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4.14 Semiquantitative PCR analysis for expression study. Steady-state mRNA level of CsF3H cDNA measured in leaves of CsF3H overexpressing transgenic lines (F10, F9 and F12b) and control tobacco. Housekeeping gene 26S rRNA was used as internal control for expression analysis.

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 26S rRNA was used as internal control. Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Black and grey bars show 26S rRNA and NtFLS transcript levels, respectively.

4.16 Transcript level of genes encoding flavonoid biosynthetic pathway enzymes NtPAL, phenylalanine ammonia lyase; NtCHS, chalcone synthase; NtCHI,
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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 min respectively at 280 nm. The standard (-)-EGC showed peak at retention time of 6.03 min (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.

4.19 (+)-Cat (a), (-)-EC (b) and (-)-EGC (c) content in NtFLS silenced transgenic lines G12, A2, B1 and E13 compared to control tobacco plant. Chromatograms showing peaks of (+)-Cat, (-)-EC and (-)-EGC at similar retention times to that of standards in NtFLS silenced transgenic lines G12 (d), A2 (e), B1 (f) and E13 (g). 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.

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4.21 Transcript level of genes encoding antioxidant pathway enzymes GR, glutathione reductase; AP, 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, 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.

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Activities of antioxidant enzymes GR, APx, CAT and GST in leaves of control and NtFLS silenced transgenic tobacco lines G12, A2, B1 and E13 (b).
Enzyme activity of GST, APx, and GR is expressed in μmoles m⁻¹g⁻¹FW and CAT is expressed in nmols m⁻¹g⁻¹FW. 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.

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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.

4.25 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.

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.
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).

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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.

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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.

4.33 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.
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 (b), F9 (c) and F12b (d) 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.

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).

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).

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).
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.

*In vitro* and *in vivo* pollen germination assays of control and *NtFLS* silenced transgenic tobacco. *In vitro* pollen germination assay of pollens from control tobacco plant (a) and from *NtFLS* silenced transgenic tobacco lines G12, A2, B1 and 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 *NtFLS* silenced transgenic tobacco lines G12, A2, B1 and 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 P < 0.05 and 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 *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 *NtFLS* silenced line 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 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 G12 line grew only nine-tenths of the way down the style (n). The tips of the pollen tubes are swollen in G12 (shown by arrow). Pollen tubes are not visible at the base of the style in G12 *NtFLS* silenced line (o) as compared to control carpels (j).
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.

4.41 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.

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).

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).

4.44 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.

4.45 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.

4.46 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.

4.47 Changes in the transcript level of genes encoding glutathione reductase (GR) (a), ascorbate peroxidase (APx) (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.

4.48 Changes in the transcript level of genes encoding glutathione reductase (GR) (a), ascorbate peroxidase (APx) (b), catalase (CAT) (c), and glutathione S-transferase (GST) (d) enzymes in tobacco roots in response to different treatments epicatechin (EC; 50 and 100 μM) and quercetin (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.