Distinct Isoprenoid Origins of cis- and trans-Zeatin Biosyntheses in Arabidopsis*

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Plants produce the common isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate (DMAPP) through the methylerythritol phosphate (MEP) pathway in plastids and the mevalonate (MVA) pathway in the cytosol. To assess which pathways contribute DMAPP for cytokinin biosynthesis, metabolites from each isoprenoid pathway were selectively labeled with 13C in Arabidopsis seedlings. Efficient 13C labeling was achieved by blocking the endogenous pathway genetically or chemically during the feed of a 13C labeled precursor specific to the MEP or MVA pathways. Liquid chromatography-mass spectrometry analysis demonstrated that the prenyl group of trans-zeatin (tZ) and isopentenyladenine is mainly produced through the MEP pathway. In comparison, a large fraction of the prenyl group of cis-zeatin (cZ) derivatives was provided by the MVA pathway. When expressed as fusion proteins with green fluorescent protein in Arabidopsis cells, four adenine phosphate-isopentenyltransferases (AtIPT1, AtIPT3, AtIPT5, and AtIPT8) were found in plastids, in agreement with the idea that the MEP pathway primarily provides DMAPP to tZ and isopentenyladenine. On the other hand, AtIPT2, a tRNA isopentenyltransferase, was detected in the cytosol. Because the prenylated adenosine moiety of tRNA is usually of the cZ type, the formation of cZ in Arabidopsis seedlings might involve the transfer of DMAPP from the MVA pathway to tRNA. Distinct origins of large proportions of DMAPP for tZ and cZ biosynthesis suggest that plants are able to separately modulate the level of these cytokinin species.

Cytokinins (CKs), a group of phytohormones, have profound physiological roles in plants, e.g. promotion of cell division, release of lateral buds from apical dominance, and delay of senescence. The biological activity, signal transduction, and metabolism of CKs have long been studied (1). However, it remains unclear how different classes of CKs are produced in plants and whether such classes of CKs play different roles in plant development. Most natural CKs are derivatives of N6-prenylated adenine. At least two CK species, trans-zeatin (tZ) and isopentenyladenine (iP), are considered to be active forms in Arabidopsis according to the specific recognition by a CK receptor that has recently been identified (2, 3). By contrast, cis-zeatin (cZ) exhibits only low or no activity to this Arabidopsis receptor (2).

In CK biosynthesis, the prenylation of AMP is catalyzed by adenylate isopentenyltransferase (adenylate-IPT; EC 2.5.1.27), which utilizes dimethylallyl diphosphate (DMAPP) as a substrate. This enzyme activity, leading to the formation of isopentenyladenine riboside monophosphate (iPRMP), was first demonstrated in slime mold, Dictyostelium discoideum (4). Thereafter, a CK biosynthesis gene, tmr, which encodes adenylate-IPT, was isolated from a crown gall-inducing bacterium, Agrobacterium tumefaciens (5, 6). Recently, the enzymatic properties of Arabidopsis adenylate-IPT-like proteins (AtIPT1, AtIPT3–AtIPT8) have been determined (7, 8). Unlike tmr, some AtIPTs preferred ADP and ATP, rather than AMP, as a substrate to produce corresponding nucleotide CKs in vitro (8, 9). Hence, we refer to these enzymes as adenosine phosphate-IPTs. Subsequent conversion of iPRMP to iP is catalyzed by 5’-nucleotidase and adenosine nucleosidase (10, 11). The conversion of iP into tZ is catalyzed by microosomal trans-hydroxylase, which is probably a P450 monooxygenase (12).

On the other hand, the tRNA-dependent CK biosynthesis pathway has also been proposed in plants, as some tRNA species contain an N6-prenylated adenine moiety, which, by hydrolysis, is capable of forming CKs (13). The prenylation of tRNA is catalyzed by tRNA isopentenyltransferase (tRNA-IPT; EC 2.5.1.8). In Arabidopsis, AtIPT2 encodes a tRNA-IPT (14). AtIPT9 is also similar to bacterial tRNA-IPT in sequence (8), though its enzyme activity has not been demonstrated. In plants, cZ and iP are generally the major components of prenylated tRNAs (13). Therefore, the prenylated tRNA has been considered as a possible source of cZ. In addition, the occurrence of zeatin cis-trans isomerase activity (15) suggests that the tRNA-mediated pathway might also contribute to the synthesis of tZ-type CKs through cZ-type CKs.

Plants have two possible biosynthesis pathways for the prenyl group of CKs, the methylerythritol phosphate (MEP) pathway in plastids and the mevalonate (MVA) pathway in the cytosol (16, 17). Both pathways supply the common isoprenoid precursors isopentenyl diphosphate and DMAPP. Although the MEP and MVA pathways exist in separate subcellular locations, there is some exchange of common precursor(s) between...
the two pathways (18, 19). Therefore, administration of a MEP pathway precursor can partially suppress the growth inhibition caused by a block in the MVA pathway and vice versa (20). As is the case with other isoprenoid biosyntheses, the MVA pathway had been considered the sole route for providing DMAPP to CKs until the MEP pathway was uncovered recently. In fact, the incorporation of $^{13}$C labeled MVA into the iP element of tRNA in vivo has been demonstrated in tobacco pith tissue (21). The incorporation of $^{13}$C labeled MVA into iP and transzeatin riboside (tZR) in vitro was also reported in the endosperm of Sechium edule seeds (22). In addition, there are some reports that indicate that CK levels are reduced in plants when the MVA pathway is limited (23–25). On the other hand, the contribution of the MEP pathway to CK biosynthesis has never been studied previously. It should be noted that the incorporation of MVA does not exclude a potential role of the MEP pathway in the biosynthesis of CKs; it has been observed that isoprene units from both MEP and MVA pathways are incorporated into a single downstream isoprenoid (19, 20).

To selectively label metabolites from the MEP and MVA pathways with $^{13}$C in vivo, we have previously performed feeding of $^{13}$C labeled 1-deoxy-D-xylulose (DX) or mevalonolactone (MVL) to Arabidopsis seedlings (19). DX is converted into the MEP pathway intermediate 1-deoxy-D-xylulose 5-phosphate by phosphorylation. Therefore, exogenous DX is able to complement the albino phenotype of the cla1-1 mutant (26), which is defective in 1-deoxy-D-xylulose-5-phosphate synthase in the MEP pathway. Similarly, the growth inhibition due to a block in the MVA pathway by mevastatin (an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase) is rescued by exogenous DX (0.8 mM) and MVL (3 mM) were fed for 15 days in Murashige-Skoog liquid media. In fact, the incorporation of $^{13}$C labeled 1-deoxy-D-xylulose (DX) or mevalonolactone (MVL) to biosynthesized through the MEP and MVA pathways in plants. We also show the biosynthesis of gibberellins, another group of phytosteroids, by gas chromatography-mass spectrometry (GC-MS) (19).

In this study, we address the biosynthesis route for the prenyl moiety of CKs using the $^{13}$C labeled tracer in Arabidopsis seedlings. Our data demonstrate that the prenyl side chains of tZ- and iP-type CKs are mainly produced through the MEP pathway, whereas a large fraction of cZ derivatives is synthesized through the MVA pathway. We also show the subcellular location of AtIPTs produced as green fluorescent protein (GFP)-fusion proteins. Based on these data, we propose a crucial role of the plastid-localized MEP pathway in CK biosynthesis, and discuss how different classes of CKs are biosynthesized through the MEP and MVA pathways in plants.

**EXPERIMENTAL PROCEDURES**

**Plant Materials, Growth Conditions, and Chemicals—**Arabidopsis thaliana ecotype Wassilewskija-2 was used for $^{13}$C labeling experiments. Feeding of [1-$^{13}$C]DX and [2-$^{13}$C]MVL was carried out in the presence of 1% sucrose as described previously (19), except that uniconazole treatment was omitted. A. thaliana Columbia-0 was used for observation of GFP fluorescence. DX and [1-$^{13}$C]DX (99% labeled) were synthesized as reported before using idiomethane and [1-$^{13}$C]-

### RESULTS

**CK Levels in Arabidopsis Seedlings—**To study the metabolic origins of the prenyl group of CKs, we planned to conduct the $^{13}$C labeling experiment that was employed previously to determine the contribution of the MEP and MVA pathways to gibberellin biosynthesis (19). To examine whether this method was feasible for CKs, we first analyzed endogenous CK levels by LC-MS in Arabidopsis seedlings under the same growth conditions, after a feeding of non-labeled DX and MVL. When DX was fed to the cla1-1 mutant to rescue its albino phenotype, all CKs analyzed were detectable by LC-MS as reported previously (33). The $^{13}$C incorporation level was calculated using the fragment ion cluster between m/z 190 and 192 after subtraction of natural $^{13}$C abundance (34). Campesterol (CAM) was eluted from the MCX column with CH$_3$Cl$_3$ (3 ml x 3), after purification of CKs. CAM was further purified by high pressure liquid chromatography and analyzed by GC-MS as reported previously (19).

**TABLE I**

| CK levels | tZRM | tZR | tZ | iP | iP | cZRM | cZ |
|-----------|------|-----|---|---|---|-----|---|
| tZRM $^{a}$ | 21.81 | 9.54 | 0.04 | 20.79 | 0.43 | 0.11 | 0.86 |
| iP | 15.80 | 3.37 | 0.99 | 15.73 | 0.33 | 0.10 | 0.89 |

$^{a}$ DX (0.8 mm) and MVL (3 mm) were fed for 15 days in Murashige-Skoog liquid media.

**Particle Bombardment—**Full-length or part of the coding regions of AtIPTs (AtIPT1, Met$^{1}$–Leu$^{27}$; AtIPT2, Met$^{1}$–Asn$^{108}$; AtIPT3, Met$^{1}$–Ser$^{47}$; AtIPT4, Met$^{1}$–Asn$^{105}$; AtIPT5, Met$^{1}$–Ser$^{47}$; AtIPT7, Met$^{1}$–Phe$^{60}$; AtIPT8, Met$^{1}$–Va$^{138}$) were fused to the amino terminus of the GFP gene, which was controlled by the cauliflower mosaic virus 35 S promoter (355-eGFP (S65T)) (28). The DNA constructs were introduced into the roots or rosette leaves of 2- or 3-week-old seedlings by particle bombardment (PDU-1000/He, Bio-Rad). Transient expression was observed by laser confocal-scanning fluorescence microscopy after overnight incubation (Fluoview IX5, Olympus).

**Stable Transformants of Arabidopsis—**Genomic DNA fragments of AtIPT3 and AtIPT7 containing the 5′-flanking regions (3.9 kb for AtIPT3, 4.1 kb for AtIPT7) and the full coding regions were ligated into pTH2 vectors (29) to produce chimeric genes fused to the amino terminus of the GFP gene. The chimeric genes were introduced into Arabidopsis by the floral dip method (30). More than five independent lines were obtained, and T3 plants were used for analysis.

**Plant Hormone Analysis—**Extraction and fractionation of CKs from Arabidopsis seedlings (3–4 g) were performed as described previously (31). The nucleotide CK fractions were further analyzed as the corresponding nucleosides after treatment with phosphatase (9). Each CK was purified by immunoaffinity columns (32) except for the addition of anti-cis-zeatin riboside (cZR) antibodies to the original protocol. After desalting, the resulting samples were dissolved in H$_2$O and analyzed with a liquid chromatography (LC)-MS system (model 2695/ZQ2000MS, Waters). CKs were separated at a flow rate of 0.25 ml/min, with the gradients of solvents A (H$_2$O, B (methanol), and C (0.1% acetic acid) set according to the following profile: 0 min, 95% A + 5% C; 1 min, 95% A + 5% C; 16 min, 45% A + 50% B + 5% C; 22 min, 25% A + 70% B + 5% C. Capillary voltage was 4.0 kV. Other conditions were described previously (9). The quantification of [M + H]$^{+}$ and +1 signals were achieved at levels between 0.1 and 100 pmol/injection using a standard curve. Abscisic acid (ABA) was purified from the methanol fraction from the MCX column (Waters) in CK purification by high performance liquid chromatography and analyzed by GC-MS as reported previously (33). The $^{13}$C incorporation level was calculated using the fragment ion cluster between m/z 190 and 192 after subtraction of natural $^{13}$C abundance (34). Campesterol (CAM) was eluted from the MCX column with CH$_3$Cl$_3$ (3 ml x 3), after purification of CKs. CAM was further purified by high pressure liquid chromatography and analyzed by GC-MS as reported previously (19).
quantification of the molecular ion and its +1 isotopomer ion by LC-MS would be possible in this system with the exception of cZ, which was detectable, but its +1 isotopomer ion was below the range of reliable quantification.

**Incorporation of \([1-13C]DX\) and \([2-13C]MVL\) into CKs—**To evaluate the role of the MEP pathway in providing DMAPP to CKs, \([1-13C]DX\) was fed to seedlings of the \(cla1-1\) mutant. Likewise, to label metabolites from the MVA pathway, \([2-13C]MVL\) was fed to wild type seedlings in the presence of mevastatin. In both systems, one \(^{13}\)C atom would be incorporated into CKs if they were produced through the MEP or MVA pathways (Fig. 1). Table II shows the levels of \(^{13}\)C incorporation into CKs determined by LC-MS. The incorporation of \([1-13C]DX\) was evident for all CKs listed in the table. For example, tZR has a molecular ion at \(m/z = 352\), whereas the corresponding ion for tZR from the \([1-13C]DX\)-treated \(cla1-1\) seedlings was observed at \(m/z = 353\). A fragment ion of tZR was detected from the \([1-13C]DX\)-treated \(cla1-1\) seedlings at \(m/z = 221\), which is 1 mass unit larger than that of authentic tZR (\(m/z = 220\)). The presence of a \(^{13}\)C atom in cis-zeatin riboside monophosphate (cZRMP) and cZR was clear in the mevastatin-treated plants fed with \([2-13C]MVL\) (Table II). Incorporation of \([2-13C]MVL\) into cZ was also indicated by an ion at \(m/z = 221\) ([M+H+1]+), which appeared more abundant than [M+H]+ of authentic cZ at \(m/z = 220\) (data not shown), but their relative intensities could not be quantified because of low levels of cZ in the samples (Table I).

Fig. 2 shows relative levels of \(^{13}\)C incorporation from \([1-13C]DX\) and \([2-13C]MVL\) into CKs, ABA, and CAM determined from the data sets given in Table II after the subtraction of natural \(^{13}\)C abundance (34). It has been reported previously (35) that the MEP pathway mainly supplies precursors to the biosynthesis of ABA, which is produced through carotenoids in plastids. By contrast, CAM is a cytosolic phytosterol that is primarily synthesized via the MVA pathway. Our data show that CKs can be classified into two groups based on the \(^{13}\)C labeling ratio from \([1-13C]DX\) and \([2-13C]MVL\): \(tZ\)- and iP-type CKs was labeled at a high rate by \([1-13C]DX\) but only at a low rate by \([2-13C]MVL\) (Fig. 2). In marked contrast, about 75% of the prenyl moiety of \(cZ\)-type CKs (cZRMP and cZR) were labeled with \([2-13C]MVL\), whereas the incorporation of \([1-13C]DX\) into cZ derivatives was significantly lower than the incorporation into \(tZ\) and iP derivatives.

As discussed previously (19), the levels of \(^{13}\)C incorporation determined across separate feeding systems must be carefully interpreted, because the ratio of \(^{13}\)C labels in products can be altered by the concentration of \(^{13}\)C precursors in the media. Thus, the values in Fig. 2 do not immediately reflect the relative contribution of each isoprenoid pathway under normal growth conditions. Nevertheless, we conclude from the LC-MS data that \(tZ\) and iP-type CKs in Arabidopsis seedlings are predominantly synthesized via the MEP pathway because of the following observations. First, the incorporation of \([2-13C]MVL\) into \(tZ\) and iP derivatives was consistently low (<20%), whereas nearly 90% of the isoprene units of CAM were labeled with \(^{13}\)C in the same sample (Fig. 2 and Table II). This observation indicates that the MVA pathway is not the primary route used to provide DMAPP to \(tZ\) and iP-type CKs. Second, the level of \(^{13}\)C incorporation into \(tZ\) and iP derivatives from \([1-13C]DX\) was as high as that into ABA, which is known to be biosynthesized mainly through the MEP pathway (35). On the other hand, we predict that the MVA pathway provides a greater proportion of DMAPP to the biosynthesis of cZ-type CKs in comparison to that of \(tZ\) and iP-type CKs, because \([2-13C]MVL\) was introduced into these CKs nearly as efficiently as it was into CAM in the same feeding experiment. This idea is also supported by the significantly lower levels of \(^{13}\)C incorporation into cZ-type CKs from \([1-13C]DX\) than that into ABA.

**Subcellular Localization of AtIPTs—**Because the prenyl group of \(tZ\) and iP-type CKs is predominantly synthesized through the MEP pathway (Fig. 2), we speculated that some IPTs should use the DMAPP produced in plastids. Compared with the bacterial enzyme tmr, all AtIPTs except for AtIPT4 have an amino-terminal extension consisting of 30–60 amino acids (7). The ChloroP 1.1 program (36) predicted that the amino-terminal regions of AtIPT1, -3, -5, and -8 might function...
to localize these AtIPTs to plastids. To obtain experimental evidence for the subcellular locations of IPT activities, we prepared DNA constructs designed to produce translational fusions of individual AtIPTs and GFP. Because some of the fusion proteins containing the full coding regions of AtIPTs tended to precipitate in cells (data not shown), the amino-terminal regions, which could function as transit peptides, were fused to GFP (for details, see “Experimental Procedures”). The plasmid constructs expressing AtIPT-GFP fusion proteins were introduced into leaf cells and root cells by particle bombardment. Stable transformants expressing AtIPT3-GFP and AtIPT7-GFP driven by their own promoters were generated. Fluorescence was observed using a confocal laser-scanning microscope. Chlorophyll autofluorescence was merged on the GFP fluorescence images. Transmission images were superimposed on the fluorescence images.

**Table II**

| CKs | Sample | Molecular ion | Mass spectrum (% relative intensity) | Fragment ion |
|-----|--------|---------------|--------------------------------------|--------------|
|     |        | [M+H]+ | +1  | +2  | [M+H−H]+ | +1  | +2  |
| tZRMP (as tZR) | standard | 352 (100) | 353 (19) | 354 (3) | 220 (54) | 221 (7) | 222 (0) |
| [1-13C]DX | 352 (3) | 353 (100) | 354 (18) | 220 (22) | 221 (59) | 222 (7) |
| [2-13C]MVL | 352 (100) | 353 (35) | 354 (5) | 220 (56) | 221 (15) | 222 (2) |
| tZR | standard | 352 (100) | 353 (19) | 354 (3) | 220 (54) | 221 (7) | 222 (0) |
| [1-13C]DX | 352 (3) | 353 (100) | 354 (18) | 220 (1) | 221 (57) | 222 (7) |
| [2-13C]MVL | 352 (100) | 353 (36) | 354 (6) | 220 (56) | 221 (16) | 222 (2) |
| iZ | standard | 220 (100) | 221 (14) | 221 (32) |
| [1-13C]DX | 220 (8) | 221 (100) |
| [2-13C]MVL | 220 (100) | 221 (32) |
| iPRMP (as iPR) | standard | 336 (100) | 337 (15) | 338 (2) | 204 (86) | 205 (11) | 206 (1) |
| [1-13C]DX | 336 (8) | 337 (100) | 338 (18) | 204 (7) | 205 (89) | 206 (11) |
| [2-13C]MVL | 336 (100) | 337 (37) | 338 (6) | 204 (93) | 205 (27) | 206 (2) |
| iPR | standard | 336 (100) | 337 (18) | 338 (2) | 204 (86) | 205 (11) | 206 (1) |
| [1-13C]DX | 336 (7) | 337 (100) | 338 (18) | 204 (8) | 205 (92) | 206 (11) |
| [2-13C]MVL | 336 (100) | 337 (27) | 338 (4) | 204 (90) | 205 (24) | 206 (3) |
| iP | standard | 204 (100) | 205 (10) |
| [1-13C]DX | 204 (27) | 205 (100) |
| [2-13C]MVL | 204 (100) | 205 (19) |
| cZRMP (as cZR) | standard | 352 (37) | 353 (12) | 354 (1) | 220 (100) | 221 (15) | 222 (0) |
| [1-13C]DX | 352 (23) | 353 (38) | 354 (6) | 220 (67) | 221 (100) | 222 (13) |
| [2-13C]MVL | 352 (13) | 353 (37) | 354 (5) | 220 (38) | 221 (100) | 222 (12) |
| cZR | standard | 352 (37) | 353 (7) | 354 (1) | 220 (100) | 221 (15) | 222 (0) |
| [1-13C]DX | 352 (19) | 353 (35) | 354 (6) | 220 (56) | 221 (100) | 222 (15) |
| [2-13C]MVL | 352 (9) | 353 (36) | 354 (6) | 220 (34) | 221 (100) | 222 (12) |

a The relative intensity represents mean value from three independent experiments, and the intensity of base ion peak was set at 100%.
b R represents a ribosyl group.c Nucleotide CKs were analyzed by LC-MS as the corresponding nucleoside forms.d [1-13C]DX (0.8 mM) was fed to the cla1-1 seedlings for 15 days.e [2-13C]MVL (3 mM) was fed to the wild type seedlings in the presence of 10 μM mevastatin for 15 days.

**Fig. 2.** Relative levels of 13C incorporation into CKs, ABA, and CAM in [1-13C]DX-fed Arabidopsis cla1-1 seedlings (A) and in [2-13C]MVL-fed wild type seedlings in the presence of mevastatin (B). The 13C incorporation level was calculated using the molecular ion cluster shown in Table II after subtraction of natural 13C abundance. 13C incorporation levels were measured in three independent experiments. Error bars indicate S.D.

**Fig. 3.** Subcellular localization of AtIPT-GFP fusion proteins in Arabidopsis. The fusion constructs expressing AtIPT1-GFP (A–C), AtIPT3-GFP (D and E), AtIPT5-GFP (G), AtIPT8-GFP (H), AtIPT4-GFP (I), AtIPT6-GFP (J), and AtIPT7-GFP (K and L) were introduced into leaf cells (A, B, D, G, J, M, N) and root cells (C, E, F, H, K) by particle bombardment. Stable transformants expressing AtIPT3-GFP (P) and AtIPT7-GFP (L) driven by their own promoters were generated. Fluorescence was observed using a confocal laser-scanning microscope. Chlorophyll autofluorescence was merged on the GFP fluorescence images in B, D, G, J, and N. Transmission images were superimposed on the fluorescence images in C, E, F, H, K, and L. Bar = 10 μm.
DNAs were introduced into both Arabidopsis leaf and root cells, and the transient expression of the chimeric genes was observed. The fluorescence of AtIPT1, AtIPT3, and AtIPT5-GFP in mesophyll cells was observed in plastids as the signal overlapped with autofluorescence derived from chlorophylls (Fig. 3, A, B, D, and G). AtIPT8-GFP exhibited the same fluorescence pattern as AtIPT1 and -3 in root cells (Fig. 3, C, E, and H). However, the fluorescence of AtIPT4 and AtIPT2-GFP was distributed in the cytosol (Fig. 3, J, M, and N). It should be noted that an identical pattern of GFP fluorescence was observed when the GFP was fused to the carboxyl terminus of AtIPT4 (data not shown). The fluorescence of AtIPT7-GFP was observed in the mitochondria (Fig. 3, J and K). To avoid mislocation of the fusion proteins because of transient expression by a strong promoter, we next generated stable transformants of Arabidopsis expressing AtIPT3 and AtIPT7-GFP controlled by their native promoters. Consequently, the pattern of fluorescence produced by both of these constructs was essentially the same as the respective transiently expressed fusion proteins (Fig. 3, compare E with F and K with L). These results strongly suggest that four adenosine phosphate-IPTs, AtIPT1, -3, -5, and -8, localize in plastids in agreement with the idea that the MEP pathway primarily provides DMAPP for tZ and iP synthesis. As for tRNA-IPT, AtIPT2-GFP localized in the cytosol (Fig. 3, M and N).

We did not examine the location of AtIPT6 and -9, because AtIPT6 does not appear to encode a functional IPT in some Arabidopsis ecotypes owing to a point mutation (8) and because the enzyme activity of AtIPT9 could not be detected (data not shown).

**DISCUSSION**

Using a $^{13}$C labeled precursor specific to the MEP and MVA pathways, we have demonstrated that both isoprenoid pathways can supply DMAPP to CKs in Arabidopsis seedlings (Fig. 2). In addition, subcellular localization of AtIPT-GFP fusion proteins has been determined in Arabidopsis cells. The results from GFP fluorescence observations suggest that IPT isoforms are distributed to multiple subcellular compartments (Fig. 4). These data now provide new insights into CK biosynthesis as discussed below.

**The Origin of DMAPP for tZ- and iP-type CKs—**The localization of several adenosine phosphate-IPTs to plastids (Fig. 3) is consistent with the role of the MEP pathway in providing DMAPP to tZ- and iP-type CKs. The dominant role of the MEP pathway in the biosynthesis of iP-type CKs is in agreement with the recent finding that overexpression of the AtIPT8/PGA22 gene, the product of which was found in plastids when expressed as a GFP fusion protein (Fig. 3), drastically increased the level of iPRMP and iP in Arabidopsis (37).

There are at least two possible explanations for the minor incorporation of [2-13C]MVL into tZ- and iP-type CKs (Fig. 2). First, as has been suggested previously, there may be some exchange of common isoprenoid precursors (e.g. isopentenyl diphosphate or DMAPP) between the cytosol and plastids. This mechanism has been proposed to explain the minor incorporation of [2-13C]MVL into ent-kaurene (a precursor for gibberellins), assuming that ent-kaurene synthesis occurs exclusively in plastids as supported by the subcellular localization of enzymes (38). However, in the case of the biosynthesis of tZ- and iP-type CKs, a small amount of $^{13}$C label from [2-13C]MVL can also be attributed to the function of another adenosine phosphate-IPT, AtIPT4, which is presumably present in the cytosol (Fig. 3J). In addition, the localization of AtIPT7-GFP in the mitochondria (Fig. 3, J–L) suggests that this enzyme may also use DMAPP from the MVA pathway, because ubiquinones, a group of isoprenoids in mitochondria, are synthesized principally through the MVA pathway (39). Therefore, a major role of the MEP pathway in the biosynthesis of tZ- and iP-type CK in Arabidopsis seedlings under the current growth conditions does not rule out a greater contribution of the MVA pathway to these CKs under different growth conditions, if the relative abundance of IPT isoforms is modulated. Thus, our present results do not contradict previous reports (22–24) indicating a role for the MVA pathway in providing DMAPP for tZ-type CKs. In this context, to fully understand the relative roles of the MEP and MVA pathways in CK biosynthesis, it will be informative to determine how individual AtIPT genes are regulated during plant development and under different environmental conditions.

Recently, Åstot et al. (24) measured the biosynthetic rates of tZRMP and iPRMP using in vivo isotope labeling and proposed an iPRMP-independent pathway for the biosynthesis of iZ-type CKs. In this model, a hydroxylated derivative of DMAPP is directly transferred to AMP (24). In light of this finding, our result showing the involvement of the MEP pathway in tZ biosynthesis is intriguing because hydroxymethylbutenyl diphosphate, the best hypothetical substrate in the iPRMP-independent pathway (24), has been known as an intermediate of the MEP pathway (Fig. 4) (40). In fact, it has been demonstrated recently (41) that TZR, an IPT from Agrobacterium, is capable of synthesizing tZRMP from hydroxymethylbutenyl diphosphate and AMP in vitro. To examine whether the iPRMP-independent pathway operates in plants, hydroxymethylbutenyl diphosphate should be tested as a substrate for AtIPT1, -3, -5, and -8 in vitro.

The Origin of DMAPP for cZ-type CKs—Based on the efficient $^{13}$C incorporation of [2-13C]MVL into cZ derivatives, relative to that into ABA and other CKs (Fig. 2), we postulate that a large portion of the prenyl group of cZ-type CKs originates from the MVA pathway. However, the level of [1-13C]DX incorporation into cZ-type CKs was also significantly higher than incorporation into CAM, a cytosolic sterol that is mainly produced through the MVA pathway. Therefore, the MEP pathway appears to supply DMAPP to cZ derivatives at a level beyond the hypothesized exchange of isoprenoid precursors (Fig. 4). Unlike the possible participation of a P450 monoxygenase in the hydroxylation of IP to form tZ (12), the conversion of iP-type CKs into their corresponding cZ derivatives has not been re-

**FIG. 4. Proposed CK biosynthesis pathway and the localization of related enzymes in Arabidopsis.** Red arrows indicate possible biosynthesis pathways for iP and tZ through the MEP pathway, and blue arrows are those proposed for cZ through the MVA pathway. Black arrows depict possible cis-trans isomerization between tZ- and cZ-type CKs. The non-filled red arrow indicates multiple steps. The non-filled black arrow indicates a predicted contribution of the MVA pathway to mitochondrial isoprenoid biosynthesis. The dashed arrows denote multiple steps. GAP, glyceraldehyde 3-phosphate; PYR, pyruvate; DMAPP, 1-deoxy-D-xylulose 5-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; iP, isopentenyl diphosphate; tZ, trans-ZEAR; cZ, cis-ZEAR; MVL, mevalonate; tZRMP and iPRMP, trans-ZEAR and isopentenyl diphosphate-derived isoprenoid monophosphates; tZS, tZ synthase; TZR, trans-ZEAR synthase.
ported. Therefore, this route cannot readily be hypothesized at the moment.

A probable explanation for the incorporation of MEP-derived DMAPP into cZ derivatives is isomerization of tZ-type CKs (Fig. 4). The presence of a cis-trans isomerase of zeatin has been reported in cell-free extracts from immature seeds of Phaseolus vulgaris (15). This enzyme catalyzes the isomerization of zeatin in both directions in favor of cZ to tZ and uses cZR and tZR as substrates as well. In our experimental conditions, the amounts of cZ-type CKs were much smaller than those of corresponding tZ-type CKs (Table 1), and the majority of tZ-type CKs were labeled with 13C in cla1-1 seedlings fed with [1-13C]DX (Fig. 2). Thus, even a small proportion of isomeriza-

tion of 14C labeled MVA into the iP element of tRNA has been pathway. In fact, our 13C labeling experiments indicated that a

dopsis of tRNA takes place in this subcellular compartment in the biosynthesis of cZ-type CKs, because the

the prenyl precursor to tZ- and iP-type CKs in contribution of the plastid-located MEP pathway in providing

modulate the level of these CK species. Further characterization of the biosynthesis pathway for cZ would be necessary to evaluate its physiological significance in plants. Additional work is required to uncover how multiple IPTs in varying subcellular compartments control the levels of different classes of CKs during plant development and in response to environ-

mental cues.

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