Research article

Transcriptome-guided identification and functional characterization of key terpene synthases involved in constitutive and methyl jasmonate-inducible volatile terpene formation in *Eremochloa ophiuroides* (Munro) Hack

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ABSTRACT

Centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.) is a warm-season turfgrass, widely planted in residential lawns and recreational fields. Here, we uncovered three major terpenes released from the shoots of *Eo*: (*E*)-β-ocimene (6%), α-muurolene (87.8%), and eremophilene (6.2%). Methyl jasmonate (MeJA) treatment increased the emission of monoterpenes, including (*E*)- and (*Z*)-β-ocimene, limonene, and myrcene, as well as sesquiterpene blends of (*E*)-caryophyllene, α-copaene, (+)-cyclosativene, and α-farnesene. RNA sequencing analysis predicted 14 putative *Eo* terpene synthase (*EoTPS*) genes, and two full-length *EoTPS* were successfully amplified: *Eo7816* (1722 bp) and *Eo6039* (1701 bp). Phylogenetic analysis revealed that *Eo7816* and *Eo6039* belonged to the clades of TPS-b and TPS-a, respectively. The *Arabidopsis* transgenic plants overexpressing *Eo7816* exclusively released (*E*)-β-ocimene (96%) with (*Z*)-β-ocimene and myrcene. In contrast, *Eo6039*-overexpressing *Arabidopsis* plants emitted significant amounts of α-muurolene (69.4%) and eremophilene (21.8%). Together, we demonstrated that the two TPSs play roles in producing major volatile terpenes in *Eo*.

1. Introduction

Eremochloa ophiuroides (*Eo*, centipedegrass) is a creeping perennial and a low-maintenance, general-purpose turf. This warm-season turfgrass is widely planted in the regions where soil fertility is poor and pH is low. It grows slowly but aggressively, forming a dense turf, and is, therefore, a favored species for residential lawns and recreational fields.

Most of *Eo* cultivars and accessions have phenotypically characteristics in tissue colors presenting red to purple colored in spikes and stolons during development (Li et al., 2018). Hence, flavonoid biosynthesis, particularly of anthocyanins, has been the focus of investigations involving the molecular regulations that determine the color changes seen in *Eo*. Flavonoid biosynthesis-related genes were recently predicted by comparative bioinformatic analyses on the tissue-specific transcriptomes of two *Eo* accessions, which represented green and red-purple colorations of the stolons and spikes, respectively. The

corresponding genes for the tissue-specific color changes were mainly chalcone synthase, dihydroflvonol-4-reductase, and flavanone-3-hydroxylase, and the expression of which were positively correlated to the accumulation of anthocyanins (Li et al., 2018).

Terpenoids are the largest class of natural products identified from plants and microbes, with known structures form more than 50,000 species (Conolly and Hill, 1991). Using isopentenyl diphosphate (IPP) as a building block, monoterpenes (C_{10}) and diterpenes (C_{20}) are biosynthesized in a plastidial 2-*C*-methyl-D-erythritol-4-phosphate pathway (MEP pathway) by mono- and diterpene synthases using geranyl pyrophosphate (GPP) and geranylgeranyl pyrophosphate (GGPP) as substrates, respectively. On the other hand, C_{15} sesquiterpenes are produced via the mevalonate pathway (MVA pathway) located in the cytosol by sesquiterpene synthases using farnesyl pyrophosphate (FPP) as a substrate (Lichtenthaler, 1999; Tholl and Lee, 2011). Terpenes are commonly released in response to abiotic or biotic stress as

phytoalexins or semiochemicals in tritrophic interactions (Abel et al., 2009; Arimura et al., 2008; Lee et al., 2015; Tholl et al., 2011). Similarly, jasmonic acid (JA) treatment induces the production and accumulation of volatile terpenes in plants (Martin et al., 2002; Rodriguez-Saona et al., 2001; Taniguchi et al., 2014). It has been known that most common problems in centipedegrass cultivation result from inadequate maintenance with excessive fertilization or irrigation. However, anthracnose disease, caused by fungi in the genus *Colletotrichum* species, has also been documented as a plant disease in centipedegrass (Crouch and Tomaso-Peterson, 2012; Fuke et al., 2006). Hence, given the physiological and ecological functions of volatile terpenes, it should be necessary to investigate the biosynthesis of volatile terpenes in *Eo*.

Here, we report the constitutive and methyl jasmonate (MeJA)-inducible volatile terpene compounds emitted from the aerial parts of *Eo*. Based on RNA sequencing analyses using control and MeJA-treated *Eo*, we found 14 transcripts of putative *TPS* genes with two full-length nucleotide fragments; the gene expression of which were induced by MeJA treatment. Ectopic overexpression of the full-length cDNAs in *Arabidopsis* plants revealed that they have catalytic activities to form multiple mono- and sesquiterpene volatiles, which constitute the major scents of the aerial tissues of *Eo*.

2. Materials and methods

2.1. Plant materials

Centipedegrass (*E. ophiuroides* [Munro] Hack.) seeds were obtained from Fukukaen Nursery (Blu co. Ltd., Nagoya, Japan) and grown in potting soil in plastic pots (depth: 15 cm, diameter: 30 cm) containing potting soil in a growth chamber for 3 months at 28 °C under a 14 h: 10 h (light: dark) photoperiod at a light intensity of 700–800 $\mu mol\,m^{-2}{\cdot}s^{-1}$. Wild-type *Arabidopsis thaliana* (Columbia-0) seedlings and transgenic plants were grown in an in-house growth room that was maintained at 22 °C, 60–70% relative humidity, and with a photoperiod of 16:8 h (light: dark) and a light intensity of 100–150 $\mu mol\,m^{-2}{\cdot}s^{-1}$. Seeds were surface-sterilized for 5 min with 70% (v/v) ethanol and for 10 min with 2% (v/v) sodium hypochlorite. The sterilized seeds were rinsed with sterile deionized water and germinated on 0.6% (w/v) phytoagar solid medium that was comprised of half-strength Murashige and Skoog (MS) medium with vitamins and 1.5% (w/v) sucrose.

2.2. Methyl jasmonate treatment, headspace volatile collection, and gas chromatography–mass spectrometry analysis

Methyl jasmonic acid (MeJA, 50 μM) and dimethylsulfoxide (DMSO 50 μM, control treatment) were foliar-sprayed on 3-month-old *E. ophiuroides* (*Eo*) plants. Ten leaves were then harvested 3 days after the treatments, and transferred into a 20 mL glass vial containing a cotton swab and a small amount of sterile distilled water. After 24 h, the headspace volatiles were collected for 60 min at 25 °C using a solid phase microextraction (SPME) fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (StableFlex 24Ga, Supelco, USA), followed by immediate injection in a GCMS-7890A/5975C system (Agilent, CA, USA). Terpene compounds were separated with a HP-5MS capillary column (0.25 mm i. d. × 30 m, 0.25 μm film thickness), with helium used as the carrier gas and maintained at a flow rate of 1 mL min^{-1}. The oven temperature was set to rise from 80 °C (3 min hold) to 150 °C at a rate of 5 °C·min^{-1} and with a thermal gradient of 10 °C·min^{-1} up to 250 °C, and then 20 °C·min^{-1} until 300 °C (3 min hold). The detailed parameters of the instrument were set according to Lee et al. (2015). The compounds were identified using Wiley 7th Edition and NIST 2008 Mass Spectral libraries, and the mass spectrometry program version 2.0 (https://chemdata.nist.gov/mass-spc/ms-search).

2.3. mRNA sequencing

Total RNA was extracted from two biological replicates of *Eo* leaves using the RNeasy Plant Mini Kit (Qiagen, Stanford, CA) according to the manufacturer's instructions. Total RNA integrity was checked using an Agilent 2100 BioAnalyzer (Agilent Technologies, CA, USA) with an RNA Integrity Number value. mRNA sequencing libraries were prepared according to the manufacturer's instructions (Illumina Truseq stranded mRNA library prep kit). mRNA was purified and fragmented from total RNA (1 μg) using poly-T oligo-attached magnetic beads over two rounds of purification. The first-strand cDNA was synthesized by using reverse transcriptase and random primers, and the final strand-specific cDNA library was created after purification and PCR-based enrichment. The quality of the amplified libraries was verified by capillary electrophoresis (BioAnalyzer, Agilent). After quantitative PCR using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), we combined libraries that had index tagged equimolar amounts in the pool. Cluster generation occurred in the flow cell on the cBot automated cluster generation system (Illumina, CA, USA). Sequencing was performed using the HISEQ 2500 sequencing system (Illumina) with a 2 × 100 bp read length.

2.4. Preprocessing and de novo transcriptome assembly

Paired-end sequence files obtained from control and MeJA-treated *Eo* were subjected to a processing step using Trimmomatic-0.32. Total preprocessed sequences from HISEQ were pooled and assembled using Trinity assembler (Grabherr et al., 2011) with default values. To remove the redundant sequences, CD-HIT-EST (Huang et al., 2010) was used with 95% sequence similarity. To confirm the assembly, the sequence reads from the MeJA-treated samples were mapped to the assembled transcriptome, which had a high mapping rate (~91%). Finally the transcripts of ≥500 bp were selected as the reference transcriptome and were subjected to functional annotation using BLASTX mapping (e-value cut-off 1e-5) against the UniProt KB (Viridiplantae) database and the Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps using Blast2GO (Conesa et al., 2005).

2.5. Identification of differentially expressed genes

Differentially expressed genes (DEGs) were determined by counting the tags from the genes in MeJA-treated *Eo*, comparing them with those of control samples, and normalizing them using the RNA Sequence Expected Maximization (RSEM) method (Li and Dewey, 2011). Initially, reads from control samples were mapped to the reference transcriptome and subjected to check the differential expression was checked using trinity utility scripts according to the stipulated instructions (http://trinityrnaseq.github.io/). Using the EdgeR statistics files, the regulated transcripts across libraries were filtered with default parameters (i.e., $1 \leq log2$ (FC), FDR < 0.01) using python scripts.

2.6. Full-length cDNA amplification and reverse transcription–polymerase chain reaction

Total RNA was isolated from *Eo* leaf tissues using the Exgene™ Plant SV mini Kit (GeneAll, Seoul, Republic of Korea), and the first-strand cDNAs were synthesized using 1 μg of total RNA with a Maxime™ RT PreMix system (iNtRON Biotechnology, Sungnam, Republic of Korea). A full-length cDNA of *Eo* terpene synthase (*EoTPS*) was amplified with the primers based on the nucleotide sequences predicted by RNAseq analysis using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). The PCRs were conducted as follows: 35 cycles at 95 °C (30 s), 55 °C (30 s), and 72 °C (2.5 min), with a final extension at 72 °C (5 min). A PCR amplicon was purified using an Expin™ Gel SV Kit (GeneAll, Seoul, Republic of Korea) and cloned into pJET1.2/blunt

vector (Thermo Fisher Scientific Inc., MA, USA). The nucleotide sequences of isolated cDNAs were confirmed in both strands and compared with those generated by RNAseq analysis. For semi-quantitative RT–PCR, the first-strand cDNAs were synthesized as described above, and a PCR was conducted as follows: 95 °C (5 min), 28 or 30 cycles of 95 °C (30 s), 55 °C (30 s), and 72 °C (1 min), with a final extension at 72 °C (5 min). The number of PCR cycles was optimized by determining the cycles, at which the amplification was in an exponential stage. The RT–PCR analyses were conducted in triplicate with biologically independent samples. *EoHIS* was used as an endogenous control gene. The oligonucleotide sequences are shown in Supplementary Table A1.

2.7. Amino acid sequence alignment and phylogenetic analysis

Amino acid sequences of EoTPSs were aligned and analyzed using DNA Star package (Laser gene, USA). Signal peptides were analyzed using the ChloroP and TargetP programs (Emanuelsson et al., 2007). The alignment was visualized using GeneDOC (http://www.psc.edu/biomed/genedoc). The selected terpene synthases used in the phylogenetic analysis were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). The details of the plant TPSs are provided in Supplementary Table A2. Phylogenetic trees were constructed using the neighbor-joining algorithm and visualized using Molecular Evolution Genetics Analysis (MEGA) version 6 (Tamura et al., 2013).

2.8. Ectopic overexpression of EoTPS in Arabidopsis

For in planta functional characterization of the EoTPSs, a full-length of *EoTPS* was introduced to pENTR™/TOPO vector using pENT™ Directional TOPO® Cloning Kits (Invitrogen, USA), followed by cloning into the pK7WG2 vector (Karimi et al., 2002) via the LR clonase reaction (a LR Clonase™ II Enzyme Mix, Invitrogen, USA) for constitutive plant expression. After sequence confirmation in both strands by an automated sequencer (Cosmogenetech, Seoul, Republic of Korea), the resulting construct was stably introduced into *A. thaliana* using an *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Transformed *Arabidopsis* seeds were selected on half-strength MS medium supplemented with 30 mg L^{-1} kanamycin and 50 mg L^{-1} cefotaxime.

2.9. Accession numbers

RNA-seq data obtained in this study have been deposited in the NCBI Sequence Read Archive (SRA) with the SRA accession number PRJNA515087 (SAMN10724124, SAMN10724125, SAMN10724126, SAMN10724127, SAMN10724128, SAMN10724129, SAMN10724130, SAMN10724131).

3. Results

3.1. Profiling of constitutive and methyl jasmonate-inducible volatile terpenes in Eo

The constitutive and methyl jasmonate (MeJA)-induced volatile terpene compounds from aerial tissues of *Eo* were analyzed using a SPME–gas chromatography–mass spectrometry (SPME–GC–MS). *Eo* leaves constitutively emitted a limited amount of mono- and sesquiterpene hydrocarbons, such as (E)-β-ocimene ($\approx$6% of total terpenes) as a major C$_{10}$ monoterpene volatile (Fig. 1A) and α-muurolene ($\approx$88% of total terpenes) as a major C$_{15}$ sesquiterpene (Fig. 2A). However, after exogenous MeJA treatment, a blend of monoterpenes was released, which included α-pinene, camphene, β-myrcene, phellandrene, limonene, α-terpinolene and (E)-β-ocimene; the last of which was a predominant monoterpene among them (Fig. 1B and Table 1). Notably, stereoisomers of β-ocimene were examined in a (E):(Z) ratio of 95:5 (6%:0.33% of total terpenes). Moreover, sesquiterpene biosynthesis was
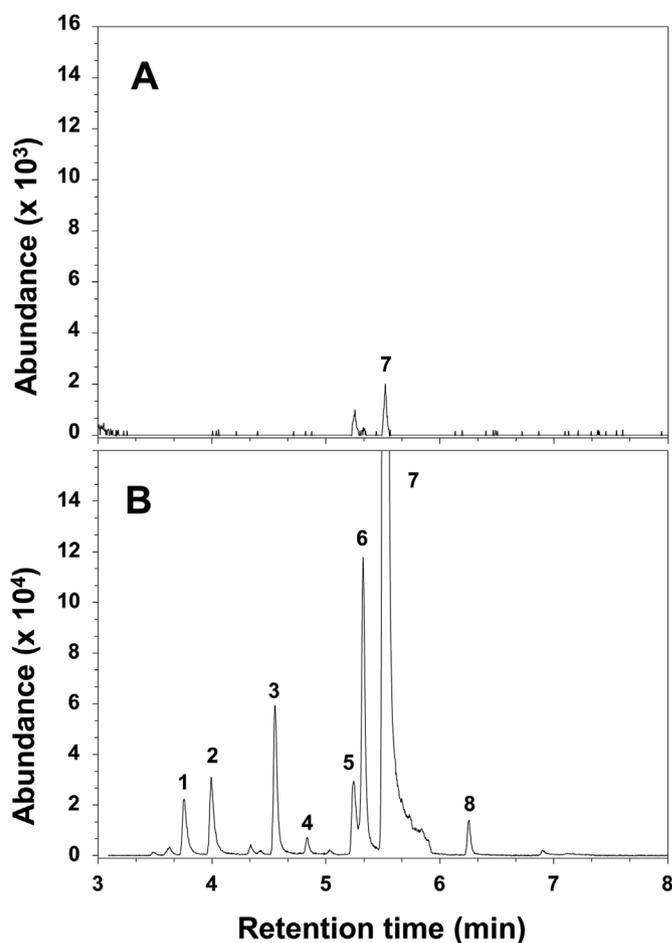

Fig. 1. Constitutive and methyl jasmonate (MeJA)-inducible monoterpenes from 3-month-old *E. ophiuroides* (*Eo*). The headspace monoterpenes were collected 3 days after the treatments of (A) control (dimethylsulfoxide, 50 μM) or (B) MeJA (50 μM) by a solid-phase microextraction (SPME) fiber and analyzed by a gas chromatography–mass spectrometry (GC–MS) using an extracted ion chromatography (EIC) mode (m/z = 93). 1, α-pinene; 2, camphene; 3, myrcene; 4, α-phellandrene; 5, limonene; 6, (Z)-β-ocimene; 7, (E)-β-ocimene; and 8, α-terpinolene.

more dramatic in the MeJA-induced terpene emission. In response to MeJA treatment, the constitutive emission of two sesquiterpenes became a complex mixture of (+)-cyclosativene, α-copaene, (E)-caryophyllene, and α-farnesene as major compounds ($\geq$13% of total terpenes) with minor constituents of elemene, zingiberene, calarene, β-farnesene, α-humulene, α-muurolene, germacrene D, eremophilene, δ-cadinene, and oxygenated caryophyllene (Fig. 2B and Table 1).

3.2. RNA sequencing and de novo assembly

Because *Eo* leaves released constitutive and MeJA-induced terpenes, we searched for the corresponding terpene synthase (*TPS*) genes. A total of 610,113,410 paired-end reads were generated from the aerial tissues of *Eo* by using the Illumina high throughput sequencing technology. After removing low quality sequences, redundant sequences, and unnecessary nucleotide sequences (e.g., primer and adapter sequences), 460,407,119 high-quality reads (75.5%) were obtained and de novo *Eo* transcriptome assembly was conducted. A total of 46,552 transcripts were assembled with a total length of 50,830,376 bp, with a maximal sequence length of 16,298 bp and a minimal sequence length of 500 bp (mean: 1091 bp) (Table 2).
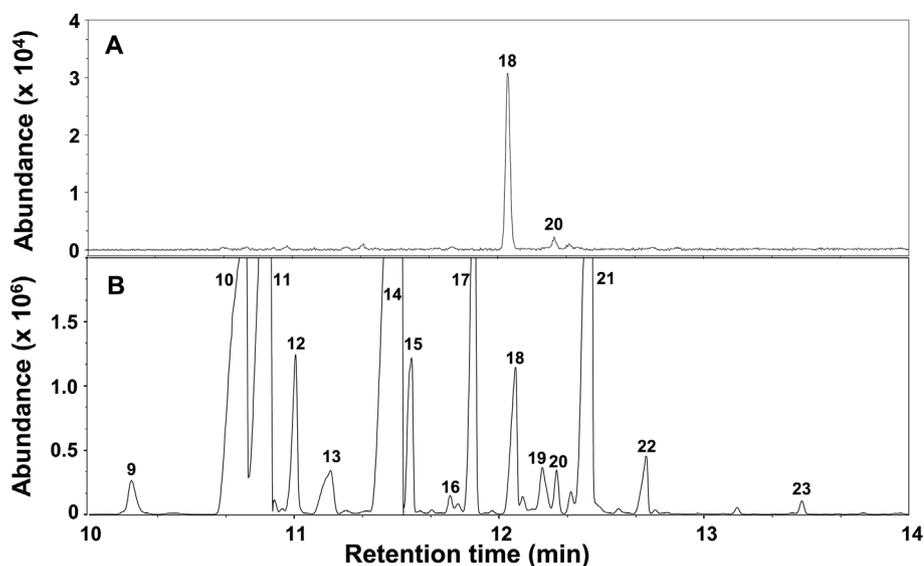
Fig. 2. Constitutive and methyl jasmonate (MeJA)-inducible sesquiterpenes from 3-month-old *Eo*. The headspace sesquiterpenes were collected 3 days after the (A) control (dimethylsulfoxide, 50 μM) or (B) MeJA (50 μM) treatments, using a solid-phase microextraction fiber and analyzed using a GC–MS analysis in EIC mode (*m/z* = 93). 9, δ-elemene; 10, (+)-cyclosativene; 11, α-copaene; 12, β-elemene; 13, zingiberene; 14, (*E*)-caryophyllene; 15, calarene; 16, β-farnesene; 17, α-humulene; 18, α-muurolene; 19, germacrene D; 20, eremophilene; 21, α-farnesene; 22, δ-cadinene; and 23, caryophyllene oxide.

3.3. Prediction of Eo terpene synthases (EoTPSs) and MeJA-inducible gene expressions

The transcripts selected for the reference transcriptome were functionally annotated by using BLASTX mapping (e-value cut-off 1e-5) against UniProt KB (Viridiplantae), GO, and KEGG using Blast2GO, and predicting a total of 14 *TPSs*. The Fragments Per Kilobase of transcripts per Million mapped reads (FPKM) values were used to differentiate the gene expression levels of the putative *TPSs* between control and MeJA-treated samples (Table 3). Eleven *TPS* transcripts were induced more than two-fold after MeJA treatment in a time-dependent manner in comparison to control samples, while transcript levels of ISGT_25263, ISGT_3519, and ISGT_31114 showed negligible change in their expression with the identical treatment (Fig. 3A and Table 3). However, the gene expressions of the other 11 transcripts were elevated more

than two-fold (up to > 9 folds for ISGT_27038) at as early as 3 days after the treatment (DAT). Three transcripts of ISGT_2018, ISGT_18791, and ISGT_3519 showed higher gene expression levels at 7 DAT than the

Table 2
Statistical parameters of the assembled transcripts.

Parameters	Value
Total RawReads	610,113,410
Total CleanReads	460,407,119
Total length of sequences (bp)	50,830,376
Total number of transcripts	46,552
Maximum sequence length (bp)	16,298
Minimum sequence length (bp)	500
Average sequence length (bp)	1091

Table 1
Relative amounts of terpenes collected from the aerial tissues of *Eremochloa ophiuroides* after control and MeJA treatments.

	Peak	Control			MeJA		
		Rt*	Compound	%**	Rt	Compound	%
MONOTERPENE	1	–	–	–	3.75	α-pinene	0.08
	2	–	–	–	3.99	camphene	0.10
	3	–	–	–	4.55	myrcene	0.17
	4	–	–	–	4.83	α-phellandrene	0.02
	5	–	–	–	5.24	limonene	0.10
	6	–	–	–	5.32	(*Z*)-β-ocimene	0.33
	7	5.67	(*E*)-β-ocimene	5.97	5.52	(*E*)-β-ocimene	6.00
	8	–			6.25	α-terpinolene	0.04
SESQUITERPENE	9	–	–	–	10.20	δ-elemene	0.93
	10	–	–	–	10.76	(+)-cyclosativene	14.55
	11	–	–	–	10.88	α-copaene	16.87
	12	–	–	–	11.00	β-elemene	2.79
	13	–	–	–	11.17	zingiberene	1.56
	14	–	–	–	11.52	(*E*)-caryophyllene	26.22
	15	–	–	–	11.57	calarene	2.72
	16	–	–	–	11.76	β-farnesene	0.29
	17	–	–	–	11.87	α-humulene	7.55
	18	12.20	α-muurolene	87.78	12.08	α-muurolene	2.94
	19	–	–	–	12.21	germacrene D	1.14
	20	12.42	eremophilene	6.25	12.28	eremophilene	0.65
	21	–	–	–	12.44	α-farnesene	13.60
	22	–	–	–	12.71	δ-cadinene	1.14
	23	–	–	–	13.48	caryophyllene oxide	0.21

-, not detected.

*, Rt, retention time (minute).

**, relative peak area in the sum of peak areas of terpenes.

Table 3

Transcript abundances of predicted terpene synthases in *Eremochloa ophiuroides* in response to MeJA treatment.

| Transcript ID	FPKM			
	Control	3 Day-MeJA	7 Day-MeJA	14 Day-MeJA
ISGT_2018	9.44	28.96	33.42	27.27
ISGT_24390	0.65	2.83	2.92	2.76
ISGT_18791	10.40	58.05	103.87	52.10
ISGT_27038	0.44	3.98	3.07	3.81
ISGT_25263	2.56	2.52	2.08	2.35
ISGT_3519	5.03	6.71	8.56	6.26
ISGT_31114	0.42	0.54	0.53	0.61
ISGT_31387	8.98	21.43	15.02	13.29
ISGT_3408	15.01	37.90	23.28	21.33
ISGT_2276	25.41	63.46	43.06	38.13
ISGT_7816	89.56	247.45	155.44	136.77
ISGT_38195	0.12	0.22	0.38	0.11
ISGT_6039	23.69	82.32	58.21	54.08
ISGT_45453	2.31	7.38	5.57	4.26
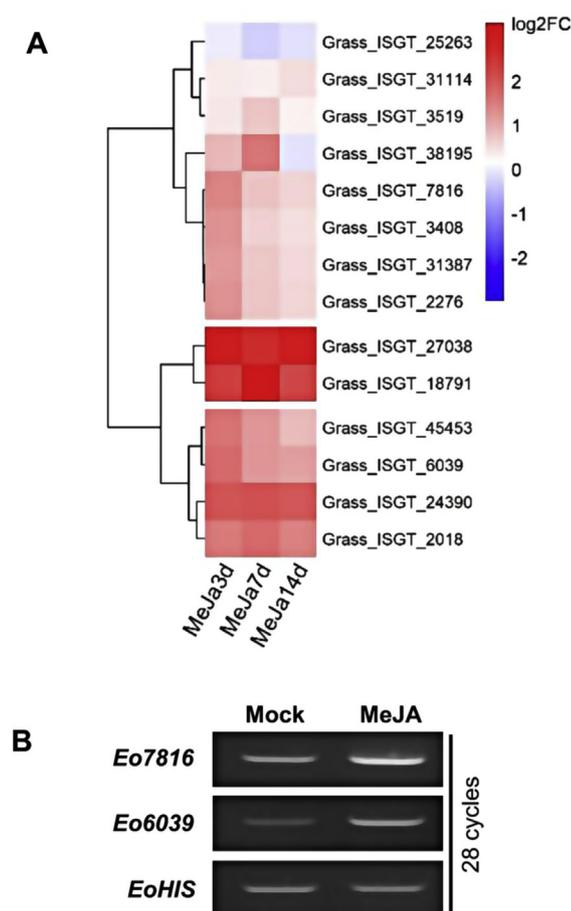
Fig. 3. Differentially expressed genes of putative *Eo* terpene synthases (*EoTPS*) in response to methyl jasmonate (MeJA). (A) Heatmap of the time-dependent expression levels of 14 *TPSs* after MeJA treatment, relative to those in the control treatment (dimethylsulfoxide). Red and blue colors represent high and low expression levels, respectively. (B) Semi-quantitative transcript abundances at 7 days after MeJA treatment (50 μM). *EoHIS, Eo* histone was used as an endogenous control gene. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

rest of them. The expression levels of *Eo27038*, *Eo24390*, and *Eo2018* were greatly induced under MeJA treatment and maintained the expression levels for 14 DAT. However, the rest of *EoTPSs* showed early induction at 3 DAT, followed by a gradual decrease in the expression

until 14 DAT (Fig. 3A and Table 3). Furthermore, we were able to obtain two putative full-length nucleotide sequences out of the 14 transcripts; *Eo6039* (1,701bp, 567 amino acid, 64.6 kD) and *Eo7816* (1722 bp, 574 amino acid, 65.7 kD). A semi-quantitative RT–PCR indicated that MeJA treatment induced high accumulation of the two *TPSs* transcripts, comparing to the control (Fig. 3B).

3.4. Phylogenetic analysis

Phylogenetic analyses using amino acid sequences deduced from the isolated full-length cDNAs were performed with known 64 known plant *TPS* genes that was retrieved from the National Center for Biotechnology Information (NCBI) non-redundant protein database (Supplementary Table A2). A phylogenetic tree showed that Eo6039 belonged to the TPS-a clade, which consists of sesquiterpene synthases, while Eo7816 was positioned on the clade of TPS-b, which mainly consists of monoterpene synthases (Fig. 4).

3.5. Amplification of full-length EoTPSs and recombinant protein production in Escherichia coli

To examine sequence characteristics, the deduced amino acid sequences of the two full-length contigs (i.e., *Eo7816* and *Eo6039*) were aligned with those of known plant TPSs. Both contigs contained an evolutionarily conserved aspartate-rich domain (e.g., DDXXD) and showed typical TPS sequences in amino acid length (Fig. 5), while the rest of the *TPS* fragments were in either partial or incomplete amino acid sequences (data not shown). Moreover, *Eo7816* was predicted to be a monoterpene synthase based on the existence of both characteristic motifs of RRX_8W motif near the signal peptide region and putative chloroplastidic targeting sequences (≈ 36 amino acids) at the N-terminal region (Fig. 5A) (Emanuelsson et al., 2007; Lee and Chappell, 2008). *Eo6039* did not have signal sequences and the RRX_8W motif, and thus it was predicted to be a sesquiterpene synthase (Fig. 5B). To obtain soluble recombinant EoTPS proteins for in vitro biochemical characterization, each *EoTPS* was overexpressed under the constitutive 35S promoter in *E. coli*. None of the *EoTPS* overexpressions were successful in the recovery of a sufficient amount of soluble proteins regardless of multiple trials with modified protein expression conditions.

3.6. Ectopic overexpression of Eo7816 in Arabidopsis

Because of the failure of obtaining soluble Eo7816 proteins, we alternatively employed in planta assays by ectopically overexpressing *Eo7816* cDNA under the constitutive 35S promoter in *A. thaliana*. The volatile terpenes emitted from leaves of transgenic *Arabidopsis* plants were analyzed using SPME–GC–MS. Introduction of an empty vector into *Arabidopsis* plants had no effect on de novo biosynthesis of terpenes (Fig. 6A), while *Eo7816*-overexpressing transgenic *Arabidopsis* plants released two isomers of β-ocimene, (*E*)-β-ocimene and (*Z*)-β-ocimene in a ratio of 96:4 with small amount of myrcene (Fig. 6B and C), which were also detected from the terpene volatiles collected from the aerial tissues of MeJA-treated *Eo* (Fig. 1B). We thus named Eo7816: a (*E*)-β-ocimene synthase (EoEβOS).

3.7. Ectopic overexpression of Eo6039 in Arabidopsis

Overexpression of *Eo6039* in *Arabidopsis* plants resulted in the emission of a wide array of sesquiterpenes, including α-muurolene, eremophilene, valencene, α-selinene, and β-elemene (Fig. 6E), many of which were also found in the volatile sesquiterpenes detected from the aerial tissues of *Eo*, while no new terpenes were emitted in the empty vector control plants (Fig. 6D). Among the emitted volatiles, α-muurolene (69.4% of total terpenes) and eremophilene (21.8% of total terpenes) were the major sesquiterpene constituents (Fig. 6F), thus Eo6039 was named: α-muurolene synthase (EoMUS). To our
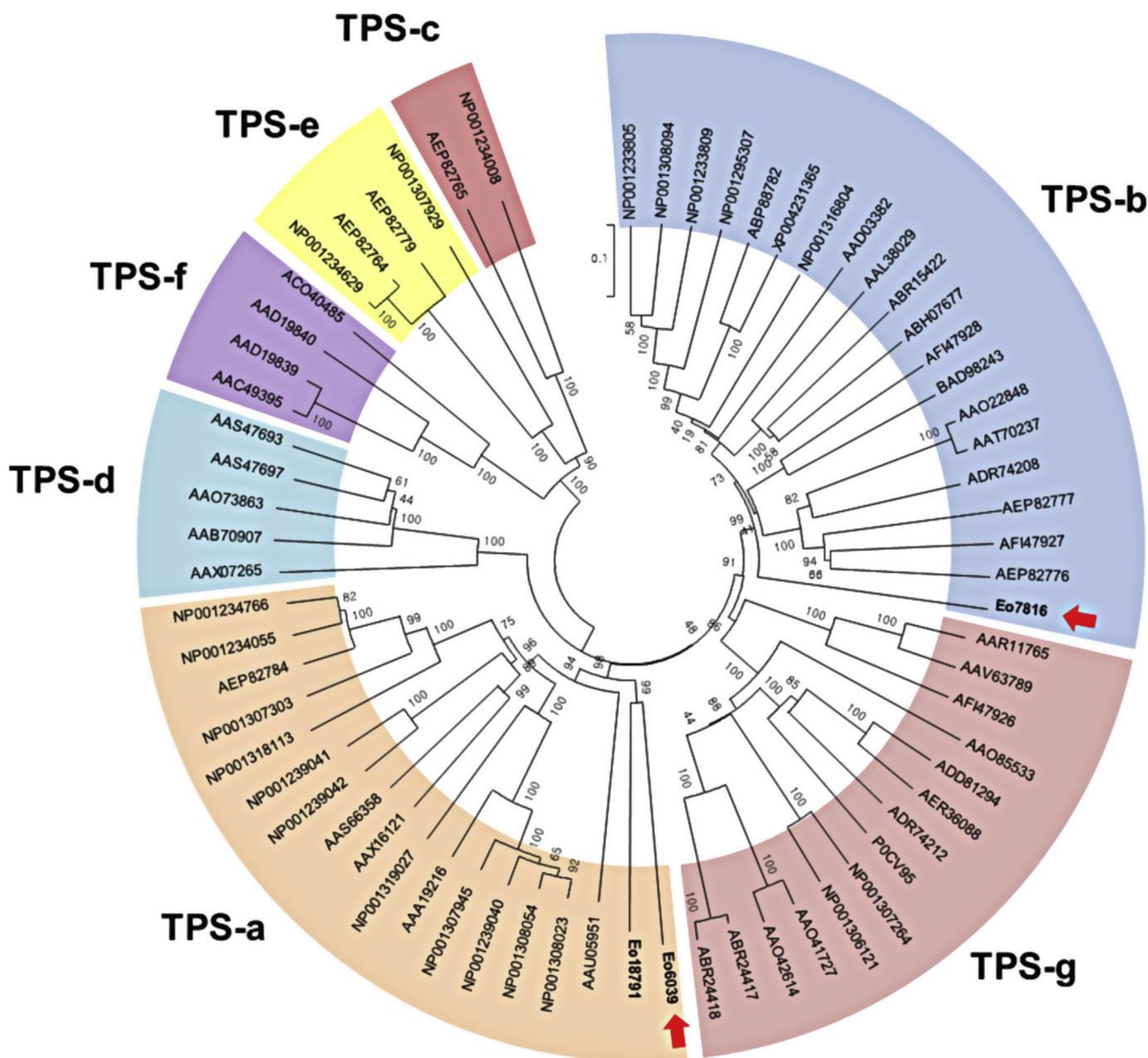
Fig. 4. Phylogenetic analysis of selected plant terpene synthases (TPSs) at the amino acid level. A phylogenetic tree was constructed using the neighbor-joining algorithm in the MEGA6 software with n = 1000 replicates for bootstrapping. Arrows in red indicates the *Eo* TPSs characterized in this study. The details of the plant TPSs are provided in Supplementary Table A2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

knowledge, this is the first report on the isolation and functional characterization of α-muurolene synthase from a plant species.

4. Discussion

We described the profile of terpene volatiles emitted constitutively and induced by MeJA treatment from the aerial tissues of *Eo*. We found that aerial tissues of *Eo* emitted two major terpene volatiles: monoterpene (*E*)-β-ocimene and sesquiterpene α-muurolene. This simple volatile emission was dramatically changed by MeJA treatment into a blend of terpene mixtures that consisted of eight components of monoterpenes, which included (*E*)- and (*Z*)-β-ocimene, and myrcene. The emission of (*E*)-β-ocimene has been documented from flowers of various plant species (Farré-Armengol et al., 2017; Knudsen et al., 1993), and its release is highly induced by insect herbivores (Arimura

et al., 2004; Paré and Tumlinson, 1999; Shen et al., 2000). It is noteworthy that aerial tissues of *Eo* emitted β-ocimene in a (*E*):(*Z*) ratio of 95:5 (6%:0.33% of total terpenes) upon MeJA treatment. Given the importance of a *E/Z* ratio of β-ocimene (97:3 and 70:30 for attraction and 60:40 for repellent of natural enemies) (Dicke et al., 1990), it should be of interest to test whether a *E/Z* ratio of β-ocimene in *Eo* will affect the tritrophic interactions when exposed to herbivory insect attacks. In addition, 13 components of sesquiterpene volatiles, including (*E*)-caryophyllene as a major compound, were produced with the identical elicitation effect. However, we cannot rule out the possibility that other mono- or sesquiterpenes were emitted in very low concentrations that were unidentifiable under our experimental conditions.

We identified 14 transcripts of putative *TPSs* in *Eo* by RNA sequencing analysis. Two full-length cDNAs (i.e., *ISGT_7816* and *ISGT_6039*) were obtained by de novo sequence assembly and
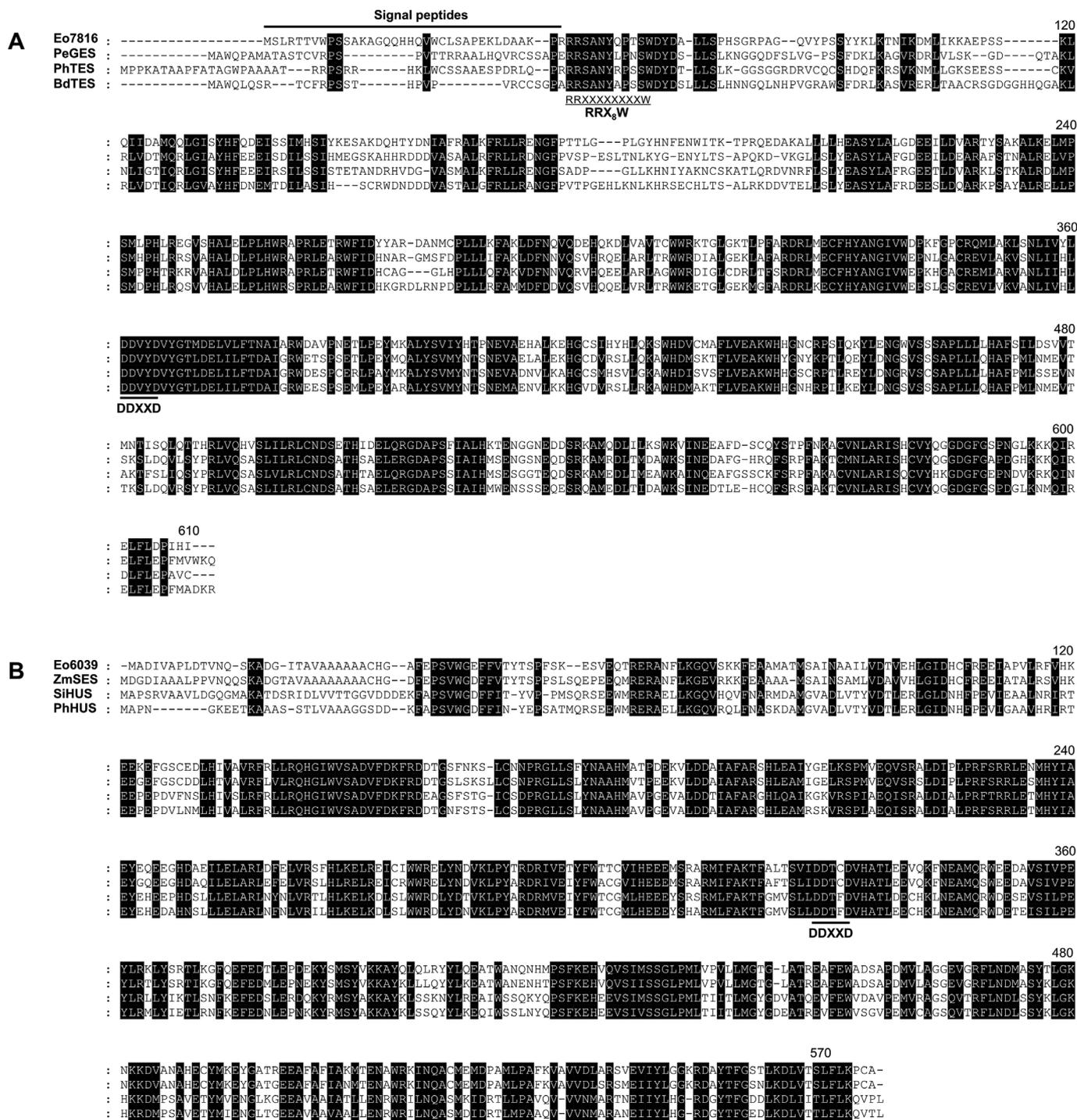
A

Fig. 5. Amino acid sequence alignments of two EoTPSs. The deduced amino acid sequences of Eo7816 (A) and Eo6039 (B) were aligned with selected plant mono- and sesquiterpene synthases, respectively. Conserved RRX₈W and DDXXD motifs are indicated. PeGES, *Phyllostachys edulis* geraniol synthase (AYD75693); PhTES, *Panicum hallii* α-terpineol synthase (XP_025576011); BdTES, *Brachypodium distachyon* α-terpineol synthase (XP_003580946); ZmSES, *Zea mays* β-selinene synthase (ATI25525); SiHUS, *Setaria italic* α-humulene synthase (XP_004975013); and PhHUS, *Panicum hallii* α-humulene synthase (XP_025825818).

functional annotations, and a functional characterization was performed by ectopically overexpressing the cDNAs in *Arabidopsis*. The transgenic *Arabidopsis* plants overexpressing *Eo7816* cDNA (*EoEβOS*) demonstrated that this gene plays a major role in producing (*E*)-β-ocimene with two minor products: (*Z*)-β-ocimene and myrcene. Similar results were observed for other (*E*)-β-ocimene synthases (Dudareva et al., 2003; Fäldt et al., 2003; Navia-Giné et al., 2009). The *E/Z* ratio of β-ocimene emitted from the *Eo7816*-overexpressing *Arabidopsis* (96:4) was comparable to that from the MeJA-treated *Eo* (95:5). On the other hand, the overexpression of *Eo6039* cDNA (*EoMUS*) in *Arabidopsis* revealed that this gene had a catalytic activity, which generated α-muurolene as a major sesquiterpene (69.4%) and eremophilene (21.8%) and other small amounts of sesquiterpenes. Although α-muurolene has been documented from essential oils and foliar scents from a wide array of plants, a cDNA encoding TPS responsible for the formation of α-muurolene as a major compound has not yet been isolated, apart from the fungal α-muurolene synthase (Cop3) from the basidiomycete (Agger et al., 2009; Wawrzyn et al., 2012). We propose
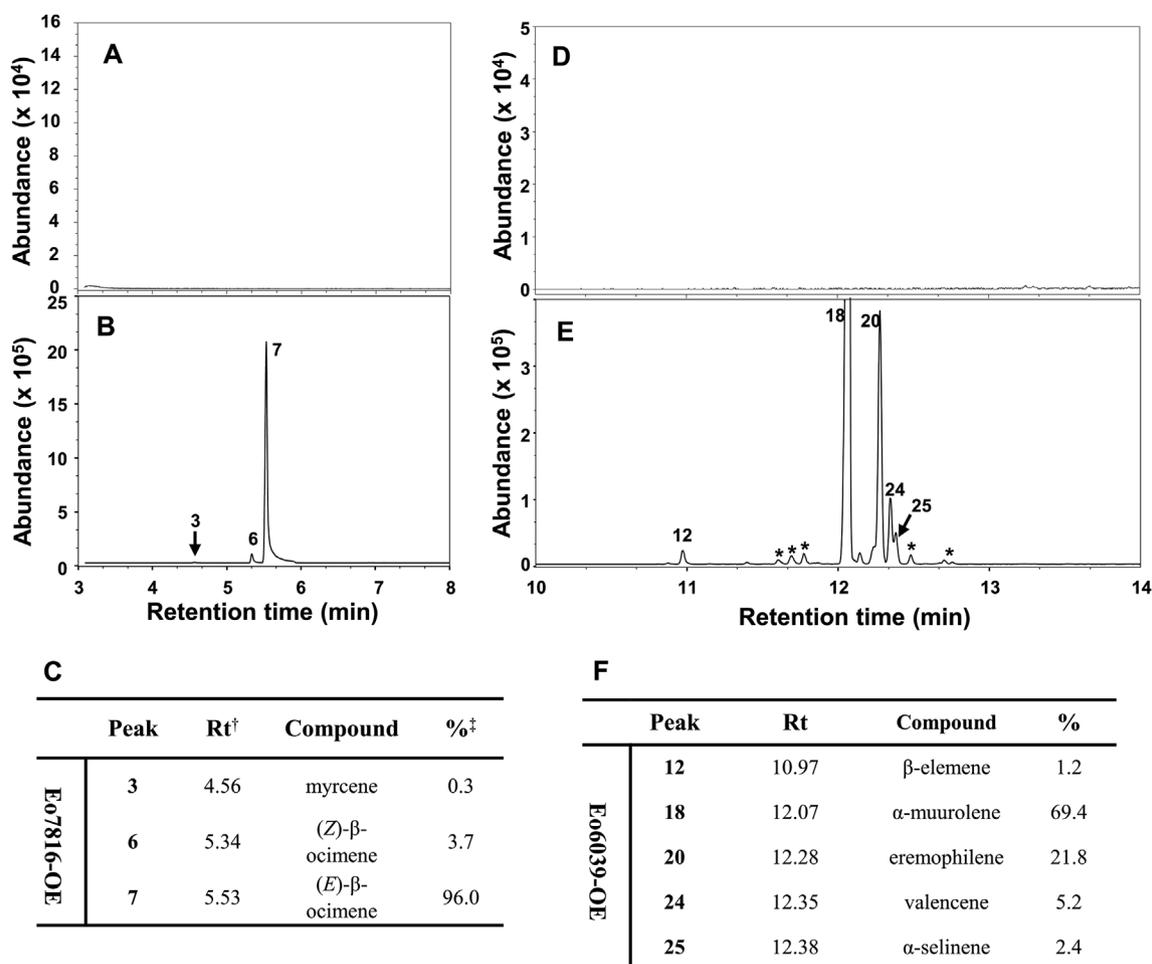
Fig. 6. In planta characterization of *EoTPSs* in *Arabidopsis* transgenic plants. A and D, empty vector-overexpressing *Arabidopsis* plants; B, Eo7816-overexpressing *Arabidopsis*; E, Eo6039-overexpressing *Arabidopsis*. C and F, relative amounts of terpenes collected from the transgenic *Arabidopsis* plants. Headspace volatile terpenes were analyzed using solid-phase microextraction–GC–MS (EIC mode, m/z = 93). *, unknown or trace amount; [†], Rt, retention time (minute); [‡], relative peak area in the total peak areas of terpenes. OE, overexpression.

that the EoMUS is the first identification and characterization of α-muurolene synthase from a plant species. An in vitro assay using purified form of EoMUS, if possible, will provide with better insights on the biochemical properties of the enzyme in the future study. With the discovery of functional EoMUS, investigation on previously unknown biological functions of α-muurolene should be another important theme to perform, which will provide a clue not only for directed breeding of centipedegrass variety with improved resistance against plant diseases (e.g., anthracnose), as well as for pharmacological application of the compound.

Since exogenous MeJA treatment is considered to mimic pathogen infection and herbivory attack (Moore et al., 2003; Vijayan et al., 1998), it is highly possible that pathogen infection or insect herbivory elevate the emissions of the volatile chemicals formed by EoEβOS and EoMUS in *Eo*. We also observed de novo emissions of a blend of sesquiterpenes after MeJA treatment [e.g., (*E*)-caryophyllene, α-farnesene, α-copaene, etc.], which suggests that *Eo* contains other functional TPSs that are responsible for the formation of corresponding volatiles. Therefore, it would be of interest to further characterize other putative EoTPSs, the expressions of which are increased by MeJA treatment.

Author contribution

S.L. and G.W.L. conceived and designed all experiments; G.W.L. and M.-S.C. performed the experiments; S.L., G.W.L., S.S.L., and B.Y.C. analyzed the data; and S.L. and G.W.L. wrote the manuscript with the assistance and approval of all authors.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.032.

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