Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis

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Edited by Enrico Coen, John Innes Centre, Norwich, United Kingdom, and approved September 14, 2006 (received for review April 29, 2006)

Cytokinins, which are central regulators of cell division and differentiation in plants, are adenine derivatives carrying an isopentenyl side chain that may be hydroxylated. Plants have two classes of isopentenyltransferases (IPTs) acting on the adenine moiety: ATP/ADP isopentenyltransferases (in *Arabidopsis thaliana*, *AtIPT1, 3, 4–8*) and tRNA IPTs (in *Arabidopsis*, *AtIPT2 and 9*). ATP/ADP IPTs are likely to be responsible for the bulk of cytokinin synthesis, whereas it is thought that *cis*-zeatin (*c*Z)-type cytokinins are produced possibly by degradation of *cis*-hydroxy isopentenyl tRNAs, which are formed by tRNA IPTs. However, these routes are largely hypothetical because of lack of *in vivo* evidence, because the critical experiment necessary to verify these routes, namely the production and analysis of mutants lacking *AtIPT*s, has not yet been described. We isolated null mutants for all members of the ATP/ADP IPT and tRNA IPT gene families in *Arabidopsis*. Notably, our work demonstrates that the *atipt1 3 5 7* quadruple mutant possesses severely decreased levels of isopentenyladenine and *trans*-zeatin (*t*Z), and their corresponding ribosides, ribotides, and glucosides, and is retarded in its growth. In contrast, these mutants possessed increased levels of *c*Z-type cytokinins. The *atipt2 9* double mutant, on the other hand, lacked isopentenyl- and *cis*-hydroxy isopentenyl-tRNA, and *c*Z-type cytokinins. These results indicate that whereas ATP/ADP IPTs are responsible for the bulk of isopentenyladenine- and *t*Z-type cytokinin synthesis, tRNA IPTs are required for *c*Z-type cytokinin production. This work clarifies the long-standing questions of the biosynthetic routes for isopentenyladenine-, *t*Z-, and *c*Z-type cytokinin production.

Since the discovery of cytokinins as inducers of plant cell division (1) and differentiation (2), they have been recognized as central regulators of plant development (3). Cytokinins also increase nutrient sink strength, delay senescence, stimulate outgrowth from lateral buds, and inhibit cell elongation (4). The important roles for cytokinins in cell division were verified by overexpression of genes for cytokinin-degrading enzymes (cytokinin oxidases, CKXs) (5–7) and by examination of single and higher-order cytokinin-receptor null mutants (8–10).

Most naturally occurring cytokinins are N^6-isopentenyladenine (iP) derivatives. iP carries an unmodified isopentenyl side chain, whereas *trans*-zeatin (*t*Z) and *cis*-zeatin (*c*Z) carry hydroxylated side chains. Cytokinins exist in free-base, riboside, and ribotide forms, with varying degrees of biological activity. Cytokinins also may be modified in several ways. For example, the *N7* and *N9* positions of the adenine moiety of cytokinins may be glucosylated to form *N*-glucosides. Alternatively, the hydroxyl group of *t*Z and *c*Z may be glucosylated or xylosylated to form zeatin-*O*-glucosides or zeatin-*O*-xylosides. *N*- and *O*-glycosides are biologically inactive (3).

Experimental evidence demonstrates that free-base cytokinins are biologically active. For example, iP (11) and *t*Z (12) were demonstrated to bind to the well characterized cytokinin receptor CRE1 and activate downstream cytokinin-dependent phosphorelay (11, 13–15). The riboside isopentenyladenosine (iPR) did not compete with radiolabeled iP for binding to a membrane fraction of *Schizosaccharomyces pombe*-expressing *CRE1* (11). Similarly, the glucosylated cytokinin, zeatin *O*-glucoside (ZOG), did not compete with radio-labeled *t*Z for binding to *E. coli* cells expressing *CRE1* (12). However, the riboside *trans*-zeatin riboside (*t*ZR) did compete with radiolabeled *t*Z for binding to *E. coli* cells expressing *CRE1*, albeit with less affinity than unlabeled *t*Z (12). In many plant species, *t*Z is more abundant and more active than *c*Z (16); however, in some species, *c*Z is an abundant and biologically active cytokinin (17, 18).

In *Dictyostelium discoideum*, *Agrobacterium tumefaciens*, and plant tissues transformed with *A. tumefaciens*, cytokinins are produced by isopentenylation of AMP. This reaction is catalyzed by adenylate isopentenyltransferases (AMP IPTs) (19–22). In plants, AtIPT4 isopentenylate ATP and ADP, forming isopentenyl ATP and isopentenyl ADP, using dimethylallyldiphosphate as the side-chain donor (23). AtIPT1 also could isopentenylate AMP (3), but the K_M for isopentenylation of AMP was significantly higher than that measured for ATP or ADP (24), indicating that isopentenylation of AMP is only a minor reaction carried out by the enzyme. The *Arabidopsis* genome encodes seven genes belonging to a plant-specific ATP/ADP IPT clade (*AtIPT1*, *AtIPT3*, *AtIPT4*, *AtIPT5*, *AtIPT6*, *AtIPT7*, and *AtIPT8*) (20, 23, 24). Overexpression of *AtIPT4* or *AtIPT8* confers cytokinin-independent shoot formation on calli (23, 25), and overexpression of *AtIPT1*, *3*, *4*, *5*, *7*, or *8* causes increased iP-type cytokinin levels *in planta* (25, 26). These results suggest that isopentenylation of ATP and ADP makes a major contribution to the control of cytokinin biosynthesis.

*t*Z ribotides are formed by the monooxygenase-catalyzed hydroxylation of the side chain of iP ribotides (27). Another pathway of *t*Z production, the iPR monophosphate-independent pathway, most likely involves transfer of the *trans*-hydroxylated isopentenyl moiety from 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate to adenosine phosphates, by the action of AtIPTs (28). However, this

Author contributions: S.T., G.S., and T. Kakimoto designed research; K.M., P.T., M.M.-K., T. Kato, D.T., and T. Kakimoto performed research; T. Kato, S.S., and S.T. contributed new reagents/analytic tools; K.M., P.T., M.M.-K., and T. Kakimoto analyzed data; and K.M., P.T., and T. Kakimoto wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviation: *c*Z, *cis*-zeatin; *c*ZR, *cis*-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenosine; *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside.

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www.pnas.org/cgi/doi/10.1073/pnas.0603522103

pathway has become less favored since the finding that AtIPT1 does not efficiently catalyze transfer of the *trans*-hydroxylated isopentenyl moiety from the likely precursor, 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate, to adenosine phosphates (26).

Each member of the ATP/ADP IPT gene family has a unique spatial expression pattern (29). Expression of *AtIPT3*, *5*, and *7* is relatively high in the vegetative organs. *AtIPT8* is expressed exclusively in reproductive organs, with highest expression in immature seeds. *AtIPT4* is expressed primarily in reproductive immature seeds. *AtIPT1* is expressed in ovules and vegetative organs. *AtIPT1*, *3*, *5*, and *7* are negatively regulated by cytokinins (29), whereas the effects of cytokinins on *AtIPT4*, *6*, and *8* have not been examined.

In tRNAs recognizing codons beginning with U, the adenine residue immediately 3′ to the anticodon may be modified to have the isopentenyl- or *cis*-hydroxy isopentenyl- side chain at the N^6 position (30). Therefore, it is possible that iP and *c*Z are produced when modified tRNAs are degraded. In bacteria, modified tRNAs are a source of low-level cytokinin production (31, 32); however, there is no experimental evidence for a tRNA-degradation route for *c*Z production in plants. tRNA IPTs catalyze isopentenylation of certain tRNAs in diverse organisms ranging from bacteria to animals and plants (33), including *Arabidopsis* (34). In the case of the bacterium *Salmonella typhimurium*, the isopentenyl chain of tRNA is hydroxylated at the cis position by the *miaE* gene product (35). It is unknown whether cis hydroxylation of isopentenylated tRNAs in plants occurs in a similar way. *Arabidopsis* has two genes (*AtIPT2* and *AtIPT9*) encoding products homologous to tRNA isopentenyltransferases (tRNA IPTs). Furthermore, *AtIPT2* has been shown to complement a yeast mutant deficient in tRNA isopentenyltransferase activities encoded by the *MOD5* gene product (34). *tRNA IPT* genes are ubiquitously expressed in *Arabidopsis* tissues (29).

As described above, isopentenylation of ATP and ADP, by ATP/ADP IPTs, is likely the key step in biosynthesis of iP- and *t*Z-type cytokinins. However, it is still possible that other pathways contribute to cytokinin production. Important questions yet to be answered include whether ATP/ADP IPTs are responsible for the bulk of iP- and *t*Z-type cytokinin production *in vivo*, and to what extent modified tRNAs contribute to the production of iP-, *t*Z-, and *c*Z-type cytokinins. To answer these questions, we isolated mutant alleles for every *ATP/ADP IPT* and *tRNA IPT*, and made several higher-order *IPT* mutant plants to clarify the role of IPTs in cytokinin biosynthesis *in planta*.

Results

Isolation of Mutants for Every *IPT*. *Arabidopsis* has seven genes for ATP/ADP IPTs (*AtIPT1*, *AtIPT3*, *AtIPT4*, *AtIPT5*, *AtIPT6*, *AtIPT7*, and *AtIPT8*) and two genes for tRNA IPTs (*AtIPT2* and *AtIPT9*). We obtained T-DNA insertion mutants for all *AtIPT*s except *AtIPT6* and *AtIPT8*. Because the T-DNA insertions were exonic for *AtIPT1*, *2*, *3*, *4*, *5*, and *7*, these likely represent null alleles. The *atipt9-1* allele contains an intronic insertion. However, a full-length *AtIPT9* transcript could not be amplified by RT-PCR (Fig. 1*B*), suggesting that the T-DNA was not spliced-out. *AtIPT6* is nonfunctional in the Wassilewskija (Ws) ecotype owing to a deletion of the 556th nucleotide relative to the translation start site, causing a frame-shift. We designate the wild type Ws line as *atipt6-1*. Because T-DNA insertion mutant was unavailable for *AtIPT8*, the *AtIPT8* gene was targeted with *35S::NPTII* flanked by upstream and downstream regions of *AtIPT8*, by homologous recombination. Thus we have collected putative null alleles for every *AtIPT* gene (Fig. 1*A*). No single mutant for an *ATP/ADP IPT* exhibited a visible phenotype. In this report, we omit allele numbers for *atipt1-1*, *atipt2-1*, *atipt 3-2*, *atipt5-2*, *atipt7-1*, and *atipt9-1*, which are Columbia ecotype, and for *atipt4-1* and *atipt6-1*, and *atipt8-1* with Ws background. For other alleles, allele numbers are shown.

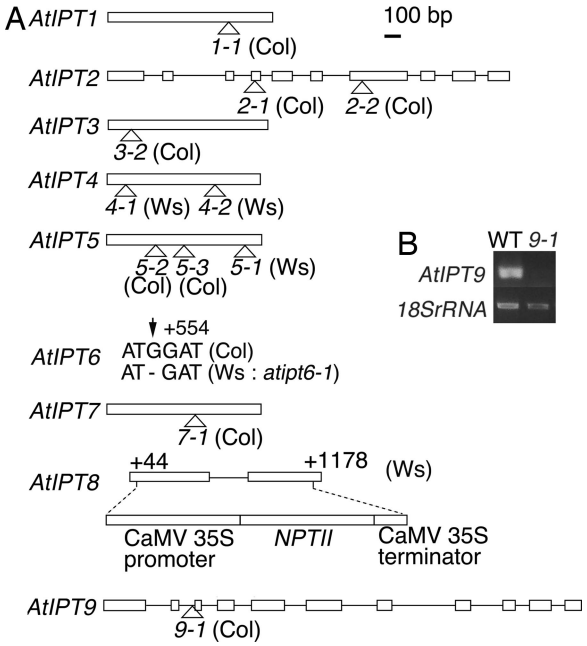

Fig. 1. Mutants of *AtIPT* genes. (*A*) Description of the mutant alleles used in this study. T-DNA insert positions within the *AtIPT1*, *2*, *3*, *4*, *5*, *7*, and *9* are represented by a triangle. The *atipt6* mutation is indicated by alignment of short sequences corresponding to the region of *AtIPT6* differing between Col and Ws. The *atipt8* mutation is represented by a schematic of the construct used for deletion of the gene by homologous recombination. Coding regions are depicted with open squares, and noncoding regions are depicted with bars. *NPTII*, *NEOMYCIN PHOSPHOTRANSFERASE II*. (*B*) Expression of *AtIPT9* in wild-type and *atipt9-1*. Because the T-DNA was inserted in an intron in the *atipt9-1* allele, expression of *AtIPT9* was examined. RT-PCR was performed on cDNA derived from Col WT or *atipt9-1* plants by using primers upstream and downstream of the T-DNA insert. The *18S rRNA* gene was used as a control. PCR cycles were 35 cycles for the *AtIPT9* gene and 20 cycles for the *18S rRNA* gene.

Growth of Mutants for ATP/ADP IPTs. The three most highly expressed genes for ATP/ADP IPTs in the vegetative phase are *AtIPT3*, *AtIPT5*, and *AtIPT7*. *AtIPT1* is also expressed in the vegetative phase at a low level. Other ATP/ADP *IPT* genes are barely detectable in vegetative organs (29). Therefore, we first made all combinations of double, triple, and quadruple mutants for *AtIPT1*, *AtIPT3*, *AtIPT5*, and *AtIPT7*. We also made multiple mutants that carried *atipt6* for selected combinations. *AtIPT6* was expressed mainly in the funiculus (data not shown). In the *atipt 1 3 5 7* quadruple mutant, *AtIPT4*, *AtIPT6*, and *AtIPT8*, which are not normally expressed in the vegetative phase, were not ectopically expressed (Fig. 6, which is published as supporting information on the PNAS web site).

There were no visible phenotypes in double mutants of any possible mutant combination for *AtIPT1*, *3*, *5*, and *7*. The *atipt 3 5 7* triple mutant (data not shown), *atipt3 5-1 6 7* (data not shown) and the *atipt1 3 5 7* quadruple mutants (Fig. 2*B*), and the *atipt1 3 5-1 6 7* quintuple mutant (data not shown) had short, thin aerial parts, and the phenotype of the latter two lines tended to be more severe. Plants heterozygous for any one of *atipt3*, *5*, and *7* and homozygous for the other two mutations appeared normal, indicating that the phenotype is closely linked to the mutations. The quadruple and quintuple *AtIPT* mutants were indistinguishable from wild type early on, but phenotypic differences become evident as they aged (Fig. 2 *A* and *B*). These mutants had fewer rosette leaves (Table 1), indicating a prolonged plastochron, appeared to have reduced shoot apical meristem size (Fig. 2 *C* and *D*), and had thin inflorescence stems. On vermiculite, flowering time was delayed in these

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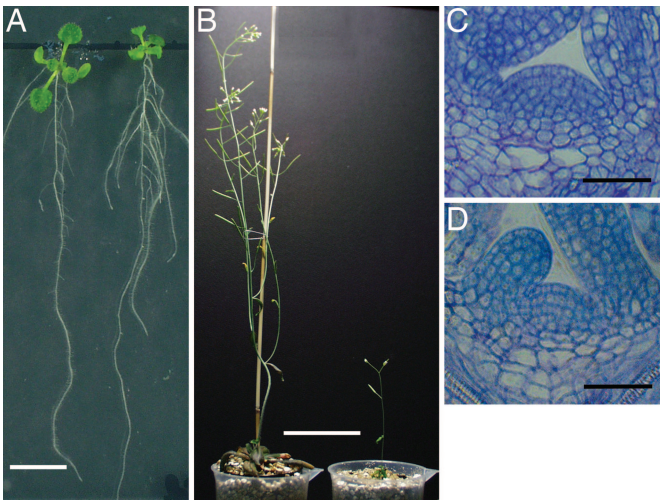

Fig. 2. Phenotypes of *atipt1 3 5 7*. (*A*) Plants grown on vertical plates for 10 days. (*B*) Plants grown on nutrient-vermiculite for 39 days. (*A* and *B Left*) Col WT. (*A* and *B Right*) *atipt1 3 5 7*. (*C* and *D*) Shoot apical meristems in 5-day-old WT Columbia (*C*) and 5-day-old *atipt1 3 5 7* (*D*). (Scale bars: *A*, 1 cm; *B*, 5 cm; *C* and *D*, 50 μm.)

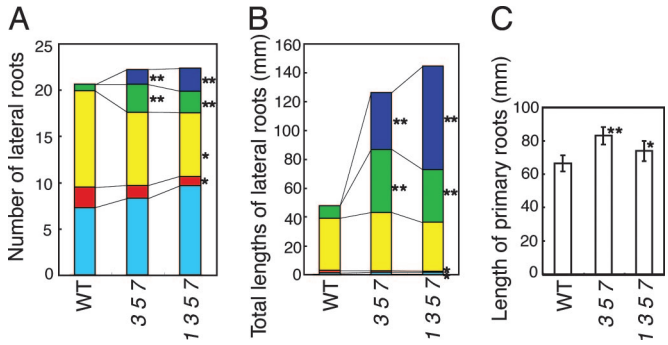

Fig. 3. Enhanced root elongation in the *atipt3 5 7* (*3 5 7*) and *atipt1 3 5 7* (*1 3 5 7*) mutants. (*A* and *B*) Average number of lateral roots (*A*) and total length of lateral roots (*B*), with individual length ranges discriminated by color. (*C*) Primary root length. Values are mean ± SD. In *A* and *B*, light blue, 0–0.5 mm; red, 0.5–1.0 mm; yellow, 1.0–10.0 mm; green, 10.0–20.0 mm; blue, >20.0 mm. At least nine plants were used for a genotype. Single and double asterisks indicate significant difference between values of mutants and WT ($0.01 < P < 0.05$ and $P < 0.01$, respectively).

multiple mutants; however, late flowering may be a secondary effect of reduced cytokinin production, because the flowering time was not delayed on nutrient agar. Some seeds were aborted, but surviving seeds were larger than those of the WT (data not shown). We also examined root growth in the *atipt3 5 7* triple and the *atipt1 3 5 7* quadruple mutants. Relative to wild type plants, both the number of lateral roots longer than 1 cm (Fig. 3*A*), and the total length of lateral roots (Fig. 3*B*) were increased in the triple and quadruple mutants. The primary roots of these mutants were slightly longer than those of the WT (Fig. 3*C*).

We also made triple and quadruple mutants of other allele combinations (*atipt1 5 6*, *atipt5 6 7*, *atipt1 3 5*, *atipt1 3 7*, *atipt1 5 7*, *atipt4 6 8*, and *atipt1 6 8*, and quadruple mutants *atipt1 3 5 6*, *atipt1 5 6 7*, and *atipt1 4 6 8*). None of these mutants had a visible phenotype when grown on plates or on soil. These mutants possessed at least 1 WT *AtIPT3*, *AtIPT5*, or *AtIPT7* gene, indicating that these three genes have redundant functions. *atipt1 4 6 8* lacked all *ATP/ADP IPT*s that were preferentially expressed in reproductive organs or their associated tissues, but still set seeds normally (data not shown). Finally, we obtained mutants that lacked all 7 *ATP/ADP IPTs* (*atipt1 3 4 5-1 6 7 8*), which were thin and small, resembling the quadruple *atipt1 3 5 7* mutant (data not shown).

Growth of Atipt Multiple Mutants Was Partially Rescued by *t*Z. To determine whether the phenotypes of higher order mutants were the consequence of a decrease in endogenous cytokinin levels, we grew mutant plants in the presence of a cytokinin, *t*Z. In the absence of cytokinins, the *atipt1 3 5 7* quadruple mutant had smaller aerial parts and longer lateral roots than WT (Fig. 4*A*), as described above. External application of *t*Z partially rescued the growth of aerial parts of the mutant (Fig. 4*B*; see Fig. 7, which is published as supporting information on the PNAS web site), and reduced the numbers of elongated lateral roots in the mutant and WT. These results suggest that the growth defect is caused by cytokinin deficiency. Higher levels of *t*Z inhibited root and shoot growth of either genotype (data not shown), consistent with normal cytokinin responses.

Table 1. Numbers of true leaves

Line (*n*)	Number (mean ± SD)
WT(Col) (5)	8.25 ± 0.41
atipt3 5 7 (10)	5.90 ± 0.32
atipt1 3 5 7 (5)	5.60 ± 0.55

Numbers of true leaves longer than 0.3 mm of 10-day-old plants were counted.

***AtIPT3-Promoter*::*AtIPT3-GFP* Complements *atipt3 5-1 6 7*.** To confirm that the phenotypes of the *atipt3 5-1 6 7* mutant are caused by mutations in these genes, we introduced *AtIPT3-GFP* driven by its native promoter (*AtIPT3p*::*AtIPT3-GFP*). The growth of *atipt3 5-1 6 7* was completely recovered by *AtIPT3p*::*AtIPT3-GFP*, indicating that the *atipt3* mutation was indeed one of the causes for the phenotypes of the quadruple mutant (Fig. 4*C*). The *AtIPT3-GFP* fluorescence patterns in WT and *atipt3 5-1 6 7* were similar (Fig. 4 *D–G*), being located in the phloem, consistent with previous reports (29, 36). A punctate fluorescence pattern was present in the cells, which were previously reported to be plastids (36). These results indicate that the *AtIPT3p*::*AtIPT3-GFP* construct complemented the *atipt3 5-1 6 7* mutant and was expressed in the same tissue with the same subcellular localization as in WT plants.

ATP/ADP IPTs Play a Crucial Role for Biosynthesis of iP-Type and *t*Z-Type Cytokinins. We determined riboside, ribotide, and glucoside forms of cytokinins in WT Columbia, *atipt1*, *atipt2*, *atipt3*, *atipt5*, *atipt7*, and *atipt9*, and in double and triple mutants of selected combinations, and in the *atipt1 3 5 7* quadruple mutant. All of these mutants are in the Columbia background. We also measured the levels of free-base cytokinins in selected lines (WT, *atipt2*, *atipt 3 5 7*, and *atipt1 3 5 7*) (Fig. 5). Similar to previous studies using cytokinin measurements, we could not discriminate between monophosphate and di- or triphosphate forms of cytokinins. Therefore, the values of isopentenyladenosine monophosphate (iPRMP) may represent the total pool of isopentenyl-AMP, isopentenyl-ADP, and isopentenyl-ATP present. Similarly, values of *t*-zeatin riboside monophosphate (*t*ZRMP) might include *t*ZRDP and *t*ZRTP.

In the single and double mutants carrying the *atipt3* mutation, the levels of iPRMP and iPR were decreased (Fig. 5*A*), but decreases in the level of *t*ZRMP or *t*-zeatin riboside (*t*ZR) were less evident (Fig. 5*B*). In the *atipt3 5 7* triple mutant and the *atipt1 3 5 7* quadruple mutant, levels of iPRMP, iPR, *t*ZRMP, *t*ZR, *t*Z-7-glucoside (*t*Z7G), *t*Z-9-glucoside (*t*Z9G), and *t*Z-O-glucoside (*t*ZOG) were all reduced to <20% of the WT values (Fig. 5*A*, *B* and *D*). The decreases in free-base iP and *t*Z in the *atipt1 3 5 7* quadruple mutant were moderate compared with the decreases in the levels of

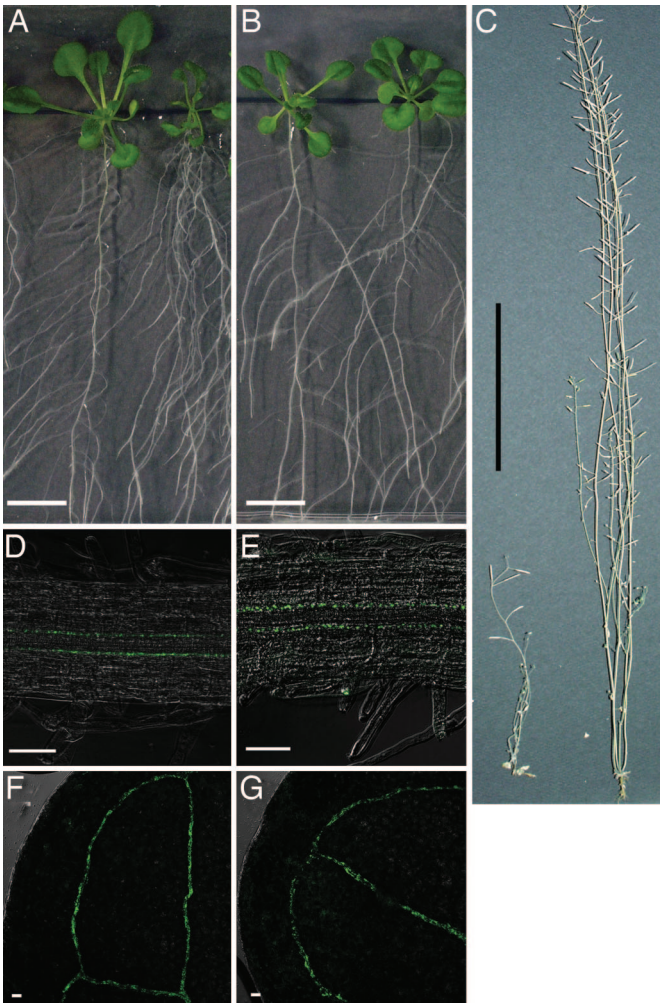

Fig. 4. Partial rescue of the *atipt1 3 5 7* quadruple mutant by external application of cytokinin and complementation of the *atipt3 5-1 6 7* mutant by *AtIPT3 promoter*::*AtIPT3-GFP*. (*A* and *B*) WT (*Left*) and *atipt1 3 5 7* (*Right*) grown without (*A*) or with (*B*) 10 nM *t*Z for 21 days. (*C Left*) *atipt3 5-1 6 7*. (*C Right*) *atipt3 5-1 6 7* carrying *AtIPT3 promoter*::*AtIPT3-GFP*. (*D–G*) Expression of *AtIPT3 promoter*::*AtIPT3-GFP* in WT root (*D*) or cotyledon (*F*), or in *atipt3 5-1 6 7* root (*E*) or cotyledon (*G*). (Scale bars: *A* and *B*, 1 cm; *C*, 10 cm; *D–G*, 50 μm.)

riboside and ribotide forms (Fig. 5*E*). Interestingly, *c*Z, *cis*-zeatin riboside (*c*ZR), and *cis*-zeatin riboside monophosphate (*c*ZRMP) were increased in mutants that possessed decreased levels of iP-type and *t*Z-type cytokinins (Fig. 5*C*). These results indicate that *c*Z-type cytokinins are produced through a different pathway than iP- and *t*Z-type cytokinins, and suggest that degradation of *c*Z is under the negative regulation of cytokinin signaling.

tRNA IPTs Are Indispensable for Biosynthesis of *c*Z-Type Cytokinins. *Arabidopsis* has two genes coding for tRNA IPT homologs, *AtIPT2* and *AtIPT9* (for review see refs. 3 and 20). Neither the *atipt2-1* nor the *atipt2-2* mutant showed a visible phenotype, but both the *atipt9* single and the *atipt2 9* double mutant often were chlorotic.

Levels of iP-type and *t*Z-type cytokinins were unaffected in *atipt2*, *atipt9*, or the *atipt2 9* double mutant (Fig. 5 *A*, *B*, *D*, and *E*). By contrast, *c*ZR and *c*ZRMP were reduced in *atipt2* and *atipt9* (Fig. 5*C*). *c*Z was also reduced in *atipt2* (Fig. 5*D*)(*c*Z was not examined in *atipt9*). *c*ZR and *c*ZRMP were undetectable in the *atipt2 9* double mutant.

Because modified tRNA is a possible source of *c*Z, we examined cytokinin levels in dephosphorylated hydrolysate of tRNA (Table 2). WT tRNA contained iPR (0.074 ng/μg tRNA) and *c*ZR (2.13 ng/μg tRNA), whereas *t*ZR was under the detection limit of 0.25 pg/μg tRNA. Levels of iPR and *c*ZR in tRNA samples were unchanged in the *atipt1 3 5 7* quadruple mutant, indicating that ATP/ADP IPTs are not involved in isopentenylation of tRNA. Interestingly, in *atipt2*, the *c*ZR-containing tRNA content was decreased to $\approx$30% of the WT level, whereas the iPR-containing tRNA level was unchanged. In contrast, in *atipt9-1*, the iPR-containing tRNA level was decreased to $<$4% of the WT level, whereas decreases in the *c*ZR-containing tRNA level were moderate.

Discussion

In the *atipt3 5 7* and the *atipt1 3 5 7* mutants, levels of iP- and *t*Z-type cytokinins, including ribosides, ribotides, and glucosides, were greatly decreased, clearly indicating that ATP/ADP IPTs are critical for the formation of iP- and *t*Z-type cytokinins. These results provided compelling evidence that cytokinins are produced by plants, arguing against the hypothesis that cytokinins could be produced exclusively by microbial symbionts (37). Decreases in free-base cytokinins in the mutants, however, were less evident. Therefore, it is possible that conversion of riboside- and/or ribotide- cytokinins to free-base cytokinins is negatively regulated by cytokinin signaling, or that deactivation or degradation of free-base, riboside, and ribotide cytokinins are differentially regulated by cytokinin signaling. Interestingly, *c*Z-type cytokinin levels were inversely correlated with iP- and *t*Z-type cytokinin levels. This indicates that *c*Z-type cytokinins are produced by a different pathway than *t*Z- and iP-type cytokinins, and that *c*Z levels are regulated by cytokinin signaling.

In *atipt1 3 5 7* seedlings, ectopic expression of the remaining ATP/ADP IPTs (*AtIPT4*, *6*, and *8*) was not detected. However, the quadruple mutant still possessed iP-type and *t*Z-type cytokinins. It is possible that these cytokinins are produced by AtIPT4, 6, and/or 8, although the undetectable expression of the corresponding transcripts for these AtIPTs in vegetative tissues argues against this hypothesis. It is possible that zeatin *cis-trans* isomerase, activity for which has been detected in *Phaseolus vulgaris* (38), produces *t*Z-type cytokinins from *c*Z-type cytokinins. However, if *cis-trans* isomerase activity is present in the quadruple mutant, the activity must be low because *t*Z-type cytokinin levels were greatly reduced compared with *c*Z-type cytokinin levels. It is also possible that degradation of tRNAs modified with a nonhydroxylated isopentenyl modification at the adenosine residue served as the source of low-levels of iP-type cytokinins, which would then be converted to *t*Z-type cytokinins. However, tRNA modification with a nonhydroxylated chain is minor compared with tRNA modification with a cis-hydroxylated side chain in *Arabidopsis* (Table 2).

None of the single and double mutants for *ATP/ADP IPT* genes exhibited a visible phenotype, and among the triple mutants, only *atipt3 5 7* exhibited visible phenotypes. This redundancy was unexpected based on the unique expression patterns detected for the *ATP/ADP IPT*s. Furthermore, the *AtIPT3*p::*AtIPT3-GFP* fusion gene complemented the defects in the *atipt3 5-1 6 7* quadruple mutant, although the transgene was expressed with a WT rather than ectopic expression pattern. This indicates that WT expression of *AtIPT3* is sufficient to complement *atipt3 5-1 6 7*. Hence, whereas every *ATP/ADP IPT* exhibits a spatially specific expression pattern, these enzymes function redundantly *in planta*. The simplest explanation for this result is that cytokinins produced by AtIPT3 is translocated between tissues.

Among the single mutants for ATP/ADP IPTs, only the *AtIPT3* mutant exhibited decreased levels of iP- and *t*Z-type cytokinins. The decreased cytokinin levels in *atipt3* plants agree with a previous report in which nitrogen-induced accumulation of cytokinins was decreased in an *atipt3* mutant (36). Because *AtIPT3* is involved in nitrate-induced cytokinin increases (36), we grew *atipt3* plants under nitrate-sufficient and nitrate-starved conditions. However,

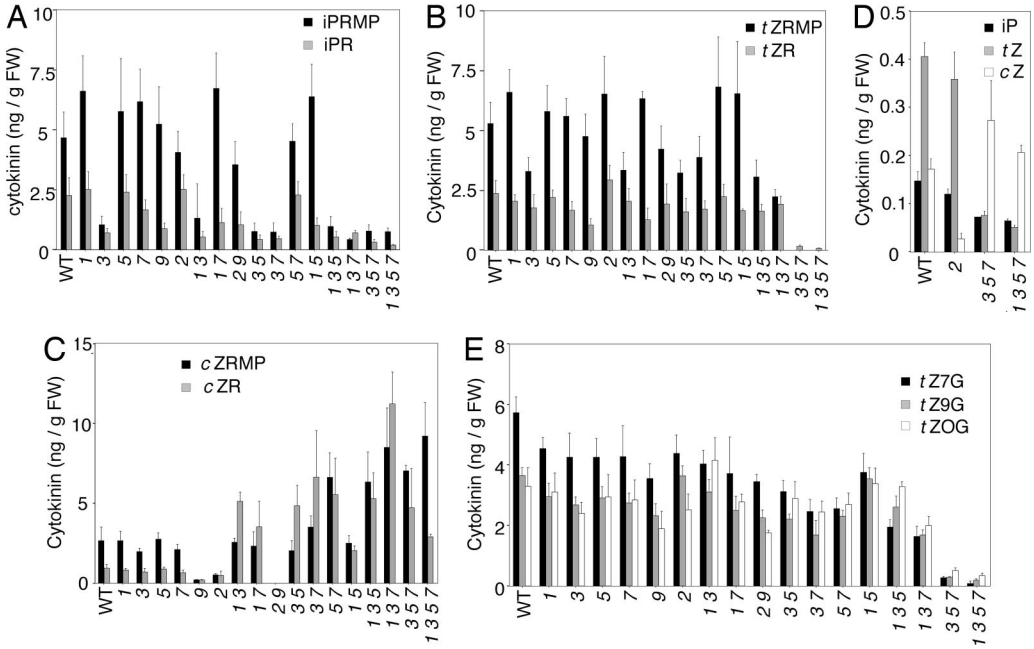

Fig. 5. Cytokinin levels in *AtIPT* mutants. Values are mean ± SD of five samples. (*A*) iPRMP (black) and iPR (gray). (*B*) *t*ZRMP (black) and *t*ZR (gray). (*C*) *c*ZRMP (black) and *c*ZR (gray). (*D*) Free-base cytokinins: iP (black), *t*Z (gray), and *c*Z (open). (*E*) Glucosylated cytokinins: *t*Z7G (black), *t*Z9G (gray), and *t*ZOG (open). Mutant names are shown as numbers (e.g., the *atipt1 atipt3* double mutant is shown as *1 3*).

we found no visible phenotypic differences between *atipt3* and wild type (data not shown) under either nutrient condition. A more detailed analysis of nitrate responses in the *atipt3* mutant is necessary to understand the role of nitrate-induced cytokinin production.

Mutants lacking *AtIPT3*, *5*, and *7* were severely inhibited in shoot growth, whereas lateral roots elongated more (Fig. 3). These phenotypes resembled those reported for cytokinin oxidase overexpressors, and agreed with previous experimental evidence that cytokinins positively regulate shoot elongation and negatively regulate root elongation (6, 7). However, a low level of cytokinin signaling is likely required for root growth, because growth of both roots and shoots was stunted in mutants lacking all cytokinin receptors (9, 39), and root growth was inhibited when a cytokinin oxidase gene was strongly expressed in roots after germination (5).

AtIPT2 and AtIPT9 resemble tRNA IPTs in their amino acid sequences (20, 23, 24), and AtIPT2 indeed suppresses the phenotypes of a *Saccharomyces cerevisiae* mutant lacking a tRNA IPT (34). We measured *c*ZR, *t*ZR, and iPR contents in tRNA hydrolysates from WT and IPT mutants. Consistent with a previous report (30), a fraction of the tRNAs from WT plants possessed the isopentenyl side chain or the *cis*-hydroxylated isopentenyl side chain (Table 2). Levels of iPR- and *c*ZR-containing tRNA were not affected in a quadruple mutant of genes representing all major ATP/ADP IPTs in the vegetative phase (*atipt1 3 5 7*) (Table 2), confirming that ATP/ADP IPTs were not involved in tRNA modification. In *Sal. typhimurium*, the isopentenyl chain of tRNA is hydroxylated at the *cis* position by the *miaE* product (35). Interestingly, in *atipt2*, the tRNA *c*ZR level was decreased to ≈30% of WT, whereas the iPR level was unchanged. In contrast, in *atipt9*, the tRNA iPR level was decreased to <4% of WT levels, whereas the *c*ZR level was only moderately decreased. Therefore it is possible that AtIPT2 transfers a *cis*-hydroxylated isopentenyl side chain to tRNA. However, in the *Sac. cerevisiae* mutant for *tRNA IPT*, *AtIPT2* expression caused isopentenylation of tRNAs in yeast, but the side chain was not hydroxylated (34). To our knowledge, the predicted precursor for *c*ZR-modified tRNA in *Arabidopsis*, *cis*-hydroxy dimethylallyl diphosphate (4-hydroxy-3-methyl-2-(*Z*)-butenyl diphosphate), has not been reported in any organism. Future work aimed at determining whether *cis*-hydroxyl dimethylallyl diphosphate exists in plants and *Sac. cereviciae*, and in which cellular compartment, will shed light on the mechanism for *c*ZR-modification of tRNAs.

Table 2. Cytokinins in tRNA

Line	*t*ZR	*c*ZR	iPR
Wild-type	ND	2.13 ± 0.28	0.0740 ± 0.0113
atipt2	ND	0.68 ± 0.10	0.0720 ± 0.0074
atipt9	ND	1.36 ± 0.31	0.0027 ± 0.0006
atipt 2 9	ND	ND	ND
atipt1 3 5 7	ND	2.26 ± 0.37	0.0685 ± 0.0073

Measurements are nanograms of riboside equivalent per microgram of tRNA). tRNA was extracted from five different cultures per line. Cytokinin contents are shown as means ± SD. ND, below detection limits.

We showed that knockout mutants for either tRNA isopentenyltransferase in *Arabidopsis*, AtIPT2 or AtIPT9, possessed decreased levels of *c*Z-type cytokinins. This, combined with the fact that *c*Z-type cytokinins were undetectable in the *atipt2 9* double mutant, clearly indicates that *AtIPT2* and *AtIPT9* are absolutely required for *c*Z-type cytokinin production (Fig. 5). The lack of detectable *c*Z in *atipt2 9* also indicates that isomerization of *t*Z to *c*Z does not occur to a detectable level. The correlation between changes in tRNA modification and decreases in *c*Z-type cytokinin levels support the idea that *c*Z-type cytokinins are derived from modified tRNA.

In summary, our data indicate that iP- and *t*Z-type cytokinins are synthesized through the action of ATP/ADP IPTs, whereas *c*Z-type cytokinins are synthesized through the action of tRNA IPTs *in planta*. As Kamínek wrote (40), creation of tRNA-independent cytokinin biosynthetic route was probably an important event during evolution to higher plants, because it made precise regulation of cytokinin biosynthesis possible.

Materials and Methods

Plant Culture and Phenotypic Analysis. *Arabidopsis* plants were grown on a solidified medium in plates or on vermiculite wetted with a

nutrient solution. Detailed methods are provided at *Supporting Materials and Methods*, which are published as supporting information on the PNAS web site

Isolation of Atipt Mutants. Plants containing the T-DNA insertion alleles of *AtIPT*s were obtained from *Arabidopsis* Biological Resource Center (www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm), the Kazusa DNA Research Institute (www.kazusa.or.jp/ja2003/english), or the *Arabidopsis* Knockout Facility at the University of Wisconsin. The ecotype of *atipt1* (SALK_020112), *atipt2-1* (SALK_042050), *atipt2-2* (SALK_019906), *atipt3-2* (KG21969), *atipt5-2* (SALK_133407), *atipt5-3* (SALK_136322), *atipt7-1* (SALK_001940), and *atipt9-1* (KG7770) is Columbia (Col). The ecotype of *atipt4-1* (Wisconsin β 12), *atipt4-2* (Wisconsin β 24), and *atipt5-1* (Wisconsin α 6) is Wassilewskija (Ws). Genotypes were determined by PCR (see *Supporting Materials and Methods*).

Homologous Recombination. Because we did not find T-DNA insertion mutants of *AtIPT8*, we targeted it by homologous recombination. The structure of the plasmid for gene targeting is described in Fig. 1. The genomic fragments of upstream (−4050 to +44) and downstream (+1,178 to +5,167) regions of *AtIPT8* were amplified by PCR and inserted upstream or downstream of the *35S promoter*::*NPT2*::*35S-terminator* in a binary vector. Primer sequences used for PCR are provided in the *Supporting Materials and Methods*. In ≈25,000 transformation events, we identified three homologous recombination events, and we used one of them for this study.

Complementation Analysis. The genomic fragment encompassing 2.01 kbp of the AtIPT3 promoter was placed upstream of the entire coding region of *AtIPT3* fused to *GFP* and cloned in a transformation vector. Then it was transformed into *atipt3 5-1 6 7* by using *Agrobacterium tumefaciens*. Detailed methods are provided at *Supporting Materials and Methods*.

RT-PCR. RNA samples were isolated from 10-day-old whole seedlings grown on plates. RT-PCR was performed as described in ref. 29. For primer sets used, see ref. 29 and *Supporting Materials and Methods*.

Cytokinin Measurements. Eighteen-day-old *Arabidopsis* plate-grown plants were used for cytokinin measurements. For measurement of riboside, ribotide, and glucoside cytokinin levels, cytokinins were purified and derivatized according to Nordström *et al.* (41) with some modifications. For free-base cytokinins, cytokinins were purified according to Bieleski (42) with some modifications. For detail, see *Supporting Materials and Methods*. Cytokinins were quantified by liquid-chromatography-positive electrospray–tandem mass spectrometry in a multiple reaction-monitoring mode.

Purification of tRNA and Quantification of Cytokinins in tRNA. tRNA was purified from 18-day old plate-grown *Arabidopsis* shoots, hydrolyzed and dephosphorylated according to Gray *et al.* (ref. 32 and references therein) with some modifications, and used for cytokinin analysis by using liquid chromatography-linked mass spectrometry (*Supporting Materials and Methods*).

We thank M. Kusumoto for sampling and purification of cytokinins and Melissa Pischke for excellent critical reading, suggestions, and editing. T. Kakimoto was supported by Grants-in-Aid for Scientific Research 15107001 (Japan Society for the Promotion of Science) and 17027017 (Ministry of Education, Sports, Science, and Technology), and Precursory Research for Embryonic Science and Technology, Japan Science and Technology. P.T. was supported by Ministry of Education, Youth and Sports of the Czech Republic Grant MSM 6198959216.

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