Title: Characterization of a sabinene synthase gene from rough lemon (*Citrus jambhiri*)

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Summary

We previously isolated two putative monoterpene synthase genes, *RlemTPS1* and *RlemTPS2*, from rough lemon (*Citrus jambhiri*) and showed that gene expression of *RlemTPS2* was induced by microbial attack. The protein product of *RlemTPS2* was obtained by using a prokaryotic expression system, and GC and GC-MS of monoterpene synthesis by RlemTPS2 determined that RlemTPS2 encodes a sabinene synthase. Sabinene has antifungal activity toward *Alternaria alternata*. Furthermore, site-directed mutagenesis identified one amino acid, Ile, located at the front of the metal ion binding motif as an important residue for the product specificity of sabinene synthase.

Key words: Citrus; Monoterpene synthase; Sabinene Abbreviations: Asn, Asparagine; GC, gas chromatography; GC-MS, GC-mass spectrometry; Ile, Isoleucine; ORF, open reading frame; TPS, terpene synthase

Introduction

Volatile compounds released from wounded or microbe-infected sites of plants are a possible source of signal molecules that induce defense systems to protect plants from tissue damage. A number of volatile compounds are already known to be plant signals that can be produced rapidly and transported through the plant body (Mur et al., 1997; Pichersky and Gershenzon, 2002). Monoterpenes are one of the universal volatile components of plants and are synthesized by various types of monoterpene synthases from geranyl pyrophosphate (GPP). The role of monoterpenes in plant defenses against herbivores and plant pathogens or as attractants for pollinators was predicted (Lewinsohn et al., 1991; Arimura et al., 2000; Pichersky and Gershenzon, 2002; Luo et al., 2004; Yamasaki et al., 2007).

Unlike most major plant species, citrus plants contain large volumes of essential oils, and most of the components of essential oils are monoterpenes (Weiss, 1997). Monoterpenes stored as essential oils are enclosed in secretory cavities surrounded by irregular parenchymatous cells in citrus leaves and fruit peels (Weiss, 1997; Choi and Sawamura, 2001). Recently, we isolated two putative monoterpene synthase genes, *RlemTPS1* and *RlemTPS2*, from rough lemon (*Citrus jambhiri*) and revealed that these genes were expressed in the epithelial tissue cells surrounding the secretory cavities (Yamasaki and Akimitsu, 2007). The properties of RlemTPS1 and RlemTPS2 as monoterpene synthases had not been clarified that time. However, gene expression of *RlemTPS2* was slightly induced by pathogen attack (Yamasaki and Akimitsu, 2007), suggesting a possibility that RlemTPS2 might play a role in the rough lemon defense system. Thus, we investigated the enzymatic properties of RlemTPS2

using GC and GC-MS, and further investigated an active-site residue responsible for product specificity of RlemTPS2 by using site-directed mutagenesis.

Materials   and   methods

Site-directed mutagenesis

The ORF encoding for the rough lemon (*Citrus jambhiri* Lush) monoterpene synthase 2 (RlemTPS2) isolated by Yamasaki and Akimitsu (2007) was used in this study. Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis kit (Stratagene, CA, USA) according to the manufacturer's instructions. The primers used were: RlemTPS2 (I346N)5, 5'-GATGTCTACAATGGTCAATGCCTTAATAACAGCAG-3'; RlemTPS2 (I346N)3, 5'-CTGCTGTTATTAAGGCATTGACCATTGTAGACATC-3'. Mutation was verified by an automated DNA sequencer.

Functional expression of RlemTPS2 in *E. coli*

The ORF of the wild type or mutated *RlemTPS2* was subcloned in frame into the pCold II vector (Takara, Shiga, Japan), and overexpression of His-tagged proteins in

E. *coli* BL21 (DE3) (Stratagene) was performed according to the manufacturer's instructions. The His-tagged proteins were purified using a HisTrap^{TM} HP column (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purified protein was dialyzed against 50 mM HEPES (pH 7.5), 5 mM DTT, 2 mM MnCl2, 10 mM MgCl2, 1 mM ascorbic acid, and 10% (w/v) glycerol and stored at -80 °C until use.

Enzyme assay

Enzyme activity was assayed by inserting about 50 μg purified TPS protein or 1 mg total protein from *E. coli* with only an empty vector into a 15 mL sealed Spelco vial (Spelco, St Louis, MO, USA) containing 1 mL of 50 mM HEPES (pH 7.5), 5 mM DTT, 2 mM MnCl2, 10 mM MgCl2, 1 mM ascorbic acid, and 10% (w/v) glycerol. The reaction was initiated by the addition of 55 μM final concentration of GPP (Echelon Biosciences, Salt Lake City, UTt, USA). After incubation at 30 °C for 1 h, the headspace above the sample was trapped for 10 min at 50 °C with a polydimethylsiloxane/divinylbenzene-coated solid-phase microextraction (SPME) fiber (Spelco) for GC or a Twister (polydimethylsiloxane-coating stir bar) (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) for GC-MS. GC analysis was performed using a Shimadzu (Kyoto, Japan) GC-2014 gas chromatograph fitted with a DB-Wax column (60 m x 0.25 mm i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA), and a flame ionization detector (FID). The oven temperature program was 60-210 °C at 2 °C/min. The collected volatile compounds were also analyzed by GC-MS [GC: Agilent Technologies, Inc. (Santa Clara, CA, USA) 6890 with a HP-5MS capillary column: 30 m x 0.25 mm i.d., 0.25 μm film thickness; MS: Agilent Technologies 5973 mass selective detector, 70 eV] equipped with a thermal desorption system (TDS), a cooled injection system (CIS), and a cold trap system (CTS) (Gerstel GmbH & Co.). The oven temperature of the GC was 40-60 °C at 5 °C/min and 60-280 °C at 2 °C/min. The compounds were identified by comparing their mass spectra to those of a database (Wiley7N) and by comparing their retention times to those of authentic compounds (Wako, Osaka, Japan). The assays were repeated independently more than three times.

Antifungal assay

Vapor treatment of fungus with sabinene (Extrasynthèse GC, Merignac, France) was performed by the methods of Gomi et al. (2003) and Yamasaki et al. (2007). Spores of the *Alternaria alternata* tangerine pathotype (isolate SH20) were vapor-treated with sabinene adjusted to the final concentration of 10, 100, or 500 μmol/L air for 6 h, and rate of spore germination and hyphal lengths of the spores were measured. The *A. alternata* tangerine pathotype is pathogenic to tangerines and their hybrids but not to rough lemon (Kohmoto et al., 1979; Kohmoto and Otani, 1991; Akimitsu et al., 2003). There is no morphological or biochemical difference between SH20 and nonpathogenic strain such as O-94 of *A. alternata*, except that SH20 produces host-selective ACT toxin (Kohmoto et al., 1979; Kohmoto et al., 1991; Kohmoto et al., 1993). Relative values (%) were calculated by comparison with the hyphal length of the mock-exposed control. The bioassays were independently repeated three times with microscopic observations of 100 spores each.

Results

Identification of RlemTPS2 as a sabinene synthase

To determine the product of GPP catalyzed by RlemTPS2, RlemTPS2 protein

was expressed in *E. coli*, and the purified RlemTPS2 protein was tested for the monoterpene synthase activity using GPP as a substrate. The catalyzed product was determined by GC and GC-MS. As shown in Fig. 1A, GC analysis demonstrated that one major and three minor products were produced by RlemTPS2. Because RlemTPS2 without substrate and total proteins from *E. coli* with an empty vector did not give these products (Fig. 1B, D), we concluded that the products were produced by enzymatic reaction of RlemTPS2. We next performed identification of the product by GC-MS. The product was identified by comparing the GC retention time and mass spectra with that of the authentic compound. As a result, the dominant product was identified as sabinene (Supplemental Fig. S1) and the minor products as α-pinene, *β*-pinene, and *β*-phellandrene, indicating that RlemTPS2 is a sabinene synthase of rough lemon.

Identification of an active-site residue responsible for product specificity

To identify an active-site residue responsible for the product specificity of RlemTPS2, we performed single amino acid substitutions in RlemTPS2. Recently, it was reported that Ile-327 located at the front of the metal ion binding DDxxD motif is an important residue for product specificity in *Salvia pomifera* sabinene synthase Sp-SabS1 (Kampranis et al., 2007). Analysis of the amino acid sequence alignment showed that the corresponding residue of Ile-346 in RlemTPS2 is different from two distinct citrus *β*-pinene synthases with Asn-346, despite high sequence identities (95-97%) among these citrus TPSs (Fig. 2A). Therefore, we converted Ile-346 to Asn in RlemTPS2 by using site-directed mutagenesis, and the product produced by RlemTPS2 (I346N) was determined by GC and GC-MS. As shown in Fig. 2B, GC analysis

demonstrated that two major products were produced by RlemTPS2 (I346N). By GC-MS analysis, one was identified as sabinene and the other newly produced major product was *β*-pinene (Supplemental Fig. S1), and the *β*-pinene was the most abundant product produced by RlemTPS2 (I346N) (Fig. 2B).

Antifungal activity of sabinene

Recently, we demonstrated that some monoterpenes have an antifungal activity toward the fungus *Alternaria alternata* (Yamasaki et al., 2007), but the effect of sabinene on *A. alternata* had not been tested yet. Thus, the effect of vapor treatment of sabinene on spore germination and hyphal growth of *A. alternata* tangerine pathotype was tested. *A. alternata* spores were treated with sabinene vapor adjusted to the final concentration of 10, 100, or 500 μmol/L air for 6 h. As a result, sabinene inhibited both spore germination and hyphal growth compared to that of untreated spores as the control in a dose-dependent manner (Fig. 3A, B).

Discussion

We previously reported that a majority of the volatile compounds released from rough lemon leaves are monoterpenes, and about 6% (the fourth largest amount) of the volatile compounds is sabinene (Yamasaki et al., 2007). In this study, we identified RlemTPS2 as a sabinene synthase of rough lemon, suggesting that sabinene emitted from rough lemon is produced by RlemTPS2. To our knowledge, this is the first report of identification of a sabinene synthase from *Citrus* species.

We identified an active-site residue (Ile-346) responsible for the product specificity of RlemTPS2 as a sabinene synthase. Ile-346 is located at the front of the metal ion binding DDxxD motif in RlemTPS2. It has been reported that the corresponding residue is also important for product specificity in *Salvia* monoterpene synthases (Kampranis et al., 2007). By substitution of Ile-346 with Asn, RlemTPS2 was converted to a *β*-pinene synthase. Although RlemTPS2 and *β*-pinene synthases of *C. limon* and *C. unshiu* show 95-97% sequence identities, the corresponding residue of Ile-346 in RlemTPS2 is Asn-346 in these citrus *β*-pinene synthases (Yamasaki and Akimitsu, 2007). This result leads us to speculate that Ile and Asn are critical residues for sabinene synthase and *β*-pinene synthase in *Citrus* species. At least, it can be seen that Ile-346 is an important residue for RlemTPS2 to work as a sabinene synthase in rough lemon. However, because catalytic specificity in TPSs may be modulated by whole structural features, further studies are needed to clarify the catalytic mechanism of TPS in rough lemon.

Sabinene had an antifungal activity toward the fungal pathogen *A. alternata*. It has been reported that sabinene has a significant growth-inhibiting effect on redwood fungal endophytes (Espinosa-Garcia and Langenheim, 1991) and is released from cultured *Cupressus lusitanica* cells after treatment with a fungal elicitor (De Alwis et al., 2008). These results suggest that sabinene may play a role in plant defense as an antifungal compound. The effect of sabinene on spore germination and hyphal growth toward *A. alternata* was similar to that of linalool (Yamasaki et al., 2007). These results suggest that sabinene and linalool act at the fungal invasion stage in rough lemon.

Various types of monoterpenes are released from wounded rough lemon leaves

(Yamasaki et al., 2007), and release of sabinene from wounded rough lemon leaves also increased about 7 times compared to that from unwounded rough lemon leaves (Yamasaki et al., unpublished data). Thus, sabinene released from the damaged site may be emitted to prevent secondary infection of fungal pathogens in rough lemon.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at XXXXXXX.

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Yamasaki Y, Kunoh H, Yamamoto H, Akimitsu K. J Gen Plant Pathol 2007;73:168-179.

Legends of figures Figure 1. Identification of RlemTPS2 enzyme. **(A,B,D)** GC profiles of products formed by RlemTPS2 with GPP (A), RlemTPS2 without GPP (B), and total proteins from *E. coli* with GPP (D). **(C)** GC profile of authentic sabinene standard. Compounds, confirmed by mass spectrometry and comparisons with authentic standards, are (1) α-pinene, (2) *β*-pinene, (3) sabinene, and (4) *β*-phellandrene. Experiments were done at least five times and showed the same results in all cases.

Figure 2. Identification of active-site residue responsible for product specificity of RlemTPS2. **(A)** Amino acid sequence alignment of the mutated region of RlemTPS2 with other monoterpene synthases. Mutated residue is indicated by an asterisk. Accession numbers of genes used this alignment are RlemTPS2 (AB266585), *C. limon* (-)-*β*-pinene synthase [Cl(-)βPINS] (AF514288), *C. unshiu β*-pinene synthase (CitMTSL62) (AB110641), *Salvia officinalis* sabinene synthase (SoSabS1) (AF051901), and *S. pomifera* sabinene synthase (SpSabS1) (DQ785794). **(B,C)** GC profiles of products formed by RlemTPS2 (I346N) (B) and wild type RlemTPS2 (C). **(D)** GC profile of authentic sabinene and *β*-pinene standards. Monoterpenes are (1) α-pinene, (2) *β*-pinene, (3) sabinene, and (4) *β*-phellandrene. Experiments were done at least three times and showed the same results in all cases.

Figure 3. Effect of sabinene on spore germination **(A)** and hyphal growth **(B)** of *A. alternata*. Hyphal lengths of spores treated with 10, 100, or 500 μmol/L air sabinene for 6 h are given as relative values (%) calculated by comparison to that of the mock-treated control (0). For (B), Values are means ± SD (n=100). Experiments were independently done three times and showed almost the same results in all cases.
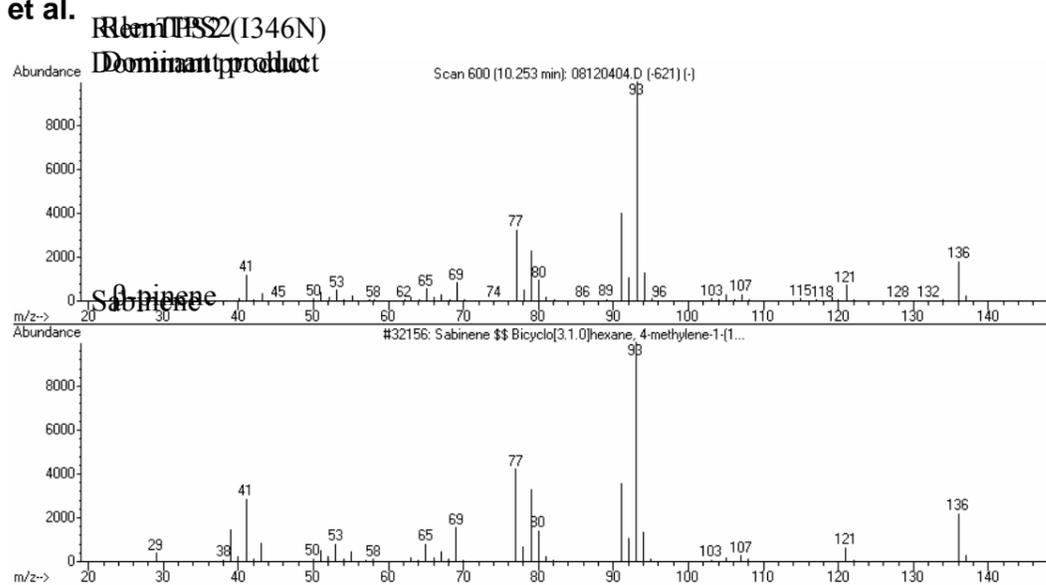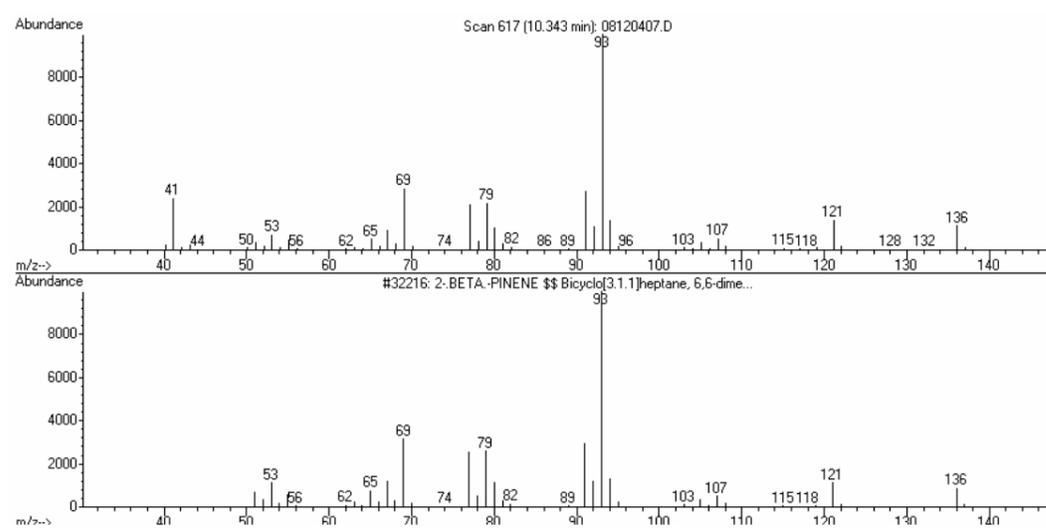
Figure S1. Mass profiles of main products and their standards. **(A)** Mass spectra of dominant product formed by RlemTPS2 (upper panel) and of authentic sabinene standard (lower panel). **(B)** Mass spectra of dominant product formed by RlemTPS2 (I346N) (upper panel) and of authentic β-pinene standard (lower panel).

Figure1

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Fig. 1

A — Intensity [μV(x10,000)] RkmTPS2 +GPP

B — Intensity [μV(x10,000)] RkmTPS2 -GPP

C — Intensity [μV(x10,000)] Sabrame

D — Intensity [μV(x10,000)] *E. coli* +GPP

Figure2

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A

RlemTPS2	(314)	FVRDRIVSNFLWTVGEKFEPQFGYFRRMSTMVIALITAVDDVYDVYGTLDELEIFTD
Cl(-)βPINS	(314)	FVRDRIVENFMWTVGSKFEPQFGYFRRMSTHVNALITAVDDVYDVYGTLEKLEIFTD
CitMTSL62	(318)	FVRDRIVENFMWTVGEKFEPQFGYFRRMSTMVNALITAVDDVYDVYGTLDELEIFTD
SoSabS1	(304)	FVRDRIVECFFWAIAAFEPHQYSYQRKMAAVIITFITIIDDVYDVYGTIEELELLTD
SpSabS1	(295)	FVRDRIVECFFWAIGAFEPHQYSYQRKMAAIIITFVTIIDDVYDVYGTLEELELFTD

B Intensity [μV(x10,000)]

RlemTPS2
(I346N)

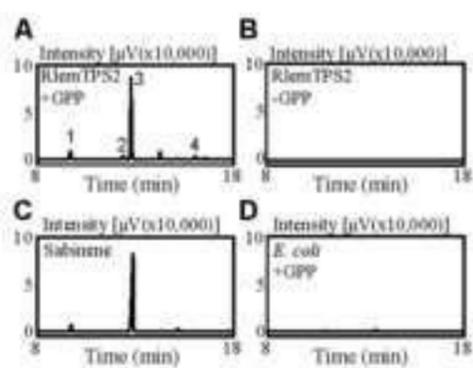C Intensity [μV(x10,000)]

RlemTPS2

D Intensity [μV(x10,000)]

β-pinene   Sabinene

Figure3

Kohzaki et al. Fig. 3

A

Relative spore germination (%)

Concentration of
sabinene (μM)

B

Relative hyphal length (%)

Concentration of
sabinene (μM)