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Characterization of the plastidial geraniol synthase from Madagascar periwinkle which initiates the monoterpenoid branch of the alkaloid pathway in internal phloem associated parenchyma

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ABSTRACT

Madagascar periwinkle (*Catharanthus roseus* [L.] G. Don, Apocynaceae) produces monoterpene indole alkaloids (MIAs), secondary metabolites of high interest due to their therapeutic value. A key step in the biosynthesis is the generation of geraniol from geranyl diphosphate (GPP) in the monoterpenoid branch of the MIA pathway. Here we report on the cloning and functional characterization of *C. roseus* geraniol synthase (CrGES). The full-length CrGES was over-expressed in *Escherichia coli* and the purified recombinant protein catalyzed the conversion of GPP into geraniol with a K_m value of 58.5 μM for GPP. *In vivo* CrGES activity was evaluated by heterologous expression in a *Saccharomyces cerevisiae* strain mutated in the farnesyl diphosphate synthase gene. Analysis of culture extracts by gas chromatography-mass spectrometry confirmed the excretion of geraniol into the growth medium. Transient transformation of *C. roseus* cells with a Yellow Fluorescent Protein-fusion construct revealed that CrGES is localized in plastid stroma and stromules. In aerial plant organs, RNA *in situ* hybridization showed specific labeling of *CrGES* transcripts in the internal phloem associated parenchyma as observed for other characterized genes involved in the early steps of MIA biosynthesis. Finally, when cultures of *Catharanthus* cells were treated with the alkaloid-inducing hormone methyl jasmonate, an increase in *CrGES* transcript levels was observed. This observation coupled with the tissue-specific expression and the subcellular compartmentalization support the idea that CrGES initiates the monoterpenoid branch of the MIA biosynthetic pathway.

1. Introduction

Madagascar periwinkle (*Catharanthus roseus* [L.] G. Don, Apocynaceae) is a pantropical medicinal plant which synthesizes a wide range of complex secondary metabolites known as monoterpene indole alkaloids (MIAs). Several of them are valuable therapeutic compounds, including monomers such as ajmalicine and serpentine used in the treatment of circulatory diseases and anxiety, and heterodimers such as vinblastine and vincristine known as powerful anticancer drugs (van der Heijden et al., 2004). Due to the pharmacological importance of these compounds, the MIA metabolic pathway has been highly studied in the whole plant and in cell suspension culture systems (Zhou et al., 2011). MIAs originate from two convergent pathways. Tryptamine (provided by the indole pathway through decarboxylation of L-tryptophan) and secologanin (provided by the monoterpenoid pathway also known as the iridoid pathway) are condensed into strictosidine, the precursor of all other MIAs (van der Heijden et al., 2004) (Supplementary Fig. S1).

Studies of fluxes in the pathways leading to the formation of MIAs by precursor feeding highlighted that the monoterpenoid branch is limiting for the biosynthesis of alkaloids in cell and tissue cultures of *C. roseus* (Oudin et al., 2007a). A key step in the

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formation of MIAs is the biosynthesis of the monoterpenoid geraniol from geranyl diphosphate (GPP).

Geraniol feeding of *C. roseus* cell (Lee-Parsons and Royce, 2006) and hairy root cultures (Morgan and Shanks, 2000) resulted in an increase in the formation of the MIAs ajmalicine and tabersonine, respectively, suggesting that the formation of geraniol is a critical step in MIA biosynthesis. To date, periwinkle geraniol synthase (GES) has not been characterized at the molecular level. The present work focuses on the cloning and functional characterization of *C. roseus* GES, the study of the corresponding gene expression in response to methyl jasmonate, the *in situ* localization of *GES* mRNA and the subcellular localization of the enzyme.

2. Results and discussion

2.1. Isolation of the C. roseus geraniol synthase (CrGES) full-length cDNA

A partial sequence of 554 bp (Genbank ID: EG558318) displaying similarities with known geraniol synthases was identified in a *C. roseus* EST database. The full-length coding sequence for CrGES (*C. roseus* geraniol synthase) was recovered by 3′ RACE, PhageWalker and GenomeWalker protocols as described in the experimental method section. The *CrGES* sequence has been deposited at NCBI under Genbank ID: JN882024.

The *CrGES* open reading frame of 1770 bp encodes a protein of 589 amino acids in length with a calculated mass of 67.7 kDa. The CrGES protein contains the highly conserved aspartate-rich motif DDxxD (positions 343–347) and the less conserved NSE/DTE motif (positions 485–496) with the consensus sequence (L,V)(V,L,A)-(N,D)D(L,I,V)x(S,T)xxxE (Supplementary Fig. S2). Both motifs are found in several terpene synthases and are involved in the fixation of the diphosphate substrate (Christianson, 2006). Amino acid sequence comparison revealed that CrGES showed similarities with terpene synthases. A high degree of similarity was found with previously characterized geraniol synthases (Supplementary Fig. S2). The highest similarity (64% of identity) was found with the geraniol synthase from *Lippia dulcis* (Genbank ID: GU136162; Yang et al., 2011). Furthermore, CrGES possessed 59% of identity with the *Ocimum basilicum* ortholog (Genbank ID: AY362553, Iijima et al., 2004), 36% with the *Cinnamomum tenuipilum* GES (Genbank ID: AJ457070, Yang et al., 2005) and 33% with the GES from *Perilla frutescens* (Genbank ID: DQ234300, Ito and Honda, 2007) and *Perilla citriodora* (Genbank ID: DQ088667, Ito and Honda, 2007).

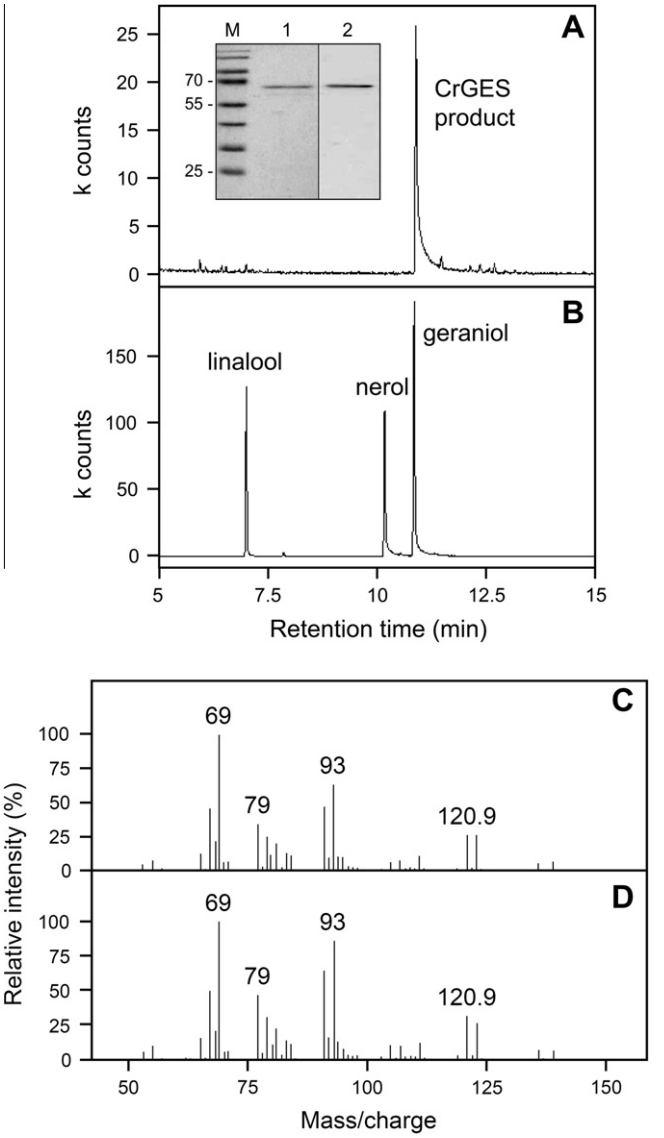

Fig. 1. Analysis of the CrGES-catalyzed reaction product. GC analysis of reaction products with the substrate geranyl diphosphate and recombinant CrGES (A) and of authentic geraniol, nerol and linalool (B). Mass spectra of authentic geraniol (C) and the peak from A (D). The inset in (A) shows the analysis of recombinant CrGES protein. The protein was separated by 10% SDS–PAGE and either stained with Coomassie Brilliant Blue (lane 1) or visualized after Western blotting using anti-His antibodies (lane 2). Sizes of relevant marker (M) bands are indicated in kDa.

2.2. Functional characterization of the purified recombinant CrGES

The close homology with previously identified geraniol synthases suggested that CrGES catalyzes the conversion of GPP into geraniol. Functional expression was thus carried out to investigate the catalytic activity. The complete *CrGES* open reading frame including the putative N-terminal plastidial targeting peptide flanked by a C-terminal His tag and an N-terminal Strep tag was expressed in *Escherichia coli* and purified by sequential Ni–NTA and Strep-tactin affinity chromatography. Analysis of the recombinant protein by SDS–PAGE and Coomassie Brilliant Blue staining or Western blotting and immunoprobing with anti-His antibodies showed the presence of one major band (Fig. 1A). Analysis of reaction products formed after incubation of the protein with GPP showed the presence of a single peak (Fig. 1A), which was identified as geraniol based on the retention time (Fig. 1B) and on the mass spectrum (Fig. 1C and D). A control reaction with boiled protein did not result in detectable products (data not shown). Under the reaction conditions employed, the protein catalyzed the conversion of GPP to geraniol with a K_m value of 58.5 μM for GPP (Supplementary Fig. S3), which is close to the apparent K_m of 55.8 μM reported for GES from the evergreen camphor tree *C. tenuipilum* assayed under similar conditions (Yang et al., 2005).

2.3. Expression of CrGES in Saccharomyces cerevisiae FPS mutants

Farnesyl diphosphate synthase (FPS) catalyzes the formation of the C_{15} product farnesyl diphosphate (FPP) by two sequential reactions: the initial condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate leading to GPP followed by the condensation of GPP with a second molecule of IPP producing FPP. It is generally accepted that FPS produces only FPP and that no GPP is released from the catalytic site of this enzyme. However, yeast mutant strains containing a mutated FPS, with lower FPS-specific activity, also produced GPP available for the synthesis of geraniol and other related monoterpenes. These compounds originate from