Herbivore-Induced Defense Response in a Model Legume. Two-Spotted Spider Mites Induce Emission of (E)-β-Ocimene and Transcript Accumulation of (E)-β-Ocimene Synthase in *Lotus japonicus*[1]

Gen-ichiro Arimura[2,3]*, Rika Ozawa[2], Soichi Kugimiya, Junji Takabayashi, and Jörg Bohlmann

Biotechnology Laboratory, Department of Botany, and Department of Forest Sciences, University of British Columbia, Vancouver V6T 1Z3, British Columbia, Canada (G.A., J.B.); Center for Ecological Research, Kyoto University, Otsu 520–2113, Japan (R.O., S.K., J.T.); and CREST of Japan Science and Technology Agency, Kawaguchi 332–0012, Japan (R.O., J.T.)

Indirect defense of plants against herbivores often involves the induced emission of volatile infochemicals including terpenoids that attract natural enemies of the herbivores. We report the isolation and characterization of a terpene synthase cDNA (*LjEβOS*) from a model legume, *Lotus japonicus*. Recombinant LjEβOS enzyme produced (E)-β-ocimene (98%) and its Z-isomer (2%). Transcripts of *LjEβOS* were induced in *L. japonicus* plants infested with two-spotted spider mites (*Tetranychus urticae*), coinciding with increasing emissions of (E)-β-ocimene as well as other volatiles, (Z)-3-hexenyl acetate and (E)-4,8-dimethyl-1,3,7-nonatriene, by the infested plants. We suggest that *LjEβOS* is involved in the herbivore-induced indirect defense response of spider mite-infested *L. japonicus* via de novo formation and emission (E)-β-ocimene. Mechanical wounding of the leaves or application of alamethicin (ALA), a potent fungal elicitor of plant volatile emission, also induced transiently increased levels of *LjEβOS* transcripts in *L. japonicus*. However, wounding or ALA did not result in elevated release of (E)-β-ocimene. Differences in volatile emissions after herbivory, mechanical wounding, or treatment with ALA suggest that neither a single mechanical wounding event nor ALA simulate the effect of herbivore activity and indicate that herbivore-induced emission of (E)-β-ocimene in *L. japonicus* involves control mechanisms in addition to up-regulation of *LjEβOS* transcripts.

The herbivore-induced emission of plant volatiles can attract carnivorous natural enemies of herbivores thus potentially protecting the plant (Paré and Tumlinson, 1999; Kessler and Baldwin, 2001; Pichersky and Gershenson, 2002). Low-molecular-weight terpenoids such as monoterpenes (C-10), sesquiterpenes (C-15), and homoterpenes (C-11 or C-16) are among the most common plant volatile compounds induced and emitted upon herbivory. These terpenoid volatiles, or in case of the homoterpenes, their precursors, are formed by the activity of families of terpenoid synthases (TPS) from polyprenyl diphosphate precursors (Bohlmann et al., 1998; Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000; Arimura et al., 2004). The acyclic monoterpene hydrocarbon compound (E)-β-ocimene is one of the most common volatile chemicals released from plants in response to herbivory (Paré and Tumlinson, 1999; Pichersky and Gershenzon, 2002). For example among the legumes, lima bean plants (*Phaseolus lunatus*) infested with two-spotted spider mites (*Tetranychus urticae*) release (E)-β-ocimene, which attracts a carnivorous natural enemy of two-spotted spider mites, predatory mites (*Phytoseiulus persimilis*; Dicke et al., 1990a, 1990b, 1999; Ozawa et al., 2000a). (E)-β-ocimene was also shown to act as a possible plant-to-plant signal which up-regulates the signaling pathway of jasmonic acid and ethylene in uninfested lima bean plants (Arimura et al., 2000, 2002). To our knowledge, *TPS* genes for the formation of (E)-β-ocimene have been cloned as cDNAs only from Arabidopsis (Fäldt et al., 2003) and from snapdragon (*Antirrhinum majus*; Dudareva et al., 2003). These ocimene synthases represent two different subfamilies of the TPS gene family (Dudareva et al., 2003).

Lotus japonicus is emerging as a model system for molecular and genetic studies in legumes due to its small genome size, its short generation times, self-compatibility, and due to the large expressed sequence tag (EST) resources and efforts toward sequencing the *L. japonicus* genome (Handberg and Stougaard, 1992;

---

[2] These authors contributed equally to the paper.

[3] Present address: Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, D-07745 Jena, Germany.

* Corresponding author; e-mail garimura@ice.mpg.de; fax 0049–3641–571202.

Endo et al., 2002). *L. japonicus* also serves as a useful system to study indirect plant-herbivore defense mechanisms in a legume. We have previously demonstrated that shoots of *L. japonicus* (ecotype Gifu B-129) infested with spider mites released a blend of volatiles, which contributed significantly to the attraction of predator mites (Ozawa et al., 2000b). We report here the cloning and functional characterization of a cDNA encoding (*E*)-β-ocimene synthase from *L. japonicus* ecotype Miyakojima MG-20. Effects of herbivory on transcript levels of (*E*)-β-ocimene synthase and increased emissions of (*E*)-β-ocimene are described. We discuss results of wound- and elicitor-induced regulation of (*E*)-β-ocimene biosynthesis at the transcriptional and emission levels.

RESULTS

Isolation of *L. japonicus* Monoterpene Synthase Full-Length cDNA *LjEβOS*

An EST clone for a putative mono-TPS (*LjTPS1*) was identified in the *L. japonicus* EST database (http://www.kazusa.or.jp/en/plant/lotus/EST/; accession no. AV409220). The subsequent BLAST search using the sequence of *LjTPS1* against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) resulted in the discovery of a transformation-competent artificial chromosome (TAC) clone (GenBank accession no. AP006119), which contains a gene with high similarity to known monoterpene synthases (*LjEβOS*). The
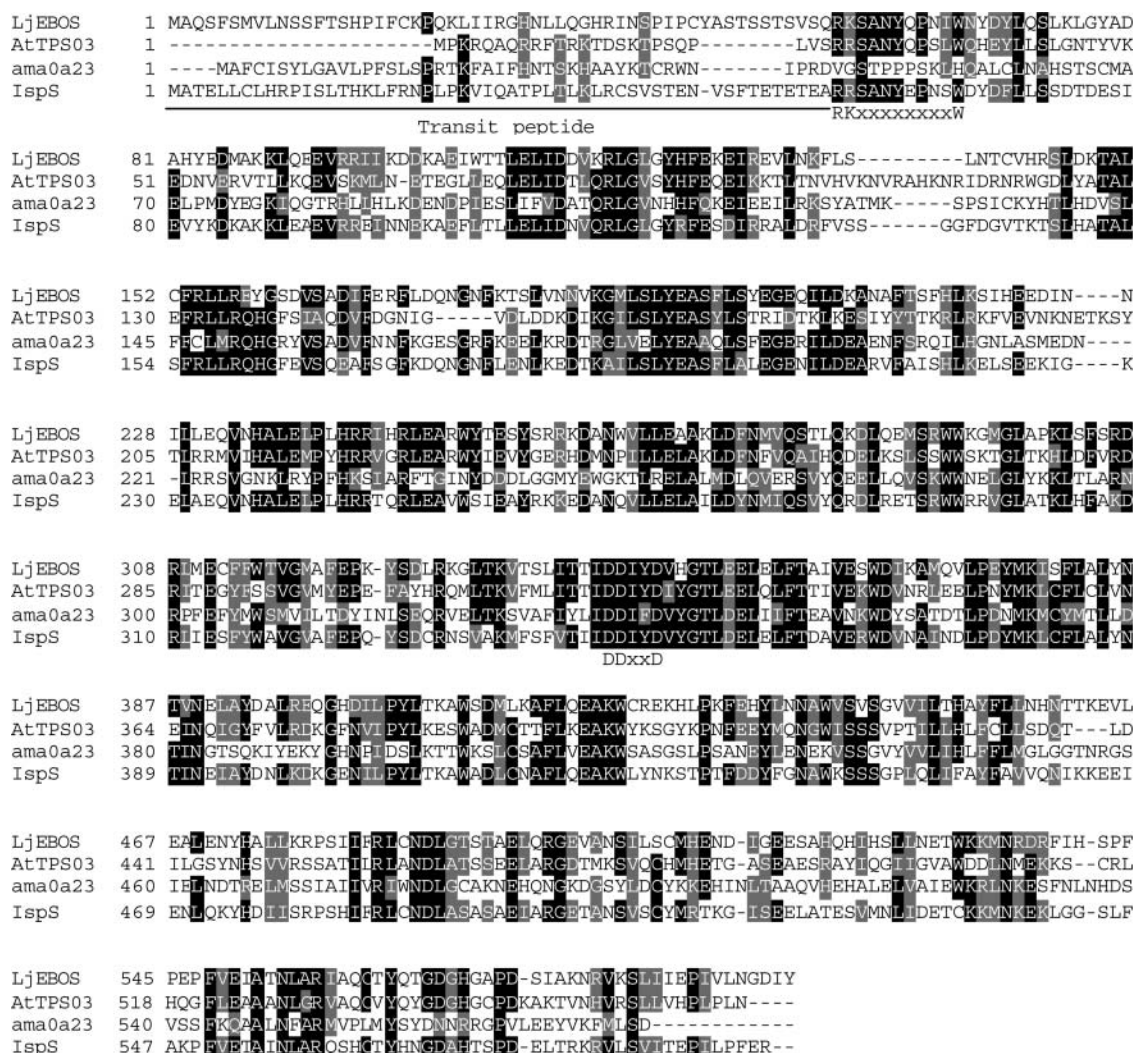
Figure 1. Amino acid sequence alignment of LjEβOS (LjEBOS), Arabidopsis (*E*)-β-ocimene synthase (AtTPS03, GenBank accession no. AY151086), snapdragon (*E*)-β-ocimene synthase (ama0a23, AY195607), and *Populus alba* × *Populus tremula* isoprene synthase (IspS, AJ294819). Amino acid residues that are identical among at least three sequences are indicated by white letters on black background. Other conserved residues are shaded in gray. Dashes indicate sequence gaps introduced to optimize the alignment. Conserved motifs R(R/K/G)x$_8$W and DDxxD are indicated.

nucleotide sequences of *LjTPS1* and *LjEβOS* share 81% identity over a length of 1,328 bp. Compared to the nucleotide sequences of the open reading frames (ORFs) of other known mono-TPSs (Bohlmann et al., 1998; Aubourg et al., 2002), the two putative *L. japonicus* mono-TPS sequences lack each approxi-

mately 400 bp from the 5′-ends of their predicted ORFs. The 5′-end of cDNA corresponding to *LjEβOS* was obtained by a rapid amplification of cDNA ends (RACE) followed by recovery of a full-length cDNA of 1,843 bp (GenBank accession no. AY575970). However, RACE did not lead to obtain the 5′-end of cDNA
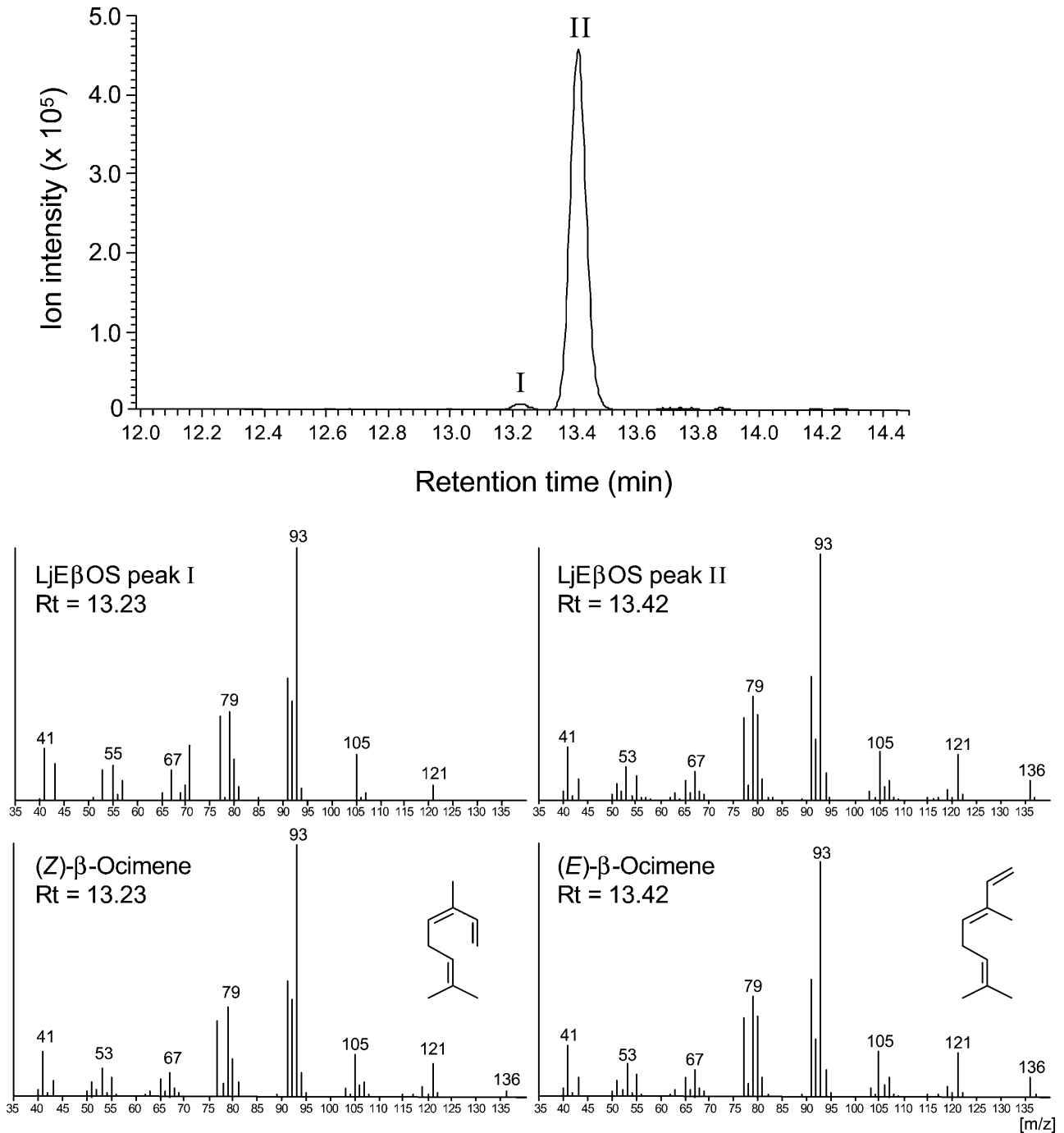
Figure 2. Expression in *E. coli* of recombinant LjEβOS protein and GC-MS analysis of monoterpene products formed by recombinant LjEβOS enzyme activity in vitro. Total ion chromatogram and mass spectra are of LjEβOS in vitro enzyme activity products with GPP as substrate. The mass spectrum of the most abundant product (Peak II) and a minor compound (Peak I), identified as (*E*)- and (*Z*)-β-ocimene, respectively, are shown together with that of the respective of authentic standards.

corresponding to *LjTPS1*, and this transcript may not be expressed in the *L. japonicus* shoots used in our research. The ORF of full-length *LjEβOS* from nucleotide 56 to nucleotide 1,843 encodes for a predicted protein of 595 amino acids, a molecular mass of approximately 68.8 kD, and a calculated pI of 6.26 (Fig. 1). The predicted protein encoded by *LjEβOS* contains a modification of the conserved motif RRx_8W motif of plant monoterpene synthase (Bohlmann et al., 1998; Williams et al., 1998) in the form of an RKx_8W motif at amino acid positions 56 to 66 from the N terminus (Fig. 1). The N-terminal region upstream of the RKx_8W motif is indicative of the presence of a plastid transit peptide. We also found the DDxxD active-site motif located at amino acids 343 to 347 from the N terminus. Based on sequence relatedness, LjEβOS is a member of the TPS-b group of angiosperm mono- and hemi-TPSs of the plant TPS-gene family (Bohlmann et al., 1998; Aubourg et al., 2002).

### Expression in *Escherichia coli* and Functional Characterization of LjEβOS

For functional identification of *LjEβOS*, the cDNA was expressed in *E. coli* strain BL21-CodonPlus(DE3) transformed with pET101/D-*LjEβOS*. For expression of LjEβOS without a transit peptide, a starting Met was introduced immediately upstream of the RKx_8W motif. Protein extracts of induced *E. coli* BL21-CodonPlus(DE3)/pET101/D-*LjEβOS* cells were assayed with three different prenyl diphosphate substrates: geranyl diphosphate (GPP), farnesyl diphosphate, and geranylgeranyl diphosphate. Assays with GPP as the substrate yielded a monoterpene hydrocarbon product profile composed of approximately 98% of (E)-β-ocimene and 2% of (Z)-β-ocimene as identified by gas chromatography-mass spectrometry (GC-MS) using authentic standards for comparison of retention times and mass spectra (Fig. 2). A control extract prepared from *E. coli* BL21(DE3) transformed with pET101/D-TOPO without the *LjEβOS* insert did not produce monoterpene hydrocarbon products (data not shown). Recombinant LjEβOS enzyme was not active with farnesyl diphosphate or geranylgeranyl diphosphate. The LjEβOS enzyme was thereby identified as (E)-β-ocimene synthase.

Curiously, the deduced protein sequence of LjEβOS has relatively low sequence similarities with two other previously identified (E)-β-ocimene synthases from Arabidopsis (At4g16740; Fäldt et al., 2003; 42% identity [ID] and 61% similarity [SI]) and from snapdragon (AY195607; Dudareva et al., 2003; 33% ID and 51% SI). In contrast, it exhibits much higher similarity with isoprene synthase, a hemiterpene synthase, from *Populus alba* × *Populus tremula* (AJ294819; Miller et al., 2001) with 55% ID and 71% SI. LjEβOS also has relatively high similarities with other angiosperm mono-TPSs, e.g. 47% ID and 65% SI with *Citrus limon* (−)-β-pinene synthase (AF514288; Lücker et al., 2002); and 46% ID and 65% SI with *C. limon* γ-terpinene

synthase (AF514286; Lücker et al., 2002). This finding supports a concept of multiple origins of (E)-β-ocimene synthases within the plant TPS-b group. The formation of (E)-β-ocimene from GPP does not involve a cyclization reaction and conceivably does not require the initial isomerization reaction of monoterpene cyclases (Wise and Croteau, 1999). It is therefore possible that (E)-β-ocimene synthases, like myrcene synthases and linalool synthase, could arise as default functions after mutations occurring in positions that are essential in monoterpene cyclization.

Spider Mites Induce Increased Transcript Levels of LjEβOS and Emissions of (E)-β-Ocimene in *L. japonicus* Plants

Emission of (E)-β-ocimene, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (Z)-3-hexenyl acetate from *L. japonicus* (ecotype Miyakojima MG-20) plants was induced 24 h after initiation of a spider mite infestation but not by a single mechanical wounding event (Fig. 3). This finding suggests that one or more herbivore-specific signals are required for the induced emission
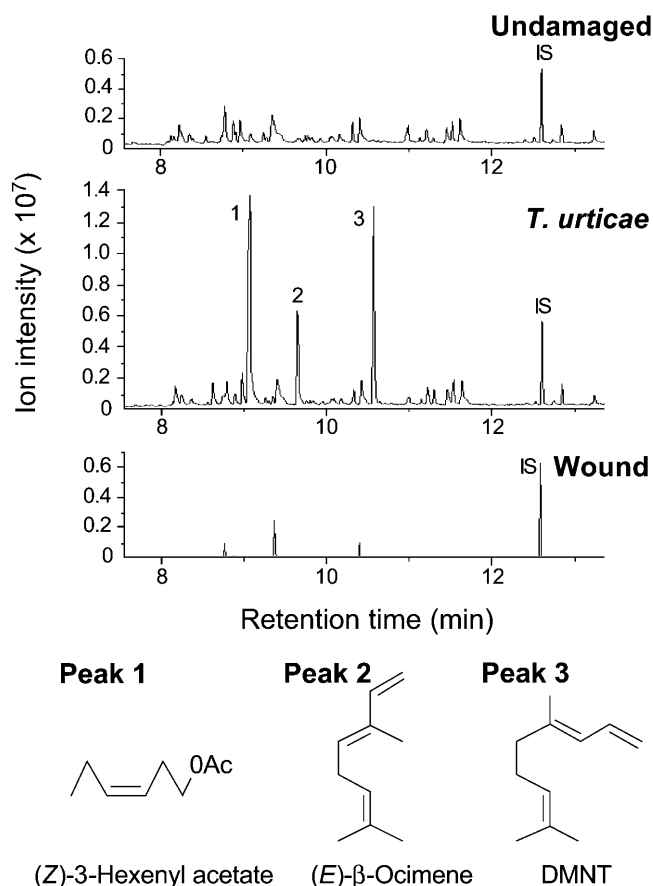
Figure 3. GC profiles of volatiles emitted from *L. japonicus* plants after 24 h of spider mite infestation or mechanical wounding compared with undamaged plants. Identification of compounds: 1, (Z)-3-hexenyl acetate; 2, (E)-β-ocimene; 3, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT); internal standard (IS), *n*-tridecane.
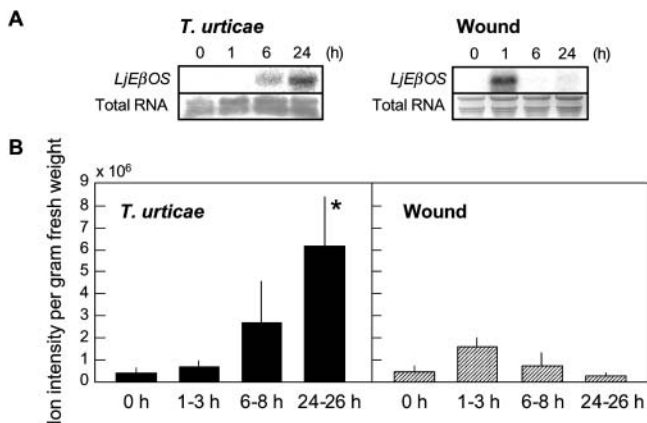
Figure 4. *LjEβOS* transcripts and (*E*)-β-ocimene emissions in *L. japonicus* plants in response to spider mite infestation or mechanical wounding. A, Northern analysis of transcripts in the *L. japonicus* shoots exposed to spider mites or wounded mechanically for 0, 1, 6, and 24 h. B, Volatiles were collected over 2 h at time points, 1 to 3 h, 6 to 8 h, and 24 to 26 h from the headspace of *L. japonicus* exposed to spider mites or wounded mechanically and subjected to GC-MS. Data represents the mean ± SE (*n* = 4–9, ion intensity of (*E*)-β-ocimene per gram fresh weight). Untreated plants served as control. A bar with asterisk is significantly different from the untreated control within each sampling interval (Dunnet's test at the 0.05 level).

of these volatiles in addition to a wound stimulus. To test if spider mite-induced emission of (*E*)-β-ocimene is the result of induced levels of *LjEβOS* transcripts and to test if differences in wound- and spider mite-induced volatile emissions are reflected in differences at the transcript level, we measured accumulation of *LjEβOS* transcript abundance as well as (*E*)-β-ocimene emissions over a time course in *L. japonicus* shoots exposed to spider mites or after wounding. Transcripts of *LjEβOS* were induced at 6 and 24 h after first contact with spider mites (Fig. 4A). We also found significantly higher emission of (*E*)-β-ocimene from spider mite-infested plants than from uninfested plants at 24 to 26 h (*P* < 0.05, Dunnet's test; Fig. 4B). For comparison with continuous feeding by spider mites, a single mechanical wounding event resulted in a very transient induction of *LjEβOS* transcripts detected at 1 h after wounding, coinciding with a slightly increased, but not significant (*P* > 0.05, Dunnet's test), higher level of (*E*)-β-ocimene emissions at 1 to 3 h after wounding.

Effects of Alamethicin on Transcripts of *LjTPS1*, *LjEβOS*, and *LjSqS*

Treatment of *L. japonicus* plants with alamethicin (ALA), an elicitor of the plant pathogenic fungus *Trichoderma viride*, induced *LjEβOS* transcripts at 1 h with a maximum in the shoots at 6 h (Fig. 5A). In contrast to the mono-TPS *LjEβOS*, transcripts of squalene synthase (*LjSqS*; AB102688; Akamine et al., 2003), the committed enzyme of the triterpenoid branch of terpenoid biosynthesis, was not induced by ALA, indicating some specificity in the response to ALA. Analyses of

transcripts by reverse transcription (RT)-PCR using a pair of *LjEβOS*-specific primers confirmed the temporal patterns of *LjEβOS*-transcripts in ALA-treated shoots (Fig. 5B). However, volatile collection analyses indicated that application of ALA to *L. japonicus* shoots did not induce a significant emission of (*E*)-β-ocimene from plants for up to 24 h (*P* > 0.05, Dunnet's test; Fig. 5C). In contrast to the *LjEβOS*-transcripts, RT-PCR using the *LjTPS1*-specific primers did not exhibit any detectable signals suggesting absence or very low levels of the *LjTPS1* transcripts in constitutive and ALA-treated plants, which is in agreement with the failure of recovering *LjTPS1* cDNA by RACE (see above).

DISCUSSION

We describe the identification, cloning, and functional characterization of a cDNA for (*E*)-β-ocimene synthase from *L. japonicus*. Increased emissions of (*E*)-β-ocimene from spider mite-infested *L. japonicus* plants seems to be associated with increased transcript levels of (*E*)-β-ocimene synthase, suggesting that herbivorous spider mites induce monoterpene volatile emissions by a mechanism that involves induced TPS gene expression. This is similar to findings in other species such as corn (*Zea mays*; Shen et al., 2000; Schnee et al., 2002) or poplar (*Populus trichocarpa* × *deltoides*; Arimura et al., 2004), where herbivore-induced emission of terpenoid volatiles is associated with induced transcript levels of the corresponding TPS genes. Dicke et al. (1990b) reported that a high ratio of the
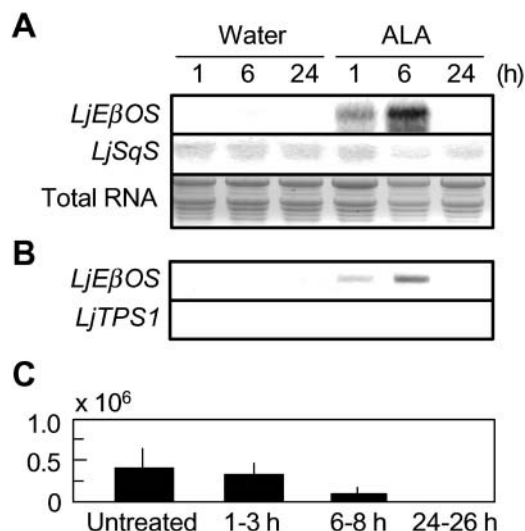
Figure 5. Effects of ALA treatment on transcripts accumulation and emission of (*E*)-β-ocimene in *L. japonicus*. A, Northern hybridization signals for *LjEβOS* and *LjSqS* in the *L. japonicus* shoots treated with water or ALA solution. B, RT-PCR analysis using the gene-specific primers of *LjEβOS* or *LjTPS1* followed the same time courses shown in A. C, (*E*)-β-Ocimene emissions from *L. japonicus* plants treated with ALA solution. Data represent the mean ± SE (*n* = 3–6, ion intensity of (*E*)-β-ocimene per gram fresh weight).

E-isomer in a mixture of (*E*)- and (*Z*)-β-ocimene (*E*/*Z* ratios of 97:3 and 70:30) results in the attraction of predator mites, which are natural enemies of spider mites, whereas a lower 60:40 *E*/*Z* ratio acts as a repellent for predator mites. Interestingly, only the *E*-isomer of β-ocimene was found in the headspace of spider mite-infested *L. japonicus* plants (Fig. 3), suggesting that the activity of LjEβOS, which almost exclusively produces (*E*)-β-ocimene (approximately 98%), leads to the emission of monoterpene volatiles in spider-mite infested *L. japonicus* that is highly enriched for one isomer of β-ocimene and could play a role in attracting carnivorous mites.

In contrast to *L. japonicus* plants infested with spider mites, only a brief induction of *LjEβOS* transcripts and a very weak emission of (*E*)-β-ocimene was found following a single mechanical wounding event of *L. japonicus* leaves (Fig. 4). One interpretation of this result is that cutting leaves with scissors may not be a good imitation of spider mite feeding. It is possible that continuous mechanical wounding, the presence of herbivore-derived elicitors, and/or a puncturing or sucking mode of damage may be required to trigger the same response with regard to volatile emission and transcript accumulation as is induced by real feeding of spider mites on *L. japonicus*.

Since the application of exogenous ALA, an ion channel-forming fungal elicitor, induced (*E*)-β-ocimene synthase transcripts in *L. japonicus* plants (Fig. 5), it is possible that not only herbivore feeding but also pathogen infections contribute to the upregulation of (*E*)-β-ocimene synthesis in *L. japonicus*. Curiously, even 24 h after treatment with ALA, the treated *L. japonicus* leaves did not produce any of the induced volatile compounds (data not shown). This is in disagreement with findings in the lima bean system where ALA elicits the emission of linalool, DMNT, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and methyl salicylate (Engelberth et al., 2001; Kunert et al., 2002). Mechanisms such as the regulation of substrate availability, in addition to induced TPS transcripts, also contribute to the control of terpenoid emissions, as was previously concluded from studies of emission of β-ocimene and myrcene in snapdragon flowers (Dudareva et al., 2003), in studies with herbivore-induced terpenoid emission in poplar leaves (Arimura et al., 2004), and in studies of terpenoid emissions in TPS-overexpressing Arabidopsis plants (Aharoni et al., 2003). It cannot be excluded that additional (*E*)-β-ocimene synthase gene(s) exist in *L. japonicus* that could contribute to regulation of herbivore-induced volatile emission in this system. For example, two myrcene synthases have been described in snapdragon with different roles in the regulation floral scent formation (Dudareva et al., 2003).

Our finding of spider mite-induced (*E*)-β-ocimene synthase transcript accumulation and (*E*)-β-ocimene emission in the *L. japonicus* MG-20 ecotype provides a foundation for future investigation of molecular genetic control of volatile emission in other ecotypes.

In contrast to MG-20 plants, the *L. japonicus* ecotype Gifu B-129 characterized by Ozawa et al. (2000b) did not show emission of (*E*)-β-ocimene after 3 d of feeding by spider mites. Both ecotypes also differ in several other traits, such as anthocyanin contents, trichome density, overall growth habit, and organ shape (Kawaguchi et al., 2001), making this a suitable system for future comparative adaptive trait analysis including an analysis of factors that determine ecotype-specific differences of herbivore-induced volatile emissions.

MATERIALS AND METHODS

Plants and Spider Mites

L. japonicus (ecotype Miyakojima MG-20) was grown in plastic pots (diameter = 8 cm, depth = 6.5 cm) each containing four plants in a growth chamber (12 h light:12 h dark, 25°C ± 1°C) for 2 months. Two-spotted spider mites (*Tetranychus urticae*) were obtained from a laboratory-maintained culture reared on kidney bean plants (*Phaseolus vulgaris* cv Nagauzuramame) in a greenhouse (25 ± 2°C).

Plant Treatment

Plants without flowers were used for each treatment. For mechanical wounding, leaves were wounded by cutting 20 leaves transversely at the widest point with scissors. ALA (100 ng/mL, Sigma-Aldrich, St. Louis) in 10 mL of water was sprayed onto four plants. Controls were sprayed with 10 mL of water. For spider mite infestation, we placed approximately 400 spider mite females on the *L. japonicus* shoots in a pot. Treatments were at 11 AM. During treatment experiments, temperature was held constant at 25°C ± 1°C and the photoperiod was 16 h light:8 h dark with light turning on at 7 AM and turning off at 11 PM.

Sequence Analysis

The putative splice sites and a potential coding region in the sequence of the TAC clone (AP006119) from *L. japonicus* chromosome 3 were predicted using the software NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/) and GeneMark.hmm (http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi). Alignments of deduced amino acid sequences were created using CLUSTALW, and visualized using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

5′-RACE and Full-Length cDNA Cloning of *LjEβOS*

Cloning of the 5′-end of *LjEβOS* was accomplished by 5′-RACE using the First Choice RLM-RACE kit (Ambion, Austin, TX) following the manufacturer's protocol. Total RNA was isolated from ALA-treated leaves by means of the method described by Wang et al. (2000). cDNA was amplified with Turbo *Pfu* polymerase (Stratagene, La Jolla, CA), using a *LjEβOS*-reverse primer (5′-GAAGCTCCAATGCATGATTCAC-3′) and a 5′-RACE outer primer (included with the kit). PCR was 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, and 60 s at 72°C, followed by 5 min at 72°C. A *LjEβOS*-reverse primer was designed from the sequence of the TAC clone (AP006119).

Total RNA from ALA-treated leaves was reverse transcribed into cDNA using Super-Script II RNase H⁻ reverse transcriptase (Invitrogen, Burlington, Canada) following the manufacturer's protocol. The cDNA was amplified by PCR using high fidelity Turbo *Pfu* polymerase with forward primer (*LjEβOS*-ORFS+CACC ATG, 5′-CACCATGAGAAAATCAGCCAATTACCAA-3′) and reverse primer (*LjEβOS*-ORFA, 5′-TTAATATATGTCTCCATTGAGAACAA-TGG-3′). The temperature program for PCR was as follows: 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, followed by 5 min at 72°C. *LjEβOS* cDNA was subcloned into pET101/D-TOPO expression vector (Invitrogen). Recombinant plasmids were transformed into *E. coli* TOP10 cells. The plasmid pET101/D-*LjEβOS* was purified, its insert sequenced, and transformed into *E. coli* BL21-CodonPlus(DE3) (Stratagene) for expression.

Functional Expression of Mono-TPS in *E. coli* and Enzyme Assay

Bacterial strain *E. coli* BL21-CodonPlus(DE3)/pET101/D-*LjEβOS* was grown to A_{600} = 0.5 at 37°C in 5 mL of Luria-Bertani medium with ampicillin at 100 μg/mL. Cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and held overnight at 20°C with shaking at 200 rpm. Cells were pelleted by centrifugation and were resuspended in 1 mL mono-TPS buffer (25 mM HEPES, pH 7.2, 7.5 mM MgCl$_2$, 20 μM MnCl$_2$, 5% glycerol). Resuspended cells were broken by sonication (Branson Sonifier 250, Branson Ultrasonic Corporation, Danbury, CT) at 5 W for 10 s. Cell extracts were cleared by centrifugation and assayed for mono-TPS activity with 55 μM GPP (Echelon Biosciences, Salt Lake City). The assay mixture (1 mL) was overlaid with 1 mL of pentane to trap volatile products. After incubation with gentle shaking at 30°C for 1 h, the pentane layer was passed through a column of equal amounts of anhydrous MgSO$_4$ and silica gel. The assay mixture was extracted with pentane (1 mL) a second time, and the pentane was passed through the column again. Samples were concentrated to <200 μL under a gentle stream of charcoal-filtered nitrogen. Extracts of *E. coli* BL21-Codon-Plus(DE3) transformed with pET101/D-TOPO plasmid without the *LjEβOS* insert, treated as described above, were used as controls.

Identification of Products of Mono-TPS Assays by GC-MS

Products of mono-TPS assays were identified and their relative ratios estimated following GC-MS analysis on an Agilent 6890 Series GC System connected to an Agilent 5973 Network Mass Selective detector (70 eV; Palo Alto, CA) using a DB-1MS capillary column (0.25 mm i.d. × 30 m with 0.25-mm film; J&W Scientific, Palo Alto, CA). Injections were splitless and the injector temperature was 200°C. The temperature program for GC was as follows: initial temperature was 40°C and immediately rose at 10°C/min to 200°C then rose at 20°C/min to a final temperature of 300°C, which was held for 1 min. The carrier gas was helium. (*E*)- and (*Z*)-β-ocimene were identified by comparisons with mass spectral libraries (ChemStation software, Hewlett-Packard, Wiley library, Palo Alto, CA) and by matching retention time with an authentic standard generously provided by Dr. John H. Borden (Simon Fraser University, Burnaby, Canada).

Northern Analysis

RNA samples (10 μg) were isolated from *L. japonicus* shoots and separated by electrophoresis through formaldehyde-agarose gels and blotted to nylon membranes (Hybond-N+, Amersham Biosciences, Piscataway, NJ). The cDNA clones for *LjEβOS* and *LjSqS* were ^{32}P-labeled by random priming (Strip-EZ DNA, Ambion). Hybridization and washes were carried out according to Arimura et al. (2004). The hybridized, labeled cDNA signals were detected using a Storm 860 phosphorimager (Amersham Biosciences). Ribosomal RNA stained with ethidium bromide was visualized (ChemiImager 5500 with AlphaEaseFC software, Alpha Innotech, San Leandro, CA).

RT-PCR Expression Analysis

Total RNA was reverse transcribed from 1 μg of total RNA, using Super-Script II RNase H⁻ reverse transcriptase following the manufacturer's protocol. The cDNAs were amplified by PCR using *Taq* polymerase (New England Biolabs, Mississauga, Canada) with 2 μL of RT-products and a pair of *LjEβOS*-specific primers (*LjEβOS*-S1, 5′-GGTAATTTCAAGACAAGTC-TTGTC-3′ and *LjEβOS*-A1, 5′-GACCCAAGCATTATTGAGATAATGC-3′) or *LjTPS1*-specific primers (*LjTPS1*-S1, 5′-GGTAATTTTGAGGCGAACCTAA-TTGG-3′ and *LjTPS1*-A1, 5′-CACCCATGCATTGTTGAGGTAGTCA-3′). The temperature program for PCR was as follows: 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C, followed by 5 min at 72°C. PCR products were separated by electrophoresis in 1.5% agarose gel including ethidium bromide and visualized by ChemiImager 5500.

Analysis of Plant Volatiles

For headspace analyses, *L. japonicus* plants in a pot were enclosed together with a piece of filter paper that contained 250 ng of tridecane in *n*-hexane, as an internal standard, in a glass container (2 L) at 25°C. The volatile compounds were drawn from the headspace of the container into a glass tube packed with Tenax TA adsorbents (100 mg, mesh 20/35) for 2 h at a flow rate of 100 mL/min. The collected volatile compounds were analyzed by GC-MS (GC: Hewlett-Packard 6890 with HP-5MS capillary column, 30 m long, 0.25 mm i.d., and 0.25-μm film thickness; MS: Hewlett-Packard 5973 mass selective detector, 70 eV) equipped with a thermal desorption cold trap injector (TCT; CP4010, Chrompack, Middelburg, The Netherlands). Headspace volatiles collected on Tenax-TA were released in the TCT themo-desorption unit at 220°C for 8 min, within a He flow. The desorbed compounds were collected in the TCT cold trap unit (SIL5CB-coated fused silica capillary) at −130°C. Flash heating of the cold trap unit provided sharp injection of the compounds into the capillary column of the gas chromatograph to which the cold trap unit was connected. The oven temperature of the GC was programmed to rise from 40°C (5-min hold) to 280°C at 15°C/min. The headspace volatiles were identified by comparing their mass spectrums and retention times with those of authentic compounds.

ACKNOWLEDGMENTS

LITERATURE CITED

Aharoni A, Giri AP, Deuerlein S, Griepink F, de Kogel WJ, Verstappen FW, Verhoeven HA, Jongsma MA, Schwab W, Bouwmeester HJ (2003) Terpenoid metabolism in wild-type and transgenic Arabidopsis plants. Plant Cell **15**: 2866–2884

Akamine S, Nakamori K, Chechetka SA, Banba M, Umehara Y, Kouchi H, Izui K, Hata S (2003) cDNA cloning, mRNA expression, and mutational analysis of the squalene synthase gene of *Lotus japonicus.* Biochim Biophys Acta **1626**: 97–101

Arimura G, Huber DPW, Bohlmann J (2004) Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa x deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (−)-germacrene D synthase, *PtdTPS1.* Plant J **37**: 603–616

Arimura G, Ozawa R, Nishioka T, Boland W, Koch T, Kühnemann F, Takabayashi J (2002) Herbivore-induced volatiles induce the emission of ethylene in neighboring lima bean plants. Plant J **29**: 87–98

Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defense genes in lima bean leaves. Nature **406**: 512–515

Aubourg S, Lecharny A, Bohlmann J (2002) Genomic analysis of the terpenoid synthase (*AtTPS*) gene family of *Arabidopsis thaliana.* Mol Genet Genomics **267**: 730–745

Bohlmann J, Meyer-Gauen G, Croteau R (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc Natl Acad Sci USA **95**: 4126–4133

Bouwmeester HJ, Verstappen FW, Posthumus MA, Dicke M (1999) Spider mite-induced (3*S*)-(*E*)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. Plant Physiol **121**: 173–180

Degenhardt J, Gershenzon J (2000) Demonstration and characterization of (*E*)-nerolidol synthase from maize: a herbivore-inducible terpene synthase participating in (3*E*)-4,8-dimethyl-1,3,7-nonatriene biosynthesis. Planta **210**: 815–822

Dicke M, Gols R, Ludeking D, Posthumus MA (1999) Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. J Chem Ecol **25**: 1907–1922

Dicke M, Sabelis MW, Takabayashi J, Bruin J, Posthumus MA (1990a) Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. J Chem Ecol **16:** 3091–3118

Dicke M, van Beek TA, Posthumus MA, Ben Dom N, van Bokhoven H, de Groot AE (1990b) Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. J Chem Ecol **16:** 381–396

Dudareva N, Martin D, Kish CM, Kolosova N, Gorenstein N, Fäldt J, Miller B, Bohlmann J (2003) (*E*)-β-Ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell **15:** 1227–1241

Endo M, Matsubara H, Kokubun T, Masuko H, Takahata Y, Tsuchiya T, Fukuda H, Demura T, Watanabe M (2002) The advantages of cDNA microarray as an effective tool for identification of reproductive organ-specific genes in a model legume, *Lotus japonicus.* FEBS Lett **514:** 229–237

Engelberth J, Koch T, Schüler G, Bachmann N, Rechtenbach J, Boland W (2001) Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiol **125:** 369–377

Fäldt J, Arimura G, Gershenzon J, Takabayashi J, Bohlmann J (2003) Functional identification of *AtTPS03* as (*E*)-β-ocimene synthase: a monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in *Arabidopsis thaliana.* Planta **216:** 745–751

Handberg K, Stougaard J (1992) *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. Plant J **2:** 487–496

Kawaguchi M, Motomura T, Imaizumi-Anraku H, Akao S, Kawasaki S (2001) Providing the basis for genomics in *Lotus japonicus*: the accessions Miyakojima and Gifu are appropriate crossing partners for genetic analyses. Mol Genet Genomics **266:** 157–166

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science **291:** 2141–2144

Kunert M, Biedermann A, Koch T, Boland W (2002) Ultrafast sampling and analysis of plant volatiles by a hand-held miniaturised GC with pre-concentration unit: kinetic and quantitative aspects of plant volatile production. J Sep Sci **25:** 677–684

Lücker J, El Tamer MK, Schwab W, Verstappen FW, van der Plas LH, Bouwmeester HJ, Verhoeven HA (2002) Monoterpene biosynthesis in lemon (*Citrus limon*). cDNA isolation and functional analysis of four monoterpene synthases. Eur J Biochem **269:** 3160–3171

Miller B, Oschinski C, Zimmer W (2001) First isolation of an isoprene synthase gene from poplar and successful expression of the gene in *Escherichia coli.* Planta **213:** 483–487

Ozawa R, Arimura G, Takabayashi J, Shimoda T, Nishioka T (2000a) Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant Cell Physiol **41:** 391–398

Ozawa R, Shimoda T, Kawaguchi M, Arimura G, Horiuchi J, Nishioka T, Takabayashi J (2000b) *Lotus japonicus* infested with herbivorous mites emits volatile compounds that attract predatory mites. J Plant Res **113:** 427–433

Paré PW, Tumlinson JH (1999) Plant volatiles as a defense against insect herbivores. Plant Physiol **121:** 325–331

Pichersky E, Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr Opin Plant Biol **5:** 237–243

Schnee C, Köllner TG, Gershenzon J, Degenhardt J (2002) The maize gene *terpene synthase 1* encodes a sesquiterpene synthase catalyzing the formation of (*E*)-β-farnesene, (*E*)-nerolidol, and (*E,E*)-farnesol after herbivore damage. Plant Physiol **130:** 2049–2060

Shen B, Zheng Z, Dooner HK (2000) A maize sesquiterpene cyclase gene induced by insect herbivory and volicitin: characterization of wild-type and mutant alleles. Proc Natl Acad Sci USA **97:** 14807–14812

Wang SX, Hunter W, Plant A (2000) Isolation and purification of functional total RNA from woody branches and needles of Sitka and white spruce. Biotechniques **28:** 292–296

Williams DC, McGarvey DJ, Katahira EJ, Croteau R (1998) Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochemistry **37:** 12213–12220

Wise ML, Croteau R (1999) Monoterpene biosynthesis. *In* DE Cane, ed, Comprehensive Natural Product Chemistry, Vol 2, Isoprenoids Including Carotenoids and Steroids. Pergamon Press, Oxford, pp 97–153