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Research Article

The Effects of Silveroxide on mRNA Level of Key Genes Involved In the Biosynthesis of RebaudiosideA in *Stevia Rebaudiana*

Alireza Mirzaei[1], Sara Dezhsetan[1], Gholamreza Kavoosi[*2], Mahdi Behnamian[3]

[1] *Department of Agronomy and Plant Breeding, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran*

[2] *Department of Biotechnology, College of Agriculture, Shiraz University, Shiraz, Iran*

[3] *Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran*

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Abstract

Stevia rebaudiana bertoni is a plant with sweetening properties. This medicinal plant is perennial and from *Asteraceae* family. Its leaves contain glycoside compounds of a sugar part and non-sugar sectors. One of the glycosides compounds is RebaudiosideA which has a greater importance in market. Several key regulating genes including copalyl diphosphate synthase (*CPPS*) (AF034545.1), geranylgeranyl diphosphate synthase (*GGDPS*) (DQ432013.3), (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (*HDS*) (FJ755689.1), UDP glucosyltranserase 85C2(*UGT85C2*)(AY345978.1), UDPglucosyltranserase–74G1 (UGT74G1) (AY345982.1) and UDP glucosyltranserase–76G1 (UGT76G1) (KC631816.1) are involved in the biosynthesis Rebaudioside A. This experiment was conducted to evaluate the effect of silver oxide Ag_2O on the mRNA level of these genes in the *stevia rebaudiana*. The experiments repeated 3 times and with concentrations of 50, 100 and 200 µM. Increasing concentrations of 50 micromoles of silver oxide up to 100 micromoles leads to an increase in the expression levels of all the studied genes. Also according to the expression profile of these genes and the results of HPLC there is a significant increase on the expression level of the genes and production of RebaudiosideA under Ag_2O treatment. In general, it was found that increasing the concentration of Ag_2O can lead to an increase in the level of mRNA for the chosen genes. On the other hand, the low expression of the genes studied under control conditions (No Treatment), compared to the treatment with Ag_2O, revealed that the treatment can lead to higher sweetener glycoside components in the Stevia leaves. The physiological assay showed that Ag_2O treatment in concentrations of 100 and 200 µM have more positive effect on chlorophyll, protein, carbohydrates and carotenoids in Stevia.

***Keywords*:** Stevia, RebaudiosideA, Key genes, Gene expression

Introduction

In recent years, the development of natural sweeteners with characteristics of: low calorific, non-cariogenic and healthier is on rise. One of the promising alternatives is diterpenoid glycosides family, especially steviol glycoside. They are usually found in leaves of *Stevia rebaudiana* (Soejarto, 2002). Effects of its therapeutic properties comes from the presence of phenolic compounds in different parts of this plant, especially in leaves and callus, and it has anti-cancer effects and possible treatment of cardiovascular and blood sugar diseases by its property absorption is considered by free radicals (Tadhani et al., 2007). In 1931, two french chemists named Bridel and Lavieille succeeded in extracting the sweetener of the plant called steviol glycoside. They obtained natural steviol glycoside by a series of non-enzymatic hydrolyzing experiments (Bridel and Lavieille, 1931).

In fact, steviol glycoside is a diterpene glycoside that has been identified in the extract of various constituents of stevia rebaudiana and is the most important sweetener in stevia (Dacomi et al., 2005). Steviol glycoside contains compounds such as DalcosideA, RibaudiosideE-A, Steviolbioside, Stevioside, etc. Currently, 34 compounds are known in steviol glycoside, from which, 8 of them are in the form of isomers. Among these compounds, RibaudiosideA and Stevioside are more important than other compounds and are recognized as dominant glycoside compounds in Stevia rebaudiana leaf extract and they have plenty of commercial significance (Chaturvedula et al., 2011). Stevia also contains protein, fiber, carbohydrates, phosphorus, iron, calcium, sodium, potassium, magnesium, zinc and vitamins A and C. These sweet compounds pass through the digestive processes without chemical

Corresponding authors E-mail:
* ghkavoosi@shirazu.ac.ir

decomposition and are therefore useful for those who need to control their blood glucose (Rita, 1997). Since metal oxides are capable of stimulating the mechanism of defense in the cell and the production of secondary metabolites, they have recently been used in culture media as Elicitors. Lobick (2008) has shown that silver oxide, through the production of active oxygen species (ROS), affects the membrane phospholipids of the microorganisms, causing their peroxidation and degradation of the cellular membrane of the microorganisms. Also silver oxide with sulfur group in the membrane of the microorganisms is replaced and creates pores in the cellular membrane of the microorganisms that cause leakage of the intracellular material of the microorganisms and their destruction (Dibrov et al., 2002). Today, metal oxides serve as an attractive candidate for the delivery of many small molecules or large biochemical molecules (Jiang et al., 2006). Among various metal oxides, silver oxide is being used for its good conductivity (Jiang et al., 2006), chemical stability (Mafune et al., 2000), catalytic activity (Jain et al., 2008) and antimicrobial property (Jeeva et al., 2014). Indeed, the discovery of these basic properties of silver oxide has led to its importance in biology and biotechnology (Jia et al., 2013). Accordingly, this study aimed to investigate the effect of silver oxide, as an elicitor, on the mRNA level of the (AF034545.1) *CPPS*, (FJ755689.1) *HDS*, (DQ432013.3) *GGDPS*, (AY345978.1) *UGT85C2*, (AY345982.1) *UGT74G1* and (KC631816.1) *UGT76G1* genes in Stevia.

Materials and Methods

Plant Growth

Very small and thin (approximately 3 mm) light brown seeds of stevia (*Stevia rebaudiana* bertoni) were purchased from Techno Kesht Shiraz Company (Shiraz, Iran). These seeds were surface-sterilized with sodium hypochlorite (5%). Then, a piece of filter paper was put into each petri dish and 5 ml of distilled water was added. The seeds were transferred onto the filter paper, with 10 seeds in each of the 32 petri dishes. About 1cm space was kept between the seeds. The dishes were covered with their lids and sealed with parafilm. After preparing the media and sealing the dishes, all petri dishes were placed in an incubator. More than 80% of the seeds germinated in the dark within five days at room temperature. The seedlings developed roots with at least 25 mm length and were then transferred into the jardiniere containing cocopeat and perlite with proportion 70 to 30, respectively. During the experimental period, the jardiniers were irrigated twice a week. The seedlings received tap water at greenhouse conditions with about 60% relative humidity for two weeks until the size of leaves reached about 10cm. Diurnal cycles at each temperature (27/24°C) were set at 16h light and 8 h dark cycles. At this state, the seedlings were treated with 50 mL of different concentrations (50, 100, 200 µM) of silveroxide, loaded onto the gelatin dispersion. The equal concentrations of gelatin dispersion were used as control conditions which continued for three days. Young leaves were harvested and frozen in liquid nitrogen for twenty-four hours after each treatment, and then stored at -70°C for the RNA extraction and real-time PCR analysis. For biochemical analysis, the young leaves were harvested and stored at 4°C.

Primer Design

Primers were designed using AlleleID7 software (Premier Bio soft Intl, Palo Alto, CA, USA) for the target genes as shown in Table 1. Elongation factor genes were used as the internal control (whose expression proved not to be influenced by metal oxides treatment) for data normalization (9, 10). Two primer pairs were designed for each individual gene, as represented in Table 1. Primers for the PCR reactions were designed to have a melting temperature of about 50°C to 55°C and to give a PCR product between 100 and 200 bp in length.

Table 1. Sequences of primers used for Real-Time PCR amplification and the expected product size.

Primer	Sequence	length of the piece on cDNA	TM
UGT85C2F	ATGATGTATTGGACACTTGCTG	166	54.2
UGT85C2R	CCTTGAGACGGATGCCTTC	166	54.7
UGT76G1F	CATCTTTCACACCAACTTCAAC	178	53.5
UGT76G1R	GCGTCGTAATTCGTCAGC	178	53.5
UGT74G1F	CCTTGGTCTCAGATGTTGTTTG	105	55
UGT74G1R	CGTCCACTCTATTACCTCTTCC	105	55
CPPSF	CTACACGGCTTCGCTTTG	113	53.1
CPPSR	GTCACATCTACTCCATCTTGC	113	53.4
GGDPSF	CGATTGGTTTGTTGTTTCAG	169	50.8
GGDPSR	GCTTCCTTGTTTAATTTCTCC	169	50.4
HDSF	TTTCTTGGCTCCGTATCG	178	50.9
HDSR	TGAGGCTACATCTGAATAGG	178	50.6
Elongation Factor-F	GATGCTTCCGACTAAACCTATGG	113	50.6
Elongation Factor-R	CACTCTTGATAACACCGACTGC	113	50.9

RNA Preparation and cDNA Synthesis

Total RNA was extracted from 100 mg of leaf material, using RNX-Plus buffer (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. The quantification of the total RNA was performed with a Nano-drop (ND 1000 spectrophotometer) at 260 nm (Thermo Fisher scientific, wilmington, DE, USA). The RNA integrity was checked by visual observation of 28S rRNA and 18S rRNA bands on

an agarose gel electrophoresis before Real-time PCR analysis (Figure 1). In order to ensure the absence of the DNA contamination, DNase treatment (Fermentas Company) was performed on the extracted RNAs. Then synthesis of the first cDNA strand synthesis was performed, using commercial kit (Fermentas company).

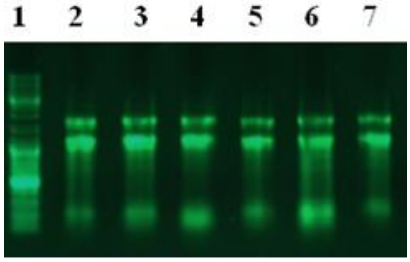


Figure 1. Agrosoe gel electrophoresis of the total RNA extraction from the Stevia leaves. 1. Gene RulerTM DNA Ladder Mix (Fermentas); RNAs extracted from leaves stevia for genes; 2. *HDS*: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; 3. *GGDPS*: geranylgeranyl diphosphate synthase; 4. *CPPS*: copalyl diphosphate synthase; 5. *UGT85C2*: UDP glucosyltranserase – 85C2; 6. *UGT74G1*: UDP glucosyltranserase – 74G1; and 7. *UGT76G1*: UDP glucosyltranserase – 76G1.

Biochemical Analysis

Chlorophyll a, b, Total chlorophyll and carotenoid content of the leaves were determined according to Arnon (1967) method. Pigments were extracted in 80% cold acetone and the absorbance of the extractions was measured spectrophotometrically at 645, 663, and 470 nm wavelength and subsequently, pigmental content were determined, based on the following standard formulas (Kichtenthaler and Wellburn, 1983) :

(1) Total chlorophyll (mg/ml) = 20.2 (A645) + 8.02 (A663)
(2) Chlorophyll a (mg/ml) = 12.7 (A663) - 2.69 (A645)
(3) Chlorophyll b (mg/ml) = 22.9 (A645) - 4.68 (A663)
(4) Carotenoid (mg/ml) = (1000A470 - 3.27[Chl a] - 104[Chl b])/227

The soluble carbohydrates were measured in a similar way of proline measurement after extraction. 0.1 ml Alcoholic extract with 3 ml anthrone were freshly prepared (150 mg anthrone+ 100 ml sulfuric acid 72%) and mixed. This solution was placed in water bath for ten minutes and the absorption rate was measured with a spectrophotometer in the wavelength of 625 nm. The amount of the soluble sugars were then calculated (Pereira et al., 1993). For protein extraction, 0.5 g of the dry explant weighed and 4 ml of buffer Tris-HCl was added. Then the samples were thoroughly vortex-mixed shaker for 20 minutes and centrifuged at 5000 rpm for 30 minutes and the upper phase, containing protein, was isolated. For measuring protein content the Bradford method was used. 0.1 ml protein extract of each sample was vortex-mixed with 5 ml of the Bradford solution and its absorbance at 595 nm was recorded (Bradford, 1976).

Real-time Quantitative PCR Analysis

Real-time PCR was performed using a line Gene K Thermal cycler (Bioer Technology Co, Hangzhou, China). The cDNA samples were diluted 1:5 by using nuclease-free water, and 5 µL of the cDNA was used for real-time PCR. The final volume for relative real-time PCR was 20 µL containing 4 pmol of each primer, 5 µL (diluted) of the first-strand cDNA and 1x SYBR Premix Ex Taq TM Π (Takara, Japan). The initial denaturing time was 5 min., followed by 40 PCR cycles consisting of 94°C for 10 s, annealing temperatures of each primer 15 s, and 72°C for 30 s. A melting curve was run after the PCR cycles followed by heating from 50 to 95°C. A proper control reaction was carried out without the reverse transcriptase treatment. For each sample, the subsequent real-time PCR reactions were performed in twice under identical conditions (Livak and Schmittgen, 2001).

Data Normalization and Quantitative PCR Verification

For real-time data analysis, the relative expression of the target gene in each sample was compared with the control sample (corresponding to the control plants) and was determined with the delta-delta Ct method (Livak and Schmittgen, 2001) with following equations:

$2^{-[\Delta Ct\ sample} - \Delta^{Ct\ control}$, $\Delta Ct = CT_{target\ gene} - CT_{internal\ control}$

The Ct for each sample was calculated using the Line-gene K software (fqdpcr ver. 4.2.00) and the Larionov (2005) method, where refers to the threshold cycle determined for each gene in the exponential phase of PCR amplification. In this analysis method, the relative expression of the target gene in the control sample was equal to one (2^0) by definition.

HPLC Analysis

Standard solution of RebaudiosideA was prepared in a mix of 30% water and 70% acetonitrile. To prepare calibration curve, a solution containing 100 µg/mL of the standard in the ratio of 30% water and 70% acetonitrile was used. In order to extract Rebaudioside-A, the *Stevia rebaudiana* leaves were pulverized and nearly 0.1 g of the powder was put in a 20-ml glass vial.

Ten ml of 30% water and 70% acetonitrile mix was added to the vial which was then vortexed. Subsequently, this crude extract was sonicated for 5 minutes. The contents of the vial were centrifuged and the supernatant was diluted 10 times with 30% water

and 70% acetonitrile mix. Finally, 10 microliters of the sample solution were injected into the column for analysis, using KNAUER – smart line manager-5000 HPLC machine. The flow rate was 1 mL per minute. HPLC column profile contains eurospher 100-5 C18 column, 2500-KNAUER Detector, column length 250 mm and measure pore 5µm. During the 14-minute period, the peak of RebaudiosideA appeared at the minute of 2.3 and the wavelength was 220 nm (Kailasam, 2011).

Results and Discussion

In this research, the effects of silver oxide on the expression pattern of the 6 key regulatory genes involved in biosynthesis of RiboidiosideA in *Stevia rebaudiana* were assayed by Real-time PCR reaction in 3 replicates with concentrations of 50, 100 and 200 µm.

mRNA Level of the *CPPS*, *GGDPS* and *HDS* Genes

The Duncan's comparison test (at 0.05%) showed that silver oxide treatments at concentrations of 100 µm caused significant changes in the mRNA level of the ***CPPS*, *GGDPS* and *HDS*** genes (Table 2).
Regarding these results, it will be determined that silver oxide treatment will have a positive effect on the increase of gene expression and production of RiboidiosideA, and the best treatment for this gene group will be at a concentration of 100 µm.

Table 2. The results of means comparison by Duncan at P<0.05 employing SPSS software version 16

GENES / Concentrations	CPPS	HDS	GGDPS	UGT76G1	UGT74G1	UGT85C2
50 µM	2.9835 B	3.1956 B	1.1573 B	3.1382 A	2.7647 A	1.9647 A
100 µM	8.6395 A	7.2388 A	3.0943 A	3.2156 A	2.9773 A	1.6463 A
200 µM	1.3942 B	1.1469 B	1.4601 B	3.3946 A	3.2573 A	2.2862 A

Expression of UGT Family Genes

As shown in Figure 2, the amount of genes expression in the treatment of silver oxide compared to the control conditions or non-treatment conditions has increased significantly.
Also increasing the concentration of silver oxide treatment leads to an increase in the expression of UGT family genes.
Generally, the highest amount of genes expression related to concentration of 200 µm. In the mode of comparison between the genes of this family, the *UGT76G1* gene showed the highest expression levels, and the *UGT85C2* gene had the lowest expression.
Regarding the changes in the expression of UGT family genes, it can be concluded that treating of silver oxide has significant and positive effect on the synthesis of stevioside and RiboidiosideA.

Chart 1. Results of expression analysis of the *CPPS*, *GGDPS* and *HDS* genes by Real-time PCR. Error bars are based on the standard error.

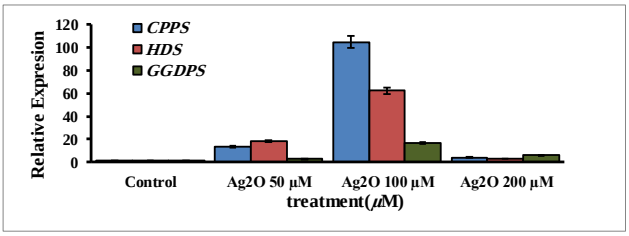
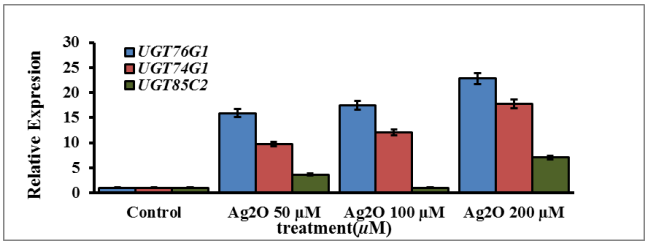


Chart 2. Results of expression analysis of the *UGT85C2*, *UGT76G1* and *UGT74G1* genes by Real-time PCR. Error bars are based on the standard error.



HPLC Analysis

The peak shown in HPLC for stevioside, RiboidiosideA, indicates the increase in the production of these active substances under the influence of silver oxide treatment (Figure 3).
As shown in Figures 2 and 3, the first peak is stevioside and the second peak is RiboidiosideA. Generally, according to the comparison of the standard peaks and silver oxide treatment, it has been shown that the production of stevioside was higher than that of RiboidiosideA, which is evident with regard to the production pathway of RiboidiosideA.

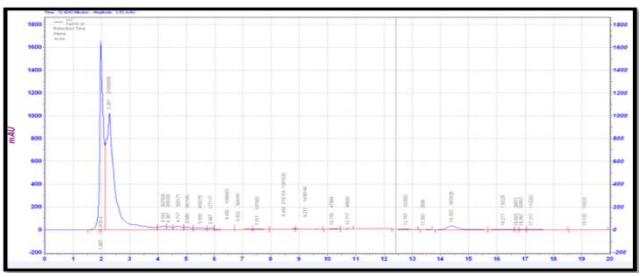


Figure 2. HPLC analysis chromatogrm for the standard solution. The first peak represents Stevioside and the second peak represents RebudiosideA.

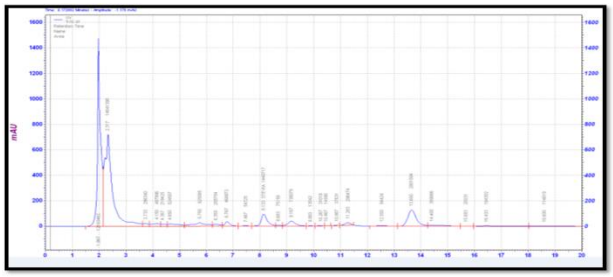


Figure 3. HPLC analysis chromatogram of silveroxide: the first peak represents Stevioside and the second peak represents RebudiosideA.

Biochemical Analysis

As shown in the table 3, treatment of the samples with silver oxide, at concentrations of 100 and 200 µm, have a more positive effect on the levels of chlorophyll, protein, carbohydrates and carotenoids. Also, the results of Duncan test at 0.05% showed a significant difference between the concentration of 50, 100 and 200 µm.

Table 3. Silver oxide impact on chlorophyll, carotenoid, carbohydrate and protein contents.

treatment	chlorophyll (mg/ml)	carotenoid (mg/ml)	protein (mg/g.D.W)	carbohydrate (mg/g.D.W)
Control	15.6±3	4.43±2	73±6	230±12
Ag2O 50 µM	19.1±8	4.36±3	102±4	274±11
Ag2O 100 µM	19.19±5	4.76±3	105±5	289±14
Ag2O 200 µM	20.09±8	5.62±2	108±3	297±13
Positive and negative numbers are related to standard deviation.				

It is known that, the transcription level of the genes involved in the pathway of ethylene signals is correlated with the defense response of the plants (Kaveh et al., 2013). Metal oxides as an inhibitor of the activity of ethylene, through the coating of the receptors, inhibits the ethylene signal pathway and affects the process of the metabolism and the expression of the associated genes (Krizkova et al., 2008). With regards to the increased level of the expression in the examined genes, it can be concluded that likely silver oxide is involved as a coating on ethylene receptors, which inactivates them. This reaction could lead to changes in the metabolic processes and expression RebaudiosideA. Similar changes have been reported in the metabolic processes of proteins in sunflowers (Krizkova et al., 2008).

The results of HPLC showed that the treatments of silver oxide leads to an increase in the production of Stevioside and RiboidiosideA in stevia. In addition, it was found that the pathway reaches closer to production of RiboidiosideA in the pathway, the activity of enzymes, is increased. Also, the amount of gene expressions synthesizing these enzymes has increased and eventually more RiboidiosideA would be expected. This uptrend suggests that treating the samples with silver oxide would have a significant effect on the expression of these genes. Also, this research suggests that the treatment with silver oxide up-regulates the amount of pigments, chlorophyll content and plant growth, which in turn could reduce the negative effects of the environmental stresses.

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