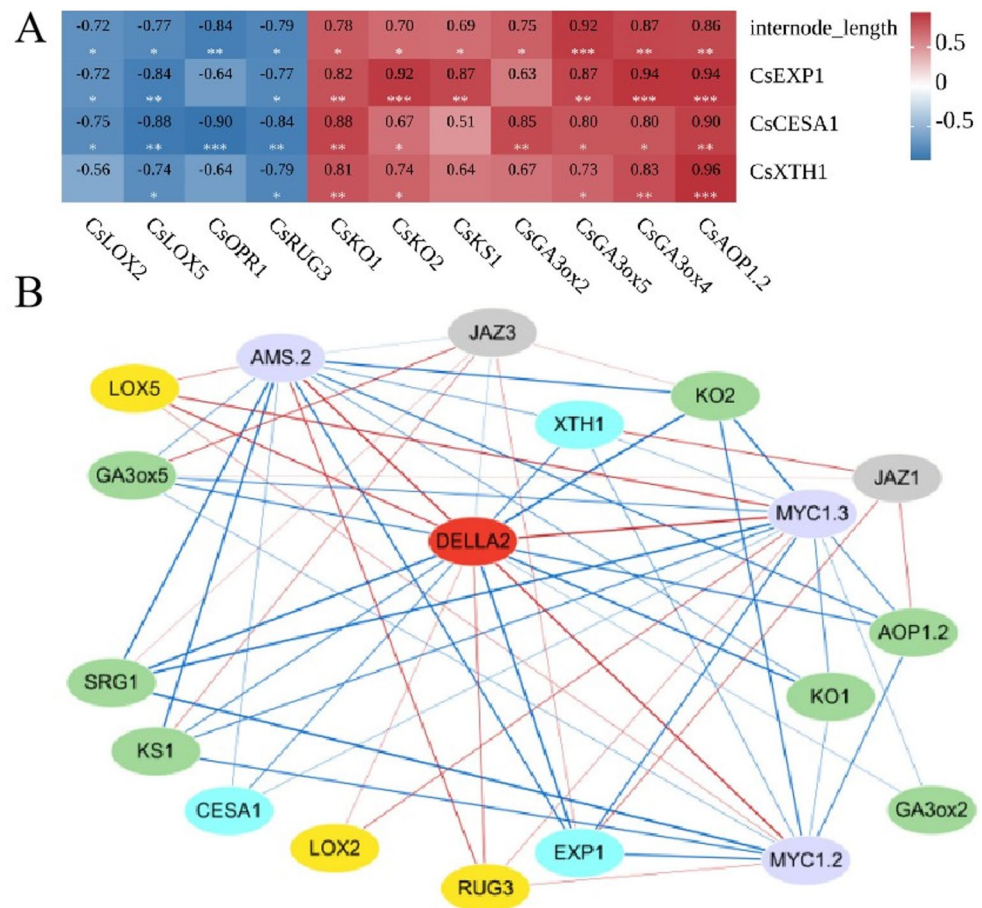


Fig. 8 The FPKM values and relative gene expression detected by qRT-PCR of 11 JA/GA biosynthetic genes in the internodes of ‘LJ43’ at 3 developmental stages. Error bars indicate standard deviation from

the mean of 3 biological replicates for the gene FPKM value (the main coordinate axis) and relative gene expression (subordinate axis), respectively

wall-related genes (Fig. 7A). During internode elongation of ‘LJ43’, the FPKM values of *CsLOX5*, *CsOPR1*, *CsLOX2* and *CsRUG3* all gradually decreased, the FPKM values of *CsKO1*, *CsKO2*, *CsGA3ox2*, *CsGA3ox4*, *CsGA3ox5*, and *CsAOP1.2* all gradually increased, while *CsKSI* increased at first and then decreased. The qRT-PCR results showed that the relative expression of these 11 biosynthetic genes were all basically consistent with the FPKM values. To be specific, the qRT-PCR results showed that the expression levels of *CsKO1*, *CsKO2*, *CsKSI*, *CsGA3ox2*, *CsGA3ox4*, *CsGA3ox5*, and *CsAOP1.2* all gradually increased, the expression of *CsLOX2*, *CsOPR1*, and *CsRUG3* all gradually

decreased, while the expression of *CsLOX5* decreased at first and then increased (Fig. 8A–C).

The co-expression Network of the FPKM Values of GA/JA Signal Transduction Genes, GA/JA Biosynthetic Genes and Cell wall-related Genes during Internode Elongation

The gene networks among the FPKM values of *CsDELLA2*, 3 *CsMYC* TFs, 2 *CsJAZ* TFs, 3 JA biosynthetic genes, 7 GA biosynthetic genes, and 3 cell wall-related genes in ‘LJ43’ were visualized in Cytoscape. The co-expression

correlations were represented by the connecting lines (edges) between genes represented. Thus, *CsDELLA2* and 3 *CsMYC* TFs were identified as 4 candidate hub genes based on their most connections in the co-expression network. However, 2 *CsJAZ* TFs had fewer connections. On the other hand, 3 JA biosynthetic genes, 7 GA biosynthetic genes and 3 cell wall-related genes showed in the network diagram also likely contributed to tea internode elongation of ‘LJ43’ (Fig. 7B).

Discussion

The tea plant is an important cash crop in China. Fresh tea leaf picking is an extremely time-consuming and labor-intensive work in tea production, and improving the mechanization level of fresh leaves picking is a key approach to promote the high-quality development of tea industry. The internode length is a major factor influencing plant height and architecture. For reducing the damaging rate of mechanically picked fresh leaves, it is necessary to moderately increase the internode length of the newly growing tea shoots (Liu et al. 2024; Zheng et al. 2023). The molecular regulatory mechanisms of plant internode elongation remain largely unclear. Thus, studying the regulatory mechanism of tea internode elongation has both theoretical and practical significance.

The Differences in the Internode Cell Length May Contribute To Different Internode Length of Two Tea Cultivars

Two cultivars ‘LJ43’ and ‘ZC102’, which have the same genetic background and significantly different internode length, were selected as experimental materials. With internode elongation, the cell diameters in the internode longitudinal-sections increased in each cultivar. Furthermore, the cell diameters and the internodes of ‘LJ43’ were both significantly greater than those of ‘ZC102’ at each developmental stage. However, there were no significant differences about the cell numbers in the cross-section between two cultivars at each stage or in each cultivar among different stages, respectively. This is consistent with existing findings that the internode elongation largely depends on cell expansion and is regulated by the biosynthesis and expansion of secondary cell wall (Oh et al. 2014; Wang et al. 2024). This study suggested that according to the correlation between the gene FPKM values and internode length at three stages of two cultivars, the transcription of 3 cell wall-related genes, including *CsEXPI*, *CsCESAI*, and *CsXTH1*, is involved in regulating tea internode elongation. Consistent with the present results, Wang et al. reported that

the transcription of *XTH* and *EXP* regulates cell wall extension, and *ZmXTH1* regulates maize internode elongation by inducing cell elongation (Wang et al. 2024).

Plant Hormones GA and JA Regulated Tea Internode Elongation

It is well known that plant hormones play key regulatory roles in cell elongation and plant internode elongation (Oh et al. 2014; Wang et al. 2024). GAs play key roles in regulating internode elongation by promoting cell division and elongation (Xue et al. 2022; He et al. 2024; Davière et al. 2014; Nagai et al. 2020), whereas JA inhibits cell and internode elongation (Wang et al. 2024). Here, we measured the levels of 7 major types of hormones, including auxins, cytokinins, ABA, ethylenes, GAs, JAs, and SAs in the internodes of the two cultivars at 2-leaf stage. The results showed that the contents of 2 kinds of active GAs (GA₁, and GA₇) in ‘LJ43’ internodes were all significantly higher than those in ‘ZC102’, while the contents of 2 kinds of active JAs (JA-Ile, and OPDA) in ‘LJ43’ were both significantly lower than that in ‘ZC102’. This is consistent with existing reports that exogenous GA₃ promoted tea internode elongation and endogenous GA significantly increased with tea bud elongation (Di et al. 2019; Li et al. 2021). Exogenous treatment with JA mimic COR inhibits internode elongation by suppressing *ZmXTH1* transcription and reducing cell size in maize (Wang et al. 2024; Ren et al. 2023). Similarly, in rice, overexpression of the cytochrome P450 gene *CYP94C2b*, which promotes JA-Ile inactivation, increased cell size and promoted internode elongation (Wang et al. 2024).

In *Arabidopsis*, AtMYC2/3/4 proteins repress the expression of secondary cell related-gene *EXP2* (Yi et al. 2020). MYC2 regulates hypocotyl elongation under red/far-red light conditions (Zhang et al. 2024; Zhu et al. 2024).

Endogenous JA and GA Pathways Were Involved in Regulating Tea Internode Elongation

The above results here suggested that both JA and GA signals might be involved in regulating tea internode elongation. To identify the candidate genes of JA and GA pathways which regulate tea internode elongation, the transcriptome sequencing was conducted. The heat map of correlation coefficients suggested that the repeatability of FPKM values within groups was relatively good and the correlation between groups was relatively high. The PCA results also indicated that the differences between groups and the replication within groups of the samples were relatively good. The co-expression venn diagram suggested that at each stage, the gene expression patterns in the two cultivars were

similar and the number of DEG uniquely expressed in LJ43 was greater than that in ZC102.

GA promotes plant growth mainly by inducing degradation of DELLA protein (Chen et al. 2023). DELLA protein can inhibit cell and hypocotyl elongation regulated by light signals (Yoshida et al. 2014; Davière and Achard 2016). Two DELLA proteins, including AtRGA (AT2G01570) and AtGAI (AT1G14920), regulate cell expansion in *Arabidopsis* hypocotyl and shoot (Wang et al. 2020; Davière and Achard 2016; Fukazawa et al. 2021; Davière et al. 2014; Sarwar et al. 2021; Guan et al. 2021). Consistent with the above existing reports, in this study, it was found that *CsDELLA2* gene, which was cloned by Yue et al. (2018) and further functionally characterized by Huan et al. (2025), is the candidate gene which regulates tea internode elongation.

This study suggested that with internode elongation of ‘LJ43’, the FPKM value of the GA receptor *CsGID1d* was highly negative correlated with the FPKM values of 3 cell wall-related genes and internode length. Moreover, both RNA-Seq and qRT-PCR results suggested that the transcription of *CsGID1d* gradually decreased (Fig. 6A, C). Similarly, previous studies reported that DELLAs promote the transcription of *GID1* gene (Qin et al. 2014). GA biosynthetic genes play key roles in regulating plant internode elongation (Fukazawa et al. 2021; Chen et al. 2023; He et al. 2024; Davière et al. 2014; Nagai et al. 2020). In this study, the results of RNA-seq and qRT-PCR both showed that 7 GA biosynthetic genes, including *CsGA3ox2*, *CsGA3ox4*, and *CsGA3ox5*, all gradually increased during internode elongation of ‘LJ43’. Furthermore, the correlation analysis and the correlation network diagram both demonstrated that 7 GA biosynthetic genes were candidate genes which regulated tea internode elongation (Fig. 7B). Numerous existing studies are consistent with the above results here. For example, *AtGA20ox1* highly expressed in the vegetative shoot, and *AtGA20ox1* and *AtGA20ox2* both promote internode elongation (Fukazawa et al. 2021). Mutations of *Atga20ox1* in *Arabidopsis* result in a dwarf phenotype and rice *Osga20ox2* mutant showed reduced plant height (Chen et al. 2023; He et al. 2024). Similarly, deep-water conditions promoted rice internode elongation by inducing *GA20ox2* transcription (Davière et al. 2014; Nagai et al. 2020).

Here, during internode elongation of ‘LJ43’, the results of qRT-PCR and RNA-seq both showed that the expression level of *CsCOI1a* gradually increased (Fig. 6B-E), and the FPKM value of *CsCOI1a* was highly positively correlated with internode length as well as the FPKM values of 3 cell wall-related genes (Fig. 6A). *CsMYC2* functions as the foremost regulatory hub in JA signaling in tea plants (Zhang et al. 2024). In this study, 3 *CsMYC* genes, including *CsMYC1.2*, *CsAMS.2* and *CsMYC1.3*, which were significantly negatively correlated with internode elongation were identified

through correlation analysis and co-expression analyses. The results of RNA-seq and qRT-PCR both suggested that with internode elongation of ‘LJ43’, the expression levels of *CsMYC1.2* and *CsMYC1.3* gradually decreased (Fig. 6B-E). The above results are consistent with existing findings. For example, Yi et al. reported that *AtMYC2/3/4* transcriptionally repressed cell wall-related genes, such as *AtEXP2*, in *Arabidopsis* (Yi et al. 2020).

Most of the JA-biosynthetic genes in tea plants have been functionally characterized in vitro due to the lack of a genetic transformation system. For example, it has been reported that the biosynthesis of JA and MeJA, the content of *CsOPR3* protein, as well as the transcriptional levels of *CsLOXs*, *CsAOS2*, *CsOPR3* and *CsJMT* genes are regulated by MeJA, JA, SA, wounding, biotic stress, cold stress and low-temperature stress in tea plants (Zhang et al. 2024). In this study, the correlation analysis suggested that with internode elongation of ‘LJ43’, there was a highly negative correlation between the FPKM values of 4 JA biosynthetic genes, including *CsLOX2*, *CsLOX5*, *CsOPR1*, and *CsRUG3*, and the internode length as well as the FPKM values of 3 cell wall-related genes (Fig. 7A). The results of RNA-seq and qRT-PCR also both suggested that the expression levels of these 4 JA biosynthetic genes were all negatively correlated with internode elongation of ‘LJ43’. Furthermore, the co-expression correlations showed that *CsLOX5*, *CsLOX2* and *CsRUG3*, which were displayed in the correlation network diagram, were candidate genes which regulate tea internode elongation (Fig. 7B).

In conclusion, this study identified candidate genes in JA and GA pathways as well as cell wall-related genes which regulate tea internode elongation. It provided a theoretical foundation for studying the molecular mechanism of plant internode elongation and suggested potential candidate genes for tea plant breeding with ideal internode length for mechanical picking.

Conclusions

This study found that in two tea cultivars ‘LJ43’ and ‘ZC102’ with different internode length, the internode parenchyma cells elongated with the internode elongation. Thus, cell elongation was associated with internode elongation during new shoot development and the variations between two cultivars. 3 cell wall-related genes, *CsEXPI*, *CsCESA1*, and *CsXTH*, likely contributed to this process. Through hormone content determination and transcriptome sequencing, it was demonstrated that endogenous JA and GA pathways were involved in regulating tea internode elongation. *CsDELLA2*, 3 *CsMYC* TFs, and 2 *CsJAZ* TFs were candidate genes involved in regulating tea internode

elongation. These genes are potential regulators and require further functional validation.

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Author Contributions X.L. (Xin Li), P.W. (Peiqiang Wang), S.Z. (Shuixing Zhu) and L.Z. (Liping Zhang) conceived and designed the research. H.S. (Hong Shen) performed the experiments and analyzed the data. Y.L. (Yang Li) analyzed the data. L.Z. wrote the manuscript. A.M. (Ahmed S. Mohamed), X.L., Y.L. and P.W. revised the manuscript. Y.M. (Yihu Mao), H.L. (Haoyang Liu), H.J. (Hongxin Jiang) and S.Z. performed the experiments. L.Z. and H.S. contributed equally. All authors read and approved the submitted version of the manuscript.

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Data Availability The raw data that are required to reproduce these findings are included in the published article and Supplementary Files.

Declarations

Conflict of interest The authors declare no conflict of interest.

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