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Original Research

Effects of life cycle and leaves location on gene expression and glycoside biosynthesis pathway in *Stevia rebaudiana* Bertoni

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Abstract: *Stevia rebaudiana* Bertoni is One of the most important biologically sourced and low-calorie sweeteners that known as "Sweet Weed". It contains steviol glycosides that they are about 200-300 times sweeter than sucrose. Tissue culture is the best method with high efficiency that can overcome to problems of traditional methods, and it is the most useful tools for studying stress tolerance mechanisms under in vitro conditions to obtain drought tolerance. In the present research, we investigated the impact of life cycle, leaves location and the harvesting time on expression of *UGT74G1* and *UGT76G1* as well as steviol glycosides accumulation. The highest gene expression of both *UGT74G1* and *UGT76G1* (207.677 and 208.396 Total Lab unit, respectively) was observed in young leaves in the second vegetative year. Also, the highest amount of stevioside accumulation (13.04) was due to the old leaves in vegetative stage which had significant differences with other effects whereas the lowest accumulation (7.47) was seen at young leaves at vegetative stage. Interestingly, the highest level of rebaudioside a production (15.74) was occurred at the young leaves at vegetative stage. There was significant differences between life cycle and leaves location on steviol glycoside production in stevia.

Key words: *Stevia rebaudiana* Bertoni; Semi-quantitative RT- PCR; HPLC; Life cycle; Leaves location; *UGT76G1*; *UGT74G1*.

Introduction

One of the most important biologically reserved and low-calorie sweeteners that known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf" is *Stevia rebaudiana* Bertoni from Asteraceae family that has been widely cultivated in the world for the sweet diterpene glycosides such as rebaudioside A, B, C, D, E, F, M, steviol bioside, dulcoside A, dulcoside C and stevioside in its leaves (1-10).

Although stevia plant is native from some of Brazil and Paraguay, but nowadays Stevia plant and stevioside that are about 200-300 times sweeter than sugar because of some applications in medicine and food industry such as anti-microbial, anti-hypertensive, anti-hyperglycemic, anti-cancerous, anti-oxidant, taste modifiers, sweetening agents and etc are being used as sweetener in South America, Asia, Japan, China, and some countries in Europe. (1, 11-17).

Among different methods for propagating Stevia, tissue culture is the best method with high efficiency that can overcome to problems of traditional methods, and it is the most useful tools for studying stress tolerance mechanisms under in vitro conditions to obtain drought tolerance (18-23).

Nowadays, many experiments were designed to measure the transcript levels of genes that involved in the biosynthesis of steviol glycosides. In the study for measuring the transcript levels of genes that involved in the biosynthesis of steviol glycosides, samples from both old and young leaves in long and short-day conditions were harvested, and the transcript levels of three UDP-dependent glycosyltransferases such as *UGT85C2*, *UGT74G1* and *UGT76G1*, were studied by using quantitative real-time polymerase chain reaction. The result showed C-13-glucoseto steviol was catalysed by *UGT85C2*, and the C-19-glucose was catalysed by *UGT74G1* and finally glycosylation of the C-3 of the glucose at the C-13 positions was catalysed by *UGT76G* (24).

Tavarini et al (2015) studied the effect of nitrate fertilizer and harvesting time on glycosides accumulation in stevia leaves. They found that stevioside accumulation was increased under 150 kg/hectare nitrate fertili-

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

Gene	Primer sequence 5´→ 3´ (forward/reverse)	Amplicon length (bp)	Accession number
UGT74G1	AATCGGGCCAACACTTCCAT/ TCGGGTCCATGTTTCACCAG	174	AY345982
UGT76G1	GACCAACAACCGCCAAGTTC/ CCCAAGAACCCATCTGGCAA	185	AY345974
β-Actin	TTGCCCTGAGGTTCTGTTCC/ ATCCGGTCAGCAATACCAGG	171	AP548026

zer treatment (25). Also, they stated that the high steviol glycoside yields occurred in long-day conditions during the spring/summer season. They concluded that "The harvest time played a key role in determining the stevia quality, influencing the rebaudioside A/stevioside ratio." Kumar et al (2011) reported that the highest level of expression for 15 key genes of steviol glycosides biosynthesis pathway was observed the leaves which were formed on third nod of stem. Also other research determined that the highest expression level of KS and CPS genes was due to the matured leaves of stevia (26-27). Brandle et al (1998) studied on amount of glycosides of stevia and concluded that their accumulation has been enhanced during the generative phase and this increasing has been continued till folding stage (28).

According to the recent peer review research work which had been done before, the most important aspect of stevia studies is amount of steviol glycosides accumulation. So, we tried to find the simple and reciprocal effects of different growth stage, harvest time and leaves location levels on gene expression and glycosides accumulation in stevia.

Materials and Methods

Plant materials

The current study, *stevia rebaudiana* Bertoni explants were provided from Zagros Bioidea Co. Razi University, Kermanshah, Iran. Stevia cultivated in department of agronomy and plant breeding, faculty of agriculture, Razi University, Kermanshah, Iran.

RNA extraction

Total RNA was extracted from fresh leaves using RNX plus™ kit (Cinnaclon) according to the manufacturer's instructions. RNA quantification was done by NanoDrop Spectrophotometer (Nanodrop®, ND-1000, Nanodrop Technologies, and Wilmington, USA). 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 *UGT74G1* and *UGT76G1* genes

Determine gene expression of *UGT74G1* and *UGT76G1* genes in stevia was done by the two-step semi-quantitative RT-PCR method. For cDNA synthesis, 10 µg of total RNA was reversely transcribed with 100 U M-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 had been 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) (29; 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 Revers 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 product of PCR was done by electrophoresis on a 1% 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 *UGT74G1* and *UGT76G1* were normalized relative to the amount of *β-Actin* expression.

HPLC analysis

The contents of stevioside and rebaudioside A were estimated in different life cycle and location leaves of stevia by the method described earlier (30). Powder of dried leaves of stevia which were from different life cycle and location, applied to extracting by 80% methanol for 4 times and after that dried in vacuo and defatted with hexane and residual extract was vacuum dried. Then extract was dissolved in acetonitrile and filtered. The chromatographic separation was obtained using a symmetry Xbridge amide column (4.6 × 150 mm, 3.5 lm, Waters, USA) at 50 °C, mobile phase involved acetonitrile:water (80:20) in isocratic elution mode with detector wavelength 210 nm. The injection volume was 10 µl with a flow rate of 0.8 ml/min. By using three independent replicates, stevioside and rebaudioside A estimation were resulted.

Statistical analysis

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

Results and discussion

Investigation of *UGT74G1* and *UGT76G1* genes expression

According to Table 2, expression of *UGT76G1* gene differed significantly in various growth stages and years. Also, there were significant differences between reciprocal effects of growth stage×year in expression level of *UGT76G1*. However, significant differences had been seen between effects of growth stage, year, growth stage×year, leaves location×year and growth

Table 2. Mean square of effect of growth stage, leaves location and year on *UGT74G1* and *UGT76G1* genes expression.

source of variation	df	Mean square	
		UGT74G1	*UGT76G1*
Growth stage (S)	1	** 287.866	**649.636
Leaves location (P)	1	^{ns}0.850	^{ns}1.681
S×P	1	^{ns}68.461	^{ns}12.611
Year (Y)	1	**288.315	**451.315
S×Y	1	**3599.938	**369.543
P×Y	1	**380.874	^{ns}30.311
S×P×Y	1	**197.092	^{ns}4.597
Error	32	31.036	11.608
Total	39		

ns= non-significant; ** = Significant differences in the levels of 0.01; * = Significant differences in the levels.

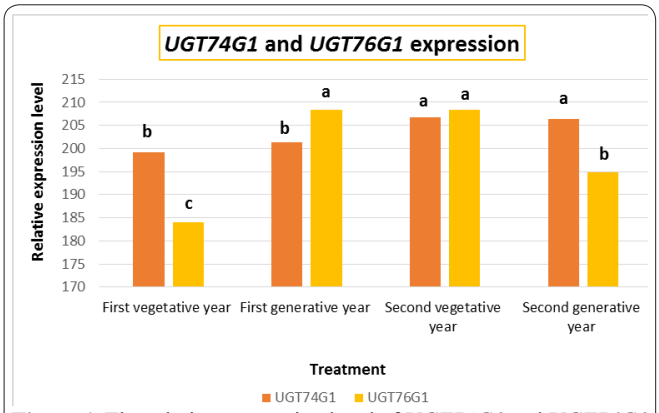

Figure 1. The relative expression level of *UGT74G1* and *UGT76G1* (related to *β-actin*) under reciprocal effects of growth stage×year. Columns with different letters indicate significant differences at P = 0.05 (Duncans test).

stage×leaves location×Year in expression pattern of *UGT74G1*. Mean comparison had been perfumed for significant effects and it have been described as follow.

As it have been shown in figure 1 and figure 2, the results of RT- PCR were normalized to the level of the housekeeping gene of *β-actin* in plants. There were significant differences between different treatments. The highest gene expression of *UGT74G1* was due to second vegetative year (207.677 Total Lab unit) which had no significant differences with expression in second generative year. Also, the lowest level of gene expression for *UGT74G1* was seen in plants in first vegetative year (199.208 Total Lab unit) that it hand no significant differences with first generative year. However, the highest level of *UGT76G1* gene expression was observed in second vegetative year (208.396 Total Lab unit) which it had no significant differences with first generative year. The lowest gene expression of *UGT76G1* was seen in First vegetative year (184.053 Total Lab unit).

Figure 3 exhibited, the highest level of *UGT76G1* expression was seen in young leaves in the second year (204.532 Total Lab unit) which had significant differences with other levels of the year×leaves location effects. Also, the lowest expression level was observed in young leaves in the first year (192.991 Total Lab unit).

The expression level of *UGT74G1* under reciprocal effects of growth stage × year × leave's location have been shown in figure 4. Based on the results, the highest gene expression was due to the young leaves in the

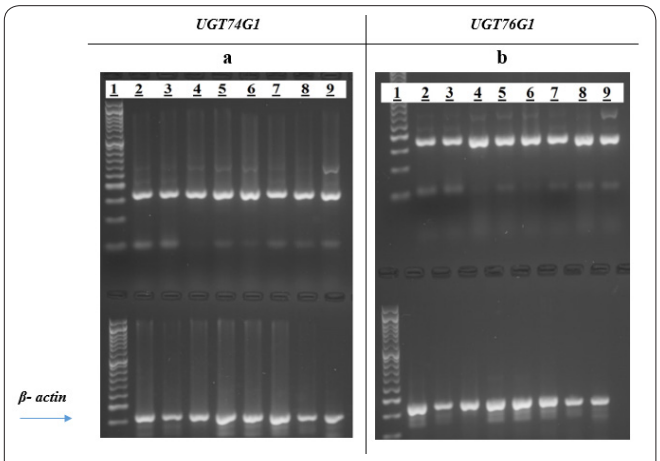

Figure 2. Semi-quantitative RT-PCR analysis of *UGT74G1* and *UGT76G1* in the leaves of *stevia rebaudiana* Bertoni in plants treated with nitrogen sources for 30 days (Where, 1= DNA ladder (50 bp); 2=Young leaves in the first vegetative year; 3 = Old leaves in the first vegetative year; 4 = Young leaves in the first generative year; 5 = Old leaves in the first generative year; 6= Young leaves in the second vegetative year; 7= Old leaves in the second vegetative year; 8= Young leaves in the second generative year; 9= Old leaves in the second generative year). *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.

second vegetative year (214.864 Total Lab unit) which had no significant differences with old leaves in the first generative year. Also, the lowest gene expression level was observed in young leaves in the first vegetative year (179.910 Total Lab unit) which had significant differences with other levels of growth stage × year × leave's location reciprocal effect.

HPLC analysis of steviol glycosides

First of all, HPLC fingerprinting was performed on the pure marker compounds, including standard of Stevioside (St) and Rebaudioside A (Re). Fingerprint patterns procured from the studied samples under different levels of simple and reciprocal effects showed significant differences between growth stage, leaves location and growth stage×leaves location for both stevioside and rebaudioside A accumulation (Table 3).

The highest amount of stevioside accumulation (13.04) was due to the old leaves at vegetative stage which had significant differences with other effects

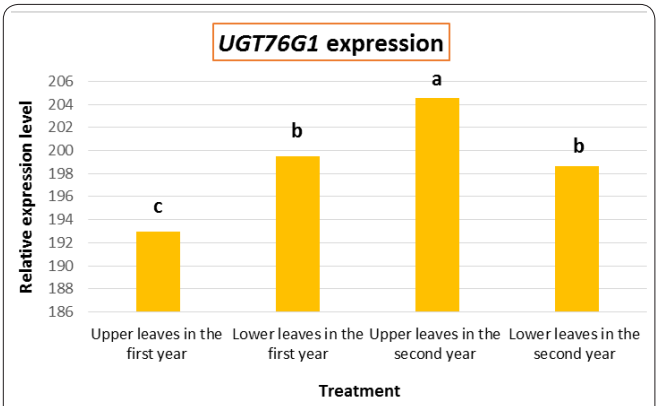

Figure 3. The relative expression level of *UGT76G1* (related to *β-actin*) under reciprocal effects of year*leaves location. Values are means ± SE of four replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (Duncans test).

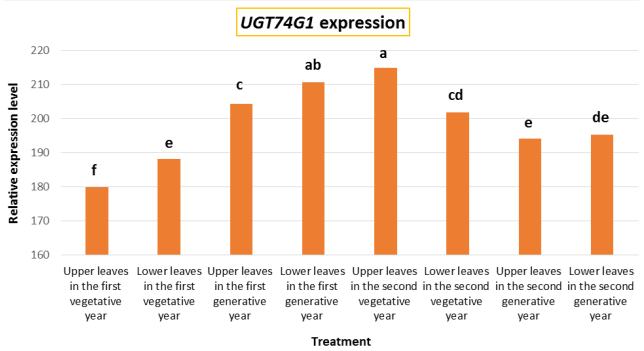

Figure 4. The relative expression level of *UGT74G1* (related to *β-actin*) under reciprocal effects of growth stage * year * leave's location. Values are means ± SE of four replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (Duncans test).

Table 3. Mean square of effect of growth stage, leaves location and year on stevioside and rebaudioside A accumulation.

source of variation	df	Mean square	
		Stevioside	Rebaudioside A
Growth stage (S)	1	**217.846	**30.173
Leaves location (P)	1	**8.773	**20.554
S×P	1	**4.327	**3.880
Year (Y)	1	^{ns}0.146	0.059^{ns}
S×Y	1	^{ns}0.105	0.001^{ns}
P×Y	1	^{ns}0.001	0.044^{ns}
S×P×Y	1	^{ns}0.155	0.008^{ns}
Error	32	0.248	0.054
Total	39		

whereas the lowest accumulation (7.47) was seen at young leaves at vegetative stage. Interestingly, the highest level of rebaudioside A production (15.74) was occurred at the young leaves at vegetative stage. However, the lowest amount of this (4.02) was seen at the old leaves at vegetative stage which was opposite trend in comparison with stevioside (Figure 5, Figure 6).

Conclusion

According to different researches, there are many factors that have major impacts on secondary metabolites accumulation. Obviously, the growth stage of plants can determine the kind and amount of secondary metabolites production. Also according to the plant needs during the life cycle, the expression of various genes could be significantly different (26, 28).

Researchers reported the effect of growth stage on various secondary metabolites and this fact had been confirmed due to our results. Variation of phenolic concentration during the growth of *Marjoram* affirm the influence of both phenological stages and climate factors on production and release of these metabolites. Also, the opposite peak of accumulation of these metabolites during the late vegetative stage reported (31). The accumulation of different metabolites during the full-flowering stage could be related to ecological roles such as intensifying antifungal defences and attracting pollinators (32). Regarding theses variations in the accumulation of secondary metabolites in plants, it could be concluded that the physiological stage of the plant affects the choice of best harvesting time.

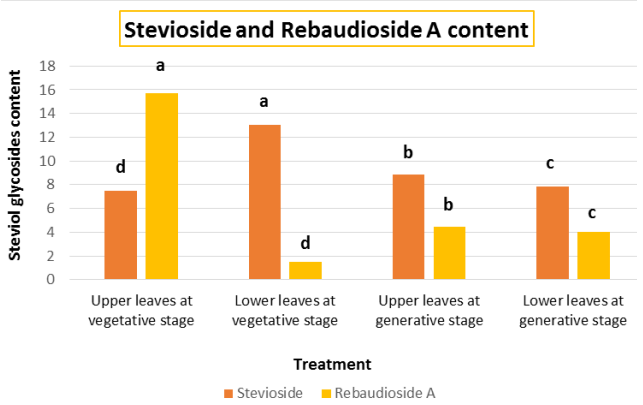

Figure 5. Steviol glycosides contents in leaf tissues of *S. rebaudiana* under different levels of growth stage*leaves location reciprocal effects.

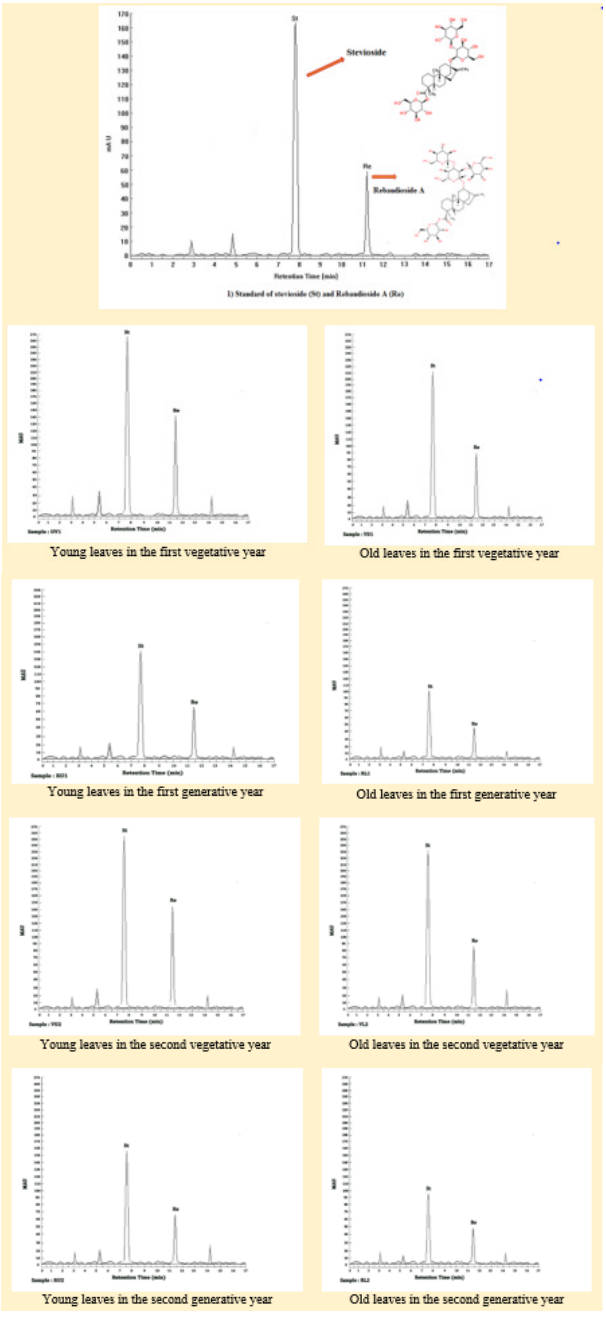

Figure 6. Representative HPLC chromatograms for quantification of stevioside and Rebaudioside A in methanolic extract of *S. rebaudiana* leaf tissues.

Studying seasonal changes in contents of phenolic compounds in *Rhus*, *Euonymus* and *Acer* leaves, Ishi

kura (1976) have found that the metabolites content per leaf changed rapidly at the early growth stages but thereafter the content was kept rather constant (33). Also, Males et al. (2003) indicated that the aerial parts of *Crithmum maritimum* collected before flowering and at the beginning of flowering had significantly different in content of metabolites in contrast with other stages (34). Ayan et al. (2007) and Verma and Kasera (2007) reported differences in metabolites productions during various stages of plant life (35-36). Also, there are many reports that they confirm the existence of various classes of secondary metabolites based on different growth stages in plants (37-38).

Whereas, the most important target of studies about stevia is enhancing steviol glycosides which cause sweet taste of the plant's leaves. In the present study, we investigated the simple and reciprocal effects of growth stage, year and leaves location on steviol glycoside accumulation. According to the present results of the present study, the highest expression for both *UGT74G1* and *UGT76G1* genes was seen in the second vegetative year which had no conformity with the results of Brandle et al (1998). Also the highest accumulation of stevioside was observed in old leaves while the highest level of *UGT76G1* gene expression was recorded in young leaves. It can suggest that the biosynthesis pathway of stevia is pretty complicated and the highest amount of products have been accumulated in developmented leaves. These results had concordance with results of research which had been performed by Kumar et al (2011). There are various reports that they showed the effect of harvest time, leaves location and growth stage on glycoside accumulation (25, 27). It is very important to find the best situation for the highest level of steviol glycoside production in Stevia.

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