Plant Biotechnol Rep (2009) 3:95–101
DOI 10.1007/s11816-008-0079-y

ORIGINAL ARTICLE

Characterization of dihydroflavonol 4-reductase cDNA in tea [*Camellia sinensis* (L.) O. Kuntze]

Kashmir Singh · Sanjay Kumar · Sudesh Kumar Yadav · Paramvir Singh Ahuja

Received: 19 March 2008 / Accepted: 17 November 2008 / Published online: 26 December 2008

Abstract Tea leaves are major source of catechins—antioxidant flavonoids. Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219) is one of the important enzymes that catalyzes the reduction of dihydroflavonols to leucoanthocyanins, a key "late" step in the biosynthesis of catechins. This manuscript reports characterization of DFR from tea (*CsDFR*) that comprised 1,413 bp full-length cDNA with ORF of 1,044 bp (115–1,158) and encoding a protein of 347 amino acids. Sequence comparison of *CsDFR* with earlier reported DFR sequences in a database indicated conservation of 69–87% among amino acid residues. In silico analysis revealed CsDFR to be a membrane-localized protein with a domain (between 16 and 218 amino acids) resembling the NAD-dependent epimerase/dehydratase family. The theoretical molecular weight and isoelectric point of the deduced amino sequence of *CsDFR* were 38.67 kDa and 6.22, respectively. Upon expression of *CsDFR* in *E. coli*, recombinant protein was found to be functional and showed specific activity of 42.85 nmol min^{-1} mg $protein^{-1}$. Expression of *CsDFR* was maximum in younger rather than older leaves. Expression was down-regulated in response to drought stress and abscisic acid, unaffected by gibberellic acid treatment, but up-regulated in response to wounding, with concomitant modulation of catechins content. This is the first report of functionality of recombinant *CsDFR* and its expression in tea.

Keywords Dihydroflavonol 4-reductase · Gene expression · Tea · Catechins · In-vitro activity

Abbreviations

DFR	Dihydroflavonol 4-reductase
DS	Drought stress
ABA	Abscisic acid
GA_3	Gibberellic acid
CsDFR	*Camellia sinensis* DFR
MtDFR	*Medicago truncatula* DFR

Introduction

The enzyme dihydroflavonol 4-reductase (DFR) catalyzes the stereospecific reduction of dihydroflavonols to leucoanthocyanidins (flavan-3,4-diol) using NADPH as a cofactor (Kristiansen and Rohde 1991; Martens et al. 2002). Depending upon the plant system, leucoanthocyanidins can be utilized for the synthesis of compounds such as flavan-3-ol [(+)-catechin] (Stafford 1990) as the likely start unit to oligomeric proanthocyanidins (Fig. 1). Catechins and proanthocyanidins are involved in plant resistance and are regarded as potential health-protecting compounds in food and feed (Harborne and Williams 2000).

DFR genes have been isolated from several higher plants (Kristiansen and Rohde 1991; Sparvoli et al. 1994; Rosati et al. 1997) and recombinant proteins produced from them have been characterized (Peters and Constabel 2002; Fischer et al. 2003). Knowledge of DFR is important to

K. Singh · S. Kumar (✉) · S. K. Yadav · P. S. Ahuja
Biotechnology Division, Institute of Himalayan Bioresource Technology, P.O. Box No. 6, Palampur 176061, Himachal Pradesh, India
e-mail: sanjayplp@rediffmail.com

Present Address:
K. Singh
Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Miedzychodzka 5, 60-371 Poznan, Poland

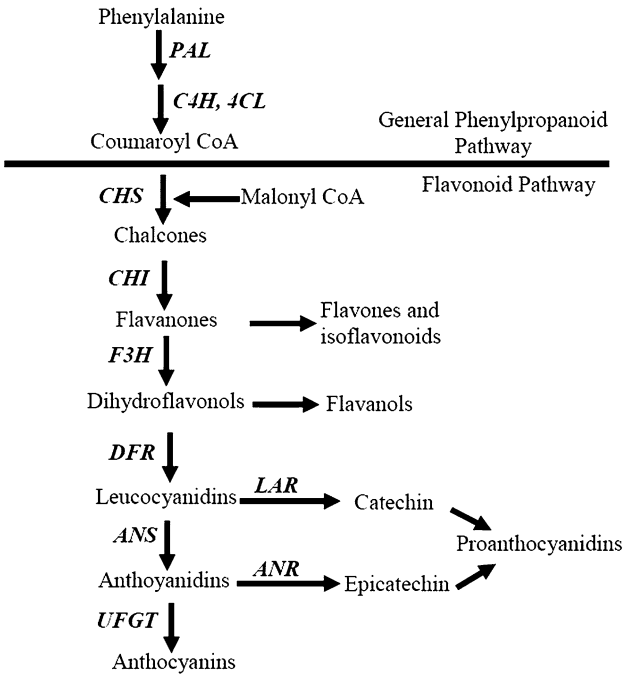

Fig. 1 Flavonoid pathway leading to biosynthesis of catechins. *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone-3-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase; *LAR*, leucoanthocyanidin reductase; *ANR*, anthocyanidin reductase

understand aspects of flavonoid biosynthesis, especially how plants regulate condensed tannins and composition and different stereochemical features of flavan-3-ols and related compounds. DFR is regarded as central enzyme of the flavonoid (FL) pathway (Fig. 1) and opens possibilities for metabolic engineering of the pathway (Forkmann and Martens 2001).

Tea is one of the most popular beverages in the world owing to its taste, stimulative effect, and health benefits. Tea (*Camellia sinensis* (L.) O. Kuntze) leaves contain extraordinary large amounts of catechins, synthesized via the flavonoid biosynthetic pathway and are responsible for imparting characteristic astringency and bitterness to black tea (Chen 2002). The amount of catechins in young leaves of tea is approximately 25–30% on a dry weight basis (Singh et al. 1999). These are water soluble polyhydroxylated flavonoids with immense medicinal properties. In animal systems, including humans, catechins are reported to prevent cancer, cardiovascular, neurodegenerative, and oxidative stress-related diseases (Bordoni et al. 2002; Levites et al. 2002; Nie et al. 2002).

A cDNA encoding *DFR* from tea has been reported (Accession number AB018685), but functionality was not evaluated and the gene was not characterized. In this work we characterized cDNA encoding DFR (*CsDFR*) from tea and showed its functionality in *E. coli*. Further, its expression in response to developmental stages and various external stimuli suggested its importance in the biosynthesis of catechins.

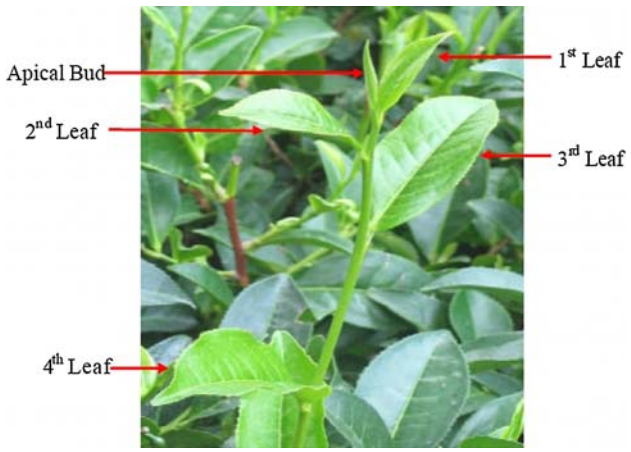

Fig. 2 Tea shoot showing leaves at different stages of development. Apical bud represents the early stage of development; leaves get gradually older from apical bud to 4th leaf

Materials and methods

Plant materials and growth conditions

This study was conducted on tea clone UPASI-10, a chinary type of tea clone (Balasaravanan et al. 2003) growing well in the tea garden of the Institute (latitude 32°06′32″N, longitude 76°33′43″E and altitude 1,300 m above mean sea level). Various experiments were performed on apical buds (AB; youngest) and on leaves at the 1st, 2nd, 3rd, and 4th positions (with reference to the apical bud). Leaves at these node positions were termed as AB, 1st, 2nd, 3rd and 4th leaf (Fig. 2). Leaves were collected during the period of active growth (July, average temperature 22.5°C). These were harvested between 9 and 10 a.m., stored immediately in liquid nitrogen, and later transferred to a −80°C freezer till further use.

Imposition of external cues

Two-year-old tea plants raised in plastic pots were housed in a plant-growth chamber (temperature 25 ± 1°C, light intensity 200 μE m^{-2} s^{-1}, RH 70–80%; Saveer Biotech, India). Drought stress (DS) was created by withholding water during the entire period of experimentation. Abscisic acid (ABA, 100 μM) and gibberellic acid (GA_3, 50 μM) were sprayed separately and also applied to the soil (Sharma and Kumar 2005). AB and the 1st leaf combined were harvested and used for various analyses. For wounding experiments, margins of third leaf were

wounded by use of a haemostat and used for various analyses. AB, and 1st and 2nd leaves were too small for creating a wound and collection of the sample.

Estimation of catechins

Catechins were quantified based on the ability of diazotized arylamine to form colored complexes with the A-ring of the catechins and not with the other polyphenolic compounds (Singh et al. 1999). Leaf tissues were ground in liquid nitrogen, extracted with chilled acetone (initially 100% and later 60%), and the combined extract was partitioned with twice the volume of petroleum benzene (*v*/*v*). The lower, aqueous acetone, layer containing catechins and other polyphenolic compounds was removed, dried, and the residue was dissolved in water to quantify catechins at A_{425} using the freshly prepared diazotized sulfanilamide. A recovery experiment was always performed to estimate the loss, and the data were taken into account when expressing the amount of catechins. Recovery of catechins varied between 83 and 90%. Analysis was repeated thrice for each sample. A standard curve for the catechins was prepared using D-(±)-catechin as standard.

Cloning of *CsDFR*

RNA was isolated from AB and 1st leaf combined using a modified guanidine hydrochloride-based method (Lal et al. 2001). Complementary DNA was synthesized using RNA preparations (2 µg) in the presence of 1.0 µg oligo(dT)$^{12-18}$ and 400 U of reverse transcriptase Superscript II (Invitrogen) after digesting with 2 U DNase I (amplification grade; Invitrogen, USA) as described earlier (Singh et al. 2004).

A primer pair, forward; 5′-GAATCCCAATCGCAAC CATATC-3′ and reverse; 5′-CGAAGAAACTCAAATTC GAGC-3′, was designed from the tea DFR sequence available in the Genbank database vide accession number AB018685. PCR was performed using 1 µL cDNA template, 0.2 µM each of forward and reverse primers, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase, and 1 × PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$) in a final volume of 25 µL. PCR was carried out on a programmable thermocycler (GeneAmp PCR system 9700; Applied Biosystems, USA) using the following cycling conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 54°C for 40 s, and 72°C for 2 min and then a final extension at 72°C for 7 min. The amplicon was cloned in pGEM-T easy vector (Promega, USA), plasmid was isolated using the Qiagen plasmid mini-isolation kit, and sequencing was performed using the BigDye terminator (version 3.1) cycle sequencing mix (Applied Biosystems) on an automated DNA sequencer (ABI Prism 310, Genetic Analyzer, Applied Biosystems). Analysis of nucleotide and deduced amino acid sequences were analyzed using various tools available at http://www.ncbi.nlm.nih.gov/, http://ca.expasy.org/, http://smart.embl-heidelberg.de/ and http://npsa-pbil.ibcp.fr/.

Recombinant protein expression, purification and measurements of enzyme activity

A primer pair (forward 5′-ATGAAAGACTCTGTT GCTTCTGCC-3′ and reverse 5′-TTAAACCTTGTTGCC ATTGACAGGG-3′) was designed to amplify the ORF of *CsDFR*, cloned into expression vector pQE-30 UA, and transformed into M15 pREP-4 competent cells (The QIAexpressionist, Germany). This vector allows in-frame cloning of PCR products resulting in a 6 × His tag attached at the N terminal end of the recombinant protein. The 6 × His tag amino acids resulted from vector sequences will increase the molecular weight of expressed protein by about 2.2 kDa. In-frame cloning of ORF of the gene with respect to the bacterial promoter was confirmed by sequencing. Expression of the recombinant protein was induced by 1 mM IPTG dissolved in sterile water and added to the liquid cell culture. Cells were harvested at different times and expression of the recombinant proteins was monitored on a SDS–polyacrylamide gel stained with Coomasie brilliant blue. The 6 × His tagged recombinant proteins were purified using Ni-NTA columns under denaturing and native conditions. The manufacturer's instructions were followed wherever necessary. DFR activity was measured according to Xie et al. (2004) with some modifications. The total reaction mixture of 1 ml contained 50 mM Tris–HCl (pH 7.0), 4 mM NADPH, 100 µM taxifolin (dihydroquercetin), and 32 µg protein samples. NADPH was added at the end and its oxidation was observed at 340 nm at 25°C for 30 min. The enzyme activity was calculated by using the extinction coefficient of NADPH, 6.22 mM^{-1} cm^{-1}.

Semi-quantitative expression analysis by RT-PCR

To study gene expression, cDNA was synthesized using RNA isolated from the different samples under examination. The resulting cDNA was used as template for PCR reaction using *CsDFR* primers (forward) 5′-GCTGAC ACGAACTTGACAC-3′, (reverse) 5′-CCACCCCACCAT ACAAAAAC-3′, and PCR conditions 94°C for 3 min for 1 cycle → 94°C for 30 s, 55°C for 40 s, 72°C for 1 min for 30 cycles → 72°C for 7 min. *26S rRNA*-based gene primers were used as internal control for expression studies (Singh et al. 2004).

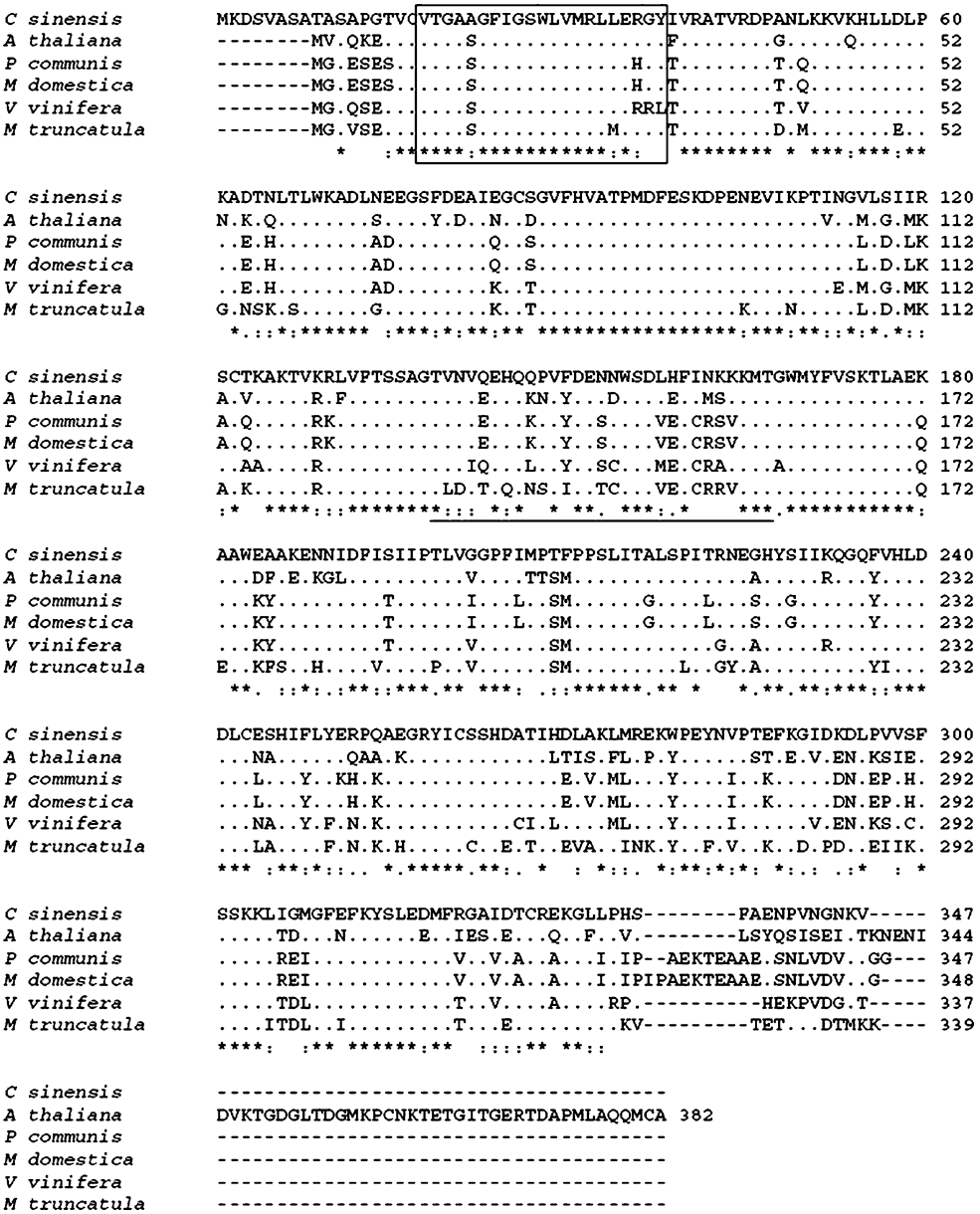

Fig. 3 Comparison of the amino acid sequences of DFRs using reported DFR sequences of *Medicago truncatula* (AAR27015), *Pyrus communis* (AAO39818), *Malus domestica* (AAD26204), *Vitis vinifera* (P51110), and *Arabidopsis thaliana* (BAD95233). *Gaps* are represented as *dashes*; *asterisks*, *colons* and *dots* indicate identical amino acid residues, conserved substitutions, and semi-conserved substitutions, respectively, in all sequences used in the alignment. The *boxed* region is a putative NADPH-binding region (Lacombe et al. 1997). The region predicted to be related to substrate specificity (Johnson et al. 2001) is *underlined*

Results and discussion

Characterization and functional validation of *CsDFR*

PCR amplified *CsDFR* (Accession number AY648027) comprised of 1,413 bp full length cDNA with ORF of 1,044 bp, starting from 115 to 1,158 encoding a protein of 347 amino acids. Northern analysis confirmed the transcript size to be approximately 1.3 kb as cloned through PCR (data not shown). Sequence comparison of deduced amino acid sequences of *CsDFR* with reported DFR sequences indicated 69–87% conservation of amino acid residues (Fig. 3). SMART domain analysis indicated amino acids at positions 16–218 had a domain resembling that of the NAD-dependent epimerase/dehydratase family. This family of proteins utilize nucleotide sugar substrates for a variety of chemical reactions involving NAD as a cofactor. The theoretical molecular weight and isoelectric point of the deduced amino sequence of *CsDFR* was 38.67 kDa and 6.22, respectively. Hydropathy analysis (Kyte and Doolittle 1982) predicted that the *CsDFR* protein contained seven putative transmembrane segments (TMs) connected by hydrophilic loops. Prediction of transmembrane domains by use of the transmembrane hidden Markov model (TMHMM) program (http://genome.cbs.dtu.dk/services/TMHMM/) suggested that the N and C hydrophilic termini were located inside the cellular compartment. This membrane topology is characteristic of many transporter proteins found in both prokaryotes and eukaryotes (Henderson 1993).The prediction of secondary

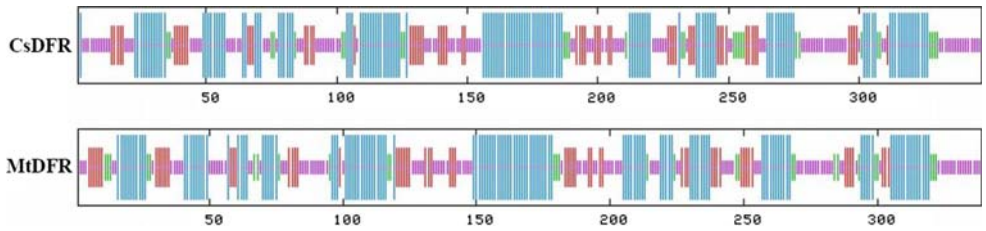

Fig. 4 Predicted secondary structure of deduced amino acid sequence of DFR from tea (*CsDFR*) by SOPMA and comparison with that of *Medicago truncatula* (*MtDFR*). *Helices*, *sheets*, *turns*, and *coils* are indicated by the *longest*, *second longest*, *second shortest*, and *shortest vertical lines*, respectively

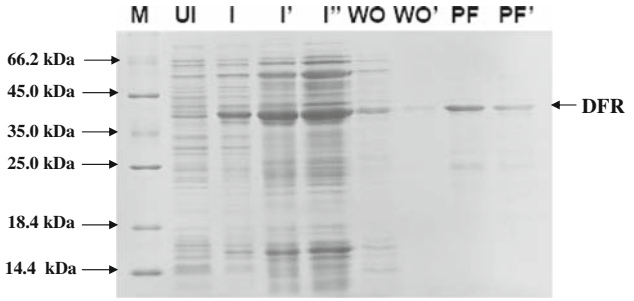

Fig. 5 Recombinant DFR protein expression and purification. *M*, molecular weight marker; *UI*, uninduced fraction; *I*, IPTG induced for 2 h; *I′*, IPTG induced for 4 h; *I″*, IPTG induced for 6 h; *WO*, first washout; *WO′*, second washout; *PF*, 1st purified fraction; *PF′*, second purified fraction

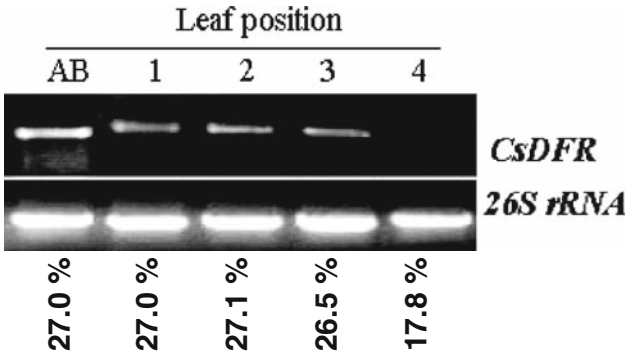

Fig. 6 Effect of leaf age on expression of *CsDFR* in the apical bud (AB) and 1st, 2nd, 3rd, and 4th leaves. Different node positions are with respect to AB. Catechin content in % g m^{-1} dry weight is indicated below each lane

structure by SOPMA (Combet et al. 2000) indicated that the deduced CsDFR contained 135 α-helices, 29 β-turns, 61 extended strands, and 122 random coils (Fig. 4). A similar secondary structure for DFR has been documented for *Medicago truncatula* (Xie et al. 2004) that comprised of 134 α-helices, 28 β-turns, 61 extended strands, and 116 random coils. Such a similar secondary structure of CsDFR with functional MtDFR suggested *CsDFR* could be a functional gene (Fig. 4).

In order to confirm if the cDNA yielded a functional protein, the entire coding region of the *CsDFR* was amplified, cloned into expression vector pQE-30 UA and expression was induced with 1 mM IPTG (Fig. 5). Recombinant protein was purified using Ni-NTA columns and PAGE analysis indicated protein size to be approximately 41 kDa, as expected from the molecular weight of DFR protein (38.67 kDa) and fusion proteins of the vector (2.2 kDa). The specific activity of the purified protein was found to be 42.85 ± 0.15 nmol min^{-1} mg^{-1} protein and was comparable with the in-vitro DFR activity reported by Xie et al. (2004).

Expression of *CsDFR*, as influenced by leaf age, across different tea clones and environmental cues

Catechin content did not show significant differences starting from apical buds to the 3rd leaf, but decreased significantly in the 4th leaf. Expression level of *CsDFR* also showed the similar pattern (Fig. 6). Earlier studies have also shown that expression of DFR was highest in young seeds and flowers, consistent with accumulation of condensed tannins and leucoanthocyanidins in these tissues (Jaakola et al. 2002; Xie et al. 2004).

External cues DS, ABA, and GA_3 treatment reduced catechins in buds and the 1st leaf combined, starting from day 2 and onwards (Fig. 7a–c). Wounding, however, enhanced the catechin content at 12 and 24 h of treatment, compared with the control (Fig. 7d). The catechin content at 48 h of treatment was almost similar to control values. The physical conditions of the wounded leaves did not permit further experimentation. The results were reproducible with three biological samples.

There was a decrease in the expression of *CsDFR* in response to DS and ABA, and it was unaffected by GA_3 treatment (Fig. 7a–c). However, up-regulation was obtained in response to wounding (Fig. 7d). DS has been found to down-regulate expression of phenylalanine ammonia-lyase (PAL) enzyme in tea (Jeyaramraja et al. 2003). ABA also led to a decrease in the activity of PAL at both molecular and biochemical levels (Ward et al. 1989; Graham and Graham 1996). A decrease in expression level of *CsDFR* in response to DS or ABA treatment could be part of the general response of tea to these stresses. GA_3

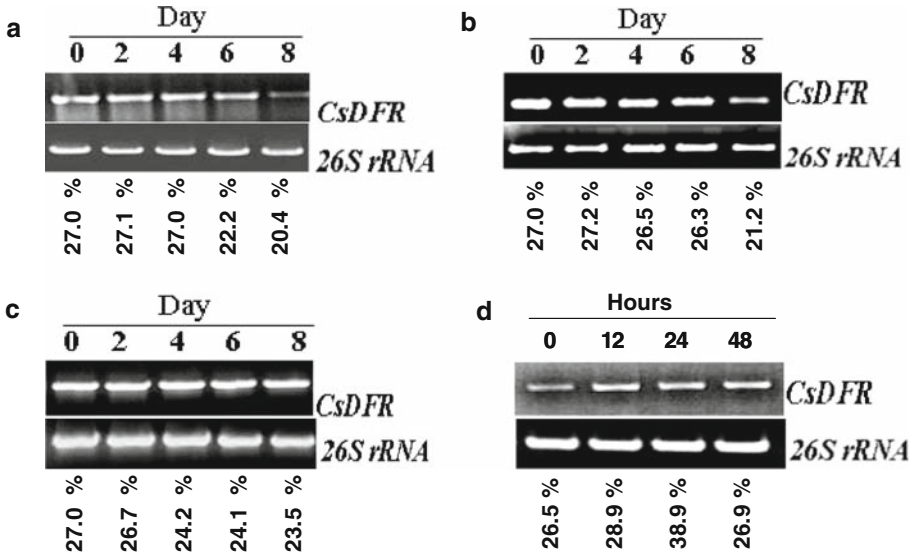

Fig. 7 Effect of **a** drought stress (DS), **b** ABA treatment (100 μM), **c** GA_3 treatment (50 μM), and **d** wounding on expression of *CsDFR* at different times. Catechin content in % g m^{-1} dry weight is indicated below each lane

has been shown to inhibit the flavonoid pathway in growing and non-growing cultures of two species of *Spirodela* sp. (Furuya and Thimann 1964) and pea (Russel and Galston 1969), at the level of chalcone synthase (CHS) in carrot (*Daucus carota*) cell cultures (Hinderer et al. 1983), and at the level of PAL in bayberry (*Myrica rubra* Bieb) (Li et al. 2003). In tea plant, GA_3 treatment up-regulated the expression of *QM like protein* (Singh et al. 2008a) and *Histone H3* (Singh et al. 2008b) transcripts but down-regulated the expression of *F3H* gene (Singh et al. 2008c). However, *CsDFR* transcripts remain unaffected but catechin content decreased with GA_3 treatment. Low catechin content might be because of down-regulation of genes next to DFR, for example *anthocyanidin synthase, leucocyandin reductase*, and *anthocyanidin reductase*.

Wounding is known to affect the flavonoid pathway by inducing *PAL* (Schulz et al. 1989; Logemann et al. 1995), *cinnamate 4-hydroxylase* (Chapple 1998), *4-coumaroyl CoA ligase* (Douglas et al. 1991), and *CHS* expression (Brignolas et al. 1995; Richard et al. 2000). Also, the catechin content was found to be increased in response to wounding in *Picea abies* (Brignolas et al. 1995). Tea also behaved the same way in that the catechin content and the transcripts of *CsDFR* increased in response to wounding at 12 h of treatment and remained at that level until 24 h. Wounding-induced enhancement of catechin content and other flavonoids could be an adaptive response to protect against tissue damage (Brignolas et al. 1995). Catechin content, however, decreased thereafter, but expression of *CsDFR* was not affected. The noted decrease in catechin content at 48 h of the treatment, compared with at 24 h of the treatment, could be because of either oxidation and/or polymerization of catechins, e.g. by polyphenol oxidase.

Acknowledgments The authors are grateful to the Council of Scientific and Industrial Research (CSIR), India, for funding this research work under the New Millennium Indian Technology Leadership Initiative (NMITLI) program entitled "Using functional genomics in plants: development and use of technologies for gene discovery and expression modulation—niche pathway engineering in tea". K. Singh is thankful to CSIR for awarding junior and senior research fellowships.

References

Balasaravanan T, Pius PK, Raj Kumar R, Muraleedharan N, Shasany AK (2003) Genetic diversity among south Indian tea germplasm (*Camellia sinensis*, *C. assamica* and *C. assamica* spp. *lasiocalyx*) using AFLP markers. Plant Sci 165:365–372

Bordoni A, Hrelia S, Angeloni C, Giordano E, Guarnieri C, Caldarera CM, Biagi PL (2002) Green tea protection of hypoxia/reoxygenation injury in cultured cardiac cells. J Nutr Biochem 13:103–111

Brignolas F, Lacroix B, Lieutier F, Sauvard D, Drouet A, Claudot AC, Yart A, Berryman AA, Christiansen E (1995) Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. Plant Physiol 109:821–827

Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu Rev Plant Physiol Plant Mol Biol 49:311–343

Chen M (2002) Tea and health—an overview. In: Zhen Y, Chen Z, Cheng S, Chen M (eds) Tea. Bioactivity and therapeutic potential. Taylor Francis, London, pp 1–16

Combet C, Blanchet C, Geourjon C, Deléage G (2000) NPS@: network protein sequence analysis. Trends Biochem Sci 25:147–150

Douglas CJ, Hauffe KD, Ites-Morales ME, Ellard M, Paszkowski U, Hahlbrock K, Dangl JL (1991) Exonic sequences are required for elicitor and light activation of a plant defense gene, but promoter sequences are sufficient for tissue specific expression. EMBO J 10:1767–1775

Fischer TC, Halbwirth H, Meisel B, Stich K, Forkmann G (2003) Molecular cloning, substrate specificity of the functionally expressed *dihydroflavonol 4-reductases* from *Malus domestica*

and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. Arch Biochem Biophys 412:223–230
Forkmann G, Martens S (2001) Metabolic engineering and applications of flavonoids. Curr Opin Biotechnol 12:155–160
Furuya M, Thimann KV (1964) The biogenesis of anthocyanins. XI. Effect of gibberellic acid in two species of Spirodela. Arch Biochem Biophys 108:109–116
Graham TL, Graham MY (1996) Signaling in soybean phenylpropanoid responses. Dissection of primary, secondary, and conditioning effects of light, wounding, and elicitor treatments. Plant Physiol 110:1123–1133
Harborne JB, Williams C (2000) Advances in flavonoid research since 1992. Phytochemistry 55:481–504
Henderson PJF (1993) The 12-transmembrane helix transporters. Curr Opin Cell Biol 5:708–751
Hinderer W, Peterson M, Seitz HU (1983) Inhibition of flavonoid biosynthesis by gibberellic acid in cell suspension cultures of *Daucus carota*. Planta 160:544–549
Jaakola L, Maatta K, Pirttila AM, Torronen R, Karenlampi S, Hahtola A (2002) Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonols levels during bilberry fruit development. Plant Physiol 130:729–739
Jeyaramraja PR, Pius PK, Kumar RR, Jayakumar D (2003) Soil moisture stress-induced alterations in bioconstituents determining tea quality. J Sci Food Agric 83:1187–1191
Johnson ET, Ryu S, Yi H, Shin B, Cheong H, Choi G (2001) Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. Plant J 25:325–333
Kristiansen KN, Rohde W (1991) Structure of the *Hordeum vulgare* gene encoding dihydroflavonol-4-reductase and molecular analysis of *ant*18 mutants blocked in flavonoid synthesis. Mol Gen Genet 230:49–59
Kyte J, Doolittle R (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132
Lacombe E, Hawkins S, Doorsselaere JV, Piquemal J, Goffner D, Poeydomenge O, Boudet AM, Grima-Pattenati J (1997) Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationship. Plant J 11:429–441
Lal L, Sahoo R, Gupta RK, Sharma P, Kumar S (2001) RNA isolation from high-phenolic tea leaves and apical buds. Plant Mol Biol Rep 19:181a–181f
Levites Y, Youdim MB, Maor G, Mandel S (2002) Attenuation of 6-hydroxydopamine (6-OHDA)-induced nuclear factorkappa B (NF-kappa B) activation and cell death by tea extracts in neuronal cultures. Biochem Pharmacol 63:21–29
Li X, Li S, Lin J (2003) Effect of GA_3 spraying on lignin and auxin contents and the correlated enzyme activities in bayberry (*Myrica rubra* Bieb.) during flower bud induction. Plant Sci 164:549–556
Logemann E, Parniske M, Hahlbrook K (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. Proc Natl Acad Sci USA 92:5905–5909
Martens S, Teeri T, Forkmann G (2002) Heterologous expression of dihydroflavonol 4-reductases from various plants. FEBS Lett 531:453–458
Nie G, Jin C, Cao Y, Shen S, Zhao B (2002) Distinct effects of tea catechins on 6-hydroxydopamine-induced apoptosis in PC12 cells. Arch Biochem Biophys 397:84–90
Peters DJ, Constabel CP (2002) Molecular analysis of herbivore induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J 32:701–712
Richard S, Lapointe G, Rutledge RG, Seguin A (2000) Induction of chalcone synthase expression in white spruce by wounding and jasmonate. Plant Cell Physiol 41:982–987
Rosati C, Cadic A, Duron M, Renou J-P, Simoneau P (1997) Molecular cloning and expression analysis of *dihydroflavonol 4-reductase* gene in flower organs of *Forsythia* 3 *intermedia*. Plant Mol Biol 35:303–311
Russel DW, Galston AW (1969) Blockage by gibberellic acid on phytochrome effects on growth, auxin responses, and flavonoid biosynthesis in etiolated pea internodes. Plant Physiol 44:1211–1216
Schulz W, Eiben HG, Hahlbrock K (1989) Expression in *Escherichia coli* of catalytically active phenylalanine ammonia-lyase from parsley. FEBS Lett 258:335–338
Sharma P, Kumar S (2005) Differential display-mediated identification of three drought-responsive expressed sequence tags in tea [*Camellia sinensis* L. (O.) Kuntze]. J Biosci 30:101–105
Singh HP, Ravindranath SD, Singh C (1999) Analysis of tea shoot catechins: Spectrophotometric quantitation and selective visualization of two-dimensional paper chromatograms using diazotized sulfanilamide. J Agric Food Chem 47:1041–1045
Singh K, Raizada J, Bhardwaj P, Ghawana S, Rani A, Singh H, Kaul K, Kumar S (2004) 26S rRNA-based internal control gene primer pair for reverse transcription-polymerase chain reaction-based quantitative expression studies in diverse plant species. Anal Biochem 335:330–333
Singh K, Paul A, Kumar S, Ahuja PS (2008a) Isolation and differential expression of *QM Like protein* homologue from tea [*Camellia sinensis* (L.) O. Kuntze]. Mol Biol Rep. doi:10.1007/s11033-008-9264-x
Singh K, Kumar S, Ahuja PS (2008b) Differential expression of *Histone H3* gene in tea [*Camellia sinensis* (L.) O. Kuntze] suggests its role in growing tissue. Mol Biol Rep. doi:10.1007/s11033-008-9211-x
Singh K, Rani A, Kumar S, Sood P, Yadav SK, Mahajan M, Singh B, Ahuja PS (2008c) An early gene of flavonoid pathway, *flavanone 3-hydroxylase*, exhibits a positive relationship with catechins content in tea [*Camellia sinensis* (L.) O. Kuntze]. Tree Physiol, in press
Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L). Plant Mol Biol 24:743–755
Stafford HA (1990) Flavonoid metabolism. CRC Press, New York
Ward EWB, Cahill DM, Bhattacharayya MK (1989) Abscisic acid suppression of phenylalanine ammonia-lyase activity and messenger RNA, and resistance of soybeans to *Phytopythora megasperma* f. sp. Glycinea. Plant Physiol 91:23–27
Xie DY, Jackson LA, Cooper JD, Ferreira D, Paiva NL (2004) Molecular and biochemical analysis of two cDNA clones encoding *dihydroflavonol-4-reductase* from *Medicago truncatula*. Plant Physiol 134:979–994