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Characterization of two geraniol synthases from *Valeriana officinalis* and *Lippia dulcis*: Similar activity but difference in subcellular localization

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ABSTRACT

Two geraniol synthases (GES), from *Valeriana officinalis* (*VoGES*) and *Lippia dulcis* (*LdGES*), were isolated and were shown to have geraniol biosynthetic activity with K_m values of 32 μM and 51 μM for GPP, respectively, upon expression in *Escherichia coli*. The *in planta* enzymatic activity and sub-cellular localization of VoGES and LdGES were characterized in stable transformed tobacco and using transient expression in *Nicotiana benthamiana*. Transgenic tobacco expressing VoGES or LdGES accumulate geraniol, oxidized geraniol compounds like geranial, geranic acid and hexose conjugates of these compounds to similar levels. Geraniol emission of leaves was lower than that of flowers, which could be related to higher levels of competing geraniol-conjugating activities in leaves. GFP-fusions of the two GES proteins show that VoGES resides (as expected) predominantly in the plastids, while LdGES import into to the plastid is clearly impaired compared to that of VoGES, resulting in both cytosolic and plastidic localization. Geraniol production by VoGES and LdGES in *N. benthamiana* was nonetheless very similar. Expression of a truncated version of *VoGES* or *LdGES* (cytosolic targeting) resulted in the accumulation of 30% less geraniol glycosides than with the plastid targeted VoGES and LdGES, suggesting that the substrate geranyl diphosphate is readily available, both in the plastids as well as in the cytosol. The potential role of GES in the engineering of the TIA pathway in heterologous hosts is discussed.

1. Introduction

Plants are estimated to produce more than 500,000 secondary metabolites of various classes (isoprenoids, phenylpropanoids, alkaloids) (Hadacek, 2002). Of these, the isoprenoids represent the largest family based on their diverse structural features which relate to numerous biological activities. Isoprenoids have been shown to affect many physiological processes such as respiration, signal transduction, cell division, membrane architecture, photosynthesis, and growth. In addition, isoprenoids have ecological significance as they play an important role in the exchange of signals between plants and between plants and microorganisms or in defense against pathogens and herbivores. Also the applications of isoprenoids in foods, cosmetics and pharmaceutical drugs make specific terpenoids interesting commerical targets.

Although isoprenoids are extraordinarily diverse, they all originate from the condensation of the universal five-carbon precursors, isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP). In higher plants, two independent pathways, located in separate intracellular compartments, are involved in the biosynthesis of IPP and DMAPP. In the cytosol, IPP is derived from the classic mevalonic acid (MVA) pathway that starts from acetyl-CoA (Porter and Spurgeon, 1981), whereas in plastids, IPP is formed from pyruvate and glyceraldehyde 3-phosphate via the methylerythritol phosphate (MEP or non-mevalonate) pathway (Eisenreich et al., 2001; Lichtenthaler, 1999). Cytosolic IPP and DMAPP are converted to farnesyl diphosphate (FPP, C15), which serves as a precursor of sesquiterpene and triterpene biosynthesis in the cytosol. In contrast, the plastidial pool of IPP/DMAPP is converted to geranyl diphosphate (GPP, C10) and geranylgeranyl diphosphate (GGPP, C20) which serve as precursors for monoterpenes, and diterpenes and tetraterpenes, respectively, in the plastid (Lange et al., 2001; McConkey et al., 2000; Tholl and Lee, 2011; Turner et al., 1999).

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Geraniol is an acyclic monoterpene alcohol that is synthesized in one step from GPP. Geraniol is a component of essential oils present in many fragrant plant species (Antonelli et al., 1997; Bakkali et al., 2008; Bayrak and Akgül, 1994; Sangwan et al., 2001; Yang et al., 2005). It has a rose-like odor and is commonly used in perfumes (Chen, 2006; Rastogi et al., 2003) and aromatic fragrance in wine (Herrero et al., 2008; Pedersen et al., 2003). Geraniol also has pharmaceutical properties, as it can inhibit the growth of human colon cancer cells (Carnesecchi et al., 2001) and interfere with membrane functions in *Candida albicans* and *Saccharomyces cerevisiae* (Bard et al., 1988). In some plant species geraniol is the precursor for terpenoid indole alkaloid (TIA) biosynthesis. For instance, in *Catharanthus roseus* the anticancer agents vinblastine and vincristine are synthesized from geraniol (monoterpene iridoid branch) and tryptophan (indole branch) in the TIA pathway.

Multiple approaches have been tested to increase TIA production. For example, overexpress gene 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase gene were shown to increase the flux towards vinblastine and vincristine in *C. roseus* hairy root (Peebles et al., 2011). Attempts to boost transcription of TIA biosynthetic genes in the hairy roots or suspension cells were only partially successful (Liu et al., 2011; Memelink and Gantet, 2007; Montiel et al., 2007). For example, ORCA3 is a jasmonate responsive transcription factor that promotes transcription of TIA biosynthesis genes (Vom Endt et al., 2007). However, when ORCA3 is overexpressed, also repressor activity is activated, which in the long term actually caused a decrease in several TIA metabolites in *C. roseus* (Peebles et al., 2009). Expression of the TIA pathway biosynthesis genes in a heterologous host may provide a way to overcome such feedback regulation problems.

The objective of the present study was the efficient production of the monoterpene geraniol as the first step in a larger program to rebuild the complete monoterpene iridoid branch of the TIA biosynthesis pathway in a heterologous host. To achieve this, a geraniol synthase (GES) was cloned from *V. officinalis* L. (Valerianananceae) (*VoGES*) and compared to the previously isolated *LdGES* from *L. dulcis* (Yang et al., 2011). Both proteins showed similar geraniol synthase activity *in vitro* and *in planta*. VoGES was subsequently used in a number of transient and stable metabolic engineering approaches to explore the possibility to reconstitute the monoterpene branch of TIA biosynthesis in tobacco.

2. Materials and methods

2.1. Cloning and sequence analysis of geraniol synthase gene

For the cloning of the geraniol synthase gene, *V. officinalis* L. (VoGES) total RNA was isolated from *V. officinalis* leaves using SV Total RNA Isolation System (Promega). Based on conserved domains of known geraniol synthases, the degenerate primers (forward primer 5′–GAYGARAAYGGIAARTTYAARGA–3′ and reverse primer 5′–CCRTAIGCRTCRAAIGTRTCRTC–3′) were designed to amplify partial cDNA fragment by reverse transcription PCR (RT-PCR). Full length sequences of the cDNAs were obtained by rapid amplification of cDNA ends (RACE).

Putative VoGES sequence was blasted against the GenBank ENTREZ database (NCBI Blast 2.2.23) (Altschul et al., 1997) and GES sequences were aligned using CLUSTALX 1.83 (Thompson et al., 1997) using standard settings. Prediction of the subcellular localization was from the targeting prediction programs PREDOTAR version 1.03 (http://urgi.versailles.inra.fr/predotar/) (Small et al., 2004) and TARGETP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2000).

2.2. Heterologous expression of VoGES and LdGES protein in E. coli

For the *in vitro* functional analysis of the putative geraniol synthase from *V. officinalis* and comparison with *LdGES*, the truncated cDNAs Δ*NVoGES* (bp 178-1785) and Δ*NLdGES* (bp 139-1755) were subcloned into the multiple cloning site of the expression vector pRSET A (Invitrogen) to yield constructs pRSET-ΔNVoGES and pRSET-ΔNLdGES. Primer sequences for PCR amplification and restriction sites for each primer are listed in Table S1. After full re-sequencing to check integrity, constructs were transformed into *E. coli* BL21 (DE3) (Invitrogen) and expression was induced by isopropyl β-D thiogalactopyranoside (IPTG) in transformed *E. coli* BL21 (DE3) cell cultures. The His-tagged proteins were isolated by passing through Ni-NTA Spin columns according to the manufacturers' recommendations (Qiagen). For quality analysis, the recombinant protein was confirmed with 12.5% (w/v) SDS-PAGE gel electrophoresis followed by Western blotting using mouse monoclonal anti-His horseradish peroxidase (HPR) conjugate antibodies (5Prime, http://www.5prime.com). Antibody binding was detected by incubation in 250 μM sodium luminol, 0.1 M Tris–HCl (pH 8.6), 3 mM H_2O_2, 67 μM *p*-coumaric acid and exposure to X-ray film.

An enzyme assay was carried out for functional characterization, using geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) as substrates. Enzymatic assays were done in 0.5 ml reaction buffer containing 50 mM Tris–HCl, 1 mM $MgCl_2$, 0.1 mM $MnCl_2$, and 10, 20, 50 or 100 μM GPP (or 62.5 μM FPP) and 0.5 μg (VoGES) and 2 μg (LdGES) of purified enzyme. The reaction mixture was incubated at 32 °C for 5 min. For quantitative analysis citronellol was added to a concentration of 50 μM as an internal standard into the reaction tube after incubation. The reaction was stopped by adding 1 volume of hexane, mixing thoroughly by vortexing and keeping on ice for 10 min. The tubes were centrifuged at 4000*g* for 10 min, and the supernatant hexane phase was collected. The extraction was repeated with hexane (0.5 ml). Then the hexane phase was collected and dehydrated and then subjected to capillary gas chromatography–flame ionizing detector (GC–FID) and gas chromatography–mass spectrometry (GC–MS, supplementary method). For the latter, the hexane extract was separated on a Agilent GC 6890 series equipped with a DB-5 capillary column (30 m × 0.25 mm, film thickness of 0.25 μm) (J&W Scientific) using nitrogen as carrier gas at a flow rate of 1.2 ml min^{-1}. The separation conditions were: split mode 1: 5, injection volume 5 μl, injector temperature 230 °C, initial oven temperature 100 °C, then linear gradient to 140 °C at a rate of 10 °C min^{-1} followed by a linear gradient to 240 °C at a rate of 35 °C min^{-1}. Amounts of geraniol formed in enzyme assays were calculated from the resultant GC/FID integral using the relative response factor with respect to the citronellol internal standard. Lineweaver–Burk plots of VoGES and LdGES activity were used to obtain the K_m values for GPP.

2.3. VoGES and LdGES subcellular localization studies

For analysis of the subcellular targeting, the coding sequences of *EGFP* were fused to the N-terminus or C-terminus of full length *VoGES* and *LdGES*. In addition a truncated version of LdGES lacking the first 46 AA (Δ*NLdGES*: bp 139-1755) and a truncated version of VoGES lacking the first 56 AA (Δ*NVoGES*: bp 178-1785) was made using standard cloning techniques and the C-terminal coding sequence of these genes was fused in frame to that of GFP. The *VoGES–GFP*, *LdGES–GFP*, *GFP–VoGES*, *GFP–LdGES*, Δ*NVoGES–GFP* and Δ*NLdGES–GFP* were cloned into impact vector pIV2A 2.1 (www.pri.wur.nl/UK/products/ImpactVector/) under control of the CaMV 35S-promoter. In addition, the truncated versions of *VoGES* and *LdGES* were provided with a heterologous plastid import signal by cloning into impact vector pIV2A 2.4 which