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Effect of Sucrose on Steviol Glycoside Biosynthesis Pathway in *Stevia rebaudiana*

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Abstract: *Stevia rebaudiana* is a well known natural sweetener. The sweetness is due to steviol glycosides, the glucosylated steviol derivatives of steviol glycoside biosynthesis pathway. In tissue culture conditions, sucrose is utilized as carbon source. In present study, we analyzed the effect of varying sucrose (1, 3 and 5%) concentrations on the genes involved in steviol glycoside biosynthesis pathway and content of steviol glycosides. The higher endogenous sucrose content in 5% sucrose treated plants than that in 3 or 1% treated plants suggested the uptake of exogenously available sucrose by plants. The transcript expression profiling of genes involved in steviol glycoside biosynthesis pathway showed an overall increase in the expression of pathway specific genes; CDPS, KS, KO, UGT85C2 and UGT76G1 in 5% sucrose treated plants compared to that in 1 and 3% treated plants. Furthermore, the quantitative estimation of steviol glycosides in leaves revealed that approximately 4.5 times higher glycosides accumulated in 5% sucrose treated plants than that in 3 and 1% treated plants. However, 1 and 5% sucrose was to be affecting stomatal and trichome density, including the germination rate in comparison to 3% sucrose. Present work thus suggests that sucrose might be acting as an enhancer of transcriptional trigger to the genes of steviol glycoside biosynthesis pathway that could positively manipulate the production of steviol glycosides.

Key words: *Stevia rebaudiana*, steviol glycoside, transcript expression, sucrose

INTRODUCTION

Stevia rebaudiana, the sweet herb of Paraguay, is one among the 150 known species of *Stevia*. It is emerging world-wide as a natural calorie-free sweetener (Debnath, 2008; Carakostas *et al*., 2008; Yadav and Guleria, 2011). The secondary metabolites known as steviol glycosides are responsible for this sweetness (Bondarev *et al*., 2003). The ability of sweetness has rendered *Stevia* a distinguishable medicinal agent and an important alternative for artificial sweeteners. These glycosides are synthesized via steviol glycoside biosynthesis pathway operating in the leaves of *Stevia;* making leaves an important commercial entity. In present times, this pathway has gained an immense interest because very less genetic information is available about it (Yadav and Guleria, 2011; Richman *et al*., 1999; Brandle and Telmer, 2007). Steviol glycoside biosynthesis pathway has sixteen steps catalyzed by different enzymes. Initial seven steps are in common with Methyl Erythritol-4-Phosphate (MEP) pathway synthesizing isoprenoids. The next four steps share similarity with gibberellic acid biosynthesis pathway. These steps have been reported to be catalyzed by enzymes GGDPS (geranylgeranyl diphosphate synthase), CDPS (copalyl diphosphate synthase), KS (kaurene synthase) and KO (kaurene oxidase). Unlike other plants, the genes encoding enzymes CDPS, KS and KO were reported to be present in duplicate functional forms in *Stevia*. Presence of two functional copies of these genes has suggested their efficient role in steviol glycoside biosynthesis pathway as well. The rest five downstream steps are specific for steviol glycoside biosynthesis pathway. These are catalyzed by KAH (kaurenoic acid-13-hydroxylase) and four UGTs (UDP-glycosyltransferases) identified as UGT85C2, UGT74G1 and UGT76G1. One UGT still remained unidentified. So, the genes encoding following enzymes KAH, UGT85C2, UGT74G1 and UGT76G1 were found significantly important for steviol glycoside biosynthesis pathway (Yadav and Guleria, 2011; Brandle and Telmer, 2007).

From times till date, studies have well documented the multifarious important functions of carbon source sucrose during plant development from their germination to

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maturity. Sucrose has been reported to enter into various metabolic pathways and initiate release of energy wherever required. In addition, sucrose also acts as gene regulator (Koch, 1996). An interesting fact regarding sucrose responsiveness is that a specific concentration of sucrose is a necessity for plant growth and survival and at the same time mere increase or decrease from the normal concentration produces adverse results. It has been suggested that plants respond to changing sucrose content by undergoing morphological and anatomical variations as well as by regulating the expression of various genes via a variety of signal transduction pathways (Koch, 1996; Smeekens, 2000; Loreti *et al*., 2001).

Thus, sucrose has been considered an important factor assisting plant growth, survival and maintenance. Keeping in view the importance of steviol glycoside biosynthesis pathway and regulatory aspects associated with exposure of sucrose, present research aims to study the influence of variable sucrose concentration on the transcript expression pattern and metabolite production of steviol glycoside biosynthesis pathway in *Stevia rebaudiana*.

MATERIALS AND METHODS

Seed germination and sucrose treatment: *Stevia* seeds were rinsed thrice in autoclaved distilled water. They were surface sterilized by soaking in 1% sodium hypochlorite for 10 min. The seeds were then again rinsed with autoclaved distilled water to remove the surfactant. The germination media used was constituted by Murashige and Skoog (MS) salts supplemented with MS vitamins solution (1000X), 0.8% agar and sucrose. The pH of the media was maintained at 5.8. It has been known for many plants that 3% sucrose is appropriate for *in vitro* plant growth and maintenance. So, *Stevia* seeds were exposed to 1 and 5% sucrose concentrations in addition to 3% sucrose concentration. The plants were treated with varying sucrose concentration by supplementing the germination media with 1, 3 and 5% sucrose. Equal number of 25 sterilized *Stevia* seeds was placed on petri plates containing the defined sucrose concentrations. These plates were placed under normal 16/8 hour light/dark tissue culture conditions. The number of germinated seeds was observed every week after germination (wag) till one month. The data is presented as mean±SD of mean value of three measurements.

Estimation of endogenous sucrose content: Endogenous sucrose was extracted by crushing 100 mg of dried sample with 80% ethanol and boiling at 95°C for 10 min. The obtained extract was vacuum dried. For sucrose estimation lyophilized samples were mixed with KBr to make pellet in pellet maker. The respective pellets were analyzed on Fourier Transform Infrared Spectroscopy (FTIR; Thermonicolet 6700 IR). The 256 scans were obtained at the resolution of 4 cm^{-1}. The band at 1051 cm^{-1} was selected for analysis as mentioned earlier (Patra *et al*., 2010). A calibration curve was prepared using sucrose as standard over a range of 1-5%.

Morphological analysis: The plants exposed to varying sucrose concentrations were analyzed for responsive morphological variations using Hitachi S-3400N SEM (scanning electron microscope). SEM has a magnification range varying from 45X-30,00,000X. For SEM analysis, third leaf from top was taken from 40 days old plants. Leaves were washed with distilled water and carbon coated using Ion Sputter. The samples were placed on the aluminium stub and dried at room temperature in a controlled environment for SEM imaging. Leaves were scanned on VP-SEM mode at desired magnifications. The number of trichomes and stomata were counted on adaxial and abaxial surface, respectively (area = 0.25 mm^2). The data is presented as Mean±SD of mean value of three measurements.

Expression analysis of steviol glycoside biosynthesis pathway gene transcripts: For evaluating the influence of variable sucrose content on steviol glycoside biosynthesis pathway, transcript analysis of genes CDPS, KS, KO, KAH, UGT85C2, UGT74G1 and UGT76G1 was carried out. Total RNA was isolated from young leaf tissues of 40 days old *Stevia* plants. Total RNA was isolated using Qiagen RNeasy Plant Minikit as per manufacturer's instructions. RNA was quantified using Nanodrop ND-1000 (Nanodrop Technologies, USA). cDNA was synthesized using 50 ng of RNA in the presence of 100 U Superscript III Reverse Transcriptase (Invitrogen), 1 μL of 10 mM dNTPs mix and 250 ng oligo-$(dT)_{12\text{-}18}$, respectively. Two μL of the synthesized cDNA was utilized as template to study the expression profile of steviol glycoside biosynthesis pathway genes via semi-quantitative reverse transcription PCR.

Gene specific primers were designed and utilized for the transcript analysis (Table 1). The 26sRNA gene expression was used as an internal control. The optimized reaction conditions were: 94°C, 5 min for 1 cycle; 94°C, 30 sec; 50-58°C, 1.5-2.5 min (gene specific annealing temperature and annealing time is mentioned in Table 1); 72°C, 1 min for 28 cycles. Amplified products were gel scanned using Alpha DigiDoc gel documentation system (Alpha Innotech, USA). Three replicates were

Table 1: PCR related details of the studied genes of steviol glycoside biosynthesis pathway

Gene name	Gene specific primer	Annealing temperature (°C)	Annealing time (min)
CDPS (Copalyl diphosphate synthase)	Forward 5'CTAGAATGAAGACCGGCTTCATC3' Reverse 5'GGATCCTCATATTACAATCTCGAACAC3	55.0	2.5
KS (Kaurene synthase)	Forward 5'AGATCTATGAATCTTTCACTATGCATC3' Reverse 5'ACTAGTTTACCTTTGTTCTTCATTTTC3'	51.0	2.5
KO (Kaurenoic acid)	Forward 5'AGATCTATGGATGCCGTCACCGG3' Reverse 5'ACTAGTTCATATCCTGGGCTTTATTATG3'	51.0	1.5
KAH (Kaurenoic acid-13 hydroxlase)	Forward 5'AGATCTATGATTCAAGTTCTAACACCG3' Reverse 5'ACTAGTTCAAACTTGATGGGGATGAAG3'	54.0	1.5
UGT85C2 (UDP glycosyltransferase)	Forward 5'AGATCTATGGATGCAATGGCTACAAC3' Reverse 5'ACTAGTCTAGTTTCTTGCTAGCACG3'	51.0	1.5
UGT74G1 (UDP glycosyltransferase)	Forward 5'AGATCTATGGCGGAACAACAAAAGATC3' Reverse 5'ACTAGTTTAAGCCTTAATTAGCTCACT3'	52.0	1.5
UGT76G1 (UDP glycosyltransferase)	Forward 5'CCATGGATGGAAAATAAAACGGAGAC3' Reverse 5'AGATCTTTACAACGATGAAATGTAAG3'	54.0	1.5

run for each gene with each sample. Relative gene expression was calculated by determining the Integrated Density Values (IDV) of gel bands using AD-1000 software.

Quantitative estimation of steviol glycosides: Leaves of sucrose treated plants were dried in open and crushed in mortar. The extraction of steviol glycosides were performed with 100 mg of each sample. Methanol: water (80:20, v/v) was used as extraction solvent. The extraction was carried out at room temperature for 12 h. The obtained extracts were filtered and dried *in vacuo*. Dried extracts were defatted with hexane and the resultant residual matter was vacuum dried. The finally obtained extract was dissolved in acetonitrile: water (80:20, v/v), filter sterilized and processed for HPLC analysis. Samples were injected into the Lichrosphere amino column using an isocratic solvent system of acetonitrile:water (80:20, v/v). The flow-rate was 0.8 mL min^{-1}. Column temperature was maintained at 25°C throughout the experiment (Jaitak *et al*., 2009). Steviol glycosides were detected at 205 nm by PAD and were identified by comparison of their retention times with that of the standards. The calibration curve was prepared by using steviol glycosides as standard over a range of 0.1-0.5 mg mL^{-1}.

RESULTS AND DISCUSSION

Sucrose has been considered as an important factor regulating growth and metabolism of plants. For the cultivation and maintenance of plants *in vitro*, 3% sucrose has been recommended optimum. In view of this, we evaluated the impact of low and high sucrose content on the leaf morphology and steviol glycoside biosynthesis pathway during normal photoperiod in *Stevia rebaudiana*.

Endogenous sucrose content of *Stevia rebaudiana* increased upon increase in sucrose exposure: In order to identify whether exogenous sucrose influencing test plants, the endogenous level of sucrose was estimated. For comparative sucrose estimation, FTIR spectroscopy was used to detect sucrose band at 1051 cm^{-1} as described earlier (Patra *et al*., 2010). Plants treated with 5% sucrose were found to contain highest sucrose content of 3.32 mg mL^{-1} than 1 or 3% treated samples which possessed 1.16 and 2.66 mg mL^{-1} sucrose content, respectively (Fig. 1). The endogenous level of sucrose documented the absorption of exogenous sucrose by treated plants. Hence, the increased content of endogenous sucrose with increase in exogenous sucrose supply suggested that plants were positively up-taking the sucrose provided via germination media.

Variation in seed germination and leaf morphology of *Stevia rebaudiana* in response to variable sucrose concentration: Various reports are present documenting the influence of varying sucrose concentration on plant growth, morphology, gene expression pattern and metabolism (Ho *et al*., 2001; Verlinden and Garcia, 2004; Rekarte-Cowie *et al*., 2008; Arsenault *et al*., 2010; Ferri *et al*., 2011). In the present study, the influence of varying sucrose on seed germination was evaluated. Equal numbers of 25 seeds in each perti plate were placed on media possessing 1, 3 and 5% sucrose. Number of germinated seeds was counted on each plate after one week of germination till four weeks. Only 3 plants were reported to grow on 1% as well as 5% sucrose plates after a period of one month. The media possessing 3% sucrose, however, showed maximum germination with 9 plants out of 25 seeds (Fig. 2a). Further, it was observed that plants grown in 3% sucrose were healthier but undergone variations in response to 1 and 5% sucrose content. It has been reported that varying sucrose level delayed seed germination. A study on *Brassica napus* has reported delayed seed germination and restrained seedling development on exposure of 167 and 333 mM sucrose. In addition, expression profile of genes involved in energy

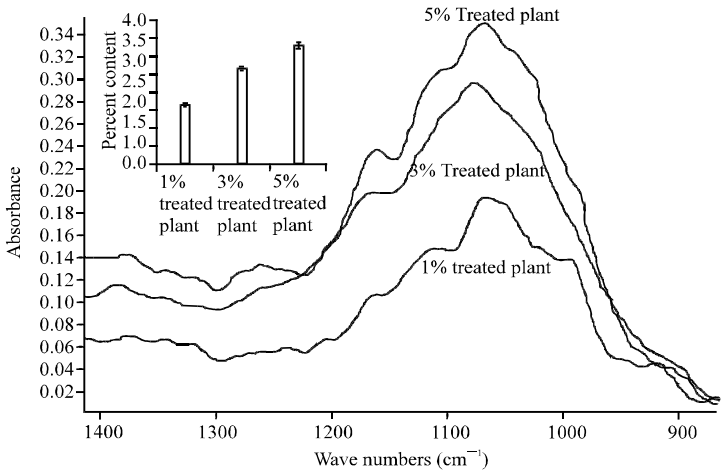

Fig. 1: FTIR absorption spectra of 1, 3 and 5% sucrose treated plant samples. The peak at 1051 cm^{-1} corresponds to the endogenous sucrose level of plant samples. In set- graph shows the percent content of endogenous sucrose in 1, 3 and 5% treated plants

metabolism has also been reported to be significantly affected with increasing sucrose (Xu *et al*., 2010). So like other plants, 3% sucrose concentration was observed as the optimum concentration for the growth of *Stevia* in tissue culture conditions.

SEM analysis was carried out to notice the morphological variations of leaves due to variability in sucrose content. It has already been revealed that plants have an unusual tendency to respond for the sucrose availability. Morphological and anatomical variations are the basic alterations that plants undergo to cope with the varying environmental conditions (Koch, 1996). In the present experiment, a difference was observed in the number and type of trichomes, as well as number and aperture movements of stomata. A comparative increase was observed in the leaf stomatal density when exposed to 1% as well as 5% sucrose concentrations. The 5% sucrose treated plants, however, possessed an overall increase in the stomatal density than that in 1 or 3% sucrose treatments (Fig. 2b). Another change was noticed in the stomatal aperture movement during these three different sucrose concentrations. In contrast to the stomata during 3% sucrose concentration, they possessed wider open apertures on exposure to 1% sucrose concentration and closed when exposed to 5% sucrose concentration (Fig. 2c). This can possibly be due to water uptake for maintaining inside-outside sucrose concentration during 1% sucrose treatments. Turgor elevation inside guard cells caused stomatal apertures to widen. On exposure to 5% sucrose concentration, stomatal density was higher and the stomatal apertures were comparatively closed possibly due to the removal of water from plant cells that decreased turgor pressure of guard cells and resulted in closure of stomata (Shimazaki *et al*., 2007). This variability in stomata suggests that exogenous sucrose was acting like an osmoticum for the treated plants. Moreover, it has already been known that varying exogenous sucrose acts like an osmoticum rather than mere a carbon source (Kaur *et al*., 2005).

Similarly, it was observed that in comparison to 3% sucrose exposed plants, 1% sucrose treated plants possessed lesser number of large, small and glandular trichomes. However, an increase was observed in the density of small trichomes than large and gland trichomes on exposure of 5% sucrose (Fig. 2d). It has been concluded from several studies that plant trichome density increases with the change in environmental conditions or stress exposure (Espigares and Peco, 1995; Gonzales *et al*., 2008). The presence of varied trichome density in 1 and 5% sucrose treated plants than that in 3% sucrose treatments documented that 1% as well as 5% sucrose concentrations has exposed plants to osmotic stress.

Expression analysis of genes of steviol glycoside biosynthesis pathway in response to sucrose variability: In order to have an account of sucrose variability on steviol glycoside biosynthesis pathway, the transcript expression of seven genes of the pathway was analyzed;

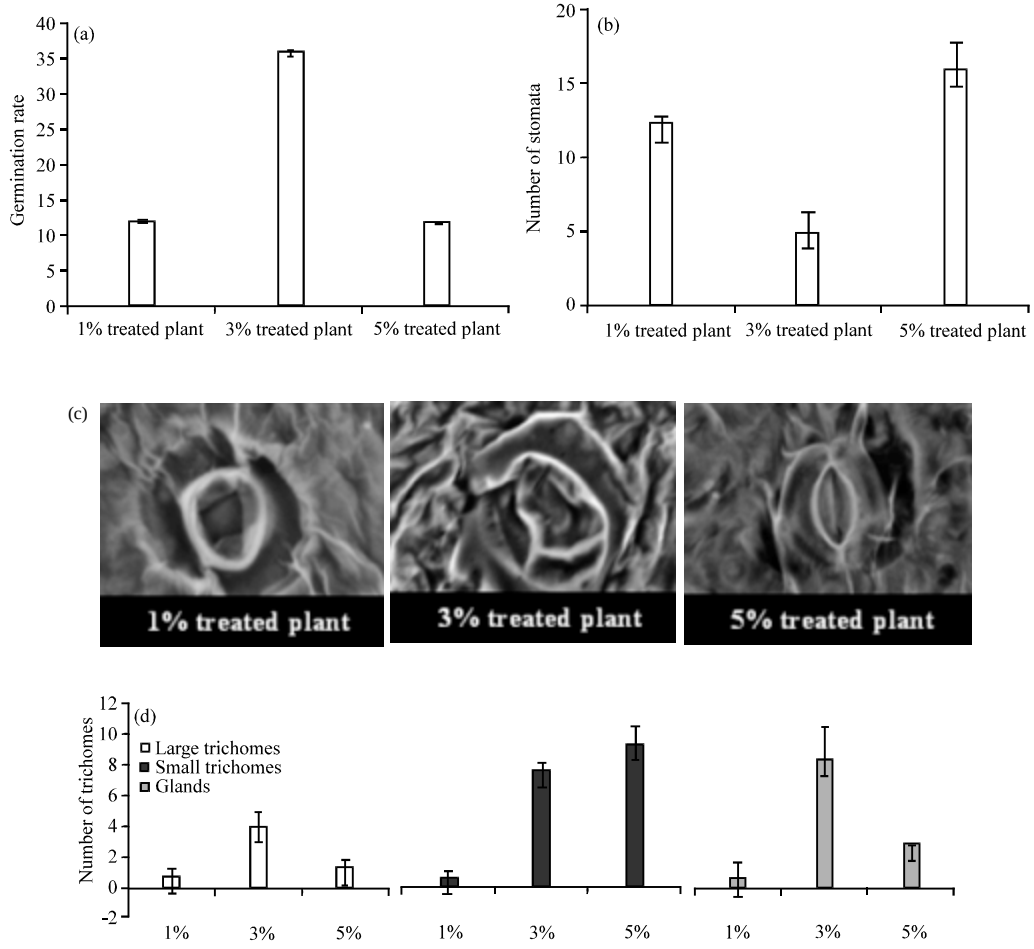

Fig. 2(a-d): Effect of varying concentration of sucrose on *Stevia rebaudiana*. a) Percent germination rate in response to 1, 3 and 5% sucrose exposure. Twenty-five seeds were sown per petriplate. The experiment was repeated thrice and data was presented as Mean± Standard Deviation (SD) of mean value of three measurements. b) Variation in stomatal density in response to 1, 3 and 5% sucrose exposure. Number of stomata was counted on area 0.25 mm^2. c) Variation in stomatal aperture movements on exposure of 1, 3 and 5% sucrose concentration as observed by SEM analysis of abaxial leaf surfaces. d) Effect of 1, 3 and 5% sucrose exposure on density of small, large and gland type of trichomes. Number of each type of trichome counted on area 0.25 mm^2

CDPS (copalyl diphosphate synthase), KS (kaurene synthase), KO (kaurene oxidase), KAH (kaurenoic acid-13-hydroxylase) and three UGTs (UDP-glycosyltransferases) UGT85C2, UGT74G1 and UGT76G1. For this, *Stevia* seeds were germinated on media supplemented with 1, 3 and 5% sucrose concentrations. Exposure of *Stevia* to variable sucrose was found to alter the transcript expression levels of steviol glycoside synthesis pathway genes in a dose dependent manner. Exposure to 1% sucrose concentration repressed the expression of most of the studied genes except UGT76G1. On the other hand, exposure of plants to 5% sucrose concentration upregulated the expression of CDPS, KS,KO, UGT85C2 and UGT76G1 genes compared to 1 and 3% sucrose exposure, except KAH and UGT74G1 which were observed to be upregulated on exposure of 3% sucrose. Taken together the changes in sucrose exposure concentration from 1 to 5%, most of the genes related to steviol glycoside pathway were upregulated (Fig. 3a). So, expression of steviol glycoside biosynthesis pathway specific genes was observed to be enhanced in response to 5% sucrose during normal photoperiod. The alteration in transcript expression profile in response to varying sucrose suggested the sucrose responsiveness of steviol

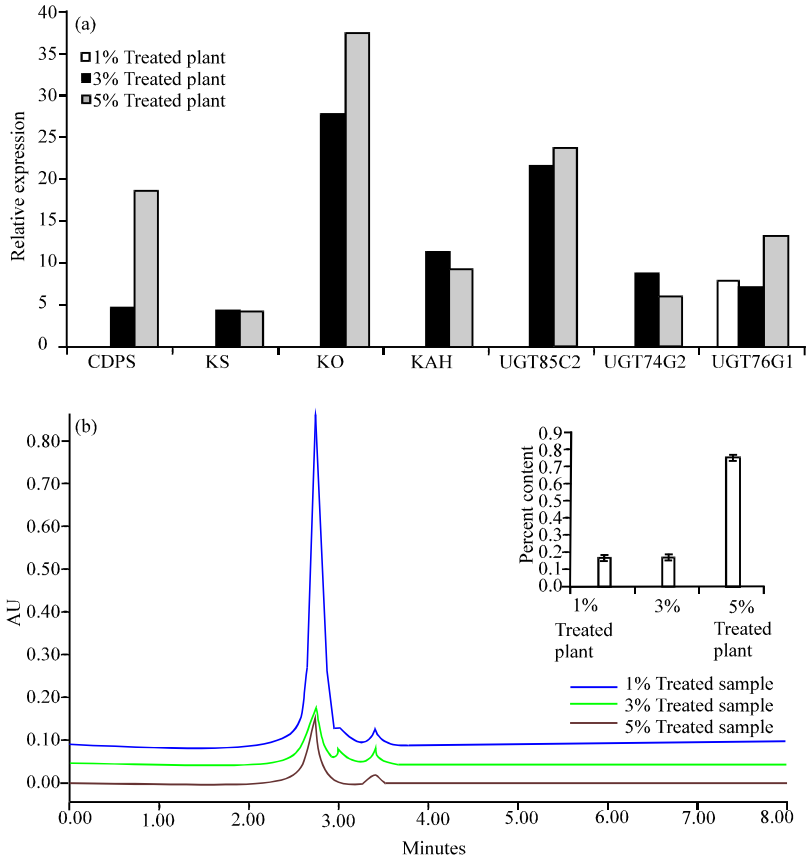

Fig. 3(a-b): Relative expression of genes encoding enzymes of steviol glycoside biosynthesis pathway. Expression of CDPS, KS, KO, KAH, UGT85C2, UGT76G1 and UGT74G1 was analyzed in *Stevia* upon exposure to 1, 3 and 5% sucrose. 26sRNA was used as internal control to equalize cDNA quantity. Each bar in the diagram corresponds to the IDV of its respective amplified band. b) HPLC chromatogram of 1, 3 and 5% sucrose treated plant samples. The absorption peak at retention time 2.75 min corresponds to the steviol glycosides of plant samples. In set- graph shows the percent content of steviol glycosides in 1, 3 and 5% treated plants

glycoside biosynthesis pathway related genes. These results were in accordance to the study carried out in sugarcane. In sugarcane, the genes associated with its sucrose content were responsive to varying sucrose treatments. Interestingly, most of these sugar responsive genes were involved in the stress response of sugarcane (Papini-Terzi *et al*., 2009).

Quantitative estimation of steviol glycosides: HPLC was carried out to quantify the steviol glycosides from plants treated with sucrose. Dried leaf samples were extracted with methanol:water and defatted with hexane. The final obtained extract was dissolved in acetonitrile:water and analyzed with HPLC (Jaitak *et al*., 2009). It was found that plants treated with 1 and 3% sucrose possessed almost equal 165 ng mL^{-1} of steviol glycosides, however, 5% sucrose treated plants showed highest content of 750 ng mL^{-1} steviol glycosides (Fig. 3b). The data thus accounts that with the increase in exposure of sucrose concentration to 5%, the transcriptional trigger was enhanced that increased the content of steviol glycosides. Till date no such report regarding the correlation of sucrose and steviol glycoside content is present. This is the first report emphasizing the proportionate increment in steviol glycosides with the enhanced sucrose levels.

In conclusion, results document that exposure of varying sucrose concentrations have imposed osmotic imbalance around *Stevia* plants. The increase in endogenous sucrose content with increase in exogenous sucrose from 1 to 5% suggests that plants were absorbing the sucrose via germination media. The plant underwent various morphological variations on 1 and 5% sucrose treatment; however, plants grown on 3% sucrose were quite healthy. Interestingly, the expression profile of genes involved in steviol glycoside biosynthesis pathway was significantly altered during sucrose variability. Increase in exogenous sucrose level significantly

enhanced the specific genes of steviol glycoside biosynthesis pathway. Furthermore, transcript alterations were favoured by the data of quantitative steviol glycoside estimations. Thus, the present data suggested the responsive nature of steviol glycoside biosynthesis pathway towards sucrose variability in *Stevia rebaudiana*. Also, exposure of *Stevia* plants to an enhanced exogenous sucrose could be a measure to significantly increase the yield of steviol glycosides.

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