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ORIGINAL ARTICLE

Meeting the challenge of stevioside production in the hairy roots of *Stevia rebaudiana* by probing the underlying process

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Abstract *Agrobacterium rhizogenes* mediated "hairy root cultures" of the renowned bio-sweetener producing plant—*Stevia rebaudiana*, were generated to explore their yet undetected biosynthetic potentials concerning the low calorie diterpene glycoside—stevioside. Four stable rhizoclones were studied under light and dark conditions showing better growth under dark. Two of these rhizoclones revealed substantially higher photosynthetic pigment accumulation under light. Evidently, capitalization on the available inter-clonal variability first time showed the stevioside synthesizing exclusivity in the SRA4 rhizoclone under light condition, while the rest failed. The dualities of the glycoside synthesizing power amongst the two photosynthetically active rhizoclones were resolved through quantitative RT-PCR analysis of UGT85C2 gene showing positive expression in the stevioside producing rhizoclone. These findings elucidated the decisive role of UGT85C2 in combination with photosynthetic proficiency of the studied hairy root cultures in regulating the biosynthetic pathway of *S. rebaudiana.*

Keywords Hairy root · Photosynthetic pigment · *Stevia rebaudiana* · Stevioside · qRT-PCR · UGT85C2 gene

Introduction

The need for biologically sourced, low-calorie sweeteners has been acknowledged globally over the last two decades owing to the increasing alertness about the detrimental effects of prolonged sugar consumption causing diverse human ailments including diabetics, obesity and cardiovascular diseases (Philippe et al. 2014). The escalating widespread consumer demand for sugar–substitutes became progressively more challenging once the adverse carcinogenic side effects of synthetic sweeteners became conspicuously discernible (Das et al. 2015) and the entire obligation predominantly turned towards nature-based alternatives (Philippe et al. 2014). At this juncture, *Stevia rebaudiana* (Asteraceae), a perennial native herb of South America, started gaining worldwide attention owing to its ability to synthesize diterpene glycosides functioning as non-toxic, high-potency, low calorie sweeteners to substitute sucrose/other synthetic sweeteners (Gasmalla et al. 2014). Two out of eight leaf derived glycosides of this plant, namely stevioside and rebaudioside-A, occupied prominent position in the relatively short list of plant-derived molecules that have yet been launched commercially (Philippe et al. 2014). The reported safety affirmation of *stevia* and *stevia* products from worldwide regulatory authorities including the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2009) conspicuously escalated its popularity and market demand which can be gauged through its inclusion in an array of food products as documented recently (Gasmalla et al. 2014; Gonzalez et al. 2014; Kumari and Chandra 2014). Its proven worth in

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fighting against tooth decay or dental caries resulted in its added efficacious use in toothpaste, chewing gum, mouth refreshers etc. (Gasmalla et al. 2014).

In addition to its distinctive sweetness trait, the plant also owns multiple therapeutic advantages owing to the unique pharmacological activities of steviol glycosides, which are exemplified by their antihyperglycemic, antihypertensive, anticancer, anti-tumor, anti-diarrheal, antimicrobial, antiviral, gastrointestinal- ulcer preventive, neuroprotective, diuretic and immunomodulatory functions (Gonzalez et al. 2014; Kumari and Chandra 2014). The therapeutic value of stevioside in stimulating the insulin signaling and at the same time antioxidant defense in both adipose tissue and the vascular wall, leading to inhibition of atherosclerotic plaque development indicated its beneficial effects in the treatment of type 2 diabetes and cardiovascular disorders (Gasmalla et al. 2014; Gonzalez et al. 2014).

In the background of such accessible facts, it is quite perceptible in recent years, that *Stevia* has attracted global attention as a genuine candidate in desperate need of biotechnological intervention to address the escalating market demand in years to come (Philippe et al. 2014; Gantait et al. 2015). Devising an alternate production source of *Stevia* metabolites through cell/tissue culture based techniques, like callus/suspension, shoot and hairy root cultures have earlier been explored by several research groups with paradoxical results (Bondarev et al. 2001; Hwang 2006; Gantait et al. 2015). Noticebly, the green calli of *S. rebaudiana* have revealed stevioside synthesizing potentials in a number of instances under the influence of light condition (Bondarev et al. 2001; Sivaram and Mukundan 2003; Jadeja et al. 2005; Ladygin et al. 2008; Janarthanam et al. 2010; Taware et al. 2010; Gupta et al. 2014; Khalil et al. 2014). On the contrary, although successful establishment of hairy roots in *S. rebaudiana* has been achieved recently (Michalec-Warzecha et al. 2016), the production of therapeutically important *Stevia* metabolites has not been established so far.

Hitherto, three reports are available concerning hairy root culture of *S. rebaudiana,* amongst which the preceding publication showed that even the light-grown green hairy roots lack the targeted diterpene glucoside synthesizing potential (Yamazaki et al. 1991). A recent report has elucidated the production of chlorogenic acid and its derivatives in the hairy roots of *S. rebaudiana* instead of stevioside (Fu et al. 2015). The adventitious root cultures of *S. rebaudiana* also showed negative outcome in terms of the targeted diterpene glucoside (Reis et al. 2011). The dualities of the reported observations relating to the affirmative and negative diterpene steviol glucoside synthesizing power of the light-grown green calli and the HR cultures of *S. rebaudiana* respectively ignited our quest to crack the underlying regulatory mechanism. The possible involvement of other specific condition beyond that of the normally envisaged photosynthetic ability was clearly indicated on the basis of the above- stated observations, which necessitated further in depth analysis for furthering the commercial feasibility of its hairy root clones. In this context, two fundamental facts attracted special attention which can be categorized as follows:

1. Steviol glycoside biosynthesis is brought about by a unique synergistic progression of the pathway from plastids through endoplasmic reticulum to final glycosylation by a rate-limiting gene (UGT85C2) in the cytoplasm (Brandle and Telmer 2007; Guleria et al. 2014), which principally governs not only the functionality of the underlying biosynthetic pathway, but also the affirmation of the product recovery (Mohamed et al. 2011).
2. It has already been universally proved that every transformation event resulting from the random insertion of *Ri* T-DNA can affect the expression of the adjacent gene within the plant genome owing to its position effect (Petit et al. 1986; Ono and Tian 2011). Accordingly, meticulous screening and selection of the desired photosynthetically active rhizoclone with positive expression of the UGT85C2 seemed essential to represent the complete functional pathway.

The decisive role of the rate-limiting gene expression in photo synthetically active green hairy root of *S. rebaudiana* towards successful stevioside synthesis has been first time elucidated through the present study, which offers ample opportunity to fulfill the challenge of hairy root based stevioside production.

Materials and methods

Plant material

Stevia rebaudiana (variety: CIM-Madhu), cultivated in Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow were used as explants source for the establishment of in vitro cultures.

Chemicals

The standards stevioside, rebaudioside A, rebaudioside C and chlorogenic acid as well as plant-growth promoters (i.e., benzyl amino purine—BAP and α-naphthaleneacetic acid—NAA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and water were purchased from Merck (Mumbai, India).

In vitro multiplication and hairy root induction

In vitro culture of *S. rebaudiana* was established using nodal explants, which were surface sterilized with 0.1 % $HgCl_2$ according to the previously reported protocol (Pandey et al. 2015). In brief, the nodal explants were inoculated on MS medium supplemented with different concentrations of BAP (0.5–2.0 mg/l) with a fixed concentration of NAA (i.e., 0.5 mg/l) for in vitro multiplication. The leaves and nodal segments from the in vitro reared and established shoots of *S. rebaudiana* were used as explants for the hairy root induction. The genetic transformation experiments were performed following the previously published protocol (Gupta et al. 2015) utilizing the A4 strain of *A. rhizogenes* (a kind gift from Prof. D. Tepfer, INRA, Versailles Cedex, France). In short, both the needle-pricking method and immersion of the cut-ends of leaf explants for 10 min in bacterial suspension (grown in liquid YMB medium, $O.D_{600} = 1.0$) were carried out according to the report of Gupta et al. (2015) for optimizing the maximum root induction rate. After 2–3 days of co-cultivation of the explants on basal MS medium with the bacterium, the explants were transferred onto the 100 mg/l of Cefotaxime (Alkem, India) containing basal MS medium under dark conditions. Similarly wounded explants without having any bacterial inoculation were also cultured under uniform conditions as controls.

Establishment of hairy roots and confirmation for *rol* genes expression

Once induced, the emerging hairy roots were individually separated as clones from each transformation event and transferred to liquid half-strength MS medium containing 100 mg/l of Cefotaxime (Alkem, India). The cultures were incubated at 25 ± 2 °C on a rotary shaker with constant agitation of 80 rpm under either continuous light 40.54 µmol photons m^{-2} s^{-1} or dark conditions. The growth indices of the hairy root clones were determined starting from 15 to 90 days of cultivation according to the previously published protocol (Pandey et al. 2015).

Extraction of DNA from the roots of normal field grown (control) plant and HR clones was done following the protocol described earlier (Pandey et al. 2014). Subsequently, the PCR amplification of the *rol* B and C genes was carried out involving independently generated hairy root clones following an earlier published procedure (Pandey et al. 2014) for confirmation of their transformed nature against the background of the untransformed control roots of the field grown plants.

Extraction of stevioside and HPLC analysis

The dried and powdered samples of the HR clones at different growth phases (15, 30, 45, 60, 75 and 90 days) were extracted with methanol for 3–4 times, dried under *vacuo* and dissolved in HPLC grade methanol prior to quantification. Likewise, the media of the corresponding HR clones were also individually extracted 3–4 times with ethylacetate, dried under *vacuo* and prepared for the HPLC analysis. The chromatographic separation was achieved using a symmetry Xbridge amide column (4.6 × 150 mm, 3.5 µm, Waters) at 50 °C, mobile phase consisted of solvent A water (20 %) and solvent B acetonitrile (80 %) in isocratic elution mode with detector wavelength 210 nm and the injection volume was 15 µl with flow rate of 1 ml/min. The quantitative analysis of stevioside (1.0 mg/ml stock solution) was based on the calibration curve.

Light microscopy

The hairy root clones (SRA4 and SRA4H1) grown under light and dark condition (75 days old) were utilized for free-hand cross-sections. The thin sections were examined and photographed by using EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc. India) at 10× and 40×.

Photosynthetic pigments and chlorophyll fluorescence

Total chlorophyll, chlorophyll a, chlorophyll b and carotenoid content were measured in 75 days old hairy root tissues of selected SRA4 and SRA4H1 rhizoclones. Tissue was kept overnight in 100 % methanol at 4 °C and quantified as described previously (Lichtenthaler and Buschmann 2001).

Chlorophyll fluorescence was measured in the selected HR clones (75 days old) using portable photosynthesis system (CIRAS-3 PP Systems, USA) attached with chlorophyll fluorescence module (CFM-3 PP Systems, USA). CFM measures chlorophyll fluorescence by using Pulse Amplitude Modulation (PAM) technique. Hairy roots were incubated in dark for 20 min before chlorophyll fluorescence measurement. For chlorophyll fluorescence measurement, hairy roots were kept in PLC3 Universal leaf cuvette (PP Systems, USA) and CO_2 concentration in cuvette chamber was maintained at 400 ppm (ambient CO_2) and cuvette air temperature was maintained at 25°C (ambient temperature). Different chlorophyll fluorescence parameters such as Fo, Fm and Fv/Fm were measured. The Fo indicates the initial minimal fluorescence emitted from a dark-adapted sample and the Fm is the maximal fluorescence measured during the first saturation pulse (9000 µmol photons m^{-2} s^{-1} for 1 s) after dark adaptation. The Fv/Fm indicates the quantum efficiency of photosynthesis. The Fv is the variable fluorescence, which represents Fm-Fo.

qRT PCR analysis of UGT85C2 gene

Total RNA was extracted from two best performing HR clones (SRA4 and SRA4H1) after 75 days cultivation under light and dark conditions using RNAISO Plus (Takara) following the manufacturer's instructions. Likewise, RNA from the leaves of 1 month old field grown plant of *S. rebaudiana* was also isolated. RNA quantification was done using NanoDrop Spectrophotometer (Nanodrop®, ND-1000, Nanodrop Technologies, and Wilmington, USA). Genomic DNA contamination was removed by treating the RNA with DNase I (Fermentas, Germany). The purified RNA (4 µg/µl) was taken for cDNA synthesis using BluePrint 1st strand cDNA synthesis kit (Takara) according to manufacturer's manual. cDNA (1 µl) was used for RT-PCR using real time gene specific primers of UGT 85C2 (Forward-5′-TTGGGTTCCGGGA ATGG-3′ and Reverse-5′-ATTGGGTTCCGGGAATGG-3′). The endogenous control (18 s rRNA) was used for normalization of the relative quantitative estimation and the primers used was forward-5′-CGTCCCTGCCCT TTGTACAC-3′ and reverse-5′-CACTTCACCGGACCAT TCAAT-3′.

Statistical analysis

All the values were expressed as mean ± SD of three independent tests in duplicates (n = 3). Mean and SD were calculated with the help of MS Office Excel version 2007.

Results and discussion

Rapid in vitro multiplication of *S. rebaudiana* through auxiliary bud proliferation was optimized as an essential prerequisite to serve as explants source of the hairy root induction studies. The best multiple shoot response could be noted with Murashige and Skoog's (MS) medium supplemented with 3 % sucrose and 1.0 mg/l BAP + 0.1 mg/l NAA, in which a maximum of 12.66 ± 0.5 shoots were produced after 3 weeks of cultivation from a single nodal segment of field grown plants (data not presented). The leaves and nodal segments from such in vitro raised plantlets were used as explants source for the genetic transformation studies, where the leaves demonstrated better susceptibility towards hairy root induction with a maximum of 86 ± 3.1 % transformation frequencies after 2–3 weeks of inoculation, whereas only 42 ± 2.3 % transformation frequency could be noted with the nodal segments after 3–4 weeks. The current observation contradicts the sole preceding report (Yamazaki et al. 1991), where only stem internodes have been successfully used to establish hairy roots in *S. rebaudiana*. In the present study, needle-pricking method was superior as compared to the immersion of cut-ends method for the maximum root induction frequency in *S. rebaudiana.* The induction of hairy roots from the leaf explant and their establishment under the light and dark conditions have been shown in Fig. 1. The compatibility threshold of the utilized explants depended upon the availability of functionally competent cells, their maturity levels and associated variations in the internal hormonal status, which could account for the observed difference in their vulnerability towards *A. rhizogenes* infection, while all other probable contributing factors (like temperature, light, strain, media etc.) were kept constant for either of the two types of tested explants (Potrykus 1990).

Growth kinetics analysis and PCR based authentication of *Rol* genes expression

The morphology and growth potential of ten individually generated rhizoclones of *S. rebaudiana*, originating from separate wound sites, demonstrated significant variation concerning the overall growth performance upon their transfer to full and half strength MS liquid medium with 3 % sucrose. Only four rhizoclones thrived steadily in half strength liquid MS medium containing 3 % sucrose, while the rest six clones ceased proliferation even after auxin treatment and/or media optimization (data not presented). Complex effect of *Ri* T-DNA interaction with host plant genome in parallel with the diversity in copy numbers and sites of integration has unanimously been accredited in creating genetic variability within the resultant rhizoclones, which credibly confers the unique opportunity of selecting the best performing hairy root clone as per the requisite decisive factor (Hobbs et al. 1990; Sheludko and Gerasymenko 2013).

The four chosen HR clones also revealed conspicuous inter-clonal variability in their morphology and growth performance as reflected in their growth period based performance analysis under both light and dark conditions (Fig. 2a, b). The growth index (GI) analysis corroborated that, irrespective of the presence or absence of light, all the established HR clones attained their maximum GI on the 60th day of cultivation in half strength liquid MS medium. Noticeably, all these clones revealed better growth potential under dark condition over that of light grown cultures (Fig. 2b), which corroborates similar observation involving hairy root cultures of *Plumbago* species (Gangopadhyay et al. 2008; Sivanesan and Jeong 2008) but contradicts the findings observed in case of *Artemisia annua* hairy root growth (Liu et al. 2002). Out of the four proficient rhizoclones, one specific clone (designated as SRA4) demonstrated the maximum growth potential at the optimum growth phase (60th day), which superseded the rest in the

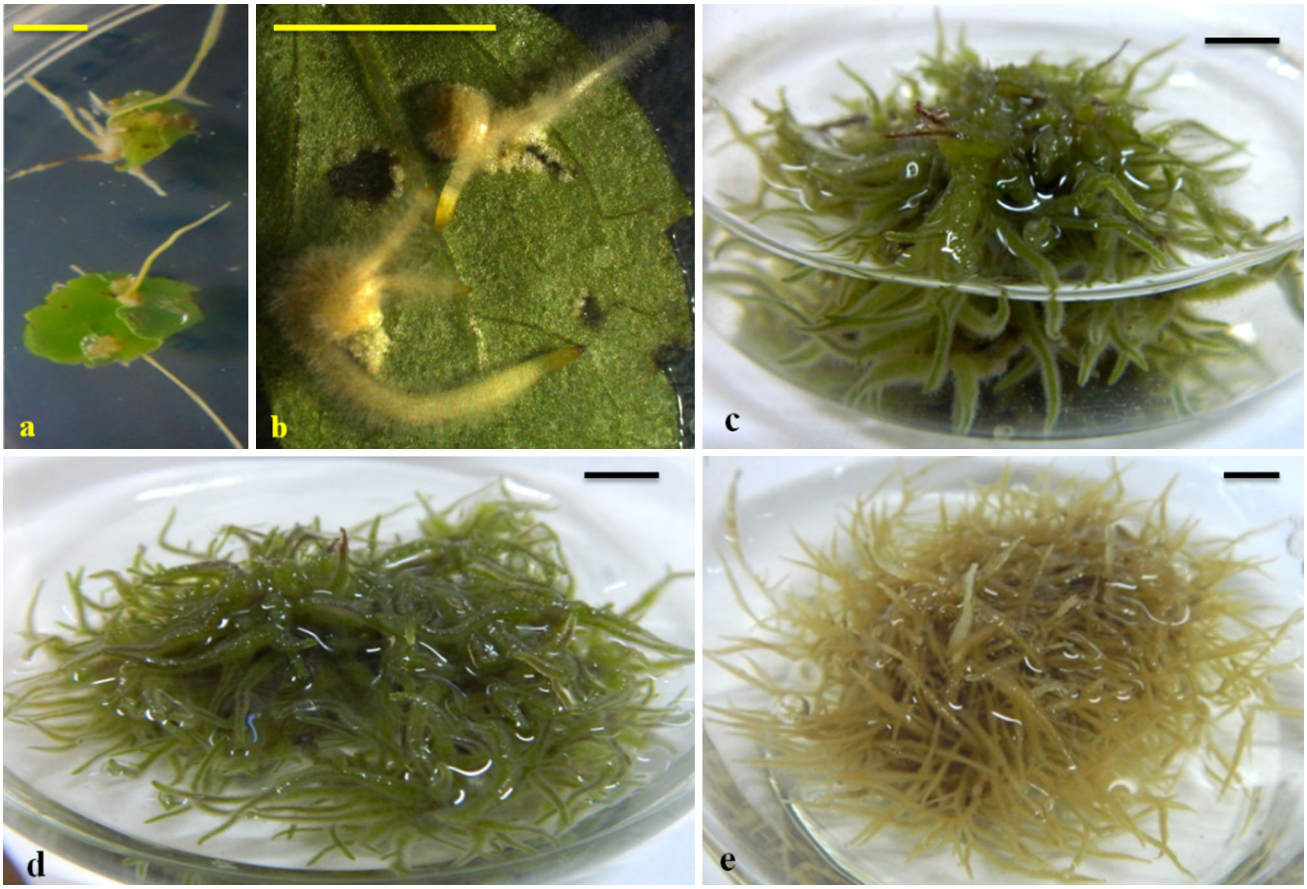


Fig. 1 Induction and establishment of *S. rebaudiana* hairy roots with A4 strain of *A. rhizogenes*. **a**, **b** Hairy root emergence from the leaf; **c**, **d** establishment of SRA4 and SRA4H1 HR clones respectively in ½ strength liquid MS medium under the light condition; **e** maintenance of SRA4 HR clone in ½ strength liquid MS medium under the dark condition (magnification **a**–**e** = 1 cm)

following reducing order: SRA4H1 > SRA4H6 > SR5 (Fig. 2a, b). It is interesting to note that out of the four flourishing rhizoclones, only two clones (SRA4 and SRA4H1) turned green under light, whereas the other two remained whitish-brown in colour even under light-grown conditions. PCR analysis exhibited the presence and expression of the *rol* B and *rol* C genes in all the four selected rhizoclones of *S. rebaudiana* (of which two have been represented in Fig. 3), signifying the integration of the *Ri* T-DNA as against their absence in the control roots of 1 month old field grown plant (Hashemi and Naghavi 2015).

Photosynthetic ability and chlorophyll fluorescence assessment

In view of the already recognized explicit role of photosynthesis on the biosynthesis of steviosides (Ladygin et al. 2008), establishment of photo-autotrophic green hairy roots of *S. rebaudiana* has been explored to address this issue. Microscopic observation of cells of dark and light grown hairy roots confirmed the presence of green cells in the two light-grown rhizoclones (SRA4 and SRA4H1) indicating the possible biosynthesis of chlorophyll (Fig. 4a–d), whereas the other two clones (SRA4H6 and SR5) didn't turn green under similar light conditions. Absorption spectra between 400 and 700 nm of methanol extract of tissues of these two green rhizoclones also confirmed the presence of photosynthetic pigments under light condition as the representative peaks were present in the region of 400–475 and 600–675 nm (Fig. 4a, c), showing the presence of chlorophyll a, chlorophyll b and carotenoids (Lichtenthaler and Buschmann 2001; Piwowarczyk et al. 2016). Amounts of total chlorophyll, chlorophyll a/b and carotenoids were higher in the light grown hairy roots than that of their dark grown counterparts as per anticipation (Fig. 5). Since Chl fluorescence has been used as a signature of photosynthesis in plants (Papageorgiou and Govindjee 2004), estimation of the same has been taken up to compare the functional status of photosynthetic apparatus in the dark-and light-grown hairy roots. Chlorophyll fluorescence parameters, such as Fo, Fm and Fv/Fm was estimated to ascertain the competence of the

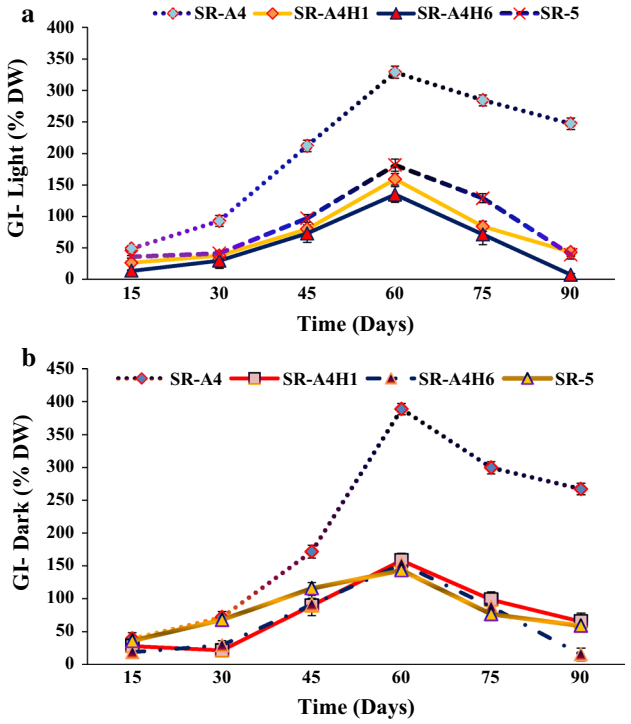


Fig. 2 Growth kinetics of four selected HR clones of at different time intervals cultured in the light (**a**) and dark (**b**). The values are mean of three replicates ± SD

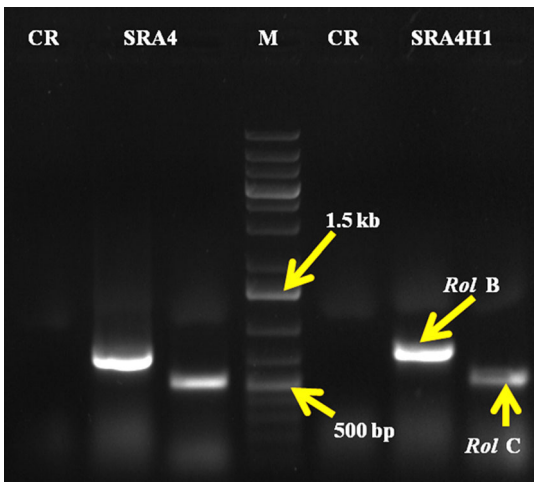


Fig. 3 PCR confirmation of *rol* genes expression in two rhizoclones (SRA4 and SRA4H1) [*rol B*—762 bp, *rol C*—539 bp, *M*-Marker (1 Kb plus—Fermentas); *CR* control root]

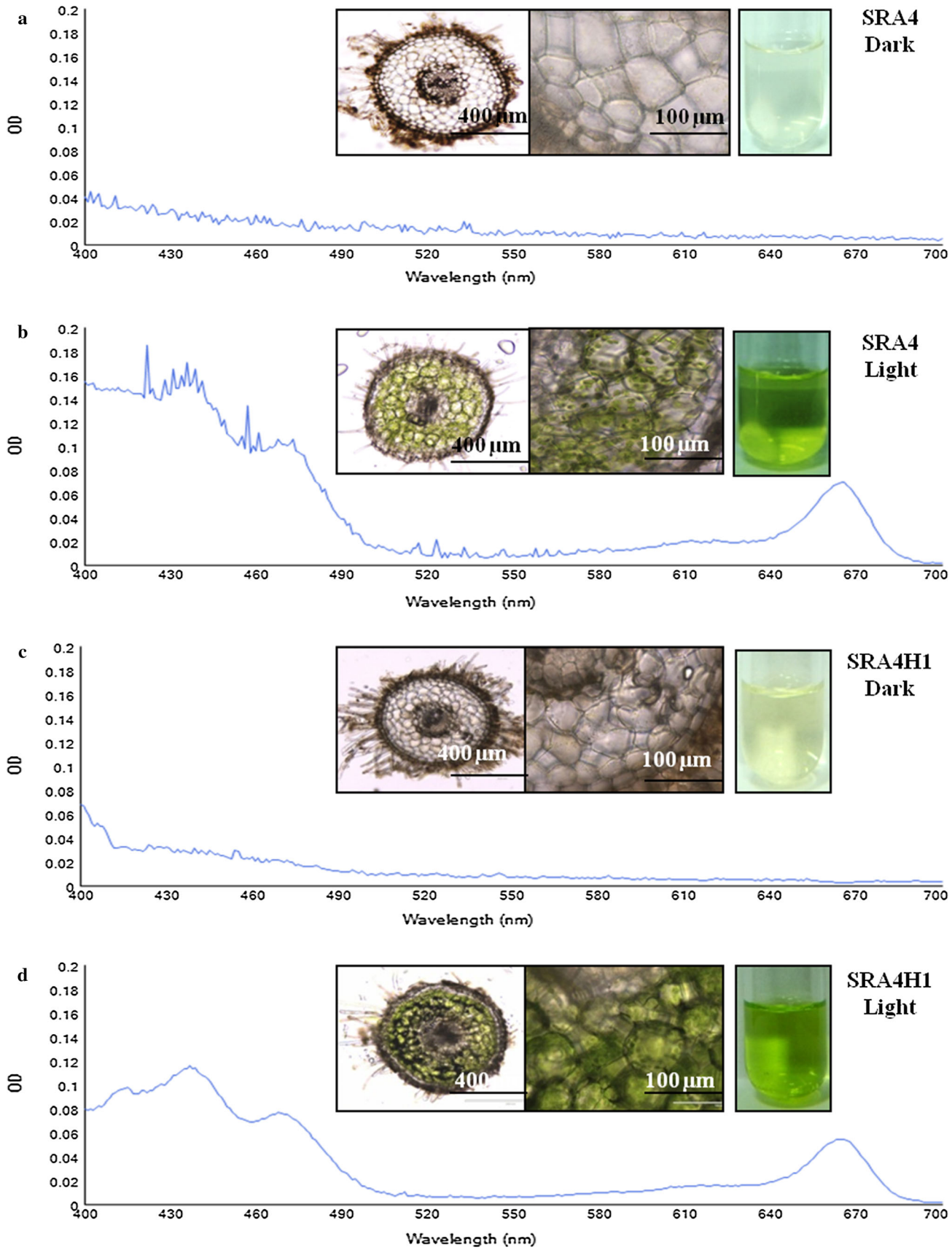
prevailing photosynthetic apparatus in the dark and light grown hairy root cultures of *S. rebaudiana* (Fig. 6a–c). Analogous perception indicating the existence of functional photosynthetic apparatus in green hairy roots has also been previously reported in diverse plant systems, which evoked fresh direction towards expanding the operating biosynthetic pathway in such promising organ cultures (Flores et al. 1993; Kino-Oka et al. 2001; Yamazaki et al. 1991).

Fig. 4 Photosynthetic pigments in dark and light grown hairy roots ▶ of *S. rebaudiana*. Absorption spectra (400–700 nm) of: **a**, **b** SRA4 rhizoclone under dark and light conditions respectively and **c**, **d** SRA4H1 rhizoclone under dark and light conditions respectively. *Insets* show microscopic pictures of cross-sections and methanolic extracts of the respective rhizoclone under dark and light grown conditions

Quantification of secondary metabolites through HPLC

The HPLC analysis of the crude methanol extracts of all the four hairy root clones (grown under light and dark conditions) were carried out with respect to four targeted molecules (i.e., stevioside, rebaudioside A and C, chlorogenic acid). The exclusive presence of stevioside could be noted in a single specific HR clone (SRA4) under light-grown cultivation process (Fig. 7b), which lacked the synthesis of the other investigated molecules (i.e., rebaudioside A and C, chlorogenic acid). The HPLC estimation of the standard stevioside revealed their best resolution at 210 nm with the retention time of 4.61 min (Fig. 7a). Alongside, the rest three clones failed to synthesize either of the targeted metabolites, in spite of the existence of the functional photosynthetic apparatus in the second (SRA4H1) green hairy roots clone. This observation clearly denotes that the presence of photosynthetic pigments is not the sole regulatory factor to steer steviol glycoside synthesis in hairy root cultures as has earlier been corroborated by Yamazaki et al. (1991). Consequent studies also corroborated the fact that although some initial steps in the process of stevioside synthesis occur in chloroplasts, the extent of development and functional activity of chloroplasts actually decides upon the final outcome of stevioside synthesis (Bondarev et al. 2001).

In the present study, the content of stevioside in the SRA4 HR clone showed a direct correlation with the cultivation time which reached its maximum after the exponential growth phase and the 75th day of cultivation proved to be optimum with subsequent declination on further prolongation of cultivation time (Fig. 8). The stevioside production profile of this particular HR clone ranged between 0.247 ± 0.011 to 1.72 ± 0.052 mg/g DW in the root tissues and 0.097 ± 0.072 to 2.12 ± 0.06 mg/l in the media (Fig. 8). Noticeably, the maximum yield of steviosides could be obtained from the medium compared to that in the HR tissues (Fig. 8), indicating the exudation of the metabolites into the surrounding growth medium, corroborating its basic advantage in the hairy root based production kinetics as elucidated previously (Cai et al. 2012). The accumulation and exudation kinetics of steviosides in the SRA4 HR clone as well as in its media successively expanded with the progression of the culture duration


a
SRA4
Dark
400 µm
100 µm
b
SRA4
Light
400 µm
100 µm
c
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d
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400
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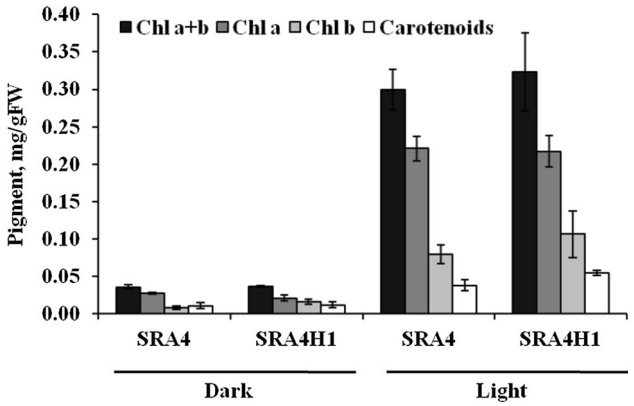

Fig. 5 Effects of light and dark on the overall content of the photosynthetic pigments (total chlorophyll, chlorophyll a, chlorophyll b and carotenoids) in the SRA4 and SRA4H1 HR clones after 75 days of cultivation. The values are mean of three replicates ± SD

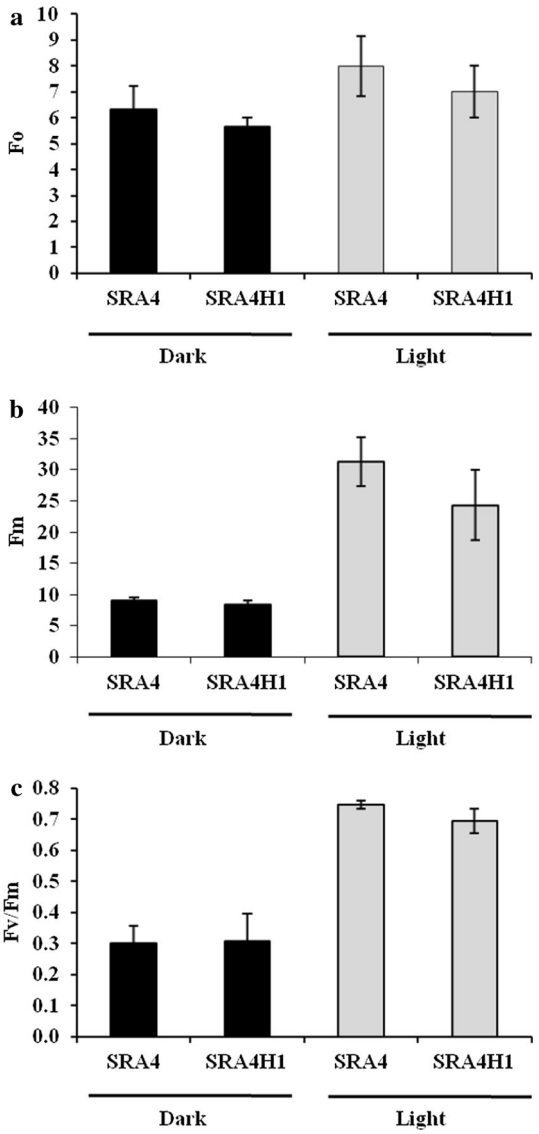

Fig. 6 Photosynthetic activities in the light and dark grown SRA4 and SRA4H1 rhizoclones after 75 days of cultivation. **a** Dark adopted initial minimum fluorescence (Fo); **b** maximal fluorescence measured during the first saturation pulse after dark adaptation (Fm); **c** quantum efficiency of photosynthesis (Fv/Fm). The values are mean of three replicates ± SD

revealing maximum exudation after the optimum production phase (Fig. 8). Contradictory result relating to the exudation dynamic of steviosides in the media was reported previously (Bondarev et al. 2001) while dealing with the suspension culture of *S. rebaudiana*. The presently observed exudation process offers the added advantage of the entire endeavor owing to its futuristic cost-effective down-stream processing back-up, which has already been abundantly evidenced for the "plant based" in vitro production of diverse secondary metabolites, prevailing over the normally encountered hurdle of feed-back inhibition process (Cai et al. 2012).

It is pertinent to highlight that the hairy root demonstrated merely 1.05 times lesser content of stevioside at its highest accumulation stage compared to that in the control leaves of 1 month old field grown plant (Fig. 8), which confers ample scope of its further enhancement through strategic optimization studies as elaborated previously (Bakkali et al. 1997; Luwanska et al. 2015).

RT-PCR of the UGT85C2 gene

The literature survey revealed that the biosynthesis of stevioside is basically localized in the plastid and it generally proceeds through the methylerythritol 4-phosphate (MEP) pathway starting with the precursor steviol (Brandle and Telmer 2007). Ample evidences indicated that the primary step in the synthesis of diterpene steviol glycosides (i.e. formation of ent- kaurenoic acid) occur only in green tissues (plastids), which ultimately gets converted into the aglycone steviol by the enzyme ent-kaurenoic acid 13-hydroxylase (KAH). However, the glycosylation of the steviol takes place in the cytoplasm by three different glucosyltransferases- UGT74G1, UGT76G1 and UGT85C2 (Brandle and Telmer 2007; Kumar et al. 2012; Yang et al. 2015). Amongst the three glucosyltransferase genes, UGT85C2 have been reported to be the rate-limiting gene of the steviol glycoside pathway, which principally governs not only the functionality of the underlying biosynthetic pathway, but also the affirmation of the product recovery (Mohamed et al. 2011).

Nevertheless, the present observation of positive biosynthetic ability of one specific green HR clone (SRA4) and at the same time failure of another one possessing similar photosynthetic potential (SRA4H1) in combination with previous reported negative results of the non-

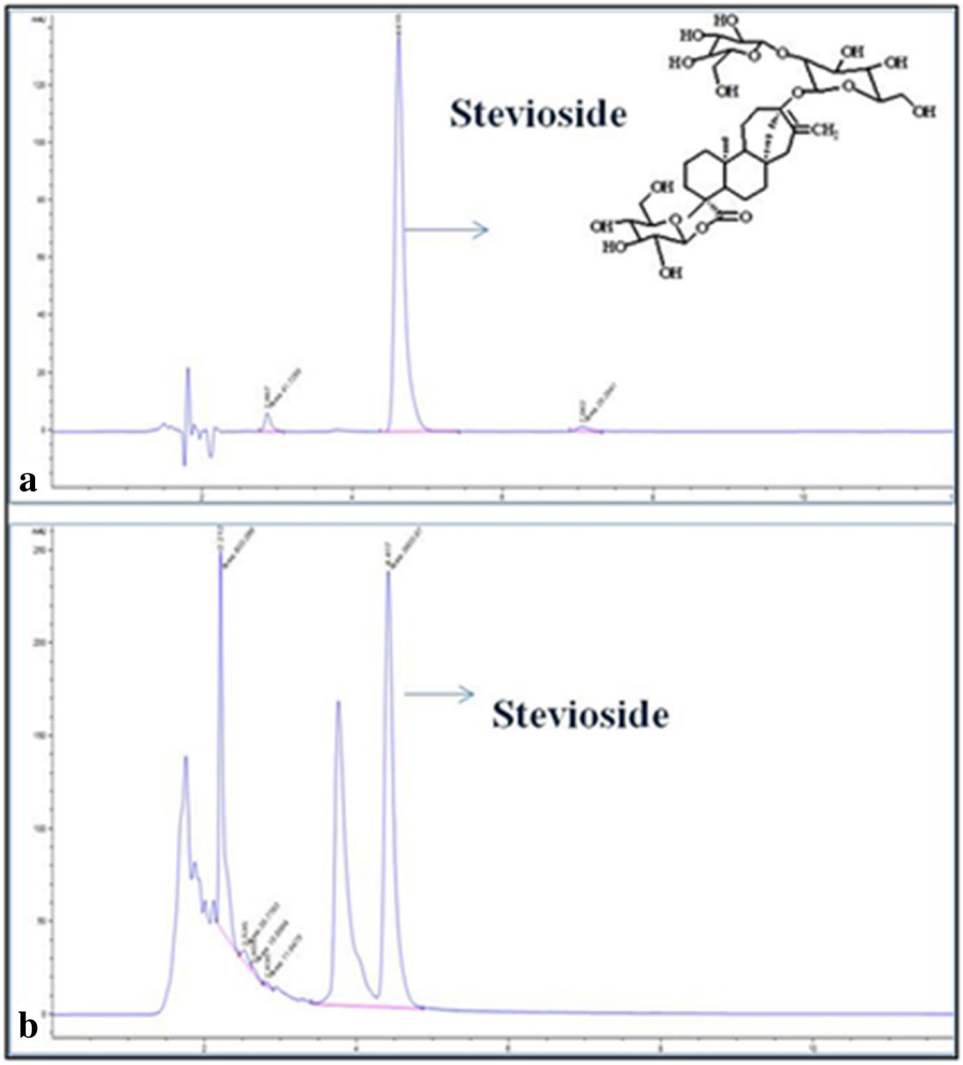

Fig. 7 Representative HPLC chromatograms for quantification of stevioside in MeOH extract of *S. rebaudiana* hairy root culture. **a** Standard of stevioside; **b** SRA4 rhizoclone

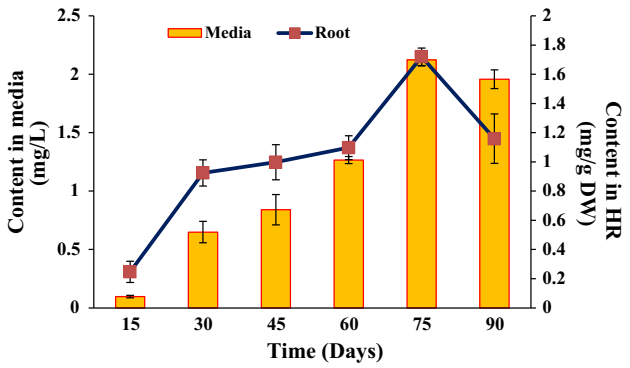

Fig. 8 Stevioside contents in the SRA4 hairy root tissues and its corresponding media at different time intervals. The secondary axis represents the content of stevioside in the HR tissues. The values are mean of three replicates ± SD

occurrence of steviol glycosides even in chlorophyll containing green hairy roots (Yamazaki et al. 1991), prompted us to investigate whether this specific rate limiting gene imparts any effect on the overall biosynthetic potential of the presently obtained hairy root clone.

The quantitative analysis revealed the expression of the selected key gene (UGT 85C2) of the metabolic pathway in the stevioside synthesizing light grown green SRA4 clone of *S. rebaudiana*, while its dark grown replicate failed to reveal any such prominent expression (Fig. 9). Compared to the control leaf of 1 month old field grown plant, this stevioside synthesizing SRA4 clone demonstrated 2.92 times lesser relative expression of the targeted gene (UGT85C2), which endorsed the comparatively lower stevioside yield in the selected HR clone over that of the control leaf. On the other hand, the second green

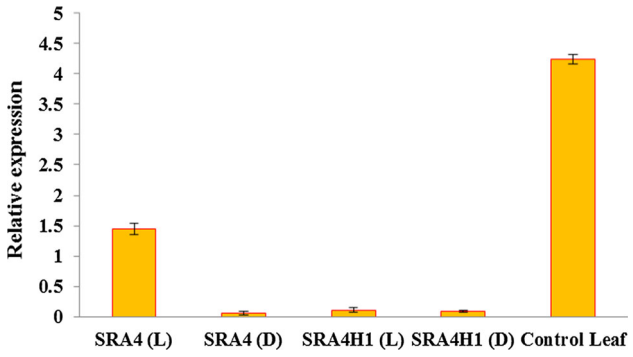

Fig. 9 RT-PCR analysis of targeted pathway gene (UGT85C2) in selected rhizoclones- SRA4 and SRA4H1 (grown in light (**L**) and dark (**D**) conditions) and in control leaf

rhizoclone (SRA4H1) possessing functional photosynthetic apparatus barely exhibited the expression of the chosen gene under the light condition (Fig. 9), suggesting that the presence of chlorophyll as well as light are not the deciding factors which regulate the production of the stevioside in the green HR clone (Fig. 9). These observations clearly indicated the potential role of plastid and cytosolic pathway-cross-talk as has been postulated earlier (Brandle and Telmer 2007). The observed decisive role of the cytosolic UGT85C2 gene further reiterates the fact that the metabolic channeling in the photosynthetically active the hairy root clone of *S. rebaudiana* could reasonably be altered owing to the site-specific insertion and the divergence in the incorporated copy numbers of Ri-T-DNA, accounting for inter-clonal variations with differential regulation process in the stevioside synthesizing potentials. Such inter-clonal diversity resulting from insertional mutagenesis has undeniably offered rewarding prospect, for the first time in *S. rebaudiana*, of selecting elite HR clone with functionally operational metabolic pathway towards stevioside synthesis, corroborating amply documented earlier reports involving several other medicinal plant species (Yoshimatsu et al. 1990; Inomata et al. 1993; Zehra et al. 1998; Halder and Jha 2015; Nayak et al. 2015).

In summary, the present study first time revealed the diterpene steviol glycoside (stevioside) synthesizing potential of a selected green hairy root clone of *S. rebaudiana* upon its cultivation under the light condition, while another green and two non-green rhizoclones failed to do so under identical growth conditions. The dualities of the glycoside synthesizing power amongst the two photosynthetically active rhizoclones were resolved through RT-PCR analysis of UGT85C2 gene, which showed positive correlation in terms of the gene expression and stevioside production potential in the SRA4 rhizoclone under light condition. These findings first time elucidated the combined influence of UGT85C2 gene expression and functionally operational photosynthetic machinery towards regulating the stevioside production in the *S. rebaudiana* hairy root cultures for its fruitful culmination.

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