THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 47, Issue of November 22, pp. 45188–45194, 2002
Printed in U.S.A.

Contribution of the Mevalonate and Methylerythritol Phosphate Pathways to the Biosynthesis of Gibberellins in *Arabidopsis**

Received for publication, August 23, 2002, and in revised form, September 12, 2002
Published, JBC Papers in Press, September 12, 2002, DOI 10.1074/jbc.M208659200

Hiroyuki Kasahara‡, Atsushi Hanada‡, Tomohisa Kuzuyama§, Motoki Takagi§, Yuji Kamiya‡ and Shinjiro Yamaguchi‡¶

From the ‡Laboratory for Cellular Growth and Development, Growth Physiology Research Group, Plant Science Center, RIKEN (The Institute of Physical and Chemical Research), Hirosawa 2-1, Wako, Saitama 351-0198, Japan and §Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

Gibberellins (GAs) are diterpene plant hormones essential for many developmental processes. Although the GA biosynthesis pathway has been well studied, our knowledge on its early stage is still limited. There are two possible routes for the biosynthesis of isoprenoids leading to GAs, the mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in plastids. To distinguish these possibilities, metabolites from each isoprenoid pathway were selectively labeled with ^{13}C in *Arabidopsis* seedlings. Efficient ^{13}C-labeling was achieved by blocking the endogenous pathway chemically or genetically during the feed of a ^{13}C-labeled precursor specific to the MVA or MEP pathways. Gas chromatography-mass spectrometry analyses demonstrated that both MVA and MEP pathways can contribute to the biosyntheses of GAs and campesterol, a cytosolic sterol, in *Arabidopsis* seedlings. While GAs are predominantly synthesized through the MEP pathway, the MVA pathway plays a major role in the biosynthesis of campesterol. Consistent with some crossover between the two pathways, phenotypic defects caused by the block of the MVA and MEP pathways were partially rescued by exogenous application of the MEP and MVA precursors, respectively. We also provide evidence to suggest that the MVA pathway still contributes to GA biosynthesis when this pathway is limiting.

Isoprenoids comprise a broad range of natural products that are synthesized by the condensation of the two precursors, isopentenyl diphosphate (IPP)[1] and dimethylallyl diphosphate (DMAPP) (1). Plants have two distinct biosynthetic routes for the formation of IPP and DMAPP, the mevalonate (MVA) pathway and the newly discovered methylerythritol phosphate (MEP) pathway (Fig. 1) (2, 3). In plants, the MVA pathway plays an essential role in the biosynthesis of sterols and sesquiterpenoids in the cytoplasm (4). On the other hand, the MEP pathway is generally responsible for the formation of carotenoids, mono- and di-terpenoids, plastoquinones, and the prenyl group of chlorophylls in plastids (3). The occurrence of two separate isoprenoid biosynthesis pathways in plants was already implicated in the 1960s from feeding experiments with $^{14}CO_2$ and [^{14}C]MVA, which showed that these substrates labeled distinct groups of terpenoids (5). The MEP pathway was first described for eubacteria (6), and enzymes catalyzing the MEP pathway have been identified mainly in *Escherichia coli* (7, 8). Although the precise reactions in the late steps of MEP pathway have still to be determined, the overall pathway in *E. coli* has recently been proposed (9). Because orthologs of each of the bacterial genes of the MEP pathway are present in *Arabidopsis thaliana* (8, 10), the same set of enzymes are likely to be involved in the MEP pathway in plants.

Gibberellins (GAs) are a class of plant hormones essential for many aspects of plant growth and development, such as seed germination, stem elongation, and flower development (11, 12). The GA biosynthesis pathway in higher plants has been studied in detail in cell-free systems from immature seeds of *Cucurbita maxima*, *Pisum sativum*, and *Phaseolus vulgaris* (13). Because ^{14}C-labeled MVA was efficiently incorporated into *ent*-kaurene (a GA precursor) in these cell-free systems, it has long been assumed that GAs are derived from MVA in plants. The GA biosynthesis pathway has also been studied extensively in a GA-producing fungus *Gibberella fujikuroi* (14). The incorporation of labeled MVA into GAs in cultured mycelia of this fungus also supported the premise that GAs are formed from MVA in this organism (15).

Several lines of evidence from recent work have indicated that *ent*-kaurene is synthesized in the plastids of plants (16–19). Therefore, the MEP pathway in plastids may play a role in providing IPP and DMAPP for *ent*-kaurene biosynthesis (Fig. 1). There is some indirect evidence to support this hypothesis. Antisense suppression of genes encoding enzymes in the *Arabidopsis* MEP pathway resulted in elevated expression of a GA-down-regulated gene, *GA4* (20), and reduced production of *ent*-kaurene (21). Recently, the biosynthetic origin of stevioside, of which the aglycone is a derivative of *ent*-kaurenoic acid, has been studied in *Stevia rebaudiana* leaves (22), where this diterpene glycoside accumulates to more than 10% of the leaf dry weight as a secondary metabolite. This high abundance allowed the ^{13}C-labeling pattern from [1-^{13}C]glucose in steviol to be determined by NMR and indicated that steviosides are synthesized through the MEP pathway. However, this method is not feasible for GAs due to their low abundance in plant tissues. Thus, to determine whether GAs are synthesized through the MEP pathway in general, a more sensitive system for detecting the labeled products is required.

To this end, we used an *Arabidopsis* albino mutant *cla1−1*, which is defective in 1-deoxy-D-xylulose 5-phosphate synthase in the MEP pathway (23). The *cla1−1* phenotype can be rescued almost completely by treatment with exogenous 1-deoxy-D-xylulose (DX), which is converted to a MEP pathway inter-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 81-48-467-9552; Fax: 81-48-462-4691; E-mail: shinjiro@postman.riken.go.jp.

[1] The abbreviations used are: IPP, isopentenyl diphosphate; GA, gibberellin; DMAPP, dimethylallyl diphosphate; DX, 1-deoxy-D-xylulose; MEP, methylerythritol phosphate; MVA, mevalonate; MVL, mevalonolactone; TMS, tetramethylsilane; GC-MS, gas chromatography-mass spectrometry; MS medium, Murashige and Skoog medium.

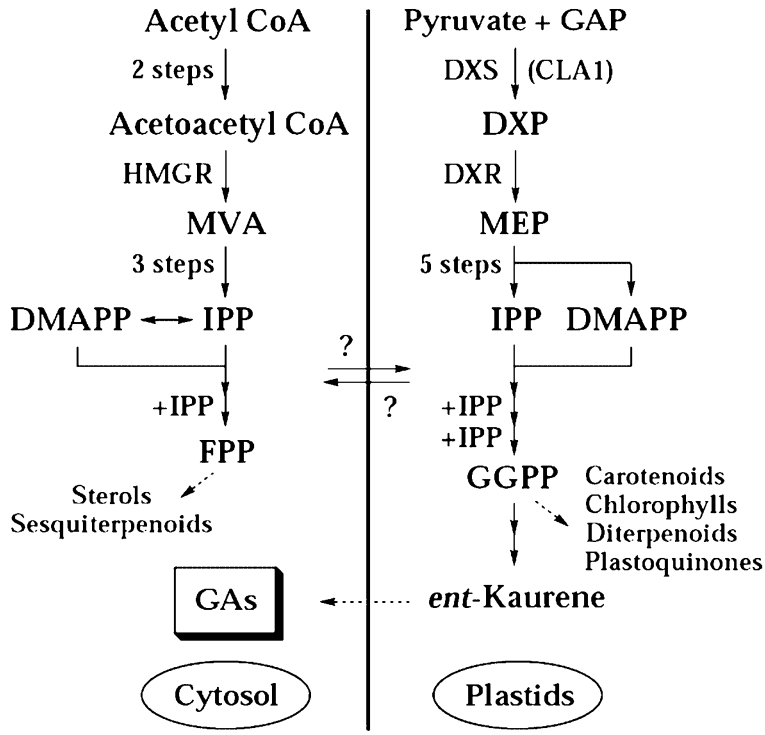

FIG. 1. **The two possible isoprenoid biosynthesis pathways leading to GAs in plants.** A cell-free system from *C. maxima* endosperm can convert MVA to *ent*-kaurene and GAs. *ent*-Kaurene is produced in plastids and then converted to GAs by following oxidation reactions in cytosol. Crossover of common isoprenoid precursors (IPP, geranylgeranyl diphosphate (GGPP), or farnesyl diphosphate (FPP)) between cytosol and plastids has been suggested in several plant species. *Dashed arrows* indicate multiple biosynthetic steps. *HMGR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *DXS*, 1-deoxy-D-xylulose 5-phosphate synthase; *DXP*, 1-deoxy-D-xylulose 5-phosphate; *DXR*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *GAP*, glyceraldehyde 3-phosphate.

mediate 1-deoxy-D-xylulose 5-phosphate in plants. This system allowed us to label the products from the MEP pathway efficiently *in vivo* using [2-^{13}C]DX. To evaluate the role of the cytosolic MVA pathway in GA biosynthesis in the same system, ^{13}C-labeled mevalonolactone (MVL) was fed to plants that were treated with mevastatin, an inhibitor of the MVA pathway. Our gas chromatography-mass spectrometry (GC-MS) analysis demonstrated that GAs are predominantly synthesized from the MEP pathway in *Arabidopsis* seedlings. However, our results also indicated a minor contribution of the MVA pathway to GA biosynthesis. Cooperation of both isoprenoid pathways was also evident for the biosynthesis of the sterol campesterol (a precursor for brassinosteroids), which is formed in the cytosol.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—*A. thaliana* ecotype Wassilewskija (WS) was used in this study. Plants were grown at 21 °C using a 16-h light/8-h dark photoperiod with cool-white illumination. Wild-type (WS-2) and the *cla1−1* mutant (24) were germinated and grown on Murashige and Skoog (MS) agar media (pH 5.7) supplemented with thiamin hydrochloride (3 $\mu g \cdot ml^{-1}$), nicotinic acid (5 $\mu g \cdot ml^{-1}$), pyridoxin hydrochloride (0.5 $\mu g \cdot ml^{-1}$), and 1 or 3% (w/v) sucrose. Liquid culture was carried out in 15 ml of MS media in 100-ml flasks on a shaker (100 rpm).

Chemicals—DX and [2-^{13}C]DX (95% labeled) were synthesized using pyruvate or [2-^{13}C]pyruvate (99% labeled, Aldrich) as previously reported (25). To determine the ^{13}C-labeling ratio of [2-^{13}C]DX, DX and [2-^{13}C]DX (*ca.* 100 ng) were converted to trimethylsilyl derivatives by heating at 70 °C with N,O-bis(trimethylsilyl)acetamide + trimethylchlorosilane + trimethylsilylimidazole (3:2:3, 50 μl, Supelco) and pyridine (50 μl) for 20 min and then analyzed by GC-MS as previously reported (26). DL-MVL and [2-^{13}C]MVL (99% labeled) were purchased from Aldrich, and mevastatin was from Sigma. *ent*-[1,7,12,18-$^{13}C_4$] Kaurene was produced from [2-^{13}C]MVA by a cell-free system prepared from *C. maxima* endosperm as previously reported (27, 28).

Feeding of [2-^{13}C]DX to the cla1-1 Mutant—The albino *cla1−1* homozygotes were selected from the progeny of *CLA1/cla1−1* plants after incubation on MS agar media for 9 days. The *cla1−1* seedlings were then transferred to MS liquid media. [2-^{13}C]DX was dissolved in H_2O, filter-sterilized, and added aseptically to the liquid culture. Twelve days after the transfer to liquid media, uniconazole (EtOH solution, final concentration 1 μM) was added, and then the plants were grown for an additional 3 days before analyzing *ent*-kaurene. GA_{12} and campesterol were analyzed without uniconazole treatment.

Feeding of [2-^{13}C]MVL to Mevastatin-treated Plants—Wild-type seedlings were grown for 5 days on MS agar media before transferring to MS liquid media. Immediately after the transfer, mevastatin (MeOH solution, final concentration 10 μM) and [2-^{13}C]MVL (filter-sterilized H_2O solution) were added aseptically to the liquid media, and the plants were grown for 9 days. *ent*-Kaurene, GA_{12}, and campesterol were analyzed as described above.

GC-MS Analysis of ent-Kaurene, GA_{12}, and Campesterol—For *ent*-kaurene analyses, seedlings (~3 g) were pulverized with a mortar and pestle chilled by liquid N_2. Powdered tissues were extracted with 80% MeOH (25 ml) overnight. The 80% MeOH extract was then partitioned against *n*-hexane (15 ml) three times, and the combined *n*-hexane fraction was evaporated to 1 ml. The *n*-hexane fraction was subjected to SiO_2 gel column chromatography (column size, 5 × 2 cm) and eluted with 15 ml of *n*-hexane. The eluate was carefully evaporated to 20 μl under gentle N_2 flow and analyzed by GC-MS. GC-MS analysis was performed on a GC-mate II mass spectrometer (JEOL, Tokyo, Japan) connected to a Agilent 6890 series GC system with a 30-m × 0.25-mm capillary column DB-5 MS (0.25-μm film thickness, J & W Scientific). GA_{12} and campesterol were extracted from 22~27-g and 0.4-g seedlings and derivatized to methyl ester and TMS ether, respectively, and analyzed as reported previously (29, 30). *ent*-Kaurene and GA_{12} were identified by Kovats retention indices (31), and full mass spectra obtained by GC-MS (29, 32).

RESULTS

Incorporation of [2-^{13}C]DX into ent-Kaurene—The *Arabidopsis cla1−1* mutant is defective in 1-deoxy-D-xylulose 5-phosphate synthase in the MEP pathway and displays a seedling-lethal albino phenotype (23, 24). Previous studies show that the *cla1−1* phenotype is in part restored when grown on agar media containing DX (23). We found that the *cla1−1* phenotype can be better rescued in liquid culture than on agar media in the presence of DX, possibly because of better uptake of the chemical by seedlings. Fig. 2*A* shows that, in the presence of 0.8–1.0 mM DX, the phenotype of the *cla1−1* plants was restored to that of the wild type. To label isoprenoids that are produced via the MEP pathway *in vivo*, the *cla1−1* mutant was treated with 1 mM [2-^{13}C]DX for 15 days. Again, the rescue of

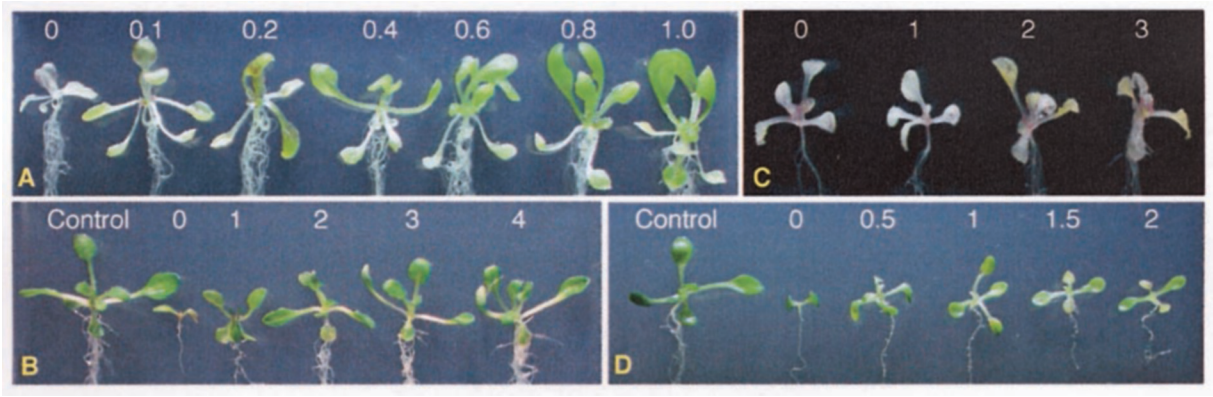

FIG. 2. ***A–D*, effect of DX and MVL on *Arabidopsis cla1−1* and mevastatin-treated wild-type seedlings.** *Numbers* indicate concentrations (mM) of DX (in *A* and *D*) or MVL (in *B* and *C*). All experiments were performed in MS liquid media supplemented with 1% sucrose. *A*, restoration of *cla1−1* seedlings by DX. The albino *cla1−1* seedlings (9 days after sowing) were incubated in MS liquid media for 10 days with DX. For wild-type, see *Control* in *B*. *B*, restoration of mevastatin-treated wild-type seedlings by MVL. WT seedlings (5 days after sowing) were incubated for 7 days in MS liquid media with MVL in the presence of mevastatin (10 μM). Control indicates the plant incubated without mevastatin and MVL. *C*, effect of MVL on *cla1−1* seedlings. The albino *cla1−1* seedlings (5 days after sowing) were incubated in MS liquid media for 3 days with MVL. *D*, effect of DX on mevastatin-treated wild-type seedlings. WT seedlings (5 days after sowing) were incubated for 5 days in MS liquid media with DX in the presence of mevastatin (10 μM). *Control* indicates the plant incubated without mevastatin and DX.

the albino phenotype nearly to wild-type confirmed that [2-^{13}C]DX was metabolized as required.

To examine the role of the MEP pathway in GA biosynthesis, we first determined the incorporation of [2-^{13}C]DX into *ent*-kaurene, a tetracyclic hydrocarbon precursor for all GAs, by GC-MS. Because *ent*-kaurene accumulates at low levels in *Arabidopsis* seedlings (data not shown), plants were treated with 1 μM uniconazole for 3 days to block *ent*-kaurene metabolism before GC-MS analysis (21). If *ent*-kaurene is synthesized from [2-^{13}C]DX through the MEP pathway, four ^{13}C atoms would be introduced at the positions shown in Fig. 3 (*Route 2*). Consistent with this prediction, the mass spectrum of *ent*-kaurene from the [2-^{13}C]DX-treated *cla1−1* plants indicated a peak at *m/z* 276, which corresponds to the molecular ion with four ^{13}C atoms per molecule (Table I). The peak at *m/z* 229 $[M-43]^+$ of non-labeled *ent*-kaurene is a fragment ion after loss of ring D (C_3H_4 and three hydrogen atoms) (Fig. 3), as previously demonstrated by deuterium-labeling experiments (32). Importantly, the corresponding ion peak from the [2-^{13}C]DX-treated plants was observed at *m/z* 232 ($[M-44]^+$). This indicates the loss of one ^{13}C label in ring D and is consistent with the predicted labeling pattern through the MEP pathway from [2-^{13}C]DX. To confirm this result, we analyzed *ent*-kaurene produced from [2-^{13}C]MVA by a cell-free system from *C. maxima* endosperm (27, 28), where ring D would not contain ^{13}C-labels (Fig. 3, *Route 3*). GC-MS showed that four ^{13}C atoms per molecule were incorporated into *ent*-kaurene and that the corresponding fragment ion was at *m/z* 233 ($[M-43]^+$). Subtraction of natural ^{13}C abundance revealed that 99% of the C_5 building blocks had been derived from [2-^{13}C]DX through the MEP pathway in the *cla1−1* seedling (33).

Incorporation of [2-^{13}C]MVL into ent-Kaurene—Mevastatin inhibits 3-hydroxy-3-methylglutaryl-CoA reductase in the MVA pathway and causes severe growth inhibition, which is restored by simultaneous application of MVL. To study the involvement of the MVA pathway in GA biosynthesis, [2-^{13}C]MVL was fed to *Arabidopsis* seedlings that were incubated with mevastatin to label MVA-derived isoprenoids (34). In our liquid culture conditions, 10 μM mevastatin was effective in inhibiting seedling growth, and this inhibitory effect was nearly completely abolished by the addition of 3 mM MVL (Fig. 2*B*).

ent-Kaurene was analyzed by GC-MS from seedlings that were treated with 10 μM mevastatin and 3 mM [2-^{13}C]MVL. In addition to the molecular ion at *m/z* 272, four isotope peaks at *m/z* 273, 274, 275, and 276 were observed, indicating that 1–4 ^{13}C labels were introduced into *ent*-kaurene (Table I). Subtraction of natural ^{13}C abundance indicated that [2-^{13}C]MVL provided 53% of the isoprene units to *ent*-kaurene under this condition. As discussed above, the mass spectrum of *ent*-kaurene produced from [2-^{13}C]MVA through the MVA pathway contains a fragment ion at *m/z* 233 ($[M-43]^+$), because ring D of *ent*-kaurene would not be labeled with ^{13}C. Considering the amount of ^{13}C incorporation (53%) calculated from the molecular ion cluster, the relative intensity of the fragment ion at *m/z* 233 confirms the incorporation of [2-^{13}C]MVL into *ent*-kaurene through the MVA pathway. These results illustrate that the MVA pathway also contributes to the biosynthesis of *ent*-kaurene in *Arabidopsis* seedlings.

Incorporation of [2-^{13}C]DX and [2-^{13}C]MVL into Campesterol—Our feeding experiments showed that both the MEP and MVA pathways can provide precursors for the biosynthesis of *ent*-kaurene. To study the contributions of these two pathways to cytosolic sterol biosynthesis in *Arabidopsis* seedlings, the incorporation of [2-^{13}C]DX and [2-^{13}C]MVL into campesterol was analyzed by GC-MS.

The mass spectrum of campesterol-TMS from the [2-^{13}C]MVL-fed seedlings showed that 98% of its isoprene units was ^{13}C-labeled (Table I). A peak at *m/z* 477, which corresponds to the molecular ion with five ^{13}C atoms per molecule, is in agreement with the expected labeling pattern because one of the six ^{13}C-labels introduced in the precursor squalene will be eliminated by C-4 demethylation during campesterol biosynthesis (Fig. 3, *Route 4*). The fragment ion peak at *m/z* 343 ($[M-129]^+$) of non-labeled campesterol-TMS is attributed to loss of C-1, -2, and -3 and a TMS-ether group of the A-ring (35), as shown in Fig. 3. The corresponding fragment ion from the [2-^{13}C]MVL-fed seedlings at *m/z* 347 ($[M-130]^+$), which indicates loss of one ^{13}C-label, is consistent with the predicted labeling pattern (Fig. 3).

To examine the role of MEP pathway in cytosolic sterol synthesis, campesterol was analyzed in the [2-^{13}C]DX-treated *cla1−1* seedlings by GC-MS. Campesterol can be labeled with ^{13}C at six positions when [3-^{13}C]IPP, originating from [2-^{13}C]DX, is incorporated (Fig. 3, *Route 1*), in contrast to the five ^{13}C atoms incorporated from [2-^{13}C]MVL. The mass spectrum of campesterol-TMS from [2-^{13}C]DX-fed *cla1−1* seedlings showed a peak at *m/z* 478 (Table I). This indicated that

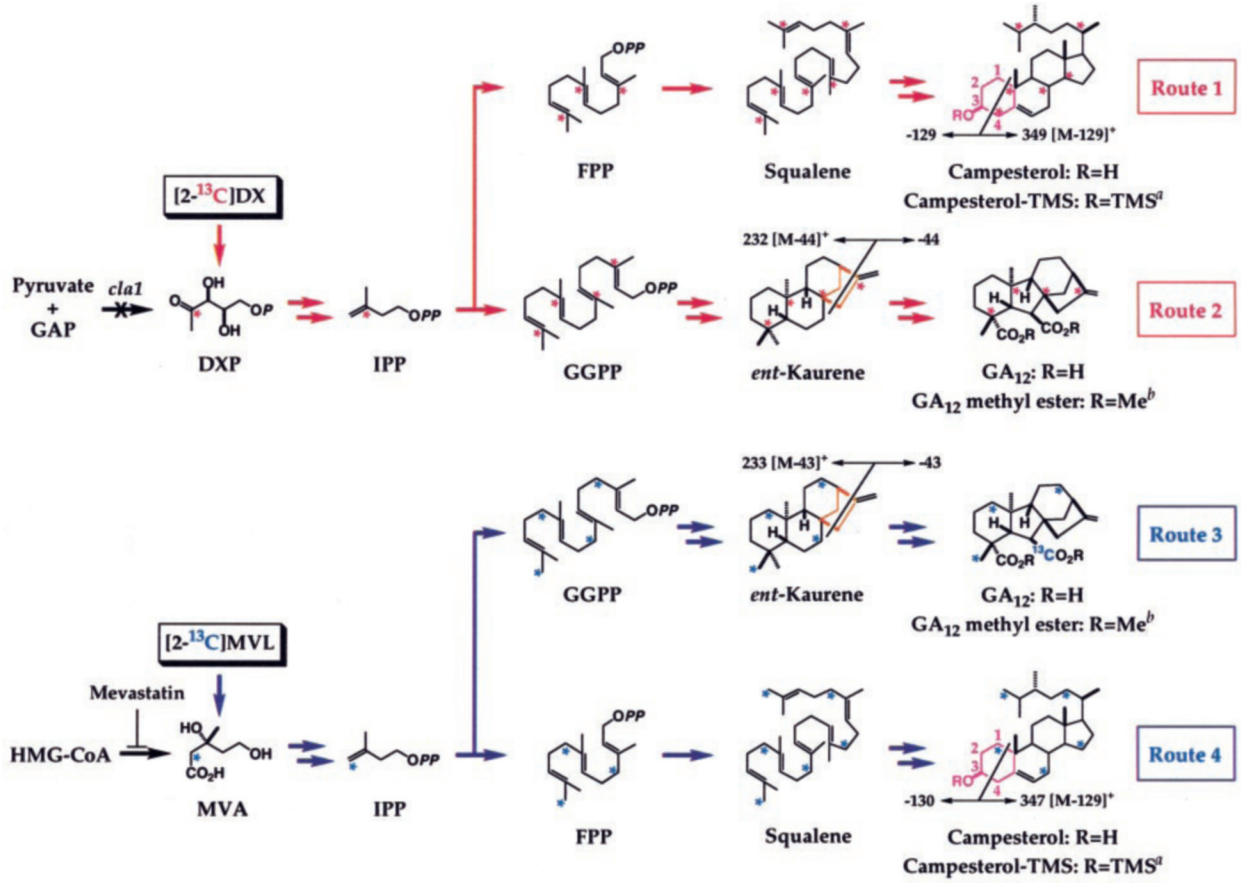

FIG. 3. **Predicted labeling patterns of *ent*-kaurene, GA_{12} and campesterol with [2-^{13}C]DX or [2-^{13}C]MVL.** *Red arrows* indicate possible metabolic conversion of [2-^{13}C]DX through the MEP pathway, and *blue arrows* are those of [2-^{13}C]MVL through the MVA pathway. *Red stars* specify positions of ^{13}C atoms from [2-^{13}C]DX, and *blue stars* specify those from [2-^{13}C]MVL. The ring D of *ent*-kaurene is shown in *orange*, the A-ring of campesterol was shown in *pink*. [a], derivatized from campesterol by trimethylsilylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. [b], derivatized from GA_{12} by methylation using diazomethane. *DXP*, 1-deoxy-D-xylulose 5-phosphate; *GGPP*, geranylgeranyl diphosphate; or *FPP*, farnesyl diphosphate; *HMG-CoA*, 3-hydroxy-3-methylglutaryl coenzyme A.

TABLE I
GC-MS data for ent-kaurene and campesterol-TMS

Feeding experiments were carried out in MS liquid media supplemented with 3% sucrose (total 15 days of incubation). *KRI*, Kovats retention index.

	KRI	Mass spectrum[a]		% Incorporation
		Molecular ion	Fragment ion	
		% relative intensity		
ent-Kaurene				
Standard	2082	272 (100) 273 (21) 274 (3)	229 (93) 230 (18) 231 (4)	
[2-^{13}C]DX/*cla1–1*[b]	2082	275 (25) 276 (98) 277 (21)	232 (100) 233 (22) 234 (3)	99
[2-^{13}C]MVL/mevastatin[c]	2082	272 (70) 273 (48) 274 (52) 27 (99) 276 (71)	229 (78) 230 (64) 231 (78) 232 (100) 233 (49)	53
[2-^{13}C]MVA/*C. maxima*	2082	276 (100) 277 (15)	232 (39) 233 (100)	
Campesterol TMS				
Standard		472 (42) 473 (16) 474 (4)	343 (100) 344 (27) 345 (4)	
[2-^{13}C]DX/*cla1–1*[b]		472 (63) 473 (65) 474 (42) 47 (23) 476 (15) 477 (13) 478 (13)	343 (88) 344 (100) 345 (89) 346 (60) 347 (40) 348 (34) 349 (33) 350 (7)	27
[2-^{13}C]MVL/mevastatin[c]		476 (6) 477 (38) 478 (14)	345 (12) 346 (43) 347 (100) 348 (26)	98

[a] The intensity of base ion peak was set as 100%.
[b] The *cla1–1* seedlings were fed with 1 mM [2-^{13}C]DX.
[c] The wild-type seedlings were fed with 3 mM [2-^{13}C]MVL in the presence of mevastatin.

a maximum of six ^{13}C atoms were incorporated per molecule, which is consistent with the incorporation of IPP/DMAPP derived from [2-^{13}C]DX into cytosolic campesterol biosynthesis. Furthermore, considering the amount of ^{13}C incorporation (27%) estimated from the molecular ion cluster, the relative abundance of the fragment ion at *m/z* 349 indicates no ^{13}C label at C-1 of the A-ring. This observation agrees with the expected labeling pattern through the MEP pathway from [2-^{13}C]DX (*Route 1*). These results indicate that the MVA pathway also contributes to the biosynthesis of campesterol in *Arabidopsis* seedlings.

Incorporation of [2-^{13}C]DX and [2-^{13}C]MVL into GA_{12}—Although *ent*-kaurene is a common intermediate for all GAs, it is also known to serve as a precursor for other diterpenoids such as stevioside in *S. rebaudiana* (22) and kaurenolides in *C. maxima* (36, 37). In *Arabidopsis*, it has not been established whether *ent*-kaurene serves as a precursor solely for GAs. To determine conclusively the role of MEP pathway in the biosyn-

TABLE II
Incorporation of [2-^{13}C]DX and [2-^{13}C]MVL into GA_{12}

Feeding experiments were carried out in MS liquid medium supplemented with 1% sucrose (total 15 days of incubation). GA_{12} was analyzed by GC-MS as a methylester derivative. *KRI*, Kovats retention index.

	KRI	Mass spectrum[a]		% Incorporation[b]
		Molecular ion	Fragment ion	
		% relative intensity		
GA_{12} methyl ester Standard	2389	360 (5) 361 (1)	300 (100) 301 (24) 302 (3)	
[2-^{13}C]DX/*cla1–1*[c]	2389	364 (2)	300 (5) 301 (12) 302 (17) 303 (34) 304 (100) 305 (28)	88
[2-^{13}C]MVL/mevastatin[d]	2389	360 (3) 361 (2)	300 (100) 301 (38) 302 (14) 303 (4)	7

[a] The intensity of base ion peak was set as 100%.
[b] Incorporation rate was calculated from fragment ion peaks at m/z 300–305 after the subtraction of natural ^{13}C abundance.
[c] The *cla1–1* seedlings were fed with 0.8 mM [2-^{13}C]DX
[d] The wild-type seedlings were fed with 3 mM [2-^{13}C]MVL in the presence of mevastatin.

thesis of GAs, we analyzed GA_{12} from the [2-^{13}C]DX-treated *cla1−1* plants by GC-MS. GA_{12} is the first GA in the GA biosynthesis pathway and is converted to biologically active forms by several oxidation steps. Because GAs accumulate to very low levels in plants and this analysis required much more tissue, we set up new liquid culture conditions to grow the plants on a larger scale. In the new system, we reduced the sucrose concentration to 1% (formerly 3%) to reduce the chance of fungal contamination.

The mass spectrum of GA_{12} methyl ester from [2-^{13}C]DX-fed *cla1−1* seedlings demonstrated the incorporation of four ^{13}C labels per molecule, illustrating the contribution of the MEP pathway to its biosynthesis. The incorporation of [2-^{13}C]DX into GA_{12} (88%), which was determined after subtraction of the natural ^{13}C abundance, was similar to that of [2-^{13}C]DX into *ent*-kaurene (87%) at the same concentration of the substrate (Tables II and III). To examine the role of the MVA pathway in GA biosynthesis, another large scale culture was carried out to test the incorporation of [2-^{13}C]MVL in the presence of mevastatin. GC-MS analysis showed that 7 and 5% of the isoprene units of GA_{12} and *ent*-kaurene, respectively, came from [2-^{13}C]MVL (Tables II and III), indicating a minor contribution of the MVA pathway to the synthesis of GA_{12} through *ent*-kaurene.

Effects of Exogenous DX and MVA on the Growth of cla1–1 or Mevastatin-treated Wild-type Seedlings—Our feeding experiments showed a minor role of the MVA pathway in *ent*-kaurene synthesis. To examine how this minor incorporation affects plant growth, we analyzed the effect of exogenous MVL on the phenotype of *cla1−1* seedlings. Fig. 2*C* shows that *cla1−1* seedlings accumulate green-yellow pigments in the presence of 1–3 mM MVL. However, the *cla1−1* albino phenotype was not fully rescued even at higher doses of MVL (data not shown). These results support our conclusion that the MVA pathway plays a minor role in the isoprenoid biosynthesis in plastids, where the major route is the MEP pathway. Likewise, we treated wild-type seedlings with varying concentrations of DX in the presence of mevastatin. Exogenous DX at 0.5 and 1 mM partially rescued the growth defect caused by mevastatin (Fig. 2*D*). However, the growth inhibition was not completely restored even at higher concentrations of DX in the media. These results are consistent with our GC-MS data showing that the MEP pathway can partially contribute to cytosolic sterol biosynthesis.

Crossover Between the Two Pathways at a Lower Dose of [2-^{13}C]DX or [2-^{13}C]MVL—Using feeding experiments with [2-^{13}C]DX or [2-^{13}C]MVL, we showed that either precursor can be incorporated into both *ent*-kaurene and campesterol with different efficiencies. These experiments were carried out at concentrations of exogenous [2-^{13}C]DX or [2-^{13}C]MVL that were sufficient to overcome the effect of the *cla1−1* mutation or mevastatin, respectively, nearly completely. To investigate the mechanism for crossover between MEP and MVA pathways, we determined the incorporation of labeled precursors when the metabolic flux through one of the pathways is limited. This set of experiments was carried out in liquid culture containing 1% sucrose.

TABLE III
Incorporation of [2-^{13}C]DX and [2-^{13}C]MVL at different concentrations into ent-kaurene and campesterol

Feeding experiments were carried out in the MS liquid medium supplemented with 1% sucrose (total 15 days of incubation).

	[2-^{13}C]DX (mM)[a]			[2-^{13}C]MVL (mM)[b]	
	0.8	0.4	0.2	3	1
	% incorporation				
ent-Kaurene	87	81	68	5	8
Campesterol	7	4	3	80	80

[a] [2-^{13}C]DX was fed to *cla1–1* seedlings.
[b] [2-^{13}C]MVL was fed to wild-type seedlings in the presence of mevastatin.

The *cla1−1* albino phenotype was rescued only partially by 0.2 and 0.4 mM DX (Fig. 2*A*). This suggests that DX is still limiting, and therefore, the MEP pathway is not saturated. Under these conditions, [2-^{13}C]DX was still incorporated into campesterol, although the incorporation was lower (3 and 4% at 0.2 and 0.4 mM, respectively; Table III) than that obtained with the higher concentration of [2-^{13}C]DX (7%). Similarly, [2-^{13}C]MVL was incorporated into *ent*-kaurene (8%; Table III) when mevastatin-treated plants were fed with 1 mM MVL, which did not fully rescue the growth inhibition by mevastatin (Fig. 2*B*). These results indicate that the incorporation of [2-^{13}C]MVL into *ent*-kaurene and that of [2-^{13}C]DX into campesterol occurs even when the MVA and MEP pathways, respectively, are limiting. Our data also showed that the concentrations of ^{13}C-labeled precursors in the media can modulate the amount of ^{13}C incorporation into the products, which is evident by the decreased incorporation of [2-^{13}C]DX into *ent*-kaurene at 0.2 mM [2-^{13}C]DX (68%) relative to that observed at 0.8 mM [2-^{13}C]DX (87%) (Table III).

DISCUSSION

Our feeding experiments using ^{13}C-labeled precursors have demonstrated that both MEP and MVA pathways can supply precursors for the biosynthesis of GAs in *Arabidopsis* seedlings. This study provides the first evidence that GAs can be synthesized through the MEP pathway. Because GAs and their precursor *ent*-kaurene are present at extremely low levels in plant tissues, we needed a sensitive method to monitor the incorporation of isotopically labeled intermediates. The use of mevastatin and the *cla1−1* mutation to block individual isoprenoid pathways allowed us to label metabolites efficiently. In stevioside biosynthesis in *S. rebaudiana*, feeding experiments with [1-^{13}C]glucose and NMR were used to show that the diterpene

moiety (a derivative of *ent*-kaurenoic acid) is synthesized through the MEP pathway. A contribution from the MVA pathway was not evident in this study. The inconsistency between this study and ours may be because of differences in plant species, tissue, or development stage, roles of the products (secondary metabolite *versus* growth regulator), or the inability to detect minor ^{13}C incorporation by NMR. Because glucose can be incorporated into both the MEP and MVA pathways, ^{13}C-labeled glucose has often been used to label isoprenoids *in vivo* (38, 39). However, it is not known whether exogenously applied ^{13}C-glucose is equally distributed to the two pathways. This would be a crucial question when both MEP and MVA pathways are involved in the biosynthesis of target compounds.

Crossover between the MEP and MVA pathways has been demonstrated in the biosynthesis of several terpenoids in other plant species using isotopically labeled DX or MVL (2, 40–42). However, in all cases, cellular concentrations of the precursor appeared to be elevated because the labeled compounds were fed to plants without reducing the endogenous levels of these precursors. By inhibiting each isoprenoid pathway and monitoring the phenotypic changes at different doses of the labeled precursors, we were able to estimate the status of isoprenoid pathways. This helped us to predict whether the feeding experiments were done under physiologically relevant conditions. Our results showed that the concentrations of [2-^{13}C]DX in the media can greatly affect the ratio of ^{13}C labels in the products (Table III). Thus, the ^{13}C incorporation determined in different feeding experiments must be carefully interpreted. When the MEP pathway is limiting in the *cla1−1* seedlings at 0.2 mM DX (judged by the incomplete recovery of the *cla1−1* phenotype; Fig. 2*A*), the incorporation of [2-^{13}C]DX into *ent*-kaurene was only 68%, whereas it increased to 87% at a higher [2-^{13}C]DX concentration. It is unclear at present whether the reduced labeling ratio at the lower dose of [2-^{13}C]DX is due to the leaky nature of the *cla1−1* mutant (*e.g.* presence of isozymes) or to the crossover from the MVA pathway. These observations indicate that the amount of ^{13}C incorporation into the product does not necessarily reflect the relative contribution of each pathway under normal growth conditions. These results also suggest that the relative contribution of each isoprenoid pathway could vary when either pathway is up- or down-regulated during plant development or in response to environmental conditions.

Nevertheless, we postulate that the MEP and MVA pathways play a major role in the biosynthesis of *ent*-kaurene and campesterol, respectively, in *Arabidopsis* seedlings. When incubated with 0.8 mM [2-^{13}C]DX, 87% of the C_5 units of *ent*-kaurene came from [2-^{13}C]DX, whereas DX fed under the same conditions provided only 7% of the isoprene units to campesterol. This demonstrates that the MEP pathway is not the primary route for the biosynthesis of campesterol. Similarly, we can conclude that the MVA pathway plays a minor role in the formation of *ent*-kaurene based on the much lower incorporation of [2-^{13}C]MVL into *ent*-kaurene relative to that into campesterol (Table III).

Our current study provides a new view regarding the crossover between two isoprenoid pathways. The incorporation of MVL into *ent*-kaurene was still observed when the growth inhibition caused by mevastatin was not fully restored by MVL. In this situation, the MVA pathway is likely to be limiting, whereas there is no obvious reason that the MEP pathway is inhibited. Therefore, it appears that [2-^{13}C]MVL can be incorporated into *ent*-kaurene against a concentration gradient. To better understand this problem, the common isoprenoid intermediate(s) (*e.g.* IPP, DMAPP, or geranylgeranyl diphosphate) responsible for the crossover between two isoprenoid pathways needs to be determined. An active uptake of IPP into isolated plastids in a facilitated-diffusion manner (43–45) implies that IPP may be involved in the precursor exchange between the MVA and MEP pathways.

It is interesting to note that the *cla1−1* phenotype was rescued only partially even at a high dose of MVL (Fig. 2*C*). This demonstrates that the MEP pathway is still required for normal plant growth even when the flux of MVA pathway is elevated. Taken together, our current data suggest that the uptake of an intermediate from the MVA pathway into the isoprenoid pathway in plastids does occur, but that this mechanism is not sufficient to fully complement the defect in the MEP pathway. This also appears to be true for the other direction, uptake from the MEP pathway to the MVA pathway. We noted that the incorporation of labeled precursors into *ent*-kaurene and campesterol was greater in our first set of experiments performed in 3% sucrose than in the second set in 1% sucrose. This observation suggests that sucrose concentrations may affect the uptake of ^{13}C-labeled substrates by plants and/or the status of two isoprenoid pathways.

In summary, our study showed that both isoprenoid pathways are involved in the biosynthesis of GAs, which are growth-promoting hormones in plants. We predict that the relative contribution of each pathway may be modulated during plant development, by environmental cues and by the status of the other pathway. Molecular genetic approaches in the model species *Arabidopsis* would be useful to uncover how each isoprenoid pathway is regulated and whether the crossover plays any role in controlling isoprenoid biosynthesis in cytoplasm and plastids.

Acknowledgments—We thank Dr. Shozo Fujioka (RIKEN) for advise on feeding experiments, Dr. Patricia León (National Autonomous University of Mexico, Mexico) for providing the *cla1−1* seeds. We also thank Drs. Peter Hedden (University of Bristol, Bristol, UK), Hiroshi Kawaide (Tokyo University of Agriculture and Technology, Tokyo), Kazunori Okada (Tokyo Gakugei University, Tokyo), Juan M. Estévez (RIKEN), Doris Albinsky (RIKEN), and Eiji Nambara (RIKEN) for helpful comments on the manuscript.

REFERENCES

1. Sacchettini, J. C., and Poulter, C. D. (1997) *Science* **277,** 1788–1789
2. Rohmer, M. (1999) *Nat. Prod. Rep.* **16,** 565–574
3. Lichtenthaler, H. K. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 47–65
4. Gray, J. C. (1987) *Adv. Bot. Res.* **14,** 25–91
5. Treharne, K. J., Mercer, E. I., and Goodwin, T. W. (1966) *Biochem. J.* **99,** 239–245
6. Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S., and Sahm, H. (1996) *J. Am. Chem. Soc.* **118,** 2564–2566
7. Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M. H., and Bacher, A. (1998) *Chem. Biol. (Lond.)* **5,** 221–233
8. Kuzuyama, T. (2002) *Biocsci. Biotechnol. Biochem.* **66,** 1619–1627
9. Rohdich, F., Hecht, S., Gärtner, K., Adam, P., Krieger, C., Amslinger, S., Arigoni, D., Bacher, A., and Eisenreich, W. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1158–1163
10. Sponsel, V. M. (2001) *J. Plant Growth Regul.* **20,** 332–345
11. Crozier, A., Kamiya, Y., Bishop, G., and Yokota, T. (2000) in *Biochemistry and Molecular Biology of Plants*, (Buchanan, B. B., Gruissem, W., and Jones, R. L., eds.) pp. 851–865, American Society of Plant Physiology, Rockville, Maryland
12. Yamaguchi, S., and Kamiya, Y. (2000) *Plant Cell Physiol.* **41,** 251–257
13. Graebe, J. E. (1987) *Annu. Rev. Plant Physiol.* **38,** 419–465
14. Hedden, P., Phillips, A. L., Rojas, M. C., Carrera, E., and Tudzynski, B. (2001) *J. Plant Growth Regul.* **20,** 319–331
15. Hanson, J. R., and White, A. F. (1969) *J. Chem. Soc.* 981–984
16. Railton, I. D., Fellows, B., and West, C. A. (1984) *Phytochemistry* **23,** 1261–1267
17. Sun, T. P., and Kamiya, Y. (1994) *Plant Cell* **6,** 1509–1518
18. Aach, H., Böse, G., and Graebe, J. E. (1995) *Planta* **197,** 333–342
19. Yamaguchi, S., Saito, T., Abe, H., Yamane, H., Murofushi, N., and Kamiya, Y. (1996) *Plant J.* **10,** 203–213
20. Estévez, J. M., Cantero, A., Reindl, A., Reichler, S., and León, P. (2001) *J. Biol. Chem.* **276,** 22901–22909
21. Okada, K., Kawaide, H., Kuzuyama, T., Seto, H., Curtis, I. S., and Kamiya, Y. (2002) *Planta* **215,** 339–344
22. Totté, N., Charon, L., Rohmer, M., Compernolle, F., Baboeuf, I., and Geuns, J. M. C. (2000) *Tetrahedron Lett.* **41,** 6407–6410
23. Estévez, J. M., Cantero, A., Romero, C., Kawaide, H., Jiménez, L. F.,

Kuzuyama, T., Seto, H., Kamiya, Y., and León, P. (2000) *Plant Physiol.* **124,** 95–104
24. Mandel, M. A., Feldmann, K. A., Herrera-Estrella, L., Rocha-Sosa, M., and León, P. (1996) *Plant J.* **9,** 649–658
25. Kuzuyama, T., Takahashi, S., Watanabe, H., and Seto, H. (1998) *Tetrahedron Lett.* **39,** 4509–4512
26. Lange, B. M., Wildung, M. R., McCaskill, D., and Croteau, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2100–2104
27. Saito, T., Abe, H., Yamane, H., Sakurai, A., Murofushi, N., Takio, K., Takahashi, N., and Kamiya, Y. (1995) *Plant Physiol.* **109,** 1239–1245
28. Graebe, J. E., Bowen, D. H., and MacMillan, J. (1972) *Planta* **102,** 261–271
29. Silverstone, A. L., Jung, H. S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T. P. (2001) *Plant Cell* **13,** 1555–1566
30. Fujioka, S., Li, J., Choi, Y.-H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., and Sakurai, A. (1997) *Plant Cell* **9,** 1951–1962
31. Gaskin, P., MacMillan, J., Firn, R. D., and Pryce, R. J. (1971) *Phytochemistry* **10,** 1155–1157
32. Gaskin, P., and MacMillan, J. (1991) *GC-MS of the Gibberellins and Related Compounds: Methodology and a Library of Spectra*, pp. 69–70, University of Bristol, Oxford
33. Rauschenbach, P., Schmidt, H. L., Simon, H., Tykva, R., and Wenzel, M. (1974) *Messung von Radioaktiven und Stabilen Isotopen*, pp. 360–366, Springer-Verlag, Heidelberg
34. Choe, S., Noguchi, T., Fujioka, S., Takatsuto, S., Tissier, C. P., Gregory, B. D., Ross, A. S., Tanaka, A., Yoshida, S., Tax, F. E., and Feldmann, K. A. (1999) *Plant Cell* **11,** 207–221
35. Goad, J. L., and Akihisa, T. (1997) *Analysis of Sterols*, pp. 158–159, Blackie Academic and Professional, London
36. Hedden, P., and Graebe, J. E. (1981) *Phytochemistry* **20,** 1011–1015
37. Blechschmidt, S., Castel, U., Gaskin, P., Hedden, P., Graebe, J. E., and MacMillan, J. (1984) *Phytochemistry* **23,** 553–558
38. Adam, K. P., and Zapp, J. (1998) *Phytochemistry* **48,** 953–959
39. Hirai, N., Yoshida, R., Todoroki, Y., and Ohigashi, H. (2000) *Biosci. Biotechnol. Biochem.* **64,** 1448–1458
40. Schwender, J., Gemünden, C., and Lichtenthaler, H. K. (2001) *Planta* **212,** 416–423
41. Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A., and Zenk, M. H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10600–10605
42. Piel, J., Donath, J., Bandemer, K., and Boland, W. (1998) *Angew. Chem. Int. Ed.* **37,** 2478–2481
43. Kreuz, K., and Kleinig, H. (1984) *Eur. J. Biochem.* **141,** 531–535
44. Bauerle, R., Lutke-Brinkhaus, F., Ortmann, B., Berger, S., and Kleinig, H. (1990) *Planta* **181,** 229–233
45. Soler, E., Clastre, M., Bantignies, B., Marigo, G., and Ambid, C. (1993) *Planta* **191,** 324–329