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Shoot-specific promoter-driven expression of *CsIPT3* and *CsCKX5* modulates leaf development in tea plants

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Abstract

Tea leaves serve as the raw material for tea, a non-alcoholic beverage cherished by populations worldwide. However, the regulatory roles of cytokinins in tea leaf development remain unclear. Here, we identified a total of 216 *CsIPT*, and 204 *CsCKX* family genes from 22 tea cultivars, and screened two genes (*CsIPT3* and *CsCKX5*) with high expression in the above-ground tissues of 'Shuchazao'. Subcellular localization revealed that both CsIPT3 and CsCKX5 were localized in the nucleus and plasma membranes. The promoters of four *CsLhc* genes with high shoot-specific expression were cloned to explore the promoter activities. Results showed that the *CsLhc11* promoter exhibited the strongest GUS activity, which was comparable with that of the 35S promoter driving *GUS* expression. Transgenic *Arabidopsis* lines overexpressing *CsIPT3* or *CsCKX5* driven by the *CsLhc11* promoter were generated. Compared to wild-type (WT) plants, shoot-specific overexpression of *CsIPT3* in *Arabidopsis* promoted cytokinin accumulation, while overexpression of *CsCKX5* inhibited cytokinin accumulation. These alterations in cytokinin levels resulted in larger, and smaller individual leaf sizes and rosette diameters, respectively. Scanning electron microscope observations further showed that shoot-specific overexpression of *CsIPT3* or *CsCKX5* in *Arabidopsis* increased, or decreased cell size and intercellular spaces. In addition, we investigated transcript abundance of six cytokinin-responsive genes, which were significantly upregulated in transgenic *Arabidopsis* lines overexpressing *CsIPT3,* and significantly downregulated in lines overexpressing *CsCKX5*, relative to WT plants. This study provided direct experimental evidence for cytokinin-mediated regulation of tea leaf development, and laid a foundation for targeted genetic improvement of tea plant leaf traits.

Keywords: Phylogenetic analysis, Expression patterns, *CsIPT3* and *CsCKX5*, Promoter activities, Transgenic *Arabidopsis* lines, Scanning electron microscope

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Introduction

Camellia sinensis (L.) O. Kuntze is an evergreen perennial woody species characterized by an outcrossing pollination mechanism. Its young leaves are used as the raw material for tea production, a non-alcoholic beverage cherished by populations worldwide[1]. The unique sensory characteristics and health benefits of tea are attributed to a rich and diverse profile of secondary metabolites present in high concentrations[2]. Leaf size and shape are important indices for tea grading, underscoring their pivotal roles in determining the quality, flavor, and appearance of the final tea products[3]. Phytohormones, as the small-molecule endogenous organic compounds synthesized through multiple key metabolic pathways, are involved in mediating plant development and responding to various stresses[4]. As a key regulator, cytokinins promote cell division and cell expansion, thereby enhancing leaf growth. Moreover, cytokinins distributed in the leaf margins play a critical role in determining the final leaf morphological traits[5,6]. However, the regulatory roles of cytokinin in tea leaf shape and size have not been reported.

Dynamic homeostasis of cytokinins in plants is predominantly governed by the coordinated interplay between isopentenyltransferase (IPT) and cytokinin oxidase/dehydrogenase (CKX) enzymes, which is tightly linked to the plant's developmental stages and external environmental stimuli[7,8]. IPTs catalyze the rate-limiting step of cytokinin biosynthesis, which are classified into adenylate-type and tRNA-type IPTs in higher plants. Adenylate-IPTs mainly mediate the biosynthesis of isopentenyladenine (iP)- and trans-zeatin (tZ)-type cytokinins, using ATP, ADP, or AMP as their preferred substrates. In contrast, tRNA-type IPTs are responsible for the production of cis-zeatin (cZ)-type cytokinins, which occur via the catalysis of isopentenylation at the N6-position of adenine residues within transfer RNA (tRNA) molecules[7,9]. LONELY GUY (LOG) proteins catalyze the conversion of inactive cytokinin nucleotides to the bioactive free-base forms[10]. Bioactive cytokinins are irreversibly catabolized by CKX, whereas they undergo reversible inactivation via glucosyl conjugation[11]. Collectively, the enzymes involved in cytokinin anabolism and catabolism form a precise regulatory network that modulates cytokinin homeostasis, thus exerting pivotal regulatory roles in plant growth and development as well as adaptive responses to various environmental stresses[12].

Previous studies have reported that leaf development was typically divided into four sequential stages, including leaf primordium initiation, the polarity establishment, the formation of leaf size and shape, and leaf senescence[13,14]. Cytokinins play essential roles in regulating the size and structure of shoot apical meristems (SAMs)[15]. For example, reduced cytokinin concentration caused by *ipt* mutation or *CKX* overexpression leads to diminished size and activity of the SAMs[16]. Moreover, cytokinins have been reported to participate in regulating leaf size[11,17]. For instance, cytokinin depletion in *ipt* mutants or transgenic lines overexpressing *CKX* decreased the expression levels of *AINTEGUMENTA (ANT)* during root

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secondary growth and early leaf proliferation, whereas *ANT* overexpression increased the size of leaves and flowers[18]. Overexpression of *CKX3* suppressed the cell proliferation and expedited the onset of cell expansion, while constitutive overexpression of *CKX1* reduced the number of leaf cells, but increased the leaf cell size[11,19]. Overexpression of *IPT* or *HvCKX2* genes was involved in mediating cell proliferation and further regulating leaf size[20]. In rice, *OsCKX3* mediated the morphogenesis of leaf pillows and negatively regulated leaf inclination angle[21]. In addition, cytokinins are closely involved in regulating leaf senescence. Transgenic tobacco overexpressing the *IPT* gene derived by the senescence-specific *SAG12* promoter significantly impedes leaf senescence and shedding[22].

Given that constitutive promoter-driven *IPT* overexpression resulted in retarded phenotype, inhibited root growth, and caused plant deformities, the application of tissue-specific promoters in plant genetic engineering represents an effective strategy to explore the specific functions of cytokinins in targeted organ development[11,23]. In this research, we systematically characterized the *CsIPT* and *CsCKX* family genes across 22 tea cultivars, performed phylogenetic analyses, and conducted expression analysis to screen for the *CsIPT* and *CsCKX* genes with high expression in aboveground tissues of tea plants. Furthermore, we identified shoot-specific promoters based on the transcript abundance of *CsLhc* genes via RNA-seq and RT-qPCR data analyses of the 'Shuchazao' cultivar. Subsequently, we generated transgenic *Arabidopsis* lines harboring *CsIPT3/CsCKX5* driven by the *CsLhc11* promoter (shoot-specific). We then compared the leaf and rosette leaf size differences between transgenic plants and WT plants. SEM was also employed to characterize variations in cell size between leaves of transgenic and WT plants. In addition, we detected the transcript abundance of six cytokinin-responsive genes in transgenic *Arabidopsis* lines overexpressing *CsIPT3/CsCKX5* derived from the *CsLhc11* promoter. This study not only provided direct experimental evidence for the regulatory mechanism of cytokinin in tea leaf development, but also laid a foundation for targeted genetic improvement of tea plant leaf-related traits.

Materials and methods

Characterization of *CsIPT* and *CsCKX* genes from 22 tea cultivars

Genome sequences and corresponding annotation files of 22 tea cultivars: 'Baiye 1' (BY1), 'Baihaozao' (BHZ), 'Danxia 5' (DX5H), 'Fuding Dabaicha' (FDDB),'Guihong 3' (GH3H), 'Huangdan' (HD), 'Huangjinya' (HJY), 'Mingke 1' (MK1), 'Jinmingzao' (JMZ),'Jinxuan' (JX), 'Longjing 43' (LJ43), 'Lingtou Dancong' (LTDC), 'Qilan 10' (QL10H), 'Rougui' (RG),'Mingshan Baihao' (MSBC), 'Shuchazao' (SCZ), 'Tieguanyin' (TGY), 'Jiaming 1' (JM1), 'Wuyi Shuixian' (WYSX), 'Yinghong 9' (YH9H), 'Zhuyeqing'(ZYQ), and 'Zijuan' (ZJ) were retrieved from the research by Chen et al.[24]. The HMM profile of the IPT domain (PF01745) downloaded from the Pfam database was used to identify *IPT* family genes from 22 tea cultivars. In addition, two core conserved domains, such as cytokinin-binding (PF09265) and FAD-linked oxidase (PF02913), were used to identify *CKX* family genes across 22 tea accessions. The Pfam, SMART, and CDD databases were applied to determine the presence or absence of the core domain(s) in all candidate sequences[25].

The tea plants of 'Shuchazao' were cultivated in Guizhou University's Tea Germplasm Resource Field (26°20′ N and 106°40′ E). All test materials in the garden were managed per standardized tea cultivation protocols, with consistent irrigation and water-fertilizer application.

Phylogenetic analysis of *IPT* and *CKX* family genes across 22 tea cultivars

The full-length protein sequences of IPTs and CKXs from the whole-genome protein databases of 22 tea cultivars and *Arabidopsis* were separately aligned using MUSCLE with default settings, respectively[26]. The alignment files were applied to construct the phylogenetic trees via the maximum-likelihood (ML) method with 1,000 bootstrap replicates in the IQTREE software package[27]. The *IPT* and *CKX* gene families were further divided into different groups based on the sequence similarity and identified *AtIPTs/AtCKXs*.

Transcript abundance of *CsIPTs* and *CsCKXs* in distinct organs and leaf stages of 'Shuchazao'

RNA-seq datasets from eight distinct organs and five leaf developmental stages of the 'Shuchazao' cultivar were obtained from the Tea Plant Information Archive (TPIA) database (https://tpia.teaplants.cn)[28]. RSEM (v1.2.12) software was utilized to quantify the transcript levels of *CsIPT* and *CsCKX* family genes, and heatmaps of the expression profiles of these two family genes were generated using MeV software[29].

Reverse transcription quantitative real-time PCR (RT-qPCR)

The isolated mRNA was converted into double-stranded cDNA using the PrimerScript RT Reagent Kit (TaKaRa). For the RT-qPCR assay, all samples were subjected to three biological replicates, each having three technical replicates to verify the reliability of the results. The $2^{-\Delta\Delta C\tau}$ method was employed to determine the transcript levels of all candidate genes[30]. The primers of all candidate genes were listed in Supplementary Table S1.

Promoter activity analysis

Approximately 2,000-bp promoter sequences of four *CsLhc* genes were obtained from the TPIA database based on the genome annotation of *C. sinensis* 'Shuchazao'. Primer_Premier 5.0 software was applied for designing the promoter-specific primers. The amplified *CsLhc* promoters from the genomic DNA were cloned into the pMD18-T cloning vector (Takara) for Sanger sequencing. The confirmed promoter fragments were then cloned into the pBI121-35s-GUS vector to replace the 35S promoter. These recombinant constructs were transformed into *A. tumefaciens* strain GV3101, and the positive transformants were used for tobacco leaf infiltration. The infiltrated tobacco leaves were incubated in darkness for 1 d, followed by 2 d under normal light conditions. After incubation, the leaves were immersed in GUS staining buffer and incubated at 37 °C for 24 h to ensure sufficient staining. The stained samples were then decolorized with 70% ethanol to eliminate chlorophyll interference[31]. Primers used for vector construction are listed in Supplementary Table S1.

For GUS quantitative analysis, approximately 0.1 g of infected leaf powder was homogenized in 1 mL of GUS extraction buffer in an Eppendorf tube and centrifuged at 12,000 r/min at 4 °C for 5 min. The supernatant was diluted 2×, 4×, and 8× (recommended), and 20 μL of each dilution was transferred to sample wells. A 200 μL aliquot of working solution was added, and the mixture was incubated at 37 °C for 15–30 min (to prevent evaporation). The absorbance at 562 nm was measured using a microplate reader, and the protein concentration was calculated based on a standard curve.

Subcellular localization of CsIPT3 and CsCKX5 proteins

The coding sequences of *CsIPT3* and *CsCKX5* were cloned into the pCAMBIA2300-GFP vector[32]. *A. tumefaciens* (GV3101) cells harboring the recombinant binary vectors were transiently transformed into tobacco leaf epidermis cells. After 2 d of incubation, GFP fluorescence signals in tobacco leaves were observed using a confocal laser scanning microscope (Leica TCS SP5-II, Leica Microsystems, Wetzlar, Germany). Primers used for subcellular localization vector construction are listed in Supplementary Table S1.

Generation of transgenic plants

Two vectors harboring *CsIPT3* and *CsCKX5* derived by the *pLhc11* promoter were introduced into *A. tumefaciens* strain 3101 by using the freeze-thaw method. After validation by PCR amplification and restriction enzyme digestion, the positive recombinant constructs were transformed into *Arabidopsis* using the floral dip method[33]. Transgenic *Arabidopsis* plants overexpressing *CsIPT3* or *CsCKX5* derived by the *pLhc11* promoter were initially screened by hygromycin resistance and PCR amplification (Supplementary Fig. S1).

Determination of cytokinin contents in transgenic *Arabidopsis* using UPLC-MS/MS

Approximately 0.1 g of pulverized leaf tissues was homogenized in 5 mL of extraction solvent consisting of isopropanol, water, and formic acid at a volume ratio of 80:19:1 (v/v). The homogenate was centrifuged at 10,000 r/min for 10 min to collect the first supernatant. Subsequently, 1 mL of dichloromethane was added to the resulting pellet for low-temperature ultrasonic-assisted extraction (30 min), followed by centrifugation under the same conditions (10,000 r/min, 10 min) to collect the second supernatant. After combining the two batches of supernatants, the mixed solution was concentrated via nitrogen-assisted vacuum evaporation at room temperature to retain only the aqueous phase, which was then brought to a constant volume of 1 mL. The concentrated solution was diluted 2-fold with methanol and filtered through a 0.22 μm organic phase filtration membrane prior to instrumental analysis. Quality control (QC) samples were prepared following the same procedure as the test samples, and all manipulations were performed on ice under light-protected conditions[34].

Waters UPLC system coupled with an AB 4,000 MS/MS detector was used for the analysis of the cytokinin component. UPLC parameters: column temperature 35 °C, flow rate 0.3 mL/min, autosampler temperature 10 °C; mobile phase (A: 0.1% formic acid aqueous solution; B: acetonitrile) with gradient elution: 0.00–0.50 min (95% A, 5% B), 0.50–1.00 min (linear to 40% A, 60% B), 1.00–3.00 min (linear to 5% A, 95% B), 3.00–5.00 min (return to 95% A, 5% B). MS parameters: curtain gas 35 psi, collision gas 7 psi, IonSpray voltage +5,500 V, ESI+ mode, ion source temperature 450 °C, Ion Source Gas 1, 40 psi, Ion Source Gas 2, 50 psi.

SEM analysis

Leaf samples collected from WT plants, *CsLhc11*::*CsIPT3*, and *CsLhc11*::*CsCKX5* transgenic *Arabidopsis* lines were subjected to fixation and dehydration following the protocol described by Sun et al.[35]. Subsequently, the treated samples were examined and imaged on a SEM under the condition of 10 kV accelerating voltage.

Results

Phylogenetic analyses of *IPT* and *CKX* gene families across 22 tea cultivars

In the present study, a total of 216 *IPT* and 204 *CKX* family genes were identified from the whole-genome protein database of 22 tea cultivars using HMM searches, respectively (Fig. 1, Supplementary Tables S2, S3). To clarify the evolutionary relationships of *IPT* genes, an unrooted ML phylogenetic tree was constructed based on full-length IPT protein sequences from the 22 tea cultivars and *Arabidopsis* (Fig. 1a). The results showed that the *IPT* gene family from 22 tea

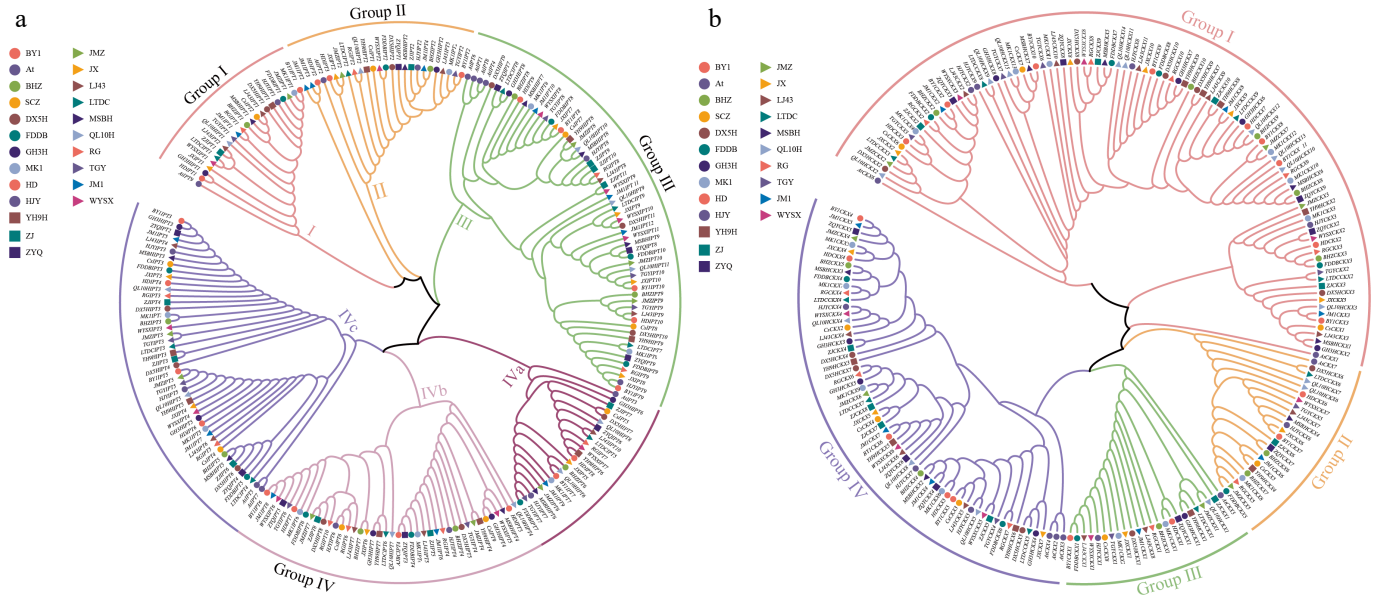

Fig. 1 Phylogenetic relationship of *IPT* and *CKX* family genes from 22 tea cultivars. (a) Phylogenetic analysis of *IPT* family genes from 22 tea cultivars. (b) Phylogenetic analysis of *CKX* family genes from 22 tea cultivars. Different shapes in various colors are used to denote distinct species. Different colored arcs represent distinct groups. The unrooted maximum-likelihood trees were inferred using IQ-TREE with 1,000 bootstrap replicates and visualized using iTOL.

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cultivars were clustered into four groups: designated groups I to IV. Among them, groups I and II comprised *tRNA-IPT* genes, with each group harboring a single-copy *tRNA-IPT* gene in all tested tea cultivars, indicating that the *tRNA-IPT* subfamily exhibits high conservation in gene copy number and distribution across tea germplasms. Groups III and IV contained the *ATP/ADP-IPT* subfamily genes from 22 tea cultivars. Notably, four *ATP/ADP-IPTs* from *Arabidopsis* clustered into a single clade, which formed a sister group to the group III *ATP/ADP-IPTs* of tea plants, suggesting that the *ATP/ADP-IPTs* in group III underwent specific expansion in tea plants. Furthermore, group IV was clustered into three subgroups (Group IVa–c). The subgroups IVa and IVc contained the *ATP/ADP-IPTs* from the 22 tea cultivars and *Arabidopsis*, while subgroups IVb only contained the *ATP/ADP-IPTs* from the tea cultivars, suggesting that the *ATP/ADP-IPT* genes were obtained from the common ancestor in tea plants or specifically lost in *Arabidopsis*. In addition, an un-rooted phylogenetic tree of the *CKX* gene family from 22 tea cultivars and *Arabidopsis* was generated (Fig. 1b). Based on the phylogeny and the identified *AtCKXs*, the tea *CKX* family genes from 22 tea cultivars were separated into four distinct groups (I, II, III, and IV). Among them, groups II and III contained a single copy gene from most species, while groups I and IV possessed two to three copies from all tea plants. These results indicated that the slight expansion of the *CKX* genes occurred in groups I and IV.

Expression profiles of *CsIPTs/CsCKXs* in various organs and the leaf stage of 'Shuchazao'

Tissue- and developmental stage-specific expression data are valuable for identifying genes involved in regulating the specific nature of each tissue at a given developmental stage[11,23]. The *IPT* and *CKX* genes mediate plant organ development by fine-tuning cytokinin homeostasis in distinct plant tissues[7,8]. To further elucidate the functional roles of *CsIPTs* and *CsCKXs* during leaf development, the transcript abundance of these two gene families across various tea plant organs was investigated using previously generated RNA-seq datasets (Fig. 2, Supplementary Table S4 and S5). The results revealed that two tRNA-IPT genes (*CsIPT1* and *CsIPT2)* were constitutively expressed across all tested organs, while the members of the ATP/ADP-IPT subfamily (*CsIPT3*-9) displayed organ-preferential expression patterns (Fig. 2a). For example, *CsIPT3* exhibited high expression levels in root, stem, mature leaf, and old leaf; *CsIPT4* was highly expressed in root and old leaf; *CsIPT5* displayed predominant expression in stems and flowers; and *CsIPT7* exhibited high expression levels in roots and stems. These results indicated that the ATP/ADP-IPT genes participated in finely regulating the development of different organs. Similarly, the expression patterns of *CsCKX* family genes were systematically investigated (Fig. 2b). The results showed that *CsCKX5* was ubiquitously expressed across all examined organs. *CsCKX6* showed high expression in root, stem, apical bud, and flower, and *CsCKX8* was highly expressed in most organs except old leaf. Notably, *CsCKX2* showed specific expression restricted to apical buds, and *CsCKX7* was specifically expressed in stems and mature leaves. Based on these distinct expression patterns, *CsIPT3* and *CsCKX5* were selected as candidate genes for subsequent functional validation experiments, and their transcript levels were further verified by RT-qPCR (Supplementary Fig. S1).

Subcellular localization of CsIPT3 and CsCKX5 proteins

To further characterize the biological functions of *CsIPT3* and *CsCKX5*, the predicted subcellular localization of their encoded proteins was experimentally verified. The CDS of *CsIPT3* and *CsCKX5* were fused with *GFP* reporter gene to generate the recombinant vectors 35S-CsIPT3 and CsCKX5-GFP vectors, which were then used for transient expression in tobacco leaf epidermal cells (Fig. 3). Confocal fluorescence observation demonstrated that the CsIPT3-GFP and CsCKX5-GFP fusion proteins were specifically localized to the nucleus and plasma membrane, whereas free GFP was dispersed uniformly across all cellular compartments, indicating that these two genes exerted their functional roles in these specific subcellular compartments.

Activity analysis of four *CsLHC* promoters from 'Shuchazao'

To further screen for the strong shoot-specific promoter, a total of 36 *CsLhc* family genes were identified from the 'Shuchazao' (Fig. 4). Phylogenetic analysis showed that the *Lhc* family genes were clustered into 13 groups, designated as groups *Lhca1-6* and *Lhcb1-7*, based on the topology and *Arabidopsis AtLhc* genes (Fig. 4a). Moreover, transcriptional abundance of *CsLhc* family genes in various organs and different developmental leaf stages was also investigated (Fig. 4b and c; Supplementary Table S6). Further analysis showed that four *CsLhc* genes (*CsLhc07*, *CsLhc11*, *CsLhc13*, and *CsLhc14*) exhibited significantly higher expression levels in leaf tissues than in other organs (Fig. 4d–g), and their transcript levels gradually increased during leaf development (Fig. 4c). These results

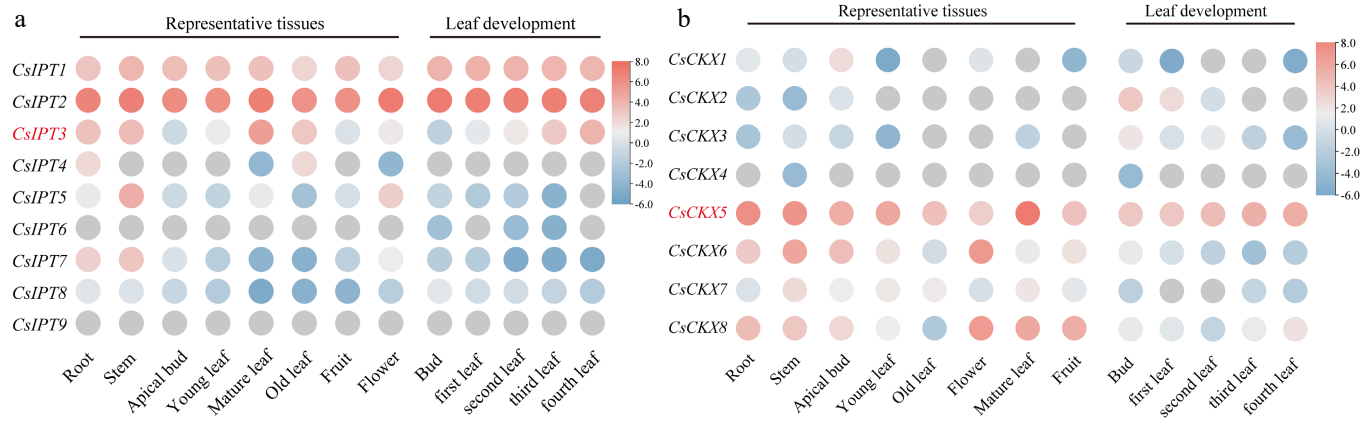

Fig. 2 Transcript levels of (a) *CsIPTs,* and (b) *CsCKXs* in various organs and different leaf stages of 'Shuchazao'. The color gradient denotes $\log_2$-converted FPKM values, and blue and red colors respectively represent low and high expression levels.

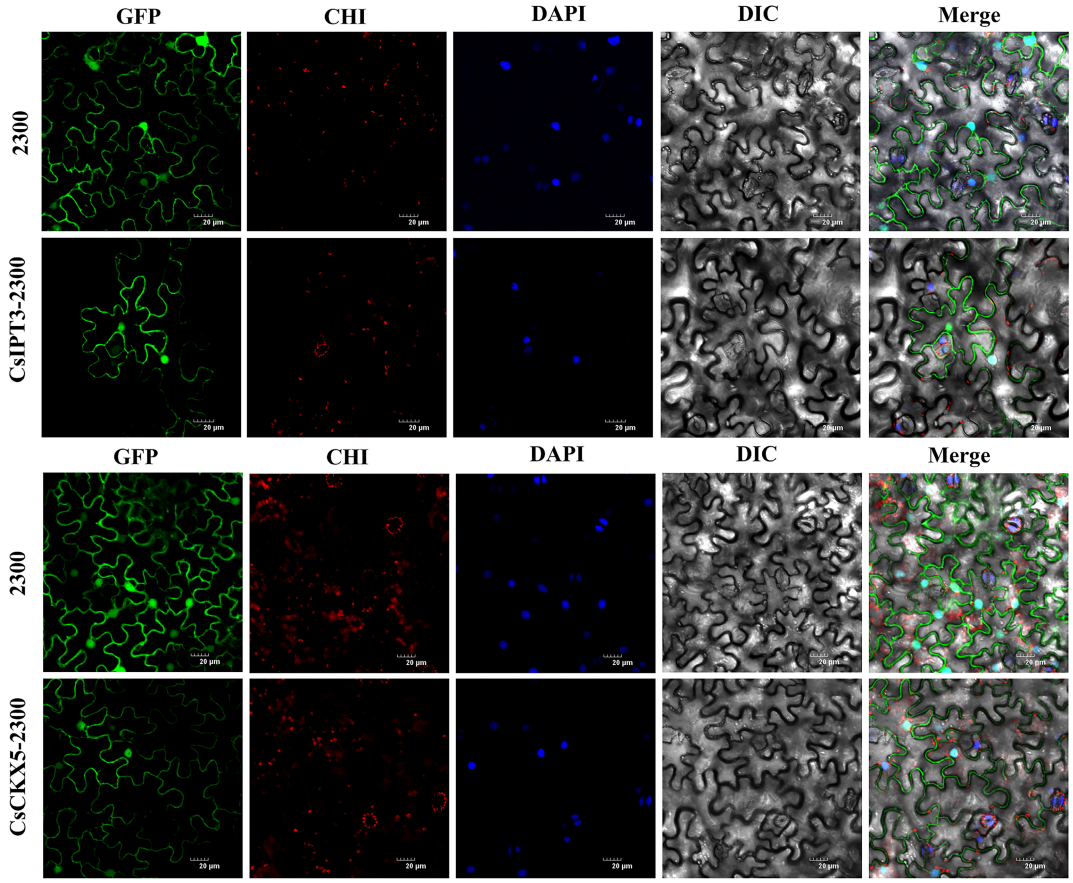

Fig. 3 Subcellular distribution of CsIPT3 and CsCKX5 proteins. Transient expression assays were performed in tobacco leaf epidermal cells, where free GFP and the 35S promoter-driven GFP-CsIPT3 and GFP-CsCKX5 fusion proteins were expressed. Laser scanning confocal microscopy was employed to detect the subcellular localization of these fusion proteins, with the scale bar set at 20 μm. Herein, the GFP channel shows the fluorescent signal of the protein-GFP fusions; the CHI channel exhibits red autofluorescence from chloroplasts, acting as a reference marker for chloroplast localization; the DAPI channel stains cell nuclei to produce blue fluorescence; the DIC channel offers differential interference contrast images that reveal cellular morphological structures; and the Merge channel is a composite of all channels, which is used to illustrate the co-localization status of the target proteins, chloroplasts, and nuclei.

suggest that these four *CsLhc* genes may play vital roles in regulating leaf developmental processes in tea plants.

To further verify leaf-specific promoter activity, the promoter fragments of these four genes were amplified via PCR, digested with *Hind III* and *Sal I* restriction enzymes, and then ligated into the pCAMBIA1391-GUS vector to construct the recombinant plant expression vector pCAMBIA1391-pCsLhcs-GUS. To assess promoter activity, the pCAMBIA1391-pCsLhcs-GUS reporter constructs were introduced into tobacco leaves via transient expression assays (Fig. 5). Compared to the control groups, GUS staining (Fig. 5a) and activity assays (Fig. 5b) revealed that the transcriptional activity of the *CsLhc11* promoter, as assessed by *GUS* reporter gene expression, was consistent with that exhibited by the 35S promoter, and both are higher than the GUS activity driven by *Lhc07*, *Lhc13*, and *Lhc14* promoters. The *Lhc07* promoter displayed stronger activity to drive *GUS* reporter genes than *Lhc13* and *Lhc14* promoters (Fig. 5a and b). The GUS activity showed no obvious differences between *Lhc13* and *Lhc14* promoters (Fig. 5a and b).

Functional characterization of *CsIPT3* or *CsCKX5* in regulating leaf morphology via transgenic *Arabidopsis*

To further investigate the regulatory effects of *CsIPT3* and *CsCKX5* in tea leaf size and shape, we generated transgenic *Arabidopsis* lines overexpressing *CsIPT3* or *CsCKX5* driven by the *CsLhc11* promoter (*pCsLhc11*::*CsIPT3-GUS* and *pCsLhc11*::*CsCKX5-GUS*, Fig. 6). In total, more than 10 independent transgenic *Arabidopsis* lines overexpressing *CsIPT3* or *CsCKX5* driven by *pCsLhc11* promoter were obtained by using PCR verification and kana-resistance selection (Supplementary Fig. S2). Transgenic plants overexpressing *IPT* display varying degrees of CK syndrome, including reduced root growth, altered apical dominance, and changes in stem and leaf growth. As shown in Fig. 6a, *CsIPT3*-overexpressing transgenic *Arabidopsis* displayed a marked increase in rosette size compared with WT plants, whereas the rosette size of *CsCKX5*-overexpressing transgenic lines was significantly smaller than that of WT plants. Furthermore, we systematically compared leaf morphological indices between transgenic lines and WT plants (Fig. 6b). The results demonstrated that the leaf length and width of *CsIPT3*-overexpressing *Arabidopsis* were increased by 29.75% and 19.56%, respectively, relative to WT plants. In contrast, the leaf length and width of *CsCKX5*-overexpressing transgenic lines were decreased by 22.46% and 19.90%, respectively, compared with the WT control (Fig. 6b).

These findings indicated that shoot-specific overexpression of *CsIPT3* promoted cytokinin biosynthesis, while overexpression of *CsCKX5* inhibited cytokinin biosynthesis, thereby positively or negatively regulating leaf size and rosette leaf area, respectively. Additionally, we quantified the contents of various cytokinin

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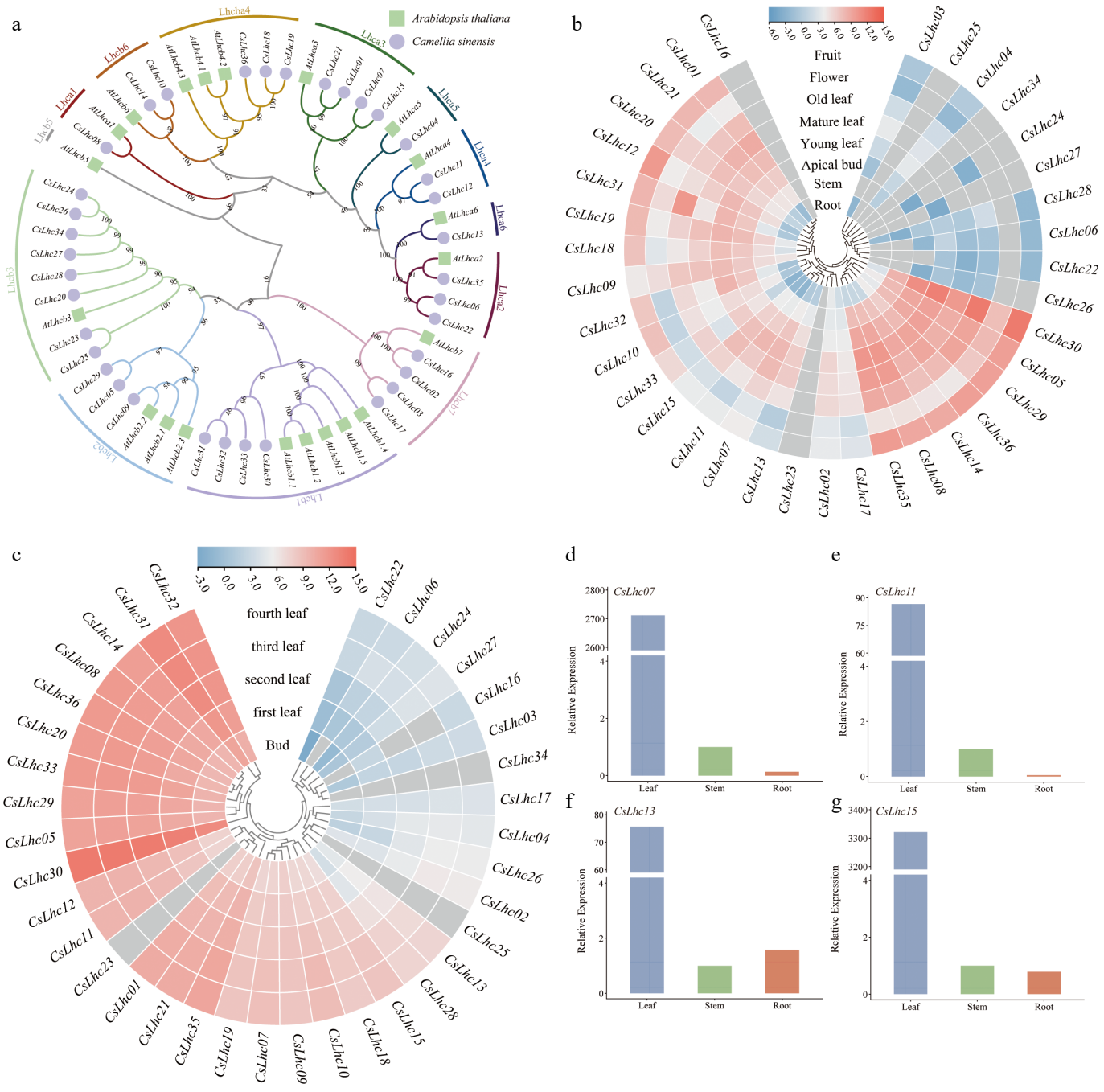

Fig. 4 Phylogenetic analysis and expression profiles of *CsLhcs* in various organs and leaf stages of 'Shuchazao'. (a) Phylogenetic analysis of *CsLhc* family genes from 'Shuchazao'; Different shapes in various colors are used to denote distinct species. Different colored arcs represent distinct groups. The unrooted phylogenetic trees were constructed and visualized using iQtree and iTOL, respectively. (b) Transcription levels of *CsLhc* family genes in various organs of 'Shuchazao'. (c) Transcription levels of *CsLhc* family genes in different leaf development stages of 'Shuchazao'. The color scale represents log_2-transformed FPKM values. Light blue- and pink-color indicated low to high expression, respectively. (d)–(g) The expression levels of four *CsLhc* genes with *CsGAPDH* used as reference gene. The $2^{-\Delta\Delta CT}$ method was applied for calculating the transcription levels of each gene. Lowercase letters are used to denote notable distinctions between transgenic *Arabidopsis* and WT plants ($p < 0.05$).

components in transgenic *Arabidopsis* and WT plants (Fig. 6c–h). The contents of ZR, ZA, T-ZA, N6-IPA, and total cytokinins (*CTK*) in *CsIPT3*-overexpressing lines were increased by 36.07%, 20.42%, 21.48%, 19.55%, and 35.74%, respectively, compared with WT plants. Conversely, the contents of these cytokinin components in *CsCKX5*-overexpressing lines were significantly decreased by 25.64%, 79.26%, 26.21%, 41.09%, and 62.18%, respectively (Fig. 6c–h).

Collectively, these results confirmed that shoot-specific overexpression of *CsIPT3* or *CsCKX5* positively and negatively regulated the accumulation of various cytokinins in leaf tissues, respectively, ultimately leading to enhanced or reduced leaf and rosette size in transgenic *Arabidopsis*. Notably, compared with WT plants, all transgenic *Arabidopsis* lines maintained normal growth and development without visible abnormal phenotypes.

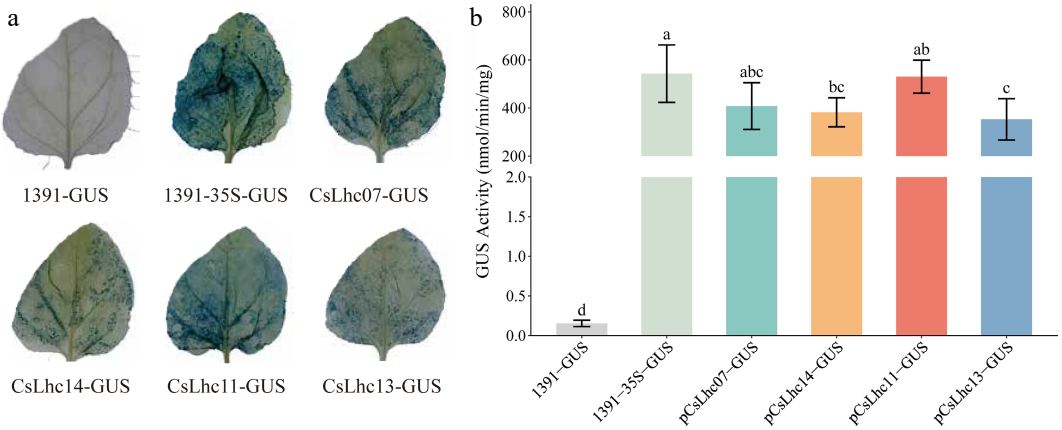

Fig. 5 The promoter activities of four *CsLhc* genes. (a) GUS staining for the transient overexpression of the *GUS* gene derived by four different *CsLhc* promoters. (b) GUS activity assay. Lowercase letters are used to denote notable distinctions between transgenic *Arabidopsis* plants and WT plants ($p < 0.05$).

Leaf cell size in transgenic *Arabidopsis* overexpressing *CsIPT3*/*CsCKX5*

Given the critical roles of cell size and cell division in plant organogenesis, we examined the epidermal cells and cross-section of young leaf of transgenic *Arabidopsis* overexpressing *CsIPT3* or *CsCKX5* by scanning electron microscopy, respectively (Fig. 7). Both leaf surfaces of transgenic *Arabidopsis* overexpressing *CsIPT3* or *CsCKX5* genes differed from the WT plants (Fig. 7a). Compared to WT plants, the epidermal cells in *CsIPT3*-overexpressing *Arabidopsis* exhibited larger, loose arrangement, and gentle wrinkling of the cell wall. In contrast, the epidermal cells in *CsCKX5*-overexpressing *Arabidopsis* showed the minimum size, prominent cell wall folds, and the most compact arrangement (Fig. 7a). These results demonstrate that cytokinin played essential roles in improving leaf water retention capacity, mechanical strength, and developmental plasticity by modulating leaf epidermal cell morphogenesis. In addition, we further investigated the leaf cross-section of *CsIPT3/CsCKX5*-overexpressing *Arabidopsis* using Scanning electron microscopy, respectively (Fig. 7b). Compared to WT plants, the leaf cross-section of *CsIPT3*-overexpressing *Arabidopsis* showed larger cells with a loose architecture, while *CsCKX5*-overexpressing transgenic plants had smaller and tightly packed leaf cells (Fig. 7b). These results indicated that specific overexpression of *CsIPT3* or *CsCKX5* genes in *Arabidopsis* leaves led to the accumulation or reduction of cytokinins, thereby increasing or decreasing the cell size and intercellular spaces in transgenic plants, and further altering leaf size.

Transcription levels of six key genes associated with leaf development

To investigate the response mechanisms underlying leaf development in transgenic plants, we explored the transcription levels of six key genes (*AtANT*, *AtTCP4*, *AtCycD3*, *AtGRF5*, *AtCDKs*, and *AtEXPA5*) associated with leaf size in transgenic *Arabidopsis* overexpressing *CsIPT3* or *CsCKX5* driven by the *CsLhc11* promoter (Fig. 8). Results showed that the transcription levels of these genes in transgenic *Arabidposis* overexpressing *CsIPT3* driven by the *CsLhc11* promoter were significantly higher than those in WT plants (Fig. 8a–f), whereas their expression levels in transgenic plants overexpressing *CsCKX5* driven by the *CsLhc11* promoter demonstrated a significant reduction relative to WT plants (Fig. 8a–f). These results were consistent with the phenotypic traits of transgenic plants.

Discussion

Tea leaf buds and young leaves represent the primary raw materials for processing non-alcoholic beverages[1]. Leaf morphology, particularly leaf size and shape, is a critical determinant of tea grading by affecting the quality, flavor, and appearance of the final product[36,37]. Notably, intact and well-developed large tea leaves are generally associated with premium quality, as their structural integrity enables retention of greater quantities of essential oils and flavor-active compounds during processing procedures. Conversely, undersized or fragmented leaves often correspond to inferior quality, since they accelerate the rate of compound extraction during brewing and result in a final flavor with diminished complexity and richness. Cytokinins are well-documented as pivotal phytohormones governing the leaf size and shape development across plant species[5,6,16]. Wu et al.[16] presented a comprehensive overview of leaf development processes, highlighting the core regulatory roles of cytokinins and their downstream signaling pathways. However, the impact of shoot-specific regulation of the cytokinin contents on leaf development in tea plants has not been reported.

In plants, the dynamic balance of cytokinins is mainly sustained by two opposing metabolic processes: *de novo* biosynthesis mediated primarily by isopentenyl transferases (IPTs) and LONELY GUY (LOG) proteins, and irreversible degradation catalyzed by cytokinin oxidases/dehydrogenases (CKXs)[7,8,10]. In this study, we totally identified 216 *CsIPT* and 204 *CsCKX* family genes from 22 tea cultivars. Phylogenetic analysis revealed that the *IPT* family genes were divided into four groups based on the topology, and identified *Arabidopsis IPTs*, which were in agreement with the previous studies[38,39]. Further comparative analysis revealed that groups I and II harbored only 1–2 copies of tRNA-IPT genes, implying that this subclass of *IPT* genes was highly evolutionarily conserved and fulfills fundamental and indispensable biological functions in tea plants. In sharp contrast, groups III and IV contained more than two copies of ATP/ADP-IPT genes, indicating that there subfamily genes have undergone significant gene expansion during the evolutionary process of tea plants, which was in agreement with the findings of Wang et al.[40]. Moreover, phylogenetic analysis of *CKX* family genes revealed that there family genes were also clustered into four groups, which is consistent with previous studies[41,42]. Our results revealed that two *CstRNA-IPTs*, and one *CsCKX* gene displayed constitutive expression patterns, indicating that these genes serve a crucial function as housekeeping genes. In contrast, the remaining

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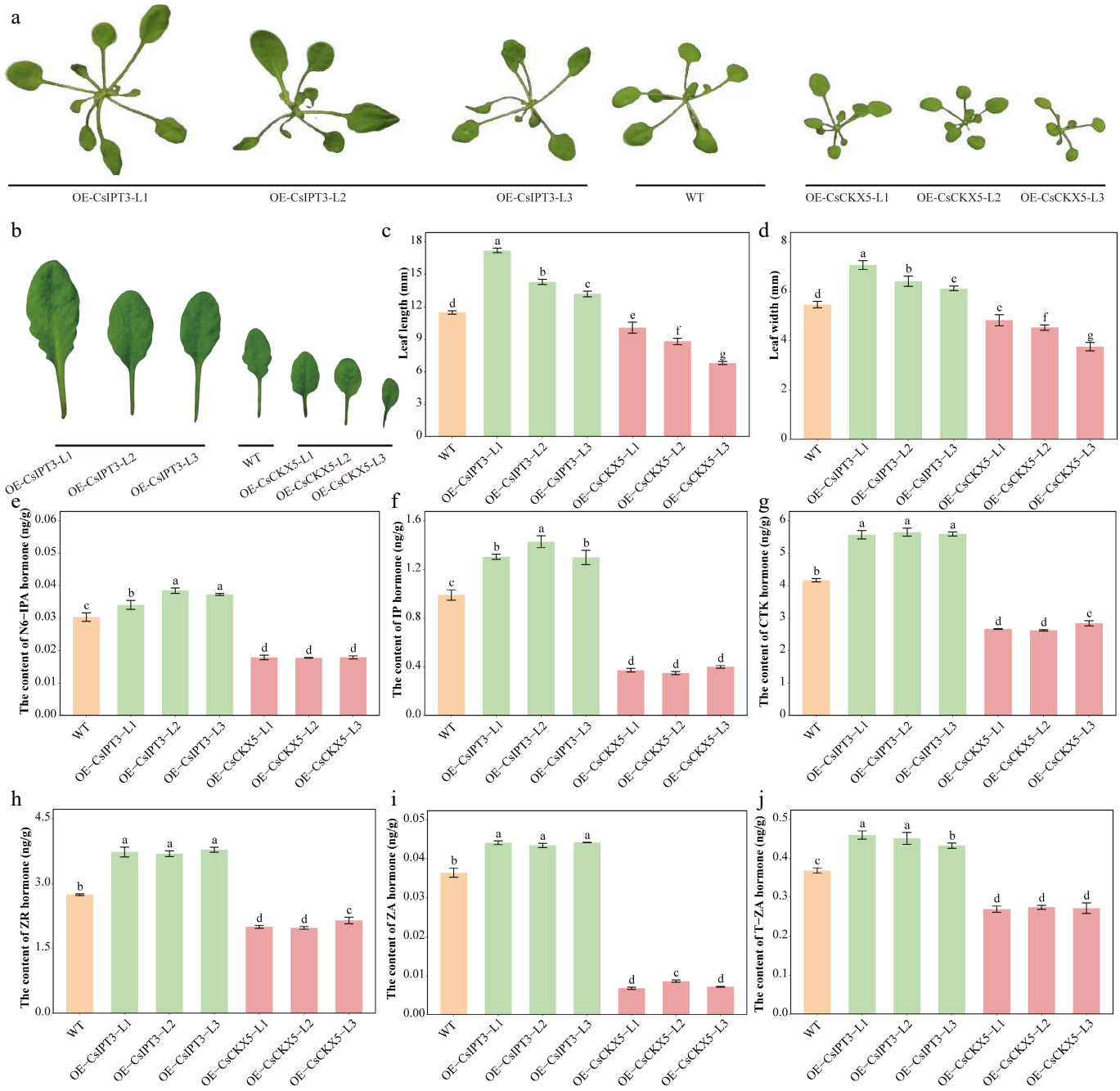

Fig. 6 Phenotype of transgenic *Arabidopsis* plants overexpressing *CsIPT3* or *CsCKX5* genes derived by the *CsLhc11* promoter. (a) The rosette size in transgenic *Arabidopsis* lines overexpressing *CsIPT3* or *CsCKX5*; (b) The leaf size in transgenic *Arabidopsis* lines overexpressing *CsIPT3* or *CsCKX5*; (c)–(h) The contents of CTK, ZA, ZR, T-ZA, iP, and N6-IPA in transgenic *Arabidopsis* lines overexpressing *CsIPT3* or *CsCKX5*. Lowercase letters are used to denote notable distinctions between transgenic *Arabidopsis* plants and WT plants ($p < 0.05$). CTK, total cytokinin; ZA, Zeatin adenine; ZR, Zeatin riboside; T-ZA, trans-zeatin adenine; iP, isopentenyl adenine, N6-IPA, N6-Isopentenyl Adenosine.

CsIPT and CsCKX genes exhibited tissue-specific expression patterns, which were in agreement with the expression patterns of *IPT* and *CKX* genes in *Arabidopsis*, strawberry, maize, and rice[11,40,43,44]. Further analysis revealed that *CsIPT3* and *CsCKX5* were expressed in multiple tissues, with their expression levels showing a gradual increase during leaf development, indicating these two genes played important roles in regulating leaf development.

Transgenic plants overexpressing *IPT* genes, derived from inducible or tissue-specific promoters, were widely used to explore the gene's regulatory roles in specific tissues and conditions with normal plant growth and development[45]. In contrast, constitutive promoter-driven *IPT* overexpression often induced excessive accumulation of endogenous cytokinins, leading to retarded growth, inhibited root development, and plant deformities[46]. Tissue-specific promoters represents a critical and effective strategy for the precise regulation of effector gene expression patterns, thereby enabling the establishment of target traits in transgenic plants. Although numerous studies revealed that cytokinins played essential roles in regulating plant organogenesis and responding to various environmental stress, how endogenous cytokinin concentration changes regulate leaf development remains incompletely elucidated[8]. Notably, no relevant studies have been reported in tea

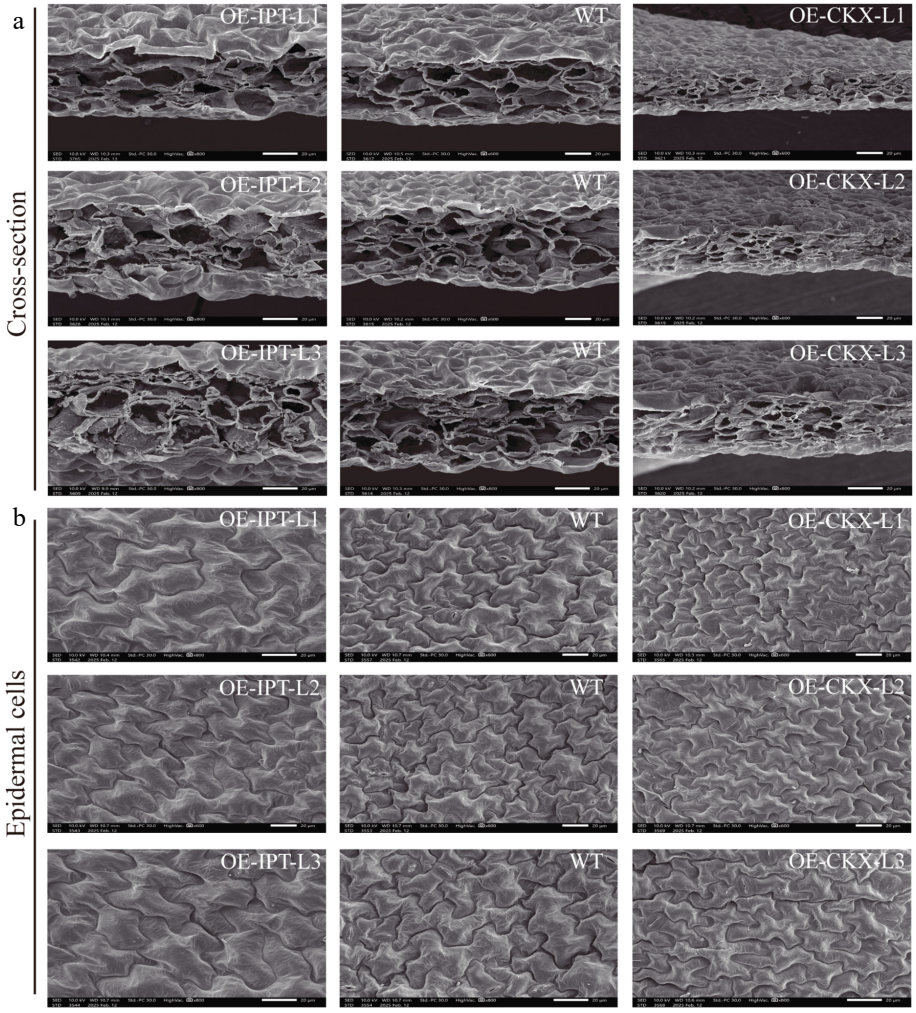

Fig. 7 The epidermal cells and cross-section of young leaf in transgenic *Arabidopsis* overexpressing *CsIPT3/CsCKX5*. (a) Epidermal cells in young leaf of transgenic *Arabidopsis* overexpressing *CsIPT3/CsCKX5* compared to WT plants; (b) Cross-section in young leaf of transgenic *Arabidopsis* overexpressing *CsIPT3/CsCKX5* compared to WT plants. WT, wild plants. OE-CsIPT3, transgenic *Arabidopsis* overexpressing *CsIPT3* driven by *CsLhc11* promoter; OE-CsCKX5, transgenic *Arabidopsis* overexpressing *CsCKX5* driven by *CsLhc11* promoter.

plants to explore the regulatory effects of specifically increasing or decreasing cytokinin levels via leaf-specific promoter-driven overexpression of *IPT* or *CKX* genes on leaf development. Thus, a total of 36 *CsLhc* family genes were identified and clustered into 13 groups, which was consistent with the findings of previous studies[47–49]. Further analysis revealed that the expression levels of four *CsLhc* genes (*CsLhc07*, *CsLhc11*, *CsLhc13*, and *CsLhc14*) in leaves were significantly higher than those in other organs, indicating that these genes might be involved in regulating leaf development. Modulation of gene promoter activity allows for the precise regulation of target gene expression in terms of temporal pattern and abundance, which enables further exploration of its biological roles during diverse physiological processes. Here, the promoter activities of four *CsLhc* genes revealed that the *pLhc11* promoter exhibited the strongest activity, suggesting its potential as a shoot-specific strong promoter in this study.

In this study, functional characterization was performed using heterologous expression systems, rather than stable genetic transformation in intact tea plants. Although heterologous expression provides preliminary insights, its inherent limitations must be acknowledged. Heterologous hosts lack the specialized cellular machinery required for authentic post-translational modifications and cannot fully reconstruct the complex gene regulatory networks in tea plants. More importantly, the absence of an efficient and stable genetic transformation system for tea plants remains a major technical bottleneck, severely restricting the *in vivo* functional validation of target genes and candidate promoters in native tea plant tissues. Thus, we generated transgenic *Arabidopsis* lines overexpressing *CsIPT3* and *CsCKX5* driven by the *Lhc11* promoter. Our results showed that shoot-specific overexpression of *CsIPT3* or *CsCKX5* specifically promoted and inhibited the accumulation of cytokinins, respectively, which significantly increased or decreased the cell size and intercellular spaces in transgenic plants, ultimately altering leaf morphology. Additionally, the expression levels of six genes associated with cell division and cell expansion in transgenic *Arabidopsis* overexpressing *CsIPT3* driven by the *Lhc11* promoter were significantly higher than those in WT, while expression levels of these genes in transgenic *Arabidopsis* overexpressing *CsCKX5* driven by the *Lhc11* promoter were dramatically lower than those in WT plants. These observations were in agreement with the previous studies. For example, transgenic *Peperomia pellucida* overexpressing the *ipt* gene driven by the *pKNOX1* promoter increased leaf and seed size[50]. Transgenic Arabidopsis overexpressing *CKX3* driven by the *ANT* promoter showed a leaf size reduced to approximately 27% of the WT, with epidermal cell number decreased to about 12% of WT levels[18]. In tea plants, Zhang et al.[51], reported that *CsIPT5.1*

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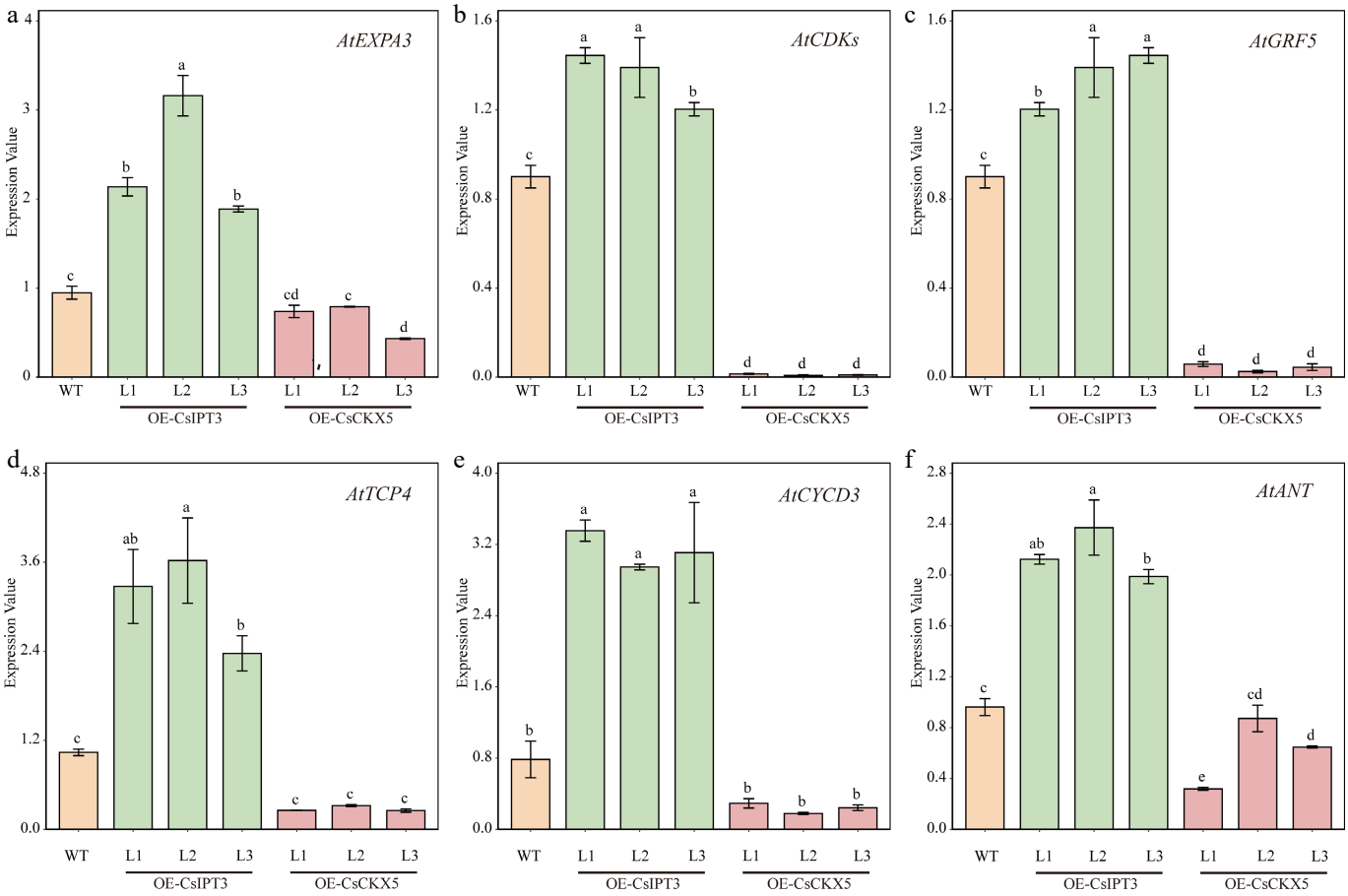

Fig. 8 Transcript abundance of six cytokinin responsive genes in transgenic *Arabidopsis* overexpressing *CsIPT3/CsCKX5* derived by the *CsLhc11* promoter. The expression levels of six candidate genes in transgenic *Arabidsopsis* lines: (a) The expression levels of *AtEXPA3* in transgenic *Arabidsopsis* lines; (b) The expression levels of *AtCDK* in transgenic *Arabidsopsis* lines; (c) The expression levels of *AtGRF5* in transgenic *Arabidsopsis* lines; (d) The expression levels of *AtTCP4* in transgenic *Arabidsopsis* lines; (e) The expression levels of *AtCYCD3* in transgenic *Arabidsopsis* lines; (f) The expression levels of *AtANT* in transgenic *Arabidsopsis* lines. Lowercase letters are used to denote notable distinctions between the control group and treatment group ($p < 0.05$).

might serve an important regulatory role during the development of the lateral bud and leaf development. Zhang et al.[52] revealed that the *CsKNOX6* gene has been reported to participate in activating the cytokinin biosynthesis and promoting the leaf bud size. The findings of the above studies were consistent with our results. Notably, constitutive overexpression of *IPT* genes systemically increased the endogenous cytokinin contents, which caused excessive branching, inhibited root development, and other unintended growth abnormalities. For instance, transgenic creeping bentgrass overexpressing *MtIPT* from *Medicago truncatula*, driven by the CaMV35S promoter, inhibited root growth, reduced stem length, and generated thinner leaves[53]. Kuderová et al.[54] revealed that long-term cytokinin overproduction inhibited primary root elongation by reducing quantitative parameters of the primary root meristem. Werner et al.[11] reported that overexpression of *AtCKX1-6* under the *CaMV35S* promoter enhanced root growth, and retarded shoot growth in the transgenic lines. Our results revealed that there is no difference in root architecture between transgenic *Arabidopsis* plants and WT plants, indicating that the tea *CsLhc11* promoter could be used as a shoot-specific promoter to explore the functional roles of certain genes in tea leaf development. Furthermore, Zhao et al.[55] reported high positive correlations between the concentrations of cytokinin and the accumulation of gallated catechin, theanine, and caffeine. This provides a direction for future research, indicating that cytokinins not only participate in regulating leaf size, but also contribute to the regulation of tea quality-related metabolites.

Conclusions

Here, we first characterized 216 *IPTs* and 204 *CKXs* from 22 tea cultivars, and further investigated the transcript abundance of these two family genes in distinct organs or leaf stages of the 'Shuchazao' cultivar. Further analysis showed that *CsIPT3* and *CsCKX5* displayed substantially higher transcriptional levels in leaf tissues than those observed in other plant organs. Subcellular localization revealed that CsIPT3 and CsCKX5 were localized in the nucleus and plasma membrane. Moreover, the transcript abundance of *CsLhc* family genes showed that four *CsLhc* genes (*CsLhc07*, *CsLhc11*, *CsLhc13*, and *CsLhc14*) displayed substantially higher transcriptional levels in leaf tissues than those observed in other plant organs. The *CsLhc11* promoter exhibited the strongest GUS activity, which was consistent with that of the 35S promoter driving *GUS*. Transgenic *Arabidopsis* lines overexpressing *CsIPT3/CsCKX5* derived by *CsLhc11* promoter were generated. Compared to WT plants, shoot-specific overexpression of *CsIPT3* or *CsCKX5* in *Arabidopsis* promoted or inhibited cytokinin accumulation, respectively, resulting in increased or decreased cell size and intercellular spaces, and consequently leading to larger or smaller individual leaf size and rosette

diameter. In addition, we also investigated the transcript abundance of six cytokinin-response genes, and found that the transcript levels of these genes in transgenic *Arabidopsis* overexpressing *CsIPT3/CsCKX5* derived from the *CsLhc11* promoter were significantly higher and lower than those of WT plants. This study not only provides direct experimental evidence for the regulatory mechanism of cytokinin in tea leaf development, but also lays a foundation for targeted genetic improvement of tea plant leaf-related traits.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Wang X; material provision: Niu S, Wang X; data analysis: Xiang F, He X; figures and manuscript construction He X, Xiang F; performed experiments: Xiang F; manuscript writing: Wang X; Xiang F. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during the current study are included in the published article and its supplementary information files.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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