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Short Communications

Isolation of a *sesquiterpene synthase* expressing in specialized epithelial cells surrounding the secretory cavities in rough lemon (*Citrus jambhiri*)

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ABSTRACT

Volatile terpenoids such as monoterpenes and sesquiterpenes play multiple roles in plant responses and are synthesized by terpene synthases (TPSs). We have previously isolated a partial *TPS* gene, *RlemTPS4*, that responds to microbial attack in rough lemon. In this study, we isolated a full length *RlemTPS4* cDNA from rough lemon. RlemTPS4 localized in the cytosol. The recombinant RlemTPS4 protein was obtained using a prokaryotic expression system and GC–MS analysis of the terpenes produced by the RlemTPS4 enzymatic reaction determined that RlemTPS4 produces some sesquiterpenes such as δ-elemene. The *RlemTPS4* gene was specifically expressed in specialized epithelial cells surrounding the oil secretory cavities in rough lemon leaf tissue.

Introduction

Volatile terpenoids, such as monoterpenes and sesquiterpenes, are one of the universal volatile components of plants and are synthesized by various types of terpene synthases (TPSs). In general, monoterpenes are synthesized from geranyl diphosphate (GPP) by TPSs *via* the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plant plastids (Tholl, 2006; Arimura et al., 2009); thus, monoterpene synthases contain a transit peptide to the plastids and are localized in the plastids (Shishido et al., 2012; Taniguchi et al., 2014a). In contrast, sesquiterpenes are synthesized from farnesyl diphosphate (FPP) by TPSs *via* the mevalonate (MVA) pathway in the cytoplasm (Tholl, 2006; Arimura et al., 2009); therefore, sesquiterpene synthases are localized in the cytoplasm (Taniguchi et al., 2014b). Almost all TPSs contain the DDxxD motif, which has been implicated in binding with divalent metal cofactors such as Mg^{2+} and Mn^{2+} (Starks et al., 1997).

Unlike most major plant species, citrus plants contain large volumes of essential oils in their leaves and fruit peels, most components of which are volatile terpenoids such as monoterpenes and sesquiterpenes (Weiss, 1997; Sawamura et al., 2001; Vekiari et al., 2002; Shimada et al., 2004). These essential oils are enclosed in oil secretory cavities surrounded by several layers of specialized epithelial cells (ECs) in citrus leaves and fruit peels (Weiss, 1997; Choi and Sawamura, 2001). Citrus plants are known to produce over twenty terpenoids in its peel and leaf (Vekiari et al., 2002; Yamasaki et al., 2007). Only a few monoterpene and sesquiterpene synthase genes have been characterized in citrus plants, although these plants produce many types of monoterpenes and sesquiterpenes. To date, excepting our previous studies, eight monoterpene synthases and two sesquiterpene synthases have been characterized in citrus plants (Maruyama et al., 2001; Lücker et al., 2002; Sharon-Asa et al., 2003; Shimada et al., 2004, 2005a,b, 2014). Thus, it may be assumed that citrus plants contain uncharacterized TPSs.

Recently, we have isolated three *monoterpene synthase* genes, *RlemTPS1* (*limonene synthase*), *RlemTPS2* (*sabinene synthase*), and *RlemTPS3* (*geraniol synthase*), from rough lemon (Yamasaki and Akimitsu, 2007; Kohzaki et al., 2009; Shishido et al., 2012). We have also isolated a partial *TPS* cDNA clone in response to the host-selective ACT-toxin from rough lemon (Shishido et al., 2012). The deduced amino acid sequence of the partial TPS has sequence

Abbreviations: GC–MS, gas chromatography–mass spectrometry; GFP, green fluorescence protein; ORF, open reading frame; UTR, untranslated region.

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homology with a sesquiterpene synthase, (−)-germacrene D synthase, from *Vitis vinifera* (Lücker et al., 2004). However, there is no direct evidence identifying the terpenoid compound(s) produced by this TPS. Therefore, in this study, we isolated a full length cDNA of the *TPS* gene and investigated the enzymatic properties of its product using GC–MS. Furthermore, we investigated the localization of expression of the *TPS* gene by *in situ* hybridization in rough lemon leaf tissues.

Materials and methods

Plant material and cloning of RlemTPS4

Young leaves (midrib length 2–3 cm) were collected from rough lemon (*Citrus jambhiri* Lush.) growing in the greenhouse and used for all experiments. A full length cDNA of the *rough lemon terpene synthase 4* (*RlemTPS4*) gene was isolated from rough lemon leaves using a SMART mRNA Amplification Kit [Clontech (Takara), Shiga, Japan]. The products of the 5′ and 3′ rapid amplification of cDNA ends (RACE) were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI sequencer. The accession number of RlemTPS4 is LC005483.

Localization of RlemTPS4

To construct *RlemTPS4-GFP*, the ORF of *RlemTPS4* without the stop codon was amplified by PCR using a forward primer (5′-TACAATTACAGTCGACATGTCTTTCGCAGTTTCAGCC-3′) with an adapted *Sal*I site (underlined) and a reverse primer (5′-CCCTTGCTCACCATGGATATTGATACAGGATTAATAAGC-3′) with an adapted *Nco*I site (underlined). The product was then subcloned into the corresponding sites of the pCaMV35S-sGFP(S65T)-NOS-3′ vector, which fused GFP to the C-terminus of RlemTPS4 in frame. The RlemTPS4-GFP fusion protein was expressed in *Nicotiana benthamiana* protoplast cells using the method previously described by Shishido et al. (2012). The GFP fluorescence was observed under a fluorescent microscope (BX51, Olympus, Tokyo, Japan) with a CCD digital camera system (DP70-SET-A, Olympus) outfitted with a GFP-specific filter unit (U-MGFPHQ, Olympus).

Functional expression of RlemTPS4 in Escherichia coli

The ORF of *RlemTPS4* was subcloned in frame into the pCold II vector (Takara), and the overexpression of His-tagged proteins in *E. coli* BL21 (Takara) was accomplished according to the manufacturer's instructions. The His-tagged proteins were purified using a HisTrap™ HP column (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purified proteins were dialyzed against an assay buffer [50 mM HEPES (pH 7.5) containing 5 mM DTT, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM ascorbic acid, and 10% (w/v) glycerol] and stored at −80 °C until use.

Enzyme assay and analysis of volatiles

The enzyme assay was performed using the method previously described by Taniguchi et al. (2014b), with minor modifications. The enzyme activity was assayed using approximately 30 μg of purified RlemTPS4 protein or 1 mg of total protein from *E. coli* with an empty vector in a 15 mL sealed Spelco vial (Spelco, Saint Louis, MO, USA) containing 1 mL of the assay buffer. The reaction was initiated by the addition of FPP (55 μM final concentration: Sigma, Saint Louis, MO, USA). After incubation at 30 °C for 1 h, the headspace above the sample was trapped for 10 min at 50 °C using a Twister (PDMS-coated stir bar: Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany). The collected volatile compounds were analyzed by GC–MS. The compounds were identified by comparing their mass spectra to those of a database (Wiley7N). The retention index (RI) of δ-elemene was calculated using the retention times of the C13–C16 alkanes using THE PHEROBASE (http://www.pherobase.com/). The assays were repeated independently at least three times. Detailed information for experimental condition of GC–MS analysis is provided in Appendix S1.

in situ hybridization

The DIG-labeled DNA probe for *RlemTPS4* was made from the 3′ region (1639–1922 bp) containing 3′-UTR using PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland), and the DIG-labeled probe for *callose synthase* (accession number AB669441) was the same as that previously used by Shishido et al. (2012). Before being used as probes, these DIG-labeled DNAs were incubated at 100 °C for 5 min and then rapidly cooled on ice.

in situ hybridization was performed using the previously described method by Yamasaki et al. (2007) with minor modifications. Detailed information for experimental condition of *in situ* hybridization is provided in Appendix S1.

Results

Isolation and localization of RlemTPS4

To isolate the full sequence of the rough lemon terpene synthase cDNA, we utilized the terpene synthase partial sequence (accession number AB669446), which had previously been obtained using subtractive PCR (Shishido et al., 2012). The isolated full length *TPS* cDNA was designated *RlemTPS4* (accession number LC005483). This gene consisted of 1922 bp containing a 1691 bp ORF (data not shown) and the deduced amino acid sequence of RlemTPS4 consisted of 563 amino acids with a calculated molecular mass of 65.1 kDa (Fig. 1A). In addition, the deduced amino acid sequence of RlemTPS4 contained a DDxxD motif, which has been implicated in binding with divalent metal cofactors (Starks et al., 1997) (Fig. 1A). BLAST analysis revealed that RlemTPS4 has high homology with sesquiterpene synthases isolated from other plants (Supplemental Fig. S1). Thus, RlemTPS4 was phylogenetically analyzed by comparing it with characterized plant sesquiterpene synthases to predict sesquiterpene(s) produced by RlemTPS4 (Degenhardt et al., 2009). As a result, the products of RlemTPS4 could not predict from the simple comparison with its amino acid sequence (Supplemental Fig. S2).

Sequence analysis of RlemTPS4 using ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted no transit peptide to the chloroplast, suggesting that RlemTPS4 works as a sesquiterpene synthase in rough lemon. However, no previous direct experimental evidence has been obtained for the localization of RlemTPS4 in cells. Therefore, we constructed a *RlemTPS4-GFP* fusion gene and transiently expressed the fusion product in *Nicotiana benthamiana* protoplast cells. As shown in Fig. 1B, RlemTPS4 localized to the cytoplasm of transformed cells. Color version of the photographs is shown in Supplemental Fig. S3. Together, these results suggest that RlemTPS4 is a putative sesquiterpene synthase in rough lemon.

Identification of RlemTPS4 as a sesquiterpene synthase

We expressed the recombinant RlemTPS4 protein in *Escherichia coli* and tested the sesquiterpene synthase activity of purified recombinant protein using FPP as a substrate to determine the product of FPP catalyzed by RlemTPS4. The catalyzed products