

# Analysis of Differentially Expressed Genes Involved in Stevioside Biosynthesis in Cultures of *Stevia Rebaudiana* Bertoni Treated with Steviol as an Immediate Precursor

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**Abstract** *Stevia rebaudiana* B. is a medicinal herb that accumulates low-caloric sweetening agents generally known as steviol glycosides among which stevioside and rebaudioside-A are predominant. These sweeteners are synthesized through the commonly known 2-C-methyl-D-erythritol-4-phosphate pathway common to steviol glycosides, gibberellic acid, pigments, and other isoprene-based secondary metabolites. The present investigation was designed to elucidate the role of steviol, an immediate precursor of sweetening agents, in the cultures of plants conducting stevioside biosynthesis in the leaf tissue. It was proposed that either the precursor or an elicitor was responsible for stimulating stevioside production in leaves. Transcript profiling of genes involved in stevioside biosynthesis was explored both in the roots and leaves of the plant and it was found that the transcript of the key enzyme kaurenoic acid hydroxylase, producing steviol, was the same in both tissue. However, when these plants were treated with the precursor steviol, most of the genes in the pathway were downregulated. Interestingly, it was found that very minute concentrations of the precursor were required to enhance the transcription level of genes of the pathway.

**Keywords** Kaurenoic acid hydroxylase · MEP pathway · Stevia · Steviol · UGTs

## Introduction

*Stevia rebaudiana* Bertoni is a medicinal plant widely used as a sweetener from ancient times in South America. The sweetening power of the herb comes from the diterpenoid glycoside generally known as steviol glycosides. The aglycon moiety in these glycosides is steviol which is glycosylated by various glycosyltransferases at the C-13 and C-19 positions to form steviol glycosides. These steviol glycosides along with other secondary metabolites like alkaloids, phenols, and flavonoids make the plant suitable for medicinal uses. Due to such metabolites, the plant and its extracts are widely used as anti-hypertensive, anti-hyperglycemic, and anti-rotavirus agents. Because of the sweetening agents the plant is also used to sweeten soft drinks, soy sauce, yogurt, and other foods in Japan, Korea, and Brazil (Tadhani and others 2007). Apart from these major glucose containing steviol glycosides, recently, Cunen and Geuns (2013) reported several other ent-kaurene glycosides which also contain rhamnose, xylose, and fructose. Moreover, they reported that steviol glycosides are also found in *Stevia phlebophylla* (Mexican origin), *Rubus suavissimus* (Chinese blackberry), and *Angelica keiskei* (Japanese perennial).

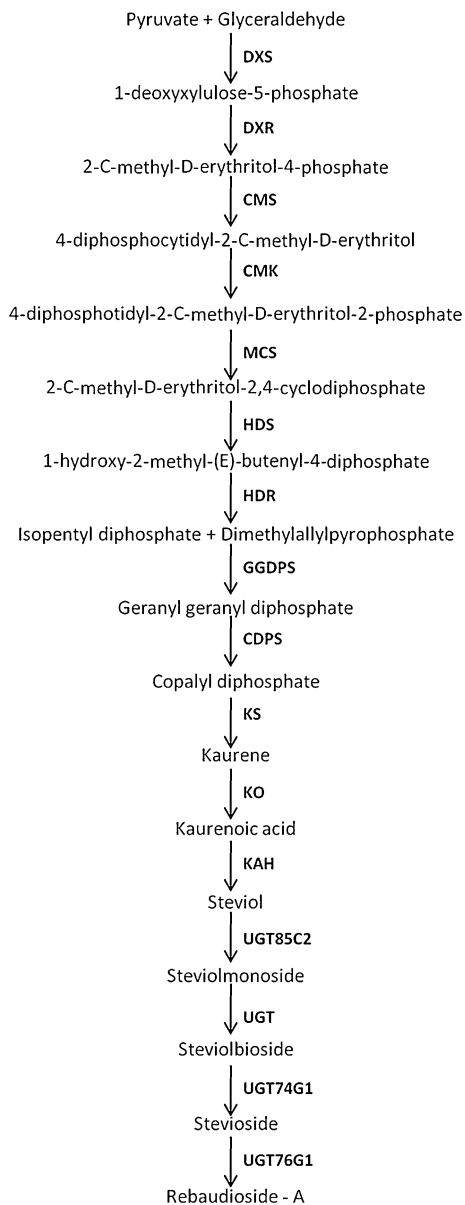
Isopentenyl diphosphate is a fundamental unit of diterpenoids and steviol glycosides and it was believed to be synthesized through the mevalonate pathway but Totte and others (2000) reported the involvement of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Fig. 1) for the synthesis of steviol glycosides. This plant has contributed its large portion of secondary metabolism to steviol

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**Fig. 1** Biosynthetic pathway for the production of steviol glycoside in *Stevia rebaudiana* Bertoni. *DXS* deoxyxylulose-5-phosphate synthase, *DXR* deoxyxylulose-5-phosphate reductase, *CMS* 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, *CMK* 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, *MCS* 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *HDS* 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate synthase, *HDR* 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate reductase, *GGDPs* geranylgeranyl diphosphate synthase, *CDPS* copalyl diphosphate synthase, *KS* kaurene synthase, *KO* kaurene oxidase, *KAH* Kaurenoic acid hydroxylase, *UGT85C2* UDP glucosyltransferase-85C2, *UGT74G1* UDP glucosyltransferase-74G1, *UGT76G1* UDP glucosyltransferase-76G1

glycoside formation. Steviol glycosides share a common pathway with gibberellic acid ( $GA_3$ ) as they are terpenoid and both are derived from the plastid and thus these glycosides are synthesized mainly in the leaf (Brandle and Telmer 2007; Richman and others 1999). However, less

than 0.1 % of steviol glycosides were found in roots also (Bondarev and others 2003). The steviol glycoside biosynthesis pathway separates from  $GA_3$  biosynthesis after the formation of kaurenoic acid. Gibberellins are synthesized via the action of kaurenoic acid oxidase whereas steviol (the precursor of steviol glycosides) is synthesized by a homotetramer enzyme kaurenoic acid hydroxylase having a subunit size of 39 kDa, after which further glycosylation of steviol takes place. An enzyme responsible for the production of steviolbioside has not yet been well characterized and thus reported as UGT in the pathway (Brandle and Telmer 2007), whereas some of the early genes *DXS* and *DXR* were well characterized from stevia (Totte and others 2003). Steviol glycoside content inside the leaf cell depends largely on genotype and environment. The sweetener is approved in many countries and now it is also recognized by the World Health Organization with an acceptable daily intake of steviol glycosides, expressed as steviol, at about 4 mg/kg of body weight.

Swanson and others (1992) studied stevioside biosynthesis in callus, root, shoots, and leaf of rooted shoot cultures of *S. rebaudiana* and found that stevioside is not synthesized in roots, shoots, or callus; moreover, it is exclusively synthesized in leaves of rooted shoots. So two hypotheses were proposed: (1) Either the precursor of stevioside is synthesized in roots and then transported to leaves or (2) the root produces some elicitor which stimulates the production of steviol glycosides in leaf. But the exact mechanism was not well understood. Furthermore, very minute quantities of precursors showed induction of steviol glycosides thus further treatments with the same precursor may reveal the co-ordination between precursor and steviol glycoside accumulation.

Steviol glycosides are synthesized in the plant through the MEP pathway. A number of researchers have identified, cloned, and characterized genes from the MEP pathway (Brandle and Telmer 2007). One of the most interesting features about such pathways is their manipulation for metabolic engineering and production of valuable secondary metabolites flashed as intermediates throughout the pathway. Moreover, these genes are differentially expressed when they are subjected to several experimental conditions. Siahsar and others (2011) reviewed the patterns of genes and enzymes in secondary pathways of medicinal plants. They concluded that the production of such metabolites requires the detailed understanding of the biosynthesis pathway and its regulation with analysis at the transcriptome, proteome, and metabolome levels. With the help of powerful techniques for gene expression analysis, it is now feasible to check the regulation of such a pathway at the transcriptome level.

The present scenario implies that the elucidation of the secondary metabolite pathway is carried out by evaluating

the up- and down-regulation, at quantitative expression levels, of the genes involved (Ghazi and others 2009). Tai and others (2009) reported that among various gene expression methods like microarray, amplified fragment length polymorphism, serial analysis of gene expression, differential display reverse transcriptase PCR, and qPCR, the most accurate and wide dynamic range of quantification can be achieved through real-time PCR (qPCR). With this aim in the center, we planned to treat a culture of *S. rebaudiana* with precursor steviol and to check the regulation

of various genes involved in steviol biosynthesis within the leaves of treated plants.

## Materials and Methods

### Culture Establishment and Treatment

Micropropagation of stevia plants was carried out and cultures were maintained according to the protocol as

**Table 1** List of primers with sequences and their respective product size as generated from primer designing tool for 3 endogenous controls and 15 target genes

Gene name	Corresponding enzyme	Primer sequence	In silico product size
<i>ACT</i>	Actin	F CGCCATCCTCCGTCTTGATCTTGC R CCGTTCGGCGGTGGTGGTAA	100
<i>UBQ</i>	Ubiquitin	F TCACTCTTGAAGTGGAGAGTTCCGA R GCCTCTGTTGGTCCGGTGGG	90
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	F CCGTGAGGTTGGAAAAAGCTGCC R TCAGTGTAACCCAAGATCCCCTTCA	97
<i>DXS</i>	Deoxyxylulose-5-phosphate synthase	F TCGAATTCGCTGGGGAGAAA R CAAGATCCTGAGTGGTGGAGGT	90
<i>DXR</i>	Deoxyxylulose-5-phosphate reductase	F GGAACGGCGCTGGTTGA R TCCCAATTGAACCAGTAGATCCC	85
<i>CMS</i>	4-Diphosphocytidyl-2-C-methyl-D-erythritol synthase	F TGACCTGTACCGGCATCCT R AGAACAAGTGACTGTAAAACCGC	109
<i>CMK</i>	4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase	F GCTGATGAGGTGGATATGAATGC R TGCCAAATCATGAAACCCATCTG	117
<i>MCS</i>	4-Diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	F ATTTCTCTCCGGCCGGTATC R AAACCATGGCCGACTCGAAA	125
<i>HDS</i>	1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate synthase	F TCGGATATTGCAGAATTCAAGCC R TCTCCAACCATAACTGTGCGT	122
<i>HDR</i>	1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate reductase	F TATCTGTTGATGCTCCGGC R CCGAACCCTTCCGGTTGTA	124
<i>GGDPS</i>	Geranyl geranyl diphosphate synthase	F CGGAAAAACACCAATCAAACCCA R AGGCGTCCAGAGCTTCATTC	123
<i>CDPS</i>	Copalyl diphosphate synthase	F AGAGACATCAACTTCCGGTGT R TTGTGTCCAAATGGTCTTCAC	118
<i>KS</i>	Kaurene synthase	F ACCAAAGAACGGATCCAAAAACT R ACTCAGGGAAACAAGGCGAT	121
<i>KO</i>	Kaurene oxidase	F AGCTATGAGACAAGCATTGGGA R CACCCATCATCGGGTCAACA	116
<i>KAH</i>	Kaurenoic acid hydroxylase	F GGAATCAGAGCCGGAGAGAT R GTCCACACAACACCGCAAAA	107
<i>UGT85C2</i>	UDP glucosyltransferase-85C2	F TCTTGAATCATCGGGCCAC R TCTCTGATTGGGATGCTCGC	108
<i>UGT74G1</i>	UDPglucosyltransferase-74G1	F ACAGTAACACCACCACCACC R GACCAACTTGTGTTGAATGTTCC	115
<i>UGT76G1</i>	UDP glucosyltransferase-76G1	F TTCACACCAACTTCAACAAACCC R ATGCGTTCGTCTTGTGGGTC	91

described by Modi and others (2012). To them, different concentrations of steviol (Sigma-Aldrich), that is 0, 50, 100, 150, and 200 nM (nanomolar), mentioned as treatments T0 (control), T1, T2, T3, and T4, respectively, were fed on the same medium on which cultures were maintained. Here steviol was dissolved in a minimum amount of dimethyl sulfoxide to make the stock of 3.1 mM. Leaves were sampled at the time of subsequent subculture. Moreover, sampling of root and leaf tissue was also carried out from the control plants to find the transcript of *kaurenoic acid hydroxylase (KAH)* gene.

#### RNA Extraction and cDNA Preparation

From the leaf samples RNA extraction was carried out by the method developed by Ghawana and others (2011) with minor manipulations. Two hundred milligrams of leaf tissue were frozen in liquid nitrogen and crushed using mortar and pestle. To this 2 ml buffer (containing 3 M sodium acetate, 0.5 M EDTA, and 10 % SDS in Tris saturated phenol) and 1 ml of DEPC (diethylpyrocarbonate) treated water were added and the slurry was kept on ice for 10 min. The solution was treated with 1/4th volume of chloroform and centrifuged at 13,000 rpm at 4 °C for 10 min. The upper aqueous phase containing RNA was carefully taken and treated with 0.6th volume of isopropanol and allowed to incubate on ice for 10 min followed by centrifugation at 13,000 rpm at 4 °C for 10 min. The final precipitation was carried out using 70 % alcohol and the RNA pellet was re-suspended in 30 µl of DEPC-treated water. Extracted RNA was quantified by Nanodrop, spectrophotometer (Thermo Scientific) and 5000 ng was used as the final concentration for the preparation of cDNA using a cDNA synthesis kit (Fermentas, Thermo Scientific).

**Fig. 2** Shoot tip explants of *Stevia rebaudiana* treated with various concentrations of steviol (T0 0 nM (control); T1 50 nM; T2 100 nM; T3 150 nM, and T4 200 nM of steviol fed to micropropagated plants of *Stevia rebaudiana*)



#### Primer Design and qPCR

All the primers for endogenous genes and gene-specific primers were designed from the primer Blast tool available at the NCBI web site using the leaf EST collection developed by Brandle and others (2002). Primer sequences for all the genes involved in stevioside biosynthesis along with their in silico product size are mentioned in Table 1. The designed primers were procured from MWG, Eurofins. With these primers qRT-PCR was performed first to study the least variable housekeeping gene and subsequently regulation of target genes of the pathway were assessed. The cycling condition was set as mentioned in the Fast SYBR Green Master Mix kit (Applied Biosystems).

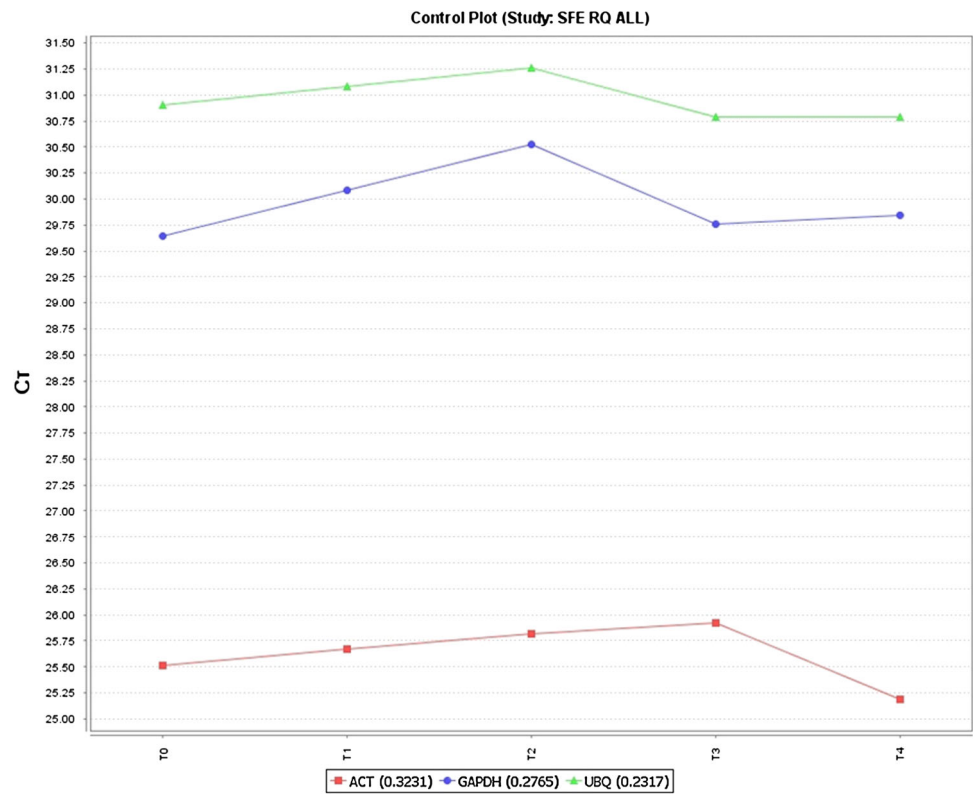
#### Relative Quantification

Relative quantification for all the targets was done with three repetitions and analyzed through the Data Assist bioinformatics tool.

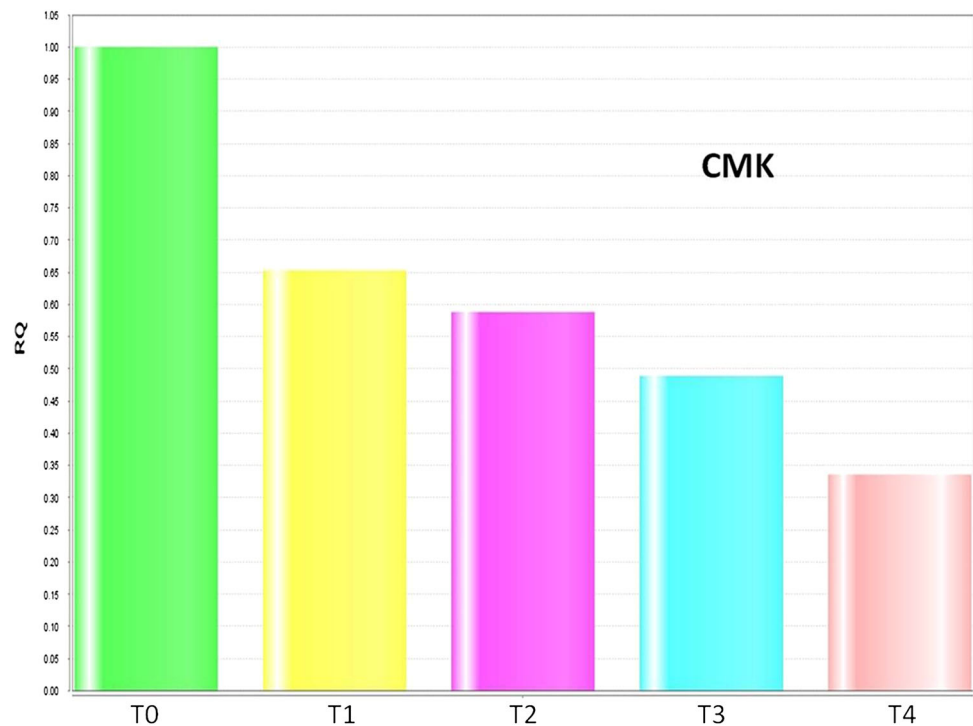
#### Results and Discussion

Homogenous cultures of *S. rebaudiana* were established in control as well as treated plants. No adverse effect of treatment was observed on plant growth and plant vigor was normal (Fig. 2). Reference genes *UBQ* and *GAPDH* were found to be the most suitable among all the treated samples (Fig. 3) and analysis for relative quantification showed that mainly *CMK* was completely downregulated (Fig. 4) as compared to control whereas *CDPS*, *HDR*, *HDS*, *KO*, and *MCS* showed a differential response within treatments (Fig. 5). However, target genes *CMS* and *GGDPS* showed upregulation (Fig. 6) as compared to

**Fig. 3** Deviation plot for the selection of endogenous control (*actin*, *ubiquitin*, and *GAPDH*) genes. (T0: 0 nM (control); T1: 50 nM; T2: 100 nM; T3: 150 nM, and T4: 200 nM of steviol fed to micropropagated plants of *Stevia rebaudiana*). Values in the box at the bottom represent standard deviation of respective endogenous control



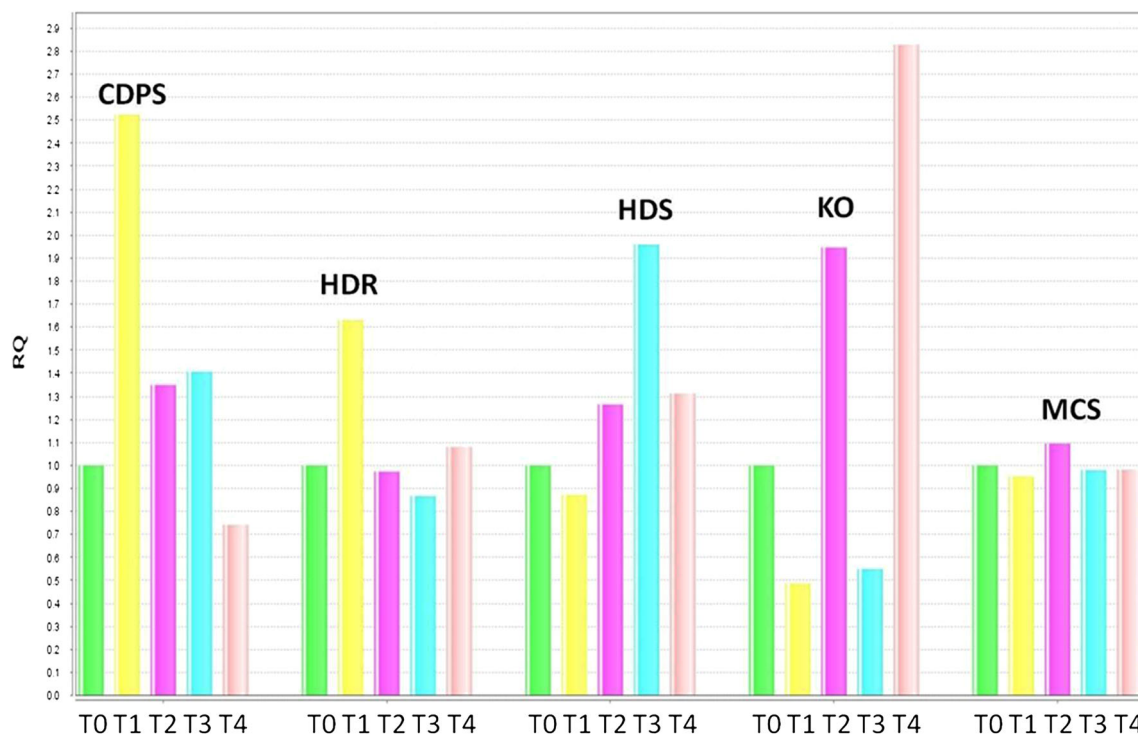
**Fig. 4** Relative quantification of *CMK* within treatments of precursor. Five bars represent one control as well as four treatments. RQ of untreated plants (control) is set as 1.0. (T0: 0 nM (control); T1: 50 nM; T2: 100 nM; T3: 150 nM, and T4: 200 nM of steviol fed to micropropagated plants of *Stevia rebaudiana*)



control (except for the second treatment in which the fold change value was slightly higher than control) and the remaining seven genes from the pathway showed upregulation in the first treatment and subsequent downregulation

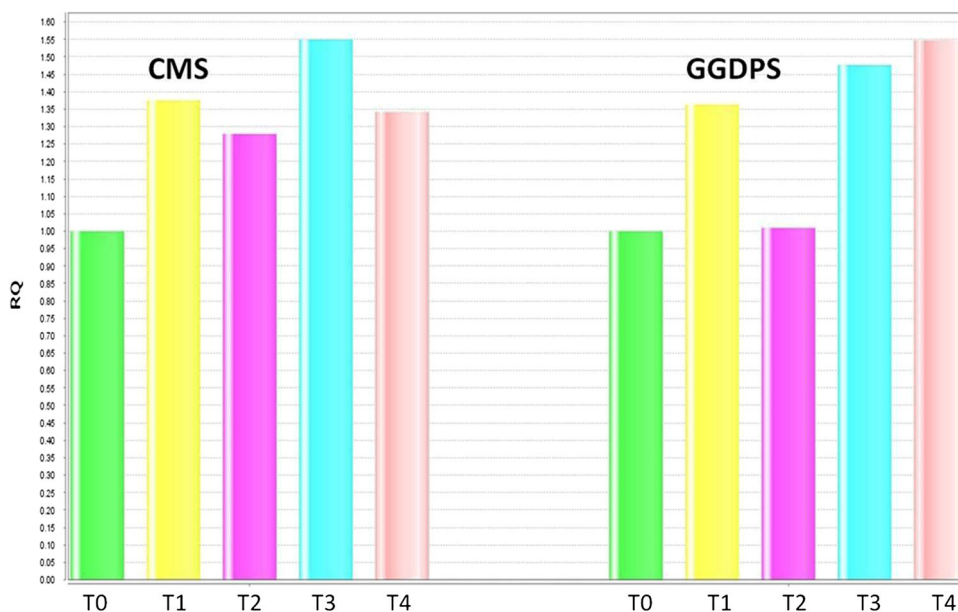
in other treatments (Fig. 7). Moreover, transcripts of *KAH* were found in both roots and leaves (Fig. 8a) but much less downregulation was observed in roots as compared to leaves (Fig. 8b). The transcripts of three predominant





**Fig. 5** Relative quantification of *CDPS*, *HDR*, *HDS*, *KO*, and *MCS* within treatments of precursor. Five bars represent one control as well as four treatments. RQ of untreated plants (control) is set as 1.0. (T0: 0 nM (control); T1: 50 nM; T2: 100 nM; T3: 150 nM, and T4: 200 nM of steviol fed to micropropagated plants of *Stevia rebaudiana*)

**Fig. 6** Relative quantification of *CMS* and *GGDPS* within treatments of precursor. Five bars represent one control as well as four treatments. RQ of untreated plants (control) is set as 1.0. (T0: 0 nM (control); T1: 50 nM; T2: 100 nM; T3: 150 nM, and T4: 200 nM of steviol fed to micropropagated plants of *Stevia rebaudiana*)

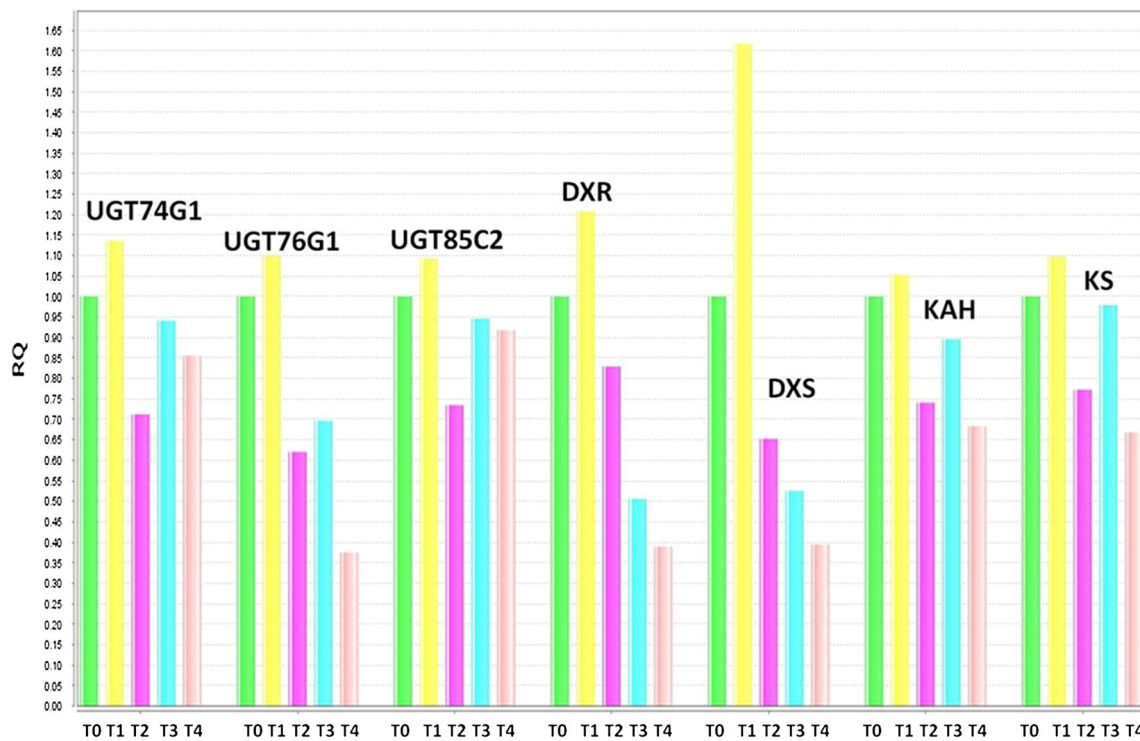


genes involved in glycosylation of steviol after *KAH* gene (*UGT85C2*, *UGT74G1*, and *UGT76G1*) were also reduced up to 30, 24, and 22 %, respectively, in roots as compared to leaves (Fig. 9).

Here it is noteworthy that the transcript of kaurenoic acid is also found in roots which suggests the possibility of the occurrence of steviol in roots and further glycosylation

may also take place in roots because the transcript level of the three enzymes involved in glycosylation was ample.

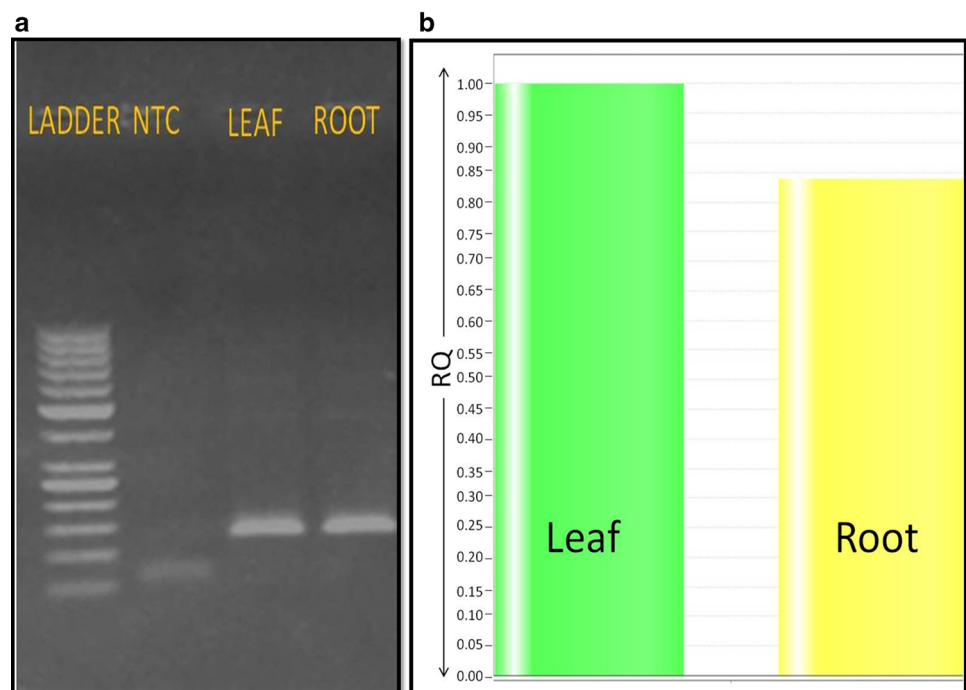
This result reveals the production of steviol glycoside in roots and supports the result obtained by Bondarev and others (2003). Seven genes from the pathway showed upregulation in the first treatment and downregulation in subsequent treatments. In the present investigation, we



**Fig. 7** Relative quantification of *UGT74G1*, *UGT76G1*, *UGT85c2*, *DXR*, *DXS*, *KAH*, and *KS* within treatments of precursor. Five bars represent one control as well as four treatments. RQ of untreated

plants (control) is set as 1.0. (T0 0 nM (control); T1 50 nM; T2 100 nM; T3 150 nM, and T4 200 nM of steviol fed to micropropagated plants of *Stevia rebaudiana*)

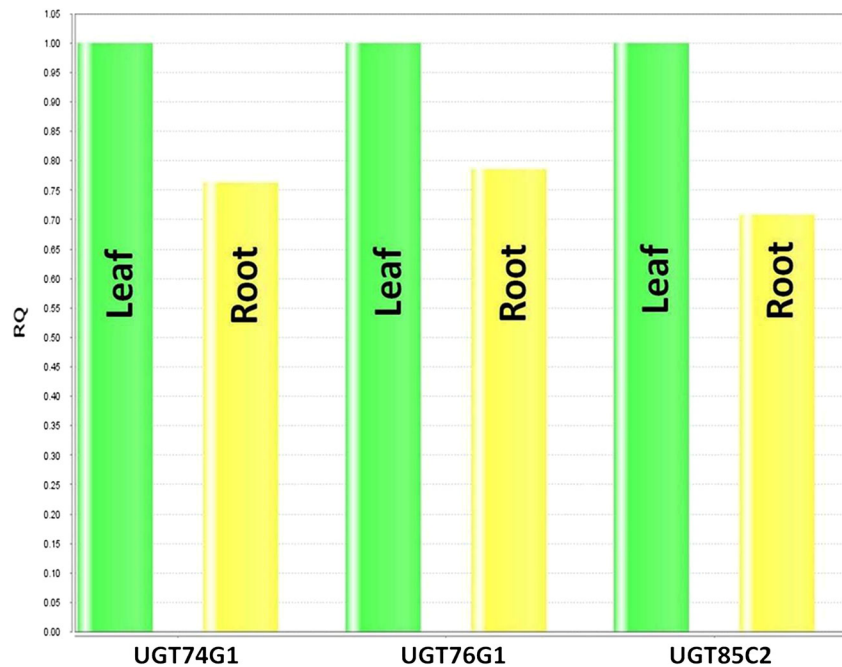
**Fig. 8** Relative transcript of kaurenoic acid hydroxylase in root and leaf tissue of *Stevia rebaudiana*; **a** result of reverse transcriptase polymerase chain reaction revealing the presence of *kaurenoic acid hydroxylase* gene expression in leaf and root tissue and **b** relative quantification of the same gene through real-time PCR



hypothesized that the steviol supplied at increasing concentrations may induce glycosylation but the result revealed that the plant promotes glycosylation only at very

low supplementation of the immediate precursor. Thus, a very low quantity of precursor stimulates steviol glycosides but it is just required to induce that, because further

**Fig. 9** Relative gene expression of genes involved in glycosylation in leaf and root of *Stevia rebaudiana*



application of precursor was unable to stimulate the enhancement. Some of the genes responded differentially to the treatments thus there may be no direct impact of precursor supply on their expression levels. Analysis of these genes in roots and leaves suggested that synthesis of steviol glycosides also occurred in roots but at much lower levels than that observed in leaves. Thus, the immediate precursor of steviol glycosides (steviol) may be synthesized in roots (because transcripts of kaurenoic acid hydroxylase and the genes involved in glycosylation were also found in the roots). This is in agreement with the result obtained by Swanson and others (1992) and there is much less chance for synthesis of any elicitor molecule which could be further transported in the leaf to stimulate as suggested.

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