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Original Research

Effects of different concentrations of mannitol on gene expression in *Stevia rebaudiana* Bertoni

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Abstract: *Stevia rebaudiana* Bertoni is one of the most important herbal sweetener plants from Asteracea family that have a lot of Steviol glycosides. Among different methods, tissue culture is the best way with high efficiency that is useful for studying stress tolerance mechanisms to obtain drought tolerance of stevia. For this purpose, different concentrations of mannitol (0, 10, 20, 30, 40, 50 mg/l) were used as various treatments in the culture medium of stevia. According to the results, the highest level of *UGT85C2* gene expression (1.181 Total lab unit) was seen in plants grown under 30 mg/l mannitol treatment and the lowest level of this gene expression (0.603 Total lab unit) was observed under 40 mg/l mannitol treatment. However, the highest level of *KO* gene expression (1.323 Total lab unit) was observed under 20 mg/l mannitol. It shows stevia growth is affected by osmotic stress. Water deficiency has a negative impact on Stevia. However, the expression of genes had increased by particular mannitol concentrations. Actually, stevia can survive under various abiotic stresses.

Key words: *Stevia rebaudiana* Bertoni; Mannitol; Semi-quantitative RT-PCR; Tissue culture; *UGT85C2; KO.*

Introduction

One of the most common environmental stresses is the drought that affecting plant growth, productivity and plant morphology or physiology (1, 2). *Stevia rebaudiana* Bertoni is one of the most important herbal sweetener plant from Asteracea family that have a lot of steviol glycosides such as rebaudioside A, B, C, D, E, F, M, steviol bioside, dulcoside A, dulcoside C and stevioside with zero calorie that have a lot of applications in medicine and food industry (3-9). Some studies reported that steviol glycosides especially steviosid, are 300 times sweeter than sucrose, depending on agricultural operations, use of organic fertilizers in a technical way and water management (10, 11). Steviol glycosides biosynthesis pathway or MEP pathway contain fifteen genes such as *KAH, DXS, DXR, CMS, CMK, MCS, HDS, HDR, GGDPS, CDPS, KS, KO, UGT, UGT76G1, UGT74G1* and *UGT85C2*. Different studies showed the importance of *KS, KO, UGT76G1, UGT74G1* and *UGT85C2* in regulating steviol glycosides content (26).

Another study that used polyethylene glycol (PEG) and NaCl as abiotic stress agent reported the concentration of steviol glycosides induced in these stress conditions, so drought and salt stress may be effective for optimizing the steviol glycoside composition (12, 13). Among different methods such as seed technique, stem cutting technique and tissue culture for propagating stevia, tissue culture is the best method with high efficiency that is useful for studying stress tolerance mechanisms under sterile and controlled environmental conditions to obtain drought tolerance (1, 3, 14-18).

Richmann et al. in 2005 reported that among out of 12 UGTs genes that exist in *Stevia rebaudiana* Bertoni, 3 UGTs such as *UGT85C2*, *UGT74G1* and *UGT76G1* have specific roles in the biosynthesis of rebaudioside A. Another study that using RT-qPCR method reported that among 3 UGTs, the *UGT85C2* gene show positive expression in the producing of stevioside in rhizoclone, and this gene in combination with photosynthetic proficiency regulate the biosynthetic pathway of *Stevia rebaudiana* in hairy root cultures (19-21).

Mannitol is one of the most important agents that used for inducing drought stress in tissue culture. A recent study showed that increasing the concentration of mannitol in medium led to decrease shoot length, internode length, and fresh weight of plants and growth rate of *S. rebaudiana* (2, 22).

Also, the present study was carried out with an objective of studying the effect of various concentration of mannitol on the expression level of genes in MEP pathway (*UGT85C2* and *KO*) of *S. rebaudiana* under in

vitro condition.

Materials and Methods

Plant materials and culture conditions

The *S. rebaudiana* plants were provided by the Zagros Bioidea Co. Razi University, Kermanshah, Iran. Stevia propagation was carried out by tissue culture. Axillary buds (1.5-2 cm in length) with two leaves were separated from the shoots of stevia and were planted on MS medium (23), with 30 g/L sucrose supplemented and different concentrations of mannitol (0, 10, 20, 30, 40 and 50 mM/l). The pH of the medium was adjusted to 5.8 and after that 8 g/L agar was added and autoclaved. Each medium contained five explants and cultures were incubated at 25 ± 1°C under 16 h light and 8 h dark photoperiod provided by cool white fluorescent lamps with 3000 Lux intensity and relative humidity 72 to 75%.

RNA extraction

The fresh leaves were used to extract total RNA using the RNX plus™ kit (Cinnaclon), according to the manufacturer's instructions. RNA quantification was done using NanoDrop Spectrophotometer (Nanodrop®, ND-1000, Nanodrop Technologies, and Wilmington, USA). Also, all RNA isolates had an OD260:OD280 between 1.8 and 2.0. Also, The RNA quality was tested by 1.0 % agarose gel electrophoresis.

Expression analysis of *UGT85C2* and *KO* genes

The two-step semi-quantitative RT-PCR method was used to determine gene expression of *UGT85C2* and *KO* genes in stevia. For cDNA synthesis, 10 µg of total RNA was reversely transcribed with 100 UM-Mulv reverse transcriptase in a total volume of 20 µL of Master Mix containing 1 µL oligo (dT)18 primer, 2 µL of 10X M-MuLV buffer, 1 µL of each dNTP and Nuclease-free Water, according to the manufacturer's recommendations (Viva 2-steps RT-PCR Kit, Vivantis, Malaysia). The *β-Actin* house-keeping gene was used as the internal control. Primers for target and *β-Actin* genes were designed using the Oligo 7 Primer Analysis Software and to achieve specific characters required for semi-quantitative polymerase chain reaction (RT-PCR) (24; Table 1). RT-PCR reactions were performed for the targets and house-keeping gene. PCR reaction mixture (25 µL) contained 2µL of cDNA, 0.5 µL of dNTPs (10 mM), 1 µL of each primer (Forward and Reverse primer), 0.32 µL of MgCl2, 2.5 µL of 10x PCR buffer and 0.5 µL of *Taq* DNA polymerase (5U/µL). PCR reaction was performed as initial denaturation at 94°C for 7 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and then a final extension at 72°C for 7 min.

The PCR products were separated by electrophoresis on a 2% agarose gel in TBE buffer. Four independent experiments were conducted. The amplicons were quantified by the Total Lab TL120 v2009 software (Nonlinear Dynamics Ltd). Which delivers quantitative estimates of the amplicon band intensities by changing them into corresponding numerical values. The expression levels of *UGT85C2 and KO genes* were normalized relative to the amount of *β-Actin* expression.

Statistical analysis

Data analysis was performed by Excel and SPSS Ver. 16 softwares. Collected data had a normal distribution, so it was used directly for statistical analysis. Also, the mean comparison was performed by Duncans multiple range test with a critical value of $P < 0.05$.

Results

The expression level of *UGT85C2* and *KO* under different concentrations of mannitol was shown in Figure 1. According to the results, the highest level of *UGT85C2* gene expression was seen in plants grown under 30 mg/l mannitol treatment (1.187 Total Lab unit) which had no significant differences with media contained 10 mg/l mannitol. On the other hand, the lowest level of this gene expression was observed under 40 mg/l mannitol treatment (0.603 Total Lab unit) which had no significant differences with 50 mg/l mannitol.

However, the highest level of *KO* gene expression was observed under 20 mg/l mannitol (1.323 Total Lab unit). On the other hand, the lowest level of this gene expression was observed under 40 mg/l mannitol treatment (0.940 Total Lab unit). There were no significant differences between 20 mg/l mannitol and other treatments except 40 mg/l mannitol treatment (Figure 2).

Discussion

According to the results, Stevia growth is affected by osmotic stress. Actually, water deficiency has a negative

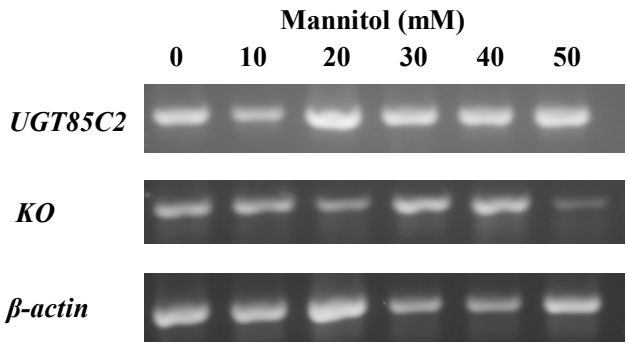

Figure 1. Expression of *UGT85C2* and *KO* in the leaves of *stevia rebaudiana* Bertoni under different concentrations of mannitol. *β-actin* was used as an internal control. The final value was the average of at least four independent experiments. Only the best pictures are shown.

Table 1. The list of primers used in RT-PCR and house-keeping genes.

Gene	Primer sequence 5´→ 3´ (forward/reverse)	Amplicon length (bp)	Accession number
UGT85C2	TTCCACACGTTCGATGAGTT / TGAAGCCACTGGAAACACTC	191	AY345978
KO	CTTGCTATCGGAAGCACAAA / ATCTTCTCTGACCCGCAAAC	186	AY364317
β-Actin	TTGCCCTGAGGTTCTGTTCC/ ATCCGGTCAGCAATACCAGG	171	AP548026

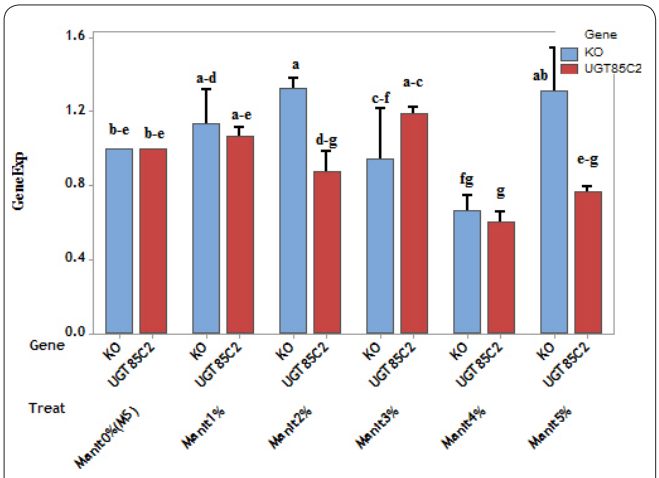

Figure 2. The relative expression level of *UGT85C2* and *KO* (related to *β-actin*) under different concentrations of mannitol. Values are means ± SE of four replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (Duncan's test).

impact on Stevia. However, the expression of genes had increased by particular mannitol concentrations. Actually, stevia can survive under various abiotic stresses, and this issue had been verified in various reports. For example, Zeng et al (2013) studied the effects of different NaCl concentrations on the growth, physiological responses, and steviol glycoside composition of Stevia *rebaudiana.* As they reported, some traits decreased and some of them increased when NaCl added to the media. Finally, it indicates that *S. rebaudiana* is moderately tolerant to salt stress. "Hypohaline soil can be utilized in the plantation of *S. rebaudiana* and may be profitable for optimizing the steviol glycoside composition". Another research reported that the concentration of steviol glycosides induced by abiotic stress such as polyethylene glycol (PEG) and NaCl (12). In another study, Pandey and Chikara, 2015, induced salinity and drought stress by adding NaCl and mannitol to MS culture medium while in this study the gene expression was studied in plants treated with different concentration of mannitol. They reported that *KAH*, *UGT85C2*, *UGT74G1* and *UGT76G1* genes were down-regulated in drought stress conditions (25). Furthermore, Hajihashemi et al, 2013, reported that expression of *UGT85C2* and *UGT76G1* genes decreased in polyethylene glycol treatment (27). Also, Yang et al, 2015, reported that expression of *KO* and *UGT74G1* genes was significantly reduced in polyethylene glycol-treated plants (28). In another study, Fallah et al, 2017, studied the effects of salinity and osmotic stress caused by the different concentrations of NaCl (0, 20, 40, 60 and 80 mM) on morphological traits, genes expression and amount of steviol glycosidws under in vitro conditions. As they reported, with increasing salinity, the values of all studied morphological traits decreased and the highest expression of *UGT74G1* and *UGT76G1* was observed in plantlets grown on MS media (with NaCl-free). Also, the lowest amounts of gene expression of these genes were seen in MS+ 60 mM NaCl (29). In another study, Kahrizi et al, 2017, studied the effect of different concentrations of KH_2PO_4 on stevia growth factors and gene expression by tissue culture methods, RT-PCR and HPLC. They reported that the best concentration of KH2PO4 for expression of *UGT74G1* was 0.00425mM KH_2PO_4 and the best one for *UGT76G1* expression was 0.017mM KH_2PO_4 (30). Also, Akbari et al, 2017, examined the effects of nitrogen caused by the different concentrations of NH_4NO_3 (0, 825 and 1650 mg/l) and KNO_3 (0, 950 and 1900 mg/l) on the expression of *UGT74G1* and *UGT76G1* genes in stevia. They reported that the highest expression of *UGT74G1* was observed in plantlets grown on MS media with 0 mg/l NH_4NO_3 and 950 mg/l KNO_3 but the highest expression of *UGT76G1* was observed in plantlets grown on MS media with 1650 mg/l NH_4NO_3 +950 mg/l KNO_3. Finally, they suggested that both the growth and the expression level of *UGT74G1* and *UGT76G1* genes were affected by the different concentrations of nitrogen sources (31, 32).

Based on this fact that the steviol glycoside pathway leads to accumulation of these metabolites in stevia, it is very important to identify the best situation for the highest level of expression of the pathway key genes.

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