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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 (2*S*)-naringenin to form (2*R*,3*R*)-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, epicatechin gallate, 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, Lambert 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 (Fe^{2+}) and ascorbate. Flavanone 3-hydroxylase catalyzes the stereospecific hydroxylation of (2*S*)-naringenin and (2*S*)-eriodictyol to form (2*R*,3*R*)-dihydrokaempferol and (2*R*,3*R*)-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

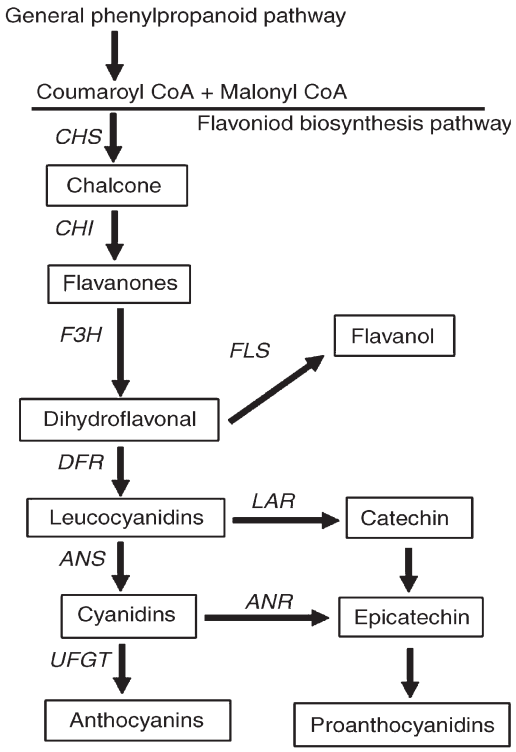

Figure 1. Flavonoid biosynthetic pathway of anthocyanins and catechins. Abbreviations: *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone-3-hydroxylase; *FLS*, flavonol synthase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase; *LAR*, leucoanthocyanidin reductase; and *ANR*, anthocyanidin reductase.

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 (λ_{max} of the colored complex). A recovery experiment was performed with each extraction to estimate the loss of catchins, 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 (GA_3) 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 GA_3 (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), GA_3 (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-^{33}PdATP, separation of PCR products on a denaturating polyacrylamide gel, elution of cDNA bands and

cloning of PCR products were performed essentially as described by Lal et al. (2001) and Sharma and Kumar (2005). The only difference was that, while performing radioactive PCR analyses, a range of arbitrary primers supplied by GenHunter Corporation (Nashville, TN) were used. The GenHunter kits used were: MessageClean to remove contaminating DNA; RNAimage for performing differential display; PCR-TRAP vector to clone PCR products after eluting and amplifying from polyacrylamide gels; and AidSeq primer set C for sequencing. *Taq* polymerase was purchased from Qiagen, and α-^{33}PdATP (specific activity, 2500 Ci mmol^{-1}) was purchased from Bhabha Atomic Research Centre, Bombay, India. We selected and analyzed only those cDNA bands that exhibited down-regulation in response to DS and GA_3 and upregulation in response to wounding compared with the control. The gene fragment showing homology with the reported *F3H* was selected to design primers for rapid amplification of cDNA ends (RACE) to clone the full-length cDNA of *CsF3H*. The primers used for 3′ RACE were GSP1 5′-GAGATGACTCGACTCGCTCGTGAGTTT-3′ and NGSP1 5′-CCGAAGAGAAGCTCCGGTTTGATATG-3′; and the primers for 5′ RACE were GSP2 5′-TAGCCTCAGACAACACCTCCAGCAACT-3′ and NGSP2 5′-CTGTAGGTCTCCGTTACAGCCCTCCAC-3′.

Plasmids were isolated with plasmid mini isolation kit (Qiagen) and sequencing was performed with BigDye terminator v 3.1 cycle sequencing mix (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 310, 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.emblheidelberg.de/). Secondary structure of the deduced CsF3H protein was predicted by SOPMA (http://npsa-pbil.ibcp.fr/).

Recombinant protein expression and purification

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 QIAexpressionist, Qiagen). The vector pQE-30 U/A allows in-frame cloning of PCR products resulting in a 6×His tag attached at the N terminal end of the recombinant protein. Directional cloning of the ORF of the gene toward the bacterial promotor and in-frame cloning with the His tag was confirmed by sequencing. Expression of the recombinant protein was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) dissolved in sterile water and added to the liquid culture. Cells were harvested at different times and expression of the recombinant proteins was checked by SDS-polyacrylamide gel electrophoresis and staining with Coomasie Brilliant Blue. The His-tagged recombinant proteins were purified by Ni-NTA column chromatography under denaturing as well as native conditions.

Measurements of enzymatic activity

The reaction mixture for F3H assay contained 50 mM Tris-HCl (pH 7.0), 429 µM naringenin, 100 nmol 2-oxoglutaric acid, 20 nmol ferrous sulfate, 2 nmol ascorbate and 60 µg enzymatic protein in a total volume of 1 ml (Latunde-Dada et al. 2001, Punyasiri et al. 2004). Various analyses were performed after a 30-min incubation at 25 °C.

Conversion of naringenin to dihydrokaempferol was confirmed by thin layer chromatography (TLC). Naringenin and dihydrokaempferol were extracted from the enzymatic reaction mixture three times with 1 ml of ethyl acetate. We used 1 mM pure naringenin as a standard and extracted flavonols were applied to a TLC plate, and the chromatogram was developed with 15% chloroform in methanol (15 ml chloroform + 85 ml methanol).

The extract in ethyl acetate was analyzed with a Shimadzu Prominence HPLC system equipped with an LC-20AT quarternary gradient pump, an SPD-M20A diode detector and a CBM-20A communication bus module. The ethyl acetate phase was dried and dissolved in 1 ml of methanol. The HPLC analysis was carried out on a reverse-phase column Zorbax (Agilent, Santa Clara, CA). Separation was performed with a linear gradient of 30:70 (v/v) acetonitrile:water for 35 min at 27 °C at a flow rate of 0.6 ml min^{-1}. Naringenin and dihydrokaempferol, which exhibited differential retention times, were monitored at 290 nm.

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′-GAGATGACTCGACTCGCTCGTGAGTTT-3′ and *CsF3H* (reverse) 5′-TAGCCTCAGACAACACCTCCAGCAACT-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 GA_3 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 GA_3 and up-regulation in response to wounding.

Among the analyzed cDNAs, a 348 bp fragment (Accession no. DN976195) exhibited significant homology with previously reported *F3H*s. 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 predicted secondary structure of CsF3H by SOPMA (Combet et al. 2000) indicated that the molecule contained 143 α-helices, 21 β-turns, 67 extended strands and 137 random coils (Figure 4), whereas the *Arabidopsis thaliana* F3H has 141 α-helices, 25 β-turns, 59 extended strands and 133 random coils. The similarity in secondary structures of CsF3H and AtF3H indicated that *CsF3H* was a functional gene (Figure 4).

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, GA_3 and wounding treatments, and the concentrations of catechins and *CsF3H* expression determined. The DS, ABA and GA_3 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 GA_3 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).

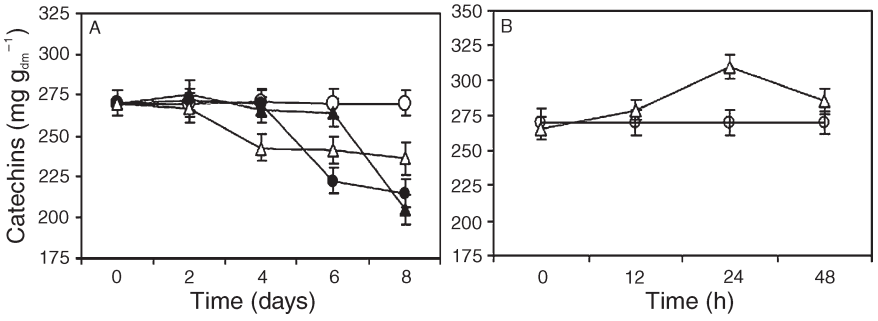

Figure 2. (A) Concentrations of catechins in response to drought (●), abscisic acid (▲), gibberellic acid (△) and control (○) treatments in tissues of the apical bud and 1st leaf combined. (B) Concentration of catechins in response to wounding of the leaf at position 3 (control, ○; and wounding, △).

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P communis          MAPATTLTSIAHEKTLQQKFVRDEDERPKVAYNDFSNEIPIISLAGIDEVEGRRAEICKK 60
M domestica         MAPATTLTSIAHEKTLQQKFVRDEDERPKVAYNDFSNEIPIISLAGIDEVEGRRGEICKK 60
C sinensis          MAPTTTLTALAEEKSLQQKFVRDEDERPKVAYNVFSNEIPVISLAGIDEIEGRRSEICRK 60
E grandiflorum      MAPS-TLTALAEEKTLQSSFIRDEDERPKVAYNQFSNEIPIISLKGLDEIDGRRAEICKK 59
V vinifera          MAPT-TLTALAGEKTLQSSFVRDEDERPKVAYNDFSNEIPVISLEGIDEVGGRRDEICRK 59
G hirsutum          MAPS-TLTALAEEKTLQASFVRDEDERPKVAYNQFSNDIPVISLAGIDDVDGKRGEICKK 59
A thaliana          MAPG-TLTELAGESKLNSKFVRDEDERPKVAYNVFSDEIPVISLAGIDDVDGKRGEICRQ 59
                    ***  *** :* *..*: .*:************ **::**:*** *:*:: *:* ***::

P communis          IVAACEDWGVFQIVDHGVDAELISEMTGLAREFFALPSEEKLRFDMSGGKKGGFIVSSHL 120
M domestica         IVAACEDWGIFQIVDHGVDAELISEMTGLAREFFALPSEEKLRFDMSGGKKGGFIVSSHL 120
C sinensis          IVEACEGWGVFQVVDHGVDANLIAEMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSHL 120
E grandiflorum      IVEACEDWGIFQVIDHGVDSDLISEMTMLAREFFALPPEEKLRFDMSGGKKGGFIVSSHL 119
V vinifera          IVEACEDWGIFQVVNHGVDSNLISEMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSHL 119
G hirsutum          IVEACEDWGVFQVVDHGVDTKLVSEMTRFAREFFALPAEEKLRFDMSGGKKGGFIVSSHL 119
A thaliana          IVEACENWGIFQVVDHGVDTNLVADMTRLARDFFALPPEDKLRFDMSGGKKGGFIVSSHL 119
                    ** ***.**:**:::****:.*:::** :**:*****.*:********************

P communis          QGEAVQDWREIVTYFSYPIRHRDYSRWPDKPEAWREVTKKYSDELMGLACKLLGVLSEAM 180
M domestica         QGEAVQDWREIVTYFSYPIRHRDYSRWPDKPEAWREVTKKYSDELMGLACKLLGVLSEAM 180
C sinensis          QGEAVQDWREIVTYFSYPIRARDYSRWPDKPEGWRAVTETYSEKLMDLACKLLEVLSEAM 180
E grandiflorum      QGEAVQDWREIMTYFSYPIKARDYSRWPDKPEAWRGVTEKYSEQLMGLACKLLEVLSEAM 179
V vinifera          QGEAVQDWREIVTYFSYPLRTRDYSRWPDKPEGWRSVTQEYSEKLMGLACKLLEVLSEAM 179
G hirsutum          QGEAVQDWREIVTYFSYPLKSRDYSRWPDKPEGWIEVTKEYSEKLMGLACKLLEVLSEAM 179
A thaliana          QGEAVQDWREIVTYFSYPVRNRDYSRWPDKPEGWVKVTEEYSERLMSLACKLLEVLSEAM 179
                    ***********:******:: ***********.*  **: **:.**.****** ******

P communis          GLDTEALTKACVDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 240
M domestica         GLDTEALTKACVDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 240
C sinensis          GLEKEALTKACVDMDQKVVINFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 240
E grandiflorum      GLEKEALTKACVDMDQKIVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 239
V vinifera          DLDKDALTNACVDMDQKVVVNFYPQCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 239
G hirsutum          GLEKEALTKACVDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 239
A thaliana          GLEKESLTNACVDMDQKIVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRD 239
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P communis          DGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATFQNPAQEAT 300
M domestica         DGKTWITVQPVEGAFVVNLGDHGHLLSNGRFKNADHQAVVNSNSSRLSIATFQNPAQEAI 300
C sinensis          GGKTWITVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNCSRLSIATFQNPAPEAT 300
E grandiflorum      GGNTWITVLPVDGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNSSRLSIATFQNPAPEAT 299
V vinifera          GGKTWITVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNHSRLSIATFQNPAPEAT 299
G hirsutum          NGKTWITVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNCSRLSIATFQNPAPDAT 299
A thaliana          NGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATFQNPAPDAT 299
                    .*:***** **:************ ***************** ************ :*

P communis          VYPLCVREGEKPILEAPITYTEMYKKKMSKDLELARLKKLAKE----QQLQD----LETA 352
M domestica         VYPLSVREGEKPILEAPITYTEMYKKKMSKDLELARLKKLAKE----QQSQD----LEKA 352
C sinensis          VYPLKIREGEKPILEEPITFADMYKRKMSKDIELAKLKKLAKEKKLLQDQQD----IEKA 356
E grandiflorum      VYPLAVREGETPVLEAPMTFAEMYRRKMSKDLELAKLKKLAKE----NELKH----VEKA 351
V vinifera          VYPLKIREGEKAVLEGPITFAEMYRRKMSKDLELARLKKLAKE----QQLQD----VEKA 351
G hirsutum          VYPLKIREGEKPILEEPITFAEMYRRKMSKDLELARLKKLAKEQ---QQLKEKEAENEKP 356
A thaliana          VYPLKVREGEKAILEEPITFAEMYKRKMGRDLELARLKKLAKE------ERD-------H 346
                    **** :****..:** *:*:::**::**.:*:***:*******       :.

P communis          KVDTKPVDDIFA 364
M domestica         KVDTKPVDDIFA 364
C sinensis          KLEIKSTDEIFA 368
E grandiflorum      KVESKPIEEIFA 363
V vinifera          KLESKPIDQILA 363
G hirsutum          KLEAKPLEEILA 368
A thaliana          KEVDKPVDQIFA 358
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Figure 3. Multiple amino acid sequence alignment of *Camellia sinensis* flavanone-3-hydroxylase (CsF3H) using ClustalW program with previously reported sequences of *Vitis vinifera* (ABM67589), *Gossypium hirsutum* (ABM64799), *Eustoma grandiflorum* (BAD34459), *Pyrus communis* (AAX89399), *Malus domestica* (Q06942) and *Arabidopsis thaliana* (AAC68584). Gaps in the amino acid sequence, identical amino acid residues, conserved substitutions and semi-conserved substitutions in all sequences used in the alignment are represented by hyphens (-), asterisks (*), colons (:) and periods (.), respectively. Gray shading indicates conserved His and Asp residues, postulated as required for ferrous ion coordination, and Arg residue of the probable binding site of 2-oxoglutarate.

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 GA_3 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 GA_3 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

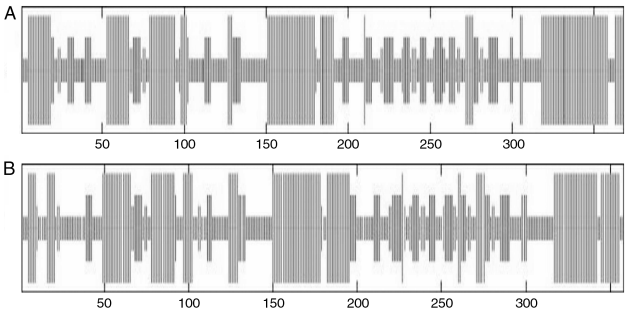

Figure 4. (A) Predicted secondary structure of *Camellia sinensis* flavanone-3-hydroxylase (CsF3H) by SOPMA and (B) comparison with that of AtF3H. Helices, sheets, turns and coils are indicated by the longest, the second longest, the second shortest and the shortest vertical lines, respectively.

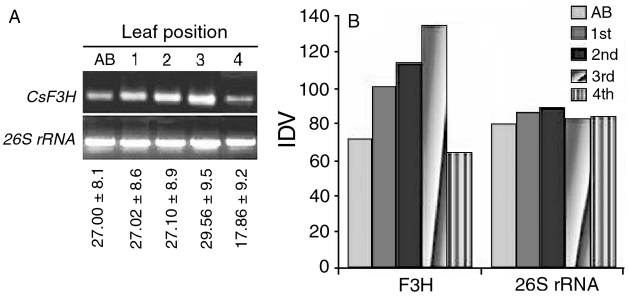

Figure 6. (A) Effects of leaf age on the expression of *CsF3H* in apical bud (AB) and the 1st, 2nd, 3rd and 4th leaf with reference to AB. Numerals below lanes indicate catechins concentration on a % (w/w) dry mass basis. Each value represents the mean ± standard deviation of three measurements. (B) 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.

carrot (*Daucus carota* L.) cell cultures (Hinderer et al. 1983), and at the level of PAL in *Myrica rubra* Lour. (Li et al. 2003). 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*

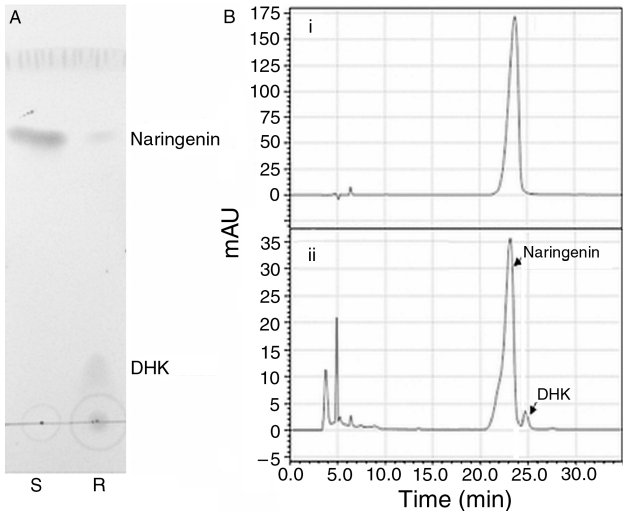

Figure 5. Formation of dihyrokaempferol (DHK) from naringenin by purified recombinant CsF3H protein as analyzed by (A) TLC (R and S represent reaction mixture and substrate, respectively) and (B) F3H enzyme activity assayed by high performance liquid chromatography: (i) pure naringenin at 290 nm and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

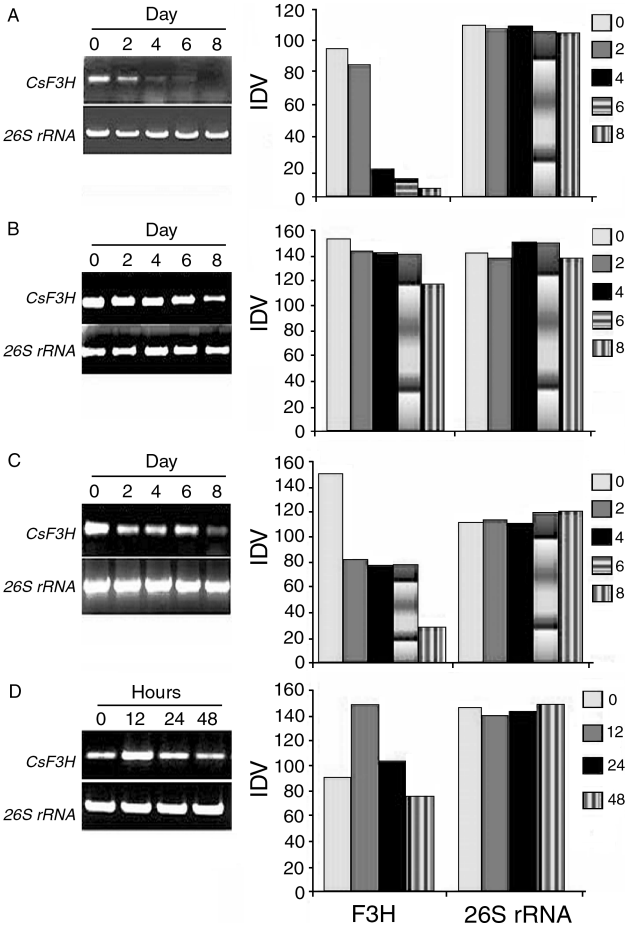

Figure 7. Effects of (A) drought, (B) ABA treatment (100 μM), (C) GA_3 treatment (50 μM) and (D) wounding on expression of *CsF3H* at different times. Bar diagram indicates intensities (integrated density values; IDV) of the amplicon as measured with Alpha DigiDoc 1000 software in response to drought, ABA treatment, GA_3 treatment and wounding. 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 GA_3 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.

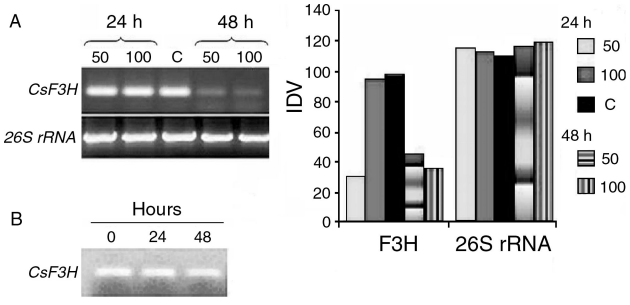

Figure 8. (A) Effects of different concentrations (50 and 100 µM) of externally supplied catechin on expression of *CsF3H* at 24 and 48 h. Bar diagram indicates intensities (integrated density values; IDV) of the amplicons as measured with Alpha DigiDoc 1000 software. The gene encoding 26S rRNA was used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

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