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 (LjEOS) from a model legume, Lotus japonicus. Recombinant LjEOS enzyme produced (E)-β-ocimene (98%) and its Z-isomer (2%). Transcripts of LjEOS 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 LjEOS 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 LjEOS 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 LjEOS transcripts.
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 LjEBOS**

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 monoterpen synthases (*LjEBOS*). The

![Figure 1. Amino acid sequence alignment of LjEBOS (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)x8W and DDxxD are indicated.](image-url)
nucleotide sequences of \( LjTPS1 \) and \( LjE\beta OS \) 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 approximately 400 bp from the 5'-ends of their predicted ORFs. The 5'-end of cDNA corresponding to \( LjE\beta OS \) 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](image-url). Expression in \( E. coli \) of recombinant \( LjE\beta OS \) protein and GC-MS analysis of monoterpene products formed by recombinant \( LjE\beta OS \) enzyme activity in vitro. Total ion chromatogram and mass spectra are of \( LjE\beta OS \) 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 \((Z)\)-\( \beta \)-ocimene, respectively, are shown together with that of the respective of authentic standards.
corresponding to \textit{LjTPS1}, and this transcript may not be expressed in the \textit{L. japonicus} shoots used in our research. The ORF of full-length \textit{LjEBOS} 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 \textit{LjEBOS} contains a modification of the conserved motif RRx8W (Fig. 1). The predicted protein of approximately 68.8 kD, and a calculated pI of 6.26 protein of 595 amino acids, a molecular mass of

Expression in \textit{Escherichia coli} and Functional Characterization of \textit{LjEBOS}

For functional identification of \textit{LjEBOS}, the cDNA was expressed in \textit{E. coli} strain BL21-CodonPlus(DE3) transformed with pET101/D-\textit{LjEBOS}. For expression of \textit{LjEBOS} without a transit peptide, a starting Met was introduced immediately upstream of the RKxW motif. Protein extracts of induced \textit{E. coli} BL21-CodonPlus(DE3)/pET101/D-\textit{LjEBOS} 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)-\textit{\beta}-ocimene and 2% of (Z)-\textit{\beta}-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 \textit{E. coli} BL21(DE3) transformed with pET101/D-TOPO without the \textit{LjEBOS} insert did not produce monoterpene hydrocarbon products (data not shown). Recombinant \textit{LjEBOS} enzyme was not active with farnesyl diphosphate or geranylgeranyl diphosphate. The \textit{LjEBOS} enzyme was thereby identified as (E)-\textit{\beta}-ocimene synthase.

Curiously, the deduced protein sequence of \textit{LjEBOS} has relatively low sequence similarities with two other previously identified (E)-\textit{\beta}-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 \textit{Populus alba} × \textit{Populus tremula} (AJ294819; Miller et al., 2001) with 55% ID and 71% SI. \textit{LjEBOS} also has relatively high similarities with other angiosperm mono-TPSs, e.g. 47% ID and 65% SI with \textit{Citrus limon} (−)-\textit{\beta}-pinene synthase (AF514288; Lücker et al., 2002); and 46% ID and 65% SI with \textit{C. limon} γ-terpinene synthase (AF514286; Lücker et al., 2002). This finding supports a concept of multiple origins of (E)-\textit{\beta}-ocimene synthases within the plant TPS-b group. The formation of (E)-\textit{\beta}-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)-\textit{\beta}-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 \textit{LjEBOS} and Emissions of (E)-\textit{\beta}-Ocimene in \textit{L. japonicus} Plants

Emission of (E)-\textit{\beta}-ocimene, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (Z)-3-hexenyl acetate from \textit{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](www.plantphysiol.org)
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 LjEBOS 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 LjEBOS 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 LjEBOS 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 LjEBOS 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.

## 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
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 up-regulation 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 (AP006139) 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. cg1). Alignments of deduced amino acid sequences were created 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 (AP006139).

Total RNA from ALA-treated leaves was reverse transcribed into cDNA using Super-Script II RTase (Invitrogen, Burlington, Canada) following the manufacturer’s protocol. The cDNA was amplified by PCR using high fidelity Turbo *Pfu* polymerase with forward primer (*LjEβOS*-ORF5+CACCAGTGAAAGAATGCCCAATACCAAA-3′) and reverse primer (*LjEβOS*-ORF6, 5′-TGAATATATGGTCTGATAGAAGAGAAT-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(DES)/pET101/D-LjEβOS was grown to *A*~0.6~ = 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 *E. coli*-mono-TPS buffer (25 mM HEPES, pH 7.2, 7.5 mM MgCl2, 20 µM MnCl2, 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 20°C for 6 h, the pentane layer was passed through a column of equal amounts of anhydrous MgSO4 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 <20 µL under a gentle stream of charcoal-filtered nitrogen. Extracts of *E. coli* BL21-CodonPlus(DES) 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) and by matching retention time with an 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) and by matching retention time with mass spectral libraries (ChemStation software, Hewlett-Packard, Wiley library, Palo Alto, CA). Injections were splitless and the injector temperature was 200°C. The oven temperature of the GC was programmed to rise from 40°C to 250°C over 1 h and then rise at 20°C/min to a final temperature of 280°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 32P-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 cleared by centrifugation and assayed for mono-TPS activity with 55 µM GPP. 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) and by matching retention time 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).

RT-PCR Expression Analysis

Total RNA was reverse transcribed from 1 µg of total RNA, using Superscript 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′-GCTAAATTTGAGACAACGTCT-TGTCC-3′ and LjEβOS-A1, 5′-GACCAACATATTGGATAGATGC-3′) or LjTPS1-specific primers (LjTPS1-S1, 5′-GCTAAATTGGAGGCGAACCTAATT-GG-3′ and LjTPS1-A1, 5′-CCCaCCGATCGATTTGGAGATGATCA-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, Middleton, The Netherlands). Headspace volatiles collected on Tenax-TA were released in the TCT thermo-desorption unit at 220°C for 8 min, within a He flow. The desorbed compounds were collected in the TCT cold trap unit (SIL5CB-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 to 40°C (5-min hold) to 280°C at 15°C/min. The headspace volatiles were identified by comparing their mass spectra and retention times with those of authentic compounds.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY409920, AP006119, AY155970, AY151086, AY159567, AJ294819, and AB102688.

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LITERATURE CITED


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