RESULTS

Flavonoids are involved in large number of plant functions. They increase antioxidant potential of plants and protect them from oxidative damage caused by environmental stresses. They also impart economical and medicinal value to several plants. Like other classes of flavonoids, flavan-3-ols are also known to possess antioxidant properties. Flavan-3-ols and other flavonoids are synthesized through common biosynthetic pathway. Flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) have been identified as important regulatory and branching point enzymes in flavonoid pathway, respectively. These two are therefore, considered to be potential candidates for use in flavan-3-ols manipulation through genetic engineering. Since tea (*Camellia sinensis*) contains highest amount of flavonoids, exploring its genes for genetic manipulation of an alternate system (*Nicotiana tabacum* L.) would be of great importance. Therefore, redirection of flavonoid pathway towards flavan-3-ols production was studied by overexpressing *CsF3H* and by silencing *NtFLS* individually in transgenic tobacco. These transgenic plants were analyzed for their influence on flavonoids and antioxidant levels. The *CsF3H* overexpressing transgenics were evaluated for their response to biotic and abiotic stress tolerance. Whereas, *NtFLS* silenced transgenic tobacco were evaluated for their influence on pollen germination and fertility. Before preparing recombinant constructs for overexpression and silencing studies, level of transcript expression of genes encoding enzymes of flavonoid pathway in tobacco was analyzed.

4.1 Expression analysis of native genes encoding enzymes of flavonoid pathway in *Nicotiana tabacum* L.

For transcript expression analysis, cDNA sequences of genes encoding enzymes of flavonoid biosynthetic pathway in tobacco (*Nicotiana tabacum* L.) i.e. phenylalanine ammonia lyase (PAL), chalcone isomerase (CHI), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), flavonol synthase (FLS), anthocyanidin synthase (ANS) were collected from NCBI. The gene specific primers for the partial fragments were designed from these sequences. For expression analysis, total RNA was isolated from tobacco leaves and first strand cDNA was prepared. Semiquantitative PCR was carried out with respective cDNA specific designed primers in
order to amplify 435 bp, 432 bp, 466 bp, 467 bp, 375 bp, 409 bp and 453 bp of \(NtPAL\), \(NtCHI\), \(NtCHS\), \(NtF3H\), \(NtDFR\), \(NtFLS\), \(NtANS\) genes respectively (Fig. 4.1).

![Image](image1.png)

Fig. 4.1 Expression level of genes encoding enzymes (a) phenylalanine ammonia lyase (\(NtPAL\)), (b) chalcone isomerase (\(NtCHI\)), (c) chalcone synthase (\(NtCHS\)), (d) flavanone 3- hydroxylase (\(NtF3H\)), (e) dihydroflavanol reductase (\(NtDFR\)), (f) flavonol synthase (\(NtFLS\)), and (g) anthocyanidin synthase (\(NtANS\)) in tobacco conducted through RT-PCR. An amplification of 435 bp, 432 bp, 466 bp, 467 bp, 375 bp, 409 bp and 453 bp was observed for \(NtPAL\), \(NtCHI\), \(NtCHS\), \(NtF3H\), \(NtDFR\), \(NtFLS\) and \(NtANS\) respectively. M, marker; 1-6, one set of primers; 1'-6', second set of primers.

PCR was carried out at annealing temperature between 50-60 °C with two set of primers for each gene. The number of cycles and the concentration of cDNA was equal in each case. PCR amplified products after elution were separately cloned in pGEM®-T Easy vector and transformed in DH5α. Colonies appeared on the selection plate were then screened out for the positive ones by colony PCR. The colony PCR for \(NtPAL\), \(NtCHI\), \(NtCHS\), \(NtF3H\), \(NtDFR\), \(NtFLS\) and \(NtANS\) is shown in Fig. 4.2.
Fig. 4.2 Colony PCR of genes encoding enzymes (a) phenylalanine ammonia lyase (NtPAL), (b) chalcone isomerase (NtCHI), (c) chalcone synthase (NtCHS), (d) flavanone 3- hydroxylase (NtF3H), (e) dihydroflavonol reductase (NtDFR), (f) flavonol synthase (NtFLS) and anthocyanidin synthase (ANS) cloned in pGEM®-T Easy vector. An amplification of 435 bp, 432 bp, 466 bp, 467 bp, 375 bp, 409 bp and 453 bp was observed for NtPAL, NtCHI, NtCHS, NtF3H, NtDFR, NtFLS and NtANS respectively.

Plasmids were isolated from the positive colonies and were sequence confirmed for the inserts by an Automated Sequencer. The partial gene sequences of NtPAL, NtCHI, NtCHS, NtF3H, NtDFR, NtFLS and NtANS showed 100% similarity with the submitted original cDNA sequences of the tobacco. Comparative transcript expression analysis indicated the differential expression of flavonoid pathway genes NtPAL, NtCHI, NtCHS, NtF3H, NtDFR, NtFLS and NtANS in tobacco (Fig. 4.3).

![Graph showing transcript expression level of genes encoding enzymes NtPAL, NtCHI, NtCHS, NtF3H, NtDFR, NtFLS and NtANS of flavonoid biosynthetic pathway in tobacco analyzed through RT-PCR.](image)

The NtFLS showed the maximum expression and NtF3H showed the least expression followed by NtANS gene of flavonoid biosynthetic pathway in tobacco. In view of this,
overexpression of \textit{NtF3H} and downregulation of \textit{NtFLS} could be a useful strategy towards improving flavan-3-ols accumulation in plants. Hence, the tobacco transgenics overexpressing \textit{Camellia sinensis CsF3H} and downregulating tobacco \textit{NtFLS} were raised and their influence on flavonoids, antioxidants and other plant functions was studied.

\section*{4.2 Preparation of overexpression construct (pCAMBIA1302 vector containing CsF3H)}

Full length cDNA of 1.12 kb \textit{CsF3H} encoding F3H was isolated from tea (\textit{Camellia Sinensis} (L) O. Kuntze) UPASI 10. Total RNA was isolated from tea plant and cDNA was prepared. PCR was run with full-length cDNA specific primers (as mentioned in material and methods section 3.2.2.1) having \textit{Bgl}II restriction site at 5’ end of forward primer and \textit{Spe}I restriction site at 5’ end of reverse primer. These primers were used to amplify \textit{CsF3H} sequence from cDNA pool. PCR amplified product was run on the gel and the band respective to \textit{CsF3H} was excised and eluted. The specificity of cDNA to \textit{CsF3H} was confirmed through sequencing. The eluted band was ligated in pGEM®-T Easy vector and transformed in DH5α competent cells. Colonies grown on selection plate were screened for the positive ones through colony PCR method (Fig. 4.4a).

![Fig. 4.4 Colony PCR of DH5α bacterial colonies containing pGEM®-T-CsF3H (a) and double digestion of isolated pGEM®-T-CsF3H with BglII and SpeI (b) produced CsF3H cDNA of 1.12 kb.](image)

Isolated plasmid (pGEM®-T-CsF3H) from positive colony was double digested with \textit{Bgl}II and \textit{Spe}I restriction enzymes. The restrict digested cDNA fragment of \textit{CsF3H} was obtained from recombinant pGEM®-T Easy vector by resolving in 0.8 % agarose gel (Fig. 4.4b). This double digested cDNA fragment of \textit{CsF3H} was ligated in a similar double digested pCAMBIA 1302 vector between cauliflower mosaic virus promoter (CaMV 35S) and NOS-
terminator. The schematic representation of pCAMBIA 1302 containing \(CsF3H\) cDNA is shown in Fig. 4.5.

![Fig. 4.5 Schematic representation of the T-DNA region of pCAMBIA 1302 containing \(CsF3H\) (1.12 kb) between restriction sites \(Bgl\)II and \(Spe\)I.](image)

The ligated product was transformed into DH5\(\alpha\) competent cells. Positive colonies grown after transformation on LB media with kanamycin were screened through colony PCR using cDNA specific primers as mentioned in material and methods (section 3.2.2.1). The appearance of band corresponding to the size of \(CsF3H\) in the agarose gel has confirmed the presence of \(CsF3H\) cDNA in pCAMBIA 1302 (Fig. 4.6a).

![Fig. 4.6 Colony PCR of DH5\(\alpha\) bacterial colonies containing pCAMBIA 1302-\(CsF3H\) (a) and double digestion of isolated pCAMBIA 1302-\(CsF3H\) with \(Bgl\)II and \(Spe\)I (b) produced \(CsF3H\) cDNA of 1.12 kb.](image)

The recombinant plasmid was isolated from the positive colony and was double digested with \(Bgl\)II and \(Spe\)I to check the presence of \(CsF3H\) cDNA in binary vector. The size of vector digested fragment was similar to size of \(CsF3H\) cDNA (Fig. 4.6b).
4.3 Preparation of RNAi construct (pFGC1008 containing NtFLS)

To prepare RNAi construct, tobacco flavonol synthase encoding cDNA (NtFLS) fragment of 233 bp was cloned in sense and antisense orientations into pFGC1008 vector. The procedure for cloning of NtFLS cDNA in RNAi construct is described in following heads. NtFLS cDNA fragments were first cloned in pGEM®-T Easy vector and then in pFGC1008.

4.3.1 Cloning of NtFLS cDNA fragment in pGEM®-T Easy vector

A 233 bp cDNA fragment of NtFLS was amplified through PCR using primers containing AscI restriction site (FLS-F1 5’-GGCGCGCCGGTTGATCATTTGTCCATAAG-3’) at 5’ end of forward primer and SwaI restriction site (FLS-R1 5’-ATTTAAATCTTTGTTGAAATCAATTATTACC-3’) at 5’ end of reverse primer to clone it in sense orientation. The same sized cDNA fragment of NtFLS was also amplified through PCR using primers containing SpeI restriction site (FLS-F2 5’-ACTAGTGTTGATCATTTGTCCATAAG-3’) at 5’ end of forward primer and BamHI restriction site (FLS-R2 5’-GGATCCCCTTTGTTGAAATCAATTATTACC-3’) at 5’ end of reverse primer to clone it in antisense orientation (Fig. 4.7a). First, these PCR amplified products were cloned in pGEM®-T Easy vector. The confirmation of cloning was done through colony PCR using same above mentioned primer sets, which was resulted in 233 bp PCR amplification product (Fig. 4.7b).

![Fig. 4.7 Cloning of NtFLS for RNAi construct preparation. PCR amplifications of tobacco flavonol synthase (NtFLS) cDNA fragment (233bp) using primers containing AscI at 5’ end of forward primer and SwaI at 5’ end of reverse primer to clone it in sense orientation, while using primers containing SpeI at 5’ end of forward primer and BamHI at 5’ end of reverse primer to clone it in antisense orientation (a). Colony PCR result confirmed the cloning of above isolated NtFLS cDNA fragments into pGEM®-T Easy vector (b).](image-url)
4.3.2 Antisense cloning of *NtFLS* partial cDNA fragment in RNAi vector

The *NtFLS* cDNA fragment was first cloned in antisense orientation in RNAi vector. For this, *SpeI* and *BamHI* restrict digested cDNA fragment of *NtFLS* was obtained from the recombinant pGEM®-T Easy vector by resolving in 1% agarose gel (Fig. 4.8a).

This double-digested cDNA fragment of *NtFLS* was ligated in antisense orientation (*NtFLS*-AS) with the similar restrict digested RNAi vector pFGC1008 (Fig. 4.8b). After transformation of the ligated product into *E. coli* (cells), confirmation of ligation was done through colony PCR using vector specific primers mentioned in materials and methods (section 3.3.2.4). The cloning of cDNA fragment of *NtFLS* in antisense orientation was confirmed through PCR amplification, where 798 bp \{control [pFGC1008 without NtFLS fragment at antisense orientation].
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(AS)] + NtFLS (AS); 565+233 bp} fragment was obtained (Fig. 4.8c). After this cloning, the recombinant plasmid pFGC1008 containing antisense \( NtFLS \) fragment [pFGC1008-NtFLS (AS)] was isolated from a positive colony and was used for sense cloning of \( NtFLS \).

4.3.3 Sense cloning of \( NtFLS \) partial cDNA fragment in RNAi vector

\( NtFLS \) partial cDNA fragment was cloned in sense orientation into RNAi vector already containing same fragment in antisense orientation. For this, \( AscI \) and \( SwaI \) restrict digested cDNA fragment of \( NtFLS \) was obtained from recombinant pGEM-T Easy vector by resolving in 1% agarose gel (Fig. 4.9a).

![Image](a)

\( NtFLS \) cDNA fragment and pFGC1008-\( NtFLS \) (AS)

\( AscI \) and \( SwaI \) digested

![Image](b)

This double-digested cDNA fragment of \( NtFLS \) was ligated in sense orientation (NtFLS-S) with the similar restrict digested pFGC1008 which already contained antisense fragment (NtFLS-AS) (Fig. 4.9a). After transformation of ligated product into \( E. coli \) (cells), confirmation of ligation was done through colony PCR using vector specific primers.
mentioned in materials and methods (section 3.3.2.5). The cloning of \textit{NtFLS} fragment in sense orientation was confirmed by PCR amplification, where 458 bp \{control [pFGC1008 without \textit{NtFLS} (S)] + \textit{NtFLS} (S); 225+233 bp\} fragment was obtained (Fig. 4.9b).

The schematic representation of RNAi construct (pFGC1008-\textit{NtFLS}) prepared is shown in Fig. 4.10a. The recombinant plasmid pFGC1008 containing sense and antisense \textit{NtFLS} fragments was isolated from a positive \textit{E. coli} colony.

![Schematic representation of RNAi construct (pFGC1008-\textit{NtFLS}).](image)

**Fig. 4.10** Schematic representation of RNAi construct (pFGC1008-\textit{NtFLS}). T-DNA region containing the inverted repeats of tobacco flavonol synthase (\textit{FLS}) cDNA fragments under the control of CaMV35S promoter (p35S) while octopine synthase (OCS) as terminator, a GUS-Intron in between sense and antisense \textit{NtFLS} cDNA fragments (\textit{NtFLS}-S and \textit{NtFLS}-AS), the T-DNA left border (LB) and right border (RB), a plant hygromycin (Hyg) resistance gene as plant selection marker (a). PCR confirmation of prepared pFGC1008-\textit{NtFLS} construct (b). PCR resulted in 458 bp (Lane 2), 798 bp (Lane 4), 1.361 bp (Lane 5) amplified products of cloned \textit{NtFLS}-S, \textit{NtFLS}-AS fragment and \textit{NtFLS} (S) + GUS + \textit{NtFLS} (AS) in pFGC1008 vector respectively. Whereas, lane 1 and lane 2 shows 225 bp and 565 bp bands as controls respectively (b). Double digestion of prepared RNAi construct with \textit{AscI} and \textit{SpeI} resulted in insert of 838 bp (c).

### 4.3.4 PCR confirmation of prepared RNAi construct

After cloning of sense and antisense cDNA fragments of \textit{NtFLS} into RNAi vector, final confirmation of prepared RNAi construct was done through PCR using vector specific primer sets (Materials and methods; section 3.3.2.4 and 3.3.2.5) and pFGC1008-\textit{NtFLS}
plasmid as template. The PCR amplification was resulted in 458 bp \{control [pFGC1008 without NtFLS (S)] + NtFLS (S); 225+233 bp\} and 798 bp \{control [pFGC1008 without NtFLS (AS)] + NtFLS (AS); 565+233 bp\} amplified products corresponding to sense and antisense cloning (Fig. 4.10b).

The whole RNAi cassette was also confirmed using the vector specific primers mentioned in material and methods (section 3.3.2.6). PCR amplification was resulted in 1.361 kb amplified product corresponding to whole RNAi cassette (Fig. 4.10b). The double digestion of prepared RNAi construct with \textit{AscI} and \textit{SpeI} produced a fragment of 838 bp [FLS-(S)+GUS+FLS(AS)], further confirm the formation of RNAi construct (Fig. 4.10c). This RNAi construct was used for tobacco transformation to silence \textit{NtFLS}.

### 4.4 Transformation of overexpression and RNAi constructs individually in \textit{A. tumifaciens}

The pCAMBIA 1302-\textit{CsF3H} and pFGC1008-\textit{NtFLS} constructs were transformed individually in \textit{Agrobacterium tumifaciens} strain LBA4404 by using triparental mating technique. The resistant colonies grown on LB media containing kanamycin and streptomycin in case of pCAMBIA 1302-\textit{CsF3H} and LB media containing chloramphenicol and streptomycin in case of pFGC1008-\textit{NtFLS} were screened via colony PCR method. The presence of band corresponding to the size of \textit{CsF3H} cDNA confirmed the presence of overexpression construct (Fig. 4.11a) and the band corresponding to the size of whole \textit{NtFLS} RNAi cassette confirmed the presence of RNAi construct in \textit{A. tumifaciens} strain (Fig. 4.11b). These confirmed positive \textit{A. tumifaciens} colonies carrying recombinant vectors were then used for tobacco transformation.

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**Fig. 4.11** Colony PCR confirmation of pCAMBIA 1302-\textit{CsF3H} (a) and pFGC1008-\textit{NtFLS} (b) in \textit{Agrobacterium} (LBA4404) strain.
4.5 Tobacco transformation with *Agrobacterium* containing overexpression construct (pCAMBIA 1302-CsF3H) and RNAi construct (pFGC1008-NtFLS).

*Agrobacterium* tumefaciens harbouring either pCAMBIA1302-CsF3H or pFGC1008-NtFLS was individually used for leaf disc transformation of tobacco (*Nicotiana tabacum* L.) to generate transgenic tobacco plants. *Agrobacterium* tumefaciens containing empty pCAMBIA 1302 (with no transgene) was used as a control for *Agrobacterium* tumefaciens harbouring pCAMBIA1302-CsF3H and empty pFGC1008 was used as a control for *Agrobacterium* tumefaciens harbouring pFGC1008-NtFLS. The controls were also used for leaf disc transformation. The leaf discs from tissue culture grown wild tobacco plants were used for *Agrobacterium* mediated transformation. 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, cefotaxime and plant selection antibiotic hygromycin (mentioned in material and methods under section 3.4.2.3) (Fig. 4.12a).

![Fig. 4.12 Agrobacterium mediated tobacco transformation.](image-url)

Empty pCAMBIA 1302 (a), pCAMBIA 1302-CsF3H (b), empty pFGC1008 (c) and pFGC1008-NtFLS (d) transformed leaf discs of tobacco placed on selection media for regeneration. Empty pCAMBIA 1302 (e), pCAMBIA 1302-CsF3H (f), empty pFGC1008 (g) and pFGC1008-NtFLS (h) transformed tobacco leaf discs on selection media under callusing phase and empty pCAMBIA 1302 (i), pCAMBIA 1302-CsF3H (j), empty pFGC1008 (k) and pFGC1008-NtFLS (l) putative transgenic tobacco plants on selection media.
After 1-2 weeks, callus was formed at the cut ends of leaf discs (Fig. 4.12b). These discs were transferred to same fresh media for shoot induction. After 3-4 weeks, hygromycin resistant well-grown plantlets at 1-2 node stage were separated and transferred to rooting media (mentioned in material and methods under section 3.4.2.4). Figure 4.12 c 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. Several transgenic tobacco lines were screened for transgene integration via genomic DNA PCR.

4.6 Screening of transgenic tobacco plants
Screening of transgenic tobacco plants for transgene insertion was done through genomic DNA PCR and the expression of transgene was analyzed through semiquantitative PCR.

4.6.1 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 F1 and F2 generation of transgenic tobacco. The transformants were screened for the presence of CsF3H cDNA using gene specific primers and for the presence of NtFLS RNAi cassette using vector specific primers. The results confirmed 20 and 10 PCR positive CsF3H overexpressing and NtFLS silenced transgenic lines in F1 generation, respectively. These PCR positive transgenic lines were allowed to grow in green house to set seeds for next generation via self pollination. The seeds of transgenic F1 plant lines were selected on MS media containing hygromycin. Resistant plants were further confirmed and found to be PCR positive for the transgene. This has confirmed the stable integration of CsF3H and NtFLS RNAi cassette in F2 progenies of transgenic tobacco. A representative figure depicting the integration of CsF3H cDNA (Fig. 4.13a) and NtFLS RNAi cassette (Fig. 4.13b) in genomic DNA of transgenic tobacco plants is shown for three and four lines, respectively. A specific band of 1.12 and 1.361 kb has indicated the introduction of CsF3H cDNA and NtFLS RNAi cassette respectively in the transformed transgenic lines.
4.6.2 Expression analysis of transgene in CsF3H overexpressing lines through semiquantitative PCR

The CsF3H overexpressing transgenic lines were evaluated for the expression of transgene through semiquantitative PCR. All PCR positive CsF3H overexpressing transgenic lines were also found to be positive for transgene expression. While in control tobacco plants, no expression was observed for the transgene (Fig. 4.14). Homozygous lines of CsF3H overexpressing transgenics from F2 progenies were kept in green house facility. Three transgenic lines i.e. F10, F9 and F12b showing the maximum expression of transgene were chosen for further analysis.

4.6.3 Reduction in NtFLS transcript expression in NtFLS Silenced transgenic lines

The PCR positive NtFLS silenced transgenic plants were further analyzed for expression of NtFLS encoding gene. The NtFLS transcript expression in control and transgenic lines was studied through semiquantitative PCR. The constitutively expressed 26S rRNA was used
as an internal standard in expression analysis. Out of 10 PCR positive *NtFLS* silenced transgenic lines, 4 lines showed significant downregulation in transcript expression of *NtFLS* gene. The two *NtFLS* silenced transgenic lines G12 and A2 showed up to 80 % decrease in expression levels of *NtFLS* gene as compared to control. In contrast, a relatively less decrease in *NtFLS* expression of about 20-22 % was found in other two *NtFLS* silenced transgenic lines B1 and E13 (Fig. 4.15).

![Figure 4.15](image.png)

Fig. 4.15 Semiquantitative PCR analysis for expression study. Steady-state mRNA level of tobacco *NtFLS* was measured in leaf tissue of *NtFLS* silenced transgenic lines (G12, A2, B1, E13) and control tobacco. Housekeeping gene 26SrRNA was used as internal control. Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Black and grey bars show 26SrRNA and *NtFLS* transcript levels, respectively.

### 4.7 Evaluation of transgenic tobacco plants

Three lines of *CsF3H* overexpressing transgenics F10, F9 and F12b and four lines of *NtFLS* silenced transgenics G12, A2, B1 and E13 were used for evaluation studies.

#### 4.7.1 Transcript expression of endogenous flavonoid biosynthetic pathway genes in transgenic tobacco plants

The influence of *CsF3H* overexpression and *NtFLS* silencing was studied on the expression of endogenous genes of flavonoid biosynthetic pathway in tobacco plants such as *NtPAL*, *NtCHS*, *NtCHI*, *NtF3H*, *NtDFR*, *NtFLS* and *NtANS*. The transcript expression was analyzed in one best line of each *CsF3H* overexpressing (line F10) and *NtFLS* silenced (line G12) transgenics. No significant change was observed in transcript level of *NtPAL*,...
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*NtCHS, NtCHI, NtF3H* genes in both *CsF3H* overexpressing (Fig. 4.16a) and *NtFLS* silenced transgenics (Fig. 4.16b) with respect to control tobacco plants. In contrast to this, genes encoding *NtDFR* and *NtANS* enzymes of flavonoid pathway showed significant increase in their expression level in both *CsF3H* overexpressing and *NtFLS* silenced transgenic lines compared to control tobacco plant. *NtDFR* showed 20.7% increase and *NtANS* showed 45.4% increase in their transcript level in *CsF3H* overexpressing transgenic line compared to control tobacco plant (Fig. 4.16a).

![Graph](image)

**Fig. 4.16** Transcript level of genes encoding flavonoid biosynthetic pathway enzymes *NtPAL*, phenylalanine ammonia lyase; *NtCHS*, chalcone synthase; *NtCHI*, chalcone isomerase; *NtF3H*, flavanone 3-hydroxylase; *NtFLS*, flavonol synthase; *NtDFR*, dihydroflavonol reductase; *NtANS*, anthocyanidin synthase in control and *CsF3H* overexpressing transgenic tobacco line F10 (a) and in control and *NtFLS* silenced transgenic line G12 (b). Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Black bars indicate 26S rRNA expression, dark grey bars indicate expression in control and light grey bars indicate expression in *CsF3H* transgenic line F10 and *NtFLS* silenced transgenic line G12.
Similarly, $NtFLS$ silenced transgenic line showed 37 % and 98 % increase in transcript level of $NtDFR$ and $NtANS$ respectively compared to control tobacco leaf (Fig. 4.16b). No significant change was observed in transcript level of $NtFLS$ in $CsF3H$ overexpressing transgenic lines, whereas $NtFLS$ silenced transgenic lines showed more than 70 % decrease in $NtFLS$ transcript level.

**4.7.2 Flavonoids content in transgenic tobacco plants**

Since $CsF3H$ is a upstream enzyme encoding gene in flavonoid pathway, therefore its upregulation might affect the flavonoids level in $CsF3H$ overexpressing transgenics. On the other side, FLS enzyme compete with dihydroflavonol 4-reductase (DFR) for the common substrate dihydroflavonols and its downregulation might also affect the flavonoids level in $NtFLS$ silenced transgenics. With this assumption, the relative amount of total flavonols, total flavan-3-ols [(catechin (Cat), epicatechin (EC) and epigallocatechin (EGC)] and anthocyanins content were determined in leaves of transgenic lines vis à vis control tobacco plants.

**4.7.2.1 Total flavonols**

The $CsF3H$ overexpressing and $NtFLS$ silenced transgenic lines were evaluated for change in total flavonol content. Three $CsF3H$ overexpressing transgenic lines F10, F9 and F12b showed decrease in their total flavonol content compared to control tobacco. But the difference in total flavonol content between control and $CsF3H$ overexpressing transgenic lines was found to be insignificant (Fig. 4.17a).

Whereas, significant decrease in total flavonol content was observed in $NtFLS$ silenced transgenic lines. The G12, A2, B1 and E13 lines showed 53 %, 38 %, 20 % and 17 % decrease in their total flavonol content respectively compared to control tobacco plants (Fig. 4.17b). The decrease was found to be maximum in transgenic lines G12, A2 followed by lines B1 and E13. Quercetin (Quer) is a major flavonol present in tobacco. Therefore, Quer content was measured in leaves of $NtFLS$ silenced transgenics and control tobacco plants. Based on HPLC analyses of leaf extract, the decrease in Quer content was observed in these transgenic lines as compared to control tobacco plant. For all $NtFLS$ silenced transgenic lines, the observed decrease in Quer content was well correlated with expression data. The G12, A2, B2 and E13 lines showed 93 %, 80 %, 27 % and 25 % reduction in their Quer content respectively as compared to control tobacco plant (Fig.
Therefore, A2 and G12 were regarded as lines with “strong” phenotype whereas B2 and E13 as “weak” phenotype.

Fig. 4.17 Total flavonols content in leaf extract of control and CsF3H overexpressing tobacco transgenic lines F10, F9 and F12b (a) and in leaf extract of control and NtFLS silenced tobacco transgenic lines G12, A2, B1 and E13 (b). Quercetin content in leaf extract of control and NtFLS silenced tobacco transgenic lines G12, A2, B1 and E13 (c). Significant differences from the control are denoted by one or two asterisk corresponding to $P < 0.05$ and $P < 0.01$, respectively, by Student’s t-test.

4.7.2.2 Flavan-3-ols content

Under flavan-3-ols, catechin (Cat), epicatechin (EC) and epigallocatechin (EGC) content was measured in leaves of CsF3H overexpressing, NtFLS silenced transgenic lines and control tobacco. The Cat content was increased to 0.23 (F10), 0.22 (F9), 0.18 (F12b) mg g$^{-1}$ FW in CsF3H overexpressing transgenic lines compared to control tobacco leaves (Fig. 4.18a). Cat content was found to be very low almost undetectable in control tobacco leaves. Similarly EC content was increased to 0.24 (F10), 0.12 (F9), 0.07 (F12b) mg g$^{-1}$ FW in CsF3H overexpressing transgenic lines compared to 0.0167 mg g$^{-1}$ FW in control tobacco (Fig. 4.18b). EGC content was increased to 0.12 (F10), 0.06 (F9) and 0.1 (F12b) mg g$^{-1}$ FW in CsF3H overexpressing transgenic lines compared to 0.05 mg g$^{-1}$ FW in control tobacco leaves (Fig. 4.18c). Thus EGC content was increased by 61.7 % (F10), 39.7 % (F9) and 28 % (F12b) in CsF3H overexpressing transgenic lines.
Fig. 4.18 (+)-Cat (a), (-)-EC (b) and (-)-EGC (c) content in CsF3H overexpressing transgenic lines F10, F9 and F12b compared to control tobacco plant. HPLC chromatogram of 0.2 mg ml\(^{-1}\) of (+)-Cat (d) and (-)-EC (e) standards showed sharp peak at retention time of 10.07 and 13.93 m respectively at 280 nm. The standard (-)-EGC showed peak at retention time of 6.03 m (Sharma et al. 2005). Chromatogram showing (+)-Cat, (-)-EC and (-)-EGC content in control (f). Cat was found to be undetectable in the control with a very less amount of EC and EGC. Chromatograms showing peaks of Cat, EC and EGC at similar retention times to that of standards in CsF3H overexpressing transgenic lines F10 (g), F9 (h) and F12b (i). Red arrows indicate the peak for Cat (catechin), black arrows indicate peak for EC (epicatechin) and green arrows indicate peak for EGC (epigallocatechin). Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test.

HPLC chromatogram of 0.2 mg ml\(^{-1}\) (+)-Cat and (-)-EC and (-)-EGC standards showed sharp peak at retention time of 10.07 m (Fig. 4.18d), 13.93 m (Fig. 4.18e) and 6.03 m (Sharma et al. 2005), respectively. UV spectra of these peaks were obtained by photodiode array detector at 280 nm wavelength. Chromatogram of (+)-Cat and (-)-EC and (-)-EGC content in control and different CsF3H overexpressing lines also showed peaks at retention times similar to standards at 280 nm (Fig. 4.18f-4.18i)

In the same way, the Cat content was increased to 0.92 (G12), 0.51 (A2), 0.7 (B1), 0.76 (E13) mg g\(^{-1}\) FW in NtFLS silenced transgenic lines (Fig. 4.19a) compared to control tobacco leaves. Cat content was found to be very low almost undetectable in control tobacco leaves.
EC content was increased to 0.038 (G12), 0.035 (A2), 0.04 (B1), 0.027 (E13) mg g\(^{-1}\) FW in \textit{NtFLS} silenced transgenic lines compared to 0.0165 mg g\(^{-1}\) FW in control tobacco leaves (Fig. 4.19b). EGC content was increased to 3.63 (G12), 0.51 (A2), 1.9 (B1) and 2.9 (E13) mg g\(^{-1}\) FW in \textit{NtFLS} silenced transgenic lines compared to 0.055 mg g\(^{-1}\) FW in control tobacco leaves. Chromatogram of (+)-Cat and (-)-EC and (-)-EGC content in control and different \textit{NtFLS} silenced transgenic lines also showed peaks at retention times similar to standards at 280 nm (Fig. 4.19d- 4.19g)

### 4.7.2.3 Anthocyanins content

Anthocyanidins form a branching point in the flavonoid pathway synthesizing anthocyanins on one side and flavan-3-ols on other side. Therefore, increase in flavan-3-ols might have affected anthocyanins level in transgenic lines. Hence its content was measured...
in transgenic lines. In contrast to flavan-3-ols level, anthocyanins content was found to be decreased in CsF3H overexpressing and NtFLS silenced transgenic lines compared to control tobacco plants. Anthocyanins content was decreased by 61.7 %, 39.7 % and 28 % in F10, F9 and F12b CsF3H overexpressing transgenic lines compared to control tobacco plants (Fig. 4.20a). Among the different CsF3H overexpressing transgenic lines, anthocyanins content was higher in F12b followed by F9 and F10. Similarly in NtFLS silenced transgenic lines G12, A2, B1 and E13, anthocyanins content was decreased by 58 %, 22 %, 29 % and 47 % respectively as compared to control tobacco plant (Fig. 4.20b). Among different NtFLS silenced transgenic lines, the anthocyanins content was higher in A2, followed by B1, E13 and G12.

![Fig. 4.20 Anthocyanin content in methanolic extracts of flowers of control and CsF3H overexpressing transgenic lines F10, F9 and F12b (a). Anthocyanin content in methanolic extracts of flowers of control and NtFLS silenced transgenic lines G12, A2, B1 and E13 (b). A_530, absorption at 530 nm; A_657, absorption at 657 nm. Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test.](image)

### 4.7.3 Antioxidant potential of transgenic tobacco plants

In addition to the observed increase in flavan-3-ols content, CsF3H overexpressing and NtFLS silenced transgenics were also studied for their in planta response on transcript levels of genes encoding enzymes of antioxidant pathway as well as activities of antioxidant enzymes such as glutathione reductase (GR), ascorbate peroxidase (APx), catalase (CAT) and glutathione-S-transferase (GST).

#### 4.7.3.1 Transcript expression of genes encoding antioxidant pathway enzymes

Transcript expression of genes encoding antioxidant pathway enzymes was studied in one line of each CsF3H overexpressing (line F10) and NtFLS silenced (line G12) transgenics
and control tobacco plants. The influence of *CsF3H* overexpression was analyzed on transcript levels of genes encoding GR, AP$_x$, CAT and GST. *CsF3H* transgenic line F10 showed induction in the expression of genes encoding GR, AP$_x$ and CAT enzymes by 21 %, 11.7 % and 33.3 % respectively, compared to control tobacco plant. While expression level of gene encoding GST was decreased by 8.5 % in *CsF3H* transgenic line compared to control tobacco plant (Fig. 4.21a).

![Fig. 4.21 Transcript level of genes encoding antioxidant pathway enzymes GR, glutathione reductase; AP$_x$, ascorbate peroxidase; CAT, catalase and GST, glutathione S-transferase in control and *CsF3H* overexpressing transgenic tobacco line F10 (a). Transcript level of genes encoding antioxidant pathway enzymes GR, AP$_x$, CAT and GST in control and *NtFLS* silenced transgenic line G12 (b). Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Black bars indicate 26S rRNA expression, dark grey bars indicate the expression in control plant and light grey bars indicate expression in *CsF3H* overexpressing and *NtFLS* silenced transgenic line.](image)

About 10 %, 21 % and 24 % increase in the expression levels of genes encoding GR, AP$_x$ and CAT enzymes was observed in *NtFLS* silenced transgenic line G12 respectively. While 65 % decrease in the expression of gene encoding GST enzyme was observed in *NtFLS* silenced transgenic line compared to control tobacco plants (Fig. 4.21b).

### 4.7.3.2 Increased activity of antioxidant enzymes in transgenic tobacco plants

To check whether the antioxidant enzymes showed transcriptional or post-transcriptional response, activity analysis of all four enzymes glutathione reductase (GR), ascorbate peroxidase (AP$_x$), catalase (CAT) and glutathione-S-transferase (GST) was conducted in leaf tissue of control, *CsF3H* overexpressing and *NtFLS* silenced tobacco. The activity of GR, AP$_x$, CAT antioxidant enzymes showed similar trend, whereas GST showed the reverse trend to that of expression pattern of their respective genes in the leaves of both *CsF3H* overexpressing and *NtFLS* silenced lines. The activity of all the four antioxidant enzymes GR, AP$_x$, CAT and GST was increased in *CsF3H* overexpressing transgenic lines.
compared to control. GR enzyme activity was observed as 1.74 (F10), 1.69 (F9), 1.55 (F12b) µmoles min\(^{-1}\)g\(^{-1}\)FW in CsF3H overexpressing transgenic lines compared to 1.097 µmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves. AP\(_x\) enzyme activity was observed as 2.46 (F10), 2.29 (F9) and 2.01 (F12b) µmoles min\(^{-1}\)g\(^{-1}\)FW in CsF3H overexpressing transgenic lines compared to 1.08 µmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves. CAT enzyme activity was observed as 0.167 (F10), 0.135 (F9) and 0.129 (F12b) nmoles min\(^{-1}\)g\(^{-1}\)FW in CsF3H overexpressing transgenic lines compared to 0.097 nmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves (Fig. 4.22a). GST enzyme activity was observed as 0.46 (F10), 0.4 (F9) and 0.35 (F12b) µmoles min\(^{-1}\)g\(^{-1}\)FW in CsF3H overexpressing transgenic lines compared to 0.29 µmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves. Hence CsF3H overexpressing transgenic line F10 showed 58.8 %, 137 %, 72.2 %, 56.8 %; line F9 showed 54 %, 113 %, 39 % 37 %, and line F12b showed 41 %, 86 %, 33 % and 21 %, increase in GR, AP\(_x\), CAT, GST activity respectively compared to control tobacco leaf (Fig. 4.22a). Maximum increase in activity of antioxidant enzymes was shown by CsF3H overexpressing transgenic line F10 followed by F9 and F12b.

Similarly, the activity of all the four antioxidant enzymes GR, AP\(_x\), CAT and GST was increased in NtFLS silenced transgenic lines compared to control. GR enzyme activity was observed as 2.44 (G12), 1.81 (A2), 1.55 (B1) and 1.24 (E13) µmoles min\(^{-1}\)g\(^{-1}\)FW in NtFLS silenced transgenic lines compared to 1.12 µmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves. AP\(_x\) enzyme activity was observed as 3.24 (G12), 2.889 (A2), 1.57 (B1) and 1.23 (E13) µmoles min\(^{-1}\)g\(^{-1}\)FW in NtFLS silenced transgenic lines compared to 1.05 µmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves. CAT enzyme activity was observed as 0.151 (G12), 0.142 (A2), 0.124 (B1) and 0.116 (E13) nmoles min\(^{-1}\)g\(^{-1}\)FW in NtFLS silenced transgenic lines compared to 0.095 nmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves (Fig. 4.22b). GST enzyme activity was observed as 0.625 (G12), 0.542 (A2), 0.458 (B1) and 0.382 (E13) µmoles min\(^{-1}\)g\(^{-1}\)FW in NtFLS silenced transgenic lines compared to 0.32 µmoles min\(^{-1}\)g\(^{-1}\)FW of control tobacco leaves. Hence concluded that NtFLS silenced transgenic line G12 showed 117 %, 208 %, 59 % and 115 %; line A2 showed 61 %, 175 %, 49 % and 87 %; line B1 showed 38 %, 50 %, 31 % and 58 % and line E13 showed 10 %, 17 %, 22 % and 15 % increase in GR, AP\(_x\), CAT and GST activity respectively compared to control tobacco leaf (Fig. 4.22a). Maximum increase in activity of all four antioxidant enzymes was shown by NtFLS silenced transgenic line G12 followed by A2, B1 and E13.
Chapter 4

Results

Fig. 4.22 Activities of antioxidant enzymes GR, glutathione reductase; AP\textsubscript{x}, ascorbate peroxidase; CAT, catalase and GST, glutathione \textit{S}-transferase in leaves of control and \textit{CsF3H} overexpressing transgenic tobacco lines F10, F9 and F12b (a). Activities of antioxidant enzymes GR, AP\textsubscript{x}, CAT and GST in leaves of control and \textit{NtFLS} silenced transgenic tobacco lines G12, A2, B1 and E13 (b). Enzyme activity of GST, AP\textsubscript{x} and GR is expressed in \(\mu\text{moles m}^{-1}\text{g}^{-1}\) FW and CAT is expressed in \(\text{nmoles m}^{-1}\text{g}^{-1}\) FW. All results are presented as mean ± SD (n=3). Significant differences from the control are denoted by one or two asterisk corresponding to \(P < 0.05\) and \(P < 0.01\), respectively, by Student's \(t\)-test.

4.7.3.3 Evaluating the effect of catechin application on \textit{in vitro} grown wild tobacco seedlings

In order to see whether the increase in antioxidant enzymes expression/activity in \textit{CsF3H} overexpressing and \textit{NtFLS} silenced transgenics could be due to an increase in flavan-3-ols content, \textit{in vitro} experiment was conducted with seven days old wild tobacco seedlings (untransformed) exposed to two different concentrations of catechin (10 \(\mu\text{M}\) and 50 \(\mu\text{M}\)) for two days. Before further experimentation, the cellular content of catechin in these exposed tobacco seedlings was determined through HPLC analysis. The tobacco seedlings exposed to 10 \(\mu\text{M}\) catechin showed 130 % increase in cellular catechin content whereas seedlings exposed to 50 \(\mu\text{M}\) catechin concentration showed about 250 % increase in catechin content as compared to untreated (wild) tobacco seedlings in which catechin content was found to be undetectable (Fig. 4.23a). HPLC chromatograms showing peaks at retention time of 10.4 and 10.7 m depict cellular concentration of catechin in 10 \(\mu\text{M}\) and 50 \(\mu\text{M}\) exposed tobacco seedlings respectively. Catechin was found to be undetectable in untreated (wild) tobacco seedlings (Fig. 4.23b-4.23d).

4.7.3.3.1 Effect of exogenous application of catechin on expression of genes encoding enzymes of flavonoid and antioxidant pathway in wild tobacco seedlings

The effect of exogenous application of catechin on transcript level of genes encoding flavonoid pathway enzymes NtPAL, NtCHS and NtF3H was studied. In this, expression of
NtPAL was found to be increased, whereas NtCHS and NtF3H expression level was found to be decreased in catechin exposed tobacco seedlings as compared to untreated control (Fig. 4.23e). The transcript level of genes encoding antioxidant enzymes GR, AP_x, CAT and GST in catechin exposed wild tobacco seedlings showed the similar trend as was observed in CsF3H overexpressing and NtFLS silenced transgenic lines. Genes encoding GR, AP_x, CAT enzymes showed an increase in their transcript expression in wild tobacco seedlings exposed to 10 µM and 50 µM catechin as compared to control. In contrast to this, the transcript level of a gene encoding GST was decreased in seedlings exposed to catechin. The decrease was less in seedlings exposed to 50 µM catechin than exposed to 10 µM catechin (Fig. 4.23f). Results suggest that catechin level is effective in modulating the transcript expression of genes encoding antioxidant enzymes.

4.7.3.3.2 Effect of exogenous application of catechin on activity of antioxidant enzymes in tobacco seedlings

The activity of antioxidant enzymes GR, AP_x, CAT and GST in *in vitro* catechin exposed wild tobacco seedlings showed similar trend as was observed in CsF3H overexpressing and NtFLS silenced transgenic lines. The activities of enzymes in wild type tobacco seedlings were higher at lower dose of catechin (10 µM) as compared to its higher dose (50 µM) exposure. The tobacco seedlings exposed to 10 µM catechin was resulted in 9 %, 26 %, 27 % and 64 % increase in GR, AP_x, CAT and GST activity respectively. Whereas 50 µM catechin exposures was resulted in 4%, 18%, and 4% increase in GR, AP_x and GST activity respectively (Fig. 4.23g). In contrast to this, CAT activity in wild tobacco seedlings was increased with lower dose of catechin exposure and decreased at its higher dose.
Fig. 4.23 Cellular content of catechin in *in vitro* catechin treated and untreated wild tobacco seedlings and its effect on the transcript level of genes encoding antioxidant and flavonoid pathway enzymes and also on their antioxidant enzyme activities. Bar diagram shows the cellular content of Cat in wild tobacco seedlings exposed to 10 and 50 µM concentrations of catechin as determined through HPLC analysis (a) Significant difference from the untreated control is denoted by one or two asterisk corresponding to $P < 0.05$ and $P < 0.01$, respectively, by Student’s $t$-test. HPLC chromatogram showing undetectable level of catechin in wild tobacco seedlings (b), HPLC chromatograms showing peaks at retention time of 10.4 and 10.7 min depicting cellular concentration of catechin in 10 µM (c) and 50 µM (d) exposed tobacco seedlings respectively. Transcript level of NtPAL, phenylalanine ammonia lyase; NtCHS, chalcone synthase; NtF3H, flavanone 3-hydroxylase in catechin treated (10 and 50 µM) and untreated wild tobacco seedlings (e). Transcript level of genes encoding antioxidant pathway enzymes GR, glutathione reductase; AP$_x$, ascorbate peroxidase; CAT, catalase and GST, glutathione S-transferase in catechin treated (10 and 50 µM) and untreated wild tobacco seedlings (f). Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Black bars indicate 26S rRNA expression, dark grey indicate untreated control, light grey bars indicate expression in wild tobacco seedlings exposed to 10 µM catechin and very light grey bars indicate expression in seedlings exposed to 50 µM catechin. Effect of exogenous application of catechin on activities of tobacco antioxidant enzymes GR, AP$_x$, CAT and GST (g). Black bars indicate activities in untreated wild tobacco seedlings, dark grey indicates activities in wild tobacco seedlings exposed to 10 µM catechin and light grey bars indicate activities in seedlings exposed to 50 µM catechin. Enzyme activity of APx, GST and GR is expressed in µmoles m$^{-2}$ g$^{-1}$ FW and CAT is expressed in nmole m$^{-2}$ g$^{-1}$ FW.
4.8 Characterization of CsF3H overexpressing transgenic tobacco

Before the effect of CsF3H overexpression was studied on plant functions, the specific activity of recombinant CsF3H protein was determined.

4.8.1 Specific activity of Camellia sinensis flavanone 3-hydroxylase (CsF3H)

Flavanone 3-hydroxylase (F3H) gene of Camellia sinensis, cloned in expression vector pQE-30 U/A was expressed using 1mM IPTG. The recombinant protein was purified under native conditions using Ni-NTA affinity chromatography as mentioned in material and methods section 3.8.4. Specific activity was measured using purified recombinant protein. CsF3H catalyses a conversion of dihydrokaemferol to naringenin in flavonoid biosynthetic pathway. The CsF3H protein exhibited activity in the presence of naringenin, oxoglutaric acid and ferrous sulfate, with a specific activity of 32 nmol min$^{-1}$ mg$^{-1}$ of protein. Formation of dihydrokaemferol from naringenin was analyzed through TLC and HPLC at 290 nm (Fig. 4.24).

![Fig. 4.24 Formation of dihyrokaempferol (DHK) from naringenin by purified recombinant CsF3H protein as analyzed by TLC (R and S represent reaction mixture and substrate, respectively) (a) and F3H enzyme activity assayed by high performance liquid chromatography (b): (i) pure naringenin at 290 nm and (ii) ethyl acetate extract of the enzymatic reaction mixture showing peaks for naringenin and DHK.](image)

4.8.2 Evaluation of CsF3H overexpressing transgenics for salt stress tolerance

Before evaluating the transgenic tobacco overexpressing CsF3H for salt stress tolerance, expression of gene encoding flavanone 3-hydroxylase (F3H) was studied against the salt stress in tea.
4.8.2.1 Expression pattern of CsF3H in response to salt stress in tea

The expression pattern of CsF3H gene encoding flavanone 3-hydroxylase (F3H) was studied in tea (Cs) leaves in response to 50 mM NaCl and 150 mM NaCl stress through semiquantitative PCR. It has been shown earlier that 3rd leaf of tea shoot has maximum expression of CsF3H than 1st leaf, 2nd leaf and apical bud (Singh et al. 2008). Therefore, transcript profiling of CsF3H was studied in 3rd leaf of tea shoot after 24 hrs, 48 hrs and 72 hrs of 0 mM, 50 mM and 150 mM NaCl stress exposure. No significant change in CsF3H expression was observed up to 24 hrs of salt stress treatment. Salt stress of 150 mM NaCl led to maximum increase in CsF3H transcript level after 48 hrs which continued after 72 hrs of stress treatment compared to untreated control. Whereas salt stress of 50 mM NaCl led to maximum increase in CsF3H transcript level after 72 hrs of stress treatment (Fig. 4.25). Hence, salt stress treatment induces the expression of CsF3H in tea leaf tissue.

![Transcript profiling of gene encoding CsF3H studied in 3rd leaf of Camellia sinensis shoot after 24 hrs, 48 hrs and 72 hrs of 0 mM, 50 mM and 150 mM NaCl stress exposure.](image)

4.8.2.2 Response of CsF3H overexpressing transgenic tobacco during normal growth vis-à-vis salt stress conditions

The effect of salt stress was studied on seedling growth and root system of control and CsF3H overexpressing transgenic tobacco lines. The seeds of control and transgenic lines after sterilization were sown on Murashige and Skoog (MS) media. The germination of transgenic seeds were found to be 1 day earlier than control tobacco seeds. After 10 days of germination, seedlings of control and CsF3H overexpressing transgenic lines were
either transferred to MS media only or to MS media supplemented with 50 mM, 150 mM and 200 mM NaCl and allowed to grow vertically for next 15 days. The control and CsF3H overexpressing transgenic seeds showed significant difference in their root system even when grown in normal MS medium without NaCl. The CsF3H overexpressing transgenic seedlings (line F10, F9 and F12b) exhibited more number of lateral roots with larger primary root length compared to control tobacco seedlings (Fig. 4.26a, 4.26e and 4.26f). The significant difference in root system was also observed between control and CsF3H transgenic tobacco seedlings (line F10, F9 and F12b) after 15 days of exposure to different concentrations of 50 mM (Fig. 4.26b), 150 mM (Fig. 4.26c) and 200 mM (Fig. 4.26d) NaCl.

![Fig. 4.26 Comparison of root system of control and CsF3H overexpressing transgenic tobacco seedlings under unstressed and salt stressed conditions. Photographs depicting root system morphology of 10 days old seedlings of control and CsF3H transgenic line F10 grown on normal MS media with 0 mM NaCl (a) and on MS media supplemented with 50 mM NaCl (b), with 150 mM NaCl (c) and with 200 mM NaCl (d) for next 15 days. Below photographs of seedlings on MS plates, bar diagram shows primary root length (e) and number of lateral roots (f) in seedlings of control and CsF3H overexpressing transgenic lines F10, F9 and F12b under 0 mM NaCl, 50 mM NaCl, 150 mM NaCl and 200 mM NaCl stress. Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test.](image)

The primary root length (Fig. 4.26e) and number of lateral roots (Fig. 4.26f) were significantly higher in seedlings of CsF3H transgenic lines F10, F9 and F12b under unstressed and 50 mM, 150 mM and 200 mM salt stress treatment compared to control tobacco seedlings. The shoot growth was also higher in seedlings of CsF3H transgenic tobacco compared to control under 50 mM and 150 mM salt treatment. However, overall
difference in shoot and root growth was more pronounced in case of F10 CsF3H transgenic seedlings exposed to 150 mM NaCl stress compared to other transgenic and control seedlings. No significant difference in shoot growth of transgenic lines was observed with 200 mM NaCl treatment compared to control.

4.8.2.3 Effect of salt stress on morphology of CsF3H overexpressing transgenic tobacco under greenhouse conditions

In order to evaluate salt stress tolerance capacity further, 45 days old control and CsF3H overexpressing transgenic tobacco were subjected to continuous salt stress of 50 mM, 150 mM and 200 mM NaCl under greenhouse conditions for next 40 days. The representative picture of control and transgenic line F10 is shown in Fig. 4.27.

Fig. 4.27 Salt stress response of control and CsF3H overexpressing transgenic potted tobacco plants. Representative photograph shows the phenotype of 45 days old control and transgenic tobacco line F10 at 0 days of 0 mM NaCl stress (a), after 25 days of 0 mM NaCl stress (b) and after 40 days of 0 mM NaCl stress (c). Photograph shows the phenotype of 45 days old control and transgenic tobacco line F10 at 0 days of 50 mM NaCl stress (d), after 25 days of 50 mM NaCl stress (e) and after 40 days of 50 mM NaCl stress (f). Photograph shows the phenotype of 45 days old control and transgenic tobacco line F10 at 0 days of 150 mM NaCl stress (g), after 25 days of 150 mM NaCl stress (h) and after 40 days of 150 mM NaCl stress (i).
4.8.2.4 Analysis of salt stress tolerance parameters in transgenic tobacco

To check the effect of salt stress on \textit{CsF3H} overexpressing transgenic tobacco, various analyses like electrolyte leakage, chlorophyll content, MDA content, \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) detection were performed in leaves of control and \textit{CsF3H} overexpressing transgenic lines F10, F9 and F12b after 40 days of salt stress.

4.8.2.4.1 Electrolyte leakage in leaves of control and \textit{CsF3H} overexpressing transgenics

The electrolyte leakage (EL) in leaves of control and \textit{CsF3H} overexpressing transgenics was studied under unstressed and salt stressed conditions. The electrolyte leakage (EL) was higher in leaves of control (28.1 %) in comparison to \textit{CsF3H} transgenic line F10 (20.9 %), F9 (21.25 %) and F12b (22.92 %) under unstressed conditions. Treatment to salt stress was resulted in an increase of percent EL. Significant difference in EL was observed in leaves of control and \textit{CsF3H} transgenic lines F10, F9 and F12b during 50 mM NaCl exposure. Similarly, leaf of control tobacco exhibited more EL (51.7 %) as compared to \textit{CsF3H} transgenic line F10 (37.9 %), F9 (38.2 %) and F12b (40.92 %) exposed to 150 mM NaCl (Fig. 4.28). The difference in EL between control and transgenic lines was found to be significant during salt stress.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig428.png}
\caption{Electrolyte leakage in leaves of control and \textit{CsF3H} overexpressing transgenic lines F10, F9 and F12b under 0 mM NaCl, 50 mM NaCl and 150 mM NaCl stress. Significant differences from the control are denoted by one or two asterisk corresponding to \(P < 0.05\) and \(P < 0.01\), respectively, by Student’s \(t\)-test.}
\end{figure}
4.8.2.4.2 Chlorophyll content in leaves of control and CsF3H overexpressing transgenics

The trend of chlorophyll content was found opposite to that of EL. The chlorophyll content was found to be higher in leaves of CsF3H overexpressing transgenic line F10 (1.40 µg ml⁻¹) followed by transgenic line F9 (1.39 µg ml⁻¹) and F12b (1.38 µg ml⁻¹) compared to control tobacco plant (1.22 µg ml⁻¹) under unstressed conditions. The transgenic lines F10, F9 and F12b showed 15 %, 14 % and 13 % increase in chlorophyll content respectively compared to control tobacco under unstressed conditions. The 50 mM NaCl stress treatment decreased chlorophyll content of control to 1.06 µg ml⁻¹ compared to 1.25 µg ml⁻¹, 1.24 µg ml⁻¹ and 1.23 µg ml⁻¹ of CsF3H overexpressing transgenic lines F10, F9 and F12b respectively. However, the effect was more pronounced with 150 mM NaCl treatment. During 150 mM NaCl stress, chlorophyll content of control was decreased to 0.45 µg ml⁻¹ and that of CsF3H transgenic lines F10, F9 and F12b to 0.89 µg ml⁻¹, 0.85 µg ml⁻¹ and 0.81 µg ml⁻¹ respectively (Fig. 4.29). But still chlorophyll content was higher in transgenic lines F10, F9 and F12b compared to control tobacco under 50 mM and 150 mM NaCl stress. Thus, the difference in chlorophyll content between control and transgenic lines was found to be significant under 50 mM as well as 150 mM NaCl stress.

Fig. 4.29 Chlorophyll content in leaves of control and CsF3H overexpressing transgenic lines F10, F9 and F12b under 0 mM NaCl, 50 mM NaCl and 150 mM NaCl stress. Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test.
4.8.2.4.3 MDA content in leaves of control and CsF3H overexpressing transgenics

Accumulation of ROS during stress treatment leads to lipid peroxidation, which was estimated in terms of malondialdehyde (MDA) content. The MDA content of control was found to be 0.90 nmol g\(^{-1}\) FW, which was higher than CsF3H transgenic line F10 (0.7 nmoles g\(^{-1}\) FW), F9 (0.71 nmoles g\(^{-1}\) FW) and F12b (0.72 nmoles g\(^{-1}\) FW) under unstressed conditions. Hence, control plants showed significantly higher 22 %, 21 % and 20 % MDA content compared to transgenic lines F10, F9 and F12b respectively under unstressed conditions. MDA content was increased during salt stress but increase was more in control than transgenic lines. The control tobacco plants showed 24 %, 23 % and 18 % higher MDA content compared to CsF3H transgenic lines F10, F9 and F12b respectively under 50 mM salt stress. While MDA content was 30 %, 26 % and 24 % higher in control compared to CsF3H overexpressing transgenic lines F10, F9 and F12b respectively under 150 mM salt stress (Fig. 4.30). Taken together, salt stress induced MDA formation to a greater extent in control plant than CsF3H transgenic lines.

![Fig. 4.30 MDA content in leaves of control and CsF3H overexpressing transgenic lines F10, F9 and F12b under 0 mM NaCl, 50 mM NaCl and 150 mM NaCl stress. Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test.](image-url)
4.8.2.4.4 Detection of $O_2^-$ and $H_2O_2$ in seedlings of control and CsF3H overexpressing transgenics

Histochemical staining with NBT and DAB was carried out in *in vitro* grown seedlings of control and CsF3H overexpressing transgenic line F10 in order to check the levels of $O_2^-$ and $H_2O_2$, respectively. The control tobacco seedlings exhibited deeper blue staining with NBT compared to CsF3H transgenics under unstressed conditions (Fig. 4.31a), 50 mM NaCl stress (Fig. 4.31b) and 150 mM NaCl stress (Fig. 4.31c). The deeper brown staining with DAB was observed in control than CsF3H transgenic seedlings under unstressed conditions (Fig. 4.31d), 50 mM NaCl stress (Fig. 4.31e) and 150 mM NaCl stress (Fig. 4.31f). Data indicates that CsF3H transgenic lines accumulated less $H_2O_2$ and $O_2^-$ under unstress as well as salt stress conditions compared to control tobacco seedlings.

![Fig. 4.31 Histochemical staining of ROS accumulation with nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) in control and CsF3H overexpressing transgenic tobacco line F10. NBT staining of *in vitro* grown seedlings of control and transgenic line F10 under unstressed conditions (a), under 50 mM NaCl stress (b), and under 150 mM NaCl stress (e). DAB staining of *in vitro* grown seedlings of control and transgenic line F10 under unstressed conditions (d), under 50 mM NaCl stress (e), and under 150 mM NaCl stress (f).](image-url)
4.8.3 Evaluation of CsF3H overexpressing transgenics for resistance against *A. solani*

The resistance of control and CsF3H transgenic lines was assessed against a fungus *Alternaria solani*. Leaves of 25 days old leaves control tobacco and CsF3H transgenic lines F10, F9 and F12b were inoculated with fungal conidial spores of *A. solani* and the lesion diameter was measured 3 days of post-inoculation (Fig. 4.32a). The CsF3H transgenic lines F10, F9 and F12b displayed smaller radial lesion diameter of 2.31, 2.43, and 2.68 mm respectively in comparison to 3.13 mm lesion diameter of control tobacco leaf (Fig. 4.32b). Thus there was 26 %, 22 % and 14 % decrease in lesion diameter in F10, F9 and F12b lines respectively as compared to control tobacco. The result suggests that CsF3H overexpression in transgenic tobacco has improved resistance to fungus *A. solani*.

![Image](image_url)

**Fig. 4.32 Reduction of *Alternaria solani* infestation symptoms in tobacco plants overexpressing CsF3H gene. Detached leaves from control and CsF3H overexpressing transgenic lines (F10, F9 and F12b) were inoculated with *A. solani* conidial spores and the lesion size was determined 3 days of post-inoculation (a). Bar diagram shows the average diameter of the expanding lesions in leaves of control and in transgenic lines F10, F9 and F12b (b). Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test.**
4.8.4 Degree of pectin methyl-esterification in control and \textit{CsF3H} overexpressing transgenic tobacco plants

Degree of pectin methyl-esterification (DPME) was determined in 10 days old untreated and salt stress treated seedlings of control and \textit{CsF3H} overexpressing transgenic tobacco. DPME was determined through ruthenium red staining and immunological methods. Also, the effect of \textit{CsF3H} overexpression was analyzed on the activity of pectin methyl esterase enzyme involved in pectin methyl de-esterification.

4.8.4.1 Decreased pectin methyl de-esterification due to lesser PME activity in \textit{CsF3H} transgenic tobacco plants

Earlier \textit{in vitro} studies have shown catechins as an endogenous inhibitors of PME activity. Therefore, we assessed here whether increase in flavan-3-ols lead to decrease in pectin methyl esterase (PME) activity in \textit{CsF3H} transgenic tobacco lines. For this, total protein extracts from control and \textit{CsF3H} transgenic lines F10, F9 and F12b were used to quantify PME activity by radial gel diffusion assay. The red zone area was observed after staining of plates with ruthenium red dye. It determines the de-esterification of pectins with pectin methyl esterase enzyme. The decrease in red zone area was observed in protein extract from \textit{CsF3H} transgenic tobacco lines compared to control tobacco extract, suggesting the decrease in PME activity of \textit{CsF3H} transgenic tobacco lines with respect to control tobacco (Fig. 4.33a). The transgenic line F10 showed maximum (16.4 \%) decrease in PME activity followed by line F9 (15.4\%) and F12b (8\%) as compared to control tobacco plant (Fig. 4.33b). The PME activity of control was set to 100 \% for calculating PME activity in \textit{CsF3H} transgenic lines. PME activity was also measured in control and \textit{CsF3H} transgenic lines under 50 mM and 150 mM NaCl stress. About 17 \% decrease in PME activity was observed for \textit{CsF3H} transgenic line F10 compared to control tobacco under 50 mM of salt stress (Fig. 4.33c, 4.33d). While there was a decrease of 31 \% in PME activity of \textit{CsF3H} transgenic line F10 compared to control tobacco under 150 mM of salt stress (Fig. 4.33c, 4.33d). PME activity of control tobacco plant under 50 and 150 mM salt stress was set to 100 \% and from there PME activity in transgenic line under stress conditions was calculated. Hence, lesser PME activity in \textit{CsF3H} transgenic tobacco has decreased the pectin methyl de-esterification rate compared to control tobacco.
Fig. 4.33 Radial gel diffusion assay for pectin methyl esterase (PME) activity measurement in leaf extract of control and CsF3H overexpressing transgenic tobacco lines F10, F9 and F12b. The red zonal area represent de-esterification of pectins observed with PME enzyme from leaf protein extract of control and transgenic lines after staining of gel plates with ruthenium red for 45 m (a). Bar diagram shows percent PME activity in leaves of control and transgenic tobacco lines F10, F9 and F12b. The PME activity of control tobacco plant was set to 100 % for calculating PME activity in transgenic lines (b). Radial gel diffusion PME assay in leaf extract of control and transgenic tobacco line F10 under 50 mM and 150 mM NaCl stress (c). Bar diagram shows percent PME activity in control and transgenic tobacco line F10 under 50 mM and 150 mM NaCl stress (d). The PME activity of control tobacco plant under 50 and 150 mM salt stress was set to 100 % for calculating PME activity in transgenic lines. Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test. C- control tobacco plant under 50 mM NaCl stress and C' - control tobacco plant under 150 mM NaCl stress. Data represent average ± SD of three biological replicates.

In order to confirm further whether decrease in PME activity has affected the pectin de-esterification in transgenic lines, microtome leaf sections of control and CsF3H transgenic seedlings were stained with ruthenium red dye under unstressed and 150 mM NaCl stress conditions. More the intensity with ruthenium red staining, higher will be the presence of de-esterified pectins in the cell wall. The more red thickening was observed in
epidermal cell wall of control tobacco seedlings (Fig. 4.34a) compared to *CsF3H* transgenic line F10 (Fig. 4.34b), line F9 (Fig. 4.34c) and line F12b (Fig. 4.34d) under unstressed conditions. The red thickened area was increased in leaf sections of seedlings exposed to 150 mM NaCl stress. The thickening was more in leaf sections of control tobacco seedlings (Fig. 4.34e) in contrast to transgenic line F10 (Fig. 4.34f), line F9 (Fig. 4.34g) and line F12b (Fig. 4.34h) exposed to 150 mM NaCl stress. Results document higher pectin de-esterification in control tobacco leaves compared to *CsF3H* transgenic leaves.

![Fig. 4.34 Degree of pectin de-esterification determined through ruthenium red staining in control and *CsF3H* overexpressing transgenic tobaccos. Ruthenium red staining of microtome leaf section of control (a), *CsF3H* overexpressing F10 (b), F9 (c) and F12b (d) transgenic tobacco seedlings under unstressed conditions. Ruthenium red staining of microtome leaf section of control (e), *CsF3H* overexpressing F10 (f), F9 (g) and F12b (h) transgenic tobacco seedlings under 150 mM NaCl stress. The more intensity of red color in control tobacco leaf under both conditions indicated higher degree of pectin de-esterification in their cell wall.](image-url)
4.8.4.2 Immunological determination of degree of pectin methyl-esterification

The degree of pectin methyl-esterification was also determined through immunological staining with monoclonal antibodies JIM5 and JIM7. These antibodies are specific for analyzing the cell wall pectin levels differing in their degree of methyl-esterification. Therefore, DPME was also determined immunologically in roots of control and CsF3H transgenic tobacco seedlings. The root portion of 2-3 mm behind the root tip was used for immunological staining. The pectic epitope of JIM5 stained low methyl esters, whereas JIM7 stained high methyl esters in cortical cells of cell wall. The root apices of control and CsF3H transgenic plants showed differential fluorescence intensity with JIM5 and JIM7. The fluorescence with JIM5 was found to be more in control plants (Fig. 4.35a) compared to transgenic line F10 (Fig. 4.35d), F9 (Fig. 4.35g) and F12b (Fig. 4.35j) under unstressed condition.

![Image of fluorescence intensity comparison with JIM5 antibody](image)

Fig. 4.35 Degree of pectin methyl-esterification determined immunologically in roots of control and CsF3H overexpressing transgenic tobacco plants. Immunolocalization of low-methyl ester pectin with JIM5 antibody in root portion of 2-3 mm behind the root tip in control tobacco seedlings under unstressed conditions (a), under 50 mM NaCl (b) and under 150 mM NaCl (c) and in transgenic tobacco seedlings F10, F9 and F12b under unstressed conditions (d,g,j), under 50 mM NaCl (e,h,k) and under 150 mM NaCl (f,i,l).

The brighter fluorescence with JIM5 was observed in roots of control tobacco seedlings (Fig. 4.35b) with respect to CsF3H transgenic F10 (Fig. 4.35e), F9 (Fig. 4.35h) and F12b (Fig. 4.35k) seedlings under 50 mM NaCl stress. More brighter fluorescence with JIM5 was
observed in roots of control tobacco seedlings (Fig. 4.35c) with respect to CsF3H transgenic F10 (Fig. 4.35f), F9 (Fig. 4.35i) and F12b (Fig. 4.35l) seedlings under 150 mM NaCl stress. Data suggests that de-esterification in the root goes on increasing with increase in salt concentration.

Since JIM7 showed the opposite behavior to JIM5, florescence with JIM7 was found to be more in transgenic line F10 (Fig. 4.36d), line F9 (Fig. 4.36g) and line F12b (Fig. 4.36h) compared to control tobacco (Fig. 4.36a) under unstressed conditions. Also florescence was more with JIM7 during control conditions in transgenic lines compared to salt stress conditions. The florescence intensity with JIM7 was higher in roots of CsF3H transgenic F10 (Fig. 4.36e), line F9 (Fig. 4.36h) and line F12b (Fig. 4.36k) compared to control tobacco (Fig. 4.36b) seedlings under 50 mM NaCl stress. Intensity of florescence was further increased with JIM7 in roots of CsF3H transgenic F10 (Fig. 4.36f), line F9 (Fig. 4.36i) and line F12b (Fig. 4.36l) compared to control tobacco (Fig. 4.36c) seedlings under 150 mM NaCl stress. Result suggests the existence of higher content of high-methyl esters pectin in transgenic roots compared to control tobacco roots.

![Fig. 4.36 Degree of pectin methyl-esterification determined immunologically in roots of control and CsF3H overexpressing transgenic tobacco plants. Immunolocalization of high-methyl ester pectin with JIM7 antibody in root portion of 2-3 mm behind the root tip in control tobacco seedlings under unstressed conditions (a), under 50 mM NaCl (b) and under 150 mM NaCl (c) and in CsF3H transgenic tobacco seedlings F10, F9 and F12b under unstressed conditions (d,g,j), under 50 mM NaCl (e,h,k) and under 150 mM NaCl (f,i,l).](image-url)
4.8.4.3 Effect of exogenous application of flavan-3-ols on the primary root length and degree of pectin methyl-esterification

The effect of flavan-3-ols on pectin methyl-esterification was further validated through *in vitro* experiment by exposing wild tobacco seedlings to 5 µM epicatechin (EC) or 5 µM epigallocatechin (EGC) under 150 mM NaCl stress. The significant increase in primary root length was found in seedlings exposed to 150 mM NaCl plus 5 µM of EC (Fig. 4.37b) and 150 mM NaCl plus 5 µM of EGC (Fig. 4.37c) compared to tobacco seedlings exposed to 150 mM NaCl stress only (Fig. 4.37a). However, increase was comparatively more in seedlings exposed to 150 mM NaCl + 5 µM EGC (Fig. 4.37d). The PME activity was also measured in flavan-3-ols exposed and unexposed tobacco seedlings. PME activity was observed to be reduced in seedlings exposed to 150 mM NaCl plus 5 µM EC or 5 µM EGC. The decrease of PME activity was 8 % and 10 % in seedlings exposed to 150 mM NaCl + 5 µM EC and 150 mM NaCl + 5 µM EGC respectively compared to tobacco seedlings exposed to 150 mM NaCl stress only (Fig. 4.37e).

To see the influence of reduced PME activity on pectin methyl-esterification, the root portion of 2-3 mm behind the root tip of flavan-3-ols exposed and unexposed tobacco seedlings were used. The root portion of tobacco seedlings exposed either to 150 mM NaCl + 5 µM EC (Fig. 4.37g) or 150 mM NaCl + 5 µM EGC (Fig. 4.37h) showed lower intensity with JIM5 compared to tobacco seedlings exposed to 150 mM NaCl stress only (Fig. 4.37f). In contrast, higher intensity with JIM7 was observed in roots of seedlings exposed to 150 mM NaCl + 5 µM EC (Fig. 4.37j) or 150 mM NaCl + 5 µM EGC (Fig. 4.37k) as compared to unexposed ones (Fig. 4.37i). Result suggests that increase in flavan-3-ols has caused the increase in degree of methyl-esterification in roots of wild tobacco seedlings.

Ruthenium red staining was also conducted for pectin de-esterification studies in leaf section of wild tobacco seedlings exposed to either 150 mM NaCl only or to 150 mM NaCl with 5 µM of EC or EGC. After staining of sections, the red thickening was found to be more in leaf cell wall of seedlings exposed to 150 mM NaCl stress only (Fig. 4.37l) compared to seedlings exposed to 150 mM NaCl + 5 µM EC (Fig. 4.37m) or 150 mM NaCl + 5 µM EGC (Fig. 4.37n), suggesting the role of flavan-3-ols in decreasing the pectin de-esterification in leaf of exposed wild tobacco seedlings.
Fig. 4.37 Effect of *in vitro* supplied flavonoids on the primary root length and degree of pectin esterification in roots of wild tobacco seedlings under 150 mM salt stress. Photographs depicting primary root system of 10 days old wild tobacco seedlings grown for next 3 days in MS media containing 150 mM NaCl alone (a), in MS media containing 150 mM NaCl and 5 µM EC (b) and in MS media containing 150 mM NaCl and 5 µM EGC (c). Primary root length of wild type seedlings grown in MS media containing 150 mM NaCl alone (W+150), in MS media containing 150 mM NaCl and 5 µM EC (W+150+5EC) and in MS media containing 150 mM NaCl and 5 µM EGC (W+150+5EGC) (d). Bar diagram shows percent PME activity in *in vitro* grown seedlings of wild tobacco exposed to MS media containing 150 mM NaCl only (W+150), to MS media containing 150 mM NaCl and 5 µM of EC (W+150+5EC) and to MS media containing 150 mM NaCl and 5 µM of EGC (W+150+5EGC) (e). Significant differences from the control (W+150) are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student’s t-test. The PME activity of wild tobacco seedlings was set to 100 % for calculating PME activity in transgenic lines. Data presented here is average ± SD of 10 tobacco seedlings. Immunolocalization of low-methyl ester pectin with JIM5 antibody in root portion of 2-3 mm behind the root tip in wild tobacco seedlings grown in MS media containing 150 mM NaCl alone (f), in MS media containing 150 mM NaCl and 5 µM EC (g) and in MS media containing 150 mM NaCl and 5 µM EGC (h). Immunolocalization of high-methyl ester pectin with JIM7 antibody in root portion of 2-3 mm behind the root tip in wild tobacco seedlings grown in MS media containing 150 mM NaCl alone (i), in MS media containing 150 mM NaCl and 5 µM EC (j) and in MS media containing 150 mM NaCl and 5 µM EGC (k). Ruthenium red staining of microtome leaf section of wild tobacco seedlings grown in MS media containing 150 mM NaCl alone (l), in MS media containing 150 mM NaCl and 5 µM EC (m) and in MS media containing 150 mM NaCl and 5 µM EGC (n).
4.9 Characterization of *NtFLS* silenced transgenic tobacco

4.9.1 Phenotypic characterization of *NtFLS* silenced tobacco

The *NtFLS* silenced transgenic tobacco lines were smaller in height to that of control plant (Fig. 4.38a). Further, silenced transgenic lines showed a delayed fruit development and yielded smaller fruits (Fig. 4.38b).

![Morphological characterization and yield parameters of *NtFLS* silenced transgenic tobacco lines compared to control. *NtFLS* silenced transgenics lines G12, A1, B1, and E13 were smaller in height as compared to control tobacco plant (*Nicotiana tabacum* cv xanthi). Flowering was delayed in *NtFLS* silenced transgenics (a). Pods derived from control flowers upon self pollination were grew to normal size. Whereas, self pollinated silenced transgenic lines G12, A2, B1, and E13 yielded smaller fruits. In *NtFLS* silenced lines pods and seed development was arrested, whereas control tobacco pods had a normal seed set (b). Pod weight in milligrams (c) and pod size (d) at equatorial cross section in millimetres of control tobacco plant and of *NtFLS* silenced transgenic tobacco lines (G12, A2, B1 and E13). Both pod weight and pod size was reduced in all silenced transgenic lines as compared to control. Values represent mean values ± SD (n=5). Significant differences from the control are denoted by one or two asterisk corresponding to $P < 0.05$ and $P < 0.01$, respectively, by Student’s $t$-test.

The small fruits (pods) of *NtFLS* silenced transgenic lines A2, G12, B1 and E13 contained very less number of seeds as compared to control tobacco (Table 4.1). This has suggested the arrest in seed set due to *FLS* silencing in tobacco. The pods of transgenic lines A2 and
G12 have produced significantly very less number of seeds. The average number of seeds per pod were 143, 300, 1010 and 1160 in G12, A2, B1 and E13 silenced transgenic lines as compared to 1417 seeds per pod of control tobacco plant. Additionally, average number of pods per plant was also very less in case of silenced transgenic lines compared to control plant. Pods per plant were found to be 4, 5, 8 and 9 for G12, A2, B1 and E13 lines respectively as compared to 11 pods for control tobacco (Table 4.1).

Table 4.1 Comparative fruit characteristics of control and *NtFLS* silenced transgenic lines. Seeds per fruit (pod) and number of pods per plant were determined in control and *NtFLS* silenced transgenic lines G12, A2, B1 and E13.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>G12</th>
<th>A2</th>
<th>B1</th>
<th>E13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds per fruit (pod)</td>
<td>1417 ± 104</td>
<td>143 ± 40</td>
<td>300 ± 50</td>
<td>1010 ± 36</td>
<td>1160 ± 53</td>
</tr>
<tr>
<td>Pods (n)</td>
<td>11 ± 0.9</td>
<td>4 ± 0.32</td>
<td>5 ± 0.4</td>
<td>8 ± 0.5</td>
<td>9 ± 0.8</td>
</tr>
</tbody>
</table>

Further, the pod size as well as pod weight was also reduced significantly in all four transgenic lines as compared to control. The silenced transgenic lines A2 and G12 showed higher decrease in pod weight and pod size as compared to pods of B1 and E13 transgenic lines and control tobacco plant. Pod weight was 35.33 mg, 59.67 mg, 107.67 mg and 110 mg for G12, A2, B1 and E13 *NtFLS* silenced transgenic lines respectively compared to 132 mg pod weight of control tobacco (Fig. 4.38c). Similarly, the pod size of G12, A2, B1 and E13 silenced transgenic lines was found to be 1.13 mm, 1.27 mm, 1.57 mm and 1.6 mm respectively as compared to 2.2 mm of control tobacco pod (Fig. 4.38d).

### 4.9.2 Pollen tube growth and fertility in *NtFLS* silenced tobacco

Pollen germination and pollen tube growth was analyzed through *in vitro* and *in vivo* experiments in *NtFLS* silenced transgenic tobacco lines. For *in vitro* experiment, pollens from control as well as silenced transgenic lines were germinated on pollen germination media. After 4 hrs of incubation, significant reduction in pollen germination percentage of all *NtFLS* transgenic lines was observed compared to control.

The representative picture of pollens of *NtFLS* silenced transgenic line G12 (Fig. 4.39b) and control (Fig. 4.39a) is shown. The pollen germination percentage was 26 % (reduced by 74 %), 37 % (reduced by 63 %), 73 % (reduced by 27 %) and 80 % (reduced by 20 %) in G12, A2, B1 and E13 silenced transgenic lines respectively as compared to control.
control (Fig. 4.39c). In addition, the pollen tube of \textit{NtFLS} silenced transgenic lines had a relatively rough surface and showed kinked and coiled shape compared to the control plant (Fig. 4.39d and 4.39e).

For \textit{in vivo} pollen germination studies, fertilized carpels of control and \textit{NtFLS} silenced \textit{G12} transgenic line were histochemically stained specifically for callose present in growing pollen tubes after 2 days of pollination. In control self-pollinated plants, pollen tubes were reached to the base of style after 2 days of pollination (Fig. 4.39f-4.39j).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_39}
\caption{\textit{In vitro} and \textit{in vivo} pollen germination assays of control and \textit{NtFLS} silenced transgenic tobacco. \textit{In vitro} pollen germination assay of pollens from control tobacco plant (a) and from \textit{NtFLS} silenced transgenic tobacco lines \textit{G12}, \textit{A2}, \textit{B1} and \textit{E13} (b) on germination media (GM) after 4 hrs of incubation. Graph depicts the germination frequency (pollen germination percentage) of pollens from control tobacco plant and from \textit{NtFLS} silenced transgenic tobacco lines \textit{G12}, \textit{A2}, \textit{B1} and \textit{E13} on GM after 4 hrs of incubation (c). Pollen germination frequency was found to be reduced in silenced transgenic lines as compared to control tobacco. Only tubes longer than half the size of pollen grains was judged as germinated. Values are mean of three replications where tube length of 50 to 100 pollen grains was measured and are represented as mean ± SD. Significant differences from the control are denoted by one or two asterisk corresponding to \textit{P} < 0.05 and \textit{P} < 0.01, respectively, by Student’s t-test. Shape and surface characteristics of pollen tubes. The foremost part of a pollen tube of control tobacco plant showed a smooth, straight shape (d), whereas pollen tube of \textit{NtFLS} silenced transgenic tobacco showed the kinked, and coiled shape (e). The arrows indicate the rough surface of the pollen tube. Histochemical staining of pollen tube growth in carpels after 2 days of pollination from control and \textit{NtFLS} silenced line \textit{G12}. Fertilized carpels were stained with aniline blue to specifically stain callose present in growing pollen tubes. Staining was conducted in control tobacco carpels after crossing with control plant pollens (f-j) and \textit{G12} line carpels after self-crossings (k-o). Callose in the pollen tubes is visible at the stigma (f and k), proliferation of pollen tube growth in the middle of the style (g, h, i and m). Pollen tubes of \textit{G12} line grew only nine-tenths of the way down the style (n). The tips of the pollen tubes are swollen in \textit{G12} (shown by arrow). Pollen tubes are not visible at the base of the style in \textit{G12} \textit{NtFLS} silenced line (o) as compared to control carpels (j).}
\end{figure}
Whereas, pollen tubes of G12 self-pollinated flowers did not grow well and did not reach to the base of style after 2 days of pollination. The NtFLS silenced line showed clear staining of callose in the stigma and absent further down, indicating the arrest in pollen tube germination. In NtFLS silenced line, the pollen tubes grew only to about nine-tenths of the way down the style, and tube tips were found somewhat swollen (Fig. 4.39k-4.39o).

4.9.3 Rescuing effect of quercetin supplement on pollen germination and fertility of NtFLS silenced transgenic lines
To confirm that the arrest in pollen germination of NtFLS silenced transgenic lines was due to lower levels of flavonol (quercetin), in vitro and in vivo experiments were performed with transgenic pollens by supplying quercetin through media. For in vitro experiments, pollens from NtFLS silenced transgenic flowers were germinated on pollen germination media that contained various concentrations of quercetin as 10 nmol, 20 nmol and 1 µM. Pollen germination rate as well as pollen tube length was found to be increased in 1 µM of quercetin supplemented medium (Fig. 4.40d) as compared to 10 nmol (Fig. 4.40b), 20 nmol (Fig. 4.40c) and without any quercetin (Fig. 4.40a) supplemented media. A little pollen germination was observed with 20 nmol quercetin compared with 10 nmol quercetin and with no quercetin supplemented media.

In vivo pollen germination assay was also performed, where tobacco flower bud at stage II (Fig. 4.40e) was exposed to pollen maturation (PM) media that contained various concentrations of quercetin as 10 nmol, 20 nmol and 1 µM. For control, buds were exposed to PM medium alone with no supplement of quercetin. Upon maturation in the media, flower buds were emasculated and pollinated. After two days of pollination, pistils from all the flowers were harvested and histochemically stained for callose staining of pollen tubes. Pollen tube growth was very efficient in flower buds exposed to 1 µM quercetin (Fig. 4.40u-4.40y) as compared to flower buds allowed to mature in media with 10 nmol (Fig. 4.40k-4.40o), 20 nmol (Fig. 4.40p-4.40t) and without any quercetin (Fig. 4.40f-4.40j). In case of 1 µM quercetin supplemented flower buds, pollen tubes were found to reach the base of styles in just 2 days of pollination. Whereas in the same time period, pollen tubes grew only a way down to style but did not reach to the end of style in case of flower buds exposed to 10 nmol, 20 nmol and no quercetin.
Fig. 4.40 *In vitro* and *in vivo* pollen germination rescue assays of *NtFLS* silenced transgenic tobacco. Effect of quercetin on *in vitro* pollen germination rate of silenced transgenic pollens. Pollens were collected from freshly dehiscent anthers of *NtFLS* silenced tobacco plant and suspended in GM containing only DMSO added to final concentration of 1 µM (a), 10 nmol quercetin (b), 20 nmol quercetin (c) and 1 µM quercetin (d). Different developmental stages of flower bud (stage I, II, III and IV) in tobacco (*Nicotiana tabacum* L.) (e). Histochemical staining of pollen tubes growth in carpels of floral buds (stage II) from silenced transgenic lines exposed to different concentrations of quercetin i.e. 10 nmol, 20 nmol and 1 µM through pollen maturation media (PM). After 2 days of pollination, carpels were stained with aniline blue to specifically stain callose present in growing pollen tubes. Pollen tube growth in self pollinated carpels of floral bud of silenced transgenic lines exposed only to PM without any treatment (f-j), carpels of floral bud exposed to PM containing 10 nmol (k-o), 20 nmol (p-t) and 1 µM (u-y) quercetin. Pollen germination is visible at stigma region (f, k, p and u). It was found to be maximum with 1 µM (u) and least in transgenic pollens with no treatment (f). The pictures g-i, l-n, q-s and v-x show proliferation of pollen tube growth in the middle of the style. The pollen tubes grew only to nine-tenths of way down the style after 2 days of pollination in case of silenced floral buds exposed to 10 nmol (l-n), 20 nmol (q-s) quercetin and with no treatment (g-i). Whereas pollen tubes are reaching the base of the style in case of floral buds exposed to 1 µM quercetin (v-x). On the other hand, in untreated buds and in buds exposed to 10 nmol and 20 nmol quercetin, no pollen tubes are seen in carpels reaching the base of style (j, o and t) within the same time period. All micrographs are of the same magnification.
4.9.4 *NtFLS* downregulation reduced endogenous free IAA content in shoot apex

Flavonols has been reported as negative regulators of auxin/free IAA transport in plants. Since *NtFLS* downregulation reduced quercetin content as well as delayed flowering, its effect was analyzed on endogenous free IAA content in apical region of the plants.

![Diagram](image)

Fig. 4.41 Endogenous free indole acetic acid (IAA) content in apical region of shoot of control and *NtFLS* silenced transgenics. Endogenous free IAA content was determined using Ultra Performance Liquid Chromatography (UPLC). UPLC chromatogram of 10 µg ml⁻¹ IAA standard showing sharp peak at retention time (RT) of 2.72 m. Absorbance spectra of this peak was measured by photodiode array detector and was observed at 222 nm (a). Chromatogram of endogenous IAA isolated from apical portion of control tobacco shoot showing peak at RT of 2.72 m (b). Chromatogram of endogenous IAA isolated from apical portion of *NtFLS* silenced transgenic line (G12) showing IAA peak at RT of 2.72 m (c). Graph depicts the endogenous content of IAA measured in apical region of control and silenced transgenic lines (G12 and A2) (d). IAA was found to be reduced in silenced transgenic lines as compared to control tobacco. The quantification was performed with three replications and is represented as mean ± SD. Significant differences from the control are denoted by one or two asterisk corresponding to P < 0.05 and P < 0.01, respectively, by Student's t-test.
Endogenous IAA content of control as well as \( NtFLS \) silenced transgenic tobacco lines were determined by ultra performance liquid chromatography (UPLC). UPLC chromatogram of 10 \( \mu g \) ml\(^{-1}\) IAA standard showed sharp peak at retention time of 2.72 m. Absorbance spectra of this peak was measured by photodiode array detector and was observed at 222 nm (Fig. 4.41a). Chromatogram of endogenous IAA isolated from apical portion of control tobacco shoot (Fig. 4.41b) and \( NtFLS \) silenced transgenics (Fig. 4.41c) also showed peak at retention time of 2.72 m. Endogenous IAA content was found to be significantly higher in apical region of control tobacco shoot as compared to strong \( NtFLS \) silenced transgenic lines, G12 and A2. IAA content was found to be 144 ng g\(^{-1}\) FW and 263 ng g\(^{-1}\) FW in G12 and A2 transgenic lines respectively as compared to 935 ng g\(^{-1}\) FW of control tobacco plant (Fig. 4.41d). Thus, \( NtFLS \) silenced lines G12 and A2 showed 85 % and 72 % reduction in their free IAA content respectively.

4.10 Evaluating the effect of flavonoids on \textit{in vitro} grown wild tobacco seedlings

In order to validate the present study, the effect of flavonoids like epicatechin (EC) and quercetin (Quer) was studied at different concentrations on growth and development as well as flavonoid and antioxidant system of \textit{in vitro} grown wild tobacco seedlings.

4.10.1 Analysis of tobacco seedlings exposed to different concentrations of EC and Quer

The effect of two flavonoids epicatechin (EC) and quercetin (Quer) was studied on \textit{in vitro} grown tobacco seedlings. These flavonoids were applied at two different concentrations of 50 and 100 \( \mu M \) to tobacco seedlings. After the treatment, root and shoot parts were separated and various analysis were performed.

4.10.1.1 Effect of EC and Quer application on flavonoid contents of tobacco seedlings

To check whether exogenously applied Quer and EC has any influence on the endogenous level of flavonoids in tobacco seedlings, total flavonoids content was estimated in shoot and root tissues. Total flavonoid content was found to be increased with the exogenous application of both Quer and EC in tobacco shoots.
Table 4.2 Total flavonoid content in shoot and root tissue of wild tobacco seedlings under untreated and epicatechin/quercetin treated conditions.

<table>
<thead>
<tr>
<th>Tissue and treatment</th>
<th>Flavonoid content (mg g^{-1} FW) *</th>
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<tbody>
<tr>
<td></td>
<td>Flavones, flavonols **</td>
<td>Flavanones ***</td>
<td>Total flavonoids</td>
</tr>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>a0.52 ± 0.01</td>
<td>a0.72 ± 0.066</td>
<td>a1.24 ± 0.076</td>
</tr>
<tr>
<td>EC-50</td>
<td>a0.57 ± 0.01</td>
<td>a0.74 ± 0.04</td>
<td>a1.31 ± 0.05</td>
</tr>
<tr>
<td>EC-100</td>
<td>a0.65 ± 0.03</td>
<td>a0.97 ± 0.005</td>
<td>a1.62 ± 0.035</td>
</tr>
<tr>
<td>Quercetin-50</td>
<td>a0.56 ± 0.02</td>
<td>a0.75 ± 0.05</td>
<td>a1.31 ± 0.07</td>
</tr>
<tr>
<td>Quercetin-100</td>
<td>a0.77 ± 0.01</td>
<td>b1.00 ± 0.02</td>
<td>b1.77 ± 0.03</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>a0.004 ± 0.0002</td>
<td>a0.97 ± 0.08</td>
<td>a0.974 ± 0.080</td>
</tr>
<tr>
<td>EC-50</td>
<td>b0.005 ± 0.0005</td>
<td>a0.96 ± 0.00</td>
<td>a0.965 ± 0.005</td>
</tr>
<tr>
<td>EC-100</td>
<td>a0.009 ± 0.0007</td>
<td>b0.85 ± 0.05</td>
<td>b0.859 ± 0.050</td>
</tr>
<tr>
<td>Quercetin-50</td>
<td>a0.007 ± 0.0004</td>
<td>a0.96 ± 0.03</td>
<td>a0.967 ± 0.030</td>
</tr>
<tr>
<td>Quercetin-100</td>
<td>b0.01 ± 0.0009</td>
<td>b0.80 ± 0.02</td>
<td>b0.81 ± 0.020</td>
</tr>
</tbody>
</table>

*: All results are presented as mean ± SD (n=3); **: Levels calculated as quercetin equivalents; ***: Levels calculated as naringenin equivalents
Different superscript letters next to values represent significant differences in the mean values of estimates at 5 % level according to LSD.

The content was further increased with the increase in dose of EC and Quer exposure. The maximum flavonoid content of 1.77 ± 0.03 mg g^{-1} FW was observed in the 100 µM Quer exposed shoot followed by 1.62 ± 0.035 mg g^{-1} FW with 100 µM EC (Table 4.2). On the other hand, total flavonoid content was decreased with exogenous application of EC and Quer in tobacco roots as compared to untreated roots. The response of root for flavonoid content was exactly reverse to that of shoot upon exogenous application of both EC and Quer. Hence, total flavonoid content was lowest in tobacco roots exposed to 100 µM EC and Quer compared to untreated roots (Table 4.2).

4.10.1.2 Effect of EC and Quer on the expression of genes encoding enzymes of flavonoid biosynthesis pathway
Since shoot and root tissue showed opposite behaviour for flavonoid content accumulation upon exogenous application of both Quer and EC, the influence of exogenously applied constituents was expected on the regulation of flavonoid biosynthesis pathway. In view of
this, expression of flavonoid biosynthesis pathway genes was monitored in tobacco shoot and root upon exposure to Quer and EC. The transcript levels of genes encoding phenylalanine ammonia lyase (NtPAL), chalcone synthase (NtCHS), chalcone isomerase (NtCHI), flavanone 3-hydroxylase (NtF3H) and flavonol synthase (NtFLS) was monitored.

Fig. 4.42 Changes in the relative transcript level of genes encoding phenylalanine ammonia lyase (NtPAL) (a), chalcone synthase (NtCHS) (b), chalcone isomerase (NtCHI) (c), flavanone 3-hydroxylase (NtF3H) (d) and flavonol synthase (NtFLS) (e) in tobacco shoots in response to 50 and 100 μM treatments of epicatechin (EC) and quercetin (Quer).
In tobacco shoot, EC exposure influenced the transcript level of genes encoding various enzymes in a concentration dependent manner. Upon exposure to 50 µM EC, the expression of genes encoding NtCHI and NtF3H was upregulated while the expression of genes encoding other three enzymes was not affected. Application of 100 µM EC has significantly increased the transcript expression level of genes encoding NtPAL, NtCHI, NtF3H and NtFLS. Interestingly, the expression of gene encoding NtCHS expression was downregulated with 100 µM EC (Fig. 4.42). The 50 µM Quer application was observed to increase transcript expression level of all genes encoding these five enzymes. The expression was further enhanced with 100 µM Quer application (Fig. 4.42).

Influence of these two flavonoids was also seen on the expression of genes encoding flavonoid biosynthetic enzymes in tobacco roots. In EC exposed tobacco roots, transcript expression of genes encoding NtCHI, NtF3H and NtFLS was decreased. While the expression of gene encoding NtPAL was unaffected with both 50 and 100 µM EC exposure compared to untreated control. Only the expression of gene encoding NtCHS was found to be upregulated with 100 µM EC applications (Fig. 4.43). Similarly, Quer exposure was also found to decrease the expression of gene encoding NtPAL in addition to gene encoding NtF3H and NtFLS. However, the expression of genes encoding NtCHS and NtCHI was found to be increased with 100 µM Quer compared to untreated control (Fig. 4.43). In general, results suggest that EC and Quer exposure enhanced the expression of genes encoding flavonoid pathway enzymes in shoots and decreased in roots of tobacco seedlings. Interestingly, the expression of gene encoding NtCHS showed reverse response in two tissues compared to genes encoding other four enzymes.
Fig. 4.43 Changes in the relative transcript level of genes encoding phenylalanine ammonia lyase (NtPAL) (a), chalcone synthase (NtCHS) (b), chalcone isomerase (NtCHI) (c), flavanone 3-hydroxylase (NtF3H) (d) and flavonol synthase (NtFLS) (e) in tobacco roots in response to 50 and 100 μM treatments of epicatechin (EC) and quercetin (Quer).

4.10.1.3 Morphological responses of tobacco seedlings exposed to EC and Quer
Tobacco seedlings grown on media supplemented with 100 μM epicatechin (EC) and quercetin (Quer) exhibited larger primary roots with no lateral and adventitious roots. Higher concentration of these flavonoids exposure was also resulted in smaller leaves and laminae (Fig. 4.44). Also, inhibition in lateral and adventitious root formation was observed with 50 μM EC but extent was lesser as compared to its higher dose. On the other hand, tobacco seedlings grown in the medium with 50 μM Quer produced no difference in
their root system as well as leaves and laminae. However, higher dose of EC and Quer application retarded the plant growth significantly (Fig. 4.44).

![Image of seedlings with different treatments](image)

Fig. 4.44 The effects of different concentrations of flavonoids application on tobacco shoot and root development. Photographs show 7-days-old seedlings grown for 21 days on 0.1 % DMSO (A), 50 μM EC (B), 100 μM EC (C), 50 μM Quer (D) and 100 μM Quer (E). Upper panel is the top view of seedlings and lower panel is the vertical view of seedlings in petri plates. EC, epicatechin; Quer, quercetin.

### 4.10.1.4 Effect of EC and Quer application on vascular system of tobacco seedlings

To assess the influence of EC and Quer on plant vascular patterning, development of vascular systems was analyzed during their exogenous application. For this, roots and leaves were analyzed through histological sections. The first nodal leaf of the seedlings grown in the presence of 100 μM EC and Quer showed increase in number of parallel vascular strands in central and petiolar region of the leaf as compared to that of untreated control. Also, these parallel vascular strands were separated from each other. The 50 μM EC and Quer exposure was also found to enhance the number of parallel vessels compared to unexposed plant. However the increase in this case was to a lesser extent compared to 100 μM exposure (Fig. 4.45A). Similarly, the effect of these flavonoids was also observed on vascular strands in the root tip. Numerous parallel vessels extended towards root apex were found only in the seedlings exposed to 100 μM EC. Other treatments did not show any effect on vessels accumulation in the root tip region (Fig. 4.45B).
Fig. 4.45 Vascular organization in flavonoid treated and untreated control tobacco plants. (A) Whole mount preparations of cleared leaf from tobacco seedlings grown on medium without flavonoids (a), with 50 μM EC (b), 100 μM EC (c), 50 μM Quer (d) and 100 μM Quer (e). Numerous parallel vessels were seen in the central and petiolar region of flavonoid treated plants. (B) Whole mount preparations of cleared primary root tips from tobacco seedlings grown on medium without flavonoids (f), 50 μM EC (g), 100 μM EC (h), 50 μM Quer (i) and 100 μM Quer (j). Arrows indicate numerous parallel vessels extended towards the root apex in 100 μM epicatechin treated tobacco seedlings. PR, petiolar region; CR, central region, RT, root tip; EC, epicatechin; Quer, quercetin.

4.10.1.5 Effect of EC and Quer on leaf anatomy of tobacco seedlings

To check the effect of flavonoids on cell size of leaf, microscopic analysis was conducted with tobacco seedlings exposed to 50 and 100 μM EC and Quer. For microscopic analysis, fixed leaves were cut with microtome and cross-sections centered with central vein were analyzed. Decrease in cell size was observed in the seedlings exposed to higher flavonoid concentrations compared to unexposed seedlings. Cell size of epidermis, palisade and spongy parenchyma revealed a shift to smaller size population upon exposure to 100 μM of both the flavonoids. However, no difference in cell size was observed with 50 μM exposure of both the flavonoids compared to unexposed control (Fig. 4.46).

Fig. 4.46 Cell size comparisons in leaf cross-sections centered with central vein. Leaf cross-section of flavonoids untreated tobacco seedling (a). Leaf cross-section of 50 μM EC (b), 100 μM EC (c), 50 μM Quer (d) and 100 μM Quer (e) treated tobacco seedling. Red bars indicate a shift towards the smaller cell size of UE, LE, PP and SP that was observed with higher concentrations of EC and Quer. UE, upper epidermis, LE, lower epidermis; PP, palisade parenchyma; SP, spongy parenchyma; EC, epicatechin; Quer, quercetin.
4.10.1.6 Effect of EC and Quer on endogenous free IAA content of tobacco seedlings

The similar morphological and anatomical responses as observed in this study have been documented previously by the use of synthetic auxin transport inhibitors. Therefore, endogenous free IAA content was determined in the shoots and roots of tobacco seedlings to see the effect of EC and Quer exposure. The endogenous free IAA content was increased in tobacco shoots upon exposure to both the flavonoids. The content was further increased with increase in dose of their treatment. The maximum free IAA content was observed in shoots exposed to 100 µM Quer followed by 100 µM EC treated shoots (Table 4.3). In tobacco roots, the endogenous free IAA was also increased upon exposure to 50 µM EC and Quer (Table 4.3). However, 100 µM exposures of both the flavonoids have decreased the free IAA content. The 50 µM quercetin treated roots showed the maximum free IAA content.

Table 4.3 Free IAA content in shoot and root of wild tobacco seedlings under untreated and epicatechin/quercetin treated conditions.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Conc. of auxin (IAA) (ng g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated control</td>
</tr>
<tr>
<td>Shoot</td>
<td>*20.5 ± 0.76</td>
</tr>
<tr>
<td>Root</td>
<td>*5.0 ± 0.4</td>
</tr>
</tbody>
</table>

* All results are presented as mean ± SD

Different superscript letters next to values represent significant differences in the mean values of estimates at 5 % level according to LSD.

4.10.1.7 Effect of EC and Quer on antioxidant potential of tobacco seedlings

Effect of EC and Quer application was studied on transcript levels of genes encoding antioxidant enzymes and activities of antioxidant enzymes such as glutathione reductase (GR), ascorbate peroxidase (APₓ), catalase (CAT) and glutathione-S-transferase (GST) in wild tobacco seedlings.

4.10.1.7.1 Transcript expression of genes encoding antioxidant pathway in EC and Quer exposed seedlings

The influence of exogenous application of Quer and EC was studied on the transcript level of genes encoding antioxidant enzymes GR, APₓ, CAT and GST in tobacco shoot and root.
In tobacco shoot, 50 µM EC exposure was found to enhance the expression of all the four enzymes GR, AP\textsubscript{x}, CAT and GST. In contrast to other enzymes, GR expression was still increased with 100 µM EC. The 50 µM Quer either decreased or has no effect on the expression of antioxidant enzymes in tobacco shoot. However, application of 100 µM Quer increased the expression of all four enzymes (Fig. 4.47).

![Image](https://example.com/image)

**Fig. 4.47** Changes in the transcript level of genes encoding glutathione reductase (GR) (a), ascorbate peroxidase (AP\textsubscript{x}) (b), catalase (CAT) (c), and glutathione S- transferase (GST) (d) enzymes in tobacco shoots in response to different treatments EC (EC; 50 and 100 µM) and Quer (Quer; 50 and 100 µM). Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Expression analysis was repeated at least three times and representative one time gel pictures are presented. Data are means of three measurements ± SD. Black and grey bars show 26S rRNA and antioxidant enzyme transcript levels, respectively. C, control; EC, epicatechin; Quer, quercetin.

Results suggest that these two flavonoids have effected transcript expression of genes encoding antioxidant enzymes. However, they were effective at different concentrations. EC was effective at lower concentration while Quer was at higher concentration.
In tobacco root, GR expression was not affected upon application of either of the flavonoids. The AP\textsubscript{x} expression was not affected by EC. But the expression of AP\textsubscript{x} was decreased upon Quer application. While GST expression was increased in tobacco root with 50 µM EC and 100 µM Quer exposures. Whereas the expression of genes encoding CAT showed reverse behaviour to that of GST (Fig. 4.48).

### 4.10.1.7.2 Antioxidant enzyme activity in EC and Quer exposed seedlings

To check whether antioxidant enzymes undergo transcriptional or post-transcriptional regulation, activity analysis of all these enzymes was conducted in tobacco shoot and root tissues exposed to epicatechin (EC) and quercetin (Quer). In tobacco shoot, activity of all
the enzymes showed similar trend to that of the transcript expression (Table 4.4). Significant increase in activity of all four enzymes GR, AP\textsubscript{x}, CAT and GST was observed with 50 µM EC exposures. Whereas 50 µM Quer exposure led to the increase in activity of AP\textsubscript{x} and GST with no significant change in the activity of GR and CAT. This has suggested the transcriptional regulation of all the antioxidant enzymes in tobacco shoot. In root, AP\textsubscript{x}, GR and GST enzyme activity pattern was found to be similar to that of respective transcript expression data with both the treatments. However, CAT activity showed the reverse trend to that of expression pattern in tobacco root (Table 4.4). This has suggested the transcriptional regulation of GR, AP\textsubscript{x} and GST and post-transcriptional regulation of CAT in tobacco roots.

**Table 4.4 Antioxidant enzyme activity in shoot and root tissue of epicatechin and quercetin exposed wild tobacco seedlings.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Control</th>
<th>Epicatechin</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>10.3 ± 0.4</td>
<td>12.9 ± 0.7</td>
<td>10.96 ± 0.6</td>
</tr>
<tr>
<td>AP\textsubscript{x}</td>
<td>54.2 ± 1.2</td>
<td>88.6 ± 1.4</td>
<td>62.9 ± 1.8</td>
</tr>
<tr>
<td>CAT</td>
<td>17.0 ± 0.8</td>
<td>22 ± 1.2</td>
<td>8.8 ± 0.2</td>
</tr>
<tr>
<td>GST</td>
<td>31.7 ± 0.9</td>
<td>50.2 ± 1.0</td>
<td>34.9 ± 0.8</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>10.96 ± 0.6</td>
<td>9.16 ± 0.4</td>
<td>11.6 ± 0.6</td>
</tr>
<tr>
<td>AP\textsubscript{x}</td>
<td>137 ± 2.5</td>
<td>152.1 ± 4.8</td>
<td>162.8 ± 6.3</td>
</tr>
<tr>
<td>CAT</td>
<td>9.2 ± 0.2</td>
<td>11.3 ± 0.4</td>
<td>13.4 ± 0.7</td>
</tr>
<tr>
<td>GST</td>
<td>14.58 ± 0.9</td>
<td>24.2 ± 1.0</td>
<td>9.99 ± 0.4</td>
</tr>
</tbody>
</table>

*Enzyme activity of AP\textsubscript{x}, GST and GR is expressed in µmoles min\textsuperscript{-1} g\textsuperscript{-1} FW, CAT is expressed in nmoles min\textsuperscript{-1} g\textsuperscript{-1} FW. All results are presented as mean ± SD (n=3). Different alphabet superscript to numeric in the same row represents significant difference in the mean values of estimates at 5% level.