Pyramiding of tea *Dihydroflavonol reductase* and *Anthocyanidin reductase* increases flavan-3-ols and improves protective ability under stress conditions in tobacco

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Abstract Tea (*Camellia sinensis*) is one of the richest sources of flavan-3-ols, an important class of flavonoids. The expression level of gene-encoded key regulatory enzymes of flavan-3-ol/anthocyanin biosynthetic pathway, dihydroflavonol 4-reductase (DFR) and anthocyanidin reductase (ANR), has been highly correlated with the flavan-3-ol contents and antioxidant activity in tea plant. In the present study, pyramiding of *CsDFR* and *CsANR* in tobacco was achieved. However, single transgenic tobacco overexpressing either *CsDFR* or *CsANR* was documented earlier. In continuation, pyramided transgenic lines were evaluated for the possible, either same or beyond, effect on flavan-3-ol accumulation and protective ability against biotic and abiotic stresses. The pyramided transgenic lines showed early flowering and improved seed yield. The transcript levels of flavan-3-ol/anthocyanin biosynthetic pathway and related genes in pyramided transgenic lines were upregulated as compared to control tobacco plants. The accumulations of flavan-3-ols were also found to be higher in pyramided transgenic lines than control tobacco

plants. In contrast, anthocyanin content was observed to be decreased in pyramided transgenic lines, while DPPH activity was higher in pyramided transgenic lines. In pyramided transgenic lines, strong protective ability against feeding by *Spodoptera litura* was documented. The seeds of pyramided transgenic lines were also found to have better germination rate under aluminum toxicity as compared to control tobacco plants. Interestingly, the synergistic effect of these two selected genes are not beyond from transgenic lines expressing either *CsDFR* and *CsANR* alone as published earlier in terms of flavan-3-ols accumulation. However, the unique flower color and better seed germination rate are some interestingly comparable differences that were reported in pyramided lines in relation to individual transgenic plants. In conclusion, the present results reveal an interesting dynamic between *CsDFR* and *CsANR* in modulating flavan-3-ol/anthocyanin levels and functional analysis of stacked *CsDFR* and *CsANR* transgenic tobacco lines.

Keywords *Camellia sinensis* · Flavan-3-ols · Transgenic tobacco · Early flowering · Antioxidants · *Spodoptera litura* · Aluminum toxicity

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Abbreviations

4CL	4-coumarate: CoA ligase
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
AN2	Anthocyanin 2
ANR1	Anthocyanidin reductase 1
ANR2	Anthocyanidin reductase 2
ANS	Anthocyanidin synthase
C4H	Chalcone-4-hydrolase

Cat	Catechin
cDNA	Complementary deoxyribonucleic acid
CHI	Chalcone isomerase
CHS	Chalcone synthase
DFR	Dihydroflavonol 4-reductase
DPPH	2,2-diphenyl-1-picryl-hydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
F3H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
MS	Murashige and Skoog medium
Nt	<i>Nicotiana tabacum</i>
PAL	Phenylalanine lyase
PCR	Polymerase chain reaction
RT	Reverse transcriptase
ROS	Reactive oxygen species
TT1	Transparent testa 1
TT2	Transparent testa 2

Fig. 1 Basic representation of flavan-3-ol/anthocyanin biosynthetic pathway in plants. The enzymes are represented in abbreviated form: *CHS* chalcone synthase, *CHI* chalcone isomerase, *DFR* dihydroflavonol 4-reductase, *F3H* flavanone-3-hydroxylase, *LAR* leucoanthocyanidin reductase, *ANR1* anthocyanin reductase 1, *ANR2* anthocyanin reductase 2, *ANS* anthocyanin synthase, *GTs* glucosyl transferase. The tea cDNA-encoded enzymes (red color) were used to be overexpressed simultaneously in transgenic tobacco plants

Introduction

Flavonoids represent the second largest group of plant secondary metabolites after alkaloids. Flavonoids are divided into several classes including anthocyanins and flavan-3-ols, and are abundant in different tissues of wide variety of plants (Kumar and Yadav 2013a; Ogo et al. 2013). The flavan-3-ols class of flavonoids is extensively investigated and involved in various biological processes including auxin transport regulation, seed development, pollen viability, and in defense responses under adverse conditions (Mahajan et al. 2011a, b, 2012; Falcone Ferreyra et al. 2012; Kumar and Yadav 2013b; Hammerbacher et al. 2014; Mahajan and Yadav 2014; Nakabayashi et al. 2014). The strong antioxidant activities exhibited by flavan-3-ols also contribute to tolerance against various environmental constraints (Mierziak et al. 2014; Nakabayashi et al. 2014). The flavan-3-ols are also well known for their interaction with proteins (Mierziak et al. 2014). In conclusion, flavan-3-ols, a class of flavonoids, are key metabolites for development and fitness of plants.

The biosynthesis of flavan-3-ols and anthocyanins is well investigated in terms of molecular genetics as well as biological mechanisms of the key regulatory enzymes in *Arabidopsis* (Dixon and Pasinetti 2010). The flavan-3-ol/anthocyanin biosynthetic pathway that leads to the production of flavan-3-ols and anthocyanins in plants is summarized in Fig. 1. The p-coumarate CoA, a product of phenylpropanoid pathway, condenses with three molecules of malonyl CoA to form dihydroflavonols using *CHS*

(chalcone synthase), *CHI* (chalcone isomerase), and *F3H* (flavanone 3-hydroxylase) enzymes (Pang et al. 2013). Dihydroflavonol 4-reductase (*DFR*) catalyzes dihydroflavonol to leucoanthocyanidins which are further converted to anthocyanidins by *ANS* (anthocyanidin synthase) enzyme (Singh et al. 2009a, b). The dihydroflavonols can also be catalyzed by *FLS* (flavonol synthase) to produce flavonols which is a separate class of flavonoids. Therefore, *DFR* is considered as a branch key enzyme that controls the carbon flux direction toward biosynthesis of both flavan-3-ols and anthocyanins or either of them (Singh et al. 2009a, b). Other key branch enzymes of this pathway are identified as leucoanthocyanidin reductase (*LAR*) and anthocyanidin reductase (*ANR*), which catalyze the formation of catechins and epicatechins from leucoanthocyanidins and anthocyanidins, respectively (Pang et al. 2013). The anthocyanidins serve as the common substrate in the biosynthesis of flavan-3-ols as well as anthocyanins. The *LAR* and *ANR* enzymes divert the carbon flux towards biosynthesis of flavan-3-ols at the cost of anthocyanins. Among these regulatory enzymes, the evidence for *LAR* function is still unclear and less convincing than it is for *DFR* and *ANR* enzymes (Pang et al. 2013).

Flavan-3-ols/anthocyanins classes of flavonoids are well known for their higher antioxidant activity and are found to offer protection against major diseases in animals and plants. In addition, flavan-3-ols/anthocyanidins are also well-characterized nutrient ingredients in a number of fruits, which have attracted the attention of researchers to

improve the antioxidant potential of plants by manipulations of flavan-3-ol contents (Wang et al. 2011; Kumar et al. 2013; Nakabayashi et al. 2014; Li et al. 2016; He et al. 2016; Rihani et al. 2017). The key rate-limiting enzymes of flavan-3-ol/anthocyanin biosynthetic pathway have been identified to allow their exploitation for metabolic engineering to improve the overall antioxidant potential of plants (Wang et al. 2011). A number of transcription factor genes have also been characterized for understanding the regulation mechanism of flavan-ol/anthocyanin biosynthetic pathway (Pang et al. 2013; Li et al. 2016; Pérez-Díaz et al. 2016; Rihani et al. 2017). Thus, this pathway is a special target for enhancement of antioxidant capacity via manipulation of flavan-3-ols in agricultural crops (Pang et al. 2013).

The metabolic engineering approach has highlighted two major strategies for the manipulations of flavan-3-ol contents in transgenic plants. The first strategy is to upregulate the pathway for flavan-3-ol production and second is to downregulate the competing branch pathways (Mahajan et al. 2011b, 2012; Yuan et al. 2012; Pang et al. 2013; Mierziak et al. 2014; Li et al. 2016; He et al. 2016; Lim et al. 2016; Pérez-Díaz et al. 2016; Rihani et al. 2017). The transgenic plants have been developed using either introduction of biosynthetic or regulatory genes from diverse plants to increase flavan-3-ol contents (Han et al. 2012; Hancock et al. 2012; Yuan et al. 2012; Kumar et al. 2013; Mahajan and Yadav 2014; Li et al. 2016; He et al. 2016; Pérez-Díaz et al. 2016; Rihani et al. 2017). The overexpression of *DFR* genes (*DFR1* and *DFR2*) from *Populus trichocarpa* in tobacco and *P. tomentosa* has resulted in the accumulation of anthocyanins and monomeric and polymeric flavan-3-ol metabolites (Huang et al. 2012). Similarly, overexpression of wheat *DFR* gene increases anthocyanin accumulation in an Arabidopsis *dfr* mutant (Shin et al. 2016). The *ANR* from *Malus domestica* has also been investigated for flavan-3-ol content in transgenic tobacco (Han et al. 2012). Similarly, transgenic tobacco overexpressing *Rosa rugosa ANR* has been documented with improved tolerance against abiotic stress (Luo et al. 2016). The engineering of flavonoid biosynthetic pathway by co-expression of biosynthetic gene (*ANR*) and regulatory gene (*PAP1*) has also provided the evidence regarding the use of combinatorial approach for enhancing flavan-3-ol contents (Xie et al. 2006). On the other hand, different strategies including RNAi have been applied to downregulate the gene(s) encoding key competing enzyme(s) of biosynthetic pathway (Mahajan et al. 2011b; Jiang et al. 2013; Mahajan and Yadav 2014; Lim et al. 2016). The suppression of endogenous *ANR* genes (*ANR1* and *ANR2*) has been documented with higher anthocyanin contents and lower polymeric flavan-3-ol content in soybean grain using redirection of metabolic flux (Kovnich

et al. 2012). The strawberry fruits with enhanced flavan-3-ol level have been achieved through downregulation of *anthocyanidin GT* gene that also relies on the concept of redirection of metabolite flux toward biosynthesis of flavan-3-ols (Griesser et al. 2008). The suppression of *DFR* in tobacco has also been achieved with white flowers with altered flavan-3-ols/anthocyanins (Lim et al. 2016).

Nowadays, attention is being centralized on flavan-3-ol/anthocyanin biosynthetic pathway of tea plant (*C. sinensis*) because of extraordinary flavan-3-ol contents in their fresh foliar tissues that are being exploited for commercial purposes (Kumar et al. 2013). The genes encoding enzymes of flavan-3-ol biosynthetic pathway have been isolated and identified (Singh et al. 2009a, b; Pang et al. 2013). In continuation of these works, we also documented that the transgenic tobacco lines either *CsDFR* or *CsANR* cDNA from tea showed higher accumulation of flavan-3-ol content, early flowering, enhanced seed yield, improved total free radical scavenging activity, lesser feeding by *S. litura*, and better tolerance against Al toxicity (Kumar et al. 2013; Kumar and Yadav 2013b). In addition, we also correlated the relationship of *CsANR* overexpression in tobacco with higher accumulation of flavan-3-ols with lesser anthocyanin levels (Kumar and Yadav 2013a). Overall, these studies collectively help establish the potential role of *CsANR* and *CsDFR* in the accumulation of flavan-3-ol/anthocyanin content in tea plant.

A recombinant biosynthetic pathway carrying a cluster of *CsF3H*, *CsDFR*, and *CsLAR* genes has been designed and studied in relation to flavan-3-ol accumulation in *E. coli* (Umar et al. 2012). However, a number of studies have been conducted on the altered flavan-3-ol/anthocyanin contents in transgenic plants overexpressing individual transgene(s). Interestingly, there is a lack of studies on the combinatorial use of many genes simultaneously from plants including *C. sinensis* to analyze for a synchronized effect on manipulations of flavan-3-ols in plant systems. Thus, the idea of generation of pyramided transgenic lines overexpressing *CsDFR* and *CsANR* was implemented for evaluation purpose in relation to flavan-3-ol accumulation and for analyzing their combinatorial influence on the improvement of overall antioxidant potential. The pyramided transgenic tobacco lines were generated by cross-breeding approach and found to show improved flavan-3-ol contents, early flowering, better seed yield with slightly higher protection response against *S. litura* as compared to tobacco plants overexpressing *CsDFR* or *CsANR* alone. The pyramided transgenic lines were also documented for better germination rate under exposure of aluminum (Al) toxicity. To the best of our knowledge, the stacking of tea genes, especially genes encoding *DFR* and *ANR*, in tobacco through cross-pollination approach is the first documentation of its own kind.

This work will also help to analyze the combinatorial effect of *CsDFR* and *CSANR* in pyramided transgenic lines over already published single transgenic lines, comparatively. This system also offers an opportunity to check the feasibility of pyramided transgenic approach for overaccumulation of flavan-3-ols over single transgenic lines. This report could be a step ahead toward establishing an alternative potential system for flavan-3-ol accumulation by genetic transformation approach in plant systems. Taking together, these findings indicate that elevated flavan-3-ol biosynthesis was mediated by simultaneous overexpression of *CsDFR* and *CsANR*, which thus improved the protective ability of transgenic tobacco plants under stress conditions.

Materials and methods

Plant materials, generation, and confirmation of pyramided transgenic tobaccos

Nicotiana tabacum vc. Xanthi was employed in all the experiments, because it is an excellent model system for transgenic generation and evaluation purposes. The transgenic tobacco plants overexpressing *CsDFR* and *CsANR* individually were generated by *Agrobacterium tumefaciens*-mediated transformation (Kumar and Yadav 2013a, b; Kumar et al. 2013). The stacking of *CsDFR* and *CsANR* in the same tobacco plant was developed using conventional breeding approach. The reciprocal crosses were obtained via fertilization of emasculated *CsDFR* (from collected seeds of D-15 homogenous transgenic lines)-overexpressing tobacco plants with pollens from *CsANR* (from collected seeds of A-05 homogenous transgenic lines)-overexpressing tobacco plants and vice versa. The D-15 (from *CsDFR* transgenic lines) and A-05 (from *CsANR* transgenic lines) lines were selected for crosses because they showed overall better response than the other generated transgenic tobacco lines (Kumar et al. 2013).

The research material was utilized from F2 generation. Pyramided transgenic F1 and F2 generation lines were selected by germination and growth on MS media with hygromycin antibiotic (50 mg/ml). Genomic DNA and semiquantitative PCR methods were used to confirm the integration and expression of *CsDFR* and *CsANR* transgenes in leaf portion of pyramided transgenic tobacco plants, respectively. The two confirmed positive pyramided transgenic lines of ♂*CsDFR* × ♀*CsANR* cross (DA-01 and DA-02) and ♂*CsANR* × ♀*CsDFR* cross (AD-01 and AD-02) were evaluated for various experiments.

RNA isolation and semiquantitative PCR

Total RNA was extracted from the leaf tissues of transgenic and control tobacco plants and was used for cDNA synthesis. RT-PCR experiments were performed (Kumar et al. 2013). The sequences of selected primers of *NtPAL*, *NtC4H*, *Nt4CL*, *NtCHS*, *NtCHI*, *NtF3H*, *NtDFR*, *NtFLS*, *NtANR1*, *NtANR2*, *NtANS*, *NtTT1*, *NtTT2*, and *NtAN2* genes, and their respective annealing temperature are shown in Table S1. The primers corresponding to 26S rRNA-based internal control were used for semiquantitative expression analysis (Singh et al. 2004).

Evaluation of transgenic lines for morphological and yield parameters

The parameters such as days for flowering time, capsules number, seed yield, and thousand seed weight (grams) were analyzed as described previously in seven different plants with similar height from each selected line of both crosses (Kumar et al. 2013). Briefly, capsules numbers were counted at full maturation, and seed yield and thousand seed weight (grams) were measured after harvesting.

Estimation of flavan-3-ol content

Samples were prepared from the leaf tissues of selected transgenic tobacco lines and control tobacco plants and used for quantification of end products of flavan-3-ol biosynthetic pathway, flavan-3-ols by HPLC method as described previously (Kumar et al. 2013). The different flavan-3-ols, namely (+)-Cat, (−)-EC, (−)-EGC, and (−)-ECG, were used as references for estimation of respective constituent and purchased from Sigma-Aldrich, USA.

Anthocyanin content estimation

Total anthocyanin content was estimated from fresh fully opened flowers of transgenic tobacco lines and control tobacco plants (Kumar and Yadav 2013a). Briefly, anthocyanins were extracted in acidic HCl and absorbance of extracts was measured at 530 nm by UV spectrophotometer. The content was calculated as cyanidin equivalents using extinction coefficient 29,500 mol^{−1}cm^{−1}.

Estimation of antioxidant potential

The antioxidant activity of the methanolic extracts from leaves of pyramided transgenic tobacco plants and control tobacco plants was measured using DPPH (diphenylpicrylhydrazyl) radical scavenging assay (Kumar et al. 2013).

Leaf disc non-choice experiment

The feeding behavior of *Spodoptera litura* (tobacco cutworms) was analyzed by preparing leaf discs as feeding material (6 cm diameter) from transgenic lines and control tobacco plants, and the experiment was performed as described earlier (Kumar et al. 2013).

Evaluation of seed germination under exposure of Aluminum toxicity

For germination study, control and transgenic tobacco seeds were surface sterilized, sown on half-strength Murashige and Skoog (MS) medium with 1 and 5 mM concentrations of Al in Petri dishes. Germination was recorded every day up to seven days to assess establishment of early seedlings under Al toxicity, and the plates were photographed.

Results

Generation and confirmation of pyramided transgenic tobacco plants overexpressing *CsDFR* and *CsANR* cDNAs

The homozygous transgenic individual tobacco lines of *CsDFR* (D-15) and *CsANR* (A-05) were selected for reciprocal crossing (Kumar et al. 2013). Seeds were harvested from mature pods of crossed lines of each combination (*CsDFR* ♂ × *CsANR* ♀ and *CsANR* ♀ × *CsDFR* ♂) and germinated on 0.8% agar medium. One-month-old 10–15 independent plants from two pyramided transgenic tobacco lines from both the combinations were acclimatized and shifted to greenhouse. There was no morphological difference between the *CsDFR/CsANR* overexpressing transgenic plants generated by reciprocal crosses with each other. Thus, there was no problem with transmission of either *CsDFR* or *CsANR* through male and female gametes. Three plants from each reciprocal cross and one plant from each of individual gene expressing transgenic line as well as control tobacco plants were used to confirm the integration and expression of *CsDFR* and *CsANR* transgenes.

The integration of *CsDFR* and *CsANR* transgenes in pyramided transgenic plants was confirmed with genomic DNA PCR using gene-specific primers. Initially, PCR with primers specific to *CsDFR* transgene confirmed the presence of *CsDFR* transgene in all selected pyramided transgenic tobacco lines as well as in selected *CsDFR* overexpressing transgenic tobacco plant. The *CsDFR* transgene was not present in selected *CsANR* overexpressing tobacco plant (Fig. 2a). Another PCR with

Fig. 2 Confirmation of pyramided transgenic tobacco plants by Genomic DNA and semiquantitative PCR and evaluation of confirmed lines for flowering time and yield parameters. **a** Genomic DNA PCR was used to confirm the integration of *CsDFR* and *CsANR* genes in genome of pyramided transgenic tobacco plants. **b** The transcript expression levels of both (*CsDFR* and *CsANR*) in transgenic tobacco lines were carried out using semiquantitative PCR. These experiments were carried out in three technical replicates. **c** The pyramided transgenic lines and control were kept in transgenic containment facility. **d** The days to flowering (days), capsule yield, seed yield, and seed weight per thousand seed (grams) in pyramided transgenic lines generated from reciprocal crosses in relation to control tobacco plants. Mean ± SD from three replications are represented

primers specific to *CsANR* transgene confirmed the presence of *CsANR* transgene in all selected pyramided transgenic tobacco lines as well as in selected *CsANR* overexpressing transgenic tobacco plant. The *CsANR* transgene was not present in selected *CsDFR* overexpressing tobacco plant (Fig. 2a). Thus, all selected tobacco plants from both crosses were found to be PCR positive for both *CsDFR* and *CsANR* transgenes.

Similarly, expressions of both *CsDFR* and *CsANR* transgenes in all selected pyramided transgenic lines were also confirmed using semiquantitative PCR. Both the transgenes were observed with their expressions in pyramided transgenic tobacco plants (Fig. 2b).

Pyramided transgenic tobacco lines overexpressing *CsDFR*-*CsANR* documented for early flowering and improved yields

The pyramided transgenic tobacco lines and control plants were morphologically analyzed for height, stem diameter, and days to flowering with significant changes. However, no significant changes in growth pattern were observed in pyramided transgenic tobacco lines as compared to control tobacco plants (Fig. 2c, d). The pyramided transgenic lines were observed for early flowering after an average of 95 (*CsDFR* ♂ × *CsANR* ♀) and 87 (*CsANR* ♀ × *CsDFR* ♂) days compared to 105 days (for control tobacco plants) after seed germination (Fig. 2d). Both reciprocal crosses did not show significant change in plant height, stem diameter, and number of leaves as compared to control tobacco plants, which were measured at the flowering time. However, the pyramided transgenic lines were documented for higher number of fruits/capsules per plant, increased seed yield per plant, and weight of seed (grams) compared to control tobacco plants. The number of fruits per plant on an average were 51.5 (*CsDFR* ♂ × *CsANR* ♀) and 55.2 (*CsANR* ♀ × *CsDFR* ♂) as compared to 40.55 in control tobacco plants (Fig. 2d). The seed yield per plant was measured as 890 (*CsDFR* ♂ × *CsANR* ♀) and 1140 (*CsANR* ♀ × *CsDFR* ♂), compared to 747 mg in control tobacco plants. Weight of thousand seeds was 94 and 88 in pyramided transgenic lines, in contrast to only 70.5 mg calculated in control tobacco plants (Fig. 2d).

Of the confirmed pyramided transgenic tobacco lines, two transgenic lines DA-01 and DA-02 (from *CsDFR* ♂ × *CsANR* ♀ cross) and AD-01 and AD-02 (from *CsANR* ♀ × *CsDFR* ♂ cross) along with control tobacco plants were subsequently used for further analysis.

Increased transcript expression of flavan-3-ol/anthocyanin biosynthetic pathway genes in pyramided transgenic tobacco plants

To check the influence of simultaneous overexpression of both *CsDFR* and *CsANR* transgenes on the transcript level of various endogenous genes of flavan-3-ol/anthocyanin biosynthetic pathway, expression analysis of the leaf tissues of DA-01, DA-02, AD-01, and AD-02 transgenic lines vis-à-vis control tobacco plants was conducted. The expression of selected flavan-3-ol/anthocyanin biosynthetic endogenous genes (*NtPAL*, *NtCHS*, *NtCHI*, *NtF3H*, *NtDFR*, *NtFLS*, *NtANR1*, *NtANR2*, *NtLAR*, and *NtANS*) was found to be upregulated in pyramided transgenic tobacco plants as compared to control tobacco plants.

The transcript expression of *NtPAL* gene was observed to be increased by 95, 117, 119, and 112% in DA-01, DA-02, AD-01, and AD-02 pyramided transgenic lines as

compared to control tobacco plants (Fig. 3a). In pyramided transgenic tobacco lines, the transcript expression of *NtCHS* gene was upregulated by approximately 52% (Fig. 3b). Similarly, transcript expression of *NtCHI* gene was also increased by 20–49% in all selected pyramided transgenic lines (Fig. 3c). The transcript expression of *NtF3H* gene was increased by 97, 110, 99, and 54% in DA-01, DA-02, AD-01, and AD-02 as compared to control tobacco plants (Fig. 3d). The transcript expression of *NtDFR* gene was observed to be upregulated in the range of 20–40% in all selected pyramided transgenic tobaccos as compared to control tobacco plants (Fig. 3e). Similarly, *NtFLS* gene was observed to be increased by 26–38% in all selected pyramided transgenic lines (Fig. 3f). The transcript expression of *NtANR1* gene was upregulated by 20, 28, 29, and 79% in selected pyramided lines with respect to control tobacco plants (Fig. 3g). The transcript expression of *NtANR2* gene was observed to be upregulated by 12–28% in pyramided transgenic lines (Fig. 3h). Transcript expression of *NtLAR* gene was upregulated by 13–21% in pyramided transgenic lines as compared to control tobacco plants (Fig. 3i). Similarly, *NtANS* gene was observed to be upregulated in pyramided transgenic lines by 12–41% (Fig. 3j). Hence, the simultaneous overexpression of both *CsDFR* and *CsANR* transgenes in pyramided transgenic tobacco plants has led to an increase in the endogenous expression of all the flavan-3-ol/anthocyanin biosynthetic pathway genes. However, individual transgenic tobacco overexpressing either *CsDFR* or *CsANR* transgene was observed to increase the expression of only *NtCHS* and *NtANR2* genes (Kumar et al. 2013).

Similarly, the transcript expressions of gene-encoded flavan-3-ol/anthocyanin-related important transcription factors (*NtTT1*, *NtTT20*, and *NtAN2*) are also analyzed in all selected pyramided transgenic lines vis-à-vis control tobacco plants. The transcript expression of *NtTT1* gene was observed to be increased by 122, 112, 125, and 167% in DA-01, DA-02, AD-01, and AD-02 as compared to control tobacco plants (Fig. 4a). The transcript expression of *NtTT20* gene was upregulated by 117–157% in pyramided transgenic lines as compared to control tobacco plants (Fig. 4b). The transcript expression of *NtAN2* was found to be upregulated by 106–154% in selected lines as compared to control tobacco plant (Fig. 4c).

Improved flavan-3-ol and altered anthocyanin contents in pyramided transgenic tobacco plants

To check the influence of simultaneous expression of both *CsDFR*/*CsANR* transgenes on flavan-3-ol accumulation in pyramided transgenic tobacco plants, Cat, EC, ECG, and EGC contents were estimated by HPLC.

Fig. 3 Relative transcript expression profiles of endogenous flavan-3-ol/anthocyanin biosynthetic pathway genes (**a** *NtPAL*, **b** *NtCHS*, **c** *NtCHI*, **d** *NtF3H*, **e** *NtDFR*, **f** *NtFLS*, **g** *NtANR1*, **h** *NtANR2*, **i** *NtLAR*, **j** *NtANS*) in pyramided transgenic tobacco lines vis-à-vis

control tobacco plants. The expression level of 26S rRNA was used as reference control for semiquantitative expression analysis, and these experiments were repeated three times. Error bars indicate SD ($n = 3$), while asterisks indicate statistical significance ($P < 0.05$)

The Cat, EC, ECG, and EGC were found to be increased in all selected transgenic lines. The level of Cat was found to be 0.3 (DA-01), 0.22 (DA-02), 0.45 (AD-01), and 0.74 (AD-02) mg g^{-1} DW in pyramided transgenic lines, while it was detected at very low level (0.007 mg g^{-1} DW) in control tobacco plants (Fig. 5a). The content of EC was estimated to be 0.41 (DA-01), 0.24 (DA-02), 0.45 (AD-01), and 0.38 (AD-02) mg g^{-1} DW, while only 0.167 mg g^{-1} DW content was reported in control tobacco plants (Fig. 5b). The ECG content was measured as 0.41 (DA-01), 0.54 (DA-02), 0.71 (AD-01), and 0.68 (AD-02) mg g^{-1} DW as compared to 0.15 mg g^{-1} DW content in control tobacco plants (Fig. 5c). The EGC content was reported as 10 (DA-01), 9.4 (DA-02), 10.8 (AD-01), and 18.1 (AD-02) mg g^{-1} DW in pyramided transgenic lines, while it was reported to be only 0.022 mg g^{-1} DW in control tobacco plants (Fig. 5d). The HPLC chromatograms indicating identified peaks for different studied flavan-3-ols (Cat, EC, ECG, and EGC) with respect to their standards are documented in Figure S1.

The difference in flower colors of pyramided transgenic lines (DA-01, DA-02, AD-01, and AD-02) from that of transgenic lines overexpressing either *CsDFR* or *CsANR* alone and control tobacco flower(s) is shown in Fig. 6. The

flowers of pyramided transgenic lines showed altered pigmentation of pink and white patches. The colors of flowers of transgenic *CsDFR* and *CsANR* were dark pink and white, respectively. The flowers of control tobacco plant were reported to be light pink in color. On the basis of this phenotypic observation, anthocyanin contents of flowers of pyramided and single transgenic tobacco plants vis-à-vis control tobacco flowers were estimated. The anthocyanin contents were estimated as 2.1 (DA-01), 2.2 (DA-02), 1.9 (AD-01), and 1.85 (AD-02) per gram fresh weight (mg/g FW) vis-à-vis 2.8 (*CsDFR*) and 1.16 (*CsANR*) mg/g FW (Fig. 6). The anthocyanin content of wild tobacco flower was also estimated as 2.55 mg/g FW (Fig. 6).

Improved antioxidant potential under the influence of *CsDFR* and *CsANR* overexpression in pyramided transgenic tobacco plants

To check the influence of higher flavan-3-ol accumulation on antioxidant activity of pyramided transgenic tobacco plants, free radical scavenging activity was measured using DPPH assay. The methanolic extracts of the lyophilized leaf samples from pyramided transgenic lines showed 79%

Fig. 4 Transcript expression levels of *NtTT1* **a**, *NtTT2*, **b** and *NtANR2* **c** in pyramided transgenic tobacco lines. The 26S rRNA was used as internal control for relative expression analysis. Error bars indicate SD ($n = 3$), while asterisks indicate statistical significance ($P < 0.05$)

(DA-01), 47% (DA-02), 96% (AD-01), and 136% (AD-02) increase in DPPH free radical scavenging activity with respect to control tobacco plants (Fig. 7).

Increased protection ability of pyramided transgenic tobacco plants against *S. litura* attack

To check the anti-feeding effect of *S. litura* on pyramided transgenic tobacco plants, the non-choice method was used as adopted previously (kumar et al. 2013). The leaf disc of pyramided transgenic lines showed lesser feeding by larvae of *S. litura* as compared to leaf discs of control tobacco plants (Fig. 8). The lesser feeding response could be due to deterrent effect of accumulated flavan-3-ols in leaf discs as offered by non-choice method.

Hence, the growth retardation and lesser survival of *S. litura* larvae were also observed when fed on leaf discs of pyramided transgenic lines as compared to control tobacco plants. Percentage growth inhibition of larvae of *S. litura* that randomly fed on leaf discs of DA-01, DA-02, AD-01, AD-02 transgenic lines were 28, 65, 28, and 52%, respectively, as compared to the growth of other larvae that non-choicely fed on leaf discs of control tobacco plants (Fig. 8). The retarded growth of *S. litura* larvae feeding on leaf discs of pyramided transgenic lines has also indicated the decreased vigor of *S. litura* larvae.

Pyramided transgenic tobacco plants showed better seed germination rate under toxic Al stress condition

To check the influence of overexpression of transgenes *CsDFR* and *CsANR* on the germination behavior of seeds under Al exposure (1 mM and 5 mM), the germination rates of the seeds of selected pyramided transgenic lines (AD-01 and DA-01), individual transgenic lines (*CsDFR* and *CsANR*), and control tobaccos were calculated 4 days after initiating imbibition of seeds. The seed germination rates are 85.8% (DA-01) and 85.7% (AD-01) compared to 79.5% (*CsDFR*), 75.2% (*CsANR*), and 74.4% in control tobaccos under exposure of 1 mM Al toxicity. At 5 mM exposure of toxic Al, the rates of seed germination were 21.6% (DA-01) and 23.0% (AD-01), 16.6% (*CsDFR*), 18.8% (*CsANR*), and 12.11% in control tobaccos (Fig. 9). Significant differences in germination were observed in seeds of pyramided transgenic lines compared to individual transgenic lines as well as control tobaccos and a combinatorial role of *CsDFR/CsANR* was confirmed in the tolerance phenotype under toxic Al exposure.

Discussion

The anthocyanin and flavan-3-ol contents share the same metabolic intermediate and represent the extensively investigated biosynthetic pathway in plants. An effective transformation system for higher accumulation of flavan-3-ol content should rely on redirection of carbon flux toward

Fig. 5 Flavan-3-ol content in pyramided transgenic lines. Cat (a), EC (B), ECG (C), and EGC (D) content in pyramided transgenic lines vis-à-vis control tobacco plants. Error bars indicate SD ($n = 3$), while asterisks indicate statistical significance ($P < 0.05$)

Fig. 6 Anthocyanin content in flowers of transgenic lines vis-à-vis control tobacco plants. The upper panel represents the colors of flowers in transgenic vis-à-vis control tobacco plants. The petals of flowers from *CsDFR* were more pinkish in color, in contrast to flowers of control tobacco plants. The petals of *CsANR* flowers were documented white in color. The petal part of all flowers from selected pyramided transgenic lines showed altered pigmentation of white and pink patches. The lower panel documents the anthocyanin content in transgenic lines vis-à-vis control tobacco plants. Error bars indicate SD ($n = 3$), while asterisks indicate statistical significance ($P < 0.05$)

biosynthesis of flavan-3-ols with higher accumulation of intermediate precursors. This could be achieved by either overexpression of genes encoding for DFR, ANR, LAR, F3H proteins, and silencing of gene encoding for FLS and

Fig. 7 DPPH free radical scavenging activity in pyramided transgenic lines vis-à-vis control tobacco plants. Error bars indicate SD ($n = 3$), while asterisks indicate statistical significance ($P < 0.05$)

ANS proteins at the point of carbon flux redirection due to their central position in the flavan-3-ol/anthocyanin biosynthetic pathway. Manipulation of flavan-3-ol/anthocyanin biosynthetic pathway has been shown to have the potential for improving flavan-3-ol content in plants (Mahajan et al. 2011b; Kumar et al. 2013; Mahajan and Yadav 2014; Hammerbacher et al. 2014; Li et al. 2016; He et al. 2016; Lim et al. 2016; Pérez-Díaz et al. 2016; Rihani et al. 2017). The transgenic flax plants overexpressing genes encoding CHS, CHI, and DFR have been crossed with transgenic flax line(s) that overexpressed the *glucosyl-transferase* (GT) gene to improve protective ability against pathogen infection (Kostyn et al. 2012; Mierziak et al. 2014). The genes encoding DFR and ANR proteins were chosen at the point of redirection based upon their key regulatory roles in flavan-3-ol/anthocyanin biosynthetic

Fig. 8 The anti-herbivore effect of pyramided transgenic tobacco lines against *S. litura*. The upper panel shows pictures of lesser feeding on leaf materials of pyramided transgenic lines by *S. litura* relative to control plants. The lower panel represents the improved relative percentage growth inhibition of *S. litura* after feeding on leaf materials from pyramided transgenic tobacco lines as compared to control. Error bars indicate SD ($n = 3$)

pathway (Singh et al. 2009a, b, Kumar et al. 2013; Kumar and Yadav 2013a, b; Shin et al. 2016; Lim et al. 2016). The overexpression of *CsDFR* and *CsANR* has been documented with improved flavan-3-ol content in transgenic tobacco plants (Kumar et al. 2013; Kumar and Yadav 2013a, b).

After growing body of knowledge, it was our goal to achieve the optimum level of flavan-3-ol accumulation with improved tolerance against biotic and abiotic stresses through pyramiding of *CsDFR* and *CsANR* in transgenic tobacco plants relative to either transgene alone. The reciprocal crossing of *CsDFR* and *CsANR* overexpressing transgenic tobacco plants generated these pyramided transgenic tobacco lines. In continuation, the pyramided transgenic tobacco plants also showed early flowering, improved capsule numbers, and thousand seed weight compared with control tobacco plants. Mahajan et al. (2011b) have reported the correlation of *NtFLS* silencing in transgenic tobacco with retarded height, delayed flowering, and lesser yield compared to control tobacco plants. In

continuation, different mutants from the same biosynthetic pathway have also been documented for their role in the overall architecture of plant, numbers of shoot/flower, and seed weight (Buer et al. 2010). Thus, the present study provides strong evidence to establish the correlation between higher accumulation of flavan-3-ol content with early flowering and improved seed yield. However, early flowering is generally considered to have negative effect on yield but overexpression of *CsDFR* and *CsANR* affects the flowering timing that can also have significant impact on post-flowering processes affecting fertility and pod development (Buer et al. 2010; Mahajan et al. 2011b; Kumar and Yadav 2013a). The other possible reason could be the modulation of phytohormone levels as experimentally proved earlier (Buer and Djordjevic 2009; Kumar et al. 2013). However, there is no significant difference in the above-said phenotypic results (early flowering, capsule numbers, and seed weight) of pyramided transgenic tobacco with respect to transgenic plants overexpressing *CsDFR* or *CsANR* alone. The reason could be that an optimal level of flavan-3-ols that is induced in individual transgenic lines alone is enough for early flowering and other changes as documented earlier. However, the flower color phenotype of pyramided transgenic lines is significantly changed as compared to transgenic plants overexpressing *CsDFR* or *CsANR* alone and control tobacco plants.

Metabolic engineering for higher accumulation of flavan-3-ols is highly coordinated with significant changes in expression levels of genes associated with the flavan-3-ols/anthocyanin biosynthetic pathway under normal and stress conditions (Han et al. 2012; Kumar et al. 2013; Mahajan and Yadav 2014; Hammerbacher et al. 2014; Li et al. 2016; Luo et al. 2016; Pérez-Díaz et al. 2016). The overexpression of apple *ANR* gene has been observed to upregulate the expressions of only *NtCHI* and *NtDFR* genes of flavan-3-ol/anthocyanin biosynthetic pathway (Han et al. 2012). The overexpression of either *CsDFR* or *CsANR* has also been reported to upregulate the expression of only *NtCHS* and *NtANR* genes (Kumar et al. 2013). Thus, these reports have suggested that overexpression of a specific transgene is only responsible for change in expression of a set of related endogenous genes. On the other hand, the most striking results of the present study are that overexpressions of *CsDFR* and *CsANR* genes have triggered significant changes in the transcript levels of endogenous flavan-3-ol/anthocyanin biosynthetic pathway genes encoding *NtPAL*, *NtCHS*, *NtCHI*, *NtF3H*, *NtDFR*, *NtFLS*, *NtANR1*, *NtANR2*, *NtLAR*, and *NtANS* proteins as well as genes encoding *NtTT1* (WIP1/Zn finger), *NtTT2* (MYB), and *NtAN2* (MYB) transcription factors. In general, like other regulators, these regulators (TT1, TT2, and AN2) could be positive target for induction of signals for higher

Fig. 9 Influence of toxic Al exposure on seed germination from seeds of transgenic tobacco lines vis-à-vis control tobacco plant. The *upper panel* documents the pictures of a 4-day-old seedlings of *CsDFR* and *CsANR* transgenic lines vis-à-vis control tobaccos under 1 and 5 mM exposure of Al toxicity. The *lower panel* represents the rate of seed germination from transgenic lines vis-à-vis control tobacco plant after 4 days of initiating imbibition of seeds

accumulation of flavan-3-ols (Hichri et al. 2011; Xu et al. 2015). These regulators activate the expression levels of genes encoding enzymes of flavan-3-ol/anthocyanin biosynthetic pathway by binding to their respective promoter sites directly or indirectly (Hichri et al. 2011; Xu et al. 2015). In advance, the expression profiles of flavan-3-ol/anthocyanin biosynthetic pathway-related gene(s) associated with either overexpression or silencing of transcription regulator(s) is a strong evidence for transcriptional regulation of flavan-3-ol/anthocyanin biosynthetic pathway (Hichri et al. 2011; Xu et al. 2015). The constitutive simultaneous expressions of *CsDFR* and *CsANR* under 35S promoter resulted in accumulation of flavan-3-ols that could worked as a part of positive feedback loop and further upregulated the expression levels of flavan-3-ol/anthocyanin biosynthetic pathway genes. The tea-specific metabolites of flavan-3-ols, Cat, EC, ECG, and EGC have been reported to be higher in transgenic lines compared to control tobacco plants. The manipulation of flavan-3-ol content has been proved to be linked with modulation of biosynthesis of anthocyanins. The anthocyanin content has been found to be altered in flowers of pyramided transgenic lines as compared to control as well as transgenic plants overexpressing single gene alone (Kumar and Yadav 2013a). Tobacco flowers have been extensively studied as models for flavan-3-ol/anthocyanin biosynthesis and petal color is the most important indicator of anthocyanin content (Nakatsuka et al. 2012; Kumar and Yadav 2013a; Zhou et al. 2013). Altered flower color has

also been reported earlier via manipulation of flavan-3-ol/anthocyanin biosynthetic pathway (Tanaka et al. 2010; Nishihara and Nakatsuka 2011; Nakatsuka et al. 2012; Kumar and Yadav 2013a; Zhou et al. 2013). The down-regulation of *Torenia NtF3'H* as well as *NtFLS* genes and simultaneous overexpression of *Gerbera DFR* gene in tobacco has been reported to increase only anthocyanin biosynthesis (Nakatsuka et al. 2007). Similarly, knock-down of *F3'H* and *F3'5'H* genes with overexpression of rose *DFR* gene in *Torenia* violet cultivar resulted in flowers with darker pink petals with elevated level of anthocyanins (Nakamura et al. 2010). This report suggested that *DFR* encoded protein might be regulatory to redirect the flavan-3-ol/anthocyanin biosynthetic pathway towards biosynthesis of anthocyanins and their precursors. The inverse correlation between anthocyanins and flavan-3-ol levels in the tobacco flowers probably reflects fine-tuning between these two branches of flavonoid biosynthetic pathway. The other important enzyme ANR protein which is also a key rate-limiting enzyme catalyzes the biosynthesis of EC (flavan-3-ols) from anthocyanidin substrate (Zhang et al. 2012). The overexpression of *ANR* encoding protein in tobacco has diverted the carbon flux toward biosynthesis of EC, monomeric flavan-3-ols instead of anthocyanin biosynthesis. So, *ANR* catalyzed reaction plays a crucial role in determining the levels of anthocyanins and EC in flower (Xie et al. 2004; Han et al. 2012; Kumar and Yadav 2013a). The white flowers with decreased anthocyanin contents have been reported in *CsANR* transgenic tobacco

plants (Kumar and Yadav 2013a; Han et al. 2012). So these studies have suggested that *CsANR* overexpression diverted the utilization of anthocyanidins toward biosynthesis of flavan-3-ols, thereby decreasing anthocyanin content. In the present study, the altered pigmentation has also been documented in flowers of transgenic lines. The anthocyanin contents in pyramided transgenic lines have also been measured at intermediate level in-between individual transgenic tobacco lines overexpressing either *CsDFR* (high) or *CsANR* (low) alone. The alterations of flower pigmentations and decrease in anthocyanin content are highly correlated with co-expression of *CsDFR* and *CsANR* in pyramided transgenic tobacco plants.

The aim of this study was also to improve antioxidant potential by increasing the flavan-3-ol content with co-expression of *CsDFR* and *CsANR* transgene simultaneously over single transgenic lines. The monomeric and polymeric forms of flavan-3-ols are best known for their antioxidant properties and their potential protective benefits against biotic and abiotic stresses (Buer et al. 2010; Falcone Ferreyra et al. 2012; Mierziak et al. 2014; Nakabayashi et al. 2014; Luo et al. 2016). The transgenic tobaccos overexpressing *CsDFR* and *CsANR* individually have also been documented for higher antioxidant properties (Kumar et al. 2013). In this study, we estimated DPPH radical scavenging activity that was found to be slightly higher than transgenic tobacco overexpressing *CsDFR* or *CsANR* transgene alone. The improved flavan-3-ol content with higher in vitro antioxidant activity has been inversely correlated with reactive oxygen-mediated cell death in *CsDFR* and *CsANR* transgenic lines as experimentally proved earlier (Kumar et al. 2013). Similarly, overexpression of *ANR* from *Rosa rugosa* has also been documented with improved tolerance to abiotic stress through elevated ROS scavenging activity (Luo et al. 2016). So, the strategy to improve the antioxidant potential of plants via overaccumulation of flavan-3-ols could be an effective strategy for tolerance against biotic and abiotic stresses (Kumar et al. 2013; Nakabayashi et al. 2014; Meng et al. 2015; Mitsunami et al. 2014; Mahajan and Yadav 2014). The flavan-3-ols play an important role in plant herbivore interaction or against pathogen infections (Porth et al. 2011; Barbehenn and Constabel 2011; Kumar et al. 2013; Mahajan and Yadav 2014; Mitsunami et al. 2014; Hammerbacher et al. 2014). Transgenic tobaccos overexpressing gene-encoded key enzymes involved in flavan-3-ol/anthocyanin biosynthetic pathway have been documented with better tolerance to biotic stresses, including herbivore (Misra et al. 2010; Kumar et al. 2013; Mitsunami et al. 2014) and fungal infection (Mahajan and Yadav 2014; Mierziak et al. 2014). So, evaluation of pyramided transgenic lines for their protective ability against feeding by *S. litura* was done. The defensive ability of pyramided

transgenic plants was found to be improved against the generalist herbivore *S. litura*. The combinatorial approach indicates that simultaneous overexpression of *CsDFR* and *CsANR* slightly improves the tolerance against feeding by *S. litura* relative to single transgene alone. The abiotic environmental constraints also induce higher accumulation of flavan-3-ols/anthocyanins in plants (Kusano et al. 2011; Yuan et al. 2012; Meng et al. 2015). The flavan-3-ol/anthocyanin metabolites scavenge the accumulated reactive oxygen species triggered by abiotic stresses. A few reports have also discussed the correlation between flavan-3-ol/anthocyanin accumulation with strong radical scavenging and tolerance to abiotic stresses such as UV-B irradiation, salt, drought, water stresses, chilling stress, and against Al toxicity (Kusano et al. 2011; Yuan et al. 2012; Kumar and Yadav 2013b; Mahajan and Yadav 2014; Nakabayashi et al. 2014; Meng et al. 2015).

In addition, extraordinary amount of flavan-3-ol content makes tea an Al-hyperaccumulator plant and encourages for assessment of aluminum toxicity as reported earlier (Osaha et al. 2011; Kumar and Yadav 2013a). In the present study, we also reported the germination rate of seeds from pyramided transgenic tobacco was found to be significantly higher under Al exposure. The higher amount of flavonoids in tea is well known for providing tolerance against Al though detoxified by potential antioxidant mechanism (Mukhopadhyay et al. 2012). Seedlings of transgenic tobacco plants overexpressing *CsDFR* and *CsANR* transgene alone relative to control were documented for better growth under exposure of Al toxicity (Kumar and Yadav 2013b). These observations confirm the usefulness of flavan-3-ols for enhancing both biotic and abiotic stress tolerance in pyramided transgenic tobacco plants. Thus, we could demonstrate that gene stacking approach is promising for flavan-3-ol production in plants. However, the optimum level of flavan-3-ol accumulation with improved tolerance against biotic and abiotic stresses through simultaneous overexpression of *CsDFR* and *CsANR* in transgenic tobacco plants is not beyond the individual transgenic lines.

Conclusions

In conclusion, this report demonstrated the coordinated expression of *CsDFR* and *CsANR* in tobacco with early flowering, flavan-3-ol accumulation, and higher DPPH scavenging activity, which not only protected against feeding of *S. litura* but also showed better seed germination under Al toxicity. Since the single transgenic lines have been studied and the work was published, it would be important here to know the significance of *CsDFR*-*CsANR*-stacked tobacco transgenic over single transgenic lines.

The overall results documented from pyramided transgenic lines were much better than those reported earlier from individual transgenic tobacco plants of either *CsDFR* or *CsANR*. In continuation, this work also emphasizes the potential role of *CsDFR*- and *CsANR*-encoded proteins in fine-tuning of anthocyanin accumulation in flower and germination rate of seeds under Al exposure. Further work is needed to investigate how the end product of flavan-3-ol/anthocyanin biosynthetic pathway is recognized by the plant cell and triggers subsequent mechanisms that control the optimum level of flavan-3-ols. In addition to stacking of *CsDFR* and *CsANR*, there is also need for identification of any potential regulatory gene(s), to design a novel strategy for maximum accumulation of antioxidant flavan-3-ol contents in agriculturally important crops including cotton, flax, maize, and rice.

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Author contribution VK and SKY conceived and designed present research. VK conducted experiments. VK and SKY analyzed data. VK wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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