RESEARCH PAPER

Diversion of carbon flux from gibberellin to steviol biosynthesis by over-expressing SrKA13H induced dwarfism and abnormality in pollen germination and seed set behaviour of transgenic Arabidopsis

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Abstract

This paper documents the engineering of Arabidopsis thaliana for the ectopic over-expression of SrKA13H (ent-kaurenoic acid-13 hydroxylase) cDNA from Stevia rebaudiana. HPLC analysis revealed the significant accumulation of steviol (1–3 μg g⁻¹ DW) in two independent transgenic Arabidopsis lines over-expressing SrKA13H compared with the control. Independent of the steviol concentrations detected, both transgenic lines showed similar reductions in endogenous bioactive gibberellins (GA₀ and GA₄). They possessed phenotypic similarity to gibberellin-deficient mutants. The reduction in endogenous gibberellin content was found to be responsible for dwarfism in the transgenics. The exogenous application of GA₃ could rescue the transgenics from dwarfism. The hypocotyl, rosette area, and stem length were all considerably reduced in the transgenics. A noteworthy decrease in pollen viability was noticed and, similarly, a retardation of 60–80% in pollen germination rate was observed. The exogenous application of steviol (0.2, 0.5, and 1.0 μg ml⁻¹) did not influence pollen germination efficiency. This has suggested that in planta formation of steviol was not responsible for the observed changes in transgenic Arabidopsis. Further, the seed yield of the transgenics was reduced by 24–48%. Hence, this study reports for the first time that over-expression of SrKA13H cDNA in Arabidopsis has diverted the gibberellin biosynthetic route towards steviol biosynthesis. The Arabidopsis transgenics showed a significant reduction in endogenous gibberellins that might be responsible for the dwarfism, and the abnormal behaviour of pollen germination and seed set.

Key words: Arabidopsis thaliana, carbon shift, ent-kaurenoic acid-13 hydroxylase, gibberellin, Stevia rebaudiana, steviol.

Introduction

Steviol glycosides are natural sweeteners obtained from the plant Stevia rebaudiana. These are tetracyclic diterpenoids and have gained scientific interest because of their fundamental and applied perspectives (Bondarev et al., 2003a; Brandle and Telmer, 2007; Yadav and Guleria, 2012). They are sweet and found to be non-mutagenic in nature (Bondarev et al., 2003b; Yadav and Guleria, 2012). Steviol glycosides have been reported to possess several medicinal applications against diseases such as diarrhea, dental caries, myocardial injury, hypertension, and, mainly, diabetes (Geuns, 2003; Zhang and Xu, 2004–2006; Hong et al., 2006; Chatsudhipong and Muanprasat, 2009). Steviol glycosides have also been approved safe and non-toxic for human consumption by FDA (FAO Fact Sheet, 2008; Yadav and Guleria, 2012). Due to the non-toxic behaviour and medicinal nature of steviol glycosides, various countries have allowed their safe use as dietary supplements and initiated their commercialization (Kim and Kinghorn, 2002; Mizutani and Tanaka, 2002; FAO Fact Sheet, 2008). The synthetic production of various analogues of steviol glycosides is even being conducted by utilizing steviol as the starting molecule (Moons et al., 2012).
Steviol glycosides are synthesized in the leaves of *Stevia* through the steviol glycoside biosynthesis pathway (Brandle and Rosa, 1992). The pathway has 16 enzyme-catalysed steps that share similarity with the methyl erythritol-4-phosphate and the gibberelin biosynthetic route. The metabolite, ent-kaurenoic acid is a common substrate of gibberelin and steviol glycoside biosynthesis (Richman et al., 1999; Brandle and Telmer, 2007). In steviol glycoside biosynthesis, ent-kaurenoic acid is converted into steviol by an enzyme that has hydrolase activity. However, the gene encoding a later enzyme has not been identified or functionally validated (Humphrey et al., 2006). *Arabidopsis thaliana* native UGTs have previously been demonstrated to glucosylate steviol, steviolmonoside, and steviolbioside and their activity resembled the activity of *UGT74G1* (19-O glucosylation activity) of *Stevia*. Further, it was reported that expression of UGTs, specific to *Stevia*, in *Arabidopsis* could partially induce the steviol glycoside biosynthesis in *Arabidopsis* (Humphrey et al., 2006). However, over-expression of UGTs would be of use only if steviol, the precursor of steviol glycosides is synthesized in *Arabidopsis*. Conversion of ent-kaurenoic acid into steviol involves hydrolysis at 13-Carbon. A putative gene *SrKA13H* (Accession number: DQ398871.3) that encodes an enzymatic protein for C-13 hydroxylation is present in Nucleotide database of the National Centre for Biotechnology Information (NCBI), but it requires experimental validation. Taking advantage of these facts, the *SrKA13H* gene was over-expressed in the heterologous plant species, *Arabidopsis thaliana*, to see its effect on the morphology and phenotype of the plants. A US patent (US 7,927,851 B2) has recently claimed the isolation and cloning of the nucleotide sequence that codes for ent-kaurenoic acid-13 hydroxylase (Brandle and Richman, 2011). BLAST analysis has revealed that the sequence mentioned in the patent and *SrKA13H* at the NCBI shared no or very limited homology. Hence, *SrKA13H* was over-expressed in *Arabidopsis thaliana* to analyse its effect on growth and development.

**Materials and methods**

**Construct preparation for SrKA13H (ent-kaurenoic acid-13 hydroxylase) over-expression**

The gene sequence of *SrKA13H* (Accession number: DQ398871.3) was obtained from the nucleotide database of *Stevia rebaudiana* at the NCBI. The binary vector pCAMBIA1302 was used to prepare an over-expression construct. For cloning, total RNA isolated from *Stevia* was used for cDNA preparation. This cDNA was used for *SrKA13H* amplification using primers, forward-5′-AGATCTTGATTCAAGTTCTAACACCG-3′, with a BgII cloning site at the 5′ end and reverse-5′-ACTAGTTCAAACTTGATGGGGATGAAG-3′ with a SpeI cloning site at the 5′ end. These restriction sites were specific to pCAMBIA1302. The amplified cDNA was first cloned in pGEMT-easy vector and restriction-digested to produce cohesive terminal ends. The cohesed ended fragment was finally cloned into the BgII and SpeI sites of pCAMBIA1302.

**Transformation of Arabidopsis thaliana**

The prepared over-expression construct for the gene *SrKA13H*, designated as pCAMBIA-SrKA13H was transformed into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating and vacuum infiltration was used to transform *Arabidopsis thaliana* ecotype *Columbia* (Becthold et al., 1993). The transformed plants were allowed to grow further and their seeds were collected. T3 seeds were screened on Murashige–Skoog (MS) medium 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 with autoclaved distilled water. The sterilized seeds were planted on MS medium, consisting of 1× MS salt, 3% sucrose, 0.7% agar, pH 5.85 and supplemented with hygromycin. 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 analysed by semi-quantitative PCR using gene-specific primers, forward 5′-ATGATTCAAGTTCTAACACCG-3′ and reverse 5′-TCAAACTTGATGGGGATGAAG-3′. The thermal profile was 94 °C, 5 min for denaturation, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s for 28 cycles, and 72 °C, 7 min for final extension. The obtained confirmed transgenics were maintained for further analysis.

**Hypocotyl elongation measurement**

The seeds of control and transgenic *Arabidopsis* were surface-sterilized as detailed above. The sterilized seeds were planted in rows on MS medium. The plates were covered with foil and kept at 4 °C for 3 d. These wrapped plates were then maintained at 22 °C for 7 d. Seven-day-old seedlings were used for the hypocotyl measurements.

**Microscopy**

The hypocotyl samples were analysed via scanning electron microscopy, using an Hitachi S-3400N SEM. Fresh hypocotyls of control and transgenics were gold-coated using ion sputter. SEM images were captured at 500 μm with 65× magnification. Pollen viability was determined by staining fresh pollen grains of control and transgenics with Alexander stain (Alexander, 1969). Images were captured at 20× magnification using the DIC mode of a Zeiss Imager M1 fluorescence microscope. Slices of control and transgenic *Arabidopsis* were mounted in a solution of glycerol/water (1:2 v/v) and viewed at 5× magnification in the DIC mode of a Zeiss Imager M1 fluorescence microscope.

**Quantitative estimation of steviol**

The accumulation of steviol was estimated in the rosette leaves of control as well as transgenic *Arabidopsis* by the method described earlier by Minne et al. (2004). Dry sample was crushed and extracted with 1 ml of methanol containing 2% (v/v) KOH for 24 h at 10 °C with continuous shaking in the dark. The extract was centrifuged and the supernatant was collected. Residue was re-extracted with 2% KOH and the supernatants were pooled together. To the collected supernatant fraction, 5 ml of distilled water was added and the mixture was extracted three times with diethyl ether for lipid removal. Each time the ether phase was removed. The pH of the aqueous phase was adjusted to 6.0 with 6 N acetic acid. The obtained phase was then extracted three times with peroxide-free diethyl ether. This time, the ether phases were pooled and dried in vacuo. The residue was dissolved in acetonitrile and filter-sterilized for HPLC analysis. The sample (10 μl) was injected into a Lichrosphere NH₂ column using the isocratic solvent system of acetonitrile:water (80:2 v/v) and maintained a flow rate 0.8 ml min⁻¹. Steviol was detected at 210 nm using a photodiode array detector.

**Estimation of endogenous gibberellin content**

The seeds of control and transgenic *Arabidopsis* were germinated on MS medium. Fresh rosette leaves of 15-d-old control and transgenic *Arabidopsis* were crushed in liquid nitrogen and immediately freeze-dried. Extraction was performed as described.
Carbon flux from gibberellin to steviol biosynthesis by SrKA13H over-expression

Results

Generation of transgenic Arabidopsis thaliana over-expressing SrKA13H

The over-expression construct corresponding to gene SrKA13H was prepared using vector pCAMBIA1302. The SrKA13H gene fragment was cloned in pCAMBIA1302 and confirmed by conducting PCR using gene-specific primers (see Supplementary Fig. S1 at JXB online). The recombinant vector was finally transformed into Agrobacterium strain LBA4404 and confirmed with PCR (see Supplementary Fig. S2 at JXB online). The Arabidopsis thaliana plants were transformed through Agrobacterium-mediated vacuum infiltration for transgenic generation. Transgens were prepared to over-express Stevia SrKA13H cDNA under the control of the constitutively expressing 35S cauliflower mosaic virus promoter. The schematic representation of T-DNA containing SrKA13H cDNA is shown in Supplementary Fig. S3A at JXB online.

The seeds of the T₀ generation were screened on MS medium supplemented with hygromycin as the selection. Plants resistant to hygromycin were maintained until the T₃ generation for further analysis. The integration of the over-expression construct T-DNA was confirmed by PCR. Genomic DNA used as the template in the PCR was extracted from rosette leaves of Arabidopsis transgenics and analysed for the presence of transgene SrKA13H and the selection gene encoding hygromycin resistance (see Supplementary Fig. S3B at JXB online). In addition, the expression of the transgene in Arabidopsis-raised transgenics and the accumulation of SrKA13H mRNA was confirmed by semi-quantitative PCR. Two lines of transgenic Arabidopsis 4KT3 and 6KT3 were observed for higher expression of SrKA13H transcript (see Supplementary Fig. S3C at JXB online). These two transgenic lines were used for further experimentation.

Phenotypic characterization of Arabidopsis transgenics over-expressing SrKA13H

The Arabidopsis transgenics over-expressing SrKA13H were observed for a change in their phenotype with respect to the control. SrKA13H over-expressing Arabidopsis transgenic line 4KT3 showed a decrease of 36% in hypocotyl length compared with the hypocotyl of control plants (Fig. 1A, B). Similarly, a decrease of 19% was noticed in the length of the hypocotyl of 6KT3 plants with respect to control plants (Fig. 1A, B). SEM analysis was conducted to determine the hypocotyl cell size. SEM images presenting the mid portion of hypocotyls of control and transgenic lines 4KT3 and 6KT3 are shown in Fig. 1C. The hypocotyls of both transgenic lines showed a significant decrease in cell size compared with the control plants. The length of hypocotyl cells of the 4KT3 transgenic line was reduced by 77% compared with that of the hypocotyl cells of control plants while, at the same time, hypocotyls of 6KT3 showed a decrease of 37% in their cell size with respect to the cell size of the control (Fig. 1D). SEM analysis revealed 4.4 ± 0.54 cells in hypocotyls of

Data analysis

The data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) was calculated for probability. P < 0.05, P <0.01 and P<0.001.

Exogenous application of GA₃

The procedure followed for the dwarf phenotype rescue experiment was similar to that described earlier by Magome et al. (2004, 2013). Briefly, seeds of the control and transgenic Arabidopsis were germinated on MS medium. Fifteen-day-old seedlings were transferred to pots and placed in a greenhouse at 22 °C. The seedlings were allowed to acclimatize for 5 d. The 20-d-old control and transgenics over-expressing SrKA13H were sprayed once with 100 µM of GA₃. The stem length of the plants was recorded after every 24 h for one week.

Pollen germination and viability assay

For the in vitro germination assay, pollen grains from the freshly opened flowers of control and Arabidopsis transgenics over-expressing SrKA13H were dehisced on a slide containing a thin layer of germination medium (GM). The GM contained 1 mM MgSO₄, 2.5 mM CaCl₂, 1 mM H₃BO₃, 1 mM KNO₃, 18% sucrose, and 0.3% agar. The samples were incubated at 28 °C at high humidity (Renata et al., 2011). The pollen germination rate was evaluated using the DIC mode of a Zeiss ImagerM1 fluorescent microscope. Pollen grains of the control and SrKA13H transgenics was stained with Alexander stain and visualized under a fluorescent microscope. The grains that acquired Alexander stain and appeared pink or reddish in colour were considered viable, whereas the pollen grains that did not take the stain and appeared green or bluish green in colour were considered to be non-viable (Alexander, 1969).

To check whether the accumulation of steviol in the transgenics was responsible for the decreased rate of pollen germination, pollen grains of control plants were germinated on GM supplemented with 0.2, 0.5, and 1.0 µg ml⁻¹ of steviol. GM without steviol was used as the control. Samples were incubated at 28 °C at high humidity and visualised as mentioned above.

earlier (Urbanova et al., 2013). Freeze-dried plant sample (30 mg) was extracted for 5 min with 1 ml of ice-cold 80% acetonitrile containing 5% formic acid. 50 pmol of [³H]-GA₁, [³H]-GA₂, [³H]-GA₃, [³H]-GA₄, [³H]-GA₅, [³H]-GA₆, [³H]-GA₇, [³H]-GA₈, [³H]-GA₉, [³H]-GA₁₀, [³H]-GA₁₂, [³H]-GA₁₃, [³H]-GA₁₅, [³H]-GA₁₆, [³H]-GA₂₀, [³H]-GA₂₆, [³H]-GA₂₉, [³H]-GA₃₁, [³H]-GA₃₅, [³H]-GA₅₃ were added to the samples as internal standard. The samples were extracted at 4 °C for 12 h and the homogenate was then centrifuged at 14 000 rpm for 10 min. The resulting pellet was re-extracted for 1 h with rotation at 4 °C. The extracts were combined and evaporated to the water phase. The joint Oasis® MCX and Oasis® HLB cartridges were activated with methanol and pre-equilibrated with 5% aqueous methanol (v/v). The evaporated samples were dissolved in 5% aqueous methanol and loaded on to joint cartridges. The joint cartridges were run to dryness, disconnected, and eluted from Oasis® HLB using methanol:diethyl ether (20:80 v/v). The eluates were evaporated under a stream of nitrogen and then dissolved in 50 µl of methanol. The final volume of dissolved sample was made up to 3 ml with 25 mM NH₄HCO₃ and loaded onto Oasis® MAX polymer-based mixed-mode columns. The columns were washed with 3 ml acetonitrile and GAs were eluted with 0.2 M formic acid in acetonitrile. The eluted GAs were evaporated to dryness and reconstituted with 50 µl of 10 mM formic acid:methanol (90:10 v/v). The prepared samples were injected into Waters UPLC™ system coupled with Xevo® TQ MS triple-stage quadrupole mass spectrometer equipped with electrospray (ESI) interface and collision cell-ScanWave™. Three independent biological samples were evaluated for GA analysis.

Carbon flux from gibberellin to steviol biosynthesis by SrKA13H over-expression

The Arabidopsis transgenics over-expressing SrKA13H were observed for a change in their phenotype with respect to the control. SrKA13H over-expressing Arabidopsis transgenic line 4KT3 showed a decrease of 36% in hypocotyl length compared with the hypocotyl of control plants (Fig. 1A, B). Similarly, a decrease of 19% was noticed in the length of the hypocotyl of 6KT3 plants with respect to control plants (Fig. 1A, B). SEM analysis was conducted to determine the hypocotyl cell size. SEM images presenting the mid portion of hypocotyls of control and transgenic lines 4KT3 and 6KT3 are shown in Fig. 1C. The hypocotyls of both transgenic lines showed a significant decrease in cell size compared with the control plants. The length of hypocotyl cells of the 4KT3 transgenic line was reduced by 77% compared with that of the hypocotyl cells of control plants while, at the same time, hypocotyls of 6KT3 showed a decrease of 37% in their cell size with respect to the cell size of the control (Fig. 1D). SEM analysis revealed 4.4 ± 0.54 cells in hypocotyls of
control plants per 500 μm. Since the cell size of transgenic hypocotyls was decreased, the number of cells per 500 μm was higher compared with the control hypocotyls at the same scale. Hence, the number of cells in hypocotyls was 7.4 ± 0.89 and 6.6 ± 0.54 in 4KT3 and 6KT3, respectively. There was no change in the total number of cells in the hypocotyls of transgenics with respect to the control.

The stem length and rosette size was reduced in transgenics compared with the control (Fig. 2A, B). The 4KT3 transgenic Arabidopsis showed a reduction of 30.5% in plant height in contrast to the control. Similarly, the 6KT3 transgenic plants had a 14.5% decrease in plant height with respect to the control plants (Fig. 2C). At flowering stage, the transgenics had fewer rosette leaves (Fig. 2D). Rosette area was also decreased in 4KT3 and 6KT3 plants by 55.3% and 44.9% compared with control plants, respectively (Fig. 2E). In addition, flowering was delayed by 12-15 d in the transgenics.

Arabidopsis transgenics accumulated steviol

In the steviol glycoside biosynthesis pathway, hydroxylation at the 13-C position of ent-kaurenoic acid leads to the synthesis of steviol. Since ent-kaurenoic acid is also one of the substrates for gibberelin biosynthesis, the expression of SrKA13H cDNA from Stevia in Arabidopsis might have catalysed the hydroxylation of ent-kaurenoic acid for the synthesis of steviol. To investigate this possibility, the quantitative estimation of steviol was conducted in the rosette leaves of control as well as transgenic plants. The standard of steviol (10 μg ml⁻¹) showed a sharp peak at a retention time (RT) of 3.26 min (see Supplementary Fig. S4A at JXB online). The extracts obtained from the rosette leaves of transgenics for steviol determination also showed peak at 3.26 RT, whereas no peak was detected for control plants (see Supplementary Fig. S4B at JXB online). The presence of the characteristic steviol peak in the extracts of transgenics has confirmed the accumulation of steviol. The endogenous content of steviol was found to be 3.21 ± 0.13 μg g⁻¹ and 1.06 ± 0.08 μg g⁻¹ in 4KT3 and 6KT3 plants, respectively (Table 1). Thus, over-expression of SrKA13H cDNA from Stevia in Arabidopsis has led to the synthesis of steviol, the precursor of steviol glycosides.

Alteration in endogenous gibberellin content of transgenics over-expressing SrKA13H

The enzyme encoded by transgene SrKA13H has acted on ent-kaurenoic acid, one of the metabolites of gibberelin biosynthesis for the synthesis of steviol. The endogenous levels of precursor and bioactive gibberellins were also estimated in
Carbon flux from gibberellin to steviol biosynthesis by SrKA13H over-expression

4KT3 and 6KT3 transgenics showed a reduction in the content of each member of the 13-hydroxy GA pathway from GA$_{53}$ to GA$_{1}$ (Table 2). The GA$_{53}$ content was decreased by 63% and 45% in the 4KT3 and 6KT3 transgenic lines, respectively. The 4KT3 line showed a 25% reduction in GA$_{19}$ and a 31% reduction in bioactive gibberellin.

Table 1. Steviol content in control and transgenic Arabidopsis over-expressing SrKA13H

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Steviol (μg g$^{-1}$ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Nil</td>
</tr>
<tr>
<td>4KT3</td>
<td>3.21 ± 0.13$^{c}$</td>
</tr>
<tr>
<td>6KT3</td>
<td>1.06 ± 0.08$^{a}$</td>
</tr>
</tbody>
</table>

Table 2. Quantification of endogenous gibberellins (pg mg$^{-1}$ dry weight) in control and transgenic Arabidopsis over-expressing SrKA13H

<table>
<thead>
<tr>
<th>Gibberellins/ genotype</th>
<th>Control</th>
<th>4KT3</th>
<th>6KT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-Hydroxylated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA$_{12}$</td>
<td>23.59 ± 0.24</td>
<td>21.27 ± 0.57$^{c}$</td>
<td>16.81 ± 0.69$^{c}$</td>
</tr>
<tr>
<td>GA$_{53}$</td>
<td>1.60 ± 0.28</td>
<td>0.60 ± 0.05$^{a}$</td>
<td>0.88 ± 0.07$^{b}$</td>
</tr>
<tr>
<td>GA$_{44}$</td>
<td>3.87 ± 0.06</td>
<td>3.91 ± 0.34</td>
<td>3.73 ± 0.93</td>
</tr>
<tr>
<td>GA$_{19}$</td>
<td>2.57 ± 0.26</td>
<td>1.94 ± 0.49</td>
<td>0.87 ± 0.05$^{c}$</td>
</tr>
<tr>
<td>GA$_{20}$</td>
<td>3.53 ± 0.4</td>
<td>5.10 ± 0.29</td>
<td>2.30 ± 0.23$^{c}$</td>
</tr>
<tr>
<td>GA$_{1}$</td>
<td>0.91 ± 0.42</td>
<td>0.632 ± 0.34</td>
<td>0.49 ± 0.16</td>
</tr>
<tr>
<td>GA$_{8}$</td>
<td>10.74 ± 0.52</td>
<td>10.32 ± 0.46</td>
<td>2.47 ± 0.21$^{c}$</td>
</tr>
</tbody>
</table>

| Non-13-hydroxylated    |         |      |      |
| GA$_{15}$              | 0.27 ± 0.02 | 0.35 ± 0.52 | 0.14 ± 0.06 |
| GA$_{14}$              | 23.31 ± 1.91 | 15.44 ± 1.07$^{b}$ | 15.98 ± 0.47$^{b}$ |
| GA$_{10}$              | 9.00 ± 0.11 | 2.97 ± 0.24$^{a}$ | 2.47 ± 0.21$^{a}$ |
| GA$_{4}$               | 6.62 ± 1.13 | 2.72 ± 1.95$^{b}$ | 3.0 ± 1.0$^{b}$ |
| GA$_{16}$              | 0.54 ± 0.13 | 0.35 ± 0.12 | 0.3 ± 0.10$^{a}$ |
| Other forms            |         |      |      |
| GA$_{1}$               | 0.05 ± 0.02 | 0.02 ± 0.01$^{a}$ | 0.03 ± 0.01$^{a}$ |
GA$_4$ with respect to the control. On the other hand, the 6KT3 transgenic line showed a 66% and a 46% reduction in GA$_19$ and GA$_4$ content, respectively. The content of deactivated gibberellin GA$_3$ was reduced by 4% in the 4KT3 transgenic line and by 77% in the 6KT3 transgenic line.

Similarly, SrKA13H transgenic lines also showed a reduction in the gibberellins of the non-13-hydroxylated pathway. The content of GA$_{24}$ was significantly reduced in both the transgenic lines. The content of the bioactive gibberellin GA$_4$ and its degradation product GA$_{34}$ was also significantly decreased in the transgens (Table 2). The GA$_4$ content was decreased by 59% and 55% in the 4KT3 and 6KT3 transgenics compared with the control. Also, compared with the control, 4KT3 and 6KT3 transgenic lines showed a reduction of 35% and 44% in their deactivated gibberellin GA$_{34}$. The levels of the bioactive form GA$_4$ was also decreased in transgenics compared with the control. A reduction of 60% and 40% in GA$_4$ was observed in the 4KT3 and 6KT3 lines, respectively.

Exogenous gibberellin application rescued the growth of transgenics over-expressing SrKA13H

In order to validate that the reduced endogenous gibberelin content was responsible for dwarfism in transgenics over-expressing SrKA13H, they were sprayed with 100 μM of GA$_3$ (Magome et al., 2004, 2013). The effect was noted after every 24h of GA$_3$ application until 120h. At 0h, plants of both the transgenic lines were observed to be shorter in length than the control plants (Fig. 3). The stem length of 4KT3 and 6KT3 plants was 50.9% and 27.5% shorter than the controls (Fig. 3). After GA$_3$ exposure, the plants of both 4KT3 and 6KT3 lines were noted to recover their stem length (Fig. 3). After 24h of GA$_3$ spray, there was only a 9% and a 3% reduction in the stem length of the 4KT3 and 6KT3 transgenics, respectively. The transgenic plants were observed to regain their height almost to that of control plants after 48h of GA$_3$ exposure (Fig. 3). Interestingly, the stem length of transgenics was comparatively higher than control plants after 72h of GA$_3$ exposure till 120h (Fig. 3). Compared with the control, an increase of 9% and 24.5% was observed in the stem length of 4KT3 and 6KT3, respectively, after 72h of GA$_3$ exposure. After 96h and 120h of GA$_3$ exposure, a 22.6% and a 29.3% increase in stem length of 4KT3 transgenic Arabidopsis was noted. Similarly, the stem length of 6KT3 transgenics was also increased by 25% and 34% after 96h and 120h of GA$_3$ spray (Fig. 3). Thus, exogenous gibberellin application was able to rescue the stem height of Arabidopsis transgenics over-expressing SrKA13H.

Pollen viability and pollen germination of transgenics over-expressing SrKA13H

The viability and germination potential of pollen grains of transgenic plants was investigated to evaluate the influence of SrKA13H over-expression on the reproductive growth of these plants. The transgenics were noted to have failure in anther dehiscence. In addition, Alexander staining was performed to check the viability of the pollen grains. Compared with the viable pollen of control plants that appeared pink in colour, most of the pollen grains in the anthers of 4KT3 plants were bluish-green. The pollen of 6KT3 plants was also stained bluish-green but the numbers of grains were lower compared with 4KT3. Thus, pollen of both the transgenic lines 4KT3 and 6KT3 showed non-viable characteristics compared with control plants (Fig. 4A).

In addition, an in vitro germination assay was conducted to evaluate the germination rate of pollen. The pollen grains of control and transgenic Arabidopsis were dehisced upon incubation on GM. By contrast to the pollen germination rate of 85.5% for control plants, the rate of pollen germination was only 20% and 40% for the 4KT3 and 6KT3 lines, respectively (Fig. 4B, C). Hence, Arabidopsis transgenics over-expressing SrKA13H had significantly lower pollen viability and germination.

Pollen germination in the presence of exogenous steviol

To check whether steviol has hindered the germination of pollen, an in vitro pollen germination assay was conducted.

Fig. 3. Growth rescue of 20-d-old Arabidopsis transgenics over-expressing SrKA13H by exogenous GA$_3$ application. The bar diagram shows the significant increase in stem height of 4KT3 and 6KT3 transgenics after 24, 48, 72, 96, and 120h of GA$_3$ spray. The transgenics were able to attain a height comparable with control plants after GA$_3$ exposure. Data are presented as mean ± standard deviation of three independent measurements. Different letters on the error bar represents a significant difference. Data having ‘b’ above the error bar is significantly different with respect to data having ‘a’ at P <0.05 and data having ‘c’ above the error bar is significantly different with respect to data having ‘a’ at P <0.01.
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for pollen of the control Arabidopsis plants on GM containing 0.2, 0.5, and 1.0 μg ml⁻¹ of steviol. In this experiment, GM without steviol served as the control. Surprisingly, there was no variability in the pollen germination rate. Pollen were observed to have an equal rate of germination on control as well as steviol-supplemented GM (Fig. 4D). Data suggested that steviol has no effect on the germination rate of pollen of Arabidopsis transgenics over-expressing SrKA13H. Further, SrKA13H transgenics exposed to GA₃ application were noted to recover their normal growth as well as their normal pollen germination behaviour.

Seed yield of transgenics over-expressing SrKA13H

Loss of pollen viability and pollen germination prompted an analysis of seed set and seed yield of SrKA13H transgenics. In this respect, microscopic imaging of siliques was conducted. The siliques of 4KT3 transgenics were found to possess significantly higher amounts of aborted seeds than the control plants. 6KT3 plants were also observed to have aborted seeds but relatively fewer than 4KT3 plants (Fig. 5A). In addition, the final seed yield of these transgenics was also analysed. The total seeds per plant were significantly lower for SrKA13H over-expressing Arabidopsis transgenics than control plants. The seed yield of 4KT3 plants was decreased by 48% compared with that of the control, whereas 6KT3 plants only showed a reduction of 24% in their seed yield with respect to the yield of the control plants (Fig. 5B).

Discussion

Steviol glycoside and gibberellin biosynthesis share a common methylyerythritol phosphate (MEP) pathway in Stevia rebaudiana (Totte et al., 2000). These routes diverge at the point where steviol is synthesized as the branching intermediate (Humphrey et al., 2006). Following steviol, a series of glycosylation reactions take place in vivo that give rise to different types of steviol glycosides. Various in vitro chemical catalysis reactions have used steviol as a precursor to synthesize novel and well-known types of steviol glycosides. The expression
of UGTs of Stevia has been proposed partially to compose the steviol glycoside biosynthesis pathway in Arabidopsis. However, due to a lack of characterization of the gene encoding for a protein catalysing hydroxylation at the 13-C position of ent-kaurenoic acid, the possibility of the synthesis of steviol in Arabidopsis was not evaluated (Humphrey et al., 2006). Hence, in the present study, Arabidopsis thaliana transgenics over-expressing SrKA13H from Stevia rebaudiana were raised and confirmed. The SrKA13H transgenics were evaluated for morphological, phenotypic, and related biochemical variations. The Arabidopsis transgenics were found to accumulate significant amounts of steviol. However, control Arabidopsis did not show steviol accumulation. Hence, the enzyme encoded by nucleotide sequence designated as SrKA13H was inferred to be responsible for the synthesis of steviol in Arabidopsis. There was no report demonstrating the synthesis of steviol in planta other than naturally synthesizing plants such as Rubus and Stevia. Hence, the present study has documented the application of genetic engineering for the synthesis of steviol in a plant system other than Rubus or Stevia rebaudiana.

Steviol glycoside and gibberellin biosynthetic routes are known to share ent-kaurenoic acid as the last metabolite in common (Kim et al., 1996; Brandle and Telmer, 2007). Hence, over-expression of SrKA13H cDNA from Stevia in Arabidopsis has catalysed the conversion of ent-kaurenoic acid to synthesize steviol. Two cytochrome P450 genes, CYP714A1 and CYP714A2, have recently been identified from Arabidopsis (Zhang et al., 2011; Nomura et al., 2013). The recombinant protein CYP714A2 was found to catalyse the conversion of ent-kaurenoic acid into steviol. In addition, Arabidopsis transgenics over-expressing CYP714A2 have been reported to have a semi-dwarf phenotype due to a reduction in their GA4 content. However, the 13-hydroxylation pathway of gibberellin biosynthesis was positively regulated and there was elevated GA1 content in these transgenics (Nomura et al., 2013). In present study, the cytochrome P450 gene SrKA13H of Stevia has been found to reduce both 13-hydroxylated as well as non-13-hydroxylated forms of gibberellins. Hence, the over-expression of SrKA13H was found to convert ent-kaurenoic acid only into steviol and not into other hydroxylated forms of gibberellins. Transgenic tomato

Fig. 5. Seed set and yield parameters of Arabidopsis transgenics over-expressing SrKA13H compared with the control. (A) Abnormal seed set was induced in 4KT3 and 6KT3 transgenics compared with the control. Seed abortion was more apparent in 4KT3 transgenics. The figure shows three replicates of control and transgenic lines. (B) Bar diagram representing the total seed yield per plant of Arabidopsis transgenics compared with the control. Seed yield was reduced in transgenics over-expressing SrKA13H compared with the control. Data are represented as the mean ± standard deviation of three independent measurements. Different letters on the error bar represents a significant difference. Data having ‘b’ above the error bar is significantly different with respect to data having ‘a’ at P < 0.05 and data having ‘c’ above the error bar is significantly different with respect to data having ‘a’ at P < 0.01.
constitutively expressing phytoene synthase cDNA has been reported to divert the gibberellin biosynthetic route towards carotenoid production. They were also reported to have a significant reduction in the accumulation of the major bioactive form of gibberellin, GA4, (Fray et al., 1995). Similarly, in the present study, the diversion from the gibberellin biosynthetic route towards steviol was found to reduce the accumulation of the bioactive gibberellins GA4, GA5, and GA7. These gibberellins have been identified as the important bioactive forms in plants. GA4 is the major bioactive gibberellin form in Arabidopsis (Yamaguchi, 2008). The dwarf and delayed-flowering 1 mutant of Arabidopsis has been reported as gibberellin-deficient due to the considerably low contents of the bioactive GA4 and GA7 forms (Magome et al., 2004). In SrKA13H over-expression transgenics, the content of bioactive gibberellins, as well as their precursors, was considerably decreased. Hence, an overall reduction in various gibberellin forms could be responsible for the observed dwarfism of SrKA13H over-expression transgenics.

Gibberellins are important endogenous plant regulators that are known to play role in plant growth and development, including hypocotyl elongation, stem length, flowering, pollen viability, and seed set (Yamaguchi, 2008). Gibberellins have been reported to regulate hypocotyl length by controlling cell elongation (Cowling and Harberd, 1999). Similarly, in the present study, reduced endogenous gibberellin was found to decrease the hypocotyl length in Arabidopsis transgenic. In the previous reports (Cowling and Harberd, 1999; Alabadi et al., 2004; Lee et al., 2012), a significant reduction in hypocotyl cell size was found to be responsible for the decrease in hypocotyl length of SrKA13H transgenics. Decreased plant height and reduced rosette area of SrKA13H transgenic Arabidopsis were features similar to gibberellin-deficient mutants. Various mutants of Arabidopsis with a lower endogenous gibberellin content have been reported to inhibit stem elongation. The ddf1 (dwarf and delayed flowering 1) mutant of Arabidopsis, raised by ectopic expression of the putative AP2 transcription factor, has been shown to have a dwarf phenotype and delayed flowering. A reduction in endogenous gibberellin was accounted responsible for such an abnormal phenotype (Magome et al., 2004). Similarly, knock-out mutants of biosynthetic genes of the gibberellin pathway, ga20ox1 and ga20ox2, have also been reported to possess a short phenotype and a smaller rosette radius, whereas, the double mutants of ga20ox1-ga20ox2 were comparatively more dwarf with a highly reduced rosette area. Reduced levels of gibberellins in these mutants has been suggested for such phenotypic variations (Rieu et al., 2008). Thus, the reduction in endogenous gibberellin content has introduced the observed phenotypic variations in Arabidopsis on SrKA13H over-expression. Further, the dwarf phenotype of transgenics over-expressing SrKA13H was reversed by the exogenous application of GA3. This has thus confirmed that the reduction in endogenous gibberellins was responsible for the observed dwarfism in transgenics. Both 4KT3 and 6KT3 Arabidopsis SrKA13H transgenic lines showed a similar reduction in bioactive gibberellins, even though the plant height of the 4KT3 plants was less than that of the 6KT3 plants, in keeping with the relative steviol concentrations. A deactivation process has been documented as an important mechanism for regulating the concentration of bioactive gibberellins (Yamaguchi, 2008). Elongated Uppermost Internode (EUI) has been identified as a gibberellin deactivation gene. The dwarf phenotype of transgenics over-expressing EUI was due to the conversion of GA4 into its deactivated form, while the eui mutant of rice was reported to have a taller phenotype due to the accumulation of a higher amount of GA4 (Zhu et al., 2006). Thus, a higher conversion rate of GA4 into its deactivated form, GA3, in the 4KT3 plants had resulted in a lower availability for its conversion into other active GA forms. On the other hand, more GA1 was available for conversion into other active GA forms in 6KT3. This could be one of the possible reasons for the overall greater reduction in plant height of 4KT3 compared with 6KT3 plants.

Gibberellins are known to influence pollen viability, pollen germination, and normal seed set behaviour. Arabidopsis transgenics over-expressing SrKA13H also possess non-viable pollen and had a reduced pollen germination rate. It is probable that seed abortion was more evident in SrKA13H transgenics. Earlier, the ga1-1 mutant of Arabidopsis was reported for the non-viability of their pollen (Goto and Pharis, 1999). Similarly, in tomato, the gibberellin-deficient mutant, ga-1, possessed sterile flowers and had abnormal seed set (Groot et al., 1987). Gibberellin deficiency has been found to be responsible for the pollen-related variations in mutants of Arabidopsis and tomato. Retarded pollen germination and seed abortion was reported in gibberellin-deficient and -insensitive Arabidopsis transgens developed by ectopic expression of pea GA2-oxidase 2 cDNA (Singh et al., 2002). Transgenic Arabidopsis over-expressing SrKA13H accumulated steviol. The pollen-related abnormalities observed in transgenics could be due to steviol formation or to modulation in the endogenous gibberellin content. To check this effect, pollen from control Arabidopsis were exposed to exogenous steviol. Surprisingly, the germination rate of control pollens was not affected. Thus, the decreased gibberellin content in SrKA13H Arabidopsis transgenics was assumed to be solely responsible for pollen sterility, impaired pollen germination rate, and abnormal seed set.

In conclusion, the present study has documented the in planta synthesis of steviol in a plant system other than Stevia. The results suggested the diversion of carbon flux from gibberellin to steviol biosynthesis upon over-expression of SrKA13H in Arabidopsis. Although such a diversion in carbon flux has an adverse effect on plant growth and development, the production of steviol through this mechanism could be very useful, at least in plants where seeds are not an economical part.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Fig. S1. Gel picture showing PCR confirmation of cloning of SrKA13H cDNA in pCAMBIA1302.

Supplementary Fig. S2. Gel picture showing PCR confirmation of transformation of recombinant vector, pCAMBIA-SrKA13H into Agrobacterium strain LBA4404.
Supplementary Fig. S3. Generation of Arabidopsis transgenics over-expressing SrKA13H and their confirmation.

Supplementary Fig. S4. Estimation of steviol accumulation in SrKA13H transgenic Arabidopsis.

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