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ORIGINAL ARTICLE

Exogenous methyl jasmonate prevents necrosis caused by mechanical wounding and increases terpenoid biosynthesis in *Panax ginseng*

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Abstract Upon mechanical wounding, plants locally induce necrosis, accumulate methyl jasmonate (MeJA) and acquire systemic resistance in nearby tissues. One-month-old in vitro grown *Panax ginseng* seedlings were treated with either 50 μM MeJA or mechanical wounding alone or a combination of both, to evaluate jasmonic acid (JA) signaling and terpene biosynthetic pathway genes along with terpenoid accumulation. After MeJA treatment, JA pathway genes, such as lipoxygenase (*PgLOX*), hydrogen peroxidase lyase (*PgHPL*), allene oxide synthase (*PgAOS*), and allene oxide cyclase (*PgAOC1*), and terpene pathway genes, such as isopentenyl diphosphate isomerase (*PgIPP*) and farnesyl diphosphate synthase (*PgFPS*), were highly expressed and resulted in the accumulation of mono- and sesquiterpenes. During mechanical wounding, *PgLOX* expression was induced relatively late after 72 h of treatment, however *PgAOC1* was not induced. This resulted in decreased production of MeJA that in turn may have lowered terpenoid production. In contrast, wounding + MeJA treatment increased *PgAOC1* and *PgLOX* gene expression earlier after 6 h and slowly promoted the production of mono- and sesquiterpenes. Furthermore, we monitored the effect of MeJA upon wounding in in vitro grown 1-month-old seedlings treated with MeJA + wounding. These result demonstrated that exogenous MeJA is able to promote recovery from the wounding effect by functioning as a long distance signal. Additionally, these results suggest that exogenous MeJA supplied at the time of mechanical wounding prevents necrosis in the ginseng leaves by increasing the production of terpenoids.

Abbreviations

JA	Jasmonic acid
MeJA	Methyl jasmonate
OGA	Oligogalacturonides
WR	Wound responsive
IPPI	Isopentenyl diphosphate isomerase
DMAPP	Dimethylallyl diphosphate
GPP	Geranyl diphosphate
FPS	Farnesyl diphosphate synthase
LOX	Lipoxygenase
HPL	Hydrogen peroxidase lyase
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
GC–MS	Gas chromatography mass spectrometry
RT-PCR	Reverse transcription PCR
qRT-PCR	Quantitative real time-reverse transcriptase polymerase chain reaction

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Introduction

Mechanical wounding is one of the major threats to the survival of all living organisms. In plants, wounding responses have been widely studied. Plants in the wild seldom escape some degree of damage from environmental stresses, such as wind, hail, sand and rain (Graham et al. 1986). When plants get wounded, each wounded cell makes competent each other for the activation of defense responses, which largely depend on the activation of specific genes (León et al. 2001), by triggering specific signal transduction pathways. In *Arabidopsis*, mechanical wounding increases the accumulation of endogenous jasmonic acid (JA) and the release of cell wall derived oligogalacturonides (OGA) that tend to activate wound responsive (WR) and jasmonate responsive (JR) genes (Rojo et al. 1998). Local release of JA at the wound site systemically increases resistance in distal parts of the plant, indicating that JA plays a role as a long distance signal. JA biosynthesis via the octadeconoid pathway starts with the release of linolenic acid from membranes and induces the production of various secondary metabolites, including volatiles (Thines et al. 2007; Chini et al. 2007; Staswick 2008). Plant damage can also remarkably increase the accumulation of volatiles, including mono- and sesquiterpenes.

Terpenes are a group of volatile compounds present at a basal level in undamaged plants. Their accumulation increases in response to various biotic and abiotic stresses. In particular, JA acts as an intermediate signal transduction molecule and increases the production of volatiles in both maize and rice (Schmelz et al. 2003; Xu et al. 2003; Cheng et al. 2007). In *Panax ginseng* C.A. Meyer, mono- and sesquiterpenes are the most important wide spread groups of secondary metabolites. The biosynthesis of terpenes through activated 5-carbon isoprenoid units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), is derived from the acetate/mevalonate or methylerythritol phosphate pathways (for review, see Chappell 1995; Gershenzon and Kreis 1999). The C5 units are condensed by prenyltransferases to form elongated linear prenyl diphosphates, such as geranyl diphosphate (GPP, a C10 compound) and farnesyl diphosphate (FPP, a C15 compound), which are the precursors of mono- and sesquiterpenes, respectively. The prenyl diphosphates in turn undergo a wide range of cyclizations and other modifications to produce the various structural types of mono- and sesquiterpenes.

Pests, including weeds, insects, diseases, and rodents, are the greatest concern of ginseng growers. It is highly probable that ginseng, as a perennial herb, is exposed to various biotic and abiotic conditions. Hence, avoiding stressful conditions while cultivating ginseng is a serious concern among ginseng farmers. In *Arabidopsis*, wounding-induced endogenous methyl jasmonate (MeJA) caused systemic resistance but failed to exhibit local resistance. There are large amounts of data showing that MeJA increases the production of terpenoids in various plants. For e.g. in *Catharanthus roseus* combined elicitor treatment is used for the enhancement of terpenoid indole alkaloids (Almagro et al. 2014). However, to date, no data exist for MeJA-dependent regulation of terpenoids in *P. ginseng*. Furthermore, no report has shown that exogenously supplied MeJA can prevent the physical local damage caused by wounding. Hence, the present study was designed to understand how plants resist wounding when exogenous MeJA is simultaneously supplied.

To this end, *P. ginseng* seedlings were treated with exogenously supplied MeJA and wounding, alone or in the combination, to investigate the role of exogenous MeJA on plant mechanical damage. To understand terpenoid regulation and the JA pathway at the molecular level, we first studied the expression profiles of various mono- and sesquiterpene biosynthetic pathway related genes, such as *PgFPS* and *PgIPPI*, and JA biosynthetic pathway related genes, such as *PgLOX*, *PgHPL*, *PgAOS*, *PgAOC1*, and *PgAOC2*. We also quantified mono- and sesquiterpene accumulation.

Materials and methods

Plant growth conditions

The *P. ginseng* seeds were obtained from the Korean Ginseng Resource Bank, South Korea. Ginseng seeds were surface sterilized in 70 % ethanol for 1 min, washed with 2 % sodium hypochlorite solution for 15 min with occasional shaking and then washed with sterile distilled water 3–5 times. After carefully dissecting the zygotic embryos, they were placed on ½ MS media (Duchefa Biochemie, The Netherlands), 3 % sucrose, and 0.7 % agar, pH 5.8 for 1 week. Later, germinated seedlings were transferred to solidified full MS media with 3 % sucrose and supplemented with gibberellic acid (30 mg/ml) at 25 °C under a 16 h photoperiod for 3 weeks.

Wounding and MeJA treatments

For wounding treatment, 1-month-old in vitro grown ginseng seedlings were wounded in all parts of the seedling using a sterile, sharp scalpel and placed in ½ MS liquid medium. For MeJA treatments, seedlings were just placed in ½ MS liquid medium containing 50 μM MeJA. For the combined treatment, seedlings were first wounded and then

placed in ½ MS liquid medium containing 50 μM MeJA. Control group seedlings were placed in ½ MS medium without undergoing wounding treatment or supplied with any MeJA. All the treated and untreated seedlings were grown at 25 °C under a 16 h photoperiod and harvested after 24, 48, 72, 96 h, 8 and 15 days. The experiments were repeated twice with similar results. For gene expression studies and terpene analysis, plants were wounded and treated as described above, harvested at different time points, immediately frozen in liquid nitrogen and stored at −70 °C for future experiments.

Gene expression analysis by RT-PCR and qRT-PCR analyses

RNA was extracted from *P. ginseng* organs that had been subjected to all experimental treatments using the RNeasy kit (Qiagen, USA), according to the manufacturer's instructions. The quality and concentration of RNA was measured using a spectrophotometer (GE Nanovalue, USA). To obtain first strand cDNA, 1 μg of total RNA was used, and cDNA was synthesized using a Power cDNA kit (Invitrogen, USA) following the supplied instructions. For semi-quantitative reverse transcription PCR (RT-PCR), the *P. ginseng* actin gene (*PgActin*) was used as a constitutively expressed control gene. The initial PCR was performed with *PgActin* primers, product levels were compared, and 100 ng of cDNA samples were used to ensure equal template concentrations. PCR was subsequently performed for 30 amplification cycles using primers specific for the genes of interest. *PgActin* gene specific primers were always included in each experiment.

We performed qRT-PCR using a real-time rotary analyzer (Rotor-Gene 6000; Corbett Life Science, Australia)

using 100 ng of cDNA in a 10 μl reaction volume, using SYBR® Green SensiMix Plus Master Mix (Quantace, England), and the gene specific primers listed in Table 1. The housekeeping gene encoding the *PgActin* protein was used as a standard for all samples. For transcript analysis of stress treated samples, untreated samples were used as negative controls. A melting point analysis of PCR products was carried out, which resulted in a single peak, indicating the presence of a single PCR product. The melting temperatures varied from 78.6 to 80 °C with no clear differences between the treatments. The PCR conditions for each of the 40 cycles were 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Fluorescence was detected and measured in the real-time reverse transcriptase PCR thermocycler, and the geometric increase of the fluorescence corresponding to an exponential increase of the product was used to determine the threshold cycle (Ct) in each reaction using the formula $2^{-\Delta\Delta Ct}$. All real time reverse transcriptase PCR experiments were performed in triplicate.

Mono- and sesquiterpene analysis by GC–MS

1 g of fresh sample was pulverized in liquid nitrogen using a mortar and pestle, and then extracted sequentially with 5 ml of hexane:ethyl acetate (85:15) twice. The extracts were combined and purified by two rounds of silica column chromatography. The extract was applied to a silica column (230 mm in length) and washed with 6 ml of fresh hexane:ethyl acetate (85:15). The collected eluent was then applied to a second silica column and washed with 3 ml of hexane:ethyl acetate (85:15) to elute hydrocarbons and oxygenated compounds. The final eluent (approximately 20 ml) was concentrated to 1–2 ml under nitrogen before analysis by GC-MS. Mono- and sesquiterpenes in the

Table 1 Genes specific primers used in the present study

Gene name	Accession no./EST no.	Specific primer sequence (5′–3′)	Tm (°C)
PgIPPI	F7GI6YX02HDDDI	F-ATGCTGGTGAAGAGGGTTTGAAG	58.0
		R-ATCCTAGACTTCTAGAATGCAGGGC	
PgFPS	DC02026E08.ab1	F-CTGAAATCCGAGCTACTCAACGA	57.0
		R-GCCATTCAATGCACCAACCA	
PgLOX	Contig02058	F-TAT ACC CGG CTG GTT TTC TG	58.0
		R-ACTTTGTTCAATTCTAAATCCGAAG	
PgHPL	PG03034B09.scf	F-CTCATAACCAGTGGTTGATAACCAC	55.5
		R-AATGCCTTTGGTGGATTCG	
PgAOS	F7GI6YX01EZ3V3	F-TTTTACAACCAAGGCCGAGAT	61.5
		R-CACTTTGTCGCACCGAGATT	
PgAOC1	C02022G06.ab1	F-CGGCGATCTTGTCCCTTT	58.2
		R-TTGCTGAAGCTTAACCTGACC	
PgAOC2	F7GI6YX02HW4Q9	F-AGCTTTTACTTCGGAGATTACGGT	60.8
		R-TTAGTGTAGTTGGAAATGGTGGC	
PgActin	DC03003B12	F-CGTGATCTTACAGATAGCTTGATGA	60.0

samples were quantified using a Thermo Finnigan DSQ GC/MS system, as described by Lee and Chappell (2008).

Results

Morphological changes in wounded ginseng leaves under MeJA treatment

Morphological changes in in vitro grown ginseng seedlings were evaluated 24, 48, 72, 96 h, 8 and 15 days after wounding alone or in combination with exogenously supplied MeJA. The seedlings subjected to wounding showed damage effects as early as 6 h post-treatment (data not shown). Upon prolonged exposure, the lesion surface areas tended to show a gradual increase 24 h after wounding. The wounded surface areas increased over time and reached a peak at 15 days, resulting in a fully necrotized phenotype. Interestingly, exogenous MeJA supplied at the time of wounding decreased necrosis and did not induce necrosis even 15 days after treatment in ginseng seedling leaves (Fig. 1).

Fig. 1 Recovery from mechanical wounding damage by exogenously supplied MeJA. Representative images of in vitro grown 1-month-old *P. ginseng* seedlings treated with 50 μM MeJA, wounding and a combination of wounding + 50 μM MeJA. *Arrows* indicate the wounding site in *P. ginseng* seedlings. Images were captured 24, 48, 72, 96 h, 8 and 15 days after treatment
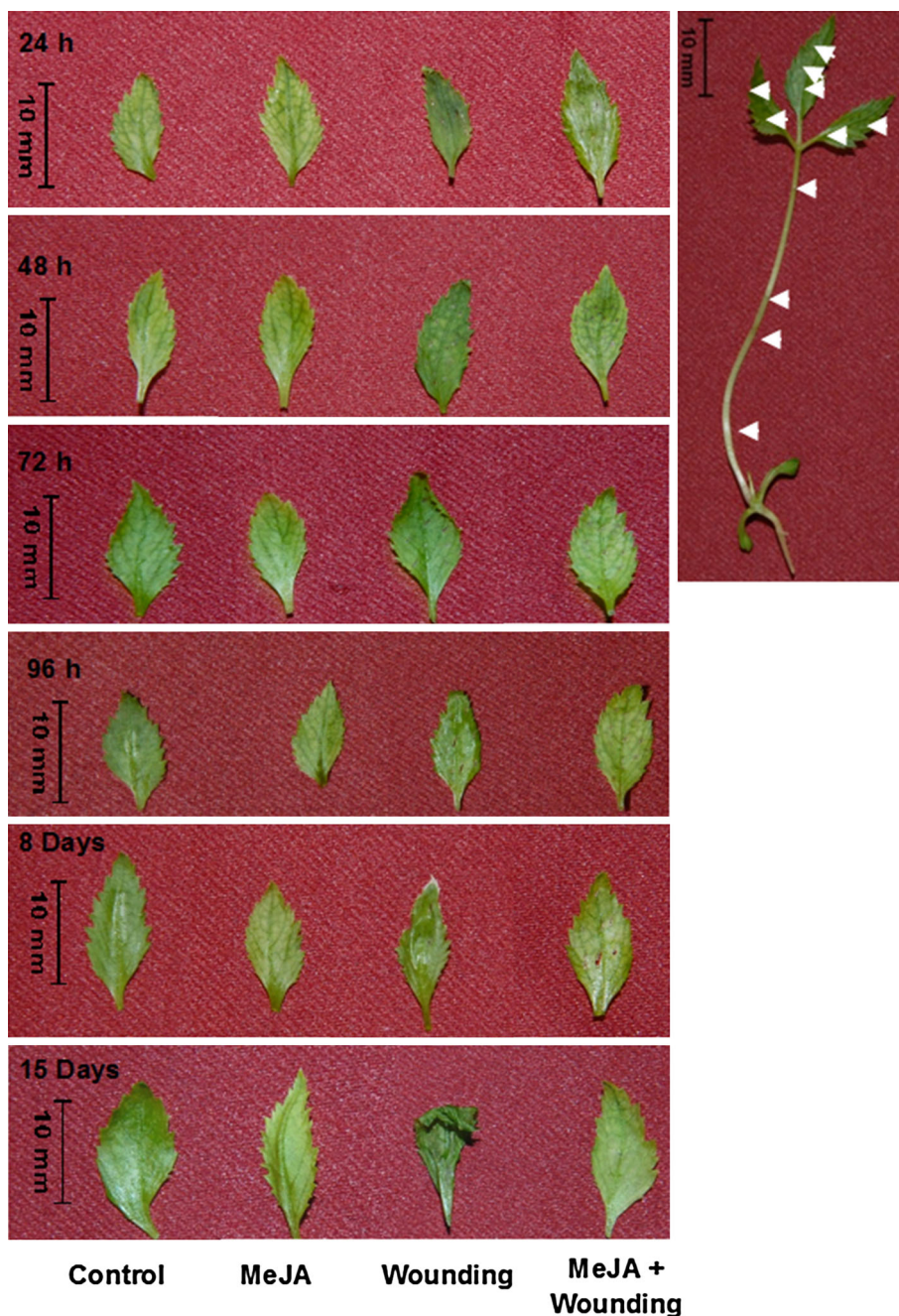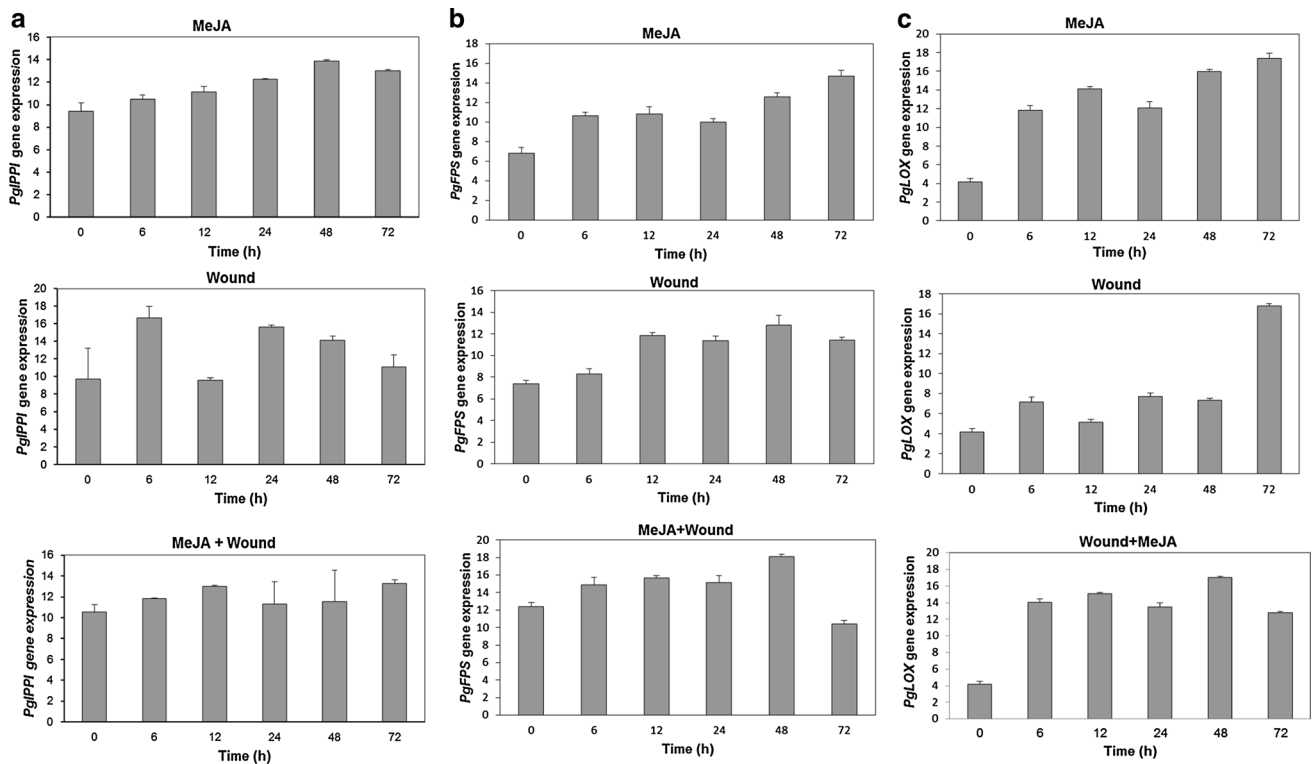
Fig. 2 Differential expression levels of terpene pathway related genes and *PgLOX* in response to MeJA, wounding and a combination of wounding + MeJA treatments. RNA was isolated from treated seedlings at the indicated times, and the expression levels of the indicated mRNAs were determined by qRT-PCR. The Ct values for the tested genes were normalized to the Ct value for β-actin and calculated relative to the calibrator using the formula $2^{ΔΔCT}$. Data represent the mean ± SD for three independent replicates
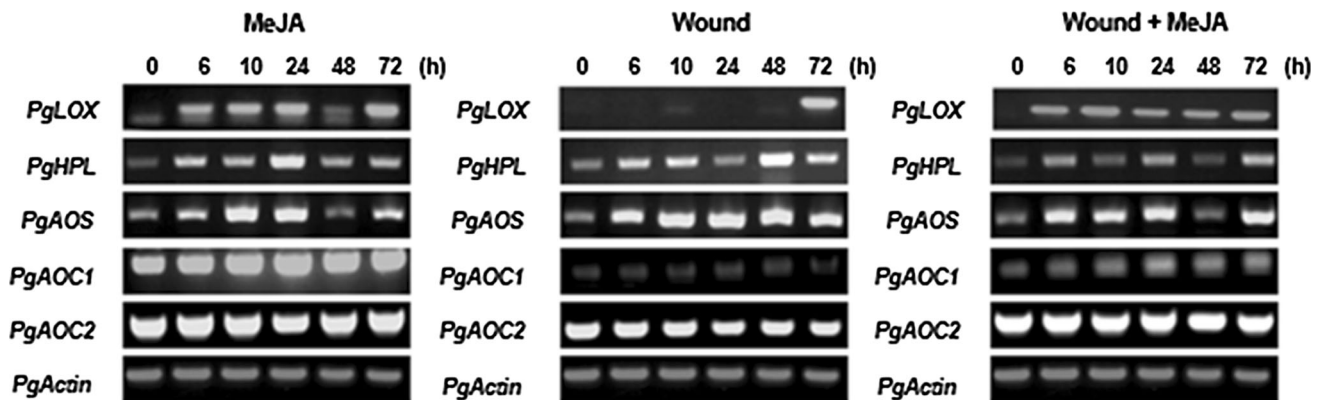

Fig. 3 Altered pattern of jasmonic acid biosynthetic gene expression in *P. ginseng* seedlings treated with MeJA, wounding and a combination of wounding + MeJA. RNA was isolated from treated seedlings at the indicated times, and the expression levels of lipoxygenase (*PgLOX*), hydrogen peroxidase lyase (*PgHPL*), allene oxide synthase (*PgAOS*), allene oxide cyclase (*PgAOC*) were determined by RT-PCR

Changes in the gene expression profiles of JA pathway and terpenoid biosynthetic genes

To determine whether terpenoid gene expression is mediated through the JA pathway, we monitored changes in terpenoid-related genes, such as *PgIPPI* and *PgFPS*, along with JA pathway genes, such as *PgLOX*, *PgHPL*, *PgAOS*, *PgAOC1* and *PgAOC2*, in in vitro grown 1-month-old ginseng seedlings treated with MeJA, or wounding and the combination of both treatments by semi-quantitative PCR and qRT-PCR (Figs. 2, 3). qRT-PCR was carried out only for certain genes like *PgLOX*, *PgIPPI* and *PgFPS* (Fig. 2). Changes in the expression patterns of genes under various stresses also proved their roles in defense mechanisms by
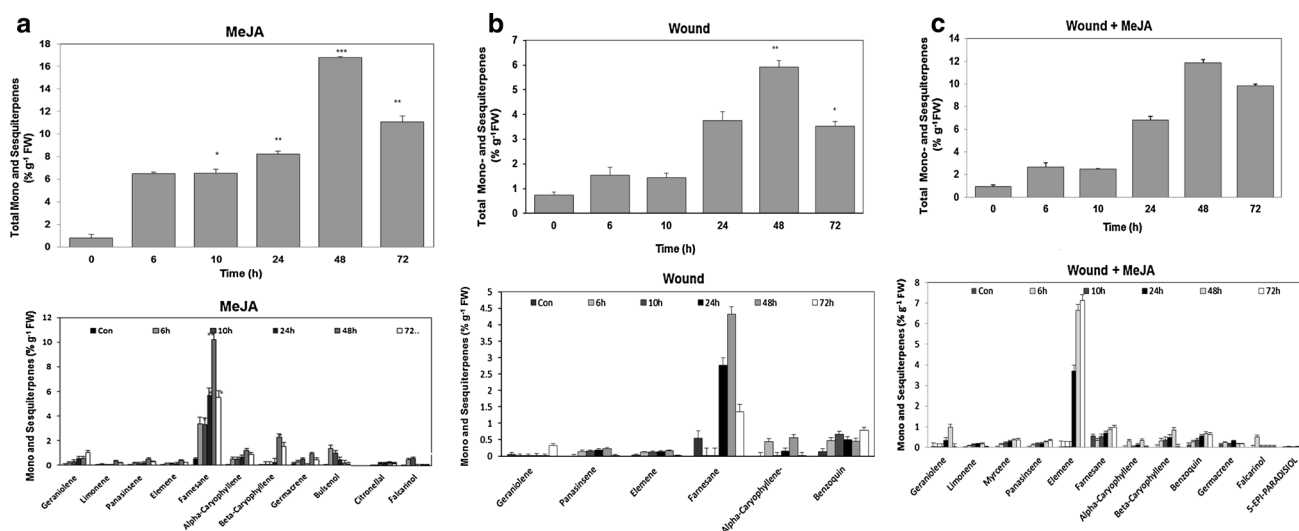
Fig. 4 Percentage of total terpenoids in *P. ginseng* seedlings treated with MeJA, wounding and a combination of wounding + MeJA. Twenty-one-day-old in vitro grown seedlings treated with 50 μM MeJA, wounding and a combination of wounding + 50 μM MeJA were collected at different time intervals for mono and sesquiterpene analysis. Means of three independent replicates were statistically analyzed and compared with controls (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) using a Student's *t* test

crosstalk between the JA and terpene pathways. Upon MeJA treatment, expression of *PgIPPI* was slightly upregulated, reached a maximum level at 48 h (13.9-fold) and declined gradually at 72 h post-treatment (Fig. 2a). After wounding, the *PgIPPI* transcripts were expressed at their highest level at 6 h (16.6-fold) and 24 h (15.6-fold), with decreased levels observed 12, 48 and 72 h post-treatment. The expression pattern of *PgIPPI* under MeJA + wounding showed a slight fluctuation compared to 0 h, and showed the highest levels of expression at 12 and 24 h. The *PgFPS* transcript increased gradually and reached a maximum 72 h (14.7-fold) after MeJA treatment (Fig. 2b). Due to the combination of wounding and MeJA, *PgFPS* transcripts started to show increased expression beginning at 6 h, reached a maximum level at 48 h and declined thereafter. The expression of *PgFPS* increased gradually, was induced 18.08-fold at 48 h and then gradually decreased in wounded leaves. MeJA increased *PgLOX* gene expression at 6 h and showed maximum accumulation with a 17.36-fold increase at 72 h (Fig. 2c). Upon wounding, *PgLOX* expression was highest at 72 h, with an average 16.78-fold increase. *PgHPL* and *PgAOS* were induced by wounding, MeJA and wounding + MeJA treatments. *PgAOC2* seemed to be upregulated by MeJA treatment, but downregulated by wounding. Interestingly, expression of *PgAOC2* seemed to be activated by MeJA treatment. However, *AOC1* did not show any significant increase or decrease in gene expression (Fig. 3).

Production of mono-and sesquiterpenes

During the 6–48 h post-treatment period, the production of total mono and sesquiterpenes increased as a result of MeJA treatment, wounding or a combination of both treatments. Ginseng seedlings treated with MeJA alone showed maximum accumulation of terpenes at 48 h (levels were higher than those of wounding alone, or in combination with wounding) (Fig. 4a). In comparison with MeJA, wounding resulted in less accumulation (5.9 %; however, this was higher than untreated controls) of mono- and sesquiterpenes (Fig. 4b). Interestingly but not surprisingly, seedlings treated with MeJA simultaneously after wounding showed slightly higher accumulations of mono- and sesquiterpenes (11.8 %) but less accumulation than in response to MeJA treatment (Fig. 4c).

Geraniolene, limonene, panasinsene, elemene, farnesane, alpha carophyllene, beta carophyllene, germacrene, bulsenol, citronellal, and falcrinol were all detectable in in vitro grown ginseng seedlings treated with MeJA. The wounding treatment resulted in less production of mono- and sesquiterpenes when compared to MeJA treatment alone or in combination. Only six different terpenes, including geraniolene, panasinsene, elemene, farnesane, alpha-carophyllene, benzoquin, were identified in wounded samples.

Discussion

The present study describes the influence of MeJA and mechanical wounding alone or in combination on volatile terpene biosynthesis. All three treatments individually resulted in an increase in terpenoid accumulation. However, each treatment accumulated different level of terpenes in the following order: MeJA > MeJA + wounding > wounding. From a biotechnological point of view, the
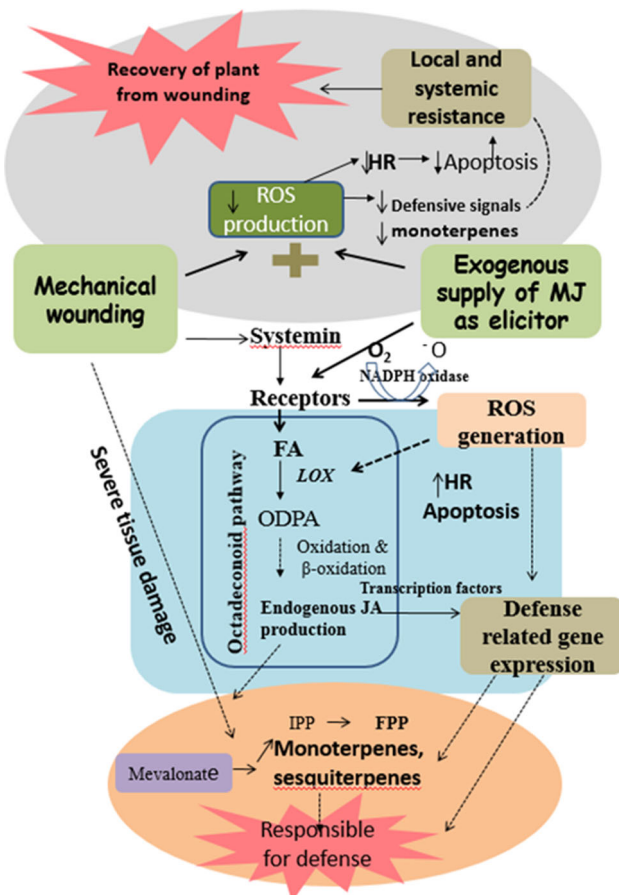
Fig. 5 Model illustrating the possible mechanism of the response to MeJA, wounding and a combination of wounding + MeJA treatments in ginseng plants. Wounding induced local MeJA which can act as a long distance signal and increase the levels of volatiles in systemic tissues, resulting in resistance in distal parts of the plants but also in severe wounding damage at the local site. In contrast, exogenous MeJA mimicked endogenous JA and resulted in local resistance, thereby preventing wounding-induced necrosis by increasing volatile emissions

relevant advantage with respect to damage recovery of using exogenous MeJA may contribute to plant wellbeing by preventing cell damage caused by mechanical wounding. The exposure of in vitro grown ginseng seedlings to wounding resulted in lower terpene levels compared to other treatments; the latter being prevalently released when ginseng seedlings were treated with MeJA in combination with wounding. However, seedlings treated with MeJA predominantly accumulated high levels of terpenes. These results demonstrate exogenous MeJA is able to help the plant recover from wounding stress by producing high levels of volatiles.

The effect of MeJA on terpene metabolite accumulation has been extensively studied in Norway spruce (Erbilgin et al. 2006; Zeneli et al. 2006). In our study, we determined the expression of terpene related genes, such as *PgIPPI* and

PgFPS, terpene metabolite accumulation and JA mediated pathway genes, like *PgLOX*, *PgHPL*, *PgAOS*, *PgAOC1* and *PgAOC2*, after treating seedlings with MeJA. Upon MeJA treatment, terpene-related gene transcripts and *PgLOX* (a key enzyme in the JA biosynthesis pathway) mRNA levels were upregulated, and resulted in the highest level of terpenoid accumulation, relative to untreated controls. The *PgHPL*, *PgAOS*, and *PgAOC1* transcripts accumulated to their highest levels as a result of this treatment. With respect to the metabolite profile, seedlings treated with MeJA accumulated significant amounts of the farnesane and beta-carophyllene sesquiterpenes and others in trace amounts, compared to controls. The largest increase in both terpenoid and gland production was observed in plants treated with JA, a ubiquitous plant hormone known to mediate defense responses to biotic and abiotic stresses. Several hundred studies have demonstrated that exogenous application of JA induces defensive responses (Heil 2008; Wasternack 2007; Farmer et al. 2003), while no data has been published on the effect of MeJA on the biosynthesis of terpenoids in *P. ginseng*.

Mechanical wounding increases endogenous MeJA at the wounded site, which can act as a long distance signal and result in systemic resistance by inducing a broad spectrum of defense-related compounds, such as volatiles, alkaloid production, trichome formation and secretion of extrafloral nectar (Heil and Ton 2008; Chini et al. 2007). Terpenoids are the largest group of plant secondary metabolites and play vital roles in plant–microbe, plant–herbivore, plant–plant, and plant–environment interactions (Chappell 1995; Mahmoud and Croteau 2002; Pichersky and Gershenzon 2002; Aharoni et al. 2003). When plants are damaged by herbivores, volatiles released from the damaged portion of the plant vaporize and induce defensive responses in neighboring tissues or in other plants. Upon wounding stress in *P. ginseng*, terpene pathway-related gene transcripts seemed to be up-regulated at different time periods. When considering the JA pathway genes, *PgLOX* showed late but strong expression 72 h after wounding. Interestingly, *PgAOC1*, a downstream gene of the JA pathway, was expressed at lower levels after wounding, compared to after MeJA treatment. Therefore, the total production of mono- and sesquiterpenes was reduced compared to MeJA and wounding + MeJA treatments. The wounded seedlings also showed increased mono- and sesquiterpenes at all-time points, however, significant increases were observed only after 24 h of treatment. Among the different type of sesquiterpenes, only farnesene showed a significant increase compared to the level of untreated controls; the other terpenes, such as geraniolene, panasinsene, elemene, alpha-carophyllene, and benzoquin, accumulated but the levels were not significantly greater than those found in untreated controls. As

a result, wounded plants showed local necrosis and failed to exhibit local resistance due to decreased MeJA production.

Panax ginseng seedlings exposed to MeJA at the time of wounding were able to recover from the damage caused by mechanical wounding, likely by inducing the production of volatiles. Furthermore, increases in *PgLOX* transcript levels after MeJA + wounding occurred much earlier than upon wounding stress and it is interesting to note that the *PgAOC1* transcript also increased when wounded plants were supplied with MeJA. This result may be due to the fact that necrosis caused by wounding stress can be recovered by increased production of MeJA. Terpene-related gene transcripts, such as *PgIPPI* and *PgFPS*, were also up-regulated at the earlier time points, indicating that MeJA is a key factor in modulating terpene genes at early times and in the accumulation of higher levels of terpenes, compared to the levels induced by wounding stress (Fig. 5).

The present results in *P. ginseng* demonstrated that necrosis caused by mechanical wounding at damaged sites can be recovered by exogenously supplied MeJA, thereby proving that MeJA acts as the long distance signal and plays a defensive role by producing systemic resistance, as suggested in other plant systems.

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