ENHANCED ANTHOCYANIN CONTENT IN TRANSGENIC TOBACCO ACHIEVED THROUGH OVEREXPRESSION OF CAMELLIA SINENSIS FLAVANONE-3-HYDROXYLASE AND DOWNREGULATION OF NICOTIANA TABACUM FLAVONOL SYNTHASE

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Abstract: Flavonoids are synthesized by phenylpropanoid pathway. They are known to participate in large number of physiological and biochemical processes in plants Anthocyanins, a class of flavonoids are the main contributors of flower color. Since genes regulating flavonoid biosynthesis pathway are available, it is possible to alter flower color by overexpressing heterologous genes or by downregulating endogenous genes. Here, in the present study, two approaches were used to engineer the flavonoid pathway in tobacco towards modifying anthocyanins content. One was through downregulation of flavonol biosynthesis in tobacco *via* post transcriptional gene silencing (PTGS) of flavonol synthase (NtFLS) encoding mRNA. Second was through overexpression of *Camellia sinensis* flavanone-3-hydroxylase (CsF3H) cDNA in tobacco. Reverse transcriptase (RT-PCR) analysis in leaf, petal and seedlings of *NtFLS* silenced and *CsF3H* overexpressed transgenic lines revealed an upregulation in expression of phenylalanine ammonia lyase (PAL), chalcone isomerase (CHI), chalcone synthase (CHS), flavanone 3- hydroxylase (F3H), dihydroflavanol reductase (DFR) and anthocyanidin synthase (ANS) genes of flavonoid pathway. This increased expression of flavonoid biosynthetic pathway genes led to enhanced anthocyanin content in flowers of *NtFLS* silenced and *CsF3H* overexpressed transgenic lines and thus modified the flower color. Hence, the present study has provided a way to understand the regulation of flavonoid pathway through gene overexpression and silencing approaches in tobacco.

Keywords: anthocyanin, flavanone 3-hydroxylase, flavonol synthase, tobacco.

Introduction: Flavonoids are the plant secondary metabolites that are involved in large number of plant functions. Among the different classes of flavonoids, anthocyanins are of great importance. They provide pigmentation to the flowers to attract the pollinators [1]. Also, novel flower color varieties are of particular interest for the ornamental industries. Anthocyanins are most abundant in berries (*Vaccinium* sp.), grapes, apples, purple cabbage, eggplant, black carrots etc. Anthocyanins have been found to exhibit higher antioxidant activities [2]. They are also known to have large number of pharmacological properties like anti-inflammatory, antimicrobial, antiartherosclerotic and anticarcinogenic [3], [4]. The anthocyanins are synthesized through general phenylpropanoid and flavonoid biosynthetic pathway. This pathway has been well studied and characterized in *Arabidopsis thaliana*, tobacco and *Medicago truncatula*, petunia, gerbera, rose, carnation, lisianthus, and torenia as well in forage legumes *Lotus corniculatus* and alfalfa [5]-[7]. The number of hydroxyl group on B ring and modification of anthocyanins with aromatic acylation affects the flower color [8]. In the flavonoid pathway, hydroxylation of naringenin results into dihydrokaemferol (DHK). This reaction is carried out by flavanone-3-hydroxylase (F3H), a member of 2-oxoglutarate-dependent dioxygenase family which is highly conserved among widely divergent plant species [9]. DHK can be further hydroxylated, either at the 3'position or at both 3' and 5' positions of the B-ring to form dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. The former hydroxylation step is catalyzed by P450 enzyme, flavonoid 3'-hydroxylase (F3'H) and later is catalyzed by flavonoid 3'5'- hydroxylase (F3'5'H), which led to uction of cyaniding and delphinidin-based anthocyanins [10]. The presence or absence of these enzymes in different plant species contributes to the petal color variations [11]. Further downstream in the main pathway, these dihydroflavonols can be used either by FLS to form flavonols or used by a series of enzymes to form anthocyanins/ PAs. Reducing FLS transcript abundance through antisense or increasing DFR transcript abundance has restored anthocyanin biosynthesis in flower limb of white petunia [12]. Similarly, introduction of an antisense *FLS* construct into *Nicotiana tabacum* has increased anthocyanin content of the petals up to three times [13]. Thus, competition between DFR and FLS for dihydroflavonol substrates commonly influences the level of anthocyanin production in plants. Among the different strategies for silencing the endogenous genes, ihpRNA based post-transcriptional gene silencing (PTGS) is the most effective [12], [14], [15].Tea (*Camellia sinensis* (L.) O. Kuntze) contains about 180–360 g kg^{-1} of tea polyphenols [16]. Almost all genes of tea flavonoid biosynthetic pathwaynamely *CsPAL*, *CsC4H*, *CsCHS*, *CsCHI*, *CsF3H*, *CsF3'5'H*, *CsDFR*, *CsLAR*, *CsANR*, *CsANS* have been cloned and characterized in order to understand their role in regulation of flavonoid

biosynthesis [17], [18]. Therefore, use of tea genes to engineer flavonoid pathway in other plants could be useful towards understanding flavonoid pathway's regulation. The schematic representation of flavonoid pathway in tobacco has been shown in Fig. 1.

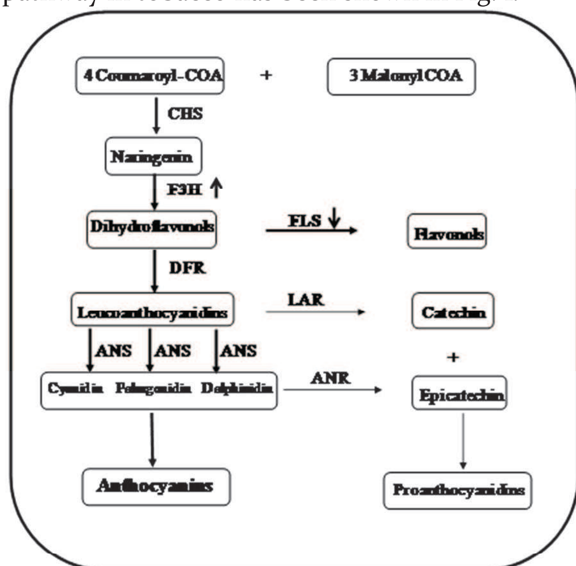

Fig. 1. Schematic overview of the flavonoid biosynthesis pathway in plants. The pathway normally active in tobacco leaves and inflorescence, leading to flavonols and anthocyanin production, is indicated by solid arrows. Whereas light arrows indicate the minor pathway in tobacco that leads to proanthocyanidins (catechin and epicatechin) synthesis.

Till yet, to the best of our knowledge no reports of anthocyanins accumulation have been documented in tobacco through overexpression of flavanone 3-hydroxylase and silencing of flavonol synthase. Therefore, the present study was planned to understand the regulation of flavonoid biosynthesis in tobacco by over expression of *F3H* from tea and downregulation of *FLS* gene. The study also aims to see the influence of these genetic engineering's on anthocyanin accumulation in tobacco.

Materials And Methods: Preparation of ihp-RNAi construct The RNAi vector pFGC1008 was used for silencing studies. The preparation of inverted hair pin (ihp)-RNAi construct. (pFGC1008-NtFLS) has been earlier discussed discussed in our research paper [19], [20].

Preparation of overexpression construct : Full length cDNA of 1.12 kb *CsF3H* encoding F3H was isolated from tea (*Camellia Sinensis* (L) O. Kuntze) UPASI 10 using full-length cDNA specific primers having *Bgl*II restriction site at 5' end of forward primer and *Spe*I restriction site at 5' end of reverse primer (forward 5'-TGGTAGATCTATGGCGCCAACAACAACGC-3'; reverse primer 5'-CTGACTAGTTCAAGCAAAAATCTCATCAGTGC-3'). The gene was under the control of CaMV 35S promoter. The amplified fragment was cloned in pGEM-T Easy vector. From pGEM-T, the *CsF3H* fragment was restrict digested with same restriction enzymes, and was cloned in pCAMBIA-1302 vector between the same restriction sites.

Plant transformation of overexpression and RNAi constructs: The prepared ihp-RNAi construct (pFGC1008-*NtFLS*) and overexpression construct (pCAMBIA 1302-*CsF3H*) construct were transformed individually in *Agrobacterium tumifaciens* strain LBA4404 using triparental mating technique. *Agrobacterium*-mediated leaf disc co-cultivation method was used for tobacco transformation [21]. *A. tumefaciens* containing empty pFGC1008 and empty pCAMBIA 1302 (with no transgene) was used as a control for silencing studies and overexpression studies respectively. The transformed plants were selected on MS medium containing 50 mg l^{-1} of hygromycin as plant selection antibiotic at 25±2°C [22]. Resistant plants were transferred to a closed greenhouse to produce seeds and for use in further analyses.

Expression analysis of flavonoid biosynthetic pathway genes: Total RNA was extracted from the leaf, petal and seedlings of T2 generation control, ihp-*NtFLS* and *CsF3H* overexpressed transgenic tobacco using RNeasy plant mini kit (Qiagen) and cDNA was synthesized according to instructors protocol (Invitrogen). This cDNA was used as a template for PCR with gene specific primers encoding enzymes of tobacco flavonoid biosynthetic pathway, phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS). The primer sequences of used for PCR amplification is given in table I. PCR was carried out under the conditions of 94 °C-4 min, 94 °C-30s, 50 to 60 °C-40s (54 °C for CHS, 57°C for CHI, F3H & FLS, 58.5 °C for DFR and 53 °C for ANS) for 25 cycles. The 26S rRNA-based gene primers were used as internal control for expression studies [23].

Estimation of anthocyanins: Extraction of anthocyanins from flowers of T2 generation control, ihp-*NtFLS* and *CsF3H* overexpressed transgenic tobacco was performed following the standard protocol [24]. To 300 mg of fresh plant material, about 1 ml of acidic methanol (1% HCl, v⁄v) was added. Samples were allowed to incubate for 18 h at room temperature with moderate shaking. Plant material was sedimented by centrifugation at room temperature for 1 min at 14,000 g. After centrifugation, 400 µl of supernatant was added to 600 µl acidic methanol. Absorption of the extracts

was determined using a UV-Vis spectrophotometer at 530 and 657 nm. Quantification of anthocyanins was performed using the following equation: $Q_{Anthocyanins} = (A_{530} - 0.25\times A_{657})\times M^{-1}$, where $Q_{Anthocyanins}$ is the amount of anthocyanins, A_{530} and A_{657} are the absorptions at the indicated wavelengths and M is the weight of the plant material in grams used for extraction.

Table I. Forward and reverse primer sequences of flavonoid pathway genes

Gene	Primer Sequences, Forward (F) and Reverse (R)
PAL	F 5'-CAAGAACGGTGGTGCTCTTC-3' and R5'-CCAGAACCAACTGCAGTACC-3'),
CHS	F 5'-GTACAACTAGTGGTGTAGACA-3' R 5'-CCAACTTCACGAAGGTGAC-3')
CHI	F 5'- CGAGTGACTATGATCTTGCC-3' and R 5'-CTGACGCGTCGGCATAGC-3')
F3H	F 5'-GGTAGTTGATCATGGTGTTGA-3'and R 5' GTTCCTGGATCAGTGTCTCG-3')
FLS	F 5'- GTCCACAACGTTGCATGGTG-3' and R 5'- CACAACTTCTCGCAGCCTC-3'
DFR	F 5'- GATGAAGCCATTCAAGGCTG-3' and R 5'-GCAGTGATTAAGCTAGGTGG-3'
ANS	F 5'-CGAGGACAAGTGCGACTTAT-3' and R 5'-GAGATTCTTACTTTCTCTTTATT-3'

Results: Tobacco transformation with *Agrobacterium* containing RNAi construct and overexpression construct

Agrobacterium tumefaciens harbouring either ihp-*NtFLS* or pCAMBIA 1302-*CsF3H* along with the controls were individually used for leaf disc transformation of tobacco (*Nicotiana tabacum* L.) to generate transgenic tobacco plants. Transformed leaf discs were transferred to co-cultivation medium with no antibiotics. After co-cultivation, leaf discs were placed on shoot selective media supplemented with bactericidal carbenicillin (100 mg l^{-1}; HiMedia, India), cefotaxime (50 mg l^{-1}; HiMedia, India) and plant selection antibiotic hygromycin (Fig. 2a). After 1-2 weeks, callus was formed at the cut ends of leaf discs (Fig. 2b). These discs were transferred to same fresh media for shoot induction. After 3-4 weeks, hygromycin (50 mg l^{-1}, Sigma) resistant well-grown plantlets at 1-2 node stage were separated and transferred to rooting media [(MS containing 1-naphthaleneacetic acid (NAA) (0.5 μM) and Benzyl amino purine (BAP) (2.5 μM)] along with hygromycin and bactericidal antibiotics carbenicillin (100 mg l^{-1}) and cefotaxime (50 mg l^{-1}) from HiMedia, India).

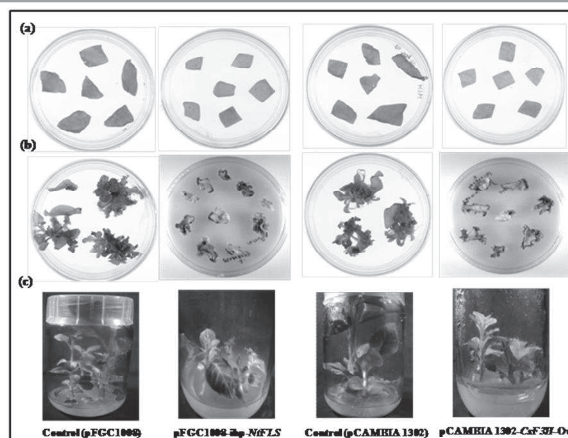

Fig. 2. Empty pCAMBIA 1302 (**a**), pCAMBIA 1302-*CsF3H*-Ov (**b**), empty pFGC1008 (**c**) and pFGC1008-*NtFLS* (**d**) transformed leaf discs placed on selection media for regeneration. Empty pCAMBIA 1302 (**e**), pCAMBIA 1302-*CsF3H*- Ov (**f**), empty pFGC1008 (**g**) and pFGC1008-ihp-*NtFLS* (**h**) transformed leaf discs on selection media under callusing phase and empty pCAMBIA 1302 (**i**), pCAMBIA 1302-*CsF3H*- Ov (**j**), empty pFGC1008 (**k**) and pFGC1008-ihp-*NtFLS* (**l**) putative transgenic plants on selection media**.** Ov, overexpressed; ihp, inverted hair pin.Figure 2c shows well developed plants after 30 days of transformation with a good quality root system. These putative transformants were then transferred to pots in green house for seed setting.

Confirmation of transgene insertion in transgenic lines through genomic DNA PCR: PCR based method was used to check the presence of transgene in the genome of all the polulations of T1 and T2 generation of transgenic tobacco. The transformants were screened for the presence of *NtFLS* RNAi cassette using vector specific primers and *CsF3H* cDNA using gene specific primers.

A band of 1.361 kb and 1.12 kb indicated the introduction of *NtFLS* RNAi cassette and *CsF3H* cDNA respectively in the transformed transgenic lines. This has confirmed the stable integration of *NtFLS* RNAi cassette and *CsF3H* cDNA T2 progenies of transgenic tobacco. A representative figure depicting the integration of *NtFLS* RNAi cassette (Fig. 3a) and *CsF3H* cDNA (Fig. 3b) in genomic DNA of transgenic tobacco plants is shown.

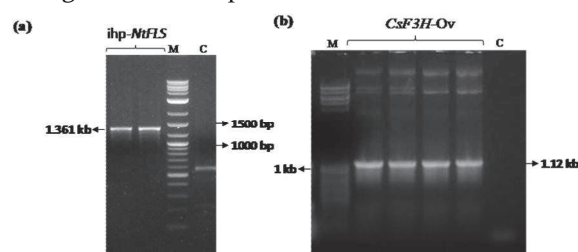

Figure 3. Genomic DNA PCR confirmation of inserted *CsF3H* in overexpressing transgenic lines **(a)**

and ihp-*NtFLS* RNAi cassette in silenced tobacco transgenic lines (**b**). C, control tobacco plants Transcript expression of endogenous flavonoid biosynthetic pathway genes in transgenic tobacco plants: The influence of *NtFLS* silencing and *CsF3H* overexpression was studied on the expression of endogenous genes of flavonoid biosynthetic pathway such as *PAL*, *CHS*, *CHI*, *F3H*, *FLS*, *DFR* and *NtANS* in leaf, petal and seedlings of transgenic and control tobacco plants. Upregulation in the transcript level of *PAL*, *CHS*, *CHI*, *F3H*, *DFR* and *ANS* genes was observed in all tissues (leaf, petal and seedling) of ihp-*NtFLS* RNAi (Fig. 4B) as well as *CsF3H* overexpressed transgenics (Fig. 4C) with respect to control tobacco plants (Fig 4A). Downstream genes encoding DFR and ANS enzymes of flavonoid pathway showed significant increase in their expression level in all tissues of both ihp-*NtFLS* RNAi and *CsF3H* overexpressed transgenics compared to control tobacco plant. In contrast to this, transcript level of *FLS* showed decrease in leaf, petal and seedlings of ihp-*NtFLS* RNAi (Fig. 4B) and *CsF3H* overexpressed transgenics (Fig. 4C) compared to control tobacco (Fig. 4A). The decrease in FLS expression was more in ihp-*NtFLS* RNAi transgenic because of antisense inhibition of FLS mRNA.

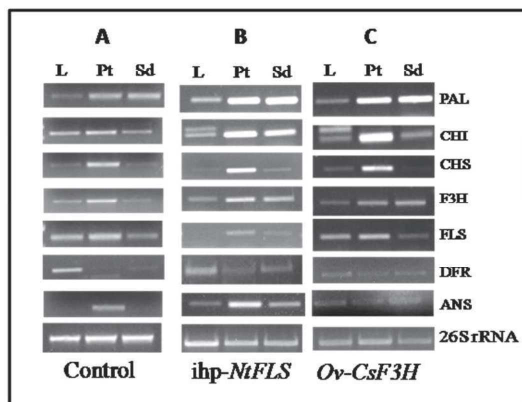

Figure 4. Transcript level of flavonoid biosynthetic pathway genes *PAL*, *CHS*, *CHI*, *F3H*, *FLS*, *DFR* and *ANS*, anthocyanidin synthase in leaf (L), petal (Pt) and seedling (Sd) of control (**A**), ihp-*NtFLS* RNAi transgenic lines (**B**) and *CsF3H* overexpressed transgenic tobacco line G12 (**C**).

Increased anthocyanins content in transgenic tobacco plants: Anthocyanidins form a branching point in the flavonoid pathway synthesizing anthocyanins on one side and flavan-3-ols on other side. Therefore, *NtFLS* silencing and *CsF3H* overexpression might affect anthocyanins level in transgenic lines. Hence its content was measured in transgenic lines. Anthocyanins content was found to more in ihp-*NtFLS* RNAi and *CsF3H* overexpressed transgenics compared to control tobacco plant (Fig. 5A) Anthocyanins content was increased by 86% and 70% in ihp-*NtFLS* RNAi and *CsF3H* overexpressed transgenics respectively compared to control tobacco plant (Fig. 5B). Amongst both transgenic lines, anthocyanins content was higher in ihp-*NtFLS* RNAi followed by *CsF3H* overexpressed transgenics and control tobacco.

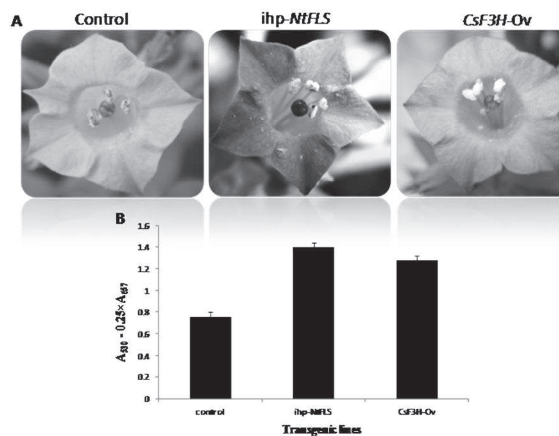

Figure 5. Pictorial representation of flower color of control, ihp-*NtFLS* RNAi and *CsF3H* overexpressed transgenic tobacco lines (**A**). Anthocyanin content in methanolic extracts of flowers of control, ihp-*NtFLS* RNAi and *CsF3H* overexpressed transgenic tobacco lines (**B**). A_{530}, absorption at 530 nm; A_{657}, absorption at 657 nm.

Discussion: Here in the present study, transgenic approaches were used to modify flower color in tobacco through downregulation of tobacco flavonol synthase and overexpression of *Camellia sinensis* flavanone 3-hydroxylase. Both strategies led to significant increase in anthocyanins content in transgenic flowers with upregulation in the expression of flavonoid biosythetic pathway genes. In the same way, metabolic engineering of flavonoid biosynthesis have successfully changed the flower or foliage colour in several ornamental species [25]. Similar strategies of overexpression or downregulation of genes encoding enzymes of flavonoid biosynthetic pathway have been used earlier to alter color of flower in several plants like petunia, rose, carnation, *Lisianthus, Nierembergia* and gerbera [26], [5]-[7]. Overexpression of petunia *CHS* and *DFR* cDNA in potato led to four fold increase in pelargonidin type anthocyanins in potato tubers. While antisense constructs of *CHS* and *DFR* resulted in decrease in anthocyanins in potato tubers [27]. Overexpression of *DFR* gene of *Populus trichocarpa* in tobacco has resulted in accumulation of anthocyanidins [28], [29]. Higher expression of *F3H* in actively growing tissues has also been reported in *Citrus paradisi* and *Petunia hybrida* L. [30]. Reducing FLS transcript abundance through antisense or increasing DFR transcript abundance has

restored anthocyanin biosyznthesis in flower limb of white petunia [12]. Similarly, introduction of an antisense *FLS* construct into *Nicotiana tabacum* and *Lasianthus* has increased anthocyanin production [12], [13]. Thus, competition between DFR and FLS for dihydroflavonol substrates commonly influences the level of anthocyanin production in plants. Similarly, chalcone isomerase (CHI) suppression has resulted in flower color change in transgenic tobacco plants [31]. Redirection of metabolic flux with suppression of endogenous *ANR* towards higher anthocyanidin production and lower PAs production has been achieved in soyabean grains [32]. Hence, the results clearly suggest that *NtFLS* silencing and *CsF3H* overexpression have diverted the potential of the flavonoid pathway in tobacco towards anthocyanins accumulation.

Conclusions: In short, the present study has provided a way for the redirection of the metabolic flux of flavonoid pathway in tobacco towards the anthocyanin production through silencing of tobacco flavonol synthase gene and overexpression of tea flavanone 3-hydroxylase gene. Hence, this study shows a better tool for enhancing anthocyanin production in plants that lack or produce less of them. Such genetic engineering's will also help in understanding the regulation of flavonoid pathway.

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