JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Induction of de Novo Volatile Terpene Biosynthesis via Cytosolic and Plastidial Pathways by Methyl Jasmonate in Foliage of *Vitis vinifera* L.

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The terpene biosynthesis in leaves of *Vitis vinifera* L. cv. Morio Muskat was studied using methyl jasmonate to induce defensive responses in vivo. The experiments demonstrated the strong activation of the de novo biosynthesis of terpenoids via the octadecanoid-signaling cascade and release of the compounds to the gas phase. Feeding experiments with [5,5-$^2H_2$]-1-deoxy-D-xylulose and [5,5-$^2H_2$]-mevalonic acid lactone allowed the investigation of the dynamic allocation of resources via the mevalonic acid and 1-deoxy-D-xylulose/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway under induced conditions and after treatment with the specific inhibitors mevastatin and fosmidomycin. The experiments reveal that monoterpenes are almost exclusively synthesized via the DOXP/MEP pathway, whereas sesquiterpenes are generated via both pathways at approximately equal rates. The biosynthesis of the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene was not affected by mevastatine or fosmidomycin.

KEYWORDS: Vitaceae; 1-deoxy-D-xylulose; mevalonic acid; inhibition studies; octadecanoid-signaling cascade; enantioselective multidimensional gas chromatography−mass spectrometry (enantio-MDGC-MS); dynamic headspace extraction

INTRODUCTION

Plants are able to communicate and interact with their environment utilizing volatiles (*1*, *2*). Due to this mechanism plants are able to attract pollinators or to influence the germination of seed and the growth of neighborhood plants (*3*). Damaged plants seem to be able to induce prophylactic defense and resistance in uninfested plants by emitting airborne volatiles such as methyl jasmonate, salicyclic acid, or ethylene (*4*, *5*). Insects are affected as well by plant volatiles in case those are closely related or even identical to chemicals from their own intraspecific communication network. Thus, plants infested with herbivores have the ability to decoy carnivores for attacking the pests by a so-called "plants' cry for help" (*1*). These defense and resistance activities afford mainly terpenes as has been demonstrated by inducing sprouts of lima bean (*Phaseolus lunatus*) with the elicitor methyl jasmonate (*2*).

It has been suggested that one of the very common volatile terpenes, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), is stored as a β-glucoside of its sesquiterpenoid precursor nerolidol and is cleaved by a β-glucosidase contained in the regurgitant of the feeding insect. DMNT is then generated by an oxidative degradation. Contrarily, it could be shown that plants synthesize the herbivore-induced volatiles de novo (*2*).

However, the constitutive and induced biosynthesis of terpenes can be accomplished by two independent pathways, the cytosolic mevalonic acid (MVA) pathway and the plastidial 1-deoxy-D-xylulose/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) route (*6*−*10*). Both biosynthetic pathways render isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the basic five-carbon building blocks of terpenoids. Generally, monoterpenes are formed via the DOXP/MEP pathway, whereas sesquiterpenes are biosynthesized via the classical MVA route. However, there is no absolute compartmental separation of the two pathways, and the extent of this cross-talk depends on the species and the physiological conditions (*11*). Examples for this cross talk vary from the exclusive utilization of plastid-derived IPP/DMAPP for germacrene D biosynthesis (*12*), over the equal utilization of both plastid- and cytosolic-derived IPP/DMAPP for the generation of β-caryophyllene (*13*), to the almost exclusive utilization of MVA-derived IPP/DMAPP with only minor spill-over of plastidial IPP units into cytosolic sterols (*14*). However, recently published findings for tobacco cells demonstrate that under rather restrictive conditions a complementation of plastidial isoprenoid synthesis by the cytosolic MVA pathway seems to be possible (*15*). The latest studies suggest the presence of a unidirectional proton symport system in plastid membranes for the export of

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10.1021/jf040421q CCC: $30.25
Published on Web 03/12/2005

specific isoprenoid intermediates involved in the metabolic cross talk between cytosolic and plastidial pathways (*16*).

It could also be shown that this cross talk can be modulated by exogenous inhibitors: The antibiotic fosmidomycin can be utilized to interfere with the DOXP/MEP pathway by inhibition of the 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase. Due to this inactivity 1-deoxy-D-xylulose 5-phosphate cannot be rearranged to the intermediate 2-C-methylerythrose 4-phosphate, which gives 2-C-methyl-D-erythritol 4-phosphate after reduction (*17*). Consequently, this terpene source is no longer available.

The MVA route can be obstructed by mevastatin, which inhibits the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. As a result the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate is restrained, which causes the breakdown of this pathway (*18*). However, little is known about the highly complex world of terpene interpathway cross talk and the induced terpene biosynthesis in the foliage of *Vitis vinifera*. Diurnal emission of terpenoid volatiles by Japanese beetle-damaged leaves of *Vitis labrusca* has been demonstrated (*23*), but the plants' reaction on interference with their biosynthesis mechanism has not been investigated yet.

Consequently, the aim of this study is to investigate cross talk and inhibition phenomena between the two terpenoid pathways. For this purpose cut stem in vivo feeding experiments with [5,5-$^2H_2$]-mevalonic acid lactone (d_2-MVL) and [5,5-$^2H_2$]-1-deoxy-D-xylulose (d_2-DOX) were conducted by inducing terminal stems of *V. vinifera* cv. Morio Muskat with methyl jasmonate. Inhibition experiments allowed us to study a possible modulation of terpenoid pathway utilization triggered by specific inhibitors.

EXPERIMENTAL PROCEDURES

Plant Material. Terminal stems and attached leaves of *V. vinifera* cv. Morio Muskat were obtained from the Research Centre Geisenheim, Department for Grapewine Breeding and Grafting (Geisenheim, Germany), in 2002−2004.

Chemicals. *cis*-Ocimene, (−)-linalool, and (±)-linalool were obtained from Fluka (Taufkirchen, Germany). β-Caryophyllene and α-humulene were obtained from Berje (Bloomfield, NJ), and fosmidomycin sodium salt was obtained from Molecular Probes (Leiden, The Netherlands), whereas mevastatin and methyl jasmonate were obtained from Sigma-Aldrich (Steinheim, Germany). [5,5-$^2H_2$]-Mevalonic acid lactone was prepared according to the method of Simpson et al. (*19*), [5,5-$^2H_2$]-1-Deoxy-D-xylulose was prepared according to the method of Jux and Boland (*20*). Spectral data of the labeled compounds were in all cases in good agreement with the data given in the references cited above.

Sample Preparation. Terminal stems of *V. vinifera* (∼4 g) were cut with razor blades and transferred into 4 mL vials containing the labeled precursors (d_2-DOX, 2 mg/mL; d_2-MVL, 3 mg/mL), inhibitors (fosmidomycin, 1.8 mM; mevastatin, 0.6 mM), and the elicitor methyl jasmonate (40 μL/100 mL). The samples were placed into the dynamic headspace equipment, and the volatile compounds were absorbed on a Tenax column for 48 h. The inhibition experiments were carried out by simultaneous addition of fosmidomycin/d_2-MVL and mevastatin/d_2-DOX, respectively, as well as by a 24 h preincubation with the inhibitors following the addition of labeled precursors.

The direct extracts were obtained after 3 days of incubation. The plant material was ground in phosphate buffer (∼20 mL) to a suspension that was extracted with pentane/diethyl ether (1:1) (3 × 30 mL). The organic fractions were combined and dried over Na_2SO_4, and the solvent was removed using a Vigreux column (water bath temperature = 42 °C).

Gas Chromatography−Mass Spectrometry (GC-MS). The GC-MS analyses of the synthesized products and dynamic headspace extracts were carried out on a Fisons Instruments GC 8065, coupled to a Fisons Instrument MD 800 mass spectrometer, equipped with a self-prepared fused silica capillary column coated with SE-52 (30 m × 0.25 mm i.d.; film thickness = 0.23 μm). GC conditions were as follows: carrier gas, helium, 69 kPa; split, 20 mL/min; injector temperature, 230 °C; oven temperature, 40 °C (5 min isothermal), then 5 °C/min to 260 °C (20 min isothermal); ion source temperature, 200 °C; mass range, 40−300 amu; EI, 70 eV. The molecular ions (M^+) and fragment ions are given as *m*/*z* with relative peak intensities in percent of the most abundant peaks.

The GC-MS analyses of the samples were also performed on a Varian GC series 3400, coupled to a Finnigan MAT Magnum mass spectrometer, equipped with a self-prepared fused silica capillary column coated with SE-52 (30 m × 0.25 mm i.d.; film thickness = 0.23 μm). GC conditions were as follows: carrier gas, helium, 10 psi; split, 20 mL/min; injector temperature, 250 °C; oven temperature, 60 °C (5 min isothermal), then 5 °C/min to 220 °C (5 min isothermal); ion source temperature, 200 °C; mass range, 50−300 amu; EI, 70 eV. The molecular ions (M^+) and fragment ions are given as *m*/*z* with relative peak intensities in percent of the most abundant peaks.

Enantioselective Multidimensional Gas Chromatography−Mass Spectrometry (Enantio-MDGC-MS). The enantio-MDGC-MS analyses were carried out on a Siemens SiChromat 2 equipped with a Live-T column switching device and a self-prepared fused silica capillary column coated with SE-52 (30 m × 0.25 mm i.d.; film thickness = 0.23 μm) as precolumn; the carrier gas was hydrogen at 2.15 bar, split = 30 mL/min, with an FID at 250 °C. The main column was a self-prepared fused silica capillary (30 m × 0.25 mm i.d.) coated with a 0.25 μm film of heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (DIAC-β-CD) (50%) in OV1701 (50%), coupled to a Perkin-Elmer ion trap detector; conditions were as follows: transfer line temperature, 250 °C; open split interface, 250 °C; ion trap manifold, 200 °C; EI, 70 eV; oven temperature program, precolumn, 60 °C (5 min isothermal), raised at 5 °C/min to 250 °C; main column, 60 °C (30 min isothermal), then 2 °C/min to 200 °C. Cut times were as follows: *trans*-β-ocimene, 9.5−12.0 min; linalool, 12.6−14.2 min; *trans*,*trans*-α-farnesene, 24.5−26.1 min.

RESULTS AND DISCUSSION

To investigate the induced biosynthesis of terpenes in *V. vinifera*, terminal stems of cv. Morio Muskat were treated with methyl jasmonate. The ability of methyl jasmonate to induce volatile production seems to be a general phenomenon and could be confirmed for *V. vinifera* as illustrated in **Figure 1**. The lower chromatogram shows the volatiles emitted from a noninduced control trapped by adsorption in a dynamic headspace apparatus for 48 h, and the upper chromatogram shows the volatiles that are emitted after jasmonate treatment under the same conditions. The experiment revealed a strong enhancement (up to 100 times) of volatile emission after induction. The three main components of the emitted volatiles were identified as the monoterpene (*E*)-β-ocimene, the homoterpene 4,8-dimethylnona-1,3,7-triene (DMNT), and the sesquiterpene (*E*,*E*)-α-farnesene. They were unequivocally identified by retention times, mass spectra, Kovats indices, and, as far as possible, co-injection of authentic standards. The homoterpene DMNT is generated from the sesquiterpene nerolidol by an oxidative degradation catalyzed by enzymes that most likely belong to the family of cytochrome P450 monooxygenases (*1*, *21*, *22*).

A blend of mono- and sesquiterpenes in minor concentrations could be identified as well (see **Figure 1**). Among them, the chiral monoterpene linalool was present as the (*S*)-configured stereoisomer as revealed by enantio-MDGC-MS analysis. The profile of the emitted volatiles is similar to that of Japanese beetle-damaged leaves of *V. labrusca* (*23*) and confirms the role of jasmonate in the wound-induced octadecanoid-signaling sequence that leads ultimately to the enhanced expression of plant defense genes including those of the mono- and ses-

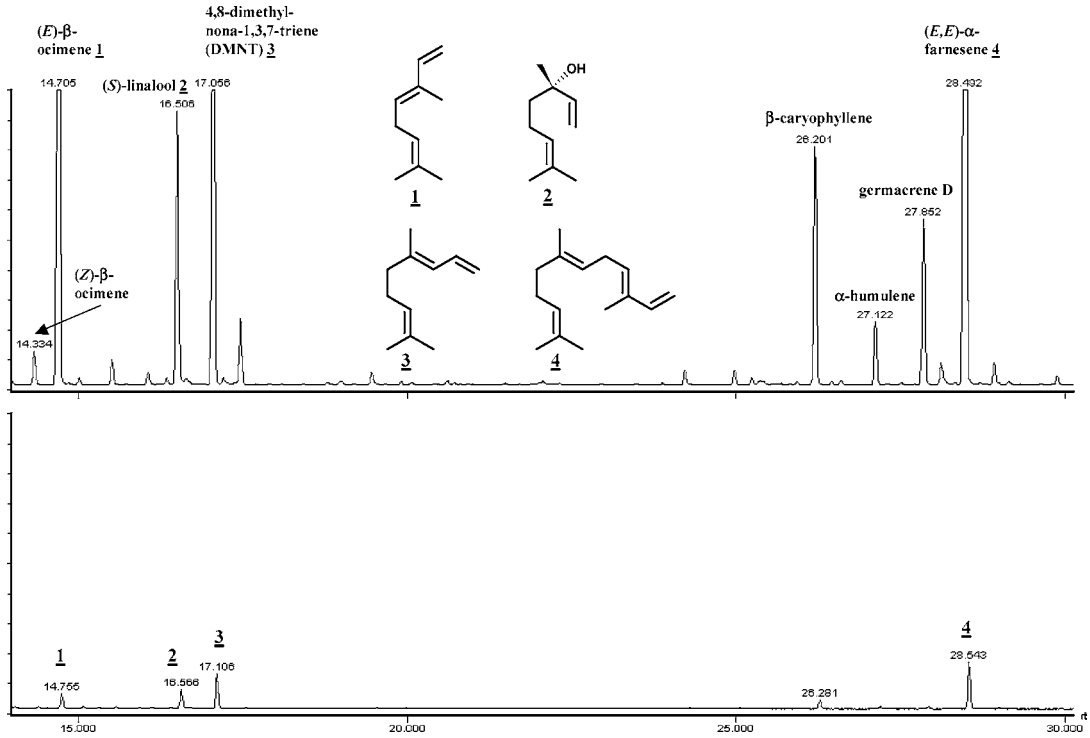

Figure 1. GC-MS anaylsis of the dynamic headspace extract of terminal stems of untreated (lower trace) and methyl jasmonate treated *V. vinifera* cv. Morio Muskat (upper trace).

Table 1. Degree of Labeling of Induced Terpenes (Methyl Jasmonate Treatment) after Preincubation of *V. vinifera* Cv. Morio Muskat Terminal Stems with [5,5-$^2H_2$]-DOX, [5,5-$^2H_2$]-MVL, [5,5-$^2H_2$]-DOX/Formidomycin, or [5,5-$^2H_2$]-MVL/Mevastatin

	labeling degree[a] (%)			
compound	[5,5-$^2H_2$]MVL	[5,5-$^2H_2$]MVL/ fosmidomycin	[5,5-$^2H_2$]DOX	[5,5-$^2H_2$]DOX/ mevastatin
plastidic compartment				
(*S*)-linalool	>1	>1	64 ± 2	5 ± 3
(*E*)-β-ocimene	1 ± 0.3	>1	74 ± 8	18 ± 3
cytosolic compartment				
(*E*,*E*)-α-farnesene	12 ± 1	8 ± 5	31 ± 10	1 ± 0.9
DMNT	13 ± 1	15 ± 4	16 ± 1	24 ± 15

[a] Relative amounts (percent) of labeled compounds of the total amounts of labeled and unlabeled compounds are shown. The labeling degree was determined by integration of the signals of the labeled compounds in the corresponding ion traces.

quiterpene biosynthesis. Nevertheless, a small biosynthetic activity of these enzymes is observed in noninduced *V. vinifera* plants as can be seen in **Figure 1**. The same results have been found for *V. labrusca* (*23*).

To investigate the pathway utilization of this induced terpenoid biosynthesis and to quantify the cross talk between the two terpenoid pathways, cut stem in vivo feeding experiments with [5,5-$^2H_2$]mevalonic acid lactone (d_2-MVL) and [5,5-$^2H_2$]-1-deoxy-D-xylulose (d_2-DOX) were conducted with methyl jasmonate induced plants. (*E*)-β-Ocimene/(*S*)-linalool and (*E*,*E*)-α-farnesene/DMNT were chosen as representatives for mono- and sesquiterpenes, respectively. The generated volatiles were directly extracted from the plant material or trapped by adsorption using a dynamic headspace apparatus as described before.

The in vivo feeding experiments with deuterium-labeled DOX revealed a good incoporation of the precursor into the monoterpenes (*E*)-β-ocimene and (*S*)-linalool with labeling degrees that exceeded 60% (see **Table 1**). Obviously, a kinase converting labeled DOX into its 5-phosphate must be highly active in *V. vinifera*. **Figure 2** shows the (*E*)-β-ocimene section of a main column chromatogram of a dynamic headspace extract and the corresponding mass spectra. The d_2- and d_4-isotopomers are well separated from the unlabeled genuine (*E*)-β-ocimene due to the inverse isotope effect of deuterium-labeled compounds in GC separations. The labeling degree can be deduced from the corresponding mass spectra: An incorporation of labeled IPP/DMAPP should cause a shift by 2 or 4 mass units, given that monoterpenes are generated from two isoprene units and that no deuterium atom is lost during the biosynthetic reaction sequence. Therefore, especially the greater fragments should be shifted by up to 4 mass units. In fact, these shifts are observable for the d_2- and d_4-isotopomers and the mass peaks 79 (shifted to 81 and 83, respectively), 93 (shifted to 95 and 97, respectively), and 121 (shifted to 123 and 125, respectively; see **Figure 2**). The fact that no d_1- or d_3-isotopomer is detectable is in agreement with the incorporation of IPP and DMAPP units that were generated from labeled DOX via the novel DOXP-pathway. The same could be observed for the mass spectra of labeled linalool (data not shown).

However, when labeled MVA was administered, only very low labeling degrees for linalool and ocimene were observed (see **Table 1**). Obviously, there is a rather limited import of mevalonate-derived IPP, DMAPP, or geranyl diphosphate (GPP)

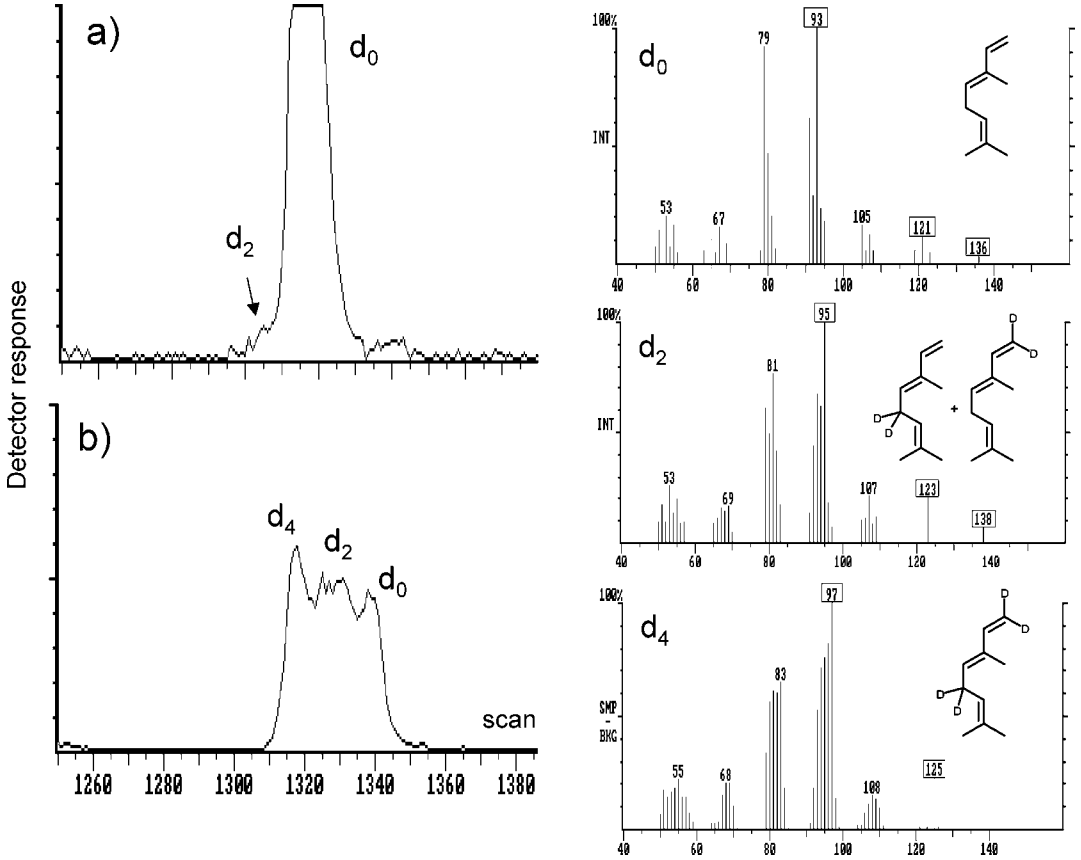

Figure 2. Main column chromatogram and MS spectra of unlabeled (d_0) and labeled (*E*)-β-ocimene (d_2, d_4) obtained from direct extraction of terminal stems of *V. vinifera* cv. Morio Muskat when [5,5-$^2H_2$]-DOX (**a**) or [5,5-$^2H_2$]-MVL (**b**) is administered.

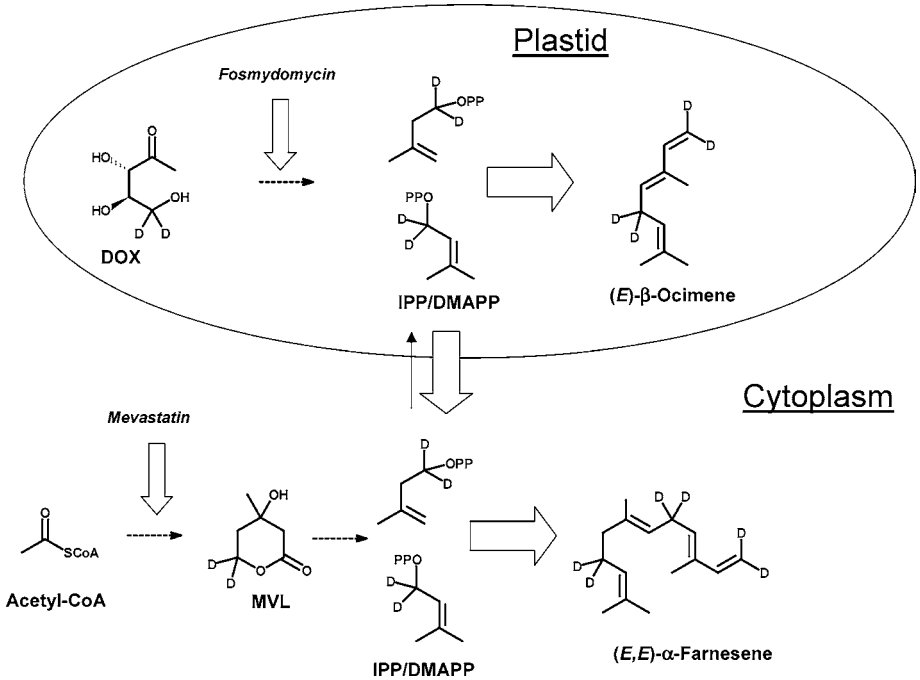

Figure 3. Model for induced terpene biosynthesis in *V. vinifera* cv. Morio Muskat leaves.

from cytosol into plastids, where the biosynthesis of monoterpenes is usually localized. This result is comparable with the situation in *V. vinifera* leaves under normal, noninduced conditions, where the monoterpene biosynthesis relies almost exclusively on the DOXP/MEP pathway (*11*).

The administration of labeled DOX and MVL revealed a good incorporation of both precursors into the sesquiterpene (*E*,*E*)-α-farnesene (**Table 1**). Thus, IPP and DMAPP utilized for biosynthesis of sesquiterpenes can be offered via the cytosolic MVA route as well as via the plastidial DOXP/MEP pathway. According to these results one can assume a transport of IPP and/or DMAPP from the plastids into the cytosol, where the biosynthesis of sesquiterpenes is usually localized. An export of geranyl diphosphate is conceivable as well. Indeed, Bick and

Lange (*16*) recently demonstrated that plastid membranes possess a unidirectional proton symport system for the export of specific isoprenoid intermediates involved in this metabolic cross talk.

The sesquiterpene-derived homoterpene DMNT showed a good incorporation of labeled DOX and MVA as well with almost equal labeling degrees (see **Table 1**).

It is remarkable that the origin of IPP/DMAPP used for sesquiterpene or sterol biosynthesis seems to be rather variable in the plant kingdom: Examples range from exclusive utilization of plastid-derived IPP/DMAPP for germacrene D biosynthesis (*12*) to almost exclusive utilization of MVA-derived IPP/DMAPP with only minor spillover of plastidial IPP units into cytosolic sterols (*14*). The results of this study for DMNT and (*E*,*E*)-α-farnesene biosynthesis lie between these two extreme cases and are comparable with sesquiterpene biosynthesis in carrot roots and leaves (*13*).

In recent studies it could be demonstrated that this allocation of resources is dynamic and can be influenced by stress factors or selective inhibitors (*1*, *2*, *15*): By blocking one of the biosynthetic terpenoid pathways, the biosynthetic activity of the noninhibited route was raised. To clarify if this dynamic allocation of resources is also active in *V. vinifera* leaves, inhibition studies were carried out by simultaneous addition of an inhibitor and labeled precursor, as well as by preinhibition and addition of the labeled precursor after 24 h. Both experimental designs led to the same results (see **Table 1**): The administration of labeled DOX after inhibition of the MVA pathway by mevastatin showed no increased incorporation into the sesquiterpene farnesene. Contrarily, the labeling degree dropped to 1%. Mevastanin, although a specific inhibitor of the MVA pathway, seems to affect the biosynthetic activity in *V. vinifera* leaves in general by nonspecific effects. That statins are able to cause some unspecific, generally toxic effects in plants is well-known (*15* and references cited therein). Although we used mevastatin at a concentration range that was applied in previous studies (*1*, *2*), causing neither bleaching nor wilting for the duration of the in vivo feeding experiments, *V. vinifera* seems to be much more susceptible to these nonspecific toxic effects. This might also explain the reduced labeling degrees for the monoterpenes linalool and ocimene when mevastatine is administered. In a similar study with lima beans (*P. lunatus*) inhibition of the MVA route by mevastatin could not increase the incorporation of d_2-DOX into the monoterpenes ocimene and linalool, thus confirming the rather low importance of the MVA pathway for the biosynthesis of monoterpenes (*2*). However, recently published findings for tobacco cells show that under rather restrictive conditions a complementation of plastidial isoprenoid synthesis by the cytosolic MVA pathway seems to be possible (*15*). Feeding labeled MVL after the inhibition of the DOXP/MEP pathway by fosmidomycin reveals no enhanced incorporation of the fed precursor into the monoterpenes, confirming again the rather low importance of the MVA pathway for the biosynthesis of monoterpenes. The biosynthesis of DMNT was not affected by mevastatin or fosmidomycin, and the incorporation of DOX was even slightly enhanced in the presence of mevastatin. Obviously, the plant is able to secure the biosynthesis of the homoterpene DMNT under stress conditions, thus ensuring the communication and interaction with its environment as well as the defense and resistance activity. These findings are in agreement with previous studies using lima beans (*1*, *2*). **Figure 3** illustrates a model for induced terpene biosynthesis in *V. vinifera* leaves.

The experiments demonstrated the strong activation of the de novo biosynthesis of terpenoids in *V. vinifera* leaves via the octadecanoid-signaling cascade and release of the compounds to the gas phase. Thus, the ability of methyl jasmonate to induce volatile production seems to be a general phenomenon and is also operative in *V. vinifera*. However, the allocation of resources via the MVA and DOXP/MEP pathways under normal and induced conditions obviously varies from one plant species to another. In edible plants and herbs such a strong influence on physiology and metabolism could affect the quality of aroma and flavor on which commercial interest is based. Moreover, the situation may be quite different for the production of terpenoids in specialized secretory tissues of terpenoid accumulators such as citrus, conifers, and essential oil plants. Thus, further investigations that are focused on fruits, vegetables, and essential oil producing plants are necessary.

ACKNOWLEDGMENT

We gratefully thank Prof. Dr. Ernst Rühl, Department of Grapewine Breeding and Grafting, Geisenheim, for making grapevines available.

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Received for review October 13, 2004. Revised manuscript received January 12, 2005. Accepted January 16, 2005.

JF040421Q