**Agrobacterium tumefaciens** increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant


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**Agrobacterium tumefaciens** infects plants and induces the formation of tumors called “crown galls” by integrating the transferred-DNA (T-DNA) region of the Ti-plasmid into the plant nuclear genome. Tumors are formed because the T-DNA encodes enzymes that modify the synthesis of two plant growth hormones, auxin and cytokinin (CK). Here, we show that a CK biosynthesis enzyme, Tmr, which is encoded by the **Agrobacterium** T-DNA region, is targeted to and functions in plastids of infected plant cells, despite having no typical plastid-targeting sequence. Evidence is provided that Tmr is an adenosine phosphate-isopentenyltransferase (IPT) that creates a new CK biosynthesis bypass by using 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) as a substrate. Unlike in the conventional CK biosynthesis pathway in plants, trans-zeatin-type CKs are produced directly without the requirement for P450 monooxygenase-mediated hydroxylation. Consistent with the plastid localization of Tmr, HMBDP is an intermediate in the methylerythritol phosphate pathway, a plastid-localized biosynthesis route for universal isoprenoid precursors. These results demonstrate that **A. tumefaciens** modifies CK biosynthesis by sending a key enzyme into plastids of the host plant to promote tumorigenesis.

Cytokinins (CKs) are a group of plant hormones essential for cell division and differentiation in plants (1). Most natural CKs, including isopentenyladenine (iP) and trans-zeatin (tZ) (Fig. 1), are derivatives of N6-prenylated adenine. (1) The prenyl side chain of CKs can be derived from the methylerythritol phosphate (MEP) pathway or the mevalonate (MVA) pathway, both of which supply common C5 units for isoprenoid biosynthesis (2, 3). The MEP pathway widely occurs in the bacterial kingdom and the plastids of plants, whereas the MVA pathway is commonly found in the cytosol of eukaryotes (2, 3). Thus, plants have the two different isoprenoid pathways in separate subcellular compartments. Recent work has shown that the majority of the prenyl side chain of iP and tZ is derived from the MEP pathway (*in Arabidopsis* seedlings (4)).

To initiate CK biosynthesis, an isoprenoid precursor is transferred to AMP, ADP or ATP by adenosine phosphate-isopentenyltransferase (IPT, EC 2.5.1.27) (Fig. 1) (5–7). At least two routes have been proposed for the formation of tZ, an active CK, in plants. In the conventional iP riboside 5′-monophosphate (iPRMP)-dependent pathway, dimethylallyl diphosphate (DMAPP) is used as the side chain precursor for iPRMP, which is then converted to tZ riboside 5′-monophosphate (tZRMP) by P450 monooxygenases (8) (CYP735A1 and CYP735A2 in *A. tumefaciens*; Fig. 1). The other proposed pathway is an iPRMP-independent bypass, in which an unidentified hydroxylated derivative of DMAPP is directly transferred to the adenine moiety (9). A candidate for this putative substrate is 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) (10), which has recently been shown to occur as an intermediate in the MEP pathway (11) (Fig. 1). However, the participation of HMBDP in CK biosynthesis has never been demonstrated in *planta*. It should also be noted that a fraction of tZ-type CKs would be produced in an iPRMP-independent manner through the isomerization of cis-zeatin (cZ)-type CKs, which are thought to be formed by the degradation of tRNA containing cZ-type prenylation (12), but the extent to which this occurs in *Arabidopsis* is unknown.

Tmr is an IPT that is encoded on the transferred-DNA (T-DNA) region of the Ti plasmid of **Agrobacterium tumefaciens**. Therefore, this bacterial enzyme functions in plant cells upon infection. Previous reports indicated that engineered expression of Tmr in plants elevated the synthesis of tZ-type CKs without a substantial increase in the production of iP derivatives (9, 13, 14). These observations imply that Tmr may be involved in the iPRMP-independent synthesis of tZ by using HMBDP in infected plant cells. In this model, however, it is unclear how Tmr recruits HMBDP as a substrate from the plastid-specific MEP pathway, because this bacterial protein does not have an apparent plastid-targeting sequence (see Fig. 6, which is published as supporting information on the PNAS web site).

To address how Tmr modifies CK biosynthesis in the host, we investigated the substrate preference and subcellular localization of Tmr in plant cells. Evidence is provided that Tmr is targeted to the plastid of infected plant cells and uses HMBDP in the MEP pathway as a major substrate. The plastid localization of Tmr explains how this bacterial enzyme utilizes the substrate in the plastid-localized isoprenoid pathway, but is surprising because it is a bacterial protein and does not have any apparent organelle-targeting sequence of plants.

**Materials and Methods**

**Plant Materials.** *Arabidopsis thaliana* ecotype Columbia was used in this study. A crown gall cell line of periwinkle V208 (*Catharanthus roseus* G. Don) (15) was cultured in hormone-free Murashige-Skoog (MS) liquid medium (16) supplemented with 3% sucrose. A wild-type cell line of periwinkle CRA was cultured in MS liquid medium supplemented with 3% sucrose and 0.5 µg/ml 2,4-dichlorophenoxyacetic acid. Both cell lines

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Abbreviations: CK, cytokinin; DMAPP, dimethylallyl diphosphate; DX, 1-deoxy-o-xylulose; HMBDP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; iP, isopentenyladenine; iPRMP, isopentenyladenine riboside 5′-monophosphate; IPT, adenosine phosphates-isopentenyltransferase; KC, 5-ketoclamazone; MEP, methylerythritol phosphate; MVA, mevalonate; tZ, trans-zeatin; tZRMP, trans-zeatin riboside 5′-monophosphate.

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Chemicals. HMBDP was synthesized by Wako Pure Chemical (Osaka). [1-13C]1-deoxy-d-xylulose (DX) (99% labeled) was prepared as reported (4).

Synthesis of [3,4-18O]DX. [3,4,18O2]DX (95% atom-labeled) was synthesized as reported (17) by using H318O (95% atom-labeled) for Sharpless asymmetric dihydroxylation of 1-benzyloxy-2(E)-penten-4-one. The structure of [3,4,18O2]DX was confirmed by its 1H and 13C NMR spectral data (18). To determine the 18O-incorporation into [3,4,18O2]DX, authentic DX and [3,4,18O2]DX were analyzed by gas chromatography-mass spectrometry after conversion to trimethylsilyl (TMS) derivatives as described (19). [3,4-18O2]DX-TMS derivative was as follows: mass spectrum (EI, 70 eV) m/z: 307 (M-43+, 20%), 218 (100), 207 (50), 147 (70), 119 (40). DX-TMS derivative was as follows: mass spectrum (EI, 70 eV) m/z: 307 (M-43+, 20%), 218 (100), 205 (50), 147 (85), 117 (40).

Synthesis of 5-Ketoclomazone (KC). KC (20) was synthesized from 2-chlorophenylmethylhydroxylamine (21). To a solution of 2-chlorophenylmethylhydroxylamine (788 mg, 5 mmol) and triethylamine (1.01 g, 10 mmol) in CH2Cl2 (10 ml) was added 2-chlorophenylmethylhydroxylamine (21). To a solution of its 1H and 13C NMR spectral data (18). To determine the 1H NMR data of KC was identical with those of authentic sample (22).

Recombinant Enzymes. The coding region of Tmr from pTi-SAKURA (23) was ligated into pQE60 (Qiagen, Valencia, CA) to express the His-tagged recombinant proteins. Detailed procedures for preparation of the recombinant proteins have been described (6).

Transgenic Arabidopsis-Expressing IPT. The coding region of Tmr and AtIPTs was ligated into the pTA7001 vector (24) to yield a construct containing the dexamethasone-inducible IPT gene (pTA-Tmr, pTA-AtIPT1, pTA-AtIPT2, pTA-AtIPT3, pTA-AtIPT4, pTA-AtIPT5, or pTA-AtIPT7). The chimeric genes were introduced into Arabidopsis by the floral dip method (25). The T3 homozygous progeny were used for all experiments.

Fig. 1. Schematic representation of isotope labeling of CK biosynthesis through the MEP pathway. Red arrows indicate the possible metabolism of [1-13C]DX (classical pathway). Blue arrows show possible conversions of [3,4-18O2]DX through HMBDP (IPRMP-independent pathway) into Cks. Dashed arrows denote multiple metabolic steps. 5-Ketoclomazone (KC) was included in all incubations to inhibit the endogenous MEP pathway. PYR, pyruvate; GAP, glyceraldehyde 3-phosphate; P, monophosphoric; PP, diphosphoric acid. CYP735A1 and CYP735A2 are cytochrome P450 monoxygenases in Arabidopsis (8).

In Vivo Labeling Experiments. Seedlings of transgenic Arabidopsis plants were grown for 5 days on Murashige-Skoog (MS)-agar media and then transferred to MS liquid media (15 ml) containing KC (1 μM; added as a 15-μl methanol solution) and isotope-labeled DX (0.8 mM). Ten days later, seedlings were treated with 30 μM dexamethasone for 24 h, then harvested for CK analysis. For in vivo labeling, V208 and CRA cells were cultured with KC (1 μM) and isotope-labeled DX (0.8 mM) in a 100-ml flask for 1 week. Wild-type strains of A. tumefaciens MAFF302307 and MAFF302376 (native to Japan) were obtained from Ministry of Agriculture, Forestry and Fisheries GenBank at the National Institute of Agrobiological Sciences (Tsukuba, Japan). Colonies of wild-type Agrobacterium were inoculated onto wounded stem tissues of tomato (Lycopersicon esculentum ‘Alisa Craig’) plants. When crown galls formed (4–6 weeks after incubation), they were injected with a mixture of 3 μl of KC (1 μM) and 30 μl of isotope-labeled DX (0.8 mM) by using a syringe. Galls were harvested 3 days after the injection for CK analysis. Extraction, purification, and analysis of Cks were performed as described (4). The 13C and 18O incorporation levels were calculated by using the molecular ion cluster ([M+H]+ and +1 to +4 isotopomers) after subtraction of natural 13C abundance (26).

CK Analysis. Extraction and determination of Cks from transgenic Arabidopsis lines overexpressing IPTs were performed as described (27).

Particle Bombardment. Full-length or partial coding regions of Tmr were fused to the amino terminus of the GFP gene, which was controlled by the cauliflower mosaic virus 35S promoter (35S–sGFP [S65T]) (28). As the control markers for plastids and mitochondria, signal peptides of Arabidopsis geranylgeranyl diphosphate synthase 1 and 6 (GGPS1 and GGPS6, respectively) (29) were fused to the amino terminus of GFP gene driven by the 35S promoter. The DNA constructs were introduced into roots of 2-week-old Arabidopsis seedlings by particle bombardment (PDU-1000/He, Bio-Rad). Transient expression was observed by using laser confocal-scanning fluorescence microscopy after overnight incubation (Fluoview IX5, Olympus, Melville, NY).

Western Analysis. About 400 mg of seedlings were homogenized in two volumes of ice-cold extraction buffer (50 mM Tris-HCl,
150 mM NaCl, pH 7.5). Intact plastids were isolated from Arabidopsis seedlings and periwinkle cultured cells as described (30, 31). The proteins were subjected to SDS/PAGE followed by Western blotting.

Results

Substrate Preference of IPTs. To investigate how the Agrobacterium Tmr modifies CK biosynthesis in plant cells, we first determined the \( K_m \) values of Tmr for DMAPP and HMBDP by using the recombinant protein produced in Escherichia coli. Table 1 shows that Tmr transferred both DMAPP and HMBDP to AMP with similar \( K_m \) values. This result suggests that Tmr may function in both the iPRMP-dependent and -independent pathways in Agrobacterium-infected plant cells. Unlike Tmr, recombinant AtIPT1 (a plastidic Arabidopsis IPT) did not use HMBDP as a substrate in combination with any of adenosine 5’-phosphates (Table 1).

There are nine IPT-related sequences in the Arabidopsis genome (5, 6). Among them, AtIPT1, -3, -4, -5, -7, and -8 are shown in Tables 2 and 3. gFW, g fresh weight.

In Vivo Labeling Experiments. Although HMBDP has been a likely intermediate in the iPRMP-independent pathway and it does serve as a substrate for Tmr (Fig. 1, Fig. 2, and Table 1), there is no direct evidence that HMBDP is a precursor for CKs in any organism in vivo. To address this problem, we designed in vivo tracer experiments using [3,4-\(^{13}C\)]DX and [1-\(^{13}C\)]DX (Fig. 1). From [3,4-\(^{13}C\)]DX, \(^{13}C\)O would be incorporated through the MEP pathway into HMBDP, but not into DMAPP (Fig. 1; see Fig. 7, which is published as supporting information on the PNAS web site). On the other hand, [1-\(^{13}C\)]DX would label both HMBDP and DMAPP with \(^{13}C\) (Fig. 1). Each of these labeled precursors was fed to Arabidopsis seedlings in the presence of KC, which blocks the endogenous MEP pathway (20, 35), to allow efficient labeling (Fig. 1).

To examine whether Tmr uses HMBDP in planta, we fed labeled DX to transgenic Arabidopsis plants expressing Tmr under the control of a dexamethasone-inducible promoter. Liquid chromatography–mass spectrometry analysis demonstrated that \(^{13}C\)O was incorporated into \( T \)-type CKs from [3,4-\(^{13}C\)]DX when Tmr was overexpressed in Arabidopsis seedlings (Fig. 34; see also Fig. 8, which is published as supporting information on the PNAS web site). However, only \(^{13}C\) from [1-\(^{13}C\)]DX, but not \(^{13}C\)O from [3,4-\(^{13}C\)]DX, was incorporated into \( T \)-derivatives without Tmr expression (Figs. 34 and 8). These results illustrate that Tmr uses HMBDP as a substrate in Arabidopsis seedlings, whereas this bypass does not occur at a detectable level without the engineered induction of Tmr in our experimental conditions. To further confirm this notion, we carried out in vivo labeling experiments using transgenic Arabi-
dipsis plants overexpressing AtIPT1. As was the case with wild-type plants, only 13C from [1-13C]DX, but not 18O from [3-18O2]DX, was incorporated into tZRMP in Arabidopsis seedlings with (pTA-Tmr) or without (pTA) overexpression of Tmr. (B) Incorporation of [3-18O2]DX or [1-13C]DX into tZRMP in Arabidopsis seedlings with (pTA-AtIPT1) or without (pTA) overexpression of AtIPT1. (C) Incorporation of [3,4-18O2]DX into tZRMP in cultured periwinkle cell lines V208 (crown gall) and CRA (wild-type). (D) A crown gall induced by inoculation of wild-type Agrobacterium on a tomato plant. [3,4-18O2]DX and KC were injected into the gall, and the 18O incorporation into tZRMP was analyzed qualitatively (see In Vivo Labeling Experiments). Values in A and C are means with SD (n = 3).

Subcellular Localization of Tmr in Arabidopsis. Because HMBDP is a metabolic intermediate in the MEP pathway, Tmr should exist in plastids to use HMBDP as a substrate. To determine the subcellular localization of Tmr, a series of Tmr-GFP fusion genes were transiently expressed in Arabidopsis root cells by particle bombardment. (Scale bar: 20 μm.) (K) Schematic representation of the fusion constructs. (K) Western blot analysis of total leaf proteins (Total) and the chloroplast fraction (Chl.) (equivalent to 1.3 μg of chlorophyll) using antibodies against Tmr or glutamine synthetase (GS). Proteins were extracted from transgenic Arabidopsis seedlings harboring pTA-Tmr with (+) or without (−) dexamethasone (DEX) treatment for 24 h. GS, and GS2, cytosolic and plastidic GS, respectively.

Fig. 3. Incorporation of stable radioisotope-labeled DX into CKs. (A) Incorporation of [1-13C]DX or [3-18O2]DX into tZRMP in Arabidopsis seedlings with (pTA-Tmr) or without (pTA) overexpression of Tmr. (B) Incorporation of [3,4-18O2]DX or [1-13C]DX into tZRMP in Arabidopsis seedlings with (pTA-AtIPT1) or without (pTA) overexpression of AtIPT1. (C) Incorporation of [3,4-18O2]DX into tZRMP in cultured periwinkle cell lines V208 (crown gall) and CRA (wild-type). (D) A crown gall induced by inoculation of wild-type Agrobacterium on a tomato plant. [3,4-18O2]DX and KC were injected into the gall, and the 18O incorporation into tZRMP was analyzed qualitatively (see In Vivo Labeling Experiments). Values in A and C are means with SD (n = 3).
igenesis, is carefully regulated. Recent work has indicated that and CK, both of which are targets of promote tumorigenesis. In uninfected plants, the ratio of auxin biosynthesis by sending a key enzyme into plastids to which destroys the organelle membrane structure (Fig. 5).

In this study, we have demonstrated that Tmr is localized to the stroma of plastids (Fig. 5A). These data indicate that Tmr is localized to the stroma of plastids in crown gall cells.

**Subcellular Localization of Tmr in Crown Gall Cells.** To examine whether Tmr is localized to plastids in crown galls, intact plastids were purified from the cell line V208 and CRA of periwinkle. Tmr was enriched in the plastid fraction relative to the total cell extract, as was the case with ferredoxin used as a plastid marker (Fig. 5A). We confirmed that marker proteins for the cytosol (phosphoenolpyruvate carboxylase), endoplasmic reticulum marker, BiP, binding protein, and mitochondria (mitochondrial aspartate aminotransferase, a mitochondrial marker) were not enriched in the plastid fraction (Fig. 5A). To assess organelle localization of Tmr, we found that Tmr is localized to the stroma of plastids in crown gall cells.

**Discussion**

In this study, we have demonstrated that Agrobacterium modifies CK biosynthesis by sending a key enzyme into plastids to promote tumorigenesis. In uninfected plants, the ratio of auxin and CK, both of which are targets of Agrobacterium for tumorigenesis, is carefully regulated. Recent work has indicated that expression of CYP735A genes is negatively regulated by auxin (8), which suggests that auxin limits the level of tZ in plants. Because elevated synthesis of both auxin and CK is essential for tumorigenesis, the use of HMBDP for tZ biosynthesis without the catalysis by CYP735As may play an important role in circumventing the hormonal homeostasis in plants. Although tZ has generally been thought as the most active CK (38, 39), it has not been entirely clear whether only tZ is active in inducing the tumor formation or whether iP also acts as an active form. Recently, we found that Arabidopsis plants do not grow normally when CYP735A genes are knocked out (K.T. and H.S., unpublished results), suggesting that tZ plays a distinguishable role from iP in plant development, and perhaps in tumorigenesis.

Although Tmr uses both DMAAPP and HMBDP with similar Kl values in vitro (Table 1), isoform tracer experiments indicated that the major substrate for Tmr was HMBDP, but not DMAAPP, in crown gall cells (Fig. 3C). Because HMBDP reductase produces isopentenyl diphosphate and DMAAPP at a ratio of 5:1 (40), the available pool size of DMAAPP for ITPs may be relatively smaller than that of HMBDP in plastids. This finding would explain why tZ-type CKs are predominantly produced in Agrobacterium-infected plant cells.

The iPRMP-independent pathway has initially been proposed in Tmr-overexpressing Arabidopsis plants, based on biased synthetic rates between iPRMP and tZRMP by using 1H2O/[3H]isopentyladenyl riboside double tracers (9). In this pioneer work, it was indicated that the iPRMP-independent synthesis of tZRMP occurs also in wild-type Arabidopsis seedlings, when hydroxylation of iPRMP was blocked by metyrapone, an inhibitor for CYP735As (9). In the present study, the incorporation of [3,4-18O2]DX into tZ-type CKs depended on expression of Tmr; the majority of tZ derivatives were labeled with 13C from [1-13C]DX, but not with 18O from [3,4-18O2]DX, in control (no Tmr expression) and AtIPT1-overexpressing Arabidopsis plants (Fig. 3A and B). In addition, only Tmr, but none of AtIPTs, caused dominant accumulation of tZ-type CK when overproduced in Arabidopsis (Fig. 2). These results indicate that the major isoprenoid precursor for tZ biosynthesis is DMAAPP, but not HMBDP, in wild-type Arabidopsis seedlings, but that the HMBDP bypass through the MEP pathway is newly created upon expression of Tmr. In this context, the identification of the iPRMP-independent tZRMP synthesis in metyrapone-treated wild-type Arabidopsis (9, 41) is unexplained; it was suggested that the isoprenoid moiety of tZRMP in treated plants came from the MVA pathway. We speculate that a fraction of tZ-type CKs in wild-type Arabidopsis might be synthesized in an iPRMP-independent manner by means of cis-trans isomerization of cis-zeatin derivatives, of which the prenyl side chain is primarily derived from the MVA pathway in Arabidopsis seedlings (9). However, the occurrence of the cis-trans isomerization of zeatin has not been demonstrated in Arabidopsis. It is therefore premature to rule out the presence of the HMBDP pathway in metyrapone-treated wild-type Arabidopsis.

The plastid targeting of Tmr is likely to be achieved by the host import machinery, because Tmr on its own was delivered to this organelle in the absence of any other Agrobacterium protein (Fig. 4). How Tmr moves into the organelle without an apparent plastid-import sequence requires further investigation. It is intriguing that the bacterium protein Tmr functions in the plastid, an organelle that has symbiotically evolved from a bacterial ancestor. The MEP pathway is widely found in prokaryotic systems (3), including Agrobacterium, suggesting that Tmr has coevolved with the MEP pathway, but not with the MVA pathway. It seems a logical strategy that Tmr produced in the plant cell is designed to function in the plastid, which provides the key substrate HMBDP through the prokaryotic isoprenoid pathway.
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