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 $^{14}$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) and dimethylallyl diphosphate (DMAPP) (1). Plants have two different 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 carotenooids, 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, Piaum 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 steviol, 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 $[1,1^{13}$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-δ-xylulose 5-phosphate synthase in the MEP pathway (23). The cla1–1 phenotype can be rescued almost completely by treatment with exogenous 1-deoxy-δ-xylulose (DX), which is converted to a MEP pathway-inter-
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 [2-13C]DX. To evaluate the role of the cytosolic MVA pathway in GA biosynthesis in the same system, [13C]-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**—Arabidopsis thaliana (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 μg ml⁻¹), nicotinic acid (5 μg ml⁻¹), pyridoxin hydrochloride (0.5 μg 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 [2-13C]DX (95% labeled) were synthesized using pyruvate or [2-13C]-pyruvate (99% labeled, Aldrich) as previously reported (25). To determine the 13C-labeling ratio of [2-13C]DX, DX and [2-13C]DX (ca. 100 ng) were converted to trimethylsilyl derivatives by heating at 70°C with N,O-bis(trimethylsilyl)acetamide + trimethylchlorosilane + trimethylsilylimidazole (3:3:2, 50 μl, Supelco) and pyridine (50 μl) for 20 min and then analyzed by GC-MS as previously reported (26). [2-13C]MVL and [2-13C]MVL (99% labeled) were purchased from Aldrich, and mevastatin was from Sigma. ent-[1,7,12,18-13C4]-Kaurene was produced from [2-13C]MVA by a cell-free system prepared from C. maxima endosperm as previously reported (27, 28).

**Feeding of [2-13C]DX to the cla1-1 Mutant**—The albino cla1-1 homozygotes were selected from the progeny of CLA/cla-1-1 plants after incubation on MS agar media for 9 days. The cla1-1 seedlings were then transferred to MS liquid media. [2-13C]DX was dissolved in H₂O, filter-sterilized, and added aseptically to the liquid culture. Twelve days after the transfer to liquid media, mevastatin (EtoH solution, final concentration 1 μM) was added, and then the plants were grown for an additional 3 days before analyzing ent-kaurene. GA₄ and campesterol were analyzed without uniconazole treatment.

Feeding of [2-13C]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 [2-13C]MVL (filter-sterilized H₂O solution) were added aseptically to the liquid media, and the plants were grown for 9 days. ent-Kaurene, GA₄, and campesterol were analyzed as described above.

**GC-MS Analysis of ent-Kaurene, GA₄, and Campesterol**—For ent-kaurene analyses, seedlings (~3 g) were pulverized with a mortar and pestle chilled by liquid N₂. 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 SiO₂ gel column chromatography (column size, 5 × 2 cm) and eluted with 15 ml of n-hexane. The elute was carefully evaporated to 20 μl under gentle N₂ flow and analyzed by GC-MS. GC-MS analysis was performed on a GC-mate II mass spectrometer (JEOL, Tokyo, Japan) connected to an 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). GA₄ 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 previously reported (29, 30). ent-Kaurene and GA₄ were identified by Kovats retention indices (31), and full mass spectra obtained by GC-MS (29, 32).

**RESULTS**

**Incorporation of [2-13C]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. 2A 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 [2-13C]DX for 15 days. Again, the rescue of...
the albino phenotype nearly to wild-type confirmed that
[2-13C]DX was metabolized as required.

To examine the role of the MEP pathway in GA biosynthesis, we first determined the incorporation of [2-13C]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 [2-13C]DX through the MEP pathway, four 13C 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 [2-13C]DX-treated clal−1 plants indicated a peak at m/z 276, which corresponds to the molecular ion with four 13C 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 (C₆H₄ and three hydrogen atoms) (Fig. 3), as previously demonstrated by deuterium-labeling experiments (32). Importantly, the corresponding ion peak from the [2-13C]DX-treated plants was observed at m/z 232 ([M-44]+). This indicates the loss of one 13C label in ring D and is consistent with the predicted labeling pattern through the MEP pathway from [2-13C]DX. To confirm this result, we analyzed ent-kaurene produced from [2-13C]MVA by a cell-free system from C. maxima endosperm (27, 28), where ring D would not contain 13C-labels (Fig. 3, Route 3). GC-MS showed that four 13C atoms per molecule were incorporated into ent-kaurene and that the corresponding fragment ion was at m/z 233 ([M-43]+). Subtraction of natural 13C abundance revealed that 99% of the C₈ building blocks had been derived from [2-13C]DX through the MEP pathway in the clal−1 seedling (33).

Incorporation of [2-13C]MVL into ent-Kaurene—Mevastatin inhibits 3-hydroxy-3-methylutanyl-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, [2-13C]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. 2B).

ent-Kaurene was analyzed by GC-MS from seedlings that were treated with 10 μM mevastatin and 3 mM [2-13C]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 13C labels were introduced into ent-kaurene (Table I). Subtraction of natural 13C abundance indicated that [2-13C]MVL provided 53% of the isoprene units to ent-kaurene under this condition. As discussed above, the mass spectrum of ent-kaurene produced from [2-13C]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 13C. Considering the amount of 13C incorporation (53%) calculated from the molecular ion cluster, the relative intensity of the fragment ion at m/z 233 confirms the incorporation of [2-13C]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 [2-13C]DX and [2-13C]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 [2-13C]DX and [2-13C]MVL into campesterol was analyzed by GC-MS.

The mass spectrum of campesterol-TMS from the [2-13C]MVL-fed seedlings showed that 98% of its isoprene units was 13C-labeled (Table I). A peak at m/z 477, which corresponds to the molecular ion with five 13C atoms per molecule, is in agreement with the expected labeling pattern because one of the six 13C-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 [2-13C]MVL-fed seedlings at m/z 347 ([M-130]+), which indicates loss of one 13C-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 [2-13C]DX-treated clal−1 seedlings by GC-MS. Campesterol can be labeled with 13C at six positions when [3-13C]IPP, originating from [2-13C]DX, is incorporated (Fig. 3, Route 1), in contrast to the five 13C atoms incorporated from [2-13C]MVL. The mass spectrum of campesterol-TMS from [2-13C]DX-fed clal−1 seedlings showed a peak at m/z 478 (Table I). This indicated that...
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 ($\text{Route 1}$). 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 $\text{ent}$-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 $\text{ent}$-kaurene serves as a precursor solely for GAs. To determine conclusively the role of MEP pathway in the biosyn-

![Fig. 3. Predicted labeling patterns of $\text{ent}$-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 $\text{ent}$-kaurene is shown in pink. $^\text{a}$, derivatized from campesterol by trimethylsilylation with N-methyl-N-trimethylsilyltrifluoroacetamide. $^\text{b}$, derivatized from GA$_{12}$ by methylation using diazomethane. $\text{DXP}$, 1-deoxy-D-xylulose 5-phosphate; $\text{GGPP}$, geranylgeranyl diphosphate; or $\text{FPP}$, farnesyl diphosphate; $\text{HMG-CoA}$, 3-hydroxy-3-methylglutaryl coenzyme A.

![TABLE I](Image)

**GC-MS data for $\text{ent}$-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 | Molecular ion | Fragment ion | % Incorporation |
|-----|---------------|--------------|----------------|
|     | Mass spectrum$^*$ | % relative intensity |     |
|     |                |              |              |
| $\text{ent}$-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) |
| $[2-^{13}$C]MVL/mevastatin$^c$ | 2082 | 272 (70) 273 (48) 274 (52) 27 (99) | 229 (78) 230 (84) 231 (78) 232 (100) |
| Campesterol TMS | | | |
| Standard | 2082 | 276 (100) 277 (15) | 232 (39) 233 (100) |
| $[2-^{13}$C]MVA/C. maxima | | | |
| $[2-^{13}$C]DX/cla1–1$^b$ | 472 (42) 473 (36) 474 (4) | 343 (100) 344 (27) 345 (4) |
| $[2-^{13}$C]MVL/mevastatin$^c$ | 476 (15) 477 (13) 478 (13) | 347 (40) 348 (34) 349 (35) 350 (7) |
| $[2-^{13}$C]MVL | 476 (6) 477 (38) 478 (14) | 345 (12) 346 (43) 347 (100) 348 (26) |

$^a$ 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.
TABLE II
Incorporation of [2-13C]DX and [2-13C]MVL into GA12

Feeding experiments were carried out in MS liquid medium supplemented with 1% sucrose (total 15 days incubation). GA12 was analyzed by GC-MS as a methylester derivative. KRI, Kovats retention index.

| KRI | Mass spectrum | Fragment ion | % Incorporation |
|-----|---------------|--------------|----------------|
| GA12 methyl ester | | | |
| Standard | 2389 | 360 (5) 361 (1) | 300 (100) 301 (24) 302 (3) |
| [2-13C]DX/cla1–1<sup>b</sup> | 2389 | 364 (2) | 300 (5) 301 (12) 302 (17) 303 (3) 304 (34) 305 (100) 305 (28) |
| [2-13C]MVL/mevastatin<sup>c</sup> | 2389 | 360 (3) 361 (2) | 300 (100) 301 (38) 302 (14) 303 (4) |
| <sup>a</sup> The intensity of base ion peak was set as 100%.<br> <sup>b</sup> Incorporation rate was calculated from fragment ion peaks at m/z 300–305 after the subtraction of natural 13C abundance.<br> <sup>c</sup> The cla1–1 seedlings were fed with 0.8 mM [2-13C]DX.

The mass spectrum of GA12 methyl ester from [2-13C]DX-fed cla1–1 seedlings demonstrated the incorporation of four 13C labels per molecule, illustrating the contribution of the MEP pathway to its biosynthesis. The incorporation of [2-13C]DX into GA12 (88%) was similar to that of [2-13C]MVL 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 [2-13C]MVL in the presence of mevastatin. GC-MS analysis showed that 7 and 5% of the isoprenoid units of GA12 and ent-kaurene, respectively, came from [2-13C]MVL (Tables II and III), indicating a minor contribution of the MVA pathway to the synthesis of GA12 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. 2C 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. 2D). 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 [2-13C]DX or [2-13C]MVL—Using feeding experiments with [2-13C]DX or [2-13C]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 [2-13C]DX or [2-13C]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.

The cla1–1 albino phenotype was rescued only partially by 0.2 and 0.4 mM DX (Fig. 2A). This suggests that DX is still limiting, and therefore, the MEP pathway is not saturated. Under these conditions, [2-13C]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 observed with the higher concentration of [2-13C]DX (7%). Similarly, [2-13C]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. 2B). These results indicate that the incorporation of [2-13C]MVL into ent-kaurene and that of [2-13C]DX into campesterol occurs even when the MVA and MEP pathways, respectively, are limiting. Our data also showed that the concentrations of 13C-labeled precursors in the media can modulate the amount of 13C incorporation into the products, which is evident by the decreased incorporation of [2-13C]DX into ent-kaurene at 0.2 mM [2-13C]DX (68%) relative to that observed at 0.8 mM [2-13C]DX (87%) (Table III).

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

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

| (2-13C)DX (mM) | (2-13C)MVL (mM) | % Incorporation |
|----------------|----------------|----------------|
| 0.8            | 3              | 87             |
| 0.4            | 3              | 81             |
| 0.2            | 3              | 68             |
| 0.1            | 3              | 5              |
| 0.03           | 3              | 8              |

<sup>d</sup> [2-13C]DX was fed to cla1–1 seedlings.

<sup>e</sup> [2-13C]MVL was fed to wild-type seedlings in the presence of mevastatin.

DISCUSSION
Our feeding experiments using 13C-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 steviolbiosynthesis in S. rebaudiana, feeding experiments with [1-13C]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 13C incorporation by NMR. Because glucose can be incorporated into both the MEP and MVA pathways, 13C-labeled glucose has often been used to label isoprenoids in vivo (38, 39). However, it is not known whether exogenously applied 13C-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 [2-13C]DX in the media can greatly affect the ratio of 13C labels in the products (Table III). Thus, the 13C 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. 2A), the incorporation of [2-13C]DX into ent-kaurene was only 68%, whereas it increased to 87% at a higher [2-13C]DX concentration. It is unclear at present whether the reduced labeling ratio at the lower dose of [2-13C]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 13C 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 [2-13C]DX, 87% of the C5 units of ent-kaurene came from [2-13C]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 [2-13C]MVL into ent-kaurene relative to that into campesterol (Table III).

Our current study provides a new view regarding the cross-over 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 [2-13C]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. 2C). 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 13C-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.

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

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