Acta Physiol Plant (2013) 35:2009–2014
DOI 10.1007/s11738-013-1226-9

SHORT COMMUNICATION

Gene transcription of steviol glycoside biosynthesis in *Stevia rebaudiana* Bertoni under polyethylene glycol, paclobutrazol and gibberellic acid treatments in vitro

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Received: 6 October 2012 / Revised: 16 January 2013 / Accepted: 16 January 2013 / Published online: 14 February 2013

Abstract This experiment was carried out to measure the transcript levels of some genes involved in biosynthesis of steviol glycosides. *Stevia rebaudiana* plants were treated with polyethylene glycol (PEG), paclobutrazol (PBZ) and gibberellic acid (GA). Using quantitative real-time polymerase chain reaction, the transcript levels of kaurene synthase (KS), kaurene oxidase (KO), kaurenoic acid hydroxylase (KAH) and three UDP-dependent glycosyltransferases, UGT85C2, UGT74G1 and UGT76G1, were studied. The transcription of *ent-KS1-1*, *ent-KAH* and *UGT74G1* were fairly stable under different treatments. The transcription of *ent-KO*, *UGT85C2* and *UGT76G1* significantly decreased by PBZ and PEG treatments. The results indicate that both PBZ and PEG treatments resulted in the same negative effect on genes transcription, which could not be reversed by GA treatment.

Keywords Drought stress · Gibberellic acid · Paclobutrazol · Real-time quantitative PCR · *Stevia rebaudiana* · Steviol glycosides

Communicated by Y. Wang.

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Abbreviations

CPPS	Copalyl diphosphate synthase
Dul	Dulcoside
GA	Gibberellic acid
GGDP	Geranylgeranyl-diphosphate
GTs	Glucosyltransferases
KO	Kaurene oxidase
KS	Kaurene synthase
KAH	Kaurenoic acid hydroxylase
PBZ	Paclobutrazol
PEG	Polyethylene glycol
SB	Steviolbioside
SVglys	Steviol glycosides
ST	Stevioside
Reb	Rebaudiosides
UGT	Uridine diphosphate-dependent glycosyltransferase

Introduction

Stevia rebaudiana (Bertoni) is well known for its high content of sweet steviol glycosides (SVglys) in its leaves (about 4–20 % of dry weight) depending on the cultivar and growth conditions (Geuns 2003). SVglys are glucosylated derivatives of the diterpenoid compound steviol. SVglys biosynthesis pathway shares some steps with Gibberellic acid (GA) biosynthesis and paclobutrazol (PBZ) block the GA biosynthesis (Fig. 1). In *Stevia*, geranylgeranyl-diphosphate (GGDP) is converted into steviol by the consecutive action of four enzymes namely copalyl diphosphate synthase (CPPS), kaurene synthase (KS), kaurene oxidase (KO) and kaurenoic acid hydroxylase (KAH) (Brandle and Telmer 2007; Fig. 1). Different

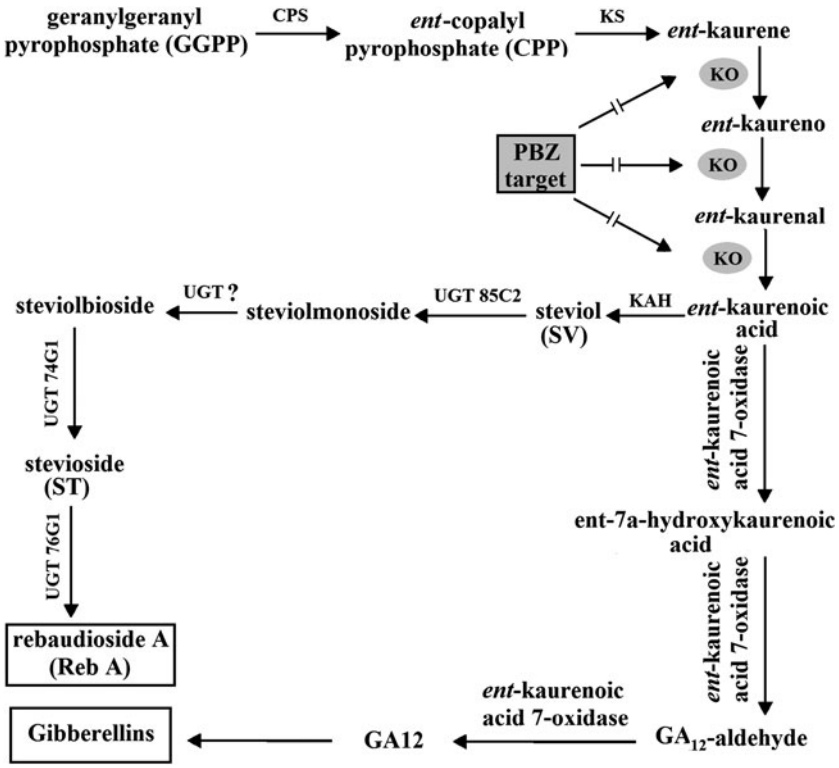

Fig. 1 The biosynthetic pathway of steviol glycosides, which shares some steps with GA biosynthesis and PBZ inhibition. *CPS* copalyl diphosphate synthase, *KS* kaurene synthase, *KO* kaurene oxidase, *KAH* kaurenoic acid 13-hydroxylase, *PBZ* paclobutrazol, *UGT* uridine diphosphate-dependent glycosyltransferase

SVglys are formed by glucosylation of steviol by specific glucosyltransferases (GTs) (Shibata et al. 1995). Stevioside (ST) and rebaudioside A (Reb A) are the major SVglys. Other SVglys present in smaller amounts are: steviolbioside (SB), Reb B, C, D, E, F and dulcoside A (Dul A) (Starrat et al. 2002).

Stevia rebaudiana originates from a semi-humid area (Paraguay and Brazil) with a yearly average rainfall of 1,500 mm. It has very little resistance to drought. Drought stress limits the appearance of leaves and hence affects SVglys accumulation (Hajihashemi et al. 2012). Simulation of drought stress with polyethylene glycols (PEG) under in vitro conditions constitutes a convenient way to study the effects of drought on the plant responses in controlled condition.

Paclobutrazol (PBZ) [(2*RS*–3*RS*)-1-4(chlorophenyl)-4,4-dimethyl-2-1,2,4-triazol-1-yl-penten-3-ol] affects the isoprenoid pathway. It inhibits GA biosynthesis by inhibiting kaurene oxidase, thus blocking the oxidation of kaurene to kaurenoic acid (Fletcher et al. 2000; Fig. 1). The growth inhibitory activity of paclobutrazol can be reversed by GA_3. It has been demonstrated that the morphological and stress protective effects of triazoles are reversed by GA_3 (Vettakkorumakankav et al. 1999). PBZ induces tolerance in plants towards environmental and chemical stresses, especially drought stress (Zhu et al. 2004).

Our previous study showed that PEG and PBZ treatments significantly reduced SVglys accumulation in *S. rebaudiana* while GA treatment slightly increased Svglys content (Hajihashemi et al. 2012). This investigation aimed to reveal a possible correlation between transcription of some genes involved in SVglys biosynthesis and SVglys contents in *S. rebaudiana*-treated with PEG in combination with PBZ and GA.

Materials and methods

The culture medium used for initiation and multiplication of *S. rebaudiana* Bertoni was MS (Murashige and Skoog 1962), which was supplemented with 30 g L^{-1} sucrose. After the pH was adjusted to 5.8, 2.9 g L^{-1} phytagel was added, after which the medium was transferred into tubes and autoclaved. Each tube contained one explant with two axillary buds without any leaves or roots. All cultures were incubated at 26 ± 1 °C under 2,500 lux derived from 2 × 120 white fluorescent lamps under a photoperiod of 16 h.

Preliminary experiments were done to select appropriate levels of PBZ (0–10 mg L^{-1}), GA (0–10 mg L^{-1}) and PEG (0–20 % w/v). The results demonstrated that 2.0 mg L^{-1} PBZ and GA, and 5 % w/v PEG were the most effective concentrations. Therefore, the subsequent experiments were done by transferring the explants to MS medium supplemented with 0 and 2.0 mg L^{-1} PBZ and/or 2.0 mg L^{-1} GA and/or 5 % w/v PEG (molecular weight 6,000). All plants were harvested after 1 month to be used for further analysis.

Total RNA of leaves was extracted using the Plant RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. For cDNA synthesis, 2.3 μg of total RNA was reversely transcribed with 50 U AMV-reverse transcriptase in a total volume of 25 μL of Master Mix containing 2.5 μM oligo $(dT)_{16}$ primer, 20 U RNase inhibitor, 1× PCR buffer, 5 mM $MgCl_2$, and 1 mM of each dNTP (GeneAmp RNA PCR kit, Roche Molecular Systems, Branchburg, NJ, USA). *β-Actin* and *18S rRNA* were used as the house-keeping genes. Primers for both target and house-keeping genes were designed using the OligoCalc software to achieve specific characters required for real-time quantitative polymerase chain reaction (RT-qPCR) (Mohamed et al. 2011; Table 1).

Real-time quantitative PCR, based on the fluorescence emitted by the amplification products in the presence of SYBR Green, facilitates quantification of the target transcript accumulation relative to the house-keeping gene transcripts taken as reference. SYBR green binds to double-stranded DNA, and upon excitation emits light. Thus, as PCR product accumulates, fluorescence increases. The reactions were prepared using the qPCR kit Master mix for SYBR Green (Promega) according to the manufacturer's protocol. The SYBR green one-step real-time RT-qPCR assay was carried out in a 96-well plate. The PCR reaction was adjusted to 10:2.5 μL RNase-free water, 0.2 μL of each primer (10 μmol L^{-1}), 5 μL SYBR green mix, 0.1 μL CXR and 2 μL of cDNA (5 ng μL^{-1}). The reactions were run in triplicate in an ABI prism 7,000 sequence detection system (ABI Prism 7000 SDS, Applied Biosystems) using the following thermal cycles: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 62 °C for 30 s. Melting curve (dissociation) analysis (60–95 °C) was done to verify amplicon specificity after 40 cycles at 95 °C for 15 s, 62 °C for 1 min and 95 °C for 15 s. The cDNA concentration used produced a CT (threshold cycle). The abundance of targeted gene transcripts was normalised to *β-Actin* and *18S rRNA* and set relative to control plants (no treatment exposure) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Data analysis of RT-qPCR results was performed using Step One software (version 2.1).

All experiments were done by using a Randomized Complete Block Design with three replications. The data were analysed by the Duncan test's SPSS (version 16) statistical package to assess significant differences (at the 5 % level) between means.

Results and discussions

The results of RT-qPCR were normalised to the level of two house-keeping genes of *β-Actin* or *18S rRNA*. As previously indicated, there are two copies of the second terpene cyclase (*ent-KS*), which are named as *ent-KS1-1* and *ent-KS22-1*. The relative transcription of *ent-CPS* and *ent-KS22-1* were very low and could not be detected in all samples. Therefore, the results were not reported in this study. Both used house-keeping genes showed similar transcription patterns. The negative control samples showed no signal amplification which indicates good sample purity. The expression patterns of samples relative to *β-Actin* or *18S rRNA* were almost similar and therefore only the values relative to *β-Actin* were reported. All samples showed the same melting curve.

Steviol glycosides are derived from the same *ent*-kaurenoid precursor as gibberellins, and *ent-KS* and *ent-KO* are involved in both gibberellin and steviol biosynthesis (Brandle and Telmer 2007). The transcription patterns of SVglys biosynthetic genes varied with different treatments. The transcription of *ent-KS1-1* and *ent-KAH* showed no significant changes under PEG, GA and PBZ treatments (Fig. 2). On the contrary, *ent-KO* transcription was significantly affected by different treatments. GA treatment

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

Gene	Primer sequence 5′→3′ (forward/reverse)	Amplicon length (bp)	Accession number
ent-KS1-1	GCTCTGATTGAACACACGATTATC/TCCTATGTAGAGTGAATCTAAGAGG	151	AF097310
ent-KO	GCTGTGATGAAGTCTCTTATTAAA/CCATAGTGGTGTCTGATGATTCAAT	162	AY364317
ent-KAH	CCATATTCACCATCCGACTTGG/GGGTAGTGAAGATCTCCTTAGC	151	Brandle and Richman (2008)
UGT85C2	TCGATGAGTTGGAGCCTAGTATT/CTAAACTGTATCCATGGAGACTC	153	AY345978
UGT74G1	TGCATGAACTGGTTAGACGATAAG/GCATCCTACTGATTCGTGTGCTA	274	AY345982
UGT76G1	GCAGCTTACTAGACCACGATC/CTCATCCACTTCACTAGTACTAC	107	AY345974
18S rRNA[a]	CCGGCGACGCATCATT/AGGCCACTATCCTACCATCGAA	59	Cloned at the laboratory
β-Actin	AGCAACTGGGATGACATGGAA/GGAGCGACACGAAGTTCATTG	65	AP548026

[a] Using specific primers designed for 18S rRNA, a segment of 400 bp of *Stevia rebaudiana* was obtained and used as a template to design primers convenient for RT-qPCR

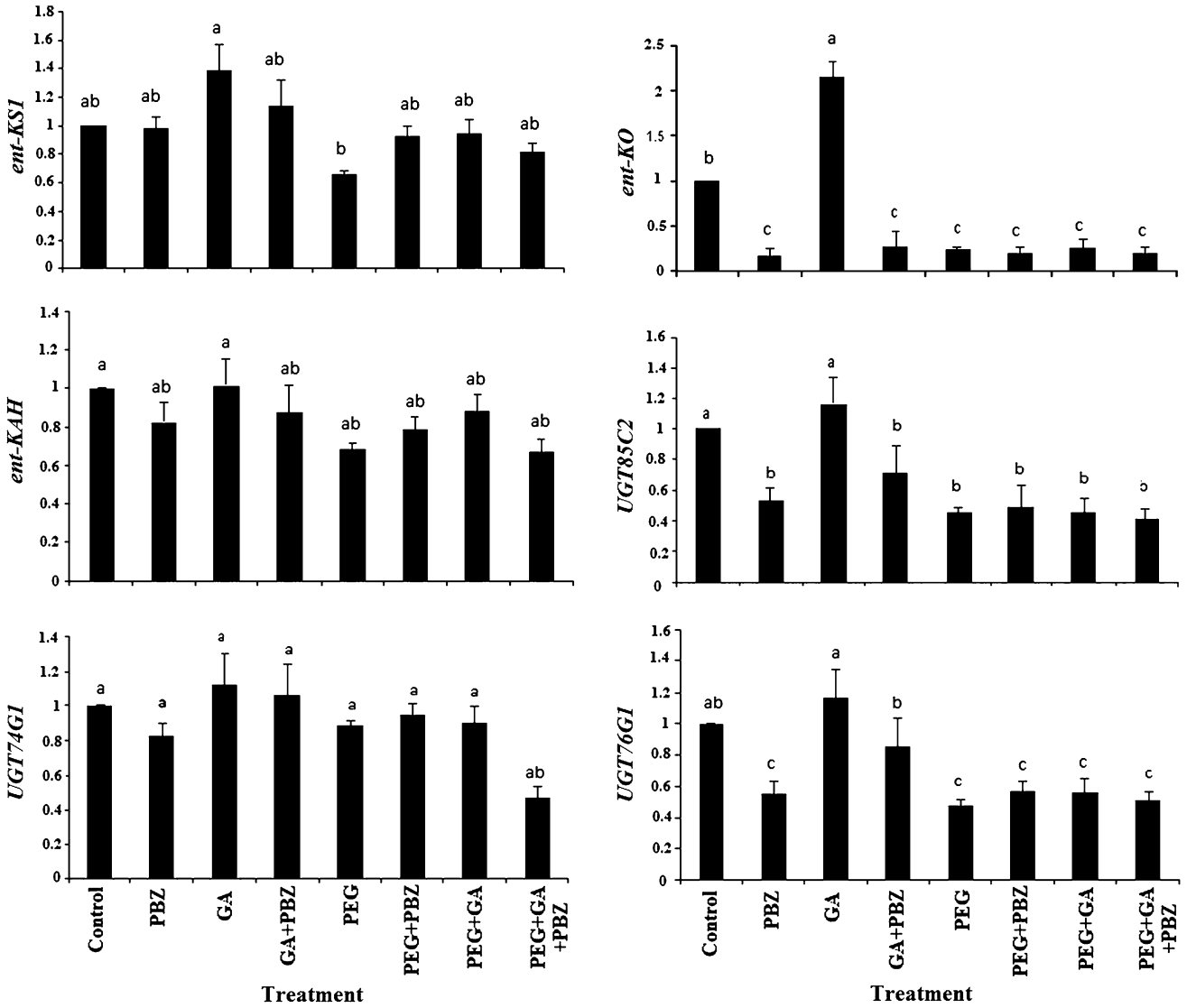

Fig. 2 The transcription of *ent-KS1-1*, *ent-KO*, *ent-KAH*, *UGT85C2*, *UGT74G1* and *UGT76G1* of *S. rebaudiana* involved in the SVglys biosynthesis, relative to that of *β-Actin* in plants subjected to paclobutrazol (*PBZ*), gibberellic acid (*GA*) and polyethylene glycol (*PEG*) treatments

alone significantly ($P \leq 0.05$) increased *ent-KO* transcription whereas the other treatments caused greatly significant reductions (Fig. 2). Opposite to our results, Kumar et al. (2012) reported no upregulation of *ent-KO* in *S. rebaudiana* in GA-treated plants. PBZ treatment resulted in about 84 % decrease in *ent-KO* transcription level. The effect of PBZ on plant growth reduction is well-known as it blocks GA biosynthesis, more specifically the pathway at the *ent*-kaurene oxidation step (Fletcher et al. 2000). In the PEG-treated plants, *ent-KO* transcription significantly ($P \leq 0.05$) decreased compared to the control plants. It is interesting that the *ent-KO* transcription pattern in PEG-treated plants was the same as PBZ and both treatments resulted in a clear inhibition. GA treatment could not reverse the negative effect of PBZ treatment on *ent-KO* transcription. *Ent-KO* is upregulated in mature leaves where it plays a role in SVglys biosynthesis (Humphrey et al. 2006). However, in *Arabidopsis*, *Cucurbita maxima* and *Pisum sativum*, *ent-KO* was found to be transcribed predominantly in developing seeds and young, rapidly growing tissues with low transcription levels in mature leaves (Helliwell et al. 2000; Davidson et al. 2004). There is a positive correlation between *ent-KO* transcription and SVglys accumulation (Kumar et al. 2012), which is supported by the results of this experiment and our previous experiments on SVglys content (Hajihashemi et al. 2012). It can be suggested that difference in *ent-KO* transcription affects SVglys content by changes in their precursor.

The glucosyltransferases *UGT74G1*, *UGT76G1* and *UGT85C2* belong to Family 1 (GT1) according to CAZy

classification (http://www.cazy.org/fam/GT1.html) which describes more than 91 distinct families. The plant CAZy Family 1 substrates include terpenoids, alkaloids, cyanogenic glucosides and glucosinolates as well as flavonoids, isoflavonoids and other phenylpropanoids (Mohamed et al. 2011). The transcription of *UGT85C2* significantly decreased in PBZ and/or GA and/or PEG treatments, with the exception of GA treatment alone (Fig. 2). The significant correlation between *UGT85C2* transcription and total SVgly accumulation suggests that the UGT85C2 enzyme, which adds a C-13-glucose to steviol to form steviolmonoside, is the rate-limiting step of the glycosylation pathway during SVgly biosynthesis (Mohamed et al. 2011). The *UGT74G1* transcription was not significantly affected by different treatments (Fig. 2). Kumar et al. (2012) reported an upregulation of *UGT74G1* in GA_3-treated *S. rebaudiana*, which is not supported by our results. The transcription of *UGT76G1* significantly decreased in PBZ treatment alone and PEG treatments with or without PBZ and/or GA (Fig. 2). GA and GA + PBZ treatments had no significant effect on *UGT76G1* transcription. Results indicated that *ent-KAH* transcription was not correlated to *UGTs*. It means that the effects of treatments and stress on genes involved in the same biosynthesis pathway can differ individually and this complicates the mechanism. It happens that the transcription patterns of different members within the same gene family considerably vary in PBZ-treated plants as was reported by Song et al. (2011) for maize.

Stevioside content obviously decreased in PBZ and PEG treatments, whereas the transcription of *UGT74G1* was not significantly affected by treatments (Hajihashemi et al. 2012). However, Reb A content and *UGT76G1* transcription both noticeably decreased under PBZ and PEG treatments. It can be concluded that the change in Reb A content is due to its precursor and *UGT76G1* transcription. Overexpression of some glycosyltransferase genes led to a significant increase in their respective glucosides (Jackson et al. 2001).

According to our previous study, the plant growth and SVglys content (%DW) in the treated plants followed this order: GA > control > GA + PBZ > PBZ and PEG (with or without PBZ and/or GA). In the present study, the transcriptions of *ent-KO*, *UGT85C2* and *UGT76G1* in the treated plants followed the same order: GA > control > GA + PBZ > PBZ and PEG (with or without PBZ and/or GA). Ceunen et al. (2012) reported that the interruption of long nights by red LED light stimulated and sustained the vegetative growth as well as the accumulation of SVglys in the leaves. *Ent-KO* is the target gene of the inhibitor PBZ but it was also inhibited in PEG-treated plants. The transcriptions of *UGT85C2* and *UGT76G1* were inhibited by both PBZ and PEG treatments.

In *S. rebaudiana*, the plant growth decreased by PBZ and PEG treatments but increased by GA treatment (Hajihashemi et al. 2012). The results indicated that inhibition of growth by both PBZ and/or PEG treatments resulted in the same negative effect on genes transcription and SVglys accumulation. Further studies are required to conclude that the gene transcription and SVglys accumulation vary positively with changes in growth.

Author contribution S. Hajihashemi wrote the manuscript, designed the experiment, ran the experiment, and analysed data. J.M.C. Geuns wrote the manuscript, designed the experiment and involved in interpretation of the results. A.A. Ehsanpour wrote the manuscript and designed the experiment.

Acknowledgments The authors would like to thank Filip Rolland, Stijn Ceunen, Amal Mohamed and Sofie Deroover for their assistance.

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