Effect of salicylic acid on stevioside and rebaudioside A production and transcription of biosynthetic genes in *in vitro* culture of *Stevia rebaudiana*

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Abstract

S. rebaudiana produces steviol glycosides including stevioside and rebaudioside A that are valuable as low calorie sweeteners. The objective of this study was to investigate the effects of salicylic acid elicitation and sampling times on the improvment of stevioside and rebaudioside A production and KA13H, UGT74G1 and UGT76G1 genes expression. The results showed that the addition of different concentrations of salicylic acid had different effects on stevioside and rebaudioside A production in a dosage-dependent manner. Among different concentrations of salicylic acid the highest amount of stevioside was 38.33 mg/g DW and the highest amount of rebaudioside A was 2.93 mg/g DW, observed 96 h after elicitation with 90 mg/L and 48 h after elicitation with 60 mg/L salicylic acid, respectively. Elicitation with salicylic acid increased KA13H and UGT74G1 genes expressions and decreased UTG76G1 gene expression. The correlation analysis indicated that, KA13H gene expression had a positive correlation with stevioside production. The UGT74G1 gene expression had a positive correlation with stevioside production and a negative correlation with rebaudioside A production. The UGT76G1 gene expression had a positive correlation with rebaudioside A and negative correlation with stevioside production. The qRT-PCR analysis indicated that, by increasing stevioside production under salicylic acid elicitation, the KA13H and UGT74G1 genes expression increased and UGT76G1 gene expression decreased.

Key words: Elicitation, Gene expression, Rebaudioside A, *Stevia rebaudiana*, Stevioside.

INTRODUCTION

Stevia rebaudiana is a perennial and natural sweetener medicinal plant belonging to the Asteraceae family (Gupta *et al*., 2015; Yoneda *et al*., 2017). *S. rebaudiana* is a traditional medicinal plant with global importance due the presence of stevioside glycosides including stevioside, rebaudioside A and dulcoside A (Khalil *et al*., 2015). Stevioside and rebaudioside A are nearly 300 times sweeter than sucrose and have been used for the treatment of diabetes, dental maladies, obesity, hypertension and cancer (Lemus-Mondaca *et al*., 2012; Mandal *et al*., 2015). Stevioside reduces blood glucose levels in patients with type II diabetes and reduces blood pressure in patients with hypertension (Bayraktar *et al*., 2016). In the stevioside and rebaudioside A synthetic pathway different enzymes are involved such as KA13H, UGT74G1 and HGT76G1 (Yoneda *et al*., 2017).

Plant cell, tissue and organ cultures are the important strategies for biomass and bioactive compounds production (Ahmad *et al*., 2013; Ali *et al*., 2013). Plant tissue culture techniques produce genetically uniform seedlings with a homogeneous secondary metabolite content at a short time under the controlled conditions.

Therefore, by using this system a more predictable and stable secondary metabolites content can be obtained, but commercially production of plant secondary metabolites remained limited (Yue *et al*., 2016). Elicitors are different exogenous and endogenous molecules that trigger plant defense responses and stimulate secondary metabolites biosynthesis by different signal transduction pathways (Zhao *et al*., 2005; Zare *et al*., 2014). Elicitors have been widely used for theinduction of secondary metabolites production in *in vitro* plant cultures. The most common elicitors used for secondary metabolites production are salicylic acid, methyl jasmonate, chitosan, yeast extract, etc (Murthy *et al*., 2014; Xu *et al*., 2015).

Signal molecules, such as salicylic acid induce a plant defense response to pathogens and insects and elicit secondary metabolites accumulation by the induction of a defense responses (Rhee *et al*., 2010; Sharma *et al*., 2015). Salicylic acid is an endogenous signal molecule, which play an important role in plant response to different stresses (Horváth *et al*., 2007). Salicylic acid is a phenolic compound naturally produced in plant in very low concentrations and has a significant impact on the various aspects of the plant life (Gharib and Hegazi, 2010). In this research, we investigated the effect of different concentrations of salicylic acid and exposure times on stevioside and rebaudioside A production and on KA13H, UGT74G1 and UGT76G1 genes expression in *S. rebaudiana* (Bert.) *in vitro* culture.

MATERIALS AND METHODS

Plant material

Stevia plant was obtained from the College of Agriculture and Natural Resource, University of Tehran, Karaj, Iran. Stem cuttings with 10-15 cm size were prepared and washed for 5 h with tap water. The cuttings were surface-sterilized with 70% (v/v) ethanol for 1 min, 5.1% (w/v) sodium hypochlorite solution for 15 min and rinsed 4 times in sterile distilled water for 6 min. For plant propagation, shoot explants with axillary buds (10-20 mm) size were prepared and transferred to the MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA and maintained in a growth chamber at 25±2 °C with 16 h photoperiod and were sub-cultured every 21 days. For equal growth, the shoots with 6-8 leaflets and without roots were selected and transferred to the MS basal medium for 15 days.

Elicitor treatment

In this study, we used different concentrations of salicylic acid as an elicitor. Stock solutions were prepared and sterilized with 0.2 µm syringe filter. Salicylic acid at concentrations of 0, 30, 60, 90 and 120 mg/L was added to MS basal medium after autoclaving. Subsequently, shoots were transferred to the elicitation MS media including 6.5 mg/L plant agar. Sampling was performed at 24, 48, 72 and 96 h after elicitation. After sampling, some of the samples were frozen in liquid nitrogen and maintained at -80 °C for gene expression analysis.

Extraction of stevioside and rebaudioside A

The stevioside and rebaudioside A extraction from shoots of elicited and control shoots was carried out by the method described by Kolb *et al*. (2001) with a few modifications. The samples were oven dried for 24 h at 50 °C. The dried samples were powdered in the porcelain dish. Then, 2 mL of 70% ethanol was added to 20 mg of the powdered sample and mixed. After that, the samples were placed for 5 h at 70 °C in water bath (Techno, England) and shaken each 5 min. Finally, the mixture was centrifuged at 14000 rpm for 10 min and supernatant transferred to a new tube for HPLC analysis and maintained at -20 °C.

HPLC analysis of stevioside and rebaudioside A

HPLC analysis was performed in a Knauer HPLC system (UV detector, Germany). A volume of 20 µL of samples was injected in a C-18 reverse-phase Tosoh column (TSKgel-ODS C-18, 5µm, 4.6 × 250 mm). The mobile phase for stevioside and rebaudioside A elution was 32% water and 68% methanol. The effluent was monitored at 210 nm, and curves of stevioside and rebaudioside A (Sigma-Aldrich Chemical Co, USA) standards were used to calculate the stevioside and rebaudioside A concentration in the samples (Serfaty *et al*., 2013).

Extraction of total RNA

After analysis of steviside and rebaudioside A production by HPLC system, the samples with the highest and lowest amounts of stevioside treated by different concentrations of salicylic acid were selected for RNA extraction. Extraction of RNA was performed with RNX-Plus kit (CinnaGen, Iran) based on the manufacturer's instructions. To evaluate the quality of RNA, the extracted RNA samples were loaded on the 1.5% agarose gel. NanoDrop 2000C spectrophotometer (Thermo Scientific, USA) was used to evaluate the quantity of RNA. The absorption ratio of 260 to 280 nm was 1.8-1.9, and the absorption of 260 to 230 nm was 1.6-1.9.

Synthesis of total cDNA

For the synthesis of the first strand of total cDNA,

Table 1. List of gene specific primers used in PCR and qRT-PCR analysis.

Gene (Accession number)	Primer sequence (forward primer, F and reverse primer, R) (5´-3´)	Amplicon length (bp)
KA13H (DQ398871.3)	F: CCTATAGAGAGGCCCTTGTGG R: TAGCCTCGTCCCTTTGTGTC	102
UGT74G1 (AY345982.1)	F: GGTAGCCTGGTGAAACATGG R: CTGGGAGCTTTCCCTCTTCT	115
UGT76G1 (AY345974.1)	F: GACGCGAACTGGAACTGTTG R: AGCCGTCGGAGGTTAAGACT	121
Actin (AF548026.1)	F: GCCCCTAGCAGCATGAAGAT R: GCACTTCCTGTGGACAATGG	162

RNA stock was prepared at 1µg/µL concentration. First, 3 µg of RNA was mixed with 1 µg/µL Oligo $(dT)_{18}$ primer (CinnaGen, Iran) and 9 µL deionized water in the 0.5 mL micro-tube and maintained at 70 °C for 5 min and was immediately transferred on ice. Then, 2 µL 10X RT buffer (CinnaGen, Iran) with 4 µL dNTP Mix 10 mM (CinnaGen, Iran) were added to the micro-tube and maintained at 37 °C for 5 min. Subsequently, 1 µL of Reverse Transcriptase enzyme (200 u/µL, CinnaGen, Iran) was added and reaction mixture incubated at 42 °C for 1 h. The reaction was stopped by heating the mixture at 70 °C for 10 min. The synthesized template cDNA was maintained at -20 °C (Japelaghi *et al*., 2012).

Primer design

In this study, the primers for KA13H, UGT74G1 and UGT76G1 genes were designed by Mandal *et al*. (2015). The actin gene was used as a house keeping gene, which was specific for *Stevia* and its primers were designed by Oligo v7.56 software (Table 1). The primers were tested by Primer Blast available in NCBI (https://www.ncbi.nlm.nih.gov) and synthesized by BioNEER Corporation (South Korea). In order to verify the authenticity of primers on cDNA, the PCR reaction was performed by combination of 7 µL deionized water, 1 µL of each forward and reverse primers, 1 µL cDNA and 10 µL Master PCR. Thermal cycles of PCR reaction included: 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 30 s and 1 cycle at 72 °C for 10 min.

Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR analysis of the expression of genes was conducted using real-time PCR (BioRad, USA). In a 15 µl reaction mixture, 1 µl of synthesized total cDNA, 7.5 µl SYBR Green Premix Ex Taq II (Takara, Japan), 0.5 µl of 10 µmol of each specific primer and 5.5 µl of nuclease free water were added. qRT-PCR conditions included: one cycle at 94 ˚C for 5 min, followed by 35 cycles of 95 ˚C for 30 s, 58 ˚C for 30 s and 72 ˚C for 30 s. Using the data obtained from the qRT-PCR device, $\Delta\Delta C_T$ was calculated (Pfaffl, 2001).

Statistical analysis

All experiments were performed as a factorial experiment based on completely randomized design with three replication. Data analyses were performed using IBM SPSS Statistics for Windows, Version 24.0 (Armonk, NY, USA). Mean comparisons were carried out using Duncan's multiple range test at a probability level of 0.05. For the comparison of the gene expression in the treatments with the highest and lowest contents of stevioside, the T-Test was used. The biological, technical and Real time PCR replications were 3, 1, and 1 respectively.

RESULTS AND DISCUSSION

Effect of salicylic acid concentrations and sampling times on stevioside

The results showed that, elicitation with different concentrations of salicylic acid had a significant effect ($p<0.05$) on stevioside production (Table 2). Elicitation with salicylic acid at 30 and 90 mg/L had a positive and significant effect on stevioside production. Addition of 30 and 90 mg/L salicylic acid to the media increased stevioside synthesis 1.12 (35.65 mg/gDW) and 1.13 (35.78 mg/gDW) fold compared to the control, respectively.. However, salicylic acid at 60 and 120 mg/L did not significantly affect stevioside production compared to the control (Figura 1A). Steviside production was affected by different sampling times (Table 2). The highest stevioside production was 35.05 mg/g DW at 96 h after elicitation, but was not significantly different from 24 and 72 h. At 96 h after elicitation the stevioside production increased 1.03, 1.14 and 1.04 fold compared to the 24, 48 and 72 h,

Table 2. ANOVA analysis of the effect of salicylic acid and sampling time on stevioside and rebaudioside A production in in *vito* cutlture of S. *rebaudiana*.

Source of variation	df	Mean of Sqaures	
		Stevioside	Rebaudioside A
Salicylic acid (SA)	4	56.91*	3.88**
Sampling time (T)	3	55.29*	1.55**
SA×T	12	21.92^{ns}	0.67**
Error	40	16.64	0.21
Coefficient of variation (%)		12.23	37.39

ns Not significant; * Significant at p < 0.05, ** Significant at p<0.01.

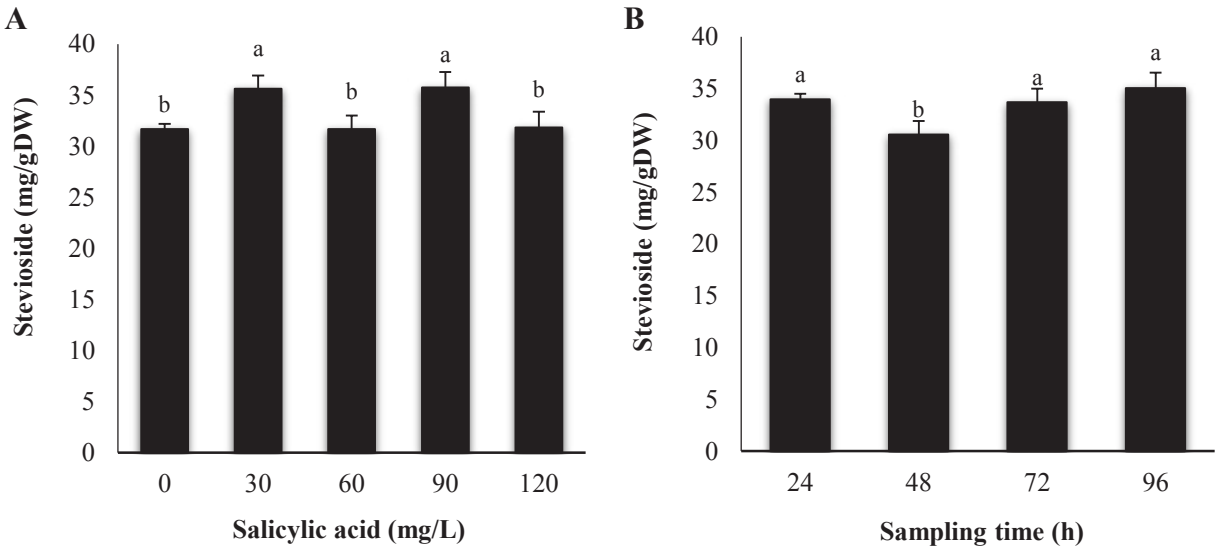

Figure 1. Effect of different concentrations of **A:** salicylic acid and **B:** sampling times on stevioside production in *in vito* cutlture of S. *rebaudiana*.

respectively (Figure 1B). In interactions of different concentrations of salicylic acid and different sampling times, the highest amount of steviside observed was 38.33 mg/g DW at 96 h after elicitation with 90 mg/L, which was 1.24 fold higher than the control (Table 3). Salicylic acid has an important role in the defense responses against biotic stresses such as microbes and herbivores, and abiotic stresses such as wounding and ozone exposure; therefore, it is used in plant *in vitro* cultures as an elicitor (Muffler *et al*., 2011). In this study, when compared to the control, the stevioside content reached 38.84 mg/g DW with the 90 mg/L concentration after 96 h. In the previous studies, the stevioside content in the *in vitro* grown plants have been reported as 13.84 mg/g DW (Bayraktar *et al*., 2016), 10.20 mg/g DW (Khalil *et al*., 2016), 82.48 µg/g DW (Aman *et al*., 2013), 2.9 mg/g DW (Dey *et al*., 2013). Reis *et al*. (2011) established successfully an adventitious root culture of *S. rebaudiana* in a roller bottle system, but no stevioside was synthesized in this culture. Pandey *et al*. (2016) reported the stevioside production as 1.72 mg/g DW in hairy root culture. Mathur and Shekhawat (2013) observed the content of stevioside as 381.03 µg/g DW in cell suspension culture. The stevioside contents reported in the intact or field-grown plants has been shown to vary to a large extent such as 1.79 mg/g (Pandey *et al*., 2016), 8.75 g/100 g (Woelwer-Rieck *et al*., 2010) and 5.8% (Gardana *et al*., 2010) and this is due to different genotypes, geographical origins, propagation methods, environmental conditions, and agronomic practices

Effect of salicylic acid and sampling time on rebaudioside A

Elicitation of *Stevia* shoots with different concentrations of salicylic acid had a significant effect on rebaudioside A production (p<0.01) (Table 2). The rebaudioside A production was increased by increasing salicylic acid concentration up to 30 mg/L, but then decreased by

Table 3. Effect of interactions of different concentrations of salicylic acid and sampling times on stevioside production in *in vito* cutlture of S. *rebaudiana*.

Salicylic acid (mg/L)	Sampling time (h)	Stevioside (mg/gDW)	Rebaudioside A (mg/gDW)
0	24	30.96±0.55	1.81±0.39^{bc}
	48	29.84±0.88	1.68±0.56^{bcd}
	72	32.63±0.60	1.48±0.35^{b-e}
	96	33.32±0.96	1.33±0.20^{c-g}
30	24	36.34±1.65	1.72±0.04^{bcd}
	48	32.61±0.39	1.86±0.52^{bc}
	72	36.48±3.57	2.29±0.08^{ab}
	96	37.19±3.69	1.01±0.13^{c-h}
60	24	30.63±0.89	1.62±0.26^{b-e}
	48	26.48±1.28	2.93±0.22^{a}
	72	33.96±2.93	1.16±0.18^{c-h}
	96	35.71±1.69	0.91±0.14^{d-h}
90	24	35.06±2.86	0.76±0.27^{e-h}
	48	37.18±4.73	0.37±0.07^{h}
	72	32.52±2.84	0.48±0.06^{gh}
	96	38.33±0.95	0.59±0.28^{f-h}
120	24	36.94±1.57	0.30±0.07^{h}
	48	26.83±0.81	1.43±0.19^{b-f}
	72	32.87±4.02	0.54±0.15^{gh}
	96	30.71±2.51	0.51±0.27^{gh}

increasing salicylic acid concentration from 30 mg/L to 120 mg/L. Generally speaking, application of 90 and 120 mg/L concentrations had an inhibitory effect on rebaudioside A synthesis. Therefore, the addition of 30 mg/L salicylic acid stimulated rebaudioside A production and increased its production 1.15 fold compared to the control. Therefore, it could be said that using 30 mg/L salicylic acid is suitable for rebaudioside A production (Table 3). The results showed that, the sampling time had a significant effect on stevioside ($p<0.01$) (Table 2). The rebaudioside A production increased until 48 h after elicitation, then showed a decrease. Among different sampling times, the highest amount (1.65 mg/g DW) of rebaudioside A was obtained 48 h after elicitation, which was 1.33, 1.38 and 1.89 fold higher than 24, 72 and 96 h, respectively (Table 3). Also, the interaction of salicylic acid concentrations and sampling times affected significantly rebaudioside A production ($p<0.01$), the highest amount of rebaudioside A production, 2.93 mg/g DW was obtained 48 h after elicitation with 60 mg/L salicylic acid, which was 1.62 fold higher than the control (Table 3). In the present study, 2.93 mg/g DW rebaudioside A was observed at 60 mg/L salicylic acid treatment after 48 h. Gupta *et al*. (2014) increased rebaudioside A from 0.07 to 0.69% in the callus culture exposed to 0.10% NaCl and from 0.56 to 1.89% in suspension culture exposed to 0.025% Na_2CO_3 on 15th day. Gupta *et al*. (2015) increased rebaudioside A from 0.07 to 1.44% in callus culture exposed to 5% PEG, from 0.56 to 1.38% in suspension culture exposed to 2.5% PEG on 15th day, and from 1.13 to 4.54% in suspension culture exposed to 5% PEG on 10th day. In another study, they cultured *in vitro* raised nodal explants of Stevia on the MS medium supplemented with 2.0 mg/L kinetin and different concentrations of NaCl (0.05-0.20%), Na_2CO_3 (0.025-0.10%), proline (2.5-10 mM) and polyethylene glycol (2.5-10%) for 4 weeks. The content of rebaudioside A was increased from 0.29 to 1.42% in *in vitro* shoots exposed to 0.05% Na_2CO_3 (Gupta *et al*., 2016).

Effect of salicylic acid on KA13H, UGT74G1 and UGT76G1 genes expression

The results showed that by 96 h elicitation with 90 mg/L salicylic acid the KA13H gene expression increased 1.04 fold, this increase was statistically significant. Also, the expression of UGT74G1 gene at 96 h after elicitation with 90 mg/L salicylic acid was 2.14 fold higher than 48 h after elicitation with 60 mg/L salicylic acid. But, the expression of UGT76G1 gene decreased 96 h after elicitation with 90 mg/L compared to the 48 h elicitation with 60 mg/L salicylic acid. Therefore,

Table 4. Correlation of KA13H, UGT74G1 and UGT76G1 genes and stevioside and rebaudioside A production in *in vito* cutlture of S. *rebaudiana*.

Characters	KA13H	UGT74G1	UGT76G1	Stevioside	Rebaudioside A
KA13H	1				
UGT74G1	0.835*	1			
UGT76G1	-0.902*	-0.701ns	1		
Stevioside	0.827*	0.852*	-0.812*	1	
Rebaudioside A	-0.806ns	-0.893*	0.822*	-0.924**	1

ns Not significant; * Significant at p < 0.05, ** Significant at p<0.01.

by increasing stevioside production under salicylic acid elicitation, the KA13H and UGT74G1 genes expression increased and UGT76G1 gene expression decreased (Figure 2). KA13H represents a branch point from the gibberellins biosynthesis considering its capacity to direct the flow of metabolites specifically towards the biosynthesis of stevioside glycosides (Humphrey *et al*., 2006). Thus, increase in its transcription implies enhanced stevioside production. Upregulation of UGT74G1 led to augmented concentration of stevioside and that of UGT76G1 led to higher concentration of rebaudioside A. Conversion of stevioside to rebaudioside A is controlled by UGT76G1 and its higher transcription ascertains increased ratio of rebaudioside A to stevioside (Richman *et al*., 2005).

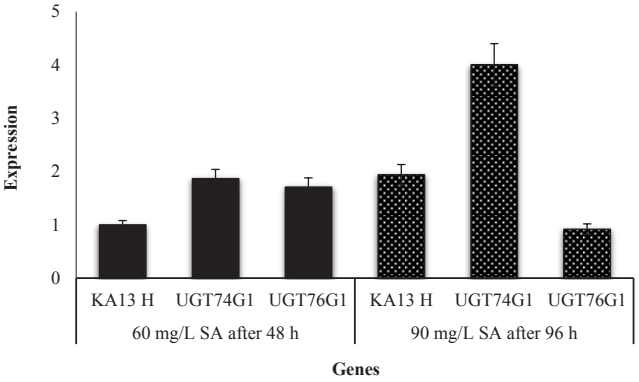

Figure 2. Effect of salicylic acid on KA13H, UGT74G1 and UGT76G1 genes expression in *in vito* cutlture of S. *rebaudiana*.

Correlation analysis of KA13H, UGT74G1 and UGT76G1 genes and stevioside and rebaudioside A production

The correlation analysis showed that, in elicitation with different concentrations of salicylic acid, the KA13H gene expression had a positive correlation with UGT74G1 gene expression and stevioside production and had a negative correlation with UGT76G1 gene expression. The UGT74G1 gene expression had a positive correlation with stevioside production and a negative correlation with rebaudioside A production. The UGT76G1 gene expression had a positive correlation with rebaudioside A and a negative correlation with stevioside production. The stevioside production had a negative correlation with rebaudioside A production (Table 4).

CONCLUSIONS

The results showed that the stevioside and rebaudioside A production are significantly affected by different concentrations of salicylic acid and sampling times. The highest amount of stevioside and rebaudioside A were 38.33 mg/g DW and 2.93 mg/g DW were obtained 96 h after elicitation with 90 mg/L concentration and 48 h after elicitation with 60 mg/L of salicylic acid , respectively. The qRT-PCR analysis indicated that by increasing stevioside production under salicylic acid elicitation, the KA13H and UGT74G1 genes expression increased and UGT76G1 gene expression decreased. Therefore, by increasing KA13H and UGT74G1 genes expression, the GT76G1 gene expression decreased.

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