Research

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Characterization of defensive cadinenes and a novel sesquiterpene synthase responsible for their biosynthesis from the invasive *Eupatorium adenophorum*

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Summary

- *Eupatorium adenophorum* is a malignant invasive plant possessing extraordinary defense potency, but its chemical weaponry and formation mechanism have not yet been extensively investigated.
- We identified six cadinene sesquiterpenes, including two volatiles (amorpha-4,7(11)-diene and (–)-amorph-4-en-7-ol) and four nonvolatiles (9-*oxo*-10,11-dehydroageraphorone, muurol-4-en-3,8-dione, 9-*oxo*-ageraphorone and 9β-hydroxy-ageraphorone), as the major constitutive and inducible chemicals of *E. adenophorum*. All cadinenes showed potent antifeedant activity against a generalist insect *Spodoptera exigua*, indicating that they have significant defensive roles.
- We cloned and functionally characterized a sesquiterpene synthase from *E. adenophorum* (EaTPS1), catalyzing the conversion of farnesyl diphosphate to amorpha-4,7(11)-diene and (–)-amorph-4-en-7-ol, which were purified from engineered *Escherichia coli* and identified by extensive nuclear magnetic resonance (NMR) spectroscopy. *EaTPS1* was highly expressed in the aboveground organs, which was congruent with the dominant distribution of cadinenes, suggesting that EaTPS1 is likely involved in cadinene biosynthesis. Mechanical wounding and methyl jasmonate negatively regulated *EaTPS1* expression but caused the release of amorpha-4,7(11)-diene and (–)-amorph-4-en-7-ol. *Nicotiana benthamiana* transiently expressing *EaTPS1* also produced amorpha-4,7(11)-diene and (–)-amorph-4-en-7-ol, and showed enhanced defense function.
- The findings presented here uncover the role and formation of the chemical defense mechanism of *E. adenophorum* – which probably contributes to the invasive success of this plant – and provide a tool for manipulating the biosynthesis of biologically active cadinene natural products.

Introduction

In complicated terrestrial communities, plants must evolve a variety of adaptive strategies to withstand biotic and abiotic stresses. One representative strategy is their capacity to synthesize an enormous variety of secondary metabolites that function as defensive substances against attacks by herbivores and pathogens (Dixon, 2001). Strikingly, a large number of terpenes have been reported to act as deterrents or toxins against mammals, insects, mollusks, fungi and bacteria, and this is therefore considered to be the primary role of terpenes in nature (Gershenzon & Dudareva, 2007). Among them are sesquiterpenes, the largest and most diversified group of natural terpenes (Holopainen & Gershenzon, 2010). For instance, volatile sesquiterpenes, as exemplified by (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), (*E*)-α-farnesene, (*E*)-β-caryophyllene and (*E*)-α-bergamotene, have been frequently reported to be involved in plant defense and can be induced by insect feeding and pathogen infection in various plants (Arimura *et al.*, 2000; Kessler & Baldwin, 2001). In addition, drimanes and eudesmanes have exhibited a wide range of defensive functions, including antifeedant, insecticidal, antibacterial, antifungal and phytotoxic properties (Jansen & de Groot, 2004; Wu *et al.*, 2006). Despite the increasing evidence supporting the idea that sesquiterpenes serve as defense chemicals, testing their genuine ecological functions in a natural setting remains challenging.

The integration of modern molecular biology and chemical ecology provides a great opportunity to improve our

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understanding of the actual roles of secondary metabolites in nature (Gershenzon & Dudareva, 2007); the genes responsible for their biosynthesis have therefore received extensive attention. The key step for sesquiterpene biosynthesis is the conversion of acyclic farnesyl diphosphate (FDP) to diverse skeletons by specific sesquiterpene synthases or cyclases (Chen *et al.*, 2011). To date, a large number of sesquiterpene synthases responsible for the formation of defensive sesquiterpenes have been cloned and functionally characterized from various plants, including cotton, maize and tomato (Chen *et al.*, 1995; Shen *et al.*, 2000; Schnee *et al.*, 2006; Bleeker *et al.*, 2012). The characterization of sesquiterpene synthase has no doubt provided much stronger evidence for sesquiterpene involvement in plant defense.

Eupatorium adenophorum, a Compositae (synonym Asteraceae) species, originates from Mexico, but it has invaded many tropical and subtropical regions world-wide, including America, eastern Australia, Europe, southwestern Africa and Asia (Feng *et al.*, 2009; Inderjit *et al.*, 2011). *Eupatorium adenophorum* invaded Yunnan province from Burma (now Myanmar) and Vietnam in the 1940s, spread rapidly into other provinces of southwestern China, and now appears in northern and eastern regions of China (Feng *et al.*, 2009). This plant inhibits the seed germination and seedling growth of native plants, becoming a dominant species and causing severe ecological damage and financial losses in invaded regions. Moreover, *E. adenophorum* is toxic to many animal species. For example, it causes pulmonary toxicity in horses, hepatic injury in mice, anorexia and photosensitization in cattle, and hepatotoxicity and cholestasis in rats (Bhardwaj *et al.*, 2001). As a result, *E. adenophorum* has been termed the 'Mexican Devil' (Zhao *et al.*, 2009; Inderjit *et al.*, 2011). However, *E. adenophorum* has also been used as a folklore medicine through application to cuts and wounds (Gaire & Subedi, 2011) and its extracts possess analgesic and anti-inflammatory properties (Mandal *et al.*, 2005; Chakravarty *et al.*, 2011).

Eupatorium adenophorum is seldom attacked by microorganisms and herbivores, except for the specialist insect *Procecidochares utilis* Stone that was first introduced into China in 1984 (Feng *et al.*, 2009), suggesting that this plant has an extraordinary defense system. Cadinene sesquiterpenes were reported to be the dominant constituents of *E. adenophorum* (He *et al.*, 2008), and they have demonstrated extensive biological functions, including contact toxicity and growth retarding activity against larvae of a noctuid species (Proksch *et al.*, 1990) and toxicity to rats (Bhardwaj *et al.*, 2001; Ouyang *et al.*, 2014), as well as aphicidal (Nong *et al.*, 2015), acaricidal (Liao *et al.*, 2014), antifungal (Kundu *et al.*, 2013), antifeedant (Shi *et al.*, 2012) and phytotoxic (Baruah *et al.*, 1994) activities. Phenolics that possessed antibacterial and phytotoxic effects have also been isolated from *E. adenophorum* (Zheng *et al.*, 2012). In addition, volatile sesquiterpenes, including (*E*)-β-caryophyllene, (*E*)-α-bergamotene and bicyclogermacrene, which play important defense functions in many plants, were released by Mexican *E. adenophorum* in much higher quantities than those observed in Indian or Chinese plants, suggesting that they might be important in defense against specialist insects in the plant's native range (Inderjit *et al.*, 2011). Therefore, cadinenes, phenolics and volatile terpenes might be the major defense chemicals of *E. adenophorum*, but direct evidence for their defensive roles and formation mechanisms are still lacking. In addition, we observed that *E. adenophorum* grown wild usually has no significant smell, but can immediately give off a horrible odor once damaged, or merely touched, thus 'warning' the attackers to stay away from the plant. In the present study, the volatile and nonvolatile defense chemicals of *E. adenophorum* were investigated, and a novel sesquiterpene synthase catalyzing the formation of defensive cadinenes was cloned and functionally characterized.

Materials and Methods

Plant treatments

Eupatorium adenophorum Spreng. (synonym *Ageratina adenophora* Spreng. King and Robinson) was propagated from seeds collected from mature *E. adenophorum* plants growing outdoors at Kunming Botanical Garden, Chinese Academy of Sciences (CAS), and grown in a glasshouse under four highly efficient full-spectrum fluorescent lamps (KDT5 type, 28 W, $\lambda \geq 0.98$) at 23°C with a 16 h : 8 h, light : dark photoperiod. Six-week-old plants, each with 6–8 leaves, were chosen and randomly divided into six groups. Each group was allocated to a different treatment and grown in a chamber to avoid cross contamination. For mechanical wounding, the 2nd and 3rd fully elongated leaves (hereafter the leaves of *E. adenophorum*) were wounded using a razor blade to punch two rows of scars on each side of the midvein. For insect feeding, four 4th instar cotton bollworms (*Helicoverpa armigera*), purchased from the Pilot-Scale Base of Bio-Pesticides (Institute of Zoology, CAS, Beijing, China) were placed on the undamaged leaves of each plant and caged with a gauze bag. After feeding for 6 h, at which time approximately 25% of the leaf area was consumed, cotton bollworms were removed from the plants (Supporting Information Fig. S1). For pathogen infection, the mechanically wounded leaves of each plant were immediately sprayed with a spore suspension of *Alternaria alternata* (isolated from the diseased leaves of *E. adenophorum*; Fig. S2) containing around 500 spores ml^{-1}. The undamaged group was used as the control. For methyl jasmonate (MJ) treatment, each plant was sprayed with 100 μM MJ (Sigma-Aldrich) in 0.1% (v/v) aqueous ethanol, and the control group was sprayed with the same amount of 0.1% (v/v) aqueous ethanol solution. Each treatment was repeated with three biological replicates.

Collection and analysis of volatile organic compounds released by *E. adenophorum*

The volatile organic compounds (VOCs) released by *E. adenophorum* were collected separately at 1, 3, 6, 12 and 24 h after different treatments using a closed-loop stripping system, as described in a study by Tholl *et al.* (2006). Each plant was placed inside a glass chamber capped with the closed loop stripping apparatus (Fig. S1). Air flow was maintained by a vacuum pump. The VOCs were collected for 30 min in 25 mg Super Q traps (Supelco; Sigma-Aldrich). Each collective column was then eluted with 300 μl *n*-hexane containing 600 ng ml^{-1} of nonane

into a sample vial for gas chromatography–mass spectrometry (GC-MS) analysis (Methods S1). A C_8–C_{40} alkane standard solution (Sigma-Aldrich) was analyzed for the calculation of retention indices (RIs) and monitoring system performance. The amount of each volatile sesquiterpene released was quantified by integration of the peak area and calibration with nonane as an internal standard because it is chemically stable and its retention time is clearly distinct from those of the VOCs released by *E. adenophorum*. The total amount of volatile sesquiterpenes released was calculated as the sum of the amounts of each detected compound.

Analysis, isolation, identification and quantification of nonvolatile compounds in *E. adenophorum*

The leaves of *E. adenophorum* were harvested at 3, 7, 14 and 21 d after various treatments and ground into powder in liquid nitrogen using a mortar. 50 mg fresh weight (FW) of each sample was extracted with acetone (3 × 10 ml) in an ultrasonic bath for 30 min. The crude extract was combined and evaporated to dryness under reduced pressure, and then suspended in 1.0 ml acetonitrile. After centrifugation at 7500 ***g*** for 10 min, the clear supernatant of each sample was analyzed by high performance liquid chromatography (HPLC) with a diode array detector (DAD) on an Agilent 1200 system equipped with a Zorbax SB-C_{18} column (5 µm, 4.6 × 250 mm; Agilent Technologies, Palo Alto, CA, USA) (Methods S2).

Approximately 10 kg of fresh *E. adenophorum* leaves were used for isolation of compounds 15–18 by silica gel column chromatography and reversed-phase semi-preparative HPLC (Methods S3). Compounds 15 (35.2 mg), 16 (4.5 mg), 17 (27.6 mg) and 18 (6.9 mg) were purified and identified by nuclear magnetic resonance (NMR) spectroscopic analysis (Methods S4).

For quantitative analysis of compounds 15–18, calibration curves were prepared for the purified compounds. The equations and correlation coefficients obtained from the linearity studies were $y = 0.0411x - 6.4352$ ($R^2 = 0.9997$), $y = 0.0641x - 4.9106$ ($R^2 = 0.9997$), $y = 0.0613x - 2.9039$ ($R^2 = 0.9999$), and $y = 0.0982x - 4.7619$ ($R^2 = 0.9997$) for compounds 15–18, respectively. The experiment for quantitative analysis was repeated with three biological replicates, and the experiment for calibration curves was repeated with five technique replicates.

Antifeedant assay of the purified compounds

A dual-choice bioassay was performed for the antifeedant assay as previously described (Shi *et al.*, 2012) (Methods S5). After feeding for 24 h, at which time the feeding area of the control leaf discs was *c.* 50%, the exact area of each consumed leaf disc was measured using 1 × 1 mm transparent coordinate paper. The insect antifeedant index (AFI) of the test compound was calculated according to the formula: AFI = ((C–T)/(C+T)) × 100%, where C and T represent the control and treated leaf areas consumed by insects. The effective concentration for a 50% feeding reduction (EC_{50}) value was determined by Probit analysis. Commercial neem oil was used as a positive control.

Cloning and functional analysis of terpene synthases from *E. adenophorum*

The degenerate primers (Table S1) were designed based on the conserved amino acid sequences of plant sesquiterpene synthases, and three specific fragments of *c.* 500 bp in length were generated from the cDNA of *E. adenophorum* leaves and sequenced. Full-length cDNA sequences were obtained by 3′ and 5′-rapid amplification of cDNA ends (RACE) using a Smart RACE cDNA amplification kit (Clontech, Tokyo, Japan).

For heterologous expression, the full-length cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) using high-fidelity PrimeSTAR DNA polymerase (Takara, Otsu, Japan), and cloned into the cold-inducible expression vector pCold TF (Takara) which contained a His-tag at the N terminus. The plasmids confirmed by DNA sequencing were then transferred into the *Escherichia coli* strain Rosetta (DE3) (Novagen, Madison, WI, USA).

The recombinant *E. coli* cells were grown to an OD_{600} of 0.6 at 37°C in Luria-Bertani (LB) liquid medium containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. Cell cultures were then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16°C for 6 h. After centrifugation at 13 400 ***g*** for 10 min at 4°C, the cell pellets were resuspended with extraction buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM imidazole and 5 mM DL-dithiothreitol (DTT), and sonicated on ice.

The soluble recombinant proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose columns (Qiagen) in wash buffer containing 20 mM imidazole, and eluted with an elution buffer containing 250 mM imidazole. The purified protein was examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and the concentration was measured using the Bradford method with albumin from bovine serum (BSA) as a standard.

The enzymatic activity assay was performed in a final volume of 200 µl containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 2 mM DTT, 20 µg purified protein, and 60 µM prenyl diphosphate substrate geranyl diphosphate (GDP), (*E,E*)-FDP, or (*E,E,E*)-geranylgeranyl diphosphate (GGDP) (Sigma-Aldrich). After incubation for 3 h at 30°C, the assay mixture was extracted with *n*-hexane by vigorous shaking, and the extract was evaporated under nitrogen gas and analyzed using GC-MS (Methods S1). At least five independent biological replicates were carried out for each *in vitro* enzyme assay.

Isolation and identification of EaTPS1 products

Seven genes in the mevalonate pathway (MVA pathway hereafter) and the *FDP synthase* (*FDPS*) gene were amplified using PrimeStar HS DNA polymerase (Takara) from pBbA5c-MevT-MBIS (Peralta-Yahya *et al.*, 2011). Three genes responsible for converting acetyl-CoA to mevalonate, including the *acetoacetyl-CoA thiolase* (*AtoB*) gene from *E. coli*, *HMG-CoA synthase* (*HMGS*) and a truncated version of the *HMG-CoA reductase* (t*HMGR*) genes from *Saccharomyces cerevisiae* with codon-optimization,

were inserted into the EcoRI and BamHI restriction sites of pBbA5c-MevT-MBIS using the In-fusion assembly kit (Takara), which resulted in a pBbA5c-MevT plasmid containing a p15A origin, a chloramphenicol resistance gene and a P_{lacUV5} promoter. Five genes responsible for converting mevalonate to FDP, including *mevalonate kinase* (*ERG1*), *phosphomevalonate kinase* (*ERG8*) and *phosphomevalonate decarboxylase* (*MVD1*) genes from *S. cerevisiae* with codon-optimization, as well as *IDP isomerase* (*IDI*) and *FDPS* genes from *E. coli*, were assembled into the PstI and SacI sites of the pBBR1MCS-1 vector (Kovach *et al.*, 1995), generating a pBBR-FDP plasmid which harbored a pBBR1 origin, a tetracycline resistance gene and a P_{lac} promoter.

The pBbA5c-MevT and pBBR-FDP plasmids were co-transformed with a pCold TF vector harboring *EaTPS1* or an empty vector (control) into BL21 (DE3) Star cells (Invitrogen). The transformants were selected on LB agar containing 34 μg ml^{-1} chloramphenicol, 10 μg ml^{-1} tetracycline and 100 μg ml^{-1} ampicillin, and then confirmed by PCR. The appropriate transformants were grown to an OD_{600} of 0.6 at 37°C and induced with 0.5 mM IPTG at 18°C for 48 h. Next, 10 l of culture was extracted with petroleum ether. After evaporation under reduced pressure at 45°C, the extract was purified by silica gel column chromatography using *n*-hexane as an eluent, yielding compounds 6 (550 mg) and 13 (35 mg) as colorless oil. Both compounds were subjected to structural identification through analysis of their NMR and MS data and optical rotations (Methods S4).

Gene expression and compound quantification

One-week-old, 2-wk-old, 3-wk-old, 4-wk-old and 6-wk-old *E. adenophorum* growing in the glasshouse, and the mature plants from a natural habitat at Kunming Botanical Garden were used for analysis of gene expression and compound quantification. Each sample was immediately frozen in liquid nitrogen and ground into powder. Total RNA was extracted using Trizol reagent (Invitrogen, USA) and examined using gel electrophoresis, and the absorbance at 260 and 280 nm was assessed using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 200 μg total RNA using TransScript One-Step gDNA Removal and cDNA synthesis SuperMix (Transgen Biotech, Beijing, China). Real-time quantitative polymerase chain reaction (qRT-PCR) was carried out on an Applied Biosystems 7500 instrument (Life Technologies) with UltraSYBR mixture reagent (with ROX; CWBiotech, Beijing, China). The *E. adenophorum* housekeeping gene *ubiquitin* (accession no. GU828011) was used as an internal standard. Primer specificity and amplification efficiency were confirmed by agarose gel electrophoresis, melting curve analysis and standard curve analysis. The relative expression level was calculated using the $\Delta\Delta C_T$ method. All experiments were performed with three independent biological replicates, each of which was carried out in three technical replicates.

Meanwhile, 100 mg FW of each powdered sample was extracted with 1 ml *n*-hexane in an ultrasonic bath for 30 min for quantification of compounds 6 and 13 in plants of different developmental stages and different organs. After centrifugation at 13 400 ***g*** for 10 min, the clear supernatant was transferred into a sample vial for GC-MS analysis (Methods S1). Calibration curves of purified compounds were prepared, and the equations and correlation coefficients were as follows: $y = 306617.56x + 1183219.38$ ($R^2 = 0.9976$) for compound 6, and $y = 714728.94x + 3433546.23$ ($R^2 = 0.9975$) for compound 13. The experiment was conducted with three independent biological replicates.

Construction, metabolic analysis and antifeedant assay of *Nicotiana benthamiana* transiently expressing *EaTPS1*

EaTPS1 was cloned into plant expression vector pEAQ-*HT* (Sainsbury *et al.*, 2009), and verified by PCR and DNA sequencing. Plasmids pEAQ-*HT* containing *EaTPS1* and empty pEAQ-*HT* (control) were separately transformed into *Agrobacterium tumefaciens* strain LBA4404 using a freeze–thaw method. Positive colonies were cultured in LB liquid media containing 25 μg ml^{-1} rifampicin, 50 μg ml^{-1} streptomycin and 50 μg ml^{-1} kanamycin at 28°C overnight to an OD_{600} of 1.2–1.8. After centrifugation at 3900 ***g*** for 5 min, *A. tumefaciens* cells were re-suspended with MMA buffer (10mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.6, 10 mM $MgCl_2$ and 100 μM acetosyringone) to a final OD_{600} of 0.4, and infiltrated into the leaves of 4- to 6-wk-old *N. benthamiana*.

After growing for 5 d, 40 g fresh leaves of infiltrated plants were harvested and ultrasonic extraction was performed with 2 × 500 ml acetone. The filtered extracts were concentrated using a rotary evaporator and partitioned with 100 ml petroleum ether three times. After concentration, the extracts were dissolved in 1 ml *n*-hexane, and the supernatants obtained by centrifugation were analyzed using GC-MS.

The fresh leaves of *N. benthamiana* harvested 5 d after infiltration were used to test antifeedant activity as described in the 'Antifeedant assay of the purified compounds' section, with modifications. Fresh leaf discs (0.9 cm in diameter) prepared from *N. benthamiana* harboring *EaTPS1* were used as the treated leaf, while leaf discs prepared from *N. benthamiana* containing empty pEAQ-*HT* were used as the control. The insect feeding index was calculated according to the formula $T/C \times 100\%$, where T and C represent the leaf areas consumed by the insect and the initial leaf areas, respectively. The experiment was repeated with 10 biological replicates.

Accession numbers

EaTPSs1–3 sequences can be found in the GenBank database under the following accession numbers: EaTPS1: MG923955; EaTPS2: MG923956; EaTPS3: MG923957.

Results

Discovery of volatile sesquiterpenes, including two cadinenes, as the dominant VOC components of *E. adenophorum*

Our field observations indicated that *E. adenophorum* growing in the wild usually has no significant smell; surprisingly, however, the plant can immediately give off an unpleasant odor upon

damage, suggesting that VOCs released by the damaged plants are likely involved in the plant defense. We therefore collected VOCs released from the plants using a headspace method and analyzed them by GC-MS. A generalist insect herbivore, cotton bollworm (*H. armigera*), which was allowed to feed on *E. adenophorum* leaves after starvation treatment, was used in a laboratory feeding test (Fig. S1). The pathogenic fungus *A. alternata* isolated from the diseased leaves of *E. adenophorum* was used in a pathogen infection test (Fig. S2). It was found that healthy undamaged (control) plants released rather limited VOCs and mainly nonterpenes, including 2-heptanone, heptanal, 2-octanone, nonanal, hexadecane, heptadecane and octadecane (Fig. 1a). After treatment with mechanical wounding, insect feeding or pathogen infection, *E. adenophorum* immediately emitted a large quantity of VOCs, especially the volatile sesquiterpenes, which accounted for 60%–73% of the total VOCs (Figs 1b, S3; Table S2). Intriguingly, two volatiles whose mass spectra showed high similarity with those of cadinenes were found, and were ultimately identified as amorpha-4,7(11)-diene (compound 6) and (−)-amorph-4-en-7-ol (compound 13) through comparison of their mass spectra and retention times with those of the purified compounds described in the 'Structural elucidation of EaTPS1 products using engineered *E. coli*' section. In addition, we also observed 12 other volatile sesquiterpenes (Fig. 1b,c). (*E*)-β-Caryophyllene (compound 1), (*E*)-β-farnesene (compound 4) and (*E*)-α-bisabolene (compound 11) were identified based on their identical mass spectra and retention times with those of commercial substances. (*E*)-α-Bergamotene (compound 2), (*Z*)-β-farnesene (compound 3), γ-curcumene (compound 5), germacrene D (compound 7), bicyclogermacrene (compound 8), β-bisabolene (compound 9), β-sesquiphellandrene (compound 10), (*E*)-α-bisabolene (compound 11), α-cedrol (compound 12) and α-bisabolol (compound 14) were tentatively identified based on the high similarity (> 90%) of their mass spectra with those in the NIST 08 library and their retention indices (RIs) with those of published data (Adams, 2007). Notably, volatile cadinenes 6 and 13, along with compounds 7 and 9, were the major volatile compounds released by the treated plants (Table S2).

The quantity (relative to nonane) of volatile sesquiterpenes released rapidly reached a maximum in mechanically wounded plants at 1 h after treatment, and little change was observed during a longer period of observation (Fig. S4). The fast response of the volatile terpenes raised the question of whether these sesquiterpenes were constitutively stored in the plants. Therefore, different organs (leaves, young stems, flowers, flower buds and roots) of untreated *E. adenophorum* were collected and extraction with *n*-hexane was performed immediately. As expected, volatile sesquiterpenes, including compounds 6 and 13, were the major VOCs in the extracts of analyzed aboveground organs, except for the roots, where no volatile sesquiterpenes were detected (Figs S4, S5).

Identification of four nonvolatile cadinenes as the constitutive and inducible chemicals of *E. adenophorum*

To determine the nonvolatile defensive chemicals present in *E. adenophorum*, we profiled the specialized compounds in the aboveground organs of plants treated with mechanical wounding, insect feeding or pathogen infection using a HPLC-DAD method. As a result, the areas of four peaks (1–4) were found to increase markedly in the chromatograms of treated plants (Fig. 2). Compounds corresponding to peaks 1–4 were then traced and isolated from the petroleum ether extract of fresh leaves of *E. adenophorum*. Based on ^{1}H and ^{13}C (including distortionless enhancement by polarization transfer (DEPT)) NMR spectroscopic analysis (Figs S6–S13), compounds 15−18 displayed 15 carbon resonances, including four methyls, suggesting that these compounds were all sesquiterpenes. Interestingly, they all exhibited an α,β-unsaturated ketone group (compound 15: δ_C197.5, 136.3, 146.8; compound 16: δ_C198.3, 136.2, 148.7; compound 17: δ_C198.0, 136.6, 141.4; compound 18: δ_C198.0, 133.3, 146.0; corresponding to C-3, C-4 and C-5 respectively). Finally, through careful comparison of their NMR data with those reported data in the literature, they were identified as four cadinene sesquiterpenes: 9-*oxo*-10,11-dehydroageraphorone (compound 15) (Bhardwaj *et al.*, 2001), muurol-4-en-3,8-dione (compound 16) (Weyerstahl *et al.*, 1997), 9-*oxo*-ageraphorone (compound 17) and 9β-hydroxy-ageraphorone (compound 18) (Bordoloi *et al.*, 1985; Shukla *et al.*, 1986) (Fig. 2c).

Subsequently, the contents of compounds 15–18 present in the control and treated plants were quantified using purified compounds as external standards (Fig. 2d–g). In *E. adenophorum* control plants, compound 15 was found to be present in larger quantities (*c.* 10 μg mg^{-1} FW) than the other compounds. In insect-fed plants, the content of compound 15 detected was as high as 21.7 ± 0.9 μg mg^{-1} FW at 14 d after treatment, which was almost double the control value (10.7 ± 0.4 μg mg^{-1} FW, $P < 0.001$). In mechanically wounded plants, the content of compound 15 increased at 7 d but decreased at 21 d after treatment. In the plants infected by *A. alternata*, no obvious change in the content of compound 15 was observed. Compound 15 was therefore assumed to be a herbivore-inducible metabolite. However, significant quantities of compounds 16–18 was inducedby all three treatments.

Cadinene sesquiterpenes showed significant antifeedant activities

To gain a better understanding of the defensive function of sesquiterpenes in *E. adenophorum*, the antifeedant activities of six cadinenes and three commercially available sesquiterpenes were tested. As shown in Table 1, it was evident that all tested compounds were potential deterrents to the cotton bollworm, with EC_{50} values ranging from 2.71 ± 0.20 μg cm^{-2} to 10.35 ± 0.60 μg cm^{-2}. Cadinenes 6, 13 and 15–18 were more active than sesquiterpenes 1, 4 and 9.

Notably, the content of compound 15 (which was detected in higher quantities than any of the other compounds) in the leaves of 6-wk-old plants was *c.* 69 times higher than its antifeedant EC_{50} value (Table 1). The content of compound 16 detected was around 2.3 times higher than its antifeedant EC_{50} value, while the content of compound 17 was comparable to its antifeedant EC_{50} value. It can therefore be concluded that cadinenes may

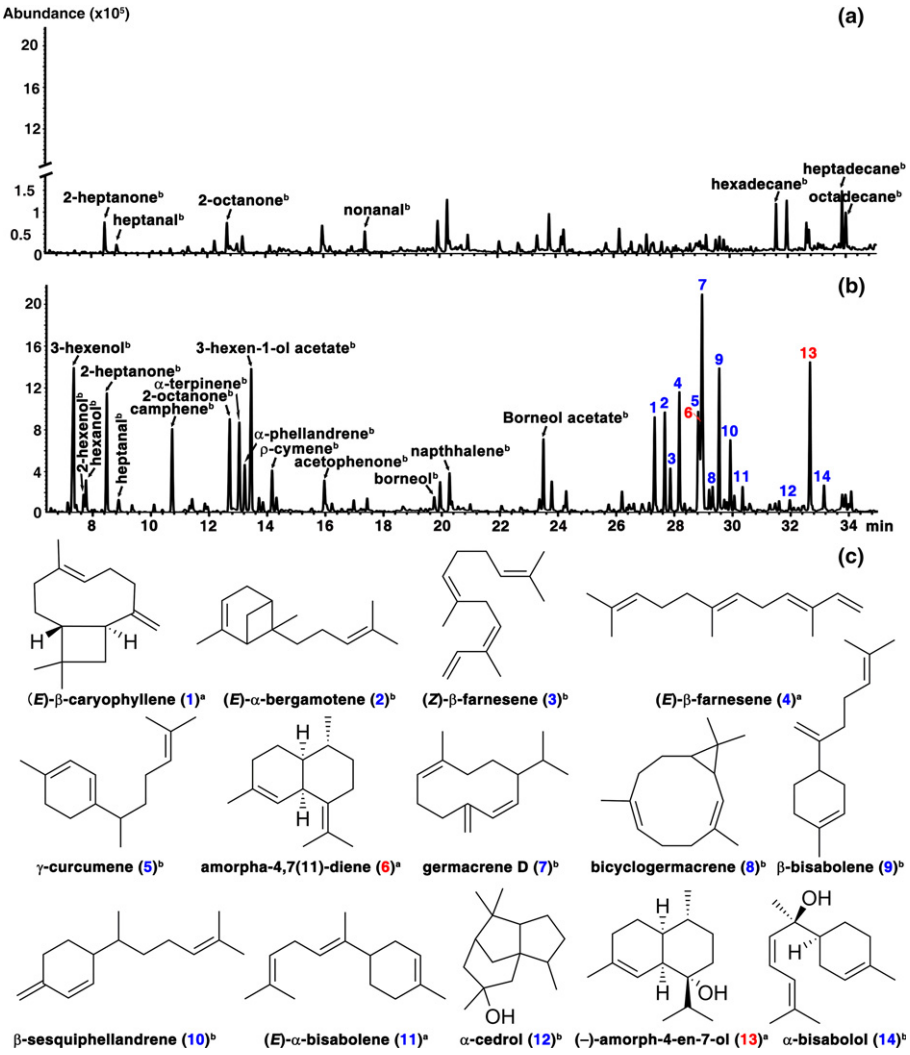

Fig. 1 Analysis of volatile organic compounds (VOCs) released by *Eupatorium adenophorum* using GC-MS. (a, b) Total ion chromatogram (TIC) of VOCs from control plants (a) and treated plants at 6 h after mechanical wounding (b). (c) Chemical structures of volatile sesquiterpenes 1–14 from the treated *E. adenophorum*. [a]Compound was identified through comparison of its retention time and mass spectrum with those of an authentic standard; [b]compound was tentatively identified through comparison of its mass spectrum and retention index with the published data.

effectively serve as defensive compounds against generalist insects in *E. adenophorum*.

Cloning and characterization of candidate sesquiterpene synthases for cadinene biosynthesis

To help understand the defensive mechanism of cadinene sesquiterpenes in *E. adenophorum*, three putative sesquiterpene synthase genes were cloned through homologous cloning and 3′- and 5′-RACE experiments. The full-length cDNA of three terpene synthase genes (*EaTPSs1–3*) was obtained from *E. adenophorum*, and their nucleotide sequences were diverse, with identity ranging from 27% to 46% (Fig. S29, Table S3). Phylogenetic analysis revealed that EaTPS1 and EaTPS2 belonged to the TPS-a clade, which was dominated by angiosperm sesquiterpene synthases (Bohlmann *et al.*, 1998). Meanwhile, EaTPS3 clustered in the TPS-g clade, which consisted of angiosperm sesquiterpene synthases and monoterpene synthases (Dudareva *et al.*, 2003) (Fig. S14; Table S4). Moreover, EaTPSs1–3 were predicted, by TargetP software (http://www.cbs.dtu.dk/services/TargetP/), to be localized in the cytosol, suggesting that they were likely sesquiterpene synthases.

For functional characterization of EaTPSs, *in vitro* enzymatic assays were carried out by incubating the substrate with purified recombinant proteins (Fig. S15). Extracts prepared from *E. coli* (same strain) with pCold TF lacking a cDNA insert served as a control, for which no terpene product was detected. The recombinant EaTPS1 protein was capable of converting (*E*,*E*)-FDP into a major product whose mass spectrum showed 94% similarity with that of γ-cadinene, along with a minor product whose mass spectrum was 87% similar to δ-cadinol (Fig. 3). The recombinant EaTPS2 protein produced at least four sesquiterpenes, which were tentatively identified as (*E*)-α-bergamotene (compound 2), (*Z*)-β-farnesene (compound 3), γ-curcumene (compound 5) and β-sesquiphellandrene (compound 10), based on the similarity between their mass spectra and those in the NIST 08 library, and between their retention indices and previously published data (Adams, 2007) (Fig. 3; Table S5). The recombinant EaTPS3 protein was able to convert FDP to (*E*)-nerolidol, which was confirmed by comparison of its retention time and mass spectrum with those of a purchased authentic nerolidol standard (mixture of isomers, Tokyo Chemical Industry, Japan) as well as comparison of its retention index with the

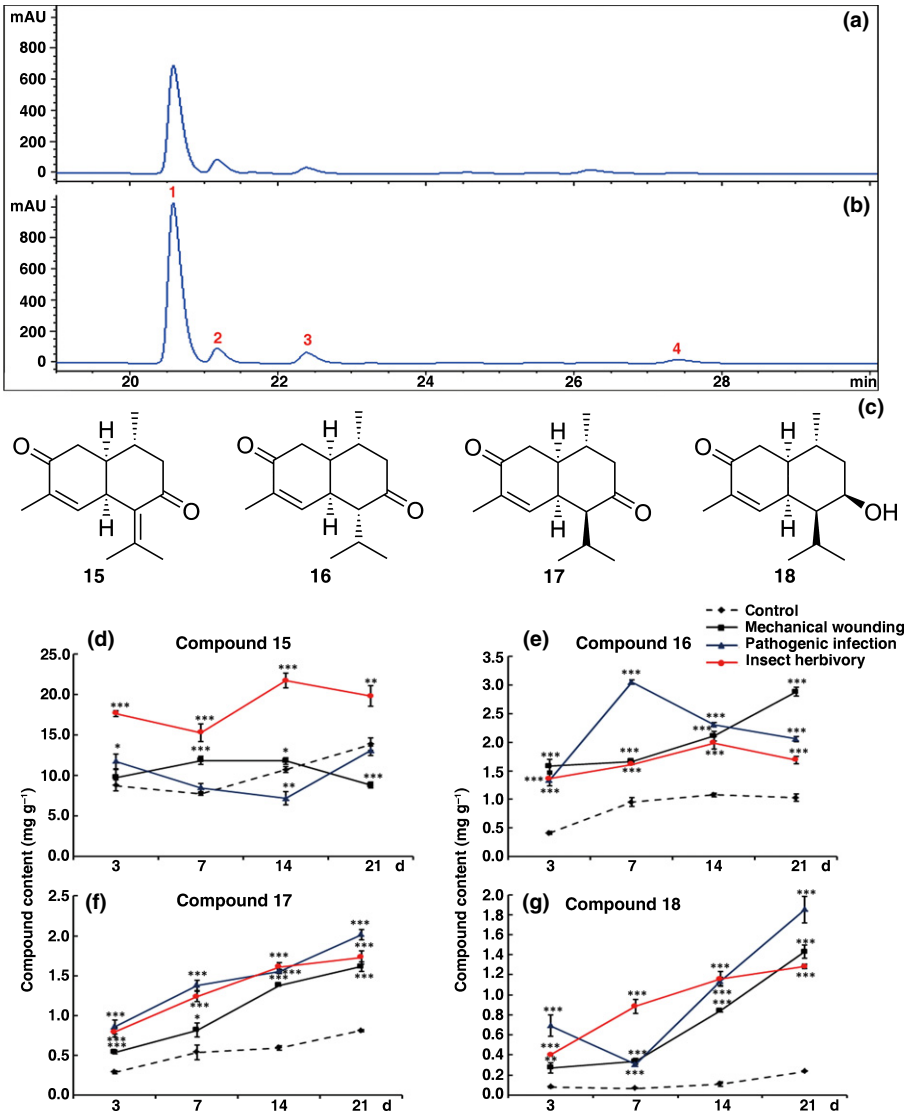

Fig. 2 Nonvolatile cadinene sesquiterpenes and their contents in *Eupatorium adenophorum*. (a, b) High performance liquid chromatography (HPLC) analysis of nonvolatile compounds in control plants (a) and the treated plants at 7 d after feeding by *Helicoverpa armigera* (b). (c) Chemical structures of compounds 15–18. (d–g) Quantitative analysis of compounds 15–18 in *E. adenophorum* after mechanical wounding, insect herbivory and pathogenic infection treatments (d, compound 15; e, compound 16; f, compound 17; g, compound 18). Data represent the SD of three independent biological replicates. Asterisks indicate significant differences from control (significant one-way ANOVA tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

published data (Adams, 2007) (Fig. 3; Table S5). None of EaTPSs1–3 could accept GDP or GGDP as a substrate, further confirming that they were sesquiterpene synthases.

Structural elucidation of EaTPS1 products using engineered *E. coli*

To elucidate the chemical structures of EaTPS1 products, the *EaTPS1* gene was introduced into *E. coli* harboring plasmids pBbA5c-MevT and pBBR-FDP in which seven genes of the MVA pathway and an FDP synthase gene were included. It was found, through comparisons of their retention times and mass spectra, that the engineered *E. coli* yielded the same products as the *in vitro* enzyme assay. Intriguingly, the product ratio of compound 6 : compound 13 was 10.29 ± 0.13 and 2.41 ± 0.75 in the *in vitro* and *in vivo* assay systems, respectively, probably due to the differences in enzyme configuration, catalytic efficiency and reaction conditions, including pH value, temperature and concentrations of substrate and cations in different systems.

After collection of 10 l of bacterial cultures, EaTPS1 products were isolated and purified by silica column chromatography to obtain compound 6 (550 mg) and compound 13 (35 mg) (Fig. 4). Their structures were identified as amorpha-4,7(11)-diene (compound 6) and (–)-amorph-4-en-7-ol (compound 13), based on their 1D (^{1}H, ^{13}C and DEPT) and 2D NMR (including ^{1}H-^{1}H correlation spectroscopy (COSY), Heteronuclear single quantum coherence (HSQC), Heteronuclear multiple-bond correlation (HMBC) and Rotating frame Overhauser effect spectroscopy (ROESY)) spectral data (Figs S17–S30) and comparison with those reported in the literature (Sanz *et al.*, 1991; Weyerstahl *et al.*, 1997).

Analysis of *EaTPS1* transcripts and cadinene contents in *E. adenophorum*

The expression patterns of *EaTPS1* in plants of different development stages and different organs were analyzed using qRT-PCR with the *ubiquitin* gene as an internal standard. Meanwhile, the

Table 1 Cadinenes and other sesquiterpenes in *Eupatorium adenophorum* and their antifeedant activity.

Compound		Antifeedant activity (EC_{50}) ($\mu g\ cm^{-2}$)	Compound content [c] ($\mu g\ cm^{-2}$)	Compound content [c] ($\mu g\ g^{-1}$ FW)
Cadinenes[a]	6	4.86 ± 0.15	0.0037 ± 0.0009	0.19 ± 0.04
	13	5.73 ± 0.61	0.0067 ± 0.0016	0.34 ± 0.08
	15	2.71 ± 0.20	186.21 ± 15.21	9407.05 ± 768.45
	16	3.58 ± 0.42	8.22 ± 1.35	415.42 ± 68.23
	17	5.13 ± 0.53	5.67 ± 0.32	286.64 ± 16.15
	18	3.18 ± 0.27	1.62 ± 0.14	81.9 ± 6.93
Other sesquiterpenes[b]	1	8.42 ± 0.22	–	–
	4	10.35 ± 0.60	–	–
	9	7.68 ± 0.78	–	–
Neem oil		2.59 ± 0.16	–	–

[a]Purified compounds (all > 95% purity in gas chromatography–mass spectrometry (GC-MS) or high performance liquid chromatography (HPLC)) and [b]commercial compounds (all > 90% in GC) were used. [c]Compound contents in the leaves of 6-wk-old plants. Standard deviation of EC_{50} values was calculated based on five biological replicates, and standard deviation of compound content was calculated based on three biological replicates.

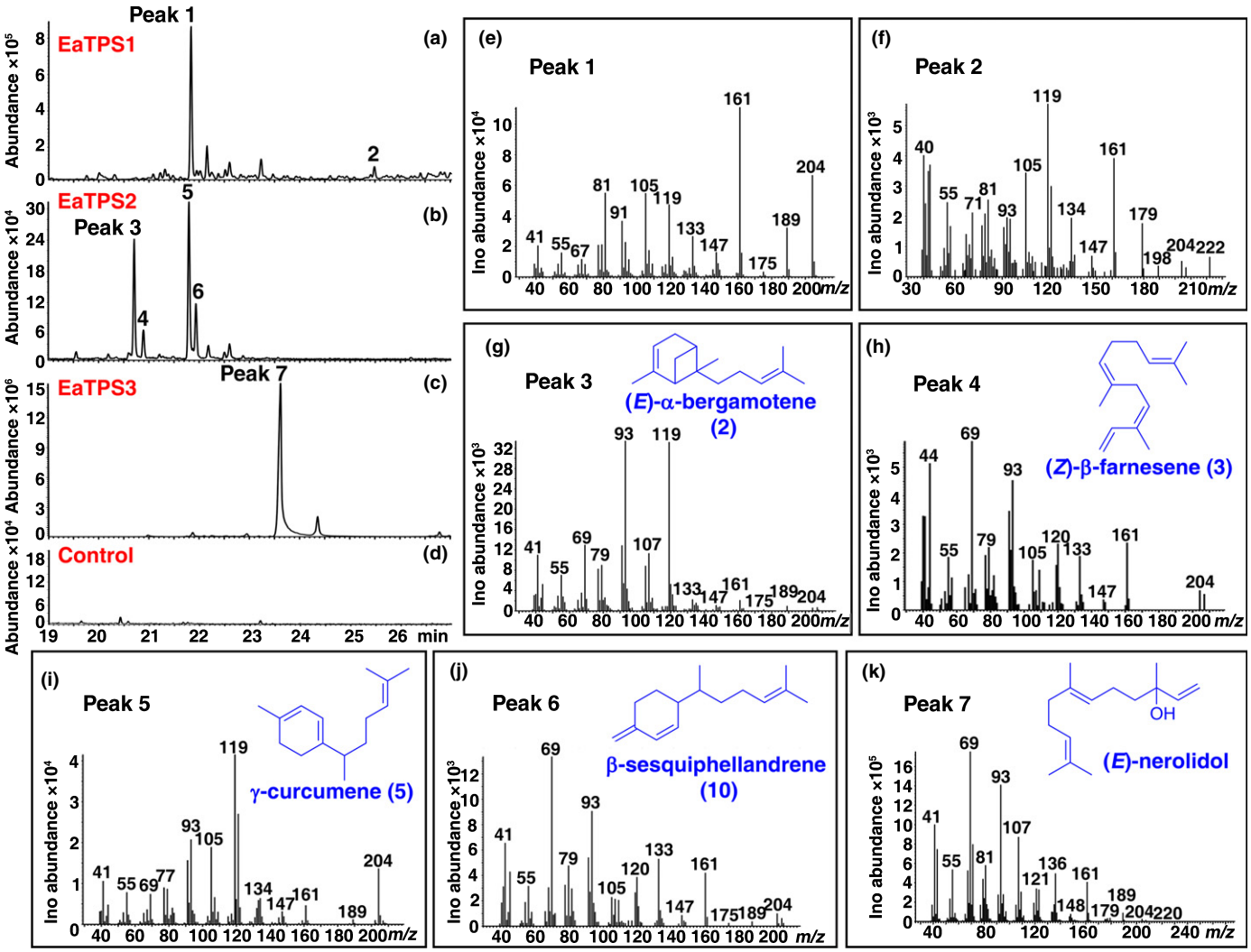

Fig. 3 Functional analysis of recombinant EaTPSs1–3 by *in vitro* enzyme assays using gas chromatography–mass spectrometry (GC-MS). (a–d) Total ion chromatogram (TIC) of the products formed by EaTPSs1–3 and an empty vector control (a, EaTPS1; b, EaTPS2; c, EaTPS3; d, control). (e–k) Mass spectra and chemical structures of the peaks presented in TIC of EaTPSs1–3 products (e, peak 1; f, peak 2; g, peak 3; h, peak 4; i, peak 5; j, peak 6; k, peak 7).

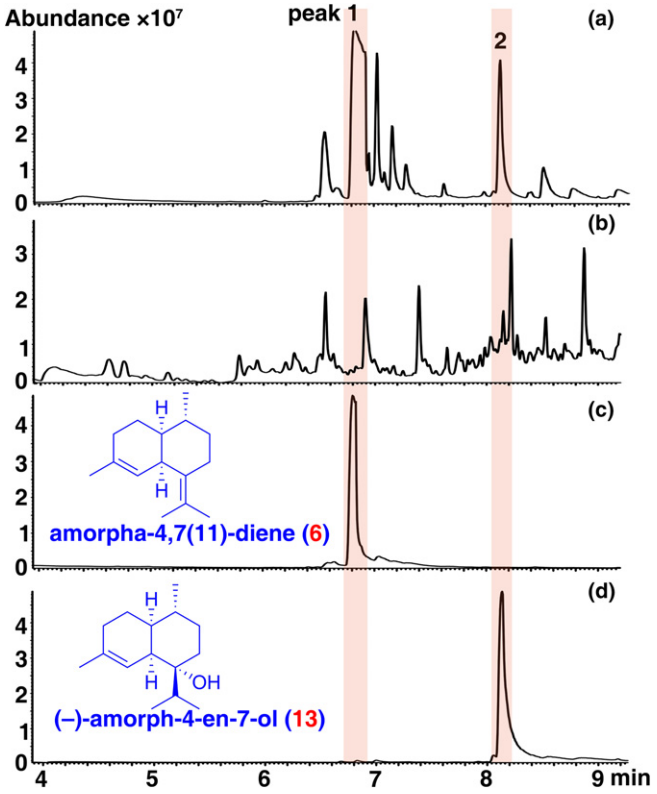

Fig. 4 Characterization of EaTPS1 products in engineered *Escherichia coli* using gas chromatography-mass spectrometry (GC-MS). (a, b) Total ion chromatogram (TIC) of the products formed by engineered *E. coli* harboring EaTPS1 (a) and control (b). (c, d) TIC and chemical structures of purified amorpha-4,7(11)-diene (compound 6) and (–)-amorph-4-en-7-ol (compound 13).

contents of compounds 6 and 13 were quantified using GC-MS with the purified compounds as external standards. It was found that *EaTPS*1 was highly expressed in the whole plants of 3-wk-old and 4-wk-old *E. adenophorum*, and in the leaves and young stems from 6-wk-old plants growing in the glasshouse, as well as the leaves, flowers and flower buds of mature plants from a natural habitat. Compound 6 (0.18–0.66 μg g^{-1} FW) and compound 13 (0.05–0.56 μg g^{-1} FW) were clearly and consistently detected in these samples, further indicating that compounds 6 and 13 were constitutively stored in the analyzed tissues of *E. adenophorum*. The *EaTPS*1 transcript level in 2-wk-old plants was found to be less abundant, and compounds 6 and 13 were both detectable only in unquantifiable trace amounts, possibly due to the latency between gene expression and compound biosynthesis. Intriguingly, *EaTPS*1 transcripts showed the lowest level in 1-wk-old plants and roots from 6-wk-old plants, which was consistent with compounds 6 and 13 being undetectable in these samples (Fig. 5). The diagnostic correlations between transcript levels and product accumulations suggested that *EaTPS1* should be responsible for the biosynthesis of compounds 6 and 13 in *E. adenophorum*.

EaTPS1 transcript levels in flowers and flower buds were lower than those in leaves and young stems (Fig. 5a). However, the contents of compounds 6 and 13 in flowers and flower buds were comparable to or even higher than those in leaves and young stems (Fig. 5b), which suggested that compounds 6 and 13 in leaves and young stems might have been partially transformed into modified downstream cadinene sesquiterpenes. In addition, we found that the transcript levels of *EaTPS1* were much higher than those of *EaTPS2* and *EaTPS3* in the analyzed samples (Fig. 5a, S31). The high transcript abundance implied that *EaTPS1* makes a major contribution to sesquiterpene biosynthesis in *E. adenophorum*, which is consistent with the high amounts of cadinene sesquiterpenes found in aerial parts of the plant.

Mechanical wounding and MJ treatments negatively regulated *EaTPS1* expression but caused the release of its products in *E. adenophorum*

Since the findings presented here have indicated that cadinenes are involved in plant defense, it was interesting to test whether *EaTPS1* expression and the release of its products could be induced by mechanical wounding and MJ treatment. The relative expression level of *EaTPS1* peaked in the control but decreased unexpectedly at 1–24 h after mechanical wounding (Fig. 6a). Mechanical wounding also resulted in the downregulation of *EaTPS2* and *EaTPS3* (Fig. S31). Nevertheless, compounds 6 and 13 were undetectable in VOCs released by the control plants, but detected in high amounts (relative to nonane) in VOCs released by the treated plants, which were maintained during the 1–24-h period after mechanical wounding (Fig. 6c), indicating that compounds 6 and 13 were constitutively stored in *E. adenophorum* and that mechanical wounding could help their release from plants. Therefore, the release and biosynthesis of these metabolites may have separate paths, even though these two cadinenes are direct products of EaTPS1.

The defense hormone MJ was then used to treat *E. adenophorum*, and it was found that the *EaTPS1* mRNA level was comparable to that of the control at 1–3 h after MJ treatment (one-way ANOVA, not significant), but declined during the 6–24-h period after MJ treatment (Fig. 6b). Compounds 6 and 13 were detected in high quantities (relative to nonane) in the VOCs released by MJ treated plants (Fig. 6d). By contrast, the transcript abundances of *EaTPS2* and *EaTPS3* were found to increase significantly after MJ treatment (Fig. S31), indicating that the regulation mechanism of *EaTPS1* was distinct from that of *EaTPS2* and *EaTPS3*.

Transient expression of *EaTPS1* in *N. benthamiana* led to the production of cadinenes and enhanced defense function

To further determine the significance of *EaTPS1* in defensive cadinene biosynthesis, transgenic *N. benthamiana* transiently expressing *EaTPS1* was constructed. Gas chromatography–mass spectrometry (GC-MS) analysis indicated two specific peaks in the extract of transgenic *N. benthamiana* leaves harvested at 5 d after infiltration with *A. tumefaciens* harboring *EaTPS1* (Fig. 7). Subsequently, the two peaks were confirmed to correspond to cadinenes 6 and 13 through comparison of their mass spectra

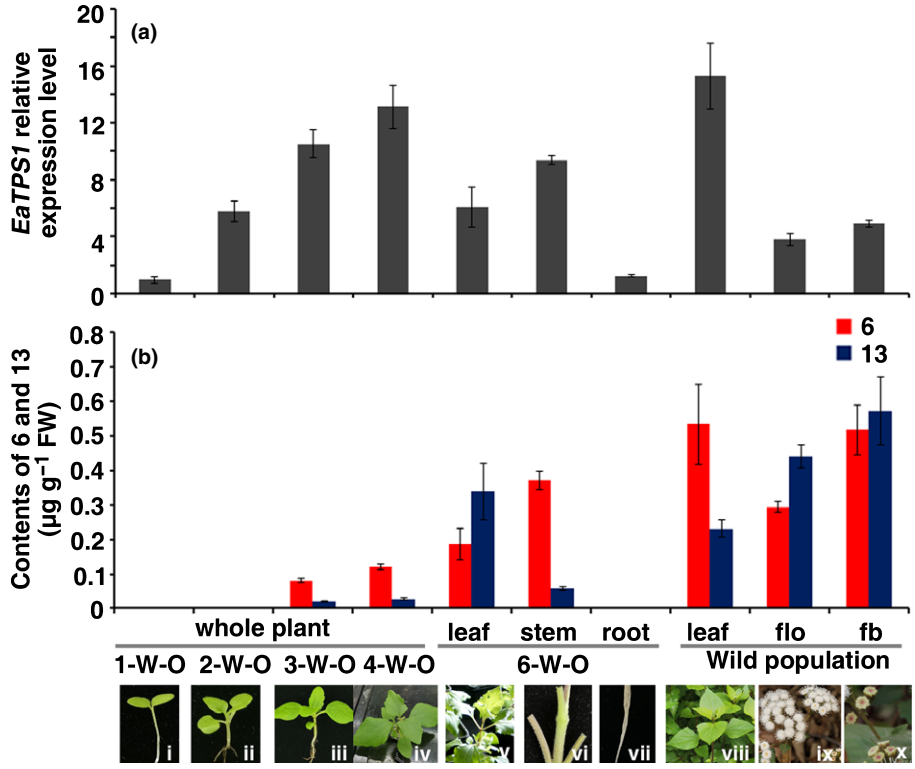

Fig. 5 Relative transcript levels of *EaTPS1* and the quantities of amorpha-4,7(11)-diene (compound 6) and (−)-amorph-4-en-7-ol (compound 13) in *Eupatorium adenophorum* at different development stages and in different organs. (a) *EaTPS1* relative transcript levels. Error bars indicate ± SD based on three independent biological replicates and three technical replicates. (b) The quantities of compounds 6 and 13. fb, flower buds; flo, flower; FW, fresh weight. i-x indicate 1-wk-old plant (1-W-O), 2-wk-old plant (2-W-O), 3-wk-old plant (3-W-O), 4-wk-old plant (4-W-O), and the leaves, young stems and roots from 6-wk-old plant (6-W-O) growing in a glasshouse, as well as the leaves, flowers and flower buds of mature plants from a natural habitat. Error bars of compound quantities represent ± SD of three independent biological replicates.

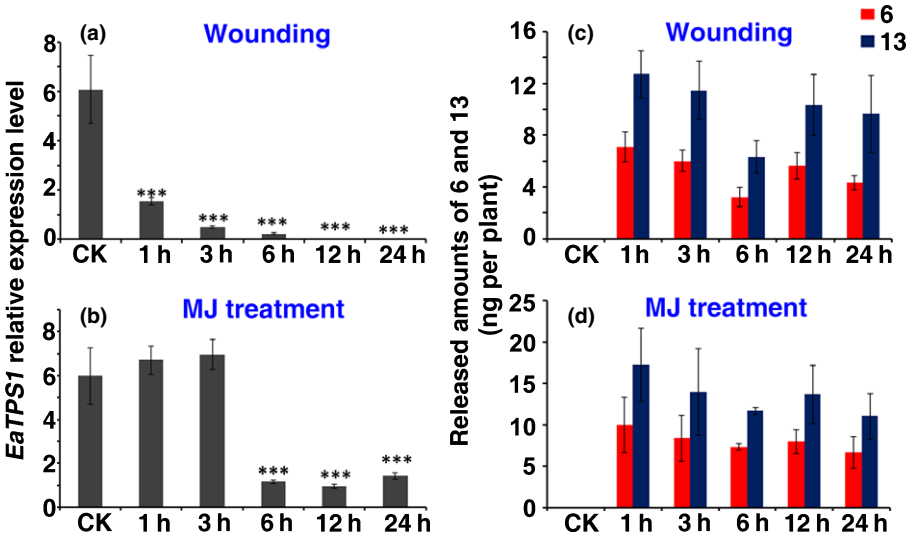

Fig. 6 Analysis of *EaTPS1* transcript levels and the quantities of its products released by *Eupatorium adenophorum* after mechanical wounding and methyl jasmonate treatment. (a, b) The relative transcript levels of *EaTPS1* in *E. adenophorum* after mechanical wounding (a) and methyl jasmonate treatment (b). (c, d) The amounts of compounds 6 and 13 released by *E. adenophorum* after mechanical wounding (c) and methyl jasmonate treatment (d). Error bars of relative transcript levels indicate ± SD based on three independent biological replicates and three technical replicates, while error bars of compound amounts represent ± SD of three independent biological replicates. Asterisks indicate significant differences according to one-way ANOVA tests (***, $P < 0.001$).

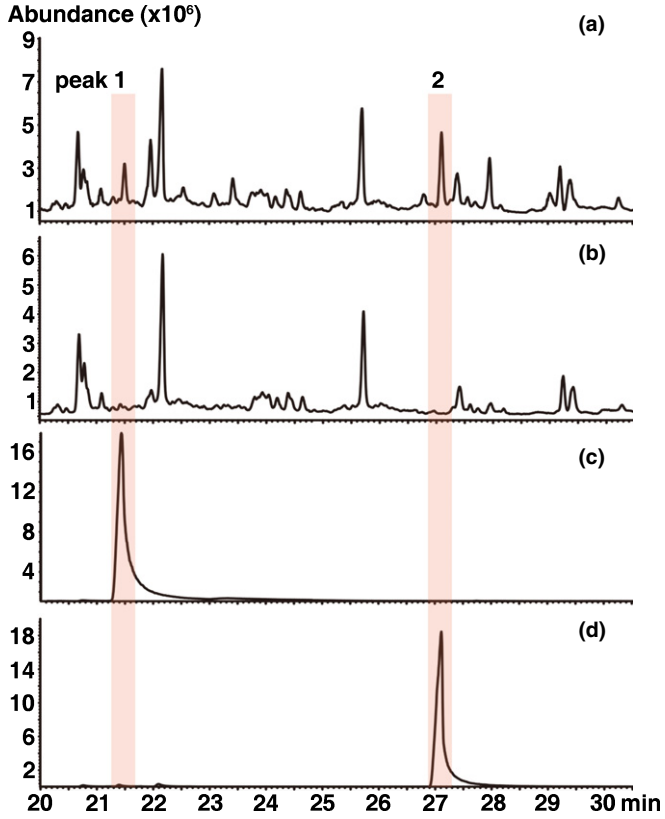

Fig. 7 Gas chromatography-mass spectrometry (GC-MS) analysis of the extracts of transgenic *Nicotiana benthamiana* transiently expressing *EaTPS1*. (a, b) TIC of the extracts of *N. benthamiana* expressing *EaTPS1* (a) and control (b). (c, d) TIC of purified amorpha-4,7(11)-diene (c) and (–)-amorph-4-en-7-ol (d).

and retention times with those of purified compounds described in the 'Structural elucidation of EaTPS1 products using engineered *E. coli*' section. It was observed that cotton bollworm tended to feed on *N. benthamiana* leaves harboring an empty vector (control) rather than those expressing *EaTPS1*, and *N. benthamiana* leaves expressing *EaTPS1* possessed a feeding index of $0.19 \pm 0.05\%$, which was less than that of the control ($0.57 \pm 0.08\%$) (Fig. S32). Consequently, transient expression of *EaTPS1* in *N. benthamiana* confirmed both the enzymatic activity and the defensive function of EaTPS1 *in planta*.

Discussion

Eupatorium adenophorum is a notoriously invasive weed that produces a large number of specialized metabolites (Feng *et al.*, 2009; Inderjit *et al.*, 2011). Here, volatile sesquiterpenes, including two cadinenes, amorpha-4,7(11)-diene (compound 6) and (–)-amorph-4-en-7-ol (compound 13), were determined to be the major VOCs stored in the aboveground organs of *E. adenophorum*, which can be released immediately after mechanical wounding, insect feeding or pathogen infection treatments. Four nonvolatile cadinenes, compounds 15–18, were determined to be the major constitutive and inducible chemicals in the aboveground organs of *E. adenophorum*. The constituent detected in the largest quantity (compound 15) was identified as a herbivore-inducible chemical, while compounds 16–18 could be induced by mechanical wounding, insect feeding or pathogen infection. Both volatile and nonvolatile cadinenes showed potent antifeedant activity against a generalist cotton bollworm, and the contents of nonvolatile cadinenes in *E. adenophorum* are sufficiently high to deter insect feeding, suggesting that these cadinenes likely serve as important defensive chemicals.

The cadinene sesquiterpenes are a subgroup of natural products whose biological importance can scarcely be overemphasized, as exemplified by artemisinin (an antimalarial drug) and gossypol (a cotton phytoalexin). In this study, we functionally characterized a sesquiterpene synthase (EaTPS1) which catalyzes FDP to form compounds 6 and 13 in *E. adenophorum*. Its activity was first determined by *in vitro* enzyme assays of the recombinant protein expressed in *E. coli*. Co-expression of *EaTPS1*, the entire MVA pathway and FDP synthase genes in *E. coli* led to significant production of compounds 6 and 13, which were isolated from engineered *E. coli* and structurally elucidated using extensive NMR spectroscopic analysis. The correlation of gene transcript levels and compound accumulations suggested that *EaTPS1* should be responsible for the biosynthesis of compounds 6 and 13 in *E. adenophorum*. Transient expression of *EaTPS1* in *N. benthamiana* also produced compounds 6 and 13, further confirming the enzymatic activity *in planta*. Moreover, *N. benthamiana* leaves transiently expressing *EaTPS1* exhibited a potent antifeedant effect against cotton bollworm, which further confirmed the defensive function of *EaTPS1* and its products.

A plausible biosynthetic pathway for cadinene biosynthesis in *E. adenophorum* was proposed (Fig. 8). From a structural point of view, amorpha-4,7(11)-diene (compound 6) shows high similarity to amorpha-4,11-diene, a critical precursor of the antimalarial drug artemisinin, which is catalyzed by an amorpha-4,11-diene synthase through a bisabolyl intermediate followed by one 1,3-hydride shift (Kim *et al.*, 2006). According to that mechanism, a common bisabolyl carbocation II arising from 1,6-closure of carbocation I would undergo a direct suprafacial 1,3-hydride shift of axial H_a-1 to C-7, resulting in the formation of carbocation III. Subsequently, the 1,10-closure leads to the production of carbocation IV with (*Z*)-decalin configuration at C-1 and C-6, which is terminated by proton elimination to generate compound 6 at C-13 or amorpha-4,11-diene at C-10 (Fig. 8). Alternatively, carbocation IV undergoes a 1,2-hydride shift and is then quenched by the addition of a water molecule to yield compound 13. Furthermore, compound 6 would undergo a series of oxygenations at C-4 and C-9 to produce compound 15, which are likely catalyzed by cytochrome P450 enzymes. Besides, the formation of compounds 16–18 should involve a reduction of the $\Delta^{7,11}$ double bond. In fact, a $\Delta^{11,13}$ double-bond reductase for artemisinin biosynthesis has been identified in *Artemisia annua* (Zhang *et al.*, 2008). Although the sequence of oxygenation and reduction still remains to be investigated, the scaffolds of compounds 15–18 are likely biosynthesized by EaTPS1 via compound 6 (Fig. 8). The identification of *EaTPS1* and construction of engineered *E. coli* and *N. benthamiana* systems in this study pave the way for an alternative method of production of biologically active cadinene-

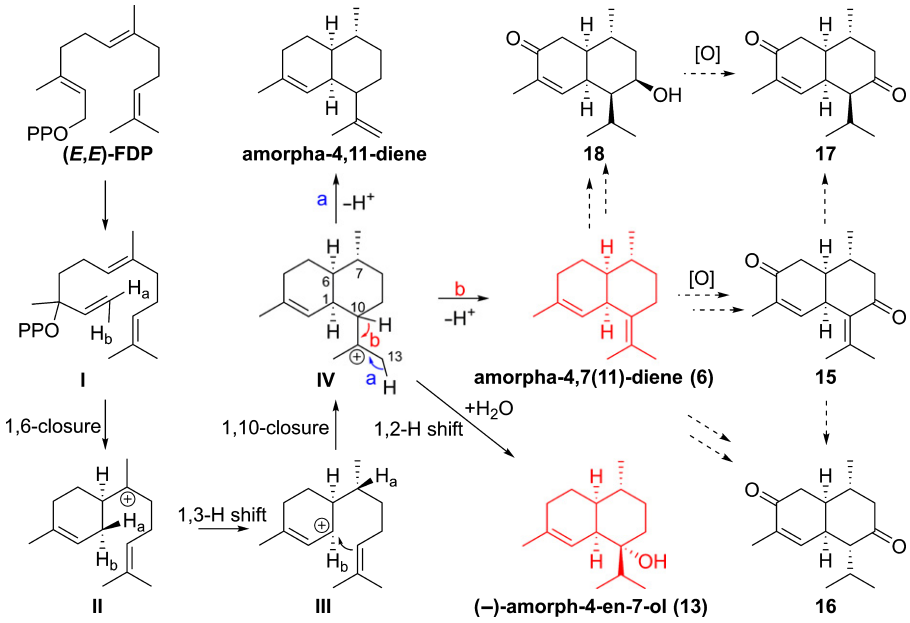

Fig. 8 Proposed formation mechanisms of cadinene sesquiterpene in *Eupatorium adenophorum* via EaTPS1.

type natural products using synthetic biology. In addition, the question of whether the products of EaTPS1 could be accepted by the hydroxylase, reductase and aldehyde dehydrogenase in the biosynthetic pathway of artemisinin to produce artemisinin derivatives is extremely interesting and worthy of further investigation.

The abundant expression of *EaTPS1* in the aboveground organs of *E. adenophorum* suggested that EaTPS1 functioned as a major sesquiterpene synthase, which was congruent with the dominant distribution of cadinenes there. *EaTPS1* expressed dominantly in leaves and young stems rather than flower buds and flowers where compounds 6 and 13 were mainly detected, which suggested that EaTPS1 products might partially undergo further modification to yield compounds 15–18 in leaves and young stems.

Intriguingly, mechanical wounding led to downregulation of the transcription of *EaTPS1* along with *EaTPS2* and *EaTPS3*, which is opposed to the general pattern, whereby mechanical wounding upregulates the expression of terpene synthases in conifers and many higher plants, including cotton and *Lotus japonicus* (Arimura *et al.*, 2004; Yang *et al.*, 2013; Celedon & Bohlmann, 2019). The defense hormone MJ also negatively regulated *EaTPS1* transcription but positively regulated the expression of *EaTPS2* and *EaTPS3*, as well as terpene synthase genes in many plants, including conifers, rice and maize (Ren *et al.*, 2016; Chen *et al.*, 2018; Celedon & Bohlmann, 2019). The regulation mechanism of *EaTPS1* is therefore distinct from *EaTPS2* and *EaTPS3* in *E. adenophorum* and terpene synthase genes in many other plants. Nevertheless, the transcription of the β-pinene synthase gene has been shown to be suppressed by mechanical wounding in *A. annua* (Lu *et al.*, 2002). In addition, abiotic stresses, including heat, drought and ozone have been reported to downregulate the expression of terpene synthase genes in tomato (*Solanum lycopersicum*), Aleppo pine (*Pinus halepensis*) and *Eucalyptus globulus* (Pazouki *et al.*, 2016; Fox *et al.*, 2018; Kanagendran *et al.*, 2018). Suppression of the transcription of *EaTPS1* and other terpene synthase genes via mechanical wounding, MJ application and abiotic stresses suggests that the plants may limit the energy and carbon flux into the biosynthesis of specialized metabolites when they confront adverse conditions.

Previous studies have demonstrated that volatile terpenes could be released from preformed storage pools or synthesized *de novo* and then emitted, and that both processes may even occur in some cases (Martin *et al.*, 2003). In the present study, the suppression of *EaTPS1* expression by mechanical wounding and MJ spraying in combination with the abundant accumulation of cadinenes in *E. adenophorum* suggested that volatile cadinenes 6 and 13 were released from stored pools. Wounding and MJ treatments could affect the structures of storage sites for compounds 6 and 13, perhaps by physical damage and changes in osmotic pressure, resulting in their release. The rapid emission of compounds 6 and 13 from storage pools after mechanical wounding and MJ treatment is a defense mechanism unique to *E. adenophorum* which probably contributes to the invasive success of this plant. Emission is a much more rapid defense response than that occurring from activation of *de novo* biosynthesis of defensive terpenes by induced terpene synthase gene expression. The response of *EaTPS1* and its products upon other elicitors and their regulation mechanism are interesting research topics to explore in the future. This work provides a model for investigating similar defensive compounds in other species and a tool for manipulating the biosynthesis of biologically active cadinenes in plants and other organisms.

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Author contributions

YLiu, S-HLi and S-HLuo planned and designed the research. YLiu, S-HLuo, JH, D-SL, YLing and QL carried out the experimental works and data analysis. YLiu, S-HLi and S-HLuo wrote the manuscript. YLiu and S-HLuo contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 *Eupatorium adenophorum* with different treatments and the collection of volatile organic compounds (VOCs) released by the plants.

Fig. S2 The mycelia and conidia of *Alternaria alternata* isolated from the diseased leaves of *Eupatorium adenophorum*.

Fig. S3 The representative gas chromatography-mass spectrometry (GC-MS) chromatograms of VOCs released by *Eupatorium adenophorum* upon different treatments.

Fig. S4 The amounts of volatile sesquiterpenes released by *Eupatorium adenophorum*.

Fig. S5 GC-MS analysis of VOCs in different organs of *Eupatorium adenophorum*.

Fig. S6 ^{1}H nuclear magnetic resonance (NMR) spectrum of 9-*oxo*-10,11-dehydroageraphorone (compound 15) recorded at 500 MHz in acetone-d_6.

Fig. S7 ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra of 9-*oxo*-10,11-dehydroageraphorone (compound 15) recorded at 125 MHz in acetone-d_6.

Fig. S8 ^{1}H NMR spectrum of muurol-4-en-3,8-dione (compound 16) recorded at 500 MHz in acetone-d_6.

Fig. S9 ^{13}C NMR and DEPT spectra of muurol-4-en-3,8-dione (compound 16) recorded at 125 MHz in acetone-d_6.

Fig. S10 ^{1}H NMR spectrum of 9-*oxo*-ageraphorone (compound 17) recorded at 400 MHz in $CDCl_3$.

Fig. S11 ^{13}C NMR and DEPT spectra of 9-*oxo*-ageraphorone (compound 17) recorded at 100 MHz in $CDCl_3$.

Fig. S12 ^{1}H NMR spectrum of 9β-hydroxy-ageraphorone (compound 18) recorded at 400 MHz in $CDCl_3$.

Fig. S13 ^{13}C NMR and DEPT spectra of 9β-hydroxy-ageraphorone (compound 18) recorded at 100 MHz in $CDCl_3$.

Fig. S14 Alignment of deduced amino acid sequences and phylogenetic analysis of EaTPSs1–3.

Fig. S15 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of recombinant EaTPSs1–3 proteins expressed in *Escherichia coli*.

Fig. S16 ^{1}H NMR spectrum of amorpha-4,7(11)-diene (compound 6) recorded at 800 MHz in acetone-d_6.

Fig. S17 ^{13}C NMR and DEPT spectra of amorpha-4,7(11)-diene (compound 6) recorded at 200 MHz in acetone-d_6.

Fig. S18 ^{1}H-^{1}H correlation spectroscopy (COSY) spectrum of amorpha-4,7(11)-diene (compound 6) recorded at 800 MHz in acetone-d_6.

Fig. S19 Heteronuclear single-quantum coherence (HSQC) spectrum of amorpha-4,7(11)-diene (compound 6) recorded at 800 MHz in acetone-d_6.

Fig. S20 Heteronuclear multiple-bond correlation (HMBC) spectrum of amorpha-4,7(11)-diene (compound 6) recorded at 800 MHz in acetone-d_6.

Fig. S21 Rotating frame Overhauser effect spectroscopy (ROESY) spectrum of amorpha-4,7(11)-diene (compound 6) recorded at 800 MHz in acetone-d_6.

Fig. S22 ^{1}H NMR spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in $CDCl_3$.

Fig. S23 ^{1}H NMR spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in acetone-d_6.

Fig. S24 ^{13}C NMR and DEPT spectra of (–)-amorph-4-en-7-ol (compound 13) recorded at 100 MHz in $CDCl_3$.

Fig. S25 ^{13}C NMR spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 100 MHz in acetone-d_6.

Fig. S26 ^{1}H-^{1}H COSY spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in $CDCl_3$.

Fig. S27 HSQC spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in $CDCl_3$.

Fig. S28 HMBC spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in $CDCl_3$.

Fig. S29 ROESY spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in $CDCl_3$.

Fig. S30 ROESY spectrum of (–)-amorph-4-en-7-ol (compound 13) recorded at 400 MHz in acetone-d_6.

Fig. S31 The relative transcript levels of *EaTPS2* and *EaTPS3* in *Eupatorium adenophorum*.

Fig. S32 The representative images for the antifeedant assay of transgenic *Nicotiana benthamiana* transiently expressing *EaTPS1* and control.

Methods S1 Conditions for GC-MS analysis.

Methods S2 Conditions for high performance liquid chromatography (HPLC) analysis.

Methods S3 Isolation of compounds 15–18.

Methods S4 ^{1}H and ^{13}C NMR data for compounds 6, 13 and 15–18.

Methods S5 Antifeedant assay of the purified compounds.

Table S1 The oligonucleotide primers used in this study.

Table S2 Relative amounts of VOCs released by *Eupatorium adenophorum* at 24 h after different treatments.

Table S3 Bioinformatic analysis of candidate sesquiterpene synthases from *Eupatorium adenophorum*.

Table S4 GenBank accession numbers of protein sequences used for phylogenetic analysis.

Table S5 The retention indices of EaTPS2 and EaTPS3 products.

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