Flavonoid 3′-hydroxylase of *Camellia nitidissima* Chi. promotes the synthesis of polyphenols better than flavonoids

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Abstract

*Camellia nitidissima* Chi. is an ornamental plant of the genus *Camellia* L. Its flowers contain a lot of flavonoids and polyphenols. Flavonoid 3′-hydroxylase (F3′H) plays an important role in the synthesis of flavonoids, polyphenols and anthocyanins. We used PCR amplification, quantitative PCR, High-performance liquid chromatography, subcellular localization, and agrobacterium-mediated leaf disk method to study the the function of *CnF3′H*. The full length of *CnF3′H* was 1859 bp (GenBank code: HQ290518.1), with an open reading frame of 1577 bp, and encoded 518 amino acid. A phylogenetic tree analysis showed that *CnF3′H* was closely related to *Camellia sinensis* L. and *C. sinensis* cultivar Zhonghuang. *CnF3′H* was expressed in flowers, leaves, fruits, sepals, petals and stamens of *C. nitidissima*, and during the flowering process the expression level in flower decreased initially and then increased. *CnF3′H* expression was significantly positive correlated with polyphenol contents in *C. nitidissima*. A *CnF3′H*-EGFP expression vector was constructed to do the subcellular localization, we found that *CnF3′H* was obviously localized in the nuclear envelope and cytomembrane. In transgenic tobacco flowers, the total polyphenol content and various polyphenol constituents were significantly increased with high *CnF3′H* expression level, while total flavonoid contents and some flavonol constituents were increased slightly. These findings suggest that *CnF3′H* promotes the synthesis of polyphenols better than flavonoids.

Keywords *Camellia nitidissima* · F3′H · Expression patterns · Functional verification · Polyphenol synthesis

Introduction

*Camellia nitidissima* Chi. was first discovered in Guangxi Province, China. It is an evergreen shrub or small tree belonging to sect. *Chrysantha* Chang in the genus *Camellia* L. of Theaceae [1]. The flowers of *C. nitidissima* have a particular golden yellow color, making it desirable as an ornamental plant that used as a genetic resource for yellow *Camellia* breeding. Studies have shown that *C. nitidissima*, like *Camellia sinensis* L., contains a large number of flavonoids and polyphenols in leaves and flowers [2, 3]. That is, not only beneficial for the plant strengthening its resistance, but may also positively affect human health [4, 5]. Flavonoids are the main component of the yellow color of *C. nitidissima* flower [6].

Flavonoid 3′-hydroxylase (F3′H) is a monoxygenase of cytochrome P450 [7, 8]. F3′H is very important for the regulation of flavonoid secondary metabolism, which uses dihydrokaempferol (DHK) as a substrate to form dihydroquercetin (DHQ) via hydroxylation [9, 10]. DHQ is an important intermediate in the synthesis of flavonols, polyphenols and anthocyanins [11, 12]. Brugliera, Barri-Revell, Holton and Mason [13] were the first to isolate the F3′H gene from *Petunia hybrida* and identified it as belonging to the CYP75B2 gene family [14, 15]. Several F3′H genes have since been identified, such as in *Arabidopsis thaliana* [16], *Ginkgo biloba* [17], *C. sinensis* [18] and *Canarium album*.
Studies on the F3′H gene of P. hybridra have shown that mutations in three regulatory sites Del, Eluta and Rosea, lead to decreased expression of F3′H, and reduce flavonoid biosynthesis [20]. Masukawa et al. [21, 22] found that the lack of F3′H contributed to pelargonidin-based anthocyanin accumulation in red radish. In expressing transgenic tobacco plants with GtF3′H of Gentiana triflora showed a slight increase in anthocyanin content and flower color intensity, and conversion of the flavonol quercetin from kaempferol [9, 23]. In sweetpotato, the dependent selection of recessive F3′H allele homozygote also altered anthocyanin composition [24]. Most studies have been shown F3′H is related to the synthesis of flavonols and anthocyanins, but few studies have been done on polyphenols synthesis of F3′H.

In C. sinensis, CsF3′H had been cloned and did some characterization [18]. It was discovered four conserved cytochrome P450-featured motifs and three F3′H-specific conserved motifs in the protein sequence of CsF3′H and CsF3′H catalyzed the naringenin, DHK and kaempferol to biosynthesize of 3′,4′ catechins, 3′,4′,5′ catechins and flavan 3-ols in tea leaves [18]. Catechins were polyphenol metabolite, which had a competitive effect with flavonols to affect the color formation of plants. In C. nitidissima, flavonols, such as quercetin-7-O-β-glucopyranoside and quercetin-3-O-glucopyranoside, were the main metabolites of the yellow flower color and CnFLS was an major regulatory gene that regulates the synthesis of flavonoids [25]. In the flavonoids pathway, DHQ synthesized by F3′H is the substrate for FLS to catalyze the synthesis of flavonols, and the interaction between F3′H and FLS affects the final plant color appearance [9, 26]. F3′H genes have not yet been reported in C. nitidissima, although they play important roles in flower color.

In this study, we cloned the full-length sequence of the CnF3′H gene from the petals of C. nitidissima and studied its expression pattern and subcellular localization. We also overexpressed CnF3′H in tobacco to verify its function. The results showed a functional diversification of the F3′H from C. nitidissima, which is different from other plants slightly. This finding is important for the study of flower color formation in C. nitidissima.

Materials and methods

Plant materials and growth conditions

Camellia nitidissima tissues were collected from the National Camellia Germplasm Resource Bank (Guangxi, China, E 108°20′53″, N 22°49′11″, 75 m above sea level) in Nanning, Guangxi Province, China. The materials were frozen in liquid nitrogen and stored at − 80 °C for later use. Nicotiana benthamiana was used in the transient transformation and stable transformation experiments. The seedlings were grown in a growth chamber (RDN-1000E, Ningbo Yang Hui Instrument Co. Ltd, China; temperature: 25 °C, humidity: 76%, illumination: 6000 Lx, light cycle: 16/8 h).

Cloning of CnF3′H

Total RNA was isolated using an RNAprep Pure Extraction Kit (DP441, Tiangen Biochemical Technology, China) and RNA integrity was determined based on 1.5% agarose gel electrophoresis analysis. A PrimeScript II 1st Strand cDNA Synthesis Kit (6210, TaKaRa, Japan) was used to synthesize the cDNA for gene cloning experiments, according to the manufacturer’s instructions. We designed a pair of specific primers (S1.1) using Primer 3 (http://www.prime r3plus.com/cgi-bin/dev/primer3plus.cgi) according to the transcriptome data. The PCR products were cloned into a T-vector (CT501, TransGen Biotech Co., Ltd, China) for sequencing. The full-length CnF3′H gene was assembled and verified based on sequence analysis.

Sequence alignment and phylogenetic analysis

BioEdit and NCBI Blast (https://blast.ncbi.nlm.nih.gov/ Blast.cgi) was used to align the sequences [27]. NCBI ORFfinder (http://www.ncbi.nlm.nih.gov/projects/orf/) was used to identify the reading frame (Wheeler et al.), and ProtParam (https://web.expasy.org/protparam/) was used to analyze the protein molecular weight and isoelectric point [28]. Amino acid sequence alignment was performed by the software of DNAMAN and the phylogenetic tree was constructed with MEGA 5.0 software, using the neighbor-joining (NJ) method and 1000 bootstrap replicates [29].

Quantitative PCR analysis of CnF3′H

We chose GAPDH as the reference gene (S1.2) and a pair of unique primers of F3′H (S1.3) for quantitative PCR analysis. A PrimeScript RT reagent Kit with gDNA Eraser (RR047, TaKaRa) was used to synthesize the first-strand cDNA. The quantitative PCR reaction system was constructed using SYBR Prime Ex Tap II (Tli RNaseH Plus) (RR420, TaKaRa). The reaction was performed on a QuantStudio® 7 Flex (Applied Biosystems, USA), and the reaction procedure was as follows: pre-denaturation at 95 °C for 30 s; 98 °C 5 s, 60 °C 34 s, 40 cycles; 95 °C 15 s, 60 °C 1 min, 95 °C 15 s. The relative expression quantity of CnF3′H was measured in different tissues and in different development periods by the 2^(-ΔΔCT) method [30].
High-performance liquid chromatography analysis

An NF555 colorimeter (Nippon Denshoku Industries Co., Ltd., Japan) was used to detect the color indicator of the petals. HPLC was used to assess the flavonoid, polyphenol and anthocyanin constituents. We first ground fresh sample (0.6 g) in liquid nitrogen, then added 5 mL of extraction solution (methanol:water:formic acid:trifluoroacetic acid = 70:27:2:1). The samples were extracted in the dark for 24 h with occasional shaking. After extraction, the samples were filtered with absorbent cotton to remove residues and passed through an organic microporous filter membrane (0.22 cm) (ANPEL Laboratory Technologies Inc., Shanghai, China). The filtrate underwent HPLC analysis.

An Agilent Technologies 1260 Infinity (Agilent Technologies, Inc., Germany) equipped with Waters SunFire C18 column (4.6 x 250 mm, 5 μm) (Waters Co., USA) was used for HPLC. The column temperature was 30 °C, the flow rate was 1.0 mL/min; and the injection volume was 10 μL. The elution procedure for polyphenols was as follows: 0–5 min, 20% B; 5–15 min, 20% up to 40% B; 15–20 min, 40% up to 60% B; 20–20.2 min, 60% down to 20% B; 20.2–24 min, 20% B. The detection wavelength of flavonoids was 350 nm. The elution procedure for polyphenols was as follows: 0–9 min, 98% down to 90.7% B; 9–15 min, 90.7% B; 15–20.5 min, 90.7% down to 85% B; 20.5–29.5 min, 85% down to 75% B; 29.5–30 min, 75% up to 98% B; 30–34 min, 98% B. The detection wavelength of polyphenols was 278 nm [25, 31].

Subcellular localization of CnF3′H protein

We designed a pair of primers (S1.4) according to the EXclone Kit instructions (exv09, Hangzhou Biogo Co. Ltd., China) for the vector construction. The overexpression vector was transformed to Agrobacterium tumefaciens GV3101 strain via the thermal shock method [32]. To perform tobacco infiltration analysis, the transformed agrobacteria were suspended in induction medium (10 mM/L MES + 10 mM/L MgCl2 + 100 mM/L acetosyringone) and injected into the N. benthamiana leaf [33]. We then detected the GFP signal 2–5 days after injection using LSM510 Meta confocal microscope (Zeiss, Germany) [34].

Tobacco transformation analysis of CnF3′H

To verify the functionality of CnF3′H, we heterologously transformed N. benthamiana by the leaf plate method. PCR primers (S1.5) were applied using T5 Direct PCR Kit (Plant) (TSE011, TSING KE Biological Technology, China) to positively identify rooting plants. The PCR procedure was as follows: pre-denaturation at 98 °C for 3 min, denaturation at 98 °C for 10 s, annealing at 65 °C for 10 s, extension at 72 °C for 1 min and 30 s, 30 cycles, extended at 72 °C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis. After the positive plants had flowered, we collected the flowers, froze them in liquid nitrogen and stored the samples at −80 °C. Quantitative PCR was used to measure the relative expression of CnF3′H with the reference gene of 18S (S1.6). The total flavonoid, total polyphenol and total anthocyanin contents in the flowers were measured by a spectrophotometric assay [2]. Individual flavonoids, anthocyanins and polyphenols in the flowers were also determined by HPLC and compared with the control group.

Results

Identification and phylogenetic analysis of CnF3′H

We obtained a fragment of 1859 bp, with an open reading frame of 1577 bp (25–1581 bp) and named it CnF3′H (GenBank HQ290518.1). This gene encoded 518 amino acids, with the molecular weight 57,030.02 Da and theoretical pI 7.76. The average hydrophaticity (GRAVY) was 0.013, indicating a hydrophobic protein. CnF3′H was a transmembrane protein containing five transmembrane regions, it was a non-secretory protein with no peptide signal. The secondary structure analysis showed that CnF3′H was mainly composed of α-helix and random coil regions, accounting for 48.26% and 35.91%, respectively (Fig. 1a). There was 78–97% amino acid sequence similarity between CnF3′H and other F3′H proteins and in the N-terminal of the amino acid sequence there were possible anchoring residues (LPPGP), while in the C-terminal contained residues of a heme-binding region (PFGAGR−RICAG), which indicated that CnF3′H belongs to the P450 super family [7, 35] (Fig. 1b).

Phylogenetic tree analysis (Fig. 1c) of the F3′H genes of the selected species showed a division into three branches. CnF3′H was closely related to C. sinensis F3′H, C. sinensis cultivar Zhonghuang F3′H, and Rhododendron × pulchrum F3′H which were all in one branch. Hevea brasiliensis F3′H, Manihot esculenta F3′H, Theobroma cacao F3′H, C. album F3′H, Ribes nigrum F3′H, Paeonia lactiflora F3′H and Paeonia suffruticosa F3′H were gathered into one branch, which was closely related to the CnF3′H branch. Helianthus annuus F3′H, Callistephus chinensis F3′H, Olea europaea var. sylvestris F3′H, Coffea arabica F3′H and Eustoma exaltatum subsp. russellianum were clustered into a single branch, which showed a more distant relationship to CnF3′H.
Expression pattern of CnF3′H in C. nitidissima

We detected the relative expression of CnF3′H in different tissues and flowers during the development of C. nitidissima (Fig. 2a) by real-time quantitative PCR. CnF3′H was found to be expressed in leaves, fruits, flowers, sepals, petals and stamens, with the highest expression level in fruits and the lowest in stamens. During the flowering process, the expression level of CnF3′H in flowers was the highest in buds 10 mm in diameter. Over the course of flowers development, the CnF3′H expression decreased rapidly and reached a minimum when the flowers were half open. After the flowers were fully bloomed, the expression level of CnF3′H increased gradually (Fig. 2b).

Next, we investigated the relationship between CnF3′H expression level and the flower color, flavonoid content and polyphenol content in the petals of C. nitidissima. The expression level of CnF3′H was negatively correlated with the yellow index (b*) of the flower color (Fig. 2c). Meanwhile, the CnF3′H expression was positively correlated with the contents of flavonoids and polyphenols. The correlation with flavonoids was not significant (Fig. 3a, b), while the correlation with polyphenols was significant (Fig. 3c, d). CnF3′H tended to promote the synthesis of polyphenols rather than flavonoids in C. nitidissima, thereby it can weaken the yellow color the flowers.

Subcellular localization of CnF3′H

Using EXclone technology, we contained CnF3′H fused to green fluorescent protein (EGFP). After transforming the epidermal cells of N. benthamiana leaves, a laser confocal microscope was used to identify the subcellular localization of CnF3′H. When excitated at 488 nm, green fluorescence signal of the empty 35 s-EGFP vector appeared in the cell nucleus, cytomembrane and cytoplasm of the cells, and the signal was dispersed throughout the whole cell (Fig. 4a), indicating that the free EGFP could be successfully expressed. In the epidermal cells containing CnF3′H-EGFP vector, green fluorescence signal appeared in the nuclear envelope and cytomembrane, and the contours were clear (Fig. 4b), indicating that CnF3′H protein was probably located on the nuclear envelope and cytomembrane.

Functional analyses of CnF3′H in tobacco

After overexpressing CnF3′H in tobacco, the gene was positively identified by PCR. No significant changes in leaf or flower color were found in positive tobacco lines.

Gerbera hybrida F3′H, PfF3′H: F3′H Populus tomentosa F3′H. A Proposed anchoring residues (LPPGP). B Residues of the active site hydrogen bond network. C Residues of the heme-binding region (PFAGRRICAG). e Phylogenetic tree analysis of CnF3′H. The F3′H gene evolutionary tree of the selected species was divided into three branches. CnF3′H was closely related to C. sinensis F3′H and C. sinensis cultivar Zhonghuang F3′H. (Color figure online)
We randomly selected six positive lines and measured the expression of CnF3’H in flowers. Compared with wild-type tobacco (WT), the CnF3’H expression levels increased significantly in flowers of positive lines, and the expression levels of CnF3’H were significantly different among the positive lines. The highest expression level (F3’H-4) was 3.9 times greater than the lowest (F3’H-6) (Fig. 5a). We also determined the contents of total flavonoid, total polyphenol and total anthocyanin in flowers of the 6 positive lines. We found the total polyphenol and total flavonoid contents in the positive lines were significantly higher than that in WT, and the total polyphenol content was obviously higher than the total flavonoid content. The total anthocyanin content could not be detected in either wild-type or positive lines (Fig. 5b).

We detected six flavonoid constituents and six polyphenol constituents in flowers of the CnF3’H positive lines by HPLC. Except for F3’H-6, with low expression level, the six polyphenol constituents (EGC, EGCG, GC, GCG, ECG and CG) in the other five lines were all significantly higher than those in WT (Fig. 5c, d). Among the flavonoids, Qu3R and Ka were not significantly increased in F3’H-17 but were significantly higher in the other five lines compared to wild-type. DHQ was significantly higher in F3’H-3 than WT and the other lines. Ru, Qu7G and Qu3G were significantly higher in the six CnF3’H positive lines (Fig. 5e, f). This indicated that CnF3’H promoted the synthesis of flavonols and polyphenols, and the promotion of polyphenol synthesis was greater than that of flavonoid synthesis. These findings show that CnF3’H preferentially promote the synthesis of polyphenols.

**Discussion**

In this study, we obtained a CnF3’H gene encoded 518 amino acids. The P450 family is an important and large gene family in plants, and is mainly involved in biosynthetic and biodegradation processes [35]. Seitz et al. [36] constructed chimeric genes and found that the area near the N-terminals of F3’H and F3’5’H determined their action characteristics, while the C-terminal determined functional differences between the enzymes. The “PPGP” sequence was determined to be a conserved sequence, and “FGAGRRICAG” was identified as the C-terminal heme-binding region necessary for cytochrome enzymes [37, 38]. We found that CnF3’H had typical F3’H characteristics (Fig. 1b). Multiple sequence alignment showed that CnF3’H was highly similar (98%) to C. sinensis F3’H (Fig. 1b), The phylogenetic tree analysis also indicated that CnF3’H was closely related to C. sinensis F3’H and C. sinensis cultivar Zhonghuang F3’H.
Fig. 3 Profiles of CnF3'H expression level and color pigments in C. nitidissima. a Profiles of CnF3'H expression level and TF (total flavonoid content). b Profiles of CnF3'H expression level and the content of Qu7G (quercetin-7-O-β-D-glucopyranoside) and Qu3G (quercetin-3-O-glucopyranoside). The expression level of CnF3'H was not significantly positively correlated with the contents of TF, Qu7G or Qu3G. c Profiles of CnF3'H expression level and TP (total phenol content). d Profiles of CnF3'H expression level and the contents of EC (epicatechin) and GCG (gallocatechin gallate). The expression level of CnF3'H was significantly positively correlated with the contents of TP, EC and GCG.

Fig. 4 Subcellular localization of CnF3'H-EGFP in leaf epidermal cells of N. benthamiana. a The green fluorescence signals of the empty 35 s-EGFP vector. There was signal in the cell nucleus, cytomembrane and cytoplasm when the cells were excited at 488 nm. b Green fluorescence signal of the CnF3'H-EGFP vector. There was signal in the nuclear envelope and cytomembrane.
Fig. 5 Content of flavonoid and polyphenol metabolites in the flowers of CnF3′H positive tobacco lines. a Relative expression of CnF3′H in the flowers of positive lines. The expression level of CnF3′H increased significantly in the flowers of positive lines. b The total flavonoid, total polyphenol and total anthocyanin contents in the flowers of positive lines. The total polyphenol and total flavonoid contents in positive lines were significantly higher than those of WT. No total anthocyanin content was detected. c Content of EGC (epigallocatechin), EGCG (epigallocatechin gallate) and GC (gallocatechin). d Content of GCG (gallocatechin gallate), ECG (epicatechin gallate) and CG (catechin gallate). The contents of EGC, EGCG, GC, GCG, ECG and CG in the other five lines (excluding F3′H-6) were significantly higher than those in WT. e Contents of Qu3R (quercetin-3-O-rutinose), Ka (kaempferol) and Ru (rutin). Qu3R and Ka in the flowers of the other five lines (excluding F3′H-17) were significantly higher than those in WT. f Contents of DHQ (dihydroquercetin), Qu7G (quercetin-7-O-β-D-glucopyranoside) and Qu3G (quercetin-3-O-glucopyranoside). DHQ was significantly higher in F3′H-3 than in WT and the other lines. Ru, Qu7G and Qu3G were significantly higher in the six positive lines than in WT.
researches have also shown that positive lines, indicating that and polyphenols. plants and is mostly related to the metabolism of flavonoids synthesis process [45]. The flavonoid content in transgenic tobacco containing the with the metabolic process of plant flavonoids. The total (Fig. 1c). In the protein sequence of CsF3'H, there were four conserved cytochrome P450-featured motifs and three F3'H-specific conserved motifs [18], these structures were also found in CnF3'H.

Subcellular localization can identify the specific location of protein expression in cells, which is important when exploring the gene function. At present, the subcellular localizations of several enzymes in the flavonoid pathway have been clarified, such as anthocyanin 5-aromatic acyltransferase [39], anthocyanidin synthase (ANS) [40], flavonoid 3-O-glucosyltransferase (UF3GT) [41] and dihydroflavonol 4-reductase (DFR) [31]. Toda et. [42] found that F3'H in soybean was located in vacuoles in the seed coat umbilicus, and Li et al. [17] suggested that GbG3'H protein might be located in the endoplasmic reticulum as a complex, according to the analysis of signal peptide (MHFLFLP-PLFFHFINSVCNPE) [43]. However, our result was not the case with the instantaneous conversion of N. benthamiana differed from the previous findings. In the current study, CnF3'H-EGFP protein was identified from the strong fluorescence signal on the nuclear envelope and cytomembrane (Fig. 3b). Thus, CnF3'H probably locates to the nuclear envelope and cytomembrane.

In this study, the expression of CnF3'H showed significant tissue specificity, which was similar to the results of previous studies. In addition, F3'H gene expression was found to be primarily related to flavonoid metabolic processes. The higher expression level of F3'H in G. biloba leaves may be related to the accumulation of secondary metabolites such as flavonoids [17]. The expression of CaF3'H in C. album was detected during fruits development and it was highest 50 days after flowering [19]. CsF3'H1 gene in tea plants regulated the flavonoid metabolism pathway, the gene was highly expressed in the young leaves in which polyphenols were concentrated [44], and it also showed the importance in the biosynthesis of catechins and flavanols in tea leaves [18]. In C. nitidissima, CnF3'H expression was proportional to polyphenol contents and not significant with flavonoids contents. It can be inferred that F3'H expression changes among plants and is mostly related to the metabolism of flavonoids and polyphenols.

After transferring CnF3'H into tobacco, we found significant increases in polyphenol and flavonol contents in positive lines, indicating that CnF3'H could indeed promote the synthesis of flavonols and polyphenols. Previous researches have also shown that F3'H gene can interfere with the metabolic process of plant flavonoids. The total flavonoid content in transgenic tobacco containing the CoF3'H gene of Chromolaena odorata was significantly increased, indicating CoF3'H promoted the synthesis of flavonoids and was one of the key genes in flavonoids synthesis process [45]. The F3'H gene of Eupatorium adenophorum Sprengel was highly homologous with the endogenous F3'H tobacco gene, the endogenous F3'H gene of tobacco was inhibited after the transformation, leading to decreased F3'H expression and decreased anthocyanin accumulation in tobacco [46]. Although there was no significant change in the color of positive transgenic lines in our study, CnF3'H was found to preferentially convert DHQ into polyphenols, which suppressed the formation of a yellow flower color. The co-regulatory effect of CnF3'H and CnFLS on the flower color requires further studies.

Conclusions

We identified one F3'H homolog from C. nitidissima (CnF3'H), and phylogenetic analysis showed that F3'Hs of Camellia species formed a clade that was close to R. pulchrum. Gene expression analysis revealed that the expression of CnF3'H was positively correlated with polyphenols but negatively with yellow coloration. Subcellular localization of CnF3'H showed a likely dual localization in the nuclear envelope and cytomembrane. Furthermore, in the transgenic tobaccos, it was found that the content of polyphenols increased significantly, while the content of flavonols increased a bit. These findings show that CnF3'H promotes the synthesis of polyphenols better than flavonoids.

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Author contributions JL finished the main experimental contents, wrote and modified the paper. FZ and TR participated in the experiment and data collection together. ZX provided the foundation and thought for the preliminary study. LJ was the architect and director of the project. YH was responsible for the experimental design, and guided the writing and modification of the paper. All authors read and agree to the final manuscript.

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Data Availability All data are presented in the manuscript. Camellia nitidissima Chi. tissues were collected from the National Camellia Germplasm Resource Bank (Guangxi, China).
Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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