An early gene of the flavonoid pathway, flavanone 3-hydroxylase, exhibits a positive relationship with the concentration of catechins in tea (Camellia sinensis)

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Summary  Tea (Camellia sinensis (L.) O. Kuntze) leaves are a major source of flavonoids that mainly belong to the flavan 3-ols or catechins. Apart from being responsible for tea quality, these compounds have medicinal properties. Flavanone 3-hydroxylase (F3H) is an abundant enzyme in tea leaves that catalyzes the stereospecific hydroxylation of (2S)-naringenin to form (2R,3R)-dihydrokaempferol. We report a full-length cDNA sequence of F3H from tea (CsF3H Accession no. AY641730). CsF3H comprised 1365 bp with an open reading frame of 1107 nt (from 43 to 1149) encoding a polypeptide of 368 amino acids. Expression of CsF3H in an expression vector in Escherichia coli yielded a functional protein with a specific activity of 32 nmol min⁻¹ mg protein⁻¹. There was a positive correlation between the concentration of catechins and CsF3H expression in leaves of different developmental stages. CsF3H expression was down-regulated in response to drought, abscisic acid and gibberellic acid treatment, but up-regulated in response to wounding. The concentration of catechins paralleled the expression data. Exposure of tea shoots to 50–100 µM catechins led to down-regulation of CsF3H expression suggesting substrate mediated feedback regulation of the gene. The strong correlation between the concentration of catechins and CsF3H expression indicates a critical role of F3H in catechin biosynthesis.

Keywords: expression analysis, F3H.

Introduction

Tea (Camellia sinensis (L.) O. Kuntze) leaves are the major source of flavonoid (FL) compounds in human nutrition. The flavan 3-ols, or catechins, are the most prominent FL compounds in tea leaves, constituting around 25–30% of the dry mass of younger tea leaves (Lin et al. 1996, Singh et al. 1999). These compounds are responsible for imparting characteristic astringency and bitterness to black tea (Chen 2002). Catechins, which is the general term for catechin, epicatechin, gallocatechin, epigallocatechin and epigallocatechin gallate, are water-soluble polyhydroxylated flavonoids possessing medicinal properties. In plants, flavonoids are categorized as secondary metabolites and are associated with an array of functions, including disease resistance. In animal systems, catechins have been implicated in preventing diseases such as cancer and neurodegenerative and other oxidative-stress-related diseases including cardiovascular diseases (Bordoni et al. 2002, Levites et al. 2002, Nie et al. 2002, Song et al. 2002, Lamberti et al. 2005).

The central pathways for flavonoid biosynthesis are highly conserved and are well characterized at the genetic, biochemical and enzymological levels in plants like Petunia hybrida hort. ex E. Vilm. and Arabidopsis thaliana (L.) Heynh. (Holton and Cornish 1995, Winkel-Shirley 2001). Flavonoids share a common 15 carbon skeleton that is formed by the condensation and decarboxylation of phenylpropanoid derivatives and malonyl-CoA units. Flavanone 3-hydroxylase (F3H), which catalyzes an early step in flavonoid metabolism leading to the formation of dihydroflavonols from flavanones (Figure 1), is classified as a soluble 2-oxoglutarate-dependent dioxygenase based on its requirements for 2-oxoglutarate, molecular oxygen, ferrous iron (Fe²⁺) and ascorbate. Flavanone 3-hydroxylase catalyzes the stereospecific hydroxylation of (2S)-naringenin and (2S)-eriodictyol to form (2R,3R)-dihydrokaempferol and (2R,3R)-dihydroquercetin, respectively (Britsch and Grisebach 1986). These dihydroflavonols serve as intermediates for the biosynthesis of flavonols, flavan 3-ols and anthocyanidins (Holton and Cornish 1995). In tea, the flavonoid pathway has been implicated in the synthesis of catechins (Mamati et al. 2006).

Punyasiri et al. (2004) reported high F3H activity in tea leaves and suggested that it is an important enzyme in the
biosynthesis of catechins in tea, however, an analysis of the gene encoding FSH was not conducted. A detailed understanding of the gene encoding F3H and its regulation is critical for metabolic engineering and to gain insight into its role in the biosynthesis of catechins. We present the first description of the full-length cloning of tea F3H (CsF3H) and demonstrate its functionality in E. coli. Expression analysis suggested that CsF3H represents a pivotal point in the regulation of the biosynthesis of catechins in tea.

Materials and methods

Plant materials and growth conditions
UPASI-10, a common China type of tea clone (Balasaravanan et al. 2003), growing in the tea garden at the Institute of Himalayan Bioresource Technology, India, was selected for study. Experiments were performed on leaves at different developmental stages including the apical bud (AB; youngest), and leaves at positions 1, 2, 3 and 4 with reference to the apical bud (hereafter 1st, 2nd, 3rd and 4th leaf, respectively). Leaves were collected between 0900 and 1000 h during the period of active growth (July, mean temperature 22.5 °C). The harvested leaves were immediately frozen in liquid nitrogen and stored at –80 °C until processed.

Estimation of catechins
Catechins were quantified as described by Singh et al. (1999). The procedure is based on the ability of diazotized aryl-amine to form colored complexes with the A-ring of catechins but not with the other polyphenolic compounds. In brief, leaves were ground in liquid nitrogen, extracted with chilled acetone (first with 100% and then with 60% acetone) and two volumes of petroleum benzene were added to the combined extract. The lower aqueous acetone layer containing catechins and other polyphenolic compounds was removed, dried and the residue dissolved in water. After reaction of the extract with freshly prepared diazotized sulfanilamide, catechins were quantified spectrophotometrically at 425 nm (λmax of the colored complex). A recovery experiment was performed with each extraction to estimate the loss of catechins, and this loss was accounted for when calculating the amount of catechins. Recovery of catechins varied between 83 and 90%. A standard curve was prepared with d-(±)-catechin.

Imposition of external cues
The flavonoid pathway is modulated by drought stress (DS), abscisic acid (ABA), gibberellic acid (GA3) and wounding in several plant systems (Furuya and Thimann 1964, Hinderer et al. 1983, Ward et al. 1989, Brignolas et al. 1995, Logemann et al. 1995, Graham and Graham 1996, Richard et al. 2000, Jeyaramraja et al. 2003, Li et al. 2003). To study the effects of external cues on the concentration of catechins and CsF3H expression, one group of 2-year-old tea plants raised in plastic pots in a plant growth chamber (temperature, 25 ± 1 °C; irradiance, 200 µmol m–2 s–1; RH, 70–80%; Saveer Biotech, India) were subjected to drought (DS) by withholding water throughout the experiment. The ABA (100 µM) and GA3 (50 µM) treatments were applied as sprays to other groups of plants and to the soil (Sharma and Kumar 2005). Apical buds and the 1st leaf were combined for the various analyses. For the wounding experiment, the margins of the 3rd leaf of another group of plants were wounded with a hemostat. The AB, 1st and 2nd leaves were too small to wound with a hemostat and hence were omitted from the wounding experiments.

Cloning of CsF3H through differential display of mRNA
Differential display (DD), a powerful technique to clone novel genes and different members of a gene family (Joshi et al. 1995, Sharma and Kumar 2005, Sharma et al. 2006), was used to clone CsF3H. Differential display was performed using total RNA isolated from control leaves and leaves subjected to DS (Day 8 of drought), GA3 (Day 8 of the treatment) and wounding (12 h after wounding) treatments. Leaves treated with ABA were omitted because DS and ABA likely modulate similar genes (Shinozaki and Yamaguchi-Shinozaki 1997). The various steps of the DD method, namely isolation of RNA, removal of contaminating DNA, cDNA synthesis, radioactive PCR using α-35PdATP, separation of PCR products on a denaturing polyacrylamide gel, elution of cDNA bands and
The open reading frame (ORF) of CsF3H was cloned into the expression vector pQE-30 U/A and transformed into M15 pREP-4 competent cells (The Qiagen, and Genetic Analyzer, Applied Biosystems) following the manufacturer’s instructions. Homology searches were conducted with the BLASTN and BLASTX algorithms (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequence was used to analyze protein families and domains with various tools available at the PROSITE database on the ExPASy proteomics server (http://ca.expasy.org/) and SMART (http://smart.embl-heidelberg.de/). Secondary structure of the deduced CsF3H protein was predicted by SOPMA (http://npsa-pbil.ibcp.fr/).

Semi-quantitative expression analysis by RT-PCR
To study gene expression, cDNA was synthesized from 2 µg of DNA-free RNA (RNA was digested with 2U amplification grade DNase I from Invitrogen, Carlsbad, CA) in the presence of 400 U reverse transcriptase Superscript II (Invitrogen) and 1.0 µg oligo (dT)12–18 (Singh et al. 2004). The resultant cDNA was used as a template for PCR. Cycling conditions were optimized to obtain amplification in the exponential phase with CsF3H (forward) 5′-GAGATGACTCGACTCGC-TGTTGAGTTT-3′ and CsF3H (reverse) 5′-TAGCCCTACA-GAACCTCCAGCAAC-3′ primers, 1 cycle of 94 °C for 3 min; 28 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s; and 1 cycle of 72 °C for 7 min. We used 26S rRNA gene primers as the internal control for expression studies (Singh et al. 2004). Gels were scanned with a gel documentation system (Alpha DigiDoc, Alpha Innotech, San Leandro, CA) and integrated density values (IDV) were calculated with AD-1000 software to determine changes in gene expression.

Substrate-mediated gene regulation
To study substrate-mediated regulation of gene expression, tea shoots (clone UPASI-10) consisting of apical bud and the three leaves closest to the apex were harvested from the field and placed in glass vials each containing 0 (control), 50 or 100 µM (±)-catechin (Sigma) for 24 and 48 h in a plant growth chamber. Apical buds and 1st leaf combined were collected at selected times and used for estimation of catechin concentration and semi-quantitative expression analysis.
Results and discussion

Cloning of functional CsF3H

The advantage of DD is that it provides an easier way of comparing transcriptomes obtained in response to various treatments than other approaches, such as analysis of subtraction suppressive libraries (Diatchenko et al. 1996). The DS, ABA and GA3 treatments decreased the concentration of catechins in tissues from the apical bud and 1st leaf combined from Day 2 of each treatment onward (Figure 2A). In contrast, wounding increased the concentration of catechins compared with the control (Figure 2B). To minimize the number of clones to be analyzed, we selected for further study only those cDNAs showing down-regulation in response to DS and GA3 and up-regulation in response to wounding.

Among the analyzed cDNAs, a 348 bp fragment (Accession no. DN976195) exhibited significant homology with previously reported F3Hs. Based on the sequence data, a full-length CsF3H was cloned by 5’ and 3’ RACE. CsF3H (Accession no. AY641730) comprised a 1365 bp full-length cDNA with an ORF of 1107 bp (from 43 to 1149). Northern analysis confirmed the transcript size was about 1.3 kb as cloned by RACE (data not shown). CsF3H showed 69–80% similarity at the amino acid level with F3Hs reported from other plant species (Figure 3). F3H catalyzes a redox reaction, and the deduced amino acids between positions 194 and 295 represent the 2-oxoglutarate-ferrous(II) oxygenase superfamily domain. Such domains are typically present at the C-terminal of the prolyl 4-hydroxylase alpha subunit (Wellmann et al. 2004). Sequence alignment analysis of F3Hs from different sources revealed conserved amino acid residues in this group of enzymes. Among the conserved residues, we identified two histidines (His218 and His276) and one aspartic acid (Asp220) as part of the putative iron-binding site, and an arginine residue (Arg286) as part of the 2-oxoglutarate binding site (Figure 3) (cf. Lukacin and Britsch 1997). The theoretical molecular mass and isoelectric point of the deduced amino acid sequence of CsF3H were 41,464.50 Da and 5.61, respectively. The pre-mass and isoelectric point of the deduced amino acid sequence (cf. Lukacin and Britsch 1997). The theoretical molecular

To confirm that the cDNA yielded a functional protein, the ORF of CsF3H was cloned into the expression vector pQE-30 U/A. The recombinant protein, eluted under non-denaturing conditions, exhibited activity in the presence of naringenin, oxoglutaric acid and ferrous sulfate, with a specific activity of 32 nmol min⁻¹ mg⁻¹ of protein. Formation of dihydrokaepferol from naringenin was demonstrated by TLC and HPLC (Figure 5).

Concentration of catechins and CsF3H expression in response to leaf age

The apical bud and leaves up to the second node position are used for tea manufacture (Jain 1999). The concentrations of catechins in the apical bud and leaves at positions 1, 2 and 3 did not differ significantly, whereas the concentration in the leaf at position 4 was significantly lower by 35%. Expression of CsF3H was similar in the apical bud and leaves at positions 1, 2 and 3, but expression was low in the leaf at position 4 (24% of that in the apical bud and 50% of that in the 3rd leaf) (Figure 6). The correlation coefficient between concentration of catechins and IDV of the amplicons had a value of 0.763, indicating a positive correlation between these parameters. Higher expression of F3H in actively growing tissues has also been reported in Citrus paradisi Macfad. and Petunia hybrid L. (Pelt et al. 2003). Such results are in agreement with earlier studies showing a positive relationship between the accumulation of the flavonoids and the activities of specific enzymes and their genes (Jaakola et al. 2002, Mamati et al. 2006).

Concentration of catechins and CsF3H expression in response to external cues

In a separate experiment, potted plants were subjected to DS, ABA, GA3 and wounding treatments, and the concentrations of catechins and CsF3H expression determined. The DS, ABA and GA3 treatments decreased the concentration of catechins in tissues of the apical bud and 1st leaf combined, starting from Day 2 and onward. By Day 8 of treatment, DS, ABA and GA3 had caused decreases of 22, 21 and 13%, respectively, in the concentrations of catechins compared with pretreatment values (Figure 2A). In contrast, wounding enhanced the concentration of catechins by 15 and 20% at 12 and 24 h after treatment, respectively, compared with control values (Figure 2B).
The concentration of catechins at 48 h after wounding had returned almost to control values. The physical condition of the wounded leaves precluded further analyses.

There was a decrease in CsF3H expression in response to the DS, ABA and GA3 treatments (Figures 7A–C), whereas up-regulation was observed in response to wounding (Figure 7D). Drought has been observed to down-regulate expression of phenylalanine ammonia-lyase (PAL) enzyme in tea (Jeyaramraja et al. 2003), and an ABA-mediated decrease in PAL activity at the molecular and biochemical levels has also been reported (Ward et al. 1989, Graham and Graham 1996). A decrease in CsF3H expression in response to DS or ABA treatment could be part of the general response of tea to stress stimuli. The role of GA3 in the regulation of plant growth and development is well known (e.g., Thomas and Sun 2004, Alvey and Harberd 2005, Swain and Singh 2005). This hormone has also been shown to inhibit the flavonoid pathway in growing and non-growing cultures of two species of *Spirodela* (Furuya and Thimann 1964) and *Pisum sativum* L. (Russel and Galston 1969), at the level of chalcone synthase (CHS) in...
Our results showed that GA$_3$ treatment resulted in the down-regulation of catechins and CsF3H transcripts, indicating that GA$_3$ has a role in the down regulation of the FL pathway at the transcriptional level.

Wounding is known to affect the FL pathway by inducing the expression of genes coding for PAL (PAL) (Schulz et al. 1989, Logemann et al. 1995), cinnamate 4-hydroxylase (C4H) (Chapple 1998), 4-coumaroyl CoA ligase (4CL) (Douglas et al. 1991) and chalcone synthetase (CHS) (Brignolas et al. 1995, Richard et al. 2000). Also, Brignolas et al. (1995) reported that the concentration of catechins increased in response to wounding in Picea abies (L.) Karst. Similarly, we found that, in tea, the concentration of catechins and CsF3H

Figure 4. (A) Predicted secondary structure of *Camellia sinensis* flavanone-3-hydroxylase (CsF3H) by SOPMA and (B) comparison with that of AtF3H. Helices, sheets, turns and coils are indicated by the longest, the second longest, the second shortest and the shortest vertical lines, respectively.

Figure 5. Formation of dihyrokaempferol (DHK) from naringenin by purified recombinant CsF3H protein as analyzed by (A) TLC (R and S represent reaction mixture and substrate, respectively) and (B) F3H enzyme activity assayed by high performance liquid chromatography: (i) pure naringenin at 290 nm and (ii) ethyl acetate extract of the enzymatic reaction mixture showing peaks for naringenin and DHK.

Figure 6. (A) Effects of leaf age on the expression of CsF3H in apical bud (AB) and the 1st, 2nd, 3rd and 4th leaf with reference to AB. Numerals below lanes indicate catechins concentration on a % (w/w) dry mass basis. Each value represents the mean ± standard deviation of three measurements. (B) Intensities (integrated density values; IDV) of the amplicon as measured with Alpha DigiDoc 1000 software. The gene encoding 26S rRNA was used as an internal control.
transcript abundance increased in response to wounding within 12 h of treatment and remained high for 24 h. Wounding-induced enhancement of catechins and other flavonoids is an adaptive response that protects against tissue damage (Brignolas et al. 1995). Correlation coefficients between the percentage increase in the concentration of catechins and the IDV of CsF3H expression in response to the DS, ABA and GA3 treatments were 0.724, 0.943 and 0.787, respectively, indicating a strong positive relationship. In contrast, in response to the wounding treatment, the correlation coefficient was only 0.137, indicating a weak positive relationship. This weak positive relationship reflects the observation that CsF3H expression was highest at 12 h after treatment, whereas the concentration of catechins continued to increase up to 24 h after wounding and then decreased to the pretreatment value.

**Catechin down regulates CsF3H expression**

Catechin, the end product of the FL pathway, was used to study substrate-mediated modulation of CsF3H expression. Catechin uptake by the cut shoot of tea was evident because the concentration in the shoot increased by 34 and 33%, after a 24-h exposure to 50 and 100 µM catechin, respectively. Thereafter, the concentration decreased compared with the 24-h treatment, but the concentrations were never less than the Day 0 control value. Expression of CsF3H was severely down-regulated in both the 50 and 100 µM catechin-treated tissues following 48 h of treatment (Figure 8A), whereas there was no change in CsF3H expression in control tissues dipped only in water (Figure 8B). These data indicate that catechin may serve as a feedback inhibitor of CsF3H expression (Britsch and Grisebach 1986, Fischer et al. 2006). Negative feedback regulation of CsF3H expression by catechins could explain the storage of catechins in the vacuole which would allow for their continuous synthesis.

![Figure 8](https://example.com/figure8.png)

**Figure 8.** (A) Effects of different concentrations (50 and 100 µM) of externally supplied catechin on expression of CsF3H at 24 and 48 h. Bar diagram indicates intensities (integrated density values; IDV) of the amplicons as measured with Alpha DigiDoc 1000 software. The gene encoding 26S rRNA was used as an internal control. (B) No change in expression of CsF3H was observed in control tea shoots treated with water.

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