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Steviol glycoside biosynthesis pathway gene expression profiling of transformed and non-transformed plant leaf tissues of *Stevia rebaudiana* (Bertoni)

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ABSTRACT

Stevia rebaudiana Bertoni, a member of the *Asteraceae* family, is recognized for its sweetened leaves, which are remarkably sweeter than sucrose by 200–300 times. This astounding property is because of steviol glycosides (SGs), a class of diterpenoid secondary metabolites that primarily consist of stevioside and rebaudioside A. These compounds are formed through a specific SG biosynthetic pathway that contains several key genes. In the present work, gene expression profiling of 15 core genes of SG biosynthetic pathway, along with metabolite analysis, was conducted in three groups of *S. rebaudiana* plants: *In vitro* regenerated non-transformed plantlets (NP), *in vitro* regenerated transformed plantlets (TP) via hairy root cultures using *Rhizobium rhizogenes* mediated transformation, and control plants (CP). Quantitative real-time polymerase chain reaction results showed that in NP and TP there was upregulation of 13 genes. Both NP and TP showed downregulated *SrDXR* and *SrCDPS* in comparison to CP, whereas *SrUGT74G1* had higher expression in NP than TP. High-performance liquid chromatography chromatographic studies on SGs showed that stevioside content followed the order TP > NP > CP. These findings demonstrate that transformation enhances SG biosynthesis and support the use of genetically modified *S. rebaudiana* lines for increased natural sweetener production. Further studies are warranted to elucidate regulatory mechanisms and optimize metabolic engineering approaches.

1. INTRODUCTION

Stevia rebaudiana (Bertoni) is a perennial shrub that belongs to the *Asteraceae* family of flowering plants. It is native to Paraguay and is noted for having a sweet taste due to a particular type of tetracyclic diterpenoids, called steviol glycosides (SGs), found in the leaves [1]. These diterpenoids are naturally occurring, low-calorie sweeteners found in three species of plants, *Angelica keiskei*, Chinese *Rubus suavissimus*, and *S. rebaudiana* [2]. SGs are primarily located in the leaves of Stevia plants, and they are later transported to other parts of the plant [3]. To date, more than 60 SGs have been found in Stevia [4,5]. The first glycoside to be extracted from Stevia leaves was Stevioside, which was then followed by the isolation of *rebA*, Dulcoside A, *rebB*, C, D, E, F, I, M, and Steviol-bioside [6].

S. rebaudiana typically contains Stevioside (5–10%), Rebaudioside A (2–5%), Rebaudioside C (1%), Dulcoside A (0.5%), and trace amounts of Rebaudiosides D, E, F (0.2%) and Steviol-bioside (0.1%) [7]. Among these, Stevioside and Rebaudioside A are the crucial SGs because they are the final products of the pathways. In addition to these secondary metabolites, plants also contain flavonoids, phenolics, and alkaloids that may have therapeutic benefits [8]. Stevia has many therapeutic properties, such as antidiabetic [9] antihypertensive, anticancer, antitumor effect [10], and antimicrobial effect [11].

Stevia is typically propagated using both seeds and shoot cuttings; however, seed propagation is less popular due to low production and germination efficiency, while shoot cutting propagation is a labor-intensive and time-consuming technique with a low success rate [12]. Optimum *in vitro* conditions are necessary for the development of an efficient method for mass propagation of Stevia. Thus, an alternative method, such as plant tissue culture, that has high efficacy, is used to raise Stevia plants to elevate the quality and quantity of SGs and further enhance the taste of *S. rebaudiana* [13,14].

In the present study, plantlets generated through two distinct tissue culture approaches, micropropagation and *R. rhizogenes*-mediated transformation, were utilized as the primary experimental material [15]. The SG biosynthesis pathway in *S. rebaudiana* synthesizes compounds responsible for the formation of the natural sweetness in the Stevia plants through a complex sequence of enzymatic reactions. It involves a series of enzymatic reactions

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mediated by approximately 15 key genes, as mentioned in Figure 1. The pathway begins with geranylgeranyl diphosphate (GGPP), which is converted by copalyl diphosphate synthase and Kaurene synthase (KS) to ent-kaurene. This is subsequently oxidized by kaurene oxidase (KO) and kaurenoic acid hydroxylase (KAH) to produce steviol, the aglycone backbone of SGs. In the final steps, UDP-glycosyltransferases (UGTs) such as UGT85C2, UGT74G1, and UGT76G1 catalyse sequential glycosylation reactions to generate stevioside and various rebaudiosides [3,16]. The coordinated activity of these 15 genes shapes the production of SGs, creating compounds such as stevioside and rebaudioside A, which are crucial to the sweetness profile of Stevia. Understanding and manipulating these genes in biotechnological applications can probably enhance the yield and composition of SGs, potentially creating sweeter and more stable stevia products for commercial use.

Recent studies have highlighted the roles of specific genes in the pathway. For example, UGT76G1 is critical for synthesizing rebaudioside A, a desirable sweetener due to its reduced bitterness compared to stevioside [17]. Modulating UGT enzyme expression can enhance specific SGs, allowing selective breeding or genetic engineering to produce high-yield, consumer-preferred sweeteners [18].

In light of the previous discussion, the present study aims to analyze the relative expression profiles of fifteen key genes involved in the SG biosynthetic pathway in *S. rebaudiana* leaf tissue. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to assess relative gene expression across two plant groups: Transformed plants (TP), non-transformed plants (NP), in comparison with *in vivo* control plants (CP) to develop a *Stevia* system exhibiting enhanced sweetness. To correlate gene expression with SG accumulation, quantitative analysis of SGs using stevioside as the reference standard was conducted via high-performance liquid chromatography (HPLC). This allowed for the comparative estimation of stevioside content among TP, NP, and CP plants, thereby validating the expression data obtained through qRT-PCR.

2. MATERIALS AND METHODS

2.1. Plant Material

S. rebaudiana Bertoni (Family: *Asteraceae*) plants were procured from Sunrise Agro Nursery for Medicinal Plants (18°35'20"N, 73°46'30"E), Wakad, Pune. These *in vivo* plants were subsequently maintained and cultivated under controlled conditions in the greenhouse facility of Dr. D.Y. Patil Biotechnology and Bioinformatics Institute, Tathawade, Pune, and served as the control group (CP) in the present study. The same plants also served as the source of explants for regeneration studies.

Specifically, internodal segments excised from CP plants were used for direct regeneration of NP through micropropagation. For the generation of TP, microshoots derived from NP lines were co-cultivated with *R. rhizogenes* to induce hairy root formation. Hairy-root-bearing microshoots were then cultured to regenerate TP plantlets.

The regeneration protocols for both NP and TP plantlets were optimized at the plant tissue culture laboratory of Dr. D.Y. Patil Biotechnology and Bioinformatics Institute, Pune, and Rise N' Shine Biotech Pvt. Ltd., Theur, Pune. The molecular confirmation of transformation through polymerase chain reaction (PCR) amplification of *rolB*, *rolC*, and *virD2* genes was also performed, which confirmed stable integration of *Ri* T-DNA into Stevia hairy roots and the corresponding regenerated plantlets [15]. These regenerated plantlets constituted the primary study [Figure 1].

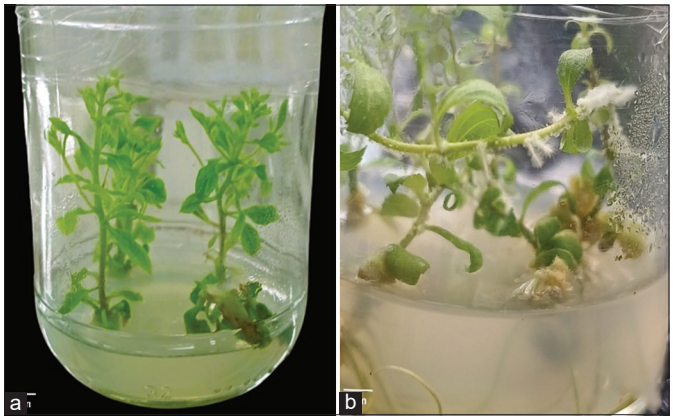

Figure 1: *Stevia rebaudiana* plant material. (a) Micropropagated plantlets; (b) *Rhizobium rhizogenes* mediated transformed plantlets [15].

2.2. Total RNA Extraction and First-Strand cDNA Synthesis

Total RNA extraction was carried out in three replications from the stevia leaf tissue of CP, NP, and TP using the Spectrum Plant Total RNA kit (SIGMA Life Sciences) by following the manufacturer's protocol.

The extracted total RNA pooled (three replications together) of each treatment that were quantified using NanoDrop spectrophotometer (Cytation 5 Multimode Microplate Reader, BioTek) and run on an agarose gel (1%, w/v) electrophoresis. The cDNA was synthesized using the pooled RNA (3 replicates/treatments) sample of treatments using the GoScript Reverse Transcription System (Promega Biotech India Pvt Ltd) by following the manufacturer's protocol.

2.3. Selection of SG Pathway Genes and Primer Designing

A total of 15 key genes involved in the SG biosynthesis pathway were selected based on their known roles in the metabolic conversion of precursors into major SGs, primarily stevioside and rebaudioside A [4]. The nucleotide sequences of all these genes were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov). The selection was based on sequence availability, functional annotation, and previous reports [4] on their involvement in SG biosynthesis in *S. rebaudiana*. Corresponding accession numbers for each gene are provided in Table 1. Primer pairs were designed using Primer-BLAST (NCBI) and Primer3Plus (https://www.primer3plus.com) under default parameters to ensure target specificity, optimal melting temperatures, and amplicon sizes suitable for qRT-PCR analysis, are mentioned in Table 1.

2.4. Gene Expression Profiling

The gene expression profiling of selected fifteen genes was performed using qRT-PCR. The two genes, *Actin* and *GAPDH* were used as a set of reference genes or internal control genes. The qRT-PCR reactions were carried out in Thermal Cycler1000 Touch CFX96 Touch™ (Bio-Rad) using iTaq Universal SYBR green Supermix (Bio-Rad). Each of the reactions was performed in triplicate, containing SYBR green Supermix (5 uL), template cDNA (1 µL), each of the primers (0.4 µL, 10 µM), and RNAse-free water (3.2 µL) with 10 µL total volume.

The qRT-PCR profile for two reference genes and eight SG pathway genes (includes *CMS*, *CMK*, *MCS*, *HDR*, *GGDPS*, *CDPS*, *HDS* and *DXS*) were as follows, 1 min (95°C), 10 s (95°C) for 40 cycles, 30 s

(56°C), 5 s (65°C), 5 s (95°C) and for other nine genes (*KO*, *KS*, *KAH*, *DXR*, *UGT76G1*, *UGT74G1* and *UGT85C2*) as follows, 1 min (95°C), 10 s (95°C) for 40 cycles, 30 s (62°C), 5 s (65°C), 5 s (95°C) for fluorescent signal recording followed by high-resolution melting curve obtained after the cycle 95°C (15 s), with constant increment in the temperature from 65°C (15 s) and 95°C (1 s). Melting curve analysis was done, and internal control genes *SrActin* and *GAPDH* were used to normalize the data individually. Finally, data were analyzed using the software, Bio-Rad CFX Maestro Version 2.3.

The fold change values from the gene expression profiling of genes in leaf tissue of CP, NP, and TP were calculated using $2^{-\Delta\Delta CT}$ method [19] and also a heatmaps plot as depicted in Figure 1 using, Multi Experiment Viewer (MeV v4.9.0) software [20]. The fold change values of each gene from NP and TP were plotted in the graph as shown in Figures 2-4.

Although CP, NP, and TP (*in vitro*-regenerated plantlets) were cultivated under distinct environmental conditions, leaf samples from both *in vivo* and *in vitro* plants were harvested after 1.5 months of growth to ensure comparable physiological status at the time of collection. The selection of *in vivo* plants as a reference for gene expression analysis aligns with prior transcriptomic studies conducted in rice and *Eucommia ulmoides* [21,22]. Quantitative gene expression data were normalized using two stable internal reference genes, *SrActin* and *GAPDH.*

2.5. HPLC Analysis

Stevioside content in the leaf samples of CP, NP, and TP was quantified using HPLC. Dried leaf powder was extracted with HPLC-grade methanol, defatted with hexane, and re-dissolved in acetonitrile. Filtrates were injected into an Agilent HPLC system equipped with a Zorbax Eclipse XDB–C18 column and detected at 204 nm. The mobile phase consisted of methanol and 0.05% o-phosphoric acid (75:25, v/v) at pH 3.15. Chromatographic data were analyzed using EZChrom Elite software. Stevioside concentrations were calculated against a standard curve and expressed in mg/g dry weight.

2.6. Statistical Analysis

The quantification studies were run in six replications for each leaf sample from CP, NP, and TP. The result was expressed as the mean value of Stevioside concentration ± standard error. Statistical Package

Table 1: Primer sequences of 15 genes of the Steviol glycosides pathway and reference genes.

Genes	Gene description	Accession no.	Primer sequence (F/R)	Temperature (°C)	Product size
SrDXS	Deoxy xylulose-5-phosphate synthase	AJ429232	F: CGACACATTGTGGTGCGTTT R: CAATTCGGGCTTCATCGGCTG	56°C	90 bp
SrDXR	Deoxy xylulose-5-phosphate reductase	AJ429233	F: GCTCGCAGGAAAAGGGATTC R: GCTCGCAGGAAAAGGGATTC	62°C	155 bp
SrCMS	4-diphosphocytidyl-2- C-methyl-D-erythritol synthase	DQ269452	F: TCAAGTTATGTCGCCCCTCAA R: TAATCGAGGATGCCGGTACA	56°C	115 bp
SrCMK	4-diphosphocytidyl-2- C-methylD-erythritol kinase	DQ269453	F: TCACACGTGCGGATAAACAA R: TACGCGGTGTTACTGGTTTG	56°C	88 bp
SrMCS	4-diphosphocytidyl-2- C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	DQ631427	F: ATTTCTCTCCGGCCGGTATC R: GATCGAAACCATGGCCGACT	56°C	130 bp
SrHDS	1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase	DQ768749	F: TTGCAATGGAGAATGCAACGG R: TTACGTGAACCACCACTCATC	56°C	104 bp
SrHDR	1-hydroxy-2-methyl-2(E)-butenyl	DQ269451	F: TGCGTAACATTCGGTTGTGG R: TCCAGACGGTTACGACACTT	56°C	71 bp
SrGGDPS	Geranyl geranyl diphosphate synthase	DQ432013	F: GCCACAAGGTGTACGGTGAA R: CGGACGATCCTGTCTTTGGA	56°C	115 bp
SrCDPS	Copalyl diphosphate synthase	AF034545	F: GGGCGAGGATGATGTTTGGA R: TGAAGCACGGCCACATAGTT	56°C	114 bp
SrKS	Kaurene Synthase	AF097310	F: CTTGACGGGGGTACTGTTGT R: AGAACCTCACCGTGTGTGAC	62°C	149 bp
SrKO	Kaurene oxidase	AY364317	F: CAACCGCAATAACCATCGGC R: GTTTGATTGGCTCCTGCGTG	62°C	154 bp
SrKAH	Kaurenoic acid hydroxylase	EU722415	F: GCCATTTCTGGGCGAAACTC R: TCCACACAACACCGCAAAAC	62°C	148 bp
SrUGT85C2	UDP glucosyltransferase – 85C2	AY345978	F: ACGGAAGCTCCTCAAAGGTC R: TGGGCCGATGGTGTAAATGT	62°C	151 bp
SrUGT74G1	UDP glucosyltransferase – 74G1	AY345982	F: TGGTGAAACATGGACCCGAA R: TTCTGGGAGCTTTCCCTCTT	62°C	147 bp
SrUGT76G1	UDP glucosyltransferase – 76G1	AY345974	F: ACAACGACCCACAAGACGAA R: CAACAGTTCCAGTTCGCGTC	62°C	153 bp
SrActin	Actin	AF548026	F: TGAAGCGTTATCATCATCTACTCA R: ATCATCGCCAGCAAACCCA	56°C	133 bp
GAPDH	Glyceraldehyde – 3 –phosphate dehydrogenase	KC669708	F: GGGGTTTGCTTTATGATTTCAGC R: AGAGCTGGAAGCACCTTTCC	56°C	126 bp

for the Social Sciences version 17 was used for the statistical analysis. To assess the significance of the mean values at ($P < 0.05$), the one-way analysis of variance (ANOVA) test was carried out, and for pairwise comparison, the *post hoc* test (Tukey's HSD) was applied.

3. RESULTS

3.1. qRT-PCR Analysis

In the present study, the expression profiling (qRT-PCR) of key genes (fifteen genes) involved in the SG biosynthetic pathway was studied from leaf tissues of *in vitro* regenerated NP, *in vitro* TP, and CP grown *in vivo*. Melt Curve Peak Temperatures (Tm) of the fifteen genes were obtained (S1).

The expression of *SrDXS* (deoxyxyulose-5-phosphate synthase) exhibited higher in *in vitro* TP (5.0-fold change, up-regulated) followed by *in vitro* regenerated NP (3.0-fold change, up-regulated), as compared to the CP as shown in [Figure 2a]. The *SrDXR* (deoxyxyulose-5-phosphate reductase) gene exhibited lower expression in TP (-3.2-fold change, down-regulated) and in NP (-25.4-fold change, highly down-regulated) compared with CP [Figure 2a].

The transcript abundance of three diphosphocytidyl related genes, *SrCMS* (4-diphosphocytidyl-2-C-methyl-d-erythritol synthase), *SrCMK* (4-Diphosphocytidyl-2-C-methyl-d-erythritol kinase) and *SrMCS* (4-diphosphocytidyl-2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase) exhibited higher expression in TP (7.4, 1.8 and 21.6-fold change respectively, up-regulated) followed by NP (5.5, 1.5 and 8.5- fold change respectively, up-regulated) when compared with CP as shown in [Figure 2b].

Furthermore, the transcript level of two 1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate synthase/reductase genes, *SrHDS* (1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate synthase) *SrHDR* (1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate reductase) exhibited the highest increase in TP (61.9 -fold change, highly up-regulated and 9.9-fold change respectively, up-regulated) and in NP (4.1 and 8.2-fold change respectively, up-regulated) as compared to CP as shown in Figure 3a.

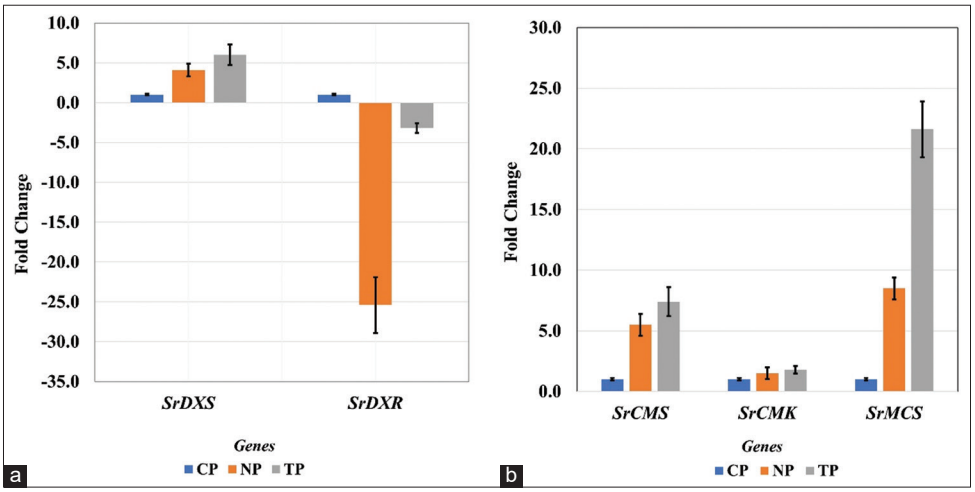

Figure 2: Gene expression profiling of Steviol glycoside biosynthesizing genes (a) *SrDXS* and *SrDXR,* (b) *SrCMS, SrCMK* and *SrMCS* in leaves tissues of transformed plantlets, non-transformed plantlets and control plants. Error bars on the top indicate standard deviation of three technical replicates. *SrActin* and *GAPDH* served as two reference genes.

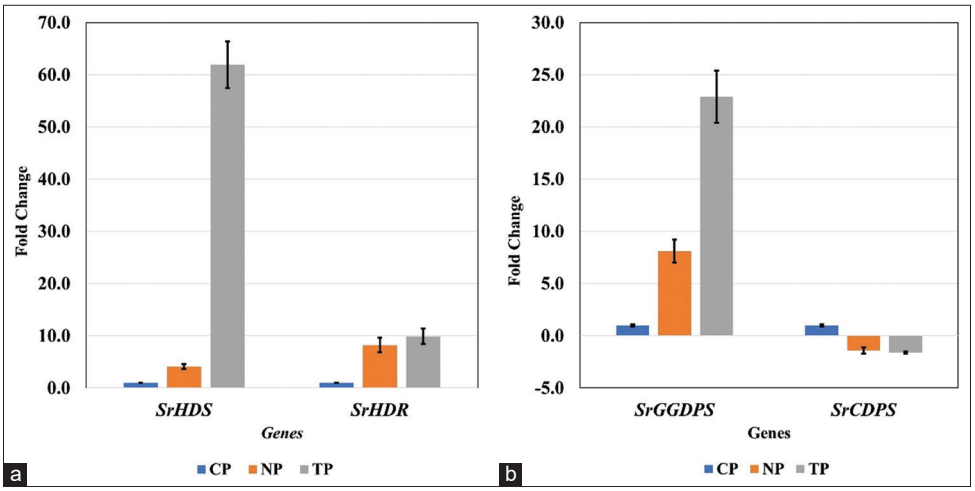

Figure 3: Gene expression profiling of Steviol Glycoside biosynthesizing genes (a) *SrHDS* and *SrHDR,* (b) *SrGGDPS* and *SrCDPS* in leaves tissues of transformed plantlets, non-transformed plantlets and control plants. Error bars on the top indicate standard deviation of three technical replicates. *SrActin* and *GAPDH* served as two reference genes.

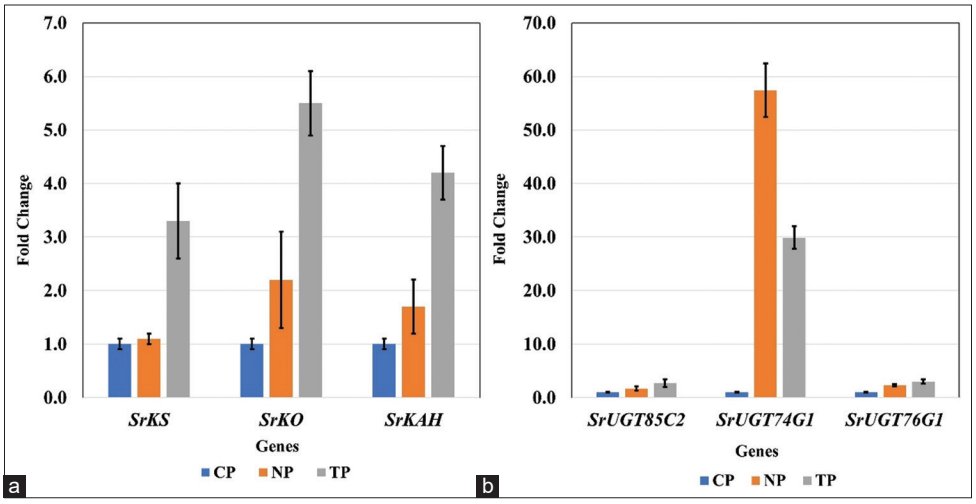

Figure 4: Gene expression profiling of Steviol glycoside biosynthesizing genes (a) *SrKS* and *SrKO* and *SrKAH,* (b) *SrUGT85C2, SrUGT74G1* and *SrUGT76G1* in leaves tissues of transformed plantlets, non-transformed plantlets and control plants. Error bars on the top indicate the standard deviation of three technical replicates. *SrActin* and *GAPDH* served as two reference genes.

Among the two diphosphate synthase genes, *SrGGDPS* (GGPP synthase) gene showed the highest expression in TP (22.9 -fold change, highly up-regulated) followed by NP (8.1-fold change, up-regulated), as compared to CP, shown in Figure 3b. Whereas in contrast, *SrCDPS* (copalyl diphosphate synthase) gene exhibited low expression in TP (−1.6- fold difference, that is down-regulated), followed by NP (1.4-fold change, down-regulated) in comparison with CP [Figure 3b].

Similarly, the transcript level of two genes, *SrKS* (KS) and *SrKO (*KO) exhibited higher in TP (3.3 and 1.1- fold increase respectively, up-regulated) followed by NP (5.5 and 2.2 -fold change respectively, up-regulated). However, the higher expression of *SrKAH* (KAH) was recorded in TP (4.2-fold change, up-regulated) and NP (1.7-fold change, up-regulated) as shown in Figure 4a.

Furthermore, among the glucosyltransferase genes, *SrUGT85C2 (*UDP glucosyltransferase-85C2) and *SrUGT76G1 (*UDP glucosyltransferase-76G) were highly expressed in TP (2.7 and 3.0 -fold difference, respectively, up-regulated) followed by NP (1.7 and 2.3-fold change, up-regulated) when compared with CP, shown in Figure 4b. However, *SrUGT74G1* (UDP glucosyltransferase-74G) gene exhibited higher expression in *in vitro* regenerated NP about 57.4-fold change (highly up-regulated), followed by *in vitro* regenerated TP, about 29.9-fold change (highly up-regulated) in comparison with CP [Figure 4b].Thus, comparatively higher expression profiling of SGs genes in *in vitro* TPs i.e., TP (plantlets regenerated from induced hairy roots *via R. rhizogenes* transformation), indicates that the modulation occurred in genes involved in the SG biosynthetic pathway might lead to enhance production and content of SGs.

3.2. HPLC Analysis

The key feature of *Stevia* is its SG accumulation. Examination of Stevioside concentrations of leaf tissues of CP, NP, and TP is carried out by HPLC. According to the results as shown in Table 2, the highest amount of stevioside was produced by *R. rhizogenes*-mediated TPs. It can be concluded that transformation increases SG contents in the leaves of stevia. One-way ANOVA shows that all the data are significant with $P < 0.05$. Tukey's HSD test, showed that all pairwise comparisons (CP vs. NP, CP vs. TP, NP vs. TP) are statistically significant ($P < 0.05$). This confirms that stevioside concentration significantly differs among

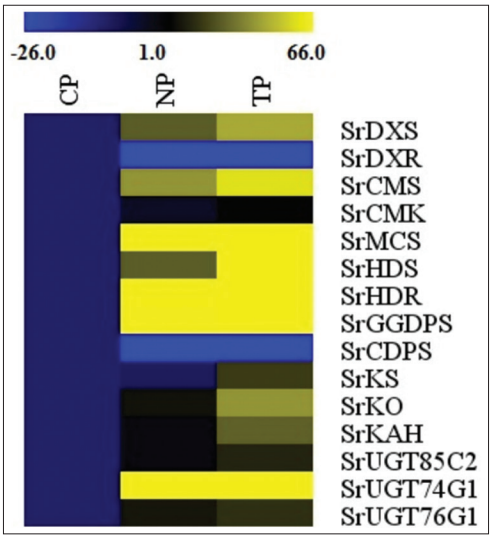

Figure 5: Heatmap representation of fifteen steviol glycoside biosynthesis pathway genes expression profile in leaf tissue of transformed plantlets, non-transformed plantlets and control plants (CP). The colour scale at the top show expression level (fold change) values. Yellow indicates a higher expression level (up-regulation), and blue indicates a lower expression level (down-regulation) of genes. For, CP the colour remains uniform throughout.

Table 2: Stevioside concentration in mg/g of the leaf extracts of the *Stevia rebaudiana* control plants CP, non-transformed plants NP, and transformed plants TP.

Sample	Retention time	Area	Area %	Height	Stevioside (mg/g) (mean±SE)
CP	6.273	193133	100.00	23026	0.024±0.02[a]
NP	6.217	317420	100.00	39609	0.042±0.01[b]
TP	6.861	304175	100.00	25401	1.67±0.13[c]

CP: Control plants, TP: Transformed plantlets, NP: Non-transformed. * Values are the mean of Stevioside concentration in different sets of plants determined by one-way ANOVA which shows data are statistically significant ($P<0.05$). *Post hoc* test of pairwise comparison was done via Tukey HSD. Different letters represent statistically significant data. The experiment was performed in 6 replicates.

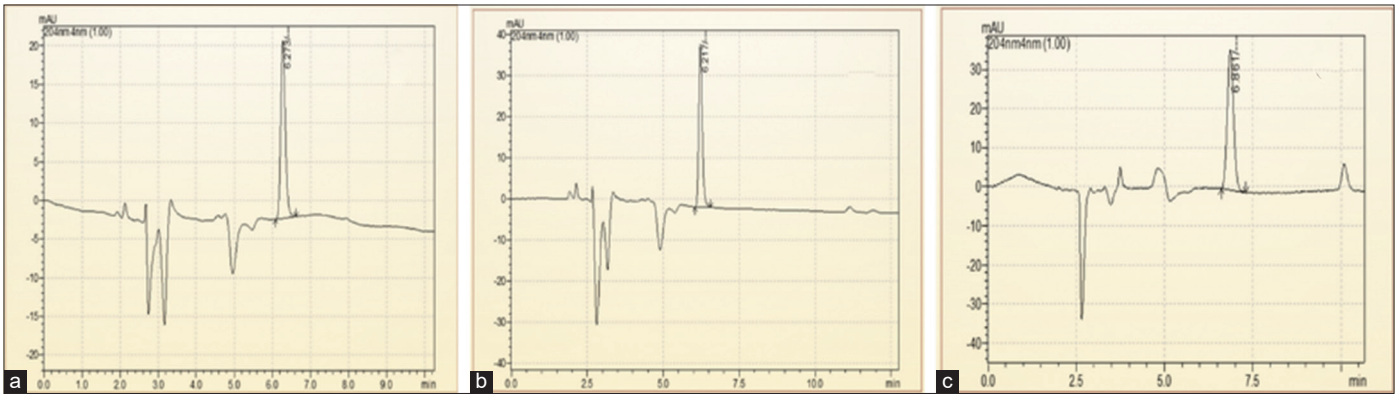

Figure 6: (a-c) High-performance liquid chromatography chromatogram of the leaf extracts of the Stevia control plants, non- transformed plantlets and transformed plantlets.

CP, NP, and TP, with TP showing the highest stevioside concentration. The corresponding HPLC chromatograms illustrating peak profiles of stevioside from CP, NP, and TP leaf extracts are presented in Figure 6.

4. DISCUSSION

In the current study, two types of transcript accumulation patterns, up-regulation and subsequently varying rates of down-regulation, were seen in the genes of the SG biosynthesis pathway in *S. rebaudiana* Bertoni, both in transformed and non-transformed lines. The highest transcript levels among the up-regulated genes were found in the leaves of the TPs, indicating that, out of the 15 pathway genes investigated in this study, *R. rhizogenes*-infected plants had superior transcript level, in general. The present findings are quite similar to the findings of Sarmiento-López, in 2020; Libik-Konieczny *et al.*, in 2020, and Sanchéz-Cordova *et al.*, in 2019 [23-25]. However, specifically, 13 genes were upregulated, and two of them were downregulated in the current study.

In our findings, higher transcript abundance (i.e., up-regulation) in three genes, *SrKS* (KS), *SrKO* (KO), and *SrKAH* (KAH) were recorded in TP and NP as compared to CP, which showed enhanced SGs content in leaves, corroborated with the earlier finding in Stevia by Zheng *et al.*, 2019, and Nasrullah *et al.*, 2023 [26,27]. The genes *SrKO* and *SrUGT74G1* show high expression, leading to an increase in Stevioside level. This finding correlates with studies by Nasrullah *et al.*, 2023 in *S. rebaudiana*, demonstrating their significant role in enhancing SG content [28].

In the present research, all NP and TP lines were propagated using the same combination of plant hormones in MS media and maintained under the same growth conditions [15], however, a higher *SrUGT74G1* expression level was noted in NP when compared with transformed lines (TP). Expression increase of *SrUGT74G1* in NP could be due to metabolic reprogramming during micropropagation stress. The lack of comparable expression in TP, even with the same culture conditions, may indicate that in the secondary metabolism of plants, the feedback regulation mechanisms responsive to the complete metabolites tend to inhibit the expression of biosynthetic pathways. For instance, Gachon *et al.*, in 2005 and Tiwari *et al.*, in 2016, discuss how glycosylation, mediated by glycosyltransferases, plays a crucial role in regulating hormone homeostasis and secondary metabolite biosynthesis [28,29].

In TP lines, higher flux through the steviol biosynthetic pathway might activate such feedback loops, leading to the downregulation of *SrUGT74G1*. Conversely, NP plants, with comparatively lower precursor flux, may not trigger these feedback mechanisms, resulting in higher expression levels of *SrUGT74G*. Another reason might be the choice of explants [30]. For the NP plants, nodal explants were used for direct shoot regeneration while, in case of TP plants, micro-shoots with hairy roots were used as explants for TP regeneration [15]. In addition, post-transcriptional regulation, including mechanisms mediated by microRNAs (miRNAs), can influence gene expression levels. Kajla *et al*, in 2023 highlight the role of miRNAs in fine-tuning the expression of genes involved in secondary metabolite biosynthesis. Such regulatory processes can lead to variations in gene expression independent of genetic transformation [31].

In the present study, two genes, *SrDXS* and *SrCDPS* indicated a downregulation, and despite that, in *in vitro* generated plants, stevioside levels were still high, as confirmed by HPLC analysis. This could be achievable due to such genes being part of a complex regulatory network in the SG pathway, which may involve a feedback mechanism, post-transcriptional regulation, and compensatory upregulation of other genes to sustain or even elevate overall SG production. The increased flux through the SG pathway in *in vitro* plantlets may trigger feedback regulation mechanisms. These mechanisms, which respond to the total metabolites, prefer to suppress the expression of biosynthetic pathways. On the other hand, *in vivo* plants, with relatively reduced precursor flux, might not activate these feedback mechanisms, resulting in varying expression levels. Moreover, the upregulation of major downstream *UGT* genes, specifically *SrUGT76G1*, could counteract the downregulation of upstream genes such as *SrCDPS* and *SrDXR*, resulting in net increase in SG accumulation. Similar trends have been reported in Stevia transformation studies, where enhanced glycosylation contributed to higher SG yields despite variations in early pathway gene expression [32,33]. Lower expression of these genes can also act as positive regulators of the pathway. The correlation between gene expression and SG quantification highlights the complex regulation of the biosynthetic pathway. While some genes exhibit downregulation, the overall metabolic flux toward stevioside biosynthesis appears to be driven by the enhanced expression of key glycosyltransferases. This underscores the potential for targeted genetic modifications to optimize SG production in Stevia [34,35].

HPLC data of the current research showed that transformed Stevia plants had higher stevioside content compared to micropropagated plants (NP), which in turn accumulated more stevioside than the *in vivo*, grown CP. This trend largely coincides with the gene expression data obtained through real-time PCR analysis in the current study. Similar findings regarding enhanced SG accumulation in *R. rhizogenes*-mediated transformed *S. rebaudiana* plantlets were reported by

Sánchez-Córdova and co-workers in (2019) [25]. The rise in stevioside content in NP as compared to the CPs might be attributed to tissue culture-induced metabolic reprogramming [36], where the controlled *in vitro* environment and synchronized developmental stage of regenerated plantlets can enhance secondary metabolite biosynthesis.

The present work mainly focused on HPLC analysis to quantify stevioside, one of the most predominant and important SGs found in *S. rebaudiana*. Stevioside is a central intermediate in the biosynthetic pathway and is frequently used as a reliable metabolic marker owing to its high accumulation in the leaf tissues. If other glycosides such as rebaudioside A were to be measured, a better picture of the metabolite spectrum would have emerged; however, the targeted analysis of stevioside alone provides a strong and representative estimate of the biosynthetic activity. This holds true for the main objective of correlating gene expression patterns with the core output of the SG biosynthesis pathway. In addition, focusing on stevioside has allowed for an accurate assessment of the impact of transformation and regeneration conditions on its accumulation.

5. CONCLUSION

In the present study, 13 out of the 15 analyzed genes involved in the SG biosynthesis pathway exhibited higher expression in the leaves of *in vitro* transformed Stevia plants (TP) compared to *in vitro* regenerated NP and CP. This upregulation suggests a positive correlation between gene expression and enhanced biosynthesis and accumulation of SGs in leaves. Furthermore, HPLC analysis confirmed that TPs demonstrated a higher stevioside content, indicating their potential reliability for improved SG production. These TP lines could serve as an alternative approach for large-scale production of SGs, facilitating their evaluation in animal trials for prospective commercial applications as a natural sweetener. SGs, being a plant-derived, low-calorie sugar substitute, could be particularly advantageous for individuals managing metabolic disorders, including high blood sugar, cardiovascular issues, and excessive weight gain. Their widespread application in the food industry may contribute to the development of healthier dietary alternatives, promoting overall well-being. This study highlights the potential for molecular-level manipulation of the SG biosynthetic pathway to enhance SG production. However, further molecular studies and pathway exploitation are essential to fully harness the biotechnological potential of *S. rebaudiana* for commercial applications.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. AVAILABILITY OF DATA AND MATERIALS

All the data pertaining to this study, are in the possession of the authors and will be supplied upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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