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Developmental Effect on Transcript Expression of Genes Encoding Enzymes for Flavan-3-ols Synthesis and its Content in Leaves and Flowers of Tea (*Camellia sinensis* (L.) O. Kuntze)

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ABSTRACT

The production of monomeric flavan-3-ols mainly comprises of catechin and epicatechin, epigallocatechin and epicatechingallate, and their polymeric form as proanthocyanidins is highly controlled during the developmental stages of leaf and flower. We studied the accumulation of monomeric flavan-3-ols, anthocyanins and proanthocyanidins during the developmental stages of leaf and flower in relation to the expression of flavan-3-ols-specific genes of tea (*Camellia sinensis*). The expression analyses of flavan-3-ols-specific genes were also studied in the presence of exogenous monomeric flavan-3-ols such as catechin and epicatechin during different development stages of both organs. We selected two developmental stages of leaf (leaf bud (LB) and old leaf (OL)) and three stages of flower development (flower bud (FB), semi-mature flower (SMF) and fully mature flower (FMF)) to assess various activities. The monomeric flavan-3-ols were highest in LB and FB and lowest in OL and FMF. The pattern of accumulation of anthocyanins was opposite to monomeric flavan-3-ols content during different development stages of the leaf and flower. In leaves, proanthocyanidins, oligomeric and/or polymeric forms of flavan-3-ols were higher in OL than in LB while in flowers, proanthocyanidins were lowest in FMF. Expression of the *DFR* gene encoding dihyroflavonol 4-reductase, the *LAR* gene encoding leucoanthocyanidin reductase and the *ANR* gene encoding anthocyanidin reductase was correlated with the accumulation of monomeric flavan-3-ols during leaf and flower development. Expression of the *ANS* gene, encoding anthocyanidin synthase, was only detected in OL, which also contained the highest anthocyanin content. Exogenous exposure of flavan-3-ols (catechin and epicatechin) modulated the expression of *DFR*, *LAR* and *ANR* in OL and LB tissues but only modulated the expression of *LAR* during all stages of flower development. This study provides an overview of new insight for monomeric flavan-3-ols biosynthesis, and their transcript regulation during leaf and flower development of *C. sinensis*.

Keywords: flower, leaf

Abbreviations: **ANR**, Anthocyanidin reductase; **ANS**, Anthocyanindin synthase; **cDNA**, complimentary DNA; **DFR**, Dihydroflavonol reductase; **ECG**, Epicatechin gallate; **EGC**, epigallocatechin; **LAR**, Leucoanthocyanidin reductase

INTRODUCTION

Flavonoids are a family of more than 9000 plant secondary metabolites (Williams and Grayer 2004; Wang *et al*. 2011). Flavonoids are classified as anthocyanins, flavonols, flavones, flavanones, and flavan-3-ols (Petroni and Tonelli 2011; Wang *et al*. 2011). Flavan-3-ols are the major flavonoids in tea (*Camellia sinensis* L.). Tea plant is reported to have various flavan-3-ols such as (+)-catechin (2,3-*trans*), (-)-epicatechin (2,3-*cis*) and their ester derivatives. Oligomeric and polymeric proanthocyanidins (syn. Condensed tannins) made up of monomeric flavan-3-ols are also reported in tea plant (Kumar *et al.* 2009). Both flavan-3-ols and proanthocyanidins are prominent secondary metabolites and are reported in different tissues such as seed coats, leaves, fruits, flowers and bark of many plant species (Marles *et al*. 2003). Their major function is to provide protection to the plant by inhibiting disease, insect feeding, and foraging by birds and other larger non-ruminant animals (Marles *et al*. 2003). The flavan-3-ols and proanthocyanidins are also deposited in the endothelial layer of seed coat in many species and are responsible for control of permeability and dormancy in seed (Debeaujon *et al*. 2000). The flavan-3-ols and proanthocyanidins are also known to contribute to the astringency of many fruits and impart taste and flavor to other plant products such as fruit juices, tea, and wine (He *et al*. 2008).

Biosynthesis of flavan-3-ols monomer shared the common steps with anthocyanins biosynthesis. Intermediates, flavan-3,4-diols (leucoanthocyanidins) and anthocyanidins are utilized for the synthesis of catechin and epicatechin by enzymes leucoanthocyanidin reductase (LAR, EC 1.17.1.3) and anthocyanidin reductase (ANR, EC 1.3.1.77) respectively (Tanner *et al*. 2003; Xie *et al*. 2003) (**Fig. 1**). Dihydroflavonol reductase (DFR, EC 1.1.1.219), an enzyme encoded by the *DFR* gene, catalyzes a NADPH-dependent reduction of dihydroflavonol to leucoanthocyanidin (Holton and Cornish 1995). Leucoanthocyanidin is a common substrate for the synthesis of flavan-3-ols and anthocyanins. Leucoanthocyanidin is reduced to catechin and oxidized to anthocyanidin by LAR and anthocyanidin synthase (ANS, EC 1.14.11.19) enzyme, respectively (Saito *et al*. 1999). The anthocyanidin is converted by ANR and UDPglucose: flavonol 3-*O*-glucosyltransferase (UFGT, EC 2.4.1.91) to epicatechin for proanthocyanidins synthesis and to cyanidin-3-glucoside for anthocyanins synthesis, respectively. The mechanism of oligomeric and polymeric proanthocyanidins (PAs) synthesis from flavan-3-ols is still not fully elucidated (**Fig. 1**).

Strategy for silencing of gene encoding DFR enzyme has been used to reduce polymeric flavan-3-ols level in transgenic root cultures of *Lotus corniculatus* (Bavage *et al*. 1997). It has been already proved that DFR enzyme is actively involved in the biosynthesis of flavan-3-ols in *C.*

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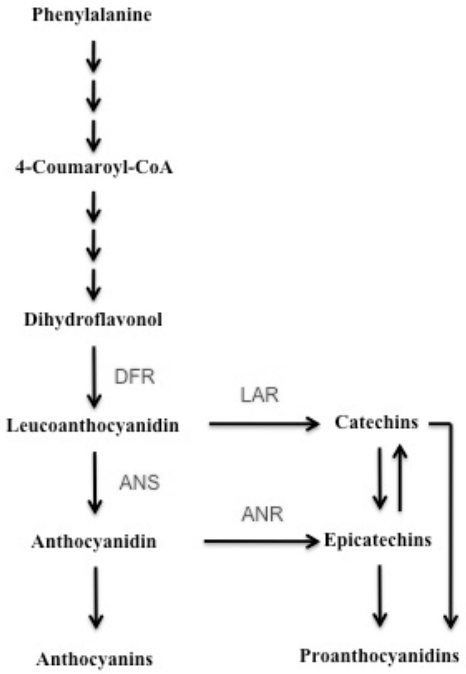

Fig. 1 A simplified representation of the flvan-3-ols pathway leading to production of anthocyanins and proanthocyanidins. Dihydroflavonol 4-reductase (DFR); Leucoanthocyanidin reductase (LAR); Anthocyanidin synthase (ANS); Anthocyanidin reductase (ANR).

sinensis (Singh *et al*. 2009a). The anthocyaninins and flavan-3-ols biosynthesis share common intermediates until leucocyanidins. Thereafter, leucoanthocyanidin dioxygenase produces anthocyanin and the leucoanthocyanidin reductase (LAR) enzyme produces flavan-3-ols. Hence, expression of genes encoding these enzymes is not only involved in anthocyanins synthesis, but also involved in polymeric flavan-3-ols biosynthesis pathway in *Arabidopsis* (Abrahams *et al*. 2003). The *ANS* suppression strategy for redirecting anthocyanins synthesis towards increased flavan-3-ols synthesis was also adopted in *Malus domestica*. LAR and ANS utilize same substrate leucoanthocyanidins for the synthesis of flavan-3ols (catechins) and anthocyanidins, respectively. Therefore, *ANS* suppression provides substrate only to LAR and that leads to synthesis of more of flavan-3-ols (Szankowski *et al*. 2009). The *ANR* and *LAR* gene due to their importance in PAs biosynthesis have been cloned and characterized from different plant species. Since ANR and LAR are key regulatory enzymes of PAs biosynthesis, genetic manipulation of genes encoding these enzymes have been found to affect PAs accumulation and composition (Tanner *et al*. 2003; Xie *et al*. 2003; Bogs *et al*. 2005; Pang *et al*. 2007; Paolocci *et al*. 2007). The expression analysis of flavonoid biosynthetic pathway genes has been studied in relation to PAs in different tissues especially leaves, flowers and fruits of plant species such as *Vitis vinifera* (Bogs *et al*. 2005), *Diospyros kaki* (Ikegami *et al*. 2005a, 2005b; Akagi *et al*. 2009; Wang *et al*. 2010), *Medicago truncatula* (Pang *et al*. 2007), *Vaccinium myrtillus* (Jaakola *et al*. 2002), *Prunus padus* (Olszewska and Kwapisz 2011) and *Saussurea medusa* (Li *et al*. 2012). These studies revealed that *DFR*, *LAR*, *ANS* and *ANR* genes encoding enzymes has been identified as regulators of flavonoids especially anthocyanins and flavan-3-ols synthesis in plants.

Here, we studied the influence of leaf and flower development on flavan-3-ols content of tea plant. We analyzed monomeric flavan-3-ols like catechin, epicatechin, epigallocatechin and epicatechingallate as well as polymeric form as PAs accumulation during different developmental stages of leaf and flower in tea. We further studied the transcript expression of various genes encoding enzymes of during leaf and flower development in tea. The transcript expression of various genes was also studied upon exposure to catechin and epicatechin.

MATERIALS AND METHODS

Materials

Tea (*C. sinensis* (L.) O. Kuntze), Kangra jat, a prevalent China type of tea clone growing in the Institute tea garden was selected for the present study. The tea clone was well maintained at the experimental farm of the Institute (Banuri tea experimental farm; 1,290 msl, 32 ^{o}N and 76 ^{o}E). Various experiments were performed with leaf and flower tissues at different stages of development. Leave bud (LB; youngest leaf), old leaf (OL; leaf at fourth position with reference to the leaf bud), flower bud (FB; condensed flower tissue), semi mature flower (SMF; half bloomed flower) and full mature flower (FMF; full bloomed flower). These tissues were collected from a 4-5 year-old tea plant during the non-dormant season (April-September) because the vegetative growth and flowering occurs during this season only in tea. All chemicals and reagents used until and unless stated in this study were of high quality and purchased from Sigma-Aldrich, St. Louis, MO, USA.

Exogenous treatment of flavan-3-ols to various tissues

Various leaf and flower tissues were treated with 50 µM catechin and 50 µM epicatechin individually. The catechin and epicatehin were dissolved individually in 100 µl dimethylsulfoxide (DMSO) and later prepared their aqueous solution (100 ml) with above said concentration. The samples were incubated in aqueous solution of catechin and epicatechin at 24°C for 24 h. The treated samples were further used for expression analysis. The samples incubated in DMSO aqueous solution (0.1%) were used as control for this study

Determination of flavan-3-ols, anthocyanins and PAs content

The flavan-3-ols were estimated following the HPLC method described earlier (Sharma *et al*. 2005). Briefly, 1 g of each sample was freeze dried and used for flavan-3-ols extraction with 70% methanol. The different flavan-3-ols content were estimated by Merck Hitachi HPLC (Darmstadt Germany) using C18 Lichrocart column (250 mm × 5 mm × 5 µm) and absorbance was read at 210 nm. Contents were determined in triplicate. Pure (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechingallate were used as standard for estimation of related constituent.

The anthocyanins and PAs were estimated according to protocol published earlier with minor modifications (Pang *et al*. 2009). For extraction of anthocyanins, 2 ml 0.1% HCl/methanol was added to 0.1 g of freeze dried samples, followed by sonication for 30 min and standing overnight at 4°C. Following centrifugation at 3,500 × *g* for 15 min, the extraction was repeated once more and the supernatants were pooled. An equal volume of water and chloroform was added to remove chlorophyll, and the absorption of the aqueous phase was recorded at 530 nm. The anthocyanins content was calculated based on the molar absorbance of cyanidin-3-*O*-glucoside.

For analysis of PAs, 0.5 g freeze-dried samples were extracted with 5 ml of extraction solution (70% acetone and 0.5% acetic acid) by vortexing, and then sonicated at room temperature for 1 h. Following centrifugation at 3,500 × *g* for 15 min, the residues were re-extracted twice as above. The pooled supernatants were then extracted three times with chloroform and once with hexane. The supernatants contained methanol-soluble PAs fraction and residues contained methanol-insoluble PAs fraction from each sample were freeze dried separately. The dried soluble PAs were suspended in extraction solution to a concentration of 3 mg/ml. The soluble PAs content was determined spectrophotometrically after reaction with DMACA reagent (0.2% w/v DMACA in methanol-3N HCl) at 640 nm. For quantification of insoluble PAs, 2 ml of butanol-HCl (95:5, v/v) was added to the dried residues and the mixture was sonicated at room temperature for 1 h, followed by centrifugation at 3,500 × *g* for 15 min. The absorption of the

supernatants was measured at 550 nm. Thereafter, samples were boiled for 1 h, cooled to room temperature, and again measured at the 550 nm. Two values were subtracted for calculation. Absorbance values were converted into PAs equivalents using a standard curve generated with procyanidin B2.

Gene expression analysis

Transcript expression analysis of various genes encoding enzymes of flavan-3-ols biosynthesis was conducted in various leaves and flowers of fresh and catechin/epicatechin treated samples. One hundred milligrams of each tissue was ground in liquid nitrogen and total RNA was isolated using Qiagen RNasy Plant Mini Kit. The complementary DNA (cDNA) was prepared according to manufacturer's protocol (Invitrogen, USA) using 2 µg of total RNA from each sample, 250 ng oligo dT12–18, 200 U of superscript III RT, and 10 mM dNTPs in a 15 µl reaction volume. Equal quantity of cDNA was used as template in PCR with *DFR*, *LAR*, *ANS*, *ANR* gene-specific primers (designed by authors): (*DFR*; forward primer 5′-GGACACTACTCGATCATAAAGCAA-3′ and reverse primer 5′-GCAAGAGTAGCTTCTTCAACTATC-3′), (*LAR*; forward primer 5′-ATCGTTCACACACGACATTTTC-3′ and reverse primer 5′-CTATCTCTGCCTACCTTTGTGA-3′), (*ANS*; forward primer 5′-ATGATAAAACCAGCAATTCAAG-3′ and reverse primer 5′-TAGCTTTTGTGATCGACTCTTT-3′) and (*ANR*; forward primer 5′-TCGAGCCCTAGCTACCAAGA-3′ and reverse primer 5′-AACTTCGGGTGTGACTGAAC-3′). Linearity between the amount of input RNA and the final PCR products was verified and confirmed by using various concentrations of RNA and temperature gradient. After standardizing the optimal amplification at exponential phase, PCR was carried out under the conditions of 94°C-4 min for 1 cycle, 94°C-30 s, 57°C (*DFR*), 58°C (*LAR*), 54°C (*ANR* and *ANS*)-30 s, 72°C-30 s for 30 cycles, and amplified product was separated on 1% agarose gel and visualized with ethidium bromide staining. The expression analysis was repeated at least three times for each sample. The 26S rRNA-based gene primers were used as internal control for gene expression studies (Singh *et al*. 2004).

RESULTS AND DISCUSSION

Developmental response of flavan-3-ols, anthocyanins and proanthocyanidins in leaf and flower of tea

The flavonoid content was reported as 25-30% of dry weight in young leaves of tea (Singh *et al*. 1999). The flavan-3-ols (major flavonoids) in tea plant were identified as epigallocatechin, epicatechingallate, epicatechin and catechin. Collectively, the measurement of all these four was named as flavan-3-ols. The flavan-3-ols were measured during different stages of selected part of tea plant. Flavan-3-ols was found as 280 and 250 mg g^{-1} dry weight of LB and OL, respectively (**Fig. 2A**). In flower, total flavan-3-ols was measured as 240, 130 and 110 mg g^{-1} dry weight of FB, SMF and FMF, respectively (**Fig. 2B**). Total flavan-3-ols was accumulated to higher levels at young stage and then declined with leaf age and flower maturation. However, total flavan-3-ols content was reported higher in leaf than flower. The flavan-3-ols content was significantly decreased from LB to OL as described earlier (Singh *et al*. 2009a, 2009b). In parallel, the flavan-3-ols accumulation was also decreased as described above from FB to FMF. The flavan-3-ols accumulation was reported higher in growing tissue of both leaf and flower as compare to old ones. The profile of flavan-3-ols accumulation was also reported significantly higher in leaf tissue than flower tissue. Thus, the accumulations of flavan-3-ols are developmentally and spatial regulated at different developmental stages of leaf and flower.

The flavan-3-ols biosynthesis shared a common flavonoid pathway with the anthocyanins biosynthesis until after flavan-3, 4-diol steps. The anthocyanins content are also potential substrate for flavan-3-ols polymerization and condensed with monomer, oligomers and polymer PAs directly or via carbocation generation by non-enzymatic oxidation

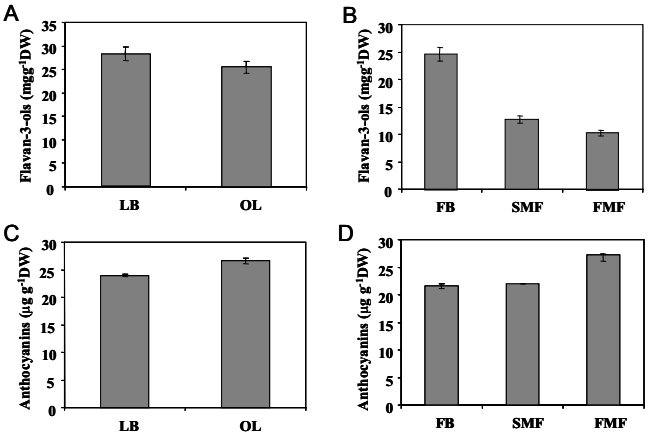

Fig. 2 Flavan-3-ols and anthocyanins content during different developmental stages of leaf and flower of tea plant. Flavan-3-ols (**A, B**) Anthocyanins (**C, D**) content in different stages of leaf and flower development of tea plant. Leaf bud, LB; Old leaf, OL; Flower bud, FB; Semi-mature flower, SMF; Fully mature flower, FMF. All results are presented as mean ± SD (n = 3). The SD values are below than 5% of their original values.

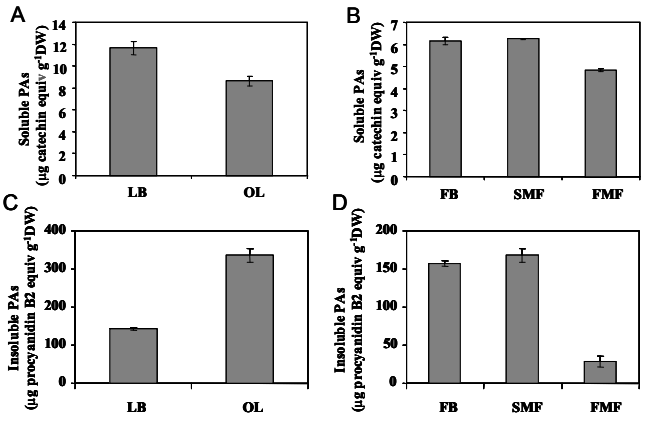

Fig. 3 Accumulation pattern of (**A, B**) soluble proanthocyanidins and (**C, D**) insoluble proanthocyanidins during different stages of leaf and flower development of tea plant. Leaf bud, LB; Old leaf, OL; Flower bud, FB; Semi-mature flower, SMF; Fully mature flower, FMF. All results are presented as mean ± SD (n = 3). The SD values are below than 5% of their original values.

(He *et al*. 2008). Thus, anthocyanins content was measured during developmental stages of leaf and flower. The anthocyanins content was quantified as 23.9 and 26.6 μg^{-1} g dry weight of LB and OL (**Fig. 2C**) While anthocyanins content in FB, SMF and FMF was quantified as 21.6, 22.1 and 26.8 μg^{-1} g dry weight (**Fig. 2D**). The accumulation pattern of anthocyanins content was increased from growing tissue to old tissue during developmental stages of leaf and flower. The pattern of anthocyanins accumulation was just opposite to flavan-3-ols accumulation during different stages of leaf and flower development. However, there was no significant difference of anthocyanins content among leaf and flower tissue. Thus, flavan-3-ols and anthocyanins biosynthesis might be differentially regulated as described earlier (Ikegami *et al*. 2007).

The flavan-3-ols act as precursors for the synthesis of their polymeric form PAs but details of synthetic mechanism remain unclear (He *et al.* 2008). Thus, PAs content was also estimated. The PAs estimation involved soluble and insoluble PAs content. The soluble PAs was estimated as 11.7 and 8.7 µg catechin equivalent g^{-1} dry weight in LB and OL, respectively (**Fig. 3A**). While the soluble PAs was estimated as 6.2, 6.2 and 4.9 µg catechin equivalent g^{-1} dry weight in FB, SMF and FMF, respectively (**Fig. 3B**). Insoluble PAs was 143.2 and 334.2 µg procyanidin B2 equi-

valent g^{-1} dry weight of LB and OL (**Fig. 3C**). In FB, SMF and FMF insoluble PAs was estimated as 157.1, 167.1 and 28.9 µg PAs B1 equivalent g^{-1} dry weight, respectively (**Fig. 3D**). The polymeric PAs, which are soluble, are composed entirely of flavan-3-ols units (Foo and Porter 1980). The pattern of soluble PAs accumulation was similar to flavan-3-ols accumulation during developmental stages of leaf and flower in tea plant. The content of insoluble PAs was reported higher in OL than LB, suggesting its accumulation followed opposite trend to that of flavan-3-ols accumulation. In flower tissue, insoluble PAs was highest in SMF and lowest in FMF. Hence, accumulation of soluble and insoluble PAs also seems to be developmentally regulated in leaf and flower tissue of tea.

Results revealed that the accumulation of flavan-3-ols, anthocyanins and PAs in leaf and flower of tea plant are under tissue and developmental stage specific regulation. The flavan-3-ols might be playing significant role during development of leaf and maturation of flower as well provide UV protection, prevent feeding by herbivores, attract the pollinators and promote the development of floral parts (Lee *et al*. 1993; Shirley *et al*. 1996; Pollastri and Tattini 2011; Wang *et al*. 2011).

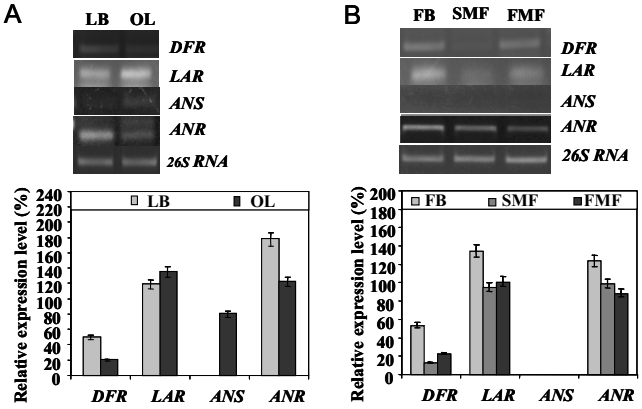

Fig. 4 Relative expression level of genes encoding enzymes Dihydroflavonol 4-reductase (DFR), Leucoanthocyanidin reductase (LAR), Anthocyanidin synthase (ANS) and Anthocyanidin reductase (ANR) during fresh tissue of leaf (**A**) and flower (**B**) development stages of tea plant. Below gel pictures, bar diagram represents the respective mean relative intensities of expression levels (n = 3) ± SD. Leaf bud, LB; Old leaf, OL.

Transcription profile of flavan-3-ols biosynthesis pathway genes during leaf and flower development in tea

DFR, LAR, ANS and ANR are identified as regulatory enzymes for the synthesis of flavan-3-ols (Xie *et al.* 2003; Tanner *et al.* 2004; Winkel 2004; Wellman *et al.* 2006; Mahajan *et al*. 2011; Pollastri and Tattini 2011; Wang *et al*. 2011). The DFR and ANS enzymes are responsible to generate precursors as leucoanthocyanidin and anthocyanidin for the biosynthesis of flavan-3-ols monomer in plants, while LAR and ANR enzymes catalyzed the production of catechin and epicatechin, respectively (Saito *et al*. 1999; Xie *et al*. 2003; Tanner *et al*. 2003). The proposed model for flavan-3-ols monomer synthesis involved LAR, ANS, and ANR enzymes, possibly along with DFR enzyme, might form a complex through which intermediate are channeled directly in to the formation of flavan-3-ols (Winkel 2004; Ashihara *et al*. 2010).

In this study, levels of transcripts of genes encoding DFR, LAR, ANR and ANS enzymes were analyzed during different developmental stages of leaf and flower. The transcript level of *DFR* and *ANR* genes were observed higher at LB (50 and 178%) than OL stage (21 and 123%). The transcript level of *LAR* gene was opposite to transcript pattern of *DFR* and *ANR* genes, higher at OL (135%) than LB stage (119%). While, the transcript level of *ANS* gene was detected only at OL stage (81%) (**Fig. 4A**). At different stages of flower, the transcript level of *DFR* and *LAR* genes were highest at FB stage (53 and 134%), declined at SMF stage (12 and 94%) and again increased at FMF stage (22 and 100%). The transcript level of *ANR* gene was highest at FB stage (123%) and then declined continuously at SMF (98%) and FMF (88%) stage. In contrast, the transcript level of *ANS* gene was not detected during different stages of flower development (**Fig. 4B**).

The transcript of *DFR* and *ANR* gene was highest in growing tissue of leaf and flower and correlated with maximal accumulation of flavan-3-ols. Higher transcript level of *DFR* and *ANR* gene in leaf compare to flower also correlated with higher level of flavan-3-ols in leaf than flower. Similarly, the role of genes encoding DFR and ANR enzymes has been discussed in different parts of various plant species in relation to flavan-3-ols monomer (Devic *et al*. 1999; Abrahams *et al*. 2003; Xie *et al*. 2003; Bogs *et al*. 2005; Singh *et al*. 2009a, 2009b). Thus the levels of transcripts of genes encoding DFR and ANR enzyme was also in good relation with flavan-3-ols accumulation in tea plant. While the relation of transcript level of *LAR* gene with flavan-3-ols accumulation was observed as tissue specific. During developmental stages of tea leaf, transcript level of *LAR* gene was not correlated with flavan-3-ols accumulation. But in tea flower, it was correlated with flavan-3-ols accumulation at different developmental stages. The poor relation of *LAR* transcript with flavan-3-ols accumulation was also reported earlier in plants (Tanner *et al*. 2003; Pang *et al*. 2007). Collectively the expression of various genes (*DFR*, *LAR* and *ANR*) was well correlation with flavan-3-ols accumulation in tissue specific manner in tea plant. The transcript of *ANS* gene was reported only at OL stage of leaf and not detected at developmental stages of flower. It was correlated with higher content of anthocyanins and insoluble PAs. The gene encoding ANS enzyme was already known to regulate the synthesis of anthocyanin and PAs in plants (Wellmann *et al*. 2006; Pang *et al*. 2007).

For different developmental stages of both leaf and flower, the transcript profile of *DFR* and *ANR* gene tended to be parallel with accumulation of flavan-3-ols. While the direct correlation of *LAR* gene transcript expression with flavan-3-ols accumulation was occurred in flower only during developmental stages. The expression of *ANS* gene was detected in OL and correlated with maximum anthocyanins content of OL. The change of flavan-3-ols accumulation in this study with expression of genes encoding DFR, LAR and ANR enzymes was consistent with studies in other plants (Devic *et al*. 1999; Abrahams *et al*. 2003; Tanner *et al*. 2003; Xie *et al*. 2003; Bogs *et al*. 2005; Fujita *et al*. 2005, 2007; Pang *et al*. 2007). Thus flavna-3-ols accumulation analyzed here showed no contradiction to other studies, suggesting the typical expression characteristics of various genes involved in flavan-3-ols accumulation.

Influence of exogenous catechin and epicatechin application on transcript expression of flavan-3-ols pathway genes during developmental stages of leaf and flower

The influence of exogenous flavan-3-ols as catechin and epicatechin on the transcript level of *DFR*, *LAR*, *ANS* and *ANR* genes was examined during leaf and flower developmental stages to understand the regulation of their biosynthetic pathway in presence of end product. The catechin (50 µM) and epicatechin (50 µM) treated samples at different stages of leaf and flower developments were used for this experiment.

The exogenous catechin application upregulated the transcript level of both *DFR* and *LAR* genes in LB by 62 and 50% and in OL by 58 and 52% relative to control samples (**Fig. 5A, 5B**). The expression of *ANS* gene was almost unaffected by catechin treatment during leaf development (**Fig. 5C**). The transcript level of *ANR* gene was remained unaffected at LB stage and only activated at OL stage

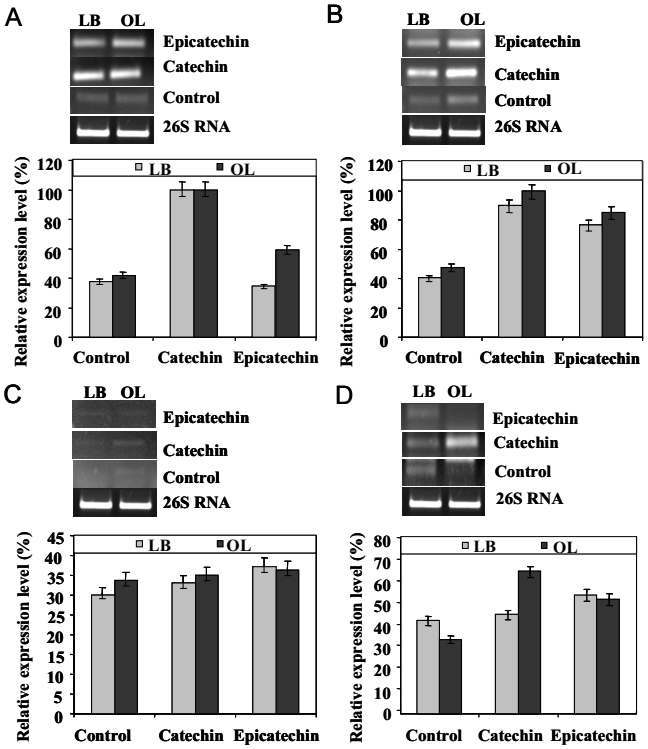

Fig. 5 Relative transcript level of genes encoding enzymes (**A**) Dihydroflavonol 4-reductase (DFR), (**B**) Leucoanthocyanidin reductase (LAR*)*, (**C**) Anthocyanidin synthase (ANS). (**D**) Anthocyanidin reductase (ANR) in 50 µM catechin and 50 µM epicatechin treated sample of leaf developmental stages of tea plant. Below gel pictures, bar diagram rep-resents the respective mean relative intensities of expression levels (n = 3) ± SD. Flower bud, FB; Semi-mature flower, SMF; Fully mature flower, FMF.

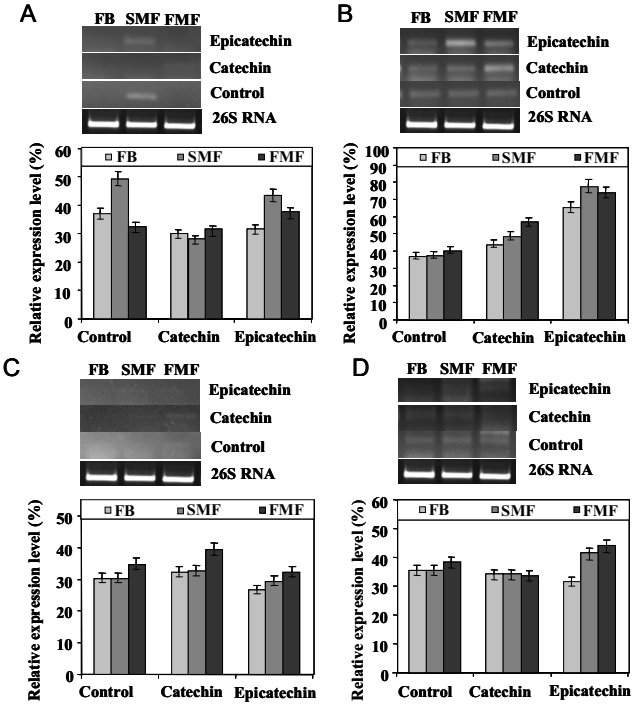

Fig. 6 Relative transcript level of genes encoding enzymes (**A**) Dihydroflavonol 4-reductase (DFR), (**B**) Leucoanthocyanidin reductase (LAR*)*, (**C**) Anthocyanidin synthase (ANS) and (**D**) Anthocyanidin reductase (ANR) in 50 µM catechin and 50 µM epicatechin treated sample of flower developmental stages of tea plant. Below gel pictures, bar diagram represents the respective mean relative intensities of expression levels (n = 3) ± SD. Flower bud, FB; Semi-mature flower, SMF; Fully mature flower, FMF.

(31%) relative to control samples (**Fig. 5D**). On the other side, the exogenous epicatechin application upregulated the transcript level of *DFR* gene at only OL stage (17%) and remained unaffected at LB stage relative to control samples (**Fig. 5A**). While the expression level of *LAR* and *ANR* genes was activated in LB by 37 and 12% and in OL by 37 and 18% relative to control samples (**Fig 5B, 5D**). Interestingly, the transcript expression of *ANS* gene was not significantly affected by exogenous epicatechin treatment during leaf development (**Fig. 5C**). These results indicated that catechin and epicatechin both individually acted as a feedback activator of *DFR*, *LAR* and *ANR* gene at transcription level during various developmental stages of leaf and flower. While there was no significant modulation of *ANS* gene expression during different developmental stages in presence of exogenous catechin and epicatechin individually. In this study, precise pattern of *DFR*, *LAR* and *ANR* gene expression demonstrated that their regulation occurs at the transcriptional level in presence of exogenous flavan-3-ols. Even the tissue specific pattern of *DFR*, *LAR* and *ANR* gene was consistent even after exogenous flavan-3-ols treatment. Thus, flavan-3-ols regulated the expression of *DFR*, *LAR* and *ANR* gene at transcriptional level.

Exogenous catechin application significantly modulated the expression of *DFR* gene and *LAR* gene during different stages of flower development (**Fig. 6A, 6B**). The expression of *DFR* gene was down regulated at FB and SMF stage by 7 and 22% and remained unaffected at FMF stage with exogenous catechin application relative to control samples (**Fig. 6A**). The transcript level of *LAR* gene was activated by exogenous catechin application at FB by 7%, SMF by 11% and FMF by 22% relative to control samples of different developmental stages of tea flower (**Fig. 6B**). The exogenous epicatechin application significantly modulated the expression of only *LAR* gene and activated their transcript level at FB stage by 28% and at the SMF stage by 40% relative to control samples of different developmental stages of flower (**Fig. 6B**). The exogenous application of catechin down-regulated the expression of *DFR* gene and upregulated the expression of *LAR* gene. While epicatechin only activated *LAR* gene expression during various stages of flower development. So, catechin and epicatechin both regulated the *LAR* gene expression at transcriptional level (**Fig. 6B**). In addition, catechin also regulated the expression of *DFR* gene expression at transcriptional level (**Fig. 6A**).

The exogenous application of catechin and epicatechin modulated the expression of flavan-3-ol pathway genes with more influence during leaf development than flower development in tea plant. They commonly regulated transcription of *DFR* and *LAR* gene in both leaf and flower development, and also regulated transcription of *ANR* gene only during leaf development. The transcriptional control of flavonoid biosynthesis has been intensively studied in *Arabidopsis thaliana* (Broun 2005; Feng *et al*. 2011). In our recent study, we found that exogenously applied flavonoids affected the transcript of genes encoding regulatory enzymes of flavonoid biosynthesis pathway in shoot and root of tobacco seedling (Mahajan *et al*. 2011).

CONCLUSION

This study provides the first detailed developmental effect on flavan-3-ols and PAs content during leaf and flower development in relation to expression of flavan-3-ols specific genes of *C. sinensis* plant. The transcript levels of *DFR*, *LAR and ANR* genes were found to be correlated with monomeric flavan-3-ols accumulation during development of leaf and flower. The exogenous application of flavan-3-ols was also found to be modulated transcript levels of *DFR*, *LAR* and *ANR* genes in leaf tissues and *LAR* gene only during flower development. This study paves the way to understanding not only the key role of the flavan-3-ols pathway genes regarding flavan-3-ols accumulation but also the regulation of their expression in presence of external flavan-3-ols.

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