Flavonoid 3'-hydroxylase of Camellia Nitidissima Chi. Can Promote the Synthesis of Polyphenols Better Than Flavonoids

Lina Jiang (jianglinayls@163.com)
Research Institute of Subtropical Forestry Chinese Academy of Forestry https://orcid.org/0000-0002-4909-0831

Zhengqi Fan
Research Institute of Subtropical Forestry, Chinese Academy of Forestry

Ran Tong
Research Institute of Subtropical Forestry Chinese Academy of Forestry

Hengfu Yin
Research Institute of Subtropical Forestry Chinese Academy of Forestry

Jiyuan Li
Research Institute of Subtropical Forestry Chinese Academy of Forestry

Xingwen Zhou
Yulin Normal University

Research Article

Keywords: Camellia nitidissima Chi., flavonoid 3'-hydroxylase, expression patterns, functional verification, polyphenol synthesis

Posted Date: February 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-222490/v1

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 cloned *CnF3'H* from the petal of *C. nitidissima* (GenBank code: HQ290518.1). The full length of *CnF3'H* was 1859 bp, 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* can promote the synthesis of polyphenols better than flavonoids.

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 (Zhang and Ren 1998). 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 showen that *C. nitidissima*, like *Camellia sinensis* L., containes a large number of flavonoids and polyphenols in its leaves and flowers(Jiang et al. 2019; Li et al. 2019b; Yu et al. 2018). That is, not only beneficial for the plant strengthening its resistance, but may also positively affect human health (Mouradov and Spangenberg 2014; Passat 2012). And flavonoids are the main component of the yellow color of *C. nitidissima* flower (Tanikawa et al. 2008; Zhou et al. 2013).

Flavonoid 3'-hydroxylase (F3'H) is a monooxygenase of cytochrome P450 (Chapple 1998; Hou et al. 2011). 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 (Nakatsuka et al. 2007; Nakatsuka et al. 2014). Dihydroquercetin is an important intermediate in the synthesis of flavonols, polyphenols and anthocyanins(Grotewold 2006; Jeong et al. 2006; Jia 2017). Brugliera et al. (Brugliera et al. 1999) were the first to isolate the *F3'H* gene from *Petunia hybrida* and identified it as belonging to the CYP75B2 gene family(Schoenbohm et al. 2000; Sirim et al. 2009). Several *F3'H* genes have since been identified, such as in *Arabidopsis thaliana* (Han et al. 2010), *Ginkgo biloba* (Li et al. 2015), *C. sinensis* (Zhou et al. 2016) and *Canarium album* (Huang et al. 2017). Studies on the *F3'H* gene of *Petunia hybrida* have shown that mutations in three regulatory sites Del, Eluta and Rosea, lead to decreased expression of *F3'H*, and reduce flavonoid biosynthesis (Lukačin et al. 2000). Masukawa et al. (Masukawa et al. 2019; Masukawa et al. 2018) 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 trifloras*howed a slight increase in anthocyanin content and flower color intensity, and conversion of the flavonol quercetin from kaempferol (Nakatsuka et al. 2007; Nakatsuka et al. 2006). In sweetpotato, the dependent selection of recessive *F3'H* allele homozygote also altered anthocyanin composition (Tanaka et al. 2019). 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 (Zhou et al. 2016). 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, dihydrokaempferol and kaempferol to biosynthesize of 3',4'-catechins, 3',4',5'-catechins and flavan 3-ols in tea leaves (Zhou et al. 2016). 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-D-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 (Zhou 2012). In the flavonoids pathway, dihydroquercetin 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 (Nakatsuka et al. 2007; Olsen et al. 2010). *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 flavonoid 3'-hydroxylase 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", 75m above sea level) in Nanning, Guangxi Province, China. The materials were frozen in liquid nitrogen and stored at -80℃ 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℃, humidity: 76%, illumination: 6000Lx, light cycle: 16/8h).

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 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.primer3plus.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 (McGinnis and Madden 2004). NCBI ORFfinder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to identify the reading frame (Wheeler et al., 2006), and ProtParam (https://web.expasy.org/protparam/) was used to analyze the protein molecular weight and isoelectric point (Wilkins et al. 1999). 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 (Thompson et al. 1994).

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 (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℃ for 30 s; 98℃ 5 s, 60℃ 34 s, 40 cycles; 95℃ 15 s, 60℃ 1 min, 95℃ 15 s. The relative expression quantity of *CnF3'H* was measured in different tissues and in different development periods by the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen 2001).

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 analysis 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 hours 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 (4.6×250 mm, 5μm) (Waters Co., USA) was used for HPLC. The column temperature was 30℃., the flow rate was 1.0 mL/min; and the injection volume was 10 μL. The elution mobile phases were A: 2% formic acid solution and B: pure acetonitrile. The elution procedure for flavonoids 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.

Subcellular Localization of CnF3'H Protein

We designed a pair of primers (S1.4) according to the EXclone Kit instructions (exv09, Hangzhou Biogle Co. Ltd, China) for the vector construction. The overexpression vector was transformed to *Agrobacterium tumefaciensGV3101* strain via the thermal shock method (Gelvin 2003). To perform tobacco infiltration analysis, the transformed agrobacteria were suspended in induction medium (10 mM/L MES+10 mM/L MgCl2+100 uM/L acetosyringone) and injected into the *Nicotiana benthamiana* leaf (Ma et al. 2012). We then detected the GFP signal 2~5 days after injection using LSM510 Meta confocal microscope (Zeiss, Germany) (Yamaguchi et al. 2005).

Tobacco Transformation Analysis of *CnF3'H*

To verify the functionality of *CnF3'H*, we heterologously transformed *Nicotiana 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℃ for 3 min, denaturation at 98℃ for 10 s, annealing at 65℃ for 10 s, extension at 72℃ for 1 min and 30 s, 30 cycles, extended at 72℃ 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℃. 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 (Jiang et al. 2019).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 57030.02 Da and theoretical *pI* 7.76. The average hydropathicity (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 (Figure 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 (PFGAGRRICAG), which indicated that CnF3'H belongs to the P450 super family (Chapple 1998; He et al. 2008) (Figure 1b).

Phylogenetic tree analysis (Figure 1c) of the *F3'H* genes of the selected species showed a division into 3 branches. *CnF3'H* was closely related to *C. sinensisF3'H*, *C. sinensis* cultivar Zhonghuang *F3'H*, and *Rhododendron* x *pulchrumF3'H* which were all in one branch. *Hevea brasiliensis F3'H, Manihot esculenta F3'H, Theobroma cacao F3'H, Canarium 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* (Figure 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 and the expression in fruits was nearly 7.8 times that 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 (Figure 2b).

We next 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 (Figure 2c). Meanwhile, the *CnF3'H* expression was positively correlated with the contents of flavonoids and polyphenols. The correlation with flavonoids was not significant (Figure 3ab), while the correlation with polyphenols was significant (Figure 3cd). *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 *Nicotiana 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 35s-EGFP vector appeared in the cell nucleus, cytomembrane and cytoplasm of the cells, and the signal was dispersed throughout the whole cell (Figure 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 (Figure 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. We randomly selected 6 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) (Figure 5a). We also determined the total flavonoid content, total polyphenol content and total anthocyanin content 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 wild-type tobacco, 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 (Figure 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 6 polyphenol constituents (EGC, EGCG, GC, GCG, ECG and CG) in the other 5 lines were all significantly higher than those in wild-type tobacco (Figure 5c, d). Among the flavonoids, Qu3R and Ka were not significantly increased in F3'H-17 but were significantly higher in the other 5 lines compared to wild-type. DHQ was significantly higher in F3'H-3 than wild-type tobacco and the other lines. Ru, Qu7G and Qu3G were significantly higher in the 6 *CnF3'H* positive lines (Figure 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 biodetoxification processes (He et al. 2008). Seitz et al. (Seitz et al. 2007) 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 (Murakami et al. 1994; Yamazaki et al. 1993). We founed that CnF3'H had typical F3'H characteristics (Figure 1b). Multiple sequence alignment showed that CnF3'H was highly similar (98%) to *C. sinensis* F3'H (Figure 1b), The phylogenetic tree analysis also indicated that *CnF3'H* was closely related to *C. sinensisF3'H* and *C. sinensis* cultivar Zhonghuang *F3'H* (Figure 1c). In the protein sequence of CsF3'H, there were four conserved cytochrome P450-featured motifs and three F3'H-specific conserved motifs (Zhou et al. 2016), 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 (He et al. 2015a),

anthocyanidin synthase (ANS) (Wang et al. 2010), flavonoid 3-O-glucosyltransferase (UF3GT) (Li et al. 2019a) and dihydroflavonol 4-reductase (DFR) (Jiang et al. 2020). Toda et al. (Toda et al. 2002) found that F3'H in soybean was located in vacuoles in the seed coat umbilicus, and Li et al. (Li et al. 2015) suggested that GbG3'H protein might be located in the endoplasmic reticulum as a complex, according to the analysis of signal peptide (MHLFLPPLFFFHINSVCNPE) (Stafford 1974). However, our result was not the case with the instantaneous conversion of *Nicotiana 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 (Figure 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 *Ginkgo biloba* leaves may be related to the accumulation of secondary metabolites such as flavonoids (Li et al. 2015). The expression of *CaF3'H* in *Canarium album* was detected during fruits development and it was highest 50 days after flowering (Huang et al. 2017). *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 (Jiang et al. 2013), and it also showed the importance in the biosynthesis of catechins and flavanols in tea leaves (Zhou et al. 2016). In *C. nitidissima*, *CnF3'H* expression was proportional to polyphenol contents and not significant with flavonoids contents. It can be infered 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 significative 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(He et al. 2015b). 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 (Zhang et al. 2009). Although there was no significant change in the color of positive transgenic lines in our study, *CnF3'H* was found to preferentially convert dihydroquercetin 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 study.

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 *Rhododendron* x *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* promote the synthesis of polyphenols better than flavonoids.

Declarations

Acknowledgments

The authors thank the members of the National Forestry and Grassland Administration Economic Forest Product Quality Supervision, Inspection and Testing Center (Hangzhou) for their assistance in condition establishment and detection of high-performance liquid phase.

Author contribution

Jiang Lina finished the main experimental contentc, wrote and modified the paper. Fan Zhengqi and Tong Ran participated in the experiment and data collection together. Zhou Xingwen provided the foundation and thought for the preliminary study. Li Jiyuan was the architect and director of the project. Yin Hengfu was responsible for the experimental design, and guided the writing and modification of the paper. All authors read and agree to the final manuscript.

Funding

This work was supported by the National Key R&D Program of China (2019YFD1001005), the special funds for basic scientific research expenses of public welfare research institutes of the Chinese academy of forestry (CAFYBB2017ZF001), the National key projects for international scientific and technological innovation cooperation among governments (2016YFE0126100), the National Natural Science Foundation of China (No. 31860228), the Key projects of Natural Science Foundation of the Guangxi Zhuang Autonomous Region (2018GXNSFDA281007), and Scientific Research Foundation of Yulin Normal University for high-level talents (G2018025).

Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Brugliera, F., Barri-Rewell, G., Holton, T. A.,Mason, J. G. (1999) Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of *Petunia hybrida*. Plant J 19 (4):441-451. http://dx.doi.org/10.1046/j.1365-313X.1999.00539.x
2. Chapple, C. (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annual review of plant biology 49 (1):311-343. http://dx.doi.org/10.1146/annurev.arplant.49.1.311
3. Gelvin, S. B. (2003) Agrobacterium-mediated plant transformation: the biology behind the “gene-jockeying” tool. Microbiol Mol Biol Rev 67 (1):16-37. http://dx.doi.org/10.1128/MMBR.67.1.16-37.2003
4. Grotewold, E. (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57:761-780. http://dx.doi.org/10.1146/annurev.arplant.57.032905.105248
5. Han, Y., Vimolmangkang, S., Soria-Guerra, R. E., Rosales-Mendoza, S., Zheng, D., Lygin, A. V.,Korban, S. S. (2010) Ectopic expression of apple F3'H genes contributes to anthocyanin accumulation in the Arabidopsis tt7 mutant grown under nitrogen stress. Plant Physiol 153 (2):806-820. http://dx.doi.org/10.1104/pp.109.152801
6. He, F., Chen, W. K., Yu, K. J., Ji, X. N., Duan, C. Q., Reeves, M. J.,Wang, J. (2015a) Molecular and biochemical characterization of the UDP-glucose: Anthocyanin 5-O-glucosyltransferase from Vitis amurensis. Phytochemistry 117:363-372. http://dx.doi.org/10.1016/j.phytochem.2015.06.023
7. He, H., Pan, H., Zhang, N.,He, L. (2015b) Chromolaena odorata Flavonoid 3'-hydroxylase Gene Cloning and Its Expression in Tobacco. Acta Agronomica Sinica 41 (3):479-486. http://dx.doi.org/10.3724/SP.J.1006.2015.00479
8. He, L., Zhao, S.,Hu, Z. (2008) Gene and function research progress of plant cytochrome P450s. Pharmaceutical biotechnology 15 (2):142. http://dx.doi.org/10.3724/SP.J.1005.2008.01026
9. Hou, J., Tong, L., Cui, G., Xu, Z.,Li, Y. (2011) Research advances of plant flavonoid 3'-hydroxylase (F3'H) gene. Plant Physiol J 47:641-647. http://dx.doi.org/CNKI:SUN:ZWSL.0.2011-07-005
10. Huang, M., Wen, Z., Chi, Y., Peng, Z.,Chen, Q. (2017) Molecular Cloning and Expression Analysis of Flavonoids 3'-hydroxylase (CaF3'H) in *Canarium album*. Molecular Plant Breeding 15 (3):839-847. http://dx.doi.org/CNKI:SUN:FZZW.0.2017-03-008
11. Jeong, S., Goto-Yamamoto, N., Hashizume, K.,Esaka, M. (2006) Expression of the flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase genes and flavonoid composition in grape (*Vitis vinifera*). Plant Sci 170 (1):61-69. http://dx.doi.org/10.1016/j.plantsci.2005.07.025
12. Jia, Z. (2017) Studies on roles of flavonoid quercetin and transcription factor ATMYB44 in induction and regulation of defense responses in *Arabidopsis thaliana*. Nanjing Agricultural University, Nanjing

13. Jiang, L., Fan, Z., Tong, R., Zhou, X., Li, J.,Yin, H. (2020) Functional Diversification of the Dihydroflavonol 4-Reductase from Camellia nitidissima Chi. in the Control of Polyphenol Biosynthesis. Genes 11 (11):1341. http://dx.doi.org/10.3390/genes11111341
14. Jiang, L., Li, J., Tong, R., He, L., Zhang, L., Li, Z.,Huang, X. (2019) Relationship between flower color and important cellular environment elemental factors in yellow camellia. Guihaia 39 (12):1605-1612. http://dx.doi.org/10.11931/guihaia.gxzw201809019
15. Jiang, X., Liu, Y., Li, W., Zhao, L., Meng, F., Wang, Y., Tan, H., Yang, H., Wei, C.,Wan, X. (2013) Tissue-specific, development-dependent phenolic compounds accumulation profile and gene expression pattern in tea plant [*Camellia sinensis*]. PLoS ONE 8 (4). http://dx.doi.org/10.1371/journal.pone.0062315
16. Li, H., Liu, J., Jin, W.,Liang, Z. (2019a) Cloning, subcellular localization and spatio-temporal expression analysis of a flavonoid 3-O-glucosyltransferase gene (SmUF3GT) in *Salvia miltiorrhiza*. China Journal of Chinese Materia Medica 44 (10):2038-2045. http://dx.doi.org/10.19540/j.cnki.cjcmm.20190301.009
17. Li, L., Cheng, H., Chen, X.,Cheng, S. (2015) Molecular cloning, characterization and expression of flavonoid 3'hydroxylase-like protein gene from *Ginkgo biloba*. Acta Horticulturae Sinica 42:643-654. http://dx.doi.org/CNKI:SUN:YYXB.0.2015-04-005
18. Li, X., Wang, J., Sun, Z., Wang, J., Yin, H., Fan, Z.,Li, J. (2019b) Flavonoid components in flowers from three species of section Chrysantha Chang in Camellia. Guihaia 39 (7):917-924. http://dx.doi.org/10.11931/guihaia.gxzw201809009
19. Livak, K. J.,Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25 (4):402-408. http://dx.doi.org/10.1006/meth.2001.1262
20. Lukačin, R., Urbanke, C., Gröning, I.,Matern, U. (2000) The monomeric polypeptide comprises the functional flavanone 3β-hydroxylase from Petunia hybrida. FEBS Lett 467 (2-3):353-358. http://dx.doi.org/10.1016/s0014-5793(00)01116-9
21. Ma, L., Lukasik, E., Gawehns, F.,Takken, F. L. (2012) The use of agroinfiltration for transient expression of plant resistance and fungal effector proteins in *Nicotiana benthamiana* leaves. Methods in molecular biology 835:61-74. http://dx.doi.org/10.1007/978-1-61779-501-5_4
22. Masukawa, T., Cheon, K.-S., Mizuta, D., Kadowaki, M., Nakatsuka, A.,Kobayashi, N. (2019) Development of mutant RsF3'H allele-based marker for selection of purple and red root in radish (*Raphanus sativus* L. var. longipinnatus LH Bailey). Euphytica 215 (7):119. http://dx.doi.org/10.1007/s10681-019-2442-1
23. Masukawa, T., Cheon, K.-S., Mizuta, D., Nakatsuka, A.,Kobayashi, N. (2018) Insertion of a Retrotransposon into a Flavonoid 3'-hydroxylase Homolog Confers the Red Root Character in the Radish (*Raphanus sativus* L. var. longipinnatus LH Bailey). The Horticulture Journal 87 (1):89-96. http://dx.doi.org/10.2503/hortj.OKD-075

24. McGinnis, S.,Madden, T. L. (2004) BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32 (suppl_2):W20-W25. http://dx.doi.org/10.1093/nar/gkh435
25. Mouradov, A.,Spangenberg, G. (2014) Flavonoids: a metabolic network mediating plants adaptation to their real estate. Front Plant Sci 5:620. http://dx.doi.org/10.3389/fpls.2014.00620
26. Murakami, K., Mihara, K.,Omura, T. (1994) The transmembrane region of microsomal cytochrome P450 identified as the endoplasmic reticulum retention signal. The Journal of Biochemistry 116 (1):164-175. http://dx.doi.org/10.1016/0165-022X(94)90059-0
27. Nakatsuka, T., Abe, Y., Kakizaki, Y., Yamamura, S.,Nishihara, M. (2007) Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. Plant Cell Reports 26 (11):1951-1959. http://dx.doi.org/10.1007/s00299-007-0401-0
28. Nakatsuka, T., Nishihara, M., Mishiba, K.,Yamamura, S. (2006) Heterologous Expression of Two Gentian Cytochrome P450 Genes can Modulate the Intensity of Flower Pigmentation in Transgenic Tobacco Plants. Molecular Breeding 17 (2):91-99. http://dx.doi.org/10.1007/s11032-005-2520-z
29. Nakatsuka, T., Sasaki, N.,Nishihara, M. (2014) Transcriptional regulators of flavonoid biosynthesis and their application to flower color modification in Japanese gentians. Plant Biotechnol 31:389-399. http://dx.doi.org/10.5511/plantbiotechnology.14.0731a
30. Olsen, K. M., Hehn, A., Jugdé, H., Slimestad, R., Larbat, R., Bourgaud, F.,Lillo, C. (2010) Identification and characterisation of CYP75A31, a new flavonoid 3'5'-hydroxylase, isolated from *Solanum lycopersicum*. BMC plant biology 10 (1):21. http://dx.doi.org/10.1186/1471-2229-10-21
31. Passat, D. N. (2012) Interactions of black and green tea water extracts with antibiotics activity in local urinary isolated *Escherichia coli*. Journal of Al-Nahrain University 15 (3):134-142. http://dx.doi.org/10.22401/JNUS.15.3.19
32. Schoenbohm, C., Martens, S., Eder, C., Forkmann, G.,Weisshaar, B. (2000) Identification of the Arabidopsis thaliana flavonoid 3'-hydroxylase gene and functional expression of the encoded P450 enzyme. Biological chemistry 381 (8):749-753. http://dx.doi.org/10.1515/BC.2000.095
33. Seitz, C., Ameres, S.,Forkmann, G. (2007) Identification of the molecular basis for the functional difference between flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase. FEBS Lett 581 (18):3429-3434. http://dx.doi.org/10.1016/j.febslet.2007.06.045
34. Sirim, D., Wagner, F., Lisitsa, A.,Pleiss, J. (2009) The Cytochrome P450 Engineering Database: integration of biochemical properties. Bmc Biochemistry 10. http://dx.doi.org/10.1186/1471-2091-10-27
35. Stafford, H. (1974) Possible multienzyme complexes regulating the formation of C6-C3 phenolic compounds and lignins in higher plants. Recent advances in Phytochemistry 8:53-79. http://dx.doi.org/10.1016/B978-0-12-612408-8.50009-3
36. Tanaka, M., Sakai, T.,Takahata, Y. (2019) Allele dosage–dependent selection of recessive F3’H allele homozygote altered anthocyanin composition in sweetpotato. Molecular Breeding 39 (11):1-12. http://dx.doi.org/10.1007/s11032-019-1062-8

37. Tanikawa, N., Kashiwabara, T., Hokura, A., Abe, T., Shibata, M.,Nakayama, M. (2008) A peculiar yellow flower coloration of camellia using aluminum-flavonoid interaction. J Jpn Soc Hortic Sci 77 (4):402-407. http://dx.doi.org/10.2503/jjshs1.77.402
38. Thompson, J. D., Higgins, D. G.,Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22 (22):4673-4680. http://dx.doi.org/10.1093/nar/22.22.4673
39. Toda, K., Yang, D., Yamanaka, N., Watanabe, S., Harada, K.,Takahashi, R. (2002) A single-base deletion in soybean flavonoid 3'-hydroxylase gene is associated with gray pubescence color. Plant molecular biology 50 (2):187-196. http://dx.doi.org/10.1023/A:1016087221334
40. Wang, H., Wang, W., Zhang, P., Pan, Q., Zhan, J.,Huang, W. (2010) Gene transcript accumulation, tissue and subcellular localization of anthocyanidin synthase (ANS) in developing grape berries. Plant Sci 179 (1-2):103-113. http://dx.doi.org/10.1016/j.plantsci.2010.04.002
41. Wilkins, M. R., Gasteiger, E., Gooley, A. A., Herbert, B. R., Molloy, M. P., Binz, P.-A., Ou, K., Sanchez, J.-C., Bairoch, A.,Williams, K. L. (1999) High-throughput mass spectrometric discovery of protein post-translational modifications. J Mol Biol 289 (3):645-657. http://dx.doi.org/10.1006/jmbi.1999.2794
42. Yamaguchi, M., Sasaki, T., Sivaguru, M., Yamamoto, Y., Osawa, H., Ahn, S. J.,Matsumoto, H. (2005) Evidence for the plasma membrane localization of Al-activated malate transporter (ALMT1). Plant Cell Physiol 46 (5):812-816. http://dx.doi.org/10.1093/pcp/pci083
43. Yamazaki, S., Sato, K., Suhara, K., Sakaguchi, M., Mihara, K.,Omura, T. (1993) Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. The Journal of Biochemistry 114 (5):652-657. http://dx.doi.org/10.1007/s00601-013-0691-4
44. Yu, J., Mo, J., Chen, Q., Xu, H.,Liu, B. (2018) Study on HPLC Fingerprint of Polyphenols in Camellia nitidissima Chi. Guangxi Sciences 25 (6):238-244. http://dx.doi.org/10.13656/j.cnki.gxkx.20181225.012
45. Zhang, H.,Ren, S. (1998) Flora of China. Vol. 49 (Theaceae). Science Press, Beijing, China
46. Zhang, S., Guo, H., Pei, X., Li, C.,Cheng, H. (2009) Expression of flavonoid 3'-hydrolyase from Eupatorium adenophorum in tobacco. Scientia Agricultura Sinica 42 (12):4182-4186. http://dx.doi.org/10.3864/j.issn.0578-1752.2009.12.008
47. Zhou, T. S., Zhou, R., Yu, Y. B., Xiao, Y., Li, D. H., Xiao, B., Yu, O.,Yang, Y. J. (2016) Cloning and characterization of a flavonoid 3'-hydroxylase gene from tea plant (*Camellia sinensis*). International Journal of Molecular Sciences 17 (2):261. http://dx.doi.org/10.3390/ijms17020261
48. Zhou, X. (2012) Clonging and function reasearch of pigment genes from *Camallia nitidissma*. Chinese Academy of Forestry Sciences, Beijing
49. Zhou, X., Li, J., Yin, H., Fan, Z.,Li, X. (2013) Cloning of Camellia nitidissima flavonol synthase cDNA and construction of sense, RNA interference expression vectors. Bull Bot Res 33 (1):58-65. http://dx.doi.org/CNKI:SUN:MBZW.0.2013-01-009

Figures

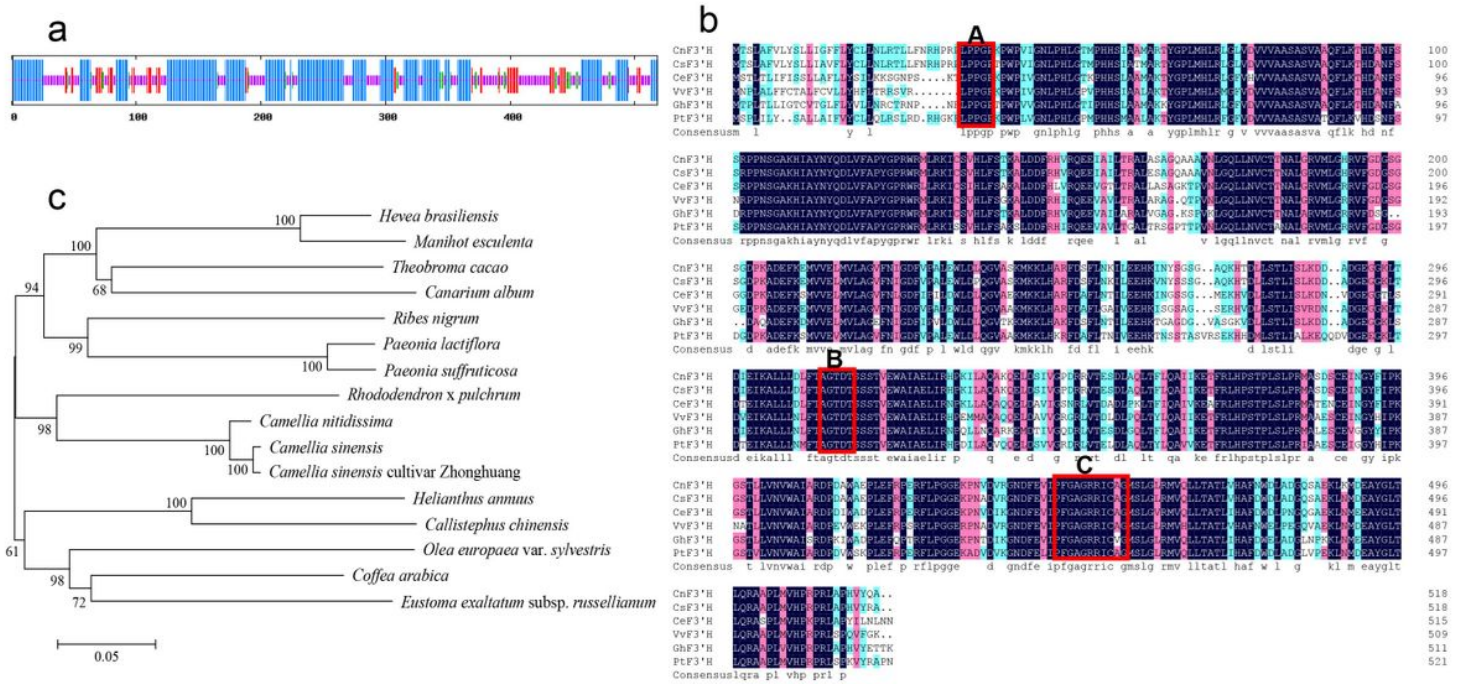

Figure 1

Amino acid alignment and phylogenetic analysis of CnF3'H a, Predicted secondary structure of the CnF3'H protein. alpha helix (blue); random coil (purple); extended strand (red); beta turn (green). CnF3'H was mainly composed of α -helix and random coil. b, Multiple sequence alignment of CnF3'H and homologous proteins. CnF3'H: Camellia nitidissima F3'H, CsF3'H: Camellia sinensis F3'H, CeF3'H: Coffea eugenioides F3'H, VvF3'H: Vitis vinifera F3'H, GhF3'H: Gerbera hybrida F3'H, PtF3'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 (PFGAGRRICAG). c, Phylogenetic tree analysis of CnF3'H. The F3'H gene evolutionary tree of the selected species was divided into 3 branches. CnF3'H was closely related to C. sinensis F3'H and C. sinensis cultivar Zhonghuang F3'H.

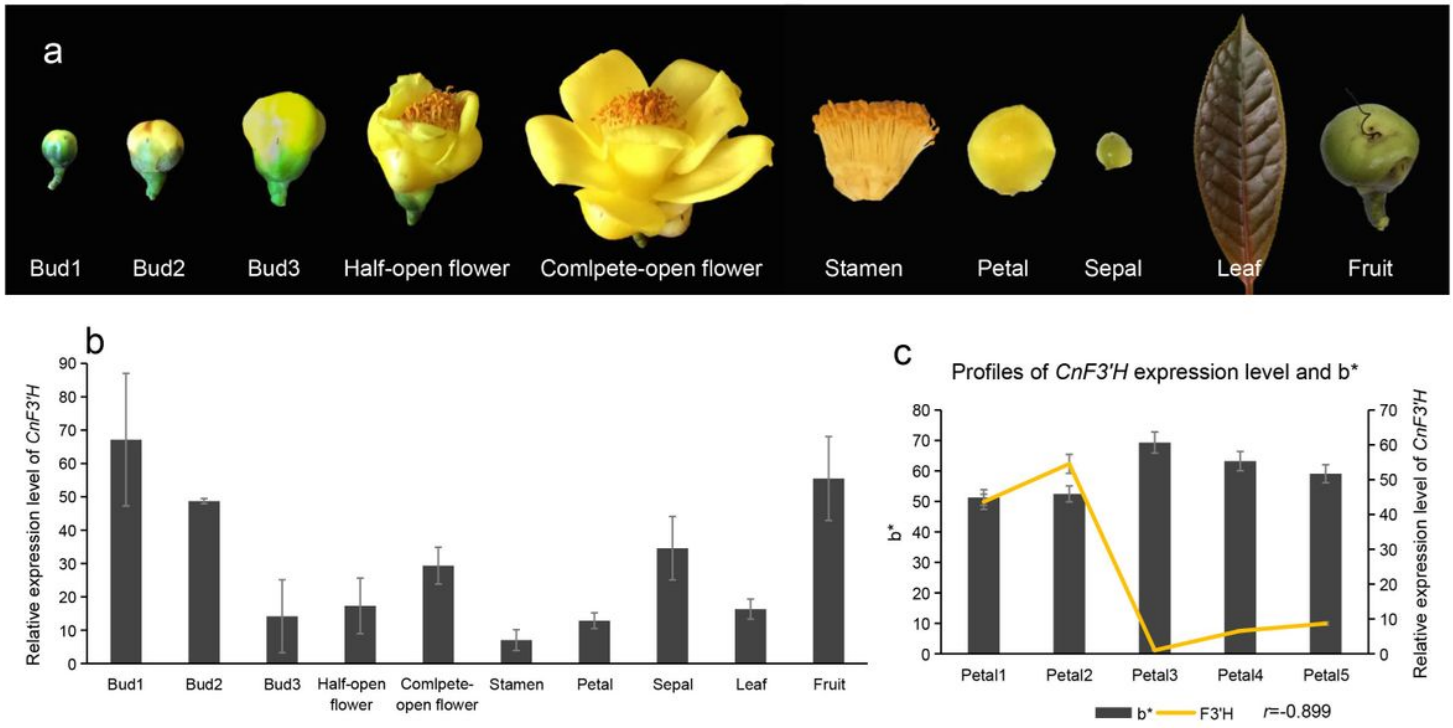

Figure 2

Relative expression level of CnF3'H in C. nitidissima a, Ten tissues of C. nitidissima. Bud1, buds 10 cm in diameter. Bud2, buds 20 cm in diameter. Bud3, buds 30 cm in diameter. b, Relative expression of CnF3'H in different C. nitidissima tissues. The highest expression was in fruits and the lowest was in stamens. Over the course of 5 flower stages, the relative expression level of CnF3'H decreased initially and then increased. c, Profiles of CnF3'H expression level and the color (b*) in petals of 5 stages. The expression level of CnF3'H was negatively correlated with the color (b*).

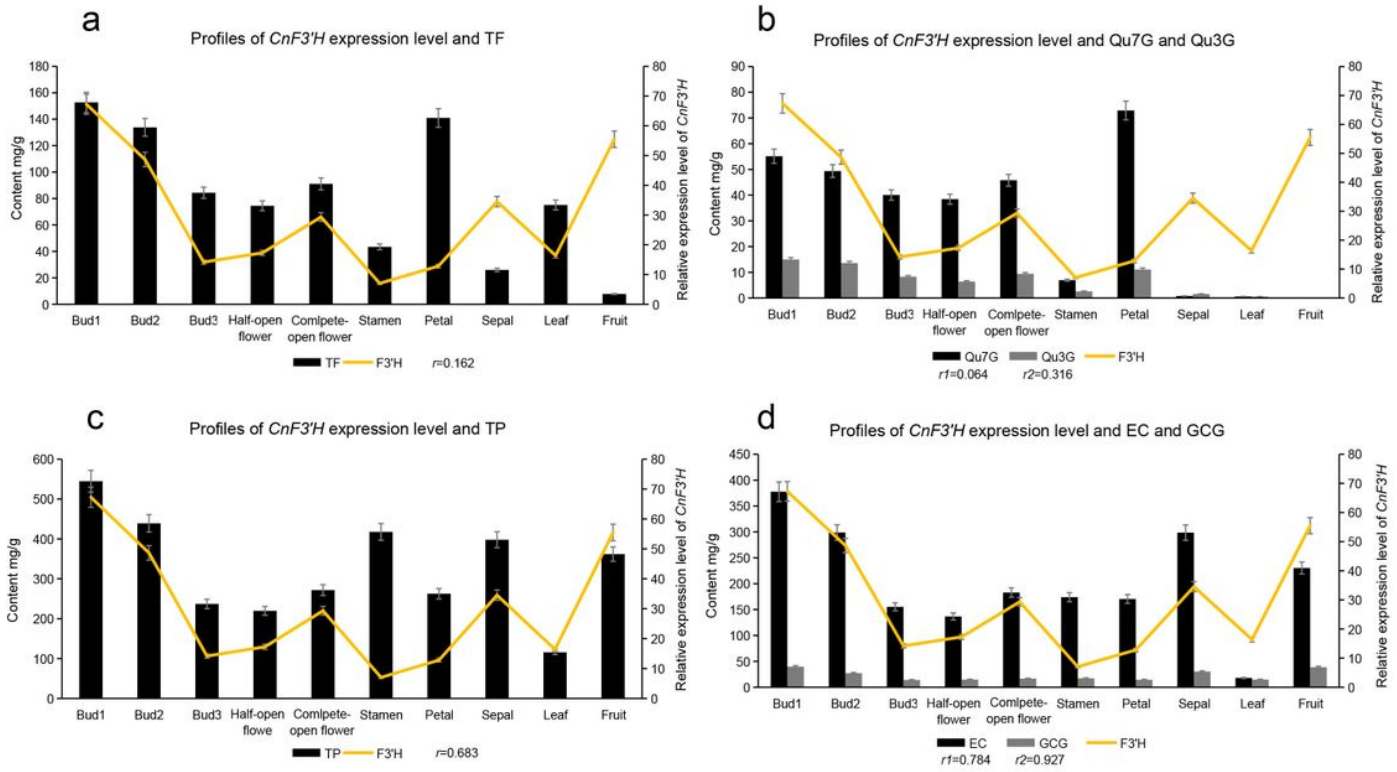

Figure 3

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 polyphenol 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.

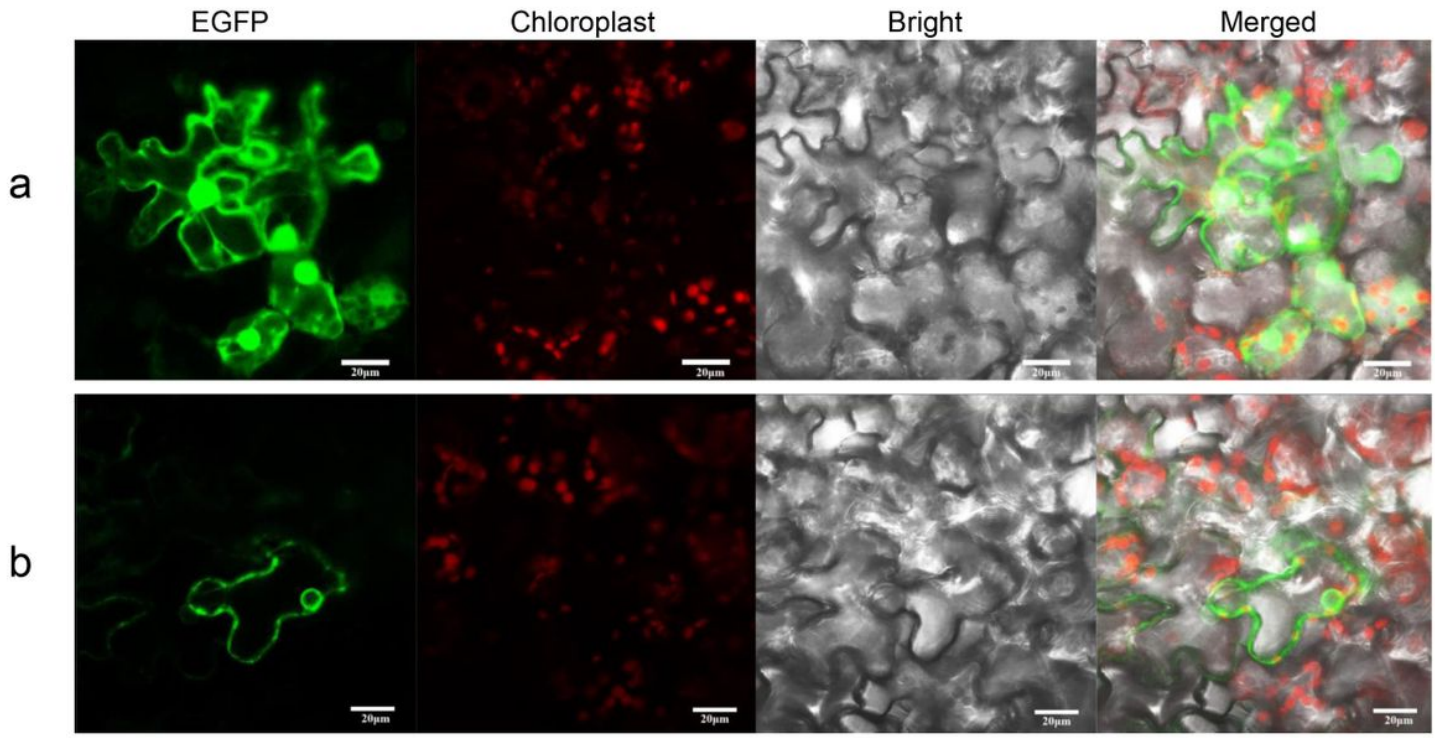

Figure 4

Subcellular localization of CnF3'H-EGFP in leaf epidermal cells of Nicotiana benthamiana a, The green fluorescence signals of the empty 35s-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.

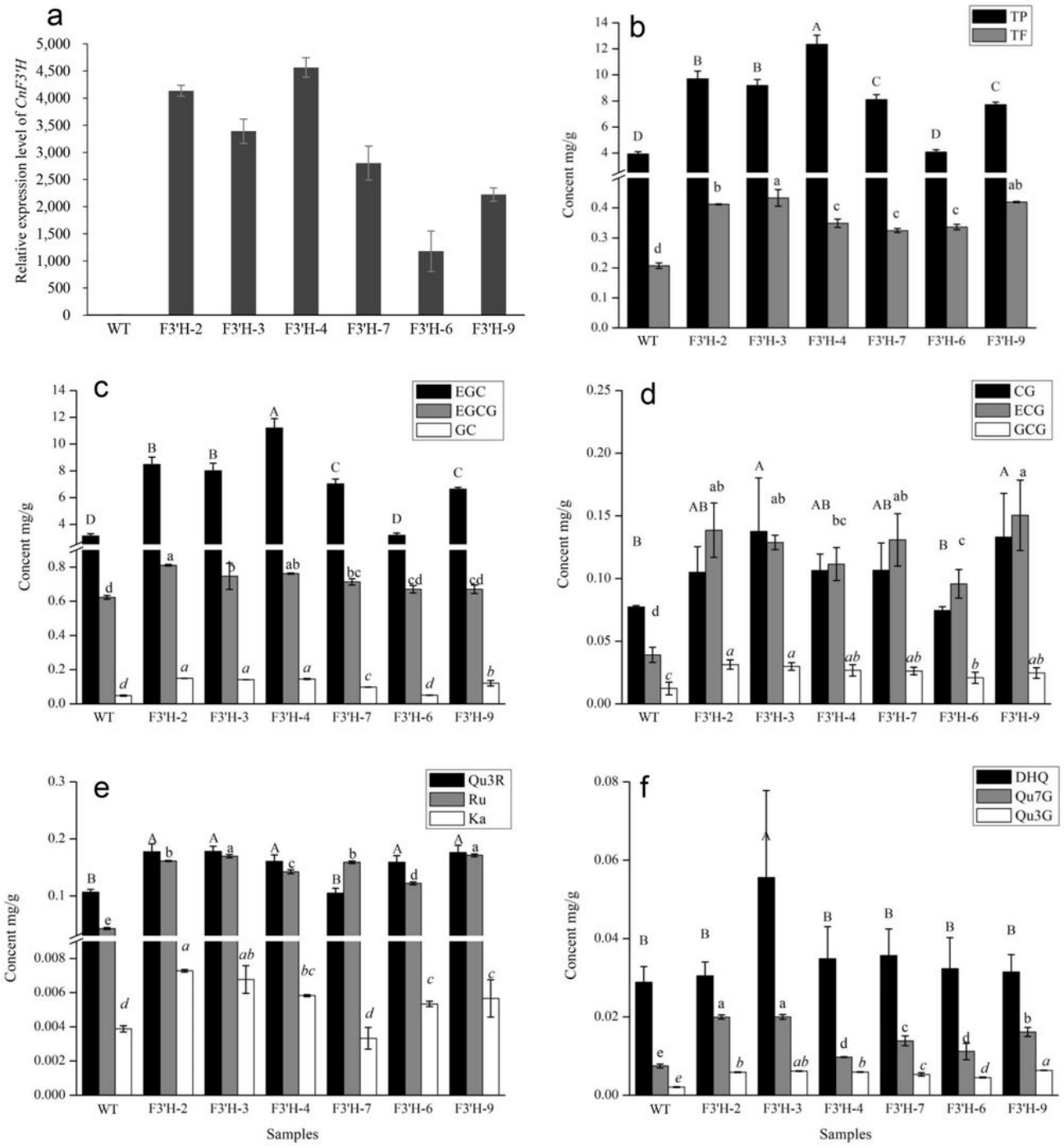

Figure 5

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 wild-type tobacco. 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 5 lines (excluding F3'H-6) were significantly higher than those in wild type tobacco. e, Contents of Qu3R (quercetin-3-O-rutinose), Ka (kaempferol) and Ru (rutin). Qu3R and Ka in the flowers of the other 5 lines (excluding F3'H-17) were significantly higher than those in wild-type tobacco. 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 wild-type tobacco and the other lines. Ru, Qu7G and Qu3G were significantly higher in the 6 positive lines than in wild-type tobacco.