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Biosynthesis of sesquiterpenes in grape berry exocarp of *Vitis vinifera* L.: evidence for a transport of farnesyl diphosphate precursors from plastids to the cytosol

Bianca May[1], **B. Markus Lange**[2], and **Matthias Wüst**[1,*]

[1]University of Bonn, Department of Nutrition and Food Sciences, Bioanalytics, Endenicher Allee 11-13, D-53115 Bonn, Germany

[2]Institute of Biological Chemistry and M.J. Murdock Metabolomics Laboratory, Washington State University, Pullman, WA 99164-6340, USA

Abstract

The participation of the mevalonic acid (MVA) and 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol-4-phosphate (DOXP/MEP) pathways in sesquiterpene biosynthesis of grape berries was investigated. There is an increasing interest in this class of terpenoids, since the oxygenated sesquiterpene rotundone was identified as the peppery aroma impact compound in Australian Shiraz wines. To investigate precursor supply pathway utilization, *in vivo* feeding experiments were performed with the deuterium labeled, pathway specific, precursors [5,5-$^2H_2$]-1-deoxy-D-xylulose and [5,5-$^2H_2$]-mevalonic acid lactone. Head Space-Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) analysis of the generated volatile metabolites demonstrated that *de novo* sesquiterpene biosynthesis is mainly located in the grape berry exocarp (skin), with no detectable activity in the mesocarp (flesh) of the Lemberger variety. Interestingly, precursors from both the (primarily) cytosolic MVA and plastidial DOXP/MEP pathways were incorporated into grape sesquiterpenes in the varieties Lemberger, Gewürztraminer and Syrah. Our labeling data provide evidence for a homogenous, cytosolic pool of precursors for sesquiterpene biosynthesis, indicating that a transport of precursors occurs mostly from plastids to the cytosol. The labeling patterns of the sesquiterpene germacrene D were in agreement with a cyclization mechanism analogous to that of a previously cloned enantioselective (*R*)-germacrene D synthase from *Solidago canadensis*. This observation was subsequently confirmed by enantioselective GC-MS analysis demonstrating the exclusive presence of (*R*)-germacrene D, and not the (*S*)-enantiomer, in grape berries.

Keywords

Grapevine; Monoterpenes; Diterpenes; 1-Deoxy-D-xylulose; Mevalonic acid; Head space; Solid Phase-Microextration; Gas Chromatography-Mass Spectrometry; Deuterium Labeling

*Corresponding author: Matthias Wüst, University of Bonn, Department of Nutrition and Food Sciences, Bioanalytics, Endenicher Allee 11-13, D-53115 Bonn, Germany; Phone: ++49-228-732361, Fax: ++49-228-733499, matthias.wuest@uni-bonn.de.

1. Introduction

Terpenoids belong to one of the largest classes of plant metabolites with numerous biological functions, including defense, growth, reproduction, and sensorial attributes (Borg-Karlson, 1990; Köllner et al., 2009; Matich et al., 2003; Price et al., 1990; Schmiderer et al., 2010). Monoterpenes contribute distinctively to the sensorial character of aromatic grape varieties such as Muscat, Gewürztraminer or Riesling (Marais, 1987; Park et al., 1991; Rapp, 1990). Attributes like floral, fruity and citrus can be assigned to the monoterpenes linalool, geraniol, nerol and citronellol, respectively. In addition, C_{13}-norisoprenoids, degradation products of carotenoids, are also considered to be key odorants (Baumes et al., 2002; Mendes-Pinto, 2009). For example, β-damascenone is often described as having a stewed apple, flowery or honey-like aroma, while β-ionone imparts a violet and raspberry-like note. Aroma-active sesquiterpenes are known to occur in various plant species (Haring et al., 1972; Kjeldsen et al., 2003) but, traditionally, relatively little attention has been given to this terpene class in grapevine. However, the sesquiterpene ketone rodundone was recently identified as the key odorant responsible for the peppery attributes of Australian Shiraz wines (Wood et al., 2008), which has led to a growing interest in this class of volatiles. Grapevine flowers produce several sesquiterpenoids with potential roles as attractants for pollinators (Buchbauer et al., 1994, Buchbauer et al., 1995; Lücker et al., 2004; Martin et al., 2009). Furthermore, several sesquiterpene hydrocarbons, most prominently a-ylangene, p-bourbonene, p-caryophyllene, a-humulene and germacrene D, and oxygenated sesquiterpenes, such as farnesol, nerolidol or y-eudesmol, have been described as grape berry and wine constituents (Alves et al., 2005;; Coelho et al., 2006; Robinson et al., 2011; Schreier et al., 1976). An investigation of the profile of grape berry volatiles revealed that their release is developmentally regulated (e.g., increase of sesquiterpenes with a cadinane backbone during ripening) and differed considerably between several varieties (May and Wüst, 2012). The majority of sesquiterpenes was found to be present in grape berry exocarp (skin), whereas only minor amounts were detectable in mesocarp (flesh) at the stage of full ripeness.

While it has been demonstrated that sesquiterpenes are selectively accumulated in the cuticular wax layer of the exocarp (May and Wüst 2011), the site of their biosynthesis and the utilized biochemical pathways are still unknown. *In vivo* feeding experiments performed with grape berries of the Muscat Ottonel cultivar demonstrated that monoterpenes are almost exclusively synthesized via the DOXP/MEP pathway (Luan and Wüst, 2002), with little if any incorporation of an MVA pathway intermediate. However, the precursor pathway contribution for sesquiterpene synthesis and its localization within the grape berries is still unknown. In some plants, both the primarily cytosolic MVA and the plastidial DOXP/MEP pathway can be involved in the formation of the universal terpenoid pathway intermediates isopentenyl diphosphate (IPP) and dimethylallyl diposphate (DMAPP) (Hemmerlin et al. 2012). This phenomenon, which likely involves an exchange of terpenoid pathway intermediates between cytoplasm and plastids, has been termed “metabolic cross talk”. This exchange and the related compartmentalization in monoterpene and sesquiterpene biosynthesis in plants has been recently reviewed by Gutensohn et al. (2013). To evaluate the origin of precursors for grape berry sesquiterpenes, *in vivo* feeding experiments were performed using [5,5-$^2H_2$]-mevalonic acid lactone ($^2H_2$-MVL) and [5,5-$^2H_2$]-1-deoxy-D-xylulose ($^2H_2$-DOX) with the neutral variety Lemberger and the floral/Muscat-type variety Gewürztraminer. Neutral grape varieties are not dependent upon monoterpenes for their flavor. This classification has been introduced by Strauss et al. (1986). By integrating our previous results on monoterpene biosynthesis (Luan and Wüst, 2002) with those reported here on sesquiterpene precursor pathways, we are providing a more detailed model of precursor utilization in grape berries.

2. Results and Discussion

2.1. Experimental design and analysis of sesquiterpene labeling patterns

To investigate pathway utilization in sesquiterpene biosynthesis, a Muscat/floral variety with high monoterpene content (Gewürztraminer) and a neutral variety (Lemberger) were chosen. Head Space-Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) analysis of volatiles released at full berry ripeness indicated that the profile of Lemberger is dominated by bicyclic sesquiterpenes (in order of elution: α-ylangene (**2**), an unknown volatile (**a**), guaia-6,9-diene (**3**), selina-4,6-diene (**4**), germacrene D (**5**) as major sesquiterpene, epi-zonarene (**6**), γ-cadinene (**7**), an unknown volatile (**b**), and β-calacorene (**8**)) (Fig. 1; Fig. 2A). In contrast, acyclic sesquiterpenes predominated in Gewürztraminer (in order of elution: (*E,E*)-β-farnesene (**9**), (*E,Z*)-α-farnesene (**10**) and (*E,E*)-α-farnesene (**11**) as the major sesquiterpene), with small amounts of copaene (**14**), **5**, **7**, cubebol (**12**) and an unknown volatile (**c**) (Fig. 1; Fig. 2B). In both varieties, the monoterpene geraniol and the volatile diterpene 13-epi-manoyl oxide (**13**) were detected as well (Fig. 2).

To evaluate the relative contribution of the MVA and DOXP/MEP pathways for sesquiterpene precursor biosynthesis, [5,5-$^2H_2$]-mevalonic acid lactone ($^2H_2$-MVL) or [5,5-$^2H_2$]-1-deoxy-D-xylulose ($^2H_2$-DOX) were injected into the mesocarp (flesh) of intact grape berries or isolated exocarp (skin), and emitted volatiles were subsequently analyzed by HS-SPME-GC-MS. This technique was the method of choice because it can be performed rapidly and at small scale, and previous studies had shown that grape berries emit sesquiterpene hydrocarbons into the head space (May and Wüst, 2012). Even without an absolute quantification of the emitted volatiles we were still able to calculate the percentage of deuterium label incorporation into volatiles (with respect to the unlabeled genuine compound), which provides essential information about pathway utilization.

Since sesquiterpenes are synthesized from one unit of DMAPP and two units of IPP, each of which can harbor up to two deuterium atoms from either $^2H_2$-MVL or $^2H_2$-DOX, the final product may contain up to six deuterium atoms. The degree of deuterium incorporation can be deduced from the corresponding mass spectra, which is facilitated by the fact that genuine und deuterium-labeled sesquiterpenes can be chromatographically separated by GC due to inverse isotope effects (Matucha et al., 1991). Since the signal corresponding to the molecular ion ($M^{\bullet+}$) of sesquiterpene hydrocarbons is generally low, the ion signal of the characteristic fragment after in-source elimination of an isopropyl group was used for quantification. It is important to note that this fragmentation does not lead to a loss of deuterium.

After administering $^2H_2$-MVL or $^2H_2$-DOX, a deuterium labeling was detected for all major sesquiterpene hydrocarbons in the headspace of all investigated grape cultivars (see Fig. 3 for Lemberger). Feeding with $^2H_2$-DOX and $^2H_2$-MVL leads to an incorporation of label into the same positions in IPP and DMAPP, and the sesquiterpene end products thus have the same deuterium labeling patterns. Most genuine sesquiterpene hydrocarbons with a molecular ion at m/z=204 showed a mass shift by six mass units to m/z=210, which is in agreement with the incorporation of three units of $^2H_2$-IPP/$^2H_2$-DMAPP (Fig. 3A, B). Nevertheless, d_4- and d_2-labeled sesquiterpene hydrocarbons were detectable as well (Supplementary Fig. 1). Interestingly, guaia-6,9-diene incorporated only five deuterium atoms (Fig. 3C), which can be explained by the elimination of deuterium during the terpene synthase-catalyzed cyclization reaction. A reaction pathway to guaia-6,9-diene has been proposed by Steele et al. (1998). However, the obtained labeling pattern that is in agreement with our MS-data can be best rationalized by a cyclization mechanism that combines early cyclization steps from (*R*)-germacrene D formation (Schmidt et al., 1999), that leads to a

germacrene C intermediate, and the last cyclization steps to guaia-6,9-diene according to Steele et al. (1998, Supplementary Fig. 2).

2.2 Injection of labeled precursors into mesocarp of intact grape berries

$^2H_2$-MVL or $^2H_2$-DOX were injected directly into mesocarp of intact grape berries (Lemberger variety) and, after an incubation time of 48 hours, berries were peeled and exocarp and mesocarp analyzed separately by HS-SPME-GC-MS. Label was not incorporated to detectable levels into mesocarp sesquiterpenes from any substrate. In contrast, the injection of precursors into the mesocarp yielded a high degree of labeling in exocarp-derived sesquiterpenes (Table 1), indicating that labeled precursors or end products must have traveled from the mesocarp to the exocarp. It is unknown whether this intriguing translocation is achieved by an active transport mechanism or by simple diffusion. These observations are in agreement with previous metabolite profiling studies, where almost no sesquiterpene hydrocarbons were detectable in the mesocarp, whereas these metabolites were found to be present in exocarp (May and Wüst, 2011). An incorporation of $^2H_2$-DOX, but not $^2H_2$-MVL, into monoterpenes was detectable in both mesocarp (Supplementary Fig. 3) and exocarp (Table 1), which is also consistent with prior studies that demonstrated the presence of monoterpenes in both tissue types (Luan and Wüst, 2002). The diterpene 13-epi-manoyl oxide was found to be labeled following incubation with $^2H_2$-DOX (low incorporation), but not when $^2H_2$-MVL was proffered (Table 1).

2.3 Incubation of isolated grape berry exocarp with labeled precursors

Our preliminary experiments demonstrated that sesquiterpenes in grapevine are synthesized in the fruit exocarp and all follow-up experiments were thus performed with excised exocarp tissue. In experiments performed with grape of the Lemberger cultivar in 2010, the incorporation rates for $^2H_2$-MVL into sesquiterpenes were higher than those obtained with $^2H_2$-DOX, while in a repeated set of experiments (2012) the incorporation rates for both precursors into sesquiterpenes was comparable (Fig. 4). These results indicate that the MVA and DOXP/MEP pathways both contribute to sesquiterpene biosynthesis in the Lemberger cultivar. The highest incorporation rates were detected for germacrene D (**5**), the major sesquiterpene of the Lemberger variety, and an unidentified sesquiterpene with a retention index of 1432, regardless of which precursor was proffered to fruit skins (Fig. 4). Deuterium label from $^2H_2$-DOX was incorporated at low rates (3 %) into the diterpene 13-epi-manoyl oxide (13), while incorporation of label from $^2H_2$-MVL was very low (< 0.5 %) (Supplementary Fig. 4).

Label from both $^2H_2$-MVL and $^2H_2$-DOX was readily incorporated into sesquiterpenes in the Gewürztraminer variety (Fig. 5). Very similar incorporation rates of deuterium label from $^2H_2$-MVL were observed for (*E,E*)-β-farnesene (**9**), (*E,Z*)-α-farnesene (**10**), (*E,E*)-α-farnesene (**11**) and cubebol (**12**), while label from $^2H_2$-DOX was preferentially incorporated into **9**, **10** and **11**, with lower incorporation rates into **12** (Fig. 5). Equal rates of both precursors were incorporated into sesquiterpenes of the variety Syrah, as well. Comparable to the variety Lemberger, highest incorporation rates were achieved for germacrene D and the unidentified sesquiterpene with a retention index of 1432 (Fig. 6).

2.4 Evidence for a transport of farnesyl diphosphate precursors from plastids to the cytosol

Plastidial synthesis of (*Z,Z*)-farnesyl diphosphate and derived sesquiterpenes has been demonstrated to occur in tomato (Sallaud et al., 2009) but, at this point in time, the available evidence indicates that sesquiterpene synthases encoded in the grape genome localize to the cytosol and use (*E,E*)-farnesyl diphosphate as substrate (Martin et al., 2012). The labeling data reported here demonstrate that pools of both cytosolic and plastidial IPP/DMAPP

(synthesized via the MVA and DOXP/MEP pathways, respectively) can serve as precursors for sesquiterpene biosynthesis in grapevine fruit exocarp. These results are comparable to those obtained with foliage of *Vitis vinifera* L., were both pathways are used at equal rates in jasmonate-induced sesquiterpene biosynthesis (Hampel et al., 2005). Bick and Lange (2003) characterized a unidirectional proton symport system for the export of isoprenoid intermediates from plastids to cytosol. Isolated chloroplasts from spinach, kale and Indian mustard were demonstrated to be capable of an efficient export of IPP and geranyl diphosphate, while a much less efficient transport was detected for farnesyl diphosphate and DMAPP, and almost none for geranylgeranyl diphosphate and mevalonate (Bick and Lange, 2003). An import of IPP by plastids isolated from cell cultures of *Vitis vinifera* cv. Muscat de Frontignan was demonstrated by Soler et al. (1993). The genes encoding these transporters have remained elusive.

Interestingly, we observed a linear relationship between the incorporation rates of $^{2}H_{2}$-MVL and $^{2}H_{2}$-DOX into grape sesquiterpenes, which appeared to be independent of the variety (Lemberger, Gewürztraminer or Syrah), the structural class of emitted volatiles (primarily bicyclic sesquiterpenes in Lemberger and Syrah; primarily acyclic sesquiterpenes in Gewürztraminer) and harvest year (feeding experiments with the Lemberger variety in 2010 and 2012) (Fig. 7). Taken together, our results provide evidence that an exchange process generates a homogenous, cytosolic pool of IPP/DMAPP from both cytosolic and plastidial precursors, which is then further metabolized into sesquiterpenes.

The monoterpene geraniol (**1**) was efficiently labeled when $^{2}H_{2}$-DOX was fed to grape berries of the Lemberger variety, but no incorporation of label was detectable in feeding experiments with $^{2}H_{2}$-MVL (Table 1). In a previous study based on feeding experiments with identical deuterium labeled precursors, we demonstrated that several grape monoterpenes are almost exclusively synthesized by the DOXP/MEP pathway (Luan and Wüst, 2002). Labeling of the diterpene 13-epi-manoyl oxide (**13**) was low but clearly detectable with $^{2}H_{2}$-DOX (Supplementary Fig. 4), whereas labeling from $^{2}H_{2}$-MVL was detectable but below the limit of quantification. Since monoterpene synthases (including the functionally characterized geraniol synthase) and diterpene synthases of grape are predicted to be localized to plastids (Martin et al., 2010), there appears to be only a very limited import of cytosolic IPP/DMAPP into plastids for the biosynthesis of mono- and diterpenes, indicating that a transport of precursors occurs primarily in the direction from plastids to the cytosol.

2.5. Stereochemistry of germacrene D

Germacrene D is a widespread chiral sesquiterpene hydrocarbon. The gape berry moth (*Paralobesia viteana* (Clemens)) perceives terpenoids, including germacrene D, emitted from *Vitis* sp. to locate its primary host (Cha et al., 2011). The stereochemistry of germacrene D influences its biological activity, including its effects on herbivors. For example, Stranden et al. (2002) showed that (*S*)-germacrene D elicits a tenfold stronger effect on a receptor neuron of the cotton bollworm moth *Helicoverpa armigera* than its enantiomer. The gene encoding germacrene D synthase in grape has been characterized (Martin et al., 2010), but the stereochemistry of germacrene D formed by this enzyme and emitted by grape berries has not yet been investigated. Deuterium-labeled germacrene D, synthesized in grape berries after administration of $^{2}H_{2}$-DOX or $^{2}H_{2}$-MVA fragmented to a prominent ion at m/z = 167 (Fig. 8A, B), which was identical to the fragmentation pattern observed for the (R)-enantiomer in *Solidago canadensis* (Steliopoulos et al., 2002). Enantioselective GC-MS confirmed the exclusive presence of the (*R*)-enantiomer in grape berries (Fig. 8C). This finding is interesting because the (*R*)-enantiomer is known to occur widely in early vascular

plants such as liverworts, but was thought to be less prevalent in angiosperms (König et al., 1996).

3. Conclusions

The analytical and labeling data obtained so far indicate that sesquiterpene biosynthesis and accumulation in grape berries is restricted to the exocarp and that both routes (MVA and MEP/DOXP) are utilized for sesquiterpene biosynthesis. However Rohmer (1999) has already stated in a remarkable review that "overfeeding of metabolites such as MVA or DX, which are not accumulated by cells, does not represent normal physiological growth conditions. High concentrations of a precursor might activate the corresponding metabolic pathway and consequently lead to an overestimation of its contribution to the production of an isoprenoid." Hence, pathway utilization in *V. vinifera* berries might be different under normal physiological conditions. Supplementary experimental techniques like 13C/12C-IRMS at natural abundance level (Jux et al. 2001, Bartram et al. 2006) and feeding more 'neutral' precursor such as labeled glucose (for a review see Schuhr et al. 2003) are available and are to be applied to investigate in more detail the metabolic cross talk in sesquiterpene biosynthesis in *V. vinifera*. Transporters implicated in the excretion of monoterpenes and diterpenes have recently been characterized (Jasinski et al., 2001; Wang et al., 2013), but the intriguing transport system responsible for the exchange of terpenoid pathway intermediates across the plastidial envelope membrane remains to be discovered.

The localization of sesquiterpenes in the exocarp is consistent with their increased release with prolonged skin contact times during fermentation into wine (Caputi et al., 2011). However, it has also been shown that inclusion of stems and leafs in grape must increases the content of the sesquiterpene rotundone (Capone et al., 2012), and further studies into the roles of these organs in the winemaking process are thus warranted.

4. Experimental

4.1. Plant material

Experiments were performed with the varieties Lemberger, obtained from the Institute of Grapevine Breeding, Geisenheim Research Centre/Germany, and Gewürztraminer and Syrah, which were obtained from the Institute of Crop Science and Resource Conservation (INRES) of the University of Bonn. The variety Lemberger was sampled in 2010 and 2012 at full fruit ripeness. The Gewürztraminer and Syrah varieties were sampled at full fruit ripeness in 2012. Golden rod (*Soligado canadensis*), which was used as a source of germacrene D enantiomers, was field-collected near Bonn (Germany) in autumn 2011.

4.2. Chemicals

[5,5-$^2H_2$]-1-deoxy-D-xylulose was synthesized according to Meyer et al. (2004), while [5,5-$^2H_2$]-mevalonic acid lactone was prepared according to Simpson et al. (1997). The spectral data of the synthesized compounds were in agreement with the data given in the original references.

4.3. Methods

Incubation of intact grapes—An aqueous solution of each precursor (10 μL, 10 mg/L) was injected into the pulp of intact grape berries (Lemberger variety). The microliter syringe was pierced through the receptacle into the pulp. After an incubation time of 48 hours, grapes were peeled, and mesocarp and exocarp were separated and analyzed separately. Sodium chloride was added to the mesocarp tissue until saturation to increase the volatility of the analytes.

Incubation of grape berry exocarp—Grapes were peeled and remaining mesocarp was removed manually from the exocarp tissue. An aqueous solution of each precursor (100 μL, 1 mg/L) was pipetted into a 10 mL headspace vial. Subsequently, approximately 1 cm^2 of the isolated skin was placed with the inner side on the solution to allow a direct uptake of the labeled compounds. The outer wax layer of the skin was not wetted, so that the emission of volatiles was not impaired. The skins were incubated for 48 hours at room temperature. The following HS-SPME-GC-MS measurement was performed directly out of the headspace vial without any further preparation.

SPME-Sampling—A 85 μm polyacrylate fiber, from Supelco (Bellefonte, PA, USA) was used for solid phase microextraction (SPME) of volatiles emitted from grape samples. After an equilibration period at 60 °C for 30 min, the SPME fiber was exposed to the sample headspace for 10 minutes. The mesocarp samples were agitated throughout the incubation at 400 rpm. Subsequently, the analytes were thermally desorbed in the GC-injection port.

GC-MS-analysis—GC-MS-analysis was performed using a Varian 450 GC coupled to a Varian 240 MS ion trap mass spectrometer (Palo Alto, CA, United States). The injection port was set to 220 °C. Splitless injection was used and the split valve was opened after 3 min. Separation was achieved using a DB5 column (Varian, length 30 m, 0.25 mm i.d., film thickness = 0.25 μm). The carrier gas (helium, 5.0) was set to 1 mL/min (constant flow). The column temperature program started at 35°C for 3 min, and was increased to 250°C at a rate of 5°C/min. The transfer line temperature was set to 260°C, the ion source to 150°C. An internal EI-ionization (70 eV) was performed and mass spectra were recorded in the range of m/z 35-350 (full scan), at a scan rate of 0.64 sec/scan. Sesquiterpenes were identified by comparison of the retention times and mass spectra with standard substances or by comparing their mass spectra and Kovats retention indices using the Massfinder® library (Hochmuth Scientific Consulting, Hamburg, Germany). Incorporation rates were calculated by dividing the peak area of the labeled compound (A_1) by the peak area of the genuine compound (A_g) multiplied by 100% i.e. (A_1/A_g)*100%.

Enantioselective GC-MS—Enantioselective GC-MS analysis was performed on the GC-MS system as described above with a fused silica capillary (30 m × 0.25 mm i.d.) coated with 20 % heptakis-(2,3-di-Omethyl-6-O-tert-butyldimethylsilyl)-b-cyclodextrin in BGB 15 (film thickness 0.25 μm) as stationary phase. Separation was achieved with the following temperature program: starting temperature was set at 60 °C for 3 min, and was then increased to 160°C with a rate of 1°C/min. The final temperature was held for further 10 min. All other GC/MS parameters were equal to those listed above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Results demonstrate the *de novo* sesquiterpenes biosynthesis in grape berry exocarp.
- Precursors from both pathways were used for sesquiterpene biosynthesis.
- Cross talk occurred primarily in the direction from plastids to the cytosol.
- Linear relationship between incorporation rates achieved for both pathways.
- Labeling data and enantio-GC-MS analysis confirmed the presence of (R)-germacrene D.

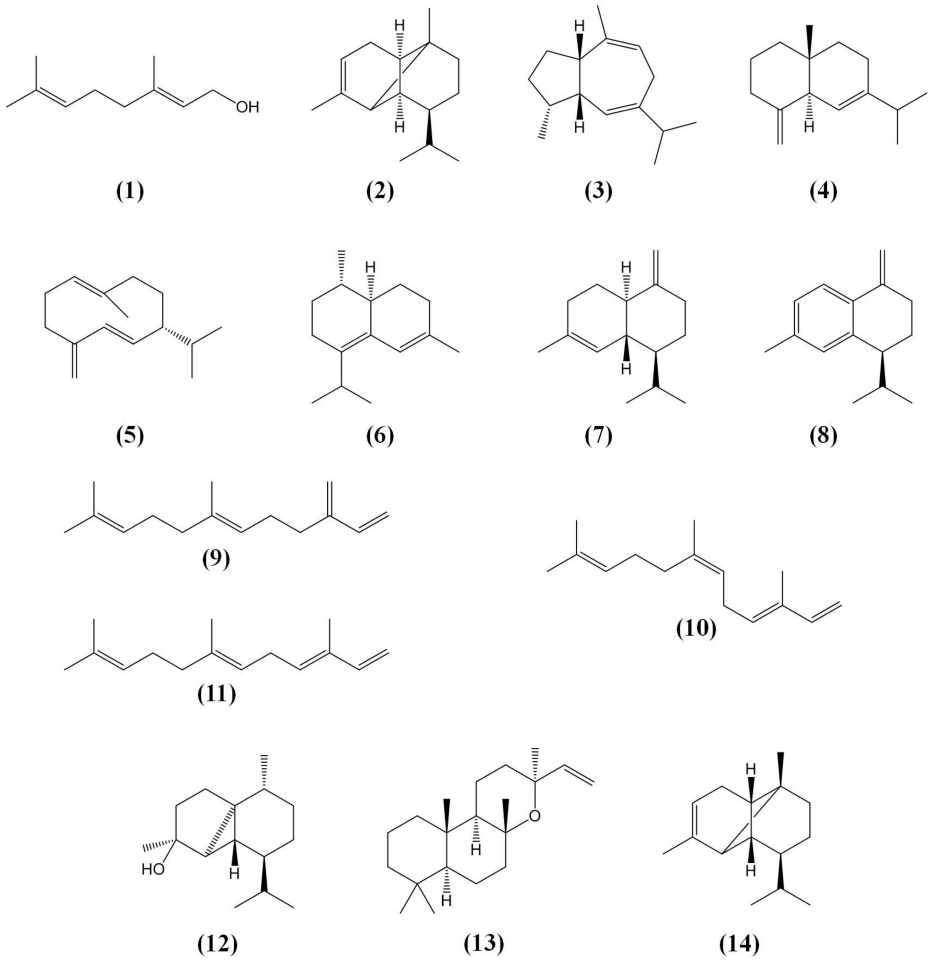

Fig. 1.

Structures of grape volatile terpenoids (absolute stereochemistry not identified with the exception of (*R*)-germacrene D (**5**)).

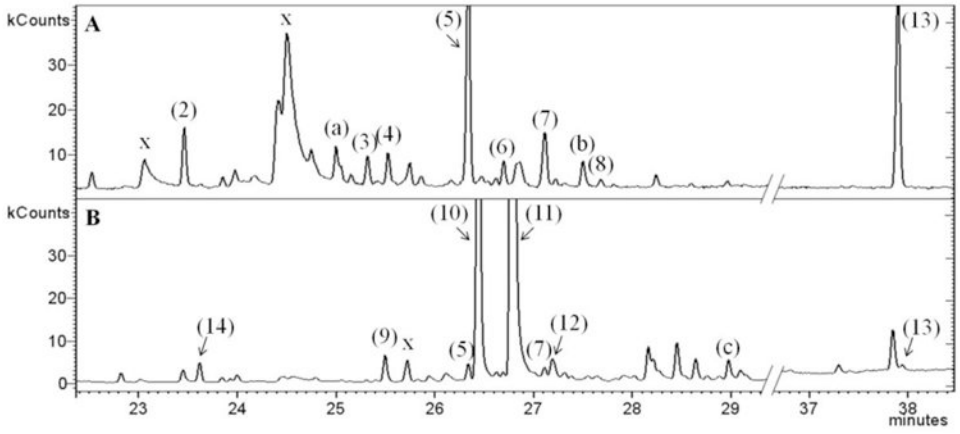

Fig. 2.

HS-SPME-GC-MS total ion chromatograms of volatiles released from grape berries of the varieties of Lemberger (A) and Gewürztraminer (B). Sesquiterpenes are numbered according to Fig. 1. Peaks labeled with “a, b, or c” belong to volatiles with sesquiterpene like mass spectra. Peaks labeled with “x” were assigned to SPME fiber bleeding.

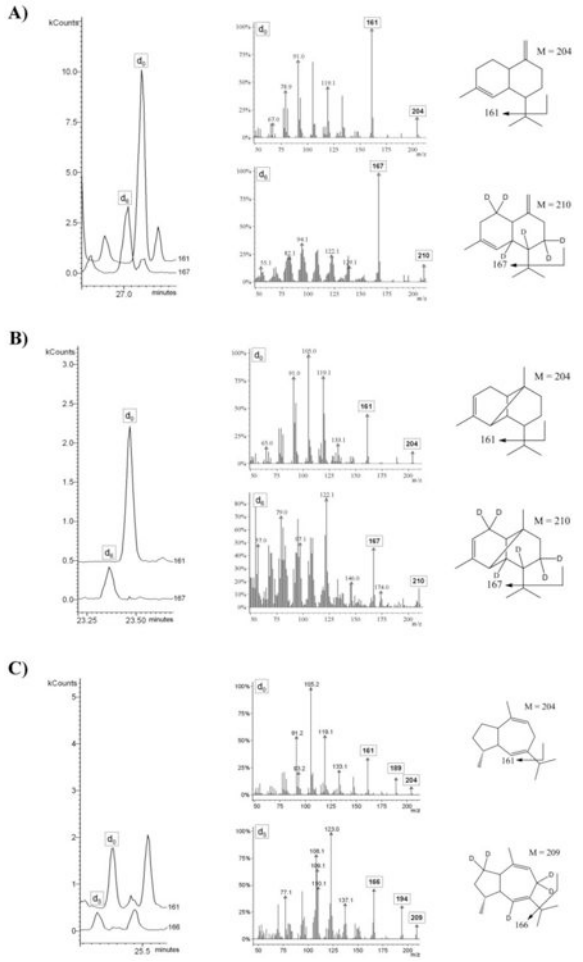

Fig. 3.

HS-SPME-GC-MS single ion monitoring chromatograms (left panel) and MS spectra of genuine and deuterium-labeled sesquiterpenes (center panel) after administration of [5,5-$^{2}H_2$]-1-deoxy-D-xylulose to isolated exocarp of gape berries (Lemberger cultivar). Expected labeling patterns are depicted for the sesquiterpenes (A) γ-cadinene, (B) α-ylangene and (C) guaia-6,9-diene (right panel). Essentially identical labeling patterns were observed in feeding experiments with [5,5-$^{2}H_2$]-mevalonic acid lactone.

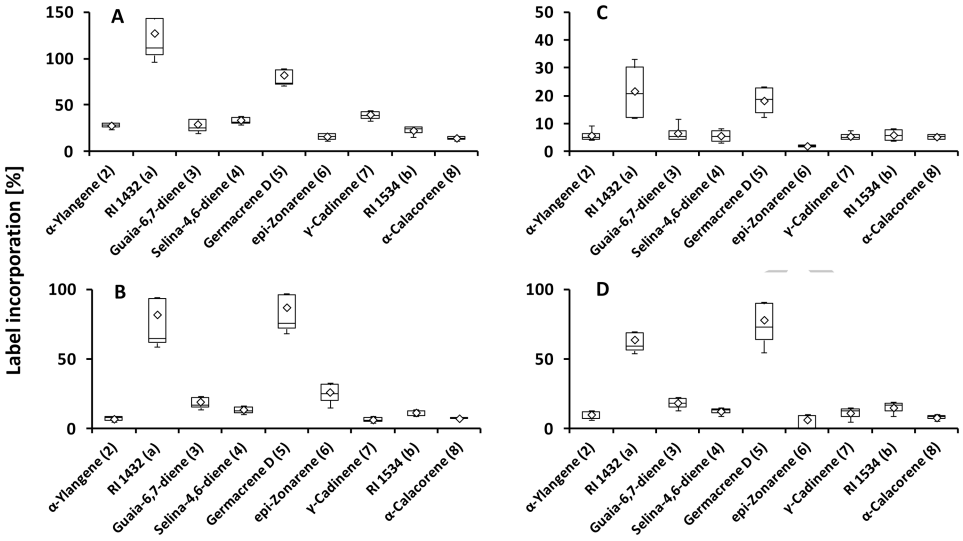

Fig. 4.

Incorporation of deuterium label from terpenoid precursors, administered to isolated fruit exocarp of the Lemberger variety (harvested in 2010 (A and C) and 2012 (B and D)), into sesquiterpene volatiles (n=4). [5,5-$^{2}H_{2}$]-Mevalonic acid lactone (A and B) or [5,5-$^{2}H_{2}$]-1-deoxy-D-xylulose (C and D) were used as precursors incorporated via the MVA and DOXP/MEP pathways, respectively. Sesquiterpenes are numbered according to Fig. 1. Box-whisker plots show the 25th (bottom) and 75th (top) percentile, separated by the median. The mean is depicted by a diamond. The lower whisker corresponds to the bottom percentile minus the minimum value, while the upper whisker indicates the maximum value minus the top percentile.

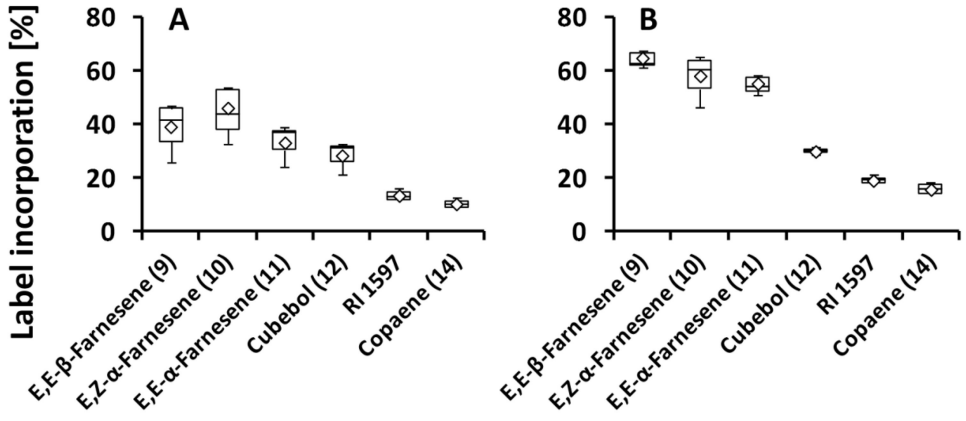

Fig. 5.

Incorporation of deuterium label from terpenoid precursors, administered to isolated fruit exocarp of the Gewürztraminer variety (harvested in 2012), into sesquiterpene volatiles (n=3). [5,5-$^{2}H_{2}$]-Mevalonic acid lactone (A) or [5,5-$^{2}H_{2}$]-1-deoxy-D-xylulose (*B*) were used as precursors incorporated via the MVA and DOXP/MEP pathways, respectively. Sesquiterpenes are numbered according to Fig. 1. Box-whisker plots show the 25th (bottom) and 75th (top) percentile, separated by the median. The mean is depicted by a diamond. The lower whisker corresponds to the bottom percentile minus the minimum value, while the upper whisker indicates the maximum value minus the top percentile.

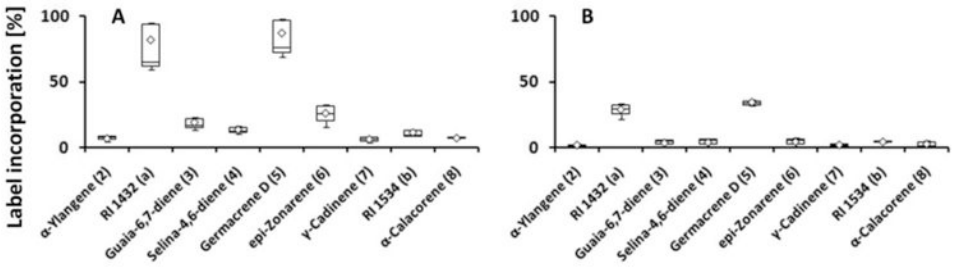

Fig. 6.

Incorporation of deuterium label from terpenoid precursors, administered to isolated fruit exocarp of the Syrah variety (harvested in 2012), into sesquiterpene volatiles (n=3). [5,5-$^2H_2$]-Mevalonic acid lactone (A) or [5,5-$^2H_2$]-1-deoxy-D-xylulose (*B*) were used as precursors incorporated via the MVA and DOXP/MEP pathways, respectively. Sesquiterpenes are numbered according to Fig. 1. Box-whisker plots show the 25th (bottom) and 75th (top) percentile, separated by the median. The mean is depicted by a diamond. The lower whisker corresponds to the bottom percentile minus the minimum value, while the upper whisker indicates the maximum value minus the top percentile.

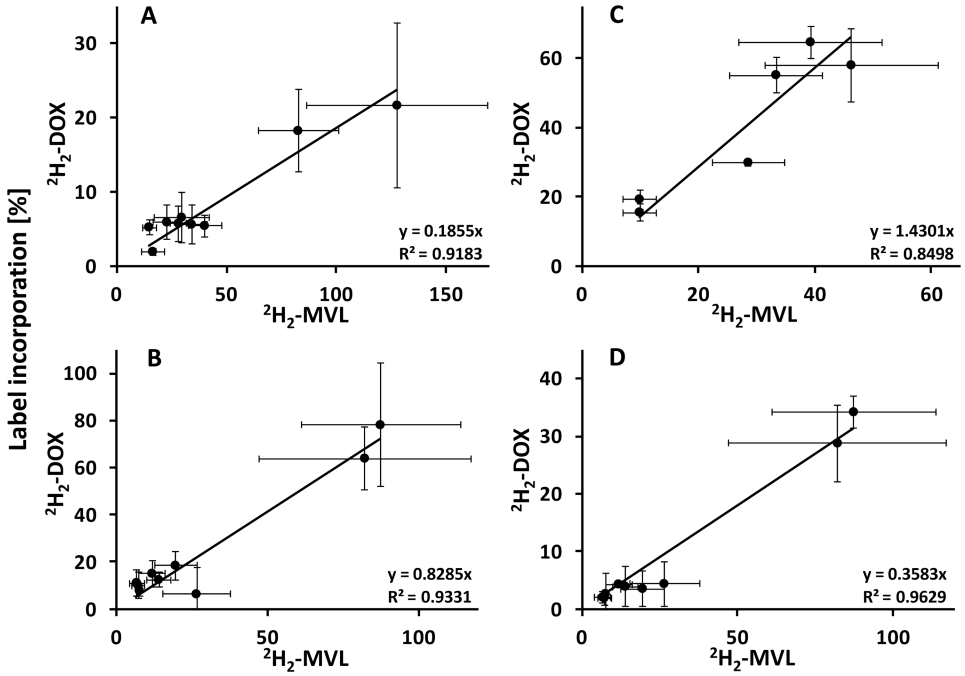

Fig. 7.

Correlation analysis of deuterium label incorporation from [5,5-$^2H_2$]-mevalonic acid lactone and [5,5-$^2H_2$]-1-deoxy-D-xylulose into grape berry sesquiterpenes of the varieties Lemberger (harvested in 2010 (A) or 2012 (B)), Gewürztraminer (C) and Syrah (D). Mean ± SD (n=3-4) is shown for the following sesquiterpenes: α-ylangene (**2**), an unknown volatile (**a**), guaia-6,9-diene (**3**), selina-4,6-diene (**4**), germacrene D (**5**) as major sesquiterpene, epi-zonarene (**6**), γ-cadinene (**7**), an unknown volatile (**b**) and β-calacorene (**8**)) of the varieties Lemberger and Syrah; (*E*)- β-farnesene (**9**), (*E,Z*)-α-farnesene (**10**) and (*E,E*)- α-farnesene (**11**) as the major sesquiterpene), cubebol (**12**), an unknown volatile (**c**) and copaene (**14**) of the Gewürztraminer variety (labeling of sesquiterpenes according to Fig. 1).

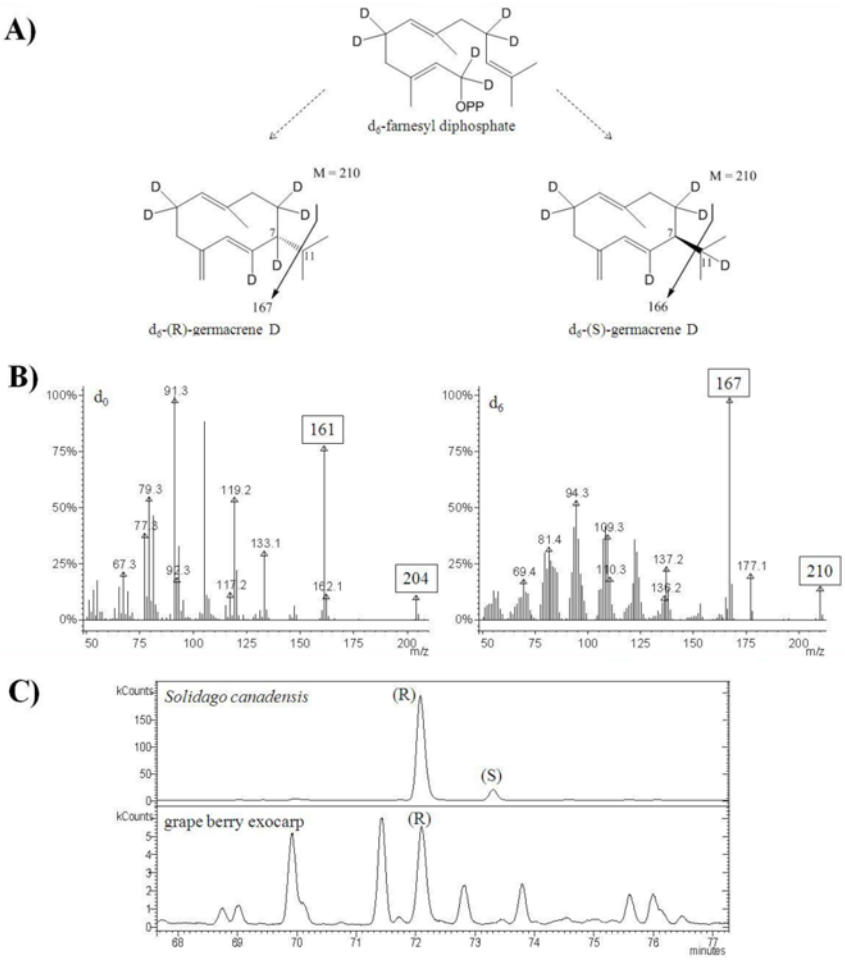

Fig. 8.

Identification of germacrene D enantiomers in volatiles emitted from grape berries (Lemberger cultivar). (A) Postulated incorporation of labeled precursors according to the enzymatic cyclization mechanism in *Solidago canadensis* (Schmidt et al., 1999). (B) MS spectra of genuine and deuterium-labeled germacrene. (C) Separation and identification of germacrene D enantiomers by enantio-GC/MS analysis of *Solidago canadensis* (reference) and *Vitis vinifera* cv. Lemberger (Syrah contained the same germacrene D enantiomer as Lemberger).

Table 1

Incorporation of [5,5-$^2H_2$]-mevalonic acid lactone ($^2H_2$-MVL) and [5,5-$^2H_2$]-1-deoxy-D-xylulose ($^2H_2$-DOX) into terpene volatiles released from intact grape berries (exocarp) of *Vitis vinifera* cv. Lemberger when labeled precursors were directly injected into mesocarp. The values were obtained from a typical experiment with grapes from the vintage 2010. Incorporation rates were calculated by dividing the peak area of the labeled compound (A_l) by the peak area of the genuine compound (A_g) multiplied by 100% i.e. (A_l/A_g)*100%. Thus, incorporation rates higher than 100% indicate that the labeled compound is present at a higher concentration than the genuine compound.

Peak[1]	Compound	Identification[2]	Diagnostic ions for quantitation (m/z)	Precursor inco $^2H_2$-MVL	rporation [%] $^2H_2$-DOX
	Monoterpenes				
1	Geraniol	a	69/71	n.d.	79
	Sesquiterpenes				
2	α-Ylangene	b	161/167	41	6
a	Unknown (RI 1432)	e	189/195	139	20
3	Guaia-6,9-diene	b	161/166	35	n.q.
4	Selina-4,6-diene	c	161/166	48	5
5	Germacrene D	a	161/167	46	n.q.
6	*epi*-Zonarene	b	161/166	16	3
7	γ-Cadinene	b	161/167	47	4
b	Unknown (RI 1534)	e	122/125	33	6
8	β-Calacorene	b	142/146	n.q.	n.q.
	Diterpenes				
13	13-epi-Manoyl oxide	d	257/265	n.d.	3

n.d., not detectable
n.q., not quantifiable
RI, retention index on a DB5 column

[1] numbers according to Fig. 1

[2] a: identified using standard substances, b: identified by mass spectrum and retention index of the massfinder® library, c: tentatively identified by mass spectrum and retention index of the massfinder® library, d: tentatively identified by mass spectrum and retention index of: R. P. Adams, *Identification of essential oil components by gas chromatography/mass spectroscopy*, Allured Publishing Corporation, Carol Stream, 1995, e: unknown