Molecular and Biochemical Characterization of Two 4-Coumarate: Coa Ligase Genes in Tea Plant (*Camellia Sinensis*)

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Abstract

Tea is rich in flavonoids benefiting human health. Lignin is essential for tea plant growth. Both flavonoids and lignin defend plants from stresses. The biosynthesis of lignin and flavonoids shares a key intermediate, p-coumaroyl-CoA, which is formed from p-coumaric acid catalyzed by p-coumaric acid: CoA ligase (4CL). Herein, we reported two *4CL* paralogs from tea plant, *Cs4CL1* and *Cs4CL2*, which were a member of class I and II, respectively. *Cs4CL1* was mainly expressed in roots and stems, while *Cs4CL2* was mainly expressed in leaves. The promoter of *Cs4CL1* had AC, light and stress-inducible (LSI), and meristem-specific elements, while that of *Cs4CL2* had AC and LSI elements only. Moreover, the promoter of *Cs4CL1* had two more stress-inducible elements than *Cs4CL2* had and the two promoters had six different light-inducible elements. These features suggested their differences in their responses to environmental conditions. Three stress treatments indicated that the expression of *Cs4CL1* was sensitive to mechanical wounding, while the expression of *Cs4CL2* was UV-B-inducible. Enzymatic assay showed that both recombinant Cs4CL1 and Cs4CL2 transformed p-coumaric acid, ferulic acid and caffeic acid to their corresponding CoA ethers. Kinetic analysis indicated that the recombinant Cs4CL1 preferred to catalyze caffeic acid, while the recombinant Cs4CL2 favored to catalyze p-coumaric acid. The overexpression of both *Cs4CL1* and *Cs4CL2* increased the levels of chlorogenic acid and total lignin in transgenic tobacco seedlings. In addition, the overexpression of *Cs4CL2* increased the levels of three flavonoid compounds. These findings indicate the differences of Cs4CL1 and Cs4CL2 in the phenylpropanoid metabolism.

Introduction

Phenylpropanoids play important roles in plant growth and defense against stresses and (Dixon et al. 2013; Vogt 2010). The biosynthetic pathway of plant phenylpropanoids starts from the shikimate pathway and branches into lignin, flavonoids, hydroxycinnamoyl esters, and other phenolics (Weisshaar and Jenkins 1998; Grace and Logan 2000; Dixon et al. 2013; Vogt 2010). Lignin is the second most abundant biopolymer next to cellulose and plays crucial role in sustaining the structural integrity of cell wall and stiffness and strength of plant stem (Boerjan et al. 2003; Grima-Pettenati and Goffner 1999; Whetten and Sederoff 1995). Additionally, lignin is essential for transporting water and solutes in plants and defending plants from pathogen attack (Grima-Pettenati and Goffner 1999; Whetten and Sederoff 1995; Boerjan et al. 2003). Flavonoids, the biggest group of plant polyphenols, play important roles in plant's interaction with different environmental stresses and plant pollination. In general flavonoids compounds can protect plants from damages caused by UV irradiation-insects, and pathogens. Furthermore, flavonoids are potent nutrients to provide important healthy benefits to humans, such as antioxidative activity (Koes et al. 1994; Winkel-Shirley 2001, 2002) .

Flavonoids and lignin are derived from the general phenylpropanoid pathway starting with phenylalanine or tyrosine, two products of the shikimate pathway. The three entry enzymes are phenylalanine ammonia lyase (PAL) (Camm and Towers 1973; Wanner et al. 1995) that catalyzes L-phenylalanine to produce cinnamic acid, cinnamate 4-hydroxylase (C4H) that converts cinnamic acid to p-coumaric acid, and 4-coumarate-CoA ligase (4CL) that transforms p-coumaric acid to 4-coumaroyl-CoA in the presence of CoA (Bell-Lelong et al. 1997; Schilmiller et al. 2009) (Hu et al. 1998; Schilmiller et al. 2009) (Fig. 1 A). Tyrosine ammonia lyase (TAL) transforms L-tyrosine to p-coumaric acid (Camm and Towers 1969).

In plant, 4CL has been characterized to be a small family that plays diverse catalytic roles involved in the metabolic pathways toward flavonoids and lignin (Hamberger and Hahlbrock 2004; Cukovica et al. 2001; Hu et al. 1998) (Figure1 A). *Arabidopsis thaliana* includes more than four 4CL isoforms (Li et al. 2015b; Ehlting et al. 1999b) . *Populus trichocarpa* was reported to have 17 4CL isoforms (Rui et al. 2010). Rice was reported to have five 4CL members (Jinshan et al. 2011). In all investigated plants, 4CL homologs are classified into two classes, class I and class II. On the one hand, 4CL members in class I have been characterized to preferably involve in the biosynthesis of lignin metabolism and wounding responses. Examples of members include At4CL1 from Arabidopsis (Li et al. 2015a), Pta4CL from *P. trichocarpa* (Shi et al. 2010), and Os4CL3, Os4CL4 and Os4CL5 from rice(Gui et al. 2011), and Gm4CL1 and Gm4CL2 from soybean (Lindermayr et al. 2002b). These 4CL members transform caffeic acid and ferulic acid to form their CoA ester toward to lignin and their expression is induced by mechanical wounding (Soltani et al. 2006). Gm4CL1 and Gm4CL2 were demonstrated to transform p-coumarate, caffeate, ferulate, and

sinapate to their corresponding CoA esters with a high affinity (Lindermayr et al. 2002a). On the other hand, the class II members have been characterized to preferably associate with the biosynthesis of flavonoids and their transcription is induced by UV irritation (Cukovica et al. 2001; Lindermayr et al. 2002b; Gui et al. 2011; Hamberger and Hahlbrock 2004; Hu et al. 1998; Costa et al. 2005). Examples include *At4CL3* from Arabidopsis (Ehlting et al. 1999b) , *Os4CL2* from rice(Gui et al. 2011), and Gm4CL3 and Gm4CL4 from soybean (Sun et al. 2013), and Pt4CL2 from *P. trichocarpa* (Harding et al. 2002). At4CL3 protein was reported to preferably transform 4-coumaric acid to coumaroyl-CoA (Ehlting et al. 1999a; B and K 2004). The recombinant Gm4CL3 and Gm4CL4 were demonstrated to transform 4-coumarate and caffeate to their corresponding CoA esters (Lindermayr et al. 2002a).

Manipulation of *4CL* genes have been reported to alter either lignin or flavonoid biosynthesis. An ectopic expression of anti-4CL (*At4CL1*) in *A. thaliana* plants reduced the lignin contents via a significant decrease of S-lignin (Lee et al. 1997). An overexpression of *Pt4CL1* in tobacco plants increased the lignin by 25% in xylem of stem (Lu et al. 2004). A class II *4CL* gene of loblolly pine, *Pinta4CL3*, was overexpressed in *P. trichocarpa* to obtain multiple transgenic plants (Chen et al. 2014). Metabolic profiling indicated the significant increase of hydroxycinnamoyl-quinate/shikimate esters, caffeoyl-quinate/shikimate esters, and cinnamoyl-quinate in leaves of transgenic Populus plants. In addition, the levels of kaempferol, rutin (quercetin-3-O-rutinoside) and quercetin-3-O-glucuronide were increased in transgenic leaves. By contrast, the overexpression of *Pinta4CL3* did not alter the levels of coniferyl or sinapyl alcohol toward the formation of lignin. Recently, the differential roles of class I and II members gained genetic evidence from *Arabidopsis*. The knockout of *At4CL1* (Class I type) was reported to reduce lignin by 20%, while the knockout of *At4CL3* (Class II type) was reported to decrease the anthocyanin content by 70% (Li et al. 2015a).

Tea plant is rich in flavonoids and other polyphenols with high health benefits to human health (Sueoka et al. 2001; Liu et al. 2012; Jiang et al. 2015). Tea is one of most popular nonalcoholic beverages worldwide. To date, the biosynthesis of tea flavonoids has gained intensive studies in both genomics and functional genomes(Wei et al. 2018). Most of pathway genes, such as *F3'5'H*, *DFR*, *ANS*, *ANR*, *LAR*, *UGT*, *GST,* and others, have been functionally characterized to understand their roles associated with the high production of flavonoid molecules (Wang et al. 2014; Wang et al. 2018; Liu et al. 2019; Zhao et al. 2017). On the one hand, these accomplishments greatly enhance the understanding of the biosynthesis of tea flavonoids. On the other hand, these studies indicate that the functional characterization of the entry pathway genes of tea phenylpropanoids, such as PAL, C4H, and 4CL, is essential to determine the pathway activities from phenylalanine or tyrosine to the downstream flavonoids. Herein, we report the functional characterization of two tea *4CL* genes, *Cs4CL1* and *Cs4CL2*. Molecular and phylogenetic analysis indicated that *Cs4CL1* belonged to class I and was highly expressed in roots and stems, while *Cs4CL2* was clustered in class II and was highly expressed in buds and juvenile leaves. Transgenic, metabolic, and biochemical data indicated that Cs4CL1 seemed to be relatively associated with the biosynthesis of lignin, while Cs4CL2 appeared to be preferably involved in the biosynthesis of flavonoids.

Materials And Methods

Plant materials

More than 1000 5-year old shrubs of Shu Chazaotea (*Camellia sinensis var. sinensis cv.* "Shu Chazao") are grown in the research station at Anhui Agricultural University for different research purposes. In middle spring, shoot buds, the, 1st, 2nd, and 3rd young leaves on new sprouts, fully expended mature leaves, young stems, and roots were collected in liquid nitrogen and then stored at -80ºC until experimental uses. *Nicotiana tabacum* cv. "G28" grown in the green house and *N. benthamiana* grown in the growth chamber were used for genetic transformation and for the subcellular protein localization experiments, respectively. The growth chamber was set up with a constant temperature at 28±3 ºC and a constant 12/12-h (light/dark) photoperiod with a light intensity of 150-200 μmol m^{-2} s^{-1}.

Gene cloning and phylogenetic analysis

Total RNA was isolated from plant tissues using an RNAiso-mate for Plant Tissue Kit (Takara, Dalian, China) according to the manufacturer's protocol. The first strand cDNAs were synthesized using a PrimeScript® RT Reagent Kit (Takara, Dalian, China).

Based on available cDNA sequence of *Cs4CL1*, forward and reverse primer pairs were design through Primer Premier 5 software according to the ORF (open read frame) sequences (Supplementary TableS1). To clone the full length of *Cs4CL2*, according to an EST sequence of *Cs4CL2*, primer pairs (Supplementary Table S1) were designed to for a RACE PCR. The ORFs of *Cs4CL1* and *Cs4CL2* were amplified with a polymerization chain reaction (PCR) program, which consisted of 98 ºC for 30s, 29 cycles of 98 ºC for 10s, 60 ºC for 20s, and 72 ºC for 30s, and then a 10 min extension at 72 ºC. The amplified PCR products were ligated to the PMD19-T vector and then sequenced at BGI (http://www.genomics.cn/index) for accuracy confirmation.

The resulting ORF sequences were deduced to obtain amino acid sequences that were used for alignment analysis performed with DNAman software (Zhao et al. 2013) . A phylogenetic tree was developed using Cs4CL1, Cs4CL2 and other 21 4CL sequences and using MEGA6.1 software. The Gene-Bank IDs of the 4CLs were listed in supplementary Table S2. The tree nodes were evaluated with a bootstrap for 1000 replicates and the evolutionary distances were estimated with a p-distance method.

Cloning and sequence analysis of *Cs4CL1* and *Cs4CL2* promoters

The cloning method of promoters followed a genome-walker method according to the Genome-Walker Kit (Clontech). The primers (Table S1) were designed for PCR to clone 1000 nucleotides of promoter sequences. The amplified PCR products were cloned to plasmid pEASY-T1 and then sequenced at BGI to confirm the accuracy. Identification of binding elements in promoter sequences was completed using an online promoter analysis tool at PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) and PlantPan (http://plantpan.itps.ncku.edu.tw/).

Construction of binary vectors and plant transformation

The OFRs of *Cs4CL1* and *Cs4CL2* were cloned to the binary vector PCB2004 under the control of a 35S-promoter using the Gateway® Cloning System (Invitrogen, Carlsbad, CA) (Lei et al. 2007) . PCR primers were designed with an addition of attB at the both end. The PCR program was as following: 98 ºC for 30s, followed by 30 cycles of 98 ºC for 10s, 60 ºC for 20s, and 72 ºC for 30s, and then a 10 min extension at 72 ºC. The PCR products were purified and then cloned to the entry vector pDONR207 using the Gateway® BP Clonase® Enzyme mix (Invitrogen, Carlsbad, CA). After sequencing confirmed the accuracy of the inserts in the recombinant pDONR207 plasmids, ORFs were cloned to the PCB2004 binary vector via an exchange reaction using Gateway® LR Clonase™ enzyme (Invitrogen, Carlsbad, CA). Two recombinant plasmids, PCB2004-Cs4CL1 and –Cs4CL2, were introduced into *Agrobacterium tumefaciens* strain EHA105 for tobacco transformation. The positive EHA105 colonies were selected on agar-solidified medium containing 50 mg/L kanamycin. The transformation of tobacco plants was completed via a leaf disc method (Li et al. 2017) . The positive transgenic plants were screened on agar-solidified MS medium supplemented with 25 mg/L of phosphinothricin. Multiple phosphinothricin-resistant plants were verified to be transgenic by genomic DNA-based PCR and RT-PCR, and then planted in pot soil in the green house.

Subcellular localization

The stop code of the ORFs of both *Cs4CL1* and *Cs4CL2* were removed by PCR and then the fragments were cloned to the pGWB5 vector, in which each was fused at the 5'end of an *EGFP* reporter gene using a Gateway cloning system as described above. Two recombinant constructs, pGWB5-Cs4CL1-EGFP and pGWB5-Cs4CL2-EGFP, were obtained and then introduced into *A. tumefaciens* EHA105. The positive colonies were activated and then used to infect leaves of 45 days old *N. benthamiana*. After infection for 48h, leaves were used to examine the EGFP fluorescence under an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan).

Quantitative reverse transcription PCR (RT-qPCR) and semi-quantitative RT-PCR analysis for gene expression in different tea tissues, treatments, and transgenic tobacco plants

RT-qPCR was performed gene expression profiling in different tissues using SYBR-Green PCR Mastermix (Invitrogen, Carlsbad, CA) on a CFX96™ instrument (Bio-RAD, California, USA) following the manufacturer's instructions. Gene-specific primer pairs and thermal programs for *Cs4CL1* and *Cs4CL2* (Table S1) were designed for PCR. *ACTIN* was used as reference. Amplified products were monitored using an optical reaction module and amplified values were normalized against the *ACTIN* gene

expression (Pang et al. 2007) . For identification of positive transgenic tobacco lines, semi-quantitative RT-PCR was completed as previously described (Zhao et al. 2013) .

Heterologous expression in *Escherichia coli* and recombinant protein purification

The ORFs of both *Cs4CL1* and *Cs4CL2* were ligated to a pet-SUMO vector (Life technology biology, USA) with a T4-ligase according to the manufacturer's protocol. The resulting positive Cs4CL1-petSUMO and Cs4CL2-petSUMO plasmids were transformed into competent *E. coli* BL21. Positive colonies were obtained after screening on agar-solidified Luria-Bertani (LB) medium supplemented with 100 μg/ml kanamycin. One positive colony for each construct was inoculated in 200 mL liquid LB medium supplemented with 100 μg/ml kanamycin in a 500 ml E-flask. The flasks were placed on a rotary shaker with a speed of 250 rpm at 37 °C. After the concentrations of suspension cultures reached 1.00 at 600 nm, IPTG was added to the suspension cultures to a final concentration of 0.2 mM. The temperature was then changed to 28 °C to induce protein expression. After 24 hrs of induction, suspension cultures were transferred to tubes for 10 min of centrifugation at 5000 rpm to harvest *E. coli*. Crude proteins were extracted as described previously (Zhao et al. 2017) . Recombinant Cs4CL1 and Cs4CL2 were purified with an affinity chromatography consisting of Ni resin (New England Biolabs, MA, USA). Two buffers were used for affinity chromatography. Buffer A consisted of 20 mM pH 7.4 Tris-HCl, 200 mM NaCl. Buffer B was composed of buffer A and 100 mM imidazole. Column was first washed and equilibrated with buffer A. After crude protein extracts were loaded onto the column, buffer A was used to elute all unbound proteins. Then, buffer B was used to elute recombinant Cs4CL1 and Cs4CL2, which were examined with electrophoresis on a 12% SDS polyacrylamide gel and staining of Coomassie brilliant blue.

Enzymatic assays

Enzymatic assay was performed in order to characterize the catalytic activity of Cs4CL1 and Cs4CL2 according to a published protocol (Knobloch and Hahlbrock, 1975). In addition, minor modifications were added for these two enzymes. Briefly, prior to optimize reaction conditions, we tested enzymatic activity with three different substrates in a 500 μl mixture volume. The reaction mixture contained 0.5 M pH 7.5 phosphate buffer, 0.3 mM coenzyme A (CoA), 5 mM ATP, 5 mM MgC12, 5 μg purified recombinant enzyme, and 0.4 mM substrates (p-coumaric acid, caffeic acid and ferulic acid) in a 1.5 ml tube. The reactions were incubated at 30 °C for 30 min and then terminated with the addition of 50 μl methanol. After the reactions were centrifuged for 10 min at 12,000 rpm, the supernatants were transferred to new tubes for immediate HPLC analysis. To further optimize pH and temperature values of Cs4CL1 and Cs4CL2, 4-coumaric acid was used for assays, while other conditions were not changed. To estimate Km and Vmax values, reactions were carried out in a 2 ml reaction volume containing 0.5 M pH7.5 PBS buffer, 0.03 to 0.3 mM CoA, 5 mM ATP, 0.04-0.4 mM substrates (p-coumaric acid, caffeic acid and ferulic acid), 2-20 μg recombinant Cs4CL1 and Cs4CL2 protein. Finally, the Km and Vmax values of Cs4CL1 and Cs4CL2 were calculated according to Lineweaver-Burk plots.

HPLC analysis of enzymatic reaction products

The enzymatic reaction products were analyzed using a Shimadzu LC20-AT system with a full wavelength detection. The elution of products was monitored at 333 nm for p-coumaroyl-CoA, 350 nm for caffeoyl-CoA, and 346 nm for feruloyl-CoA. The mobile phase consisted of solvents A (0.5% acetic acetate in double deionized water) and B (100% HPLC-grade acetonitrile). A gradient elution program used consisted of 30–90% solvent B from 0 to 23 min and 90–30% buffer B from 23 to 29 min, and then followed by a 10 min column washing with 30% buffer B.

Analysis of total lignin in transgenic and wild-type tobacco plants

Lignin analysis was carried out by following the Syros method (Halpin et al. 1994) . Brifely, roots, stems, and leaves were harvested from 90-day old plants grown in the pot soil, pooled together, and then ground into fine powder in liquid nitrogen. One hundred mg of powder was suspended in 1 ml 50 % ethyl alcohol (in water) in a 2 ml tube. After 3 hours of extraction at 80 °C, 1 m1 methanol was added into the mixture, which was incubated for 1 hr at 80 °C. Tubes were centrifuged at 12,000 rpm for 10 min to obtain supernatant and residue phases. The supernatant was removed to a waste container. The remaining residues were fully dried at 60°C in an oven. Ten-milligram of dry powder was weighed to a 1.5 ml tube, suspended in 5 mL 25% (w/w)

acetyl bromide in acetic acid, and treated for 30 min at 70 °C. The mixtures were added with 0.2 mL 70% perchloric acid in water, mixed thoroughly, and then continuously treated at 70°C for 30 min. After cooling to the room temperature, tubes were centrifuged at 3000 g for 15 min to obtain supernatants and residues. The supernatant of each treatment was pipetted into a new 50 ml tube, which was added with 5 mL of 2 M NaOH. Then, glacial acetic acid was immediately added to the mixture to adjust the final volume to 25 ml, followed by mixing thoroughly. One ml of mixture was used to measure absorbent value at 280 nm on UV-visible Hitach U-5100 spectrophotometer (Hitach, Tokyo, Japan). According to a previously published method (Piquemal et al. 2002) , the absorbent values were converted to the lignin content in samples.

Extraction of polyphenolic compounds from transgenic and wild-type tobacco plants

Leaves from 90-day old wild-type, *Cs4CL1* and *Cs4CL2* transgenic tobacco plants were collected to analyze polyphenols. Phenolic compounds were extracted using the following procedure. Samples were ground into fine powder in liquid nitrogen. Powdered samples (150 mg) were suspended in 1 ml 80% methanol: 1% hydrochloric acid in a 1.5 ml tube. Tubes were completely vortexed and then placed in the room temperature for 20 min, followed by 10 min of centrifugation at 12000 rpm. The supernatant was transferred to a new tube. This extraction was repeated once to obtain a final volume of 2 ml extract for each sample. All extracts were then filtered through a 0.22 μm membrane into a new tube prior to UPLC-MS analysis described below.

Analysis of metabolites by UPLC-MS/MS

Products from Cs4CL1 and Cs4CL2 assays and polyphenolic metabolites extracted from wild type and *Cs4CL1* and *Cs4CL2* transgenic tobacco plants were analyzed using ultra-high-performance liquid chromatography (UPLC)-MS/MS analysis on an Agilent LC-MS system (Palo Alto, CA, USA). Compounds were separated in an Agilent 20RBAX RRHD Eclipse Plus C18 column (particle size: 1.8μm, length: 100 mm, and internal diameter: 2.1 mm Palo Alto, CA, USA). The column oven, mobile gradient, and electrospray ionization technique were as described previously (Jiang et al. 2013a) .

Results

Cloning of two *4CL* genes from tea plants

We used different tea organs that were pooled together to construct a cDNA library for RNA sequencing to clone *4CL* genes from tea plant. After sequence assembly, a *4CL* EST sequence was annotated from the contigs. Based on this sequence, a 3’ and 5’ rapid application of cDNA ends (RACE) was completed to amply a full length cDNA, namely *Cs4CL1* (KY615680). Sequence analysis revealed that the open read frame (ORF) of *Cs4CL1* included 1629 bp nucleotides, which were deduced to encode 543 amino acids. The molecular mass and the isoelectric point (IP) of Cs4CLs were predicted to be 59.55 KD and 5.72, respectively. Sequence analysis indicated that Cs4CL1 was different from the reported the *4CL* sequence (ABA40922.1) curated in the GenBank. Next, based on the sequence of ABA40922.1, we designed gene special primers and used PCR to amplify the full length of a cDNA from “Shu Chazao”, the sequence of which was 95% identical to ABA40922.1. a. We named this cDNA as *Cs4CL2* (KY615679). The ORF of *Cs4CL2* was 1713bp nucleotides, which were deduced to encode 570 amino acids with a molecular mass of 61.4KD and IP at 5.74. An amino acid sequence comparison showed that Cs4CL1 and Cs4CL2 shared 61% identity (Figure S1). In addition, Cs4CL1 had 66% identity to *Arabidopsis thaliana* 4CL1 (At4CL1) and Cs4CL2 had 68% identity to the At4CL3 (Figure S1). In addition, the analysis of functional domains revealed that both Cs4CL1 and 2 had the AMP binding domain BOX I (SSGTTGLPKGV) and catalytic domain BOX II (GEICIRG) (Figure S1). These features suggested that Cs4CL1 and Cs4CL2 might catalyze the coenzyme CoA ligation reaction in tea plants.

A phylogenic tree was constructed with amino acid sequences of Cs4CL1, Cs4CL2 and 19 other 4CL homologs from different plants. The resulting tree was obviously clustered into two subgroups, class I and class II. Cs4CL1 was clustered in the Class I subgroup associated with the formation of lignin and the Cs4CL2 was clustered in the Class II subgroup associated with the biosynthesis of flavonoids (Fig. 1B). This result suggests that Cs4CL1 and Cs4CL2 might diverge in their functions.

Features of regulatory elements in promoters of *Cs4CL1* and *Cs4CL2*

The genomic sequences of tea plants was recently published (Xia et al. 2017) . This advantage allowed us to use genome-walker technology to clone the promoters of *Cs4CL1* and *Cs4CL2*. As a result, 1537 bp nucleotides at the upstream of the *Cs4CL1* ORF was obtained, and 987 bp nucleotides at upstream of the *Cs4CL2* ORF was cloned. Further sequence analysis via Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PlantPan(http://plantpan2.itps.ncku.edu.tw/) identified four types of different regulatory elements, including AC-like elements, light responsive elements, meristem specific elements, and defense and stress relative elements. The results showed that the *Cs4CL1* promoter contained one AC-like element (MYB transcription factors binding sites) and the *Cs4CL2* promoter included five AC-like elements (Figure 2A). The two promoters contained nine light responsible elements (LRE). The LREs of Cs4CL1 included AAAC, BOX4, G-BOX, CGA, GT1, I-BOX, MNF1, SP1 and TCT-MOTIF (Figure 2A). The LREs of Cs4CL2 included BOX4, BOX1, CAG, GATA, GT1, I-BOX, SP1, TCCC, and TCT. Only three, BOX4, 1-BOX, and SP1, were shared by two genes. The *Cs4CL1* promoter had two meristem specific elements CAT-BOX and a CCGTCCC-BOX, while the *Cs4CL2* did not have. The *Cs4CL1* and *Cs4CL2* promoters had eight and two defense and stress relative elements, respectively (Fig. 2A).

Expression profiles in different tissues and three different treatments

The expression profiles of two genes in seven tea tissues were featured with qRT-PCR analysis. The resulting data indicated that the expression levels of *Cs4CL1* were the highest in roots, followed by stems, mature leaves, and young leaves and buds (Fig. 2B). By contrast, a relatively opposite expression trend of *Cs4CL2* was observed in these tissues, the highest in buds, followed by the 2nd, 1st 3rd and mature leaves, then by stems and roots (Fig. 2 B). These data suggest that two genes might be differentially associated with the biosynthesis of phenylpropanoids in tea tissues.

UV-B, mechanical wounding, and cold treatments were designed to understand their effects on the expression of the two genes in young leaves (Fig. 2C). RT-qPCR was completed to understand gene expression levels in treatments of 1-hr UV-B and 6-hr wounding and 6-hr 4°C. The results showed that the expression of the two genes differentially responded to the three treatments. In the 60 min treatment of UV-B irritation, the expression of *Cs4CL2* quickly increased in 15 min and decreased at 30, 45, and 60 min, while the expression levels of *Cs4CL1* slightly decreased in the first 45 min and then increased slightly at 60 min. During the 6-hr wounding treatment, the data indicated that the expression levels of *Cs4CL1* reduced in the first 30 min and then increased from 0.5 to 6 hrs, while the *Cs4CL2* did not transcriptionally respond to this treatment. During the 6-hr cold treatment, the expression levels of *Cs4CL2* did not change significantly at five time points tested, while the expression levels of *Cs4CL1* decreased either significantly or slightly.

Recombinant Cs4CL1 and Cs4CL2 uses three hydroxycinnamate substrates

Recombinant proteins were used to test different substrates to understand the catalytic activity of two enzymes. The ORFs of Cs4CL1 and Cs4CL2 were cloned into the prokaryotic expression vector Pet-SUMO and then expressed to induce their recombinant proteins in *E. coli*. The recombinant Cs4CL1 and Cs4CL2 were further purified by a His-Tag-column system (Figure S2). A mixture of three hydroxycinnamate molecules, p-coumaric acid, ferulic acid and caffeic acid, was used as substrates for this catalytic test. HPLC analysis showed that the enzymatic incubations of both the recombinant Cs4CL1 and Cs4CL2 with the three compounds produced new compound peaks (Fig. 3 A and B), while these compounds were not formed from the negative control (Fig. 3C). Further UPLC-MS/MS indicated that three peaks had the mass charge of ratios 912, 930, and 944 $[m/z]^-$, which were corresponding to coumaroyl-CoA (CM-CoA), caffeoyl-CoA (CF-CoA), and feruloyl-CoA (FR-CoA). MS/MS fragmentation analysis identified two main fragments 428 and 407 m/z from 912 $[m/z]^-$, 428 and 423 m/z from 930 $[m/z]^-$, and 428 and 437 m/z from 944 $[m/z]^-$ (Fig. 3D). These main fragments confirmed that the three peaks were CM-CoA, FR-CoA, and CF-CoA. Moreover, these data demonstrated that two enzymes used three hydroxycinnamate molecules as substrates

Optimization of pH and temperature values and kinetic analysis

The reaction temperatures and pH values were tested from 0ºC to 60ºC and from pH5.5 to 8.0 with 4-coumarate, respectively. The resulting data showed that the temperature optimum of two enzymes was 50ºC (Figure S3). The optima of pH values for

Cs4CL1 and Cs4CL2 were pH6.5 and pH7.5, respectively (Fig. S3).

The kinetics of Cs4CL1 and Cs4CL2 were estimated in the condition of optimum pH and temperature. Three substrates tested were p-coumaric(CM), ferulic (FR) and caffeic (CF) acids. Double reciprocal plots established with different concentrations showed that the catalysis of Cs4CL1 and Cs4CL2 followed the Michaelis-Menten kinetics (Figure S4). The Km values of the two enzymes to these substrates were estimated to be 8.86-19.99 (Table 1). Based on the Km values, two enzymes preferred to the three substrates in order of CF, FR, and then CM. The relative maximum velocity values were estimated, indicating that the relative catalytic rate of CM, FR and CF was in the order of 100%, 75%, 55% for both enzymes. Based on Kcat values calculated, the orders of the turnover rates were CF, CM, and FR for Cs4CL1 and CM, FR, and CF for Cs4CL2.

Subcellular localization of Cs4CL1 and Cs4CL2

Cs4CL1 and Cs4CL2 were fused at N-terminal of EGFP in the vector PGWB5 for subcellular localization analysis. In addition, EGFP and an ER marker protein were used as positive controls. Confocal analysis of infected leaves of *N. benthamiana* indicated that both the Cs4CL1-EGFP and Cs4CL2-EGFP fusion proteins were localized in the cytoplasm of epidermal cells (Fig. 4A).

Overexpression of both *Cs4CL1* and *Cs4CL2 in tobacco plants increases lignin and polyphenolic compounds*

The ORFs of both *Cs4CL1* and *Cs4CL2* were overexpressed in tobacco plants. Three transgenic lines for each gene (Fig. 4B) was selected for analysis of lignin, flavonoids, and other polyphenolic compound. In comparison with wild-type plants, no phenotypic alterations were observed in transgenic plants (Fig. 4B). The estimation of the total lignin from all tissues showed that the overexpression of both *Cs4CL1* and *Cs4CL2* increased the contents in transgenic plants compared with wild-type plants (Fig. 5). UPLC-MS analysis identified quercetin-3-O-glucoside (quercitrin), quercetin-3-O-rutinoside (rutin), kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, and chlorogenic acid (Jiang et al. 2013b) . Compared to the wild-type, the contents of chlorogenic acid were increased 2.5 to 3.0 folds and 2.8-3.5 folds in *Cs4CL1* and *Cs4CL2* transgenic plants, respectively (Table 2). The contents of quercetin-3-O-rutinoside were slightly or obviously increased in seedlings of three *Cs4CL1* transgenic plants, while were significantly increased in seedlings of three *Cs4CL2* transgenic ones. Due to the lack of standard samples, peak areas of kaemferaol-3-O-rutinoside and kaempferol-3-O-glucoside were used to compare their levels in transgenic and wild-type seedlings. The resulting data indicated that these four compounds were significantly increased 1.4-4.7 fold in levels in *Cs4CL2* transgenic plants compared with wild-type ones (Table 2). In comparison, the levels of kaemferaol-3-O-rutinoside and kaempferol-3-O-glucoside were only slightly increased in *Cs4CL1* transgenic plants.

Discussion

Tea plant is highly rich in plant phenylpropanoids (Dixon et al. 2013; Weisshaar and Jenkins 1998; Jiang et al. 2013b). Although the late pathway genes toward flavan-3-ols and anthocyanins have been intensively studied the characterization of tea polyphenolic biosynthesis (Liu et al. 2012; Wang et al. 2019), the entry pathway genes from phenylalanine to 4-coumaroyl CoA remain for functional characterization to understand their roles in the biosynthesis of flavonoids, lignin, and other phenolic acids such as chlorogenic acid reported here. Although 4CL, a key enzyme that catalyzes one key entry step toward the biosynthesis of both lignin and flavonoids, is well characterized in other plants (Cukovica et al. 2001), homologs in tea plant remain for functional characterization to understand their metabolic roles involved in lignin and flavonoids pathways. In Arabidopsis, two types of 4CL isoforms have been identified to involve in the formation of phenylpropanoids. One subgroup is classified as class I, such as At4CL1 that is preferably involved in the biosynthesis of lignin. The other subgroup is classified as class II, such as At4CL3 that is involved in the biosynthesis of flavonoids. These two classes are further characterized to be functional differentiation in gene expression patterns in different tissues different kinetics on substrates, and various responses to environmental stresses (Hamberger and Hahlbrock 2004; Li et al. 2015a). In the tea genome, we also identified two *4CL* isomers, *Cs4CL1* and *Cs4CL2*. *Cs4CL1* is a class I member (Fig. 1 B) highly expressed in roots and stems (Fig. 2B), which are rich in lignin, while *Cs4CL2* is a class II member (Fig. 1 B) highly expressed in young leaves (Fig. 2B), which are rich in

flavonoids. Because, to date, no tea mutants are available and it is impossible for tea genetic transformation, we used *in vitro* enzyme assays and tobacco plants to test their functions. Although enzymatic assays indicated that two enzymes could catalyze three hydroxycinnamate substrates (Fig. 3 and Table 1) and their overexpression in tobacco plants increased the contents of lignin (Fig. 5), metabolic analysis revealed their differential effects on the levels of three flavonols. In addition, the responses of two genes to UV-B irradiation, wounding, and cold treatments were different (Fig. 2 C). These data indicate that two isomers likely play various roles in the biosynthesis of tea phenylpropanoids associated with stresses. Tea plant is an important beverage crop due to its richness in flavonoids. Our findings provide insight into the differential roles of two 4CL members in the entry pathway steps of tea flavonoid biosynthesis. These findings are anticipated to enhance the metabolic engineering for value-increased beverage products in the future.

Declarations

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.

Authors' contributions:

M L, L G, and T X conceived the experiments. M L, L G, Y W, X J and Y L performed the experiments. M L and D X prepared the manuscript. All authors read and approved the manuscript.

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Tables

Table 1 Kinetic values of Cs4CL1 and Cs4CL2 to three substrates

Isoform	Substrate	Km(μM)	Relative Vmax (% of coumarate)	Relative Vmax/Km (μM^{-1})
Cs4CL1	4-Coumarate	19.99	100	5
	Ferulate	15.98	75	4.68
	Caffeate	8.86	54	6.04
Cs4CL2	4-Coumarate	16.08	100	6.22
	Ferulate	14.64	75	5.26
	Caffeate	12.61	55	4.38

Table 2. Comparison of the levels of three flavonoid compounds and chlorogenic acid in leaves of *Cs4CL1* and *Cs4CL2* transgenic tobacco plants

Genotypes	WT	Cs4CL1 transgenic plants			Cs4CL2 transgenic plants		
	Multiple plants	line2	line3	line4	line2	line5	line8
Contents (mg/g, fresh weight)							
Rutin (quercetin-3-*O*-rutinoside)	0.35±0.05	0.47±0.23	0.48±0.10	0.67±0.10*	0.81±0.12*	0.75±0.14*	1.03±0.31*
Chlorogenic acid	0.51±0.04	2.00±0.12**	2.26±0.27*	1.83±0.26*	1.90±0.15*	1.89±0.17*	2.27±0.16*
Levels (peak area values)							
K-3-*O*-rutinoside	7557±1941	7521±531	7692±1122	11611±1139*	12173±2071*	10626±535*	10287±250*
K-3-*O*-glucoside	111±34	178±76	242±62*	244±72*	238±113*	170±16*	271±84*

Figures

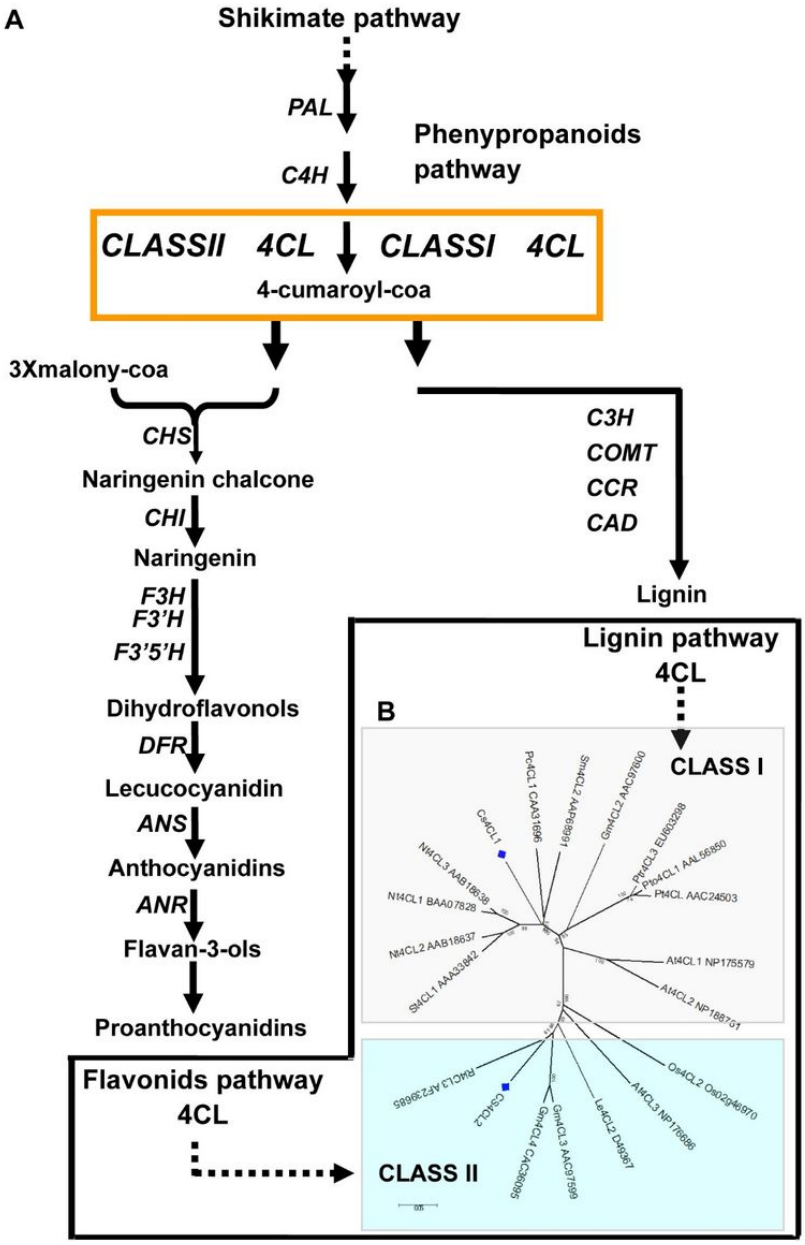

Figure 1

The step catalyzed by 4CL toward the biosynthesis of flavonoids and lignin and a phylogenetic tree of the 4CL protein family. A, Biosynthetic pathways start from the general phenylpropanoid pathway to flavonoids and lignin. Class I and class II 4CL enzymes are shown to catalyze the step toward flavonoids and lignin. B, A phylogenic tree was constructed with amino acid sequences of Cs4CL1, Cs4CL2 and other 18 4CL proteins. The tree shows that Class I subgroup 4CL and Class II subgroup 4CL differentially catalyze the step toward flavonoid and lignin.

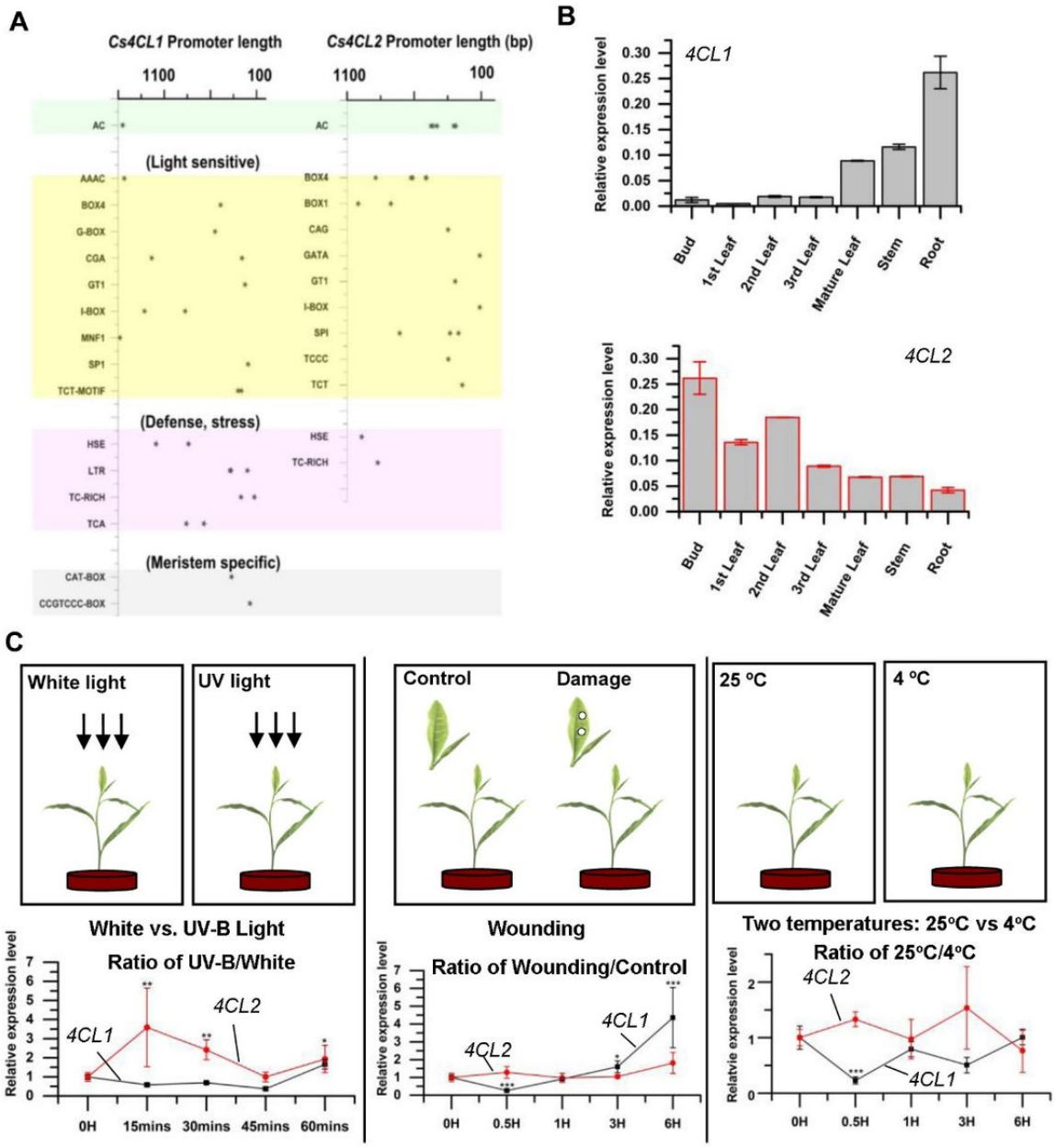

Figure 2

Promoter analysis and expression patterns of Cs4CL1 and Cs4CL2. A, Schemes show the locations of MYB-putative binding sites (AC-elements), light sensitive responsive elements, defense and stresses responsive elements, and meristem specific expressing sites in 1000 bp upstream sequence of Cs4CL1 and Cs4CL2 open reading frames. Promoter analysis was completed with the software PlantPan 3.0. B, Relative expressing profiles of Cs4CL1 and Cs4CL2 were estimated in the buds, 1st, 2nd, 3rd and mature leaves, stems, and roots by qRT-PCR. C, Real time qRT-PCR data shows effects of white and UV-B light, mechanical wounding, and two temperatures on the expression of Cs4CL1 and Cs4CL2. In the three compared conditions, the relative expression levels of each gene were indicated with ratios of expression values obtained from UV-B and white light, from wounding and control, and from 4oC and 25oC.

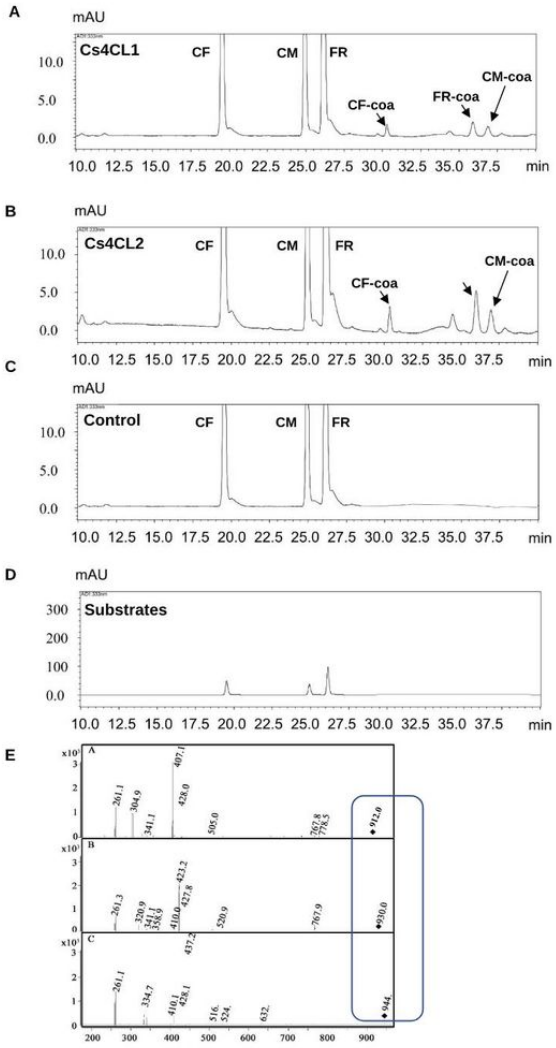

Figure 3

HPLC and MS profiles showing the conversion of p-coumaric acid, ferulic acid, and caffeic acid to corresponding products by recombinant Cs4CL1 and Cs4CL2. A, HPLC peak profiles show that the recombinant Cs4CL1 converts p-coumaric acid (CM), ferulic acid (FR), and caffeic acid (CF) to p-coumaric acid-CoA (CF-coa), ferulic acid-CoA (FR-coa), and caffeic acid-CoA (CM-coa), respectively. B, HPLC peak profiles show that the recombinant Cs4CL2 converts p-coumaric acid (CM), ferulic acid (FR), and caffeic acid (CF) to p-coumaric acid-CoA (CF-coa), ferulic acid-CoA (FR-coa), and caffeic acid-CoA (CM-coa), respectively. C, HPLC peak profiles show p-coumaric acid, ferulic acid, and caffeic acid as controls. D, Tandem mass fragment profiles of p-coumaric acid-CoA (CM-coa), ferulic acid-CoA (FR-coa), and caffeic acid-CoA (CF-coa).

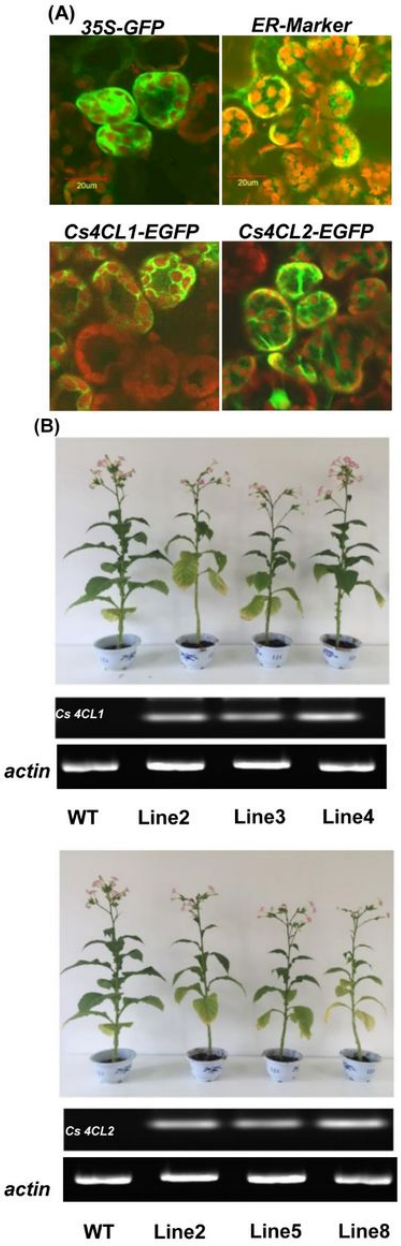

Figure 4

Subcellular localization of Cs4CL1 and Cs4CL2 and their overexpression in tobacco plants. A, confocal microscopy images indicate subcellular localization of GFP alone, ER-localized-marker controls, and fused 4CL1-GFP and 4CL2-GFP in epidermal cells of leaves of Nicotiana benthamiana. B, Images show phenotypes of 75 days old wild-type and transgenic Nicotiana tabacum plants of Cs4CL1 and Cs4CL2. For each gene, three transgenic plants are selected for this comparison. RT-PCR images show the overexpression of Cs4CL1 and Cs4CL2 in transgenic plants.

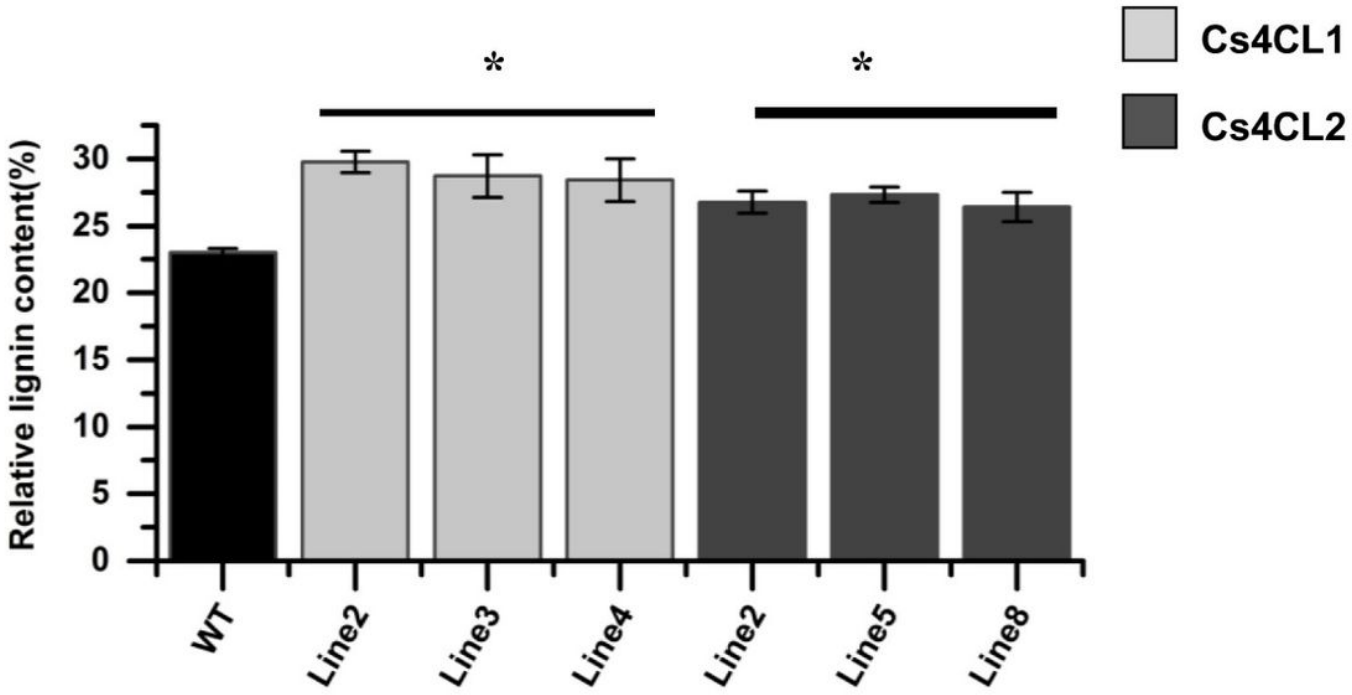

Figure 5

Increase of lignin in Cs4CL1 and Cs4CL2 in transgenic tobacco plants. Lignin contents were measured for wild-type, three Cs4CL1 transgenic, and three Cs4CL2 transgenic plants. “*” denotes the significant differences (P-value less than 0.05) in the lignin contents between transgenic and wild-type plants.

Supplementary Files

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