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Biosynthesis of Mono- and Sesquiterpenes in Strawberry Fruits and Foliage: ^2H Labeling Studies

DANIELA HAMPEL,† ARMIN MOSANDL,† AND MATTHIAS WÜST*,§

Institut für Lebensmittelchemie, Johann Wolfgang Goethe-Universität, Marie-Curie-Strasse 9, D-60439 Frankfurt/Main, Germany, and Department of Life Technologies, University of Applied Sciences Valais, Route du Rawyl 47, CH-1950 Sion 2, Switzerland

The biosynthesis of the monoterpene (*S*)-linalool and the sesquiterpene *trans*-(*S*)-nerolidol in fruits of *Fragaria* × *ananassa* Duch. cv. Eros and Florence and of the monoterpene (−)-α-pinene in *Fragaria vesca* was investigated by 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). The feeding experiments indicate that (*S*)-linalool and *trans*-(*S*)-nerolidol in *Fragaria* × *ananassa* Duch. and (−)-α-pinene in *F. vesca* are exclusively synthesized via the cytosolic mevalonic acid pathway without any contribution from the plastidial 1-deoxy-D-xylulose/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) route. Inhibition experiments revealed that even the presence of mevastatin, an export of plastid-derived isopentyl diphosphate/dimethylallyl diphosphate, cannot be induced. However, the enantioselective analysis shows that in *Fragaria* × *ananassa* Duch. cv. Eros and Florence both linalool enantiomers are present and that only (*S*)-linalool is labeled after administration of d_2-MVL. Therefore, the origin of (*R*)-linalool in these fruits remains unknown. Contrarily, in *Fragaria* × *ananassa* Duch. foliage (*R*)-linalool is the dominant enantiomer. Feeding experiments revealed an incorporation of d_2-MVL and d_2-DOX at equal rates exclusively into (*S*)-linalool. Only in *F. vesca* foliage, where (*R*)-linalool is present at high enantiomeric purity (ee > 90%), is a de novo biosynthesis of the (*R*)-enantiomer via the DOXP/MEP pathway detectable. These results demonstrate a complex intraplant variation of (*R*)- and (S)-linalool biosynthesis via the cytosolic and plastidial route.

KEYWORDS: Rosaceae; fruit flavor; terpenes; non-mevalonate pathway; stir bar sorptive extraction (SBSE); thermal desorption−enantioselective multidimensional gas chromatography−mass spectrometry (TD-enantio-MDGC-MS)

INTRODUCTION

Strawberry, a member of the Rosaceae family (genus *Fragaria*), is a very popular fruit that is mainly consumed due to its pleasant taste. More than 360 components contribute to the typical strawberry flavor (*1*−*6*). Among them, terpenoids can reach concentrations up to 20% of the total volatiles (*7*). However, until recently little was known about their biosynthesis in the fruit. A substantial advance in this field has been made by Aharoni et al. (*8*) by cloning and functional expression of a *Fragaria ananassa* nerolidol synthase 1 (*FaNES1*) and a *Fragaria vesca* pinene synthase (*FvPINS*). The recombinant *FaNES1* enzyme generated (*S*)-linalool and *trans*-(*S*)-nerolidol from geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), respectively. It could be demonstrated by green fluorescent protein (GFP) localization experiments that *FvPINS* does not contain a targeting signal at its N terminus and that *FaNES1* localizes to the cytosol because of deletions and the introduction of a premature stop codon, which excludes the plastid targeting sequence from the coding region. Because biosynthesis of mevalonic acid (MVA) is expected to occur in the cytosol, whereas the biosynthesis of 1-deoxy-D-xylulose phosphate is expected to occur in the plastid (*9*−*15*), the generation of *FaNES1*- and *FvPINS*-derived mono- and sesquiterpenes should rely exclusively on isopentyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP) that are generated via the cytosolic MVA pathway given the absence of a possible export of plastidial IPP/DMAPP. To verify this, we have investigated the biosynthesis of the monoterpene linalool and the sesquiterpene *trans*-(*S*)-nerolidol in fruits and foliage of *Fragaria* × *ananassa* Duch. cv. Eros and Florence and the biosynthesis of the monoterpene (−)-α-pinene in fruits and foliage of *Fragaria vesca* by 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). Furthermore, feeding experiments following the inhibition of the respective pathways with mevastatin and fosmidomycin were conducted to investigate a possible cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis.

* Corresponding author (telephone +41-27-606-86-54; fax +41-27-606-85-15; e-mail matthias.wuest@hevs.ch).
† Johann Wolfgang Goethe-Universität.
§ University of Applied Sciences Valais.

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EXPERIMENTAL PROCEDURES

Plant Material. Ripe fruits and foliage of *Fragaria* × *ananassa* Duch. cv. Eros and Florence were obtained from the Research Center Geisenheim (Geisenheim, Germany) in June and July 2004, whereas *F. vesca* and the foliage of *Fragaria semperflorens* Duch. Ser. were obtained from our own harvest in June 2004.

Chemicals. (±)-Linalool, (−)-linalool, and (−)-α-pinene were obtained from Fluka (Taufkirchen, Germany), whereas geraniol, *trans*-nerolidol, and terpinolene were obtained from Roth (Karlsruhe, Germany). *trans*,*trans*-α-Farnesol was derived from Aldrich (Steinheim, Germany). [5,5-$^2H_2$]Mevalonic acid lactone was prepared according to the method of Simpson et al. (*25*), and [5,5-$^2H_2$]-1-deoxy-D-xylulose was prepared according to the method of Jux and Boland (*26*). Spectral data of the labeled compounds were in all cases in good agreement with the data given in the references cited above. Fosmidomycin sodium salt was obtained from Molecular Probes (Leiden, The Netherlands), whereas mevastatin was obtained from Sigma Aldrich (Steinheim, Germany).

Sample Preparation. Strawberry fruits and foliage were cut into equal pieces (fruits, approximately 10 × 3 × 3 mm; foliage, approximately 10 × 3 mm). One gram of the plant material (*F. vesca*, 0.5 g; foliage, 0.2 g) was incubated with 1 mL (*F. vesca*, 0.5 mL; foliage, 2 mL) of an aqueous feeding solution (d_2-MVL, 2 mg/mL; d_2-DOX, 2 mg/mL) for 4 days at room temperature in darkness. After removal of the feeding solution, the pieces were ground in 2 mL of phosphate buffer (pH 7) to a suspension (*F. vesca*, 1 mL; foliage, 1.5 mL), which was used for stir bar sorptive extraction (SBSE) analysis. Blank experiments were performed by grinding pieces in pure buffer. Geraniol and *trans*,*trans*-α-farnesol (*F. vesca*, terpinolene) were added as internal standards. Each experiment was repeated at least three times. The inhibition experiments were carried out by preincubation with an aqueous inhibition solution that contained fosmidomycin or mevastatin at 100 μM. The chosen concentrations of inhibitors had no effect on tissue appearance for the duration of the experiments. After 24 h, the inhibition solutions were removed followed by the addition of labeled precursors according to the feeding experiments described above.

SBSE Sampling. A stir bar consisting of a magnetic core sealed inside a glass tube with a length of 1.2 cm and an outer diameter of 1.2 mm and coated with 55 μL of poly(dimethylsiloxane) (PDMS) was used. Stir bars are manufactured and offered by Gerstel (Mülheim/Ruhr, Germany). The SBSE conditions were as follows: stirring time 30 min at room temperature at ∼400 rpm.

^{1}H NMR. The spectra of the synthesized products were recorded on a Bruker AMX 300, 300 MHz, at room temperature in $CDCl_3$/TMS or D_2O.

Gas Chromatography−Mass Spectrometry (GC-MS). The GC-MS analyses of the synthesized products 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 at 69 kPa; split, 20 mL/min; injector temperature, 230 °C; oven temperature, 40 °C (5 min isothermal), then raised at 5 °C/min to 260 °C (20 min isothermal); ion source temperature, 200 °C; mass range, 40−300 amu; EI, 70 eV.

Thermal Desorption−Enantioselective Multidimensional Gas Chromatography−Mass Spectrometry (TD-Enantio-MDGC-MS). The TD-enantio-MDGC-MS system (*27*) consists of a Gerstel TDS thermal desorption system, mounted on a Siemens SiChromat 2−8, with two independent column oven programs and a live T-switching device, coupled to the transfer line of a Finnigan MAT ITD 800 mass spectrometer using an open split interface. For the thermal desorption the following conditions were applied: desorption temperature program, 10 °C, raised at 60 °C/min to 250 °C, 2 min isothermal (6.0 min); flow mode, TDS; splitless; transfer line temperature, 250 °C. A Gerstel CIS-3 PTV injector was used for cryogenic focusing of the released analytes.

The PTV was cooled to −150 °C using liquid nitrogen. The PTV was programmed from −150 °C at 12 °C/s to 250 °C, 2 min isothermal (2.5 min). Flow mode CAS was splitless (1 min). The liner was filled with Tenax TA (Alltech, Deerfield, IL).

GC conditions were as follows: precolumn, self-prepared fused silica capillary coated with SE-52 (30 m × 0.25 mm i.d.; film thickness = 0.23 μm); carrier gas, helium, 1.9 bar; detector, FID, 250 °C. The main column was a self-prepared fused silica capillary (30 m × 0.25 mm i.d.) coated with a 0.23 μm film of 4‰ heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (DIME-β-CD) (30%) in SE-52 (70%): detector, ITD 800; transfer line temperature, 250 °C; open split interface, 250 °C; helium sweeping flow, 1 mL/min; ion trap manifold, 200 °C; EI, 70 eV; oven temperature program, precolumn, 60 °C (5 min isothermal), raised at 3 °C/min to 250 °C (20 min isothermal); main column, 60 °C (30 min isothermal), then raised 2 °C/min to 200 °C (15 min isothermal). Cut times were as follows: (−)-α-pinene, terpinolene, (*R/S*)-linalool, 21.0−23.8 min; geraniol, 30.2−32.0 min; *trans*-(*S*)-nerolidol, 44.5−46.3 min; *trans*,**trans**-α-farnesene, 50.9−52.7 min.

Table 1. Concentrations (Nanograms per Gram of Plant Material) of Genuine and Labeled (−)-α-Pinene, (*R*)- and (*S*)-Linalool, and *trans*-(*S*)-Nerolidol As Determined by SBSE after Administration of d_2-MVL[a]

	genotype		
terpene	*Fragaria* × ananassa cv. Eros	*Fragaria* × ananassa cv. Florence	*Fragaria vesca*
d_0-(−)-α-pinene	nd	nd	3500 (820)
$d_{2/4}$-(−)-α-pinene	nd	nd	34 (9)
d_0-(*R*)-linalool	8 (3)	4 (0.1)	tr
d_0-(*S*)-linalool	32 (0.2)	16 (2)	tr
$d_{2/4}$-(*S*)-linalool	14 (2)	6 (2)	nd
$d_{2/4}$-(*R*)-linalool	nd	nd	nd
d_0-*trans*-(*S*)-nerolidol	55 (26)	1180 (250)	nd
$d_{2/4/6}$-*trans*-(*S*)-nerolidol	390 (83)	254 (94)	nd

[a] Results are given as average of three independent determinations. Standard deviation is shown in parentheses. nd, not detectable; nq, not quantifiable; tr, trace.

RESULTS AND DISCUSSION

To investigate the terpene biosynthesis in strawberry fruit, *Fragaria* × *ananassa* Duch. cv. Eros and Florence and *F. vesca* were used for the feeding experiments. The volatiles, extracted by SBSE, were analyzed by TD-enantio-MDGC-MS, allowing the simultaneous detection of genuine and labeled terpenes. In the fruits of the cultivated species Eros and Florence (*R*)- and (*S*)-linalool and *trans*-(*S*)-nerolidol could be identified by co-injection of commercial standards, retention times, and mass spectra. In contrast, the fruits of the wild species *F. vesca* contained linalool only in traces, whereas *trans*-(*S*)-nerolidol was not detectable. (−)-α-Pinene was the main monoterpene in *F. vesca*, whereas no sesquiterpene could be identified in the fruit (see **Table 1**). This observation is good agreement with previous studies (*8*, *28*). As shown in **Table 1**, the concentration of the genuine sesquiterpene was clearly higher in the fruits of cultivars Eros and Florence when compared to the genuine monoterpene.

The fruit tissues were directly incubated with an aqueous solution of labeled MVL or DOX. Following the incubation, the plant material was homogenized using a mortar and pestle and the volatiles were extracted by SBSE as described above. The feeding experiments revealed an incorporation of d_2-MVL into (*S*)-linalool and *trans*-(*S*)-nerolidol in the fruit tissue in both cultivated species Eros and Florence. **Figures 1** and **2** show the SBSE-enantio-MDCG-MS analysis of (*S*)-linalool and *trans*-(*S*)-nerolidol after administration of labeled MVL to *Fragaria*

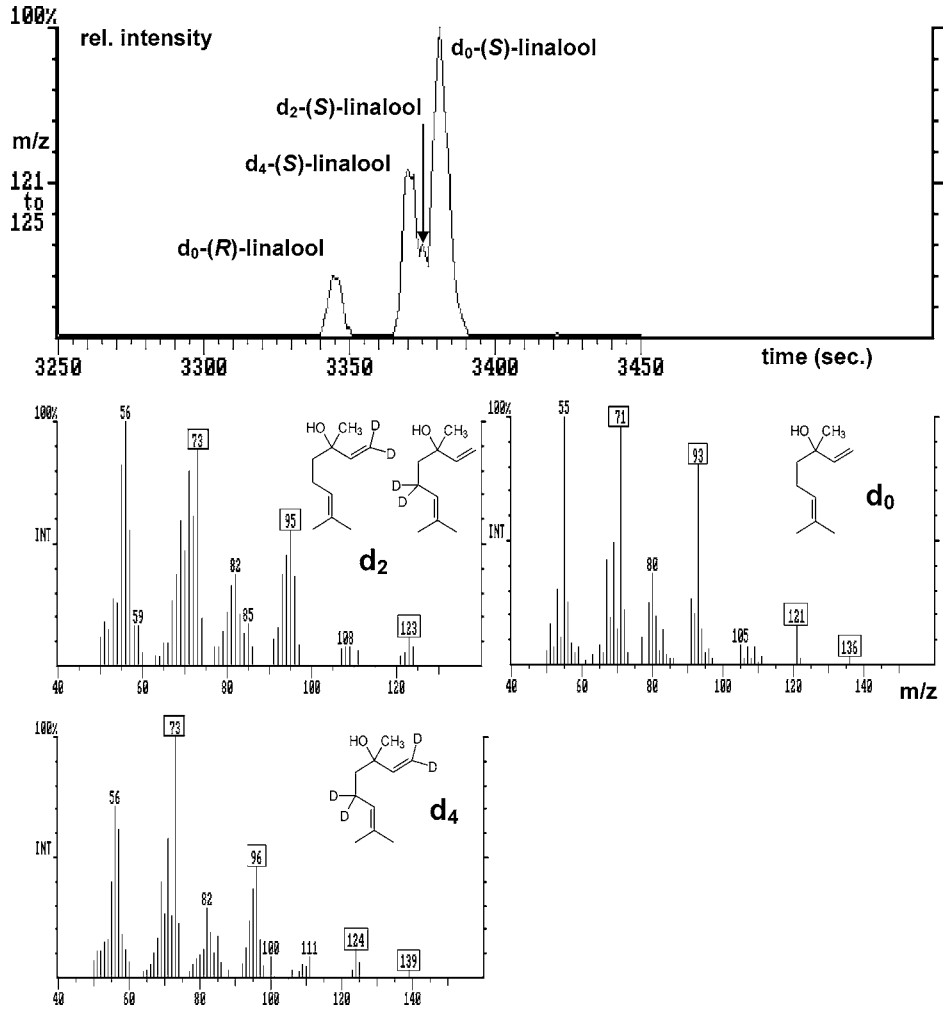

Figure 1. Main column chromatogram and MS spectra of unlabeled and labeled (*S*)-linalool obtained from the SBSE of fruit tissue of *Fragaria* × *ananassa* Duch. cv. Florence when [5,5-$^2H_2$]MVL is administered.

× *ananassa* Duch. cv. Florence. Besides genuine unlabeled (*R*/*S*)-linalool, labeled d_2- and d_4-(*S*)-linalool are clearly detectable and can be separated from unlabeled linalool due to the inverse isotope effect of deuterium-labeled compounds in GC.

Surprisingly, no incorporation of labeled MVL into (*R*)-linalool was detectable. It is conceivable that (*R*)-linalool is only synthesized during an early fruit ripening stage and that the expression of the corresponding (*R*)-linalool synthase is completely down-regulated when the fruit attains the fully ripe stage.

The administration of labeled MVL resulted also in an incorporation into *trans*-(*S*)-nerolidol (**Figure 2**). Sesquiterpenes are assembled by two IPP molecules and one DMAPP, so a shift up to 6 mass units should be visible for higher mass fragments and is indeed observable: the mass peaks at *m*/*z* 161 and 189 are shifted to *m*/*z* 167 and 195, respectively.

F. vesca also showed an incorporation of labeled MVL into (−)-α-pinene (see **Table 1**). The administration of labeled DOX led in no case to any detectable incorporation. Obviously, the biosynthesis of the investigated mono- and sesquiterpenes in strawberry fruits relies exclusively on the cytosolic mevalonate pathway. These results are in perfect agreement with the GFP localization experiments of the corresponding cytosolic enzymes FaNES1 and FvPINS (*8*) and demonstrate that an export of plastid-derived IPP/DMAPP does not occur under normal conditions in ripe fruits. This finding does not support the concept of an export of plastid-derived IPP/DMAPP, which had been demonstrated previously by several labeling studies (*16*−*21*). Another question addresses the origin of the geranyl diphosphate (GPP) substrate used for monoterpene biosynthesis. Thus far, the experimental evidence points to a plastidial localization in plants. Because the DOXP/MEP pathway was ruled out as a source of GPP, one could hypothesize that a cytosolic GPP synthase is present in strawberry (unless IPP and DMAPP could be transported into plastids and converted to GPP and the GPP transported back to the cytosol). Alternatively, cytosolic farnesyl diphosphate synthase (FPP) could release small amounts of GPP, which could then be used by monoterpene and sesquiterpene synthases. However, recent findings demonstrated the dual targeting of GPP synthase into plastids and in the cytosol (*31*).

To verify if an export of plastid-derived IPP/DMAPP can be induced, inhibition experiments were carried out utilizing the inhibitors mevastatin and fosmidomycin. Feeding labeled DOX following the inhibition of the cytosolic pathway by mevastatin (*24*) revealed no incorporation of the precursor into mono- and sesquiterpenes in all three species. Thus, an export of plastid-derived IPP/DMAPP does not occur in ripe strawberry fruits, even when the MVA route is blocked. This result is opposed to recent experiments with various plant species that provided sound evidence for a unidirectional transport of isoprenoid intermediates from the plastids to the cytosol (*22*, *29*).

Feeding labeled MVL after the specific inhibition of the DOXP/MEP pathway by fosmidomycin (*23*) revealed a strong inhibition of MVL incorporation into (*S*)-linalool and *trans*-

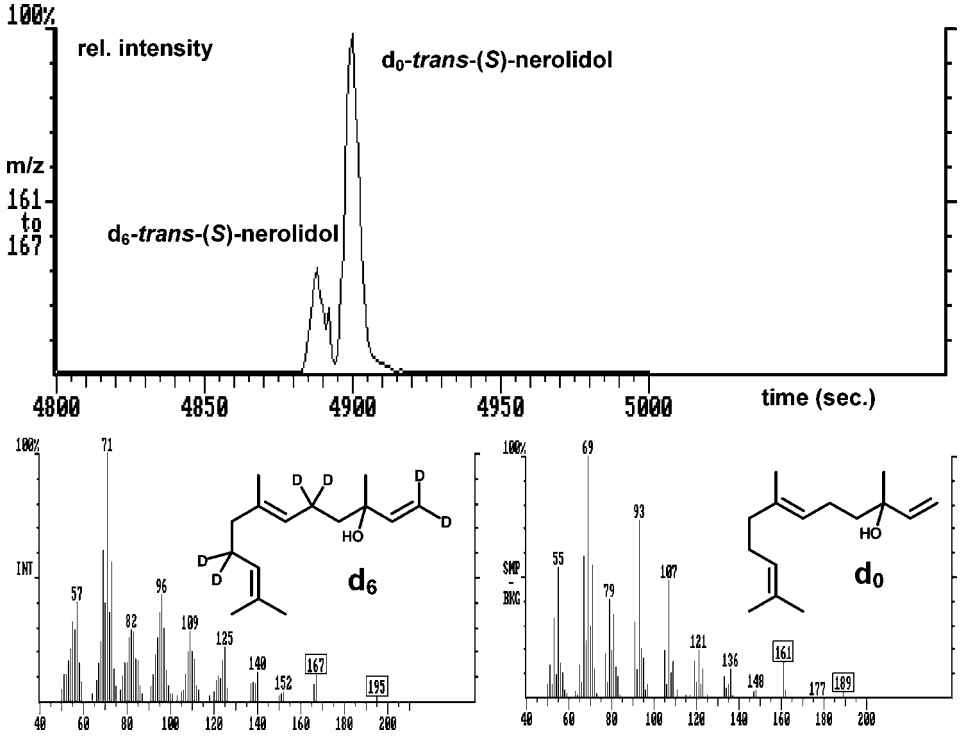

Figure 2. Main column chromatogram and MS spectra of unlabeled and labeled *trans*-(*S*)-nerolidol obtained from the SBSE of fruit tissue of *Fragaria* × *ananassa* Duch. cv. Florence when [5,5-$^2H_2$]MVL is administered.

Table 2. Enantiomeric Distribution (Percent) of (*R*)- and (*S*)-Linalool in Fruit and Foliage of Different *Fragaria* Species[a]

genotype	plant tissue	(*R*)-linalool	(*S*)-linalool
F. × ananassa Duch.	fruit	16 (1)	84 (1)
	foliage	60 (4)	40 (4)
F. semperflorens Duch. Ser.	fruit	67 (1)	33 (1)
	foliage	86 (1)	14 (1)
F. vesca	fruit	nd	nd
	foliage	96 (1)	4 (1)

[a] Standard deviation is shown in parentheses. nd, not detectable.

(*S*)-nerolidol in the cultivated species. The incorporation into (−)-α-pinene in *F. vesca* ceased completely. Fosmidomycin, although a specific inhibitor of the DOXP/MEP pathway, seems to affect the biosynthetic activity in strawberry fruits in general by nonspecific effects at the applied concentration.

The feeding experiments demonstrated that labeled MVL was exclusively incorporated into (*S*)-linalool, although ∼16% of linalool in fruit tissue of the cultivated species was (*R*)-configured (see **Table 2**). Therefore, the biosynthetic origin of (*R*)-linalool in these fruits remained unknown. To follow up the question of the appearance of (*R*)-linalool within the fruit, the biosynthetic capabilities of the foliage tissue of different *Fragaria* species (*Fragaria* × *ananassa* Duch., *F. semperflorens* Duch. Ser., *F. vesca)* were investigated as well. A comparison between the fruit and the foliage concerning the enantiomeric distribution of linalool was carried out followed by feeding experiments with foliage tissues. **Table 2** shows the enantiomeric distribution of linalool in different fruit and foliage tissues. Significant differences were observed between fruit and foliage within the same species. In contrast to the fruit, the foliage generally contains (*R*)-linalool as the main enantiomer. In *F. vesca* even >95% of linalool is (*R*)-configured.

Feeding labeled MVL to the foliage of different species revealed an incorporation into at least one of the linalool enantiomers (see **Table 3**). The exact quantification of the labeled metabolites using an internal standard was not performed because the principal aim was to demonstrate pathway activities in a qualitative manner. The results are thus reported in a semiquantitative manner by giving the relative amounts (percent) of labeled compounds of the total amounts of labeled and unlabeled compounds as is frequently done in similar labeling studies (*30*). *F. semperflorens* Duch. Ser. incorporated the precursor into both enantiomers of linalool, whereas *Fragaria* × *ananassa* Duch. as well as *F. vesca* showed only an incorporation into (*S*)-linalool or (*R*)-linalool, respectively. Genuine *trans*-(*S*)-nerolidol was detectable in only trace amounts in *Fragaria* × *ananassa* Duch. Surprisingly, the exogenously supplied labeled MVL led to a strong increase in nerolidol biosynthesis in *F. semperflorens* Duch. Ser. and *Fragaria* × *ananassa* Duch. as indicated by high labeling degrees: up to 87% of total nerolidol was deuterium labeled in *Fragaria* × *ananassa* Duch. The administration of labeled DOX gave results that were qualitatively comparable with those of the MVL administration. However, the labeled sesquiterpene *trans*-(*S*)-nerolidol was detectable in *Fragaria* × *ananassa* Duch. in only

Table 3. Degree of Labeling after Incubation of Foliage of Different *Fragaria* Species with [5,5-$^2H_2$]-DOX or [5,5-$^2H_2$]-MVL[a]

genotype	precursor	labeling degree (%) (*R*)-linalool	(*S*)-linalool	*trans*-(*S*)-nerolidol
F. × ananassa Duch.	[5,5-$^2H_2$]DOX	nd	2.0 (0.5)	>1
	[5,5-$^2H_2$]MVL	nd	2.4 (0.5)	87 (8)
F. semperflorens	[5,5-$^2H_2$]DOX	>1	>1	nd
Duch. Ser.	[5,5-$^2H_2$]MVL	1.0 (0.6)	2.1 (0.7)	50 (4)
F. vesca	[5,5-$^2H_2$]DOX	2.1 (0.1)	nd	nd
	[5,5-$^2H_2$]MVL	>1	nd	nd

[a] Shown are relative amounts (percent) of labeled compounds of the total amounts of labeled and unlabeled compounds. Standard deviation is shown in parentheses. nd, not detectable.

trace amounts. These results demonstrate a complex intraplant variation of (*R*)- and (*S*)-linalool biosynthesis via the cytosolic and plastidial route. Unlike in the fruit tissue, in the foliage tissue monoterpenes are formed via the cytosolic MVA route as well as via the plastidial DOXP/MEP pathway. These results give evidence for a cross talk between the cytosolic and the plastidial isoprenoid pathways in strawberry foliage.

In conclusion, the experiments demonstrated an independent de novo biosynthesis of terpenoids in fruit tissue and foliage of different *Fragaria* species. Whereas in the fruit tissue terpenoids are exclusively synthesized via the cytosolic MVA pathway, in the foliage both biosynthetic pathways are utilized for biosynthesis of mono- and sesquiterpenes. However, the origin of (*R*)-linalool in the fruits remains unknown. It is well-known that during fruit development both physical and morphological changes are often a result of changes in enzyme levels and activities. It is therefore conceivable that (*R*)-linalool is synthesized only during an early fruit ripening stage and that the expression of the corresponding (*R*)-linalool synthase is completely down-regulated when the fruit attains the fully ripe stage. For this reason a more detailed feeding study that includes the utilization of strawberry fruit and foliage tissues throughout the ripening process is mandatory to clarify the origin of (*R*)-linalool in strawberry fruits and is currently in hand.

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