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A functional (*E*)-*4-hydroxy-3-methylbut-2-enyl diphosphate reductase* exhibits diurnal regulation of expression in *Stevia rebaudiana* (Bertoni)

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ABSTRACT

The leaves of stevia [*Stevia rebaudiana* (Bertoni)] are a rich source of steviol glycosides that are used as non-calorific sweetener in many countries around the world. Steviol moiety of steviol glycosides is synthesized via plastidial 2*C*-methyl-D-erythritol 4-phosphate pathway, where (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) is the key enzyme. HDR catalyzes the simultaneous conversion of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate into five carbon isoprenoid units, isopentenyl diphosphate and dimethylallyl diphosphate. Stevia *HDR* (*SrHDR*) successfully rescued *HDR* lethal mutant strain MG1655 ara<>ispH upon genetic complementation, suggesting *SrHDR* to encode a functional protein. The gene exhibited diurnal variation in expression. To identify the possible regulatory elements, upstream region of the gene was cloned and putative *cis*-acting elements were detected by *in silico* analysis. Electrophoretic mobility shift assay, using a putative light responsive element GATA showed the binding of nuclear proteins (NP) isolated from leaves during light period of the day, but not with the NP from leaves during the dark period. Data suggested the involvement of GATA box in light mediated gene regulation of *SrHDR* in stevia.

1. Introduction

Isoprenoids are low molecular-mass natural products synthesized by plants. Stevia accumulates as high as 20% (w/w) of diterpenoid isoprenoids called steviol glycosides (SGs) in the leaf tissue (Kinghorn and Soejarto, 1985; Phillips, 1987; Starratt et al., 2002). SGs are the glucosylated derivates of diterpenoid alcohol steviol and are approximately 300 times sweeter than common table sugar (Geuns, 2003; Kennelly, 2002). SGs are non-calorific sweeteners and exhibit antioxidant, anti-tumor, anti-hypertensive, anti-hyperglycemic, immunomodulatory, anti-rotavirus and anti-inflammatory properties (for review see Madan et al., 2010).

The 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway plays a central role in the biosynthesis of compounds such as isoprene, monoterpenes, sesquiterpenes, diterpenes, tetraterpenes and carotenoids (Lichtenthaler et al., 1997a,b). The MEP pathway is the single source of essential isoprenoids in the majority of pathogenic bacteria and for the apicoplast subgroup of pathogenic protozoa (Rohdich et al., 2005). Therefore, the enzymes of the pathway are considered potential targets for prevention and treatment of diseases such as tuberculosis and malaria (Missinou et al., 2002). Experiments using labeled glucose revealed the involvement of MEP pathway in the formation of diterpene skeleton of SGs (Totté et al., 2000). The (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR; EC: 1.17.1.2) is a key enzyme in the last step of the MEP pathway (Fig. 1) that catalyzes the synthesis of isoprenoid units isopentenyl diphosphate and dimethylallyl diphosphate in a ratio of 5:1 (Adam et al., 2002). Several reports suggested a correlation between the activity of HDR and synthesis of secondary metabolites. HDR activity was shown to be essential for isoprenoid biosynthesis in *Escherichia coli* (McAteer et al., 2001). Transgenic *Arabidopsis thaliana* plants co-over-expressing *taxadiene synthase* and *HDR* showed a twofold increase in taxadiene production (Botella-Pavía et al., 2004). *HDR* is also shown to be involved in camptothecin (terpenoid indole alkaloid) biosynthesis in *Camptotheca acuminata* (Wang et al., 2008). *HDR* was found to be over-expressed in the isoprenoid-rich glandular trichomes of mint leaves (Cunningham et al., 2000). High transcript levels of *HDR* in the resin-producing stem wood of

Abbreviations: HDR, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; *SrHDR*, *Stevia rebaudiana HDR*; NP, nuclear proteins; MEP, 2*C*-methyl-D-erythritol 4-phosphate; EMSA, electrophoretic mobility shift assay; SGs, steviol glycosides; IRGA, Infra-red gas analyzer; MEGA, Molecular Evolutionary Genetics Analysis; SOPMA, Self-Optimized Prediction Method with Alignment; PLACE, Plant *Cis*-acting Regulatory DNA Elements; LB, Luria Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; cDNA, complementary DNA; *E. coli*, *Escherichia coli*; *A. thaliana*, *Arabidopsis thaliana*; MCT, 4-(cytidine 5′ diphospho)-2*C*-methyl-D-erythritol synthase; CMK, 4-(cytidine 5′ diphospho)-2*C*-methyl-D-erythritol kinase; *SrMDS*, *Stevia rebaudiana* 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; bp, base pair; *RBCS*, *ribulose-1,5-bisphosphate carboxylase*; *CAB*, *chlorophyll a/b binding protein*; *GAP*, *glyceraldydyde-3-phosphate dehydrogenase.*

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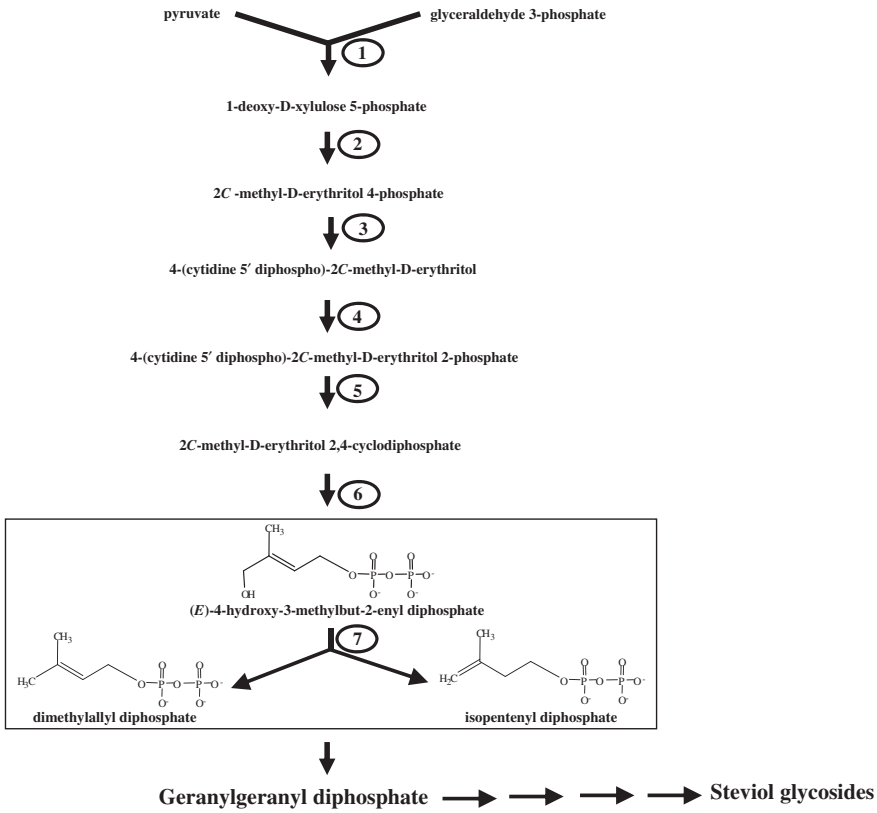

Fig. 1. SGs biosynthesis pathway in stevia, adapted from Kumar et al. (2012a). SrHDR [(*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase] catalyzes conversion of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate into isopentenyl diphosphate and dimethylallyl diphosphate (shown in the box). Encircled numbers represent enzyme catalyzing the corresponding reaction step as follows: 1. DXS: 1-deoxy-D-xylulose 5-phospate synthase; 2. DXR: 1-deoxy-D-xylulose 5-phospate reductoisomerase; 3. MCT: 4-(cytidine 5′ diphospho)-2C-methyl-D-erythritol synthase; 4. CMK: 4-(cytidine 5′ diphospho)-2C-methyl-D-erythritol kinase; 5. MDS: 2C-methyl-D-erytrithol 2,4-cyclodiphosphate synthase; 6. HDS: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase and 7. HDR: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase.

Pinus taeda further supported the participation of *HDR* in the secondary metabolism (Kim et al., 2008). *HDR* over-expression resulted in enhanced geranylgeranyl diphosphate derived products during (i) tomato fruit ripening, and (ii) seedling de-etiolation in *A. thaliana* (Botella-Pavía et al., 2004). Silencing of *HDR* in *Nicotiana benthamiana* led to an albino phenotype (Page et al., 2004). The *A. thaliana* null mutants for *HDR* also exhibited albino phenotype indicating the involvement of *HDR* in chloroplast development and photosynthetic pigment biosynthesis (Gutierrez-Nava et al., 2004; Hsieh and Goodman, 2005).

Previously, cloning of *SrHDR* from stevia and its expression regulation in different organs of the plant and in response to various phytohormones was reported (Kumar et al., 2012a). The gene was found to be highly expressed in leaf tissue, consistent with the data that SGs biosynthesis took place in the leaves. However, neither functional validation of *SrHDR* nor the bioinformatics analysis of the gene was reported. After thorough bioinformatics analysis, the present work functionally validated the gene through complementation assay in *E. coli HDR* mutant strain MG1655 ara<>ispH, studied expression regulation, and identified the putative *cis*-acting element in the promoter region of the gene that exhibited protein binding. For the first time, the present work showed the involvement of GATA box in light mediated regulation of *SrHDR*.

2. Materials and methods

2.1. Plant material and growth conditions

Plants of *Stevia rebaudiana*, well maintained at an experimental farm of the institute (32°06′20″ N latitude; 76°33′29″ E longitude; 1300 m amsl), were chosen for various studies. Diurnal variation in gene expression was studied in the 3rd leaf (leaf position with reference to the apical leaf as the 1st leaf) harvested at 06:00 h, 10:00 h and 20:00 h, frozen immediately in liquid nitrogen and stored at −80 °C, until further use. Light intensities in the field were measured using a light meter housed on an infra-red gas analyzer (IRGA; LI-6400; Li-COR Instruments, USA).

2.2. Bioinformatics analysis

Theoretical isoelectric point and molecular weight of deduced protein were calculated with the ExPASy compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) 5.0 program (http://www.megasoftware.net) based on the neighbor-joining method (Saitou and Nei, 1987; Tamura et al., 2011). The reliability of the tree was measured by bootstrap analysis with 1000 replicates (Felsenstein, 1985). Secondary structures of deduced amino acid sequences were predicted by Self-Optimized Prediction Method with Alignment (SOPMA) analysis (http://npsa-pbil.ibcp.fr/) (Geourjon and Deleage, 1995). Putative *cis*-acting elements were identified by searching the Plant *Cis*-acting Regulatory DNA Elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE) (Higo et al., 1999).

2.3. Complementation assay

Complementation assay was performed in the *E. coli* mutant strain MG1655 ara<>ispH in which *HDR* was deleted and conditionally complemented through integration of *HDR* at the *araBAD* locus under the control of arabinose-inducible *araBAD* promoter (McAteer