Original research article

Overexpression of *CsDFR* and *CsANR* enhanced flavonoids accumulation and antioxidant potential of roots in tobacco

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Abstract: Flavonoids are widespread throughout the plant kingdom and present in different parts of plants. Tea (*Camellia sinensis*) is well known for very high content of flavonoids especially flavan-3-ols antioxidants and is an aluminium (Al) accumulator plant. Dihydroflavonol 4-reductase (DFR) and anthocyanidin reductase (ANR) are known to be regulatory enzymes of flavonoid biosynthetic pathway. In this study, cDNA encoding DFR (*CsDFR*) and ANR (*CsANR*) from tea were overexpressed individually in tobacco to check their influence on accumulation of flavonoid contents and antioxidant potential in roots of transgenic tobacco. Root morphological features, such as total volume and the number of lateral roots were improved in *CsDFR* and *CsANR* overexpressing tobacco plants relative to control tobacco plants. Both types of transgenic showed higher content of flavonoids and proanthocyanidins and lower content of anthocyanins in the roots compared to roots of control tobacco. Among flavan-3-ols, only epigallocatechin was observed in the roots and its content was higher in *CsDFR* and *CsANR* overexpressing tobacco as compared to control tobacco. Expression of genes encoding various other enzymes of flavonoid pathway like Phenylalanine ammonia-lyase, Chalcone isomerase, Flavanol synthase and Anthocyanin synthase was increased in roots of *CsDFR* and *CsANR* overexpressing tobacco plants as compared to control tobacco. The antioxidant potential of root portion of *CsDFR* and *CsANR* transgenic tobacco plants was found to be increased as indicated by enhanced total free radical scavenging activity and tolerance against Al toxicity. Taken together, these changes in roots of *CsDFR* and *CsANR* transgenic tobacco provided tolerance to aluminum toxicity.

Keywords: flavonoids, *CsDFR*, *CsANR*, root, tobacco, transgenic

Abbreviations: cDNA, complementary DNA; ANR, anthocyanidin reductase; ANS, anthocyanin synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; DMACA, 4-dimethylaminocinnamical -dehyde; DPPH, diphenyl-1-pikryl-hydrazyl; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine lyase; RT-PCR, reverse transcriptase PCR

Introduction

Flavonoids, a class of polyphenolics compound are majorly classified as anthocyanins, flavonols and flavan-3-ols in plants (Routboul et al. 2012). These flavonoids play multidirectional role in growth and development of plant. The important roles of flavonoids are associated with transport of phytohormone auxin, defense, allelopathy, attraction of pollinators, pollen viability and protection from UV light and root growth (Buer et al. 2010; Osawa et al. 2011). Flavonoids biosynthetic pathway is well understood and majority of enzymes and their encoding genes have been identified and characterized from several plants (Tian et al. 2008).

Flavonoids especially flavan-3-ols biosynthesis is ubiquitous throughout plant kingdom and their accumulation is tissue or certain cell type specific. Some plants such as *Camellia sinensis*, *Coffeia arabica*, *Malus x domestica*, *Vitis vinifera* and *Vaccinium myrtillus* contain high levels of flavan-3-ols in either leaf or fruit portion and both, attributed to agronomic and economical values of these plants (Punyasiri et al. 2004; Farah and Donangelo 2006; Pfeiffer et al. 2006; Hokkanen et al. 2009). In contrast, flavonoids are also present in root portion of plants. They are not only present along the

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length of a root, but also accumulated at the root tip and in root cap cells (Hassan and Mathesius 2012). The flavan-3-ols seems less abundant among root polyphenols and studied only in some plants *Centuria maculosa, Rumex acetosa*, *Zea mays*, *Fagopyrum esculentum, Fragaria ananassa* and *M. domestica* (Bais et al. 2006; Tolrà et al. 2005; Kalinova et al. 2007; Tolrà et al. 2009; Hoffmann et al. 2011 Osawa et al. 2011). The flavan-3-ols has also been reported to facilitate the expansion of epidermis cells away from Al during root elongation in *Cinnamomum camphora* (Osawa et al. 2011). The flavan-3-ols is secondary metabolite characteristic of *Rosaceae* and studied with respect to their pharmaceutical relevance in root (Hoffmann et al. 2011). Flavan-3-ols are also identified as constituent of root exudates (Hassan and Mathesius 2012). The presence of flavan-3-ols in root portion or root exudates and both are associated with chelation of cations, establishment of mycorrhizas, pathogen defense and allelopathic action (Walker et al. 2003; Hassan and Mathesius 2012). Flavan-3-ols in root also help to establish biotic as well as abiotic interaction in several plants (Tolrà et al. 2005; Bais et al. 2006; Osawa et al. 2011). So, flavonoids especially flavan-3-ols in root portion of various plant species are responsible for various physiological, biochemical processes and improved allelopathic interaction as well as improved tolerance against Al toxicity.

These flavonoids shared common biosynthetic pathway and synthesized through phenylpropanoid pathway; precursors for the biosynthesis of these flavonoids are malonyl-CoA and p-coumaroyl-CoA, which are derived from carbohydrate metabolism and phenylpropanoid pathway, respectively. This pathway is initiated by the enzymatic step catalyzed by chalcone synthase (CHS, EC 2.3.1.74) resulting in chalcone, which is further converted by chalcone isomerase (CHI, EC 5.5.1.6) to naringenin. The naringenin is subsequently hydroxylated to dihydroflavonols by flavanone-3-hydroxylase (F3H, EC 1.14.11.9). Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219) enzyme catalyzes the stereospecific reduction of dihydroflavonols to leucoanthocyanidins (flavan-3, 4-diols) using NADPH as a cofactor. Leucoanthocyanidins is reduced to catechin a monomeric flavan-3-ols and oxidized to anthocyanidins by leucoanthocyanidin reductase (LAR, EC 1.17.1.3) and anthocyanidin synthase (ANS, EC 1.14.11.19) enzymes respectively. The anthocyanidins is converted by anthocyanidin reductase (ANR, EC 1.3.1.77) and UDPglucose: flavonol 3-O-glucosyltransferase (UFGT, EC 2.4.1.91) to epicatechin a monomeric flavan-3-ols and to cyanidin-3-glucoside for anthocyanins formation, respectively. Furthermore, monomeric flavan-3-ols as catechin and epicatechin act as precursors for proanthocyanidins synthesis.

Tea (*C. sinensis*) is an Al accumulator plant because of its very high content of flavonoids especially flavan-3-ols antioxidants. Recently, enzymes and corresponding genes of flavonoid pathway have been identified and characterized from tea (Punyasiri et al. 2004, Singh et al. 2008, Singh et al. 2009a; b). Among them *CsDFR* and *CsANR* genes encoding enzymes have been reported as NADPH dependent enzymes and have their expression correlation with flavonoids accumulation (Singh et al. 2009a; b; Kumar and Yadav 2012). The DFR and ANR enzymes have also been characterized from a number of other plant species (Shimada et al. 2005; Shen et al. 2006; Piero et al. 2006; Paolocci et al. 2007). Transgenic plants raised with some of these genes were analyzed primarily for change in flavonoids content in leaf or shoot part (Xie et al. 2004; Takahashi et al. 2006; Xie et al. 2006; Kovinich et al. 2011 Han et al. 2012; Kumar and Yadav 2013; Kumar et al. 2013b). However, influence of overexpression of such genes encoding DFR and ANR enzymes on flavonoids accumulation and antioxidant potential in plant roots as well as their role in root growth under normal conditions and tolerance against Al toxicity is lacking. Here, we report the overexpression of tea Cs*DFR* and *CsANR* genes individually under the control of a CaMV 35S promoter in tobacco that resulted in improved root morphology, flavonoids accumulation and enhanced antioxidant potential of root portion and provided better growth under Al toxicity.

Materials and Methods

Plant material, plasmid construct, transformation and transgenic confirmation

The cDNAs from tea *CsDFR* (GenBank accession no. AY64027) and *CsANR* (GenBank accession no. AY641729) were isolated and cloned into pCAMBIA 1302 plasmid (Cambia, Australia) between *Nco* I and *Bgl* II restriction sites. This was resulted in the formation of recombinant construct pCAMBIA-*CsDFR* and pCAMBIA-*CsANR* and these were transferred into *Agrobacterium* by triparental mating. The *Agrobacterium tumefaciens* stain LBA4404 harboring pCAMBIA-*CsDFR* and *CsANR* constructs individually were used for leaf disc transformation of tobacco (*Nicotiana tabacum* cv *Xanthi* nc). Regenerated shoots were transferred to fresh media. Rooted plants on hygromycin selection (50 mg/ml) were screened for the presence of *CsDFR* and *CsANR* transgene by carrying out PCR with genomic DNA and gene specific primers. The reaction conditions were as follow: 94 °C for 1 min, and then 35 cycles

of 30 s at 94 °C, 30 s at 54 °C (for *CsDFR*) and 58 °C (for *CsANR*), and 2 min at 72 °C. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. T_1 transformants were self-pollinated and the seeds obtained from T_1 were analyzed for segregation by germinating on half strength Murashige and Skoog medium supplemented with hygromycin (50 mg/ml). The expression of tea *CsDFR* and *CsANR* was also checked in transgenic tobacco plants. Thirty days old plants (five plants of each transgenic) were transferred in pots containing the manure, sand and soil (1: 1: 2 ratios) in the greenhouse. Tobacco plants transformed with vector alone were used as control in all described experiments. All chemicals and reagents used until and unless stated in this study were of high quality and purchased from Sigma-Aldrich, USA.

Measurement of root morphological features

Transgenic and control tobacco plants were harvested at full maturation (86 days) and the shoots were separated from the roots. Plant roots were extensively washed with fine stream of water to remove soils without damaging them and measured root volume by measuring the amount of water it displaced in a cylinder. Roots and shoots were dried and total root and shoot dry mass were measured. To evaluate the root branching data, root length, root number and root diameter at different branching levels were also measured in *CsDFR* and *CsANR* overexpressing tobacco vis-à-vis control tobacco plants. First set of nodal roots elongating from lowermost node of crown area of each tobacco plant was also photographed.

Expression analysis of flavonoid biosynthetic pathway genes

Semi-quantitative RT-PCR analysis was performed to check the influence of overexpressed *CsDFR* and *CsANR* on the endogenous expression levels of flavonoid biosynthetic pathway genes in tobacco roots. For this, total RNA was extracted from roots of control and transgenic plants using RNeasy Plant Mini Kit (Qiagen, Germany). First stand cDNA was prepared using 1 µg of total RNA using SuperScript III Reverse Transcriptase according to manufacturer's protocol (Invitrogen, USA). Equal quantity of cDNA was used as template in PCR with gene-specific primer sets to see the expression of *NtPAL*, *NtCHS*, *NtCHI*, *NtF3H*, *NtFLS*, *NtDFR, NtANR1, NtANR2* and *NtANS* genes. The sequence of various primers as used earlier is shown in table no-01 (Kumar and Yadav 2013). Linearity between the amount of input RNA and the final PCR products was verified and confirmed. 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, 52 to 58 °C-40 s (52 °C for *NtANS, NtANR1 & NtANR2*, 54 °C for *NtCHS* and 58 °C for *NtDFR*, *NtPAL, NtCHI, NtF3H & NtFLS*)-30 s, and 72 °C-30 s for 30 cycles. The expression of *CsDFR* and *CsANR* was also checked in transgenic tobacco plants as well as control tobacco plants. The amplified products were separated on 1 % agarose gel and visualized with ethidium bromide staining. The 26S rRNA-based gene primers were used as internal control for gene expression studies (Singh et al. 2004).

Table 1. Sequences of primers used in this study

Gene	Sequences of primers (5'-3')
CsANR	Forward - TTAAACCTTGTTGCCATTGACAGG
	Reverse -TCAATTCTTCAAAATCCCCTTAGCCT
CsDFR	Forward - ATGAAAGACTCTGTTGCTTCTGCC
	Reverse - TTAAACCTTGTTGCCATTGACAGG
NtPAL	Forward - CAAGAACGGTGGTGCTCTTC
	Reverse - CCAGAACCAACTGCAGTACC
NtCHS	Forward - GTACAACTAGTGGTGTAGACA
	Reverse - CCAACTTCACGAAGGTGAC
NtCHI	Forward – GAAATCCGATCCAGTGA
	Reverse – CAACGTTGACAACATCAGGC
NtF3H	Forward – GGTAGTTGATCATGGTGTTGA
	Reverse – GTTCCTGGATCAGTGTCTCG
NtDFR	Forward – GATGAAGCCATTCATTCAAGGCTC
	Reverse – GCAGTGATTAAGCTAGGTGG
NtFLS	Forward – GTCCACAACGTTGCATGGTG
	Reverse – CACAACTTCTCGCAGCCTC
NtANR1	Forward – CATTTGACTTTCCCAAACGC
	Reverse – ATTGGGCTTTTGAGTTGTGC
NtANR2	Forward – TGTTCCCACTTGGGATGATA
	Reverse – TGCACCTATACTCTGTTAGTGGC
NtANS	Forward – CGAGGACAAGTGCGACTTAT
	Reverse – GAGATTCTTACTTTCTCTTTATT

DMACA staining for evaluation of flavonoid accumulation in root

Histochemical test was done following the method described earlier (Pang et al. 2007). Roots of *CsDFR* and *CsANR* transgenic as well as control tobacco were collected, extensively washed and soaked in ethanol-glacial acetic acid (GAA) solution (3: 1, v/v) for discolorations. Then roots were stained for about 20 min with 0.1 % DMACA in methanol: 6N HCl (1: 1, v/v), and then rinsed with several changes of 70 %

(v/v) ethanol and photographed. DMACA staining was also applied on 7 days old seedlings to check the accumulation of flavonoids in root tips of *CsDFR* and *CsANR* transgenic as well as control tobacco plants.

Estimation of total flavonoids, proanthocyanidins and anthocyanins content

The total flavonoids, proanthocyanidins, anthocyanins and tea specific flavan-3-ols were estimated in root portion of control as well as transgenic tobacco. For flavonoids and proanthocyanidins, one-gram freeze dry root sample was mixed with 20 ml methanol and sonicated without heat treatment for 45 min at 35 kHz. The methanolic extract was obtained by evaporation using a rotary vacuum evaporator and resuspended in methanol to make 50 mg/ml stock solutions for further experiments as described earlier (Kumar et al. 2013a). For flavonoids estimation, 0.5 ml of 2 % $AlCl_3$ ethanol solution was added to 0.5 ml volume of 0.1 mg/ml of each root-extracted samples. After one hour at room temperature, the absorbance was measured at 420 nm using the Hewlett Packard UV-VS spectrophotometer. Flavonoids content was expressed as mg/g quercetin acid equivalent. For determination of proanthocyanidins, volume of 0.5 ml of 0.1 mg/ml of root extract solutions was mixed with 3 ml of 4 % vanillin- methanol solution and 1.5 ml hydrochloric acid. The mixture was allowed to stand for 15 minute and absorbance was measured at 500 nm using the Hewlett Packard UV-VS spectrophotometer. Proanthocyanidins content was expressed as mg/g catechin equivalent.

Anthocyanins content was estimated as described earlier (Pang et al. 2007). For extraction of anthocyanins, 2-3 ml 0.1 % HCl/methanol was added to 100 mg powdered root-extracted samples, followed by sonication for 30 min and standing overnight at 4 °C. Following centrifugation at 2,500 g for 10 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 using the Hewlett Packard UV-VS spectrophotometer. Anthocyanins content was calculated based on the molar absorbance of cyanidin-3-*O*- glycoside.

Estimation of flavan-3-ols

Flavan-3-ols were analyzed in the root portion of control as well as transgenic tobacco following the previously described method (Mahajan et al. 2012). Briefly, 1 gram of freeze-dried root was used for flavan-3-ols extraction with 70 % methanol. Flavan-3-ols were measured by using Merck Hitachi HPLC (Darmstadt, Germany) with C18 Licrocart column (250 mm x 5 mm x 5 µm) and absorbance was read at 210 nm. The (+)-Catechin (Cat), (-)-Epicatechin (EC) and (-)-Epigallocatechin (EGC) from Sigma-Aldrich, USA were used as standards for estimation of respective constituent. Only EGC was detected in root of tobacco. EGC content was measured as µg/10 mg dried weight in transgenic lines as well as control tobacco plants.

Estimation of DPPH radical activity

DPPH free radical scavenging assay was performed as described earlier (Joshi et al. 2011). The initial absorbance of DPPH in methanol was measured using spectrophotometer at 517 nm until the absorbance remained constant. A total of 50 µl of methanolic extract was added to 1950 µl of 0.1 mM methanolic DPPH solution. The mixture was incubated at room temperature for 30 min before the change in absorbance at 517 nm was observed.

Evaluation for tolerance against Al toxicity

7 days old fresh seedlings of transgenic as well as control tobacco plants were evaluated for tolerance against toxic Al level. Al was applied in the form of $AlCl_3$. Fresh seedlings of transgenic vis-à-vis control tobacco at stage of 1-1.2 cm root length were transferred to the MS plates containing 1 and 5 mM Al concentration. Seedlings were allowed to grow for next 14 days and photographed.

Results

Generation of transgenic tobacco overexpressing CsDFR and CsANR

The transgenic tobaccos overexpressing *CsDFR* and *CsANR* cDNA under the control of CaMV 35S promoter were developed through *Agrobacterium tumefaciens*-mediated transformation. A schematic representation of pCMABIA-*CsDFR*/pCAMBIA-*CsANR* construct is shown in Fig. 1A. The regenerated plantlets were screened on MS plates containing hygromycin (50 mg/ml). Total of five independent plants overexpressing *CsDFR* and *CsANR* were selected and shifted to green house containment facility. The integration of *CsDFR* and *CsANR* was confirmed by total genomic DNA PCR method. Semi-quantitative PCR analysis was conducted to confirm the expression of transgene in transgenic lines. To make easy presentation, the picture showing single lines of *CsDFR* and *CsANR* with respect to control representing confirmation by genomic DNA-PCR and semiquantitative PCR method (Fig. 1B).

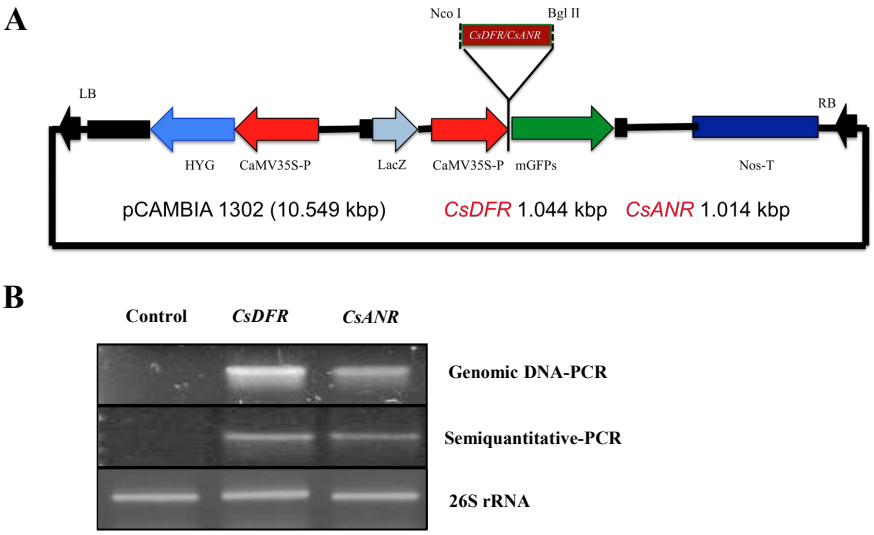

Fig. 1. Generation and confirmation of transgenic tobacco overexpressing cDNAs *CsDFR* and *CsANR* from tea. (A) Schematic representation of pCAMBIA1302 harboring *CsDFR* or *CsANR* cDNA (B) Genomic DNA PCR and semi quantitative PCR data of one of the representative line of each *CsDFR* and *CsANR* overexpressing tobacco. The lower panel represents the equal expression of housekeeping 26S rRNA gene.

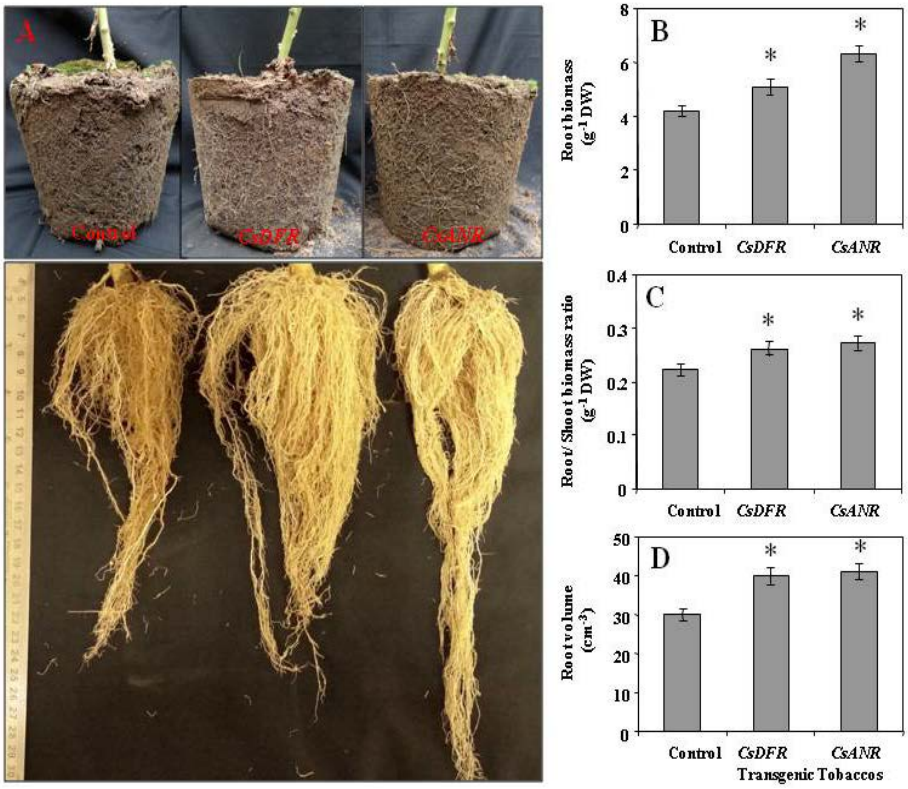

Fig. 2. Characterization of roots of tobacco plants overexpressing *CsDFR* and *CsANR* vis-à-vis control plant. (A) Morphological comparison of the root system of *CsDFR* and *CsANR* transgenic and control tobacco plants. Root biomass (B), Root-to-shoot biomass ratio (C) and Root volume (D) of plants overexpressing *CsDFR* and *CsANR* as compared to control tobacco plants after 86 days of their growth. Data represents mean values ± SD (n =3). Statistical significance is indicated as (*) for P<0.05.

Influence of CsDFR and CsANR overexpression on root morphology of tobacco

The root morphology plays a significant role in the absorption of water and nutrients. To study the morphological features of root of transgenic plants vis-à -vis control tobacco plants, parameters such as dry mass, volume, root to s hoot ratio, number, length and diameter were analyzed. All these parameters were improved in transgenic plants relative to control tobacco plants.

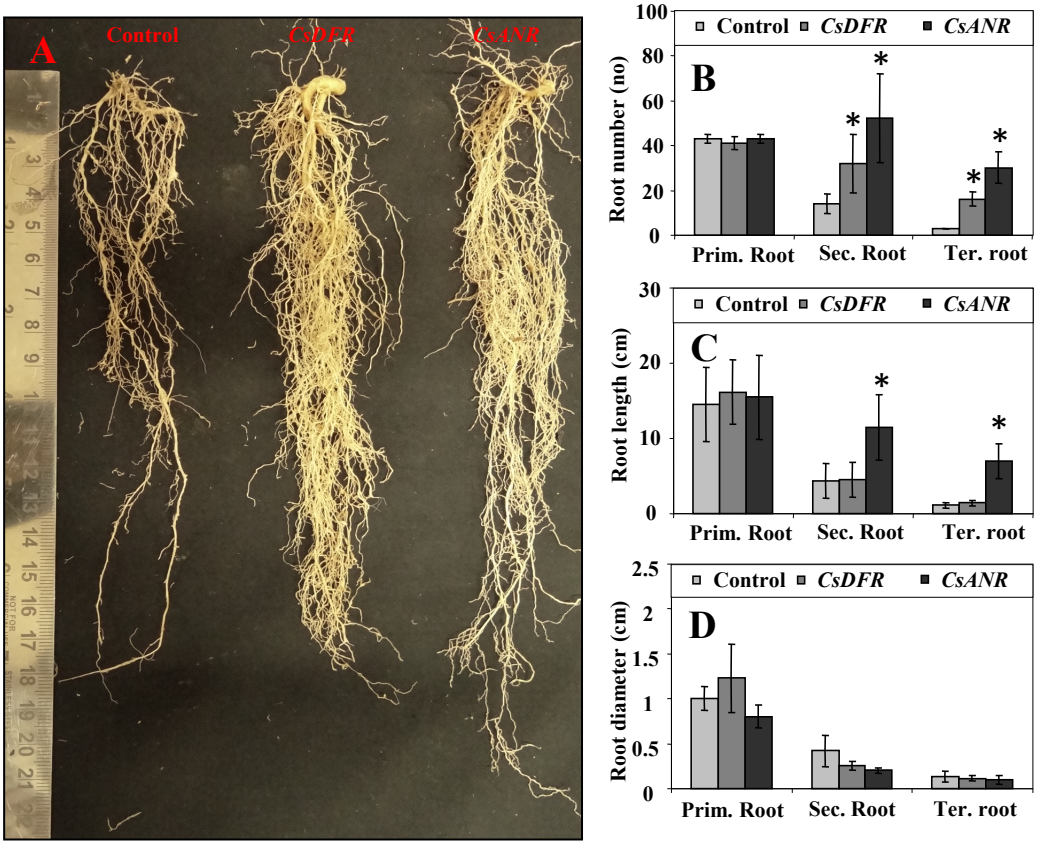

Fig. 3. Root architecture of tobacco plants overexpressing *CsDFR* and *CsANR* vis-à-vis control plant at different branching levels. (A) Pictures of individual first set of nodal roots elongating from lowermost node of crown of *CsDFR* and *CsANR* transgenic tobacco vis-à-vis control plants. The number (B), length (C) and diameter (D) of roots at different branching level of plants overexpressing *CsDFR* and *CsANR* vis-à-vis control tobacco plants. Data represents mean values ± SD (n =3). Statistical significance is indicated as (*) for P<0.05.

A larger root system of transgenic plants was clearly visible in soil grown plants (Fig. 2A). Root biomass as dry weight (DW) was estimated as 5.1 and 6.2 grams (g) in *CsDFR* and *CsANR* transgenic tobacco plants as compared to 4.2 g in control tobacco plants (Fig. 2B). Root to shoot biomass ratio was estimated as 0.27 and 0.26 in *CsDFR* and *CsANR* transgenic tobacco plants relative to 0.22 in control tobacco plants (Fig. 2C). Root volume was estimated as 40 and 41 cm^3 in *CsDFR* and *CsANR* transgenic tobacco as compared to 30 cm^3 in control tobacco plants (Fig 2D). The root number, length and diameter were also evaluated at different branching level. The influence of *CsDFR* and *CsANR* overexpression were significantly seen at individual crown roots with lateral roots (Fig. 3A). At secondary branching level, numbers of root were increased to 32 and 16 in *CsDFR* and *CsANR* transgenic tobacco as compared to 14 in control tobacco. Root numbers were increased to 16 and 30 in *CsDFR* and *CsANR* transgenic tobacco as compared to 3 in control tobacco at tertiary branching level of root (Fig. 3B). The root lengths at secondary and tertiary level of branching in *CsANR* were increased to 11.4 and 7 cm as compared to 4.43 and 1.1 cm in control tobacco plants. The root lengths of *CsDFR* transgenic tobaccos were not significantly increased as compared to control tobacco plants (Fig. 3C). While the diameters of root were not significantly changed in both types of transgenic (Fig. 3D).

Influence of CsDFR and CsANR overexpression on transcript expression of flavonoid biosynthetic pathway genes and flavonoids content in root of transgenic tobacco

The transcript expression analysis of flavonoid biosynthetic pathway genes such as *NtPAL*, *NtCHS*, *NtCHI*, *NtF3H, NtDFR*, *NtFLS, NtANR1, NtANR2* and *NtANS* indicates the functionality of this pathway in tobacco roots. So, the influence of *CsDFR* and *CsANR* overexpression on the transcript levels of various flavonoid biosynthetic pathway genes was tested in roots. The transcripts level of *NtPAL*, *NtCHI*

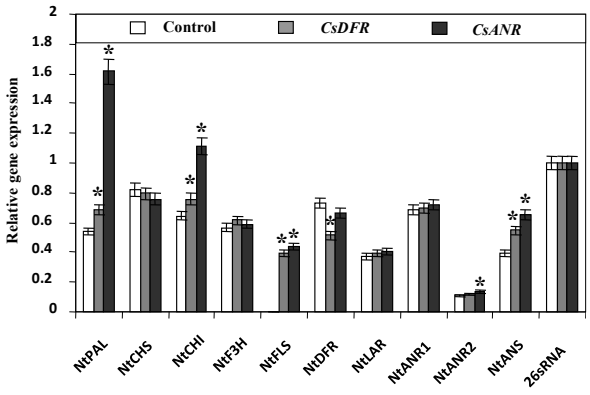

Fig. 4. Relative gene expression levels of flavonoid biosynthetic genes in roots of *CsDFR* and *CsANR* overexpressing and control tobacco plants. *PAL* Phenylalanine ammonia-lyase, *CHS* Chalcone synthase, *CHI* Chalcone isomerase, *F3H* Flavanone-3β-hydroxylase, *DFR* Dihydroflavonol 4-reductase, *FLS* Flavonol synthase, *LAR* Leucoanthocyanidin reductase, *ANR1* Anthocyanin reductase 1, *ANR2* Anthocyanin reductase 2, *ANS* Anthocyanin synthase. Data represents mean values ± SD (n =3). Statistical significance is indicated as (*) for P<0.05.

and *NtANS* was increased in roots of *CsDFR* and *CsANR* transgenic tobacco plants as compared to control tobacco plants (Fig. 4). The expression of *NtPAL* was upregulated by 35 % and 216 % in roots of *CsDFR* and *CsANR* transgenic tobacco plants relative to control tobacco plants. The expression of *CHI* was increased by 17 % and 72 % in roots of *CsDFR* and *CsANR* transgenic tobacco plants relative to control tobacco plants. Also, transcript level of *NtANS* was increased by 39 % and 66 % in roots of *CsDFR* and *CsANR* tobacco compared to control tobacco plants. The transcript level of *NtFLS* was not detected in roots of control tobacco plants. However, expression of *FLS* was activated in root portion of *CsDFR* and *CsANR* transgenic tobacco plants (Fig. 4). There was no significant change in expression of *NtCHS*, *NtF3H, NtLAR* and *NtANR1* in roots of *CsDFR* and *CsANR* transgenic tobacco plants relative to control tobacco plants. The transcript expression of *NtANR2* was increased only in roots of *CsANR* transgenic tobacco plants, while no significant change was observed in root of *CsDFR* transgenic tobacco plants as compared to control tobacco plants. The expression of *NtDFR* was decreased by 30 % and 10 % in roots of *CsDFR* and *CsANR* transgenic tobacco plants respectively compared to control tobacco plants (Fig. 4).

The modulation of expression of flavonoid biosynthetic pathway genes was emphasized to estimate flavonoids, proanthocyanidins and anthocyanins content in root portion of *CsDFR* and *CsANR* overexpressing tobacco plants as well as control tobacco plants. Staining with dimethylaminocinnamaldehyde (DMACA) reagent reflected more dark brown color in the roots of *CsDFR* and *CsANR* overexpressing tobacco than the controls, suggested the presence of more proanthocyanidins or precursors flavan-3-ols (Fig. 5A). Flavonoids content was estimated as 32.81 mg per 100 g DW in root of control tobacco plants, while estimated as 42.97 and 48.56 mg 100 g^{-1} DW in *CsDFR* and *CsANR* transgenic tobacco plants respectively (Fig. 5B). Proanthocyanidins content was estimated as 12.81 and 15.43 mg 100 g^{-1} DW in root of *CsDFR* and *CsANR* transgenic tobacco plants as compared to 11.36 mg 100 g^{-1} DW in control tobacco plants (Fig. 5C). While anthocyanins content was found to be decreased in both types of transgenics. Anthocyanins content was estimated as 4.58 µg g^{-1} DW in root of control tobacco plants, while reported as 2.36 and 3.96 µg g^{-1} DW in root of *CsDFR* and *CsANR* transgenic tobacco plants respectively (Fig. 5D). Analysis of flavan-3-ols in root of tobacco could identify EGC. The EGC was estimated as 16 and 34 µg 10 mg^{-1} DW in roots of *CsDFR* and *CsANR* overexpressing tobacco respectively, as compared to 3 µg 10 mg^{-1} DW in roots of control tobacco (Fig. 5E). The accumulation of flavonoids was also found higher in root tip of seedlings from *CsDFR* and *CsANR* compared to control as revealed by DMACA staining (Fig. 5F).

Overexpression of CsDFR and CsANR enhanced antioxidant potential of roots

To evaluate the influence of accumulated flavonoids on the antioxidant potential of root portion of plant, total scavenging activity of root extract of control as well as *CsDFR* and *CsANR* transgenic tobacco plants was carried by using DPPH radical assay method. The total scavenging activity was monitored as percentage inhibition of DPPH by the root extract of control as well as *CsDFR* and *CsANR* transgenic tobacco plants. The percentage inhibition of DPPH was estimated as 14.7 % and 14.5 % in root extract of *CsDFR* and *CsANR* tobacco plants compared to 6.25 % in root extract of control tobacco plants (Fig. 6), suggesting increase in antioxidant potential of tobacco root due to overexpression of these two cDNAs from tea.

Overexpression of CsDFR and CsANR provided tolerance against toxic Al stress

The tolerance ability of *CsDFR* and *CsANR* transgenic tobacco seedlings was tested against Al toxicity. Overexpression of *CsDFR* and *CsANR* provided

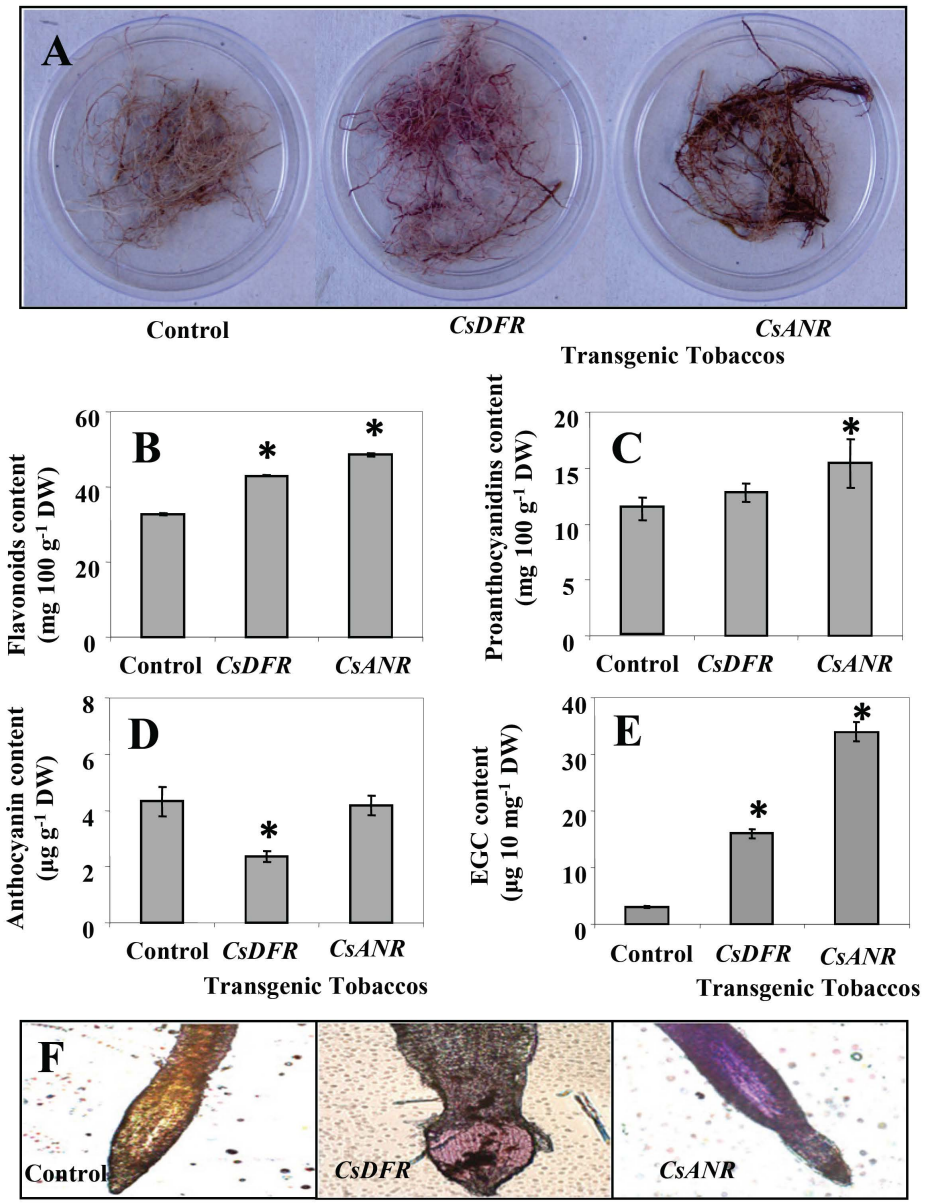

Fig. 5. Tobacco plants overexpressing *CsDFR* and *CsANR* show alteration in flavonoids content in root portion. (A) Histochemical staining of root revealed higher accumulation of flavan-3-ols/proanthocyanidins content. Total flavonoids (B), total proanthocyanidins (C), anthocyanins content (D) and epigallocatechin (EGC) (E) in roots of *CsDFR* and *CsANR* overexpressing tobacco and control tobacco plants. (F) The higher accumulation of flavonoids in root tip of 7 days old fresh *CsDFR* and *CsANR* seedlings relative to control seedlings revealed by DMACA staining. Data represents mean values ± SD (n =3). Statistical significance is indicated as (*) for $P<0.05$.

tolerance to transgenic tobacco against Al toxicity (Fig. 7). Seven days old seedlings were used to evaluate Al toxicity of two concentrations 1 and 5 mM for 14 days. At 1 mM Al, seedlings of *CsDFR* and *CsANR* transgenic tobacco show better growth as compared to control tobacco plants. At 5 mM Al, growth of the control seedlings was severely affected while transgenics still grow healthier.

Discussion

Tea (*Camellia sinensis*) is one of the highest producers of flavonoids among plants. Studies on understanding the expression and activity of flavo-

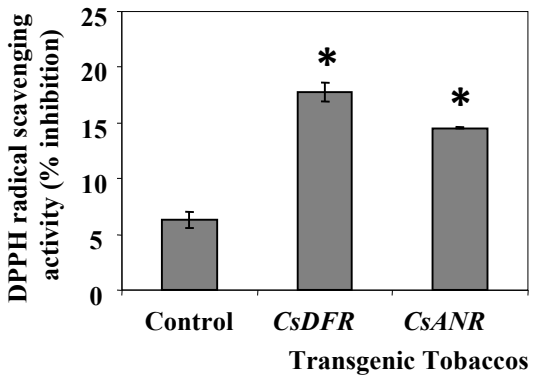

Fig. 6. Root extract from *CsDFR* and *CsANR* overexpressing tobacco showed higher DPPH radical scavenging activity compared to control tobacco plants. Data represents mean values ± SD (n =3). Statistical significance is indicated as (*) for P<0.05.

noid biosynthesis has been conducted in relation to flavonoids content in tea plant (Punyasiri et al. 2004, Singh et al. 2008, Singh et al. 2009a; b). They have suggested the important regulatory role of DFR and ANR enzymes in the flavonoids biosynthesis. Hence, we selected *CsDFR* and *CsANR* cDNAs to raise transgenic for analyzing their influence on flavonoids content in tobacco roots.

Tobacco plants overexpressing CsDFR and CsANR showed improved root morphological features

Recent advances have revealed that flavonoids play important role in many aspects of the adaptation. They are particularly crucial for plant growth and development in response to environment challenges (Treutter et al. 2006). Flavonoids play important roles in regulating plant growth and development throughout the life cycle, including seed germination, root branching and reproduction (Shirley 1998, Brown et al. 2001; Schijlen et al. 2007). Overexpression of *CsDFR* and *CsANR* has increased the root proliferation in tobacco analyzed in the form of highly branched root system, increased in root biomass and volume. Transgenics showed higher root-to-shoot biomass ratio. Thus, these changes improved the root surface area and might be responsible for better minerals absorption. The enlarged root system is very important for providing tolerance against stresses (Werner et al. 2010). Plants with larger root systems also increase their ability to survive under nutrient deficiency (Coque and Gallais 2006). Flavonoid pathway mutants were affected at various traits including alteration to root growth, lateral root density, root hair development and length (Buer et al. 2010). The flavonoids-mediated changes in root architecture will provide more resilient responses to environmental changes.

Overexpression of CsDFR and CsANR modulated transcript expression of flavonoid biosynthetic pathway genes and flavonoids content in root of transgenic tobacco

The diversion of flux between branches of the flavonoid pathway has been observed earlier by using metabolic engineering strategy (Xie et al. 2004; Besseau et al. 2007; Nakatsuka et al. 2007; Kovinich et al. 2011). To check the influence of overexpression of *CsDFR* and *CsANR* on diversion of flux between braches of the flavonoid pathway at molecular level, expression analysis of some flavonoid biosynthetic pathway genes was carried out in transgenic tobacco. The transcript levels of *NtPAL*, *NtCHI*, *NtFLS* and

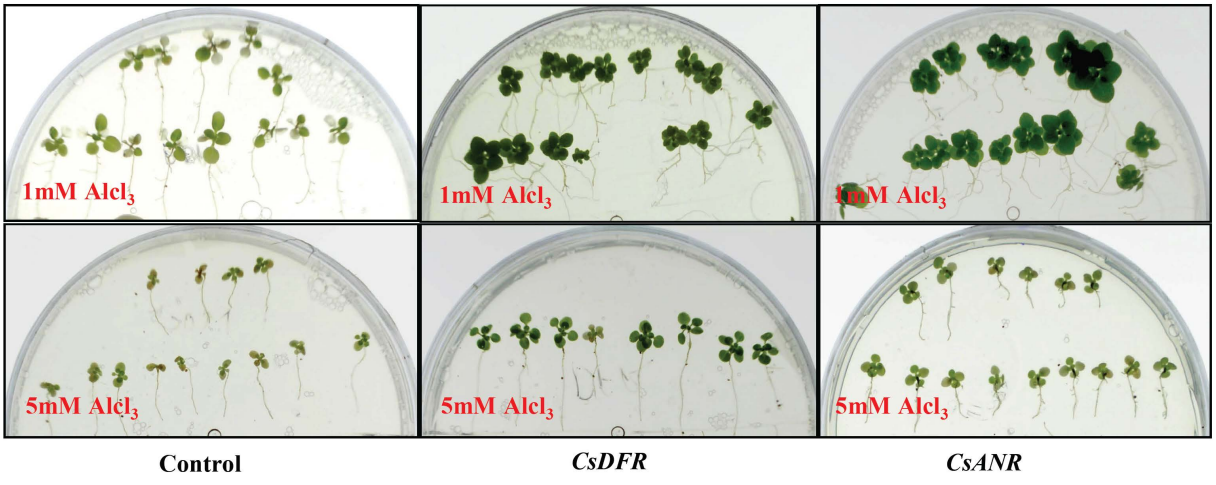

Fig. 7. Better growth of seedlings of *CsDFR* and *CsANR* transgenic plants relative to control after 14 days of aluminum exposure.

NtANS genes were upregulated in root of both transgenic tobacco overexpressing *CsDFR* and *CsANR*. The modulation of flavonid biosynthetic pathway genes expression has earlier been reported upon overexpression/silencing of genes from same pathway in different parts of plant except root portion (Xie et al. 2004; Takahashi et al. 2006; Xie et al. 2006; Kovinich et al. 2011).

Dark brown colors of roots from *CsDFR* and *CsANR* transgenic tobacco by DMACA staining, confirmed presence of polymeric proanthocyanidins, or precursors flavan-3-ols. Total flavonoids, total proanthocyanidins and flavan-3-ols as epigallocatechin were found higher and anthocyanins content was lower in root portion of *CsDFR* and *CsANR* transgenic as compared to control tobacco plants. Thus, the decreased anthocyanins and increased flavan-3-ols/proanthocyanidins indicate the diversion of carbon flux towards flavan-3-ols/proanthocyanidin biosynthesis in roots of *CsDFR* and *CsANR* overexpressing tobacco. Similar diversion has earlier been reported in plant shoots (Han et al. 2012; Kumar et al. 2013b). Also, the ectopic expression of apple *MdANR* gene has been reported for higher proanthocyanidins and lower anthocyanins in tobacco flower (Han et al. 2012). But the ectopic expression of *PtrDFR1* has been reported to increase anthocyanins content, while ectopic expression of *PtrDFR2* did not make any significant change in anthocyanins content of tobacco flower (Huang et al. 2012). These two DFR proteins of same orthology were reported for different responses on flavonoids. While the roots of transgenic tobacco overexpressing *CsDFR* in this study showed decrease in anthocyanins content and increase in flavonoids content.

Different flavan-3-ols are known to be present in roots of *Fragaria* (strawberry), *Malus* (apple), *Rosa* (rose), *Pyrus* (pear) and *Prunus* (plum) (Hoffmann et al. 2011). In fresh leaves of tea plant, major flavan-3-ols are GC, EGC, Cat, EC, EGCG, and ECG. But Only EC was detected in root of tea plants (Liu et al. 2009). Interestingly, only EGC was detected in tobacco root and its level was found to be increased in *CsDFR* and *CsANR* overexpressing tobacco as compared to control tobacco. This has documented that overexpression of *CsDFR* and *CsANR* has increased the capacity of tobacco root to synthesized flavan-3-ols in the form of EGC. Flavonoids accumulated at the root tip and in root cap cells are known as potential regulator of auxin transport (Hassan and Mathesius 2012). The flavonoid-deficient mutants *tt4*, *tt5* and *tt6* defective in CHS, CHI and F3H enzymes of flavonoid biosynthetic pathway respectively have been reported to alter auxin tran sport rate and defective in lateral root formation and gravitropism (Brown et al. 2001; Buer et al. 2010). In present study, the flavonoids are also accumulated at root tip due to the influence of *CsDFR* and *CsANR* overexpression in transgenic tobacco.

Thus the modulation in flavonoids content and in the level of expression of flavonoids pathway genes due to overexpression of *CsDFR* and *CsANR* transgenes have proved a coordinated control of transcription of flavonoid biosynthetic pathway in root portion of tobacco leading to increase in production of flavonoids.

Increased flavonoids enhanced antioxidant potential of CsDFR and CsANR tobacco

Flavonoids are involved in various biochemical and physiological activities in roots of various plant species. Flavonols are identified as natural inhibitor of auxin transport and influenced auxin-controlled developmental process of root such as lateral root growth and nodule formation in *Medicago truncatula* (Imin et al. 2007). On the other hand, flavan-3-ols and their polymeric condensation product, proanthocyanidins behave as antioxidant via several mechanisms as scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes (Aron and Kennedy 2008; Tolrà et al. 2009). The secretions of flavan-3-ols from *Centuria maculosa* provide protection against pathogen attacks and show allelopathic effect (Bais et al. 2006). Their strong antioxidant and free radical scavenging characters make them ideal to protect plant or its portion against oxidative damage mediated by environmental cues. Thus, improved antioxidant potential of roots of tobacco could be due to increase in flavonoids of tobacco overexpressing *CsDFR* and *CsANR*.

Overexpresison of CsDFR and CsANR provided tolerance against Al toxicity

In acid soils, toxic levels of Al has reduced crop yield through root growth inhibition resulting in poor nutrient and water uptake. The major symptom of Al injury is the inhibition of root elongation (Eticha et al. 2005; Wang and Kao 2007). Flavonoids are reported to be involved in providing tolerance to plants against Al toxicity (Tolrà et al. 2005; Morita et al. 2008; Tolrà et al. 2009; Duressa et al. 2010). The transient proliferation of cells accumulating proanthocyanidins on the epidermal apex have contributed to Al resistant root elongation in Camphor tree (Osawa et al. 2011). The flavonoids provided Al-tolerance by two mechanisms as Al chelation and antioxidation (Tolrà et al. 2005; Morita et al. 2008). The high content of flavonoids in tea is known to provide tolerance to Al by detoxification through antioxidation mechanism

(Tolrà et al. 2005; Morita et al. 2008; Mukhopadyay et al. 2012). Hence, the increase in flavonoids content of root of transgenic tobacco seedlings overexpressing *CsDFR* and *CsANR* gene from tea has provided tolerance against toxic Al level.

In conclusions, we generated *CsDFR* and *CsANR* transgenic tobacco with improved root morphology, increased flavonoids, and proanthocyanidins content. The increased flavonoids and proanthocyanidins content might be responsible for higher DPPH radical scavenging activity in root portion of *CsDFR* and *CsANR* overexpressing tobacco. This genetic manipulation has provided tolerance against Al toxicity and may prove to be an effective technology for agricultural improvement.

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