Arabidopsis CYP735A1 and CYP735A2 Encode Cytokinin Hydrolases That Catalyze the Biosynthesis of trans-Zeatin*

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Cytokinins (CKs), a group of phytohormones, are adenine derivatives that carry either an isoprene-derived or an aromatic side chain at the N6 terminus. trans-Zeatin (tZ), an isoprenoid CK, is assumed to play a central physiological role because of its general occurrence and high activity in bioassays. Although hydroxylation of isopentenyladenine-type CKs is a key step of tZ biosynthesis, the catalyzing enzyme has not been characterized yet. Here we demonstrate that CYP735A1 and CYP735A2 are cytochrome P450 monooxygenases (P450s) that catalyze the biosynthesis of tZ. We identified the genes from Arabidopsis using an adenosine phosphate-isopentenyltransferase (AtIPT) P450 expression system in yeast. Co-expression of AtIPT4 and CYP735A1 enabled yeast to excrete tZ and the nucleosides to the culture medium. In vitro, both CYP735A1 and CYP735A2 preferentially utilized isopentenyladenine nucleotides rather than the nucleoside and free base forms and produced tZ nucleotides but not the cis-isomer. The expression of CYP735A1 and CYP735A2 was differentially regulated in terms of organ specificity and response to CK. Root-specific induction of CYP735A2 expression by CK suggests that the trans-hydroxylation is involved in the regulation of CK metabolism and signaling in roots.

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TZS, an agrobacterial IPT (14). However, HMBDP was identified as a metabolic intermediate of the methylerythritol phosphate ( MEP) pathway (15), which occurs in bacteria and plastids (16). Selective labeling experiments using 13C-labeled precursors specific for either the MEP or MVA pathway demonstrated that the isoprenoid side chain of iP and tZ predominantly originates from the MEP pathway, whereas a large fraction of the cZ side chain is derived from the MVA pathway (17). However, the utilizing efficiency of Arabidopsis IPT was much lower for HMBDP than for dimethylallyl diphosphate in vitro (18). Thus, the physiological significance of the iPRMP-independent pathway remains obscure to date. Although several routes might contribute to tZ production in higher plants, the physiological importance and functional differentiation of the possible pathways is unclear. As a first step toward an understanding of the trans-hydroxylation step of iP-type CKs in the iPRMP-dependent pathway, we attempted to identify CK hydroxylase genes in Arabidopsis. Here we demonstrate that CYP735A encode CK trans-hydroxylases that catalyze the biosynthesis of tZ and discuss the physiological role of the trans-hydroxylation in the regulation of CK metabolism and signaling.
trans-Zeatin Biosynthesis in Arabidopsis

**Table I**

| Plasmids | CK levels |
|----------|-----------|
|          | iP | iPR | tZ | tZR | cZ | cZR | DZ | DZR |
| pYES-lacZ/Esc-ATR1-IPT4* | 8.26 | 3.80 | ND | ND | ND | ND | ND | ND |
| pYES-CYP735A1/pESC-ATR1-IPT4 | 3.71 | 1.75 | 3.46 | 2.07 | 0.02 | 0.01 | 1.89 | 1.71 |
| pYES-CYP735A2/pESC-ATR1-IPT4 | 5.91 | 2.91 | 2.85 | 1.32 | 0.06 | 0.02 | 0.71 | 0.51 |

* Control plasmid.

**Expression vectors for CYP735A1 or CYP735A2, respectively.

ZQ2000MS; Waters) on a reverse-phase column (Symmetry C18, 5 μm, 2.1 mm × 150 mm; Waters) at a flow rate of 0.25 ml/min in 0.1% acetic acid with a linear gradient of methanol (0% for 1 min, 0–50% for 15 min, and 50–70% for 6 min). CKs were quantified in the selected ion recording mode. Monitored ions for each CK species were as follows: m/z 220 and 352 for tZR and cZ riboside, m/z 136 and 220 for Tz and cZ, m/z 222 and 354 for DZ riboside (DZR), m/z 136 and 222 for DZ, m/z 204 and 336 for iP, m/z 136 and 204 for iP, and m/z 357 for [2H3]tZR. Other conditions for MS analysis were as described previously (8).

Preparation of Microsomal Fraction—Yeast cells harboring pESC-ATR1 and pYES-CYP735As were cultured in 500 ml of medium under inductive conditions as described above. The microsomal fraction was prepared by the method of Venkateswarlu et al. (21), except that the buffer A did not contain reduced glutathione. After ultracentrifugation, the pellet was further washed once with the buffer A. The final preparation of microsomal pellet was resuspended in the buffer A. Protein concentration was determined with protein assay (Bio-Rad) using bovine serum albumin as the standard. The P450 content was estimated by the method of Omura and Sato (22).

Synthesis of iP Nucleotides—iPRMP, iPRDP, and iPRTP were synthesized enzymatically with IPTs (AtIPT1 and TZS) from dimethylallyl diphosphate and with AMP, ADP, and ATP, respectively, and purified by anion exchange chromatography using a MonoQ column as described previously (8). iPRMP was purchased from Apex Organics (Devon, UK).

**Enzyme Assay**—Microsomal proteins were incubated in 40 μl of reaction mixture (100 mM sodium phosphate, 10% sucrose, iP-type CK, 3 mM NADPH, 1 mg/ml bovine serum albumin, pH 7.5) at 20 °C (model 600/717plus/PDA996; Waters) as described previously (23). The reaction mixture (100 mM sodium phosphate, 10% sucrose, iP-type CK, 3 mM NADPH, 1 mg/ml bovine serum albumin, pH 7.5) at 20 °C (model 600/717plus/PDA996; Waters) as described previously (23). The reaction mixture was dried and redissolved in 50% for 15 min, then diluted to 500. The capillary voltage was 4 kV. The supernatant was passed through a reverse-phase column (Oasis HLB, 10 mg/1 cc; Waters) pre-equilibrated with 1 ml of 20% acetic acid. After centrifugation, the supernatant was further washed once with the buffer A. The final preparation of microsomal pellet was resuspended in the buffer A. Protein concentration was determined with protein assay (Bio-Rad) using bovine serum albumin as the standard. The P450 content was estimated by the method of Omura and Sato (22).

**RESULTS**

**Isolation of Arabidopsis P450 Genes Involved in tZ Biosynthesis Using the AtIPT4/P450 Co-expression System**—To identify gene(s) that catalyze tZ biosynthesis, we designed a screening method using an AtIPT4/P450 co-expression system in yeast. When AtIPT4, which catalyzes an initial step of the biosynthesis of iP nucleotides, was expressed in yeast, iP and iPR but not tZ and tZR were excreted into the culture medium (Table I, pYES-lacZ/Esc-ATR1-IPT4). Thus, AtIPT4 can synthesize iP nucleotides in yeast cells, whereas authentic yeast enzymes transform iP nucleotides into the corresponding nucleosides and free bases. However, the hydroxylation activity required for tZ production is lacking. We reasoned that if the appropriate P450 were co-expressed with AtIPT4 in yeast, tZ-type CKs could be expected to be synthesized. To construct such cell lines, we used two expression vectors, one carrying AtIPT4 and the other carrying one of a series of Arabidopsis P450s. To gain electrophoretic efficiency from NADPH to the plant P450s in yeast, an Arabidopsis NADPH-P450 reductase ATR1 was also expressed in the yeast cells in all cases (19).

**The Arabidopsis genome contains more than 270 genes for P450s (Refs. 26–28; arabidopsis-p450.biotech.uu.nl; biobase.e.dk/P450/p450.shtml). For further experiments, we chose genes that appeared closely related to CK metabolism, either because they encoded homologues of P450s known to catalyze the trans-hydroxylation of the terminal-methyl group in isoprenoid compounds (A12g45560, A12g45580, A12g45550, and A13g52997) (29), because they were regulated by CKs (At1g-67110) (30) or because they were induced in a CK-overproducing mutant (At1g13710) (31). Their homologues (At5g38450, At2g46960, At2g46950, At4g27710, At5g09970, At1g01190, At1g26180, and At1g17410) and some others (At1g16410, At1g16400, At3g26220, At5g36220, At4g37310, At4g00360, At2g27690, At2g23180, and At2g34500) were also chosen. We expressed these P450s in the yeast system and found that the supernatants of cultures expressing CYP735A1 (At1g13710) and CYP735A2 (At1g67110) contained tZ and tZR at similar levels as iP and iPR (Table I). Significant amounts of DZ and DZR and traces of cZ and cZ riboside were also detected in both culture media (Table I).

**CYP735A1 and CYP735A2** (formerly named CYP709A1 and CYP709A2, respectively) are members of the CYP735A subfamily in Arabidopsis and share 79% identity at the amino acid level. CYP735A2 was first described as a CK-inducible gene by a DNA microarray analysis (30). In a phylogenetic tree of Arabidopsis P450 genes, the CYP709B subfamily was a sister

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group of the CYP735A subfamily (biobase.dk/P450/p450.shtml). It consisted of three genes, CYP709B1 (At2g46960), CYP709B2 (At2g46950), and CYP709B3 (At4g27710), which showed 35–40% identity with the CYP735A at the amino acid level. We tested the ability of the CYP709B to hydroxylate CKs in the yeast system, but no secretion of tZ-type CKs was detected (data not shown).

CYP735A1 and CYP735A2 Utilize iP Nucleotides as Substrates—Microsomal fractions prepared from yeast co-expressing ATR1 and CYP735A1 or CYP735A2 were used to measure the enzymatic activity in vitro. When iP nucleotides were used as substrates, efficient conversion to tZ-type CKs was detected (Fig. 2A). iPRMP and iPRDP were processed more efficiently than iPRTP by both CYP735As. The conversion efficiency of iP and iPR was less than 10% of that of iPRTP (Fig. 2A). These results strongly suggest that the CYP735As function as iP nucleotide hydroxylases in vivo.

Although the activity was almost abolished when NADPH was absent from the reaction mixture, the presence of NADH partially restore the activity (Table II). This suggests that the reaction was not completely dependent on NADPH-P450 reductase, and NADH could act as an electron donor probably via other reduction systems such as a NADH-cytochrome b5 reductase/cytochrome b5 system (32). The substantial inhibition of the reaction by metyrapone was coincided with the sensitivity to this compound of the CK hydroxylase detected in the microsomal fraction of cauliflower (13). CYP735A1 was more sensitive to metyrapone than CYP735A2 (Table II).

In our routine method of measuring P450 activity, we dephosphorylated the primary products with phosphatase and monitored the nucleosides because of the difficulties to separate nucleotides on reverse-phase columns and the low sensitivity for nucleotides of our MS analysis. To confirm the nature of the primary product, the reaction products generated by CYP735A1 with iPRMP as a substrate were prepared on a large scale and were purified. The retention time determined by HPLC (Fig. 2B) and the spectrum obtained by LC-MS (Fig. 2C) showed that the primary product was iZRMP. We obtained a similar result with CYP735A2 (data not shown).

CYP735A1 and CYP735A2 Catalyze trans-Hydroxylation in Vitro—In the culture medium of yeasts co-expressing AtIPT/ CYP735A, tZ-type and DZ-type CKs were also detected (Table I). To establish whether CYP735As catalyze the formation of cis-isomers and/or the reduction of the tZ side chain in vitro, we analyzed the reaction products by LC-MS. When iPRMP was used as substrate, both CYP735As mainly produced the trans-isomer; production of the cis-isomer occurred at a negligible level (Fig. 3, m/z 352). Although a small peak was detected at m/z 354, it was an unrelated peak because the retention time of the peak (13.3 min) did not coincide with that of the DZR standard (13.1 min; Fig. 3, m/z 354). When iZRMP was incubated with total soluble proteins prepared from the yeast transformants, production of DZR 5′-monophosphate was detected. However, this conversion was not CYP735A-dependent (data not shown). We concluded that the production of tZ- and DZ-type CKs in the yeast expression system was due to endogenous enzymatic activities of the yeast cells and that CYP735As catalyze the stereo-specific formation of tZ nucleotides.

Kinetic Parameters of CYP735A1 and CYP735A2—The contents of P450s in the microsomes were estimated to be 0.062 nmol/mg of microsomal protein for CYP735A1 by analysis of CO difference spectra (Fig. 4). The content of CYP735A2 could not be determined because of its low expression level in yeast (data not shown). We determined the kinetic parameters of CYP735A1 and CYP735A2 for the iP nucleotides (Table III). Of all substrates tested, the Km values of CYP735A1 and CYP735A2 for iPRMP were the smallest, whereas those for iPRTP were the largest. CYP735A2 exhibited higher substrate affinities than CYP735A1 in all cases. The Km values for iP and iPR could not be determined because of the low activities with these substrates. Furthermore, comparisons of the catalytic
for DZR (\(10\) pmol of each compound were used as standard). The reactions strongly suggested that CYP735A1 and CYP735A2 were incubated in water or iP, and accumulation of the CYP735A1 transcript was transient; it reached a maximum after 30–60 min and decreased to the initial level after 4 h (data not shown). In both genes, a strong enhancement of mRNA accumulation was also observed when tZ instead of iP was applied (Fig. 6). CYP735A1 

DISCUSSION

In this study, we demonstrated that the Arabidopsis CYP735A1 and CYP735A2 encode CK hydroxylases that catalyze tZ biosynthesis via the iPMP-dependent pathway. In vitro analysis revealed that the stereo-specific trans-hydroxylation occurs at the nucleotide level. So far, hydroxylating activities of iP-type CKs have been examined only at the base (iP) and nucleoside (iPR) levels in microsomal fractions of cauliflower (13). Therefore it is uncertain whether the cauliflower enzymes also act on nucleotides. We attempted to determine CK hydroxylase activity in microsomal fractions prepared from Arabidopsis roots with various iP-type CKs but could not detect any. This was probably due to the low level of CK hydroxylase in the preparation (data not shown).

Although CK bases and nucleosides are regarded as the physiologically active form and the translocation form, respectively (3), the role of the nucleotides remains to be clarified. If the conversion to tZ-type CKs is a nucleotide-specific process in planta, one of the roles of the CK nucleotides might be to form a metabolic pool for the side chain hydroxylation. iP nucleotides are primary products of the CK biosynthetic reaction (6, 7), indicating that hydroxylation occurs at an early stage of CK metabolism. Such a metabolic hierarchy might be important to maintain the homeostasis between iP- and tZ-type CKs.

The stereo specificity of the tZ formation catalyzed by CYP735As indicates that an unknown system must be involved in cis-hydroxylation. This is also supported by analyses using \(^{13}\)C-labeled precursors specific to the MEP or MVA pathways, which demonstrated distinct isoprenoid sources in the biosyntheses of tZ and cZ (17). At present, it is thought that the hydroxylation of the prenyl moiety of iP residues that are contained in some tRNA species, and their subsequent release by tRNA degradation is a major pathway for cZ biosynthesis (35) (Fig. 1). miaE is a cZ biosynthesis gene in Salmonella typhimurium (36); so far, no miaE homologues have been found in plant genomes.

The fact that CYP735As and IPTs are differentially regulated by CKs and auxin is intriguing. In Arabidopsis, the ac-
and CYP735A2

AtIPT5

accumulation of the transcripts of CYP735A1 iP. After 1 h, total RNA was extracted, and the accumulation of transcripts of CYP735A1 iP. After 1 h, total RNA was extracted, and the accumulation of tran

plants grown for 4 weeks were separated and treated with water or 5 μM of various phytohormones, and roots were harvested after 1 h. Accumulation of transcripts of CYP735A1, CYP735A2, ARRE5 (CK-responsive control), and UBQ10 (CK-insensitive control) was analyzed by quantitative real time PCR. The data shown are the means with S.D. (n = 3).

Very recently, it has been reported that auxin also negatively regulated tZ biosynthesis via the iPRMP-independent pathway (39). Treatment of Arabidopsis seedlings with 1-naphthalenacetic acid greatly reduced the accumulation of tZRMP but not that of iPRMP (39). Although biochemical nature of the iPRMP-independent pathway has not been elucidated, these lines of evidence suggest that auxin is an important factor that regulates the both iPRMP-dependent and iPRMP-independent pathways.

Little is known about the physiological significance of CK side chain hydroxylation. In other phytohormones, hydroxylation reactions catalyzed by P450s participate in biosynthesis (40–43), inactivation (44), and catabolism (45, 46). Hydroxylation is not essential for CK catabolism because CKX cleaves side chains from iP as well as tZ (47) (Fig. 1). The modification might be involved in reversible inactivations of CKs by CK O-glucosyltransferases (3, 48). The O-glucosides are biologically inactive, insensitive to CKX, and reversibly converted to the active form by β-glucosidase (49) (Fig. 1). CK-dependent induction of CYP735A expression (Figs. 5 and 6) might cause a change in the metabolic flow that leads to storage of excess CKs in an inactive form. It would be interesting to see whether genes for the O-glucosyltransferases are also regulated by CKs.

The trans-hydroxylation might be involved also in the regulation of the long range translocation of CKs from roots to shoots via the xylem. This transport plays a role in signaling of nitrogen nutrition (23), adventitious root formation (50), and shoot branching (51). tZ-type CKs such as tZR are the dominant CKs in xylem exudates (23, 52). Therefore, predominant expression of CYP735As in roots and root-specific induction of CYP735A2 by CK (Fig. 5) appears consistent with the idea. On the other hand, leaf exudates contained mainly iP-type CKs (53). Thus, trans-hydroxylation may be important for the compartmentalization of the CK species and may control the direction of translocation.

| Enzyme | Substrate | $K_m$ μM | $V_{max}$ nmol/min/mg protein | $k_{cat}$ min$^{-1}$ | $k_{cat}/K_m$ s$^{-1}$ μM$^{-1}$ |
|--------|-----------|---------|-----------------------------|-------------------|-------------------|
| CYP735A1 | iPRMP | 1.76 ± 0.25$^b$ | 2.16 ± 0.09$^b$ | 34.8 ± 1.4 | 2.3 × 10$^3$ |
| CYP735A1 | iPRDP | 2.28 ± 0.17 | 1.44 ± 0.04 | 23.2 ± 0.6 | 7.5 × 10$^2$ |
| CYP735A1 | iPRTP | 8.32 ± 0.26 | 0.586 ± 0.010 | 9.3 ± 0.2 | 2.5 × 10$^{-1}$ |
| CYP735A2 | iPRMP | 0.39 ± 0.03 | 0.087 ± 0.007 | ND | ND |
| CYP735A2 | iPRDP | 0.76 ± 0.08 | 0.109 ± 0.004 | ND | ND |
| CYP735A2 | iPRTP | 4.9 ± 0.9 | 0.051 ± 0.010 | ND | ND |

$^a$ Microsomal fractions were prepared from yeasts harboring pESC-ATR1/pYES-CYP735As.

$^b$ The concentration of iPRMP, iPRDP, or iPRTP in the assays was varied from 0.05 to 10, 0.05 to 20, or 0.5 to 20 μM, respectively.

$^c$ The amount of microsomal protein.

$^d$ ND, not determined.

**Fig. 5. Tissue specificity and CK responsiveness of CYP735A1 and CYP735A2 transcript accumulation.** Organs of Arabidopsis plants grown for 4 weeks were separated and treated with water or 5 μM iP. After 1 h, total RNA was extracted, and the accumulation of transcripts of CYP735A1, CYP735A2, ARRE5 (CK-responsive control), and UBQ10 (CK-insensitive control) was analyzed by quantitative real time PCR. The data shown are the means with S.D. (n = 3).

**Fig. 6. Expression of CYP735A1 and CYP735A2 in roots treated with various phytohormones.** Arabidopsis seedlings grown for 12 days were sprayed with 5 μM of various phytohormones, and roots were harvested after 1 h. Accumulation of transcripts of CYP735A1, CYP735A2, and UBQ10 (as a control) were analyzed by quantitative real time PCR. The data shown are the means with S.D. (n = 3). Con, control (sprayed with 0.05% Me2SO); IAA, indole-3-acetic acid; ABA, abscisic acid; GA, gibberellic acid; BR, brassinolide. *, p < 0.05; **, p < 0.01; ***, p < 0.001 Student's t test.
trans-Zeatin Biosynthesis in Arabidopsis

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