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Methyl jasmonate-induced compositional changes of volatile organic compounds in *Polygonum minus* leaves

Reyhaneh Rahnamaie-Tajadod, Hoe-Han Goh*, Normah Mohd Noor

Institute of Systems Biology, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia

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ABSTRACT

Polygonum minus Huds. is a medicinal aromatic plant rich in terpenes, aldehydes, and phenolic compounds. Methyl jasmonate (MeJA) is a plant signaling molecule commonly applied to elicit stress responses to produce plant secondary metabolites. In this study, the effects of exogenous MeJA treatment on the composition of volatile organic compounds (VOCs) in *P. minus* leaves were investigated by using a metabolomic approach. Time-course changes in the leaf composition of VOCs on days 1, 3, and 5 after MeJA treatment were analyzed through solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC–MS). The VOCs found in MeJA-elicited leaves were similar to those found in mock-treated leaves but varied in quantity at different time points. We focused our analysis on the content and composition of monoterpenes, sesquiterpenes, and green leaf volatiles (GLVs) within the leaf samples. Our results suggest that MeJA enhances the activity of biosynthetic pathways for aldehydes and terpenes in *P. minus*. Hence, the production of aromatic compounds in this medicinal herb can be increased by MeJA elicitation. Furthermore, the relationship between MeJA elicitation and terpene biosynthesis in *P. minus* was shown through SPME–GC–MS analysis of VOCs combined with transcriptomic analysis of MeJA-elicited *P. minus* leaves from our previous study.

1. Introduction

Plant interactions with the environment lead to the accumulation of diverse natural products that are believed to influence plant fitness (Bourgaud et al., 2001). These natural products are secondary metabolites that mostly function as defense molecules against adverse conditions and play a role in plant communications with other plants, animals, and/or microbes (Spinelli et al., 2011).

Volatile organic compounds (VOCs) are a large group of secondary metabolites. VOCs play a role in mediating plant responses to stress, defense, and reproduction (Pierik et al., 2014). VOCs help plants attract pollinators and seed dispersers, defend against herbivores and pathogens (below- and above-ground), protect against fungi and parasites, and protect against abiotic stresses, such as extreme light and temperature (Dudareva et al., 2013). Terpenoids, phenylpropanoids, and C6 green-leaf volatiles (GLVs) are the dominant compounds among the VOCs in many plants (Holopainen and Gershenzon, 2010; ul Hassan et al., 2015).

Elicitation is the most practical way of inducing the biosynthesis of various defense metabolites in plants or tissue cultures (Angelova et al., 2006; Namdeo, 2007). Elicitation using chemicals related to plant signaling molecules is the most effective way to increase secondary metabolite production (Hussain et al., 2012). Jasmonates (JAs), which comprise jasmonic acid (JA) and its volatile methyl ester (methyl jasmonate, MeJA), are plant signaling molecules commonly applied as elicitors (Pauwels et al., 2009). Most studies, such as those in Norway spruce (Martin et al., 2003), Scots pine, and evergreen oak (Semiz et al., 2012), have focused on the emissions compared to the content of VOCs after MeJA elicitation. This is in contrast to studies on the MeJA-elicited roots of *P. minus* (Ismail et al., 2011) and exogenous application of JAs to stimulate the production of bioactive compounds, such as terpenes in *Centella asiatica* (Tugizimana et al., 2015), anthocyanin in *Vitis vinifera*(Tassoni et al., 2012), rosmarinic acid in *Mentha piperita* (Krzyzanowska et al., 2012), bacoside A in *Bacopa monnieri*(Sharma et al., 2013), andrographolide in *Andrographis paniculata* (Sharma et al., 2015), and plumbagin in the hairy roots of *Plumbago indica* (Gangopadhyay et al., 2011).

Abbreviations: GC–MS, gas chromatography–mass spectrometry; GLV, green leaf volatile; HD, hydrodistillation; HS, headspace; JA, jasmonate; MeJA, methyl jasmonate; MT, monoterpene; MVA, mevalonate pathway; PCA, principal component analysis; PDMS, polydimethylsiloxane; PLS-DA, partial least squares-discriminant analysis; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SPME, solid-phase microextraction; ST, sesquiterpene; VIP, variable importance in projection; VOC, volatile organic compound

* Corresponding author at: Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.
E-mail address: gohhh@ukm.edu.my (H.-H. Goh).

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Polygonum minus Huds. (syn. *Persicaria minor*), commonly known as kesum in Malaysia (Bulkill, 1966), belongs to the Polygonaceae family (Bunawan et al., 2011). The essential oil of *P. minus* leaves contains high levels of terpenes (Baharum et al., 2010), aldehydes (Yaacob, 1990), and phenolic compounds (Maizura et al., 2011). *P. minus* is known for its medicinal properties and traditional usages (Wan-Ibrahim et al., 2010). Decoctions of *P. minus* leaves and seeds are used for digestive disorders, while its oil is used in aromatherapy and for dandruff treatment (Vikram et al., 2014). *P. minus* is reported to possess antioxidant (Huda-Faujan et al., 2007), antimicrobial (Uyub et al., 2010), and anticancer (Ahmad et al., 2018) properties. In an effort to understand the production of secondary metabolites in *P. minus*, a recent leaf and root transcriptomic study identified key genes involved in the biosynthesis of phenylpropanoids and flavonoids (Loke et al., 2017).

The advent of analytical tools in metabolomics has improved our ability to measure time-course changes of many metabolites in response to various environmental stimuli. The complexity of VOCs can now be analyzed using the headspace (HS) solid-phase microextraction (SPME) method coupled with the gas chromatography-mass spectrometry (GC–MS) approach (Lee et al., 2013). Tamogami et al. (2015) showed MeJA-induced emissions of volatile methyl (E)-2-hexenoate in *Achyranthes bidentata* through SPME-GC–MS. The induction of volatile compounds upon MeJA application on grape berry pulp was also determined by the HS-SPME-GC–MS technique (Wang et al., 2017). A similar approach was taken in *P. minus* to study the temperature effects on the leaf compositions of VOCs and flavonoids in different plant populations (Goh et al., 2016).

The integration of metabolomic and transcriptomic data helps in the discovery of defense-related genes and compounds under elicitation (Kliebenstein, 2012). This integrative approach was previously applied to investigate the biosynthesis of diterpenoid tanshinones and coumarins in *Salvia miltiorrhiza* and *Peucedanum praeruptorum* (Gao et al., 2014; Zhao et al., 2015), respectively. Furthermore, novel genes involved in the JA-mediated accumulation of secondary metabolites were also identified from *S. miltiorrhiza* (Ge et al., 2015). Hirai et al. (2004) and Jia et al. (2016) studied the changes in the transcriptomes and metabolomes of *Arabidopsis thaliana* and *Astragalus membranaceus*, respectively, under different stresses to identify key pathways in the metabolic regulation of stress responses. Moreover, this integrative analysis in *Salvia pomifera* provided insights into the isoprenoid biosynthetic pathway through the identification of key enzymes involved in terpene biosynthesis (Trikka et al., 2015).

An integrative approach is also informative for studying MeJA-induced gene expression and the accumulation of secondary metabolites in *P. minus*. Previously, we investigated the transcriptome-wide response in *P. minus* leaves within 24 h of MeJA elicitation using an RNA-sequencing approach (Rahnamaie-Tajadod et al., 2017). Here, we study the metabolic response of *P. minus* leaves to MeJA elicitation through HS-SPME-GC–MS. Elicited leaves were sampled at different time points to determine compositional changes in secondary metabolites. This was integrated with a transcriptomic study to better understand the transcriptional regulation of the biosynthesis of terpenes, particularly sesquiterpenes (STs). This integrative analysis also provides potential gene targets for metabolic bioengineering, such as that of flavonoid production (Ku Bahaudin et al., 2018), to improve the production of useful VOCs in *P. minus*. Furthermore, this report complements recent efforts regarding the functional characterization of *P. minus* terpene synthesis (Ee et al., 2013; Ker et al., 2017; Rusdi et al., 2018; Tan and Othman, 2012).

2. Materials and Methods

2.1. Plant materials

Cuttings of *Polygonum minus* plants were sampled from the Genting Highland (3° 25′ 42.18″ N, 101° 47′ 21.45″ E), Malaysia and were propagated using compost soil in a controlled environment chamber (A1000 Conviron, Canada) at 22/16 °C day/nighttime temperatures under 12 h light/dark photoperiods with a light intensity of 170 ± 20 μmol m^{-2} s^{-1} at 75% relative humidity. After forty-five days, nine plants were sprayed with a distilled water solution of 150 μM MeJA (Sigma-Aldrich, USA) and 0.01% (v/v) Tween 20 (Sigma-Aldrich, USA) (Khairudin et al., 2014). The control plants were mock treated with distilled water containing 0.01% Tween 20. The treated and mock-treated plants were grown separately in different growth chambers.

A total of five expanded young leaves from the apical parts of different individual plants were collected independently to avoid the confounding effects of wounding (Sharma et al., 2013) and were pooled as one biological replicate at 24, 72, and 120 h (days 1, 3, and 5, respectively) after treatment for both the control and MeJA-treated samples. For each time point, at least three biological replicates were prepared from both control and treated conditions. The harvested leaf samples were flash frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. SPME extraction of volatiles

Approximately 300 mg of fresh leaves were ground in liquid nitrogen and then placed in a 20 mL headspace glass vial (flat bottom, Perkin Elmer, USA). (R)-(+)-limonene (4.5 mM) (Sigma-Aldrich, USA) was added as an internal standard to the ground leaves, and the vial was then tightly covered with a PTFE/silicon septum cap (Watson et al., 2015).

To compare the effects of elicitation on the composition of VOCs, the SPME method was applied using 100 μm of polydimethylsiloxane (PDMS) fiber (Supelco, USA) to extract the volatile metabolites (Huang et al., 2011). PDMS fibers provide high sensitivity of detection, fast sampling, and good reproducibility (Ma et al., 2012). For desorption, the fiber was exposed to the sample headspace by inserting the fiber through the septum, and the vial with the exposed fiber was incubated in a water bath at 45 °C for 20 min. After incubation, the fiber with trapped volatiles was thermally desorbed by injecting the fiber into the GC–MS injector at 250 °C for 10 min (Ahmad et al., 2014). Three biological replicates with three technical replicates for each treatment were analyzed for each time point.

The separation of volatiles was achieved with a Clarus 600 T series GC–MS device (Perkin Elmer, USA) equipped with a 30 m × 0.25 mm × 0.25 μm Elite-5 MS column (Perkin Elmer, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. According to Goh et al. (2016), the column temperature was increased from 40 °C to 220 °C at a rate of 4 °C/min, the injector temperature was 250 °C, the injection volume was 1 μL, and the transfer temperature was 280 °C. Mass spectrometry parameters were as follows: EI mode, with ion source, 180 °C; electron energy, 70 eV and scan range, 50–600 mass units.

2.3. Compound identification

All peaks from each sample generated by TuboMass workstation were tabulated into a single table in Microsoft Excel. Compound identification was based on the National Institute of Standards and Technology (NIST, version 2.0) library data and the Wiley Registry 8th Edition database of mass spectra (https://www.sisweb.com/software/wiley-registry.htm) with literature information. The identification of volatiles was based on the name of the compounds after filtering the match and reverse match with cut-off values of 700. To normalize the data and quantify the relative proportions of constituents, calculations were based on the relative quantification method with peak area normalization against the internal standard.

2.4. Multivariate data analysis

A multivariate statistical analysis was carried out through SIMCA-P

+ (version 12.0, Umetrics, Sweden) software using the Pareto scaling (square root of the standard deviation) method. A principal component analysis (PCA) algorithm using an unsupervised clustering method was used to map data points for visualizing general clustering and outliers between observations. Supervised partial least square discriminant analysis (PLS-DA) using the regression method was used to represent PCA regression analogies and to assess the covariance between the sample classifications (Steingass et al., 2015).

2.5. Reverse transcription quantitative PCR validation

A total of six candidate genes related to ST synthases were chosen based on previous transcriptome data (Rahnamaie-Tajadod et al., 2017), which was deposited at DDBJ/EMBL/GenBank under the accession GEFX00000000. Using PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software, primers were designed to amplify short regions of each gene, ranging from 75 to 200 bp (Supplementary Table S1). Total RNA was extracted using the modified Lopez-Gomez method (Abdul-Rahman et al., 2017; Lopez-Gomez and Gomez-Lim, 1992) from at least three biological replicates and was treated using the DNA-free™ DNase clean-up Kit (Huntingdon, UK) followed by cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA) according to the manufacturer's instructions.

Reverse transcription quantitative PCR (RT-qPCR) was performed using the iTaq Universal SYBR® Green SuperMix kit and the iQ™5 Real-Time PCR detection System (Bio-Rad, Hercules, CA). Amplification was performed according to Rahnamaie-Tajadod et al. (2017) using three independent biological replicates of the control and treated samples, each with three technical replicates. The relative fold change calculation was based on the comparative C_T ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak, 2008), normalized to the C_T values of the reference gene CDPK (Calcium-Dependent Protein Kinase, comp58469_c0_seq3).

2.6. Author contributions

Conceived and designed the experiments: RRT, HHG. Performed the experiments: RRT. Analyzed the data: RRT, HHG. Wrote the manuscript: RRT, HHG, NMN.

3. Results

3.1. Effects of MeJA treatments on the overall compositions of VOCs in P. minus leaves

A total of 44 compounds were detected from *P. minus* leaves in which all samples contained similar terpenes, alkanes, alkenes, and green leaf volatiles (GLVs) (Table 1). However, the composition of the compounds changed across different time points. In the control plants, sesquiterpenes (STs) were the most abundant (55%) of all VOCs, represented by 24 compounds, followed by 6 (14%) monoterpenes (MTs); 10 (23%) GLVs; 2 (5%) alkanes, one homoterpene, and one alkene. The largest family of VOCs in the control *P. minus* leaves was terpenes (70%). The most abundant STs were β-farnesene and β-caryophyllene, accounting for 56% of the total STs. α-Pinene and bornyl acetate comprised almost 89% of the total MTs; aldehydes decanal and dodecanal comprised almost 98% of the total GLVs, whereas undecane comprised nearly 80% of the total alkanes.

3.2. Effects of MeJA treatment on sesquiterpenes

GC–MS analysis showed that the production of STs increased approximately 8.6-fold by MeJA one day after treatment compared with the control plants (Supplementary File 1). Hence, STs, which comprised 7.4% of the mean peak area in the control samples, increased to 64.6% of the total terpenes after MeJA treatment (Supplementary File 1). Moreover, the amount of β-farnesene and β-caryophyllene increased more than 9- and 8-fold, respectively, after 24 h of MeJA treatment (Fig. 1) compared with the control plants.

3.3. Effects of MeJA treatment on monoterpenes

The amount of total MTs also increased (more than sevenfold) (Supplementary File 1) within the first 24 h after treatment compared with the control. As shown in Table 1, α-pinene and bornyl acetate were the most abundant constitutively produced MTs, with 8- and 9-fold increases, respectively, 24 h after MeJA treatment (Fig. 2). The other volatile MTs induced by MeJA included β-ocimene, β-pinene, and terpinolene with a greatest increase of 12-fold (Fig. 2). Notably, myrcene did not show significant MeJA induction.

3.4. Effects of MeJA treatment on GLVs

In *P. minus* leaves, we identified ten GLV compounds, including two alcohols and eight aldehydes. There was a great effect of MeJA treatment on the composition of GLV compounds, as the total amount of GLVs increased more than 10-fold under MeJA treatment (Supplementary Fig. 2).

In the control *P. minus* leaves, the aromatic volatiles with the highest amounts were decanal and dodecanal. The aldehydes decanal and dodecanal were also the principal GLVs (51.4% and 46.6% of total GLVs, respectively), showing approximately 10-fold increases within 24 h of MeJA treatment (Table 1). Interestingly, 2-hexenal increased more than 16-fold after 24 h of MeJA treatment, followed by 1-decanol, nonanal, hexanal, and tetradecanal (Fig. 3). All GLVs showed significant upregulation of six- to 16-fold, which drastically declined to less than 2.5-fold from day 3 onwards.

3.5. Effect of MeJA treatment at late time points

The VOC compositions on days 3 and 5 in the control plants were dominated by similar compounds as for the day 1 control plants (Table 1). MeJA treatment significantly increased the amounts of 19 and 13 STs on days 3 and 5, respectively, in which β-farnesene, β-caryophyllene, α-bergamotene, α-panasinsen, valencene, farnesol, 4,11-selinadiene, aromadendr-1-ene, β-elemene, caryophyllene oxide, α-selinene, and isocaryophyllene were the common 12 significantly increased STs (Table 1). Furthermore, increased ST production on day 1 was reduced from 8.7-fold to 1.8- and 1.5-fold on days 3 and 5, respectively, after MeJA treatment (Fig. 1).

Among the six MTs, bornyl acetate was the only MT with significantly greater amounts on day 3 and similarly for terpinolene on day 5 (Fig. 2). Enhanced MTs in MeJA-treated samples were reduced to 2.2- and 1.4-fold on days 3 and 5, respectively, compared to 7.9-fold after 24 h (Supplementary File 1).

On the other hand, seven and six GLVs were significantly more abundant on days 3 and 5 for the MeJA-treated samples, with 2.1- and 1.5-fold increases, respectively, compared to a 10-fold increase on day 1 (Supplementary File 1). The six GLVs that were higher since day 1 included decanal, dodecanal, tetradecanal, undecanal, 1-decanol, and nonanal (Table 1, Fig. 3).

3.6. Multivariate analysis

The final peak lists after filtering and normalization were used to evaluate the metabolic responses of *P. minus* leaves to MeJA elicitation through multivariate analysis. Unsupervised PCA analysis did not show distinct clustering of samples in the principal component space, which was dominated by the great differences on day 1 for the MeJA-treated samples (Supplementary Fig. 3). Therefore, supervised PLS-DA analysis was conducted to generate a better sample separation (Fig. 4).

The PLS-DA plot shows clear separation between controls and treated samples, especially at 24 h, with samples of different time points

Table 1
Metabolites identified from *P. minus* leaves by SPME-GC–MS analysis.

No	Compound [a]	Retention Indices [b]	Composition[c]					
			Control			MeJA-Treated		
			Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
	Alkane							
1	Undecane	1101	0.551	0.163	0.092	**3.005**	**0.198**	**0.145**
2	Nonane	900	0.144	0.061	0.047	**1.460**	**0.153**	0.079
	Alkene							
3	1-Nonene	888	0.002	0.003	0.002	**0.057**	0.007	0.004
	GLV							
4	Decanal	1209	37.181	18.088	12.233	**367.036**	**37.359**	**16.553**
5	Dodecanal	1413	33.701	14.571	10.210	**346.228**	**31.875**	**16.213**
6	Tetradecanal	1614	0.416	0.270	0.203	**4.899**	**0.499**	**0.296**
7	2-Hexenal	854	0.410	0.1855	0.143	**6.924**	**0.250**	0.153
8	Hexanal	803	0.277	0.103	0.068	**3.573**	0.134	0.087
9	Undecanal	1308	0.144	0.061	0.047	**1.460**	**0.153**	**0.079**
10	1-Decanol	1274	0.139	0.112	0.076	**1.995**	**0.272**	**0.116**
11	Nonanal	1106	0.023	0.014	0.010	**0.306**	**0.033**	**0.016**
12	1-Nonanol	1173	0.008	0.005	0.006	**0.049**	0.010	0.005
13	2-Decenal	1250	0.007	0.002	0.003	**0.052**	0.005	0.002
	Terpene (monoterpene)							
14	α-Pinene	932	0.436	0.175	0.097	**3.467**	0.237	0.106
15	Bornyl acetate	1285	0.325	0.141	0.094	**3.142**	**0.353**	0.156
16	(E)-β-Ocimene	1038	0.067	0.016	0.013	**0.229**	0.020	0.013
17	β-Pinene	981	0.011	0.004	0.002	**0.071**	0.008	0.003
18	Myrcene	992	0.008	0.004	0.002	0.020	0.007	0.004
19	Terpinolene	1079	0.006	0.003	0.002	**0.074**	0.007	**0.005**
	Terpene (sesquiterpene)							
20	β-Farnesene	1457	2.496	1.033	0.516	**22.821**	**1.433**	**0.716**
21	β-Caryophyllene	1467	1.735	0.705	0.509	**13.940**	**1.322**	**0.742**
22	α-Bergamotene	1431	0.520	0.1869	0.168	**4.907**	**0.387**	**0.210**
23	α-Panasinsen	1519	0.325	0.141	0.094	**3.142**	**0.353**	**0.156**
24	Valencene	1489	0.324	0.147	0.068	**2.575**	**0.292**	**0.130**
25	Farnesol	1544	0.280	0.190	0.147	**2.722**	**0.449**	**0.253**
26	4,11-Selinadiene	1498	0.268	0.075	0.045	**1.914**	**0.167**	**0.102**
27	Aromadendr-1-ene	1457	0.258	0.073	0.043	**1.859**	**0.204**	**0.087**
28	β-Elemene	1393	0.224	0.089	0.053	**1.746**	**0.167**	**0.104**
29	Caryophyllene oxide	1573	0.207	0.117	0.087	**2.318**	**0.222**	**0.144**
30	Germacrene D	1487	0.151	0.117	0.040	**1.137**	0.185	**0.053**
31	Nerolidol	1561	0.115	0.070	0.065	**1.045**	**0.154**	0.081
32	Alloaromadedrene	1503	0.092	0.037	0.025	**0.709**	**0.094**	0.040
33	(-)-Drimenol	1770	0.060	0.064	0.053	**0.636**	0.093	0.038
34	Zingiberene	1494	0.060	0.028	0.021	**0.340**	**0.080**	0.028
35	β-Bisabolene	1507	0.058	0.028	0.024	**0.470**	**0.070**	0.032
36	α-Sesquiphellandrene	1523	0.053	0.023	0.014	**0.465**	**0.051**	0.019
37	α-Selinene	1484	0.050	0.021	0.013	**0.451**	**0.046**	**0.024**
38	Germacrene D-4-ol	1574	0.043	0.024	0.019	**0.369**	**0.040**	0.031
39	Glubulol	1585	0.040	0.011	0.015	**0.301**	0.018	0.020
40	Isocaryophyllene	1438	0.037	0.017	0.017	**0.385**	**0.042**	**0.023**
41	Copaene	1375	0.031	0.014	0.010	**0.279**	**0.027**	0.013
42	β-Himachalene	1478	0.007	0.013	0.003	**0.035**	0.007	0.005
43	Bicycloelemene	1333	0.006	0.004	0.001	**0.050**	0.004	0.002
	Terpene (homoterpene)							
44	E-DMNT	1103	0.000	0.005	0.003	**0.129**	0.008	**0.005**

[a] Identified by GC–MS; names according to the NIST mass spectral library.
[b] Experimentally determined Kováts retention indices.
[c] The composition of each component is calculated as the peak area of analyte divided by the peak area of the internal standard. The data represents the means of three biological replicates. Bolded fonts denote significant ($P < 0.05$) differences compared to the controls based on two-tailed *t*-test.

clustered separately (Fig. 4). Moreover, day 1 and day 3 treated samples were more distinct than those on day 5, which clustered closer to the control samples. This indicates that the compositions of VOCs for the day 5 treated samples were more similar to the controls, as supported by GC–MS analysis (Table 1).

The importance of the metabolites in PLS-DA models can also be evaluated by applying a variable importance in a projection (VIP) plot, which summarizes the contributions of the key variables. In the VIP plot, decanal, dodecanal, α-farnesene, 2-hexanal, and ß-caryophyllene contributed significantly to the model with VIP > 1.0 (Supplementary Fig. 4). Therefore, this is consistent with the observation that MeJA elicitation stimulated a significant increase in the level of the volatiles by triggering changes in the biosynthesis of secondary metabolites and culminating in metabolomic alterations in *P. minus* leaves.

3.7. Metabolomic and transcriptomic integration for study of terpene biosynthesis

We combined metabolomic profiling of VOCs from current analysis with previously reported transcriptomic analysis to assess any relationship between the JA pathway and terpene biosynthesis in *P. minus*. We hypothesized that the accumulation of VOCs in *P. minus* leaves is linked to the increased levels of transcripts for genes involved in the biosynthetic pathways.

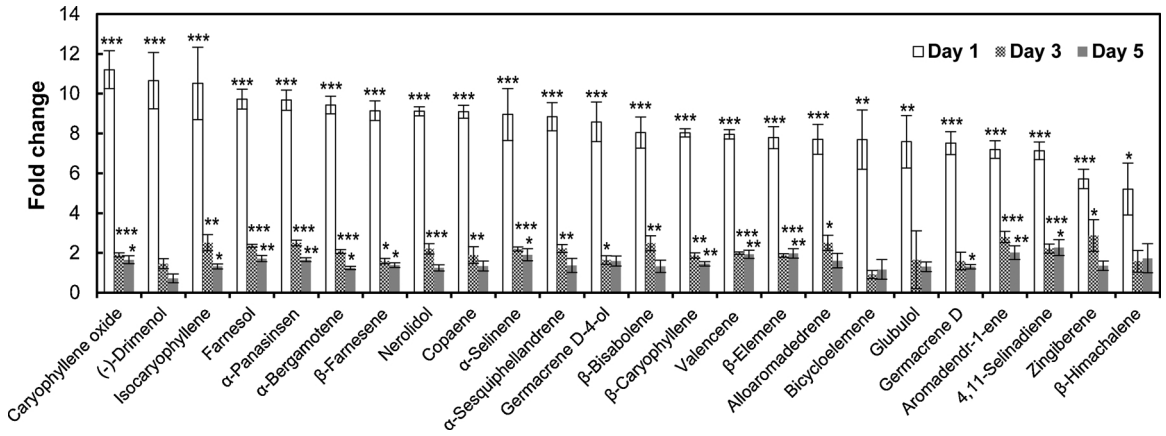

Fig. 1. MeJA-induced accumulation of sesquiterpenes in treated samples relative to the controls at three different time points. Fold change is calculated based on the ratios between the averages of the treated and control samples. Error bars represent combined standard errors from three biological replicates of the control and treated samples. Two-tailed *t*-test based on relative peak area % of three biological replicates: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

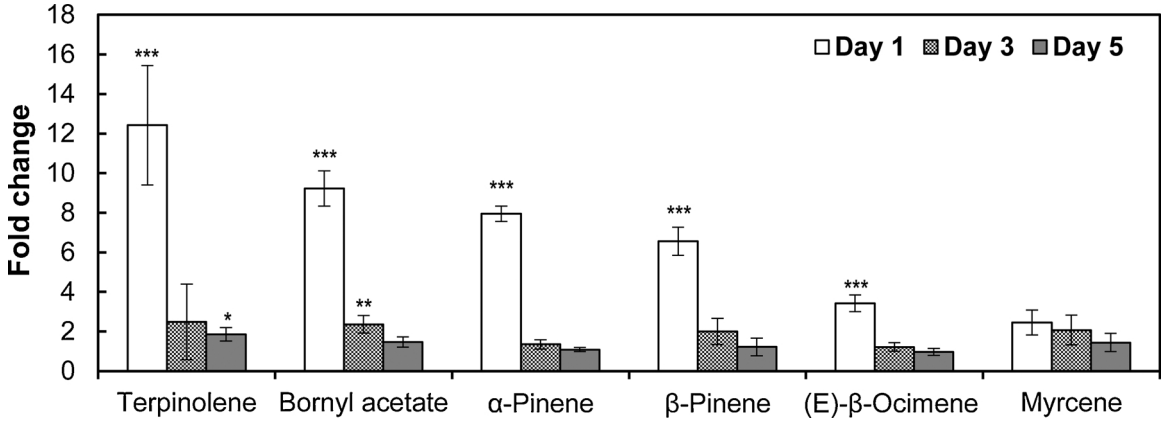

Fig. 2. MeJA-induced accumulation of monoterpenes in treated samples relative to the controls at three different time points. Fold change is calculated based on the ratios between the averages of treated and control samples. Error bars represent combined standard errors from three biological replicates of the control and treated samples. Two-tailed *t*-test based on relative peak area % of three biological replicates: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Based on metabolomic experiments conducted in this study, we focused on the genes in pathways related to terpene biosynthesis. According to Rahnamaie-Tajadod et al. (2017), a total of 94 *P. minus* transcripts (Supplementary File 2) were predicted to encode enzymes involved in the main steps of the terpene backbone precursor biosynthesis pathway (KEGG entry 00900). In plants, two biosynthetic pathways are responsible for terpene biosynthesis: the mevalonate pathway (MVA) and the non-mevalonate pathway (MEP). Hence, we targeted genes participating in the upstream isoprenoid precursors of the MEP and MVA biosynthetic pathways. Fig. 5 was constructed based

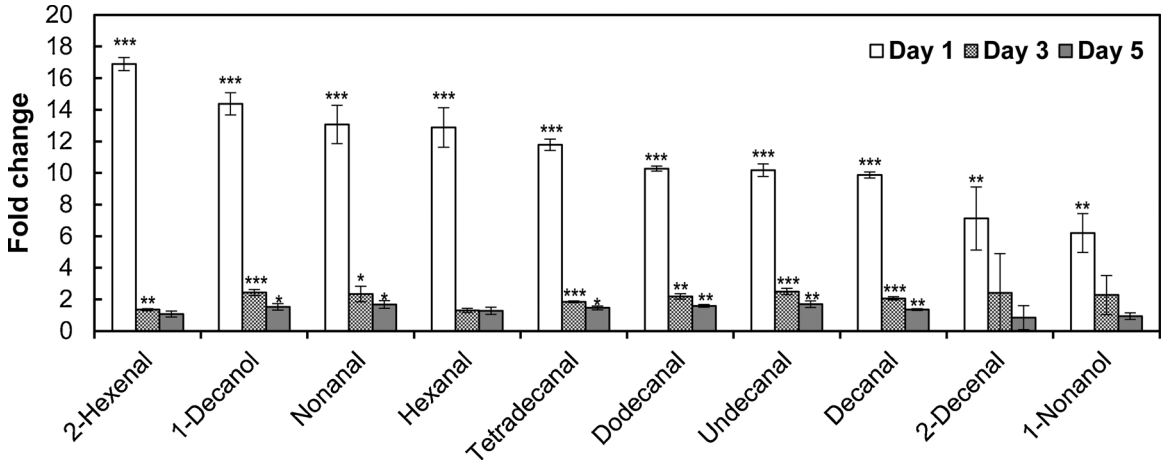

Fig. 3. MeJA-induced accumulation of green leaf volatiles in treated samples relative to the controls at three different time points. Fold change is calculated based on the ratios between the averages of treated and control samples. Error bars represent combined standard errors from three biological replicates of the control and treated samples. Two-tailed *t*-test based on relative peak area % of three biological replicates: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

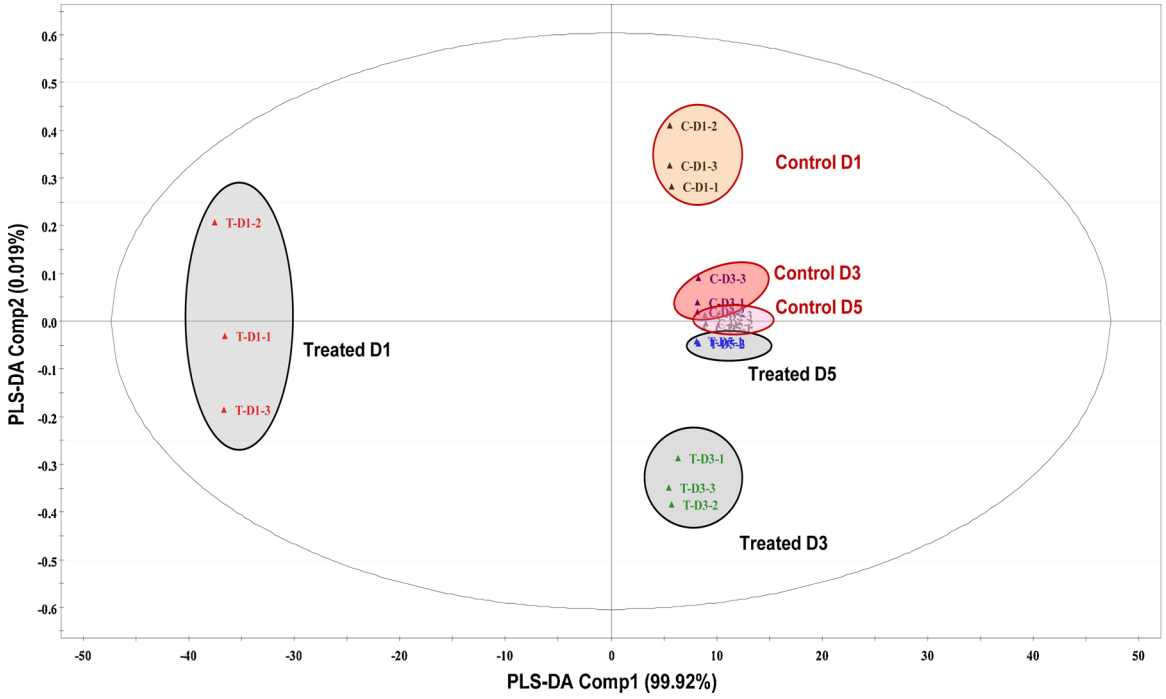

Fig. 4. PLS-DA score plot showing a clear separation between the controls and treated leaf samples at different time points after MeJA treatment. Each group is represented by three biological replicates. D: day.

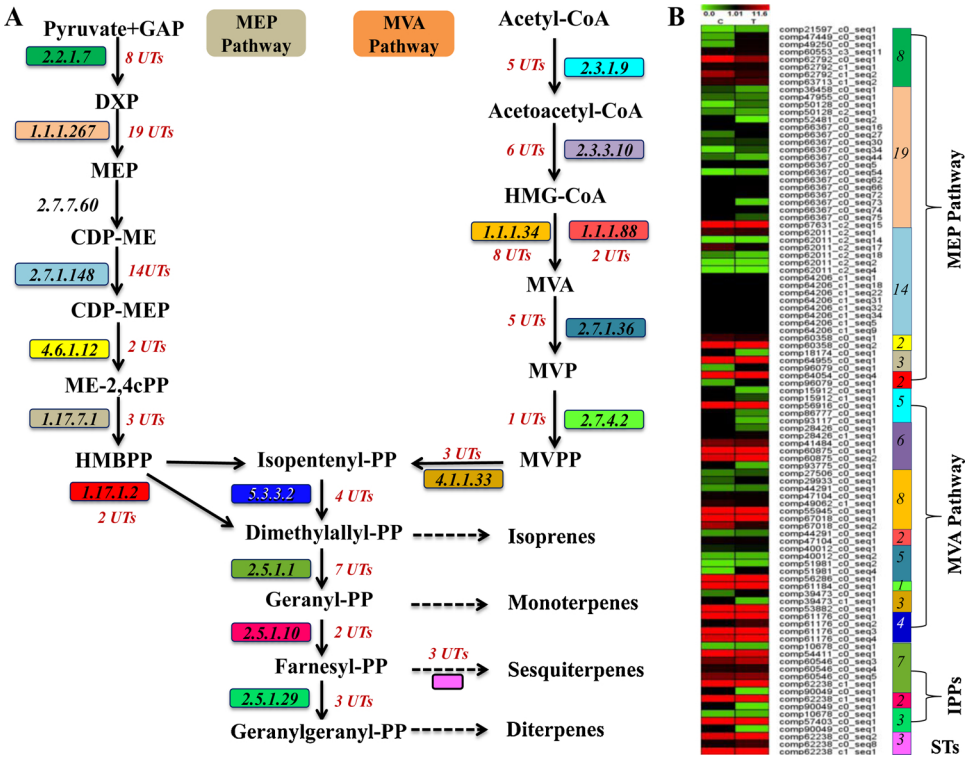

Fig. 5. Transcriptional effect of MeJA on the pathway of terpenoid biosynthesis. (A) Biosynthetic pathway for terpenoids, beginning with MVE and MEP pathways and leading to isopentenyl diphosphate (IPP) precursors. Each colored box represents mapped transcripts (UTs) to the respective enzymes. (B) Expression heatmap of *P. minus* UTs, which are putatively involved in the MVA and MEP pathways of terpenoid biosynthesis in leaves. The color bar at the top of the heatmap figure shows the color intensity according to normalized transcript abundance (FPKM). C: control; T: treated. GAP: D-glyceraldehyde-3-phosphate; DXP: 1-deoxy-D-xylulose-5-phosphate; MEP: 2-C-methyl-D-erythritol 4-phosphate; CDP-ME: 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol phosphate; ME-2,4cPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP: 4-hydroxy-3-methylbut-2-enyl diphosphate; CoA: coenzyme A; HMG: 3-hydroxy-3-methylglutaryl; MVA: mevalonate; MVP: mevalonate-5-phosphate; MVPP: mevalonate-5-diphosphate; PP: pyrophosphate.

on the canonical pathway involved in terpene biosynthesis as referenced from the KEGG database and using results from previous transcriptomics analysis (Rahnamaie-Tajadod et al., 2017). Fig. 5A shows the enzymes in the MEP and MVA biosynthetic pathways and the number of transcripts encoding these enzymes based on transcriptomic analysis.

The first enzyme/protein of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXP, EC: 2.2.1.7), is encoded by eight transcripts (Fig. 5). Another important enzyme of the pathway, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HMBPP, EC: 1.17.1.2), is encoded by two transcripts. On the other hand, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA, EC: 1.1.1.34) of the MVA pathway is encoded by eight transcripts. Most of the transcripts that were annotated to genes in the MVA pathway exhibited a significant increase in expression levels at 24 h, while most of the transcripts related to the MEP pathway showed differential expression

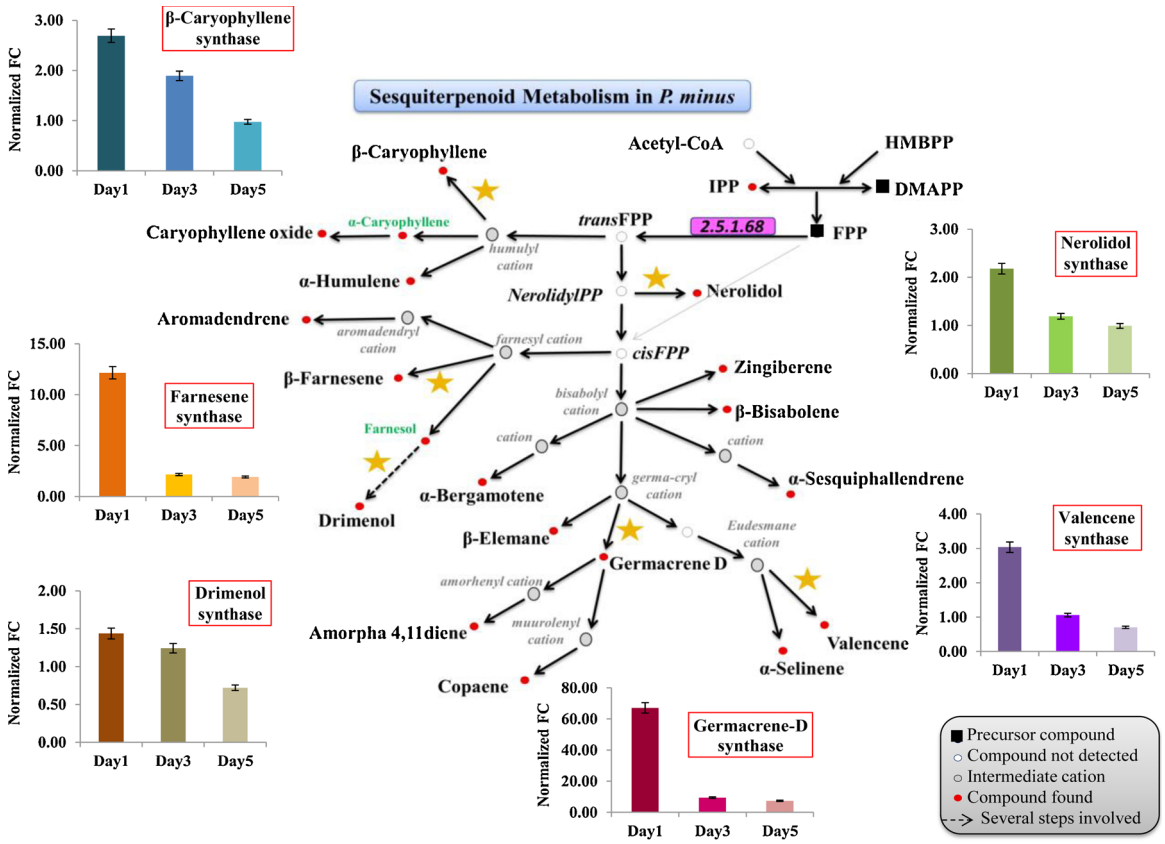

Fig. 6. Transcriptional effect of MeJA on the pathway of sesquiterpenoid biosynthesis in *P. minus*. Time course MeJA effects on transcript levels of six candidate sesquiterpene synthases between treated and control samples are depicted in the bar graphs. The pathway was built based on in-house datasets. All data are the means of three biological replicates, with error bars indicating standard errors. D: day, FC: fold change. Stars represent ST synthases analyzed by RT-qPCR. HMBPP: 4-hydroxy-3-methylbut-2-enyl diphosphate; DMAPP: 4-hydroxy-3-methylbut-2-en-1-yl diphosphate; IPP: isopentenyl diphosphate; FPP: farnesyl diphosphate.

with either upregulation or downregulation (Supplementary File 2, Fig. 5B). The upregulation of the MEP pathway may be related to ST biosynthesis.

ST biosynthesis typically consists of an initial cyclization of farnesyl pyrophosphate (FPP) that yields various ST products. The transcripts encoding the FPP synthase genes were significantly upregulated. Transcript comp62238_c0_seq2, coding for FPP synthase, was one of the highly expressed isoprenyl pyrophosphate synthases (IPPSs) (Fig. 5B). Among the IPPSs, the expression of transcripts associated with geranyl diphosphate (GPP) synthase and geranylgeranyl diphosphate (GGPP) synthase genes did not show significant differential expression with MeJA elicitation.

A total of six candidates of ST synthases in the *P. minus* ST biosynthetic pathway were selected to follow their expression patterns after MeJA treatment over the three time points (Fig. 6), including farnesene synthase (comp65731_c0_seq6), β-caryophyllene synthase (comp58342_c0_seq1), germacrene-D synthase (comp64628_c0_seq1), valencene synthase (comp45619_c0_seq2), nerolidol synthase (comp57437_c1_seq1), and drimenol synthase (comp57437_c1_seq4). RT-qPCR analysis showed that the expression of all candidate genes was upregulated 24 h after MeJA elicitation followed by a decrease in expression at later sampling times (Fig. 6), which agrees with GC–MS analysis.

4. Discussion

4.1. MeJA induces rapid compositional changes in P. minus leaf VOCs

In the current study, we compared the effects of exogenously applied MeJA across three time points on the induction of compositional changes in *P. minus* leaf VOCs. Overall, the MeJA-induced increases in the accumulation of VOCs exhibited the strongest effects within 24 h, which was more than four times greater than those of day 3 and more than six times greater than those of day 5. Over the time course of the MeJA treatment, the highly induced volatiles decreased drastically after day 1. This suggests an early response to MeJA with the biosynthesis of VOCs within 24 h, and the subsequent decrease in content might be due to the release of VOCs from leaf tissues. With a similar experimental design, Ismail et al. (2011) previously reported on the stimulatory effect of 150 μM JA on *P. minus* roots, showing a decline in terpene levels over a period of 3 days, which agreed with our results.

In a previous study, characteristic volatile compounds from *P. minus* were identified using two extraction techniques, hydrodistillation (HD) and SPME (Ahmad et al., 2014). In comparison with the HD extraction technique, SPME requires minimal sample preparation and instrument setup when coupled with GC–MS and can detect more volatile compounds. In addition, Goh et al. (2016) applied multivariate statistical methods to SPME–GC–MS data from highland and lowland populations of *P. minus* to build a classification model based on volatile compounds and concluded that highland *P. minus* plants produced terpenes and flavonoids in greater abundance than lowland plants. Current HS-SPME-GC–MS analysis showed that exogenous MeJA treatment had profound effects on the composition of VOCs.

4.2. MeJA elicitation mainly affected the contents of aldehydes and sesquiterpenes

Multivariate statistical methods enabled the separation of changes

in VOC composition between the three different time points after elicitation. Using unsupervised PCA (Supplementary Fig. 3), volatile compositions after one day of MeJA elicitation varied from those after 3 and 5 days. However, differentiation of volatile compositions in leaves harvested between day 1, day 3 and day 5 after MeJA elicitation was more apparent using a supervised PLS-DA method, which helps to identify the VOCs that contributed to the separation between samples based on VIP coefficients. The aldehydes, decanal and dodecanal, as well as the sesquiterpenes, β-farnesene and β-caryophyllene, were among the significant discriminatory components due to their increased abundances after MeJA treatment.

Aldehydes belong to GLVs, which also consist of alcohols and esters, are important compounds that contribute to the characteristic aromas of fruits and leaves (Schwab et al., 2008). GLVs have been shown to be produced in plant defense responses to pathogens or herbivory infection (Matsui, 2006; Stumpe and Feussner, 2006). The study on *Pinus sylvestris* and *Quercus ilex* leaves indicated that exogenously applied MeJA also caused increased emissions of GLVs (Semiz et al., 2012). High abundances of decanal and dodecanal in *P. minus* leaves agree with results reported by Yaacob (1990), Baharum et al. (2010), and Goh et al. (2016), which are the major components in *P. minus* essential oil. The ratios between decanal and dodecanal compositions were shown to be significantly affected by temperature, with higher relative amounts of aldehydes under lower temperatures (Goh et al., 2016). However, the biological significance of this observation remains to be explored. On the other hand, the rapid increase in 2-hexenal, which possesses high antibacterial properties even at low concentrations (Croft et al., 1993), supports its important role related to biotic stress (Kunishima et al., 2016).

Terpenes represent one of the largest groups of secondary metabolites with many volatile constituents and play an important role in plant-pathogen, plant-insect, and plant-plant interactions (Cheng et al., 2007a). Abiotic and biotic stresses can elicit the emission of a spectrum of volatiles in different plant species, such as MTs and STs (Niinemets, 2010). The MeJA-mediated production of terpenes may be associated with their role in protecting plants against both biotic and abiotic stresses, thus conferring adaptation to different environmental conditions (Peñuelas and Munné-Bosch, 2005). It has been reported that MT α-pinene displayed immunostimulatory activity (Kedzia et al., 1998), and bornyl acetate was shown to possess analgesic and anti-inflammatory activities (Wu et al., 2004). Therefore, it is desirable to increase the amount of such beneficial terpenes in herbal products.

In our study, MeJA triggered increased contents of volatile terpenes, particularly at 24 h after elicitation (Supplementary Fig. 1). Among the volatile groups, the accumulation of terpene metabolites in *P. minus* leaves shows that they could be the main players in the defense response. This observed effect of MeJA on increasing terpenes agrees with the MeJA response in other plant species, such as Norway Spruce, *Quercus ilex* and *Pinus sylvestris* (Filella et al., 2006; Martin et al., 2003; Semiz et al., 2012). Semiz et al. (2012) showed increased emissions of terpenes in *Pinus sylvestris* leaves treated with MeJA. MeJA-triggered accumulations of the antioxidant terpinolene were also observed in grapes in response to UV-B radiation (Gil et al., 2012). Moreover, the homoterpene (E)-DMNT content of *P. minus* was significantly induced by MeJA, which was observed in MeJA-treated *Quercus ilex* (Semiz et al., 2012).

Our findings are also in agreement with other studies, which support the concept that JAs play a key role in the biosynthesis of sesquiterpenes. In *Artemisia annua*, MeJA noticeably increased the expression of genes in the artemisinin biosynthetic pathway, subsequently resulting in increased accumulations of artemisinin (Maes et al., 2011). The induction of ST production by exogenously applied MeJA was demonstrated in *Aquilaria* cell suspension cultures (Ito et al., 2005; Xu et al., 2016). Ismail et al. (2011) reported that the production of STs was also strongly elicited by JA in *P. minus* roots.

Among the STs produced in *P. minus* leaves, β-farnesene, an attractant for insect enemies such as parasitoid wasps on citrus aphids, *Diaphorina citri* (Hijaz et al., 2013), is the most abundant compound induced by MeJA (Table 1). The amount of β-farnesene accumulations increased approximately 35%, accounting for nearly one-third of the total STs. β-caryophyllene, the common ST with different pharmacological activities (Galdino et al., 2012), was the second most abundant compound following MeJA elicitation. Caryophyllene and farnesene are associated with stress-induced volatiles in a number of angiosperms (Huang et al., 2012; Pettersson, 2007). Therefore, these volatiles could be target volatiles to monitor stress responses in plants.

Farnesyl pyrophosphate (FPP) is an important metabolic intermediate in the mevalonate pathway, which serves as a precursor for the biosynthesis of all plant sesquiterpenes that contribute to plant defense responses (Singh and Sharma, 2015). STs have important defense and health properties for plants and humans, respectively (Chadwick et al., 2013), and it is shown that STs are the main terpenes in *P. minus* leaves (Ahmad et al., 2014). Most identified terpenes in this study are in the ST category (Fig. 6). The ST biosynthesis pathway involves the modification of ST synthase products, which produces diverse compounds that exhibit different bioactivities (Zook et al., 1996).

4.3. Integrative analysis reveals MeJA-induced transcriptional changes related to terpene biosynthesis

Previous *P. minus* transcriptomics analyses focused on the biosynthesis of phenylpropanoids and flavonoids (Loke et al., 2017). Through transcriptional and metabolite profiling of *P. minus* leaves in the current study, we provide a comprehensive analysis of transcripts related to terpene biosynthesis in response to MeJA elicitation. The combination of metabolic and transcript datasets provides insight into the molecular mechanism of stimulated terpene biosynthesis in MeJA-elicited *P. minus* leaves. Although the precise mechanisms of how JAs trigger increased terpene production are still elusive, there is evidence that JAs induce the expression of terpene synthase-like genes. Fäldt et al. (2003) reported an enhancement in the transcript levels of the AtTPS03 gene, which encodes an enzyme that catalyzes the synthesis of (E)-β-ocimene, in JA-sprayed *Arabidopsis thaliana* leaves (Fäldt et al., 2003). Moreover, the exogenous application of MeJA to rice (*Oryza sativa*) has also been reported to increase transcript levels of sesquiterpene synthases, such as OSTPS3, a gene encoding a β-caryophyllene synthase (Cheng et al., 2007b). In *P. minus* leaves, it seems that MeJA affected the activity or transcription of terpene enzymes, resulting in changes in the relative abundances of terpenes.

The expression level of the six selected ST synthase genes analyzed using RT-qPCR across different time points showed consistent induction by MeJA treatment within 24 h. However, terpene synthase was not found among the differentially expressed proteins in a recent quantitative proteomics informed by transcriptomics study of MeJA-induced *P. minus* leaves (Aizat et al., 2018a, b). This can be due to limited sensitivity in the nontargeted shotgun proteomics approach with only 751 identified proteins. Nevertheless, MeJA-induced upregulation of terpene synthases supports earlier reports that jasmonate induced the biosynthesis of terpenes in *P. minus* roots (Ismail et al., 2011). This shows that VOC compositional changes from exogenous MeJA elicitation, particularly for terpenes with an accumulation of sesquiterpene content above normal levels, are associated with elevated transcripts of ST synthases.

This study suggests that MeJA serves as a key signaling compound for the induction of GLVs and terpenes, particularly sesquiterpenes, likely as a defense response in *P. minus* leaves. MeJA-induced rapid increases in VOCs might be useful for improving *P. minus* resistance to pathogens and herbivores. However, the timing and dose effects of exogenous MeJA on shifts in carbon allocations between defense and growth will need further study.

5. Conclusion

This is the first study of MeJA-induced volatiles in *P. minus* leaves using an integrated metabolomic and transcriptomic approach, which leads us to a better understanding of exogenous MeJA elicitation on the production of *P. minus* leaf volatiles. The SPME-GC–MS study with multivariate statistical analysis illustrated time-course changes in VOCs that occurred after elicitation via data clustering. Our results indicate that MeJA elicitation appears to mainly affect the synthesis of aldehydes and sesquiterpenes compared to other classes of volatiles. Therefore, the MeJA induction of volatiles in *P. minus* provides an opportunity for the discovery of genes involved in the accumulation of induced terpenes and GLVs, which are important for the characteristic pungent smell of *P. minus*. The present study also opens an avenue to conduct further investigations into gene-metabolite networks, targeted enzyme analysis, and biotechnological applications.

CRediT authorship contribution statement

Reyhaneh Rahnamaie-Tajadod: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Hoe-Han Goh:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing - review & editing. **Normah Mohd Noor:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.152994.

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