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Overexpression of a glycosyltransferase gene *SrUGT74G1* from *Stevia* improved growth and yield of transgenic *Arabidopsis* by catechin accumulation

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Abstract Steviol glycoside and gibberellin biosynthetic routes are known as divergent branches of a common origin in *Stevia*. A UDP-glycosyltransferase encoded by *SrUGT74G1* catalyses the conversion of steviolbioside into stevioside in *Stevia rebaudiana* leaves. In the present study, transgenic *Arabidopsis thaliana* overexpressing *SrUGT74G1* cDNA from *Stevia* were developed to check the probability of stevioside biosynthesis in them. However, stevioside accumulation was not evident in transgenics. Also, the transgenic *Arabidopsis* showed no change in GA_3 content on *SrUGT74G1* overexpression. Surprisingly, significant accumulation of catechin was noticed in transgenics. The transgenics showed a considerable increase in shoot length, root length and rosette area. An increase in free radical scavenging activity of transgenics was noticed. Moreover, the seed yield of transgenics was also increased by 6–15 % than control. Additionally, variation in trichome branching pattern on leaf surface of transgenics was observed. The trichome branching pattern was also validated by exogenous catechin exposure (10, 50, 100 ng ml^{-1}) to control plants. Hence, present study reports the probable role of *SrUGT74G1* from *Stevia* in catechin accumulation of transgenic *Arabidopsis thaliana.* Thus, detailed study in present perspective has revealed the role of *Stevia SrUGT74G1* gene in trichome branching pattern, improved vegetative growth, scavenging potential and seed yield by catechin accumulation in transgenic *Arabidopsis*.

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Introduction

Steviol glycoside biosynthesis occurs in the leaves of plant *Stevia rebaudiana* [1]. Stevioside and rebaudioside A have been considered two important metabolites of this biosynthetic route. This pathway is known to be constituted of sixteen enzyme catalyzed steps. Among these 16 steps, initial 7 steps are common with methylerythritol-4-phosphate pathway [2]. The next 4 steps giving rise to ent-kaurenoic acid share similarity with gibberellin biosynthetic route [3]. As a result, steviol glycoside and gibberellin biosynthetic pathways have been considered as divergent branches of common route in methylerythritol-4-phosphate pathway [4]. However, the genetic regulation of steviol glycoside biosynthesis is still not known [5]. Only last 5 steps succeeding ent-kaurenoic acid have been considered specific to steviol glycoside biosynthesis [6]. These steps are catalyzed by enzymes, ent-kaurenoic acid-13 hydroxylase and three UDP-glycosyltransferases encoded by genes *SrKA13H* and three *SrUGTs* (*SrUGT85C2*, *SrUGT74G1* and *SrUGT76G1*), respectively. However, enzyme catalyzing one of the intermediate step even in this section of pathway is still not elucidated. Among these pathway specific enzymes, UDP-glycosyltransferase encoded by gene *SrUGT74G1* catalyses the synthesis of stevioside by glycosylating steviolbioside at C-19 position [3].

Earlier, *Arabidopsis* protein extract has been documented to mimic SrUGT74G1 enzyme like activity. More than 100 of the identified *Arabidopsis* UGTs have shown C-19 glycosylation activity like SrUGT74G1. Thus, *UGTs* showing 19-glcosylation activity have been considered

ubiquitous in plant kingdom that could possibly recognize and glucosylate any number of plant metabolites [7]. This study has further reported that ectopic expression of *Stevia UGTs* could partially synthesize steviol glycosides [7]. However, these conclusions were drawn on the basis of in vitro protein assays. Moreover, *in planta* synthesis of any of the steviol glycoside in any heterologous plant species has not been reported.

Taking advantage of following facts, *Arabidopsis thaliana* was engineered in present study for the ectopic overexpression of *SrUGT74G1* cDNA from *Stevia*. This has helped to evaluate the probability of *in planta* stevioside production in heterologous plant species on *SrUGT74G1* overexpression. Developed transgenics did not produce stevioside. But these plants were observed for catechin accumulation. Therefore, plants were also observed for the influence of *SrUGT74G1* overexpression on their phenotype, growth behavior and yield parameters.

Materials and methods

Construct preparation for *SrUGT74G1* overexpression

The gene sequence of *SrUGT74G1* (Accession number-AY345982.1) was obtained from Nucleotide database of *Stevia rebaudiana* at NCBI. Overexpression construct was prepared by using the binary vector pCAMBIA1302. For cloning, total RNA isolated from the leaves of *Stevia* was used for cDNA preparation. This cDNA was used for *SrUGT74G1* amplification using primers, Forward-5′AGATCTATGGCGGAACAACAAAAGATC3′, with a Bgl II cloning site at 5′ end and Reverse-5′ACTAGTTTAAGCCTTAATTAGCTCACT3′, with a Spe I cloning site at 5′ end. These restriction sites are specific to vector pCAMBIA1302. The amplified cDNA was initially cloned in pGEMT-easy vector and restriction digested to produce staggered terminal ends. The staggered ends fragment was finally cloned between the Bgl II and Spe I sites of vector pCAMBIA1302.

Transformation of *Arabidopsis thaliana*

The prepared overexpression construct for the gene *SrUGT74G1*, designated as pCAMBIA-*SrUGT74G1* was transformed into *Agrobacterium tumefaciens* strain *LBA4404* by triparental mating. Vacuum infiltration was used to transform *Arabidopsis thaliana* with prepared recombinant *Agrobacterium* cell culture [8]. The transformed plants were allowed to grow further and their seeds were collected. T_0 seeds were screened on germination medium (GM) constituting of 1X Murashige and Skoog (MS) salt, 3 % sucrose, 0.7 % agar, pH 5.85 and supplemented with 25 mg l^{-1} of hygromycin antibiotic. Seeds were washed with absolute alcohol and 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 planted on GM supplemented with hygromycin antibiotic. Resistant plants were transferred to pots and allowed to set seeds 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 internal control for cDNA quantification during semi-quantitative PCR analysis [9]. The obtained confirmed transgenics were maintained for further analysis.

Quantitative estimation of stevioside

The stevioside content was estimated in the rosette leaves of control as well as transgenic *Arabidopsis* by the method described earlier [10]. 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 microliter of sample was injected to Lichrosphere NH_2 column 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 photodiode array detector.

Estimation of endogenous gibberellin (GA_3) content

GA_3, one of the biologically active forms of gibberellin [11] was estimated in control and transgenic *Arabidopsis* overexpressing *SrUGT74G1* by following protocol mentioned earlier [12]. 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 Lichrosphere C_{18} column 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 photodiode array detector.

Flavan-3-ols analysis by DMACA (dimethyaminocinnamaldehyde) staining

The control as well as transgenic *Arabidopsis* overexpressing *SrUGT74G1* was histochemically analyzed for flavan-3-ols accumulation via DMACA (dimethyaminocinnamaldehyde) staining. Leaves were decolorized in ethanol:glacial acetic acid (3:1, v/v) for 24 h. The decolorized tissue was stained in 0.3 % DMACA in cold mix of methanol and 6 M HCl (1:1, v/v) for 20–30 min. The stained tissue was rinsed several times with 70 % ethanol and imaged.

Flavan-3-ols quantification by HPLC

Flavan-3-ols (catechin, epicatechin, epigallocatechin gallate) were estimated in leaf tissues of control and transgenic *Arabidopsis* following the method described earlier [13]. Briefly, leaf samples were crushed and dried. Dried sample was extracted thrice with 2.5 ml of 70 % aqueous methanol followed by 1.5 ml of 70 % aqueous methanol. This extract was centrifuged at 4,000 rpm for 10 min. Supernatant was collected and volume was made up to 5 ml by 70 % methanol. The extract was filtered and injected to Lichrosphere C_{18} reverse phase column. The mobile phase adopted for catechin was acetonitrile/0.1 % ortho-phosphoric acid in water (w/v) with a flow rate of 1 ml min^{-1}. Flavan-3-ols were detected at 280 nm using photodiode array detector.

Anthocyanins estimation

Anthocyanins were extracted from the leaves of control and transgenic *Arabidopsis* following the protocol described earlier [14]. The fresh plant tissue was homogenized in 1 ml of acidic methanol (1 % HCl, v/v). Samples were incubated for 18 h at room temperature under moderate shaking and centrifuged. The obtained supernatant (400 µl) was added to 600 µl of acidic methanol. Absorption of the reaction mix was determined using UV–Vis spectrophotometer at 530 and 657 nm. Quantification of anthocyanins was performed by following the equation: $Q_{Anthocyanins} = (A_{530} - 0.25 \times A_{657})/M$, where $Q_{Anthocyanins}$ is the amount of anthocyanins, A_{530} and A_{657} are the absorptions at the indicated wavelengths and M is the weight of the plant material used for extraction. All samples were measured as triplicates in two independent biological replicates.

Estimation of free radical scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was performed as reported earlier [15]. Briefly, initial absorbance of DPPH in methanol was measured by spectrophotometer at 517 nm until consistency in absorbance was attained. Extract (50 µl) was added to 1,950 µl of 0.1 mM methanolic DPPH solution. The mixture was incubated at room temperature for 30 min before the change in absorbance at 517 nm was observed and percent inhibition was calculated.

Microscopy

The leaf samples were analyzed via scanning electron microscopy, using Hitachi S-3400 N SEM. Fresh leaves of 15 days old control and transgenic *Arabidopsis* seedlings were gold coated using ion sputter. SEM images were captured at 1 mm with 30× magnification.

To check whether accumulation of catechin was responsible for variation in trichome branching, control seeds were germinated on GM supplemented with 10, 50 and 100 ng ml^{-1} of catechin. GM without catechin served as control. After 15 days, fresh leaves of control as well as catechin treated seedlings were analyzed via SEM.

Pollen viability assay

Pollen viability was determined by staining fresh pollen grains of control and transgenics with Alexander stain [16]. Images were captured at 20× magnification using DIC mode of Zeiss ImagerM1 Fluorescent microscope.

Root gravitropic response assay

To assess root gravitropic responses, surface sterilized seeds of control as well as transgenic *Arabidopsis* were planted on GM and vernalized at 4 °C for 48 h. The plates were then shifted to constant white light. Seedlings were later transferred to fresh GM plates maintaining the same orientation and allowed to grow vertically for further 2–3 days. The plates were then turned at 90° angle and maintained as such for another 2–3 days under the same growth conditions. These seedlings were then used for scoring the orientation of root growth.

Results

Generation of *Arabidopsis* transgenic overexpressing *SrUGT74G1* cDNA from *Stevia*

The *SrUGT74G1* nucleotide sequence retrieved from NCBI (Accession number-AY345982.1) was employed for making overexpression construct. The binary vector pCAMBIA1302 was used as backbone for construct preparation. Briefly, *SrUGT74G1* cDNA of size 1,395 bp was amplified from *Stevia* and cloned in pGEMT-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 pGEMT-easy vector. The fragment was then cloned in pCAMBIA1302 and finally transformed into *Agrobacterium* strain *LBA4404*.

The *Arabidopsis thaliana* plants were transformed through *Agrobacterium* mediated vacuum infiltration for transgenic generation. The transgenics were prepared to overexpress *SrUGT74G1* cDNA from *Stevia* under the control of a constitutively expressing 35S cauliflower mosaic virus promoter. The schematic representation of T-DNA containing *SrUGT74G1* 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 *SrUGT74G1* and selection gene encoding hygromycin using genomic DNA as template, extracted from the rosette leaves of *Arabidopsis* transgenics (Fig. 1b). Further, the accumulation of *SrUGT74G1* mRNA was confirmed by semi-quantitative PCR. Three lines of transgenic *Arabidopsis* 3T3, 1T3 and 12T3 showed higher expression of *SrUGT74G1* transcript (Fig. 1c). These three transgenic lines were utilized for further experimental analysis.

Transgenics overexpressing *SrUGT74G1* do not accumulate stevioside

In steviol glycoside biosynthesis pathway, enzyme encoded by gene *SrUGT74G1* catalyses the glycosylation of steviolbioside at C-19 position to synthesize stevioside. To investigate the probability of stevioside synthesis in transgenic *Arabidopsis*, quantitative estimation of stevioside was conducted in the rosette leaves of control as well as transgenics. Extraction was performed using 80 % methanol and estimation was done by HPLC [10]. The standard of stevioside (10 μg ml^{-1}) showed a sharp peak at retention time (RT) 8.079 min. The absorption spectrum of stevioside was estimated using photodiode array detector at a wavelength of 205 nm. The extracts obtained from rosette leaves of control as well as transgenics did not show a detectable peak at RT 8.079 (Fig. 2a). Thus, overexpression of *SrUGT74G1* cDNA from *Stevia* was not able to synthesize stevioside in transgenic *Arabidopsis*.

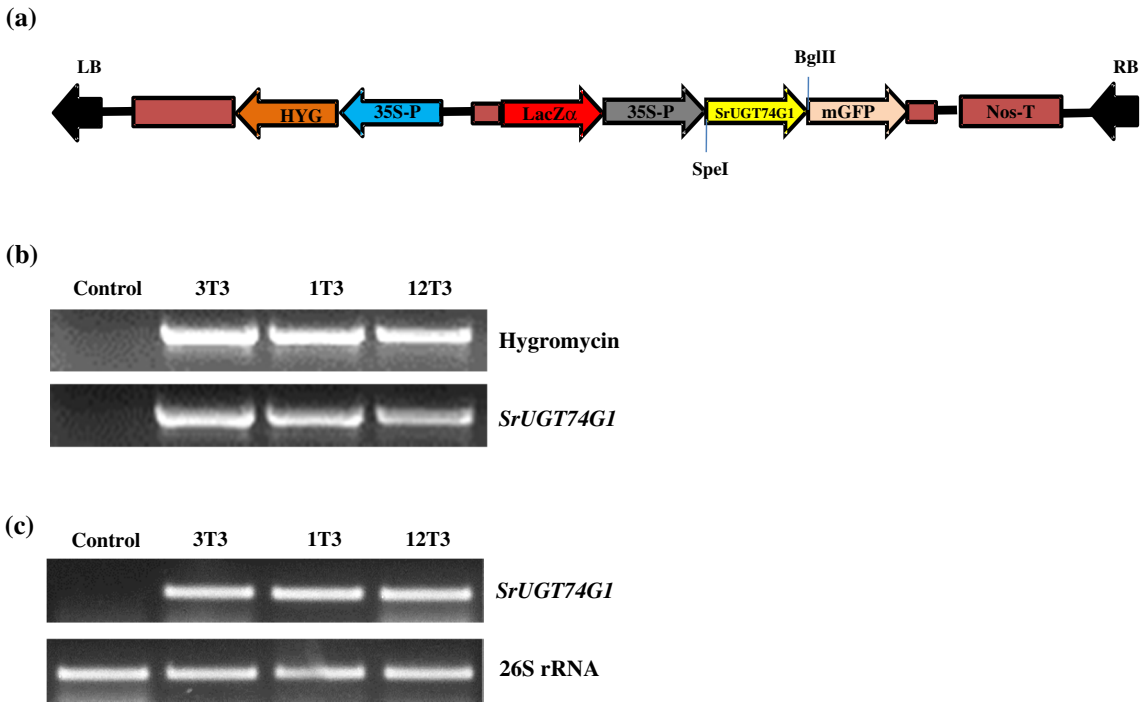

Fig. 1 Generation of transgenic *Arabidopsis* overexpressing *SrUGT74G1* and their confirmation. **a** Diagrammatic representation of T-DNA region of *SrUGT74G1* overexpression construct. The expression was regulated by the constitutively expressing promoter 35S CaMV. **b** Confirmation of integration of T-DNA region of construct in the genomic region of transgenic *Arabidopsis*. *Gel pictures* show the integration of genes encoding hygromycin resistance and *SrUGT74G1* in genomic DNA of 3T3, 1T3 and 12T3 transgenic *Arabidopsis* lines. **c** Semi-quantitative PCR analysis of *SrUGT74G1* transcript in 3T3, 1T3 and 12T3 transgenic lines. 26S rRNA was employed as internal control for cDNA quantification

Overexpression of *SrUGT74G1* does not affect gibberellin accumulation

The level of endogenous bioactive gibberellin GA_3 was also estimated in transgenic *Arabidopsis*. GA_3 was extracted from the rosette leaves of control and transgenic plants with diethyl ether and quantified via HPLC [12]. The standard of GA_3 (10 μg ml^{-1}) was detected using photodiode array detector at a wavelength of 208 nm and showed a peak at RT 2.79 min (Fig. 2b). Extracts obtained for gibberellin quantification from control and transgenic *Arabidopsis* lines also showed peak at same RT, 2.79 min (Fig. 2c). However, least variation was noticed in the GA_3 content of control and transgenic *Arabidopsis* overexpressing *SrUGT74G1*. The gibberellin content was found to be 113 ± 23 ng g^{-1} in control *Arabidopsis*. While the estimated content was 104 ± 5, 105 ± 11 and 116 ± 16 ng g^{-1} in 3T3, 1T3 and 12T3 transgenic lines, respectively (Fig. 2c). Hence, overexpression of *SrUGT74G1*, a gene belonging to *Stevia* has no influence on the accumulation of endogenous bioactive gibberellin GA_3 in *Arabidopsis* transgenics.

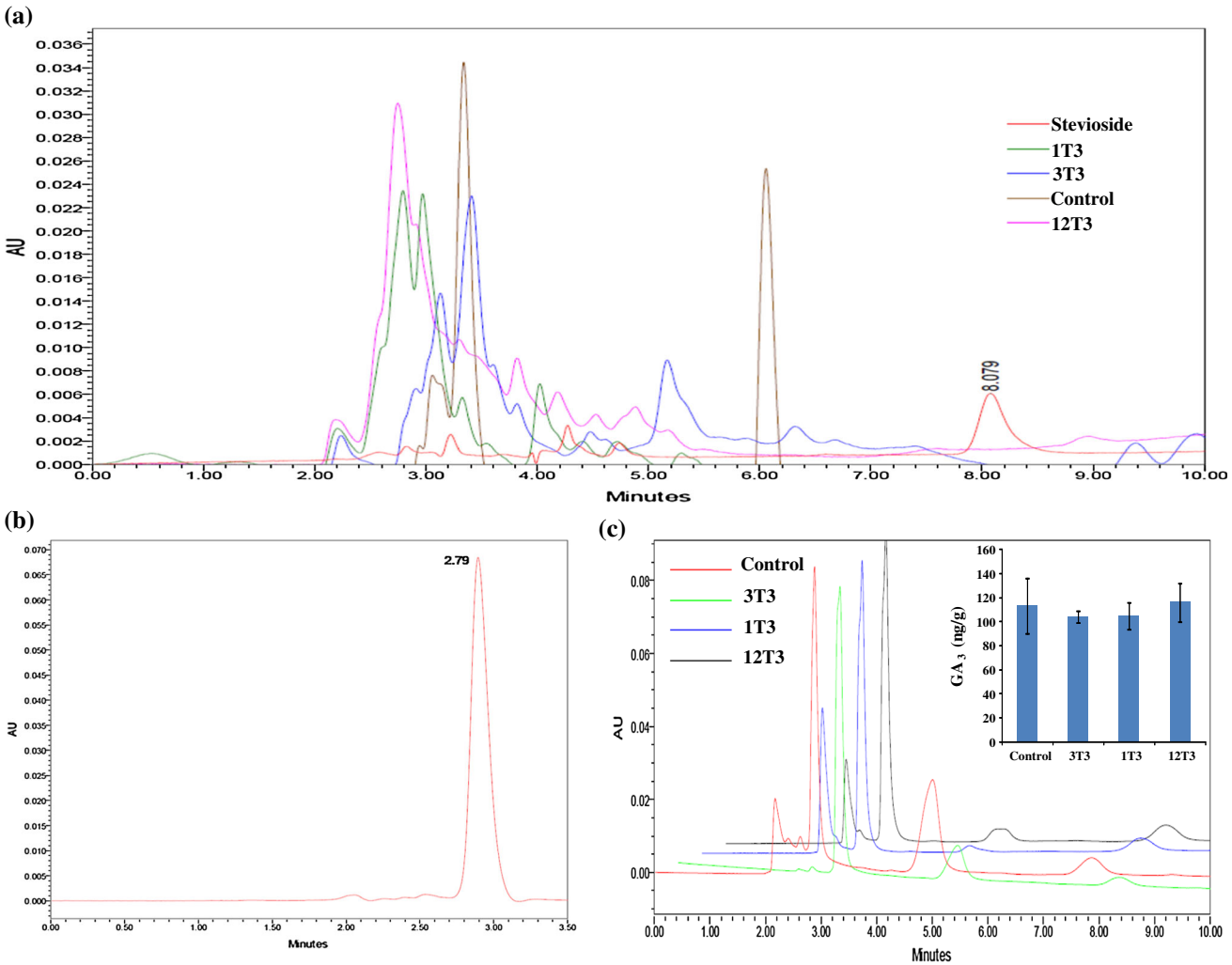

Fig. 2 Estimation of stevioside accumulation and endogenous gibberellin GA_3 content in *Arabidopsis* transgenics and control. **a** The overlapped HPLC chromatogram for control and *SrUGT74G1* transgenic lines. Absorption spectrum was observed at 205 nm and detected using photodiode array detector. HPLC chromatogram of stevioside standard, 10 μg ml^{-1} showed sharp peak at retention time (RT) 8.079 min. Control as well as transgenics overexpressing *SrUGT74G1* showed no peak at RT 8.079 min. (**b**) HPLC chromatogram of GA_3 standard, 10 μg ml^{-1} showed sharp peak at RT 2.79 min. Absorption spectra was observed at 208 nm and detected using photodiode array detector. (**c**) The normalized HPLC chromatogram showed the presence of peak at RT 2.79 min for control as well as 3T3, 1T3 and 12T3 transgenic lines. The *inset bar diagram* depicts endogenous gibberellin content in control and transgenic lines. No variation was noticed in gibberellin content of control and transgenic *Arabidopsis*. Data represented as mean ± standard deviation of three independent measurements

Arabidopsis transgenics overexpressing *SrUGT74G1* accumulate catechin, a flavan-3-ol

Glycosylation is one of the important modifications required for the synthesis of flavonoids [17]. The transgenics overexpressing *SrUGT74G1* were hence, analyzed for flavonoids estimation. DMACA (dimethyaminocinnamaldehyde) was employed to visualize the flavan-3-ols content in the rosette leaves of control and transgenic lines, 3T3, 1T3 and 12T3, respectively. DMACA reacts with flavan-3-ols and generate a blue chromophore, indicating flavan-3-ol intensity. The leaves of transgenic lines 3T3, 1T3 and 12T3 attained higher intensity of blue color in contrast to control (Fig. 3a). Further, HPLC quantification of flavan-3-ols was conducted. Flavan-3-ols comprise of mainly catechin, epicatechin and epigallocatechin gallate. The standards of catechin, epicatechin and epigallocatechin gallate (10 μg ml^{-1}) were detected as a peak at retention time (RT) 12.07, 25.7 and 31.2 min, respectively using photodiode array detector at a wavelength of 280 nm (Fig. 3b). The accumulation of catechin was noticed in the extracts of transgenics compared to control. Extracts obtained for flavan-3-ol quantification from transgenic *Arabidopsis* showed detectable peak only at same RT, 12.07 min. On the other hand, no detectable peak was observed in the extract of control at RT corresponding to catechin. There were no peaks in transgenic and control at two other RTs (Fig. 3c). The content of catechin was

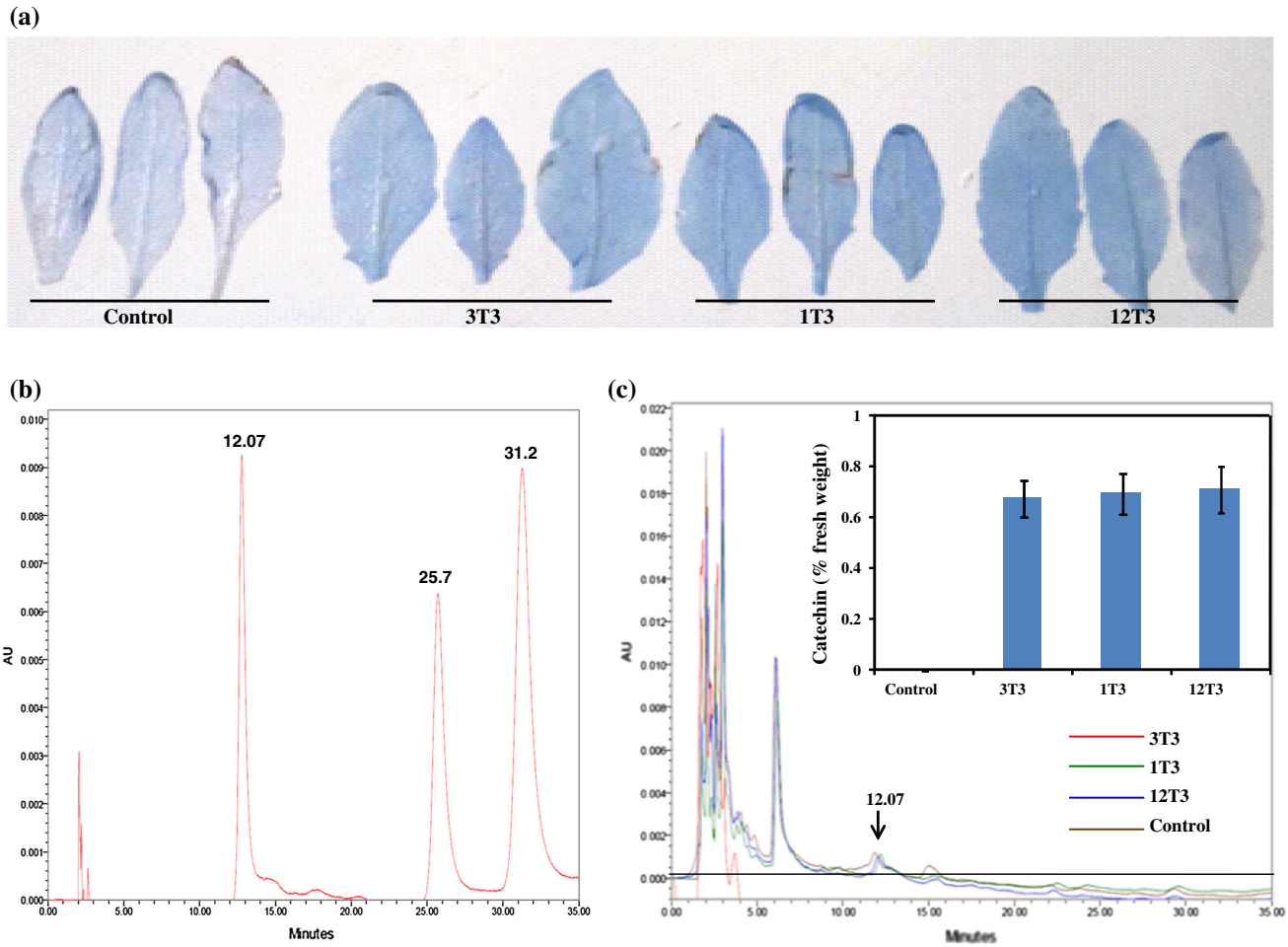

Fig. 3 Estimation of flavan-3-ols in *Arabidopsis* transgenics overexpressing *SrUGT74G1* in comparison to control. **a** DMACA (dimethyaminocinnamaldehyde) staining of leaf samples of control as well as 3T3, 1T3 and 12T3 transgenic lines. Leaf of transgenics retained higher intensity of *blue color* as compared to leaf of control plants. **b** HPLC chromatogram of catechin, epicatechin and epigallocatechin gallate standard, 10 μg ml^{-1} showed sharp peak at RT 12.07, 25.7 and 31.2 min, respectively. Absorption spectrum was observed at 280 nm and detected using photodiode array detector. **c** The overlapped HPLC chromatogram for control and *SrUGT74G1* transgenic lines. The transgenic lines 3T3, 1T3 and 12T3 showed peak at RT 12.07 corresponding to catechin. Control showed no peak at RT 12.07. However, control as well as transgenics showed no peak at RT 25.7 and 31.2 min corresponding to epicatechin and epigallocatechin gallate, respectively. The *inset bar diagram* represents catechin content in transgenic lines vis-à-vis control. Catechin was accumulated in transgenics in considerable amounts compared to control. (Color figure online)

estimated to be 0.67, 0.69 and 0.71 % fresh weight of leaves of transgenic lines 3T3, 1T3 and 12T3 respectively (Fig. 3c). However, peaks corresponding to epicatechin and epigallocatechin gallate were not distinguished in any of the samples (Fig. 3c). So, overexpression of *SrUGT74G1* gene has led to the accumulation of catechin in *Arabidopsis* transgenics.

Transgenics show reduction in anthocyanin content

Further, the influence of *SrUGT74G1* overexpression was evaluated on the content of anthocyanins. Anthocyanins were photometrically estimated in the leaves of control as well as transgenic *Arabidopsis* [15]. The anthocyanin content was significantly reduced in transgenics than control. A reduction of 53 % in anthocyanin accumulation was evident in *Arabidopsis* transgenic line 3T3 in contrast to control. Similarly, the lines 1T3 and 12T3 showed a decrease of 63 and 51 %, respectively in anthocyanin content than control (Fig. 4a). Hence, the overexpression of gene *SrUGT74G1* from *Stevia* was found to down-regulate total anthocyanin content in *Arabidopsis*.

Transgenics overexpressing *SrUGT74G1* possess better free radical scavenging activity

Flavanoids have been known to improve the antioxidant potential of plants [18]. So, the free radical scavenging potential of control as well as transgenic *Arabidopsis* was estimated using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay by preparing methanolic extract of rosette leaves. The methanolic extract of transgenic *Arabidopsis* showed an increase in DPPH free radical scavenging activity in comparison to control extracts. The free radical scavenging potential of transgenic lines 3T3, 1T3 and 12T3 was increased by 15, 40 and 64 % than control, respectively (Fig. 4b).

Transgenics show variation in trichome branching pattern

The 15 days old seedlings of control and transgenics overexpressing *SrUGT74G1* were used for SEM analysis of rosette leaves (Fig. 5a). The trichomes on the adaxial surface of rosette leaves of control plant were predominantly three branched. However, the transgenic *Arabidopsis* overexpressing *SrUGT74G1* additionally possessed trichomes with two and four branches. SEM micrographs of adaxial surface of rosette leaves of 3T3 and 1T3 transgenics showed that majority of trichomes were two, three and four branched. Similarly, the trichomes of 12T3 transgenics were observed to be three and four branched (Fig. 5a).

To check whether accumulation of catechin was responsible for the observed variation in trichome branch number in transgenics, trichome branching pattern was analyzed in control non-transgenic *Arabidopsis* by exogenous application of catechins. For this, seeds of control *Arabidopsis* were germinated on GM in the presence of 10, 50 and 100 ng ml^{-1} of catechin. GM without catechin was used as control. The leaf surface of seedlings grown in absence of catechin showed only three branched trichomes. Surprisingly, variability was noticed in the trichome branching on the leaf surface of seedlings germinated on GM supplemented with catechin. While the observed trichomes were one, two, three and four branched on

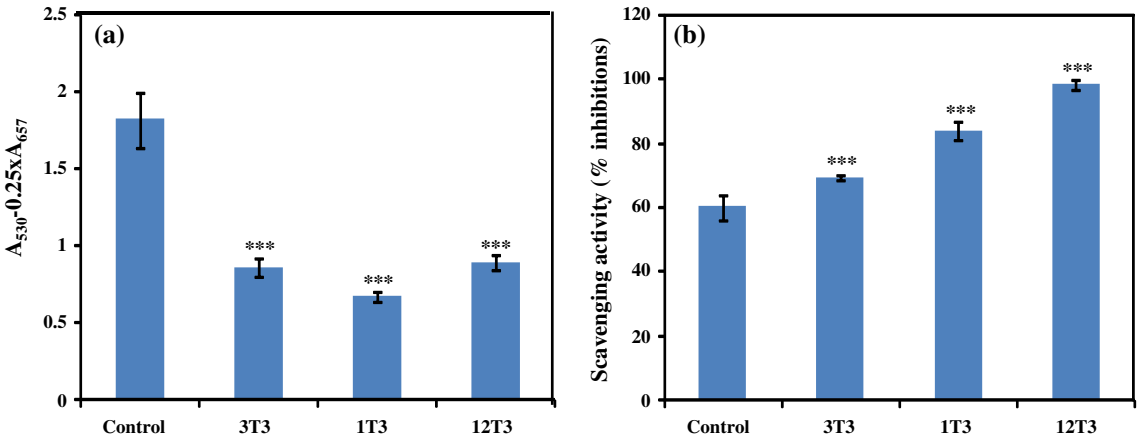

Fig. 4 **a** Influence of *SrUGT74G1* overexpression on anthocyanin accumulation. *Bar diagram* shows total anthocyanin content in control and transgenic lines 3T3, 1T3 and 12T3. Anthocyanin content was significantly reduced in 3T3, 1T3 and 12T3 transgenics compared to control. Data represented as mean ± standard deviation of three independent measurements, (***$P < 0.001$). **b** *Bar diagram* shows the scavenging potential estimated in control and transgenic overexpressing *SrUGT74G1*. The free radical scavenging activity was significantly enhanced in 3T3, 1T3 and 12T3 transgenics compared to control. Data represented as mean ± standard deviation of three independent measurements, (***$P < 0.001$)

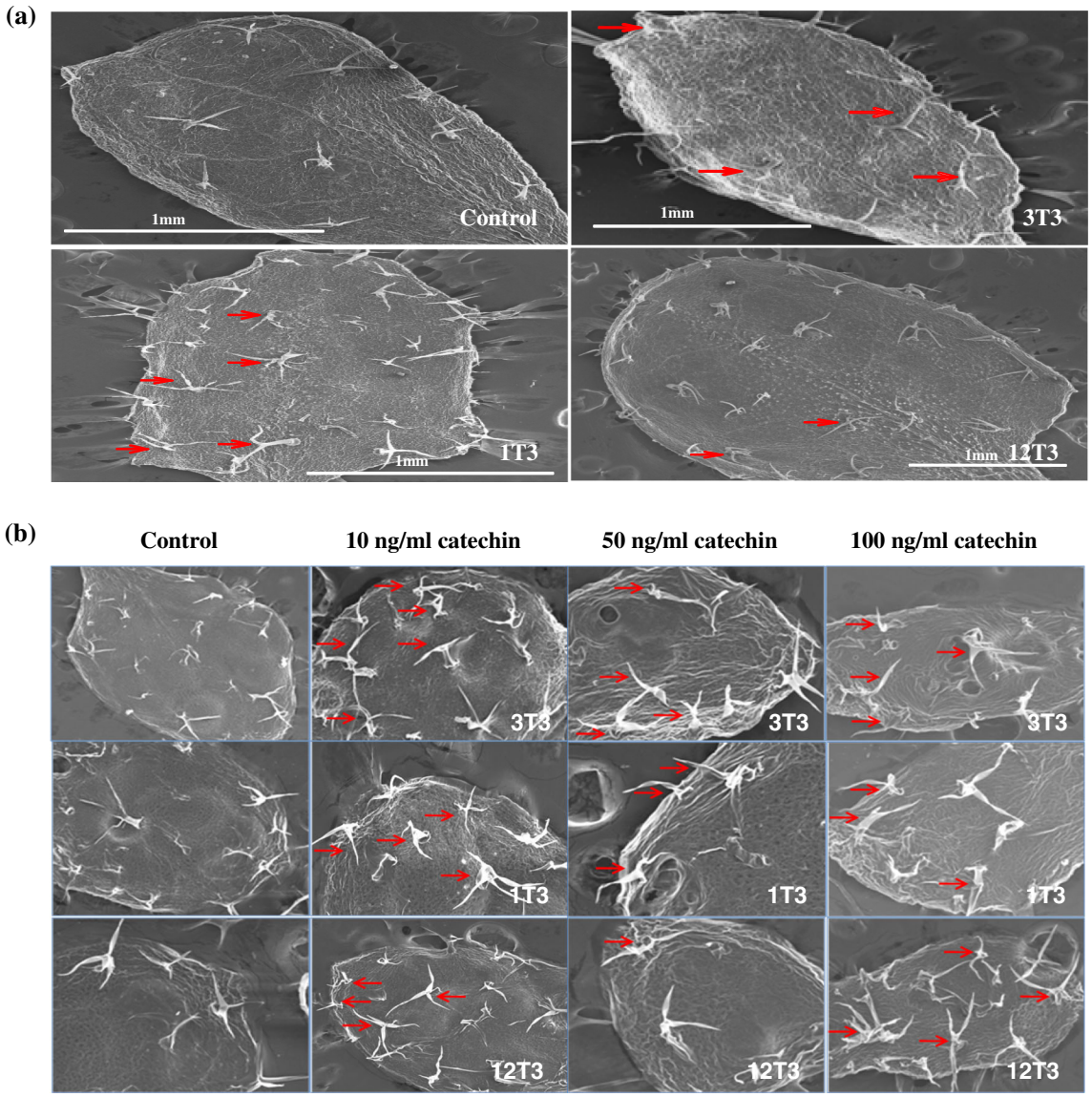

Fig. 5 Effect of *SrUGT74G1* overexpression on trichome branching of *Arabidopsis* transgenics. **a** Scanning electron microscope (SEM) images of adaxial leaf surface of control and 3T3, 1T3 and 12T3 transgenic lines showed altered branching pattern of trichomes in transgenics than control. The trichomes were three branched on the surface of control leaves. While, the trichomes of transgenic lines were observed to be two, three and four branched. **b** Effect of exogenous catechin was evaluated on the branching pattern of trichomes. Germination medium (GM) was exogenously supplemented with 10, 50 and 100 ng ml^{-1} of catechin. GM without catechin supplement served as control. The leaf trichomes were one, two, three and four branched on catechin exposure. Three replicates of each treatment type are shown

adaxial surface of rosette leaves upon catechin exposure (Fig. 5b). Thus, catechin seems to be influencing the trichome branching pattern of *Arabidopsis* transgenics upon overexpression of *SrUGT74G1*.

Phenotypic characterization of transgenic *Arabidopsis* overexpressing *SrUGT74G1*

The *Arabidopsis* transgenics overexpressing *SrUGT74G1* were observed for change in their phenotype with respect to control. Fifteen days old seedlings of control and transgenics were evaluated for morphological variations. The shoot and root length was significantly increased in transgenics compared to control (Fig. 6a). The 3T3, 1T3 and 12T3 transgenic showed a considerable elevation in shoot length by 122, 159 and 270 %, respectively than control (Fig. 6b). Likewise, the root length was also increased by 49, 86 and 97 % than control in seedlings of 3T3, 1T3 and 12T3 transgenics, respectively (Fig. 6c).

The phenotypic changes were further noticed in well grown 1 month old transgenics overexpressing *SrUGT74G1* compared to control. The rosette size was increased in

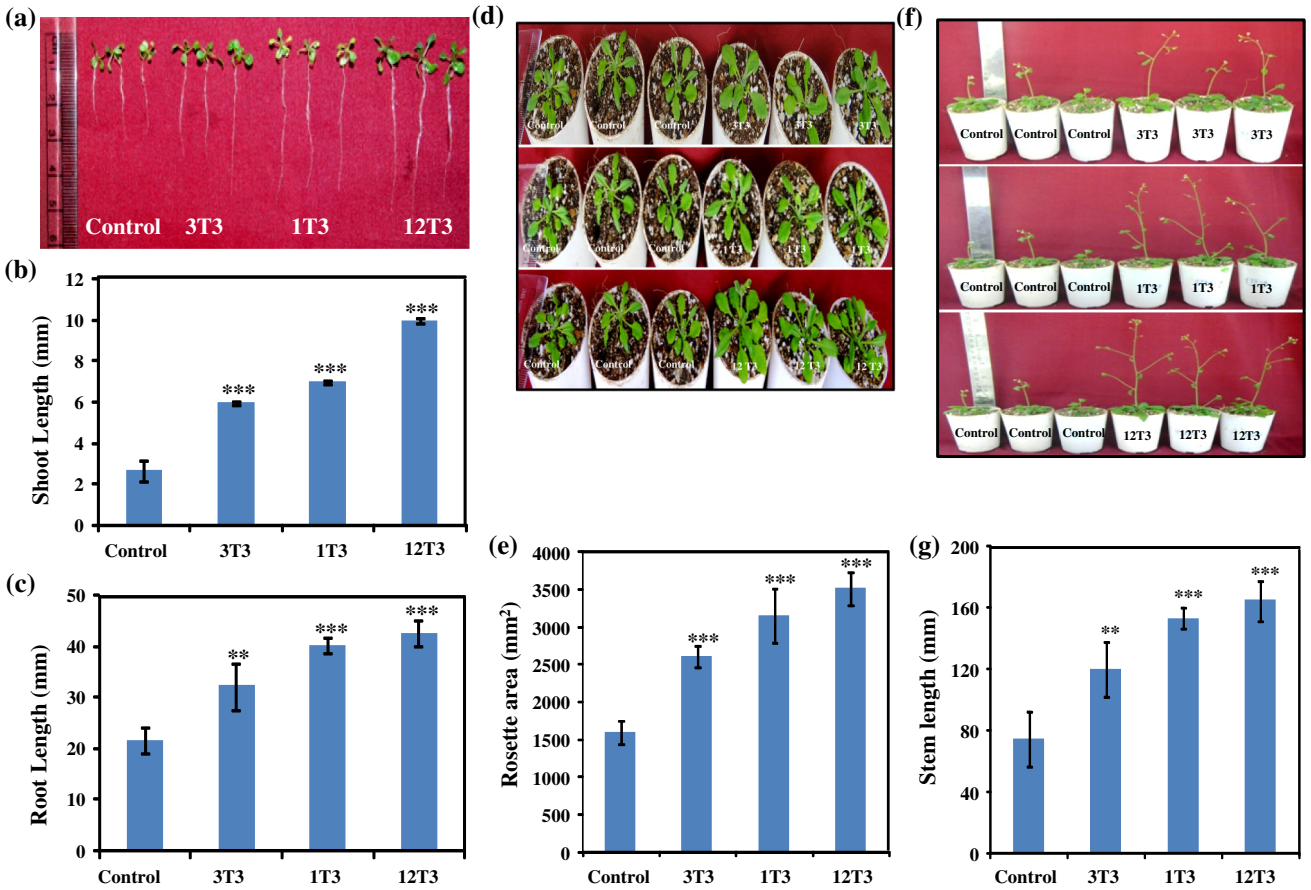

Fig. 6 Morphological characterization of *Arabidopsis* transgenics overexpressing *SrUGT74G1* compared to control plants. **a** Pictures show the variation in shoot and root height of 15 days old seedling of 3T3, 1T3 and 12T3 transgenic lines in comparison to control. **b** *Bar diagram* shows a significant increase in shoot length of 3T3, 1T3 and 12T3 transgenic lines compared to control. Data is presented as mean ± standard deviation of three independent measurements, (***$P < 0.001$). **c** *Bar diagram* represents a significant increase in root length of 3T3, 1T3 and 12T3 transgenic lines compared to control. Data is presented as mean ± standard deviation of three independent measurements, (***$P < 0.001$, **$P < 0.01$). **d** Rosette area of soil grown control and *SrUGT74G1* transgenic lines. **e** *Bar diagram* shows a significant increase in rosette area of 3T3, 1T3 and 12T3 transgenic lines compared to control. Data is presented as mean ± standard deviation of three independent measurements, (***$P < 0.001$). **f** Pictures show variation in plant height of transgenics overexpressing *SrUGT74G1* in comparison to control. **g** Stem length of control and *Arabidopsis* transgenic lines, 3T3, 1T3 and 12T3. Data is presented as mean ± standard deviation of three independent measurements, (***$P < 0.001$)

transgenics compared to control (Fig. 6d). The rosette area was found to be increased by 64, 97 and 120 % in 3T3, 1T3 and 12T3 transgenics respectively in comparison to control (Fig. 6e).Further, the stem height was also increased in transgenics overexpressing *SrUGT74G1* (Fig. 6f). The 3T3 transgenic *Arabidopsis* showed an elevation of 60 % in plant height in contrast to control. Similarly, the 1T3 and 12T3 transgenic plants were noticed to possess 105 and 239 % increase in plant height with respect to control, respectively (Fig. 6g).

Pollen viability, root gravitropic responses and seed yield

Pollen viability was assessed by Alexander staining. In Alexander staining, the grains that acquire stain and appear pink or reddish in color are considered viable, whereas the pollens that do not take stain and appear green or bluish green are non-viable [16]. It was found that pollens of control as well as the transgenic lines overexpressing *SrUGT74G1* stained reddish/pink in color (Fig. 7a). Appearance of red/pink color revealed the viability of pollens. So, accumulation of catechin in transgenic *Arabidopsis* overexpressing a *Stevia SrUGT74G1* has no affect on pollen germination. Further, the final seed yield of transgenics was also analyzed with respect to control. The total seeds per plant were significantly higher for *SrUGT74G1* overexpressing *Arabidopsis* transgenics than control. The seed yield of 3T3 plants was increased by 6 % than control. Whereas 1T3 and 12T3 plants showed an enhancement of 13 and 15 %, respectively in their seed yield with respect to the yield of control plants (Fig. 7b).

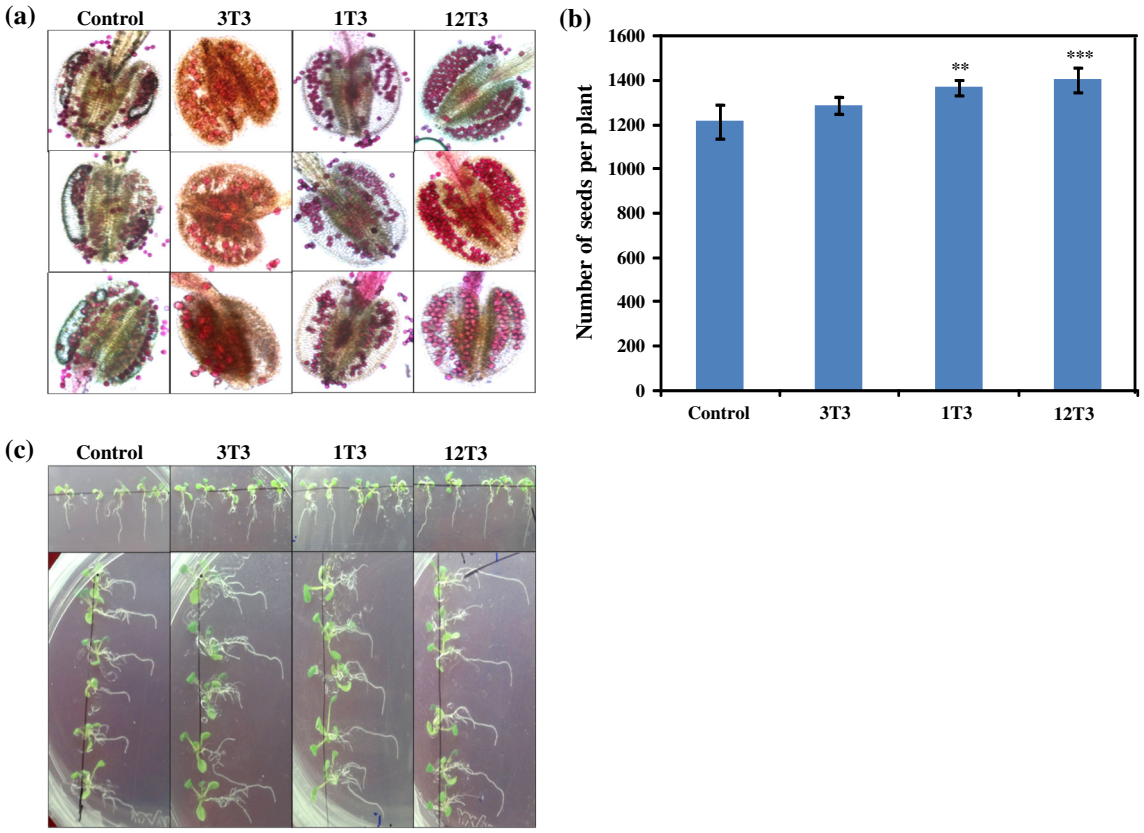

Fig. 7 Pollen viability, seed yield and root gravitropic responses of *Arabidopsis* transgenics overexpressing *SrUGT74G1* compared to control. **a** Pollen viability was evaluated using Alexander staining. *Pink stained pollens* show viability, whereas *green/bluish-green colored pollens* represent non-viability. The pollen of control as well transgenic lines showed pollen viability. Each *row* shows three replicates of control as well as transgenic lines. **b** *Bar diagram* presents the total seed yield per plant of *Arabidopsis* transgenics compared to control. Seed yield was increased in transgenics overexpressing *SrUGT74G1* than control. Data is represented as mean ± standard deviation of three independent measurements, (***$P < 0.001$, **$P < 0.01$). **c** Root gravitropic responses were evaluated by performing gravitropic assays. The roots of control as well as transgenic lines 3T3, 1T3 and 12T3 showed effective responses to gravity. (Color figure online)

Similarly, root gravitropic assay was performed to evaluate the response of transgenic roots for gravity. It was found that roots of control as well as transgenic lines 3T3, 1T3 and 12T3 respond to the degree of orientation of plate. When plates were kept horizontal, the roots were heading downward by the effect of gravity. On orientation of plate at 90°, the roots were likewise re-oriented at an angle of 90° (Fig. 7c). Thus, the assay suggested that roots of transgenic *Arabidopsis* overexpressing *SrUGT74G1* were responding to the force of gravity just like root of control plants.

Discussion

Steviol glycoside biosynthesis has been given immense attention because of its two important calorie free metabolites, stevioside and rebaudioside A. The synthesis of stevioside involves glycosylation of steviolbioside at C-19 position by the activity of enzyme encoded by gene *SrUGT74G1*. Similarly, rebaudioside A is synthesized by the C-13 glycosylation of stevioside by the enzyme encoded by gene *SrUGT76G1*. Both the enzymes are encoded by a single gene family designated as UDP-glycosyltransferases (*UGTs*) [3]. *UGTs* comprises of 69 distinct families, among which family 1 involves genes that encode enzymes catalyzing the glycosylation of small molecules. Further, *UGTs* have been considered multi-substrate acting enzymes [19]. An interesting fact regarding the gene *SrUGT74G1* is that heterologous plant species including *Arabidopsis* and tobacco have been known to possess *UGTs* that also show C-19 glycosylation activity. Moreover, it has been mentioned that these *UGTs* have the ability to glycosylate a variety of plant metabolites [7]. However, as far as activity of enzyme encoded by *SrUGT74G1* is concerned, it has only been identified to catalyze glycosylation of steviolbioside [3]. In order to identify whether overexpression of *SrUGT74G1* could

synthesize stevioside in heterologous plant species or some other plant metabolite, transgenic *Arabidopsis* overexpressing *SrUGT74G1* cDNA from *Stevia* were raised and characterized.

The transgenic *Arabidopsis* overexpressing *SrUGT74G1* were not able to synthesize stevioside. Hence, it can be inferred that the presence of all the steviol glycoside biosynthesis pathway related enzymes in one plant system is essential for the synthesis of stevioside in species other than *Stevia*. Steviol glycoside biosynthesis has been identified as divergence of gibberellin biosynthetic route [3]. The transgenics overexpressing *SrUGT74G1*, however, showed no variation in gibberellin accumulation. Hence, overexpression of *SrUGT74G1* cDNA from *Stevia* has not altered gibberellin content in transgenics.

Flavan-3-ols, an important class of secondary metabolites is known to play important role in plant growth and survival [20]. Similar to steviol glycosides biosynthesis, flavan-3-ols biosynthesis pathway also involves glycosylation as an essential process [17]. In the present study, higher accumulation of flavan-3-ols was evident in transgenic *Arabidopsis* overexpressing *SrUGT74G1* cDNA from *Stevia* than control. There was significant accumulation of catechin and decrease in anthocyanin content of transgenics in comparison to control. These findings suggested the possible role of *SrUGT74G1* in flavan-3-ols biosynthesis. Since flavan-3-ols particularly catechins were not detected in the non-transgenic *Arabidopsis*, the occurrence of catechins in *SrUGT74G1* overexpressing transgenic *Arabidopsis* suggested the importance of glycosylation plausibly in the formation or stability of catechins. Earlier, *Arabidopsis* knockout mutants of *ugt78d2* and *ugt79b1*, family 1 glycosyltransferases, have also been reported for decrease in total anthocyanin content. These studies have documented the role of proteins encoded by genes, *UGT78D2* and *UGT79B1* in stable anthocyanin accumulation [21, 22]. Hence, qualitative as well as quantitative evaluation for flavan-3-ols in transgenics overexpressing *SrUGT74G1* has suggested the important role of this gene in regulating flavonoids biosynthesis by affecting anthocyanin accumulation. The transgenics, moreover, showed higher free radical scavenging activity. This could be due to high levels of flavan-3-ols particularly catechins. Flavonoids and catechins have earlier been documented as a potential antioxidant molecules and free radical scavengers in plant systems [18, 23, 24].

Exposure of plants to low doses of catechin has induced growth in *Arabidopsis* [25]. An increase in primary root length of *Arabidopsis* on exogenous exposure of catechin has been reported. Catechin has also been demonstrated to regulate cellular expansion in *Arabidopsis* [26]. Similarly, the observed improvement in shoot–root length and rosette area of transgenics overexpressing *SrUGT74G1* could be due to increase in the catechins content.

The *Arabidopsis* transgenics overexpressing *SrUGT74G1* showed two, three and four branched trichomes. However, control *Arabidopsis* possessed only three branched trichomes. Earlier, transgenics with suppressed expression of *AtMYB103*, member of *R2R3 MYB* gene family have shown such morphology of trichomes. It has been documented that *AtMYB103* has negatively regulated the endo-reduplication process during trichome initiation and expansion. The variation in DNA content has caused alteration on cell size and hence, increased/decreased the number of trichome branches [27]. Flavonoids have also been known to regulate cell division during growth and development of plants [28]. Also, exogenous exposure of catechin has been reported to regulate cellular expansion in *Arabidopsis* [26]. Hence, accumulation of catechin in transgenic *Arabidopsis* overexpressing *SrUGT74G1* might have affected the cell cycle during trichome initiation and expansion. The variation in trichome branching was validated by treating the seeds of control *Arabidopsis* with exogenous supplement of catechins. As expected, trichomes on leaf of catechin treated *Arabidopsis* were one, two, three and four branched. Hence, accumulation of catechin in *SrUGT74G1* transgenics might have altered cellular expansion as well as branching during trichome formation.

Various reports have documented that altered expression of *UGTs* in *Arabidopsis* have caused pollen sterility and loss of root gravity response. *Arabidopsis* transgenics downregulating *UGT74B1* were reported for partial sterility [29]. While *Arabidopsis* transgenics overexpressing *PsUGT1* were documented to show altered root development. Also, the roots of these transgenics were unable to respond to gravity [28]. However, the overexpression of *SrUGT74G1* has not affected pollen viability in *Arabidopsis* transgenics. Rather the transgenics have been observed for improved seed yield than control. Also, *SrUGT74G1* transgenic *Arabidopsis* have possessed increase in root length without obviating the response of roots to gravity. The root response of these transgenic was very much similar to the control plants.

Conclusion

Overall, present study has documented for the first time a probable role of *Stevia SrUGT74G1* gene in plant growth improvement by catechin accumulation. The overexpression of *SrUGT74G1* gene alone could not synthesize any steviol glycosides. Catechin accumulation might also be responsible for better free radical scavenging potential and yield of the developed *Arabidopsis* transgenics. The altered trichome branching pattern by regulation of cell cycle during trichome initiation and expansion process could also be mediated by catechin accumulation in transgenic *Arabidopsis* overexpressing *SrUGT74G1* gene.

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