Plant Cell Rep (2014) 33:393–400
DOI 10.1007/s00299-013-1538-7

ORIGINAL PAPER

Both the mevalonate and the non-mevalonate pathways are involved in ginsenoside biosynthesis

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Received: 28 February 2013 / Revised: 17 October 2013 / Accepted: 4 November 2013 / Published online: 19 November 2013

Abstract

Key Message **When one of them was inhibited, the two pathways could compensate with each other to guarantee normal growth. Moreover, the sterol biosynthesis inhibitor miconazole could enhance ginsenoside level.**

Abstract Ginsenosides, a kind of triterpenoid saponins derived from isopentenyl pyrophosphate (IPP), represent the main pharmacologically active constituents of ginseng. In plants, two pathways contribute to IPP biosynthesis, namely, the mevalonate pathway in cytosol and the non-mevalonate pathway in plastids. This motivates biologists to clarify the roles of the two pathways in biosynthesis of IPP-derived compounds. Here, we demonstrated that both pathways are involved in ginsenoside biosynthesis, based on the analysis of the effects from suppressing either or both of the pathways on ginsenoside accumulation in *Panax ginseng* hairy roots with mevinolin and fosmidomycin as specific inhibitors for the mevalonate and the non-mevalonate pathways, respectively. Furthermore, the sterol biosynthesis inhibitor miconazole could enhance ginsenoside levels in the hairy roots. These results shed some light on the way toward better understanding of ginsenoside biosynthesis.

Keywords Ginsenoside biosynthesis · Hairy roots · Mevalonate pathway · Non-mevalonate pathway

Communicated by Q. Zhao.

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Introduction

Ginsenosides, the major bioactive components in ginseng, are of growing interest due to their prominent pharmacological functions such as anti-cancer, anti-fatigue, and anti-diabetes activities. Their presence at low level in plants and their tissue and cell cultures motivates a deeper understanding of their biosynthesis. Ginsenosides are triterpenoid saponins. It has been widely accepted that terpenoids are synthesized from the precursor isopentenyl pyrophosphate (IPP) (Dubey et al. 2003). For ginsenosides, the proposed biosynthesis pathway is described in Fig. 1.

Presently, it has been well established that IPP is biosynthesized in plants from both the MVA and the non-MVA (also named as MEP or DOXP) pathways, which are located in cytosol and plastids, respectively (Hampel et al. 2005). The MVA pathway was initially revealed in the 1950s. However, the discovery of MEP pathway was a more recent event (in 1990s) (Lombard and Moreira 2011). Since then, it has attracted increasing attention from botanists to determine roles of the two pathways in the biosynthesis of some high-valued IPP-derived metabolites. Substantial progress has been achieved in this respect. In plants, sterols, sesquiterpenes and ubiquinones are considered to be synthesized predominantly via the MVA pathway, while hemi-, mono-, sesqui- and di-terpenes, along with carotenoids and phytol chain of chlorophyll, are synthesized mainly from the non-MVA pathway (Dubey et al. 2003). It has been observed that some compounds are synthesized exclusively through only one of the pathways, e.g., 2-methyl-3-buten-2-ol synthesis via the MEP pathway in needles of *Pinus ponderosa* (Zeidler and Lichtenthaler 2001), terpinolene and myrcene via MEP pathway in *Daucus carota* L.

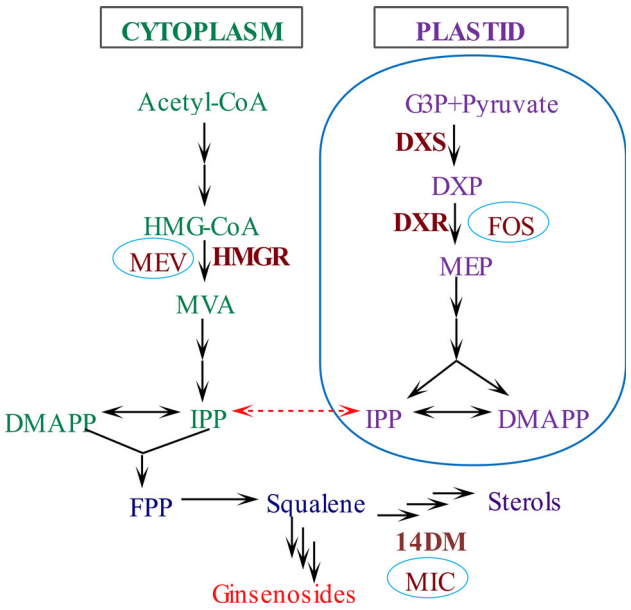

Fig. 1 The generalized biosynthesis pathway leading to ginsenosides via IPP and points of inhibition for the inhibitors used in this study. Inhibitors are shown in *oval boxes* and the enzymes affected are shown in *bold*. *Dotted lines* indicate exchange of precursors between cellular compartments. *HMG-CoA* hydroxymethylglutaryl CoA, *MEV* mevinolin, *HMGR* HMG-CoA reductase, *MVA* mevalonic acid, *DMAPP* dimethylallyl diphosphate, *IPP* isopentenyl diphosphate, *FPP* farnesyl diphosphate, *MIC* miconazole, *14DM* 14a-demethylase, *G3P* glyceraldehyde-3-phosphate, *DXP* deoxyxylulose 5-phosphate, *DXR* DXP reductoisomerase, *FOS* fosmidomycin, *DXS* DXP synthase, *MEP* methylerythritol 4-phosphate

(Hampel et al. 2005), (−)-*R*-pinene and (*S*)-linalool in raspberry fruits via the MVA pathway (Hampel et al. 2007), and hydrocarbon, botryococcene, and tetramethylsqualene via the MEP pathway in the *Botryococcus braunii* race B (Sato et al. 2003). However, in some cases both pathways play important roles, e.g., artemisinin in *Artemisia annua* L. (Towler and Weathers 2007), dolichols in *Coluria geoides* (Skorupinska-Tudek et al. 2008), gibberellins in *Arabidopsis thaliana* (Kasahara et al. 2002), and polyisoprenoids in *Eucommia ulmoides* (Bamba et al. 2010). Nevertheless, the question of whether both pathways or only one of them furnishes ginsenosides remains to be answered.

Hairy roots, induced by infection of plants with *Agrobacterium rhizogenes*, were recommended as an excellent system for many research fields relative to plants including metabolomics and genomics, due to their genetic stability, rapid growth, and ease of maintenance (Talano et al. 2012; Guillon et al. 2006). Here, we take advantage of *Panax ginseng* hairy root culture system to provide evidences indicating that ginsenosides are synthesized through both the MVA and the MEP pathways, more preferably through the former.

Materials and methods

Hairy root-inhibitors coculture assays

Hairy roots of *P. ginseng* used in this study were previously established in our laboratory and maintained by subculture on solid 1/2 MS medium at 25 °C and 4-week intervals (Liang et al. 2009). Stock solutions for inhibitors and relative solvents were filter sterilized (0.22 μm) into sterile containers and stored at 4 °C. FOS (Hubei Kechuang Medicine Co., Ltd, Wuhan, China) was dissolved in dH_2O. For better solubility, MEV (Sigma M2147) was hydrolyzed to mevinolin salt using a reported method (Rodrígues-Concepción and Gruissem 1999). Miconazole (MIC) (50 mM, Sigma M3512) was dissolved in DMSO (Sigma D8779). Aliquots of stock solutions were added into experimental medium (1/2 MS medium with 5 % sucrose, pH 5.8 prior to autoclaving at 120 °C for 20 min). Inhibitors and corresponding control solvents were added into the medium together with the inocula. Unless otherwise stated, tips (∼1 cm, 0.5 g) of hairy roots cultured in liquid 1/2 MS medium were used as inocula; each culture was incubated in a 100-ml experimental medium at 120 rpm and 25 °C in dark.

Hairy roots were harvested and gently blotted on filter paper. After blotted fresh weights (FW) were recorded, hairy roots were placed into pre-weighed stainless steel pans in a 60 °C oven for 3 days to determine dry weights (DW).

Ginsenoside and phytosterol analysis

Total ginsenoside level analysis was performed according to a protocol optimized in our laboratory, which was described as follows. Three replicates of dried hairy root powder (2 g) was dispersed in 70 % EtOH (v/v, 80 ml) and sonicated at 40 kHz and 40 °C for 30 min. The mixture was filtered and then the filtrate was evaporated to produce ginsenoside fraction, which was then dissolved in 10-ml dH_2O. Ten ml of ethyl ether was added into the aqueous phase to remove interfering chemicals. Afterwards, ginsenosides were extracted three times from the aqueous phase with an equal volume of H_2O-saturated *n*-butanol. The final obtained butanol solution was concentrated under vacuum and freeze-dried as ginsenoside extract. The extract was redissolved in 1-ml chromogenic agent [0.2 ml 5 % vanillin glacial acetic acid solution (w/v) and 0.8 ml perchloric acid]. The extract solution was incubated in a water bath at 60 °C for 15 min, and subsequently in an ice bath for 10 min, followed by addition of 5-ml glacial acetic acid. Total ginsenoside levels were calculated based on the OD_{544} of the final mixture. Ginsenoside Re was used as standard, and the resulting regression equation was

$OD_{544} = 3.3806X$ (X the concentration of ginsenoside, mg/ml).

Ginsenoside Rg_1, Re, and Rb_1 were quantitatively analyzed by HPLC. Ginsenosides were extracted as mentioned above and analyzed on a Waters C18 (4.6 × 250 mm, 5 μm) analytical column (Waters, USA) at 203 nm. Elution was carried out with 90 % CH_3CN at 0.8 ml/min. The contents of ginsenosides were calculated from the ratio of the peak area of the respective compound to that of the standard.

Freeze-dried hairy root powder (0.2 g) was suspended in 1-ml acetic anhydride, shaken at 60 °C for 6 h, and centrifuged at 10,000*g* for 20 min. The suspension was used for sterol analysis by a reported colorimetric method (Gao et al. 2001).

Enzyme activity analysis

Fresh hairy roots (2 g) cocultured with MEV were homogenized with 6-ml Buffer A (sucrose 3.423 %, KCl 0.3728 %, $K_2HPO_4 \cdot 3H_2O$ 0.913 %, EDTA 1.117 %, pH 7.2); the homogenate was centrifuged at 12,000*g* for 30 min; the resulting suspension was recentrifuged at 100,000*g* for 60 min. The obtained pellet was further homogenized with 750-μl Buffer B (dithiothreitol 0.3804 % in Buffer A) and 250-μl glycerol; the homogenate was incubated in a water bath at 37 °C for 60 min and diluted by addition of 2-ml Buffer B, then centrifuged at 100,000*g* for 60 min. Finally, the resulting suspension was used as crude HMGR for activity analysis by HMG-CoA Reductase Assay kit (Sigma) according to the attached procedure.

Fresh hairy roots (1 g) cocultured with FOS were homogenized with Buffer C (Tris–HCl 50 mM, mercaptoethanol 10 mM, polyvinylpyrrolidone 10 g/l, pH 7.5); the homogenate was centrifuged at 10,000*g* for 20 min; the resulting suspension was used as crude DXR. DXR activity was analyzed based on the decreased in A_{340} signal resulted from oxidation of NADPH. DXP and NADPH were incubated at 30 °C with 150-mM Tris–HCl (pH 7.5), 5-mM $MgCl_2$, 1-mM $MnCl_2$, and 4-mM DTT, prior to initiation of the reaction with DXR extract.

Protein was quantified using BCA Protein Assay kit (Peking Kangwei Shiji Biotechnology Co., Ltd, Peking, China).

Gene expression analysis by quantitative real-time PCR

Total RNA was isolated from the hairy roots with RNAiso Reagent (TaKaRa Dalian Biotechnology Co., Ltd.) according to the manufacturer's protocol. Primers for dammarenediol synthase (GenBank Accession no. AB265170) were designed as forward primer (F: 5′-GACACCACATACCAACAAGA-3′) and reverse primer (R: 5′-CCGATTTATTAT-TTATTTGAGAG-3′). PCR reaction was run using the Qiagen Quantitect SYBR Green PCR system according to the manufacturer's protocol. A denaturation step at 95 °C for 15 min was run prior to the amplification in three steps: denaturation at 95 °C for 15 s; annealing at 55 °C for 15 s; 20 s of extension at 72 °C, with a total of 40 cycles. The gene expression level of treated roots relative to the untreated roots was calculated by the comparative C_T method $\left(2^{-\Delta\Delta C_{(T)}}\right)$ with *Actin* as an internal control (Schmittgen and Livak 2008).

Statistical analysis

Data were analyzed with ANOVA followed by Dunnett's test using the software Sigma-Stat 3.5.

Results

Effects of inhibitors on growth and ginsenoside accumulation

It has been reported that very low levels of inhibitor could cause great inhibition on cell growth. Hence, to determine a suitable inhibitor concentration, we evaluated the effects of the concentrations of MEV and FOS on the hairy root growth. As shown in Fig. 2, the inhibition extent was dependent on the levels of the inhibitors. When the concentration increased to 60 μM, inhibition on growth began to be observed. Lower than this concentration threshold, neither of the inhibitors has an obvious negative effect on growth. To eliminate the growth difference on ginsenoside accumulation, the concentration of 50 μM was selected for the following experiment.

To eliminate the effects of the initial ginsenoside contents on the following result analysis, the newly developed tips of 1-week-old hairy roots were used as inocula. This is because that ginsenosides are secondary metabolites and mainly synthesized in the older hairy roots. With the presence of MEV, ginsenosides accumulation levels were reduced by up to 57.94 %, and up to 42.17 % with FOS (Fig. 3). HPLC analysis also showed that ginsenoside Rb_1, Re, and Rg_1 levels were significantly decreased by the inhibitors (Fig. 4). As expected, ginsenoside biosynthesis was almost completely silenced under the presence of both inhibitors (Fig. 3). Although the inhibitor concentration (50 μM) was lower than the above-proposed threshold, the hairy root growth was also affected (Fig. 5). Biomass or age difference could be the cause. However, the biomass difference was excluded, because reducing the older hairy root biomass did not lead to growth inhibition with either of the inhibitors at 50 μM. It has been suggested that younger cells are more sensitive to inhibitors than older ones (Heintze et al. 1990; Hemmerlin and Bach 1998; Towler and Weathers 2007). This also might account for the observation in this study. As

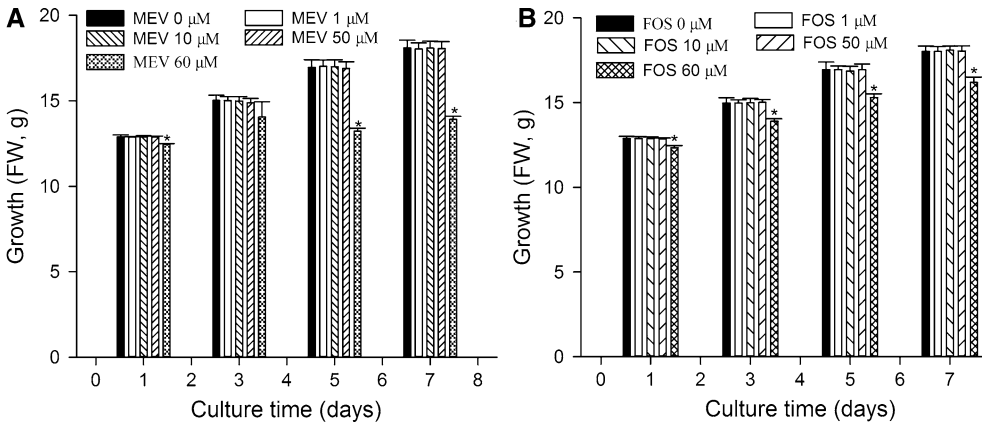

Fig. 2 Effects of MEV (**a**) and FOS (**b**) with different concentrations on growth of the hairy roots. Three-week-old hairy roots of 12.5 g were cocultured with the inhibitors in 200-ml medium for 1 week. Each histogram represents the mean level (±standard error) from triplicate culture. An *asterisk* above the *bar* indicates the individual lines showing a statistical difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leq 0.05$

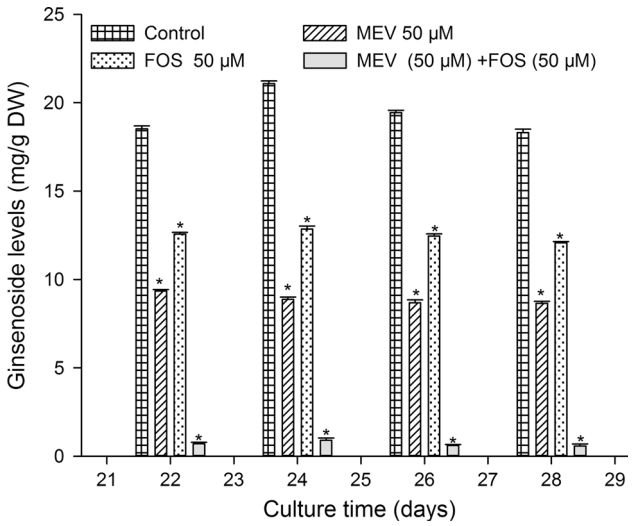

Fig. 3 Effects of MEV and FOS on ginsenoside levels of the hairy roots. Each histogram represents the mean level (±standard error) from triplicate cultures. An *asterisk* above the *bar* indicates the individual lines showing a statistical difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leq 0.05$

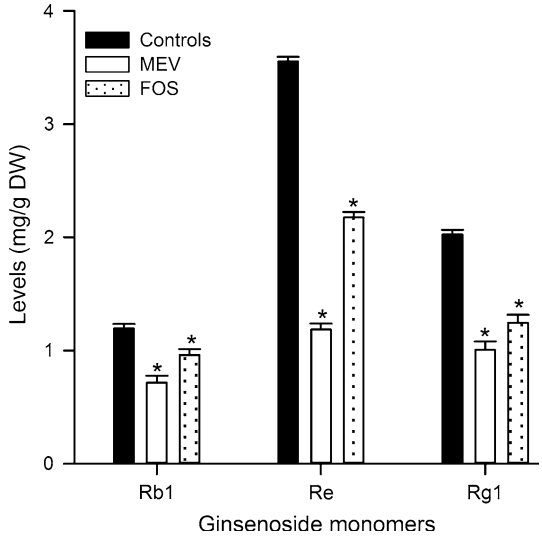

Fig. 4 Effects of MEV and FOS on levels of ginsenoside Rb_1, Re, and Rg_1. Each histogram represents the mean level (±standard error) from triplicate cultures. Data from FOS + MEV are not shown, because the levels were not detectable. An *asterisk* above the *bar* indicates the individual lines showing a statistical difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leq 0.05$

observed in a previous study (Hemmerlin et al. 2003), FOS was a less effective growth inhibitor than MEV in this work. Thickened root phenotype was observed in several studies (Kasahara et al. 2002; Towler and Weathers 2007; Rodrígues-Concepción et al. 2004). However, here no obvious phenotype difference was observed between the inhibitor-treated hairy roots and the controls (Fig. 6).

Effects of inhibitors on HMGR and DXP activities

To provide further supporting data, HMGR and DXP activities were tested in hairy roots cocultured with MEV and FOS, respectively. As predicted, HMGR and DXP activities were significantly decreased (Fig. 7). Dammarenediol synthase gene is considered one of the most important genes involved in ginsenoside biosynthesis, therefoe its expression was analyzed by qPCR. The results suggested that the expression of this gene was also down-regulated under the presence of the inhibitors (Fig. 8).

Effects of MIC on ginsenoside accumulation

MIC can suppress sterol biosynthesis by inhibiting sterol 14a-demethylases (Zarn et al. 2003). Ginsenoside

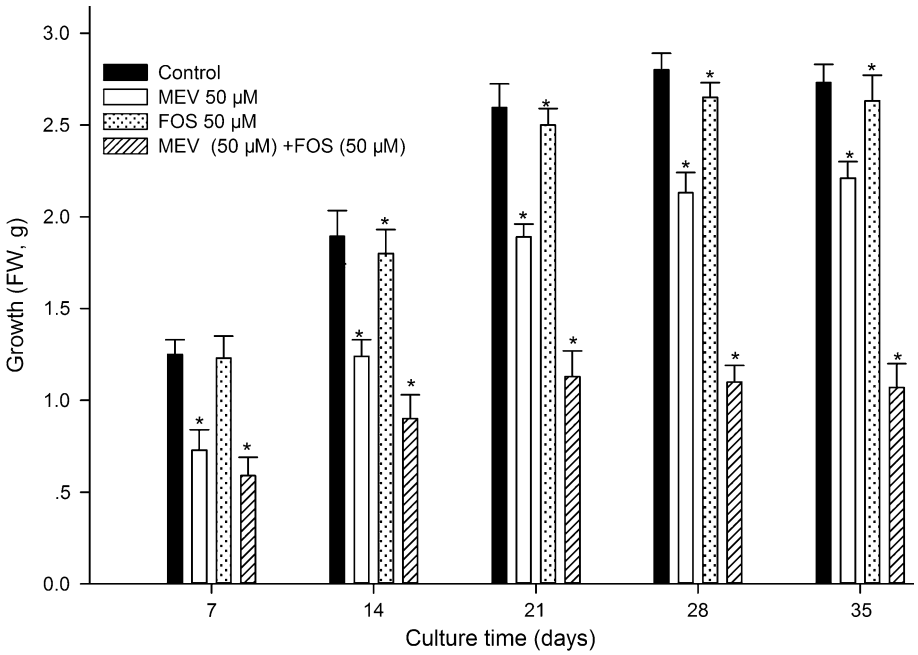

Fig. 5 Effects of MEV and FOS on hairy root growth. Each histogram represents the mean level (±standard error) from triplicate cultures. An *asterisk* above the *bar* indicates the individual lines showing a statistical difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leq 0.05$

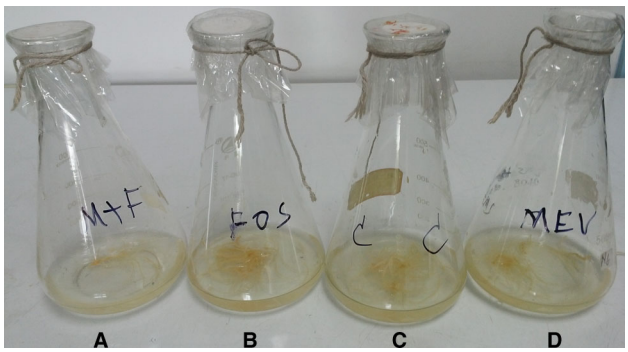

Fig. 6 Representative pictures of hairy roots cultured with inhibitors for 21 days. **a** MEV + FOS, **b** FOS, **c** control, and **d** MEV. Both inhibitors were present at 50 μM

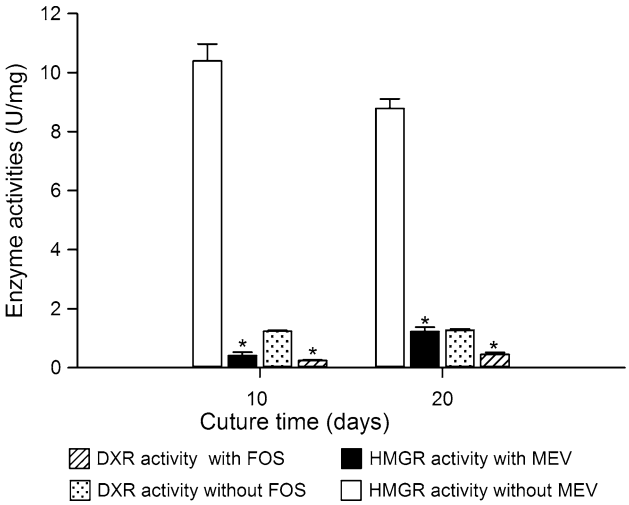

Fig. 7 Effects of MEV (50 μM) and FOS (50 μM) on HMGR and DXR enzyme activity in *P. ginseng* hairy roots. Each histogram represents the mean level (±standard error) from triplicate cultures. An *asterisk* above the *bar* indicates the individual lines showing a statistical difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leq 0.05$

accumulation was significantly enhanced by MIC (Fig. 9). When cocultured with MIC for 24 days, the hairy roots can accumulate ginsenoside at a level more than two times that of control. Due to its water insolubility, MIC was dissolved in DMSO. Therefore, control cultures with DMSO were established. It is noteworthy that the solvent also promotes ginsenoside biosynthesis. Moreover, the effect of MIC on phytosterol accumulation was assessed. As predicted, the phytosterol contents were down-regulated by MIC (Fig. 9).

Discussion

The cytosolic MVA and the plastidic MEP pathways constitute the two arms in biosynthesis of IPP. From an evolutionary point of view, MVA pathway is considered to be native in eukaryotes, while MEP pathway is native in bacteria. Plants and other plastid-containing eukaryotes obtained MEP pathway by the transfer of genes from the original cyanobacterial endosymbiont, from which the plastids originated (Lombard and Moreira 2011). It has been shown that some compounds are synthesized exclusively from one of the pathways, but others are from both. Here, we employed inhibitors (MEV and FOS) specific to each pathway to elucidate the roles of both the MVA and the MEP pathways in ginsenoside biosynthesis using *P. ginseng* hairy roots as experimental biomaterials. It has been well documented that MEV, majorly originated from

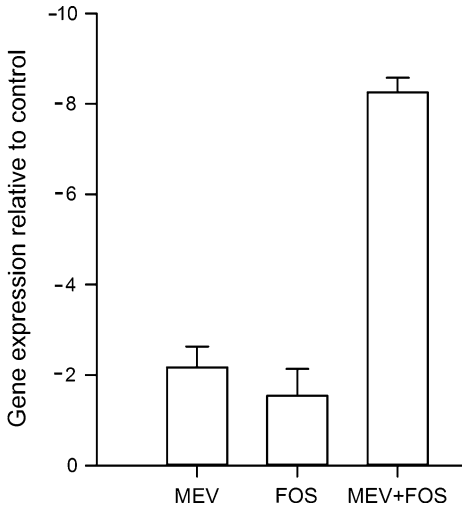

Fig. 8 Quantitative real-time PCR analysis on expression of dammarenediol synthase gene responsive to stress of the inhibitors in 20-day-old *P. ginseng* hairy roots. Both inhibitors are present at 50 μM. Each histogram represents the mean level (±standard error) from triplicate cultures. The relative gene expression levels were calculated by the comparative C_T method. The fold change due to treatment was expressed as $-\left(1/2^{-\Delta\Delta C_{(T)}}\right)$

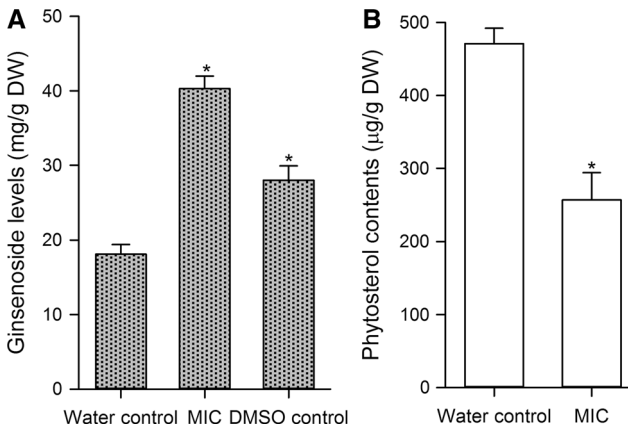

Fig. 9 Effects of sterol inhibitor MIC on ginsenoside (**a**) and phytosterol (**b**) levels in the hairy roots. Hairy roots cocultured with 50-μM MIC for 24 days were used for the analysis. Each histogram represents the mean level (±standard error) from triplicate cultures. An *asterisk* above the *bar* indicates the individual lines showing a statistical difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leq 0.05$

fungi, could function as a MVA pathway-specific inhibitor via suppression of the rate-limiting enzyme HMGR (Ormeno et al. 2007). FOS, originally isolated from *Streptomyces lavendulae* could serve as a MEP pathway-specific inhibitor by interaction with the enzyme DXP (Bach et al. 1990; Hemmerlin et al. 2003). Both inhibitors have proved to be powerful reverse tools in studies relative to the corresponding pathways (Towler and Weathers 2007; Hemmerlin and Bach 1998; Masse et al. 2004).

In this study, hairy roots exposed to either of the inhibitors at higher concentrations showed retarded growth. However, there were no obvious negative effects on the hairy root growth with inhibitors at lower concentrations (≤50 μM). However, under the presence of both inhibitors at lower concentrations, growth was significantly inhibited (Figs. 5, 6). Together with the almost complete blockage of ginsenoside biosynthesis in the presence of both inhibitors at lower concentrations, this implied that commutative compensation between the MVA and the MEP pathways could guarantee a normal growth for the hairy roots. Consequently, we could speculate that the inhibitors, when present at higher concentrations, might have other negative effects on hairy root growth. Side effects were also hypothesized with FOS (Hemmerlin et al. 2003). Down-regulation of plant growth by both inhibitors has been observed in a wide range of studies (Towler and Weathers 2007). It was also reported that complete blockage of MVA pathway could lead to suppression of gametophyte development (Suzuki et al. 2009).

Down-regulation of ginsenoside levels in the hairy roots by either MEV or FOS demonstrated that both the MVA and the MEP pathways contribute to ginsenoside anabolism. Although these two pathways are considered to be separated in different compartment (MVA in cytosol and MEP in plastids), cross-talk between them has been observed (Hemmerlin et al. 2003). In terms of ginsenoside biosynthesis, the above data are not enough to justify the cross-talk between the two pathways. The reason is that it is still unclear whether the ginsenoside synthetic pathway, especially the downstream part, is localized in the compartment with MVA pathway or MEP pathway or with both. For better understanding of ginsenoside biosynthesis, this aspect is clearly worth more effort.

Our observation that ginsenoside biosynthesis could operate under blockage in either of the pathways, but was completely silenced by inhibition of both, excluded the possibility of the existence of another pathway leading to IPP in ginsenoside biosynthesis. In addition, the extent of inhibition in ginsenoside synthesis with MEV was greater than that with FOS, indicating that the MVA pathway plays a more important role in ginsenoside biosynthesis.

Sterol biosynthetic pathway is a competitor to that of ginsenoside. This study showed that sterol pathway blockage by MIC-enhanced ginsenoside biosynthesis in the hairy roots. This was consistent with the previous research where antisense suppression of sterol biosynthesis resulted in improved ginsenoside levels (Liang et al. 2009). However, phytosterol level in the hairy roots is not more than 1 mg/g; it appears that this sterol level is not enough to quantitatively explain the ~15 mg/g up-regulation in ginsenoside level (Fig. 9). As MIC was previously reported to enhance cell permeability (Sreedhara Swamy et al. 1974), alternatively

the enhancement of ginsenoside levels might mainly be resulted from membrane permeabilization leading to enhanced substrate uptake and product formation. On the other hand, we could think that phytosterols in organisms are also consumed as building blocks or intermediates for metabolism, or digested. The stimulatory effect of DMSO on ginsenoside production was unpredicted. It was also shown that MIC could improve artemisinin biosynthesis in *Artemisia annua* L. (Towler and Weathers 2007). As DMSO was also reported as a cell permeabilization chemical (Cai et al. 2012), enhancement of cell permeability could be the one possible explanation for the enhancement of ginsenoside level. The effects of DMSO on plant secondary metabolism, like ginsenoside and artemisinin, should be further studied to determine its mechanism of action and possible utility in overproduction of valuable secondary metabolites.

In summary, this work provides some clues indicating that ginsenosides are synthesized from both the MVA and the MEP pathways. This study will be helpful for better understanding of ginsenoside biosynthesis. Presently, there are lots of ginseng transcriptome data in public databases, and most of the corresponding genes involved in both pathways have been discovered from these dataset based on several previous *P. ginseng* transcriptome studies (Chen et al. 2011; Jung et al. 2003; Kim et al. 2006; Li et al. 2013; Sathiyamoorthy et al. 2010a, b). Indubitably, these data will contribute to dissecting both pathways in *P. ginseng*. Nevertheless, more work remains to be carried out relative to both pathways in *P. ginseng*. The questions of how the MVA and the MEP pathways are fine-tuned to meet metabolism requirement, and how these two pathways cooperate in biosynthesis of ginsenosides and other compounds remain to be solved.

Acknowledgments This work has been supported by funds from National High Technology Research and Development Program 863 (2013AA102604-3), National Natural Science Foundation of China and Fundamental (31270337), Specialized Research Fund for the Doctoral Program of Higher Education of China (20120061110038), and Fund for Undergraduate Innovation Training Program of Jilin University.

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