

AtIPT3 is a Key Determinant of Nitrate-Dependent Cytokinin Biosynthesis in *Arabidopsis*

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We analyzed the spatial expression pattern of *Arabidopsis thaliana* adenosine phosphates-isopentenyltransferase genes (*AtIPT1*, *AtIPT3* to *AtIPT8*) and the effect of inorganic nitrogen sources on their regulation. In mature plants, the *AtIPTs* were differentially expressed in various tissues including the roots, leaves, stems, flowers and siliques. In transgenic seedlings expressing a gene for green fluorescent protein (GFP) driven by the *AtIPT* promoters, *AtIPT1::GFP* was predominantly expressed in the vascular stele of the roots, *AtIPT3::GFP* was in the phloem companion cells, *AtIPT5::GFP* was in the lateral root primordium and pericycle, and *AtIPT7::GFP* was in both the vascular stele and the phloem companion cells of the roots. In a long-term treatment, the accumulation level of *AtIPT5* transcript was correlated with the concentrations of NO_3^- and NH_4^+ in the growth medium. However, under nitrogen-limited conditions, *AtIPT3* expression was rapidly induced by NO_3^- in the seedlings accompanying the accumulation of cytokinins, whereas *AtIPT5* expression was little affected. The NO_3^- -dependent accumulation of both the *AtIPT3* transcript and the cytokinins was markedly reduced in a *Ds* transposon-insertion mutant of *AtIPT3*. These results suggest that nitrogen availability differentially regulates expression of *AtIPT3* and *AtIPT5*, and that *AtIPT3* is a key determinant of cytokinin biosynthesis in response to rapid changes in the availability of NO_3^- .

Keywords: Ammonium ions — *Arabidopsis* — Cytokinin — Isopentenyltransferase — Nitrate ions.

Abbreviations: *AtIPT*, *Arabidopsis thaliana* adenosine phosphates-isopentenyltransferase genes; CK, cytokinin; *cZ*, *cis*-zeatin; GFP, green fluorescent protein; *iP*, isopentenyladenine; *iPR*, isopentenyladenine riboside; *iPRMP*, isopentenyladenine riboside 5'-monophosphate; *tZ*, *trans*-zeatin; *tZR*, *trans*-zeatin riboside; *tZRMP*, *trans*-zeatin riboside 5'-monophosphate.

Introduction

The availability of inorganic nitrogen sources is an important limiting factor for plant growth and development. Plants

constantly sense differences in nitrogen availability, and modulate their own metabolism and developmental program to adapt to their changing environment (Zhang and Forde 1998, Linkohr et al. 2002, Stitt et al. 2002). This ability to orchestrate changes throughout the whole plant calls for an integrated network of intracellular, intercellular and inter-organ signaling of nitrogen availability. In terms of the signaling molecule, NO_3^- itself functions as a primary signal to activate the expression of the assimilatory genes and the related genes (Stitt 1999, Wang et al. 2000). In general, the NO_3^- -specific response occurs rapidly without de novo synthesis of protein. On the other hand, some genes are regulated by multiple nitrogen sources, NO_3^- , NH_4^+ and/or amino acids (Sugiyama and Sakakibara 2002). Thus, plants appear to employ multiple routes for communication of nitrogen signals.

Recent studies have revealed some novel signaling molecules involved in the propagation of nitrogen signals (Forde 2002, Takei et al. 2002). One of those signaling molecules, cytokinin (CK) has been suggested as a pivotal signaling substance communicating nitrogen availability from root to shoot via the xylem vessels (Simpson et al. 1982, Takei et al. 2002). Supplementing NO_3^- has been shown to induce the rapid accumulation of CKs in the roots of barley (Samuelson and Larsson 1993), maize (Sakakibara et al. 1998, Takei et al. 2001a) and *Arabidopsis thaliana* (Takei et al. 2002). In maize xylem vessels, the translocation rate of *trans*-zeatin riboside (*tZR*) increases markedly in response to the presence of NO_3^- supplement (Takei et al. 2001b). These results suggest that NO_3^- substantially affects the activity of de novo CK biosynthesis and its translocation. In maize, expression of *ZmRR1*, a CK-responsive gene, is induced not only by NO_3^- but also by NH_4^+ , implying that CK biosynthesis could be regulated by both nitrogen sources (Sakakibara et al. 1998, Sakakibara et al. 1999). On the other hand, CK is also suggested to function as a systemic signal, translocated via the phloem. It is suggested that elevation of sucrose content in the phloem, regulated by day length regime, triggers the synthesis and translocation of CK from root to leaves via the xylem and from leaves to apical meristem via the phloem, facilitating flower initiation (Bernier et al. 1993).

In *Arabidopsis*, seven genes for adenosine phosphates-isopentenyltransferase (*AtIPT1* and *AtIPT3* to *AtIPT8*) have

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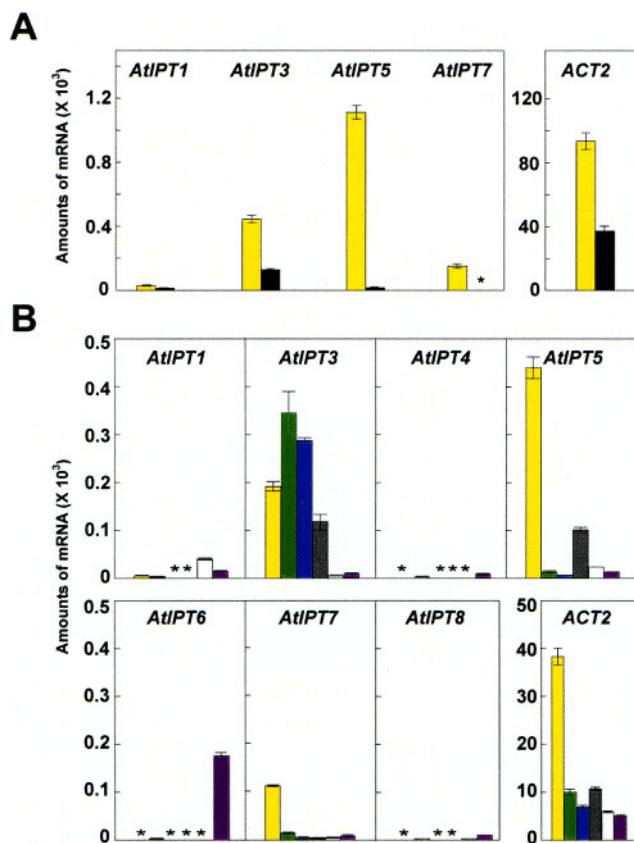


Fig. 1 Expression patterns of *AtIPTs* in *Arabidopsis*. (A) Accumulation patterns of *AtIPT* transcripts in roots and shoots of the seedlings. Total RNA prepared from roots (yellow bar) and shoots (black bar) of 2-week-old seedlings grown on MGRL agar medium with 1% sucrose was subjected to quantitative real-time PCR. (B) Accumulation patterns of *AtIPT* transcripts in various organs of the mature plants. Total RNA prepared from roots (yellow bar), rosette leaves (green bar), cauline leaves (blue bar), stems (grey bar), flowers (white bar) and siliques (violet bar) of mature plants grown hydroponically with MGRL medium for 6 weeks was subjected to quantitative real-time PCR. Other detail conditions are described in the Materials and Methods. Accumulation levels of the transcripts are given as the copy number of mRNA per 1 ng total RNA. Real-time PCR was performed in triplicate, and the mean values with SD are shown. *, Content less than the quantifiable limit (<5 mRNA molecules in 1 ng total RNA). *Actin2* (*ACT2*) was used as an internal standard.

been identified as CK biosynthesis genes (Kakimoto 2001, Takei et al. 2001b). The high level of redundancy suggests that the *AtIPTs* could be functionally differentiated in terms of the temporal and spatial patterns of expression as well as in response to environmental conditions. To understand the physiological function of CKs in nutrient signal transduction, detailed analysis of the regulatory mechanisms of the *AtIPTs* in terms of nutrient availability is clearly important.

In this study, we characterized the spatial expression patterns and nutritional regulation of the *AtIPTs*, and found that *AtIPT3* and *AtIPT5* are differentially regulated by nitrogen

availability. Analysis of a *Ds* transposon-insertion mutant showed that *AtIPT3* is a possible determinant of CK biosynthesis in response to rapid changes in the availability of NO_3^- . We discuss the physiological significance of the regulation and spatial expression of *AtIPT3* and *AtIPT5* for nitrogen signaling pathways.

Results

Spatial expression patterns of AtIPTs

To evaluate the levels of *AtIPTs* (*AtIPT1* and *AtIPT3* to *AtIPT8*) expression in different tissues, total RNAs were prepared from various tissues of 2-week-old seedlings and mature plants. Accumulation levels of the *AtIPTs* transcripts were analyzed by a quantitative real-time PCR method (Fig. 1). An actin gene, *ACT2*, (An et al. 1996) was used as an internal standard. In the seedlings (Fig. 1A), the transcripts of *AtIPT1*, *AtIPT3*, *AtIPT5* and *AtIPT7* were detected in roots, and that of *AtIPT5* was the most abundant. The accumulation of the *AtIPTs* transcripts in shoots was relatively low, but the *AtIPT3* transcript was most common. Accumulation levels of transcripts of *AtIPT4*, *AtIPT6* and *AtIPT8* were below the quantifiable limit in either organ at this developmental stage. Conversely, in mature hydroponically grown plants, the *AtIPTs* were differentially expressed in various tissues (Fig. 1B). Accumulation of *AtIPT1* transcript was always extremely low, but was more abundant in flowers than other tissues. *AtIPT3* transcript was detected in all organs, but only at low levels in the reproductive organs, flowers or siliques. In contrast to the seedlings, *AtIPT3* transcript was more abundant in the photosynthetic organs (rosette and cauline leaves) than the roots. *AtIPT5* was strongly expressed in the roots and stems, and weakly in other organs. The transcripts of *AtIPT6* were detected most abundantly in the siliques. *AtIPT7* predominantly expressed in the roots. So far as we were able to determine, the accumulation level of the transcripts of *AtIPT4* and *AtIPT8* was extremely low in those organs. These results demonstrate that expression of the *AtIPTs* is spatially differentiated, and that CK can be synthesized in various parts of the plant body.

For a more detailed analysis, we generated transgenic *Arabidopsis* carrying *AtIPT* promoter::green fluorescent protein (GFP) chimeric genes (*AtIPT::GFP*). Histochemical analysis of the GFP fluorescence was carried out with the T3 seedlings (Fig. 2). The fluorescence of GFP in *Arabidopsis* expressing *AtIPT1::GFP* was observed in the elongation area and vascular stele of the primary roots (Fig. 2A, B); in *Arabidopsis* expressing *AtIPT3::GFP* fluorescence was observed in the phloem companion cells throughout the whole seedling (Fig. 2C–G) whereas a recent study using *Arabidopsis* expressing *AtIPT3::beta-glucuronidase* suggested a more generalized staining pattern i.e. the phloem and the pericycle tissues (Miyawaki et al. 2004). Fluorescence of GFP in *Arabidopsis* expressing *AtIPT3::GFP* was also observed in the basal stele of young lateral roots, in which the vascular system is immature (Fig. 2D, E). Strong

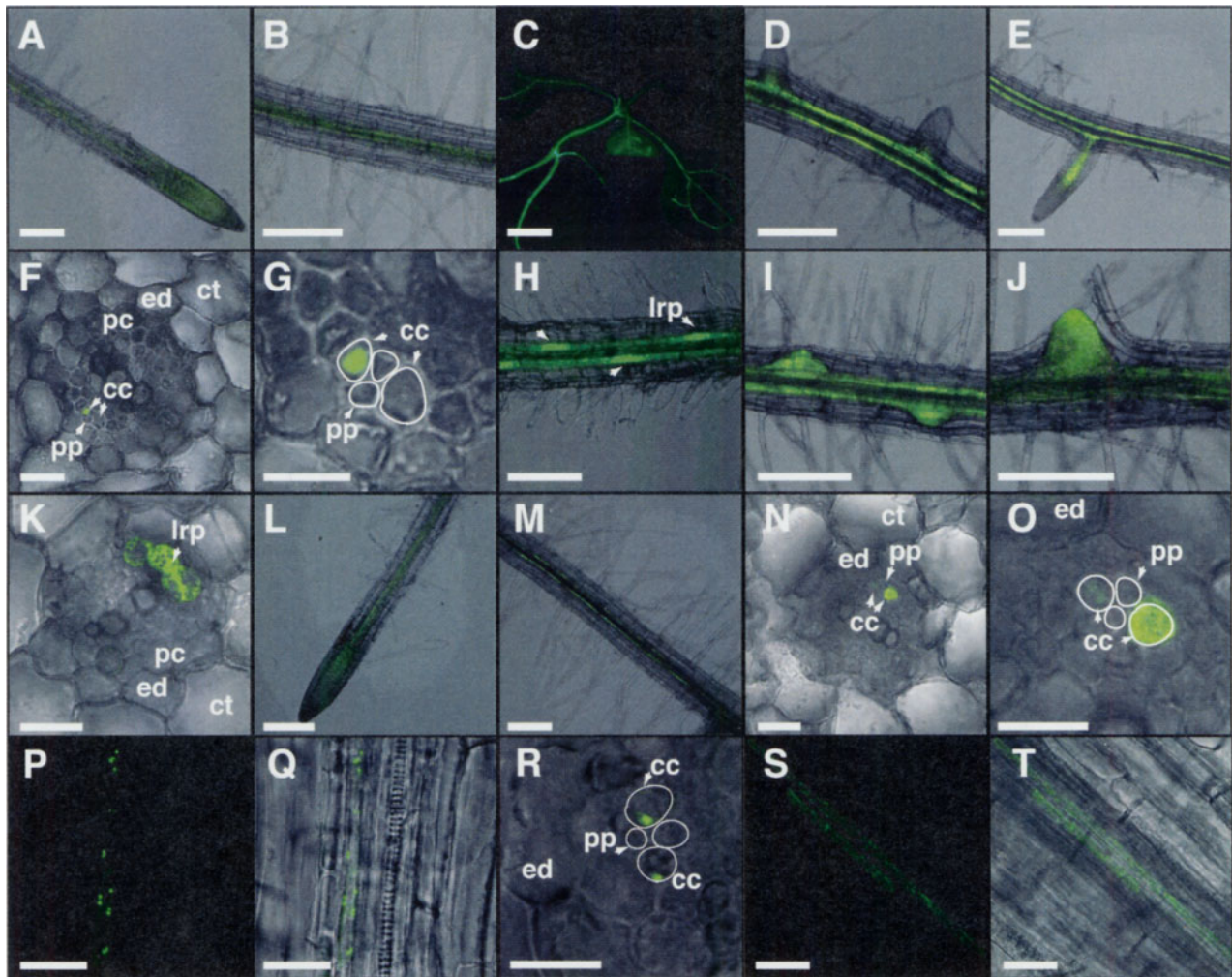


Fig. 2 Fluorescence patterns of GFP expressed under control of *AtIPT* promoters in *Arabidopsis*. (A, B) *AtIPT1::GFP* transgenic plants. Root tip (A) and basal area (B) of the primary root. (C–G) *AtIPT3::GFP* transgenic plants. Seedling (C), emerging lateral roots (D, E), and root cross-sections (F, G). G is an enlargement of F to show the cell profile. (H–K) *AtIPT5::GFP* transgenic plants. Primary roots (H–J) and root cross-section (K). Different stages of the emerging lateral roots are shown in H, I and J. K is a cross-section of the stage shown in H. (L–O) *AtIPT7::GFP* transgenic plants. Root tip (L) and basal area (M) of the primary root and the cross-section (N, O). O is an enlargement of N to show the cell profile. (P–R) Primary roots (P, Q) of *AtIPT3::AtIPT3-GFP* transgenic plants expressing *AtIPT3-GFP* fusion protein driven by the native promoter, and the cross-section (R). Q is a superimposition of the transmission image on P. (S, T) *AtIPT7::AtIPT7-GFP* transgenic plants expressing *AtIPT7-GFP* fusion protein driven by the native promoter. T is a superimposition of the transmission image on S. cc, companion cells; pp, protophloem, ct, cortex; ed, endodermis; lrp, lateral root primordium; pc, pericycle. Bars (A, B, D, E, H, I, J, L, M), 100 μ m; (C), 3 mm; (F, K, N, P, Q, S, T), 10 μ m; (G, O, R), 5 μ m.

fluorescence in *AtIPT5::GFP* was detected in the lateral root primordium and the pericycle (Fig. 2H, K) and the emerging lateral roots (Fig. 2I, J), suggesting that *AtIPT5* is involved in the early stages of lateral root development. *AtIPT7::GFP* fluorescence was observed both in the vascular stele of the elongation area (Fig. 2L) and in the root phloem companion cells (Fig. 2M–O). The fluorescence of *AtIPT7::GFP* in the elongation area appeared not to overlap with that of *AtIPT1::GFP* (Fig. 2A, L). These observations suggest that the CKs are mainly synthesized in the tissues and cells in which the fluorescence was observed.

It is reported that some GFP proteins expressed in phloem could be symplastically moved to the neighbor cells and sink tissues (Imlau et al. 1999). To reduce a possibility of movement of the GFP polypeptides in the transgenic plants, we also observed the GFP fluorescence in the transgenic plants expressing *AtIPT3-GFP* and *AtIPT7-GFP* fusion genes driven by the native promoters. Since *AtIPT3* and *AtIPT7* are localized in the plastids and the mitochondria, respectively (Kasahara et al. 2004), most of the fusion proteins are thought to stay on-site of the translation. In those plants, fluorescence of GFP was observed in restricted cells (Fig. 2P, Q, S, T). In further obser-

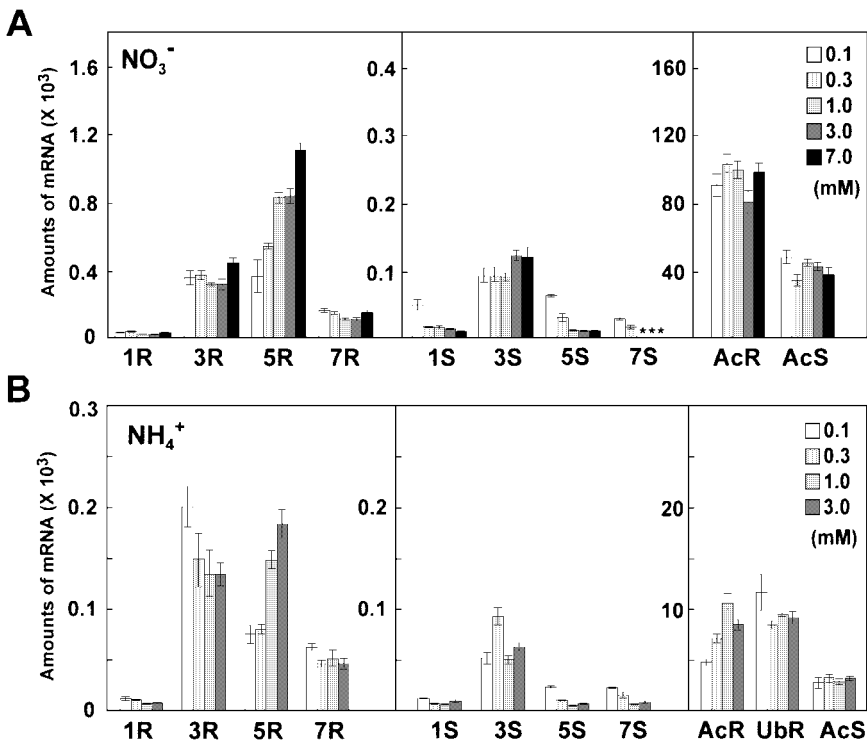


Fig. 3 Effect of the concentration of NO_3^- (A) and NH_4^+ (B) in the growth medium on the expression of *AtIPTs*. *Arabidopsis* seedlings were grown for 11 d after germination on MGRL medium containing 1% sucrose and the indicated concentration of NO_3^- (A) and NH_4^+ (B). Other details of conditions are described in the Materials and Methods. Total RNAs prepared from the roots and shoots were subjected to quantitative real-time PCR. Accumulation levels of the transcripts are given as the copy number of mRNA per 1 ng total RNA. Real-time PCR was performed in triplicate, and the mean values with SD are shown. 1R, *AtIPT1* in roots; 3R, *AtIPT3* in roots; 5R, *AtIPT5* in roots; 7R, *AtIPT7* in roots; 1S, *AtIPT1* in shoots; 3S, *AtIPT3* in shoots; 5S, *AtIPT5* in shoots; 7S, *AtIPT7* in shoots; AcR, *ACT2* in roots; AcS, *ACT2* in shoots; UbR, *UBQ10* in roots. *, Content less than the quantifiable limit (<5 mRNA molecules per 1 ng total RNA).

vation of their cross-sectioned tissues, the fluorescence of *AtIPT3-GFP* was detected in the phloem companion cells (Fig. 2R) whereas that of *AtIPT7-GFP* could not due to the low intensity of the fluorescence.

Effect of availability of inorganic nitrogen sources on *AtIPT* expressions

To determine the effect of inorganic nitrogen sources on the expression of *AtIPTs*, the accumulation levels of the transcripts were analyzed (Fig. 3). *Arabidopsis* seedlings were grown for 11 d after germination on agarose plates with MGRL salt that contained various concentrations of NO_3^- or NH_4^+ as indicated in the figures. Total RNAs were extracted from roots and shoots of the seedlings, and the amounts of mRNA of each gene were analyzed by a quantitative real-time PCR method. In roots, the accumulation of *AtIPT5* transcript (column 5R) was positively correlated with the concentration of NO_3^- and NH_4^+ (Fig. 3A, B, respectively). The expression level of *AtIPTs* in NH_4^+ -grown roots was lower than that in NO_3^- -grown ones. The *AtIPT3* expression in roots was decreased under a higher concentration of NH_4^+ in the growth media. Conversely, in shoots the accumulation of *AtIPT1*, *AtIPT5* and *AtIPT7* transcripts was negatively correlated with the concentration of NO_3^- and NH_4^+ (Fig. 3A, B, columns 1S, 5S, 7S). Unexpectedly, the accumulation of the *ACT2* transcript used as a control correlated positively with the concentration of NH_4^+ (Fig. 3B, column AcR). Therefore, we used a ubiquitin gene, *UBQ10* (Callis et al. 1995), as another control; the accumulation level of its transcript did not change (Fig. 3B, column UbR). These

results demonstrate that the expression level of *AtIPT5* in roots is positively correlated with the availability of inorganic nitrogen sources, and that this availability confers a distinct effect on the accumulation of *AtIPT5* transcript in roots and shoots.

Rapid accumulation of *AtIPT3* transcript and cytokinins in response to NO_3^-

In nitrogen-limited *Arabidopsis*, NO_3^- feeding caused a rapid accumulation of CKs in the roots (Takei et al. 2002). To understand the short-term effect of nitrogen sources on the expression of *AtIPTs*, changes in the accumulation level of *AtIPTs* transcripts in roots during induction by NO_3^- or NH_4^+ were monitored by a quantitative real-time PCR method (Fig. 4A). When NO_3^- was re-supplied to nitrogen-limited seedlings, *AtIPT3* transcript rapidly accumulated within 1 h with a similar kinetics to the nitrate reductase gene (*Nia1*). The manner of *AtIPT3* induction corresponded well with that of the accumulation of tZR and the 5'-monophosphate (tZMP) in roots (Fig. 4B). Supplementing NH_4^+ did not induce *AtIPT3* expression. Although positive correlations between the accumulation of *AtIPT5* transcript and the availability of NO_3^- and NH_4^+ were observed in the long-term treatments (Fig. 3), the short-term response of *AtIPT5* to the nitrogen sources was different. NH_4^+ -supplement did not affect the accumulation of *AtIPT5* transcript, and NO_3^- -supplement had only a slight positive effect (1.6-fold) on that of *AtIPT5* transcript comparing the NaCl control during the period of observation (Fig. 4A). The accumulation levels of *AtIPT1* and *AtIPT7* transcripts did not change significantly during this period (data not shown).

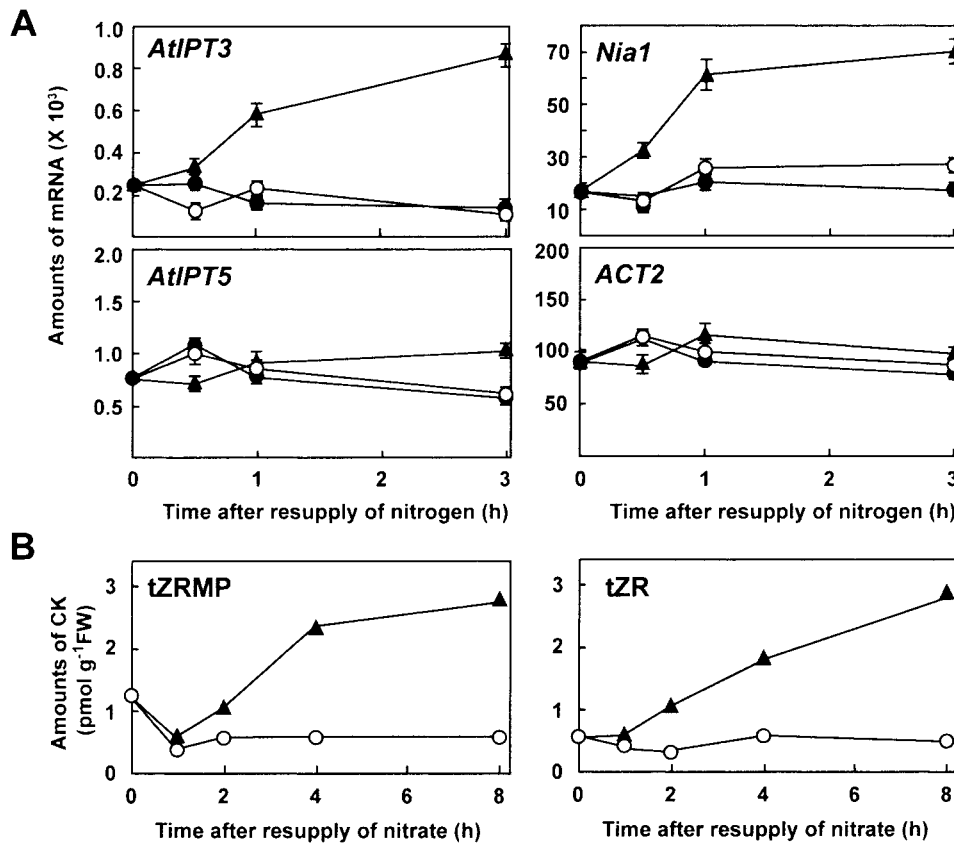


Fig. 4 Effect of re-supply of inorganic nitrogen sources on *AtIPT3* and *AtIPT5* expressions, and on CK accumulation. (A) Accumulation of *AtIPT3* transcript by NO₃⁻. *Arabidopsis* seedlings were grown on MGRL agar plates containing 1% sucrose for 14 d after germination with a reduced level of nitrogen (0.3 mM NO₃⁻). To wash out the remaining nitrogen sources, roots of the seedlings were put into the nitrogen-free MGRL solution and incubated for 1 h with leaving at rest. Next, the seedlings were transferred to 10 mM NaNO₃ (closed triangle), 10 mM NH₄Cl (closed circle) or 10 mM NaCl (open circle) solution and root tissues were harvested at the indicated times. Other conditions are described in the Materials and Methods. Total RNA prepared from the roots was subjected to quantitative real-time PCR. Accumulation levels of the transcripts are given as the copy number of mRNA per 1 ng total RNA. Real-time PCR was performed in triplicate, and the mean values with SD are shown. *Nia1* and *ACT2* were used as internal controls. (B) Accumulation of CK during the NO₃⁻ induction. Seedlings grown under limited nitrogen sources were sprayed with a solution of 50 mM KNO₃ (closed triangle) or 50 mM KCl (open circle). The root tissues were harvested at the indicated times. CK fractions were extracted and quantified. Only the results of *tZRMP* and *tZR* are indicated.

Putting together the long-term and short-term effects of nitrogen sources, it is suggested that *AtIPT3* and *AtIPT5* can respond to nitrogen availability but are distinctly regulated in terms of nitrogen specificity and temporal pattern of response. Furthermore, it is also suggested that *AtIPT3*, rather than *AtIPT5*, mainly contributes the CK accumulation in roots in response to rapid changes in NO₃⁻ availability.

Analysis of the *Ds* transposon-insertion mutant of *AtIPT3*

To examine the contribution of *AtIPT3* to the NO₃⁻-dependent accumulation of CKs, we used a *Ds* transposon-insertion mutant of *AtIPT3* from the *Arabidopsis* *Ac/Ds*-transposon tag-line bio-resource library of the RIKEN Genomic Sciences Center (Kuromori et al. 2004). In line number 15-0348-1, the transposon DNA was inserted at position +14 base pairs from the putative transcription start site proposed from the full-length cDNA sequence (accession number AY125508)

(Fig. 5A) (Seki et al. 2002). We designated the mutant as *ipt3-1*. The *ipt3-1* homozygote (*ipt3-1/ipt3-1*) was segregated in the F3 line (Fig. 5B). No obvious phenotypic effect was observed when homozygous plants were grown on Rockfiber blocks with MGRL medium (data not shown).

To evaluate the effect of the insertion, total RNA was prepared from the seedlings of wild type and the *ipt3-1/ipt3-1* homozygote that had been treated with KNO₃ or KCl for the indicated times, and their *AtIPT* expression was monitored (Fig. 6). In the *ipt3-1/ipt3-1* plants, the basal accumulation level of *AtIPT3* transcript in roots was markedly reduced (to less than one-twentieth) and NO₃⁻-dependent induction of the expression was almost diminished. On the other hand, *AtIPT5* transcript was slightly and transiently accumulated in wild-type plants 3 h after NO₃⁻ treatment. Interestingly, a substantial accumulation of *AtIPT5* transcript occurred in roots of the *ipt3-1/ipt3-1* plants, and the level was maintained for over 24 h. The

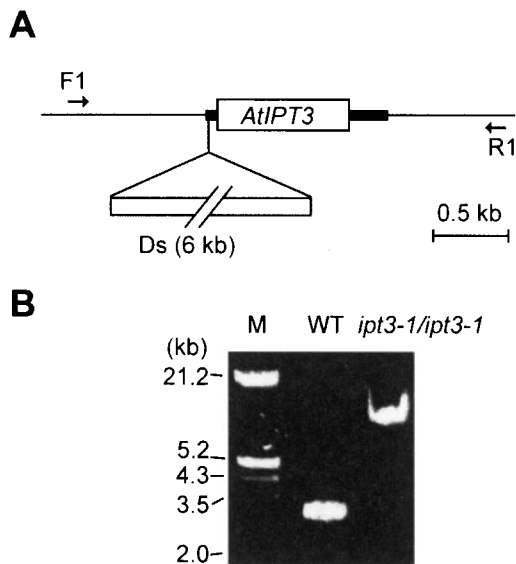


Fig. 5 *Ds* transposon-insertion mutant of the *AtIPT3*. (A) Schematic representation of the genomic *AtIPT3*, in which the rectangle indicates the exon and the thick bars indicate the 5'- and 3'-untranslated regions. (B) Segregation of the homozygous line of the mutant (referred to as *ipt3-1/ipt3-1*). Genomic DNAs prepared from wild-type (WT, DS15) and homozygous (*ipt3-1/ipt3-1*) plants were analyzed by PCR with the indicated primers (see Fig. 5A, F1 and R1). The PCR products were analyzed by agarose-gel electrophoresis. M, molecular size markers.

expression pattern of *AtIPT1* was not affected in the mutant. In wild-type plants, the expression of *AtIPT7* was repressed by NO_3^- during the first 12 h of treatment, but was partially restored in the *ipt3-1/ipt3-1* plants.

We further analyzed the CK content of wild-type and *ipt3-1/ipt3-1* plants. Roots and shoots were separately harvested 9 h after NO_3^- -treatment, and CKs were extracted and measured (Tables 1, 2). In wild-type plants, the contents of *trans*-zeatin (tZ), tZR and tZRMP were markedly increased by NO_3^- -treatment in both roots and shoots, whereas *cis*-zeatin (cZ)-type species were unchanged or decreased. Isopentenyladenine (iP)-type CKs, such as iP riboside (iPR) and iPR 5'-monophosphate (iPRMP), were also significantly increased in shoots. Conversely, in *ipt3-1/ipt3-1* plants, the NO_3^- -dependent accumulation of iP-type and tZ-type CK species was markedly reduced or diminished whereas accumulation of cZ-type species was not affected. These results strongly suggest that *AtIPT3* plays a major role in the rapid response of CK biosynthesis, especially for tZ and iP-type CKs, to the availability of NO_3^- .

Discussion

In this study, we characterized the regulatory manner of *Arabidopsis* CK biosynthesis genes, *AtIPTs*, focusing on the spatial distribution and response to nitrogen availability. Our data clearly demonstrate that nitrogen availability tightly regu-

lates CK biosynthesis. Although some characterizations of the *AtIPTs* have been reported very recently (Miyawaki et al. 2004), our analyses provide some new insights into the regulation.

Quantitative analysis of accumulation of *AtIPT* transcripts (Fig. 1) and histochemical analysis of GFP fluorescence in various tissues (Fig. 2) enable us to discuss the detail of their spatial differentiation and contribution for CK production. Our study suggests that *AtIPT5* and *AtIPT3* play a dominant role for CK production in roots and *AtIPT3* does in shoots, although their relative contributions can be changed by the plant's nutritional status (Fig. 3). Under long-term nitrogen-sufficient conditions, CK biosynthesis is suggested to occur mainly in the lateral root primordium, pericycle and phloem companion cells in roots and in the phloem companion cells in shoots, and when the nutritional supply is limited the contribution of *AtIPT5* for CK production in the roots is decreased and that of *AtIPT1*, *AtIPT5* and *AtIPT7* in the shoots is increased. It has recently been demonstrated that *AtIPT1*, *AtIPT3*, *AtIPT5* and *AtIPT8* localize in the plastids and *AtIPT7* in the mitochondria (Kasahara et al. 2004). Thus, the major compartment of CK biosynthesis is the plastids but the relative contribution of mitochondria to synthesis is increased when the nitrogen sources are limited.

The analysis with *AtIPT::GFP* transgenic plants also suggests that some *AtIPTs* appear to function in the production both of a local signal and of a long-distance signal. The expression of *AtIPT5* in the lateral root primordium and pericycle appears to contribute to provide CKs both to induce cell division in the expressing cell and its immediate neighbors and to transport them to xylem vessels, via the metaxylem, for long-distance translocation. *AtIPT3* and *AtIPT7* are expressed in the phloem companion cells, also implying a function in long-distance signaling via the vascular systems and paracrine signaling to the neighbors.

Detailed analyses of the *AtIPT3* and *AtIPT5* expression patterns in relation to nutritional regulation revealed that nitrogen-availability differentially regulates their expression: *AtIPT3* rapidly and specifically responds to NO_3^- availability under nitrogen-limited conditions and *AtIPT5* responds to both NO_3^- and NH_4^+ under long-term treatment (Fig. 3, 4). The dual response system regulating CK biosynthesis might be important in allowing plants to cope with changes in the nitrogen status. It is intriguing to know how *AtIPT3* and *AtIPT5* are functionally differentiated or share roles in the production of CK in response to nitrogen sources for regulating plant development because the two genes are expressed in clearly distinct tissues (Fig. 2). It is well known that the long-term exposure of roots with nitrogen-excess or nitrogen-limited status severely affects the root morphological architecture (i.e. number and/or length of lateral roots). Further analysis using disruption mutants of *AtIPT3* and *AtIPT5* is clearly required to elucidate their physiological roles.

Analyses of the transposon-inserted mutant *ipt3-1* clearly demonstrated that *AtIPT3* is a key determinant of NO_3^- -

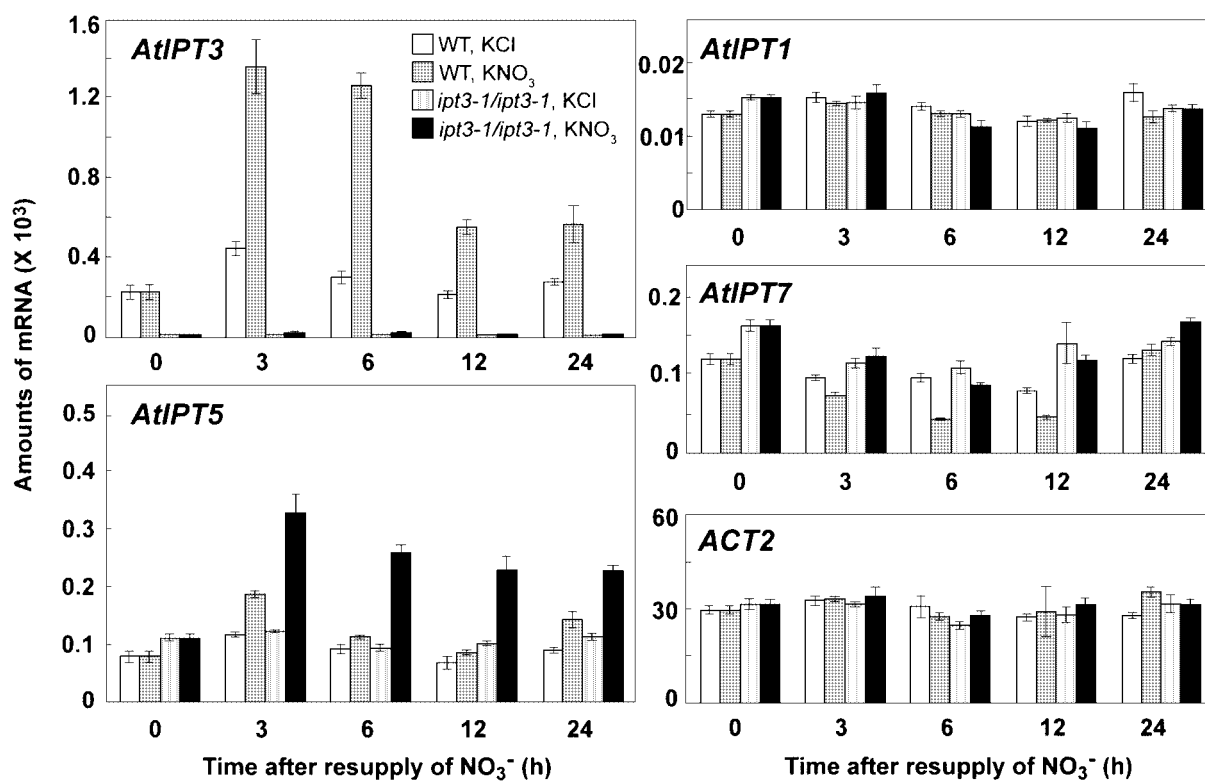


Fig. 6 Analysis of accumulation of *AtIPTs* transcripts in NO_3^- induction in *ipt3-1* homozygous line. Wild-type (WT, DS15) and *ipt3-1/ipt3-1* *Arabidopsis* seedlings were grown on MGRL agar plates containing 1% sucrose for 14 d after germination with a reduced level of nitrogen (0.3 mM NO_3^-). To wash out the remaining nitrogen sources, roots of the seedlings were put into the nitrogen-free MGRL solution and incubated for 1 h with leaving at rest. Next, the seedlings were transferred to 10 mM KNO_3 (WT, dark-grey bar; *ipt3-1/ipt3-1*, black bar) or 10 mM KCl (WT, white bar; *ipt3-1/ipt3-1*, light-grey bar) solution and harvested at the indicated times. Other details of conditions are described in the Materials and Methods. Total RNA was prepared from the roots and the accumulation levels of the transcripts were analyzed by a quantitative real-time PCR. Accumulation levels of the transcripts are given as the copy number of mRNA per 1 ng total RNA. Real-time PCR was performed in triplicate, and the mean values with SD are shown.

responsive CK biosynthesis. Although the expression of *AtIPT5* was correlated to nitrogen availability in the long-term treatment, it was little induced by NO_3^- in the short-term and did not correspond to CK accumulation in wild-type plants (Fig. 4, 6). However, in *ipt3-1/ipt3-1* plants, *AtIPT5* transcript could accumulate substantially in response to the re-supply of NO_3^- (Fig. 6). This alternative response appears to be reflected in the faint NO_3^- -dependent accumulation of tZ-type CK in roots and shoots of *ipt3-1/ipt3-1* plants (Table 1, 2). A recent report implies that *AtIPT5* expression can be up-regulated by auxin and repressed by CK (Miyawaki et al. 2004). In wild-type plants, CKs produced by *AtIPT3* might repress the expression of *AtIPT5*, which is potentially responsive to rapid changes in NO_3^- -availability.

It should be cautioned that the GFP fluorescence does not always correspond to the site of CK production because some GFPs could be symplastically moved to sink tissues (Imlau et al. 1999). Although the fluorescence of *AtIPT3::GFP* and *AtIPT5::GFP* were observed in basal stele of young lateral roots (Fig. 2E) and meristem of emerging lateral roots (Fig. 2J),

respectively, we could not exclude the effect of movement of GFP proteins at present, because the GFP fluorescence in basal stele of young lateral roots was not observed in *AtIPT3::AtIPT3-GFP* plants (data not shown). Similarly, the accumulation level of *AtIPT* transcripts does not always correspond to the activity of CK production because CK production is controlled by the precise balance between the biosynthesis and catabolism. Further analyses of the protein accumulation and regulation of other CK metabolic genes will finally elucidate physiological contribution of the *AtIPTs* to nitrogen-dependent CK production.

The similar kinetics of the NO_3^- -responsive induction of *AtIPT3* to those of *Nial* leads us to speculate on a common regulatory NO_3^- -response mechanism. The minor effect on induction caused by treatment with cycloheximide supports this idea (Miyawaki et al. 2004). In the NO_3^- -specific activation of transcription, it was demonstrated that a putative 12-bp cis-element, AT-rich cluster followed by A(C/G)TCA, was necessary for NO_3^- -responsive transcription (Hwang et al. 1997). When we searched the consensus sequence in the promoter

Table 1 CK contents ^a in wild-type (WT) and *ipt3-1/ipt3-1* roots ^b

CKs (pmol g (FW) ⁻¹)	WT		<i>ipt3-1/ipt3-1</i>			
	KCl	KNO ₃	KCl	KNO ₃		
iPRMP ^c	2.06±0.58	1.65±0.14	1.64±0.28	1.48±0.21		
iPR	0.54±0.16	1.09±0.60	0.42±0.24	0.24±0.02		
iP	2.02±0.21	4.13±1.68	1.25±0.62	1.03±0.37		
tZRMP	0.70±0.04	4.16±0.42	inc*** ^d	0.39±0.03	0.86±0.12	inc***
tZR	0.47±0.05	4.94±0.44	inc***	0.30±0.09	1.17±0.18	inc***
tZ	1.28±0.20	4.08±0.52	inc***	0.94±0.34	1.07±0.14	
tZ9G	6.59±0.57	7.60±0.62	inc*	4.07±1.39	3.44±0.49	
cZRMP	1.40±0.15	0.83±0.07	dec***	1.10±0.10	0.84±0.12	dec**
cZR	0.71±0.08	0.60±0.12		0.64±0.25	0.38±0.03	
cZ	1.49±0.08	1.43±0.26		1.40±0.49	0.74±0.16	dec*

^a Mean value from five or six replicate samples with SD.

^b After treatment of wild-type (WT) and *ipt3-1/ipt3-1 Arabidopsis* seedlings with 10 mM KNO₃ or KCl for 9 h, the roots and the shoots were separately harvested. Next, the CK content in the roots was analyzed as described in the Materials and Methods section. The growth conditions of the seedlings were the same as those in Fig. 6.

^c iP, isopentenyladenine; iPR, iP riboside; iPRMP, iPR 5'-monophosphate; tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5'-monophosphate; tZ9G, tZ 9-glucoside; cZ, *cis*-zeatin; cZR, cZ riboside; cZRMP, cZR 5'-monophosphate.

^d Asterisks indicate that contents of the compound were significantly increased (inc) or decreased (dec) between KCl- and KNO₃-treatments (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, Student's *t*-test).

Table 2 CK contents ^a in wild-type (WT) and *ipt3-1/ipt3-1* shoots ^b

CKs (pmol (g FW) ⁻¹)	WT		<i>ipt3-1/ipt3-1</i>			
	KCl	KNO ₃	KCl	KNO ₃		
iPRMP ^c	1.59±0.49	8.61±1.36	inc*** ^d	1.19±0.13	1.82±0.19	inc***
iPR	0.12±0.04	0.49±0.13	inc**	0.36±0.07	0.24±0.03	dec*
iP	1.98±0.45	1.17±0.29	dec*	2.47±0.60	2.80±1.86	
tZRMP	0.14±0.03	14.53±1.10	inc***	0.07±0.00	2.25±0.27	inc***
tZR	0.47±0.90	8.34±1.56	inc***	0.06±0.02	1.63±0.26	inc***
tZ	0.60±0.08	3.16±0.28	inc***	0.66±0.10	1.08±0.21	inc**
tZ9G	6.30±0.83	5.27±0.31	dec*	4.99±0.71	4.03±0.72	
cZRMP	1.11±0.07	0.56±0.05	dec***	0.99±0.08	0.38±0.04	dec***
cZR	0.63±0.14	0.44±0.09	dec*	0.85±0.09	0.49±0.15	dec**
cZ	1.12±0.20	0.38±0.08	dec***	1.04±0.24	0.81±0.43	

^a Mean value from five replicate samples with SD.

^b After treatment of wild-type (WT) and *ipt3-1/ipt3-1 Arabidopsis* seedlings with 10 mM KNO₃ or KCl for 9 h, the roots and the shoots were separately harvested. Next, the CK content in the shoots was analyzed as described in the Materials and Methods section. The growth conditions of the seedlings were same as those in Fig. 6.

^c iP, isopentenyladenine; iPR, iP riboside; iPRMP, iPR 5'-monophosphate; tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5'-monophosphate; tZ9G, tZ 9-glucoside; cZ, *cis*-zeatin; cZR, cZ riboside; cZRMP, cZR 5'-monophosphate.

^d Asterisks indicate that contents of the compound were significantly increased (inc) or decreased (dec) between KCl- and KNO₃-treatments (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, Student's *t*-test).

region of the *AtIPT3*, several candidates were found at positions -1,195 to -1,184 (ATAATTCACTCA) and -1,482 to -1,470 (TAACAATAAGTCA) from the putative transcription start site. Although their positions are distant from the start

point, these sequences might nevertheless be involved in NO₃⁻-responsive transcription.

It has previously been thought that a CK-mediated nutritional signal is transmitted predominantly via the xylem ves-

sels. However, our results strongly suggest that such signals can be also mediated via the phloem. The nutritional status of the phloem interior or the proximal space of companion cells in the vascular stele would be important to regulate the biosynthesis of CK in response to rapid changes in NO_3^- -availability. Analyses of the concentration of CKs in xylem and phloem saps of *Arabidopsis* and of the changes in the contents by the nitrogen sources will help us understand the long-distance signaling system for nutritional status.

Materials and Methods

Plant material and growth conditions

A. thaliana ecotype Columbia was grown on agar plates with MGRL salt (Fujiwara et al. 1992) supplemented with 1% sucrose at 22°C, at an intensity of $70 \mu\text{E m}^{-2}\text{s}^{-1}$ and a photoperiod of 16 h light/8 h dark. In the experiments on the effect of NH_4^+ availability, MES-KOH (pH 5.7) was added to the medium at 10 mM as a buffer. For growth on Rockfiber block (Nittobo, Tokyo) or hydroponic growth, MGRL salt nutrition was used without modification.

Quantitative real-time PCR

Total RNA was prepared by RNeasy® Plant Mini Kit (Qiagen) with RNase-free DNase I (Qiagen). cDNA was synthesized using SuperScript II RT (Invitrogen) with oligo(dT)₁₂₋₁₈ primers. Accumulation levels of the aimed transcripts were analyzed by a real-time PCR method, with ABI PRISM 7000 Sequence Detection System (Applied Biosystems) monitoring the amplification with the SYBR-Green I dye (Applied Biosystems). The primers for PCR were designed using Primer Express software (Applied Biosystems) and checked for the specific product formation by polyacrylamide gel analysis. The sequences of the primers used for PCR were: 5'-AGAGATCAACGAATCAGATTACGT-3' and 5'-ATGACGCCGAGGAGATGGT-3' for *AtIPT1*; 5'-CATGGCGAATCTCTCCATTGA-3' and 5'-AGTTGGAACTCCCAACGATGA-3' for *AtIPT3*; 5'-CACCACGAATCAATCGACAT-3' and 5'-GAAATTCTGCCGCTGTGACTT-3' for *AtIPT4*; 5'-AGGATTTTCAGCGTGAAGCAA-3' and 5'-CTATGATCGGGACACGGTCTCT-3' for *AtIPT5*; 5'-CCCACTGCATGAGCAAGGA-3' and 5'-GTGTCCGCAAGAACCGGAAC-3' for *AtIPT6*; 5'-AACCTAACGGCCACCCAGTA-3' and 5'-TGTTGTTGCTGAGAGTTTCGA-3' for *AtIPT7*; 5'-TTCCCATGACTACCGTTTGCA-3' and 5'-CGATTGAGAGGCATGACTTGC-3' for *AtIPT8*; 5'-TTGTTTGTTCATTTCCCTTTTGG-3' and 5'-GCAGACGTAAGTAAAAACCCAGAGA-3' for *actin 2 (ACT2)*; 5'-AACTTTGGTGGTTGTGTTTTGG-3' and 5'-TCGACTTGTTCATTAGAAAGAAAGAGATAA-3' for *ubiquitin 10 (Ubiq10)*; and 5'-CGCCATTATCCCACCATGA-3' and 5'-GGAGAAGGAACGAGAGGAGTTT-3' for *Nia1*. In each case, plasmid DNA containing the corresponding cDNAs was used as a template to generate a calibration curve.

Plant transformation and transgenic plant analysis

Promoter regions of *AtIPTs* were amplified by PCR with the *A. thaliana* genome DNA as a template. The primer sequences were: 5'-GCGGTGCGAGGTTAGATGAAGCGATGAC-3' and 5'-GCGGATCTTAGCTAACAACAACAACACGG-3' for *AtIPT1*, 5'-GCGCTCAGGAGTTAAACAGATTGGAGATG-3' and 5'-GCGGGATCCAGATATCTTCATGATCATG-3' for *AtIPT3*, 5'-GCGGTCGACTTTATAGACCCAGATGGATTG-3' and 5'-GCGGGATCCTCTTATAAAAACTCCTTAAC-3' for *AtIPT5*, and 5'-GCGGTCGACTTGATGTGAAAGACAATCCATC-3' and 5'-GCGGGATCCGATGATTGACTTTTTTTGTTGGG-3' for *AtIPT7*. The lengths of the amplified DNA frag-

ments were: 4 kb (*AtIPT1*), 3.9 kb (*AtIPT3*), 3.7 kb (*AtIPT5*), and 4.1 kb (*AtIPT7*). The DNA was ligated into the *SalI/BamHI* site of pBI-sGFP (Chiu et al. 1996). *A. thaliana* plants were transformed with the plasmids by the floral dip method (Clough and Bent 1998). More than five independent lines were obtained for each construct, and their T3 plants were used for analyses. Stable transformants expressing *AtIPT3-GFP* and *AtIPT7-GFP* fusion proteins driven by the native promoters were generated previously (Kasahara et al. 2004). The transgenic plants were grown for approximately 2 weeks on MGRL agarose medium with 1% sucrose. The expression pattern of each gene was observed by laser confocal-scanning fluorescence microscopy (Fluoview IX5, Olympus). For the histochemical analysis of GFP, plant tissues were embedded in 5% agar and sectioned with a microslicer (DTK-1000, Dosaka EM, Kyoto).

Transposon insertion mutant of *AtIPT3*

A *Ds* transposon-insertion mutant of *AtIPT3* was picked up from the *Arabidopsis Ac/Ds*-transposon tag-line database which is built by RIKEN Genomic Sciences Center (Kuromori et al. 2004). The parental DS15 line (ecotype Nossen) (Kuromori et al. 2004) was used as a wild type for the mutant analysis. Evaluation of the insertion of the transposon was carried out by PCR with primers 5'-GCTCGCAAGCCAAAT-TACATTCCAATAGC-3' (F1) and 5'-TTCTTCGTTTTGTTCCGACTCAAAGTGGT-3' (R1). Genomic DNA was prepared by DNeasy® Plant Mini Kit (Qiagen).

CK analysis

Extraction and fractionation of CKs was performed as described previously (Dobrev and Kamínek 2002). The CK fractions were purified further using immuno-affinity columns (Takei et al. 2001a, Takei et al. 2001b). After desalting, the resulting samples were dissolved in 0.005% acetic acid and analyzed with a liquid chromatography-MS system (model 2695/ZQ2000MS, Waters) using an ODS column (Symmetry C₁₈, 3.5 μm , 2.1×150 mm, Waters) as described previously (Yonekura-Sakakibara et al. 2004).

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