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Overexpression of *SrUGT85C2* from *Stevia* reduced growth and yield of transgenic *Arabidopsis* by influencing plastidial MEP pathway

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ABSTRACT

The transcript expression of a gene *SrUGT85C2* has been documented for direct relation with steviol glycoside content in *Stevia* plant. Steviol glycoside and gibberellin biosynthetic routes are divergent branches of methyl erythritol-4 phosphate (MEP) pathway. So, *SrUGT85C2* might be an influencing gibberellin content. Hence in the present study, transgenic *Arabidopsis thaliana* overexpressing *SrUGT85C2* cDNA from *Stevia rebaudiana* was developed to check its effect on gibberellin accumulation and related plant growth parameters. The developed transgenics showed a noteworthy decrease of 78–83% in GA_3 content. Moreover, the transgenics showed a gibberellin deficient phenotype comprising stunted hypocotyl length, reduced shoot growth and a significant fall in relative water content. Transgenics also showed 17–37 and 64–76% reduction in chlorophyll a and chlorophyll b contents, respectively. Reduction in photosynthetic pigments could be responsible for the noticed significant decrease in plant biomass. Like steviol glycoside and gibberellin biosynthesis, chlorophyll biosynthesis also occurs from the precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) of MEP pathway in the plastids. The observed downregulated expression of genes encoding MEP pathway enzymes geranyl geranyl diphosphate synthase (GGDPS), copalyl diphosphate synthase (CDPS), kaurenoic acid oxidase (KAO), chlorophyll synthetase and chlorophyll a oxygenase in transgenics overexpressing *SrUGT85C2* might be responsible for the reduction in gibberellins as well as chlorophyll. This study has documented for the first time the regulatory role of *SrUGT85C2* in the biosynthesis of steviol glycoside, gibberellins and chlorophyll.

1. Introduction

Steviol glycosides are the ent-kaurene diterpene secondary metabolites reported from *Stevia rebaudiana* (Yadav and Guleria, 2012). These are synthesized in the plastids of leaves by steviol glycoside biosynthesis pathway (Bondarev et al., 2003/4). The pathway has been known to originate from pyruvate and glyceraldehyde-4-phosphate. Similar substrates have been identified as the starting molecules of methyl-erythritol-4 phosphate (MEP) pathway (Wanke et al., 2001). MEP pathway also operates in the plastid of plants (Jomaa et al., 1999). As a result, steviol glycoside biosynthesis pathway has been identified as the branching route of MEP pathway (Wanke et al., 2001). In addition to steviol glycoside biosynthesis, gibberellin and chlorophyll biosyntheses are also derived from the same MEP pathway (Gomez-Roldan et al., 2008; Umehara et al., 2008). Hence, steviol glycoside biosynthesis route has been considered as divergence along with gibberellin biosynthesis of MEP pathway (Richman et al., 1999).

In addition to the steps that are in common with MEP and gibberellin pathway, steviol glycoside biosynthesis pathway possesses five specific steps (Yadav and Guleria, 2012). The first pathway specific step involves the synthesis of steviol from ent-kaurenoic acid catalyzed by an enzyme encoded by gene *SrKA13H* (Brandle and Telmer, 2007). The rest of the steps involve the subsequent glycosylation of steviol into rebaudioside A and are catalyzed by four *SrUGT* encoded enzymes. However, only three *SrUGTs* have been identified so far (Brandle and Telmer, 2007). At the second pathway specific step, an enzyme encoded by *SrUGT85C2* catalyzes the glycosylation of steviol into steviolmonoside (Brandle and Telmer, 2007). An enzyme encoded by *SrUGT74G1* and *SrUGT76G1* has been known to glucosylate steviolbioside and stevioside at C-19 and C-13 positions giving rise to stevioside and rebaudioside A, respectively (Brandle and Telmer, 2007). These glycosylations have been identified important for triggering the synthesis of steviol glycosides. However, a significant correlation has been identified between the transcript expression of gene *SrUGT85C2* and total accumulation of steviol glycosides (Mohamed et al., 2011). Since steviol glycoside and gibberellin biosynthetic routes share a common origin, the expression levels of genes involved in the steviol glycoside pathway might have some influence on the accumulation of gibberellins. Hence, present study was conducted with the aim of evaluating the response of *SrUGT85C2* overexpression on gibberellin accumulation as well as on the growth and development of raised *Arabidopsis thaliana* transgenics.

Abbreviations: CDPS, copalyl diphosphate synthase; DMAPP, dimethylallyl diphosphate; GGDPS, geranyl geranyl diphosphate synthase; GM, germination medium; IPP, isopentenyl diphosphate; KOA, kaurenoic acid oxidase; MEP, methyl-erythritol-4 phosphate; MS, Murashige Skoog; RWC, relative water content.

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2. Materials and methods

2.1. Construct preparation for SrUGT85C2 overexpression

The nucleotide sequence of *SrUGT85C2* (Accession number — AY345978.1) was obtained from Nucleotide database of *S. rebaudiana* at NCBI. Overexpression construct was prepared by using the binary vector pCAMBIA1302. Total RNA isolated from the leaves of *S. rebaudiana* was used for cDNA preparation. This cDNA was used for *SrUGT85C2* amplification using primers, Forward-5′ AGATCTATGGATGCAATGGCTACA AC 3′, with a Bgl II cloning site at 5′ end and Reverse-5′ ACTAGTTCTA GTTTCTTGCTAGCACG 3′, with a Spe I cloning site at 5′ end. These restriction sites were specific to vector pCAMBIA1302. The amplified cDNA was initially cloned in the pGEM-T easy vector and restriction digested to produce staggered terminal ends. The staggered end fragment was finally cloned between the Bgl II and Spe I sites of vector pCAMBIA1302.

2.2. Transformation of A. thaliana

The prepared overexpression construct for the gene *SrUGT85C2*, designated as pCAMBIA-*SrUGT85C2* was transformed into *Agrobacterium tumefaciens* strain *LBA4404* by triparental mating. Vacuum infiltration was used to transform *A. thaliana* with prepared recombinant *Agrobacterium* cell culture (Bechtold et al., 1993). The transformed plants were allowed to grow further and their seeds were collected. T_0 seeds were screened on a germination medium (GM) constituting of 1× Murashige Skoog (MS) salt, 3% sucrose, 0.7% agar, pH 5.85 and supplemented with 25 mg l^{-1} of hygromycin antibiotic. The seeds were washed with an absolute alcohol and the surface sterilized with 0.01% mercuric chloride. The sterilant was removed from the seeds by washing twice with autoclaved distilled water. The sterilized seeds were placed on GM supplemented with hygromycin antibiotic. Resistant plants were transferred to pots and allowed to set seed by self pollination. The transgenics were confirmed for transgene integration by conducting PCR using gene specific primers. Further, the accumulation of transcript mRNA was analyzed by semi-quantitative PCR using gene specific primers. The primers corresponding to gene 26S rRNA was employed as an internal control for cDNA quantification during semi-quantitative PCR analysis (Singh et al., 2004). The confirmed transgenics were maintained for further analysis.

2.3. Hypocotyl elongation measurement

The seeds of control and transgenic *A. thaliana* were surface sterilized as mentioned above. The sterilized seeds were placed in rows on MS medium. The plates were covered with foil and kept at 4 °C for three days. These wrapped plates were then maintained at 22 °C for seven days. Seven-day old seedlings were employed for hypocotyl measurements. The measurements were performed using three independent biological replicates.

2.4. Relative water content measurement

Relative water content (RWC, %) of control as well as transgenic *A. thaliana* overexpressing *SrUGT85C2* was calculated using the formula — (FW — DW) / (TW — DW). Fresh weight (FW) was obtained by harvesting and weighing freshly detached rosette leaves of control as well as transgenic *A. thaliana*. Turgid weight (TW) was attained by incubating cut rosettes in de-ionized water for 16 h at room temperature. TW was estimated by weighing the plant material after removing excess water by soaking with absorbent paper. Rosette dry weight (DW) was obtained after drying the turgid rosette leaves at 75 °C in a dry oven (Bouchabke et al., 2008). The RWC measurements were performed using three independent biological replicates.

2.5. Estimation of stevioside

The accumulation of stevioside was estimated in the rosette leaves of control as well as transgenic *A. thaliana* by the method described earlier (Jaitak et al., 2009). Samples were crushed and extracted overnight with 80% methanol at room temperature. Extract was filtered and the filtrate was dried in vacuo. The dried extract was defatted with hexane and residual extract was vacuum dried. The resulting extract was dissolved in acetonitrile and filter-sterilized for HPLC analysis. Ten microliters of sample was injected to Waters Spherisorb® 10 μm NH_2 column (Waters, USA) using an isocratic solvent system of acetonitrile:water (80:20) at a flow rate of 0.8 ml min^{-1}. Stevioside was detected at 205 nm on a photodiode array detector. The stevioside estimation was performed using three independent biological replicates.

2.6. Estimation of endogenous gibberellin (GA_3) content

Gibberellin (GA_3) content was estimated in control and transgenic *A. thaliana* overexpressing *SrUGT85C2* by following the protocol mentioned earlier (Hedden and Phillips, 2000; Kelen et al., 2004). Briefly, fresh tissue was homogenized with 70% methanol and stirred overnight at 4 °C. The extract was filtered and dried in vacuo. The pH of obtained residue was maintained to 8.5 with 0.1 M phosphate buffer and partitioned thrice with ethyl acetate. After removal of ethyl acetate phase, the pH of aqueous phase was adjusted to 2.5 with 1 N HCl. The obtained solution was partitioned thrice with diethyl ether. The ether phases were pooled together and vacuum dried. The obtained residue was dissolved in acetonitrile. The extract was filtered and subjected to HPLC. The sample (10 μl) was injected into Lichrospher®100 RP-18e column (Waters, USA) using the isocratic solvent system of acetonitrile: water (26:74) supplemented with 30 mM phosphoric acid (pH 4.0). The flow rate was maintained at 0.8 ml min^{-1}. GA_3 was detected at 208 nm using a photodiode array detector. The estimation was performed using three independent biological replicates.

2.7. Determination of chlorophyll content

Chlorophyll content of control as well as transgenic *A. thaliana* overexpressing *SrUGT85C2* was determined following the method described earlier (Chory, 1992). Rosette leaves of plants were collected and crushed in 80% acetone. The mixture was rapidly shaken in the dark for 30 min. The debris was pelleted down by centrifugation and supernatant was used for spectrophotometric determination. The optical densities of the supernatant were determined at 645 (A_{645}) and 663 nm (A_{663}) using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher USA). Chlorophyll a and b contents were calculated using MacKinney's specific absorption coefficients (MacKinney, 1941). The equations employed for the calculation were:

$$\begin{aligned}\text{Chlorophyll a} &= 12.7(A_{663}) - 2.69(A_{645})\\ \text{Chlorophyll b} &= 22.9(A_{645}) - 4.48(A_{663})\end{aligned}.$$

The content measurements were performed using three independent biological replicates.

2.8. Expression analysis of MEP pathway genes

Total RNA was isolated from the 100 mg rosette leaves of control and transgenic *A. thaliana* using Qiagen RNeasy Plant Mini kit according to the manufacturer's instructions. First strand cDNA synthesis was conducted using Superscript III RT (Invitrogen) with 1 mg of total RNA and oligodT primers. Equal quantity of synthesized cDNA was employed to evaluate the transcript expression of genes geranyl geranyl diphosphate synthase (*GGDPS*), copalyl diphosphate synthase (*CDPS*), kaurenoic acid oxidase (*KAO*), chlorophyll synthetase and chlorophyll a oxygenase. These genes are involved in the biosynthesis of gibberellins and

chlorophylls. The level of 26S rRNA amplified in each sample with standard primers was employed as an internal control (Singh et al., 2004).

3. Results

3.1. Generation of A. thaliana transgenic overexpressing SrUGT85C2 cDNA from S. rebaudiana

The *SrUGT85C2* nucleotide sequence retrieved from NCBI (Accession number — AY345978.1) was used for preparing the overexpression construct. The binary vector pCAMBIA1302 was used as backbone for construct preparation. Briefly, *SrUGT85C2* cDNA of size 1458 bp was amplified from *S. rebaudiana* and cloned in the pGEM-T easy vector. The primers used to amplify cDNA were supplemented with sites of restriction enzymes Spe I and Bgl II, specific to cloning sites present in pCAMBIA1302. By making use of these restriction enzymes, cohesive end fragment was digested out from the pGEM-T easy vector. The fragment was then cloned in pCAMBIA1302 and transformed into *Agrobacterium* strain *LBA4404*.

The *A. thaliana* plants were transformed through *Agrobacterium* mediated vacuum infiltration for transgenic generation. The transgenics were prepared to overexpress *S. rebaudiana SrUGT85C2* cDNA under the control of a constitutive 35S cauliflower mosaic virus promoter. The schematic representation of T-DNA containing *SrUGT85C2* cDNA is shown in Fig. 1A.

The seeds of T_0 generation obtained after transformation were screened on GM supplemented with hygromycin as selection. Plants resistant to hygromycin were maintained till T_3 generation for further analysis. The integration of T-DNA of overexpression construct was confirmed by PCR mediated analysis of the transgene *SrUGT85C2* and selection gene encoding hygromycin resistance. In this PCR, genomic DNA extracted from the rosette leaves of *A. thaliana* transgenics was used as template (Fig. 1B). Further, the accumulation of *SrUGT85C2* mRNA was confirmed by semi-quantitative PCR. Two lines of transgenic *A. thaliana* T-138 and T-434 showing good expression of *SrUGT85C2* transcript were utilized for further experimental analysis (Fig. 1C).

3.2. A. thaliana transgenics overexpressing SrUGT85C2 show reduced hypocotyls

The seeds of control and transgenics overexpressing *SrUGT85C2* were placed on MS medium and maintained under complete dark conditions. Seven-day old seedlings were used for the measurement of hypocotyl length. It was noticed that one of *SrUGT85C2* overexpressing *A. thaliana* transgenic line T-138 showed a decrease of 64% in hypocotyl length in comparison to control hypocotyl (Figs. 2A, B). Similarly, a decrease of 48% was noticed in the length of hypocotyl of T-434 plants with respect to control plants (Figs. 2A, B). Hence, overexpression of *SrUGT85C2* cDNA from *S. rebaudiana* has significantly decreased the hypocotyl length.

3.3. Phenotypic characterization of A. thaliana transgenics overexpressing SrUGT85C2

The *A. thaliana* transgenics overexpressing *SrUGT85C2* were observed for change in their phenotype with respect to control. Fifteen-day old seedlings of control and transgenic plants were evaluated for morphological variations. The shoot length was significantly reduced in transgenics compared to control. However, no variation was noticed in the root length. The transgenics were observed to be pale green in color compared to the dark green texture of control plants (Fig. 2C). The T-138 transgenic *A. thaliana* showed a reduction of 47% in their shoot length compared to control. Similarly, the T-434 transgenic plants were noticed to possess a 33% decrease in shoot length with respect to control plants (Fig. 2D). Further, the transgenics showed a significant reduction in fresh biomass in comparison to control. The fresh biomass was decreased by 47% and 54% in T-138 and T-434 transgenics compared to control (Fig. 2E). Likewise, the RWC was also reduced in T-138 and T-434 plants by 21% with respect to control (Fig. 2F). Moreover, the rosette area was also decreased in transgenics compared to control (Fig. 2G). A reduction of 77% and 56% in rosette area was noticed in T-138 and T-434 plants with respect to control plants, respectively (Fig. 2H).

3.4. Transgenics overexpressing SrUGT85C2 do not accumulate steviolbioside and stevioside

In steviol glycoside biosynthesis pathway, an enzyme encoded by gene *SrUGT85C2* catalyzes the glycosylation of steviol into steviolmonoside. Steviolmonoside has been known to possess least stability and immediately converted into steviolbioside. Furthermore in the in vitro assay, protein extract of *A. thaliana* has been shown to glucosylate steviolbioside into stevioside (Humphrey et al., 2006). Hence to investigate the synthesis of steviolbioside and stevioside in transgenic *A. thaliana*, quantitative estimation of steviolbioside and stevioside was conducted in the rosette leaves of control as well as transgenics (Jaitak et al., 2009). The standard of steviolbioside and stevioside (10 μg ml^{-1}) showed a sharp peak at retention times (RTs) 6.59 and 8.079 min, respectively. The absorption spectrum of steviolbioside and stevioside was obtained using a photodiode array detector at a wavelength of 205 nm. The extracts obtained from rosette leaves of control as well as *SrUGT85C2* transgenics

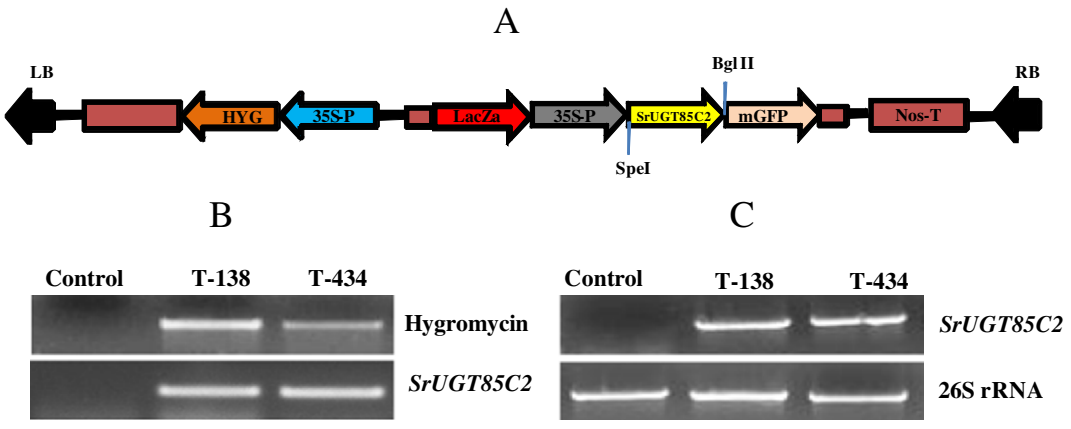

Fig. 1. Generation of transgenic *A. thaliana* overexpressing *SrUGT85C2* and their confirmation. Diagrammatic representation of T-DNA region of *SrUGT85C2* overexpression construct. The expression was regulated by the constitutively expressing promoter 35S CaMV (A); confirmation of integration of T-DNA region of construct in the genomic region of transgenic *A. thaliana* through PCR. Gel pictures show the presence of genes for hygromycin resistance and *SrUGT85C2* in T-138 and T-434 transgenics *A. thaliana* lines (B); semi-quantitative PCR analysis of *SrUGT85C2* transcript in T-138 and T-434 transgenic lines. 26S rRNA was employed as an internal control for cDNA quantification (C).

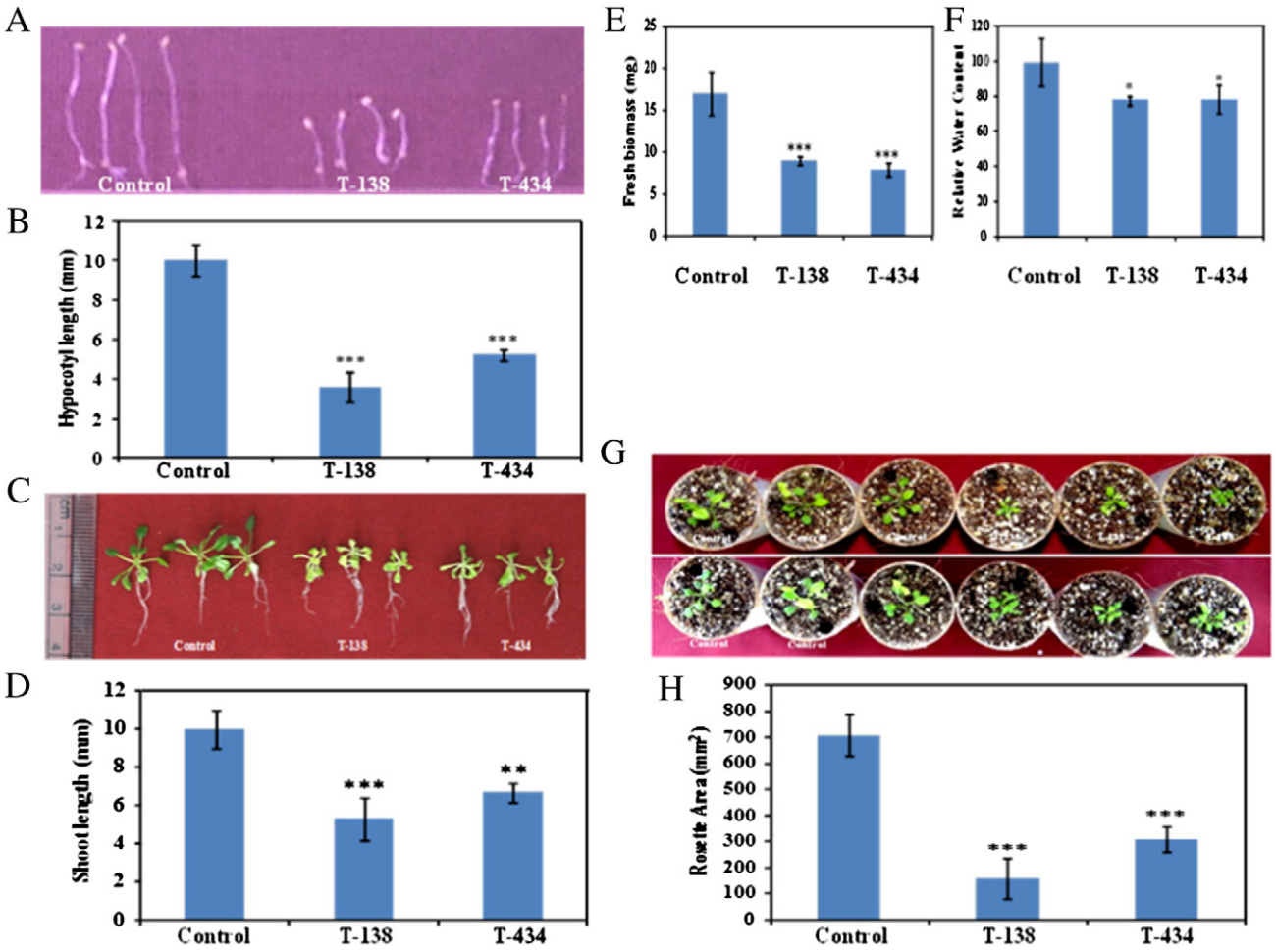

Fig. 2. Morphological characterization of *A. thaliana* transgenics overexpressing *SrUGT85C2* compared to control plants. Comparison of hypocotyl length of 7 day old control and transgenics overexpressing *SrUGT85C2* grown in dark conditions. The T-138 and T-434 transgenics possessed shorter hypocotyls than control plants (A); hypocotyl length. Each bar represents mean hypocotyl lengths from four independent measurements. Error bar represents ± standard error (***, $P < 0.001$; **, $P < 0.01$) (B); pictures show the variation in shoot height of 15 day old seedling of T-138 and T-434 transgenic lines in comparison to control (C); bar diagram shows a significant increase in shoot length of T-138 and T-434 transgenic lines compared to control. Data is presented as mean ± standard deviation of three independent measurements (***, $P < 0.001$; **, $P < 0.01$) (D); bar diagram represents a significant decrease in fresh biomass of T-138 and T-434 transgenic lines compared to control. Data is presented as mean ± standard deviation of three independent measurements (***, $P < 0.001$) (E); bar diagram represents a reduction in relative water content of T-138 and T-434 transgenic lines compared to control. Data is presented as mean ± standard deviation of three independent measurements (*, $P < 0.05$) (F); rosette area of soil grown control plants and *SrUGT85C2* transgenic lines (G); bar diagram showing a significant decrease in rosette area of T-138 and T-434 transgenic lines compared to control. Data was presented as mean ± standard deviation of three independent measurements (***, $P < 0.001$) (H).

A. thaliana did not show a detectable peak at RTs 6.59 and 8.079 (Fig. 3A). Thus, overexpression of *SrUGT85C2* cDNA from *S. rebaudiana* was not able to synthesize steviolbioside and stevioside in transgenic *A. thaliana*.

3.5. Overexpression of SrUGT85C2 reduced GA_3 accumulation

The level of endogenous bioactive gibberellin GA_3 was also estimated in transgenic *A. thaliana* overexpressing *SrUGT85C2*. GA_3 was extracted from the rosette leaves of control and transgenic plants with diethyl ether and quantified via HPLC (Kelen et al., 2004). The standard of GA_3 (10 μg ml^{-1}) was detected using a photodiode array detector at a wavelength of 208 nm and showed a peak at RT 2.79 min (Fig. 3B). Extracts obtained for gibberellin quantification from control and transgenic *A. thaliana* lines also showed a peak at the same RT 2.79 min (Fig. 3B). However, the content of GA_3 was relatively higher in control *A. thaliana* in comparison to the transgenic *A. thaliana* overexpressing *SrUGT85C2*. The gibberellin content was found to be 90.05 ng g^{-1} in control *A. thaliana* leaves. While, the content was significantly decreased in the case of T-138 and T-434 plants and showed an accumulation of 20.05 ng g^{-1} and 15.28 ng g^{-1}, respectively (Fig. 3C). Hence, overexpression of *SrUGT85C2*, a gene belonging to *S. rebaudiana*, in T-138 and T-434 *A. thaliana* transgenics has led to the reduction in their gibberellin accumulation by 78% and 83% in comparison to the control, respectively.

3.6. Reduction in chlorophyll content of transgenics overexpressing SrUGT85C2

The transgenics were observed to be pale green in color than control plants. Hence, chlorophyll a and chlorophyll b contents were estimated in control as well as in transgenic plants. Chlorophyll content was extracted with 80% acetone and spectrophotometrically estimated. The transgenic extracts showed a reduction in absorbance at wavelengths of 645 and 663 nm in contrast to control extract (Fig. 4A). Likewise a 37 and 17% decrease was noticed in chlorophyll a content of T-138 and T-434 transgenics compared to control, respectively (Fig. 4B). The chlorophyll b content was reduced by 76 and 64% in T-138 and T-434 transgenics than in control, respectively (Fig. 4C). Hence, overexpression of *SrUGT85C2* from *S. rebaudiana* in transgenic *A. thaliana* has decreased their chlorophyll content as well.

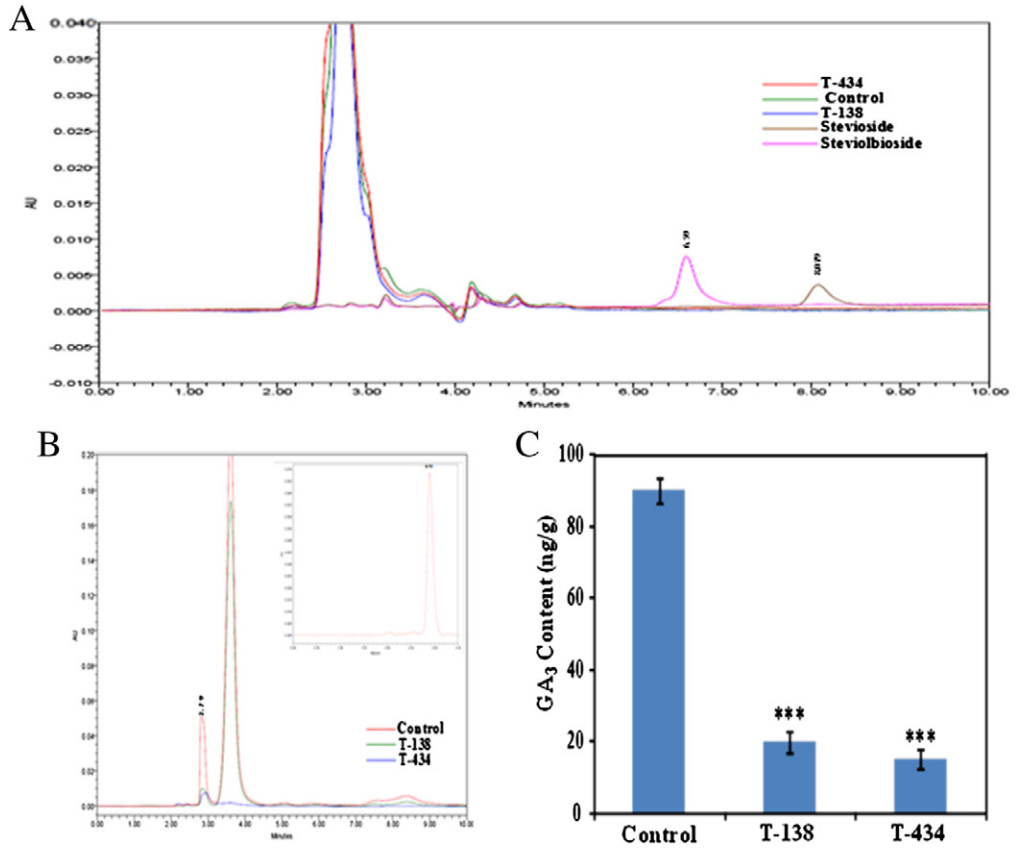

Fig. 3. Steviol glycoside accumulation and endogenous gibberellin GA_3 content in *A. thaliana* transgenics and control. The overlapped HPLC chromatogram of steviol glycoside accumulation for control and *SrUGT85C2* transgenic lines. Absorption spectrum was observed at 205 nm and detected using a photodiode array detector. HPLC chromatogram of steviolbioside and stevioside standard, 10 μg ml^{-1} showed a sharp peak at retention times (RTs) 6.59 and 8.079 min, respectively. Control as well as transgenics overexpressing *SrUGT85C2* showed no peak at RTs 6.59 and 8.079 min, respectively (A); the inset represents HPLC chromatogram of GA_3 standard, 10 μg ml^{-1} showing a sharp peak at RT 2.79 min. Absorption spectrum was observed at 208 nm and detected using a photodiode array detector. The overlapped HPLC chromatogram showed the presence of peak at RT 2.79 min for control as well as for T-138 and T-434 transgenic lines (B); bar diagram showing endogenous gibberellin content in control and transgenic lines. Gibberellin content was significantly reduced in T-138 and T-434 transgenics compared to control. Data represented as mean ± standard deviation of three independent measurements (***, $P < 0.001$) (C).

3.7. Decreased mRNA accumulation of MEP pathway genes involved in gibberellin and chlorophyll biosyntheses

The transgenic *A. thaliana* overexpressing *SrUGT85C2* showed a reduction in gibberellin and chlorophyll contents. Therefore, transgenics were analyzed to see the influence on the expression of genes of MEP pathway involved in gibberellin and chlorophyll syntheses (Fig. 5A). The GGDPS enzyme encoded by gene *GGDPS* is common for both gibberellin and chlorophyll biosyntheses (Fig. 5A). A decrease of 54% in mRNA accumulation of *GGDPS* was noticed in T-138 transgenics. However, the transgenic T-414 showed a insignificant decrease in *GGDPS* expression (Fig. 5B). Followed by GGDPS, CDPS and KAO enzymes encoded by genes *CDPS* and *KAO* are specifically involved in gibberellin biosynthesis. The transgenics showed a significant decrease in the mRNA accumulation of *CDPS* compared to control. The *CDPS* gene expression was relatively decreased by 81 and 98% in T-138 and T-414 transgenic lines than in control, respectively (Fig. 5C). Similarly, both the transgenic lines T-138 and T-414 showed a 33 and 37% reduction in *KAO* mRNA accumulation (Fig. 5D). Hence, overexpression of gene *SrUGT85C2* was observed to downregulate expression of the genes involved in gibberellin biosynthesis. Likewise, the transgenics also showed downregulation in expression of gene encoding enzymes of chlorophyll biosynthesis such as chlorophyll synthetase and chlorophyll a oxygenase. The mRNA accumulation of chlorophyll synthetase was decreased by 71 and 74% in T-138 and T-414 transgenic *Arabidopsis*, respectively (Fig. 5E). Similarly, an 80 and 94% reduction in relative expression of chlorophyll a oxygenase was noticed in transgenics T-138 and T-414 compared to control, respectively (Fig. 5F). Therefore, overexpression of *SrUGT85C2* cDNA from *Stevia* was also found to significantly reduce the expression of chlorophyll biosynthesis genes as well.

4. Discussion

Steviol glycosides are tetracyclic diterpenes synthesized from the precursor ent-kaurenoic acid in plastids of *S. rebaudiana*. The ent-kaurene skeleton of diterpene glycosides in chloroplast has been identified to be formed by the methyl-erythritol-4 phosphate (MEP) pathway (Totte et al., 2003). MEP pathway is also responsible for the synthesis of plastid isoprenoids that includes plant hormones and photosynthetic pigments (Sauret-Gueto et al., 2006). Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the two important precursors of MEP pathway from where all isoprenoids are derived. IPP and DMAPP give rise to geranyl geranyl diphosphate (GGPP) that acts as a precursor for the synthesis of gibberellins and chlorophylls (Sauret-Gueto et al., 2006). The initial steps of steviol glycoside biosynthetic route involve the synthesis of IPP and DMAPP that shares similarity with MEP pathway. Further, steviol glycoside and gibberellin biosynthetic routes have been considered as the diverging branches of MEP pathway in *S. rebaudiana* (Totte et al., 2000).

The biosynthesis of steviol glycosides is composed of sixteen enzyme catalyzed steps. The downstream last five steps have been considered specific to steviol glycoside biosynthesis. However, only four genes encoding the enzymes of these last of steviol glycoside biosynthesis have been identified (Yadav and Guleria, 2012). These genes include *SrKA13H*, *SrUGT85C2*, *SrUGT74G1* and *SrUGT76G1*. SrKA13H catalyzes the conversion of ent-kaurenoic acid into steviol. The rest of SrUGTs

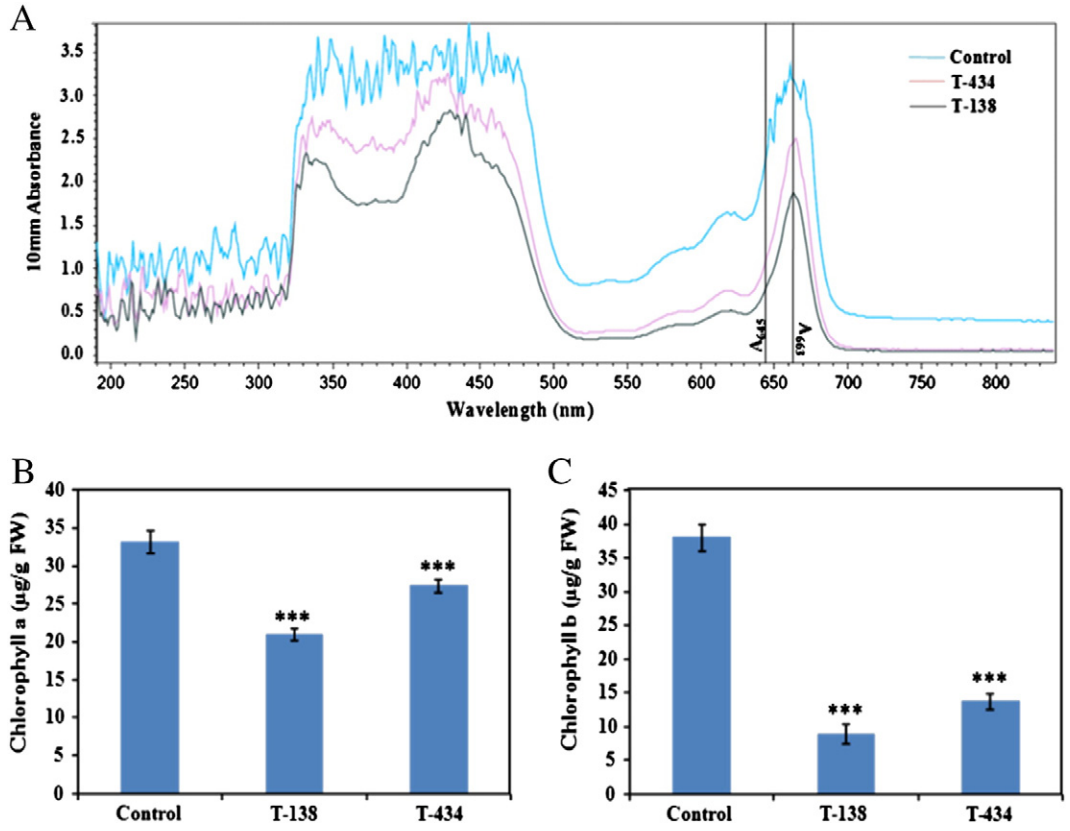

Fig. 4. Influence of *SrUGT85C2* overexpression on chlorophyll accumulation. UV–visible absorption spectra showing the absorbance of control as well as transgenic extracts at 645 and 663 nm (A); bar diagram shows chlorophyll a content in control and transgenic lines T-138 and T-434. Chlorophyll a content was significantly reduced in transgenics compared to control. Data represented as mean ± standard deviation of three independent measurements (***, $P < 0.001$) (B); bar diagram shows chlorophyll b content in control and transgenic lines T-138 and T-434. Chlorophyll b content was also significantly decreased in transgenics compared to control. Data represented as mean ± standard deviation of three independent measurements (***, $P < 0.001$) (C).

catalyze the subsequent glycosylation of steviol into rebaudioside A. SrUGT85C2 is the first UGT enzyme that initiates the glycosylation process by converting steviol into steviolmonoside (Brandle and Telmer, 2007). The gene *SrUGT85C2* has been considered important and noticed to show a significant correlation with the accumulation of steviol glycosides (Mohamed et al., 2011). Earlier, in vitro assays of total protein isolated from *A. thaliana* overexpressing *SrUGT85C2* have demonstrated that the protein retained its native activity in plant system other than *S. rebaudiana* (Humphrey et al., 2006). However, the transgenics overexpressing *SrUGT85C2* in the present study were not able to synthesize either steviolbioside or stevioside. Hence, it can be inferred that in vivo presence of steviol is necessary for the enzyme SrUGT85C2 to synthesize stevioside in plant species other than *S. rebaudiana*.

Since steviol glycoside biosynthesis diverges from gibberellin biosynthetic route at ent-kaurenoic acid metabolite (Brandle and Telmer, 2007; Kim et al., 1996), transgenics overexpressing *SrUGT85C2* were evaluated for gibberellin accumulation. Interestingly, the transgenics showed a significant reduction in gibberellin accumulation than control. Studies have reported a reduction in gibberellin content even on overexpression of genes unrelated to gibberellin biosynthesis pathway. Overexpression of *A. thaliana DREB1A* gene in soybean has been demonstrated to decrease the accumulation of gibberellins (Suo et al., 2012). Similarly, overexpression of a homeobox gene *NTH15* in tobacco has also documented a reduction in gibberellin content (Tanaka-Ueguchi et al., 1998). Gibberellins are endogenous plant hormones that are known to play an important role in plant growth and development. Gibberellins have been known to regulate plant morphological features as hypocotyl elongation, stem length, leaf expansion and rosette area (Yamaguchi, 2008). In the present study, the transgenics overexpressing *SrUGT85C2* showed a reduced hypocotyl and stem length. Likewise, the rosette area was also decreased in the transgenics. Earlier, various gibberellin deficient mutants have been reported to show such phenotypes. The transgenic soybean overexpressing *AtDREB1A* has been reported to show dwarfism and decreased rosette area. These phenotypic changes in soybean have been documented to be due to the reduction in gibberellin content (Suo et al., 2012). The *ddf1* (*dwarf and delayed flowering 1*) mutant of *A. thaliana* overexpressing putative AP2 transcription factor has also been reported to show severe dwarfism. Reduction in gibberellin was again accounted responsible for such abnormal phenotype (Magome et al., 2004). Likewise, a decrease in gibberellin content on silencing of gibberellin biosynthetic genes *ga20ox1* and *ga20ox2* has also been documented to reduce stem height and smaller rosette area (Rieu et al., 2008). Thus, the reduction in GA_3 content seems to be responsible for these phenotypic variations in *A. thaliana* transgenics overexpressing *SrUGT85C2* similar to gibberellin deficient mutant.

Previous studies have shown the opposite trend for endogenous gibberellin and chlorophyll contents. As *AtDREB1A*-overexpressing soybean transgenics have been reported to decrease gibberellin levels by upregulating the expression of GA-20-oxidase and GA-3-oxidase genes. These transgenics were observed for dark green coloration of plant leaves due to an increase in their chlorophyll content (Suo et al., 2012). Similarly, transgenic tobacco overexpressing AtGA20-ox has shown an increase in gibberellin accumulation and a decrease in chlorophyll content (Biemelt et al., 2004). Gibberellin and chlorophyll are the end products of a common MEP pathway. So, a increase/decrease in gene expression of gibberellin biosynthetic pathway could leave substrates unreacted that in turn decrease/increase the substrate usage for chlorophyll biosynthesis. However in transgenic *A. thaliana* overexpressing *SrUGT85C2*, chlorophyll content was also reduced in addition to the reduction in gibberellin levels. Further, reduction in chlorophyll pigment could be responsible for the observed decrease in biomass of transgenics (Biemelt et al., 2004). Reduction in both gibberellin and

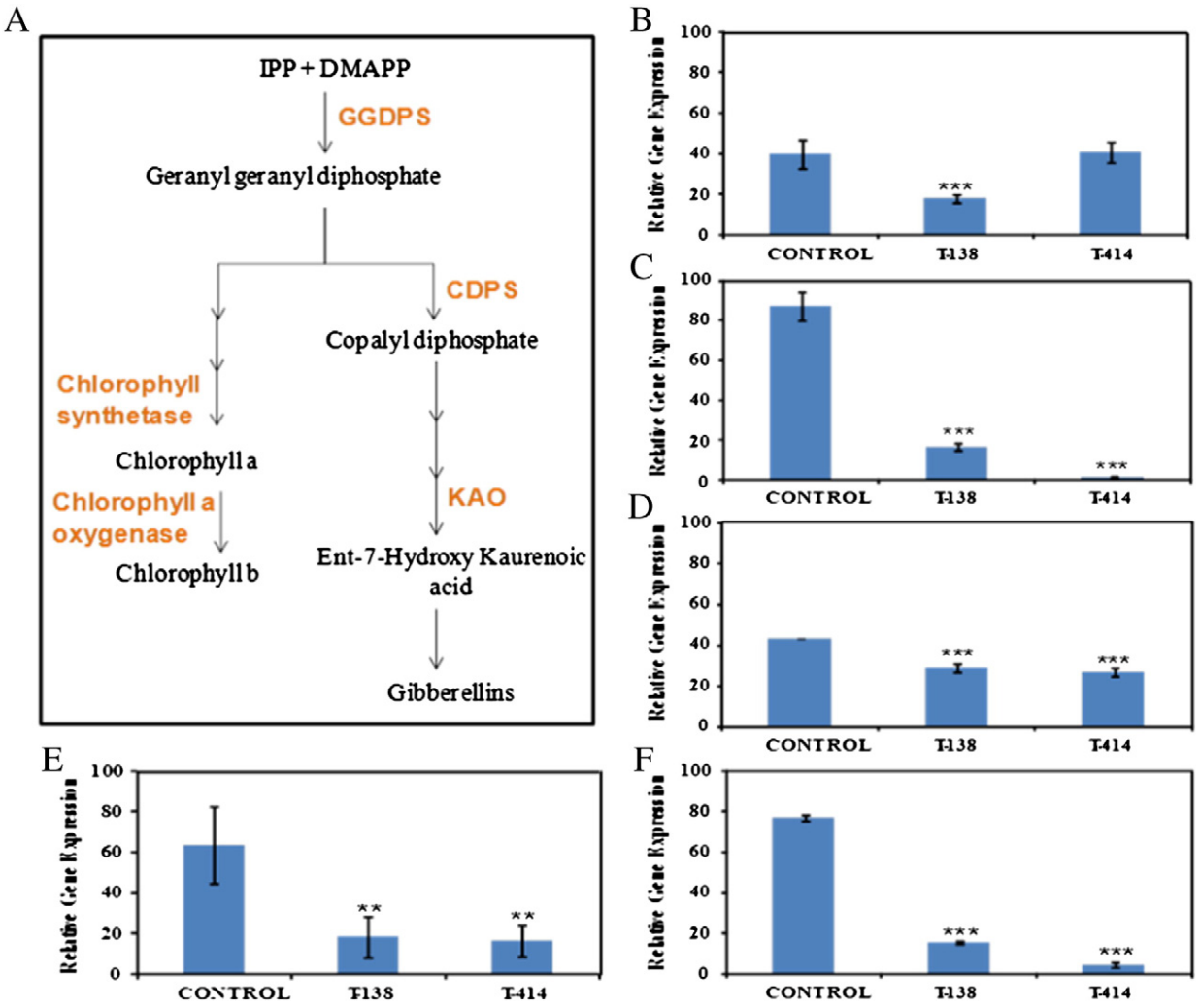

Fig. 5. Influence of *SrUGT85C2* overexpression on expression of MEP pathway genes involved in gibberellin and chlorophyll biosyntheses. MEP pathway showing steps of gibberellin and chlorophyll biosyntheses, where geranyl geranyl diphosphate synthase (GGDPS), copalyl diphosphate synthase (CDPS), kaurenoic acid oxidase (KAO), chlorophyll synthetase and chlorophyll a oxygenase (A); bar diagram shows a relative mRNA expression of genes in control and transgenic T-138 and T-414 line encoding enzymes, GGDPS (B); CDPS (C); KAO (D); chlorophyll synthetase (E); and chlorophyll a oxygenase (F). The mRNA expression was significantly reduced in transgenics compared to control. Data represented as mean ± standard deviation of three independent measurements (**, $P < 0.01$; ***, $P < 0.001$).

chlorophyll contents has further suggested the possibility of transgene influence on certain common metabolites of MEP pathway. The down-regulation in the expression of gene encoding enzymes of MEP pathway in transgenic *A. thaliana* overexpressing *SrUGT85C2* could be responsible for the observed decrease in gibberellin and chlorophyll. Thus, it has suggested the negative influence of *SrUGT85C2* overexpression in *A. thaliana* on the downstream steps of MEP pathway. Earlier, knockout mutants of several genes of MEP pathway have been reported to possess alterations in chloroplast development, thus showed an albino phenotype (Gutierrez-Nava et al., 2004; Mandel et al., 1996). Hence, the overexpression of *SrUGT85C2* in *A. thaliana* might have downregulated the metabolite(s) of MEP pathway where from thereafter synthesis of gibberellin and chlorophyll originates.

MEP pathway is also operated in pathogenic bacteria for IPP and DMAPP biosynthesis. Hence, the elucidation of occurrence of MEP pathway in plants has opened a possibility to employ it for the development of strategies against pathogens (Rodriguez-Concepcion et al., 2004). Presently, MEP pathway is being targeted to understand its regulation and importance in plants. Understanding and development of inhibitors for MEP pathway enzymes have opened the way for the establishment of broad spectrum herbicides and drugs (Cordoba et al., 2009). In conclusion, overexpression of *SrUGT85C2* in transgenic *A. thaliana* has decreased two most important end products gibberellins and chlorophyll of MEP pathway. This has made plants dwarf and decreased the overall biomass. Present study also documents the role of *SrUGT85C2* in gibberellin regulation, in addition to its significance towards steviol glycoside accumulation.

Conflict of interest

Authors declare that there is no conflict of interest.

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