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Research article

Enhanced production of steviol glycosides in mycorrhizal plants: A concerted effect of arbuscular mycorrhizal symbiosis on transcription of biosynthetic genes

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ABSTRACT

Stevia rebaudiana (Bertoni) produces steviol glycosides (SGs) – stevioside (stev) and rebaudioside-A (reb-A) that are valued as low calorie sweeteners. Inoculation with arbuscular mycorrhizal fungi (AMF) augments SGs production, though the effect of this interaction on SGs biosynthesis has not been studied at molecular level. In this study transcription profiles of eleven key genes grouped under three stages of the SGs biosynthesis pathway were compared. The transcript analysis showed upregulation of genes encoding 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway enzymes viz.,1-deoxy-D-xylulose 5-phospate synthase (DXS), 1-deoxy-D-xylulose 5-phospate reductoisomerase (DXR) and 2-C-methyl-D-erytrithol 2,4-cyclodiphosphate synthase (MDS) in mycorrhizal (M) plants. Zn and Mn are imperative for the expression of MDS and their enhanced uptake in M plants could be responsible for the increased transcription of MDS. Furthermore, in the second stage of SGs biosynthesis pathway, mycorrhization enhanced the transcription of copalyl diphosphate synthase (CPPS) and kaurenoic acid hydroxylase (KAH). Their expression is decisive for SGs biosynthesis as CPPS regulates flow of metabolites towards synthesis of kaurenoid precursors and KAH directs these towards steviol synthesis instead of gibberellins. In the third stage glucosylation of steviol to reb-A by four specific uridine diphosphate (UDP)-dependent glycosyltransferases (UGTs) occurs. While higher transcription of all the three characterized UGTs in M plants explains augmented production of SGs; higher transcript levels of *UGT76G1*, specifically improved reb-A to stev ratio implying increased sweetness. The work signifies that AM symbiosis upregulates the transcription of all eleven SGs biosynthesis genes as a result of improved nutrition and enhanced sugar concentration due to increased photosynthesis in M plants.

1. Introduction

Stevia rebaudiana (Bertoni) finds an unparalleled use in the medicinal world due to singular properties of its diterpenoid steviol glycosides (SGs) — stevioside (stev) and rebaudioside-A (reb-A) that are nearly 300 times sweeter than sucrose (Lemus-Mondaca et al., 2011). These have been used for the treatment of diabetes, dental maladies, obesity, hypertension and cancer (Geuns, 2003) hence, many efforts have been propounded to increase the concentration of SGs. While certain physiological parameters such as photoperiodism are known to affect the concentration of SGs (Ceunen et al., 2012; Ceunen and Geuns, 2013; Yang et al., 2015) on the other hand, mutualistic associations such as arbuscular mycorrhizal (AM) symbiosis offer a potent, economically conducive approach to encounter the growing demand of SGs (Mandal et al., 2013). Despite of several studies connoting the potential of AMF to augment the secondary metabolite production in commercially important medicinal plants (Kapoor et al., 2002a,b, 2004, 2007; Copetta et al., 2006); their role in manipulating the biosynthesis pathways of these metabolites have not been dissected at molecular level. In a first of such attempts, it was recently reported that AM symbiosis upregulates the expression of crucial artemisinin biosynthesis genes (Mandal et al., 2014). However, more studies need to be done to optimize the mechanism based on which AM symbiosis functions to increase the secondary metabolite production.

Biosynthesis of SGs takes place in leaves (Brandle et al., 1998) in a seventeen steps process that can be categorized under three

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stages. SGs biosynthesis commences with the conversion of pyruvate and glyceraldehyde-3-phosphate to isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in eight sequential reactions forming the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Wanke et al., 2001; McGarvey and Croteau, 1995). 1-deoxy-D-xylulose 5-phospate synthase (DXS) and 1-deoxy-D-xylulose 5-phospate reductoisomerase (DXR) play a central role in regulation of MEP pathway (Cordoba et al., 2009). These enzymes catalyse the condensation of pyruvate and glyceraldehyde-3-phosphate into MEP (Kuzuyama et al., 2000; Takahasi et al., 1998). 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS) is an important control point in SGs biosynthesis (Kumar et al., 2012b) as it could bind to intermediates of the pathway and control the metabolite flux by feedback regulation (Kemp et al., 2005).

The second stage of the SGs biosynthesis pathway begins with a condensation reaction catalysed by important branch point enzyme geranylgeranyl diphosphate synthase (GGDPS) to form geranylgeranyl diphosphate (GGDP) which is a common precursor for the biosynthesis of several diterpenoids (Okada et al., 2007). GGDP is converted to steviol by consecutive action of 4 enzymes viz., copalyl diphosphate synthase (CPPS), kaurene synthase (KS), kaurene oxidase (KO) and kaurenoic acid hydroxylase (KAH) (Brandle and Telmer, 2007). Biosynthesis pathways of SGs and gibberellins share the same kaurenoid precursor and GGDPS, CPPS, KS and KO are all involved in the biosynthesis of both gibberellins and SGs (Kumar et al., 2012a; Richman et al., 2005). Since, CPPS is crucial for the conversion of GGDP to copalyl diphosphate (CDP) it is therefore, believed to be a control point for the flow of metabolites (Silverstone et al., 1997). Kaurene is produced from CDP in an ionization dependent cyclization by KS (Brandle and Telmer, 2007). It is important for the synthesis of SGs since *KS* is present in duplicates only in *S. rebaudiana* (Richman et al., 1999). KO catalyzes oxidation of kaurene to form kaurenoic acid (Humphrey et al., 2006). The divergence of steviol biosynthesis from gibberellin biosynthesis occurs with the hydroxylation of kaurenoic acid into steviol catalysed by KAH (Hanson and white, 1968; Kim et al., 1996). This is the first specific step towards SGs biosynthesis (Brandle and Telmer, 2007).

In the third stage glucosylation of steviol to reb-A by four specific uridine diphosphate (UDP)-dependent glycosyltransferases (UGTs) occurs. However, in *S. rebaudiana* only three have been identified so far, namely, UGT85C2, UGT74G1 and UGT76G1 (Brandle and Telmer, 2007). These UGTs transfer sugar molecules from activated donor to an acceptor molecule and thus sugar concentration influences the glucosylation of ent-kaurene that form the SGs (Richman et al., 2005). Mohamed et al. (2011) suggested that glucosylation of steviol by UGT85C2 is a rate limiting step. Both UGT74G1 and UGT76G1 are necessary for the synthesis of stev and reb-A, respectively (Humphrey et al., 2006).

With the knowledge of above, effect of AMF inoculation on transcript profiles of eleven enzymes crucial for SGs biosynthesis was studied. The present work is complementary to the previous study where it was established that AM symbiosis augments the production of SGs through nutritional and non-nutritional mechanisms (Mandal et al., 2013).

2. Material and methods

2.1. Experimental design

Stem cuttings of *S. rebaudiana* var. Cimmeethi, a high yielding variety, were procured from Central Institute of Medicinal and Aromatic Plants (CIMAP), Pantnagar, India. Root induction in stem cuttings was achieved by hormone treatment in autoclaved sterile soil. Plants of uniform size were transplanted to pots (14′ X 13″) with 13 kg of autoclaved soil in each. Before transplantation the soil was autoclaved twice for 1 h at 121 °C and 15 psi on alternate days to kill resilient spores, if any. The soil used was a sandy loam soil (sand 14.7%, silt 35.5% and clay 22.8%) with the following chemical characteristics: pH (H_2O) 7.6, EC 0.12 Sm^{-1} at 32 °C; organic C 1.12%; total N 0.49%; and available P, K, Na, Mg, Ca, Zn, and Cu were 11.1, 55, 61.3, 45, 150, 55, and 22 mg kg^{-1}, respectively.

The experiment comprised of two treatments viz., plants without mycorrhizal inoculation represented non-mycorrhizal (NM) control plants, and plants inoculated with AMF *Rhizophagus intraradices* (N.C. Schenck& G.S. Sm.) C. Walker & A. Schüßler represented the mycorrhizal (M) plants. Each treatment consisted of two plants per pot replicated ten times. The experiment consisted of 20 pots arranged in a completely randomized block design. Plants were grown in the months of February to July under natural conditions of growth at Delhi India. The mean temperature at the time of experiment ranged between 25° C and 40 °C; and relative humidity between 50% and 70%. Soil moisture was maintained by watering the plants twice every week with normal tap water. Estimation of all the parameters was carried out at full maturity i.e. 120 days post inoculation when the concentration of SGs is reported to be maximum (Brandle et al., 1998).

2.2. Inoculation and colonization by AMF

The starter inoculum of *R. intraradices* (accession number CMCCWep319) was procured from The Energy and Resources Institute (TERI, New Delhi, India). Details of mass cultivation procedure have been published previously (Kapoor et al., 2007). Approximately 10 g of AMF inoculum containing soil and chopped roots was applied just below the roots of M plants at the time of transplantation.

Roots of *S. rebaudiana* were cleared in KOH and stained with trypan blue (Phillips and Hayman, 1970) to confirm mycorrhization. Percent root colonization (87%) was estimated microscopically following Biermann and Linderman (1981).

2.3. Mineral nutrient analysis

Analysis of mineral nutrients (P, Mg, K, Cu, Zn, Mn, and Fe) was carried out using oven dried leaf samples. Dried leaves were ground and approximately 0.2 g of each sample was digested in tri-acid mixture. The acid digest was allowed to cool for some time and diluted to 50 ml using double distilled water. A reagent blank was prepared by following the whole extraction procedure without a sample. Phosphorus concentration from the digested samples was determined following molybdate blue method (Allen, 1989) at 700 nm using a UV–vis spectrophotometer (Beckman Coulter DU®730). The concentrations of Mg, K, Cu, Zn, Mn, and Fe in the samples were measured following Allen (1989) using atomic absorption spectrophotometer (Shimadzu AA-130).

2.4. Estimation of net photosynthetic rate and total sugars

Measurement of net photosynthetic rate (P_N) was performed on sunny days at 11:00 a.m. with fully expanded leaves using IRGA (Infra Red Gas Analyzer, LI-COR 6400 Portable Photosynthesis System, Lincoln, Nebraska, USA). IRGA was calibrated prior to carrying out measurements and also approximately after every 30 min during the measurement period. Each leaf was enclosed in the gas exchange chamber for 60 s. P_N measured by IRGA was recorded six times for each treatment.

Concentration of total sugars in the fresh leaves of *S. rebaudiana* was determined following the protocol described by Yemm and