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Overexpression of Tomato *Prosystemin* (*LePS*) Enhances Pest Resistance and the Production of Tanshinones in *Salvia miltiorrhiza* Bunge

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Supporting Information

ABSTRACT: Tanshinones are a group of active diterpenes with pharmacological properties that are widely used in the treatment of cardiovascular diseases. Jasmonate (JA) acts as an elicitor to enhance tanshinone biosynthesis in *Salvia miltiorrhiza*. However, because of high labor costs and undesirable chemical characteristics, the use of JA elicitation is still in the experimental stage. In our experiments, the overexpression of *Lycopersicon esculentum* (tomato) *Prosystemin* (*LePS*) in transgenic plants of *S. miltiorrhiza* increased their JA concentrations, significantly enhanced the production of tanshinone, and activated the expression of key genes in the tanshinone biosynthesis pathway. Meanwhile, the relative levels of metabolites related to defense such as sterols, terpenes, and phenolic acids were also increased in our OEP lines. In addition, when the larvae of cotton bollworms (*Heliothis armigera*) were fed with leaves from transgenic lines, their mortality rates rose by nearly 4-fold when compared to that of larvae exposed to leaves from the nontransformed wild type. Our study provides a new strategy for genetic engineering by which tanshinone production and pest resistance can be improved in *S. miltiorrhiza.* This is accomplished by simulating the wounding signal that increases the endogenous levels of JA.

KEYWORDS: *Salvia miltiorrhiza, tanshinone accumulation, insect resistance, wound signal, JA*

INTRODUCTION

Salvia miltiorrhiza Bunge (Lamiaceae) is a highly valued herb from which roots and rhizomes are obtained for use in traditional Chinese medicine to treat various cerebrovascular and cardiovascular diseases.[1,2] Lipophilic tanshinones (including tanshinone I, tanshinone IIA, dihydrotanshinone I, and cryptotanshinone) belong to the diterpene quinone group and are major active constituents in *S. miltiorrhiza*.[3] Tanshinones are beneficial for the treatment of myocardial infarction, atherosclerosis, hyperlipidemia, and stroke because of their anti-inflammatory, antioxidant, and antitumor properties.[4] Because these compounds have significant pharmacological activities, the clinical demand for *S. miltiorrhiza* is increasing. However, these components are naturally present in plants only in small amounts, and pest infestations and more extensive cultivation have led to a serious degradation of quality for that species during the past two decades.[5]

Plants respond physiologically and morphologically to stress elicitors and adapt their environment by increasing the production of secondary metabolites. Therefore, researchers have tried to exploit diverse elicitors to induce the production of important active compounds in economically valuable plants.[6] Among those elicitors, jasmonate (JA) is a novel defense phytohormone that is commonly used to alter the biosynthesis of alkaloids, terpenoids, and phenylpropanoids.[7,8] In *S. miltiorrhiza*, tanshinone production can be markedly induced by treatment with exogenous JA.[9,10] Although this type of elicitation has great potential, such an approach is still in the experimental stage because of high labor costs and undesirable chemical characteristics (i.e., low solubility and effumability) that can lead to difficulties in field operations. Therefore, increasing endogenous JA levels by genetic engineering could be an effective alternative strategy.

Systemin, an 18 amino acid peptide signal derived from the C-terminal region of its precursor prosystemin (PS),[11,12] was originally purified from wounded tomato leaves (*Lycopersicon esculentum*) because of its reported role in defense responses.[13] Overexpression of *PS* induces the accumulation of protease inhibitors (PIs), which degrade essential amino acids in the herbivore midgut.[14] Conversely, transgenic tomato plants that express antisense *PS* are defective in their expression of PI genes, making them more susceptible to damage by insect pests.[15] Moreover, tomatoes that overexpress *PS* produce more volatile organic compounds and other secondary metabolites.[16,17]

Systemin and JA work together in the same signaling pathway to activate the expression of defense-related genes.[18,19] Genetic analyses of tomato mutants have demonstrated that both components are necessary for wound-induced systemic

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responses.[20−22] Mutant plants that are defective in either JA biosynthesis/perception or systemin functioning are compromised in their resistance to pests or mechanical wounding.[23,24] Briefly, when tissues are damaged by such stressors, systemin is released from the wound site and the biosynthesis of JA from linolenic acid is activated via the octadecanoid pathway. In the chloroplasts, sequential enzymes involved in the initial steps of JA biosynthesis catalyze the production of 12-oxo-phytodienoic acid (OPDA), which is then transported to the peroxisome to yield JA by several peroxisomal enzymes. As a mobile signal, JA is perceived by its receptor SCF^{COI1}complex to activate the downstream expression of JA-induced defense genes. This leads to the production of chemicals for defensive metabolism and the initial defense response. Therefore, we speculated that the overexpression of tomato *Prosystemin* (*LePS*) in *S. miltorrhiza* could induce a wounding response by triggering JA synthesis to improve pest resistance and promote a greater accumulation of tanshinones.

Here, we compared the degree of pest resistance and concentrations of tanshinones between wild-type (WT) *S. miltiorrhiza* and transgenic plants that overexpressed *LePS*. We found that both resistance and tanshinone levels, as well as JA concentrations, were significantly elevated in the transgenic lines. Our study results provide a novel strategy for enhancing insect resistance in *S. miltiorrhiza* and demonstrate that genetic engineering techniques can be applied to improve the production of tanshinones by regulating endogenous JA levels.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Sterile *S. miltiorrhiza* plantlets were cultured on a Murashige and Skoog (MS) basal medium, as described by Yan.[25] Conditions included 25 ± 2 °C, 60% relative humidity, and a 16/8 h photoperiod. After 30 days of culturing, the seedlings were used for *Agrobacterium*-mediated transformation.

DNA and RNA Extraction and RT-PCR. Total genomic DNA was isolated from transgenic and nontransformed *S. miltiorrhiza* plants using an E.Z.N.A. Plant DNA Kit (Omega Biotek, GA, USA) as recommended by the manufacturer. The total RNA was extracted from fresh tomato leaves (*Lycopersicon esculentum*) using RNAiso Plus (TaKaRa, Dalian, China), and genomic DNA was removed with DNase I (TaKaRa). The first-strand cDNA was synthesized with an RT reagent kit (TaKaRa) according to the manufacture's protocol.

Vector Construction and Transformation. The open reading frame of tomato *Prosystemin* (*LePS*; GenBank accession number M84801.1) was cloned from tomato cDNA and inserted into the pMD19-T simple vector (TaKaRa). It was then verified with sequencing by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

The amplified *LePS* coding region with *Spe I* and *Bst II* sites (Figure S1) was ligated into the pCAMBIA1302 vector to construct recombinant vector pOE-LePS. The pOE-LePS plasmid was transferred into *Agrobacterium tumefaciens EHA*105 by the freeze−thaw method.[26] After transformation, explants cocultured with *A. tumefaciens* were transferred to an MS selection medium containing 1 mg L^{-1} naphthalene acetic acid, 10 mg L^{-1} 6-benzyl-aminopurine, 100 mg L^{-1} hygromycin B (HygB), and 400 mg L^{-1} cefotaxime (cef). They were transferred to fresh selection media in 10 day intervals. Developing shoots were excised and placed on a $^1/_2$-MS selection medium supplemented with 10 mg L^{-1} HygB and 200 mg L^{-1} cef for root induction. A well-developed root system usually formed within 2 weeks, and plantlets were propagated through several generations to expand the culture in the MS basic medium.

PCR Detection, Semiquantitative PCR, and Real-Time qPCR Analysis. LePS-F/R primers (Table S1) were used to amplify *LePS* from the genomic DNA of WT and transgenic *S. miltiorrhiza*. Conditions for PCR amplifications included an initial 5 min at 94 °C and then 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C, followed by a final elongation for 10 min at 72 °C. The target fragments were 603 bp long. The pOE-LePS vector served as the positive control, whereas genomic DNA from WT plants was the negative control.

The mixture for semiquantitative PCR comprised 125 ng of cDNA, 0.5 U Taq polymerase, 4 mM $MgCl_2$, 10 pmol of RT-LePS-F/R primers, and 0.4 mM dNTP's. Amplification conditions included 3 min at 94 °C and then 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C, followed by a final extension for 10 min at 72 °C. Housekeeping gene elongation factor (EF) was used as a loading control, and loading was estimated by staining the gel with ethidium bromide (1 μg mL^{-1}). In all of the negative-control reactions, cDNA templates were replaced with sterile water to check for the absence of contaminants. The gels were photographed with FluorChem software (ProteinSimple, USA), and the grayscale was calculated with that software.

Real-time qPCR analysis was conducted on an IQ5 thermocycler (Bio-Rad, USA). Each reaction was performed with 5 μL of a 1:10 (v/v) dilution of the first-strand cDNA. The 2× SYBR Green PCR Master Mix (TaKaRa, Japan) was employed by strictly following the manufacturer's instructions and using each primer at a concentration of 200 nM (Table S1) in a total volume of 20 μL. Cycling conditions were 10 s at 94 °C, followed by 35 cycles for 10 s at 94 °C and 20 s at 60 °C. A dissociation curve was added to check for amplification specificity. The fragment of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was amplified as an internal control to calibrate the relative expression. Nuclease-free water was added as the negative control, and three PCR reaction replicates were used. The expression data were evaluated by the C_T method for analysis.[27] All gene-specific primers for PCR are shown in Table S1.

Extraction and Determination of Jasmonate Concentrations. Levels of JA were investigated by culturing 0.5 g samples of fresh leaves from transgenic plants and WT control plants. After the tissues were ground in liquid nitrogen, 3 mL of 10% trichloroacetic acid was added, and the mixtures were stored overnight at −20 °C. The extracts were then centrifuged (8000 rpm for 1 h at 4 °C), and the upper layer was discarded. The precipitate was diluted with a 5 mL solution containing 2.7 g of urea and 0.2 g of 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate. After centrifugation (8000 rpm for 15 min), the upper layers were retained for our extractions, which were measured in triplicate. Endogenous JAs were measured in both transgenic and WT plants using a Plant Jasmonic Acid ELISA Kit (RapidBio, USA) according to the manufacturer's protocol. A set of calibration standards were assayed to produce a standard curve of optical density (OD) versus JA concentration at 0, 125, 250, 500, 1000, and 2000 pmol L^{-1}. The level of JA in each sample was determined by comparing OD values with the standard curve. The intensity of the final reaction color was measured spectrophotometrically at 450 nm to calculate the final JA concentration.

Insect Bioassay. Cotton bollworm (*Heliothis armigera*) larvae were kindly supplied by the Plant Protection Research Institute, Chinese Academy of Agricultural Science, for our insect-resistance bioassays, which were performed according to the method of Meng.[28] Fully expanded young leaves were detached from transgenic lines and WT and then placed in plates containing wet filter paper. Each leaf was inoculated with five third-instar larvae. After 5 days of exposure, leaf damage and insect mortality were evaluated. The level of damage was classified along a four-point scale: I, damage covering <10% of the entire leaf area; II, 10 to 25%; III, >25 to 50%; and IV, damage to >50% of the leaf area. The rate of insect mortality was used to represent the degree of resistance by the transgenic plants. Three replicates were used to compare the transformed lines to the WT control.

Metabolomic Analyses. Leaves and roots from six biological repeats of OEP lines and nontransgenic wild-type control lines were collected and extracted with alcohol for metabolomic analyses. The analysis was performed on an ISQ Trace Ultra (Thermo Fisher Scientific, MA, USA) system. A DB-5 ms capillary column (15 m × 0.25 mm, 0.25 μm thickness; Agilent Technologies, CA, USA) was

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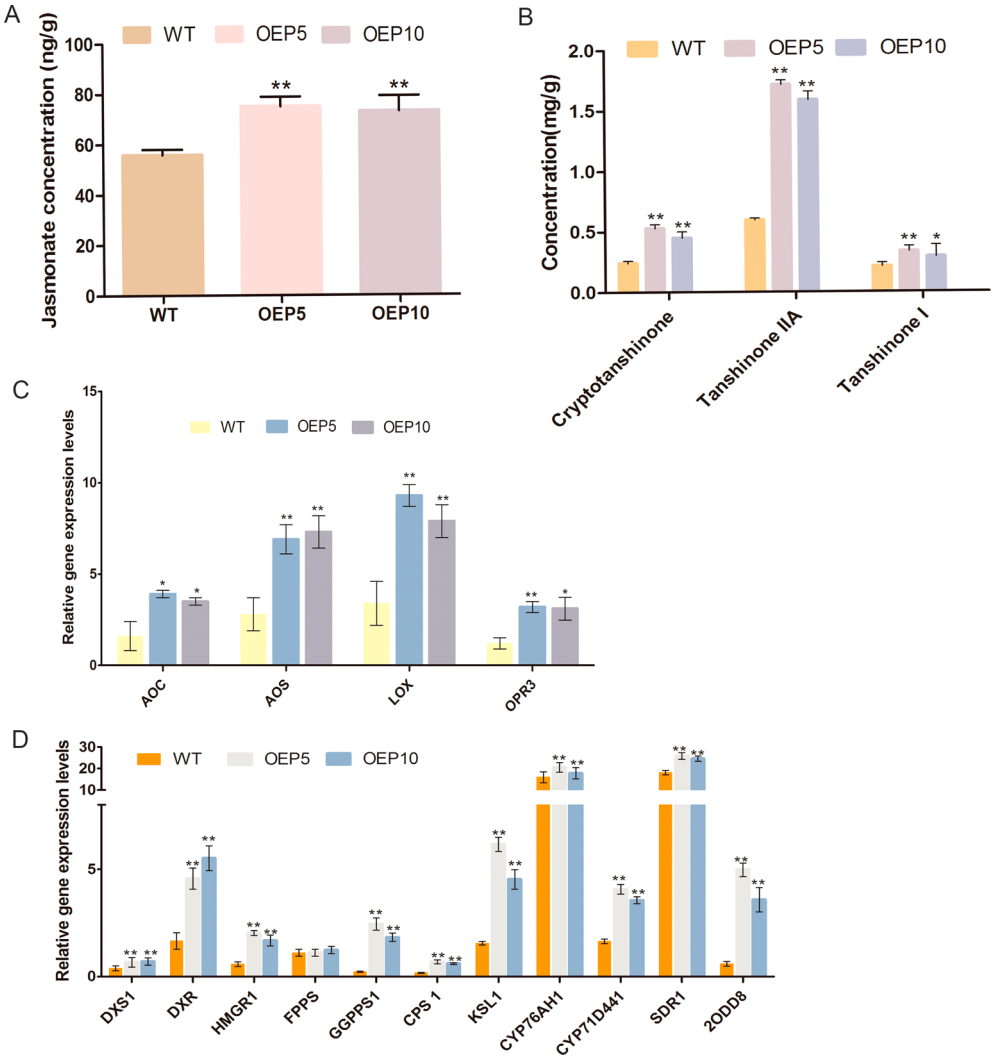

Figure 1. Concentration of JA (A) in leaf extracts and tanshinones (B) in root extracts from nontransformed WT control and transgenic lines OEP5 and OEP10. Relative expression levels of genes involved in JA (C) and tanshinone (D) biosynthesis pathways of the WT control and transgenic lines OEP5 and OEP10. Values are significantly different at $P < 0.01$ (*) and $P < 0.05$ (**). All data are the means of three replicates; error bars indicate SD.

used for separation. The initial oven temperature was 150 °C for 1 min, and the temperature was subsequently increased to 280 °C at 2 °C min^{-1} and held at 280 °C for 5 min. Helium was used as the carrier gas at a constant flow rate of 0.6 mL min^{-1} with a 20:1 split injection ratio.

The transfer line and ion source temperatures were 250 and 280 °C, respectively. Ionization was carried out in electron impact ionization (EI) mode at 70 eV. Mass spectra were acquired using a full scan monitoring mode with a mass scan range of 50 to 500 m/z. Metabolite identifications were based on their matches against mass spectra recorded at the NIST/EPA/NIH 2014 Mass Spectral Library (Upgrade) (Wiley Technology, NJ, USA).

The normalized values (GC/MS and LC/MS spectral data) were used with the SIMCA-P+ software package (version 14.0, Umetrics, Umea, Sweden) as variables and then mean-centered and pareto-scaled prior to multivariate statistical analysis. Principal component analysis (PCA) and PLS-DA analysis were applied to analyze the spectral data to separate the leaves of OEP from the leaves of WT control lines and the roots of OEP from the WT control lines, respectively.

The dried roots and leaves of transgenic plants and WT control plants were extracted for LC/MS analyses as described by Zhang et al.[29] Supernatant (10 μL) was applied to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The mass spectra were acquired using a Bruker Esquire 6000 ion trap instrument with an ESI source (Bruker Daltonics, Inc., USA). HPLC was performed on a Phenomenex C_{18} column (5 μm, 250 mm × 4.6 mm) at a flow rate of 1.0 mL min^{-1}, and PDA was utilized to detect UV−visible absorption at 280 nm. Metabolites were identified on the basis of retention times, UV spectra, and mass fragmentation via MS analysis by comparing them with authentic standards and reported data.[30] To calculate the concentrations of tanshinones accurately, three major tanshinones—tanshinone I, tanshinone IIA, and cryptotanshinone—were quantified with authentic standard. All standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Statistical Analysis. All experimental data presented here were the means of at least three independent replicates, and comparisons between transgenic and WT plants were performed using one-way ANOVA with Duncan's multiple range tests with SPSS software (standard release version 13.0 for Windows).

RESULTS

Molecular Detection of Transgenic *S. miltiorrhiza* Plants Overexpressing *LePS*. We used PCR methods with genomic DNA to confirm that *LePS* was integrated into 11 putative transgenic lines of *S. miltiorrhiza* (Figure S1). Semiquantitative RT-PCR and real-time PCR analyses

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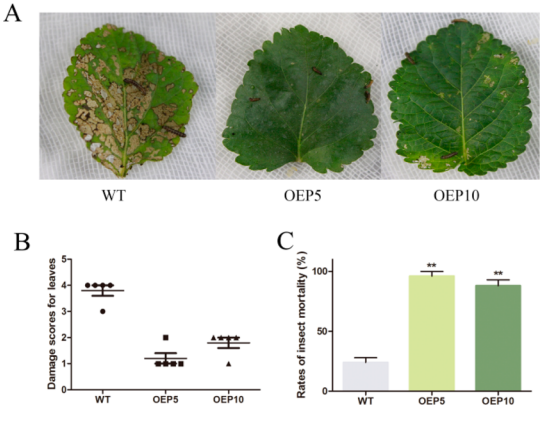

Figure 2. Insect bioassay on leaves detached from nontransformed WT control and transgenic lines OEP5 and OEP10. (A) Photographs represent typical leaf damage. (B) Damage scores for leaves from WT and transgenic plants. (C) Rates of insect mortality for cotton bollworm larvae feeding on leaves from WT and transgenic plants. **, values are significantly different at $P < 0.01$. All data are the means of three replicates; error bars indicate the SD.

demonstrated that this gene was obviously expressed at the transcriptional level in lines OEP3, OEP5, OEP10, and OEP19 (Figure S1). Because expression was highest in OEP5 and OEP10 when compared to the nontransformed WT control, we selected those lines for further examination.

Transgenic *S. miltiorrhiza* Plants Have Higher Concentrations of Endogenous JA and an Enhanced Expression of JA Biosynthesis Genes. Wound signals induce defensible biological processes that protect plants by activating the synthesis of endogenous jasmonates. This defense response is catalyzed by a series of enzymes including lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), and 12-oxophytodienoate reductase 3 (OPR3), which are involved in the octadecanoid pathway.[31,32] The endogenous JA contents of fresh leaves from OEP and WT lines were determined by ELISA. On the basis of the OD values of our samples, JA concentrations were significantly higher for the transgenic OEP lines, i.e., 75.26 ng g^{-1} for OEP5 and 72.36 ng g^{-1} for OPE10 versus 56.78 ng g^{-1} for the WT (Figure 1A).

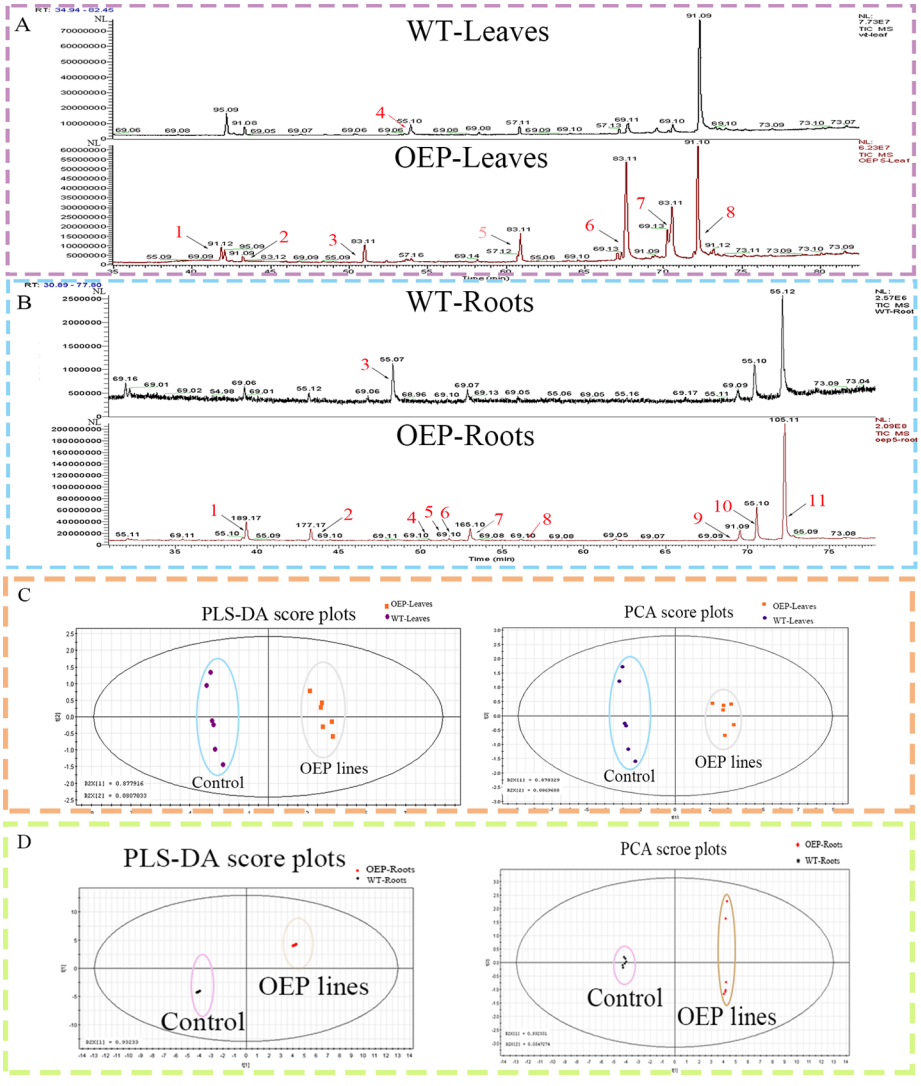

Figure 3. GC/MS chromatogram depicting metabolite peaks of leaves (A) and roots (B) of OEP and the WT control. The peaks labeled by m/z and indicated in red font stand for metabolites that are different between OEP and WT control lines. OPLS-DA and PCA score plots based on GC/MS data of leaves (C) and roots (D) of OEP and WT control lines.

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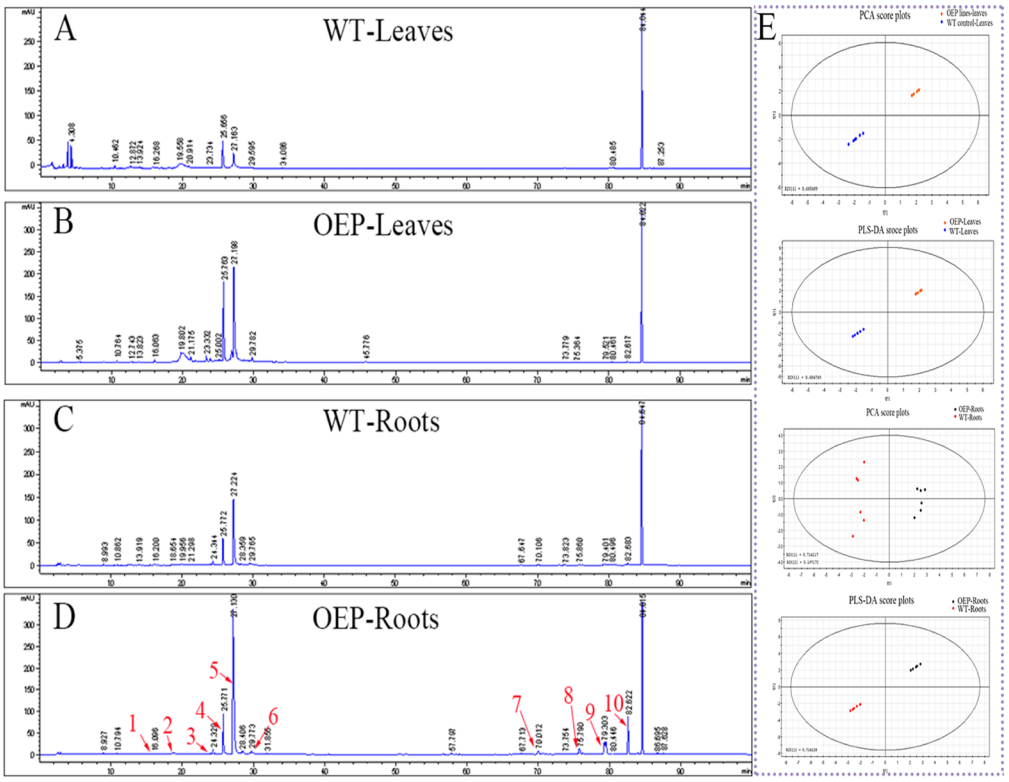

Figure 4. LC/MS chromatogram depicting metabolite peaks of leaves of WT (A) and OEP lines (B) and the metabolite peaks of roots of WT (C) and OEP lines (D). OPLS-DA and PCA score plots based on LC/MS data of OEP and WT control lines (E). The peaks labeled by the remaining time and indicated by red font stand for metabolites that are different between OEP and the WT control lines.

Table 1. Differential Metabolites in Leaves between OEP Lines and Controls Based on GC/MS Data (VIP > 1.0)[a]

differential metabolites	retention time (min)	formula	molecular weight	VIP[b]	MS fragment ions (m/z)	change fold (OEP/W)[c]
9,12-octadecadieneoic acid (linoleic acid)	42.06 ± 0.02	$C_{18}H_{32}O_2$	280	1.07	67, 55, 79, 82, 81	12.96**
9,12,15-all-*cis*-octadecatrienoic acid (linolenic acid)	43.02 ± 0.14	$C_{18}H_{30}O_2$	278	1.06	177, 149, 161, 121, 133	2.02*
2,5-octadecadiynoic acid, methy ester	50.99 ± 0.03	$C_{19}H_{30}O_2$	290	1.16	83, 93, 91, 55, 79	
3-methoxy-estra-2,5(10)-dien-17-ol	53.02 ± 0.10	$C_{19}H_{28}O_2$	288	1.03	83, 55, 117, 105, 53	16.89*
cholestan-3-ol,2-methylene	60.9 ± 0.09	$C_{28}H_{48}O$	400	1.05	57, 71, 85, 55, 83	3.21*
stigmasterol	67.65 ± 0.05	$C_{29}H_{48}O$	412	1.26	83, 137, 55, 69, 81	7.17*
r-sitosterol	70.54 ± 0.01	$C_{29}H_{50}O$	414	1.27	83, 55, 137, 143, 84	7.72**
g-sitosterol	72.21 ± 0.08	$C_{29}H_{50}O$	414	1.36	105, 91, 144, 81, 55	8.45**

[a]All metabolites are identified by the NIST/EPA/NIH 2014 Mass Spectral Library (similarity >70%). Values are significantly different at $P < 0.01$ (*) and $P < 0.05$ (**). All data are the means of six replicates. [b]VIP: Variable importance obtained from PLS-DA analysis. [c]OEP/W > 1 indicates a relatively higher concentration present in OEP lines, and OEP/W < 1 means a relatively lower concentration as compared to the controls; OEP, OEP lines; W, wild-type control.

These results indicated that the overexpression of *LePS* improved endogenous JA accumulations by simulating wound signaling that promoted the production of JA.

We also monitored the transcript levels of fresh leaves of OEP and WT lines for genes involved in the JA biosynthesis pathway and found that the expressions of *LOX*, *AOC*, *AOS*, and *OPR3* were upregulated by more than 2-fold in OEP lines when compared to the WT control (Figure 1C).

Transgenic *S. miltiorrhiza* Plants Show Enhanced Resistance to Cotton Bollworm. Systemin is a primary signal molecule involved in the response to pest attacks and mechanical wounding. To determine whether the overexpression of *LePS* in *S. miltiorrhiza* could enhance its insect resistance, we exposed leaves from both transgenic plants and WT control plants to cotton bollworm larvae (Figure 2A). On the fifth day of treatment, the mean damage score for the WT was 3.80 versus 1.80 for OEP5 and 1.20 for OEP10 (Figure 2B). This suggested that the overexpression of *LePS* led to enhanced insect resistance. In addition, the mortality rate was 3.67- to 4.00-fold higher when insects were fed with leaves from transgenic plants than from the WT (Figure 2C). This finding further confirmed the inhibitory effect of *LePS* overexpression on larval activity.

Metabolic Analyses between OEP and WT Lines. GC/MS and LC/MS were performed to determine the metabolic difference between OEP lines and WT lines. On the basis of the GC/MS and LC/MS data (Figures 3A,B and 4A–D), PCA and PLS-DA analysis were carried out and the results areclearly clustered into two categories that showed significant differences in the metabolic components of the two lines (Figures 3C,D

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Table 2. Differential Metabolites in Roots between OEP Lines and Controls Based on GC/MS Data (VIP > 1.0)[a]

differential metabolites	retention time (min)	formula	molecular weight	VIP[b]	MS fragment ions (m/z)	change fold (OEP/W)[c]
9,12-octadecadienoic acid (Z,Z)-, methyl ester, oxidized	39.09 ± 0.03	$C_{19}H_34O_2$	294	1.05	81, 76, 95, 55, 71	3.04*
ferrgnoid	43.3 ± 0.12	$C_{20}H_{30}O$	286	1.23	55, 69, 91, 286, 98	2.20*
17-octadecynoic acid	48.29 ± 0.10	$C_{18}H_{32}O$	264	1.03	55, 57, 70, 56, 149	5.19*
14-isopropylpodocarpa-8,11,13-triene-7β,13-diol	50.6 ± 0.07	$C_{20}H_{30}O_2$	302	1.06	91, 115, 171, 187, 128	13.14*
isotanshinone II	51.36 ± 0.05	$C_{19}H_{18}O_3$	294	1.08	77, 62, 261, 294, 74	8.16**
9,12-octadecadienal	51.80	$C_{18}H_{32}O$	264	1.06	117, 161, 149, 164, 121	10.23**
tanshinone IIA	52.88 ± 0.03	$C_{19}H_{18}O_3$	294	1.15	73, 147, 281, 221, 99	16.31**
cryptotanshinone	56.14 ± 0.20	$C_{19}H_{20}O_3$	296	1.20	253, 152, 128, 143, 296	5.91**
campasterol	69.54 ± 0.20	$C_{28}H_{48}O$	400	1.21	105, 91, 107, 145, 95	13.83**
stigmastero	70.56 ± 0.09	$C_{29}H_{48}O$	412	1.16	91, 73, 119, 399, 412	9.35**
sitosterol	72.27 ± 0.22	$C_{29}H_{50}O$	414	1.04	105, 69, 67, 81, 95	9.32**

[a]All metabolites identified by NIST/EPA/NIH 2014 Mass Spectral Library (similarity >70%). Values are significantly different at $P < 0.01$ (*) and $P < 0.05$ (**). All data are means of six replicates. [b]VIP: Variable importance obtained from PLS-DA analysis. [c]OEP/W > 1 indicates a relatively higher concentration present in OEP lines, and OEP/W < 1 means a relatively lower concentration as compared to the controls; OEP, OEP lines; W, wild-type control.

Table 3. Differential Metabolites between OEP Lines and Controls Based on LC/MS Data[a]

differential metabolites	retention time (min)	VIP[b]	$[M - H]^-$ (m/z)	$MS^{(n)}$ fragment ions (m/z)	change fold (OEP/W)[c]
protocatechuic aldehyde[d,e]	16.10 ± 0.14	1.08	137	113, 100	2.71*[d], 0.97[e]
ferulic acid[d]	19.80 ± 0.34	1.23	193	150, 179	0.98*[d]
cinnamic acid[d,e]	24.32 ± 0.01	1.21	147	132, 112	1.74*[d], 1.06*[e]
rosmarinic acid[d,e]	25.62 ± 0.11	1.21	359	161, 197, 179	5.54**[d], 1.30**[e]
salvianolic acid B[d,e]	27.72 ± 0.18	1.19	717	519, 339	1.95*[d], 1.56*[e]
iaosalvianolic acid B[d,e]	29.86 ± 0.72	1.02	717	519	2.76*[d], 2.95*[e]
unknown[e]	70.08 ± 0.11	1.01	310	284, 252	2.09*[e]
tanshinone I[e]	75.84 ± 0.22	1.34	277	249, 231	3.32**[e]
cryptotanshinone[e]	79.49 ± 0.08	1.26	297	279, 251	6.18**[e]
tanshinone IIA[e]	82.62 ± 0.06	1.10	279	261, 233	18.13**[e]

[a]Values are significantly different at $P < 0.01$ (*) and $P < 0.05$ (**). All data are the means of six replicates. [b]VIP: Variable importance obtained from PLS-DA, analysis. [c]OEP/W > 1 indicates a relatively higher concentration present in OEP lines, and OEP/W < 1 means a relatively lower concentration as compared to the controls; OEP, OEP lines; W, wild-type controls. [d]Metabolites were detected in leaves. [e]Metabolites were detected in roots.

and 4E). Among those different metabolites, we identified 23 metabolites from leaves and roots listed in Tables 1, 2, and 3 that contained long-chain fatty acids and their derivates (i.e., linoleic acid, linolenic acid, and octadecadiynoic acid ester), sterols (i.e., stigmasterol, sitosterol, and campasterol), terpenoids (i.e., tanshinone I and isotanshinone II), and organic acids (i.e., cinnamic acid, rosmarinic acid, and salvianolic acid B). Those results demonstrated that the overexpression of *LePS* might influence the secondary metabolism of fatty acids, terpenes, and phenolic acid.

We further determined the concentrations of tanahinone I, tanshinone IIA, and cryptotanshinon using HPLC. In the dry roots of OEP lines, the concentrations of tanahinone I, tanshinone IIA, and cryptotanshinone were significantly increased (Figure 1B), the yields of tanshinone I, tanshinone IIA, and cryptotanshinone were 0.43, 1.71, and 0.53 mg g^{-1} for OEP5; 0.39, 1.59, and 0.49 mg g^{-1} for OEP10; and 0.18, 0.59, and 0.24 mg g^{-1} for the WT, respectively. This means that the overexpression of *LePS* increased the production of tanshinone I by 2.17- to 2.39-fold, that of tanshinone IIA by 2.69- to 2.89-fold, and that of cryptotanshinone by 2.04- to 2.21-fold. Therefore, overexpressing *LePS* appears to be an effective strategy for improving the synthesis and accumulation of tanshinones.

Expression of Genes Involved in Tanshinone Biosynthesis. All terpenoids, including tanshinones, are derived from their precursor isopentenyl (IPP) and its isomer dimethylallyl diphosphate (DMAPP). They are produced by two biosynthesis pathways: the mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in the plastids. Metabolic flow in the MVA pathway is controlled by key enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and l-deoxy-D-xylulose 5-phosphate synthase (DXS) is the rate-limiting enzyme in the MEP pathway. Both IPP and DMAPP are further catalyzed by enzymes geranylgeranyl pyrophosphate (GPPS), farnesyl diphosphatesynthase (FPPS), geranylgeranyl pyrophosphate (GGPPS), copalyl diphosphatesynthase (CPS), kaurene synthase-like cyclase (KSL), and newly found CYP enzymes CYP76AH1 and CYP71D441 to form tanshinones. Additionally, the oxygenases (i.e., 2-oxo-glutatatedependent dioxygenases (2ODDs)) and dehydrogenases (i.e., short-chain alcohol dehydrogenases (SDRs)) may also participate in tanshinone production.[5,33]

To examine how the expression of genes related to tanshinone biosynthesis is influenced in transgenic lines, we monitored relative transcript levels for *DXS1*, *DXR HMGR1*, *FPPS*, *GGPPS1*, *CPS1*, *KSL1*, *CYP76AH1*, *CYP71D441*, *SDR1*, and *2ODD8* in roots of OEP and WT lines. Our internal control was the housekeeping *GAPDH*. Relative gene

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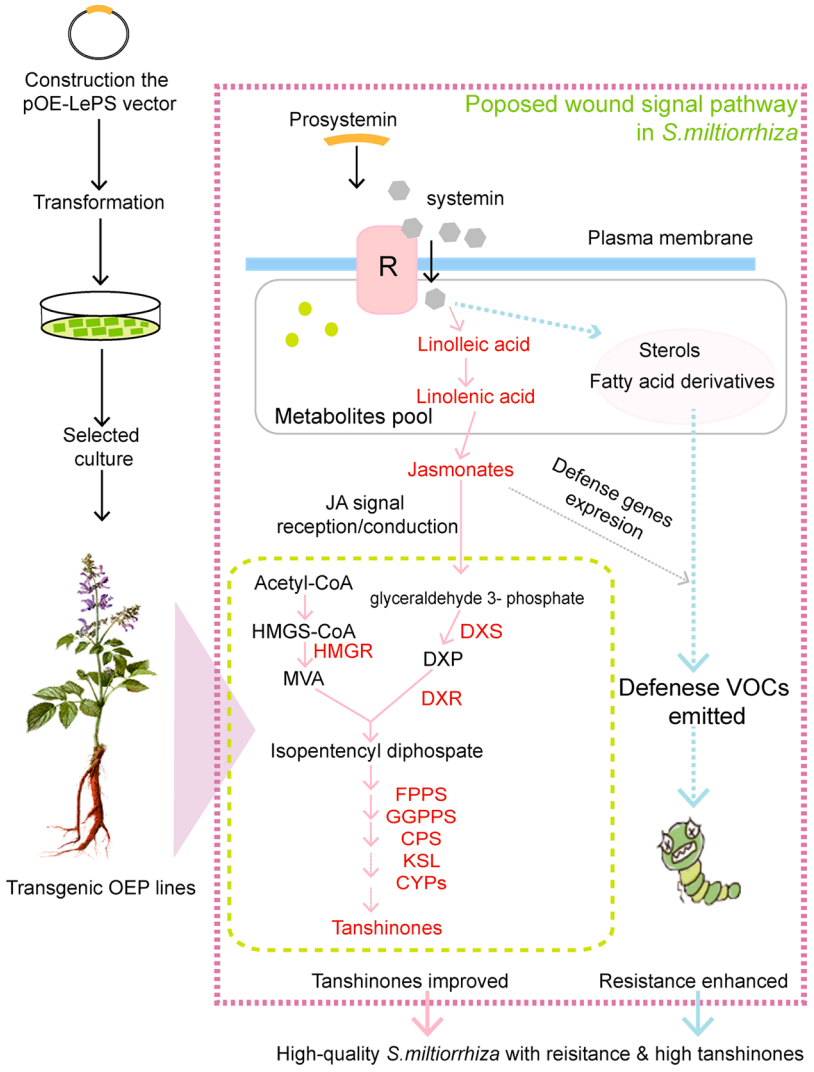

Figure 5. Proposed model for the systemin/JA-signaled response in *Salvia miltiorrhiza*. A recombinant vector was introduced into plants. After selected culture, transgenic lines were obtained. The overexpression of *Prosystemin* leads to systemin cleavage and binding to the proposed receptor (R) in the plasma membrane, triggering JA biosynthesis. Here, JA is catalyzed from linolenic acid by a series of enzymes, including lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). A high JA level activates the expression of genes involved in tanshinone biosynthesis in both MAV and MEP pathways. Meanwhile, systemin/JA could active wound signaling and emit some defense-related chemicals to participate in the defense response. They also might induce defense-related genes against insect feeding via other signaling pathways. In the diagram, red font indicates that levels of genes or chemicals are enhanced.

expression levels were increased by 2- to 4-fold for *DXS1*, *DXR*, *HMGR1* *CPS1*, *KSL1*, *CYP76AH1*, *CYP71D441*, and *SDR1* and by approximately 10-fold for *GGPPS*1 and *2ODD8* (Figure 1D). However, expression rose only slightly for *FPPS* in the transgenics when compared to the WT.

DISCUSSION

Enhancing Endogenous JA Levels in Transgenic *S. miltiorrhiza* Plants Is a Green and Efficient Approach for Introducing Resistance to Insect Pests. *S. miltiorrhiza* is a well-known and widely planted medicinal herb in China and is becoming a major crop plant. However, pests and diseases can cause yield losses of approximately 15 to 45%, making this species less amenable for commercialization. Currently, chemical reagents are the primary tool for preventing plant damage, and little has been done to explore the pathogenic mechanism and develop and antipest germplasm for this species via genetic engineering.[34]

Transgenic lines of tomato and tobacco that overexpress tomato *Prosystemin* have heightened resistance to insects and pathogens.[16,35] When we overexpressed tomato *LePS* in *S. miltiorrhiza*, the OEP lines also showed greater resistance to cotton bollworm larvae. In our data (Tables 1−3), the metabolites related to plant defense were increased significantly in our OEP lines, which might be the cause of enhancement of the antipest ability with the overexpression of *LePS*. Among them, phytosterols (i.e., sitosterol and stigmasterol) might play a role in the plant defense process;[36] some volatile fatty acid derivates (i.e., linoleic acid, 9,12-linoleic acid, and 2,5-methyl-octadecadiynoic acid ester) might produce plant hormones that might participate in the defense signal pathway;[37] and phenolic acids (i.e., cinnamic acid, rosmarinic acid, and salvianolic acid) play roles in the plant resistance of herbivores.[49] Additionally, the transgenic plants did not differ from the nontransformed WT in phenotype when grown in the greenhouse. Therefore, our data indicate that it is possible to manipulate the genome of

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this valuable herb and introduce a new property without negatively affecting other morphological characters. Such an approach might also be used to improve the germplasms and exploit useful traits of other traditional medicinal plants, thereby increasing their yields while also enhancing their resistance to insect pests.

Tanshinone Accumulations Can Be Feasibly Improved through the Heterologous Expression of *LePS* in *S. miltiorrhiza*. As an important compound in *S. miltiorrhiza*, tanshinone can ameliorate cardiac dysfunction, upregulate antioxidant systems, display cytoprotective activity, and be very beneficial for the treatment of Parkinson's disease.[38]

Previously, tanshinone production was most commonly stimulated by using various efficient elicitors in *S. mitiorrhiza* hair root. Those elictors included biotic factors[39,40] such as yeast extract and the endophytic fungus *Streptomyces pactum Act12* and *Trichoderma atroviride D16*, respectively, phytohormones such as salicylic acid, abscisic acid, and JA;[10,41,42] or heavy metal ions such as Co^{2+}, Ag^{+}, and Cd^{2+}.[43] Other research groups have overexpressed genes for rate-limiting enzymes in the tanshinone biosynthesis pathway and have also demonstrated that the genetic manipulation of *S. mitiorrhiza* can enhance tanshinone accumulations.[5,44]

In contrast to those efforts presented above, we have provided a new integrated means for improving levels of tanshinones by exploiting the response of that species to stimulate wounding treatment, i.e., through the overexpression of *LePS*. In OEP lines, relative levels of tanshinones as well as their precursor ferruginol were increased significantly compared to nontransformed WT control. This suggested that the wound signal can be recognized successfully by these plants and activate the downstream responses, including the production and accumulation of tanshinones. Importantly, *DXR1* and *HMGR1* were both upregulated in the OEP lines, which are key enzymes in the MEP and MVA pathway, respectively. Overexpression of *SmDXR* and *SmHMGR* could significantly enhance the yield of tanshinones in transgenic hair root lines, and the enhancement was more evident in the coexpressed of *SmDXR* and *SmHMGR* transgenic lines.[44,45] Besides the genes involved in the upstream tanshinone biosynthesis pathway, other genes involved in the downstream were also significantly upregulated. It suggested that the overexpression of *LePS* might activate the most genes in the whole tanshinone biosynthesis pathway.

Tanshinone Biosynthesis Is Regulated through the Systemin/JA Signal Pathway. When *Prosystemin* is expressed in tomato, the wound signaling pathway is initiated through mitogen-activated protein kinases, and the synthesis of JA is catalyzed by a series of enzymes including LOX, AOS, AOC, and OPR3, which are involved in the octadecanoid pathway.[46] Our investigation also showed that, when compared to the control, JA and its biosynthesis precursors were significantly enhanced and the key genes involved in the JA biosynthesis pathway were upregulated in the OEP lines. This provided evidence that the overexpression of *LePS* could generate a wound signal and proved that systemin was correctly cleaved. Also, our data suggested that tomato and *S. miltiorrhiza* share a similar wound signaling pathway such that the *S. miltiorrhiza* receptor can recognize tomato systemin and activate the downstream response. Furthermore, as a strong elicitor, JA induces tanshinone production that could be mediated by transcriptional regulator *SmJAZs*,[30,47] which conducts the JA signal and regulates the biosynthesis of tanshinone and Sal b by interaction with their target transcription factors, such as MYC2a/b.[48]

Briefly, systemin initiates the wounding signal pathway and activates JA biosynthesis. Then systemin and JA both act as signal molecules in the wound signal pathway that leads to plant defense mechanism wounding. And in this process, JA could play an important role in active defense genes expression and stimulate the synthesis of defense-related metabolites[36,37] to work together in the defense system. In conclusion, we developed a model for the systemin/JA-signaled response in *S. miltiorrhiza* (Figure 5). Overexpression of tomato *Prosystemin* in *S. miltiorrhiza* induced JA biosynthesis via the octadecanoid pathway. High JA concentrations then activated the tanshinone biosynthesis pathway through key genes in both MVA and MEP pathways. Moreover, the overexpression of *LePS* leads to the production of volatile chemicals that work together with JA involved in plant defense. Meanwhile, systermin and JA also appear to induce the expression of defense genes in response to various pests, such as cotton bollworm larvae. Our study provides a new strategy for genetic engineering by which tanshinone production and pest resistance can be improved in *S. miltiorrhiza*. This is accomplished by simulating the wounding signal that increases endogenous levels of JA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02844.

Recombinant vector pOE-LePS from T-DNA schematic and PCR results in OEP and WT lines. Primer sequences used in this study. (PDF)

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Notes

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■ ABBREVIATIONS USED

2ODD, 2-oxo-glutatatedependent dioxygenases; AOC, allene oxide cyclase; AOS, allene oxide synthase; cef, cefotaxime; *CPS*, copalyl diphosphatesynthase; DMAPP, dimethylallyl diphosphate; *DXR*, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *DXS*, l-deoxy-D-xylulose 5-phosphate synthase; *EF*, elongation factor; *FPPS*, farnesyl diphosphatesynthase; *GAPDH*, glycer-

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aldehyde-3-phosphate dehydrogenase; *GGPPS*, geranylgeranyl pyrophosphate; *HMGR*, 3-hydroxy-3-methylglutaryl-CoA reductase; HygB, hygromycin B; IPP, isopentenyl; JA, jasmonate; *KSL*, Kaurene synthase-like; LOX, lipoxygenase; MEP, methylerythritol phosphate; MVA, mevalonate; OPDA, 12-oxo-phytodienoic acid; OPR3, 12-oxophytodienoate reductase 3; *SDR*, short-chain alcohol dehydrogenases; WT, wild-type

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