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ORIGINAL ARTICLE

Steviol glycosides correlation to genes transcription revealed in gibberellin and paclobutrazol-treated *Stevia rebaudiana*

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Abstract *Stevia rebaudiana* contains steviol glycosides (SVglys) responsible for its sweet taste. The knowledge of some shared steps between gibberellin (GA) and SVglys biosynthesis made it imperative to study the effect of paclobutrazol (PBZ; a GA biosynthesis inhibitor) and GA itself on SVglys accumulation and transcription of their correlated genes. Plants were treated with GA and PBZ (10 mg L^{-1}) in controlled greenhouse conditions. The results showed that GA and PBZ treatments increased or decreased, respectively, stevioside, rebaudioside A, B, C, F, rubusoside, steviolbioside, dulcoside A, and total SVglys contents. The rebaudioside A/stevioside ratio is considered as a parameter to measure the quality of *Stevia* extract. PBZ treatment increased the rebaudioside A/stevioside ratio which means less aftertaste bitterness of *Stevia* extracts. GA treatment had no significant effect on this ratio. The transcription of *ent-KO,* ent-*KS1*, *ent–KAH*, *UGT76G1*, *UGT85C2* and *UGT74G1* were maximally increased by GA and minimally by PBZ treatments, respectively. This might explain the higher or lower accumulation of SVglys by treatments with GA or PBZ, respectively. The results revealed the correlation between gene transcription and SVglys accumulation which is worth to study in depth to produce *Stevia* with higher SVglys and sweeter taste.

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Introduction

The sweet *Stevia rebaudiana* (Bertoni) plant accumulates the diterpenic steviol glycosides (SVglys). There are more than 30 SVglys in *S. rebaudiana* with different concentrations (Ceunen and Geuns 2013; Wölwer-Rieck 2012). The most abundant SVglys in *Stevia* are stevioside (ST) and rebaudioside A (Reb A). Reb A, with a sweeter taste than ST, is used in drinks and foods (Soejarto 2002; Tanaka 1997). The Reb A/ST ratio is considered as a parameter to determine the quality of the *Stevia* extract taste (Ceunen and Geuns 2013; Yadav et al. 2011). Other SVglys consist of Reb C–F, dulcoside A (Dul A), steviolbioside (SB) in a total amount of 1–2% (Geuns 2003; Makapugay et al. 1984; Starratt et al. 2002). In *Stevia,* the enzymes of kaurene synthase (KS), kaurene oxidase (KO, a P450 monooxygenase) and kaurenoic acid 13-hydroxylase (KAH) convert copalyl pyrophosphate into steviol (SV). Then, specific glucosyltransferases form different SVglys by SV glucosylation (GTs; Figs. 1, 4) (Brandle and Telmer 2007; Shibata et al. 1995).

S. rebaudiana is currently considered as an alternative substitute for sucrose. Tribes from Brazil and Paraguay used *S. rebaudiana* as medicinal tea for years (Singh and Rao 2005). SVglys are introduced as a food additive in many countries. Highly purified SVglys and Reb A received the generally recognized as safe status in the United State of America (Wölwer-Rieck 2012). Antioxidant activities (Ahmad et al. 2010; Hajihashemi and Geuns 2013), anti-carcinogenic, anti-hyperglycaemic (Dey et al.

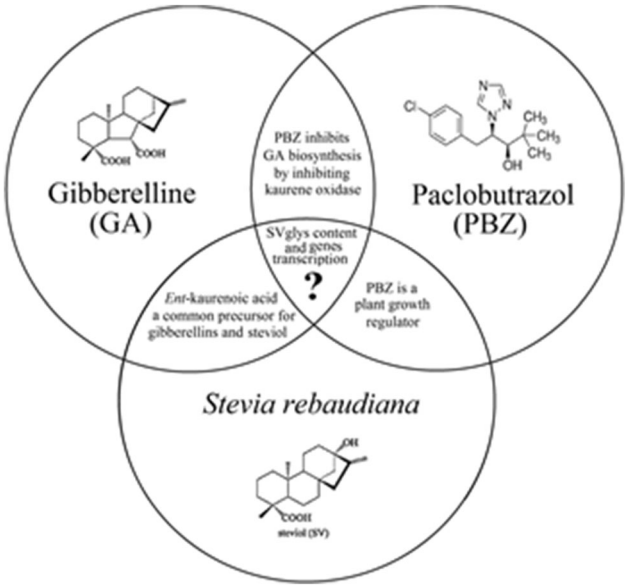

Fig. 1 A diagram represents the correlation between gibberellin, paclobutrazol and steviol glycosides

2013), anti-pathogenic (Fazal et al. 2011) and anti-hypersensitive properties (Liu et al. 2003) have been reported for SVglys.

Both SV and GA have a tetra-cyclic diterpene skeleton (Fig. 1). *Ent-KO* and *ent-KS* function in SVglys biosynthesis as well as GA biosynthesis and *ent*-kaurenoic acid is a common precursor for them (Figs. 1, 4). *Ent-KO* is up-regulated in mature *Stevia* leaves (Humphrey et al. 2006) whereas in *Cucurbita maxima*, *Arabidopsis* and *Pisum sativum*, *ent-KO* transcription is low in mature leaves (Davidson et al. 2005; Helliwell et al. 1999).

Triazole fungicides (e.g. paclobutrazol, hexaconazole- and propiconazole), with plant growth regulating side-effects, inhibit GA biosynthesis (Fletcher et al. 2010; Hedden and Graebe 1985). Paclobutrazol (PBZ) affects the isoprenoid pathway and blocks GA biosynthesis at the *ent-KO* step by inhibiting oxidation of kaurene to kaurenoic acid (Fletcher et al. 2010; Sankar et al. 2007).

S. rebaudiana is a new introduced crop and its physiological properties have not been studied extensively. Some steps are common between the SVglys and GAs biosynthesis pathway. PBZ blocks the GA biosynthesis (Figs. 1, 4). According to this knowledge, the present study has been designed to unravel the mechanism by which the vital step of SVglys biosynthesis in *S. rebaudiana* is regulated by GA and PBZ treatments and to study the possible correlation to genes transcription as well. The aim of this study was to (1) measure SVglys contents, (2) measure the transcription of some genes involved in GAs and SV biosynthesis, (3) and characterize the correlation between SVglys contents and the transcription of the genes analyzed.

Materials and methods

In vitro *S. rebaudiana* plants were provided by the Laboratory of Functional Biology, KULeuven, Leuven, Belgium. Plants were propagated by tissue culture technique. After 2 months, plants with a similar size were cultured into pots (1.5 L) filled with a mixture of vermiculite and perlite. The plants were grown under controlled greenhouse conditions with a photoperiod of 16 h light and 8 h dark. The air temperature ranged from 22 ± 4 °C during the day to 15 ± 3 °C during the night. The humidity level was between 40 and 60%. The irrigation was done every other day and the plants were fertilized by a nutrient solution (pH 6.0) every week. The plants (6 months old) were irrigated with 0 (distilled water), and 10 mg L^{-1} of PBZ and GA solutions. PBZ and GA treatments were carried out every 4 days, for 1 month. Two days later, the newly emerged leaves of the treatments were harvested for further analysis. Leaves were immediately frozen in liquid nitrogen and stored in a freezer at −80 °C. Some of the harvested leaves were freeze-dried for SVglys analysis. Plant culture and treatments were repeated thrice at different times. In each experiment, there were six replicates per treatment.

Water extraction of SVglys

The freeze-dried leaves (20 mg) were extracted three times by boiling for 15 min in 300 µL HPLC quality water. After centrifugation, the pellet was re-extracted twice with 300 µL HPLC quality water. The supernatants were mixed and centrifuged to remove particles (Ceunen et al. 2012). The samples were not purified to avoid possible losses. The samples were stored at −80 °C for further analysis.

Analysis of SVglys

The amount of SVglys was determined according to Ceunen et al. (2012). Twenty µL of extract was injected in the HPLC (Shimadzu, Deurne, Belgium) with two Grace Alltima C_{18} columns in series. The solvent flow was 1 mL min^{-1} and the gradient of acetonitrile (AcCN): 1.0 mM phosphoric acid was as described by Ceunen et al. (2012). Different SVglys (Reb A, ST, Reb B, Reb C, Reb F, Dul A, SB, Rub) were measured. The standard of Reb A with purity >99% was used to quantify the results (Geuns 2010). The SVglys amounts were reported as percent of the leaf dry mass (LDM).

Analysis of gene transcription using RT-q PCR

RNA extraction of frozen leaves was done by the RNA Mini Kit. For cDNA synthesis, a Master Mix containing

Table 1 List of primers used in RT-qPCR and house-keeping genes. Kaurene synthase (*ent-KS*), kaurene oxidase (*ent-KO*), kaurenoic acid hydroxylase (*ent-KAH*), UDP-dependent glycosyltransferases of *UGT85C2*, *UGT74G1* and *UGT76G1*

Gene	Primer sequence 5′ → 3′ (forward/reverse)	Amplicon length	Accession number
ent-KS1-1	GCTCTGATTGAACACACGATTATC/TCCTATGTAGAGTGAATCTAAGAGG	151 bp	AF097310
ent-KO	GCTGTGATGAAGTCTCTTATTAAA/CCATAGTGGTGTCTGATGATTCAAT	162 bp	AY364317
ent-KAH	CCATATTCACCATCCGACTTGG/GGGTAGTGAAGATCTCCTTAGC	151 bp	Brandle and Richman (2008)
UGT85C2	TCGATGAGTTGGAGCCTAGTATT/CTAAACTGTATCCATGGAGACTC	153 bp	AY345978
UGT74G1	TGCATGAACTGGTTAGACGATAAG/GCATCCTACTGATTCGTGTGCTA	274 bp	AY345982
UGT76G1	GCAGCTTACTAGACCACGATC/CTCATCCACTTCACTAGTACTAC	107 bp	AY345974
18S rRNA[a]	CCGGCGACGCATCATT/AGGCCACTATCCTACCATCGAA	59 bp	Cloned at the laboratory
β-Actin	AGCAACTGGGATGACATGGAA/GGAGCGACACGAAGTTCATTG	65 bp	AF518026

[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-q PCR

2.3 µg of total RNA was prepared in a total volume of 25 µL (Hajihashemi et al. 2013). The house-keeping genes of *β-Actin* and *18S rRNA* were used for quantification. The OligoCalc software was used to design primers (RT-qPCR; Table 1) (Hajihashemi et al. 2013).

Real-time PCR is based on the fluorescence signal emitted by SYBR Green I bound to the PCR product. House-keeping gene transcripts were taken as a reference. The qPCR kit Master Mix was used to prepare SYBR Green reaction mix. The SYBR green one-step RT-qPCR assay was done in a 96-well plate. The reaction mixture of PCR was composed of 2 µL of cDNA (5 ng μL^{-1}) in a 10 µL volume. The reactions were done in an ABI prism 7000 SDS as described by Hajihashemi et al. (2013). Obtained data were analysed according to Step One software (Version 2.1).

Statistical analysis

A Randomized Complete Block Design was used for all experiments with three biological and technical replications. The Duncan test (SPSS, version 16) was used to assess significant differences (at the 5% level) between means. The results of statistical analysis are shown by superscript letters to reveal significant differences.

Results

Figure 2 shows that PBZ induced shorter plants with smaller internodes while GA treatment resulted in longer plants. To compare the effect of GA and PBZ on *Stevia*, first we measured the SVglys contents. The accumulation of SVglys in leaves of *Stevia* decreased with about 53% by PBZ treatment (Fig. 3). ST, Reb A, Reb B, C, F, Dul A, Rub, and SB amounts were less than those of control plants. *S. rebaudiana* usually accumulates mainly ST,

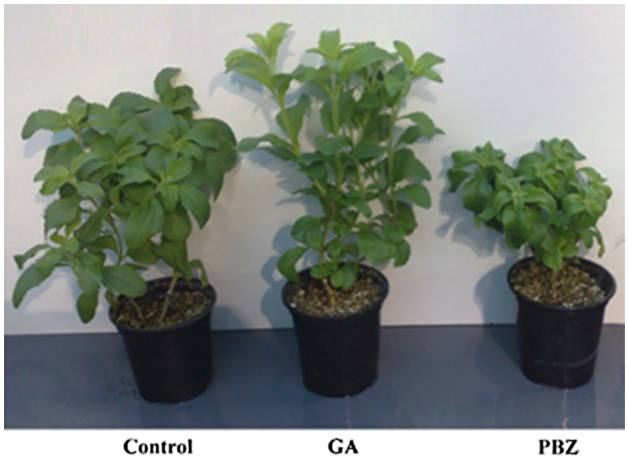

Fig. 2 *Stevia rebaudiana* plants under different treatments of gibberellin (GA) and paclobutrazol (PBZ) at 0 (control plant) and 10 mg L^{-1} concentrations

about three times more than Reb A (ST/Reb A ratio about 3.33). PBZ treatment decreased ST and Reb A content by about 60 and 37%, respectively. PBZ treatment led to a significant increase of the Reb A/ST ratio. GA treatment significantly increased the amount of SVglys by about 20% leading to increases of the different SVglys: ST, Reb A, Reb B, C, F, Dul A, Rub, and SB (Fig. 3). However, GA treatment had no significant effect on the Reb A/ST ratio.

In the next step of the study, the RT-q PCR analysis on treated plants was performed. The RT-q PCR results were normalized to the level of *18S rRNA* and *β-Actin*. The results relative to *β-Actin* were reported because the expression patterns of samples relative to *18S rRNA* and *β-Actin* were almost the same. The same melting curve was obtained for all samples. Samples were good purifies because no signal was observed in the negative control samples.

The *ent-KS1*, *ent-KO*, *ent–KAH* genes are involved in the biosynthesis of both SV and GA. The transcription of

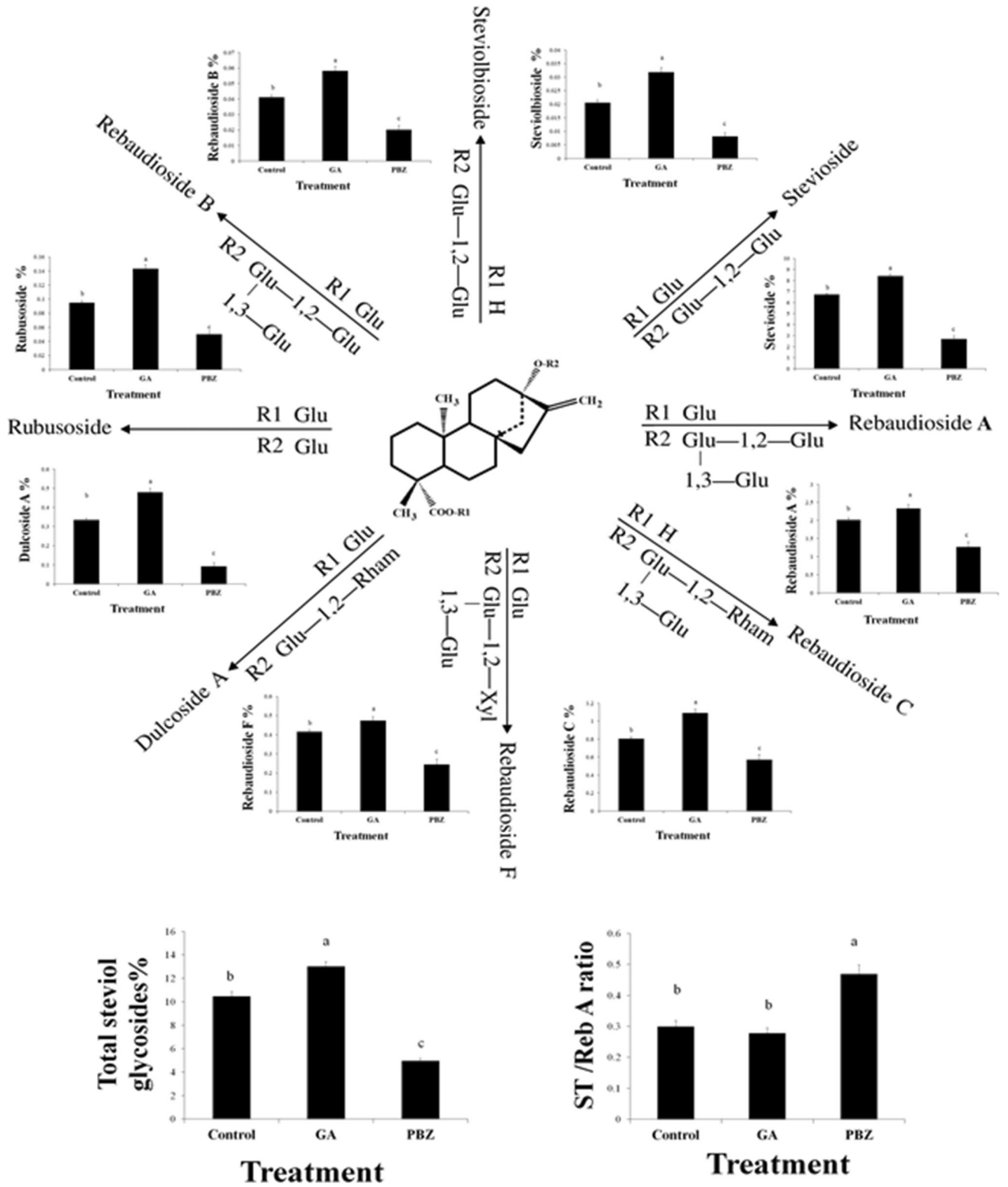

Fig. 3 SVglys content (% LDM) in leaves in *S. rebaudiana* treated with gibberellin (GA) and paclobutrazol (PBZ; mean ± standard deviation). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p < 0.05$

ent-KS1, *ent-KO* and *ent–KAH* were significantly decreased by PBZ treatment by about 22, 82 and 19%, respectively (Fig. 4). On the contrary, GA treatment significantly increased the transcription of *ent-KS1*, *ent-KO* and *ent–KAH* by about 19, 133 and 20%, respectively (Fig. 4). The transcription of *UGT74G1*, *UGT76G1* and

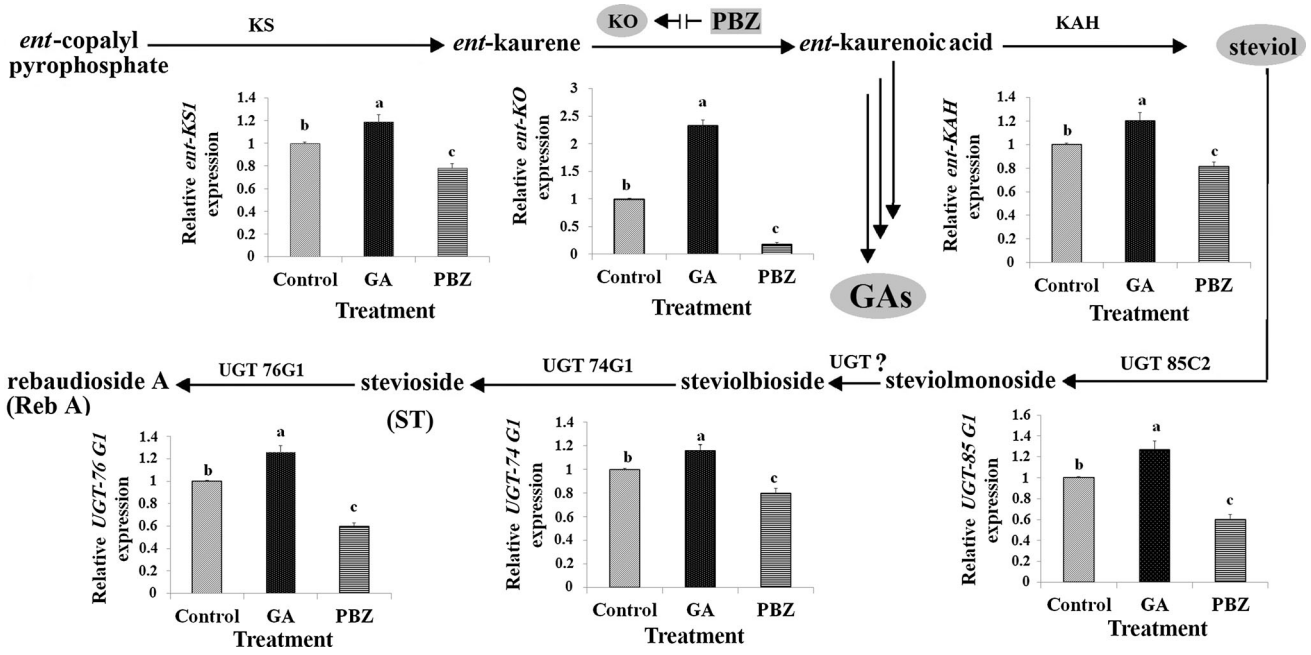

Fig. 4 The biosynthetic pathway of steviol glycosides. The transcription of *ent-KS1*, *ent-KO*, *ent-KAH*, *UGT85C2, UGT74G1* and *UGT76G1* of *S. rebaudiana* involved in the SVglys biosynthesis, relative to that of *β-Actin* in plants subjected to gibberellin (GA) and paclobutrazol (PBZ) treatments. kaurene synthase (KS), kaurene oxidase (KO), kaurenoic acid hydroxylase (KAH), UDP-dependent glycosyltransfrases (UGT). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p < 0.05$

UGT85C was significantly decreased by PBZ treatment by about 20, 40 and 40%, respectively (Fig. 4). GA application increased the transcription of *UGT74G1*, *UGT76G1* and *UGT85C* by about 16, 26 and 27%, respectively (Fig. 4). The most reduction or increase in gene transcription of six analyzed genes was observed with *ent-KO* by PBZ or GA treatments, respectively.

Discussion

S. rebaudina has a huge economic potential because of its SVglys accumulation. Since many years, SVglys products have been used as a sweetener in different countries (Ceunen and Geuns 2013; Geuns 2003; Woelwer-Rieck et al. 2010). A critical point in *S. rebaudiana* is to study the elements which may affect SVglys accumulation. *Ent*-copapalyl pyrophosphate serves as an intermediate for biosynthesis of steviol and different SVglys are formed by glucosylation of steviol (Brandle and Telmer 2007; Ceunen and Geuns 2013; Geuns 2003), hence analysis and regulation of genes in the SVglys biosynthesis assumes a central position in the manipulation of SVglys contents in *S. rebaudiana*. Previous studies showed the important role of *ent-KS*, *ent-KO* and GTs genes in regulating SVglys contents (Richman et al. 2005; Humphrey et al. 2006; Kumar et al. 2012). The knowledge of some shared steps in biosynthesis pathway of GA and SVglys made it imperative to study the effect of a GA biosynthesis inhibitor (PBZ) and GA itself on SVglys accumulation in the present study. Interestingly, the obtained results showed that GA and PBZ treatments resulted in positive and negative effects, respectively, on ST, Reb A, Reb B, C, F, Dul A, Rub and SB contents. Also, GA and PBZ treatments significantly increased and decreased, respectively, the total SVglys content. ST has three units of D-glucose attached to a steviol ring, whereas, Reb A has four of these units (Kennelly 2002; Starrat et al. 2002; Ceunen and Geuns 2013). This difference makes Reb A sweeter than ST. Commercially, it is worth to study the production of *Stevia* plants with higher Reb A than ST content or to make a selection of such plants with less aftertaste bitterness. It is valuable to report that PBZ increased the Reb A/ST ratio which means a sweeter taste of *Stevia* leaves extract of PBZ-treated plants while GA treatment had no effect on this ratio. Development of new varieties of *S. rebaudiana* with a higher content of Reb-A and a reduced content of ST is the aim of plant breeders (Ceunen and Geuns 2013; Dacome et al. 2005; Yadav et al. 2011).

It was interesting to reveal the correlation between SVglys content with the transcription of some key genes involved in the SVglys biosynthesis pathway. PBZ is a critical limiting treatment, adversely affecting GA biosynthesis (Fletcher et al. 2010; Sankar et al. 2007).

Evidently, the negative effect of PBZ on SVglys was observed at this study. The finding that PBZ reduced SVglys contents while GA increased them is reasonable, as PBZ negatively down-regulate but GA up-regulated the transcription of six involved genes in SVglys biosynthesis of *ent-KS1*, *ent-KO*, *ent–KAH*, *UGT74G1*, *UGT76G1* and *UGT85C2*. The transcript levels of *ent-KAH* and UGTs showed a linear correlation during vegetative growth (Ceunen and Geuns 2013). The inhibition or stimulation of *ent-KS1* and *ent-KO* transcriptions with PBZ and GA treatments, respectively, suggest a correlation between SVglys contents and their *ent*-kaurenoic acid precursor. GA is derived from the same *ent*-kaurenoid precursor as SVglys, and *ent-KS* and *ent-KO* are involved in their biosynthesis (Brandle and Telmer 2007). Kumar et al. (2012) reported a positive correlation between SVglys accumulation and *ent-KO* transcription, which is supported by the results of the present experiment. According to Fig. 2, the plant height decreased in PBZ-treated plants and increased in GA-treated plant which reflects the down-regulated and up-regulated transcription of *ent-KS* and *ent-KO* genes involved in GA biosynthesis pathway. The negative effect of PBZ on plant growth is correlated to GA biosynthesis inhibition, more specifically at the *ent-KO* step (Fletcher et al. 2010; Sankar et al. 2007). Based on a previous study (Hajihashemi et al. 2013), *ent-KO* transcription significantly decreased in the PBZ-treated plants while it increased in GA-treated plants which also supports the results of the present study. Hajihashemi et al. (2013) similarly reported that the transcription of *ent-KS1* decreased in PBZ-treated plants under in vitro conditions. On the contrary, the result of the previous study on *ent-KAH* transcription does not support the result of this study on PBZ treatment. That study showed that GA treatment had no significant effect on *ent-KS* and *ent-KAH* transcriptions under in vitro culture conditions (Hajihashemi et al. 2013) while in the present study, their transcriptions significantly increased by GA treatment. The observed differences about the transcription levels of *ent-KS1* and *ent-KAH* under in vitro culture conditions (Hajihashemi et al. 2013) and greenhouse conditions might be correlated to different parameters such as plant age, growth conditions, concentration of treatment solutions and others which need more investigation. Both *ent-KS* and *ent-KO* are highly expressed in mature leaves in *S. rebaudiana* while they are only highly expressed in the very young, actively growing leaves in other plants (Humphrey et al. 2006; Richman et al. 1999). A linear correlation was observed between low SVglys content with the very low transcription of *ent-KS*, *ent-KO*, and UGTs in *S. rebaudiana* roots (Humphrey et al. 2006; Richman et al. 1999) which is in according to the results of PBZ treatment in this study. It is suggested that the expression of the *ent-KS* and *ent-KO* genes is mainly limited to GA biosynthesis occurring in the root tip (Richman et al. 1999).

According to the CAZy classification, the glucosyltransferases of *UGT74G1*, *UGT76G1* and *UGT85C2* belong to Family 1 (GT1). Terpenoids, alkaloids, cyanogenic glucosides, glucosinolates, flavonoids, isoflavonoids and other phenylpropanoids belong to the plant CAZy Family 1 (Mohamed et al. 2011). Our results showed that the transcription of *UGT85C2* and *UGT76G1* significantly decreased by PBZ treatment which corroborates results of Hajihashemi et al. (2013). The *UGT85C2* enzyme adds a C-13-glucose to SV to form steviolmonoside. The correlation between transcription of *UGT85C2* and total SVgly contents suggests *UGT85C2* as a rate-limiting step in SVgly biosynthesis (Mohamed et al. 2011). *UGT76G1* is suggested to regulate the synthesis of ST and Reb A (Richman et al. 2005; Richman et al. 1999). In this study, the transcription of *UGT74G1* significantly decreased by PBZ treatment, which is different from in vitro studies (Hajihashemi et al. 2013). In this study, GA treatment increased the transcription of *UGT74G1*, *UGT76G1* and *UGT85C2* contrary to the results from in vitro conditions.

It is known that plant responses to treatments are associated with the plant species, concentration and time of treatments and environmental conditions (Chaves et al. 2002). Hajihashemi et al. (2013) treated *Stevia* plant by adding 2 mg L^{-1} GA and PBZ to MS culture medium containing explants with just two auxiliary buds without roots and leaves while in the present study 6 month old plants were treated with 10 mg L^{-1} of GA and PBZ. Changes in SVglys contents and the transcription of genes were shown to be dependent of growth stage (Ceunen et al. 2012; Ceunen and Geuns 2013; Yang et al. 2015). The harvesting time of *Stevia* also affected the quality and yield of SVglys in leaves (Pal et al. 2014). In conclusion, it can be strongly suggested that the *Stevia* response to GA and PBZ was influenced not only by the treatment itself but also by the concentration of the substances administered, the growth conditions, and the plant growing stage which had a major influence on SVglys accumulation and some genes involved. PBZ inhibit GA production by blocking *ent-KO* step. The results showed the highest reduction and increse in *ent-KO* transcription by PBZ and GA treatments, respectively. The linear correlation observed between *ent-KO* expression with five others studied genes suggests one possible mode of regulation involves metabolon formation (Ceunen and Geuns 2013). Another important point might be the positive effect of PBZ treatment on the Reb A/ST ratio which resulted in a better taste of the *Stevia* extract. It is an important issue to do more research on the production of *S. rebaudiana* with a sweeter taste and less bitter taste via bioengineering.

The response of *S. rebaudiana* (Bertoni) Bertoni to different treatments outlined in this study confirms that SVglys accumulation is affected by PBZ and GA treatments closely related to the transcription of the genes involved. Six genes of *ent-KS1*, *ent-KO*, *ent–KAH*, *UGT74G1*, *UGT76G1* and *UGT85C2* were transcribed maximally by GA and minimally by PBZ treatments which resulted in a greater accumulation of SVglys by GA treatment and a smaller one by PBZ treatment, respectively. The transcription of genes involved in the same biosynthesis pathway can differ individually depending upon the growth (in vitro vs. greenhouse). This complicates the explanation of the involved mechanisms. *Ent-KO* transcription changed by PBZ and GA treatments in both this study and the previous study (Hajihashemi et al. 2013). It might be suggested to classify the genes involved in the SVglys biosynthesis pathway into two groups of early and late induced genes. The gene of *ent-KO* can be classified as the early induced genes in response to PBZ and GA treatments. With increasing concentrations of PBZ and GA, the other genes of the pathway become engaged (e.g. *ent-KS1*, *ent–KAH*, *UGT74G1*, *UGT76G1* and *UGT85C2*) which might be classified as late induced genes. Overall, these findings provide new insights into the complicated response mechanisms in *Stevia* plants. Moreover, it should be considered that these studies open new perspectives for the study of other genes involved in GAs and SVglys biosynthesisin *S. rebaudiana* to reveal the mechanism of gene expression regulation by GA and PBZ treatments. Another valuable result obtained in the present study is the increased Reb A/ST ratio in PBZ-treated plants resulting in a sweeter taste of the *Stevia* extracts.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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